1 THE EFFECT OF THE AROMATASE INHIBITOR, ARIMIDEX AND TAMOXIFEN ON TUMOUR GROWTH IN A NUDE MOUSE MODEL FOR POSTMENOPAUSAL BREAST CANCER

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We have previously established a model for postmenopausal, hormone-dependent, breast cancer in nude mice. In the present study, we use this model to determine the effects on the tumour growth of the new nonsteroidal aromatase inhibitor arimidex, the antioestrogen, tamoxifen, and the combination of arimidex and tamoxifen. MCF-7 human breast carcinoma cells transfected with the aromatase gene are inoculated in Matrigel into 4 sites (3×10^6 cells/site) in ovariectomized mice. The animals were injected with androstenedione (0.1 mg/mouse day^{-1} s.c.), substrate for aromatization to oestrogens. After 3 weeks, the mice were assigned to groups of 5 mice and injected with the arimidex 5μg day^{-1} s.c., tamoxifen 3 μg day^{-1} s.c., arimidex 5 μg + tamoxifen 3 μg day^{-1} s.c. or vehicle (control). After 5 weeks of treatment, the animals were killed and tumours were weighed. The mean tumour weights were 743.33 ± 157.36 (s.e.) mg for controls, 452.86 ± 78.88 (s.e.) mg for arimidex 5 μg, 661.88 ± 58.31 (s.e.) mg for tamoxifen 3 μg and 412.75 ± 90.20 (s.e.) mg for arimidex 5 μg + tamoxifen 3 μg. The mean tumour weights of arimidex 5 μg, arimidex 5 μg + tamoxifen 3 μg treatment groups were significantly less than for the control group ($P < 0.05$), the combination of arimidex 5 μg + tamoxifen 3 μg was significantly more effective in inhibiting tumour growth than tamoxifen 3 μg ($P < 0.05$). In addition, the mean uterine weights were 68.25 ± 13.98 (s.e.) mg control, 28.00 ± 7.16 (s.e.) mg arimidex 5 μg, 62.75 ± 4.50 (s.e.) mg tamoxifen 3 μg 48.00 ± 5.04 (s.e.) mg arimidex 5 μg + tamoxifen 3 μg. The mean uterine weight of arimidex 5 μg treatment group was significantly less than for the control group or the tamoxifen 3 μg group ($P < 0.05$). This suggests that arimidex and the combination of arimidex and tamoxifen could be more effective than tamoxifen for blocking oestrogen stimulated growth in breast cancer patients. (Supported by NIH grant CA-62483).

2 ABNORMAL METABOLISM OF GAMMA-LINOLENIC ACID BY HUMAN BREAST EPITHELIAL CELLS

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There are many proponents for the use of gamma linolenic acid (GLA) in the treatment of psoriasis and arthritis, and, in the form of evening primrose oil, GLA has also been shown to be effective in the relief of mastalgia (Holland and Gateley, 1994). The mechanism of action is unclear, but it is believed to be related to the conversion of the GLA to arachidonic acid and thence to prostaglandins. This belief is supported by the finding that dietary supplementation with GLA results in increased arachidonic acid release from human neutophils (Chilton-Lopez et al., 1996) and increased prostaglandin synthesis in rat aorta (Quoc and Pascaud, 1996). The aim of this study was to elucidate the fate of supplementary GLA in human breast cells.

MCF7 human breast epithelial carcinoma cells were cultured and differentiated by the method outlined previously (Tyers et al, 1995) after which the culture medium was supplemented with either evening primrose oil (EPO) which contains approximately 10% GLA and 75% linoleic acid, or pure GLA (30mL oil per 10mL culture medium). The linoleic and gamma-linolenic acid content of the cells was then determined after 12, 24, 48 and 72 h. by derivatization of the fatty acids to fatty acid methyl esters using the method of Metcalfe and Schmitz (1961) followed by GLC analysis. The fatty acid content of the culture medium was determined by a similar method. All procedures were performed in triplicate and results are expressed as the mean ± s.e.m.

Supplementation of the culture medium with pure GLA resulted in a five-fold increase of GLA content, whilst supplementation with EPO resulted in a four fold increase in linoleic acid (LA), but no significant effect on GLA content.

Cells exposed to the raised GLA paradoxically showed an initial, significant (41± 6%) reduction in GLA content which rose to normal values by 72 hours ($p<0.025$, ANOVA); there was also a significant (39 ± 11%) increase in LA content ($p<0.01$, ANOVA). Exposure to raised LA also caused a significant increase in intracellular LA (27 ± 5%; $p<0.01$, ANOVA), and a significant increase in GLA content (64 ± 29%; $p<0.025$, ANOVA). Exposure to both media resulted in the appearance of additional unidentified mioities, both within the cells and within the supernatant medium, which were probably fatty acid breakdown products.

In the cells exposed to cell culture medium supplemented with evening primrose oil, the observed increase in GLA content is probably dependent upon the increase in intracellular LA, the LA being metabolized to GLA by the enzyme delta-6-saturase. In cells exposed to culture medium supplemented with pure GLA however, the finding of raised intracellular LA and decreased intracellular GLA suggests that the excess GLA shifts the equilibrium of the metabolic pathway such that the GLA is metabolized in a retrograde manner to LA. This finding thus suggests that delta-6-saturase is a reversible enzyme. As the concentration of LA increases, the normal equilibrium is restored and the intracellular content of GLA returns to normal.

If the process observed here in human breast cancer cells also occurs in normal breast epithelial cells, these results suggest that the ratio of LA to GLA in evening primrose oil may be important in its actions in the relief of mastalgia, and therefore attempts to potentiate the efficacy of evening primrose oil by addition of extra GLA, or the use of borage oil (also called starflower oil), which contains approximately 26% GLA but very little LA, instead of evening primrose oil, may be of little extra benefit, and may even delay the onset of any therapeutic effect.

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3 TPA-INDUCED TRANSLOCATION OF PROTEIN KINASE C β_1- AND β_2-ISOTYPES OF HUMAN MONOCYTES

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Human monocytes have been found to contain α and β PKC isotypes in the cytosol and membrane (Chang et al 1993).
Exposure of human monocytes to 100nM 12-tetradecanoyl phorbol-13-acetate (TPA) for a time interval of 5 minutes, was found to translocate and equally distributed PKC β_1 and β_2 to the membrane and cytoskeleton. This was easily detected using ECL western blotting reagents (Amersham, UK).
We have been investigating the translocation time dependent of PKC in human monocytes, on stimulation with TPA and two daphnane esters, resiniferatoxin (RX) and resinferanol-9, 13, 14-orthophenacetate (RO), which are non tumour promoters.
Human monocytes were prepared from human venous blood obtained from healthy donors. Human mononuclear cells (HMNC) were then produced by centrifugation over Histopaque 1077 (sigma) at 400g x 20 min. Monocytes were isolated by adherence to plastic petri dishes as described previously by Pawan (1994). Non adherent cells were aspirated off and adherent cells gently scraped from the walls of the petri dishes and resuspended in the medium prior to stimulation with phorbol esters and homogenisation.
The monocytes were greater than 95% viable as determined by trypan blue exclusion method.
Aliquots of human monocytes were exposed to various concentrations (1-1000nM) of TPA, RX and RO for a time interval of 5 minutes. Cytosolic, membrane and cytoskeleton fractions of each sample were produced as described by Kiley et al (1994).
TPA concentrations (< 100nM) lacked the ability to activate the PKC while, high concentrations (> 100nM) and long exposure periods either caused depletion or down-regulation of PKC β. RX and RO failed to activate the PKC and this result may be consistent with the fact that RX is a non-promoting diterpene in Berenblum-type tumour-promoting experiments (Aldolf et al 1982).
Table 1 shows that TPA is the most potent and the only compound which activates and translocates PKC β_1 and β_2.

Table 1 - Translocation of PKC β_1 and β_2 isotypes in human monocytes

Phorbol ester	Dose (nM)	Translocation
TPA	100	✓
RX	1 - 1000	×
RO	1 - 1000	×

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Chang Z L et al (1993) Immunol. 80, 360-366
Kiley S C et al (1994) Carcinogenesis 15, 319-324

4 APOLIPOPROTEIN E ISOFORMS ASSOCIATED WITH HEREDITARY RETINAL DEGENERATIONS: IMPLICATIONS FOR THERAPY

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Retinitis pigmentosa (RP) is a family of hereditary retinal degenerations characterised by progressive night blindness and tunnel vision. It is known that the photoreceptor cells, which degenerate in RP, are unusually rich in a long-chain polyunsaturated fatty acid, docosahexaenoic acid (DHA; C22:6, n-3), and experimental animals deprived of DHA or its precursors in their diet show decreased visual performance.

We have been investigating the various ways in which DHA is supplied to the retina, to see if a defective supply could underlie some forms of RP. Plasma lipids are transported in the form of lipoproteins which interact with target tissues via the apo B,E receptor, so we have screened the RP population for defective apolipoprotein B (apo B) or apolipoprotein E (apo E). Apo E occurs in three isoforms, E2, E3, and E4, thus there are 6 phenotypes, E2/2, E2/3, E2/4, E3/3, E3/4 and E4/4. The less common E2 and E4 isoforms are dysfunctional: E2 does not bind to the B,E receptor and is associated with Type III hyperlipidaemia, and E4 is associated with Alzheimer's disease.

Huq et al (1993) reported that E2/2 and E4/4 are significantly elevated in the RP population, and that several patients have variants of apo E which behave anomalously on gel electrophoresis or isoelectric focusing after cysteamine modification, so may be new mutations in this protein.

We determined the phenotypes of 90 patients attending the RP Clinic at the Western Infirmary, Glasgow. The data (Table 1) confirm Huq's finding of a high incidence of the E2/2 and E4/4 isoforms in RP.

Table 1 Incidence of apo E phenotypes in the Scottish RP population compared to Scottish controls

	E2/2	E2/3	E2/4	E3/3	E3/4	E4/4
This study (n = 90)	5	15	3	48	14	5
%	6	17	3	53	16	6
Huq (1993) (n = 100)	4	20	1	44	23	8
%	4	20	1	44	23	8
Controls (n = 400)	2	51	11	233	99	4
%	1	12	2	59	25	1

The possibility of new mutations in apo E from 6 patients with unusual isoelectric focusing patterns (including patient I.C., reported by Huq, 1993) was investigated using polymerase chain reaction (PCR) amplification of exon 3 which codes for the majority of the apo E gene. No new mutations were found.

In conclusion, we find a higher incidence of apo E2/E2 and E4/E4 phenotypes in RP, but no evidence for new mutations. It is likely that the dysfunctional apo E isoforms are contributing to the aetiology of the disease (which is probably polygenic in these individuals) but are not the main cause. In any case, therapy which enhances the supply of the PUFA to the retina would appear to be indicated in RP.

Supported by the W.H.Ross Foundation (Scotland) for the Study of Prevention of Blindness. We are grateful to R.Tate and C.Hissitt for technical support and D. Bedford for advice.

Huq, L., et al (1993) Lipids 28: 995-998.

5 AN INVESTIGATION OF THE EFFECTS OF (1→3)-β-D-GLUCAN ON AN IN-VITRO GUINEA-PIG PERFUSED LUNG MODEL

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The (1→3)-β-D-glucans are glucose polymers found in the cell walls of moulds, fungi and bacteria. They have recently come into focus as possible mediators of the respiratory symptoms observed on inhalation of organic dusts, such as cotton and grain, and are currently under investigation. Exposure of guinea pigs to water soluble glucans can evoke an inflammatory response, with an influx of neutrophils into the lungs, Rylander (1994). However, the water insoluble glucans appear not to influence the number of inflammatory cells found in the lung, Fogelmark et al (1992). The aim of the present study was to determine whether the (1→3)-β-D-glucans have any effect on another facet of the respiratory symptoms - airway reactivity.

After sacrifice, the trachea and lungs from male Dunkin-Hartley guinea pigs (250-500g) were removed into Krebs solution. The lungs were separated into right and left lobes, and then perfused with Krebs via the bronchi (perfusion rate 5ml min^{-1}). After equilibration, dose response (DR) profiles were established using the bronchoconstrictor methacholine. The lung was then perfused with glucan (from bakers yeast), at a predetermined concentration (3, 10, 50 or 100μg ml^{-1}) for 1h. After reperfusion with Krebs the DR profile was redetermined immediately and 1h after exposure.

The results in Figure 1 show that glucan (50μg ml^{-1}) causes hyporesponsiveness in the perfused lung. There was a significant decrease in response at all methacholine concentrations which was sustained over the 1h period. At glucan exposures of 10μg ml^{-1} and 100μg ml^{-1} there is also a decrease in response. However, significance is only observed at the lower methacholine concentrations (1μg and 3μg methacholine for 10μg ml^{-1}; 1μg methacholine for 100μg ml^{-1}). A glucan exposure of 3μg ml^{-1} elicits no significant change in the methacholine induced constriction.

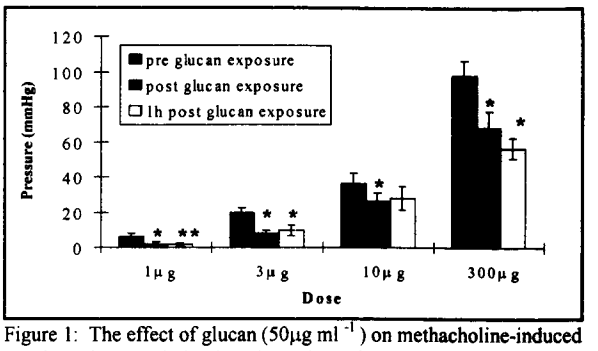

Figure 1: The effect of glucan (50μg ml^{-1}) on methacholine-induced bronchoconstriction in guinea pig perfused lung. Results are means±s.e.m, n=6, * P<0.05, ** P=0.05

This study indicates that (1→3)-β-D-glucans have a direct effect on the lungs, at least *in vitro*. The hyporesponsiveness and decrease in maximal response to methacholine suggests that glucan may cause a change in the efficacy of the methacholine / M3 receptor complex, possibly through the 2nd messenger system.

PJN is in receipt of a grant from the British Cotton Growing Association.

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6 EFFECT OF DAPSONE ON ANAPHYLACTIC RESPONSES OF SENSITIZED LUNG STRIPS

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Dapsone is a sulphone used to treat leprosy, tuberculosis and malaria. It has antiinflammatory properties and has been used for rheumatoid disorders and may have potential in chronic asthma (Berlow et al. 1991). In this study we investigate whether dapsone inhibits the anaphylactic response of sensitized lung strips to antigen.

Guinea-pigs (male, 250-350g) were sensitized with ovalbumin (OA, 10μg and aluminium hydroxide 100mg in saline). 21 days later they were killed and the lungs removed. Parenchymal strips (4cm x 5mm) were cut from the margins of the lower lobes and superfused at 37°C with Krebs-bicarbonate solution (5ml/min) pregassed with 5%CO_2 in O_2. Isometric tension was initially set at 1g. Paired tissues from the same animal were set up , one as the control and the other superfused with dapsone (100μM) throughout. Injections into the superfusion fluid of submaximal bolus doses of histamine (1μg), carbachol (3μg) and OA (20μG) produced contractions.

Dapsone had no effect on the peak responses to histamine (absence 0.14±0.06g, presence 0.14±0.01g, n=4) or carbachol (absence 0.07±0.005g, presence 0.08±0.003g). This indicates a lack of antagonism of histamine H_1 and muscarinic M_3 receptors at this concentration. The contraction to OA in the absence of dapsone peaked at 0.13±0.03g and was relatively prolonged, returning to baseline at 75.5±3.5min. In the presence of dapsone, the peak response (0.13±0.05g) was unaltered, but there was a rapid return to baseline by 13.3±2.1min This suggests that dapsone inhibits the mediator(s) responsible for the secondary part of the response, such as prostaglandins or leukotrienes. Experiments were next performed with mepyramine (10μM) present throughout in both tissues to inhibit histamine H_1 receptors. Responses to histamine were abolished. Responses to carbachol were again virtually identical in the absence (0.06±0.004g) and presence of dapsone (0.058±0.008g, n=4). OA caused a prolonged contraction in the absence of dapsone, peaking at 0.21±0.03g and returning to baseline at 105±11.9min. In the presence of dapsone, the peak was not significantly reduced (0.18±0.02g) but the response was significantly shortened to under 20min. Next, mepyramine and indomethacin (1μM), to additionally inhibit cyclooxygenase, were present throughout. Carbachol produced similar responses in the absence (0.04±0.007g) and presence of dapsone (0.03±0.01g, n=4). The OA response in the absence of dapsone (peak, 0.16±0.03g) was sustained, recovery to 50% being 85.9±21.6min and was presumably due to the leukotriene products of 5'-lipoxygenase. Dapsone did not significantly affect the peak (0.09±0.03g) response to OA which was sustained with 50% recovery at 41.5±19.8min.

Finally, the experiments were repeated in the presence of the 5'-lipoxygenase inhibitor, 2-(1-thienyl)ethyl 3,4-dihydroxybenzylidene-cyanoacetate (1μM), and mepyramine. Carbachol was not affected by dapsone (0.05±0.01 and 0.044±0.01g, n=4). The response to OA in the absence of dapsone, presumably due to cyclooxygenase products, peaked at 0.17±0.06g and remained elevated to 76.5±3.3% at 60min. In the presence of dapsone, the OA peak was unaltered (0.125±0.04g) but there was a rapid decline to baseline by 20min.

This study has shown that dapsone shortens the anaphylactic response of lung strips to OA, probably by inhibition of cyclooxygenase products released from mast cells.

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7 THE ROLE OF HISTAMINE ON MYOFIBROBLAST CONTRACTILITY AND ITS ROLE IN WOUND HEALING

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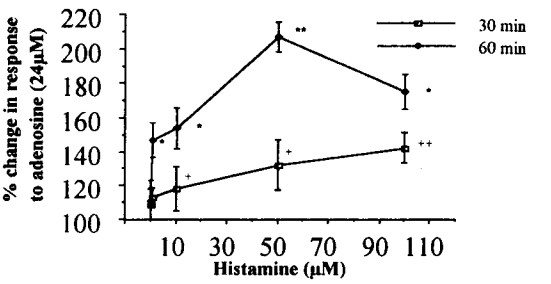
Following skin injury, inflammation occurs mediated via a variety of agents one of which is histamine. The major source of such histamine is thought to be from pre-formed stores in the mast cells which are commonly found in the loose, connective tissue fascia underlying the lower regions of skin. The release of histamine occurs via degranulation of the mast cells which is a rapid process, Majno & Joris (1996). In the fascia are fibroblasts which may induce wound contraction and the purpose of this study was to investigate whether histamine could influence their contractile abilities.

Strips (1x2cm, n=8) of similar areas of the dorsal subcutaneous fascia (SF) from male, Hooded Lister rats (250-300g) were placed under 2g tension in an organ bath arranged for tissue superfusion. The degree of contractility of each preparation was established for 12, 24, & 48μM adenosine and then the responses were re-examined following incubation with histamine (1-100μM) for both 30 and 60 minutes.

The results (Fig.1) show that the adenosine response was potentiated by incubation with all the concentations of histamine tested on SF. Even a concentration as low as 1μM after 60 min incubation produced this effect.

The process of wound healing is an integrated, temporally orchestrated phenomenon of a series of complex interactions of many factors. Some factors are known, others remain uncertain and the significance of each factor at some stages may predominate over others. Although in this study histamine potentiated a contractile effect others have recently reported it to have metabolic effects on synovial fibroblasts, inducing the secretion of hyaluronic acid and proliferation of fibroblasts via H_1 and H_2 receptors, Nagata et al (1992). Although it is accepted that there is a complex interaction of various factors within a wound, this experiment has clearly shown that at least in the adenosine primed state, histamine may have a role in the overall healing process by activating fibroblasts to promote wound contraction and so aid the healing process.

Fig. 1: The influence of incubations of rat superficial fascia with histamine on the contractile response to adenosine (24μM). Histamine (1-100μM) incubation for both 30 and 60 min increased the responses in a concentration- and duration of incubation-dependent manner. + and ++ (against the control). * & ** (between the two durations), [P<0.05 & 0.01 respectively, Student's paired t-test, n=8].



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It is now generally accepted that in the vast majority of natural habitats, be they associated with environmental sites, industrial plant, manufactured products or the tissues of animals and plants, microorganisms are found attached to surfaces rather than as free-swimming planktonic organisms. The latter are only prevalent in highly nutritious situations where all essential nutrients are in abundance. Whilst such conditions have been traditionally favoured for laboratory studies, they create populations of bacteria which are growing at growth rates close to their physiological maximum, where metabolism is wasteful and inefficient, and where the phenotype, adopted by the cells, is unrepresentative of in-vivo/in-situ. Planktonic growth is favoured under these conditions since the proximity of nutritionally inert surfaces, or indeed other cells, reduces the availability of nutrients. Conversely in most environmental situations the scarcity of essential nutrients substantially reduces the growth rate of the cells, increases competitiveness, and forces the cells to adopt phenotypes which reflect the prevailing physico-chemical environment. Often this includes attachment to a surface, prior to growth and the formation of a biofilm.

Attachment is mediated through physico-chemical interactions between the substratum and components of the bacterial envelope. This is subsequently reinforced by an overproduction of exopolymers which cement the organisms in place. In harsh environments biofilm cells have a competitive advantage over their planktonic counterparts since, (i) they are protected against desiccation and phagocytosis by an exopolymer slime-layer (glycocalyx) (ii) they gain access to increased quantities of organic nutrients, which become concentrated at solid:liquid interfaces, and inorganic salts which are bound within the glycocalyx, (iii) they are able to moderate their own micro-environment with respect to pH and oxygen tension, (iv) the close proximity of other cells facilitates co-operative activities such as cross-feeding, and (v) the relatively high cell density promotes genetic exchange and renders advantageous the production of extracellular factors such as enzymes, siderophores, bacteriocins. Biofilms are therefore often regarded as being highly evolved, functional consortia of microbial cells, having physiologies and metabolic capabilities that are greater than those of the component species.

A large number of microbial species are able to survive and grow not only within the general environment but also within a debilitated animal host. These opportunistic pathogens, together with a minority of species that are obligated to derive nutrients and water solely from animal hosts, require a highly intimate contact with the host tissues in order to obtain nutrition. The outcome of any infective process is either peaceful co-existence between host and microbe, the elimination of the microbe from the hosts tissues or the death of the host. This outcome reflects a balance between the ability of the microbes to obtain nutrients and multiply within the host, and the ability of the host to kill/remove the infecting cells. These are very similar criteria to those which determine which microorganisms are able to colonise various environmental eco-systems. Accordingly many of the attributes possessed by biofilm communities are also essential for growth within an animal or plant host. Firm attachment allows the microbe to resist physical removal by fluid dynamic forces associated not only with river beds and streams but also the flow of air, blood, mucin, urine and gut lumen contents. The expression of specialist, adhesins enable the bacteria to attach more avidly to particular tissues. Protection, by the glycocalyx, against phagocytosis includes not only ingestion by protozoa but also white blood cells and macrophages. Cell density mediated responses of the microbial community, within the attached micro-colonies, trigger the production of extracellular factors such as proteases, siderophores, lipases and exopolymers, many of which are regarded as virulence determinants. The facilitating event for all of these pathogenic adaptations is firm attachment to a surface.

9 EFFECT OF INHIBITION OF PROTEIN SYNTHESIS AND CELL DIVISION ON THE POST-ANTIBIOTIC EFFECT OF CIPROFLOXACIN ON *ESCHERICHIA COLI*

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Ciprofloxacin (Cip), like many other antibacterials, continues to inhibit cell division after drug removal (Chin & Neu 1987). This post antibiotic effect (PAE) can be viewed as an expression of the time required for repair before exponential cell division resumes. The clinical value of PAEs lies in their potential to lengthen dosage intervals.

Quinolones bind to the bacterial DNA gyrase-DNA complex and disrupt DNA supercoiling (Shen et al 1989), but the subsequent events leading to cell death are less clear. Modern quinolones require protein synthesis together with active cell division for at least part of their activity (Smith & Lewin, 1988). We report experiments to determine PAEs in bacteria treated with ciprofloxacin under conditions that inhibit protein synthesis or cell division.

Overnight nutrient broth (NB) grown cultures of *Escherichia coli* AB1157 were diluted 1 in 50 into nutrient broth containing 0.02 mg L^{-1} Cip, with or without the presence of the protein synthesis inhibitor chloramphenicol (Cm) at 20 mg L^{-1}. Cultures were also exposed to Cip in phosphate buffered saline (PBS) to inhibit cell division. After 3 h at 37°C, cells were recovered by filtration, washed twice with NB, resuspended in NB and maintained at 37°C. Viable counts on nutrient agar (Figs. 1 and 2) allowed PAEs to be calculated as the differences in time taken for the viable counts of drug-exposed and control cultures to increase ten-fold.

Control cultures grown in drug-free NB for 3 h were diluted 1 in 100 to give the uninhibited growth rate (1). From this it was possible to calculate that Cip in NB (2) resulted in a PAE of 96 min, with Cm in NB (3) giving a similar PAE of 105 min. The presence of Cm increased the PAE of Cip to 183 min (4). Control cells suspended in PBS for 3 h (5) recovered at the same rate as NB-grown controls (1). The PAE of cells exposed to Cip in PBS (6) was 104 min when compared to the PBS control (5), or 113 min when compared to the NB control (1).

Fig. 1

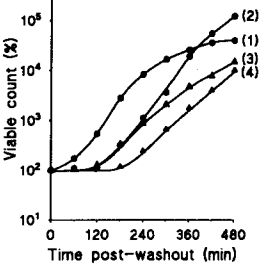
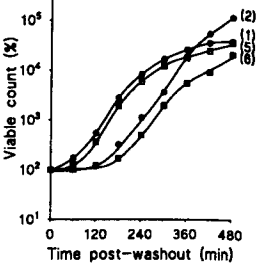


Fig. 2



Figures: Recovery of *E. coli* after drug exposure. Viable counts are expressed as a percentage of the respective initial post-washout count.

Concurrent inhibition of protein synthesis during the action of ciprofloxacin would appear to extend the PAE of the latter. However, inhibition of cell division during Cip exposure has little effect on the Cip PAE. These observations could be of clinical importance when quinolones are co-administered with antibacterials that inhibit protein synthesis, and may contribute to an understanding of the precise mechanism of action of quinolones.

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10 *N*-ACYL HOMOSERINE LACTONES AND BACTERIAL BIOFILMS

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N-Acyl Homoserine Lactones (AHLs) are quorum sensing molecules used by a wide variety of Gram-negative bacteria to sense population size (Salmond *et al.*, 1995). When present at sufficient concentrations, these AHLs regulate a number of virulence determinants and secondary metabolites and help to coordinate a population response (Salmond *et al.*, 1995). Such bacterial processes are fundamental to the interaction of bacteria with each other, their environment and with the host. At the molecular level AHLs have been shown to activate transcription of DNA through global regulators such as *LuxI/R*, *LasI/R* and *VsmI/R* (Winson *et al.*, 1995). Consequently, there has been much speculation as to whether global regulatory systems such as these play a part in the attachment of bacteria to surfaces and/or the maintenance and persistence of bacterial biofilms.

Pseudomonas aeruginosa PA01 is known to produce at least 4 chemically distinct AHLs, namely; butanoyl (BHL), hexanoyl (HHL), oxohexanoyl (OHHL) and oxododecanoyl (odDHL) (Winson *et al.*, 1995). Kinetics of attachment and strength of attachment of PA01 to chemically clean glass coupons were compared to those of two mutant strains, PA0-R1 and PAN067, defective in their ability to produce odDHL, and BHL and HHL respectively. In some instances the growth media of the two mutant strains was supplemented with the missing AHLs. These were chemically synthesised and kindly provided by Dr Eberhard (Ithaca College, New York, USA). Bacterial attachment was quantified using the methods of Allison and Sutherland (1987) whilst strength of attachment was assessed by performing a plate succession exercise according to the methods of Eginton *et al* (1995).

The level of bacterial attachment varied not only with strain but also with addition of the missing AHLs. Strain PA0-R1 showed a two-fold decrease in the number of attached cells after 5h incubation compared to PA01, the level of which was restored by the addition of exogenous HHL. By comparison, strain PAN067 showed a 2.5-fold increase in the number of cells attached relative to PA01. However, on addition of BHL the number of cells attached to the slide dropped to a level slightly below that of PA01. Results suggested that these two AHLs have different effects on the cell surface of *P. aeruginosa* whereby HHL positively promotes attachment whereas BHL has a negative effect on attachment. Indeed, cell surface analysis of the two mutant strains grown in the absence or presence of their cognitive AHL showed both qualitative and quantitative differences in outer membrane protein profiles compared to the parent strain.

Strength of attachment was also shown to be influenced by AHL composition and presence. Whereas strain PAN067 was more easily removed from a surface than PA01 but increased in attachment strength following addition of BHL, strength of attachment of strain PA0-R1 was weakened by the addition of HHL. The results suggest a complex series of interactions between different AHLs, each of which have a separate role in biofilm formation and physiology. From the data generated it is proposed that, for *P. aeruginosa* attachment to glass, HHL is involved in the initial stages of attachment whilst BHL has a positive role in biofilm persistence and maintenance.

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11 THE EFFECT OF TEAR FILM CONDITIONING ON BACTERIAL ADHESION TO CONTACT LENS MATERIALS

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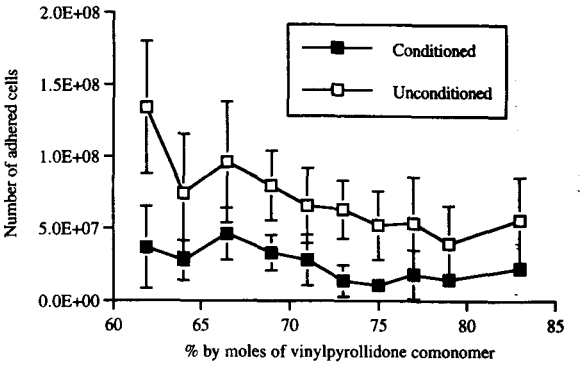
Microbial keratitis represents a major clinical complication to contact lens wearers, and the organism most frequently isolated from the corneal ulcers is *Pseudomonas aeruginosa* (Koidou-Tsiligianni et al 1989). Studies have reported the effects of lens material formulation on microbial adhesion (Cook et al 1993), and the effect of tear film conditioning (an important precursor to bacterial adhesion) has been investigated in studies using pre-worn lenses (Boles et al 1992). However, the effect of controlled *in vitro* conditioning of contact lens materials on bacterial adhesion has received little consideration. This communication describes an *in vitro* study to investigate the effect of conditioning on bacterial adhesion to contact lens materials.

An ophthalmic clinical isolate of *Pseudomonas aeruginosa*, kindly provided by Moorfields Eye Hospital, was cultured on Trypticase Soy Agar and subcultured in Trypticase Soy Broth for 14 h at 37°C on an orbital shaker (120 rpm). Bacteria were harvested by centrifugation (20 min, 2100 g), washed twice and resuspended to $1x10^8$ cells/mL in phosphate buffered saline (PBS). High water content, non-ionic (FDA class II) contact lens materials based on vinylpyrrolidone/methyl methacrylate were prepared and 6 mm disks were cut from the sheets of hydrogel. Some disks were incubated for 1 h at 37°C in artificial human tear film (conditioned), which was prepared according to the method described by Mirejovsky et al (1991). Lens material samples (both conditioned and clean) were placed into 24-well assay plates. Bacterial suspension (2 mL) was added and the materials were incubated at 37°C on an orbital shaker (120 rpm). After 4 h the materials were removed to fresh wells and rinsed 10 times in PBS. Bacterial ATP was extracted by lysis in 1% w/v trichloroacetic acid with 2 mM EDTA in water. Following 10-fold dilution in TRIS-acetate buffer containing 2 mM EDTA, ATP was quantified using a bioluminescent ATP assay kit (Bio-Orbit, Finland) with a luminometer (Amersham, UK). Cell number was calculated using a previously constructed calibration plot.

Figure 1. shows that bacterial adhesion to a range of FDA class II contact lens materials which had been previously conditioned in artificial human tears was lower (by approximately 50%) than to clean materials of the same type.

Figure 1. (n=6, mean±sd)



Conditioning lens materials with tear film lipids and proteins can lower bacterial adhesion, compared to clean materials. This is in agreement with in vivo studies, which report reduced bacterial adhesion to human-worn lenses compared to unworn lenses (Boles et al 1992). Since contact lenses are exposed to tear film for extended periods, it is essential that in vitro bacterial adhesion studies should be carried out on conditioned materials if they are to be clinically relevant.

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12 IDENTIFICATION AND ANTIBIOTIC SENSITIVITIES OF BACTERIA ISOLATED FROM ORTHOPAEDIC IMPLANTS FOLLOWING REVISION HIP SURGERY

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Bacterial infection, whether real or suspected is a frequent cause of failure in hip joint replacements. Antibiotic treatment to prevent such infection includes the use of antibiotic impregnated acrylic bone cement and the intravenous administration of antibiotics peroperatively. Failure of such antibiotic treatment can lead to multiple hip replacement operations with concurrent patient trauma and cost to the health service. In many instances of revision hip surgery, although the bacteriological report is negative, slime is apparent on the removed prosthesis. This failure to detect the presence of bacteria may be due to the fastidious culture requirements of anaerobic bacteria, which have been shown to be frequently involved in joint infection (Brook & Frazier 1993) The aims of this study were, therefore, to isolate and identify bacteria from revision hip prostheses and to determine the sensitivity of these isolates to a range of antibiotics.

Prosthetic hip implants removed at the time of surgery were immediately placed in an anaerobic jar and subsequently transferred to an anaerobic cabinet to ensure that anaerobic conditions were rapidly achieved and maintained throughout sample processing. Mild ultrasonication (5 min) in Ringers solution (25% v/v) was used to dislodge bacteria attached to the implants which were then isolated and identified using standard culture techniques. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for a range of antibiotics against these isolates were determined as described previously (Gorman 1991).

Quantifiable numbers of bacteria were cultured from 19 of the 76 retrieved samples examined (25%, Table 1). Previous studies have reported infection from 2% (Barrack & Harris 1993)-15% (Lachiewicz et al 1996) of cases. The anaerobic bacterium, *Propionibacterium acnes*, was present alone or in association with Gram positive cocci in 10 instances (53%). The previously reported isolation rate for anaerobes range from 0% (Barrack & Harris 1993)-8% (Maderazo et al 1988). Within and between strain variation in MIC and MBC values were apparent for each of the antibiotics assessed.

Table 1. Bacteria isolated from orthopaedic implants following revision hip surgery.

Infecting organism (s)	Number of implants
Gram positive coccus (single isolate)	6
Gram positive cocci (2 isolates)	3
P. acnes	7
P. acnes & Gram positive coccus	3

This study has shown that immediate transfer of prosthetic implants to an anaerobic atmosphere followed by mild ultrasonication of the implant will significantly increase the detection rate of both aerobic and anaerobic bacteria, thereby improving post-operative antibiotic therapy and reducing further implant infection.

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13 IN-VITRO ENCRUSTATION AND MICROBIAL COLONISATION OF POLY(HYDROXYETHYLMETHACRYLATE)—CHITOSAN SEQUENTIAL INTERPENETRATING NETWORKS

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Deposition of infection-induced crystalline material, principally ammonium magnesium phosphate and calcium phosphate, onto medical devices is a common urological problem. These encrusted deposits may damage bladder mucosa, cause blockage of urine flow and may necessitate device removal (Tunney et al. 1996).
It has been suggested that biodegradable polymers may reduce encrustation due to surface degradation, thereby preventing a build-up of encrustation on their surface. Chitosan is a biodegradable polysaccharide which is currently being investigated as a potential biomaterial by our group. Hydrogels based on the polyhydroxyethylmethacrylate (pHEMA) are currently used in many biomedical applications due to their lubricity, however, these do not improve resistance of medical devices to encrustation (Hedelin et al. 1991). Therefore, the combination of these two biomaterials, pHEMA and chitosan, may yield composites that combine the advantageous properties of each polymer.

Initially, HEMA was polymerised by mixing with the initiator (azobisisobutyronitrile, 0.5%) and incubating at 60°C for 18 h, after which, the polymeric films were freeze-dried. The sequential interpenetrating polymer networks (s-IPN) were prepared by incubating pHEMA films in a chitosan solution (2% w/v) for 24 hours at 37°C. The films containing pHEMA and chitosan were then removed and blotted dry prior to cross-linking the chitosan component by immersion in glutaraldehyde solution (0.125 or 0.25% v/v), for 4, 24 or 96 hours. sIPNs were then rinsed with 10mM sodium borohydride to neutralise unreacted glutaraldehyde. The resistances of the s-IPNs, polyurethane (PU, as a control) and pHEMA to encrustation were evaluated using an *in vitro* model (Tunney et al. 1996). Quantification of encrustation, as calcium and magnesium, on the surface of each material was performed using an Atomic Absorption Spectrophotometry. Secondly, the resistance of each material to bacterial adhesion was evaluated as previously reported (Jones et al. 1997), using a strain of *Escherichia coli* isolated from a retrieved ureteral stent.

Table 1. Mass of calcium and magnesium ($\mu g/cm^2 \pm$ s.d.) on each biomaterial after six weeks immersion in the encrustation model.

Biomaterial	Ca ($\mu g/cm^2$)	Mg ($\mu g/cm^2$)
PU	264.32±18.18	76.37±2.69
HEMA	248.68±11.24	74.22±6.25
H-C 1	231.86±16.36	70.99±3.82
H-C 2	213.64±26.27	69.79±6.25
H-C 3	215.82±22.07	70.36±7.42
H-C 4	199.59±21.48	62.94±6.3

(H-C 1 is a HEMA-Chitosan s-IPN cross-linked with 0.125% glutaraldehyde for 4 hours, H-C 2 cross-linked with 0.125% for 96 hours, H-C 3 with 0.25% for 4 hours and H-C 4 with 0.25% for 96 hours).

s-IPNs that had been cross-linked with 0.25% glutaraldehyde for 96 hours demonstrated significantly lower levels of encrusted deposits than PU and pHEMA. The amount of encrustation on all other materials was statistically equivalent. In the microbial adhesion assay, significantly lower numbers of viable bacteria were found on the surfaces of each composite compared to the control materials (PU and pHEMA). In light of these findings, it can be postulated that pHEMA-Chitosan s-IPNs may be useful for reducing device-related infections and encrustation in the urinary tract.

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14 THE INFLUENCE OF HEXETIDINE ON *CANDIDA ALBICANS* VIRULENCE FACTORS

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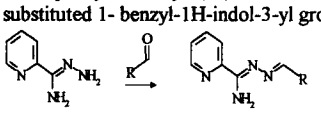
Mamolo et al (1992, 1996) have published the preparation and antimycobacterial activity of a set of 2-pyridylamidrazones (1). The key structural feature which they identified for antimycobacterial activity was hydrophobic substituent on the benzylidene, preferably at the 2-position. Examination of their published results indicates a rough correlation between lipophilicity and activity, the more lipophilic compounds being generally more active. Electron-withdrawing substituents this such as cyano and nitro were found to be injurious to activity. The most active compounds included a 2-chlorophenyl moiety (1a), 2-bromophenyl (1 b) and various substituted 1- benzyl-1H-indol-3-yl groups (1c).



1a R = 2-ClPh
1b R = 2-BrPh
1c R = Aryl-1H-indol-3-yl

This communication describes the synthesis of a set of related compounds in an automated fashion and in a purity sufficient for initial biological screening. A large set of 2-pyridinecarboxyamidrazone derivatives (1) were prepared by heating 2-pyridinecarboxyamidrazone with a slight excess of the corresponding aldehyde in ethanol at reflux. These encompassed a wide range of functional groups. A significant proportion was chosen to give benzylidenes substituted at the 2-position and imparting an overall lipophilicity greater than or equal to the most active compounds obtained by previous workers. The reactants and solvents were dispensed automatically and the products, which were usually crystalline, were isolated by robotic trituration with a suitable solvent. The purities of material thus obtained were typically greater than 85%, the only contaminant usually detected being the aldehyde. Such a result is perfectly adequate for initial screening purposes whilst allowing a much greater range of new chemical entities to be investigated for biological testing than could formerly be obtained by traditional hand crafted means.
The chemical entities obtained were tested against *mycobacterium fortuitum* which has resistance towards isoniazid. The initial screen involved simply the observation of a zone of inhibition of growth of the organism. Those compounds showing activity at this stage were investigated in greater detail and minimum inhibitory concentrations were obtained. Four of the most active previously published compounds were included. At least six new entities were observed to be much more active than the published compounds.

Mamolo M.G. et al (1992) Il Pharmaco, **47**, 1055-1066
Mamolo M.G. et al (1996) Il Pharmaco, **51**, 65-70

15 AUTOMATED SYNTHESIS AND ANTIMYCOBACTERIAL ACTIVITY OF A SERIES OF 2-PYRIDYLCARBOXYAMIDRAZONES

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Microbial colonisation of host tissue or medical device is a product of the complex interaction between microorganism and surface. Considered a critical event in microbial pathogenesis, initial attachment by a microorganism can progress to colonisation and, if unhindered, ultimately infection. Treatment of buccal epithelial cells (BECs) or *Candida albicans* blastospores with non-antibiotic, antimicrobial agents has been shown to significantly decrease viable blastospore adherence to BECs (Gorman et al 1996). In light of this, the ex vivo anti-adherence properties of the non-antibiotic antimicrobial agent hexetidine (as Oraldene®) were investigated. Furthermore, the influences of hexetidine on *C. albicans* morphogenesis and viability were also examined.

BEC samples were obtained from 12 healthy adult volunteers before and at pre-determined times after use of Oraldene® (0.1% hexetidine) mouthwash. Acridine orange-stained *C. albicans* blastospores were mixed with each BEC sample and incubated at 37°C for 2 hours. After this time, fluorescence microscopy was employed to count non-viable (green) and viable (orange) blastospores adherent to 150 BECs from each collection time for each volunteer. Frequency distribution profiles were constructed and viable or non-viable adherence was expressed as area under the curve (AUC) of the profile (Jones and Gorman 1997). Statistical differences in AUC of control and treated populations were performed using a Mann-Whitney U-test ($p<0.05$ denoting significance). Adherence of viable blastospores to BECs was significantly reduced up to and including 4 hours after oral rinsing; at 24 hours post-rinsing, blastospore adherence had returned to pre-rinse levels (table 1).

The effects of hexetidine on in vitro morphogenesis of *C. albicans* were performed as previously described (Jones 1995). *C. albicans* blastospores, cultured at 25°C for 18 h, were treated with phosphate buffered saline (PBS) or hexetidine (0.1%, 0.05%, 0.01% or 0.005%, as Oraldene®) for periods of 10, 30 and 60s. Following rinsing with PBS, blastospores were incubated at 37°C. Microscopic examination of blastospores up to 5 h post-treatment revealed a significant inhibitory influence of hexetidine on % morphogenesis and subsequent hyphal length. Concentrations which failed to totally inhibit morphogenesis did, however, significantly delay its onset.

To examine the effect of hexetidine on *C. albicans* viability, stationary phase yeast cells were exposed to PBS or hexetidine concentrations as described above for periods of 10, 30 and 60s and 5 min. Yeast suspensions were then serially decimally diluted to sub MIC, dilutions were plated out onto Sabouraud Dextrose agar and viable counts were determined following incubation at 37°C for 24 hours. A significant cidal action was exhibited by hexetidine and this was both concentration and treatment time dependent.

Table 1 Mean (±sd) AUC for viable adherent blastospores

Sample time	Control	Treatment
15 minutes	90.64 ± 3.12	77.06 ± 3.73
4 hours	82.39 ± 7.06	62.03 ± 3.19
24 hours	82.14 ± 8.16	84.39 ± 5.60

As Oraldene® mouthwash, hexetidine has the ability to alter the *Candida albicans* virulence factors of adherence to buccal epithelial cells, blastospore morphogenesis and viability. Therefore, with this broad first line of defence, delivery of hexetidine to the oral cavity in a suitable form may reduce or prevent oral colonisation by potentially pathogenic microorganisms.

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Jones, D.S. and Gorman, S.P. (1997) Eur. J. Pharm. Sci. **5**: 43-49
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16 MULTIDRUG RESISTANCE: EXPRESSION, MECHANISMS AND CIRCUMVENTION IN-VIVO AND IN-VITRO, IN YEAST, MICE AND MAN

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Drug resistant tumour cells are born survivors. They have achieved this status by developing, expressing and exploiting a variety of strategies to evade the cytotoxic effects of antitumour drugs. Drug resistance has been identified following exposure to all classes of chemotherapeutic agents and this is true of their use as antibacterial, antifungal, antiviral and antiparasitic treatments, as well as anticancer therapies. This phenomenon is widely recognized clinically and has been studied in the laboratory using model systems. Typically, resistant cell lines have been established by exposure of cells to a single chemotherapeutic agent. When the selecting drug is a natural product, cells frequently acquire simultaneous cross-resistance to a broad spectrum of agents with neither obvious structural homology nor common targets. Such cells are described as displaying a multidrug resistance or MDR phenotype. Drugs encompassed by this experimental phenotype are almost always natural products and their semisynthetic derivatives, including the Vinca alkaloids, anthracyclines and epipodophyllotoxins. The one notable exception apparently being camptothecin which specifically targets topoisomerase I. One implication of this involvement of natural products is that resistant cells may merely exploit mechanisms that already exist with some 'normal' cellular function. MDR may result from structural changes at the plasma membrane or within the cytoplasm, cellular compartments or nucleus. MDR is a multifactorial phenomenon and associated molecular mechanisms include alterations in detoxification and DNA repair pathways, decreases in drug-target affinity, modified or inappropriate targeting of proteins and accelerated exclusion of drugs.

Major changes identified in MDR cells include a decreased accumulation of cytotoxic drugs, changes in activity or expression of certain cellular proteins, as well as in physiological alterations. The finding that a particular protein or physiological change is not detected in a specific cell line or tumour population should be considered as evidence of the heterogeneity
of responses associated with this MDR phenotype and its multifactorial nature rather than ruling out its significance in some MDR mechanisms.

A number of proteins can be overexpressed in MDR cells. The first identified was a 170-kDa transmembrane glycoprotein called P-glycoprotein (Pgp), the product of the MDR1 gene. This protein is strongly homologous to a family of ATP-binding cassette (ABC) protein membrane transporters which translocate proteins, such as STE6 which in yeast transports a small peptide a factor and HlyB which in E. coli transports haemolysin. Although Pgp-mediated resistance is often used synonymously with MDR, there are also non-MDR1-expressing drug resistant cells. MDR is also now known to be conferred by the 190-kDa multidrug resistance protein (MRP), encoded by the MRP gene. Overexpression of at least two other members of this ABC family has been identified in MDR cells, LRP a 110-kDa protein found primarily in lysosomes, which turns out to be the human homologue of the major vault protein and, recently, the transporter associated with antigen processing (TAP). Interestingly, the cystic fibrosis transmembrane conductance regulator has also been shown to mediate an MDR phenotype. Other proteins variously overexpressed in MDR include glutathione S-transferase pi, catalase and a subunit of the vacuolar H^+-ATPase, while topoisomerase II, but not topoisomerase I expression appears down-regulated in certain tumour cells, which has been ascribed to an 'alternate' form of non-Pgp-mediated MDR (at-MDR).

Many of the initial experimental studies investigating Pgp-associated MDR used cell lines express orders of resistance in the

hundreds or thousands. Levels of resistance in non-Pgp expressing resistant sublines have generally been lower. This may relate to the more recent emphasis on developing models expressing clinically-relevant levels of resistance or to the fact that high levels of resistance mediated by non-Pgp-associated mechanisms are more difficult to achieve. What determines whether a cell will acquire drug resistance through increased expression of either Pgp or MRP or any of the other mechanisms identified is not yet known. Indeed there is current interest in evaluating the sequential expression of the various 'markers' of MDR as resistance develops, since , for example, overexpression of the two major MDR associated proteins is not mutually exclusive. This knowledge may prove of clinical relevance and could be valuable in designing strategies for overcoming or circumventing MDR. Whilst first generation resistance modulators, generally drugs originally developed for other therapeutic indications, often proved to be highly active reversers in-vitro, their use in the clinic has proved disappointing, but they have at least indicated the potential for resistance reversal. Second generation molecules are now being developed, designed to possess a higher affinity for Pgp and some look interesting in laboratory studies. Indeed, efforts are being focused on identifying normal physiological substrates for the various drug-resistance associated proteins which should contribute to the development of more appropriate agents to reverse this MDR phenotype. A more unusual role for resistance modulators or so-called 'chemosensitisers' was suggested recently, when verapamil was shown to suppress the emergence of Pgp-mediated MDR in cultured myeloma cells. Interestingly, other phenotypic changes also occurred resulting in hypersensitivity to the nitrosoureas as a result of the functional loss of the DNA repair enzyme O^6-methylguanine-DNA-methyltransferase. In this respect it is salutary to remember that MDR can be very efficiently circumvented by using one of the wide range of antitumour drugs not implicated in the MDR phenotype including, for example, the alkylating agents, antimetabolites, nitrosoureas and cisplatin. An alternative approach to resistance reversal has been to use antisense oligonucleotides. With improvements in the delivery of antisense oligonucleotide technology, this approach may prove valuable in reversing both MRP- and Pgp-mediated MDR.

Although this pleotropic MDR is considered one of the major obstacles to the successful treatment of
clinical cancers, the precise role of these various proteins in clinical MDR remains unclear. Despite laboratory evidence that Pgp and MRP can confer the MDR phenotype, aberrant expression of either or both of these proteins or even of their respective mRNAs, does not by any means always correlate with the clinical status of the patient or the outcome of therapy. Attempts to circumvent clinical MDR with Pgp 'modulators' have proved generally disappointing and certainly have not produced uniform results, although with the new generation of these modulating agents more encouraging data may be forthcoming. Some of these inconsistencies no doubt stem from difficulties in working with clinical specimens and in detecting these various MDR-associated proteins, as well as other biochemical and pharmacological factors contributing to resistance. In this regard the recent consensus recommendations for detecting Pgp-associated MDR in patients' tumours represent a step forward and provide a paradigm for investigations of other resistance-associated proteins. Such studies will undoubtedly be assisted by the availability of new antibodies and improved methodologies applicable, for example, to study expression of genes in paraffin-embedded tissues. Practically, clinical samples need to be evaluated by a battery of tests including those for mdr1/Pgp, MRP, LRP and other resistance associated parameters, since a negative result for one MDR-associated protein does not preclude the involvement of another – since clinical drug resistance is generally multifactorial.

MDR is now a well documented fact, the more we learn about it, the more we realize exactly how devious drug resistant cells can be. However, with our increasing knowledge, more potent and more selective drugs are being sought and designed. In another area we can directly exploit this resistance phenotype by generating drug resistant bone marrow to facilitate the safe development of aggressive chemotherapy regimens – so-called gene replacement therapy. As we approach the third millennium, I am optimistic about the availability of novel clinically-effective compounds for addition to or substitution in combinations of previously identified antitumour agents, which together with certain new therapeutic strategies will prove useful against previously refractory cancers.

17 PARASITE RESISTANCE TO THERAPY

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Parasitic protozoa and helminths are responsible for several of the major diseases of man and his domesticated livestock and chemotherapy is currently the main line of defence.

Malaria caused by plasmodia protozoa is one of the major health concerns in the tropics and sub-tropics i.e. two billion people reside in malarial regions and over two million deaths are reported each year. In parts of the world rnultidrug resistance is common and the reporting of drug resistant malaria appears to be exceeding the rate of new synthetic antimalarial development. A continuing review of recommendations for prophylaxis and standby treatments of malaria in travellers is becoming crucial. Resistance to other important parasitic protozoa is emerging e.g. *Entamoeba histolytica, Trypanosoma brucei brucei, Leishmania* and *Giardia duodenalis.*

Drug resistance amongst parasitic nematode heiminths is becoming an extremely serious obstacle to the sheep industry worldwide and, for example, in South Africa anthelmintic treatment is not regarded as an effective control strategy. Furthermore, for the three main anthelmintic groups the resistant alleles are naturally present in low frequency in the nematode population. Thus, no new mutations are required via selection pressure and the resistant alleles will become dominant.

Research into the resistance mechanisms in parasites has historically lagged behind equivalent bacterial and cancer studies. However, molecular biology and protein chemistry have now provided the easily available tools to investigate resistance mechanisms. For example, resistance in parasitic protozoa can be divided into a number of familiar groupings that include:-

a) Loss of drug transporter e.g. arsenials in African trypanosomes

b) Increased export of drugs by P-glycoproteins and ATPases e.g. multidrug resistance (MDR) pfmdrl alleles in malaria

c) Loss of drug activation e.g. metronidazole resistance in Trichomonas and Giaridia.

d)Alteration of drug target e.g. in dihydrofolate reductase and thymidylate synthase in Plasmodium species.

Research into resistance of veterinary important parasitic nematodes to the benzimidazoles (BZs) has shown that resistance is closely associated with structural alterations in cytoskeletal β-tubulin and the free living nematode *Caenorhabditis elegans* is providing a useful resistance biological model e.g. by heterologous expression of parasite BZ resistant/sensitive alleles and in-vitro mutated β-tubulin gene constructs.

Parasitic helminth glutathione *S*-transferases (GSTs) and lipid binding proteins (LBPs) have also been associated with drug resistance and research is currently underway to resolve the crystal structure of these potentially important proteins.

Growing drug resistance in parasites is, without doubt, a major health and economic issue. However, understanding the molecular mechanisms of parasite resistance is, fortunately, a very active research field and it can be envisaged that the powerful tools of molecular biology, genetics and mathematical modelling will provide future opportunities for tipping the balance against the parasite.

18 INCREASE IN MUTATION FREQUENCY TO NALIDIXIC ACID RESISTANCE INDUCED BY GROWTH IN LIQUID MEDIUM CONTAINING SUB-LETHAL CONCENTRATIONS OF DRUG

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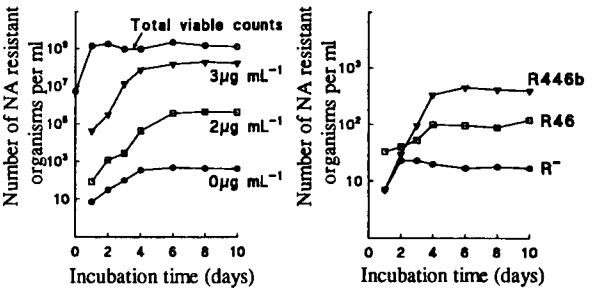
The antibacterial drug, nalidixic acid (NA), the archetypal 4-quinolone, damages bacterial DNA and induces mutagenic error-prone DNA repair (Piddock & Wise, 1987). This study reports the effects of exposure to sub-inhibitory concentrations of NA in liquid growth medium on the occurrence of NA resistance in *Escherichia coli*. The effect of mutator plasmids (Ambler & Pinney, 1995) on the development of resistance has also been investigated.

1.5 mL of overnight nutrient broth (NB) cultures of *E. coli* AB1157 and derivatives of this strain carrying the mutator plasmids R46 or R446b (Pinney 1980) were diluted in 150 mL NB containing 0, 2 or 3 μg mL^{-1} NA. Cultures were incubated at 37°C for ten days. Samples were removed at 24 or 48 hour intervals, diluted in NB and titered for nalidixic acid resistant mutants by diluting and plating on nutrient agar containing 16 μg mL^{-1} NA (four times the minimum inhibitory concentration). Plates were incubated for 2 days at 37°C. Total viable counts were also determined on drug-free nutrient agar.

Growth of *E. coli* AB1157 in NB containing 2 or 3 μg mL^{-1} NA led to 17- and 500-fold increases respectively in the number of NA resistant mutants recovered after 10 days incubation, compared to control cultures grown in drug-free broth. Plasmid R446b enhanced this effect. The numbers of NA resistant cells present in cultures of AB1157(R446b) after 10 days incubation in 2 or 3 μg mL^{-1} NA were 1 x 10^3- and 4 x 10^5-fold greater respectively than in control cultures grown in drug-free broth (Fig. 1). Plasmid R46 gave similar results (data not shown). Carriage of R46 and R446b also increased the number of NA resistant mutants after growth in drug-free broth: 6-fold by R46 and 20-fold by R446b (Fig. 2).

Fig. 1. Effect of growth in 0 (●), 2 (□), or 3 (▽) μg mL^{-1} NA on NA resistance in *E. coli* (R446b)

Fig. 2. Effect of plasmid carriage on NA resistance in *E. coli* grown in drug-free broth



These results show that growth in the presence of sub-inhibitory concentrations of NA significantly increases the frequency of bacterial resistance to this antibacterial drug. They may therefore have important implications in the clinical management of bacterial infections, suggesting that inappropriate dosage regimens could lead to the development of resistant strains. The presence of mutator plasmids further increases the frequency at which bacteria become resistant.

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Piddock, L.J.V., Wise, R. (1987) FEMS Microbiol. Lett. 41:289-294
Pinney, R.J. (1980) Mutat. Res. 72:155-159

19 INCREASE IN MUTATION FREQUENCY TO NALIDIXIC ACID RESISTANCE INDUCED BY GROWTH ON SOLID MEDIA CONTAINING INHIBITORY OR LETHAL CONCENTRATIONS OF DRUG

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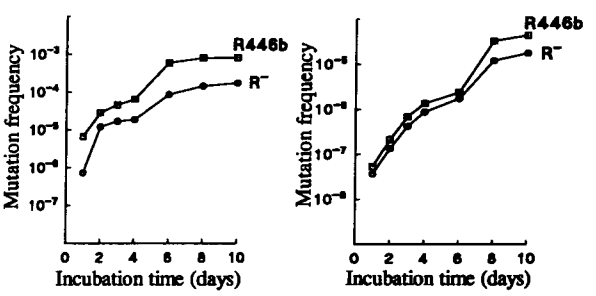
Increased frequency of mutation to lactose utilisation has been demonstrated when strains of *Escherichia coli* that cannot metabolise lactose were plated on media containing lactose as sole carbon source (Cairns et al, 1988). "Adaptive mutations" of this type are said to occur only in selected genes after the application of non-lethal selection in non-growing cultures. The antibacterial drug, nalidixic acid (NA) damages DNA and induces mutagenic error-prone DNA repair (Piddock & Wise, 1987). This study reports the generation of, what might be considered "adaptive" NA resistant mutants in *E. coli* AB1157 by growth on solid medium containing NA, and the effect that mutator plasmid R446b (Pinney, 1980) has on the frequency at which mutants appear.

0.1 mL volumes of overnight nutrient-broth grown cultures of *E. coli* AB1157 and AB1157(R446b) were plated onto nutrient agar containing NA at 4 or 8 μg mL^{-1} (one or two times MIC, with the latter concentration being bactericidal). These plates were incubated for 10 days at 37°C and the number of visible colonies on each plate scored at 24 or 48 hour intervals. Total viable counts of the plated cultures were also determined on drug-free nutrient agar. Mutation frequencies were expressed as ratios of the number of visible NA resistant clones to the number of cells plated.

The number of visible NA resistant colonies of strain AB1157 increased with time on media containing both 4 and 8 μg mL^{-1} NA (Figs. 1 and 2), giving 250- and 500-fold increases respectively in apparent mutation frequencies to NA resistance. The strain carrying plasmid R446b gave similar results, with the plasmid-mediated mutator effect increasing mutation frequency 5- and 2.5-fold respectively after 10 days. The NA resistance phenotype was confirmed by streaking onto nutrient agar containing 4, 8, 16 or 32 μg mL^{-1} NA. Colonies of strains AB1157 and AB1157(R446b) isolated on media containing 4 μg mL^{-1} NA

Fig. 1. Effect of plasmid carriage on mutation to resistance to 4 μg mL^{-1} NA

Fig. 2. Effect of plasmid carriage on mutation to resistance to 8μg mL^{-1} NA



retained resistance to this concentration on restreaking, but did not grow on higher concentrations of NA. Colonies of both strains isolated in the presence of 8 μg mL^{-1} NA, grew when restreaked onto nutrient agar containing 4, 8, 16 or 32 μg mL^{-1} NA.

These results suggest that the presence of NA induces mutational resistance to itself, with a mutator plasmid enhancing the effect. Since plasmid-mediated resistance *per se* to quinolones is unknown, the data may have important implications on our understanding of how mutational resistance to these clinically important antibacterial drugs develops.

Cairns, J. et al (1988) Nature 335:142-145
Piddock, L.J.V., Wise, R. (1987) FEMS Microbiol. Lett. 41:289-294
Pinney, R.J. (1980) Mutat. Res. 72:155-159

20 RAPID DEVELOPMENT OF RESISTANCE TO β_1-ADRENOCEPTOR STIMULATION IN RABBIT ISOLATED ILEUM

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β_1-Adrenoceptors have been shown to undergo desensitization with continuous exposure to agonists. This has been widely measured as reduced radioligand binding (Lefkowitz et al 1990), but there are fewer studies which demonstrate loss of functional sensitivity and these are mainly confined to cardiac responses. For example, we have shown that chronic treatment of rats with the β agonist isoprenaline (ISO) causes loss of sensitivity to isoprenaline of their isolated atria (Martin & Broadley,1994). Similarly, incubation of isolated atria with ISO causes loss of sensitivity after washout (Herepath & Broadley, 1990). If desensitization occurs rapidly, the response to the agonist should decline in its continued presence. With cardiac tissues the positive inotropic effect of the agonist might fade naturally, which would be difficult to distinguish from desensitization. In the present study, we used the β_1-adrenoceptor-mediated inhibition of rabbit ileum in which any desensitization would be revealed as a recovery of spontaneous contractions in the continued presence of agonist; opposing any fade.

New Zealand White rabbits (male, 1-3kg) were killed by anaesthetic overdose (urethane or halothane/nitrous oxide). The small intestine was removed and segments (2-3cm) set up in organ baths containing Tyrodes solution (37°C) gassed with air. Spontaneous contractions were recorded isotonically. A cumulative concentration-response curve (CRC) for (±)-isoprenaline sulphate (or chloride) was obtained. It was washed out and 10^{-6}M ISO was added and left in the bath for 1h. The bath was then washed thrice and a second CRC established.

In the presence of ISO (10^{-6}M), spontaneous activity was maximally inhibited, but there was a slow recovery of 82±5% at 45min and 87±3% at 1h (n=4), indicating desensitization of the β_1-adrenoceptors. Further evidence for desensitization was the leftwards shift of the second CRC to ISO (dose-ratio 25±13 and reduced maximum (82±5%). To determine whether the recovery of spontaneous contractions was due to oxidation of the ISO, experiments were repeated in the presence of an antioxidant, L-ascorbic acid (10^{-5}M). The rate of recovery was unchanged at 82±3% and 96±4 (n=3) at 45 and 60min, respectively, and there was a 36±32-fold shift of the second CRC with a reduced maximum (65±28%). To eliminate the possibility that recovery of spontaneous contractions was due to removal of ISO by extraneuronal uptake, experiments were repeated in the additional presence of metanephrine (10^{-5}M) an inhibitor of extraneuronal uptake. Recovery of 91±7 and 100±0% occured by 45 and 60min and there was still a significant 52±9 shift ($P<0.05$, n=4) of the second CRC to ISO, with a maximum response of 56±5%. Finally, a CRC for the P_1-purinoceptor-mediated inhibition of the ileum by adenosine before and after the 1h incubation with ISO was not displaced to the right (Dose-ratio 0.83±0.4, n=3).

Thus, the recovery of contractions in the presence of ISO appears to be due to desensitization of β_1-adrenoceptors with no cross-desensitization to the P_1-purinoceptor. The rabbit ileum is a useful model for further examination of β-adrenoceptor desensitization.

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Lefkowitz RJ, Hausdorff WP & Caron MG (1990) Trends Pharmacol Sci 11, 190-194.

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21 TOLERANCE TO THE CNS DEPRESSANT ACTION OF AMINOGLUTETHIMIDE

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Tolerance to the CNS effects of the aromatase inhibitor, aminoglutethimide (AG) occurs on repeated administration in breast cancer patients receiving the drug (Lonning & Kvinnsland 1988). This phenomenon has been reproduced in mice (Ahmad & Nicholls 1990). However the mechanism for the tolerance is not clear. As the drug is a phenobarbitone -like inducer of hepatic enzymes (Damanhouri et al 1987), tolerance may arise as a result of autoinduction of AG metabolism. To evaluate this, the pharmacokinetics of AG after repeated administration have been examined in male albino mice (25g).

The animals received either vehicle (1% carboxymethyl cellulose) or AG (100 mg kg^{-1}) suspended in vehicle (orally), daily for 14 days. On day 15, a time when tolerance is established (Ahmad & Nicholls 1990), a challenge dose of AG (50 mg kg^{-1}) was administered orally. At various times up to 12h after dosing, blood was collected under terminal anaesthesia. At death (1h time point) brains were also removed. AG was assayed in plasma and brain by HPLC. Pharmacokinetic parameters were calculated on the basis of a single compartment model.

From the results presented in Table 1, it may be observed that daily administration (14 days) of AG (100 mg kg^{-1}) significantly altered the $t_{0.05}$ and C_{max} (decreased) and total clearance (increased) of the challenge dose of AG on day 15. Volume of distribution and T_{max} were significantly lowered by the pretreatment, the extent of this was relatively smal (control: treated being 1:0.8).

This pretreatment protocol has been found to abolish several lines of indices of AG (50 mg kg^{-1})-induced CNS depression (Ahmad & Nicholls 1990). It is therefore unlikely that the low order of the pharmacokinetic change induced by repeated AG dosing can completely explain the development tolerance. Earlier studies (Ahmad & Nicholls 1989) on the development of cross tolerance to the hypnotic effect of AG in mice concluded that CNS tolerance to AG may be dependent on a functional mechanism. The findings of the present study would not be inconsistent with this view.

Table 1. Pharmacokinetic parameters of AG (50 mg kg^{-1}, p.o.) on day 15 in mice receiving AG (100 mg kg^{-1},p.o.) daily for 14 days.

	Vehicle	AG
$t_{0.05}$ (h)	3.6 ± 0.4	2.3 ± 0.2*
CL_T (mL min^{-1})	0.16 ± 0.02	0.30 ± 0.02*
V (mL)	50.0 ± 1.8	57.0 ± 10.0
C_{max}(μg mL^{-1})	20.8 ± 0.7	16.0 ± 0.2
T_{max} (h)	0.9 ± 0.1	0.8 ± 0.1
Brain (μg mL^{-1}, 1h)	25.3 ±2.6	20.5 ± 1.8

*P<0.01 vehicle vs AG

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22 EVIDENCE FOR P-GLYCOPROTEIN-ASSOCIATED, BUT NOT MRP-MEDIATED RESISTANCE TO VINFLUNINE (F 12158), A NOVEL FLUORINATED VINCA ALKALOID, WITH MARKED PRECLINICAL IN-VIVO ANTITUMOUR ACTIVITY

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Vinflunine or 20', 20'-difluoro-3, 4'-dihydrovinorelbine, is a novel *Vinca* alkaloid synthesised by reaction of vinorelbine in superacidic media and characterised by superior *in vivo* activity against experimental murine and human xenografted tumours (Kruczynski et al 1997b), to that of vinorelbine. Vinflunine which appears to function as a definite inhibitor of tubulin with mitotic-arresting properties, exhibits quantitatively different tubulin binding properties to the classic *Vinca* alkaloids (Barret et al 1997; Kruczynski et al 1997a). Cytotoxicity of vinflunine proved dependent both on concentration and duration of exposure, inducing an exponential-plateau shaped dose response effect, like other tubulin-interacting agents. There was a very high correlation coefficient for the IC_{50} cytotoxicity values of vinflunine, relative to vinblastine, in a panel of eight human tumour cell lines, comparable to that noted comparing vinorelbine and vinblastine. These data are consistent with all these compounds having a similar intracellular target. Evaluating vinflunine cytotoxicity in a series of sublines expressing either P-glycoprotein(Pgp)-mediated multidrug resistance (MDR) or non-Pgp-associated MDR, provided evidence of cross resistance in all the Pgp-MDR sublines. However, full sensitivity was retained against sublines expressing a non-Pgp-MDR phenotype in which resistance was variously mediated by overexpression of the other drug transporter MRP, alterations in the level/activity of topoisomerase II and/or increased drug detoxification enzymes. Although the four Pgp-MDR subline all proved cross resistant to each of the *Vinca* alkaloids tested, this was to varying extents and notably three proved least cross-resistant to vinflunine. Further evidence of Pgp-mediated resistance to vinflunine was provided by the highly efficient reversal of vinflunine resistance by the addition of the modulator, verapamil. Consistent with this was the finding that whilst markedly reduced uptake of vinflunine was characteristic of the Adriamycin-selected Pgp-MDR P388 cells, this could be augmented by verapamil addition. Overall our *in vivo* and *in vitro* data, provide some evidence of a mechanism of action for vinflunine differing in certain respects, mainly quantitatively, relative to vinorelbine and even more so relative to the classic *Vincas*. It remains to be established whether these differences account for the superior spectrum of *in vivo* preclinical activity identified for vinflunine and whether this novel fluorinated *Vinca* alkaloid, vinflunine, can improve on and widen the major clinical activity already identified with vinorelbine (Johnson et al 1996).

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23 THE DESIGN OF CHIRAL DRUG SEPARATIONS IN CAPILLARY ELECTROPHORESIS USING FT-NMR

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During the past decade increasing regulatory pressure on the pharmaceutical industry now requires all new chiral drugs to be accompanied by pharmacological/ toxicological data on each enantiomer and, if a racemate is to be marketed, on the racemic mixture itself. Since approximately 30% of new drugs are chiral this has led the industry to focus on developing pure, single-enantiomeric drugs that possess the optimum therapeutic activity with minimal side effects and toxicity. For a single enantiomer to be licensed, however, the Regulatory Authorities require a justification for the particular enantiomer selected ('eutomer'), plus data on the opposite antipode ('distomer') that may be present as an impurity.

Thus the successful development of a new chiral drug presents a significant challenge to analytical scientists – not only for developing enantioselective separations and analyses, but also for handling the very high analytical workload involved. Since the physical properties of enantiomeric pairs are by definition identical (except for rotation of plane-polarised light), strategies for chiral separation in GC, HPLC, and capillary electrophoresis (CE) involve introducing a spatially selective chiral dimension. In HPLC the chiral ligand is immobilised in a chiral column (or used as a mobile phase additive), but the rationale for selecting the ligand for a specific chiral separation is complicated by the fact that there are at least 5 different modes of chiral separation, with more than 100 columns commercially available. Given the expense of chiral columns for direct enantioseparation, there is increasing interest in CE, where an inexpensive chiral ligand can be readily introduced as an additive to the run-buffer. Among others, cyclodextrin (CD) derivatives, which have been widely used as column packing materials in HPLC, are being vigorously explored as low-cost CE additives for the design of highly selective enantioseparations. Both in CE and in HPLC there is need for a rational basis for selecting a particular CD from the large number of charged and uncharged CDs available – all of them based on derivatives of the native molecules, α-CD, β-CD and γ-CD. This has led to the exploration of high-field FT-NMR as a preliminary screen for chiral recognition of drug enantiomers by a CD, as an aid to method development both in CE and HPLC.

For a model drug such as oxamniquine or amlodipine (each with a single stereogenic centre), at 400 MHz the resonance signals of some of the protons in each of the two enantiomers are seen to split after addition of equimolar concentrations of a particular CD to the sample tube. This shift non-equivalence may also be accompanied by displacement of the resonance signal (singlet or multiplet) upfield or downfield as a so-called 'shift displacement'. These shift parameters have been examined for a range of CDs, including hydroxyethyl-β-CD, hydroxypropyl-β-CD, the native CDs themselves and anionically charged CDs. The enantioseparation observed in CE for these CDs shows a good correlation with the shifts recorded in FT-NMR.

Moreover, studies of the through-space interactions between the chiral drug and a CD using the rotating frame version of the Nuclear Overhauser Effect (ROESY), supported by spin-lattice relaxation data, permit information on the mechanism of chiral recognition to be deduced. Thus, it is clear that the anionic β-CDs (carboxymethyl-β-CD and sulphobutylether-β-CD) display different mechanisms of interaction with these enantiomers, since ionic phenomena play a significant role. Although the present work is at an early stage of development and is by no means widely generalised, it is clear that the orthogonal nature of the information yielded by FT-NMR studies on chiral separations offers another option for screening CDs as part of method development, and for probing the enantioselective recognition method itself.

24 HPLC/NMR DETERMINATION OF THE RATE OF 5-AMINOLEVULINIC ACID DECOMPOSITION AND IDENTIFICATION OF BREAKDOWN PRODUCTS IN INTRAVENOUS FORMULATION MEDIA

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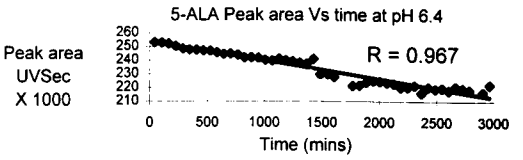
5-Aminolevulinic acid (5-ALA) is used in the photodynamic treatment of skin malignancies where it is applied topically (Roberts & Cairnduff 1995). It is recognised that it would prove more effective if administered intravenously. A neutral/alkaline formulation has been proposed but the rate of decomposition of 5-ALA in this formulation media and the identity of breakdown products must be determined. The decomposition of 5-ALA is documented (Franck & Stratmann 1981). A variety of reaction conditions did not affect the basic non-enzymatic formation of two main products a dihydropyrazine and a porphobilinogen in the ratio 10:1. HPLC with diode array detection was used to monitor the rate of decomposition of 5-ALA and NMR to confirm the identity of the decomposition in the new formulation. Formulations in media pH of 5.0 and 6.4 were used. 1 g of 5-ALA were dissolved in 50ml of each of the two formulation solutions.

The gradient HPLC system used two eluents 'A' and 'B'. Eluent A was water while eluent B was acetonitrile. Both contained 1% of 2.5 M sulphuric acid. The gradient consisted of 4 minutes at 1% 'B' raised to 40% 'B' over 6 minutes and maintained at 40 % for 4 minutes, (Waters et al 1995). 10 µl of pH 5.0 and 6.4 formulations of 5-ALA were run on the gradient HPLC system every hour for 50 hours. NMR spectra were recorded on a Bruker 500 MHz instrument at room temperature. For kinetic studies 1 g of 5-ALA was dissolved in D_2O and the pH adjusted to 6.8 using NaOD. Spectra were recorded immediately and after 96 hours. Three peaks are identified in the chromatograms of both formulations, 5-ALA at 2.5 minutes while a secondary peak at 12.5 minutes increases with time and corresponds to the main decomposition product. Further small peaks may be the porphobilinogen and related products. The 5-ALA peak has a shoulder which is attributed to 5-ALA being a zwitterion resulting in an equilibrium that disturbs the chromatography. The figure shows the fall of peak area of 5-ALA to be linear over the time period, although overall the reaction would be expected to be first order.



The estimated half life of 5-ALA from the regression line is 156 hours or 4.2% degraded after 12 hours. At pH 6.4 the rate of formation of the dihydropyrazine is ten times the rate at pH 5.0.

NMR spectroscopy confirms the structure of 5-ALA in neutral/alkaline solutions. In the buffered solutions the spectra show the formation of a additional product corresponding to the condensed dimer. This confirms the rate of reaction and structure of the principal product.

These results were useful in defining the optimum time scale for preparation and administration of 5-ALA injection solution.

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25 SOME DIFFICULTIES IN THE SEPARATION OF FREE AND BOUND FORMS OF BUPIVACAINE IN PLASMA USING ULTRAFILTRATION

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The extent of protein binding has a strong bearing on the activity of a drug and it was of interest to determine the free and bound forms of bupivacaine following its admininistration as an epidural block in children. There are three methods which are applicable to the measurement of the free and bound forms of a drug: equilibrium dialysis, ultrafiltration and use of HPLC with restricted access phases. All of these methods suffer from drawbacks. Ultrafiltration using membranes included in a microcentrifuge tube provides a simple, cheap and rapid means of separating free and protein bound forms of a drug. Although, as in all methods, the question remains as to how much the separation process perturbs the equilibrium between the free and bound forms of a drug.

In the current study samples of plasma *ca* 0.4 ml were obtained at various time intervals after administration of bupivacaine as a single dose (2mg/kg). Total levels of bupivacaine were determined in plasma by addition of 100 ng of pentycaine (internal standard) to 200 µl of plasma followed by addition of 200 µl of 0.5 M NaOH. The sample was extracted with ethyl acetate, the solvent was removed, the residue was redissolved in 0.5 ml of 0.1 M phosphate buffer at pH 3.0.The sample was passed through a SCX solid phase extraction cartridge and eluted with 1 M ammonia in methanol. It was then blown to dryness and dissolved in ethyl acetate (100 µl) for analysis by GC-MS as described previously (Tahraoui *et al*, 1996). For analysis of the free bupivacaine an aliquot of plasma 200 µl was diluted with 20 µl of 1 M phosphate buffer 1 M pH 7.4 and was centrifuged in a Centricon-10 ultrafiltration unit. The ultrafiltrate was collected and 10 ng of pentycaine internal standard were added. The sample was then processed and analysed as described above. The ultrafiltration procedure used was similar to that reported previously (Arvidsson and Eklund, 1995) in which losses of > 10 % of analyte were reported from buffer solutions. In our studies we found that losses of > 95 % were obtained when a solution containing 100 ng of bupivacaine in 200 µl of buffer was filtered through the polysulphone membrane. This is perhaps not suprising since bupivacaine has a pKa value of 8.1 and will still be highly lipophilic at pH 7.4. However, it is difficult to account for the differences from the earlier study. The recovery was improved by filtering 200 ng lignocaine through the membrane to condition it prior to loading the buffer containing bupivacaine. Under these circumstances recoveries were 65.2± 9.5 % (n=3). Although the method was not perfect some measurements were carried out. Table 1 shows typical free and bound concentrations of bupivacaine in plasma

Table 1 Free and bound bupivacaine in two patients.

Patient 1 time point (min)	Total bup. ng/ml	Free bup. ng/ml	Patient 2 time point (min)	Total Bup. ng/ml	FreeBup. ng/ml
15	434.8	20.6	15	771.0	51.6
30	795.1	23.7	30	974.9	22.8
45	565.3	31.3	45	623.4	17.4
60	623.6	28.4	60	320.9	13.4
120	763.9	19.1	120	413.8	17.0
			240	371.8	12.8

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26 INVESTIGATION OF THE THERMORHEOLOGY OF POLOXAMERS USING OSCILLATORY RHEOMETRY

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Poloxamers are block co-polymers of polyoxyethylene and polyoxypropylene which exhibit reversible thermal gelation. However, there is limited information available concerning the thermorheological properties of aqueous poloxamer gels. Therefore, this study examined the thermorheological properties of gel systems containing poloxamer blends of different molecular weights were using oscillatory rheometry.

Solutions of poloxamers were prepared using the cold method (Schmolka, 1972) with a total poloxamer content of 25 or 35%w/w. In each case the poloxamer content was composed of the two different grades (Lutrol® F127, m.wt. 9840-14600 and Lutrol® F68, m.wt. 7680-9510) in various proportions; 100/0, 90/10, 80/20, 70/30, 60/40, 50/50 (% Lutrol® F127/% Lutrol® F68). Oscillatory measurements were performed using a Carri-Med CSL^2 -100 rheometer with a 2cm diameter stainless steel parallel plate geometry (1mm plate gap). The linear viscoelastic region (LVR) of each sample was investigated by torque sweep. A strain of 7.5 x 10^{-4} rad was found to lie within the LVR for all samples and was selected for all subsequent analyses. Frequency sweep analysis was performed at 20°C under controlled strain over a frequency range from 0.01 - 1.0 Hz. Thermal analysis was performed under controlled strain at a frequency of 0.53 Hz over a temperature range from 5 to 40°C.

In samples containing a total poloxamer content of 25%w/w, those in which the Lutrol® F127 proportion was 80% or less did not form a gel when warmed to room temperature. With a total poloxamer content of 35%w/w all samples formed a gel when warmed to room temperature with the exception of the sample containing 50% of each of the two poloxamer grades. Samples which did not form a gel at room temperature were not analysed. In frequency sweep analysis the storage modulus of each sample was observed to increase gradually with increasing frequency of oscillation. The dynamic viscosity and loss tangent of each sample were seen to decrease gradually with increasing frequency of oscillation. For samples containing a total poloxamer content of 25%w/w a reduction in the proportion of Lutrol® F127 from 100 to 90% produced a significant reduction in the storage modulus whilst increasing the loss tangent (one-way Analysis of Variance, $p<0.05$ denoting significance). Similarly, for samples containing a total poloxamer content of 35%w/w each incremental decrease in the proportion of Lutrol® F127 between 100 and 60% produced a significant reduction in the storage modulus whilst increasing the loss tangent ($p<0.05$). Samples with a total poloxamer content of 35%w/w had significantly higher storage moduli than those with a total poloxamer content of 25%w/w with the same proportion of each of the two grades (two-way ANOVA, $p<0.05$ denoting significance). Temperature sweep analyses were used to identify the sol-gel transition temperature for each of the samples. At this temperature gelation produces a sudden increase in structure identified by a sharp increase in the storage modulus. Decreased proportions of Lutrol® F127 produced a significant increase in the sol-gel transition temperature. In samples with a total poloxamer content of 35%w/w, the sol-gel transition temperature was significantly lower than those with a total poloxamer content of 25%w/w with the same proportion of each of the two grades.

Poloxamers are used as topical drug delivery platforms. However, processing and storage conditions will affect their rheology. In addition, following clinical application these formulations would be expected to experience temperature changes and stresses which affect their rheological properties (Jones et al. 1997). This study has illustrated the use of oscillatory rheometry to investigate these properties and provide information useful to the formulator.

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27 THE IDENTIFICATION OF DRUGS BY APPLICATION OF THE SIX PEAK METHOD TO NEAR INFRARED SPECTROSCOPY

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The near infrared (NIR) spectrum of a material is rich in chemical and physical information and hence ideally suited for the identification of drugs. Chemometric methods such as Mahalonobis Distances, Principal Component Analysis and Correlation Spectral Matching have all been used in the past for identification of raw materials and tablets (Mark & Tunnel 1985; Gemperline et al 1989), however they all suffer from considerable complexity and sensitivity to spectral distortion. In this work the simpler six peak method commonly used in the mid-infrared region (Curry et al 1967) has been investigated for NIR spectroscopy.

The second derivative NIR spectra of approximately 300 drugs were recorded and used to set up a database of the six most intense negative peaks. Various batches of 12 drugs were treated as unknown samples and were run against the database in a blind manner, including examples that were both present and absent from the database. Peaks were placed in order of relative intensity with ± 1 nm permitted for identification based upon peak wavelength.

Table 1 shows some typical results. Test compound 1 produced a full six peak match for penicillamine. The next nearest match based on the most intense peak was aspirin but it can be clearly distinguished by differences in the other peaks. Lactose was identified as the next test compound based upon a match of the first five peaks. For nine of the other test compounds identification was established after three peaks at the most. After three peaks if there were no suggested candidates the test compound was deemed to be not included, such as test compound 3.

Sample	Peaks					
	1	2	3	4	5	6
Test compound 1	2397.9	2286.7	1692.6	1738.4	2448.5	1184.7
Penicillamine	2398.0	2286.7	1692.7	1738.5	2448.7	1184.8
Aspirin	2399.5	1656.3	2254.5	2136.2	2477.4	1127.8
Test compound 2	1933.9	2255.4	2318.6	2091.6	2279.8	1449.3
Lactose	1934.1	2255.5	2319.0	2091.8	2280.0	2474.9
Demeclocycline	1927.3	1661.7	1520.5	2477.1	2437.5	2249.0
Test compound 3	2415.1	2323.5	2286.2	2446.0	2350.3	1642.9
Butobarbitone	2430.3	2477.2	2265.1	2291.0	2368.8	1688.9
Chlorpromazine	2433.6	1667.6	2351.5	2157.5	2249.9	2387.1

Table 1. Unknown test samples and the best two matches produced from the database based upon the six most intense peaks.

Physical effects such as particle size produced variation in relative peak intensity and in peak order but not in peak wavelength. Different batches of samples of similar particle size produced no effect on peak order.

Thus the NIR six peak method proves to be a rapid and reliable identification method for pharmaceuticals.

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28 PARTICLE SIZE ANALYSIS OF PHARMACEUTICAL POWDERS USING NEAR INFRARED SPECTROSCOPY

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Particle-size analysis of pharmaceutical powders is typically achieved by sieving methods, laser diffraction analysis and electrical zone sensing. Though these methods are time consuming the latter two do provide accurate particle-size information which can be used as reference methods to calibrate other techniques such as near-infrared (NIR) spectroscopy (Ciurczak et al 1986). Kortum (1969) has shown that, for particles comparable in size to that of the wavelength used, the reflectance, R, is inversely proportional to the mean particle size, d_{50}:

$$R \propto 1 / d_{50}$$

The application of NIR spectroscopy to particle size analysis of three pharmaceutical powders, aspirin, lactose monohydrate and paracetamol has been investigated. Using a single batch of each material, between 5 and 7 discrete particle size fractions were obtained for each by: air-jet sieving (paracetamol); mechanical sieving (lactose monohydrate) and grinding and mixing (aspirin). The mean particle size for each fraction was determined by forward angle laser light scattering (FALLS) using a Malvern 2600C particle-sizer. The NIR spectrum for each fraction was also determined by FT-NIR spectroscopy, using a Buhler NIR VIS spectrometer, with 6 scan accumulation. All spectra exhibited multiplicative scattering with non-uniform baselines. Correlations between the reflectance at any one wavelength with reciprocal mean particle size were poor. Much better results could be obtained by fitting a polynomial to each spectrum to represent the baseline. A minimum of 9 data points of high reflectance (approximating to the baseline) were chosen across the NIR spectrum and fitted to a fourth order polynomial.

		Reciprocal mean particle size/ NIR correlation coefficient, r				
Powder	Particle size fractions	c_4	c_3	c_2	c_1	c_0
Lactose monohydrate	7	0.9987	0.9986	0.9986	0.9986	0.881
Aspirin	5	0.9880	0.9863	0.9842	0.9842	0.984
Paracetamol	6	0.9800	0.9779	0.9593	0.9925	0.869

Significant correlations ($p < 0.05$) between the reciprocal mean particle size as measured by FALLS and each of the coefficients c_1 to c_4 were obtained. The baseline shape clearly contains important particle size information.
These findings indicate that NIR reflectance spectroscopy can be used to rapidly measure the mean particle size of pharmaceutical powders.

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29 PROGRESS TOWARDS THE COMPUTER PREDICTION OF POLYMORPHISM

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Changes in the polymorphic form of a pharmaceutical during processing and storage can be disastrous for quality control, and hence there is considerable interest in establishing all the possible crystal structures of the drug molecule. Recently the first steps have been taken towards the prediction of the crystal structures, and hence polymorphism, of rigid organic molecules, based on the assumption that the experimental structure corresponds to the global minimum in the static lattice energy, with any other minima which are close in energy being possible polymorphs. Initial work concentrated on generating sufficient hypothetical crystal structures as starting points for lattice energy minimisation to be reasonably confident that the global minimum would be found (Gavezzotti 1997). The searches are generally successful in finding the experimental crystal structure, but they often found a variety of unknown structures of similar, even lower, lattice energy than the known structure, suggesting a large number of possible polymorphs.

A major problem in the interpretation of the lattice energies of the hypothetical structures is the uncertainty which arises from the use of empirical models for forces between the molecules. We have developed the use of realistic models for the electrostatic forces in crystal structure modelling, based on an accurate representation of the quantum mechanical charge distribution of the molecule (Willock et al. 1995). This gives a considerably improved description of the orientation dependence of the packing forces, particularly hydrogen bonding and the stacking of aromatic rings (Coombes et al. 1996).

Using these state-of-the-art models for the lattice energy, we are running crystal structure prediction calculations on a range of molecules, exemplified by allopurinol, uracil (Price & Wibley 1997) and alloxan (Coombes et al. 1997). The accumulating results show that current methods are capable of predicting the known crystal structure of some molecules and showing that other polymorphs are unlikely. However, for many molecules there are many possible crystal structures, within the energy range of possible polymorphs. Thus, improvements in the model potential give more accurate reproduction of the known crystal structures, and more confidence in the relative lattice energies, but confirm that it is common for even hydrogen bonding molecules to be able to pack in a wide variety of energetically plausible crystal structures. Since these hypothetical structures are unknown (or at least no crystal structure has been published), this raises the question as to what further kinetic and thermodynamic factors must be considered for the computer prediction of possible polymorphs.

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30 HOW MANY POLYMORPHS HAS SULPHATHIAZOLE? PROPOSALS FOR REPORTING CRYSTALLOGRAPHIC DATA OF POLYMORPHS

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It has been known since 1941 that sulphathiazole is polymorphic. Four well described forms are now recognised in the pharmaceutical literature, as set out by Burger and Dialer (1983) and by Anwar, Tarling and Barnes (1989). There are also 4 detailed published crystal structure determinations. During the preparation of the polymorphs we found that the crystal structures and the polymorphs described in the literature do not correspond. In particular, the material of commerce is not represented by the structure of Kruger and Gafner (1972) as has been assumed for 25 years. We believed originally that the structure reported by Babilev (1987) was that of Polymorph II (mp 197^0). On examination, the crystal structure of the latter proved to be novel. It is monoclinic, space group $P2_1/n$, with 4 molecules in the unit cell and dimensions a 10.439, b 15.267, c 15.326 A and β 91.060^0. This then suggested that Babilev's polymorph was novel. It is more dense (1.60) than any other, so we attempted to prepare it by flotation of sulphathiazole mixtures in CCl_4 (d = 1.59); by subjecting other polymorphs to high pressure at elevated temperatures to induce transformation; and by crystallising from various solvents and from aqueous and mixed aqueous systems at low temperatures in presence of surfactants and dyestuffs. Eventually it was determined that Babilev's structure was that of the material of commerce. Kruger and Gagner's structures are of similar but distinguishable polymorphs. We have determined the structure of Polymorph I (mp 204^0) in order to obtain better hydrogen bond distances for the spectra-structure investigations. There is a remarkable coincidence in the cell dimensions so that the structure can be described equally well as $P2_1/n$ or $P2_1/c$. The confusion over the number of polymorphs has arisen because of the failure to record physical properties of the crystal sample examined or adequate description of crystallisation conditions. Some proposals to address this will be presented. The surprising conclusion from these results is that the dissolution and transformation behaviour of a common material known for nearly 60 years may not be certain.

Sulphathiazole crystallizes erratically from solution, usually as mixtures of polymorphs and often in a wide range of habits. Its X-ray powder patterns are notoriously variable (Anwar, Tarling and Barnes (1989)) and calculated patterns show poor coincidence with reality. Hot-stage microscopy and D.S.C. are often irreproducible. Vibrational spectra may fail to distinguish clearly between polymorphs. We have evidence from all these methods of at least four additional polymorphs and have isolated and determined the unit cells of two of these. These extraneous polymorphs may account for some of the problems of identification. We have also obtained solid state NMR spectra to confirm the polymorphic status of samples. Despite carrying out over one hundred crystallisation experiments, many of which confirm previous work, we still cannot provide guaranteed recipes for producing single polymorphs.

FT-Raman, infrared and near infrared spectra in both the first overtone (4000-8000cm^{-1}) and second overtone (8000-12000cm^{-1}) regions have been obtained of sulphathiazole polymorphs and of their deuterated versions and of other sulpha drugs in order to carry out multi-2-dimensional vibrational correlation analysis. This has previously been used for interpreting the near infrared spectra of mixtures, but it has not been generally realised that it is widely applicable, for example to the interpretation of the near infrared or Raman spectra of polymorphs. One of the goals of this part of the work is to relate polymorphs via their hydrogen bonding networks using graph set analysis in order to understand the nature of polymorphic transformation.

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31 TURNING DRUGS INTO EFFICIENT MEDICINES: THE NEED FOR HIGH FUNCTIONALITY EXCIPIENTS AND THE ROLE OF PHYSICOCHEMICAL RESEARCH IN PROVIDING THEM

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The primary objectives of a dosage form are the delivery of the correct amount of a bioactive principle in a safe and effective manner. Achieving these objectives invariably requires formulation of drugs with appropriate excipients. In the design of medicines for oral delivery, which has long been one of the most acceptable routes for drug therapy, excipients play an increasingly important role as drug delivery requirements become more demanding.

For example, in immediate release tablet preparations, it is essential to provide robust yet fast-disintegrating compacts which can be formulated without delay and manufactured simply and quickly. Tableting excipients thus require enhanced functionality and, of these types of material, microcrystalline cellulose is capable of confers a higher tablet strength and disintegration than any other diluent excipient. However, it is now known that MCC
loses a significantt amount of compactibility (up to 50%) as a result of aqueous granulation. This loss of functionality has been found due to a phenomenon known as quasi-hornification by silicification of microcrystalline cellulose molecules become hydrogen-bonded, causing a macroscopic densification and loss of surface. An understanding of the physico-chemical interactions has led to the prevention or reduction of quasi-homification by silicification of microcrystalline cellulose. Silicified microcrystalline cellulose retains compressibility following wet granulation and has improved compressibility in direct compression tablet production over conventional mictocrystalline cellulose.

Other research in this field of polysaccharide physico-chemical interactions has led to the development. of sustained release tablets what can be produced using the direct compression technique as well as following wet granulation. Direct Compression Sustained Release (DCSR) tablets produce the required pharmacokinetic profiles as the result of a synergistic interaction between a heteropolysaccharide and a homopolysaccharide in the presence. of water. The hydrogen bonding between these materials can be further manipulated by incorporation of smaller molecules including, in some cases, ionic species. This universal sustained release excipient system is capable of first and zero order release profiling and is also flexible enough to allow chronotherapeutic release profiles to be generated. Although the excipient is simple to use and produce, the complex nature of the physico-chemical interactions which occur allow close control of concentration–time release profiles to be achieved. For example, this excipient technology has recently been successfully used as the basis for an ANDA filing with USFDA for a sustained release form of nifedipine which is the active ingredient in Procardia XL that uses a complex osmotically-driven pump technology to achieve the same release profiling.

32 CHARACTERISATION OF THE PHYSICAL AND CHEMICAL INTERACTION OF A FREEZE DRIED PEPTIDE WITH WATER USING ISOTHERMAL MICROCALORIMETRY

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The interaction of water with a lyophilized decapeptide was studied using high sensitivity isothermal microcalorimetry. The decapeptide is susceptible to water that can effect both its physical state (glass to plastic transition) and its chemical instability (ring closure and epimerization). Knowing the effect of water thus is a major determinant in the strategies used in the formulation and processing of proteins and peptides. Microcalorimetry provides a rapid and very sensitive method to measure water vapor affects on peptides in the solid state.

Sample sizes of 2-4 mg are placed in the ampoule of a commercially available perfusion cell. Water partial pressure above the solid sample is controlled by mixing streams of dry and water-saturated nitrogen at closely controlled flow rates. In the isothermal microcalorimeter (Thermal Activity Monitor, Thermometric AB, Jarfalla, Sweden) heat flows associated with reactions occurring in the calorimetric cell are monitored as a function of time. Heat flow is a measure of rate and the area under the curve measures the enthalpy associated with the reaction. In these studies heat flow can reflect water sorption, physical transformation, or chemical instability. Studies were made at 25, 45 and 55°C at 0.03 to 0.80 water partial pressures. Background heats were determined using empty cells. Desorption of the water is determined using only dry nitrogen. In the first 10 hours of all studies, the area under the desorption curve is essentially the same as the area involved in water sorption indicating that no detectable chemical reaction has occurred.

Using data at early times, isotherms were constructed by plotting corrected area under sorption curves per gram of decapeptide as a function of water partial pressure. The shapes of the curves were typical of Type II BET adsorption behavior. The sorption of water was essentially independent of temperature. Assuming that the interaction follows a BET adsorption isotherm, an equation can be used to relate area under the curve (Q_{int}) to the partial pressure, x.

$$Q_{\text{int}} = \frac{CV_m\left[H_1 x + \left(H_L - H_1\right)x^2\right]}{(1-x)(1-x+Cx)}$$

H_1 is the heat of interaction between water vapor and solid, H_L is the heat of condensation of water (both in J/mole water), V_m is the number of moles of water/g solid needed to form a monolayer (which can be a measure of surface area), and C is a constant,

$$C = e^{\frac{(H_1 - H_L)}{RT}}$$

When the experimental data are least squares fit to the equation, the fitted parameters show H_1 to be slightly larger but close to H_L and V_m to be about 0.0025 (5% water).

Using microcalorimetric studies it is possible to predict the amount of water associated with decapeptide at any temperature and relative humidity. The relevant constants for H_1 and V_m are 49,304 and 2.6E-3 at 25°; 48,937 and 2.76E-3 at 45°; and 49,454 and 2.6E-3 at 55°. The error in H_1 is about 1% and about 2% in V_m.

When the decapeptide reacts with water vapor at 80% relative humidity and 55°C for about one day, a slowly declining heat flow-time relationship is found. HPLC assay indicates the formation of about 6.7 % of cyclized product over this time.

33 PROCESS CONTROL DURING FLUID BED GRANULATION - THE IMPORTANCE OF HUMIDITY CONTROL

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The robustness of fluid bed granulation processes is dependent on the ease of achieving a critical dynamic equilibrium between water addition and evaporation. Factors affecting granule wetting, such as spray rate, quantity of granulating fluid and atomising pressure are relatively easy to control. Drying rates are affected by the drying capacity of the inlet air to the granulator (i.e. air temperature, flow rate and humidity). Drying air temperatures and flow rates are generally well controlled, although the importance of controlling inlet air humidity is frequently overlooked.

The granulation process studied is intrinsically non-robust due to the nature of its formulation. It has a high binder content (> 30%w/w) and the drug characteristics are not conducive to effective granule growth (high dose, hydrophobic, low median particle size). In addition, the process is taken close to the limit of overwetting in order to achieve the desired median particle size of > 300 microns. Controlled granule growth is essential as overwetting of the formulation produces an irrecoverable 'dough'.

Production scale granulation trials have been conducted using a Glatt WST200 fluid bed granulator/drier. No humidity control was applied to the inlet air supply to this machine. The water content of the drying air was therefore governed by the prevailing atmospheric conditions.

Initial granulation trials (4 batches) were successfully conducted using a defined set of process parameters. Granule growth appeared well controlled and the desired particle size was achieved following addition of 20kg of water. The process operating window appeared wide, since up to 30kg of water could be added before reaching the limit of overwetting (close to bed collapse). The drying air humidity during these trials was 6%RH at 45°C.

During subsequent granulation trials the drying air humidity was higher, 13%RH at 45°C. Granule growth was more rapid than seen during initial trials. The first batch appeared overwet after addition of 16kg of water and bed collapse occured after addition of 17.5kg water. A second batch behaved similarly and spraying was stopped after addition of 15kg water, in order to prevent bed collapse.

Granule water contents calculated from the wetting and drying rates from the two series of trials are shown in Table 1. These data show that the granule water content at bed collapse (17.5kg water added) was similar to that at the limit of the process identified during the initial trials (30kg water added).

Table 1 Water contents of granules manufactured under different drying air humidity conditions

Trial No	Drying air humidity at 45°C (%RH)	Cumulative water content drying air (kg)	Water sprayed (kg)	Granule water content (%w/w)	Comment
1	6	11.9	20	9.6	end-point
			30	10.6	overwet
2	13	26.2	16	10.7	overwet
			17.5	11.0	bed collapse

The data clearly demonstrates the influence of drying air humidity on the operating window for this process and underlines the importance of humidity control on the reliability of fluid bed granulation processes.

Subsequently a dehumidification unit has been fitted to the air handling system supplying the granulator, which controls air humidity to 5% ± 3%RH. More than 20 batches have been successfully manufactured, demonstrating that the process is now reliable.

34 EXPANDED HANSEN SOLUBILITY PARAMETER APPROACH. PARACETAMOL AND CITRIC ACID IN INDIVIDUAL SOLVENTS

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In this study, two solubility parameter models are compared using as dependent variables the logarithm of the mole fraction solubility $\ln X_2$ and $\ln\alpha/U$, the latter having originally been used in the extended Hansen method (Martin et al. 1981).

Experimental mole fraction solubilities are determined in pure solvents as described by Bustamante et al. (1989). For paracetamol, when $\ln X_2$ is used as the dependent variable, all the solvents fit the four parameter model with a 0.05 confidence level. To obtain a higher confidence level, a statistical weight of 0.001 must be applied to values of benzene and chloroform. For citric acid, assigning a weight of 1 to each of the 14 solvents used except for two solvents, led to valid coefficients in the four parameter model. For the two drugs tested, the four parameter model appears to better describe solubility phenomena than the three parameter model. In the three parameter model, maximum interaction is reached when the partial parameters of the solute and the solvent are equal. In the four parameter model, maximum interaction is reached when the products representing the acid-base interaction of the solute with the solvent ($\delta_{2a}.\delta_{1b}$ and $\delta_{1a}.\delta_{2b}$) are large and the product representing the self-association tendency of the solvent ($\delta_{1a}.\delta_{1b}$) is small. This model provides a better interpretation for hydrogen bonding than the δ_h parameter of Hansen. In Table 1, the partial solubility parameters of paracetamol and citric acid determined from the models giving statistically significant regression coefficients are compared with the values calculated from different group contribution methods estimating the partial $\delta_{d,p,h}$ values. For both drugs, the total solubility parameters are within the same range than the predicted values for both variables.

Table 1. Comparison between experimental solubility parameters ($MPa^{1/2}$) and predicted parameters calculated from group contribution methods.

Variable	Weight = 1 except for	Confidence level	δ_d	δ_p	δ_h	δ_a	δ_b	δ_t
		------ PARACETAMOL ------						
$\ln X_2$	Chloroform, benzene	<0.001	16.6	13.8	17.5	20.0	7.7	27.8
$\ln X_2$	none	0.05	16.6	14.3	18.4	22.3	7.6	28.6
Group contribution		Van Krevelen	21.1	8.5	15.0	---	---	27.3
methods		Hansen and Beerbower	20.7	11.5	12.8	---	---	26.9
		------ CITRIC ACID ------						
$\ln\alpha/U$	Chloroform, 1,2 propanediol	<0.001	16.5	13.9	18.1	18.2	9.0	28.2
$\ln X_2$	Chloroform, glycerol	<0.001	16.2	13.5	17.3	17.2	8.7	27.4
Group contribution		Van Krevelen	18.8	7.3	20.4	---	---	28.7
methods		Hansen and Beerbower	21.0	8.2	22.4	---	---	31.8

The Lewis acid properties of citric acid are stronger than its basic property. Paracetamol shows the same trend. Since it also shows proton acceptor ability, it can also interact with acidic solvents. Thus, its solubility is higher in alcohols and glycols than in the weakly acid chloroform. The polarity parameter obtained for paracetamol is high. This is consistent with its higher solubility in solvents of higher polarity. Because dispersion parameters of different drugs and solvents are quite similar, the difference in the solubility of paracetamol and citric acid is mainly due to the difference of polarity, and particularly of hydrogen bonding ability. The four parameter model provides very reasonable partial solubility parameters with the set of solvents selected.

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35 AN INVESTIGATION OF EXPERIMENTAL VARIABLES DURING TRIBOELECTRIFICATON STUDIES ON POWDERS

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Triboelectrification of powders is a complex process involving particle-substrate and particle-particle interactions and may cause adhesion/cohesion problems during processing (Carter et al 1992). Characterization of powders by charge propensity and decay properties provides useful information to the pharmaceutical industry. This work investigates a practical technique for measurement of charge on powder samples and the effects of selected process variables. Lactose BP (Lactochem) was sieve fractionated, air-jet sieved and sized by optical microscopy, and glass spheres (106-125μm) were used as a model system. Glass sphere samples (0.5g to 2.0g) (n=5) were agitated at 40% relative humidity in 0.1 or 0.5 litre stainless steel (SS) and 0.1 litre Perspex (P) (poly(methylmethacrylate), ICI) cylindrical vessels for 5 minutes on a Turbula mixer (Glen Creston) at 30rpm and then poured in a reproducible manner into a Faraday well to measure charge (Mackin et al 1993). Investigations with lactose sieve fractions were similarly undertaken with only the 0.1 litre SS vessel. In all cases, adhesion (g m^{-2}) inside the vessel after charging was determined.

Table 1. Specific charge (nC g^{-1}) for 106-125 μm glass spheres.

Vessel	mean charge values nC g^{-1}, (%coefficient of variation) [mean adhesion g m^{-2}] mass 0.5g	1.0g	2.0g
SS (0.1L)	-0.02(34.0) [4.2]	-0.03(58.0) [3.4]	-0.02(53.0) [4.6]
SS (0.5L)	-0.58(11.8) [2.6]	-0.34(27.9) [6.5]	-0.36(35.5) [5.3]
P (0.1L)	-1.35(33.7)[35.7]	-0.28(32.7)[35.6]	-0.04(47.2)[77.3]

Table 1 shows specific charge to be influenced by the nature and volume of the charging vessel. The effect of decrease in charge with increased sample mass is significant ($p<0.05$) for P but not SS and is attributed to greater particle-particle interactions in P due to adhesion.

Table 2. Specific charge (nC g^{-1}) for lactose in SS (0.1 litre).

median size (μm,10-90%)	mean charge values nC g^{-1}, (%coefficient of variation) [mean adhesion g m^{-2}] mass 0.5g	1.0g	2.0g
48(35-67)	-2.08(16.7)[6.6]	-1.70(17.4)[7.1]	-1.61(9.1)[25.9]
84(61-116)	-0.33(66.2)[4.8]	-0.26(41.3)[4.2]	+0.09(39.5)[5.8]
115(83-157)	-0.42(84.8)[1.8]	-0.29(39.7)[4.2]	-0.01(21.4)[2.1]
148(101-210)	-0.18(42.5)[1.1]	-0.22(41.2)[1.2]	-0.18(30.4)[2.3]
186(106-240)	-0.16(77.2)[1.2]	-0.11(51.8)[1.4]	-0.25(17.1)[2.1]
240(178-305)	-0.14(45.1)[1.7]	-1.16(35.4)[2.5]	-0.21 (8.3)[2.5]

Table 2 shows specific charge and charge variation generally decrease with increased sample mass for lactose <148μm, and greater adhesion with these samples will provide greater particle-particle interactions. Above 148μm these effects are not seen. Thus, the charging of powders has been shown to be influenced by the sample mass and volume of the charging vessel where particle interactions are significant compared to particle/contact surface interactions. It is important to consider these variables when undertaking charge characterization procedures.

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36 THE CHARACTERISATION OF AN AMORPHOUS DRUG USING MODULATED TEMPERATURE DIFFERENTIAL SCANNING CALORIMETRY

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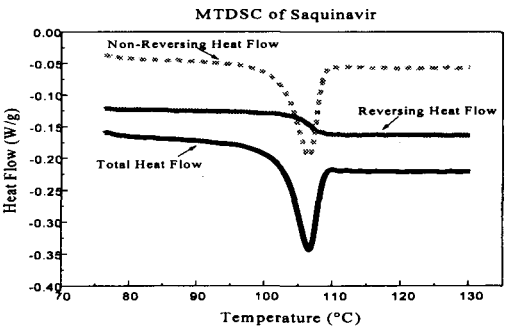
Modulated temperature differential scanning calorimetry (MTDSC) is a novel thermoanalytical technique which involves the superimposition of a sinusoidal heating signal on the conventional DSC linear programme. By measuring the response to both the underlying heating signal and the oscillation, it is possible to separate simultaneous thermal events (Reading et al 1993). The total heat flow response is separated into a reversing signal (related to changes in heat capacity, such as are seen in glass transition phenomena) and a non-reversing signal (related to kinetic events such as the endothermic relaxation which is frequently seen superimposed on glass transitions).

MTDSC has been widely used in the polymer sciences, although the use of the technique in the pharmaceutical sciences is in its infancy (Coleman and Craig, 1996). We report here on the novel use of the technique as a means of characterising the glass transitional behaviour of an amorphous drug, the protease inhibitor Saquinavir, particularly in terms of examining the effects of humidity on the glass transition.

Saquinavir was obtained from Roche Pharmaceuticals and used as received. Samples were stored over silica gel or at 76% RH for 24 hours at 20°C. Thermogravimetric analysis (High-Res TGA 2950, TA Instruments) was used to determine the water content of the samples, using a sample size of 2mg and a heating rate of 10°C/min. MTDSC studies were carried out using a DSC 2920 Modulated DSC, TA Instruments, at an underlying heating rate of 2°C/minute with a modulation amplitude of ±0.159°C and a period of 30 seconds. The sample size used was 2mg and TA instruments aluminium hermetic DSC pans were used throughout. Each experiment was repeated five times.

A typical MTDSC response for Saquinavir stored over silica gel (0.7% water) is shown in Figure 1. It was observed that the total heat flow signal, which is equivalent to a conventional DSC trace, showed an endotherm superimposed upon a shift in baseline. Examination of the separated signals, however, clearly indicates that the response is composed of two separate thermal transitions, corresponding to the glass transition and the endothermic relaxation. The value of the glass transition (Tg) was measured as 107.0 ± 0.4°C and the relaxation enthalpy as 5.1 ± 0.2J/g (onset temperature of 102.5°C ± 0.3°C). Storage at 76% RH (2.4% water) resulted in a lowering of Tg to 75.2 ± 0.4°C. As such plasticizing effects are associated with the stability of amorphous systems, the ability to identify and quantify the glass transition and relaxation endotherm are of considerable practical significance. The study has therefore demonstrated that MTDSC is a potentially highly useful technique for the characterisation of amorphous drug substances.

Figure 1: MTDSC response of Saquinavir



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37 EFFECTS OF CRYSTALLISATION CONDITIONS ON THE MORPHOLOGY OF LACTOSE INTENDED FOR USE AS A CARRIER FOR DRY POWDER INHALERS

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α-Lactose monohydrate has been widely used as a carrier for inhalation aerosols. The carrier morphology is believed to affect the delivery of the drug. For example, increasing the surface smoothness of lactose has been claimed to increase the respirable fraction of salbutamol sulphate (Ganderton, 1992). α-Lactose monohydrate is usually prepared by crystallisation from aqueous solutions. However, little work has been done to control morphological properties such as particle shape and surface smoothness, all of which may change with the conditions of crystallisation. Thus, in the present work, the effects of crystallisation conditions on the morphology of lactose were investigated with a view to preparing lactose particles that may have a preferred morphology for drug delivery by dry powder aerosols.

Lactose was crystallised from aqueous solutions until the majority of crystals were grown to 63-90 μm. The crystals were then filtered and washed with water-ethanol mixtures. The crystallisation was carried out at different supersaturations, temperatures and in the presence of different water-miscible organic solvents. Lactose crystals were characterised using optical microscopic image analysis, scanning electron microscopy and an air permeation method. The particle shape and surface smoothness were quantified by elongation ratio (the ratio of the length to width of particle's projected image), shape factor ($\frac{4\pi\ area}{perimeter^2}$) and rugosity (the ratio between the specific surface areas measured by air permeation and microscopy).

The majority of lactose crystals were either tomahawk-shaped or pyramidal after crystallisation at an initial concentration between 33-43%, w/w. When lactose concentration was increased to 50% w/w, most crystals became prismatic. When the concentration reached 60% w/w, elongated cuboidal crystals were prepared. Higher initial lactose concentrations also tended to result in the crystallisation of more elongated particles. For example, an initial lactose concentration of ≥50% (w/w) resulted in the production of lactose crystals with a significantly higher (ANOVA, $p<0.001$) value (1.78±0.48, n=208) of elongation ratio than 1.39±0.28 (n=371) of the crystals prepared from a concentration of 43% w/w. Crystallisation at 40°C was shown to prepare lactose crystals with a more regular shape and smoother surface than those crystallised at 0°C. For example, lactose particles crystallised at 40°C had a shape factor of 0.73±0.11 (n=766), which was significantly higher ($p<0.01$) than 0.69±0.12 (n=541) of the crystals prepared at 0°C. Addition of 10% (v/v) different water-miscible organic solvents to the mother liquor resulted in different morphology of lactose crystals. Lactose crystals prepared in the presence of ethanol and acetone had an elongation ratio of 1.25 ± 0.19 and 1.35 ± 0.23, respectively, both of which were significantly ($p<0.001$) lower than 1.82 ± 0.23 of the crystals prepared in the presence of glycerine. Further, lactose prepared in the presence of glycerine had a smaller specific surface area (709 $cm^2\ g^{-1}$) than those of either ethanol (771 $cm^2\ g^{-1}$) or acetone (1057 $cm^2\ g^{-1}$) although they had similar particle size. These results suggest that glycerine resulted in the production of lactose crystals with a more elongated shape and smoother surface, in comparison to ethanol or acetone. Therefore, the particle morphology of lactose changes with crystallisation conditions. In order to achieve efficient and reproducible drug delivery by dry powder aerosols such morphological properties of the carrier particles should be carefully controlled.

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38 AB INITIO STRUCTURE DETERMINATION OF POLYMORPH II OF SULPHATHIAZOLE FROM POWDER X-RAY DIFFRACTION DATA

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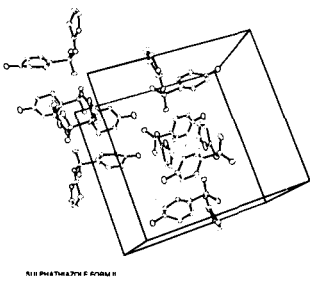
A knowledge of the crystal structure is highly important as it enables one to understand, manipulate, and engineer the bulk properties of solids. The crystalline forms of the antibacterial sulphathiazole have been studied extensively and this drug is now an important model for polymorphism. Although four distinct polymorphs have been identified (Anwar et al 1989), the structure of form II is unknown. Elucidation of its structure would fill an important gap for this model compound. Single crystal X-ray diffraction remains the definitive method for structure determination. However, it is not possible to recrystallise form II to produce crystals of suitable quality for such studies, and one is forced to use a polycrystalline sample. *Ab initio* structure determination from powder diffraction data is extremely challenging due to severe peak overlap in the pattern, and the compression of the three dimensional information into one dimension. Structures solved from powder data have generally been restricted to simple inorganic or organometallic compounds (Langford and Louer 1996). We present here preliminarily results for the crystal structure of form II of sulphathiazole which has been solved *ab initio* from powder diffraction data.

Polymorph II of sulphathiazole was recrystallised from water at 100°C as described by Anwar et al (1989). The powder pattern was collected using synchrotron radiation using a wavelength of 1.1996 nm at Daresbury Laboratory. The lattice parameters were determined using the program *ITO* (Visser 1969). Form II crystallises in the space group $P2_1/n$ with a = 14.321 Å, b = 15.268 Å, c = 10.446 Å and β = 91.057°. Whole pattern decomposition was performed using MPROFIL5 (Rwp = 11.84%) and structure determination was attempted using a number of different packages. All initial attempts led to failure. Success was achieved after adopting a systematic strategy in which the fitting of the fluctuating background was varied, different peak profiles were considered, and the amount of data used in the determination was also varied. This approach yielded a dimer trial structure within the asymmetric unit with the Direct Methods program SIRPOW92 (Cascarano et al 1992).

The trial structure was distorted but was improved by taking the coordinates of the two sulphur atoms from the dimer unit and regenerating the whole structure using SHELXL93 (Sheldrick, 1990) via a difference Fourier calculation (R_1 = 0.3115 for 2160 unique reflections with the temperature factors unrefined). The structure is shown below. It is anticipated that this trial structure can be further improved by performing Rietveld refinement.



In conclusion, the structure of form II of sulphathiasole has been solved *ab initio* from powder data. This is a remarkable achievement given the complexity of the molecule. Furthermore, our experience has led us to develop a strategy that is expected to be useful for solving other structures from powder diffraction data.

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39 STRUCTURE, CHEMISTRY AND STABILISATION IN POLYMORPHIC SYSTEMS: THE CASES OF L-GLUTAMIC ACID AND SULPHATHIAZOLE

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The phenomenon of polymorphism whereby a molecule can adopt more than one crystal structure is well known in both inorganic and molecular crystal chemistry. In the pharmaceutical and specialty chemicals industries it is of vital importance in the context of patent protection, process development and product specification since different polymorphs possess different physical properties. The appearance of a particular polymorph during a crystallisation process is determined by a combination of structural and kinetic processes as first recognised by Ostwald in his 'Law of Stages' which dates from 1897. This simply states that upon crystallsation a system will adopt the crystal structure which leads of the smallest loss in free energy and that these crystals will subsequently transform stagewise to the most stable crystal structure. For example, in the case of L-glutamic acid this implies that the least stable α phase will appear first followed by transformation to the β structure. Sulphathiazole, however, has four polymorphs which according to their known relative stabilities should appear (at room temperature) in the order I→II→IV→III. This paper reports combined modelling and experimental studies of these systems in which a structurally based approach is derived for the selection of solvents and additives designed to stabilise certain desired polymorphic forms. Central to this methodology are the concepts of tailor made additives for controlling crystal growth (Davey (1991)) and the realisation that in order to stabilise a metastable structure the crystallisation of the more stable polymorphs must be inhibited (Davey et al (1997)).

Using a combination of Bernstein and Etter's (Bernstein et al (1995)) 'Graph Set Analysis' and Kitaigordskii's (Kit) 'Aufbau Principle' the crystal chemistry and hydrogen bonding patterns in the polymorphic structures have been compared and the differences and similarities highlighted. This information when combined with crystal morphology data makes it possible to relate these differences to the fastest growth directions of the crystal. This simple approach links kinetics to structure and so enables the design of additives and solvents which interact selectively with desired polymorphic forms.

The polymorphism of L-glutamic acid is based on molecular conformation and this paper demonstrates the successful design of additives which discriminate between structures by mimicking a particular molecular conformation (Davey et al (1997)). In the case of sulphathiazole polymorphism is based on a more complex array of packings arising from intermolecular dimer formation. The approach presented here offers a rational explanation for the observed solvent dependence of polymorph appearance as well as the selection of a new additive capable of stabilising form I of sulphathiazole in acqueous solutions.

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40 MULTIFUNCTIONAL POLYMERS AS SAFE PENETRATION ENHANCERS FOR MUCOSAL PEPTIDE ABSORPTION

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The peroral route of peptide and protein administration is currently the greatest challenge in developing suitable dosage forms, because it offers the highest ease of application to the patient. However, particular difficulties are met in designing effective delivery systems for gastro-intestinal application. The challenge to achieve sufficient peptide drug absorption is to overcome the very efficient absorption barriers of the gastro-intestinal tract. As a new strategy two classes of mucoadhesive polymers, namely crosslinked poly(acrylic acid) derivatives as carbomer and polycarbophil as well as chitosans have been shown to be able to positively influence the intestinal barriers and to improve peptide drug absorption.

At first the potency of mucoadhesive excipients to inhibit intestinal proteases has been evaluated. Among the different mucoadhesive polymers investigated, uniquely the poly(acrylates) polycarbophil and carbomer 934P were able to inhibit the activities of trypsin, α-chymotrypsin, carboxypeptidase A and B as well as cytosolic leucine aminopeptidase. Carbomer was found to be more efficient in reducing proteolytic activity than polycarbophil. These polymers were able to deprive Ca^{2+} and Zn^{2+}, from the enzyme structures, thereby inhibiting their activities. Chitosan salts were unable to deactivate luminal enzymes as trypsin.

The effects of the mucoadhesive polymers Carbomer, chitosan glutamate and chitosan hydrochloride were studied in an in-vitro model (Caco-2 cell monolayers) with respect to their ability to enhance paracellular intestinal peptide drug absorption. The polymer preparations were applied to the apical side of Caco-2 cell monolayers and the effects on transepithelial electrical resistance (TEER), paracellular transport of FITC-dextran Mw 4,400 (FD-4) and [^{14}C]-mannitol were measured. Paracellular transport of FD-4 was additionally visualized by means of confocal laser scanning microscopy (CLSM). In concentrations up to 1% (w/v) carbomer and 1.5% (w/v) chitosan salts substantial, but reversible lowering of TEER was found. Opening of the tight junctions of the Caco-2 cell monolayers after apical incubation of all mucoadhesive polymers used could be clearly visualized by CLSM and significant paracellular transport of both FD-4 and [^{14}C]-mannitol across Caco-2 cell monolayers could be detected.

To study the peptide drug absorption in-vivo, two different types of mucoadhesive polymers including the fast dispersing freeze-dried sodium salt of carbomer 934P (FNaC934P) were investigated in-vivo in rats. The nonapeptide drug buserelin was applied intraduodenally in control buffer, 0.5% (w/v) carbomer, 0.5% (w/v) FNaC934P, and 1.5% (w/v) chitosan hydrochloride. As a result it was found that all polymer preparations showed a statistically significant improvement of buserelin absorption compared to the control solution. The absolute bioavailabilities for the different polymer preparations were: control, 0.1%; 0.5% FNaC934P, 0.6%; 0.5% carbomer, 2.0% and 1.5% chitosan hydrochloride, 5.1%.

Development of a peptide drug delivery system has shown that such a system based on poly(acrylate) derivatives for both local deactivation of the main luminal enzymes and improving intestinal membrane permeability by increasing paracellular transport should consist of two phases. The first phase should contain a suitable polymer which is able to rapidly dissolve (or swell) in order to locally deactivate the luminal enzymes and to open the tight junctions. The second phase should contain the peptide drug and should protect it against degradation during the time the first phase exerts its activity. Because deactivation of luminal enzymes and opening of tight junctions are time-dependent processes, the peptide should be released in a burst after a lag-time of about 30 min. First experimental results show that, with N-α-benzoyl-L-arginine ethyl ester (BAEE) as model substrate for trypsin, incorporated together with carbomer in suitable fatty masses (second phase) and in combination with a fast dispersing poly(acrylate) formulation based on FNaC934P (first phase), a rapid release of BAEE is achieved with an optimal preservation against tryptic degradation.

41 DIFFERENTIAL PERMEATION OF PROPRANOLOL ENANTIOMERS ACROSS HUMAN SKIN IN-VITRO AS A RESULT OF STEREOSELECTIVE SORPTION/DESORPTION

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Chirality continues to be a topical issue in the field of pharmaceuticals. An estimated 50% of all currently prescribed drugs are racemates and the majority of NCEs are also chiral. Regulatory authorities generally favour the administration of single stereoisomers wherever this is practicable. It was realised that common excipient materials used in drug formulations were chiral and were potentially capable of stereo-selective binding/release by analogy with the action of certain chiral chromatographic stationary phases. Some evidence to suggest that differential release could indeed occur if the formulation contained a racemic drug has been reported (Vakily et al, 1995). We propose that this phenomenon represents a potential method to achieve *deliberate* selective administration of enantiomers, by formulating a racemate with an enantioselective sorbent which maximally binds the distomer causing it to be retained within the formulation. On the other hand, the eutomer is bound more weakly and consequently more able to diffuse from the formulation (Heard et al, 1997).

We recently reported that significant differences in the release of propranolol enantiomers can be obtained in terms of tablet dissolution (Suedee and Heard, 1997) and diffusion across dimethylpolysiloxane (Heard and Suedee, 1996) when certain derivatives of cellulose, e.g. *tris*(3,5 dimethylphenyl carbamate) (CDPC) are formulated with racemic propranolol. Binding of S-propranolol was found to be greater and consequently the rates of release and permeation were greater for R-propranolol. In the current study, mixtures of racemic propranolol and CDPC were applied to excised human skin and the permeation rates of R- and S-propranolol determined.

Full thickness human skin (ex cosmetic surgery) was cut into 1 inch squares, following the removal of subcutaneous fat. Specimens were mounted in Franz diffusion cells and 2.5ml of pH 7.4 PBS was added to the receptor phases, which were continually stirred. Cells were placed in a water bath maintained at 37°C and after 1hr, 1ml of donor phase was added (1mg propranolol/ml pre-equilibrated with 10mg CDPC). Samples were taken at appropriate timepoints and analysed by chiral HPLC (column Daicel OD-R, mobile phase 60% 0.5N sodium perchlorate, 40% acetonitrile).

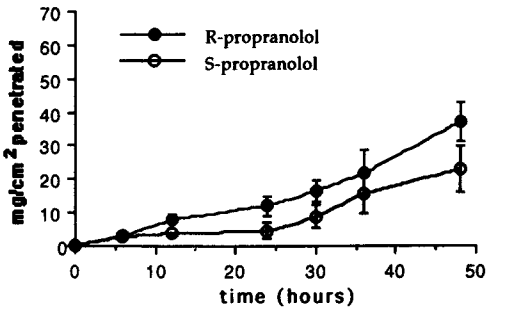

Figure 1 Permeation of R- and S-propranolol in the presence of cellulose *tris*(3,5-dimethylphenyl carbamate) across human skin from PBS pH 7.4 at 32°C, (n=6±SE)

Results showed that under these conditions the flux of R-propranolol was significantly greater than that of S-propranolol (Figure 1), and demonstrate that differential sorption/desorption can result in stereoselective penetration when racemates are applied topically.

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42 POLYMERIC PALMITOYL GLYCOL CHITOSAN (GP) VESICLES - A NEW DRUG DELIVERY SYSTEM

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The modification of glycol chitosan by reaction with palmitic acid N-hydroxysuccinimide ester results in the production of a new polymer palmitoyl glycol chitosan (GP) bearing hydrophobic pendant units. On hydration of GP in the presence of cholesterol a micro- or nano-particulate drug delivery system (Uchegbu, 1997) capable of entrapping the hydrophilic aqueous marker 5(6)-carboxyfluorescein (CF) is produced (Table 1). Nanoparticulate systems were prepared by hydrating polymer/ lipid mixtures at 70°C, followed by sonication, thus eliminating the need for organic solvents. Solulan C24 (poly-24-oxyethylene cholesteryl ether) was added to some formulations as a steric stabiliser. Freeze fracture electron microscopy (FFEM) confirmed the unilamellar vesicular structure of GP nanoparticles and a largely spherical vesicular population was seen. Different levels of modification as calculated from the original ratio of hydrophobic (palmitoyl) to hydrophilic (chitosan) units resulted in two forms of GP – GP41 (low hydrophobic modification) and GP21 (high hydrophobic modification).

Table 1: CF loaded GP vesicles

PARTICLES	SIZE	% CF ENCAPSULATION
GP21, cholesterol	34.6μm	7.4%
GP21, cholesterol, Solulan C24	30.7μm	4.6%
GP21, cholesterol, Solulan C24	325nm	6.9%
GP41, cholesterol, Solulan C24	333nm	4.6%

Freeze drying GP41, cholesterol formulations resulted in an increase in particle size (determined by photon correlation spectroscopy), increasing from 407nm to 1.87μm. The zeta potential (determined by measuring electrophoretic mobility) of the particles was unaffected by freeze-drying (–3.95 before freeze drying and –4.58 after freeze drying).

GP polymeric vesicles are being evaluated as a gene delivery system; hence the interaction of GP41 particles with DNA was studied. pEGFPC1 (+ CMV promoter) plasmids were incubated with GP41 formulations and the size and zeta potential of the complexes determined (Figure 1)

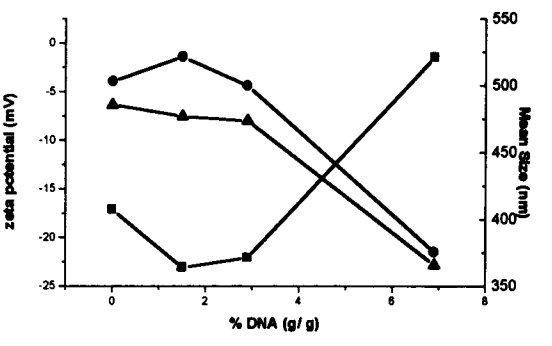

Figure 1: Size and zeta potential of GP41, DNA mixtures, ● = zeta potential of GP41, cholesterol (10:4 weight ratio) + DNA, ▲ = zeta potential of GP41, cholesterol, Solulan C24 (10: 4: 3 weight ratio) + DNA, ■ = size of GP41, cholesterol (10: 4 weight ratio) + DNA

There was evidence of DNA charge neutralisation by GP41 vesicles at low DNA levels and an increase in particle size as DNA levels increased. GP41 vesicles were biocompatible with a panel of cell lines – A2780, A549 and A431. Haemocompatibility data will also be presented.

I.F. Uchegbu, Patent Application Number 9706195.6

43 NONIONIC OIL-IN-WATER MICROEMULSIONS: THE EFFECT OF OIL INCORPORATION

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Microemulsions are clear, low viscosity, infinitely stable oil and water dispersions. An understanding of the way in which oils are incorporated into oil-in-water (o/w) microemulsions (m/e) would be invaluable in explaining how drugs are solubilised within these systems. The present study examines the way in which oils are incorporated into a surfactant monolayer at the air-water interface. Ethyl ester oils; ethyl butyrate (EB), ethyl caprylate (EC) or ethyl oleate (EO), were added (40 µl) to the surface of micellar solutions of the nonionic surfactants N,N-dimethyldodecylamine-N-oxide (C12AO) and polyoxyethylene dodecyl ether (C12EO). Any change in surface tension in the presence of oil was measured using the Wilhelmy plate technique.

Table 1. Surface tension of micellar solutions in the presence of ethyl ester oils.

Oils	Surface tensions (mN/m)	
	C12AO	C12EO
	33	34
ethyl butyrate	25	28
ethyl caprylate	26	31
ethyl oleate	29	32

From Table 1 it can be seen that there is a direct relationship between the molecular volume of oil and reduction in surface tension, in that the smallest molecular volume oil, EB, reduced surface tension to the greatest extent, and the largest oil, EO, reduced it the least. This result suggests that EB penetrates the surfactant monolayers to the larger amount.

The effect of the nature of oil on the molecular structure of the m/e prepared from the above components was investigated by means of total intensity light scattering (tils) experiments using a Malvern 4700c series laser light scatterer at 25°C. Analysis of the tils data showed that as the amount of oil present increased the number of surfactant molecules comprising the m/e droplet decreased; this decrease was most marked in the EB containing m/e (Fig. 1) suggesting that the oil is behaving as if it were a cosurfactant and penetrating the interfacial surfactant monolayer and that this penetration is greatest in the case of EB. This observation supports the surface tension experiments. These results may aid in the understanding of how drugs are incorporated into m/e.

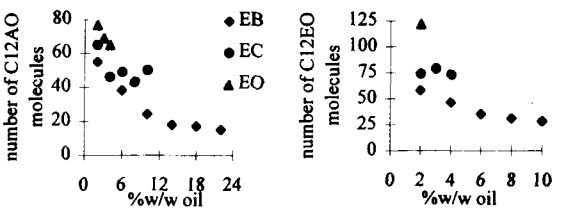

Fig. 1. Variation in surfactant molecules a) C12AO and b) C12EO with various % w/w ethyl esters.

44 PRELIMINARY BIOLOGICAL EVALUATION OF POLYAMINODENDRIMERS

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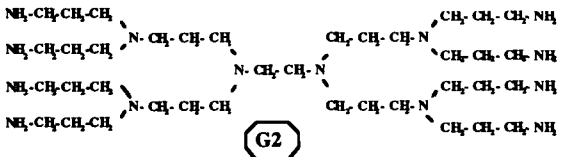
Introduction

Dendrimers are macromolecules which have a well-defined, highly branched structure radiating from a central core. The surface has a large number of reactive end groups (Tomalia et al 1985). Because of their small size, and uniformity, dendrimers have potential as nanoparticulate drug carriers. In theory, drugs can either be covalently bound to the surface or trapped within the internal cavity. To select dendrimers candidates with real potential for *in-vivo* use, it is essential to evaluate their basic biological properties ie. biocompatibility and toxicology (Duncan & Malik 1996). Here we have studied a series of novel polyaminodendrimers of increasing size and number of surface groups which have the following structure.



We have examined their membrane activity (red blood cell lysis), cytotoxicity against B16F10 melanoma cells, and also their ability to increase transport across the gut *in vitro*.

Materials and Methods

Four generations of polyaminodendrimers based on an ethylenediamine core were studied; G1-G5 of Mw 233 to 3,486 daltons, and also a propylene diamine core (G6). Membrane activity of the dendrimers was determined using a haemolysis assay. Dendrimers were incubated with rat red blood cells for 1h; and the haemoglobin release measured spectrophotometrically. Cytotoxicity was determined by incubating dendrimers with B16F10 cells (murine melanoma) for 72h ; and cellular viability was assessed using MTT assay (Sgouras & Duncan 1990). Rat everted gut sacs were incubated with glucose in the presence of dendrimers for 30 min, and the uptake of glucose measured by the glucose oxidase assay (Pato et al 1994).

Results and Discussion

All dendrimers caused red blood cell lysis at concentrations above 1 mg /mL. The lysis was concentration-dependent, but not generation-dependent. Dendrimers did not show cytotoxicity against B16F10 murine melanoma cell lines over the concentration range used, except in the case of G5 at the highest concentration (0.1 mg/mL).This observation was consistent with the gut sac experiment where G5 showed a concentration-dependent effect on glucose accumulation. This study, and previous studies with PAMAM dendrimers, demonstrate the importance of early biological testing in determining the potential of these novel macromolecules for *in vivo* use. Cationic dendrimers can be toxic. Toxicity is greatest with higher Mw and concentration.

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45 SPRAY-DRIED COMBINATIONS OF BIODEGRADABLE POLYMERS TO MODULATE DRUG RELEASE

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Spray-dried biodegradable poly(DL-lactide) (PDLA) microspheres frequently exhibit considerable 'burst' release, particularly at high drug loadings. Use of slowly degrading polymers results in a reduced 'burst' but with a protracted terminal release phase. The permeability of these polymers can be increased by incorporating channelling agents (Latha and Sapna, 1993). An alternative approach whereby the release of rifampicin, as a model drug, has been modulated by varying the proportions of low and moderate molecular weight PDLA was examined. This technique, which avoids the use of potentially toxic release-controlling excipients, has previously been applied to films and solvent-evaporated microspheres (Bodmeier et al., 1989) with a PDLA of MW 120,000. However, this approach has hitherto not been examined using spray-drying. Microspheres containing 20% w/w rifampicin were prepared by spray-drying a solution (3% w/v total solid) of combinations of Resomer® R104 (MW 2000) and R202H (MW 9000) in the % ratios 90:10, 80:20, 60:40, 56:44, 52:48, 48:52, 44:56, 40:60, 20:80 and 10:90 through the nozzle of a spray-drier (Buchi model B191): inlet temp. 40°C; flow rate 600 NL/h. Dissolution studies in pH 7.4 phosphate buffer were performed at different temperatures comparing two methods: shaking bath (method A) and USP paddle (method B). Surface morphology and thermal behaviour before and after dissolution (method A) were examined using SEM and DSC respectively.

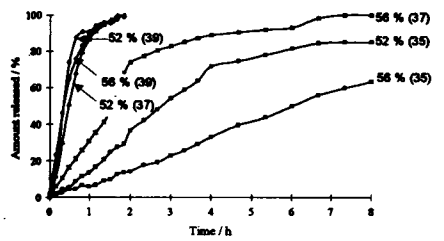

Figure 1. Release profiles using method B at two levels of R202H (temperatures in °C in parentheses)

Method B achieved similiar but dramatically faster dissolution profiles compared with method A. This was attributed to the considerably greater agitation afforded by the former. Dissolution studies showed a dramatic change in drug release particularly between 52 and 56 w/w R202H (Figure 1). Furthermore, drug release rate showed a remarkable dependence on temperature (Figure 1) as illustrated by the microspheres containing 56% R202H which approximated to zero order (r^2=0.969), first order (r^2=0.981) and biexponential release (r^2=0.991) at 35, 37 and 39°C respectively. DSC gave slight, but progressive increases in melting onsets between 10 and 90 % R202H of 55.3-58.7°C before dissolution However the corresponding values, most notably for 52 and 56% R202H, were 34 and 51°C after dissolution (7 days) respectively. The reduced onset of melting when 52% R202H was used was attributed to the hydrolysis of R104, indicated by a broad endotherm corresponding to oligomeric fractions of this polymer. With 56% R202H, a secondary peak superimposed on the R104 oligomeric peak corresponding to higher MW R202H, indicated preservation of matrix integrity. The proposed mechanism of polymer hydrolysis, and softening of the microsphere matrix was supported by SEM after dissolution. From SEM, the absence of surface roughening or reduction in microsphere size ruled out erosion as a significant contribution to the release. These data highlight the criticality of matrix composition and the role of temperature to the release mechanism. Moreover, the utility of low MW PDLA fractions to modulate release i.e. reduce 'burst' whilst maintaining acceptable release from spray-dried biodegradable particles is highlighted.

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46 TARGETTING OF HYALURONAN TO TUMOURS

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Most cytotoxic drugs have very little inherent targeting to the pathological site and as a consequense give serious side effects when used systemically for the treatment of cancer. Expression of receptors for the endogenous polysaccharide hyaluronan (HA) by some tumour cells forms a basis for a novel site directed cancer therapy. The normal site of HA clearance from the circulation is via high affinity receptors in the liver that, contrary to some tumour cells (Gustafson 1997, Samuelsson & Gustafson 1997) also recognize chondroitin sulphate (CS). In order to see if HA can be used as a vehicle for antitumour drugs we have studied targeting of intravenous HA to solid tumours in rats.

The tissue distribution of ^{125}I-labeled HA (Gustafson et al 1994), injected intravenously in Wistar-FU rats carrying tumours from a nitroso-guanidine induced tumour cell-line inoculated in one hind leg, was studied by scintigraphy of live animals and γ-counting of tissues obtained at autopsy. In some cases the animals were pretreated with unlabeled CS.

The labeled HA localized mainly in the liver, but a significant amount was also found to associate specifically with tumour tissue with a wet weight adjusted tumour/control (muscle) tissue (T/C) ratio of 11.4±0.4 (n=3, p=0.006). To reduce liver uptake, some rats were given CS 30 seconds before the labeled HA. This led to increased radioactivity in tumours and a T/C ratio of 16.2±2.5 (n=4, p<0.001) at 18-20 h after injection. Using CS pretreatment, significant targeting with reduced liver uptake (T/C ratio of 8.8±0.9, n=4, p<0.001)was seen using also a tracer dose of ^{125}I-labeled HA. If the CS was given 1 h prior to injection of label no significant targeting of tracer doses was observed at 70 min after injection of label and liver radioactivity was not decreased (n=3). Increasing the dose of CS given 30 seconds prior to label from 200- to 1000-fold the amount of labeled HA, significantly increased the amount of specifically targeted radioactivity from 20 105 cpm/g (n=3) to 44 169 cpm /g (n=3; p=0.006) and significantly decreased the liver uptake (p=0.009).

The present study shows that differences in specificity between HA binding sites can be exploited to reduce uptake of the polysaccharide in healthy tissue without negatively affecting targeting to solid tumours. Similar findings of reduced liver uptake and increased targeting of labeled HA to site of pathology by preinjection of CS was also found in balloon catheter-damaged rat carotid arteries (Gustafson et al 1996). The biocompatability of HA is excellent and providing care is taken so that drug association does not interfere with the biological properties of the polymer, targeting of HA/drug complexes to accessible unoccupied HA binding sites could be a way for more efficient drug treatment with reduced side effects.

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47 IN-VITRO RELEASE OF SODIUM SALICYLATE FROM WATER-IN-OIL PHOSPHOLIPID MICROEMULSIONS

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Amphiphilic molecules have a tendency to form aggregates spontaneously, called micelles, follwoing dissolution in organic solvents or oil. The micelle core is capable of solubilizing a relatively high amount of water. These systems with such characteristics are also called water-in-oil microemulsions and could be used as novel drug delivery systems for topical and intramuscular administrations, due to the inherent properties such as transparency, thermodynamic stability and small particle size.

In this study, partial phase diagrams were constructed for systems composed of 2% w/w sodium salicylate solution/lecithin/alcohol/ isopropyl myristate by titration method (Aboofazeli, 1994) at various surfactant / cosurfactant ratios ($R_{s/c}$). Two commercially available soybean lecithins, namely Epikuron 200 and Epikuron 170 (phosphatidylcholine purity of 94% and 68-72%, respectively) were used as surfactants.Three short chain alcohols (namely, n-propanol, 2-propanol and n-butanol) were investigated as cosurfactants. Phase diagrams showed the area of existence of a stable isotropic region to be along the surfactant/oil axis (ie, reverse microemulsion area) in all systems regardless of the $R_{s/c}$. Although no remarkable difference was observed between the phase diagrams produced by two types of lecithin, the extent of the isotropic regions was found to be dependent upon both the nature of the cosurfactant and $R_{s/c}$.

Sodium salicylate (ionizable model drug) containing microemulsions prepared were then examined for their drug releasing ability. For this purpose, static typed diffusion cells were employed. The receiver compartment of each cell was filled with 45 ml distilled water and the donor compartment with 2 ml microemulsion sample or a test solution. A 5 cm^2 section of abdominal skin from male hairless rats with an average weight of 220±30 g was excised immediately before the skin permeation experiments. The cells were sampled initially at 30 min and then 1 hr intervals over a period of 12 hrs in order to achieve constant absorption rate. The setup was kept at room temperature with continuous stirring and a sink condition was always maintained in the receiver compartment. The amount of sodium salicylate released from the microemulsions was measured spectrophotometrically at 296 nm and the accumulated release was calculated using calibration curve.

The results obtained from the release studies showed that systems with 7 and 9% water (containing 2 wt% drug) were not capable of releasing their drug content with a lower rate, compared with the control sample (ie, drug solution). This could be suggested to be due to the absence of microemulsion droplets. In most cases, however, samples with more than 9% water were found to release their drug at a significantly ($p<0.05$) slower rate compared with the control sample. Among the investigated samples, those with n-propanol, $R_{s/c}$ of 1.5:1 and 11-20 wt% water contents showed a relatively low absorption rate.

In conclusion, it is suggested that microemulsions could open up a new horizon for sustained and controlled delivery of drugs, in particular peptides and proteins, and should be considered as future drug delivery systems.

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48 SYSTEMIC AND LOCAL IMMUNE RESPONSES FOLLOWING ORAL AND NASAL DELIVERY OF MICROSPHERES OF DIFFERENT SIZES

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A main objective in the development of new vaccines is the induction of both systemic and mucosal immunity, following mucosal delivery of antigens. Due to its easier accessibility, the nasal cavity is a potential alternative to parenteral and oral routes for the delivery of immunogenic substances and we have shown particle uptake by the nasal mucosa (Almeida & Alpar, 1996). Particle size dependant uptake is an area of some controversy (Jani et al 1990) and this study was carried out to examine the effect of particle size and delivery route on systemic and mucosal responses.

Cholera toxin B subunit (CTB), a potent immunogen, was adsorbed to the surface of mono-sized surfactant-free latex microspheres, 0.2μm (ms1) and 1.0μm (ms2). Release profiles were performed in phosphate buffer (pH 7.5) and a bicinchoninic acid assay was used to measure protein. Groups of Balb/c mice (n=4) were dosed with a dose equivalent to 10μg CTB by oral (i.g.) and nasal (i.n.) routes on day 1 and boosted on day 22 (Table 1). An intramuscular (i.m.) dose of CTB in Freund's Incomplete Adjuvant (FIA) was given on day 1. Serum and salivary samples were taken on days 21 and 42 and gut wash samples on day 42. The samples were analysed for anti-CTB antibodies using an ELISA method.

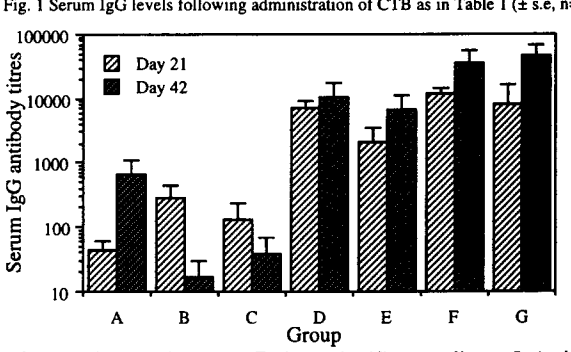
Table 1 Treatment groups and IgA levels detected in mucosal samples (n=4).

Group	Treatment	Relative IgA gut wash titre	Salivary IgA Absorbance Day 21	Day 42
A	free i.g.	1.003	n.d.	0.008
B	ms1 i.g.	50.506	n.d.	0.007
C	ms2 i.g.	12.409	n.d.	0.004
D	free i.n.	12.736	0.009	0.204
E	ms1 i.n.	2.723	n.d.	0.070
F	ms2 i.n.	5.334	0.156	0.153
G	i.m. (FIA)	0.441	0.015	n.d.

n.d. not detected

CTB loadings were similar for both microsphere sizes (1% w/w) and release was faster for ms1 than ms2 after 20 h (18% and 9% respectively) with both preparations releasing 40% of loaded CTB after 70 h. High but variable levels of IgA were detected in the gut wash samples with all groups demonstrating higher levels than the i.m. control. Serum IgG titres were elevated in all groups receiving CTB by the nasal route compared with oral ($p<0.05$) with titres induced by the larger particles not significantly different from the i.m. control (Fig. 1) and greater than free antigen and the smaller microspheres.

Fig. 1 Serum IgG levels following administration of CTB as in Table 1 (± s.e, n=4).



After 21 days, only group F show significant salivary IgA titres ($p<0.05$) whereas after boosting all groups with nasally delivered antigen show significant IgA titres demonstrating a disseminated mucosal immune response. There are also low but detectable levels found after oral delivery. This study illustrates the difference in mucosal responses dependant on particle size and route of delivery, with micron-sized particles providing the most promising responses.

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49 LIPOPHILIC DRUG MODELS FOR SCINTIGRAPHIC EVALUATION OF DRUG DELIVERY SYSTEMS

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Gamma scintigraphy is an established method for the in-vivo evaluation of drug-delivery systems. The limitations of the technique lie in the select number of radionuclides, suitable for administration to human subjects, which can be imaged by gamma camera. Many studies are performed using $^{99}Tc^{m}$ technetium or ^{111}In radiopharmaceuticals which are well established in diagnostic nuclear medicine. These radionuclides are 'foreign' in terms of the structures of drug molecules and it is therefore not possible to use the technique to study drug biodistribution. In general, for the study of drug delivery systems, chelates or complexes of technetium or indium are incorporated by association within a component of the formulation, or used as probes which allow the behaviour of drugs to be predicted.

The majority of radiopharmaceuticals exist in the form of hydrophilic, polar molecules, and therefore provide models for water-soluble drugs. For the study of oil-based formulations containing lipophilic drugs, alternative models must be considered (Wilson et al., 1997). The present study examined a number of lipophilic radiopharmaceuticals, in order to assess their suitability as drug models in an emulsion comprising castor oil and water. Radiopharmaceuticals investigated were $^{99}Tc^{m}$-exametazime (Ceretec, Amersham International), $^{99}Tc^{m}$-hexakis-methoxyisbutyle isonitrile (Cardiolite, Du Pont), ^{111}In-Indium oxine (Amersham International), and three other $^{99}Tc^{m}$ hexakis isonitrile derivatives (*t*-butyl, *n*-butyl and *cyclo*hexyl) synthesised in the laboratory. Rates and extents of partitioning in an emulsified system comprising castor oil and water have been assessed. Radiochemical purity determinations on the radiopharmaceuticals were performed where appropriate by HPLC using a reversed phase column. In order to achieve some simulation of the *in-vivo* conditions encountered by formulations on administration, serum albumin was incorporated into the aqueous phase, at 0.5% and at 5% concentrations.

All compounds showed a high degree of lipophilicity. Active compounds introduced into the castor oil phase generally remained associated with the oil although Ceretec did show some losses into the aqueous phase. HPLC analysis showed this activity to be present as $^{99}Tc^{m}$-pertechnetate, with no evidence of the primary or secondary complex. Indium oxine demonstrated a high affinity for the oil phase, but in the presence of serum albumin 5.0% in the aqueous phase, some loss from the oil phase did occur. None of the technetium compounds behaved differently in the presence or absence of serum proteins. Active compounds introduced into the aqueous phase migrated at different rates into the castor oil. Ceretec demonstrated the fastest rate but again was shown to be unstable and $^{99}Tc^{m}$-pertechnetate was detected on HPLC analysis. The stability was improved by increasing the tin (II) concentration of the formulation by the addition of stannous pyrophosphate. Of the isonitriles, *n*-butyl showed the highest affinity

All compounds studied have potential as models for lipophilic drug formulations. The use of Ceretec is limited by instability problems to studies of short duration (<30 minutes). The use of ^{111}In indium oxine may be limited in situations where formulations come into contact with high concentrations of protein in solution. This is probably caused by a disassociation of the indium from the oxine in the presence of other ligands on the protein molecule with higher affinity constants for indium (Kaempfer 1987). Isonitriles appear highly stable chemically, and remain associated with the oil phase for long periods of time (>2 hours). *t*-butyl and methoxy*iso*butyl isonitriles both have an established use in myocardial imaging and have therefore been evaluated toxicologically. The other isonitrile compounds have similar properties, but have not hitherto been used in human subjects. Isonitriles offer great potential as lipophilic drug models but toxicological evaluation of some derivatives is necessary.

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Today, there is a greatly increased interest in the study of bioactive natural products as potential drugs, and strategies in this type of research is quckly changing. To discover such bioactive substances it is important for academic research to focus on methodological aspects.

This lecture will present our research strategy and some recent results concerning natural products with anti-inflammatory activity. Important aspects of this research include: development of fractionation protocols and separation methods that permit isolation of minute quantities of bioactive substances from plant biomass with complex chemical composition; development of selection strategies for plant materials based on biodiversity, ethnomedicine and chemotaxonomy; structure determination of isolated substances; and characterisation of biological (pharmacological) properties of isolated substances on cell (human neutrophil) and enzyme (COX- 1, COX-2, elastase) levels. Research activities are also focussed on how to dereplicate, i.e. to avoid the repetitive isolation of common and well-known structures with known biological activity and the emergence of artefacts and interference with the bioassay applied.

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51 FEVERFEW (*TANACETUM PARTHENIUM*): BIOLOGICAL AND THERAPEUTIC ACTIVITY

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Feverfew (*Tanacetum parthenium*) has been used therapeutically since antiquity and there are many anecdotal claims of efficacy. It is a plant in which there has been a resurgence of interest in recent years.

Feverfew is a strongly aromatic plant. It grows about 50 cm tall, has green Chrysanthemum-like leaves and in summer displays daisy-like flowers in clusters. They have yellow centres and radiating white florets. It is often found growing in the wild. Over the years several other names have been given to the plant, including: federfoy, featherfoil, bachelor's buttons. nosebleed, midsummer daisy, and mutterkraut (mother herb). The latter related to ancient reports of efficacy in treatment of various "female problems".

It is a plant for which a great many medicinal properties have been claimed, and these can be divided into ancient uses (agues, fever, headache, stomach ache, insect bites, toothache, vertigo, difficulties in labour, threatened miscarriage, regulation of menstruation) and more recent uses (migraine, arthritis). There are some reported adverse effects of the plant; these include contact dermatitis (particularly evident in growers of the plant) and mouth ulceration.

In the 1980s, anecdotal claims of the efficacy of feverfew in migraine and arthritis appeared regularly in newspaper articles, and several books were written on the subject. The level of interest was such that scientists started to take an interest, and three clinical trials were performed to formally test the efficacy of feverfew in these conditions. It turned out that in migraine good evidence was obtained to substantiate the claims of benefit. In two separate trials significant benefit was seen in terms of reduction in frequency and severity of migraine attacks. A third trial in rheumatoid arthritis failed to demonstrate any benefit in this condition, but it should be noted that the patients studied were also unresponsive to other, more conventional therapies. Consequent to the publication of these clinical trials, feverfew has found a place in Martindale's pharmacopoeia.

Several investigations have been performed to obtain information on the mode of action of the herb. Extracts of feverfew were shown to inhibit release of serotonin from storage granules in blood platelets and this may be relevant to the mode of action of the herb in migraine. There is good evidence that serotonin is involved in precipitating migraine attacks and platelets contain more than 95% of the serotonin normally present in blood. This serotonin can be released from platelets when they are stimulated, and feverfew extracts inhibit this release. Feverfew extracts also inhibit release of certain proteins from storage granules in activated leucocytes. This is also of interest because the proteins that are released are found in synovial fluid in rheumatoid arthritis and are thought to contribute to synovial damage.

The constituent of feverfew that is most likely to be responsible for these effects is parthenolide. This is an amphiphilic sesquiterpenelactone with an α-methylenebutyrolactone function that undergoes Michael addition-type reactions with intracellular sulphydryl groups. It must be emphasized, however, that it is by no means certain that feverfew's therapeutic mode of action is via inhibition of granule release. Other mechanisms may come into play. Also we await absolute confirmation of the identity of the constituent(s) responsible for its therapeutics effects.

In view of the current interest in feverfew it may be useful to comment on the commercial preparations that are available, and how these compare with the feverfew that was used in clinical trials. The clinical trials were all performed using a species of *Tanacetum parthenium* indigenous to the UK. Further, the trial material had three important characteristics: 1) it was derived from feverfew plants that had been authenticated by qualified botanists; 2) it consisted of pure leaf material; 3) its parthenolide content was defined: trial feverfew capsules each contained between 250 and about 800μg parthenolide. Unfortunately, most of the commercial preparations that have been analysed do not conform with the trial material, at least as regards parthenolide content. Out of 22 commercial preparations that were analysed, only four preparations contained amounts comparable with that in the clinical trial material, another 10 contained much smaller amounts, and in 8 parthenolide was undetectable. Clearly, better standardization of commercial preparations is required.

52 EVALUATION OF *GLYCYRRHIZA GLABRA* USED IN THE TREATMENT OF PSORIASIS

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Psoriasis is a common and complex disease affecting 2-3% of the Caucasian population. It is a single disease which has several morphological variants, *Psoriasis vulgaris* being the most common, it is characterised by areas of skin becoming thickened, scaly and covered by erythematous plaques, due to a considerably shortened cell cycle and cell turnover rate, Ilzuka,H. & Takahashi,H. (1993). The stolons of *Glycyrrhiza glabra* (L.) have been employed in pharmacy since 2100 BC., and have been used for the treatment of arthritis and various inflammatory conditions. The extracts of *Glycyrrhiza glabra* have been extensively studied to verify passed claims and investigate the mechanism of action of the constituents responsible for them.

An active compound, X, (structure yet to be fully elucidated) was successfully extracted and purified using a bioassay guided technique, it has been confirmed as not being glycyrrhetinic acid. The ethyl acetate extract was chromtaographed on a silica gel column (eluting with chloroform → methanol), the active fraction was further isolated using a sorbisil column and purified on an alumia gel column (LH20). The bioassay guided technique used involved determination of the inhibition of chemically induced erythema on mouse ears, Evans,F.J., & Schmidt,J. (1979). The ED0 (dose required to produce a 50% inhibition to erythema response) of the crude ethyl acetate extract was 15µg. Compound X has an ED50 of 1µg, at 6 hours after the application of the irritant, 12-O-tetradecanoyl phorbol-13-acetate (TPA). Inhibition of TPA induced erythema could be due to the interactions of compound X with the intracellular receptor for TPA, protein kinase C (PKC). The PKC enzyme plays a regulatory role in epidermal proliferation and differentiation, its activity and distribution differs in psoriatic lesions and healthy skin. Compound X was also tested for inhibition of TPA and ADP(adenosine diphosphate) induced platelet aggregation. Blood platelets release inflammatory mediators in response to external damage affecting the proliferation of cells. An ether phospholipid known as PAF (platelet activating factor) is readily found in psoriatic lesions. At 100µg blood platelet aggregation was inhibited by X by 40% and 36% when induced by TPA and ADP respectively. At 10µg inhibition was 30%(TPA) and 15%(ADP), and at 1µg 20%(TPA) and 10%(ADP). TPA's effects on platelets are not identical to those produced by drugs or ADP. TPA- induced aggregation of blood platelets is reported to be associated with the activation of Ca^{2+} - dependent protein kinase C, which is found in high levels within blood platelets. ADP aggregation is activated by the presence of a primary receptor on the surface of the non-activated platelet. Inhibition of ADP induced platelet aggregation may be due to the inhibition of the primary receptor or the enzyme cylcoxygenase.

At present it appears that compound X is capable of inhibiting two different pathways of platelet aggregation. Future work will be to elucidate the structure of compound X and test its anti-inflammatory effects further using cell culture techniques and physically induced erythema tests.

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53 INHIBITION OF ERYTHROCYTE ACETYLCHOLINESTERASE BY DROPLET COUNTER-CURRENT CHROMATOGRAPHY FRACTIONS OF *SALVIA LAVANDULAEFOLIA* OIL

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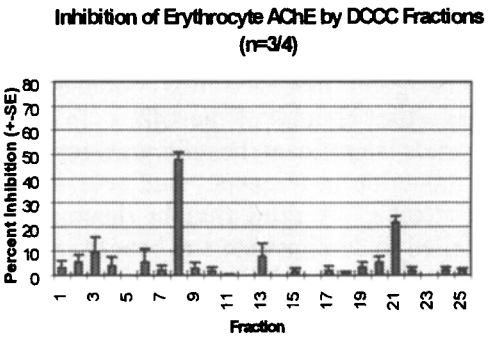
Alzheimer's Disease (AD) presents increasing social and economic problems for healthcare of the elderly in industrialised society. Loss of the neurotransmitter acetylcholine (ACh) is implicitly involved in the underlying pathologies of AD and may contribute substantially to associated memory dysfunction. Current therapy is based on the synthetic chemical tacrine, which inhibits breakdown of ACh by inhibition of the enzyme acetylcholinesterase (AChE), but has toxic side effects.

Sage (*Salvia officinalis*) is reported to 'enhance the memory' in English herbals dating from the sixteenth century. Preliminary screening of essential oils of *S.officinalis* and *S.lavandulaefolia* caused inhibition of human brain striatal AChE, (Perry et al., 1996). *S. lavandulaefolia* was selected over *S. officinalis* for further investigation since the latter contains thujone which is toxic.

Droplet Counter-Current Chromatography (DCCC) of *S. lavandulaefolia* essential oil using ascending mode of hexane:ethyl acetate:nitromethane:methanol 8:2:2:3 was carried out. Fractions were monitored by TLC and like fractions were bulked yielding 25 fractions. Fractions (1µl/ml) were tested for inhibition of human erythrocyte AChE using a modified version of the colorimetric method of Ellman et al (1961). Results are shown in the Figure and indicate that fraction 8 contains active components. The isolation and elucidation of compounds from this fraction is in progress.

This anticholinesterase activity of *S.lavandulaefolia* oil may be relevant to the treatment of AD as an alternative AChE inhibitor.

FIGURE



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54 BETULINIC ACID AND BETULIN AT UP TO 10 μM CONCENTRATION HAVE NO INHIBITORY EFFECTS ON MELAN-A LINE

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Betulinic acid has been identified as a promising melanoma-specific cytotoxic agent (ED_{50} 1-5μg ml^{-1}, approximately 2-5μM) through bioassay-guided fractionation of the extracts of stem bark of *Ziziphus mauritiana* Lam. (Pisha et al 1995). In the present study, the cytotoxic effect of betulinic acid and its alcoholic derivative betulin on melan-a cells, a non-tumorigenic pigmented mouse melanocyte line (Bennett et al 1987) was investigated.

Subconfluent melan-a cells were subcultured and $0.6x10^4$cells/well were inoculated into 96-well plates containing compounds dissolved in DMSO/H_2O. The final concentrations of the tested compounds were 1 and 10μM, with final DMSO concentrations of 0.001 and 0.01% respectively. Incubations were conducted with or without 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA), a phorbol ester capable of stimulating melanocyte proliferation. Plates were incubated for 4 days before they were subjected to SRB assay for cell number (Skehan et al 1990). Absorbance at 550nm of the treated wells was compared with that of untreated wells. One way ANOVA and Dunnett's comparison were employed to determine the significance of any difference (Table 1). Similar results were obtained in a replicate experiment. DMSO at concentrations of 0.01% or less had no significant effect on the cell growth rate (Table 2).

Betulinic acid and betulin showed no significant effect on inhibition of melan-a cell growth either in the presence or absence of TPA. It is thus postulated that betulinic acid is a cytotoxic agent specific to malignant melanoma cells, as it did not have an inhibitory effect on the growth of non-tumorigenic melanocytes.

Table 1. Effect of betulinic acid and betulin on the growth of melan-a cells; cell number expressed as % of control (n = 6)

	betulinic acid		betulin	
	1μM	10μM	1μM	10μM
TPA 0nM[1]	99±3	101±10	103±9	106±9
TPA 20nM[2]	98±8	98±8	108±7	106±3

[1]100% = $0.95x10^4$cells/well; [2]100% = $2.17x10^4$cells/well.

Table 2. Effect of DMSO on melan-a cells (n = 6).

concentration	10%	5%	1%	0.1%	0.01%	.001%
% of control	34±8*	37±2*	74±8*	86±6*	100±7	102±7

* $P<0.05$

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55 THE CLINICAL EVALUATION OF *THUJA OCCIDENTALIS* IN THE TREATMENT OF VERRUCA PEDIS

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The Human Papilloma Virus (HPV), the cause of wart/verruca, represents a group of highly specialised DNA viruses displaying a remarkable host and target-cell specificity. They induce papillomatous proliferation in skin and mucosa via specific differentiation of the genome-harbouring cell. No reproducible method has yet been developed for HPV propagation *in vitro*, which consequently has numerous studies concerning the infectivity in man. This has impeded research into the biological properties of the virus and immunological responses to wart infection (Zur Hausen, 1980).

Verruca pedis is a common condition seen in podiatric and dermatological clinics. Radical therapies are used but can rarely be evaluated successfully, hence not giving positive indications of therapeutic results.

It has been demonstrated that *Thuja occidentalis* possesses anti-viral properties which have been successful on herpes simplex (I) virus (Beuscher, 1986).The aim of this presentation is to evaluate *Thuja* occ. on verruca pedis.

Methanolic extract was obtained by Soxhlet extraction. The crude extract was evaporated to dryness under reduced pressure at 40°C and the dried crude extract was dissolved in 70% methanol and used with chiropody felt cavity pad for the clinical study.

Thirty patients with verruca pedis were randomly selected, according to inclusion and exclusion criteria. Ethical approval was not required as a pharmaceutical preparation in current use was being evaluated. The extract was applied weekly for three weeks. Patients were assessed after one month and again after three months and finally after a further six months.

In the majority of cases, resolution occurred by the time of the first assessment with no recurrence by the time of the final assessment. The findings of the study confirm the presence of anti-viral compounds in *Thuja* occ. and demonstrate that these have an effect on verruca pedis.

Research is now being concentrated on the isolation and purification of compounds present in the *Thuja* extract and the development of a tissue culture system for HPV propagation to facilitate *in vitro* investigation.

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56 EVALUATION OF CRYPTOLEPINE AND SOME ANALOGUES AS LEADS TO SELECTIVE ANTIMALARIAL AGENTS

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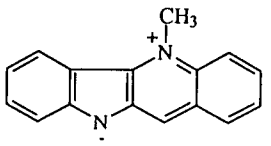
The roots of Cryptolepis sanguinolenta, a West African shrub are used traditionally in the treatment of malaria and contain cryptolepine (I), an indoloquinoline alkaloid which has potent antiplasmodial activity and is also a DNA intercalator (Kirby et al., 1995). In addition cryptolepine, like quinine, inhibits the formation of β-haematin in cell-free systems (Wright et al., 1996). Since DNA intercalating activity is likely to be associated with cytotoxicity, we have prepared a number of cryptolepine analogues in order to determine whether antiplasmodial activity can be separated from DNA-intercalating properties.



I

Isatin was condensed with N-acetyl indoxyl to give quindoline-11-COOH which was then decarboxylated to quindoline. Cryptolepine was prepared by methylation of quindoline with dimethylsulphate and was then nitrated to yield a mono-nitro derivative. In vitro antiplasmodial activity was determined by measuring Plasmodium falciparum (K1) lactate dehydrogenase activity (Makler et al., 1993); DNA intercalation was determined using spectrophotometric and thermal denaturation techniques and inhibition of β-haematin formation was determined using methodology adapted from that of Egan et al. (1994).

The antiplasmodial activity of cryptolepine (IC50 = 0.68 μM) was similar to that for chloroquine (IC50 = 0.44 μM), but quindoline and quindoline-11-COOH were only weakly active (IC50's > 200 μM). Nitro-cryptolepine retained antiplasmodial activity (IC50 > 6.9 μM) although this was 10-fold less potent than that of cryptolepine; however, unlike cryptolepine this compound was not able to intercalate with DNA but did inhibit β-haematin formation. These findings suggest that it may be possible to prepare cryptolepine analogues with greater selectivity against malaria parasites than the parent compound.

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57 ELECTRORHEOLOGICAL BEHAVIOUR OF MICROCRYSTALLINE CELLULOSE IN SILICONE OIL

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First described extensively by Winslow (1949), electrorheology (ER) is the rapid and reversible increase in viscosity exhibited by certain suspensions of solid particles in electrically non-conducting liquids upon application of an electric field. We believe that this phenomenon, which has shown considerable promise in a range of mechanical engineering applications, could have potential pharmaceutical uses in controlled drug delivery or as a means of preventing sedimentation in suspensions.

A controlled stress rheometer (CSL^2 100, TA Instruments) was specially modified to allow the application of an electric field across test fluids. A concentric cylinder arrangement was chosen as the gap is uniform and therefore provides constant field conditions. Although a parallel plate configuration fits these criteria, the shear rate is non-uniform across its diameter. Preliminary studies on microcrystalline cellulose (MCC, Avicel® PH101) in 100 cS silicone oil (Aldrich) have shown that, in the absence of an electric field, these fluids behave in a Newtonian manner ($\sigma = \eta\gamma$, where σ is the shear stress, η is the viscosity and γ is the shear rate). When a continuous dc electric field is applied and the fluid is sheared in a direction perpendicular to the fluid, the relationship between the shear stress, σ, and the shear rate, γ, can be described by the Bingham model ($\sigma = \sigma_y + \eta_B\gamma$, where σ_y is the yield stress and η_B is the Bingham viscosity). The effects of particle size, concentration and moisture content on the development of the yield stress have been investigated together with the influence of an applied electric field. At the concentrations tested, we have shown that, for sieve fractions of MCC < 45 μm, no ER response was produced and the fluid behaved in a Newtonian manner. However, an ER effect was obtained for the sieve fractions 45-63 and 90-125 μm with yield stresses of 0.78 (± 0.03) and 10.90 (± 0.05) respectively. As the suspensions were formulated to contain approximately the same number of particles, we concluded that particle size has a positive effect on the ER response over the range studied. An investigation of the effect of particle concentration on ER effect showed no response in dilute suspensions of ER fluids (below 5 % w/w). However, at higher concentrations, the ER response was found to increase exponentially with increasing concentration.

Most ER fluids require the presence of water in the range 5 to 10 % by weight to activate or enhance performance (Winslow 1949). In general, it is thought that these systems are capable of ionic conductance and that water may either provide a suitable medium for the ionic transportation of charge when an electric field is applied, or directly provide the source of mobile charge carriers to form regions of charge separation. In the present study, decreasing the moisture content in the powder component was found to decrease the ER effect. Increasing the applied voltage from 50 to 750 V/mm resulted in a significant increase in the yield stress. At 50 V/mm, for a 10 % w/w suspension of < 45 μm Avicel PH101, the Bingham yield stress was 0.51 Pa (± 0.22), compared to 23.64 Pa (± 0.38) at 750 V/mm. Microscopic observation of these fluids indicated that particle chains or fibrils formed, spanning the electrode gap on application of an electric field; the fibrils increased in diameter with increasing electric field. The existence of such fibrils is thought to be of paramount importance in the ER phenomenon, as they are presumed to provide the mechanical links which lead to the development of a yield stress.

Winslow, W.M. (1949) J. Appl. Phys. 1137-1140

We thank Hoechst Marion Roussel for funding this studentship (to J.L.D.) and Dr. D. Jordan for his continuing interest in these studies.

58 DETERMINATION OF SURFACE THERMODYNAMIC PROPERTIES OF UNMILLED AND MILLED SALBUTAMOL SULPHATE BY INVERSE GAS CHROMATOGRAPHY (IGC)

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Consistency and uniformity of raw materials for dry powder inhalers (DPIs) are crucial for efficient product performance. However, the complexity of particle formation operations leads to variations in the properties of nominally equivalent batches. Also, as materials are rarely crystallised to provide optimum size, milling by micronisation is common, which can cause uncontrolled degrees of disruption of the crystallised structure with amorphous regions on the particle surface (Buckton et al., 1988). As routine analytical techniques do not identify such phenomena, variations are often revealed during impaired secondary processing or product performance.

In this study, IGC is used to probe any changes in surface properties of salbutamol sulphate on micronisation. IGC involves packing a sample into a silanised glass GC column, fitted to a GC apparatus (Hewlett Packard 5880A) and injecting small amounts of a range of liquid probes with differing polarities. From analysis of probe retention times and volumes, surface thermodynamic properties of the powders can be determined (Ticehurst et al., 1994).

Two unprocessed batches (U1 and U2) and one micronised U1 batch (M1) of salbutamol sulphate (GlaxoWellcome) were analysed using several routine solid state powder measurement techniques including DSC, TGA, Karl-Fischer, specific surface area analysis, helium pycnometry, XRPD and FT-Raman. No significant differences between the samples were observed. However, when analysed by IGC, different surface adsorption properties for the three samples were obtained (see Table 1).

Batch U2 has a more energetic surface than batch U1 both in terms of its dispersive (γ_s^d) and specific ($-\Delta G_A^{SP}$) components of surface free energy. Micronisation has increased γ_s^d implying that the surface of M1 has a more energetic surface for non-polar surface interactions. The specific interaction ($-\Delta G_A^{SP}$) of the acidic polar probe (chloroform) has also increased, in contrast to the amphoteric (acetone) and basic (tetrahydrofuran) polar probes. This suggests stronger basic (electron donor) interactions at the surface of M1, resulting from exposure of more basic functional groups at particle surface, after particle breakage on micronisation.

Batch	γ_s^d (mNm^{-1})	$-\Delta G_A^{SP}$ (kJmol^{-1}) Chloro-form	Acetone	Diethyl-ether	Tetrahy-drofuran
U1	47.01	1.48	9.69	7.27	9.08
U2	65.39	1.61	10.45	8.59	9.72
M1	55.20	1.78	7.79	5.13	6.34

Table 1 - Surface properties of salbutamol sulphate batches U1, U2 and M2.

These results demonstrate that IGC can detect differences in surface thermodynamic properties, a feature of importance for DPIs, particularly as these differences remained undetected by routine testing procedures.

Buckton et al., Int. J. Pharm. (1988) 47: 121-128
Ticehurst et al., Int. J. Pharm. (1994) 111: 241-249

59 VARIATION IN SURFACE THERMODYNAMIC PROPERTIES OF ALTERNATIVE CHIRAL FORMS OF MANNITOL AS DETECTED BY INVERSE GAS CHROMATOGRAPHY (IGC)

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Inverse gas chromatography (IGC) has been successfully used to detect batch to batch variation in pharmaceutical excipients in terms of surface energetic properties (Ticehurst et al., 1994).

This study investigates the use of IGC as a tool to detect changes in surface chemistry associated with enantiomeric and racemic structures of the model compound mannitol, as these may influence secondary processing behaviour and end product characteristics.

Several analytical techniques, including x-ray powder diffraction (XRPD) using a Siemens D5000 x-ray diffractometer (Siemens, Germany) were performed on two samples of mannitol, i.e. A and B (BDH Chemicals Ltd., Poole, UK). XRPD scans were carried out between 2-72° 2θ at a rate of 1° (2θ) min^{-1} by a copper Kα radiation source (λ = 1.542Å). The two XRPD profiles matched those retrieved from the Cambridge Structural data base (Cambridge Crystallographic Data Centre, UK) and theoretical XRPD profiles generated using Cerius^2 molecular modelling software (M.S.I., Cambridge) for the enantiomer mannitol Dβ (sample A) and of the racemate mannitol DL (sample B).

IGC was carried out at 30°C on each sample using a range of probes, with a nitrogen carrier gas flow rate of 10 ml.min^{-1} . For IGC measurements, a suitably calibrated HP5880a gas chromatograph was used (Hewlett Packard, USA) and chromatographic data used to calculate γ_s^d (dispersive component of surface free energy) and ΔG_a^{sp} (specific component of the free energy of adsorption) (see Tables 1 and 2).

Sample	γ_s^d
A	54.28 (0.89)
B	65.9 (0.27)

Table 1 γ_s^d (% error), mJm^{-2} obtained from samples A and B

Probe	Sample A	Sample B
Ethylacetate	5.336 (0.011)	6.342 (0.115)
THF	4.809 (0.001)	5.412 (0.130)
Ethanol	7.390 (0.010)	9.105 (0.124)
1-Propanol	8.532 (0.002)	10.043 (0.105)

Table 2 ΔG_a^{sp} (SD), Kjmol^{-1} for samples A and B

Tables 1 and 2 show significant differences in the γ_s^d and ΔG_a^{sp} values between the samples, with both parameters higher for B than A. Calculated values indicate that sample B, i.e. the DL form, will be more reactive than sample A, i.e. the Dβ form, towards non-polar and polar molecules. The results displayed here illustrate that the thermodynamic surface properties of different optical forms of pharmaceutical powders can be distinguished and quantified by IGC. The consequences of these alternative surface properties on secondary processing are under investigation.

Ticehurst et al. (1994) Int.J.Pharm., 111: 241-249.

60 THE EFFECT OF POLYSORBATES ON THE RHEOLOGICAL PROPERTIES OF MUCUS GEL

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Mucus is a viscoelastic fluid which covers most epithelial surfaces. The rheological properties of mucus are mainly governed by a high molecular weight, strand-like glycoprotein called *'mucin'* and is dependent on the formation of a gel. The mucus layer has been identified as a barrier to drug absorption since it promotes the formation of an unstirred water layer adjacent to epithelial cell membranes. Mucus thinning agents, with the ability to reduce the rheological properties of mucus gels, have been found to enhance the absorption of otherwise poorly absorbable drugs (O'Hagan et al., 1990).

The aim of this study was to investigate the effect of four polysorbate (PS) surfactants including PS 20, 40, 60 and 80 on mucus structure as reflected by their effects on mucus rheology to assess their potential effects on drug absorption.

Purified mucus gel was prepared by the method described by El-Hariri et. al. (1989). Concentrated solutions of the test compounds were mixed with the mucus to give an 8% w/w mucus containing 20 mM of each polysorbate. The gels were incubated at 4°C for 4 h after which time rheological measurements were carried out using an oscillatory shear technique in a Carri-Med controlled stress rheometer. The elastic modulus, G' and the loss modulus, G" were measured using 4 cm parallel plate geometry at 20°C over a frequency range of 0.1 to 10 Hz at an amplitude of four milliradians. The results were expressed as percent changes in the G' and G" of mucus induced by polysorbates at 0.1 and 10 Hz in comparison with values obtained in water as the control (Table 1).

Table 1. Percentage changes in the storage and loss moduli of porcine gastric mucus (PGM) treated with various polysorbate surfactants at 4'C compared to water as the control (n=6).

Test	G' (Pa)		G'' (Pa)	
compound	0.1 Hz	10 Hz	0.1 Hz	10 Hz
PS 20	-35	-21	-25	-13
PS 40	-15	-9	-12	-4
PS 60	-29	-19	-23	-13
PS 80	-35	-23	-28	-16

It was found that the four polysorbates tested decreased both G' and G" of mucus samples significantly ($p<0.05$) relating to the values obtained in water except for G" at 10 Hz of PS40. The mechanism by which surfactants disturb the mucus structure is not fully understood, nonetheless, they could possibly affect the mucus gel properties by causing depletion of the glycoprotein constituents such as non-mucin proteins and mucin associated lipids. It can be concluded that muces structure can be disrupted by polysorbates and since these compounds have shown moderate effects on membranes (Sajadi Tabassi *et. al.*, 1994) they could be used as absorption enhancers of poorly absorbed drugs such as peptides and proteins.

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El-Hariri, L.M. et. al. (1989) J. Pharm. Pharmacol. 41:75P
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61 INFLUENCE OF MOLECULAR WEIGHT AND SUBSTITUTION TYPE ON DYNAMIC VAPOUR SORPTION OF CELLULOSE ETHER POLYMERS

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Non-ionic cellulose ethers are extensively used in hydrophilic matrices. The interaction of water with these polymers plays an important role in hydration, gel formation and subsequently drug release. Thermal analysis of water uptake into polymer discs failed to differentiate between substitution types of cellulose ethers (Ford & Mitchell 1995) but identified some differences in the water distribution within their gels (McCrystal et al 1997). This study investigates the water sorption/desorption properties of cellulose ethers in an attempt to discriminate between their molecular weights and substitution types.

Hydroxypropylmethylcellulose (HPMC; Methocels K100LV, K4M, K15M, K100M, E4M, F4M) and Methylcellulose (Methocel A4M) were obtained from DOW Chemicals, USA. Dry powders (20.83±1.73mg; particle size: 90-125μm) were analysed on a dynamic vapour sorption apparatus (DVS), (Surface Measurement Systems, U.K.). The sorption/desorption of water vapour as a function of relative humidity (0-90% RH) was studied at 25°C. For each sample the mass of moisture taken up (or released) per unit mass of dry powder was plotted against the relative humidity (RH) to produce a moisture sorption (or desorption) isotherm.

All samples showed Type II isotherms (Shaw 1992) irrespective of molecular weight and substitution type. The molecular weight of the polymer had little effect on the moisture sorption/ desorption behaviour of the polymers (Fig 1). On the other hand, substitution level appeared to affect their water sorption/ desorption characteristics (Fig 2). HPMC K4M sorbed more water at all RH values compared to the other three substitution types. This study has highlighted differences between the cellulose ethers which were not previously distinguished (Ford & Mitchell 1995), and may be related to the methoxyl substituent level in these polymers. This hydrophobic group is at its lowest level of percentage substitution in the K-grades of HPMC.

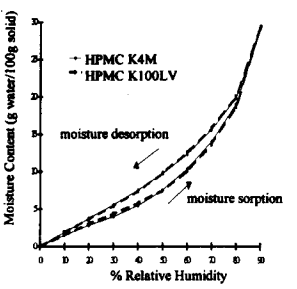

Figure 1: Moisture Sorption/ Desorption profiles for HPMC K4M and HPMC K100LV

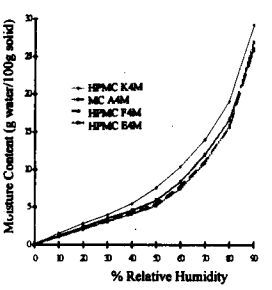

Figure 2: Moisture Sorption profiles for HPMC K4M, E4M, F4M and MC A4M

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62 AMPHIPHILIC POLY(ETHYLENE GLYCOL) REDUCES CATIONIC LIPOSOME ZETA (ξ) POTENTIAL AND ENHANCES RESISTANCE TO PLASMA PROTEIN AND LIPOPROTEIN-INDUCED DESTABILIZATION

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Liposomes formulated with cationic lipids (cationic liposomes) are currently being evaluated for their ability to deliver therapeutic nucleic acids (plasmid gene therapy, antisense oligonucleotides) via the endosomic pathway. Such liposomes are formulated with a cationic lipid for complexation with the polyanionic nucleic acid, and a pH sensitive, low transition temperature lipid or phospholipid which can destabilize the endosomal membrane, thus effecting release of the nucleic acid into the cytosol. A major problem confronting the utilization of cationic liposomes in vivo is their non-specific and potentially destabilizing interaction with anionic molecules in the body. The parenteral administration of cationic liposomes into the bloodstream will result in their interaction with negatively charged plasma lipoproteins and proteins.

The presence of amphiphilic cholesterol-poly(ethylene glycol) (PEG) or phosphatidylethanolamine-PEG in anionic liposomes containing myristic acid has been shown to significantly mask the zeta (ζ) potential in a PEG-chain length-dependent manner that is independent of the lipophilic anchor, and to protect against plasma-induced liposome aggregation [Yoshioka (1991)]. The ability of amphiphilic PEG to reduce liposomal ζ potential is consistent with the movement of the hydrodynamic plane away from the charge-bearing plane of the liposome. The presence of DPPE-PEG_{2000} in cationic liposomes containing DOTAP results in a significant reduction in zeta potential that is associated with increased stability towards serum albumin, IgG and salivary mucin [Phillips & Heydari,(1996)]. In the present study the effect of amphiphilic PEG chain length and concentration on the stability of cationic liposomes formulated with DOTAP and containing plasmid DNA in the presence of lipoproteins and plasma has been evaluated.

Small unilamellar cationic liposomes (100-110 nm diameter) prepared from dioleoylphosphatidylethanolamine (DOPE) and the cationic lipid dioleoyltrimethylammonium propane (DOTAP) (1:1 mol ratio) had a ζ potential of +34 mV. The surface charge of cationic liposomes containing either 10 mol% amphiphilic dipalmitoylphosphatidylethanolamine-poly(ethylene glycol)$_{2000}$ (DPPE-PEG_{2000}) or DPPE-PEG_{5000} was completely masked, with ζ potentials of -1 to -5 mV. Incubation of cationic liposomes with micellar DPPE-PEG_{2000} or DPPE-PEG_{5000} (10-20 mol%) for 30 minutes at 20°C also resulted in complete masking of cationic liposome zeta potential. Human plasma (50% v/v) or purified human lipoproteins (HDL, LDL, VLDL; 1 mg/ml apoprotein) induced the aggregation of cationic liposomes within several minutes: the presence of amphiphilic DPPE-PEG significantly enhanced stability. Although plasmid DNA was readily incorporated in cationic liposomes (efficiency of incorporation 65-75%), the presence of amphiphilic PEG at 10 mol% reduced the efficiency of incorporation (efficiency of incorporation 5-7%). Grafting of amphiphilic PEG to cationic liposome-plasmid DNA complexes resulted in an efficient masking of ζ potential and maintained efficient DNA incorporation. Aggregation of cationic liposomes by plasma or lipoproteins was associated with the liberation of incorporated plasmid DNA, whereas plasmid DNA was not liberated from cationic liposomes containing amphiphilic PEG.

These results clearly show that the incorporation or insertion of amphiphilic PEG in cationic liposomes masks the positive charge of the cationic lipid, and significantly reduces plasma lipoprotein/protein aggregation.

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63 SOLID-STATE CHARACTERISATION OF SPRAY DRIED PROTEIN/SUGAR SYSTEMS

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Debate exists over the exact mechanism by which sugars stabilise proteins in the solid-state. One theory suggests that by maintaining the protein in an amorphous glassy sugar matrix, the physical hindrance encountered by the protein functions to stabilise it (Carpenter et al., 1993). In order to prolong stability, it follows that the nature of the sugar/protein interaction is important as is the maintenance of the sugar in an amorphous form without any recrystallisation. It has been demonstrated that spray dried lactose is produced initially in an amorphous form which readily reverts to a stable crystalline form at relative humidity >52% (Byron et al., 1996). We report the effect of co-spray drying with different proteins on the physical stability of lactose. Preparations of bovine liver catalase (Sigma Chemical Co.) and bovine pancreatic insulin (Sigma Chemical Co.) have been spray dried in the presence of lactose, (feed liquor composition: 4% protein (w/v), 4% lactose (w/v); catalase in potassium phosphate buffer pH 7, 0.05M, insulin in sodium acetate buffer pH 2.4, 0.2M), and then stored over phosphorous pentoxide. The spray dried powders (Buchi 190) were tested for crystallinity by x-ray powder diffraction (Siemens Model D5000) and hygroscopicity by moisture sorption on a moisture balance (CI Electronics, CISORP). While no extensive crystallinity was evident in powders kept over phosphorous pentoxide the insulin/lactose powder underwent significant irreversible re-crystallisations as the relative humidity was increased from 50% to 80% on the moisture balance, but the catalase/lactose powder did not display the same behaviour. This implies that catalase is able to overcome the tendency for spray dried lactose to recrystallise at higher humidities, whereas insulin does not. However, after one week exposure to relative humidity of 75% both the insulin/lactose and catalase/lactose preparations showed significant crystallinity, characteristic of lactose. Thus it appears that the ability of catalase to prevent lactose recrystallisation is lost on prolonged exposure to moisture. This suggests that each protein/sugar system requires individual characterisation to identify an optimal formulation, and to establish the relationship between physical stability and biochemical activity.

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64 DIELECTRIC ANALYSIS OF GLYCERYL MONOOLEATE LIQUID CRYSTALLINE SYSTEMS

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Glyceryl monoolein is a bioadhesive lipid which forms lamellar (L_α), cubic (C), and reversed hexagonal (H_{II}) phases in water, depending on concentration and temperature (Larsson 1989; Geraghty et al 1996). The objective of this investigation was to assess the potential use of dielectric analysis for studying these materials with a view to developing the technique as a means of characterising liquid crystalline systems.

GMOrphic-80 glyceryl monoolein (Eastman Chemical Co.) was used; four gels containing 15%, 20%, 30% and 35% w/w water were prepared. Microscopic observations were performed using an Olympus differential interference contrast (DIC) microscope. Dielectric measurements were carried out using a Novocontrol GmbH spectrometer.

Microscopic examination confirmed that the 15% and 20% water systems were lamellar phase, while the 30 and 35% systems were cubic phase at room temperature. Figure 1 shows real permittivity (ϵ) spectra of the gels at 23 °C. The plots exhibit marked differences between the responses of the lamellar and cubic structures. For the cubic structures, a non-dispersive bulk dielectric response was observed with higher conductance than for the lamellar systems, indicative of the bicontinuous structure of this phase allowing continuous charge movement through the material. This is in contrast to the lamellar phase, whereby the bilayers form a barrier to charge transport.

The data may also be modelled in terms of an equivalent circuit, as shown in Figure 1. This is essentially a modified generalised Maxwell-Wagner equivalent circuit, based on the work of Hill & Pickup (1985). This circuit consists of a fractional power law dispersive capacitance, C_s, representing a layer of lipid formed at the electrode surface, in series with a parallel RC circuit corresponding to the bulk processes (C_b and R_b). A series connection (C_2 and R_2) was employed to model the additional polarisation behaviour seen in the lamellar phase which may be associated with interlamellar water. The circuit was found to yield an excellent fit to the experimental data (continuous curves shown in Figure 1) and the values of selected associated parameters are given in Table 1.

Figure 1: Real permittivity spectra of GMOrphic 80/water systems, with equivalent circuit diagram

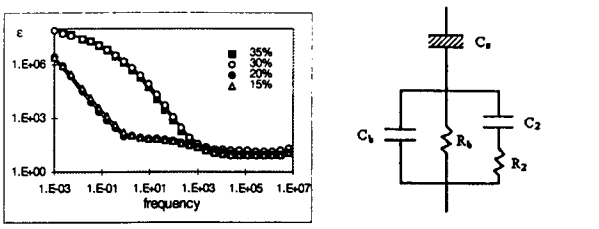

Table 1: Dielectric fitting parameters corresponding to GMOrphic 80/water systems

% w/w water	R_b (Ω)	C_b (pF)	R_2 (Ω)	C_2 (pF)
15	$1.0x10^6$	47.1	$5.3x10^6$	329
20	$1.3x10^6$	45.6	$7.2x10^6$	279
30	$1.1x10^4$	54.8	17.5	17.1
35	$7.0x10^3$	31.5	2.4	31.4

We wish to thank the EPSRC for a postdoctoral fellowship for Dr He and Drs Geraghty and Collett for their considerable contribution.

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Hill, R. M., Pickup, C. (1985) J. Mater. Sci. 2: 4431-4444
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65 OSCILLATORY AND THERMORHEOLOGICAL CHARACTERISATION OF ALGINATE/MUCIN MIXES

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Alginates are block copolymers of mannuronic (M) and guluronic (G) acid which previous studies have indicated may have mucoadhesive properties (e.g. Smart et al 1984). Examination of the rheological synergy between mucin/polymer mixtures may be used as an indicator of the bioadhesive behaviour (Hassan & Gallo 1990), hence the objective of this study was to correlate the chemical composition of a series of sodium alginates to their synergistic interaction with mucin.

A range of sodium alginates (SF60L, 56.2%M; LF120L, 55%M; SF/LF40, 37%M; SF120, 30.6%M; SF200, 31%M; Pronova Biopolymer a.s.) were prepared as 2%w/w solutions. 15%w/w porcine gastric mucin type III (Sigma) was prepared in de-ionised water. The samples were left to equilibrate for 24 hours at 4°C. The viscoelastic properties of the mucin, alginates and mixes of the two at the concentrations given above were assessed using a controlled stress rheometer (Carri-med CSL 500, TA instruments). Frequency scans (0.01 to 10 Hz) were performed at 25°C at a set displacement of 10^4 rad, while temperature scans were performed from 10°C to 90°C and back to 10°C at a rate of 4°C/min. and at a frequency of 1 Hz.

The storage moduli of the five alginate samples alone were very low (max. 269 Pa) and no clear correlation with the mannuronic acid content was observed. Similarly, the storage modulus of 15%w/w mucin alone yielded a low elastic response (Figure 1). However, there was a marked increase in the elastic moduli of the mixed systems compared to mucin alone (representative spectra given in Figure 1). The increase was in the order SF60L>LF120L>SF/LF40 >SF200>SF120. This is in accordance with the content of the mannuronic acid component in the alginate polymer.

Figure 2 shows the effects of heating the mucin and mucin/alginate mixes on the rheological properties. A small increase in elastic modulus was seen for the mucin alone at approximately 80°C, as previously reported by Craig & Tamburic (1997). This increase was considerably more marked for the alginate mixes, with the same rank order seen between the different alginate samples as for the isothermal studies. No such increase was seen for alginate samples alone.

Figure 1: Frequency dependent rheological behaviour of mucin and representative mucin/alginate mixes

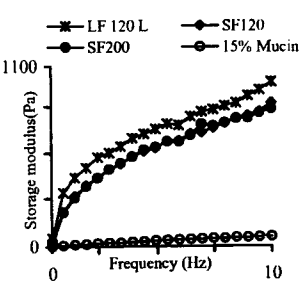
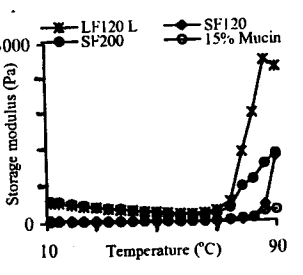


Figure 2: Temperature dependent rheological behaviour of mucin and representative mucin/alginate mixes



In conclusion, both the isothermal frequency scans and the temperature sweeps indicate that there is an interaction between mucin and sodium alginate samples, this synergy being more marked for the high M ratio systems. In addition, the study indicates that frequency sweeps and thermorheological investigations may be used as a means of studying mucin/bioadhesive interactions.

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Smart, J.D., et al. (1984) J.Pharm. Pharmacol. 36: 295-299
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66 THE POTENTIAL OF SURFACE MODIFICATION TO IMPROVE WETTING, DISSOLUTION AND ELECTROSTATIC CHARGE OF A HYDROPHOBIC DRUG

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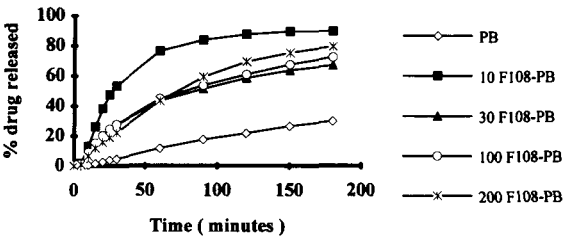
Poor wetting characteristics of hydrophobic drugs may lead to difficulties in dissolution and bioavailability. Further difficulties often arise during powder processing due to accumulation of electrostatic charge that may result in problems with powder and drug content uniformity. Previous work, Nguyen et al (1997), showed the potential of surface modification by adsorption of hydrophilic polymers to improve dissolution and electrostatic charge of a hydrophobic drug. In this work a hydrophobic drug (PB) was equilibrated with Synperonic F108 solutions (10-200 mg l^{-1}) at 25°C for 24 hours. The treated PB was filtered and dried to constant weight prior to dissolution testing in phosphate buffer pH 6.4; electrostatic charge measurement after triboelectrification in a stainless steel mixing vessel agitated for 10 minutes using a Turbula mixer; contact angle (θ°) with water using sessile drop technique. The mean specific charge (nC g^{-1}) of 5 determinations (standard deviation) were measured in a Faraday well connected to an electrometer and are shown in Table 1. The mean percentage drug release from 6 hard gelatin capsules (size 3) containing 50 mg of each sample are shown in Figure 1. PB charged highly negatively whereas F108 had a low positive value . PB samples modified by adsorbed poloxamer showed considerable reduction in charge. Figure 1 shows that all the treated PB samples had better dissolution properties than untreated PB, however the sample prepared at the lowest F108 treatment concentration (10 mg l^{-1}) had the highest dissolution, although this sample had the smallest amount adsorbed and the highest θ°(Table 1).The results confirm the potential of this technique to improve wetting, dissolution and electrostatic charge of hydrophobic materials. Preliminary surface analyse results (XPS) provided an estimate of adsorbed layer thickness which increased with increasing F108 treatment concentration. These results correlate with θ° and specific charge data, however the effect of F108 on dissolution properties requires futher investigation with particular reference to polymer orientation and coverage at the drug surface.

Table 1 : Specific charge values, adsorbed layer thickness and θ°

	A	B	C	θ°	Specific charge nC g^{-1}
F108					+0.39 (0.05)
PB				106	-21.35 (3.39)
F108-PB	10	0.40	0.53	60	-25.96 (0.76)
	30	0.89		51	-14.90 (6.04)
	100	1.40	0.93	40	-3.10 (0.73)
	200	1.44	1.87	32	-1.18 (0.50)

A : Initial concentration of F108 (mg l^{-1})
B : Adsorbed amount of F108 (mg g^{-1})
C : F108 adsorbed layer thickness (nm)

Figure 1 : Dissolution profiles of PB and PB treated with F108



Nguyen, H. et al, (1997), Proc. Pharm. Tech. Conf., 16, 3, 47-53

67 PREDICTING THE MELTING POINT OF HYDRATE INCLUSION COMPOUNDS

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Hydrate inclusion compounds appear to mirror the hydration structures of collagen peptides and other biological macromolecules (Bella et al., 1995). Theories of anaesthesia involving hydrate inclusion compounds (e.g. Pauling, 1961) have been dismissed and may be regarded as controversial, but there is still the exciting prospect that structured water may play a significant biological role in ligand-receptor interactions.

We have constructed a databank of the physicochemical properties of all 120 guest molecules which form clathrate hydrates, and used this to establish correlations between the melting point of the hydrate and properties of the guest molecules. All these molecules form clathrates of either the structure-I or structure-II type, ranging in melting point from -60°C (oxygen) to 22°C (tetrahydrofuran). The guest molecules are mainly non-polar and interact with the water framework primarily by van der Waals forces. The molecules range in size from argon to n-butane. Those with maximum van der Waals diameter < 5.5 Å form structure-I clathrate hydrates and those with diameter in the range 5.5 to 7 Å form structure-II clathrate hydrates (Jeffrey, 1984).

We have established linear relationships between the melting point of the clathrate and the boiling point of the guest molecule for both clathrate hydrate structures. The graph (Fig. 1) below shows the regression line for Equation 1 for structure-II clathrates. Standard errors are shown in brackets. This equation can be used in parallel with molecular modeling studies of the guest inside the host cavities to identify new molecules which will form thermally stable clathrates.

Eqn 1 : **m.p. = 7.27 b.p. - 71.30** $R^2 = 84\%$;
St.Err : (1.17) (1.55) (9.66) F = 1.49;

As Eqn 1 requires that the boiling point of the guest molecule be known, we are now developing similar predictive equations involving only fundamental terms which can be calculated from the structure of the guest molecule. The equation for our current best regression is shown as Eqn 2.

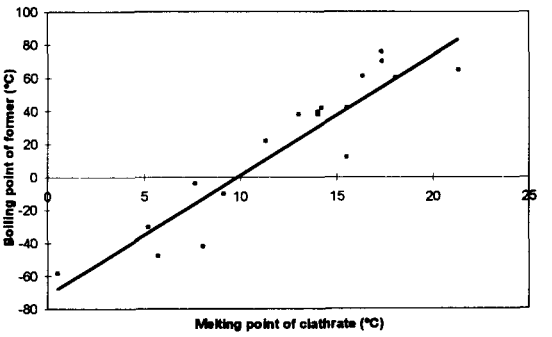

Fig. 1 : Regression on boiling point of guest for structure-II clathrates. (see Eqn 1)

Eqn 2 : m.p. = 0.6 Dipole + 271 Volume - 0.02 M.Mass - 0.6 L.Diam. $R^2 = 62\%$;
St.Err: (1.59) (0.10) (32.54) (0.003) (0.11) F = 3.4;

There are no significant correlations between the parameters, the largest being 0.26 between molecular mass and large diameter of the guest. We are confident that we can refine this system to generate reliable prediction methods using only easily calculable structural terms. Using the results of these statistical analyses and molecular modeling studies, together with knowledge gained from practical work, the aim is to prepare clathrates which are stable at physiological temperature and to evaluate their biological significance.

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68 AN INNOVATIVE TECHNIQUE FOR THE MEASUREMENT OF CONTACT ANGLE OF α-LACTOSE MONOHYDRATE

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BURROWS R. AND TICEHURST M., PFIZER CENTRAL RESEARCH, SANDWICH KENT

The Dynamic Angle Tester (DAT 1100 from Fibro Systems ab) for the contact angle measurement of liquids on solids has been reviewed. It is based on the sessile drop technique but unlike previous sessile drop experiments, does not require compaction of the solid nor presaturation of the sample with the test liquid. The DAT is fully automated and the angles measured are hence not operator dependent. Contact angles of three liquids: bromonaphthalene (Bro), formamide (For) and water on lactose were measured by the DAT and compared with those using the Wilhelmy plate technique (DCA-312). Past studies (Buckton et al 1995), showed that angles measured with the DCA are subject to error due to the surface roughness of the sample. A correction factor of 1.78 in all perimeter terms was used to account for the underestimated contact angles. For the DCA, compacts and powder coated slides were used and for the DAT, powder was brushed onto the flat surfaced holder sprayed with adhesive. The contact angles and standard deviation ( ) are shown in Table 1.

Table 1 shows the contact angles obtained from the DCA and DAT.

	Liquid	DCA/compacts	DCA/stuck-on	DAT/stuck-on
Measured angles	Bro	0 (0)	22 (4)	61 (1)
	For	1 (0)	68 (4)	128 (4)
	Water	41 (2)	89 (4)	110 (3)
Corrected angles	Bro	55	59	-
	For	56	78	-
	Water	65	90	-

The DCA gave low contact angles which after undergoing correction were brought in line with the DAT values hence confirming the relevance of correcting contact angles obtained from the DCA. It is unclear why the contact angle of formamide on lactose measured by the DAT is significantly higher than the corresponding corrected DCA values. The surface energy data (shown in Table 2) was determined by the geometric-mean equation (Fowkes 1964) using the corrected contact angles; bromonaphthalene as the apolar liquid, formamide and water as polar liquids.

Table 2 shows the surface energy data of lactose using bromonaphthalene/formamide and bromonaphthalene/water.

Technique/Sample	Liquid	γ^d (mJm^{-2})	γ^p (mJm^{-2})
DCA/ compacts	For	27	8
	Water	27	15
DCA/ stuck-on	For	25	1
powder	Water	25	3
DAT/ stuck-on	For	25	20
powder	Water	25	0

The values for the dispersive surface energies (γ^d) are essentially identical for all techniques, due to the similarity of the contact angle values produced with bromonaphthalene. The polar surface energy terms (γ^p) however are variable due to substantial differences in measured contact angles for the polar liquids in the three techniques. The differences in measured values arise from artifacts in the experiment, such as the fact that the sessile drop will be placed on a small region of surface which will be rough and potentially composed of both lactose and adhesive; these effects can maximise hysteresis. The artifacts for the Wilhelmy plate approach have been discussed elsewhere (Buckton et al 1995). In conclusion, numerous techniques are available which each give precise contact angle measurements for powders, but a true accurate assessment remains elusive.

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69 MEASUREMENT OF ALTERATIONS IN SURFACE ENERGY CHARACTERISTICS DUE TO DIFFERENT DRYING PROCESSES

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The physicochemical analysis of powders has become very important in monitoring surface energy changes due to differences in processing between batches of the same formulation. A poorly soluble drug, Saquinavir (Roche - 0.22g/100ml in water) was subjected to three different drying processes: 1) A dry batch heated to 160°C in a tray oven for 2 hours. 2) Wetted thoroughly and dried in a tray oven at 160°C for 2 hours. 3) Wetted thoroughly and dried in a vacuum oven at 80°C at 200mBar for 4 hours. 30g of each batch were prepared and wetting was achieved using 90mls of distilled water.

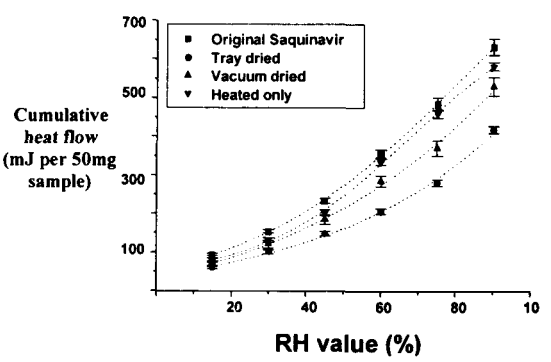
Two methods of surface analysis, inverse gas chromatography (IGC) and microcalorimetry, were used. The IGC, at infinite dilution, gave similar non-polar (γ_S^D) and basic (K_D) components of surface energy between batches and the original sample, but the acidic nature (K_A) of the powder was found to have altered most noticeably with batches that were treated primarily with water.

Table 1. IGC surface energy results.

	γ_S^D	K_A	K_D
Original Saquinavir	44.0	0.0353	0.193
Heated only	37.1	0.0278	0.207
Tray dried	38.7	0.0217	0.209
Vacuum dried	38.5	0.0196	0.208

The microcalorimetry (fig.1) was also able to readily distinguish between the batches, each having different adsorption isotherms. Since there was little alteration in the batches surface area using BET liquid nitrogen analysis, any changes in the heat flow are due to changes in the surface energy of the powder.

Figure 1. Microcalorimetric adsorption isotherms.



In conclusion, it has been shown that different drying processes are able to influence the surface energy profile of a given drug quite markedly. In this case, Saquinavir's low solubility (<0.7%) ensures that only a negligible amount dissolves and recrystallises, thus the reason for this change in surface nature requires further investigation. Both the methods used to analyse the surface chemistry have been sensitive enough to be able to distinguish between the four samples tested. It is clear that one must be able to appreciate, measure and, if possible, control the surface energy throughout the various development stages of a drug.

70 THE INFLUENCE OF HEATING ON WATER LOSS (DRYING) OF COLLAPSED AMORPHOUS LACTOSE

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Collapse phenomena in amorphous systems have been described as diffusion-controlled consequences of a structural relaxation, and the plasticising effect of water on Tg is accepted as being a critical influencing factor (Levine and Slade, 1987). The different forms of water present in amorphous lactose samples which were collapsed to different extents and the ease of loss of water on drying were investigated in this study.

Samples of amorphous lactose were exposed to 50%RH (after drying at 0%RH / 25° C for 4hours) in an automated humidity controlled microbalance system (DVS , Surface Measurement Systems Ltd.) for varying lengths of time in order to induce varying degrees of collapse, see Buckton and Darcy (1996). All the samples were amorphous on removal from the 50%RH environment. The samples were then investigated using differential scanning calorimetry using ca 3mg and scanning from 25-250°C and thermogravimetric analysis using similar conditions. Typical responses for a collapsed sample are shown in fig.1. The DSC exotherm at 70°C was shown to be recrystallisation of the collapsed material (uncollapsed amorphous lactose recrystallises at 187°C). Multiple endotherms were observed between 100°C and 150°C. Different proportions of α and β lactose were produced depending on the extent of collapse of the sample before introducing it to the DSC. This indicates an effect of the extent of collapse on the crystal form obtained on recrystallisation. TGA revealed three distinct regions of water loss for the collapsed samples. One of these was the loss of hydrate water at 150°C, and the two other peaks at around 80°C and 110°C, are thought to be the loss of sorbed water on recrystallisation of the collapsed sample. The magnitude of these peaks decreased and the monohydrate peak increased as the extent of collapse increased.

Water which is trapped in the collapsed material is only released if recrystallisation is induced, which happened here on heating the sample. This water will not be released during "loss on drying" experiments unless sufficiently high temperatures are reached. Thus, samples which appear to be dry may contain substantial amounts of water in collapsed regions, which can subsequently be released into the product, risking spoilage.

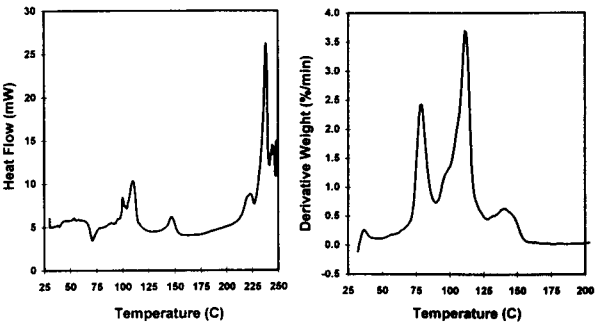

Fig.1. Typical DSC and TGA responses for collapsed amorphous lactose.

Levine H. and Slade L., (1987) In Franks,F.(Ed.), Water Science Reviews, Vol.3, Cambridge University Press, pp.79-185.
Buckton G. and Darcy P. (1996) Int.J.Pharm., 136:141-146.

71 VARIABLE TEMPERATURE FT-RAMAN SPECTROSCOPIC STUDIES OF α,α-TREHALOSE

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α,α-Trehalose is a non-reducing disaccharide increasingly used in pharmaceutical formulations. Since trehalose can be obtained in a variety of forms-anhydrous amorphous, anhydrous crystalline, crystalline dihydrate, glass, an understanding of the environmental and experimental conditions required to obtain and maintain these different forms is important.

DSC studies of thermal behaviour in the crystalline dihydrate (Shafizadeh and Susott, 1973) showed that an open system facilitated free evaporation of crystalline water producing broad dehydration endotherms and an amorphous glass which liquefies, recrystallises and melts at 215°C. A closed system gave a sharp dehydration with subsequent formation of anhydrous crystals melting at 215°C.

In the present study variable temperature FT-Raman spectroscopy (FRA 106 module, IFS 66 optics bench, Bruker) was used to follow the conversion between the different forms of trehalose and to characterise the structural rearrangements of the reported thermal events. Raman spectroscopy is particularly valuable as it is sensitive to molecular conformational changes and spectra are not masked by a strong water signal which is a weak Raman scatterer.

Spectra were recorded from samples mounted in a heating chamber accessory and were acquired at 10°C intervals from 40 to 180°C (500 scans, 4cm^{-1} resolution). Each temperature gave spectral differences indicating continuous molecular rearrangement. Representative spectra obtained at 40 and 90°C, corresponding to the reference spectra of the crystalline dihydrate and anhydrous amorphous forms respectively (Figure 2), and 160°C, believed to be an anhydrous crystalline form, over the wavenumber range 1500-1300cm^{-1} are presented in Figure 1.

Clear differences are apparent with the spectra obtained at 40 and 160°C being characteristic of crystalline forms as peaks are sharp and clearly defined. The spectrum recorded at 90 °C contains broad and poorly defined features, characteristic of an amorphous material.

This study has shown that interconversion between the different forms of trehalose, which can be monitored in situ by FT-Raman spectroscopy, is a continuous change. Findings have implications when defining processing conditions for trehalose formulations.

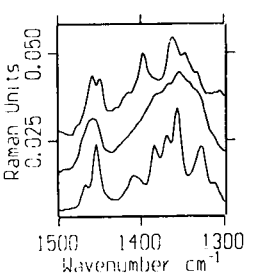

Figure 1 FT-Raman spectra of crystalline trehalose dihydrate acquired at elevated temperatures. Bottom to top: 40, 90 and 160 °C.

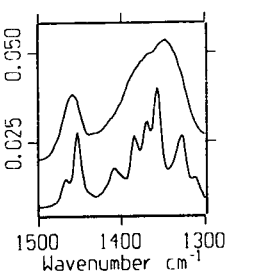

Figure 2 Reference FT-Raman spectra of trehalose. Crystalline dihydrate (bottom) and anhydrous amorphous (top).

Shafizadeh, F. and Susott, R.A., (1973) J. Org. Chem. 38 (21) 3710-3715.

72 ABSORPTION OF POLOXAMER ON POLYSTYRENE SPHERES IN RELATION TO ELECTROSTATIC PROPERTIES

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Electrostatic charging of powders can be altered by the physical adsorption of polymers onto the surface of the particles (Cassidy et al, 1996, Cassidy and Rowley, 1997a).The resultant specific charge of modified particles appears to be adsorbate type and concentration dependent. In addition, the molecular weight of polymer (poloxamer) influences the charge reduction phenomenon (Cassidy and Rowley, 1997b). Investigation of the factors that influence the amount, extent and mechanism of polymer uptake on particles will improve the understanding of the charge reduction properties. An investigation of the adsorption of block copolymers of the PEO-PPO-PEO type onto a model particulate system, polystyrene spheres (PS, mean diameter approximately 50μm) has been undertaken. The copolymers, poloxamers (PLX) employed were Synperonics (ICI) L62, L64 and F68 with HLB values of 7, 15 and 29 respectively. PS was equilibrated in aqueous solutions of PLX of known concentration, separated from solution and allowed to dry to constant weight prior to charging studies. The supernatants were analysed for PLX by UV spectroscopy using a standardised iodine complexation technique, and the amount adsorbed calculated by a solution depletion method based on the difference between the initial and final (equilibrium) copolymer concentrations. The propensity for electrostatic charge accumulation of PS and those modified with PLX following triboelectrification in a stainless steel cyclone (Cassidy and Rowley, 1997a) is examined. The mean specific charge (Q/M) and σ_{n-1} for five representatives of each sample treated with four concentrations of L62 is presented in Table 1 together with the amounts adsorbed for this polymer. The uptake of PLX is the mean of three values equilibrated with the same initial concentration of L62.

Table 1 Specific charge of PS and L62 modified PS

Sample	Q/M (nC g^{-1})	Amount Adsorbed (mole/g x10^{-7} PS)
PS (50μm)	-28.9(5.4)	
PS+L62 10mg l^{-1}	-31.0(3.9)	0.7(0.1)
PS+L62 30mg l^{-1}	-37.5(10.6)	1.3(0.5)
PS+L62 100mg l^{-1}	-22.8(9.1)	2.5(1.1)
PS+L62 200mg l^{-1}	-18.9(3.5)	2.8(0.5)

PS charged electronegatively against a stainless steel contact surface. There was no significant reduction in magnitude of specific charge of PS treated with 10, 30 or 100mg l^{-1} L62. However, the charge reduction of PS modified with 200mg l^{-1} L62 was significant ($p<0.05$, comparing mean values by t-test).The amount adsorbed in mole g^{-1} of PS is greater for L62 than other PLX (isotherms not shown). This increase in polymer uptake may be a result of a decrease in PEO chain length and therefore lower HLB for this polymer when comparing with PLX with similar PPO content. The inability of the copolymer to significantly reduce electrostatic charge is contrary to previous findings where smaller amounts of other PLX adsorbed onto PS have produced particles with very low specific charge (Cassidy and Rowley, 1997b). Comparison of the adsorption and electrostatic charging results of PS modified with L64 and F68, in combination with surface analysis work will help to elucidate if indeed the length of PEO chain affects the adsorption process and hence alteration of charging properties.

Cassidy,O. et al (1996)Eur. J. Pharm. Sci. 4: S65
Cassidy,O. and Rowley G.(1997a)Proc. Pharm. Tech. Conf.16:5-11
Cassidy,O. and Rowley G.(1997b)Proc. Pharm. Tech. Conf.16:29-35

73 A RHEOLOGICAL EVALUATION OF THE MUCOADHESIVE/MUCUS INTERACTION: THE EFFECT OF MUCOADHESIVE TYPE

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The ability of mucoadhesive polymers to produce a large increase in the resistance to deformation when incorporated into a mucus gel, relative to when the mucus gel and test materials are evaluated separately at the same concentration, has been reported in several previous studies (e.g. Hassan & Gallo 1990, Mortazavi et al 1992).

It has been proposed that this phenomenon, termed rheological synergism, can be used as a measure of the strength of the mucoadhesive interaction. In this investigation rheological synergism was investigated for 16 putative mucoadhesive materials by dynamic oscillatory rheology.

Fresh pig stomachs were scraped, and the mucus gel obtained homogenised using the procedure described by Madsen et al (1996). 1.5g samples of mucus were mixed with 1.5g of the test polymer gel using a concentration found in preliminary studies to give a pronounced synergy effect. The mix was then adjusted to pH 6.2 and made to 4.5 g with water. Samples of the test polymer gel and mucus alone were diluted to the same concentration as that present in the mix and evaluated. All the samples were allowed to equilibrate at 4°C overnight then at 15°C for 5 min prior to testing in a Carri-Med CSL 100 Rheometer using a 4 cm parallel plate and a 0.5 mm gap. After determining the linear viscoelastic region in a torque sweep at 1 Hz, the rheological behaviour was evaluated using a frequency sweep between 10-0.1Hz. Changes in the mean storage modulus (G'), loss modulus (G''), and loss tangent (tan δ) were found and the rheological synergism (ΔG' and ΔG'' values, the difference between the moduli of the mix and the components of the mix evaluated separately at the same concentration) determined. The 'relative' rheological synergy values, the ΔG' and ΔG'' values divided by the sum of the moduli for the individual components, was also calculated.

Rheological synergism was evident for a range of materials with known mucoadhesive properties giving mechanical spectra between that of cross-linked and physically entangled systems. This effect was most marked with materials known to be of high mucoadhesive strength, such as the poly(acrylic acid) based polymers and carrageenans, with the exception only of sodium carboxymethylcellulose and a high molecular weight poly(ethylene oxide). The rank order of materials differed when rheological synergy and 'relative' rheological synergy values were calculated, as some materials when tested alone were found to be strongly viscoelastic, with large moduli.

It was concluded that macromolecules possessing numerous hydrogen bond forming groups and an open expanded network in the test environment gave pronounced rheological synergism, and the relevance of this to mucoadhesion studies will be investigated in further work. This study also confirmed the advantages of dynamic oscillatory rheology over simple viscosity measurement in the study of these systems.

Hassan, E.E., Gallo, J.M. (1990) Pharm. Res. 7: 491-495
Madsen, F., et al (1996) Pharm. Sci. 2: 1-4
Mortazavi, .S.A. et al (1992) Int. J. Pharm. 83: 221-225

74 LECITHIN-BASED NON-AQUEOUS POLAR SOLVENT MICROEMULSIONS

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Microemulsions (ie oil-water dispersions, stabilised by a surfactant and possibly a cosurfactant) have been widely investigated for a range of applications, most recently because of their ability to incorporate drugs of varying properties into a one phase system. Although much emphasis has been placed on establishing the drug delivery potential of oil and water-based microemulsions, little work has examined non-aqueous solvent-based systems, yet such microemulsions would have the advantage of incorporating drugs that would not ordinarily be stable or soluble in aqueous-based systems.

Pseudo-ternary phase diagrams have been constructed for systems comprising non-aqueous polar solvents-lecithin-*n*-butanol-isopropylmyristate (IPM) at a lecithin:*n*-butanol mixing weight ratio of 1:1. The polar, non-aqueous solvents used were ethylene glycol, propylene glycol, triethylene glycol, and tetraethylene glycol. As lecithin (Ovothin 200; Lucas Meyer) when used as a sole surfactant does not form microemulsions over a sufficiently wide range of compositions, butanol was added as a cosurfactant to facilitate this process. The resultant pseudo-ternary phase diagrams all showed a large one phase region stretching from the oil-rich to the water-rich portion with a two phase region at low surfactant concentrations. This result is in marked contrast to the corresponding aqueous-based systems in which a much smaller microemulsion region is located in the oil-rich part of the phase diagram (Aboofazeli and Lawrence, 1993).

Photon correlation spectroscopy studies (PCS) performed using a Malvern 4700c series laser light scatterer at a surfactant:cosurfactant concentration of 25%w/w in the oil-rich portion of the phase diagram did not reveal the presence of microemulsion droplets in any of the systems investigated, while corresponding studies in the water-rich region of the phase diagram, demonstrated the presence of microemulsion droplets only in systems containing ethylene glycol and propylene glycol (Table). This 'apparent' absence of microemulsion droplets may be due either to the presence of a cosolvent system, or more likely, to an optical (or contrast) matching phenomenon in which the microemulsion droplets appear invisible due to their possession of a refractive index very similar to that of the continuous phase. This was substantiated by mathematical modelling of the light scattering data. Although further experiments (eg neutron scattering or NMR self-diffusion measurements) are required to unambiguously confirm the hypothesis, it is the first time such a phenomenon has thought to have been observed in the polar solvent-rich portion of the phase diagram.

Table PCS results for non-aqueous polar solvent microemulsions

	Droplet size (nm) (%w/w oil)					
Polar solvent	0	1	2	3	4	8
Ethylene glycol	4.7	5.9	6.5	8.4	10.0	—
Propylene glycol	6.5	—	6.5	—	N/S	N/S

N/S no particles observed

Aboofazeli, R., Lawrence, M.J. (1993) Int. J. Pharm. 93, 161-175.

75 MEMBRANE DISRUPTION INDUCED BY PHOSPHOLIPID AND SURFACTANT MIXED AGGREGATES: THE INFLUENCE OF PARTICLE SIZE AND AGGREGATE TYPE

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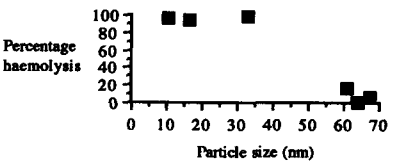
Mixed micelles of lipids and surfactants have been used as drug delivery systems for insoluble drugs. Taxol, for example, has been formulated using such a system for intravenous use (Alkan-Onyuksel et al 1994). If the drug is administered intravenously within mixed micelles then, upon dilution with the blood, the surfactant and phospholipid micellar system is transformed into vesicles containing the solubilised drug. Few data exist correlating the physical characteristics of the mixed aggregate with potential toxic effects of mixed aggregates on biological membranes. The combination of different ratios of egg phosphatidylcholine (egg PC) and surfactant results in mixed aggregates of various sizes, depending on whether they are mixed micellar, vesicular or some intermediate structure (Vinson et al 1989). Thus the aim of this study was to determine the size of mixed aggregates of egg PC and the nonionic surfactant, Brij 96, by a light scattering technique using photon correlation spectroscopy (PCS). The haemolytic effects of the mixed aggregates were determined and compared to their physical characteristics.

Mixed aggregates of egg PC : Brij 96 were prepared by hydrating films of egg PC, prepared by rotary evaporation, with solutions of Brij 96 in McIlvaine's buffer at the egg PC : Brij 96 molar ratios of 0.0, 0.1, 0.2, 0.6, 1.0 and 2.0 : 1.0. The mixtures were sonicated continuously for 90 min using a Soniprobe (fitted with a 1/18" microtip) and centrifuged at 6,200 g, at 4°C, for 60 min. The supernatant was then removed for examination by PCS at 25°C, at an angle of 90° using a Malvern 4700c light scattering instrument equipped with a 75 mW Argon laser beam (λ = 488 nm). The external phase of the sample was McIlvaine's buffer (viscosity: 0.891, refractive index: 1.33). The toxicity of the samples to erythrocytes was examined by mixing aliquots (0.2 mL) of sample with 0.2 mL of washed erythrocytes and incubating for 30 min at 20°C before the unlysed erythrocytes were removed by centrifuging for 15 s in a microcentrifuge. Aliquots (0.2 mL) of the supernatants were added to 3 mL Drabkin's reagent and the solution analysed spectrophotometrically at 540 nm. The percentage haemolysis induced by the samples was calculated with reference to a positive control. Transmission electron micrographs were taken of the samples after negative staining with 1% v/v ammonium molybdenum.

Fig. Percentage haemolysis induced by sonicated mixed aggregates of egg PC : 1.0 mM Brij 96 (mean ± SD, n=9) as a function of the particle size (90°) of the mixed aggregates.



Brij 96 alone (10.5 nm) and the 0.1 and 0.2 : 1.0 egg PC : Brij 96 mixed aggregates, with sizes of 33.2 nm or less, were 100% haemolytic (Fig). A minimum ratio of 0.6 egg PC : 1.0 Brij 96 was required to reduce the haemolytic activity of the surfactant to less than 20% of a postitve control. The aggregates with reduced toxicity had a particle size of 61.0 nm or above. Electron micrographs of the samples showed that the reduction of toxicity at 0.6 : 1.0 ratio, and the increase in size, correlated with a change in the type of mixed aggregate from a micellar to a lamellar system. One interpretation of the data is that within the lamellar systems less free surfactant is available for incorporation into the erythrocyte membrane.

Alkan-Onyuksel, H. et al (1994) Pharm. Res., 11: 206-212
Vinson, P.K. et al (1989) Biophys. J., 56: 669-681

76 THE EFFECT OF LUBRICANTS ON THE PROCESS OF CAPSULE FILLING

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Inclusion of a lubricant into the capsule powder formulation is essential for reproducible capsule filling, especially for high speed automatic filling machines. However, the amount and type of lubricant needed differs according to the host powder used. Avicel PH101 (Av.101) and PH102 (Av.102), maize starch (M. St.), starch 1500 (St.1500), lactose fine (F.Lact.) and coarse (C.Lact.) grades 230 and 80 mesh respectively; were used as host powders in this study.

An annular shear cell (Bristol, Technigraphic) was used to measure the effect of two lubricants, magnesium stearate (Mg St.) and glyceryl monostearate (GMS), on the flow properties of host powders. Flow factor (FF), angle of internal friction (θ) and angle of wall friction (ϕ) were measured as described in (Tan and Newton, 1990 a,b). Carr compressibility index (CCI) was measured using a Tap Density volumeter (Copley). The powder blends were filled in an intermittent automatic capsule filling machine (Zanasi AZ5). The coefficient of variation (CV) was calculated from the weights of 20 capsules.

Both lubricants had different effects on the powder flow properties as measured by both FF and CCI. However, CCI only changed slightly with the addition of lubricants. Both angles (θ and ϕ) decreased rapidly after the addition of 0.5% Mg St. and 1.0% GMS then ceased to decrease with higher concentrations of both. Mg St. decreased both angles more than GMS.

The addition of lubricants also affected the weight uniformity of the capsules. Av.101 and Av.102, C.Lact. and St.1500 were less sensitive to the changes in the concentration of both lubricants and produced more uniform capsule fills. Blends with CCI >32% gave high CV when filled into capsules while powders with CCI <30% showed good capsule filling performance (figure 1); this value could be set as an optimum CCI value for reproducible capsule filling. However, FF showed poor correlation with CV (figure 2) and did not give an indication of the capsule filling performance.

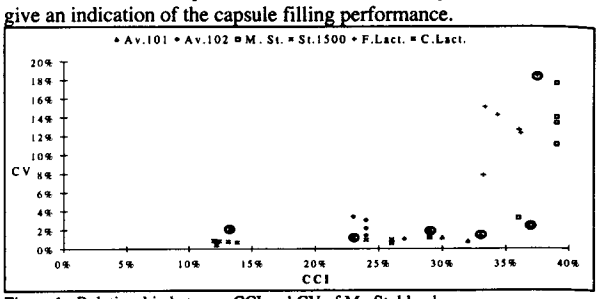

Figure 1: Relationship between CCI and CV of Mg St. blends.

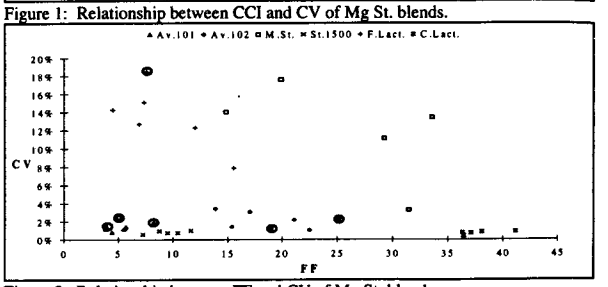

Figure 2: Relationship between FF and CV of Mg St. blends.

** Circled symbols indicate unlubricated blends.

Tan, S.B. and Newton, J.M., Int.J.Pharm., 61: 145-155, 1990a.
Tan, S.B. and Newton, J.M., Int.J.Pharm., 64: 227-234, 1990b.

77 THE EFFECT OF POLYHYDRIC ALCOHOLS AND SUGARS ON THE HEAT DENATURATION OF LYOPHILISED HEN EGG LYSOZYME

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Sugars have been demonstrated to protect proteins against loss of stability during drying and to inhibit heat coagulation (Smith et al, 1979). Recently sugars and polyols have been used routinely with lyophilised proteins as cryo and lyoprotectants (Borchardt et al, 1996). In this study, the effect of various sugars and polyhydric alcohols on the heat denaturation of previously lyophilised hen egg lysozyme (HEL) is investigated. A high sensitivity differential scanning calorimeter (Microcal MCS-DSC, Microcal Inc. USA) was used to measure the denaturation temperature (transition mid-point, Tm) and transition enthalpy (ΔH), and thus obtain a quantitative estimate of the degree of stabilisation afforded by the additives. All additives were prepared to a concentration of 20%w/v. HEL (3mg ml^{-1}) solutions alone and containing additives were heated at 1°C min^{-1} in 100mM acetate (pH 3.8) between 20 and 120°C followed by cooling to 25°C and reheating at the same rate. The results obtained are listed in Table 1. Typical Tm and ΔH values for lysozyme alone were obtained (Cooper, 1992). All the additives investigated with the exception of ethylene glycol stabilised the heat denaturation of HEL as observed by an increase in the Tm. In each case the magnitude of the stabilising effect (ΔTm) was dependent on the nature of the sugar or polyol. For the sugars examined ΔTm ranged from +2.6°C (maltose) to +4.4°C (glucose) and a mean ΔH of 478.8 kJ mol^{-1}. Addition of a polyol led to a rise in the Tm ranging between +2.4°C (glycerol) to +4.6°C (sorbitol) thus indicating that both the sugars and polyhydric alcohols investigated increased the thermostability of HEL in solution. The reversibility of the stabilising effects observed was also examined by further cooling and reheating the samples. In each case the resultant transition profile was greatly affected and ΔH was reduced by up to 25% as compared to 5% with HEL alone. Thus the stabilising effect of the additives was dependent on the protein maintaining its folded conformation. Increasing the protein concentration resulted in little or no changes in Tm and reversibility, confirming HEL is monomeric in nature and undergoes a two-stage unfolding transition. Such data provide useful knowledge when selecting cryo or lyoprotectants for protein formulations.

Additive (20%w/v)	Tm (°C)	ΔH (kJ mol^{-1})
HEL alone	75.8	479.1
HEL + Maltose	78.4	543.9
HEL + Fructose	79.0	369.4
HEL + Galactose	79.6	581.6
HEL + Glucose	80.2	493.7
HEL + Maltitol	79.4	497.9
HEL + Adonitol	79.6	531.4
HEL + Glycerol	78.2	615.0
HEL + Ethylene glycol	64.1	543.9
HEL + Xylitol	79.5	514.6
HEL + Sorbitol	80.4	581.6

Table 1. Tm and ΔH data for HEL and HEL-additive systems

Borchardt, R.T. et al. (1996) J. Pharm. Sci. 85 : 873-877.
Cooper, A. (1992) Am. Chem. Soc. 114 : 9208-9209.
Smith, M.B. et al. (1979) Am. Chem. Soc. 18 : 5191-5196.

78 MECHANISMS OF DRUG RELEASE FROM THIXOTROPIC TRIGLYCERIDE GEL SYSTEMS, FOR FILLING INTO HARD GELATIN CAPSULES

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Liquid fill formulations for hard gelatin capsules can be based on simple Newtonian liquids or more complex thermosoftened or thixotropic systems. Since the reintroduction of liquid filling, thixotropic systems have not been investigated as extensively as thermosoftened systems, in terms of material characterization, drug release and release mechanisms. Of the limited work on release mechanisms from thixotropic systems Walters et al (1994) concluded that release from propantheline-Miglyol 829-Aerosil 200 gel systems was a diffusion process with some surface erosion. The aim of this work was to investigate the mechanisms of release from a range of drug-triglyceride-silicon dioxide gel formulations.

The actives, with different aqueous solubility, were isoniazid > sulphaguanidine > tolbutamide (Sigma, UK) and the excipients used for gel preparation were the triglycerides: Miglyol 810, 818, 829 (Huls, UK) and silicon dioxide: Aerosil 200 (Degussa, UK).

The drug-triglyceride-silicon dioxide gel systems were prepared and filled into hard gelatin capsules using the standardized mechanical process of Ellison et al (1996). After 24 hours storage at room temperature six capsules were tested using the B.P. beaker/basket dissolution apparatus with 1000 ml distilled water at 37°C and agitation rates of 60 rpm, 120 rpm and 180 rpm. Dissolution profiles were obtained over 150 minutes. Penetration rate of aqueous media was determined using a calibrated cylindrical tube, sealed at one end, containing the drug-gel formulation which was equilibrated at 37°C. An aqueous solution of methylene blue was placed on the gel surface and the time for liquid penetration through the gel was determined.

At 60 rpm, the drug release profiles for all the active formulations demonstrated an initial linear burst effect, for 20 to 40 minutes followed by a dramatic decrease to a slower linear release rate. The time for the initial burst effect decreased and release rate increased with an increase in agitation rate for all the formulations, however there was little effect of agitation rate on the slower rate phase for the isoniazid formulations. The slower rate phase increased slightly for the sulphaguanidine formulations at 120 rpm and for the tolbutamide formulations at both 120 rpm and 180 rpm, indicating an effect of drug solubility on diffusion from the gel. Liquid penetration results showed that aqueous media penetration rate was also related to drug aqueous solubility i.e. isoniazid > sulphaguanidine > tolbutamide. The release kinetics of the slower release phase, for all formulations showed a good correlation to linearity (>0.99) for the Higuchi plot (1961) and showed a reasonable correlation for first order kinetics (>0.9).

The mechanism of release from triglyceride gel systems is a two stage process. An increase in agitation rate increased the initial release rate, which provided evidence for an erosion controlled process with drug release from the gel surface. The slower release phase is diffusion controlled by the non-rigid gel matrix created by the hydrophilic network of silicon dioxide in the gel. The effect of agitation on the slower phase rate was minimal, and thus provides evidence for control by diffusion through the gel rather than transfer across a stationary film surrounding the gel.

Walters, P.A. (1994), Ph.D thesis, University of Sunderland
Ellison, M.J.H. et al (1996), Proc. Pharm. Tech. Conf., **15**(2), 102-110
Higuchi, W.I. (1961), J. Pharm. Sci., **50**, 874-876

79 A STRUCTURAL INVESTIGATION OF THE PHASE TRANSITIONS OF CAFFEINE

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Crystalline modification of pharmaceutical powders is important when considering the formulation of dosage forms. Hydrous caffeine dehydrates in ambient conditions to an anhydrous β-phase which converts at high temperature to a second anhydrous α-phase. Consequently, discrimination of the various solid state phases is required. These transformations have been investigated using FT-Raman spectroscopy and x-ray crystal structure analysis.

Beta-phase caffeine (Lot 79F0469) was obtained from Sigma Chemicals Ltd., (purity = 99.6%). Crystals of caffeine hydrate were obtained by slow recrystallisation from water and stored in a sealed vessel at 75%RH in the presence of an NaCl saturated solution at room temperature. Alpha phase material was formed by sublimation in a sealed tube.

Single crystal diffraction data were collected with a Stoe STADI4 four circle diffractometer and the x-ray powder data (XRPD) collected with Stoe STADIP and Siemens D5000 diffractometers. Structure determination was accomplished with SHELXS-86 and SHELXL-93 crystallographic software. Stoe STADIP Rietveld software was used for restricted refinement of the lattice parameters of the hydrate and beta anhydrous phase, and water occupancy of the hydrate phase.

Transition in the molecular environment of caffeine samples during dehydration was detected using FT-Raman spectroscopy with a fitted environmental chamber at 30, 35 and 40°C. Measurements of 200 scans were obtained at 5 minute intervals over the wavenumber range 50-3500 cm^{-1} at a spectral resolution of 4 cm^{-1}. The accumulated spectra were recorded using a Bruker IFS66 IR bench with an FRA 106 FT Raman module attachment using Nd:YAG excitation of 1064nm.

Single crystal analysis of caffeine hydrate confirms, more accurately, the basic structure reported by Sutor (1958a). The hydrate O1 to purine N9 hydrogen bond distance is 2.816(4) Å and the degree of occupancy of the hydrate site is 0.84(1), in contrast to 0.91(11) determined by Rietveld refinement. The instability of the hydrate arises from the weakness of its intermolecular bonds in contrast to the strong intermolecular H-bonds of its analogues theophylline monohydrate (Sutor 1958b) and 1,7,9-trimethyl-2,6-purinedione monohydrate (Parvez & Ferguson 1994).

A trigonal lattice was identified from single crystal analysis of the α-anhydrous phase which proved to be a very weak x-ray scatterer. The x-ray powder diffractograms of α and β-caffeine indicate that only a slight relaxation of the crystal packing is required for the polymorphic transformation. Profile refinement of the β-phase triclinic lattice reveals only a slight deviation in lattice parameters from those of the trigonal α-polymorph.

The Raman spectra of caffeine observed during dehydration show changes in spectroscopic behaviour of vibrational bands in the region 3300 to 2700 cm^{-1}. The relatively large intensity increase of the C=O stretch observed at 1656 cm^{-1} signifies a modification in environment of a carbonyl group. The results indicate the formation of a weak CH....O intermolecular interaction which is evident in the trigonal crystal structure of α-phase caffeine. Additionally, the lattice vibrations occurring in the region <200 cm^{-1} change dramatically as the hydrated lattice is destroyed, indicating the formation of a new, distinct solid state form which is also observed by x-ray diffraction. The concomitant disappearance of the water libration at 890 cm^{-1} is also observed. These effects suggest molecular realignment associated with corresponding loss of water of crystallisation, as well as providing structural evidence for the facile loss of crystalline water from caffeine hydrate.

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80 THE EFFECT OF RELATIVE HUMIDITY ON MILLED AND SPRAY DRIED NEDOCROMIL SODIUM

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An ideal drug powder, for use in dry powder inhalers has particles in the respirable size range (<6μm), which are not adhesive, cohesive or static. The particles should be stable in most atmospheric conditions, flow well and disperse readily from the liberation device (Timsina et al, 1994).
In this study, spray drying has been investigated as a means of controlling the particle size and morphology of nedocromil sodium and the effects of moisture on spray dried and milled powder assessed.
A 10%w/v aqueous solution of nedocromil sodium was spray dried using a Mini Büchi 190 spray dryer. The operating conditions, adapted from Chawla et al, (1994), were: pump flow rate, 7ml/min; inlet temperature, 200°C; outlet temperature, 115°C; nozzle size, 0.7mm. Samples of milled and spray dried powder were placed in petri dishes and exposed to varied relative humidities (0, 4, 12, 23, 35, 76 and 96%) prepared with saturated salt solutions for 2 weeks. The thermal behaviour of the samples was studied using Thermogravimetric Analysis (TGA, TA Instruments), at a heating rate of 10°C/min, and the surface morphology of the powders were examined by Scanning Electron Microscopy (SEM, Phillips XL20).
SEM micrographs indicated that the milled powder was crystalline with agglomerated particles of 5μm mean size. Spray drying produced discrete, spherical particles in the size range 1μm to 5μm. The appearance of milled and spray dried samples did not change after storage at humidities less than 76%. On storage at 76 and 96% RH, spray dried and milled particles appeared to fuse, having no distinct shape and roughened surfaces. At 96%RH, the sample visibly began to deliquesce, however, SEM revealed that the spray dried material had recrystallised.
At ambient conditions, the thermogram obtained from the TGA of milled nedocromil sodium, indicated that water escapes from the crystalline milled form in two distinct phases. Both stages of weight loss were attributed to be the loss of different forms of hydrate. The thermogram obtained with the spray dried sample at ambient conditions, displayed a steady escape of water from the sample in one distinct phase. These results are thought to be due to the difference in water binding mechanisms of these two samples.
Samples stored under different conditions gave different TGA profiles, (figure 1). The % weight loss increased with increasing humidity. In the case of the milled samples, between 12 and 76%RH, the thermograms have a similar profile suggesting no additional moisture uptake at these humidities. In the case of the spray dried samples, the thermograms differed, indicating that water loss occurred in one distinct phase and the % weight loss varied at different relative humidities. However, the thermograms at 23% and 76%RH, resembled those for the milled drug, indicating that crystallisation had occurred. These studies show that spray drying produces particles in the respirable size range and that these particles are less stable on exposure to moisture, compared to the milled drug.

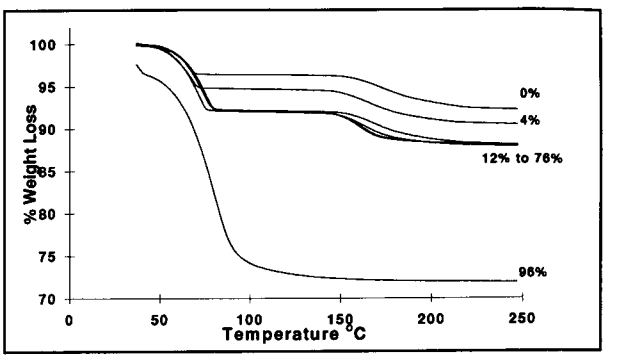

Figure 1: Effect of relative humidity on milled nedocromil sodium.

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81 IN-VITRO AND IN-VIVO EVALUATION OF TABLETS OF A DIRECTLY COMPRESSIBLE FORM OF PARACETAMOL

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As part of our research programme on the process and material optimization in tablet. manufacturing, we investigate interactions at the solid-liquid interface as an approach to manipulate the powder characteristics of large dose drugs to convert them to a directly compressible form.
In the present work, we report on the in-vitro and in-vivo performance of tablets prepared by direct compression of solvent-treated paracetamol.
Paracetamol was treated with a pre-selected solvent under controlled conditions. Changes in crystal morphology (scanning electron Xerography), flowability and compressional properties as well as dissolution rate of paracetamol were assessed.
The solvent treated material was directly compressed into tablets after mixing with 20% Avicel and 5% starch. Samples of the test tablets and commercial brands of paracetamol tablets were subjected to comparative routine in-vitro testing and a long term stability program.
Further, the bioavailability of the test tablets was compared to that of Panadol® tablets in 12 healthy male volunteers using HPLC.
The results indicated that solvent treatment of paracetamol resulted in changes in crystal morphology accompanied by improved flowability and compressional properties and no adverse effect on dissolution rate.
In-vitro evaluation data indicated that test tablets conformed to the requirements of the tests performed. Accelerated stability testing for twelve weeks indicated good performance of the test tablets. Results of the in-vivo study showed that the mean relative bioavailability of the test tablets was 121.7% and 125.5% based on mean values of $AUC_{0\rightarrow 24h}$ and $AUC_{0\rightarrow\infty}$, respectively, pointing to bioequivalence of the test tablets and Panadol®.
In conclusion, provided that the solvent and conditions are carefully selected, our solvent treatment technique can be considered a simple, reproducible and economical approach capable of converting powdered drugs with poor physico-technical characteristics into directly compressible materials. These can be used in the production of superior quality tablets.

82 THE EFFECT OF PARTICLE SIZE DISTRIBUTION ON IN-VITRO DEPOSITION OF SALMETEROL XINAFOATE FROM A DRY POWDER INHALER MIX

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Dry powder inhaler systems are often composed of an ordered mix of micronised active attached to a larger sized carrier particle, e.g. lactose, which imparts good flow properties. It was demonstrated by Kassem (1990) that the separation of active/carrier occurred due to the turbulent air stream provided by the inspiratory flow, and the design of the device. The interparticulate forces will therefore have an effect on the detachment of the active from the carrier particle, and subsequently on lung deposition. Electrostatic forces play a very important role in the bonding of particles and Staniforth et al (1982) demonstrated that these forces affect ordered mix stability. The charging tendency of a material is determined by several factors, as shown by Bailey (1984) including particle characteristics such as size, size distribution and surface area. Bennett et al (1996) reported a decrease in specific charge of lactose 63-90μm as the proportion of lactose <10μm was increased from 0% to 30% W/w in the binary mix.

In this work 0.5% salmeterol xinafoate was mixed with binary mixes of crystalline α-lactose monohydrate (Borculo Whey), containing 63-90μm, with <10μm size fraction at concentrations of 0%, 5%, 10% and 15% W/w. Formulations of the same composition were made where salmeterol xinafoate was charged against a stainless steel surface prior to mixing.

Each mix was hand filled into two Rotadisks™, 12.5mg (±1.5mg) in each pocket, for use with a diskhaler, and the *in vitro* deposition studies using a cascade impactor. The four pockets of each formulation were pierced, and each dose drawn through the cascade impactor by a three second pulse of air, at 60 l min^{-1}. The results are reported as the mean % of the respirable fraction of salmeterol from two Rotadisks™.

An increase in respirable fraction was observed for salmeterol as the concentration of <10μm lactose particles is increased from 0% to 10%W/w, for both charged and uncharged systems, however at 15%W/w there was a decrease.

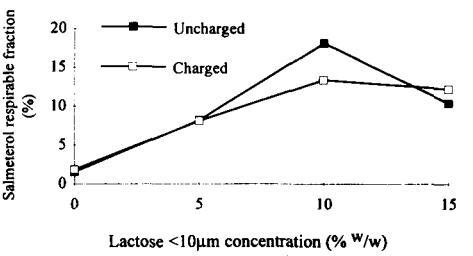

Figure 1: Change in salmeterol respirable fraction with increase in lactose <10μm size fraction.

The increase in respirable fraction with increase in <10μm lactose is attributed to a reduction in electrostatic forces in the active/carrier mix, since Bennett et al (1996) had demonstrated a reduction in specific charge of lactose binary mixes as the concentration of <10μm increased. However further investigations are underway to substantiate the role of <10μm lactose particles on the distribution of salmeterol xinafoate, and ultimately the % respirable fraction.

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83 LECTINS IN DRUG DELIVERY - AN EVALUATION OF THE IRRITANCY OF TWO LECTINS

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Lectins are proteins or glycoproteins of non-immune origin capable of binding to one or more specific sugar residues. Certain lectins have been reported to be mitogenic, inflammatory or cytotoxic (Shier 1979). The potential for using lectins as a means of 'anchoring' a drug delivery system to the mucosal surfaces of the eye has been investigated (Nicholls et al 1996a), and the lectins from *Solanum tuberosum* and *Helix pomatia*, shown particular promise. In a preliminary study the acute toxicity of these lectins, in terms of their potential to cause inflammation and tissue necrosis, was investigated in anaesthetised rabbits over a 3 h period (Nicholls et al 1996b). This study has now been extended to 6 h in conscious animals.

Five male New Zealand white rabbits from the same litter had their backs shaved 24 hours prior to the study. Anaesthesia was induced by the injection of sodium thiopentone (40mg Kg^{-1}) into a marginal ear vein. Evans blue solution (0.5 mL, 2%) was then administered by this route to provide an indication of increased vascular permeability. The lectin solutions were prepared in normal saline at a range of concentrations from 50 to 500 μg ml^{-1} and sterilised by filtration. Solutions of 1% carageenan and 20% ethanol were used as positive controls and normal saline as a negative control. 50 μl volumes of the test solutions were injected intradermally at 18 sites across each rabbits' back. The rabbits were allowed to recover from anaesthesia and observed every hour for any signs of behaviour indicative of localised irritation. The development of an inflammatory weal and Evans Blue infiltration into the injection area was also monitored. The rabbits were killed 6 h later with an overdose of anaesthetic. The skin from the injection site was removed and histological sections prepared. These sections were examined for signs of inflammation, such as heterophil migration, oedema or tissue damage.

Unlike the positive controls, there was no evidence of oedema or Evans blue infiltration with any of the lectin solutions. The rabbits also did not display any signs of discomfort such as scratching or continued grooming throughout the experiment. Histological examination of the injection sites revealed even fewer heterophils than that observed in the previous unconscious rabbit study.

It can be concluded that these lectins demonstrate minimal inflammatory properties, and will therefore be taken forward for formulation and in-vivo studies.

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84 A QUANTITATIVE IN-VIVO EVALUATION OF LECTIN BINDING TO THE ORAL MUCOSA OF THE RAT

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Lectins are ubiquitous natural proteins or glycoproteins of non-immune origin that bind reversibly and specifically to carbohydrates moieties of complex glycocongugates. They have been shown to bind, and be retained, on human buccal cells in-vitro (Nantwi et al 1997) and in-vivo (Gibbons & Dankers, 1983). The aim of this study was to determine the quantity and duration of lectin binding to the oral epithelia *in vivo* using a rat model, in order to investigate their potential for extending drug delivery to the oral cavity.

Two lectins, from *Canavalia ensiformis* and *Triticum vulgaris,* that have been shown to bind avidly to both rat and human oral mucosae in-vitro (Nantwi et al 1997), were used in this study. Technetium-99m labelled lectins were prepared using a DTPA conjugation technique as described by Nantwi et al (1996). The final labelled lectin solution was concentrated by centrifugation. Rats were briefly anaesthetised with halothane to allow application of 10 µL of the solution containing either 233 µg of the *Canavalia ensiformis,* or 217 µg of the *Triticum vulgaris* lectin. The labelled lectin solution was placed into the buccal pouch between the lower incisors, gingiva and buccal mucosa. The rats were kept for set time intervals, during which they were allowed free access to water, then sacrificed, and the lower buccal cavity mucosal tissue (LBM) and tongue dissected out and monitored for bound lectin. All experiments were completed in triplicate, and results calculated as % applied dose retained (± sd).

Binding of *Canavalia ensiformis* lectin to rat buccal tissue was evident 30 min (2.31 ± 0.18 % on the LBM and 2.15 ± 0.27 % on the tongue) and 60 min (2.04 ± 0.24 % ov the LBM and 2.30 ± 0.66 % on the tongue) after application. Much higher levels of *Triticum vulgaris* lectin were evident after 30 min (13.57 ± 1.94 % on the LBM, and 13.04 ± 0.98 % on the tongue) and lectin retention was evident 2 hours after application (15.11 ± 1.78 % on the LBM, and 14.41 ± 2.00 % on the tongue). Approximately 25% of the *Canavalia ensiformis* lectin was recovered from the stomach 10 min after administration.

This study is the first quantitative assessment of lectin binding within the oral cavity and indicates that retention occurs for a least a 2 h period, confirming the work of Gibbons and Dankers (1983). This study considered only two areas within the oral cavity, and significant binding would be expected in the other, less accessible, areas such as the gingiva and hard palate. Appropriate mouth-rinsing techniques to expose all the surfaces within the oral cavity to the lectin solution during clinical use should further enhance binding. The potential of these lectins for inclusion into drug delivery systems that allow prolonged retention within the oral cavity is thus demonstrated in this study.

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85 APPLICATION OF ARTIFICIAL NEURAL NETWORKS AND MULTIVARIATE REGRESSION TO SOLID DOSAGE FORM OPTIMIZATION

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The development of any dosage form requires the optimization of the many formulation and processing variables. The development procedure can be more efficient if the physico-chemical system could be represented by mathematical models. Artificial Neural Networks (ANNs) (Hussain et al. 1991; Richardson et al. 1997) have been proposed as an alternative to multivariate regression for such optimization. This study presents a rigorous comparison of an optimized ANN with an optimized polynomial in modelling the characteristics of a direct compression tablet as a function of formulation variables and compaction force.

A total of 27 formulations of Paracetomol tablets were manufactured by direct compression. The independent variables were the concentrations of the disintegrant (1, 2 & 4 %w/w) and lubricant (0.1, 1.0, 5.0%), and the compaction force (2.5, 5, 10 kN). The dependent variables monitored were the tablet weight variation, thickness, hardness, tensile strength, disintegration time, impact friability, erosion friability and dissolution rate.

The data were divided randomly into a training set (22 formulations) and a validation set (5 formulations). The ANNs employed in the study were feed-forward perceptrons with a topology consisting of 3 input neurons, one or two hidden layers with a variable number of nodes, and 8 output neurons. The optimum topology was selected by testing various architectures using the validation data set. The topology yielding the minimum estimated generalization error (Murtoniemi et al. 1994) consisted of one hidden layer with 3 or 4 nodes. The optimum regression model was developed using the backwards elimination procedure. The procedure yielded 8 regression equations, one for each dependent variable Finally the optimum tablet formulation and compaction force that yields the maximum dissolution rate was determined for both the ANN and the regression models using a maximization algorithm.

The estimated generalization errors based on the predicted values for the validation data set for both the ANN and the regression models are given in Table 1. Although there is some variation in the estimated error from one variable to the next between the two methodologies, the overall error in the predictions (Regression = 7.10 %, ANN = 7.97 %) was found not to be significantly different. The optimum tablet formulations obtained were also quite similar. Such a comparison, however, may not be fair, since with the ANN a single model characterizes the relationship between the independent variables and *all* of the dependent variables. In contrast, the regression model consists of 8 independent equations, one for each of the dependent variables.

Table 1. Average percentage deviation between predicted and observed values for the regression models and the ANN (3:3:8 configuration) .

	weight	thickness	hardness	tensile
Regression	1.55	1.01	1.85	13.80
ANN	1.16	1.29	6.63	9.99

	disintegration	erosion friab.	impact friab.	dissolution
Regression	15.36	6.43	6.87	9.89
ANN	9.74	11.88	13.69	9.34

In summary, a rigorous comparison between ANNs and regression for modelling the chracteristics of a direct compression tablet has been carried out. The ANNs have been found to be equally effective as the regression approach but no better. It is possible that ANNs may prove to be better for problems with a more complex hypersurface.

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86 THE CLEARANCE OF SODIUM CHLORATE BY IN-VITRO MODELS OF CONTINUOUS HAEMOFILTRATION AND HAEMODIALYSIS QUANTIFIED USING A MODIFIED FTIR METHOD

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Sodium chlorate ($NaClO_3$), a common herbicide, accounted for 113 deaths as a result of poisoning in England and Wales between 1945 and 1989 (Casey & Vale 1994). Chlorate poisoning is characterised by methaemoglobin formation and death during the early stage of intoxication is due to anoxia, although haemolysis and coagulation disturbances followed by renal insufficiency often determine the clinical outcome of such cases. Following the admission of a patient with acute renal failure resulting from acute chlorate poisoning to the intensive care unit (RSCH) it was clear that no method was available for the rapid and accurate quantification of serum chlorate levels. In addition, although the patient was placed on continuous haemofiltration (HF), the efficiency of such continuous renal replacement therapies in the removal of chlorate was unknown. The detection of chlorate in plasma and urine has previously been based on a method dependent upon the ability of metallic zinc to reduce chlorate to chloride which can be determined amperometrically (Steffen & Wetzel 1993). This technique, however, takes 24 hours to complete and is, therefore, inappropriate for the detection of serum chlorate levels in a patient requiring immediate therapy. We have applied a Fourier transform infra-red (FTIR) spectrometry technique, developed for the quantitative analysis of $NaClO_3$ during the industrial production of perchlorate (Kargosha et al 1995), to the assessment of chlorate in Tyrode buffer, 3.5% human albumin solution in Tyrode and human plasma. This method of analysis has been used to determine the efficiency of two commonly used methods of CRRT (continuous HF and haemodialysis (HD)) at clearing chlorate using an in-vitro model of each process. A BSM22SC Hospal blood safety module was used to regulate the flow of carrier fluid (3.5% human albumin solution in Tyrodes buffer containing a starting concentration of 5% w/v $NaClO_3$) at 100mL/min from a bell jar representing the patients body (37°C) through a hollow fibre Gambro® polyamide FH66 HF membrane or FH66D HD membrane. During HF the ultrafiltrate rate (UFR) was adjusted to 15 or 25 mL/min via a peristaltic pump. Fluid removed was replaced with Tyrode buffer prior to recirculation. During HD Haemovex dialysis fluid was circulated in a counter current fashion against the flow of carrier fluid at a rate (Qd) of 1 or 2L/hr. Serial pre and post-membrane and ultrafiltrate/dialysate samples were collected. Each sample was scanned (x4) using an Perkin Elmer 1720 FTIR fitted with a zinc selenide ATR crystal. A validated peak height method ($970cm^{-1}$) employing standard curves (linear range 0.2 to 7% w/v; %RSD ± 0.64) was used to obtain $NaClO_3$ concentrations allowing sieving coefficients (S) and filter clearances (Fcl) to be calculated (Table 1).

Table 1. S and Fcl of sodium chlorate during HF and HD

	Qd/UFR	S ±SD	Fcl (L/hr)
HD	1L/hr	0.72±0.02	0.754
	2L/hr	0.62±0.01	1.272
HF	15mL/min	1.02±0.03*	0.918
	25mL/min	1.00±0.03*	1.503

n=5 except * where n =4

As can be seen from the S and Fcl obtained, the convective forces associated with HF at a UFR of 25mL/min cleared chlorate from the system most rapidly. HD also cleared chlorate via diffusion, but at a slower rate approximately equivalent to HF at a UFR of 15mL/min. This work shows that chlorate levels can be rapidly and accurately assessed (in both buffer and plasma) using the FTIR technique presented here and that CRRT can be used to clear chlorate in cases of acute poisoning, although HF at high UFR's appears to be the most efficient process. Further work is underway to investigate the in-vitro clearance of chlorate from whole blood.

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87 CYTOKINE CLEARANCE FROM SEPTIC PATIENTS DURING HAEMOFILTRATION WITH HOLLOW FIBRE POLYACRYLONITRILE MEMBRANES

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Multiple organ dysfunction syndrome is currently the major cause of death in patients with sepsis. Human sepsis is associated with the systemic release of a number of pro-inflammatory mediators including cytokines. Various methods are used to combat individual organ failure including continuous renal replacement therapies (CRRT) such as haemofiltration (HF), and haemodiafiltration (HDF). Recently, attention has focused on the possibility of using CRRT for the elimination of the inflammatory mediators, particularly cytokines, from the circulation of septic patients. Previous studies into cytokine removal by CRRT have been contradictory due to differences in the techniques used i.e. HF or HDF, patients presenting condition or the fact that sampling times were often separated by a matter of hours whereas the half-life of many of the cytokines can be measured in minutes (Bellomo et al 1993; Hoffmann et al 1995). We report the preliminary findings of an ongoing project to examine the clearance of pro- and anti-inflammatory cytokine levels of septic patients at regular intervals over the first 24 hours of HF. Patients were selected on the basis of diagnosed acute renal failure and sepsis. Each patient (2 from a target of 6) was haemofiltered using a Hospal® Prisma blood pump incorporating a Hospal® M100 polyacrylonitrile (PAN) membrane (MW cut-off 20kD). A venous blood sample was taken before the initiation of HF to obtain baseline levels of each cytokine studied. Simultaneous pre- and post-filter blood samples (6mL) and ultrafiltrate (UFR) samples (10mL) were taken 5, 10, 20, 30, 60, 90 and 120 min and then 12 and 24 hours after initiation of HF. Each blood sample was centrifuged in heparinised tubes to separate the plasma. Aliquots of plasma and UFR were analysed for the concentration of the pro-inflammatory cytokines TNF-α, IL-1β, IL-8 and the anti-inflammatory IL-4, using the Quantikine™ immunoassay kits. The assessment of cytokine levels in UFR from two patients have failed to show any elimination of TNF-α, IL-1β or IL-4 during the first 24 hours of HF despite elevated serum levels. This was supported by the fact that differences between pre- and post-filter cytokine levels were either absent or small enough that they may be attributable to binding to the PAN or proteins adsorbed onto the membrane (Barrera et al 1992). Like previous studies employing polyamide membranes (Hoffmann et al 1995) our study failed to show any elimination of TNF-α despite having a molecular weight of 17kD. This may be due to membrane characteristics e.g. hydrophobicity or net charge, or that this cytokine may exist as a 51kD trimer, raising it above the membrane cut-off point. However, IL-8 was eliminated from both patients with UFR levels reaching a maximum of 31 and 180 pg/mL compared with plasma concentrations 275 and 465 pg/mL respectively. The elevated clearance of IL-8 compared with the other cytokines investigated may be due to its relatively low molecular weight (8kD). IL-8 has previously been shown to be cleared by HD using flat plate PAN membranes but in minimal concentrations (<11 pg/mL) (Bellomo et al 1995). The more efficient elimination of IL-8 by the convective forces associated with HF using hollow fibre membranes may be clinically significant as this cytokine has been shown to correlate with increased mortality in septic patients (Casey et al 1993).

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88 RADIOPHARMACEUTICALS FOR DETECTION OF ADULT RESPIRATORY DISTRESS SYNDROME

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Adult respiratory distress syndrome (ARDS) is a recognised and common problem in hospital intensive care units. It is an exaggerated acute inflammation of the lung which can be incited by a wide variety of pulmonary and non-pulmonary conditions.The condition is associated with a high mortality rate and is characterised by increased lung permeability to water and proteins (Green 1987)

Early diagnosis and monitoring of patients with ARDS is difficult and symptoms are often quite advanced before detection is possible. A nuclear medicine technique has shown considerable potential in the early demonstration of the condition, based on comparison of blood cell bound and protein bound radioactivity ratios over the heart and lungs (Rocker 1988) This allows the calculation what some authors have termed a protein accumulation index (PAI) which gives a high specificity and sensitivity for ARDS.

The technique requires specific radiolabelling of circulating blood cells, and circulating plasma proteins with appropriate radiopharmaceuticals, and detection of their radioactive emissions using suitable probes. Two different radionuclides with different characteristic gamma-ray emission spectra are necessary since the method requires simultaneous detection of the two radiolabelled species. In general, blood cells are radiolabelled with sodium ($^{99}Tc^{m}$) pertechnetate in the presence of an appropriate reducing agent, while proteins, in particular transferrin, are labelled with indium ($^{113}In^{m}$ or ^{111}In) chloride.

Sodium ($^{99}Tc^{m}$) pertechnetate, being generator produced, is readily available. However, maintenance of a supply of indium (^{111}In) chloride is not cost effective unless indium is also required for other purposes. The present study seeks to evaluate the suitability of other combinations of radiopharmaceuticals which would permit the provision of a diagnostic service at any time.

For many years, haematological studies in nuclear medicine have been performed using chromium compounds, in particular sodium (^{51}Cr) chromate and (^{51}Cr) chromic chloride. A combination of chromium (^{51}Cr) blood cells and technetium ($^{99}Tc^{m}$) proteins fulfils the requirements in terms of the gamma-ray emission spectra, and experiments have been performed to assess the response of the probe system used to this combination of radionuclides.

The detector system comprised four caesium iodide photodiode detectors and amplifier/discriminator unit (Mediscint, Oakfield instruments, Oxford) interfaced to an Elonex 486 IBM personal computer. Experiments were performed to assess the sensitivity, collimation, depth detection characteristics, the ability to discriminate between the two radionuclides, and the effects of attenuation and scatter. For the latter, a water-filled phantom was constructed, with radioactive sources suspended in the water at different depths to simulate the effects of soft tissue attenuation in clinical use.

Results indicated that there was an approximately tenfold difference in sensitivity between the detection of technetium and that of chromium, but count rates from chromium were still sufficiently high at permitted dose levels to allow adequate detection. In air, the radiation of chromium was poorly collimated, with significant counts being detected beyond an arc 60° from the plane normal to the detector. increasing the shielding thickness from 1.5 to 4.5 mm to a depth of 1 cm from the face of the collimator improved this situation. Significant improvements in detection characteristics were also observed when the sources were immersed in the attenuating medium.

Results obtained are sufficiently encouraging to suggest that clinical evaluations should be undertaken

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89 CAVEOLAE-MEDIATED ENDOCYTOSIS OF NATIVE ALBUMIN BY EPITHELIAL CELLS: SIGNIFICANCE TO ABSORPTION PROCESSES IN THE PULMONARY CAPILLARY BARRIER

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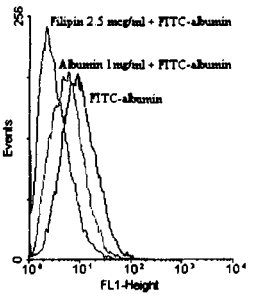
Introduction. Considerable effort is directed to exploiting the pulmonary route of administration for the systemic delivery of therapeutic peptides and polypeptides. With increasing molecular size passive diffusion via paracellular pathways becomes restrictive and vesicular trafficking mechanisms in the absorption process become quantitatively more important. In the lung periphery systemic absorption will occur across pulmonary epithelial (comprised 95% with respect to surface area by thin squamous alveolar type I epithelial cells) and capillary endothelial barriers. Caveolae are nonclathrin-coated membrane invaginations which can pinch off from plasma membranes to form flask-shaped vesicles (approximate diameter 80-100 nm) within the cytoplasm. Caveolae are morphologically most prominent in endothelial cells but also occur in the alveolar epithelial type I cell. Endothelial caveolae have been shown to transport native and modified albumins by specific receptor-mediated processes, however, it has been suggested that caveolae may serve different functions in the epithelial cell. (Lisanti et al 1994). The **objective** of this study was to determine if an epithelial cell known to possess high densities of caveolae, MA104, can actively endocytose albumin by a specific caveolae-mediated process.

Methods. MA104 cells (African green monkey kidney epithelial cells) were cultured in DMEM supplemented with 10% Foetal Bovine Serum (FBS) and cultured to 100% monolayer confluency in standard 24 well tissue culture plates. At the time of experimentation the cells were washed to remove all traces of FBS and incubated in DMEM with one of the following treatments: FITC-albumin (0.5 mg/ml); Filipin (0.5 to 2.5 μg/ml) +/- FITC-albumin; albumin (1 to 2 mg/ml) +/- FITC-albumin; phorbol-12-myristate-13-acetate (1 μM PMA) +/- FITC-albumin; indomethacin (400 μM) +/- FITC-albumin. After 30 min incubation with FITC-albumin the cells were washed and trypsinised and for each treatment well 1 x 10^4 cells (with replicates) were analysed by fluorescence activated cell sorting (FACS). Cell and modulator autofluorescence were accounted for in the analysis.

Results. Figure 1. shows the concentration-related inhibition of FITC-albumin uptake by the cholesterol binding agent filipin, and the competitive ligand mediated inhibition with native albumin, inhibition shifting the histogram of fluorescence per cell to a lower level. Cholesterol is important for both structure and function of caveolae with filipin decreasing endocytosis of FITC-albumin upto 76%, albumin competition decreasing uptake by 58%, the protein kinase C modulator, PMA, decreasing uptake by 37%, and indomethacin decreasing uptake by 66%. No cell toxicity was observed during the above treatments.

FIGURE 1



Conclusion. The modulators used in this study are recognised caveolae inhibitors (Smart et al 1995) either shown to be specific or whose mechanism of action has yet to be fully elucidated. We have demonstrated that epithelial caveolae endocytose albumin through a specific receptor-mediated event. Type I alveolar epithelial caveolae may function in a parallel manner and possess other receptors that potentially mediating polypeptide transport.

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90 CYTOCHROME P450 REGULATION BY THE IMMUNOSTIMULATOR LICOPID

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Licopid (*N*-acetyl-D-glucosaminyl-(b-4)-*N*-acetylmuramyl-L-alanyl-disoglutamine) is a synthetic analogue of a glycopeptide obtained from the cell wall of *Lactobacillus bulgaricus*. Licopid is water-soluble and non-toxic; the LD50 value in mice is 7 g kg^{-1}, compared with 625 mg kg^{-1} for muroctasin. We have demonstrated that both licopid and its des-glucosaminyl analogue are effective regulators of both cytochrome P450 enzymes and the immune system.

As well as their immunostimulant activity, muramyl dipeptides have anti-cancer, anti-septicaemic and hepatoprotective effects. They have been shown to be anti-inflammatory and anti-allergic. Muramyl dipeptides have also been shown to have effects on the central nervous system (hypnotic, antinociceptive, anxiolytic, thermoregulatory), to decrease blood pressure, to activate the detoxifying effects of the liver, and to enhance the activity of insulin. This multiplicity of pharmacological activity suggests that the muramyl dipeptides are natural effective regulators of many biological functions.

We have investigated the properties of licopid and the des-glucosaminyl analogue as substrates for P450 enzymes. Both compounds exhibited type I optical difference spectra in oxidized liver microsomes from phenobarbital-induced rats (licopid: E_{max} 390 nm, E_{min} 421 nm, K_s 0.1 mM; muramyldipeptide: E_{max} 380 nm, E_{min} 419 nm, K_s 0.001 mM). Normally, there is an inverse relationship between immune and P450 activities but licopid was found to stimulate both systems. Licopid increased the metabolism of alcohol, aldehyde and a number of xenobiotics including cyclophosphane. In clinical studies, licopid reduced blood levels of bilirubin, urea and creatinine. We suggest that the detoxifying effect of licopid will be useful for the therapy of hepatic encephalopathy. We suggest that the multiple adaptive activity of licopid may be stimulated by its direct interaction with histones of chromatin and activation of the adaptive expression of stress-responsive genes; licopid-histone binding has also been demonstrated (V. A. Nesmeyanov, personal communication).

91 THE EFFECT OF OMEPRAZOLE ON CLARITHROMYCIN PHARMACOKINETICS IN MAN

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The mechanism by which omeprazole markedly potentiates antimicrobial therapy against the human gastric pathogen *Helicobacter pylori* is as yet undefined. The aim of this study was to investigate the effect of omeprazole on the pharmacokinetics of clarithromycin (CLA) and its active metabolite hydroxyclarithromycin (CLAOH) in plasma, saliva and gastric juice in human volunteers.

Seven healthy volunteers took part in the placebo-controlled, randomised, double-blind crossover study. Clarithromycin (500 mg) was administered to the volunteers intravenously while they were receiving placebo or omeprazole (40 mg) twice daily for 5 days. There was a washout period of 5 days between the placebo and omeprazole dosing. Plasma, saliva and gastric juice samples were collected at intervals for periods of 8 hr, 4 hr and 4 hr respectively. Gastric juice was aspirated *via* the nasogastric or orogastric route and pyloric losses were assessed by the phenol red recovery method. The pH of gastric juice samples was adjusted to 7.0 using aqueous sodium hydroxide solution (50 mmol) and all samples were stored at -70°C prior to their analysis for CLA and CLAOH content by HPLC. Pharmacokinetic parameters were calculated using standard techniques.

The plasma pharmacokinetics of CLA and its active hydroxylated metabolite were not significantly changed by omeprazole co-administration and both of the compounds were easily detected in saliva and gastric juice under either placebo or omeprazole dosing conditions.

Treatment with omeprazole resulted in no significant changes in the salivary concentrations of CLA and CLAOH but it is possible that if the mouth acts as a reservoir for *H. pylori* that such salivary concentrations may be of significance.

CLA and CLAOH were found in this study to concentrate in gastric juice when compared to plasma in both placebo and omeprazole groups although the co-administration of the proton-pump inhibitor did not alter the gastric juice AUC_{0-t}, C_{max} or CL_g parameters. The gastric transfer of clarithromycin is likely to result from a combination of the high lipophilicity of the drug and potential active secretion into gastric juice resulting in high gastric juice concentrations compared with plasma.

The results of these studies have demonstrated the transport of CLA and CLAOH into saliva and gastric juice and these observations may be significant in the eradications of gastric *H. pylori* infections.

Clarithromycin (CLA) and hydroxyclarithromycin (CLAOH) pharmacokinetics in plasma, saliva and gastric juice.

		Placebo	Omeprazole
Plasma (CLA)	AUC_{0-8} (μmol.h.l^{-1})	18.1 ± 3.3	19.0 ± 5.1
	CL_p ($l.h^{-1}$)	31.1 ± 7.5	30.6 ± 8.4
	MRT (h)	2.9 ± 0.2	3.0 ± 0.2
Plasma (CLAOH)	AUC_{0-8} (μmol.h.l^{-1})	4.1 ± 2.5	4.1 ± 2.7
	C_{max} (μmol.l^{-1})	0.7 ± 0.3	0.7 ± 0.4
Saliva (CLA)	AUC_s (μmol.h.l^{-1})	8.5 ± 2.9	8.2 ± 1.7
	C_{max} (μmol.l^{-1})	4.9 ± 3.2	4.0 ± 1.2
Saliva (CLAOH)	AUC_s (μmol.h.l^{-1})	2.3 ± 0.7	2.5 ± 0.9
	C_{max} (μmol.l^{-1})	0.8 ± 0.2	0.8 ± 0.3
Gastric (CLA)	AUC_g (μmol.h.l^{-1})	25.0 ± 14.7	30.7 ± 4.8
Juice	C_{max} (μmol.l^{-1})	11.4 ± 6.2	17.7 ± 5.1
	CL_g ($l.h^{-1}$)	0.2 ± 0.1	0.2 ± 0.1
Gastric (CLAOH)	AUC_g (μmol.h.l^{-1})	3.8 ± 2.6	2.6 ± 1.7
Juice	C_{max} (μmol.l^{-1})	2.9 ± 2.3	1.9 ± 1.3

92 THE EFFECT OF OMEPRAZOLE ON DRUG ABSORPTION IN MAN

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Currently used *Helicobacter pylori* eradication regimens fail to cure 5-20% of patients (van der Hulst et al 1996) partly due to the failure of anti-*H. pylori* agents to reach the bacteria in effective concentrations. In subjects who received olive oil, test meals were retained in the stomach whilst on the left lateral position (Boulby et al 1997). We applied this finding to investigate the effect of omeprazole on gastric permeability and the absorption of drugs from the stomach.

Ten healthy *H. pylori* negative volunteers took part in the placebo-controlled, randomised, single blind crossover study. After an overnight fast, each volunteer received a test meal whilst in the left lateral position. The test meals which contained the compounds investigated were vegetable soup/water, 125:125ml (control) or soup/water/olive oil, 100:100:50ml. The drugs used were amoxycillin (Amo) 1g and metronidazole (Met) 400mg whilst mannitol (Man) 5g was used as a marker of gastric emptying. Each volunteer was given placebo or omeprazole (Om) 20mg twice daily for 5 days before the oil containing meal was given. The tests were carried out at 1 week intervals. Plasma samples were collected at intervals for 3 hr and were analysed for drug content by HPLC. AUC values were calculated and compared using Wilcoxon's signed rank test.

The absorption of Man, Amo and Met were significantly lower after ingesting the oil meals indicating reduced gastric emptying ($AUC_{0\text{-}180}$ & $AUC_{0\text{-}60}$). The absorption of Amo and Man was significantly reduced by Om administration ($AUC_{0\text{-}180}$) when compared with placebo. The most likely explanantion for this observation is due to reduced gastric emptying caused by Om. Ionic trapping of Amo by Om (which increases gastric pH) could also have contributed to the lower absorption of Amo. However, gastric emptying was only effectively delayed for 1 hr in 60% of the volunteers as indicated by mannitol absorption (marker of the gastric emptying).

Om and the administration of a fat meal reduce gastric emptying. The absorption of Amo and Met from the stomach and the effect of the reduced gastric emptying observed in this study on gastric drug absorption could not be easily determined.

Area under the plasma concentration-time curve

		$AUC_{0\text{-}180}$ (mg.min.l^{-1}) mean ± sd	p-value	$AUC_{0\text{-}60}$ (mg.min.l^{-1}) mean ± sd	p-value
Man	A^a	6436 ± 1291	$^{ab}p = 0.005$	1203 ± 664	$^{ab}p = 0.001$
	B^b	3382 ± 2370	$^{ac}p = 0.001$	445 ± 403	$^{ac}p = 0.005$
	C^c	1869 ± 1596	$^{bc}p = 0.05$	280 ± 359	$^{bc}p > 0.05$
Amox	A^d	1309 ± 226	$^{de}p = 0.003$	217 ± 133	$^{de}p = 0.002$
	B^e	658 ± 488	$^{df}p = 0.001$	91 ± 90	$^{df}p = 0.005$
	C^f	275 ± 246	$^{ef}p = 0.019$	46 ± 59	$^{ef}p > 0.05$
Met	A^g	930 ± 147	$^{gh}p = 0.002$	233 ± 97	$^{gh}p = 0.001$
	B^h	469 ± 301	$^{gi}p = 0.001$	92 ± 83	$^{gi}p = 0.002$
	C^i	358 ± 216	$^{hi}p > 0.05$	61 ± 66	$^{hi}p > 0.05$

A, control; B, oil/placebo; C, oil/omeprazole

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93 TAZOCIN CLEARANCE BY AN IN-VITRO MODEL OF HAEMOFILTRATION QUANTIFIED USING A NEW HPLC METHOD

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Tazocin is a preparation containing both Piperacillin (Pn) and Tazobactam (Tz). Pn is susceptible to hydrolysis by a range of β–lactamases including plasmid mediated enzymes. Tz, a triazolylmethyl penicillanic acid derivative is a potent and irreversible inhibitor of β-lactamases. Administering the two drugs in combination therefore results in an increased spectrum of activity against a range of Gram +ve and Gram-ve bacteria. Pn and Tz are both extensively removed by the kidney so that clearance is reduced in patients with renal failure. However, the structural and pharmacokinetic characteristics of Tz and Pn, i.e. low molecular weight (300.3 and 517.6 kDa respectively), high water solubility and low volume of distribution suggest that both components may be lost from a patient receiving continuous renal replacement therapies such as haemofiltration (HF). Any such loss may necessitate adjustments to dosage regimes to maintain therapeutic levels. Alternatively, if one component is cleared at a faster rate problems associated with toxicity and sub-optimal efficiency may occur. The aim of this study was two-fold. Firstly to develop an HPLC system for the quantification of both Pn and Tz using a single isocratic method. Previously quantification of the two compounds required separate isocratic methods or gradient elution (Ocampo et al 1989). Secondly to use the assay developed to investigate the removal of each component by an in-vitro model of continuous HF. Briefly, a peristaltic pump (Hospal BSM22SC blood safety module) was used to regulate the flow of carrier fluid (3.5% human albumin solution in Tyrode buffer) at 100mL/min through a hollow fibre polyacylonitrile membrane (Multiflow 60; Hospal®). A heated bell jar was used to represent the patients body. Ultrafiltration rates (UFR's) were set at 15 or 25 mL/min throughout the course of the experiments using a peristaltic pump. Fluid removed as ultrafiltrate was replaced with Tyrode buffer.

Pre and post-filter circulating fluid was sampled serially together with ultrafiltrate. Protein was removed by centrifugation using an Amicon ultrafiltration unit (10kD MW cut off). Concentrations were determined using a novel HPLC method employing a 250 x 4.6mm propylamine column (Hichrom) and a mobile phase consisting of 15% methanol in phosphate buffer (pH3) at a flow rate of 1mL/min. A wavelength of 220nm was used for detection. Capacity factor values (k') of 1.7 and 2.1 were obtained for Tz and Pn respectively with a resolution factor of 2.4. Concentrations of Pn and Tz were determined by reference to standard curves (regression coefficient of 1.000 and 0.999, linear range 0.1 to 100μg/mL with a coefficient of variance of 1.94 and 1.92 respectively), plotted against time and used to calculate sieving coefficients (S) and filter clearances (Fcl) of each compound (Table 1).

Table 1. S and Fcl of Tazocin and Piperacillin during HF

Compound	UFR (mL/min)	Mean S ±SD	Mean Fcl ±SD (L/hr)
Tazobactam	15	0.98±0.16	0.91±0.15
Tazobactam	25	0.98±0.04	1.50±0.07
Piperacillin	15	0.92±0.09	0.86±0.08
Piperacillin	25	0.89±0.07	1.31±0.12

n=5 in all cases. Values compared using Mann-Whitney U test.

A significant difference was found between both the S and Fcl of Tz and Pn at UFR's of 25mL/min ($p<0.05$ in both cases) but not at the lower UFR of 15mL/min. These results suggest that during HF at high UFR's the systemic ratio of Piperacillin to Tazobactam is altered. Such changes may result in decreased efficiency of the preparation. This fact should be taken into account when administering Tazocin to patients on continuous HF.

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94 INFLUENCE OF CHIRALITY ON THE PHARMACOKINETICS OF AMINOGLUTETHIMIDE IN BREAST CANCER PATIENTS

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Aminoglutethimide (AG) was the first widely used aromatase inhibitor for the palliative management of advanced breast cancer in postmenopausal women. Despite the development of newer more potent and selective inhibitors, AG is still regularly used (Alshowaier & Nicholls 1997). The drug is used as a racemate although the R-enantiomer possesses the greater activity (2-3 times more potent than the racemate), whereas S-AG has very little action even at 10-fold higher concentrations (Graves & Salhanick 1979). In the absence of pharmacokinetic data of the enantiomers, it was considered useful to examine the plasma levels and urinary excretion of R- and S-AG following a dose of racemic AG.

Six postmenopausal women with advanced breast cancer received a single dose of AG (500mg, Orimeten). Plasma samples were taken at various times up to 24h and urine was collected for 48h. The AG enantiomers were assayed after extraction by the method of Alshowaier et al (1995).

For each enantiomer, plasma levels could be described by a single compartment model. Over the first 24h, plasma levels of R-AG were about 1.5 times higher than those of S-AG. There were significant ($P<0.05$) differences in C_{max}, 3.8(1.5) and 3.3(0.9)μg/ml, and $AUC_{0-\infty}$ 79.9(25.8) and 47.1(14.5)mgh/L (mean ± SEM) for R- and S-AG respectively. However, there were no significant differences in T_{max} 1.4(0.7) and 1.3(0.5)h, $t_{0.5}$ 16.8(9.2) and 11.1(3.7)h and V/F 77.0(31.1) and 85.8(27.6)L for R- and S-AG respectively.

There was a significant ($P<0.05$) difference in the 48h urinary excretion of the enantiomers 15.4(8.1) and 26.3(9.2)% of the dose as R- and S-AG respectively. Renal clearance (L/h) of S-AG (3.2±1.7) was significantly ($P<0.05$) greater than that of R-AG (1.4±0.8). However, there was no significant difference in metabolic clearance for the enantiomers. It is considered that the plasma level-based pharmacokinetic differences between the two enantiomers are related to the differences in their renal clearance. Overall these differences are small and it is concluded that they only contribute marginally to the activity of the racemic drug as an aromatase inhibitor in breast cancer patients.

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95 THE RELATIVE BIOAVAILABILITY OF SALBUTAMOL TO THE LUNG FOLLOWING NEBULISED THERAPY

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We have previously reported a method to determine the relative lung bioavailability of salbutamol to the lung and body following inhalation by both metered dose and dry powder inhalers. This method uses urinary excretion data at 0.5hrs and 0-24hrs post-inhalation, respectively (Hindle & Chrystyn, 1992). We have now extended this method to nebulised salbutamol.

The urinary excretion of salbutamol following oral (O), oral with 5g activated charcoal (O+C), metered dose inhaler (MDI) and MDI with 5g activated charcoal (MDI+C) have been compared. For each study a 100μg dose was taken at 0, 2, 4, 6, and 8 minutes and urine samples were collected at 0 and 30 minutes post (first) dose. Ten healthy volunteers whose mean (s.d.) age and weight was 28.8(6.9)yrs and 69.1(10.3)Kg completed the study. No salbutamol was excreted in the urine following O+C. The mean (s.d.) salbutamol excreted 0-30 minutes following O, MDI and MDI+C was 0.41(0.50), 11.01(3.58) and 11.66(3.49)μg respectively. These results indicate that oral absorption of salbutamol is insignificant in the first 30 minutes when the dose is given over 8 minutes. Thus, the urinary salbutamol excretion method is valid to use when administration is over a prolonged period e.g. nebulisation.

Urinary salbutamol excretion following oral (O), MDI, nebuliser (NEB) and nebuliser with 25g activated charcoal (NEB+C) has been determined. The nebuliser system used was a Micro-Neb III (Lifecare Hospital Supplies, UK) chamber attached to a Medix AC 2000 Hi-Flo compressor (Medix Ltd, UK). 5 x 100μg doses were swallowed or inhaled at 1.25 minute intervals for O and MDI and 2.5mg of salbutamol was added to the chamber for NEB and NEB+C and inhaled over 5 minutes. A nose clip prevented nasal breathing and all exhaled air passed through a filter to retain the expired salbutamol. Urine samples were collected at 0 and 30 minutes post dose. Eight healthy volunteers whose mean (s.d.) age and weight was 33.6(9.4)yrs and 68.9(5.6)Kg gave written consent for the study. The mean(s.d.) 0-30 minute salbutamol urinary excretion following O, MDI, NEB and NEB+C was 0.59(0.78), 12.72(3.13), 15.37(5.21) and 16.73(4.67)μg respectively. The mean(s.d.) amount of salbutamol left in the nebuliser and exhaled to the environment after nebulisation for NEB was 1326.53(108.78) and 507.44(56.49)μg and for NEB+C was 1228.99(117.75) and 538.44(102.42)μg respectively. Thus the mean(s.d.) dose available for inhalation was 666.03(102.06) and 732.67(98.53)μg. Expressed as a percentage of the dose available for inhalation, the mean(s.d.) salbutamol excreted in the urine during the first 30 minutes post dose for O, MDI, NEB and NEB+C was 0.12(0.16), 2.55(0.63), 2.28(0.53) and 2.31(0.66)% respectively. The mean difference (95% confidence interval) for the MDI compared to NEB and NEB+C was 0.27(-0.54,1.08) and 0.24(-0.63,1.11)% respectively. Similar values comparing NEB to NEB+C were -0.03(-0.25,0.19)%. These comparisons indicate no significant differences.

The results indicate that the majority of the dose available for nebulisation either remained in the chamber or was exhaled to the environment. The 30 minute urinary excretion values, when normalised for the dose delivered, indicate similar delivery of drug to the lung following inhalation by MDI, when using a trained MDI technique, and a nebuliser.

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96 COMPARISON OF THE URINARY EXCRETION OF SODIUM CROMOGLYCATE (SCG) FOLLOWING INHALATION USING DIFFERENT METHODS

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Drugs for the treatment and prophylaxis of asthma are delivered to the lung by inhalation and this is facilitated by the use of pressurised metered dose inhalers (MDIs), MDIs with spacer devices and dry powder inhalers (DPIs). The deposition pattern of drug in the respiratory tract is determined by a complex interaction between the device, the aerosol characteristics and the patient's inhalation technique (Newman 1995). Large spacer devices (like Fisonair®) have the beneficial effect of allowing time for the droplet size of the aerosol to decrease and do not require co-ordination between inspiration and actuation. These two factors increase the potential for more drug to be deposited into the lungs and reduce oropharyngeal deposition. Dry powder inhalers (DPIs) are also easy to use because they are breath actuated.

We have recently reported a high performance liquid chromatography assay together with a method to determine the relative lung bioavailability of SCG after inhaled administration using urinary excretion (Aswania et.al., 1997). We have used this method to compare the urinary SCG excretion following inhalation of SCG from an Intal® 5mg (MDI), Intal® 5mg MDI + Fisonair® 750ml spacer device (MDI+SPC) and Intal® 20mg Spincap® using a Spinhaler® (DPI). Ethical committee approval was obtained and all subjects gave written informed consent. Each volunteer inhaled either 4x5mg SCG from MDI, 4x5mg from MDI+SPC or 20mg from DPI. The order of administration was randomised and each subject was crossed over after 7 days. Urine was collected at 0.0, 0.5, 1.0 and up to 24 h post-inhalation. 7 (3 female) healthy volunteers whose mean (s.d.) age and weight was 32.0 (7.8) years and 72.8 (9.7) kg, completed the study. The mean (s.d.) urinary excretion of SCG is shown in table 1 and the statistical analysis is described in table 2.

Table 1. Mean (s.d.) SCG excreted post inhalation

	Mean (s.d.) µg SCG renally excreted		
	0.0 - 0.5 hr	0.0 - 1.0 hr	0.0 - 24 hr
MDI	38.3 (14.6)	78.1 (29.0)	387.5 (140.8)
MDI + SPC	245.6 (132.5)	384.7 (171.9)	1314.0 (767.0)
DPI	160.3 (95.8)	341.9 (195.6)	1347.0 (645.0)

Table 2. Statistical analysis

	Mean difference (95% Confidence interval) µg		
h. post dose	MDI vs. MDI+SPC	MDI vs. DPI	MDI+SPC vs. DPI
0.0 - 0.5	-207.4 (-333.4, -81.4)	-122.1 (-214.5, -29.7)	85.3 (-77.7, 248.3)
0.0 - 1.0	-306.6 (-475.9,-137.4)	-263.9 (-441.4, -86.4)	42.8 (-199.7, 285.2)
0.0 - 24	-926.0 (-1644, -209)	-960.0 (-1486, -433)	-33.0 (-1149, 1083)

The Intal® MDI with the Fisonair® spacer and the Intal® Spinhaler® both deliver more drug to the lung than the Intal® MDI. The statistical analysis of the results for the Intal® MDI when used with the Fisonair® spacer and the Intal® Spinhaler® indicates that the two are bioequivalent.

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97 THE RELATIVE BIOAVAILABILITY OF NEDOCROMIL SODIUM FOLLOWING ORAL AND INHALED DOSING

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Nedocromil sodium (NCS) used topically by inhalation in the treatment of asthma (Orr, 1991) is poorly absorbed from the gastrointestinal tract (2-3% of the oral dose). Greater than 80% of an intravenous dose is excreted in the urine and 88% of the total urinary excretion occurs within 90 min after an intravenous dose (Neale, et.al, 1987). These data suggested that urinary excretion of NCS may be used as an indirect estimate of the in-vivo lung deposition and hence similar to methods we have described for salbutamol (Hindle & Chrystyn, 1992) and sodium cromoglycate (Aswania, et.al, 1997).

We have previously reported a simple reversed-phase ion-pair high performance liquid chromatographic (HPLC) assay with solid phase extraction to quantify sodium cromoglycate (SCG) in the urine. This HPLC method used NCS as the internal standard (Aswania, et.al, 1997). Thus this method could be suitable for the measurement of urinary NCS excretion. NCS was extracted from urine using a 100mg phenyl cartridge (Isolute, Jones Chromatography, UK), then quantified on a 5µ Spherisorb C_8 25cm x 4mm i.d. stationary phase. The mobile phase was methanol : 0.045M phosphate buffer : 0.5M dodecyl triethyl ammonium phosphate (550:447.6:2.4 v/v), adjusted to pH 2.3 at a flow rate of 0.85 ml min^{-1}. UV detection at 256nm was used and the internal standard was SCG.

The mean (SD) absolute recovery of NCS from urine over the concentration range 0.075-3.0 µg ml^{-1} was 90.4 (4.3)%. The mean (SD) intra-assay accuracy and precision for urine samples over the same concentration range was 99.9 (1.56) and 7.05 (4.95)%, respectively. These values for inter-assay accuracy and precision were 102.4 (4.08) and 10.53 (2.66)%, respectively. The assay was linear over the concentration range investigated with r≥0.9997.

In 10 healthy volunteers the mean (SD) amount of NCS excreted in the urine at 0.5 and 24h post inhalation of 8mg NCS (4 puffs from a Tilade® inhaler) were 41.0 (19.5) and 319.87 (138.1)µg, respectively. In contrast those values after oral administration of 8mg of NCS were 2.07 (2.17) and 74.43 (58.8)µg, respectively. The rate of urinary NCS excretion during the 24h period post inhalation and oral dosing is shown in Fig 1.

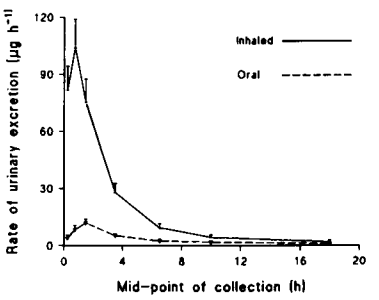

Fig 1. Mean rate of urinary excretion of NCS during 24h collection period

Urinary excretion of NCS offers the potential to determine the relative bioavailability to the lung following inhalation.

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98 DETERMINATION OF ANTIGLIADIN ANTIBODIES IN SALIVA FROM COELIAC PATIENTS BY ELISA-BASED ASSAY

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Coeliac disease is a permanent intolerance to dietary gliadin (alcohol soluble protein fraction of wheat gluten) resulting in small intestinal villous atrophy with consequent malabsorption and malnutrition (Trier 1991) and the presence of high levels of sera antigliadin antibodies of IGA class (Engstrom et al 1992). A Gluten-free diet is life-long treatment for such patients. Diagnostic procedure is a long lasting and highly invasive procedure including at least three duodenal biopsies (Meeuwise 1970).

The main aim of our work was development of a specific ELISA-based assay for detection and quantification of antigliadin antibodies in saliva from coeliac patients that can be used in diagnostic procedures.

Saliva from 12 coeliac patients on a gluten-free diet, as well as saliva from a control group of 12 healthy human subjects was collected without stimulation through filter paper in tubes with an enzyme inhibitor (Benzamidine-HCl 6 mmol L^{-1} in ε-amino capronic acid 30 mmol L^{-1} in phosphate buffer pH 7.4) and samples were examined using an ELISA assay with wheat gliadin (commercial and extracted by ourselves) as antigen and prolamins from maize and rice as negative control. Secondary antibody was rabbit antihuman IgA + goat antirabbit IgG (H + L) labelled with horseradish peroxidase (Bio-Rad). Specific substrate for peroxidase was fast OPD (Sigma). Developed colour was measured at 492 nm in a microplate reader Titertek Multiscan (Flow Laboratories). Results showed the presence of high levels of IgA antigliadin antibodies in all investigated saliva from coeliac patients and very good correlation with clinical status. The proposed technique can be used in diagnostic procedures and in monitoring of the clinical status and dietary treatment for patients on gluten-free diets.

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99 A RAPID HPLC METHOD FOR THE DETERMINATION OF LAMOTRIGINE IN SMALL SAMPLE VOLUMES OF PLASMA

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Lamotrigine is a new and effective antiepileptic drug of use in the management of partial and tonic-clonic seizures. A number of hplc assays are available for the measurement of lamotrigine in plasma (e.g. Meyler et al 1993; Sinz & Remmel 1991). Several of these methods involve large volumes of organic solvents, lengthy extraction procedures and poor recovery. The present work describes a simple, rapid, selective and reproducible reversed-phase hplc micro-assay of the determination of lamotrigine in plasma.

The drug was extracted from 100µl of plasma with chloroform: isopropanol (95:5%, v/v) in the presence of 100µl of phosphate buffer (10mM). The extract was evaporated and the residue was reconstituted with mobile phase and injected onto the hplc system. The drug and the internal standard (chloramphenicol) were eluted from a Symmetry C_{18} stainless steel column at ambient temperature with a mobile phase consisting of 0.01M potassium phosphate: acetonitrile:methanol (70:20:10%, v/v), adjusted to pH 6.7, at a flow rate of 1.3ml/min, and the eluate was monitored at 214nm. Retention times were 4.0 min (lamotrigine) and 6.65 min (chloramphenicol). Quantitation was achieved by measurement of the peak-area ratio of the drug to the internal standard and the lower limit of detection for lamotrigine in plasma was 20ng/ml. The intraday precision ranged from 3.34 to 6.12% CV and the interday precision ranged from 2.15 to 8.34% CV. The absolute and relative recoveries of lamotrigine ranged from 86.93 to 90.71% and from 95.18 to 107.13% respectively. None of the commonly used antiepileptic drugs or their main metabolites interfered with the assay.

The method was applied to the determination of lamotrigine in plasma collected at various times, after an oral dose (18.6mg/kg), from a group of 6 New Zealand White rabbits. C_{max} values of 5.11±0.69µg/ml were reached in 0.94±0.48h. The $t_{0.5}$ was 5.5±1.65 h and the $AUC_{0-\infty}$ was 23.9±6.05µg h/ml. This reliable micro-method would have application in pharmacokinetic studies of lamotrigine where only small sample sizes are available e.g. paediatric patients.

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100 PHARMACOKINETICS AND URINARY EXCRETION OF CYCLOPHOSPHAMIDE IN BONE MARROW TRANSPLANT PATIENTS: A LINK TO HAEMORRHAGIC CYSTITIS

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Cyclophosphamide (CP) is often employed at high dose as part of the immunosuppressive conditioning regimens in bone marrow transplant (BMT) recipients. However, one of its major dose-limiting side effects is haemorrhagic cystitis (HC; Cox 1979). This has been attributed to a high urinary concentration of acrolein, a urotoxic metabolite of CP, resulting from altered pharmacokinetics of CP (Friedman et al 1979). As part of an investigation of CP-induced HC, the present study has examined the pharmacokinetics and urinary excretion of CP in 16 patients (9 males and 7 females) with leukaemia or aplastic anaemia who received 50mg/kg of this drug by 1-hour infusion in an immunosuppressive regimen prior to BMT. This dose was repeated 24, 48 and 72h after dosing was initiated, however, blood samples were collected at several time intervals only within the first 24h after the first dose. Urine was also collected during this interval and CP was analyzed in plasma and urine by capillary gas chromatography with thermionic specific N-P detection. The concentration-time data of CP exhibited the characteristics of a two-compartment open model quite well. The mean (SEM) values of α, β, k_{12}, k_{21}, k_{10}, V_C, V_{SS}, AUC, total clearance, and mean residence time observed were 1.29 (0.31) h^{-1}, 0.17 (0.03) h^{-1}, 0.48 (0.16) h^{-1}, 0.34 (0.09) h^{-1}, 0.64 0.17) h^{-1}, 0.51 (0.15) L\kg, 0.67 (0.13) L/kg, 657.4 (208.5) mg.h/L, 0.14 (0.02) L/h.kg, and 5.88 (1.12) h, respectively. The rate of urinary excretion of unchanged CP in 24h was 17.5 (4.4) mg/h and the renal clearance was 0.0188 (0.002) L/h.kg. Of the 16 patients included in this study, only two developed HC. By comparing the data obtained for each of these two patients to that generated for the remaining patients who did not develop HC, it was found that, while the plasma-derived pharmacokinetic parameters of these two patients did not differ significantly from those of the other 14 (non-HC) patients, the HC patients excreted 4.4 to 5.6 times ($p<0.01$) more CP than did the non-HC patients. It is noteworthy that this increase in excretion of CP in the patients who developed HC occurred principally within the first eight hours of CP administration.

If this finding is corroborated in further studies, it may be possible to apply the phenomenon of an abnormally high urinary excretion of CP as an index of increased risk of developing HC.

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101 PLASMA AND SALIVA LEVELS OF BECLAMIDE IN MAN AFTER A SINGLE ORAL DOSE

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Beclamide (N-benzyl-3-chloropropionamide) is an anticonvulsant that has been successfully used in the management of behavioural disorders associated with epilepsy (Sharpe et al 1958). Although the drug is no longer marketed, interest in it and related compounds continues as both the drug and a novel analogue display a very useful degree of antiaggressive activity without sedation (Darmani et al 1990). The present study describes the plasma and salivary levels of the drug following a single dose. The plasma data were used to estimate various pharmacokinetic parameters.

Eight healthy drug- and alcohol-free adult male volunteers (22-32 yr) swallowed beclamide (2x500mg Nydrane tablets, Lipha Pharmaceuticals) after an overnight fast. Blood samples by forearm venepuncture were taken pre-dosing and at various times up to 8h post-dosing. Unstimulated saliva was also collected for 5 min at each time point. Belcamide in plasma and saliva was assayed by hplc using hexobarbitone as internal standard. The samples, adjusted to pH 7.4, were extracted with diethyl ether. The latter was evaporated and the residue dissolved in methanol for chromatography (250x4.5mm, 5µm Apex ODS column; mobile phase acetonitrile/water, 22.5/77.5 v/v containing 0.14g KH_2PO_4/dL; ambient temperature; flow rate, 2mL/min; detection at 215nm; retention times, 8.8 min beclamide, 14.0 min hexobarbitone). Binding of beclamide to plasma was assessed by equilibrium dialysis.

Peak plasma levels, reached within 1-3h, ranged from 10.0-19.4µg/mL. For absorption, the mean $t_{0.5}$ was 0.7h and the mean lag phase was 0.2h. The terminal phase of the plasma profile was mono-exponential. Assuming a single compartment for distribution and using F=1.0 (obtained from ^{14}C-beclamide studies in man; Nicholls et al 1979), the following parameters were estimated:- elimination $t_{0.5}$, 3.3±0.7h; apparent volume of distribution, 57.5±9.9L; plasma clearance, 12.5±3.4mL/min. Over the range of plasma levels found, 61.8-69.9% of beclamide was bound to plasma protein. Peak saliva levels (5.1±1.6µg/mL) were reached within 1-3h. Good correlation between salivary concentrations of the drug with both total (r=0.84) and free (r=0.98) plasma beclamide was obtained. In view of the simplicity of the hplc assay, the mild overt pharmacological effects of beclamide and the close correlation of its concentrations in plasma and saliva, it is proposed that the drug may be a useful tool for studies of factors affecting the salivary excretion of drugs.

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102 INHIBITION OF AROMATASE ($P450_{AROM}$) BY SOME 1-(BENZOFURAN-2-YLMETHYL) IMIDAZOLES

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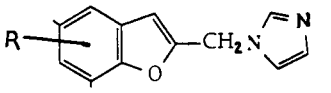
Aromatase ($P450_{AROM}$) catalyses the final step in the steroidogenic pathway for the synthesis of oestrogens. A high proportion of breast tumours in postmenopausal women is dependent on oestrogen for growth and after surgery oestrogen deprivation strategies are used to oppose development of metastases. Aromatase inhibitors have been used in the clinic following relapse on tamoxifen therapy (Brodie 1994). Several potent reversible non-steroidal inhibitors of the enzyme are known which are more specific inhibitors of the target enzyme (see Miller 1996) than the well established aminoglutethimide (AG) without its undesirable side effects. We describe here some 1-(benzofuran-2-ylmethyl) imidazoles (**1-5**) as inhibitors of the enzyme.

Incubation mixtures, (0.5ml) in triplicate, containing [1β,^{3}H]-androstenedione (24Ci mmol^{-1}, final concentration 0.5μM, 10μl), NADPH-generating system (50μl) in phosphate buffer (50mM, pH7.4) and with and without inhibitor in ethanol (10μl) were warmed to 37°C and then placental microsomes (0.452mgml^{-1} 20μl) added. After 6 min incubation an aliquot (300μl) was added to activated charcoal (1%, 900μl) and mercuric choloride (1mM, 300μl), mixed thoroughly for 15 min and then centrifuged (2000xg) for 20 min. Aliquots (500μl) of the supernatant were dispersed in scintillation fluid ('Hisafe') and counted for ^{3}H on a liquid scintillation counter.

The 1-(benzofuran-2-ylmethyl-imidazoles, **1-5**, were about 4-7 fold more potent than AG (Table 1), the most potent being **2** and **3**. Mono substitution of a 5-Cl or 5-Br increases activity over the unsubstituted compound **1** whereas 5,7-disubstitution has little effect on activity.

1-[(Benzofuran-2-yl) phenylmethyl] imidazoles, **6**, are potent inhibitors (IC_{50} *ca* 10nM) of $P450_{AROM}$ (Whomsley et al 1993).

Table 1. Inhibition of $P450_{AROM}$ by some imidazoles



Compound	IC_{50}(μM) (±S.D.)*
AG	28.69 ± 1.65
1 : R = H	7.32 ± 0.12
2 : R = 5-Cl	3.9 ± 0.02
3 : R = 5-Br	3.91 ± 0.08
4 : R = 5,7-DiCl	6.25 ± 0.3
5 : R = 5,7-DiBr	8.06 ± 0.1

*Androstenedione = 0.5μM. Values are the mean of 3 determinations each in triplicate.

Using a model of aromatase derived from homology modelling (Laughton et al 1993) we, with Dr. C. Laughton, have modelled **6**. The phenyl and benzofuran could be equally accommodated either along the binding site for the steroid backbone or in the hydrophobic cavity below the A-ring. Apparently removal of a phenyl group from **6** to give **5** considerably reduces activity by *ca* 3 orders through either of these hydrophobic interactions.

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103 SOME 1-(BENZOFURAN-2-YLMETHYL) IMIDAZOLES AS INHIBITORS OF 17α-HYDROXYLASE: 17,20-LYASE(P450 17) AND THEIR SPECIFICITY PATTERNS

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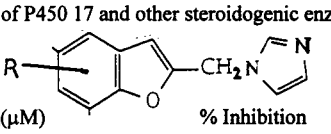
Therapies for prostatic cancer to remove androgen support are directed at suppression of synthesis of testicular testosterone (orchidectomy, LH-RH agonists, inhibitors of P450 17) and blocking the combination of androgen with its receptors (anti-androgens) (Grayhack et al 1987; Denis 1991). Androstenedione is produced from progesterone by P450 17 and then converted to testosterone and finally dihydrotestosterone responsible for prostatic growth. Ketoconazole, an inhibitor of P450 17, has been used clinically at high oral doses but frequent dosing is required and gastrointestinal and hepatic side effects appear (Vanden Bossche & Moereels 1994). A need for a less toxic P450 17 inhibitor exists and we have synthesised several novel 1-(benzofuran-2-ylmethyl)imidazoles and examined their inhibitory profiles towards human testicular P450 17, and enzymes involved in cortisol production i.e. CSCC ($P450_{scc}$), 21-hydroxylase ($P450_{c21}$) and 11β-hydroxylase ($P450_{11\beta}$).

P450 17: Incubations contained [1,2,6,7-^{3}H]17α-hydroxy-progesterone (6μM) NADPH generating system (50mM, 50μl), with or without inhibitor in ethanol (10μl) and testicular microsomal enzyme (0.162mgml^{-1}) in phosphate buffer (50mM, pH7.4). After 30 min at 37°C the steroids were extracted and run on TLC plates using $CHCl_3$:C_6H_{12}:CH_3COOEt:CH_3OH (80:10:10:4) with 17α-hydroxyprogesterone, androstenedione and testosterone. The relevant spots were counted for ^{3}H. $P450_{c21}$:[4-^{14}C]progesterone (2.12μM), rat adrenal enzyme (10,000xg fraction; 1.38mgml^{-1}) were used for 10 min. $P450_{11\beta}$: [1,2,6,7-^{3}H] 21-hydroxy progesterone (1μM) and bovine adrenal enzyme (10,000xg fraction (0.21mgml^{-1}) for 15 min.

Table 1. Inhibition of P450 17 and other steroidogenic enzymes



Compound	IC_{50}(μM) $P450_{17}$	% Inhibition $P450_{c21}$	% Inhibition $P450_{scc}$	IC_{50}(μM) $P450_{11\beta}$
1 : R=5-Br	0.38	32.5(52.1)	21.9(2.9)	0.5
2 : R=5,7-DiBr	0.19	57.0(45.5)	13.8(0.91)	138.4
3 : R=5-Cl	0.23	21.0(20.3)	31.7(2.5)	0.5
4 : R=5,7-DiCl	0.18	57.7(43.8)	10.2(0.63)	81
Ketoconazole	0.03	7.9	95.9	90.9

$P450_{scc}$: [26,27-^{3}H]25-hydroxycholesterol (24.81μM) and bovine adrenal enzyme (10,000xg fraction 0.7mgml^{-1}) for 30 min. Compounds **2**, **3** and **4** are 6-7 fold and **1** 13-fold less potent than KC as inhibitors of P450 17 (Table 1) and overall less potent towards $P450_{scc}$ and more potent against $P450_{c21}$ and $P450_{11\beta}$ (except for **2**). Potential side effects on cortisol production by comparison with KC when administered on a theoretical equi-potent dose for P450 17 inhibition were calculated from (P450 17 IC_{50} compound/P450 17 IC_{50} KC) x (P450 % inhibition compound/P450 % inhibition KC) where a value of <1 shows greater selectivity than KC (parentheses in Table 1). Compounds **2** and **4** were more selective towards $P450_{scc}$ but less selective towards $P450_{11\beta}$ than ketoconazole.

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104 OESTROGEN-INDUCED RELAXATION IN CALCIUM-DEPLETED AORTA

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Oestrogens have been shown to reduce cardiovascular morbidity and mortality (Henderson & Paganini-Hill, 1991); this may be related to their vasodilator actions. Several mechanisms have been proposed to explain relaxation in different blood vessels including inhibition of protein kinase C (Magness et al., 1989) and reduction in calcium (Ca^{++}) influx (Shan et al., 1994). However, we noted oestrogen-induced relaxation of aorta in a Ca^{++}-free solution in which calcium influx is unlikely to occur (Babaei *et al.* 1995). In the present experiments we compared the ability of oestrogens and other agents of diverse structure and mechanism of action to relax contractions elicited by $PGF_{2\alpha}$ receptor activation at the membrane or by stimulation of an intracellular component of the contraction-activating system, protein kinase C, by a phorbol ester, phorbol dibutyrate (PDB).

Aortic rings 3-4mm wide were prepared from male Hooded Lister rats (250-350g), set up in Krebs' solution containing 10μM indomethacin under 2g tension (37°C, 95% O_2, 5% CO_2) and contracted with $PGF_{2\alpha}$ (10μM), an approximately EC_{80} concentration giving a response of 1.77 ± 0.03g. When contraction was stable a range of agents was applied for 40 mins: β-oestradiol (β-EST, 1-20μM), 17α-ethinyl oestradiol (ET-EST, 1-20μM), tamoxifen (TXF, 1-20μM), progesterone (PROG, 1-20μM), bisindolylmaleimide, a specific protein kinase C inhibitor (BIM, 1-10μM) or nifedipine, a calcium channel blocker (NIF, 0.01-0.1μM). Only one agent was used in each tissue. All agents caused dose-related relaxation, expressed as % reversal of contraction (mean ± SE) (Table 1). No vehicle effects were observed at the concentrations used. NIF and ET-EST caused 100% relaxation but BIM was relatively ineffective.

To ascertain whether these agents relaxed aorta in the absence of Ca^{++} when contraction was stimulated by activation of protein kinase C, tissues were incubated in Ca-free Krebs' containing 100μM EDTA for 120 mins. Phorbol dibutyrate (PDB, 40nM) was added to the tissue; a stable tension of 0.83 + 0.03g was produced.

Table 1. Relaxation (%) of contraction induced by $PGF_{2\alpha}$ (10μM) in normal Krebs' solution or phorbol dibutyrate (40nM) in Ca^{++}-free solution. **Significantly different from relevant group. P<0.001.

Relaxant	$PGF_{2\alpha}$	(n)	PDB (Ca++-free)	(n)
β-EST(20μM)	57.5 ± 4.2	(10)	52.6 ± 3.6	(17)
ET-EST(20μM)	100	(4)	66.0 ± 7.0**	(12)
TXF(20μM)	30.7 ± 9.9	(6)	36.9 ± 4.4	(8)
PROG(20μM)	49.3 ± 4.5	(7)	45.3 ± 5.0	(8)
BIM(1μM)	10.0 ± 1.8	(4)	100**	(4)
NIF(0.1μM)	100	(5)	-3.6 ± 4.6**	(14)

β-EST, ET-EST, TXF, PROG, BIM and NIF, at the above concentrations were applied for 40 mins. All agents, except NIF, produced concentration related relaxation. BIM, a highly selective inhibitor of protein kinase C, caused 100% relaxation. β-EST, TXF and PROG relaxed the tissue to the same extent as in normal Krebs' but relaxation by the synthetic oestrogen ET-EST was reduced. NIF was completely ineffective and caused no relaxation. We conclude that in rat aorta a substantial component of oestrogen-induced relaxation is independent of Ca^{++} channel blockade.

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105 DRUG DISCRIMINATION STUDIES USING THE NOVEL, ORALLY-ACTIVE ENKEPHALIN CATABOLISM INHIBITOR RB1000

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RB 1000 is the first orally active mixed inhibitor of enkephalin degradation that is able to cross the blood brain barrier. Its effects at doses producing strong analgesic responses (Roques et. al., 1997) were investigated using an operant drug discrimination paradigm. The ability of morphine (MP) to act as a discriminative cue in rats is well established (Colpaert, 1978). Male Wistar rats (270-350, n=10) were trained in a food-reinforced two lever choice task to select an appropriate lever depending on whether they had been injected with MP or vehicle before the training session. When at selection criteria, RB 1000 was tested for its ability to replace or "generalise" to the trained MP cue by inducing a MP appropriate lever selection. A separate group of rats (n=10) was trained to determine whether RB 1000 could act as a discriminative cue in its own right.

The group of rats receiving MP 3.0 mg/kg i.p (10 minutes before the session) acquired the discrimination in 48.4 ± 5.05 sessions (range 24-77 sessions) and were utilised in generalisation testing. Those rats receiving RB 1000 10 mg/kg i.p or its vehicle (10 minutes before the session) failed to acquire any discrimination between the treatments after 80 training sessions. Table 1 depicts the results of the generalisation study. A dose dependent increase in MP lever selection was observed with increasing doses of MP. Administration of RB 1000 at 0, 5, 10 and 20 mg/kg, however, caused no notable drug lever responding. Animals treated at the highest dose of RB 1000 (50 mg/kg) failed to respond to either of the presented levers. Under these conditions RB 1000 at the doses showing lever responding, did not generalise to the MP stimulus. The inability of the enkephalins protected by RB 1000 to generate a discriminative cue or generalise to MP suggests that this analgesic drug may have a low abuse liability.

Drug	Dose ($mgkg^{-1}$)	% Choosing MP lever (1)	Error Score (2)
MP	0	0	10.0 ± 0
	0.15	33.3	6.7 ± 1.9
	0.75	50.0	5.0 ± 2.0
	2.25	71.4	2.9 ± 1.7*
	3.0	66.7	3.3 ± 1.9*
	3.75	100.0	0.0 ± 0.0*
RB1000	0.0	0.0	10.0 ± 0.0
	5	0.0	10.0 ± 0.0
	10	12.5	8.75 ± 1.2
	20	0.0	10.0 ± 0.0
	50	nr	nr

Table 1. (1) Percentage of animals in that session selecting the drug lever. (2) Mean ± SEM number of responses on vehicle lever before drug lever was pressed 10 times. Animals selecting the vehicle lever only scored 10. *P< 0.005 one way ANOVA with Dunnetts post hoc test. (n = 5-9; nr = no response)

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106 EFFECTS OF COMBINATIONS OF ROXATIDINE AND AN_{12} ON EXPERIMENTAL STOMACH ULCERS AND GASTRIC ACID SECRETION IN RATS

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In the last decade the pharmacotherapy of gastric ulcer disease has improved with the use of new generations of H_2-blockers, anti-Helicobacter agents, etc. (Ivanov et al 1996; Scholtholt et al 1988). In 1993, Suzuki et al observed that some antipsychotic agents inhibit experimental ulcerogenesis. On the basis of our previous pharmacological studies, a combination of H_2-blocker roxatidine, synthesized at CPRI, and the potential antidepressant drug, 4-(4-bromophenyl)-2-methyl-1,2,3,4-tetrahydro-isoquinoline (code name AN_{12}) was selected for investigation (Ichikawa et al 1994; Mondeshka et al 1994). The activities of these combinations were tested on : water-immersion stress-induced lesions (according to Takagi et al 1964); indomethacin-induced lesions (according to Satoh et al 1981) and gastric acid secretion (by the method of Shay et al 1945). The combinations tested orally (roxatidine 12.5 mg kg^{-1} plus AN_{12} 1 or 2 mg kg^{-1}) significantly inhibited the development of stress-induced ulcers and this effect was similar to that of roxatidine alone (100 mg kg^{-1}). The concomitant application of both substances at doses: roxatidine (25 or 50 mg kg^{-1}) and AN_{12} (1 mg kg^{-1}p.o.) caused more changes in the parameters tested compared with those induced only by roxatidine at 25 mg kg^{-1} (Table 1). The advantages of the combinations were: high antiulcer and antacid activity; synergistic effect of the components (additive for the stomach juice) and a considerable decrease in dosage of H_2-blocker roxatidine. The antiulcer combination (roxatidine plus AN_{12}) may thus be a potential candidate for the treatment of ulcer disease. Further pharmacodynamic and toxicological studies are in progress to clarify the action of this combination.

Table 1. Effects of the combination of roxatidine and AN_{12} (25:1 mg kg^{-1}); AN_{12} alone (1 mg kg^{-1}) and roxatidine alone (25 mg kg^{-1}) on the gastric juice in rats, n=20 in each group, mean ± s.e.m.):

Treatment	Gastric juice mL/5h	Initial pH	Acid output (meq H^+/5h
Control	2.90 ± 0.32	2.33 ± 0.06	84.22 ± 5.4
Combination	1.06 ± 0.40	3.33 ± 0.80	42.61 ± 6.2
AN_{12}	1.99 ± 0.42	2.53 ± 0.14	55.10 ± 8.50
Control	2.57 ± 1.00	2.53 ± 0.22	102.0 ± 20.10
Roxatidine	1.82 ± 0.90	3.25 ± 0.58**	83.34 ± 11.20

*P<0.02; **P<0.001

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107 EFFECTS OF OREN-GEDOKU-TO AND UNSEI-IN, CHINESE TRADITIONAL MEDICINES, ON INTERLEUKIN-8 AND SUPEROXIDE DISMUTASE IN RATS

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The Chinese traditional blended medicine Oren-gedoku-to contains four components: Coptis Rhizome, Scutellaria Root, Phellodendron Bark, and Gardenia Fruit. Another Chinese traditional blended medicine, Unsei-in, contains Rehamannia Root,Japanese Angelica Root, Cnidium Rhizome,and Peony Root, as well as each component of Oren-gedoku-to. These traditional medicines have been clinically used for many years for the treatment of various diseases, such as Behcet's disease and rheumatic arthritis, but they are mainly supported by clinical experience in China and Japan. There are already numerous clinical reports on Oren-gedoku-to and Unsei- in. The present study was undertaken to clarify the anti-inflammatory effects of Oren-gedoku-to and Unsei-in, using acetic acid-induced inflammation in the rat, with respect to IL-8 production and SOD.

The following drugs were used: Oren-gedoku-to, Unsei-in, and ketoprofen were donated by the respective companies. The other materials were obtained from commercial sources: acetic acid and the Rat IL-8 kit and the SOD Wako test.

These medicines reduced IL-8 production: that of the Oren-gedoku-to group was 32.4 pg mL-1, and that of the Unsei-in group was 36.9 pg mL-1; these values were significantly different from that of the control group (53.8 pg mL-1). Serum SOD was more increased in the acetic acid-induced group than in the normal group ($P<0.05$), but these drugs did not show any effects on the serum SOD .

The process of inflammation can be broken down into three phases: the first phase features increased capillary permeability; the second phase is represented by the migration of leukocytes; and the proliferation of connective tissue is the hallmark of the third phase. Our results suggested that these medicines can be considered to exert their anti-inflammatory effects mainly on the first and second phases, because they inhibit capillary pearmeability, and because they reduce IL-8 production and increase SOD. Our laboratory has just begun studying IL-8. The present study utilized CINC (cytokine-induced neutrophil chemoattractant), which is the rat counterpart of human growth-regulated gene product.

108 A COMPARISON OF THE ANTI-ASTHMATIC EFFECTS OF FRUSEMIDE ON GUINEA-PIG PERFUSED HALF LUNGS AND PARENCHYMAL STRIPS IN-VITRO

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Much work has been undertaken suggesting that inhaled frusemide is of value in the management of asthma, particularly in patients suffering from the 'extrinsic' exercise and allergen - induced disease (O'Connor et al 1994). Although it has been demonstrated that the drug reduces bronchoconstriction induced by agents such as sodium metabisulphite (O'Connor et al 1994) and adenosine -5- monophosphate (Paesa et al 1990) in asthmatic subjects, it does not affect contractions of human and bovine isolated upper airway smooth muscle in vitro induced by hypertonic saline, histamine and potassium chloride (Knox & Ajao 1990). The mechanism by which its bronchoprotective action is mediated is unclear. The aim of the present study was to determine whether the antibronchoconstrictor action of frusemide could be detected in vitro using isolated preparations of more peripheral sites of the lung, namely the parenchymal lung strip and perfused half lung of the guinea pig.

Male Dunkin - Hartley guinea - pigs (250 - 500g) were killed by cervical dislocation and the respiratory tract was dissected in its entirety from the animal. The lungs were separated and either perfused through the main bronchus at a constant flow rate of 5ml min^{-1} with Krebs solution (PL) or one parenchymal strip (PS) was cut from the edge of each of the right and left upper lobes. The strips were then suspended in aerated Krebs solution. In both cases the temperature was set at 37°c Dose - response (DR) profiles to histamine were then established for the PL (rise in perfusion pressure, pp), PS (isotonic contraction) in the presence and absence of frusemide (10^{-5}M). The profiles were analysed pre - and post - frusemide exposure (Table 1) where results are means +/- s.e.m. (n=4); *P < 0.05.

Table 1. Effect of frusemide (10^{-5}M) on histamine - induced bronchoconstriction in guinea - pig perfused lung and parenchymal strips

	Histamine dose (µg)	Pre -	Post - frusemide
PL (pp mmHg)	1	8.0 +/- 1.2	3.3 +/- 2..2*
	3	23.6 +/- 3.8	13.3 +/- 1.5*
	10	49.1 +/- 12.0	25.5 +/- 7.7
PS	1	23.2 +/- 20.1	11.3 +/- 18.5
(% max. response)	3	31.5 +/- 17.5	13.2 +/- 12.5
	10	40.0 +/- 19.2	20.1 +/- 12.9

The data illustrate that the anti - bronchoconstrictor effect of frusemide is well demonstrated in the perfused half lung preparation. This suggests that the anti - asthmatic effect of the drug is exerted on divisions of the bronchial tree lower than those examined by Knox & Ajao (1990). However, it will be observed that frusemide was unable to influence the action of histamine on the parenchymal strip. This could indicate that this preparation may not be suitable for further studies of the anti - asthmatic actions of frusemide.

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109 THE EFFECT OF LIPOPOLYSACCHARIDE (LPS) AND β 1,3-D-GLUCAN EITHER ALONE OR IN COMBINATION ON MAST CELLS ISOLATED FROM GUINEA-PIG LUNG: A PRELIMINARY STUDY

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Mast cells are known to play a central role in a range of inflammatory disorders including airway hyperresponsitivity. They are often regarded as being responsible for the immediate effects seen on lung function typified by bronchial asthma (Pearce 1990). Organic dusts and their components are known to be responsible for a range of allergic responses (Rylander and Jacobs 1994). However, the important constituents of such dusts and their effect on lung mast cells are not well understood.LPS (endotoxin) is a component of Gram-negative bacterial cell membranes while β 1,3 -D-Glucan is a fungal cell wall component, both are found in organic dusts. They have been implicated in airway hyperresponsiveness although their mechanisms of action and effects on various immunological responses are poorly defined.The present study aims to examine the effects of LPS and β 1,3 -D-Glucan either alone or in combination, on mast cell enriched cell isolates with measurement of histamine release as a marker of activation.

Lung mast cells from male Dunkin - Hartley guinea pigs (250-300g) were obtained by a standard enzymic dispersion procedure using collagenase (Ali and Pearce 1985). The cell isolate typically contained 4-5% mast cells as calculated using alcian blue staining, with a 90% active cell population when stained with Trypan blue. A soluble Glucan and LPS , from Sigma, were incubated for 30 mins at 37°C with the cell isolate, in a final volume of 0.3ml containing 10 to 20 x 10^3 mast cells. Calcium Ionophore A23187 was employed as a positive control. Supernatants (SN) were removed by centrifugation and the cell pellet (CP) resuspended. Levels of histamine in both the CP and SN were then examined using a modified OPT fluorescent assay based on the method of Shore (1959). Spontaneous release was typically less than 10%.

Table 1: Histamine release from guinea pig lung tissue - preliminary results of an ongoing study (n=3, values ± SEM)

Stimulus	Histamine Release %
A23187 10µM	28.9 ± 2.1
Glucan 1µg/ml	0.7 ± 0.6
LPS 1µg/ml	6.3 ± 1.9
Glucan 1µg/ml + LPS 1µg/ml	15.5 ± 4.2

From Table 1 it can be seen that Glucan (1µg/ml) was ineffective, while a small but definite release of histamine occurred under the influence of LPS. In combination there was an approximate two to three fold greater release of histamine than with LPS alone. This may reflect an important synergy relevant to occupational asthma arising from inhaled organic dusts.

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110 AIRWAY RESPONSIVENESS AND INFLAMMATION IN GUINEA-PIGS FOLLOWING THE INHALATION OF *ESCHERICHIA COLI* LIPOPOLYSACCHARIDE

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Endotoxins are lipopolysaccharide (LPS)-protein complexes located on the outer membrane of Gram-negative bacteria (Raetz 1990). When inhaled as a component of various organic dusts, they are associated with the development of organic dust toxic syndrome (ODTS), a lung condition identified in workers in a variety of occupational environments (Rylander & Vesterlund 1982). As part of a study of ODTS, it was found that airway hyperresponsiveness to carbachol occurred 1h after inhalation of LPS by guinea pigs (Broadley et al 1993). The present investigation extends this observation by examining airway responses to other bronchoconstrictors and identifying inflammatory signs following inhalation of LPS.

Male guinea pigs (400g) inhaled an aerosol (50% particles < 5.8 microns) of a 20μg/ml solution of E.coli serotype 026:B6 LPS for 1h. Responses (changes in specific airway conductance) to inhaled aerosols of bronchoconstrictor (methacholine, U46619 - a thromboxane A_2 analogue, 5HT) solutions for 1 min were measured before and at several times after exposure to LPS by whole body plethysmography and expressed as % change from baseline. Inflammation was assessed by measuring cell content and type in bronchoalveolar lavage fluid (BALF); protein and surfactant were also measured in BALF (Richards & Curtis 1984).

One h after exposure to LPS, there was an increase in airway responsiveness to methacholine. This was no longer present at 4h. At 24-48h, the lungs were hyporesponsive to methacholine but by 72h were normoresponsive. Reactivity to U46619 was significantly increased at 1, 4 and 24h after LPS exposure but the responsiveness had returned to pre-LPS levels by 48-72h. Hyperresponsiveness to 5HT occurred at 1h but this was short-lived and followed by a phase of hyporesponsiveness to this bronchoconstrictor evident at 72h.

Following inhalation of LPS, the concentration of neutrophils in BALF had significantly increased by 1h. However, this did not reach a maximum until 4h. Over the remaining period of observation (up to 72h) numbers of these cells steadily declined. Macrophages showed an increase 4h after LPS inhalation but did not become maximally elevated until 24h. Thereafter the numbers of these cells declined. No increase was observed in either eosinophil number, extracellular protein, surfactant or wet:dry weight lung ratio at any time during the experimental period of 72h.

The different time-course of reactivity changes and cellular influx allow the conclusion that inflammation is not directly associated with hyperresponsiveness of the airway to bronchoconstrictors after exposure of the lung to LPS.

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111 RELAXANT ACTIONS OF OESTROGENS ON ISOLATED PORTAL VEIN PREPARATIONS

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Epidemiological evidence suggests oestrogens have protective effects on cardiovascular morbidity and mortality (Henderson & Paganini-Hill, 1991) which may involve their vascular relaxant effects. Shan et al (1994) suggested that relaxation involves inhibition of calcium (Ca^{++}) influx but other mechanisms, such as protein kinase C inhibition (Magness et al, 1989), have been proposed. In the present experiments we investigated the actions of natural and synthetic oestrogens in rat portal vein, a tissue exclusively dependent on extracellular Ca^{++} for contractile activity.

Portal veins from male Hooded Lister rats (250-350g) were set up in Krebs' solution containing 10μM indomethacin under 0.5g tension (37°C, 95% O_2, 5% CO_2). A control concentration-response curve to KCl (2-128mM) or $PGF_{2\alpha}$ (2-100μM) was constructed and repeated after incubation with 17β (βEST 2-16μM) or 17α oestradiol (α EST, 4-20μM), diethylstilboestrol (DEST, 1-4μM) or vehicle (60% ethanol/40% water) for 20 mins. No vehicle effects were observed. All agents displaced both KCl and $PGF_{2\alpha}$ concentration-response curves to the right with significant reduction in E_{max}.

The extent of oestrogen-induced relaxation was compared in tissues contracted submaximally by KCl (32mM) or $PGF_{2\alpha}$ (32μM) to obtain an apparent IC_{50} for each agent, defined as the oestrogen concentration required to inhibit 50% contraction. Data are expressed as mean ±95% confidence limits and analysed by Student's unpaired t-test, $n \geq 4$. DESB appeared to be the most effective relaxant in this tissue (Table 1).

Table 1. IC_{50} values for oestrogens obtained in KCl (32mM) or $PGF_{2\alpha}$ (32μM) or Ca^{++} (5mM) contracted tissues. Values are mean ± CL. **Significantly different from βEST $P<0.01$. $n \geq 4$.

Oestrogen	KCl	$PGF_{2\alpha}$	Ca^{++}
βEST (μM)	3.3 ± 0.3	6.5 ± 1.1	6.6 ± 1.2
αEST (μM)	12.1 ± 4.6**	9.3 ± 0.9**	7.8 ± 0.9
DESB (μM)	1.5 ± 0.6**	0.6 ± 0.15**	1.7 ± 0.2**

To investigate potential Ca^{++} channel blocking activity of the oestrogens cumulative concentration-response curves to Ca^{++} (0.01-10mM) were obtained in Ca^{++}-free depolarising Krebs' solution containing 32mM KCl and the effect of βEST (4-16μM), αEST (4-16μM) and DESB (2-8μM) compared with that of the calcium channel blocker, nifedipine (NIF, 1-10nM). All oestrogens and NIF caused similar rightward shifts in the Ca^{++} concentration-response curve and significant depression of E_{max}. Apparent IC_{50} values for βEST, αEST and DEST obtained for submaximal Ca^{++}-induced contractions (5mM) as described above, are shown in Table 1. The value for NIF was 6.8 ± 0.5nM.

Oestrogen-induced relaxation appeared very similar to that of NIF, a calcium channel blocker, in portal vein. However, Babaei et al (this meeting) show that oestrogens relaxed Ca^{++}-depleted aorta when NIF was ineffective. The results suggest that conflicting reports on the mechanism of oestrogen action may be related to variations in Ca^{++} mobilisation in different blood vessels.

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112 POSITIVE EFFECTS OF LOSARTAN IN LABORATORY TESTS INDICATIVE OF ANXIOLYTIC-LIKE ACTIVITY AND THE IMPORTANCE OF ANIMAL STRAIN

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We have previously reported that the non-peptide, selective AT_1 angiotensin-receptor antagonist losartan produces behavioural changes in mice: when administered subcutaneously at a dose of 20 mg.kg^{-1}, losartan induced effects in the forced swim test indicative of antidepressant activity (Gard et al, 1994). The aim of these experiments was to investigate the effects of losartan in two other rodent behavioural tests, namely the elevated plus-maze and the light/dark aversion test, both of which have been used in studies of potential anxiolytic agents (Pellow et al, 1985 & Costall et al, 1989 respectively); diazepam was used as a positive control.

The elevated plus-maze consists of an elevated cruciform runway, the four arms of which are 10 cm wide and 50 cm long when used with rats and 8 x 15 cm when used with mice. The outer limits of two of the opposing arms are also enclose by walls (40 cm high for rats and 15 cm high for mice), but are open-topped. The animal is placed in the centre of the maze and has a natural tendency to move into one of the enclosed arms; anxiolytic drugs increase the amount of time spent on the open runways.

The light aversion test is performed by placing mice into the dark area of an observational field (45 x 27 cm), two thirds of which is painted white and is illuminated brightly and one third of which is screened from ambient light and illuminated by red light. The mouse is able to cross between the areas by movement through a small hole in the dividing partition. The natural tendency of the mouse is to spend more time in the dark portion; anxiolytic drugs increase the amount of time spent in the light.

In male Wistar rats (100-150g), 30 min. intraperitoneal pretreatment with losartan (5mg.kg^{-1}; n=8) caused an approximately 5-fold increase in the amount of time spent in the open arms of the maze during the 5 min. test period, compared to vehicle control (n=10; $p<0.01$, Student's 't'). Compared with animals receiving vehicle control (n=8), 15 min. intraperitoneal pretreatment with diazepam (2mg.kg^{-1}; n=10) caused an approximately 8-fold increase in the percentage time spent in the open arms ($p<0.02$, Student's 't').

Similarly in male BKW mice (25 - 30g), 30 min. intraperitoneal pretreatment with losartan (10mg.kg^{-1}; n=8) increased the percentage time spent in the open arms of the maze by approximately 250 percent, compared to vehicle control (n=8, $p<0.01$). Administration of diazepam (1mg.kg^{-1} i.p.; n=8) caused a 300 percent increase in the amount of time spent in the open arms, compared with animals receiving vehicle control (n=8, $p<0.02$). In male CD mice (23 - 30g) there were no significant effects of either losartan (10 or 20mg.kg^{-1}; n=8 in both cases) or diazepam (up to 2.5mg.kg^{-1}; n=8) on the amount of time spent in the open arms.

In the light dark aversion test, compared to vehicle control, there were no significant effects of losartan (0.1 mg.kg^{-1} p.o., n=6 nor 1 mg.kg^{-1} i.p., n=5) in CD strain mice, but oral pretreatment with losartan (1mg.kg^{-1}; n=5) to BKW strain mice caused a 50% increase in the amount of time spent in the light area, compared to animals receiving vehicle control (n=5, $p<0.05$, Student's 't').

These findings support and extend the work of Barnes et al (1990) who, using BKW mice, showed that oral losartan (0.1mg.kg-1) produced effects indicative of potential anxiolytic activity in the light-dark aversion test. Taken together, the results suggest that angiotensin II may be involved in the aetiology and treatment of anxiety and that losartan may be useful as an anxiolytic, with an efficacy of the same order of magnitude as that of diazepam. The present findings indicate, however, that experimental data from some experimental studies using losartan are significantly influenced by the strain of animal chosen, although the reasons for these strain differences are unclear.

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113 CAVEOLATION AND CAVEOLIN EXPRESSION IN GUINEA-PIG LUNGS FOLLOWING INDUCTION OF LUNG INFLAMMATION

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Introduction. Caveolae are nonclathrin-coated membrane invaginations which can pinch off from plasma membranes to form flask-shaped vesicles within the cytoplasm. Caveolae are morphologically most prominent in endothelial cells but also occur at a reduced density in alveolar epithelial type I cells. As in the endothelial cell, caveolae in the type I cell possess a characteristic cytoplasmic protein coat of caveolin (Campbell et al 1996) which, unlike clathrin, remains attached as the caveolae invagination buds from the plasma membrane to form a vesicle. Functions for caveolae may include sequestration and transport of macromolecules, internalisation of small molecules and ions by the process of potocytosis, and the fulfilment of signal transduction processes for the cell (Lisanti et al 1994). Early morphometric electron microscopy (EM) studies (Defouw 1983) reported pulmonary oedema to be associated with a 200-300% increase in the density of caveolae plasma membrane invaginations and cytoplasmic vesicles in the pulmonary capillary endothelial, and the type I alveolar epithelial, cell without concomitant changes in vesicle size or cell thickness. Recently, a chemokine receptor has been shown localised to caveolae in endothelial cells (Chaudhuri et al 1997). We **hypothesize** that increased caveolation is regulated by specific pro-inflammatory signals and that caveolae have an active functional role in the inflammatory process. The **objective** of this study was to determine morphologically whether increased caveolation occurs in established models of lung inflammation, and to examine for associated changes in the expression of cytoplasmic coat protein, caveolin.

Methods. Male Dunkin-Hartley guinea pigs (350-450 g ; n=4 each treatment) were placed in a chamber and exposed to one of the following treatments: endotoxin (20 mcg/ml) for 1 hr; ovalbumin (0.01% for 1 hr) at 14 days post sensitisation; ozone (0.3-0.75 ppm) for 2 hrs. Guinea pig lungs were harvested at: 1 hr or 24 hrs after endotoxin exposure; 1 hr after ovalbumin exposure; and 2 hrs or 24 hrs after ozone exposure. For EM examination lung tissue was fixed, embedded, stained and sectioned using standard methodologies. Remaining lung tissue was processed through homogenisation and cell lysis for SDS-PAGE leading to Western blot analysis for caveolin.

Results. There was no significant increase in total protein of guinea pig lungs after endotoxin, ovalbumin and ozone exposure compared to appropriate saline or air-exposed controls. Treatments resulted in a substantial increase in the density of caveolae invagination / vesicularisation in both the type I alveolar epithelial, and the pulmonary capillary endothelial, cell. Western blot analysis (mean data ± s.d. shown below) showed no significant difference in caveolin expression between treatment and respective control groups (caveolin units per 20 mcg total protein lung homogenate): Endotoxin 1 hr - 22.9 ± 3.31 *vs* Saline control 1 hr - 18.7 ± 4.12; Endotoxin 24 hr - 22.4 ± 2.74 *vs* Saline control 24 hr - 22.6 ± 1.75; Ozone 2 hr - 9.46 ± 3.08 *vs* Air control 2 hr - 7.15 ± 1.87; Ozone 24 hr - 8.95 ± 0.54 *vs* Air control 24 hr - 7.30 ± 1.44; Ovalbumin - 5.45 ± 1.79 *vs* Saline control - 5.05 ± 4.46.

Comment and Conclusion. This study has demonstrated that induction of lung inflammation by a variety of agents results in increased caveolation in the alveolar- pulmonary capillary barrier which is not associated with increased caveolin expression. At the gross organ level sufficient caveolin protein must therefore be available within plasma membrane domains and / or within the trans-golgi network to accomodate such a biophysical response. This report provides corroborative biophysical evidence to support the hypothesis of a role for caveolae in inflammation. Ongoing cellular studies are addressing impact of pro-inflammatory mediators upon caveolation and caveolae signal transduction function.

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114 ANTIBACTERIAL ACTION OF NITROFURANTOIN AGAINST *ESCHERICHIA COLI* WHEN PROTEIN SYNTHESIS, RNA SYNTHESIS, OR CELL DIVISION ARE INHIBITED

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Both quinolone and nitrofuran antibacterials bind to DNA causing strand breaks. Their precise bactericidal actions are unknown, but modern fluoroquinolones exhibit three mechanisms of activity referred to as A, B and C. Mechanism A is abolished if cells cannot divide, or synthesise RNA or protein. Mechanism B occurs in the absence of RNA or protein synthesis or cell division. Mechanism C also occurs in cells that cannot divide but requires RNA and protein synthesis (Smith & Lewin, 1988). We report experiments to determine whether the bactericidal activity of a nitrofuran is similarly affected.

Log phase cells of *Escherichia coli* AB1157 grown in nutrient broth (NB) were exposed to nitrofurantoin (Nf) at 200 μg mL^{-1} in NB. Rifampicin (Rf) at 160 μg mL^{-1} and chloramphenicol (Cm) at 20 μg mL^{-1} were used to inhibit RNA or protein synthesis respectively. NB-grown log phase cells were also washed twice in phosphate buffered (normal) saline (PBS) and resuspended in PBS. This inhibits cell division but allows RNA and protein synthesis to continue. Bacteria were suspended in incubation mixtures (IM; Table 1) at initial viable counts of 10^6 organisms mL^{-1}. Survival was determined by diluting in NB and plating on nutrient agar.

Table 1. Incubation mixtures.

	Antibiotic added at	
Nº IM	0 hours	2 hours
1 NB	-	-
2 NB	Nf	-
3 NB	Cm	-
4 NB	Rf	-
5 NB	Nf	Cm
6 NB	Nf	Rf
7 NB	Cm	Nf
8 NB	Rf	Nf
9 PBS	-	-
10 PBS	Nf	-

From Fig. 1 the following conclusions can be drawn. Cells did not grow or die in NB containing Cm (3) or Rf (4), nor in PBS (9). The addition of Cm (5) or Rf (6) after 2 h. did not affect the bactericidal activity of Nf (2). Pre-exposure to Rf (8) or Cm (7) delayed the onset of Nf-induced lethality by 1 or 2 h. respectively. However, once death commenced, the rate was similar to that of the Nf control (2). Incubation in PBS (10) increased the lethal effect of Nf over that seen in NB (2).

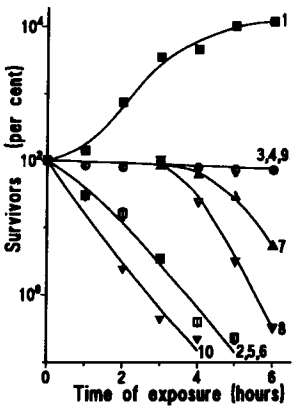

Fig. 1. Survival of *E. coli* AB1157

These results contrast to those obtained with the quinolones. Mechanism A is abolished in PBS whereas the activity of Nf does not require cell division. Mechanisms A and C are abolished by Cm or Rf due to the requirement for inducible protein synthesis (Howard et al., 1993), whereas Nf activity is delayed by pre-exposure to Cm (7) or Rf (8) but then proceeds at the same rate as control (2). Thus, unlike the quinolones, the bactericidal activity of nitrofurantoin is independent of RNA and protein syntheses and of cell division.

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115 A NOVEL COULTER COUNTER METHOD FOR DETERMINATION OF MICROBIAL ADHERENCE TO BIOMATERIALS

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Rapid growth has occurred in the use of medical devices concurrent with developments in polymer science, engineering and clinical care. Unfortunately, attendant with this increased device use is an increasing number of problems. Medical device-related infection is initiated by adherence of infecting microorganisms to the biomaterial surface. Microbial adherence is influenced by factors such as surface charge and hydrophobicity associated with the microorganism and biomaterial (Tunney et al 1996). Device-related infection may lead to patient morbidity and mortality, therefore, improvements in biomaterials are required to reduce or prevent microbial adherence. Methods based on radioisotopic measurement, direct microscopic counting, image analysis and viable counting techniques have been employed to determine the number of microbial cells adhering to surfaces (Gorman et al 1986). As disadvantages are associated with each of these methods, we have examined the use of an electronic particle counter to determine microbial adherence to biomaterials. This method is based on a similar technique described by us in respect of microbial adherence to mucosal epithelial cells (Gorman et al 1986). *Candida albicans* is a frequent isolate from biofilm adherent to PVC endotracheal tubes (Gorman et al 1993). A clinical isolate of *C. albicans* was employed as the test organism. In addition to PVC, polyurethane (PU) and silicone were used as test materials. The particle counter employed was the Coulter® Multisizer II.

Candida albicans was grown to stationary phase, washed twice to remove traces of broth and resuspended to OD_{540} 0.5 with Isoton®, a filtered isotonic electrolyte solution. This was diluted 1 in 100 in Isoton®. The test material of defined surface area was placed in a sterile McCartney bottle and 20 ml of diluted suspension was added. Dynamic conditions were provided by placing the bottle and contents on a bench-top rotary mixer (20 revolutions per minute). Samples (50 μl) from the suspension were removed hourly for analysis in the Multisizer fitted with a 50 μm orifice.

The number of cells per ml in suspensions incubated with material was expressed as % of control suspensions without material. Tabulated results show the number of *C. albicans* adhering to each material, expressed as a % of initial inoculum adhering per cm^2 of material (± s.d.).

Table 1 *C. albicans* adherence to biomaterials

Time (min.)	PVC	Silicone	PU
60	0.451±0.028	0.816±0.050	0.954±0.063
120	0.880±0.009	1.001±0.043	1.271±0.113
180	1.270±0.081	1.354±0.094	1.367±0.061
300	1.968±0.160	1.736±0.084	1.980±0.137

An increase in adherence to the materials was observed over time. Adherence to PU was significantly higher than to silicone or PVC ($p<0.05$).

These results show that use of the Coulter® Counter in this manner provides a rapid and reproducible method to quantify microbial adherence to biomaterials, allowing optimisation of medical device surfaces.

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116 AUTOMATED SYNTHESIS AND ANTIMYCOBACTERIAL ACTIVITY OF A SERIES OF 2-HETEROARYLCARBOXYAMIDRAZONES

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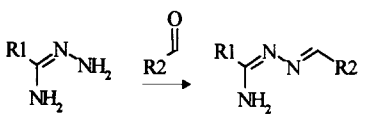
Mamolo et al (1992, 1993a, 1993b, 1996) have published the preparation and antimycobacterial activity of various heteroarylcarboxyamidrazones. These results combined with our experience of this class of compounds led us to expect significant antimycobacterial activity from lipophilic 2-pyrazinecarboxyamidrazone and 2-quinolylcarboxyamidrazone derivatives. To that end a large set of 2-pyrazinecarboxyamidrazone and 2-quinolylcarboxyamidrazone derivatives have been prepared in an automated fashion and in a purity sufficient for initial biological screening.



R1 = 2-Pyrazinyl
2-Quinolyl
R2 = various aryl groups

Heteroarylcarboxyamidrazone derivatives were prepared by heating the corresponding 2-heteroarylcarboxyamidrazone with a slight excess of the corresponding aldehyde in ethanol at reflux. These encompassed a wide range of functional groups. A significant proportion was chosen to give benzylidenes substituted at the 2-position and imparting an overall lipophilicity greater than or equal to the most active compounds obtained by previous workers. The reactants and solvents were dispensed automatically and the products, which were usually crystalline, were isolated by robotic trituration with a suitable solvent. The purities of material thus obtained were typically greater than 85%, the only contaminant usually detected being the aldehyde. Such a result is perfectly adequate for initial screening purposes whilst allowing a much greater range of new chemical entities to be investigated for biological testing than could formerly be obtained by traditional hand crafted means. The chemical entities obtained were tested against *mycobacterium fortuitum* which has resistance towards isoniazid. The initial screen involved simply the observation of a zone of inhibition of growth of the organism. In contrast to the results obtained by us for the corresponding set of 2-pyridylcarboxyamidrazone derivatives, none of the compounds in this study was found to have significant antimycobacterial activity. The implications of these results on the SAR of antimycobacterial activity in this class will be discussed.

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117 IMPROVEMENT IN THE HEALING RATE OF RADIATION-INDUCED WOUNDS USING A TOPICAL FORMULATION CONTAINING GLA, VITAMIN E AND DEXPANTHENOL

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Polyunsaturated fatty acids of n-6 series in PUFAs such as those in Evening Primrose Oil (EPO) are essential dietary constituents for maintenance of skin integrity and wound healing (Ref. 1). PUFAs are subject to spoilage and if inadequately protected, form oxidised product which are irritant and have off odours (Ref. 2). Topical formulations containing tocopherols and dexpanthenol (pro-vitamin B5) and EPO have now been shown not only to have good redox stability, but also to have enhanced wound healing and skin penetration properties. The contribution to wound healing for each component has been demonstrated *in vivo*. Time to healing in β-irradiated pig skin (35 Grays to an area of approximately 2.4cm) was assessed by a clinical rating score using a hydroalcoholic stearate (Hydrogel) base as control:-

Formulation	Time to Healing, days, (SE)	
Hydrogel control	43	(8)
Hydrogel + Vit. B5 and E	42	(3)
Hydrogel and EPO	25	(3)
Hydrogel and EPO & Vits E and B5	21	(4)
Hydrogel and MSM	35	(4)
Hydrogel and EPO and MSM	19	(2)
Hydrogel and EPO and Fenclofenac	23	(5)
Commercial Cream	35	
Commercial Cream and EPO	26	(6)
No treatment	34	(4)

In Franz cell tests, greater transdermal penetration has been demonstrated for Methyl Sulphonyl Methane and Fenclofenac in formulations containing EPO and Vitamins E and Pro B5 than in formulations containing saturated fatty acid triglycerides. It is concluded that optimal transdermal absorption and wound healing are achieved in a base containing a combination of an n-6 PUFA and Vitamins B5 and E, and a source of organically combined sulphur (MSM) or a re-vascularising agent (Fenclofenac).

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118 INVESTIGATION OF IBUPROFEN-XYLITOL SOLID DISPERSION SYSTEMS

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The concept of making solid dispersions of hydrophobic drugs in hydrophilic carriers has been studied for over thirty years. The advantage these systems possess is a marked improvement in dissolution rate, which can lead to a higher rate and degree of absorption of lipophilic drugs. However, this formulation approach is problematic due to the inherent instability of many such systems, (Ford, 1986). Here, we have examined the potential for preparation of solid dispersions of ibuprofen (IB) with xylitol (XYL). Ibuprofen (Berck Pharmaceuticals) was used as the model drug, owing to its poor aqueous solubility. Xylitol (Sigma-Aldrich Co Ltd) was selected as the model hydrophilic carrier due to its low toxicity, physiological acceptance and high aqueous solubility.

Solid dispersions of IB and XYL (1:1, 1:3, and 3:1 w/w) were prepared using a solvent evaporation technique and the melt method. For the coevaporates, drug and sugar were dissolved in the minimum volume of methanol at 40°C before evaporation of the solvent under vacuum at 50°C. Fusion samples were prepared at 130°C before crash-cooling in liquid nitrogen. Dispersions were desiccated over P_2O_5 for 24 hours then characterised by DSC and x-ray powder diffraction (XRPD). The solubility parameters of the drug and carrier were calculated using the Hansen Parameter Group Contribution Method (Barton, 1983).

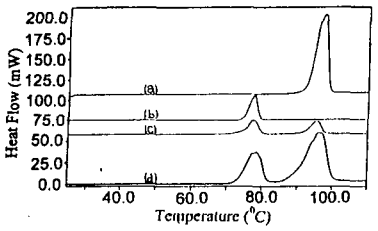

Figure 1: DSC traces of; (a) pure xylitol; (b) pure ibuprofen; (c) a 1:1 coevaporate of xylitol and ibuprofen (d) a 1:1 fusion sample of xylitol and ibuprofen.

All compositions of the fusion mixtures showed two distinct phases in the molten state, suggesting that the two substances are not miscible. XRPD of the solidified samples showed both components to be present as separate phases, with no change in their crystal structure nor any indication of an interaction. DSC of all samples (Fig. 1) showed melting endotherms of IB (75°C) and XYL (93°C), with replicate samples from different compositions showing varying proportions of drug and sugar, supporting the hypothesis that the two components are not miscible. This was confirmed by adding XYL to an IB melt maintained at 120°C; even 0.1% XYL was immiscible with IB. DSC analysis of the coevaporates showed that for each system, samples gave major differences in heats of fusion for both components. This was due to heterogeneity in the samples induced by the poor miscibility. The XRPD spectrum of the 3:1 XYL:IB coevaporate showed diffraction peaks characteristic of XYL but none of IB. In a second sample from the same coevaporate the reverse was shown. These results illustrate that IB and XYL do not cocrystallise, thus creating isolated domains of sugar and drug. Neither the drug or carrier appear to maintain the supersaturation of the other in solution, so the two components crystallise out at different rates. The large difference in calculated values of total solubility parameter between xylitol (38MPa$^{1/2}$) and ibuprofen (20MPa$^{1/2}$) partially explains the incompatibility of the two components.

Ibuprofen and xylitol show no physical or chemical interaction. In order to produce a stable soiid dispersion, a "bridging" agent may be needed which has appropriate solubility characteristics in both drug and carrier i.e. a surfactant with a suitable HLB value.

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119 MOLECULARLY IMPRINTED POLYMERS AS NOVEL EXCIPIENTS IN CONTROLLED RELEASE SYSTEMS

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Polymeric systems that allow the controlled release of a drug are well established. Through the use of molecular imprinting the current study aims to develop a molecularly specific polymeric release system to augment current techniques, Ashardy (1991).

Molecular imprinting is an effective method for introducing sites capable of specific or near-specific molecular recognition into an otherwise uniform matrix. Molecules can be successfully imprinted using non-covalent interactions, such as hydrogen bonding, ionic forces and hydrophobic interactions, to form a pre-polymerisation complex that is ultimately fixed within a cross-linked polymer. Subsequent removal of the imprint molecule leaves a reciprocal site capable of molecular recognition. Such molecularly imprinted polymers (MIPs) have been used as high efficiency chiral HPLC stationary phases, antibody mimics in immunoassays, chemosensory adjuncts and catalysts, Mosbach and Ramstrom (1996). The proposed system utilizes MIPs as highly selective, stereoisomeric differentiating excipients operating within a controlled environment. Since the hydrogen bonding is the significant factor influencing the ability of a MIP to differentiate between its imprint and other closely related molecules, it is important that the hydrogen bonding potential of its immediate surroundings is controlled, Nicholls et al. (1995). Low molecular weight, strongly hydrogen bonding solvents have the effect of negating selectivity. In this study, the MIPs were embedded within a transdermal adhesive thus providing a polymeric, low hydrogen bonding environment within which selectivity can occur.

Anti-cinchonidine and anti-propranolol MIPs were prepared using chloroform as the solvent, ethylene glycol dimethacrylate as the crosslinker and methacrylic acid as the functional monomer. After ultra sonic degassing under vacuum and nitrogen sparging, radical initiated polymerisation was carried out in sealed vials using standard conditions, O'Shannessy et al (1989). After 18 hours the polymers were removed from the reaction vials, coarsely hand ground and dried. This material was then wet balled milled in an all-porcelain system prior to 45μm screening. Imprint removal was achieved by successive washes in acetonitrile containing 10% glacial acetic acid. The final rinse was assayed for the imprint molecule to ensure maximum imprint removal.

The cinchonidine MIP was complexed to either cinchonidine or its diastereoisomer cinchonine and then embedded within a transdermal adhesive (National Starch 387-2051 and 387-2287). Dissolution from this system showed stereoselective release when compared to a control, non-imprinted polymer system.

The propranolol MIP was similarly complexed to its imprint and embedded in an adhesive. Release from this composite was observed across a silicone membrane in a Franz type diffusion experiment. By the addition of modifying agents to the adhesive, selective reterdation of propranolol release could be carefully controlled.

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120 IN-VIVO EVALUATION OF BIODEGRADABLE NORETHINDRONE IMPLANTS

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There has been considerable interest in the potential of tissue compatible biodegradable polymers for the implantation of antifertility drugs. Biodegradable implants are known to be suitable for controlled release of drugs [Langer et. al.(1984),Salthouse et. al.(1981)].The advantage of a long acting contraceptive drug delivery is the relative ease in use.The reliability and efficacy of a long duration therapy with a single administration makes it more acceptable. This work embodies the in-vivo evaluation of subcutaneous implants using a copolymer of Polyhydroxybutyrate-Polyhydroxyvalerate[PHB-PHV](80-20%) capable of providing controlled release of a fertility control drug for an extended period of time.The copolymer is known to degrade continuously at the site of implantation and the biocompatability is also known to be good [Gould et. al.(1987).

Norethindrone acetate implants were implanted subcutaneously in female rabbits.Serum Norethindrone acetate levels were determined by HPLC in rabbits which fluctuated between 0.95-1.5 ng/ml.In -vivo release studies were carried out in female rabbits where almost 100% of the payload was released within 6 months. The results revealed uniform sustained in-vivo release for up to 6 months.The in-vitro Norethindrone release was estimated using modified beaker method under sink conditions and assayed spectrophotometrically at 240 nm [Popli et.al.(1997)].These implants gave a positive in-vivo-in-vitro correlation .

No local tissue reaction was observed after implantation or removal of implants in rabbits . Temporal variations in haematological parameters of the rabbits were estimated after implantation so as to determine the compatibility of drug-polymer implant with the experimental animal.Total white cell count,total erythrocyte count and haemoglobin concentration were determined. No significant change was observed in RBC count and haemoglobin concentration when compared with placebo.A significant increase in white cell count was observed up to one month which returned to normal count.

The blood levels of estradiol and progesterone were measured by radio-immunoassay technique 60 days prior to implantation and up to 6 months after implantation at regular intervals.The estradiol and progesterone levels maintained a cyclic pattern.Five groups of 4 female rats were taken to study contraceptive effectiveness. No pregnancy was detected in rats for 6 months after implantation.The effect of the implant on the body weight of the rats was also studied in the same group and no significant change was found after implantation.These results indicate the potential of biodegradable Norethindrone implants as long acting, low dose contraceptive agents.

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121 AN INVESTIGATION INTO THE USE OF PVC/POLOXAMER BLENDS AS POTENTIAL BIOCOMPATIBLE MATERIALS

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Although poly (vinyl chloride) (PVC) is widely used in the medical device industry in a range of blood contacting applications the biocompatibility of this material is limited (Pearson (1995), Yin et al (1996)). The surface adsorption of poloxomers has been previously used to improve the compatibility of various implantable and drug delivery systems (Sheu et al (1992)). However, the long term instability of such coatings precludes their use in many circumstances. This communication describes an investigation into a series of PVC/poloxamer blends as potential novel biomaterials.

PVC/poloxamer membranes were prepared by a solvent evaporation technique by dissolving the PVC (1g) and poloxamer 407, 188 or 908 (1 or 5%w/w) in tetrahydrofuran/chloroform mixture and allowing the solvent to evaporate under controlled conditions to give a membrane of approximately 2mm thickness. The membranes were washed continuously with water until all leachates had been removed. 13mm discs were cut from each membrane and used to assess bacterial adhesion and fibroblast attachment as indicators of potential biocompatibility.

Bacterial adhesion A clinical isolate of *S. epidermidis* was grown as an overnight culture in nutrient broth. After extensive washing in sterile phosphate buffered saline (PBS) the cells were diluted to a density of $3x10^8$ cells mL^{-1} and were incubated at 37°C with the test materials for 4 h. The samples were then removed, washed with PBS and the bacterial cell biomass determined by quantifying the cellular ATP. The ATP was extracted from the cells using 0.05% trichloroacetic acid in Tris-acetate buffer and measured using the luciferase bioluminescence assay. Figure 1 shows the relative bacterial adhesion to the various substrates (Mean ± SD (n=6)).

Fibroblast Attachment. 1000 viable 3T3 mouse embryonic fibroblasts cells were plated onto PVA disks. After a 72 h growth period the disks were removed, rinsed with PBS and the adhered biomass was determined by measuring cellular ATP. The ATP was extracted by hypotonic lysis and quantified using the luciferase bioluminescence assay using a commercial ATP assay kit (LabTech. UK). Fig. 2 shows the relative attachment of the fibroblasts to the materials (Mean ± SD (n=6)).

Fig 1 Bacterial Attachment

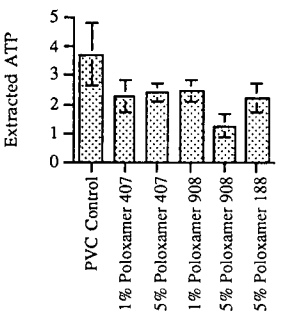
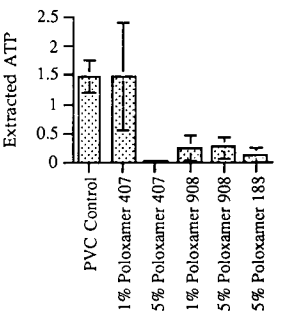


Fig 2 Fibroblast Attachment



These studies indicate that PVC/poloxamer blends may have application as novel biomaterials for use in devices in which it is desirable to minimise cellular adhesion.

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122 THE EFFECT OF A MODIFIED MOUTHPIECE ON DRUG DELIVERY FROM DRY POWDER INHALERS

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One possible strategy for improving drug delivery from a dry powder inhaler is to employ a mouthpiece on the device which directs the formulation into the mouth at an angle with a view to reducing impaction at the back of the throat. One means of assessing the respirable fraction of a drug in vitro is to determine the fine particle fraction (FPF) which is delivered to the lower stage of a twin-stage impinger (TSI) (BP 1993). The purpose of this study was to investigate the effect of mouthpiece design on FPF. Angled adapters were produced for the Cyclohaler® so that the aerosolised formulation was delivered at angles of 4.7, 6.6, 10.3 and 13° from the horizontal to the TSI. The adapters were moulded from silicone rubber and curing agent at a ratio of 10:1 (Silastic®, Dow Corning, Germany). Another mouthpiece modification involved the use of a 55 mm cylindrical glass tube (1 cm diameter) bent to obtain a radius of curvature of 1.16 cm at the end remote from the device. The formulations were prepared by mixing 0.2 g salbutamol sulphate with 13.5 g of different size ranges of lactose carrier in a Turbula mixer (Basel, Switzerland) for 2 h. Lactose (63-90 μm, Meggle, UK), 10-40 μm lactose (Lactochem, UK) and micronised lactose (5-10 μm) were employed as the carrier. The 27.4 mg powder mix was filled into a size 2 capsule and used as the dosing unit. Rotacaps® 400 were included in this study as the control formulation. There were no differences in the FPF produced by employing different angles of delivery via the mouthpiece of the Cyclohaler® when charged with Rotacaps®. The deposition of drug using a curved glass mouthpiece and a straight glass tube were very similar using with Rotacaps®. Although the FPF increased when the Lactochem lactose formulation was employed (Figure1),there was no significant difference in deposition from the curved glass and straight glass mouthpiece. However, the micronised lactose formulation showed a marked increase in FPF using the curved compared to the straight glass mouthpiece. The original device and one fitted with a straight glass mouthpiece did not produce any difference in FPF.

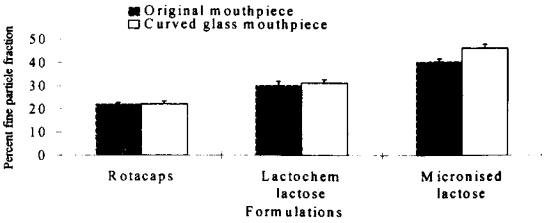

Figure 1 The effect of modified mouthpiece of the Cyclohaler on drug deposition using TSI (mean ±sd, n=6)

It can thus be concluded that both the smaller sized lactose carrier and the shape of the mouthpiece contribute to differences in FPF. Improved drug delivery might be achieved by bending the mouthpiece to an angle that is appropriate to the mouth anatomy. However, it remains to be determined whether the curved mouthpiece is acceptable to the patient since the curved mouthpiece projects into the mouth and could contact the tongue. The patient might find the insertion of such a mouthpiece projecting towards the back of the throat uncomfortable to use. However, the design of the mouthpiece might be improved by producing a mouthpiece that is straight externally with an internal air channel that is angled to induce a curved air stream. Such a design could lead to a higher peripheral airways deposition for certain formulations.

123 IN-VITRO CHALLENGE OF FLUORINATED AND NON-FLUORINATED NIOSOMES BY SIMULATED GASTROINTESTINAL FLUIDS

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The use of particulate systems may offer a means of orally administering gut labile drugs (Florence & Jani, 1993). Niosomes, non-ionic surfactant vesicles, formed from a variety of amphiphiles (Uchegbu & Florence, 1995) have been shown to increase the bioavailability of methotrexate on oral administration (Azmin et al, 1985). Vesicles formed from a fluorinated surfactant, 5-O-[3'-(F-octyl)-2'-propenyl] xylitol (**F**) (Zarif et al, 1990), which might be postulated to be more stable when challenged with bile salts and perhaps simulated GI fluids because of the hydrophobic and lipophobic nature of the fluorinated bilayers have been compared to Span 60 vesicles with respect to their degradation by components of gastrointestinal tract evaluated in vitro using simulated fluids.

Fluorinated and non-fluorinated 5(6)-carboxyfluorescein (CF) niosomes were prepared from **F** and sorbitan monostearate (Span 60) by the hydration of surfactant lipid films with CF in phosphate buffered saline (pH=7.4). The release of CF was studied in the presence of a) simulated gastric fluid (SGF) USP XX pH 1.2, b) simulated intestinal fluid (SIF) USP XX pH 7.5, and c) 2% w/v bile salt solution, pH 7.5 all at 37°C. Both types of niosomes released low levels of CF in SIF and higher levels in the presence of SGF or bile salts (Figure 1). There was no difference in the release rates of fluorinated and non-fluorinated niosomes (Figure 1). The high rate of release of CF in SGF was due to presence of a high pH gradient across the membrane in this medium. Incubation of Span 60 niosomes in SGF devoid of enzymes still resulted in high release rates. Microscopic examination of the niosomes 24 h after incubation in SGF and SIF revealed no obvious changes in niosome morphology. However, a change in Span 60 niosome ultrastructure was apparent on incubating with bile salts, and the fluorinated niosomes could no longer be visualised. This change in niosome morphology indicates that the bile salts caused a high efflux of CF by destruction of the niosome membrane, disproving our hypothesis.

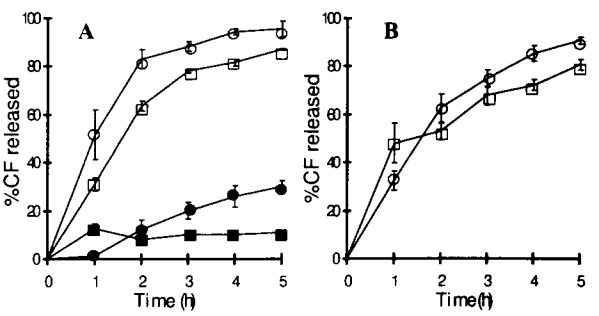

Figure 1: **[A]** CF release profile in SGF of (○) **F** niosomes and (□) Span 60 niosomes , and in SIF of (●) **F** niosomes and (■) Span 60 niosomes. **[B]** CF release profile in bile salts solution of (○) **F** niosomes and (□) Span 60 niosomes.

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124 MECHANICAL AND SURFACE PROPERTIES OF FILMS COMPOSED OF POLY(ε-CAPROLACTONE) AND POVIDONE-IODINE

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Poly(ε-caprolactone, PCL) is a biodegradable aliphatic polyester that has frequently been used for medical applications, e.g. drug delivery systems (Medlicott et al. 1992). However, one potential application of PCL is as a biodegradable coating for urinary medical devices. In this, polymer degradation will assist in the removal of adherent microorganisms and urinary encrustation and hence, would be expected to decrease the incidence of many of the current problems associated with urinary medical devices. The physical properties of the biodegradable films will affect their clinical performance. Therefore, this study examined the mechanical and surface properties of PCL films alone and composites of PCL and povidone-iodine (PVP-I).

High and low m.wt. PCL (50,000 and 4,000, respectively) were used in this study and were dissolved in dichloromethane (DCM) in a range of m. wt. proportions (100:0 to 50:50). When required, PVP-I (0.5 and 1% w/v) was dissolved in this polymeric solution. The final concentration of PCL +/- PVP-I was 10% w/v in all solutions. Films were prepared by casting the polymeric solution into glass petri dishes and allowing DCM to evaporate under controlled air flow at 25°C. The mechanical properties of film samples (5 x 1 cm) were determined using a Texture Analyser in tensile mode (5 mm s^{-1} clamp separation rate). From the resultant stress/strain plot, the ultimate tensile strength (UTS), % elongation at break (% Elong), and Young's modulus (YM) were determined. The advancing (Adv CA) and receding contact angles (Rec CA) of each polymeric sample in water were determined using a Dynamic Contact Angle analyser. coverage (SC, %) of discs by adherent organisms was also obtained using image analysis. Statistical evaluations were performed using an analysis of variance ($P<0.05$ denoting significance).

The effects of m.wt. of PCL and PVP-I content on the mechanical and surface properties of the polymeric samples were statistically evaluated using a two-way Analysis of Variance ($p<0.05$ denoting significance). Table 1. The effects of PCL m.wt ratio and % w/v PVP-I on UTS (MPa), % Elong, YM (MPa) and Adv CA of PCL films (*ratio of high to low m.wt PCL)

PCL*	PVP-I	UTS	% Elong	YM	Adv CA
100	0	16.0±1.1	193.5±10.1	7.1±1.5	89.6±1.2
100	0.5	13.5±2.1	100.1±10.6	8.0±1.0	81.2±0.3
100	1.0	13.5±1.4	97.7±15.1	4.9±1.0	77.4±1.3
80:20	0	14.4±1.2	11.54±2.6	7.6±1.9	79.6±2.7
80:20	0.5	15.4±1.0	23.98±9.1	8.0±1.6	81.1±0.7
80:20	1.0	13.9±1.0	15.24±4.5	7.5±1.6	78.2±2.2
50:50	0	11.4±0.7	3.96±0.6	8.9±2.1	76.6±1.0
50:50	0.5	12.3±0.8	4.33±0.9	9.8±0.9	76.7±1.0
50:50	1.0	7.3±1.1	4.04±1.1	6.4±0.7	76.9±3.0

Increasing film content of lower m.wt PCL significantly decreased the UTS, % elong but did not affect YM. This is attributed to the poor film forming properties of low m.wt. PCL. Introduction of low m.wt. PCL significantly decreased film Adv. contact angle. Conversely, increasing the content of PVP-I did not affect film mechanical properties, but decreased both Adv. and Rec. contact angles. The latter phenomena are attributed to the hydrophilic nature of PVP.

In conclusion, the mechanical and surface properties of PCL films are appropriate for use as coatings for urinary devices. The presence of PVP-I did not adversely affect these properties and indeed, the increased hydrophilicity of these films may increase resistance to urinary encrustation.

Medlicott, N.J. et al. (1992) Int. J. Pharm. **84** 85-89

125 TEXTURAL PROPERTIES OF SEQUENTIAL INTERPENETRATING GEL NETWORKS OF POLY(HYDROXYETHYLMETHACRYLATE) AND POLY(ACRYLIC ACID)

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Xerostomia is a clinical condition in which there is salivary gland dysfunction, resulting in dry and burning mouth, eating and speech difficulties, oral and dental infections. Treatment involves the use of lubricants, however, currently available preparations have only a limited duration of action (due to their rapid removal from the mucosal surface) and their patient acceptability is compromised due to unavailability of adequate delivery systems (Ferguson and Barker 1994). This study reports the design and mechanical characterisation of a series of gel networks designed for the treatment of xerostomia.

Synthesis of sequential interpenetrating gel networks was performed by dissolving Polyacrylic acid (PAA, Carbopol™ 974P, 0.25 0.5% w/w) in either distilled water or in a distilled water/propylene glycol (PG) mixed solvent system (70:30) with stirring and adjustment of the pH to neutrality by the addition of NaOH. Into this gel was added hydroxyethylmethacrylate (HEMA) (2.5, 5% w/w final concentration) and potassium persulphate initiator (0.06% w/w). HEMA was allowed to polymerise by incubation at 60°C for 3 h. Mechanical characterisation of the gel networks was performed in triplicate using Texture Profile Analysis (TPA, Jones et al. 1997). In brief, the analytical probe (1 cm diameter) was twice depressed into each sample at a defined rate (10 mm s^{-1}) and to a defined depth (15 mm). A delay period of 15 s was allowed between the end of the first and beginning of the second compressions. The effects of PAA, pHEMA and solvent composition were statistically evaluated using a three-way ANOVA ($p<0.05$ denoted significance).

Increased concentrations of pHEMA and PAA and inclusion of propylene glycol (PG) into the solvent system significantly increased product hardness and compressibility.

Table 1. Effect of concentration (% w/w) of pHEMA, PAA and solvent composition on the hardness (N), compressibility (Comp, Nmm) and Adhesiveness (Adh, Nmm) of formulations.

pHEMA	PAA	Solvent	Hardness	Comp	Adh
2.5	0.25	Water	0.5±0.0	5.0±0.2	2.4±0.6
2.5	0.25	Water/PG	0.4±0.0	4.8±0.1	2.0±0.3
2.5	0.50	Water	0.8±0.0	6.9±0.3	6.3±0.6
2.5	0.50	Water/PG	0.9±0.1	7.7±0.3	7.2±0.5
5.0	0.25	Water	0.6±0.1	5.3±0.3	2.2±0.2
5.0	0.25	Water/PG	0.9±0.0	5.1±0.3	3.4±0.5
5.0	0.50	Water	0.8±0.1	7.0±0.4	6.6±0.3
5.0	0.50	Water/PG	1.1±0.1	9.4±0.6	8.5±0.9

These results are due to both the effects of polymer concentration and solvent composition on gel viscosity and also to the greater compatibility of pHEMA with solvents containing propylene glycol. Increased concentration of PAA, but not pHEMA, increased formulation adhesiveness, reflecting the bioadhesive properties of PAA and the non-adhesive properties of pHEMA.

Therefore, in this study, a series of gel networks have been prepared that offer a range of mechanical properties. The final choice of formulation for clinical evaluation will involve a compromise between minimal product hardness and compressibility (to ensure ease of removal from the container and ease of application) and maximal adhesiveness (to ensure product retention) at the site of application. Candidate formulations are currently undergoing clinical evaluation.

Ferguson, M.M. & Barker, M.J. (1994). Adv. Drug Del. Rev. **13** 151-159

Jones, D.S. et al. (1997). Pharm. Res. (In press)

126 BEHAVIOUR OF OILS IN NONIONIC OIL-IN-WATER MICROEMULSIONS

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As a result of their thermodynamic stability, clarity, fluidity and dilutability, microemulsions (m/e) have been widely studied as drug delivery vehicles. However, for oil-in-water (o/w) m/e stabilized by polyoxyethylated amphiphiles changes in temperature may cause instability due to the surfactants possession of a phase inversion temperature (PIT).

Formation of o/w m/e stabilized by the nonionic surfactant, polyoxyethylene-10-oleyl ether (Brij 97) and containing either an ethyl ester [ethyl butyrate (EB), caprylate (EC), oleate (EO)] or a triglyceride [tributyrin (TR), Miglyol 812 (MG), soybean oil (SB)] as oil was established by mixing the appropriate amounts of each component, heating to 70°C and stirring until cool. Clear non-birefringent samples were classified as m/e. The larger molecular volume oils (EO, MG, SB) were incorporated to a greater extent than the smaller oils (EB, EC, TR), ie 7-10%w/w as opposed to 1-3%w/w at a surfactant concentration of 20%w/w.

PIT studies were performed on the m/e by heating and determining the temperature at the onset of turbidity (Fig. 1). M/e containing the larger molecular volume oils showed, after an initial fall, an increase in PIT upon increasing oil concentration. In contrast m/e containing the smaller oils resulted only in a decline in the PIT to temperatures around ambient. This observation may be explained by the way in which the various oils are incorporated into the m/e (Aveyard et al 1990); oils causing an increase in the PIT (here EO, MG and SB) promote the formation of spherical m/e droplets from the slightly asymmetric Brij 97 micelles by forming a central oil core, while those oils causing a decrease in the PIT are thought to penetrate the interfacial surfactant monolayer further encouraging the formation of asymmetric aggregates. Total intensity light scattering measurements (Malvern 4700c series, 75mW argon ion laser) performed on diluted m/e (1%w/w Brij 97) showed, after an initial fall, an increase in the Rayleigh ratio at 90°(R_{90}) with increasing oil concentration for the m/e containing the larger molecular volume oils (Fig. 2). This result confirms the interpretation of the PIT studies (Oetter & Hoffmann 1989). It was not possible to perform analogous experiments on m/e containing the small oils due to their limited region of existence. These results may have important implications for the way in which drugs are incorporated into o/w m/e.

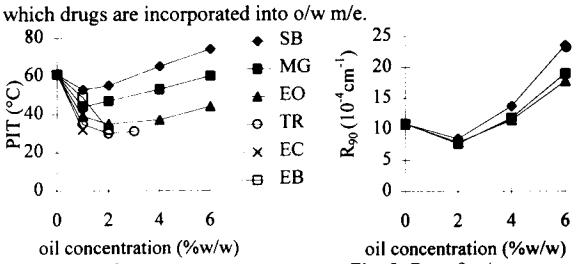

Fig. 1. PIT of m/e

Fig. 2. R_{90} of m/e

Aveyard, R. et al (1990) J. Chem. Tech. Biotechnol. 48, 161-171.
Oetter, G., Hoffmann, H. (1989) Colloids Surf. 38, 225-250.

127 THE EFFECTS OF EMULSIFIER AND DRUG (CHLORHEXIDINE) CONCENTRATIONS ON THE MECHANICAL (TEXTURAL) AND VISCOELASTIC PROPERTIES OF CREAMS

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Pharmaceutical creams are semi-solid systems that are employed for the topical administration of pharmacologically active agents. The clinical performance of semi-solid systems is dependent, in part, on their mechanical and viscoelastic properties (Jones et al. 1996). In addition, the textural properties of topical formulations will affect patient acceptability. This study examined the effects of both emulsifier and chlorhexidine concentration on the viscoelastic and textural properties of a series of cream formulations using oscillatory rheometry and texture profile analysis (TPA), respectively.

A series of creams (n=16) were prepared containing water (300g), liquid paraffin (100g) and a mixed emulsifier system composed of cetostearyl alcohol (32.4 - 57.6g) and cetrimide (3.6 - 6.4g) in a mass ratio of 9:1, and, when required chlorhexidine (CH, 2 or 5%w/w). Oscillatory properties of all formulations were investigated using a Carri-Med CSL^2-100 rheometer in conjunction with stainless steel parallel plate geometry (4cm diameter, 1.0 mm separation). Oscillatory measurements were performed at 20.0 ± 0.2°C over a frequency range from 0.01 - 1.0 Hz at a constant strain of 6.5 x 10^{-3} rad (selected from the linear viscoelastic region of the samples). TPA was performed as previously described (Jones et al. 1997), employing a 2 mm s^{-1} probe speed and 15 s delay period between compressions. The effects of mixed emulsifier concentration, and when appropriate chlorhexidine concentration, on textural and rheological parameters were statistically evaluated using a one-way and two-way Analysis of Variance (ANOVA), respectively ($p<0.05$ denoted significance).

Increased concentrations of mixed emulsifier resulted in significantly increased Hardness, Compressibility, Adhesiveness, Storage and Loss Moduli. These observations confirm the role of mixed emulsifier on the formation of a network structure within the continuous phase and hence on the formation of a semi-solid system. The effects of mixed emulsifier concentration on adhesiveness reflects the greater tack associated with increasingly semi-solid formulations. Inclusion of CH significantly decreased the above parameters in formulations containing lower concentrations of mixed emulsifiers (<11.6%), suggesting that the suspended CH reduced the integrity of network formation within the continuous phase. Above this concentration, CH had no effect on these parameters.

Table 1. Effect of concentration (% w/w) of cetostearyl alcohol (CS), cetrimide (Cet) and CH on the mean values of hardness (H, N), compressibility (Comp, Nmm), Adhesiveness (Adh, Nmm), Storage modulus (G' MPa) and Loss Modulus (G'', MPa) of representative formulations (CV<5% in all cases).

CS %	Cet %	CH %	H	Comp	Adh	G'	G''
7.4	0.8	0	0.36	4.38	2.4	1.62	0.44
7.4	0.8	2	0.21	2.66	0.99	1.9	0.30
7.4	0.8	5	0.25	3.24	0.71	1.37	0.40
10.4	1.2	0	0.66	8.68	5.61	4.62	1.21
10.4	1.2	2	0.35	4.92	2.19	2.86	0.75
10.4	1.2	5	0.35	4.77	3.27	2.71	0.84
12.4	1.4	0	0.66	9.89	5.67	4.96	1.52
12.4	1.4	2	0.59	9.52	5.73	4.78	1.53
12.4	1.4	5	0.62	9.81	5.73	4.87	1.65

The textural data also provides information concerning the patient acceptability of these formulations. Thus, as formulation hardness and compressibility increase, the ease of removal of the formulation from its container and ease of spreading on a substrate will decrease.

In conclusion, this study has shown the importance of both emulsifier and suspended drug concentration on the mechanical and rheological properties of creams. The final choice of concentrations of these agents will influence their clinical performance.

Jones, DS, et al. 1997. Int. J. Pharm. (In press)

128 THE EFFECT OF DISSOLUTION MEDIUM ON THE RELEASE OF PROPRANOLOL FROM HMPC/SODIUM CARBOXYMETHYLCELLULOSE MATRICES

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Cellulose derivatives, e.g. hydroxypropylmethylcellulose (HPMC) or sodium carboxymethylcellulose (NaCMC), are used in controlled-release tablets. Since NaCMC is ionisable, this study examines the effect of dissolution media on the release of propranolol hydrochloride from HPMC/NaCMC matrices.

Flat-faced tablets, 12.7 mm diameter, were directly compressed at 182 MNm^{-2}, to contain 160 mg propranolol (BP (<125 μm), 285 mg HPMC2208 (Methocel K4M), NaCMC (Blanose 7H4XF) or their 1:1 mixture and 0.75% magnesium stearate. Dissolution was determined using a Pharmatest dissolution tester at 285 nm in 1000 mL distilled water, 0.1M hydrochloric acid, phosphate buffer solutions pH 6.8 or pH 7.4 or borate buffer solution pH 9.4, at 37°C. The USP XXII (Apparatus 1) was used, rotating at 100 rev min^{-1}. Dissolution was also studied by a pH-change method, in 750 mL 0.1M hydrochloric acid for 2h and after the addition of 250 mL 0.2M trisodium phosphate, at pH 6.8. The means of 6 determinations were used. Data corresponding to 5 to 60% release were fitted to equation 1:

$$Q = K(t - l)^n \qquad (1)$$

where Q is the percentage of drug release at time t, K is a dissolution rate constant, l is a lag time and n is the release exponent. The value of n indicates the release mechanism (Ford et al, 1991).

The sensitivity to the media of the three types of matrices ranked as NaCMC > 1:1 NaCMC/HPMC > HPMC (Table 1). The values of n were higher for matrices containing NaCMC or NaCMC/HPMC in distilled water than in 0.1M hydrochloric acid, suggesting erosion rather than diffusion control probably due to the insolubility of NaCMC in acidic media. Propranolol release from 1:1 NaCMC/HPMC matrices into further media (Table 2) confirms a sensitivity to changes in pH. Increase in pH resulted in an increase in $T_{50\%}$ (time for 50% of drug to dissolve). The values of n concomitantly increased. The release mechanisms would be influenced by a decrease in solubility of propranolol but an increase in ionisation of NaCMC with increased pH.

Table 1: Release rates (% $min^{-1/2}$), K and n values for matrices containing 285 mg HPMC, NaCMC, or 1:1 NaCMC/HPMC

Media	Polymer	Release rate	K	n
0.1M HCl	HPMC	2.0 ± 0.1	1.11	0.65
	NaCMC	8.8 ± 0.7	1.83	0.82
	HPMC:NaCMC	3.2 ± 0.1	1.85	0.59
Distilled water	HPMC	2.6 ± 0.2	1.42	0.59
	NaCMC	3.1 ± 0.1	0.04	1.16
	HPMC:NaCMC	1.3 ± 0.1	0.14	0.81

Table 2. Estimated values of $T_{50\%}$ (h), release rates (% $min^{-1/2}$), K and n from matrices containing 285 mg of 1:1 HPMC:NaCMC

Media	$T_{50\%}$	Release rate	K	n
0.1M HCl	4.7 ± 0.2	3.2 ± 0.1	1.85	0.59
pH change	6.3 ± 0.5	3.0 ± 0.2	1.29	0.65
pH 6.8	12.4 ± 1.0	2.1 ± 0.1	0.44	0.71
pH 7.4	$14.9 \pm 0.8^{40\%}$	1.7 ± 0.1	0.47	0.68
pH 9.4	$27.6 \pm 0.5^{25\%}$	1.1 ± 0.1	0.03	1.00

Ford, J.L., Mitchell, K. et al (1991) Int. J. Pharm. 71: 95-104.

129 INVESTIGATION OF DRUG RELEASE AND USE OF PH MODIFYING EXCIPIENTS TO GIVE PH INDEPENDENT CONTROLLED RELEASE FROM HYDROXYPROPYL METHYLCELLULOSE (HPMC) MATRIX TABLETS

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A controlled release solid dosage form providing pH independent drug release was required for a novel drug substance. The drug substance, a pyrimidinylpiperazinyl substituted fluoroindole with pKa 4.7 and 8.9, showed pH dependent solubility; saturated solubility at 37°C was approximately 20 mg/ml in 0.1N hydrochloric acid (acid) and 0.2 mg/ml in simulated intestinal fluid without enzymes (SIF). The drug substance was formulated as a controlled release tablet containing hydroxypropyl methylcellulose (HPMC, Methocel®) as the matrix forming component. USP Type I dissolution apparatus was used to study the influence of matrix viscosity on drug release in acid and SIF. In acid, the % drug released from formulations containing 80% w/w Methocel E5, E15, E50 or E4M correlated with square root time, indicating diffusion controlled release. The rate of release decreased with increasing matrix viscosity. No such trend was observed in SIF and since the drug substance was approximately 10 fold less soluble in SIF than acid, it was considered that release in SIF was controlled predominantly by erosion of the tablet matrix. This finding was supported by the observation that tablets exposed to acid showed a clear outer gel layer whilst tablets in SIF exhibited a cloudy gel layer indicating precipitated drug substance and release by erosion. Fast hydration of the matrix to form an integral gel layer prior to erosion was found necessary to prevent an initial burst release of drug in SIF. Rapid tablet hydration was achieved by substitution of Methocel E4M with K4M, a faster hydrating grade of HPMC.

pH modifying excipients were used to alter drug solubility within the hydrated matrix, thereby promoting the same mechanism of drug release in acid and SIF, and giving pH independent release. To identify a suitable type and level of excipient, formulations containing 40% w/w Methocel K4M and different levels of citric acid or fumaric acid (acidic modifiers) or disodium hydrogen orthophosphate (basic modifier) were prepared as slurries in acid or SIF and the pH determined. Diffusion controlled release was considered more robust than tablet erosion and an acidic modifier was preferred. Citric acid was the most effective agent to lower slurry pH in SIF, thus potentially increasing drug solubility so that release was diffusion controlled in acid and SIF. Tablets containing 5 or 10% w/w citric acid gave pH dependent release and showed a cloudy gel layer in SIF, indicating the matrix pH was not low enough to prevent drug precipitation and therefore release in SIF was still by matrix erosion. Tablets containing 15% w/w citric acid gave pH independent release which correlated with square root time in both acid and SIF. The tablets exhibited a clear gel layer in both media, indicating that a low microenvironmental pH was achieved irrespective of the bulk medium and diffusion of dissolved drug through the gel matrix was the release controlling mechanism in both acid and SIF.

In summary, these results show that the pH-solubility profile of a novel drug substance causes the mechanism of drug release from HPMC matrix tablets to differ in acid and SIF. Inclusion of a suitable pH modifying agent stabilises microenvironmental pH and promotes the same release mechanism in each medium, thereby achieving pH independent drug release.

130 THE USE OF SOLID DISPERSIONS FOR IMPROVING THE DISSOLUTION RATE OF NITRENDIPINE TABLETS

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A reduction of the particle size and an increase of the specific surface area can enhance the dissolution rate, and the concept of solid dispersions can be applied for drugs of low aqueous solubility. Nitrendipine is a calcium channel antagonist with anthypertensive activity, practically insoluble in water (2mg/ml) which predicts its low dissolution from solid dosage form.

The aim of this study were to investigate the influence of solid dispersion tehnique application and addition of surfactants in to the dispersion itself, on dissolution rate of Nitrendipine tablets. Classic wet granulation method were also used for production of Nitrendipine tablets for comparation.

Solid dispersion of 1 part of Nitrendipine and 0.6 parts of PVP K-25 has been prepared by the solvent method (ethanol / dichlormethan mixture). Because of handling difficulties with coprecipitates itself, granules have been prepared by coating inert core such as croslinked PVP / corn starch mixture with Nitrendipine coprecipitate. The properties of tablets produced from these granules have been examined and compared with similar tablets produced by wet granulation method with water as a solvent.

For dissolution testing basket method (USPXXIII) with 1000ml of 1% sodium lauryl sulfate water solution at 37±°C and 75 rpm during 45 min was used. Sampless were assay on a UV / VIS spectrophotometer at a wavelength of 237nm in comparation with standard solution.

Physical properties of tablets prepared by both methods were similar, but better dissolution (86%) of Nitrendipine was achieved from tablets containing the solid dispersed drug. By the addition of 0.1 part of sodium laurylsulfate into the dispersion itself about 95% of dissolved drug was obtained. This was due to inadequate wetting of the dispersion units, since the drug portion represented a large surface area of hydrophobic nature. This effect was counteracted by the addition of surfactants into the dispersion itself. Croslinked PVP / corn starch mixture as insoluble core for Nitrendipine fine particles loading, permits the uniform distribution of this slightly water soluble drug and result in an increase of the specific surface area for dissolution and fast desintegration of tablets in aqueous media

131 GASTRIC RESIDENCE OF GAVISCON ADVANCE AND LIQUID GAVISCON IN HEALTHY VOLUNTEERS

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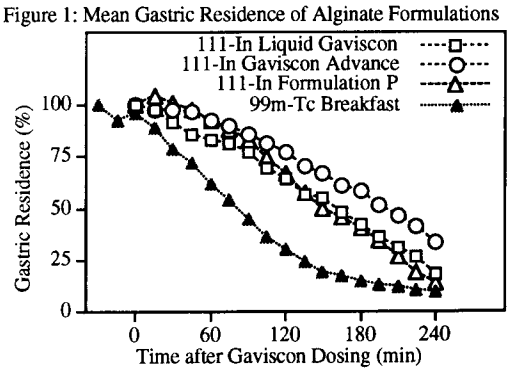
Liquid Gaviscon® is a reflux suppressant which interacts with gastric acid to form an alginate raft. The raft floats on the stomach contents to provide a physical barrier preventing acid reflux into the oesophagus. This study has investigated whether administering the same dose of alginate in a reduced volume formulation would alter the gastric formation, distribution and residence of the alginate rafts.

Using a three-way balanced cross-over study, 12 healthy male non-patient volunteers received, on three separate occasions, single doses of Liquid Gaviscon® (20 mL), Gaviscon Advance® (10 mL) and Formulation P (10 mL). Each 10 mL or 20 mL dose contained 1000 mg of sodium alginate together with calcium carbonate and either sodium bicarbonate (Liquid Gaviscon® and Formulation P) or potassium bicarbonate (Gaviscon Advance®). On each dosing occasion, the volunteers fasted overnight and at 8:30 the following morning ingested a standard ^{99m}Tc-labelled breakfast. Thirty minutes later they received a single dose of Liquid Gaviscon®, Gaviscon Advance® or Formulation P radiolabelled with a trace of ^{111}In-alginate. Gastric distribution and residence of the radioisotopes were assessed using gamma scintigraphy for a period of 4.5 hours after administration of the ^{99m}Tc-labelled breakfast (i.e. 4 hours after alginate dosing). The primary efficacy parameter for performance of the formulations was gastric residence, determined from 4 hour area under the curve (AUC) values.

Mean data are presented in Figure 1 where it is clearly evident that gastric residence of all three alginate formulations was far greater than that of the standard breakfast. Statistical analysis (2-way ANOVA) demonstrated that alginate/breakfast (^{111}In/^{99m}Tc) mean AUC ratios were 1.28 for Liquid Gaviscon®, 1.54 for Gaviscon Advance® and 1.46 for Formulation P, each ratio was significantly greater than 1. The alginate formulations also exhibited significantly different gastric distribution from that of the breakfast.

Mean proportions resident in the upper stomach were 71% for Liquid Gaviscon®, 73% for Gaviscon Advance® and 72% for Formulation P. In contrast only 53-55% of the breakfast resided in the upper part of the stomach.

Figure 1: Mean Gastric Residence of Alginate Formulations



Comparisons between the formulations showed that Gaviscon Advance® had a statistically greater retention than Liquid Gaviscon® with a mean gastric residence ratio of 1.18 (and 95% confidence limits of 1.05 and 1.28). Formulation P and Liquid Gaviscon® had statistically equivalent gastric residences. In conclusion all three alginate formulations clearly demonstrated that they formed robust rafts which resided predominantly in the upper part of the stomach and persisted for greater periods of time than the standard meal. Gaviscon Advance® demonstrated a gastric residence time statistically significantly greater than Liquid Gaviscon®.

132 RHEOLOGICAL CHARACTERISATION OF CARBOPOL GELS AS ELECTRICALLY-CONDUCTING DRUG DELIVERY INTERFACES

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Some commercially available iontophoretic drug delivery systems employ electrically-conducting gel interfaces (ECGIs). Carbopol gels are commonly used in drug delivery systems and may have applications as ECGIs. The rheological properties of gels, including those used as ECGIs, are important in their clinical performance (Jones et al 1997). This study examined the effects of various ionic species on the rheological properties of Carbopol gels in a range of solvent systems.

Two series of Carbopol gels (ETD 2050) were prepared. In the first series, the effects of solvent composition (100% water, water:ethanol 75%:25%, water:ethanol 50%:50%) and NaCl concentration (0, 200, 400 mM) on the gel rheological properties were determined. In the second series, the effects of varying concentrations (20, 100, 200 mM) of NaCl, $CaCl_2$ and a model drug, amethocaine hydrochloride, on the above properties were examined. The solvent for all gels in the latter series was a 75%:25% water/ethanol mixture. All formulations were adjusted to pH 7 using triethanolamine. Rheological properties of the gels were evaluated using a Carri-Med CSL^2-100 rheometer with 4 cm radius stainless steel parallel plate (gap width 1 mm) geometry. Oscillatory measurements were performed over a frequency range 0.01-10 Hz at 293 K, at a constant displacement of 1 $x10^{-4}$ radians from which the viscoelastic properties, elastic modulus (G'), loss modulus (G") and dynamic viscosity (η') were obtained. Statistical analyses were performed using a two-way ANOVA, $p<0.05$ denoting significant differences.

Table 1 shows the elastic moduli at 1 Hz for the first series of Carbopol gels. On addition of ethanol to the solvent systems, and in the absence of NaCl, the gels showed significantly decreased elastic and loss moduli, although the loss tangent tan δ (the ratio of loss to elastic moduli) increased, indicating a greater viscous contribution to viscoelastic behaviour.

Addition of a monovalent cation (Na^+) also significantly decreased G' and G" and gave a significant increase in tan δ, again indicating greater viscous behaviour. Optimal elastic behaviour of NaCl-containing gels was associated with a 25%/75% ethanol/water solvent.

Table 1. Storage moduli (G', Pa at 1 Hz) for first series of Carbopol ETD 2050 gels (mean±s.d.)

	% ethanol in solvent system (% v/v)		
NaCl conc.(mM)	0	25	50
0	595.2±1.6	523±7.4	474.3±3.8
200	212.6±2.4	485.6±3.1	402.2±5.6
400	192.0±1.1	449.6±1.7	387.5±4.8

Rheological parameters for Carbopol gels containing 200 mM salt and without added salt are presented in Table 2. The divalent calcium salt caused a significant lowering of G' and a significant increase in tan δ, due to decreased water availability for polymer solvation. Similar trends were observed at 20 and 100 mM salt concentrations.

Table 2. Rheological data for Carbopol gels (1 Hz, mean±s.d.)

	5% Carbopol	5% Carbopol with salt (200 mM)		
Parameter		NaCl	$CaCl_2$	Ameth. Hyd.
G' (Pa)	523±7.4	496±2.7	302.2±3.4	513.0±2.1
Tan δ	0.09±0.01	0.11±0.01	0.14±0.02	0.12±0.01
η' (Pa.s)	9.78±0.3	4.42±0.1	2.11±0.1	5.33±0.3

Addition of ionic drugs to Carbopol gels significantly reduces the elastic component of their viscoelastic behaviour although this reduction in elastic character may be offset by inclusion of ethanol in the solvent system. These findings will have important implications for the use of Carbopol gels as ECGIs.

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133 THE EFFECT OF DELIVERY ORIFICE SIZE ON THE RATE OF METRONIDAZOLE RELEASE FROM A TWO-COMPARTMENT OSMOTIC DEVICE

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A pharmaceutical development of a two-compartment osmotic delivery system (push-pull type) have been designed in order to get a zero-order rate for active ingredient release. The active ingredient quantity dissolved at 12 hours study have to be more than 75 % of content (250 mg metronidazole) and the lack time have to be less than 2 hours. The delivery orifice size effect on the release characteristics have been studied.

The available bilayer core formulation was studied by a simplified mathematical model (Pacheco et al, 1995) based on geometrical considerations which permits to know the release profiles for simulated osmotic delivery systems in order to find the excipients combination that gives the best fit to zero-order release rate that lasts more than 8 hours and release of the totality of the dose before swelling rate of the push compartment begins to slow down.

Because of active ingredient high dosage and his low osmotic pressure was necessary to get a film coating with high porosity in order to begins the fluid gel formation at active ingredient compartment by water absorption quickly. Differents coating formulations by organic process are studied. Have been checked the effect of solubility excipients on permeability film and on lack time. Permeability of the coating semipermeable membrane could then be adjusted in order to obtain the desired release rate. This could be accomplished by controlling membrane thickness and by incorporating permeability enhancers in the coating.

The osmotic device are perforated with different diameters (0.4, 0.6, 0.8, 1.0 and 2.0 mm). Delivery orifice size was mesured by electronic micrography. Have been checked if the nominal value of diameter is agree with value obtained.

Dissolution studies were made with each one diameter size at three stirring rates (50, 100 and 150 rpm) during 20 hours. The dissolved quantity of the active ingredient and the release rate (mg/h) are calculated each 15 minuts.

Basical statistical analysis was made on each group of values and the ANOVA one way for each one of variables (orifice size and stirring rate).

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134 MECHANISMS OF CONTROLLED S(+) IBUPROFEN RELEASE FROM MULTIPLE-UNIT ORAL CAPSULE FORMULATIONS

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The administration of the pharmacologically active ibuprofen isomer, S(+)ibuprofen, has therapeutic advantages over its racemic form (Klein et al 1992). There have been few published attempts to formulate the active enantiomer into a solid oral dosage form e.g. Romero and Rhodes (1993). In this study, S(+)ibuprofen (I) was formulated as a controlled release multiple-unit dosage form using encapsulated mini-matrices containing two natural hydrophilic gums to control the drug release rate, together with various directly compressible diluent excipients. The gums used were xanthan (X) and karaya (K) while the excipients used were spray-dried lactose (L), Encompress®(E) and Avicel® (A). In each case the drug:gum:excipient ratio was 1:1:1. The mini-matrices (4.5 mm in diameter, 30±1.0 mg) were compressed using a single punch instrumented tablet machine. The drug release behaviour and the effect of elevated temperature and relative humidity(RH) on drug release, crushing strength, friability and weight of the mini-matrices containing I, X and L were investigated.

The drug release studies from encapsulated mini-matrices were performed using the USP XXIII basket method over a 12 hour period in phosphate buffer medium pH 7.0. The results were processed according to: $M_t/M_\infty = kt^n$ where M_t/M_∞ is the fractional drug release at time t, k is a constant, and n is the release exponent. The first 60% of the release curve was quantified according to the heuristic model: $M_t/M_\infty = k_1t^m + k_2t^{2m}$ where the first term on the right hand side is the Fickian contribution (F) and the second term is the Case II relaxational contribution (R). The coefficient m is the purely Fickian diffusion exponent.

The release mechanisms from mini-matrices containing X with L, E and A were anomalous, but approached Case II transport (zero order) with n values of 0.732, 0.644 and 0.881 and release rates of 3.74, 3.69 and 5.56 %/min$^{1/2}$ respectively. The mini-matrices containing K with L, E, and A displayed Super Case II transport with n values of 1.143, 1.248 and 1.620 and release rates of 6.91, 7.79 and 10.18 %/min$^{1/2}$ respectively. Relaxational release predominated in all the mini-matrices containing K. However, in the mini-matrices containing X, the dominant mechanism depended on the excipient used. Relaxation was dominant in I:X:A matrices, while for I:X:L, Fickian diffusion was dominant in the initial stages (0-2 h) followed later by relaxational release. In the case of I:X:E, Fickian diffusion was dominant over the first 8 hours followed by polymer relaxation.

After storage of the mini-matrices (I:X:L) at 35%RH (5, 22 and 37°C) for 2 months, there was no change in the mechanisms of drug release but there were small changes in the rates of release, crushing strength, friability and weights of the mini-matrices. Isothermal storage at 22°C with different RH's (10, 35 and 80 %), had no effect on the mechanisms of release, but produced small changes in release rates. However at high RH (80%), crushing strength decreased whereas both friability and weight increased significantly.

These studies have shown that near zero-order release of S(+)ibuprofen can be achieved using encapsulated mini-matrix formulations which were relatively stable to variation in temperature and RH over a 2 month time period.

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135 CHLORHEXIDINE SORPTION INTO AND RELEASE FROM DENTAL PROSTHETIC BIOMATERIALS

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Denture stomatitis (DS) is a erythematous condition of the denture-bearing oral mucosae in which *Candida spp*, particularly *Candida albicans*, have been directly implicated. Prevention of DS may be achieved by the use of dentures containing antimicrobial agents that are released at a controlled rate directly to the inflamed mucosae. However, polymerisation of denture biomaterials in the presence of antimicrobial agents may affect their mechanical properties. Therefore, this study examined the inclusion (sorption) of chlorhexidine (CH) from aqueous and aqueous/non-aqueous solvent systems into, and subsequent release from, dental prosthetic biomaterials.

Two primary dental biomaterials, polyhydroxyethylmethacrylate (pHEMA) and polymethylmethacrylate (pMMA) and their co-polymers were employed in this study. Polymerisation of pure monomers and also monomer blends (99.5% w/w) was performed by mixing with azobisisobutyronitrile (0.5% w/w) and incubating at 60°C for 18 h. Unreacted monomers were removed from each polymeric material by washing, following which, each polymeric material was freeze-dried. Uptake of CH into each polymeric biomaterial was performed by immersing portions (2 x 1 cm) of each biomaterial in PBS (pH 7) or ethanol/PBS mixtures containing CH (10 - 100 μg mL^{-1}). At defined intervals, samples of immersion fluid were removed and the equilibrium concentration of CH quantified using UV spectroscopy at 254 nm. Release of CH from biomaterials that had previously been loaded with CH was examined into PBS (pH 7) using a shaking water bath (at 37°C). All experiments were performed, at least, in triplicate and the results shown in tables are mean values (CV < 5% in all cases).

The time required for equilibrium uptake of CH was statistically dependent on the biomaterial type and also on the composition of the immersion solvent. Minimum and maximum equilibrium uptake times were observed for pMMA (1.0 ± 0.08 h) and pHEMA (24.1 ± 1.1 h), respectively, reflecting the greater hydrophobicity of pMMA and the hydrogel properties of pHEMA. Increasing the content of pHEMA significantly increased the equilibrium uptake time. Uptake profiles of CH onto pMMA and pHFMA were Langmuirian (L-type) and Constant (C-type), respectively, and dependent of the composition of immersion fluid.

Table 1. The effects of biomaterial composition and immersion fluid type on subsequent sorption of chlorhexidine (CH)

CH Conc^n. in immersion fluid (μg/mL)	CH sorption from PBS (μg/g) into :		CH sorption from Ethanol (95%) (μg/g) into
	pMMA	pHEMA	pHEMA
10	0.07	16.95	65.04
20	0.61	74.66	117.75
35	0.95	143.20	186.59
50	1.27	272.57	310.76
100	1.37	605.11	661.94

The release of CH from pMMA was negligible over 24 h, primarily due to the its strong surface affinity for this polymer. The release of CH from pHEMA, previously immersed in absolute ethanol was greater than when immersed in PBS. Typically, CH release from pHEMA (in which sorption had occurred from PBS containing 20 and 35 μg mL^{-1} CH) was 1.26 and 2.56 μg after 12 h, respectively. Release of CH from pMMA/pHEMA co-polymers was dependent on pHEMA content.

In conclusion this study has shown the feasibility of both sorption of CH into, and subsequent release from denture prosthetic biomaterials. This strategy may be useful in the prevention of denture stomatitis.

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136 PHYSICAL CHARACTERISATION OF HEXETIDINE-LOADED POLYVINYL CHLORIDE

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Introduction of an endotracheal (ET) tube into the management of the intensive care patient increases the risk of nosocomial pneumonia (Craven et al 1990). Microbial adherence to polyvinyl chloride (PVC), the constituent material of ET tubes, is followed by colonisation of the device with associated biofilm accretion in the inner lumen of the tube (Gorman et al 1993). The non-antibiotic, antimicrobial agent hexetidine has been shown to exert an inhibitory effect on microbial adherence (McGovern et al. 1997). Hexetidine may therefore be beneficial for the prevention of ET biofilm of microbial origin. This study examined the physical properties of hexetidine-loaded PVC as candidate biomaterials for use in ET tubes.

Hexetidine (1, 5 and 10% w/w) was incorporated into medical grade PVC emulsion and material films were polymerised by heating at 160°C for 10 minutes. Hexetidine release into PBS containing Tween®20 (1% v/v) at 37°C was examined in conjunction with HPLC. Biomaterial tensile properties were examined using a TA-XT2 Texture Analyser in tensile mode (cross-head speed 5 mms^{-1}). Before and after drug release, biomaterial dynamic contact angle and microrugosity were determined using a Dynamic Contact Angle Analyser (wetting medium HPLC water) and atomic force microscopy, respectively. The effects of hexetidine loading on release rates and tensile properties were statistically evaluated using a one-way Analysis of Variance ($p<0.05$ denoting significance).

Hexetidine release from PVC was proportional to square root of time, fractional release rates for films containing 1, 5 and 10% w/w hexetidine being 0.017, 0.024 and 0.031 $hr^{-1/2}$. Increased hexetidine concentration significantly increased release rates. Material tensile strength and % elongation were found to be significantly lower with 1% and again with 5% w/w hexetidine (table 1). No difference in either of these parameters was observed between 5% and 10% w/w hexetidine. Work of failure, an indication of film toughness, was significantly reduced for hexetidine-loaded PVC, with no difference recorded between 1, 5 and 10% w/w hexetidine. Young's modulus, a measure of film stiffness, was significantly lower for 5% and 10% w/w hexetidine than films containing 1% or devoid of hexetidine. Material microrugosity increased with concentration of incorporated hexetidine both before (table 1) and after drug release. Following release (3 wk), film surfaces were found to be significantly smoother ($p<0.05$) than freshly prepared films. As hexetidine concentration increased, the advancing contact angle of film surfaces was observed to decrease significantly before (table 1) and after hexetidine release. Similarly after release, advancing contact angle of film surfaces was significantly reduced.

Table 1 Physical characteristics of hexetidine-loaded PVC

Hexetidine loading (% w/w)	Tensile strength (MPa)	Advancing contact angle (°)	Surface roughness (nm)
0	2.96±0.29	92.6±1.8	55±12
1	1.26±0.12	89.2±1.0	137±14
5	0.74±0.20	89.6±0.4	136±27
10	0.78±0.12	87.0±0.2	142±35

In this study, a series of hexetidine-loaded PVC biomaterials have been developed. However, the inclusion of hexetidine significantly reduced the mechanical performance of PVC. Therefore, a compromise will be necessary between desirable drug release (to reduce biofilm formation) and durable tensile performance.

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137 GLUCOSE-RESPONSIVE GELS BASED ON DEXTRAN COVALENTLY COUPLED WITH LECTIN TO CONTROL INSULIN DELIVERY

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The best therapy for diabetes mellitus would be one which mimics the physiological process in which the amount of therapeutic agent release can be affected according to physiological needs by a truly 'closed loop' approach, In this concept, the insulin dosage would be governed by the blood glucose level on a continuous basis. An in vitro self-regulating system has been described previously using a glucose-responsive gel based on concanavalin A (Con A) and a polysaccharide displacement mechanism independent of the drug used (Taylor 1992 & 1993). The mechanism depends on a competitive displacement of a glucose-bearing polysaccharide by free glucose from Con A. The gel structure which forms between Con A and the polysaccharide is dismantled when glucose causes this displacement. The gel viscosity falls as a result but reforms on glucose removal, thus providing the switch controlling the diffusion of drug held in a reservoir. The delivery of model solutes and insulin has been demonstrated using this system (Taylor & Tanna 1994; Tanna & Taylor 1994; Taylor et al 1995).

One of the problems encountered in the previous studies was the significant leakage of Con A during the low viscosity phases (Tanna 1996). This apparent Con A leakage was undesirable for two reasons. Firstly, due to its toxicity to the immune system and secondly the repeated action of the mechanism depends on maintaining the components in juxtaposition. For these reasons, an approach for physically or chemically restraining the components while retaining activity was investigated. The Schiffs reaction was used to couple Con A to dextran. The object was to retain the reversible receptor binding capabilities of Con A to dextran via the terminal glucose units while Con A was permanently anchored to the dextran at other points. This is a simple method for the activation of polysaccharides by oxidation with periodate to produce reactive aldehyde groups for the attachment of proteins. The Schiffs bases formed are stabilised by borohydride reduction.

Dextran of molecular weight 500,000 [D500] and 2,000,000 [D2M] was treated in this way and coupled at 10% or 20% v/v concentrations with either 5 or 10% w/v Con A. The oxidation was conducted with between 0.02M and 0.2M sodium periodate and the coupling was either at uncontrolled pH or varied using buffers between 5.9 and 8.0. Borohydride reduction was carried out with 1mg/ml sodium borohydride. Each gel formulation formulated in this way was examined for Con A retention and for glucose responsiveness in terms of differential delivery of a model solute, tartrazine. Initial studies at 20°C revealed that the efficacy of the covalent coupling was influenced by dextran molecular weight and concentration, periodate concentration, Con A concentration and pH of Con A solution. All formulations studied restricted Con A escape significantly with respect to non-coupled gels and the differences between the oxidised formulations as a function of pH were small by contrast. The delivery of insulin was demonstrated using this modified gel system in conditions of repeated glucose triggering (Fig. 1).

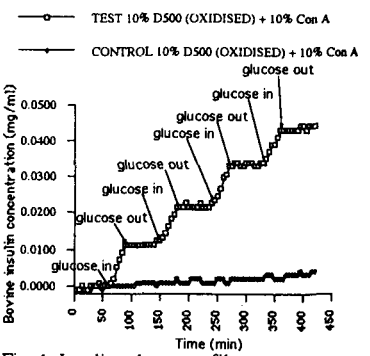

Fig. 1. Insulin release profile across a glucose-responsive gel

The performance of this novel system has been improved in terms of reduced Con A leakage. This could therefore be used to form the basis of the design of a self-regulating drug delivery device for therapeutic agents used to treat diabetes mellitus.

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138 THE OESOPHAGEAL RETENTION OF A THERMALLY SENSITIVE HYDROGEL

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The development of a mucoadhesive gel capable of adhering to the oesophageal mucosa, thus providing a protective barrier for the tissue from refluxed gastric contents, would undoubtedly be of benefit in the treatment of gastro-oesophageal reflux disease. This study examined the retention of a hydrogel, which demonstrated a temperature dependent gelation and capacity to adhere to the oesophageal mucosa.

Smart Hydrogel™, (poly(oxyethylene-b-oxypropylene-b-oxyethylene)-g-poly(acrylic acid)), is an unique interpenetrating polymer network (IPN) composed of poly(acrylic acid) and a block copolymer of poly(ethylene oxide) and poly(propylene oxide).

Gamma scintigraphy was employed as a non invasive means of monitoring the retention of the formulation in the gastrointestinal tract of three healthy male volunteers. Each volunteer received 5ml of the hydrogel by the oral route. The polymer was radiolabelled by the inclusion of ^{99m}Tc-DTPA to give an activity of 3MBq per dose. Scintigraphic imaging of the head, oesophagus and stomach of each of the volunteers was carried out over the 10 minute period immediately following administration. The administering syringe was imaged pre and post dosing in order to determine the amount of activity swallowed. The data was analysed by creating a region of interest around the oesophagus and by taking a sample of background counts, corrected for background radiation and for decay of the isotope.

Biphasic clearance of the formulation was noted with approximately 65% of the dose entering into the stomach within 5 seconds of administration. 15% of the dose displayed prolonged retention in the oesophagus. A mean value of 13% (n = 6 administrations) of the formulation was retained 10 minutes after administration.

Retention of the hydrogel in vitro was compared with hydroxypropylmethyl cellulose (Methocel K4M PREM) and Carbopol 934P using a dip coating technique. Twenty-five ml of liquid agar was poured on each side of 4 microscope slides and allowed to set. The slides were then dipped into the formulations, which had been radiolabelled with ^{99m}Tc-DTPA, and heated in an oven at 37 ^{0}C for 8 minutes. In order to simulate exposure to saliva, the slides were dipped in physiological saline (90mls) at 37 ^{0}C repeatedly at 2 minute intervals over a 10 minute period. Slides and saline were imaged using a gamma camera before rinsing and after each dip. The resulting data, after correction for background radiation and for decay of the isotope, revealed 83.5% of the hydrogel (n=3) was retained on the agar after 10 minutes. The hydroxypropylmethyl cellulose and the Carbopol 934P formulations displayed a mean retention of 89.7 % (n=3) and 92.9% (n=3) respectively. The validity of the agar plate model will be investigated in a comparison with the oesophageal retention of the three formulations in vivo.

Smart Hydrogel™ is a trademark of Gelmed Inc. USA.

139 THE ROLE OF THE ENHANCED PERMEABILITY AND RETENTION (EPR) EFFECT IN TUMOUR ACCUMULATION OF POLYMERIC ANTICANCER AGENTS

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Introduction

Macromolecules such as soluble synthetic polymers and small particles can selectively accumulate in solid tumour tissues by a mechanism called enhanced permeability and retention (EPR) effect first described by Meada et al (1986). This effect is primarily due to (a) enhanced tumour angiogenesis resulting in hypervasculature, (b) defective/ incompleteness vascular epithelial layer and (c) the absent of effective lymphatic drainage in the tumour tissues (Duncan et al, 1996). The increase vascular permeability can be visualised by the leakage of Evans Blue dye. Currently, we are studying the EPR effect in mouse tumour models, subcutaneous L1210 and B16F10 melanoma to determine the extent of EPR effect at different stages of tumour growth and understand how this correlates with pharmacological activity of macromolecular drugs. Two probes are being used to monitor EPR effect. Evans Blue and N-(2-hydroxypropyl)methacrylamide (HPMA)-doxorubicin copolymer which is currently in Phase I/II Clinical Trial (Vasey et al 1996).

Materials and Methods

The tumour models used were DBA.2 mice bearing L1210 lymphocytic leukaemia and C57 mice bearing B16F10 murine melanoma. Tumour cells injected sub-cutaneously into the back of the neck and once the tumour reached 25 mm^2 the study was initiated . Evans Blue was injected intravenously at a dose of 10mg/kg to groups of animals bearing tumours of different sizes (from 25 to 400 mm^2). An hour after administration of the dye, animals were killed and the tumour tissue was carefully removed and weighed. The dye, Evans Blue, was then extracted and assayed using a spectrophotometer at wavelength of 590nm. (Harada et al 1971). HPMA copolymer-doxorubicin was administered at doses 5-50 mg/kg and tumour uptake analysed by HPLC (Seymour et al 1994).

Results and Discussion

In the L1210 tumours, less than 0.6% of the injected dose of Evans Blue was recovered when tumour tissue weighed less than 0.6g As the tumours grew, the intratumoural concentration of the dye increased. More than 10% of the injected dye was recovered when the tumour weight reached 1.2 to 2.2g. This is consistent with valuaes seen for HPMA copolymer doxorubicin seen in solid tumours of similar size.Once tumour weight exceeded 2.2g, the accumulation of Evans Blue-albumin complex decreased.

After injection, Evans Blue immediately binds to albumin resulting in the formation of a 70 kDa complex. This study showed that as the solid tumour grew, accumulation of the complex increased, probably due to increased angiogenesis. However, above a maximum tumour size, decreased accumulation was noted which could be due to, the formation of a necrotic centre devoid of blood vessels, increased intratumoural pressure limiting extravasation or alternatively formation of a complete endothelial barrier in the mature blood vessels. Further studies using different tumour cell lines are under investigation to try to elucidate these mechanisms.

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Acknowledgements

Yee Nee Sat would like to thank University Kebangsaan Malaysia, and The University of London (ORS) for supporting her studentship.

140 ENHANCING THE INCORPORATION OF A WATER SOLUBLE DRUG INTO PLGA NANOPARTICLES

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The aim of this study was to assess various formulation parameters for the enhancement of the incorporation of a water soluble drug into submicron (<200nm) PLGA nanoparticles of low polydispersity prepared by the Interfacial Polymer Deposition (IPD) technique. Optimisation of the incorporation of water insoluble drugs into such nanoparticles has been studied by various researchers who have reported high degrees of incorporation (Guterres et al., 1995; Chacon et al.,1996). However, the IPD technique suffers from poor incorporation of water soluble drugs and there is a lack of data in the literature on approaches to enhance such incorporation. Approaches investigated in the present study included:

- effect of the pH in the aqueous phase
- conversion of the salt form of the drug to its base form
- inclusion of fatty acids and poly(methylmethacrylic) acid (PMMA)

Procaine hydrochloride was used as a model drug and was quantitated by UV spectroscopy at 292 nm. Drug incorporation efficiency was expressed as drug content (mass of drug in nanoparticles as a percentage of total mass of polymer and drug used in the formulation) (DC) and drug entrapment (mass of drug in nanoparticles as a percentage of total mass of drug added to the system) (DE). Nanoparticles were characterised in terms of their particle size (Photon Correlation Spectroscopy), surface charge (Laser Doppler Anemometry) and morphology (Transmission Electron Microscopy).

Some of the major findings are highlighted. Nanoparticles produced were of low polydispersity and ranged from 125nm-197nm.The initial preparation procedure comprising 2%w/w drug loading in its salt form and deionised water (pH5.8) as the aqueous phase demonstrated a low incorporation with a DE of 11% and a DC of 0.35%w/w only. Changing the pH of the aqueous phase to 9.3 significantly increased DE to 58% and DC to 1.27%w/w. TEM studies showed that the nanoparticles were spherical, discrete and uniform. Conversion of the drug to its dihydrate form also improved DC from 0.35%w/w to 0.92%w/w and DE from 11% to 41%. Interestingly, the nanoparticle morphology was adversely affected at high drug loadings indicating that there might exist a limit as to the amount of drug that can be incorporated into nanoparticles without particle formation and morphology being adversely influenced. Inclusion of PMMA resulted in a slight increase in DC and DE while fatty acids led to a highly significant increase in both DC and DE by promoting complex formation between the amino group of the drug and the carboxyl groups of these excipients. In conclusion, this study has shown that formulation variables can be optimised in order to enhance the incorporation of a water soluble drug into PLGA nanoparticles.

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Guterres,S.S., Fessi, H., Barrat,G., Devissaguet, J.P., and Puisieux, F. (1995), **Int. J.Pharm**., 113, 57-63.

141 THE USE OF ATOMIC FORCE MICROSCOPY TO INVESTIGATE THE PRESENCE OF ATTACHED POLYMER ON THE SURFACE OF HUMAN BUCCAL CELLS

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The atomic force microscope (AFM) is one of several types of scanning probe microscopes developed from the scanning tunnelling microscope (Binning et al 1986). AFM shows great promise for producing new kinds of morphological information with very little preparation and in an almost completely non-invasive manner. AFM provides molecular scale resolution of solid surfaces by scanning across the sample with a sharp probe, to reproduce the surface topography by following its contours. In this study the use of the AFM will be investigated as a means of examining for the presence of attached polymer on the surface of buccal cells.

Buccal cells were obtained by scraping the inner surface of the cheek of a volunteer. The cells were suspended in 10 mL of tris buffered saline (pH 7.6), washed three times and adjusted to a concentration of 48×10^4 cells per 2 mL. 5 mL isotonic saline solution containing 0.5% w/v of either polycarbophil, chitosan or Methocel 65HG was added to the cell suspension and incubated for 15 min. at 30°C. The cells were then washed three times, a drop of suspension removed, placed onto a mica surface and allowed to dry. The cells were then examined using the AFM.

Untreated buccal cells were shown to have a relatively flat surface but with many craters and indentations (Fig. 1). Cells exposed to chitosan showed large solid crystalline like structures on their surface (Fig. 2), the size of which decreased with an increased number of washes prior to examination. The chitosan treated cells showed the greatest roughness with a pronounced raised surface. Methocel 65HG treated cells showed a rippling effect over the cell surface and polycarbophil gave a slightly smoother covering which was still clearly different from the surface of untreated cells.

This investigation has showed that it is possible to obtain quality images of the buccal cell surface using the AFM, and clear evidence of polymer presence on the surface was obtained.

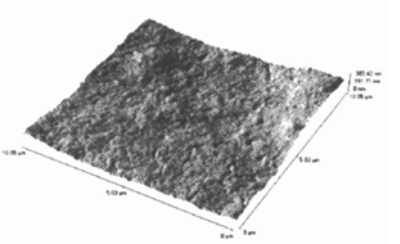

Fig. 1 Untreated buccal cells

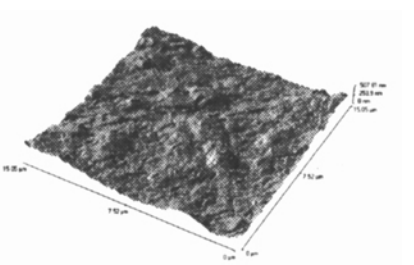

Fig. 2 Buccal cells exposed to a chitosan solution

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142 TOWARDS MALARIA PROPHYLAXIS BY TRANSDERMAL DELIVERY OF SOME ANTIMALARIAL DRUGS

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Malaria continues to be an important cause of mortality in the tropics and subtropics for both the indigenous population and traveller alike. The chemotherapy of malaria involves both blood schitzontocide and anti-relapse activity, with no single drug currently available that shows both functions. With regard to the prophylaxis of infection by the plasmodium parasite, one potentially useful drug, primaquine, has proven effective when taken orally. However, numerous advantages would exist by the administration of this and other anti-malarial drugs via the transdermal route, including enhanced pharmacokinetics and improved patient compliance. In order to obtain a suitable transdermal therapeutic system, the availability of the potential drug candidates from topically applied doses must be determined and optimised. Passive penetration of primaquine across excised human skin has been demonstrated (Thassu and Vyas, 1993) and it was recently demonstrated that the penetration of primaquine base can be enhanced across rat skin (Mayorga et al, 1996).

In this work we examined the permeation of a range of currently administered anti-malarial drugs: quinine, primaquine, mefloquine and chloroquine across excised human cadaver skin, using vehicles containing propylene glycol (PG) and Mygliol. Figure 1 shows that the flux of primaquine from a vehicle of Mygliol 812 was some x9 that from a PG vehicle. This was unexpected as PG is a recognised skin penetration enhance, whereas Mygliol does not seem to have been considered previously for this effect. This work also demonstrated much lower flux rates across human skin than the fluxe rates obtained across rat skin (Mayorga et al, 1996) and further illustrates the importance of inter-specific differences in the selection of models.

Furthermore, each of the drugs examined in this work are chiral and, with the exception of quinine, are currently administered as racemic mixtures. We have recently demonstrated that stereoisomers can, under certain conditions, penetrate the human skin at differential rates (Heard et al, 1997). This presentation will also include a discussion on the permeation rates of individual enantiomers of the drugs under test and the pharmacological consequences thereof (Heard and Brain, 1995).

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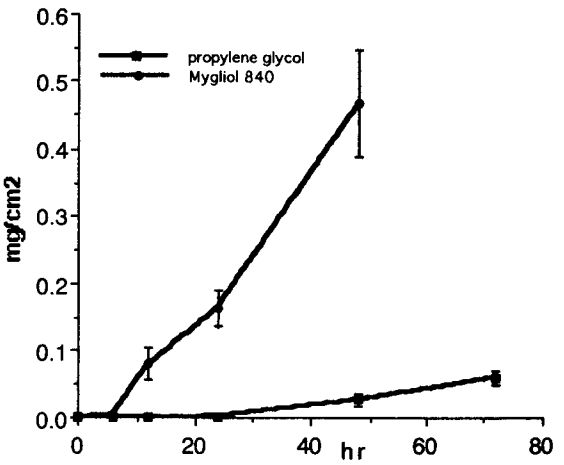
Mayorga P., Puisieux F. & Courraze G. Int. J. Pharm. 132:71-79 (1996)
Thassu D. & Vyas S.P. Drug Devel. Ind. Pharm. 19:1943-1356 (1993)

Figure 1 Permeation of primaquine base across human cadaver skin from PG and Mygliol 812 vehicles (n=6±SE)



143 ULTRASTRUCTURAL ARCHITECTURE OF PROTEIN AND DNA CONTAINING LIPOSOMES AS VISUALIZED USING CRYO-SEM

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Adequate characterisation of liposomes is necessary in order to maximise their potential as drug/gene delivery systems. Previous studies have used cryo-TEM to reveal outer convex or concave fracture surfaces, however this technique provides limited information on the deposition of incorporated substances. We describe here the use of cryo-SEM for the ultrastructural characterisation of liposomes and their associated contents.

Multilamellar vesicles (MLVs) composed of either lecithin, DMPC, or DMPC and cholesterol were prepared by mechanical dispersion methods (Bangham et al 1969). Protein-containing MLVs were prepared by re-hydrating the lipid films with 1% w/v ovalbumin at either 25 or 100°C. Small unilamellar vesicles (SUVs) were prepared from MLVs by probe sonication. Lambda phage DNA-containing LUVs were prepared using cholesterol, lecithin and dihexyldodecyldimethyl ammonium chloride (DAM). DNA-containing LUVs were also prepared by the reverse-phase evaporation method (Szoka et al 1978). Further samples of pre-formed liposomes were agitated in a DNA solution (1% w/v in PBS) in order to produce liposomes with DNA exclusively on the outer suface. Liposome suspensions were concentrated by centrifugation, snap-frozen in slushy nitrogen and fractured at -196°C in a Polaron LT7400 Cryoprep system (Fisons). Liposomes and control solutions were etched by subliming under vacuum at -90°C, sputter coated with gold and viewed with a Jeol 6310 scanning electron microscope utilizing a cooled cryostage (-196°C).

Cross-sectional images of fractured liposomes reveal the inner architecture of the lipid layers and their associated contents, allowing analysis of surface and internal topography (Fig 1).

The images obtained of MLVs reveal arrays of inner layers and their associated contents which may be observed in 3-D using stereo-micrographs. Ovalbumin was seen deposited as a net-like matrix within liposomes and could be clearly seen both within the aqueous cores and between the lamella (Fig. 2). Protein containing MLVs prepared at 100°C were more distorted, and the outer lamellae had a reticulated structure, with a mesh of compact protein networks visible within the cavities. Very dense networks of DNA could be seen packed into the central compartments of liposomes. In pre-formed liposomes to which DNA was subsequently added, coiled protrusions were visible on the liposomal outer surface.

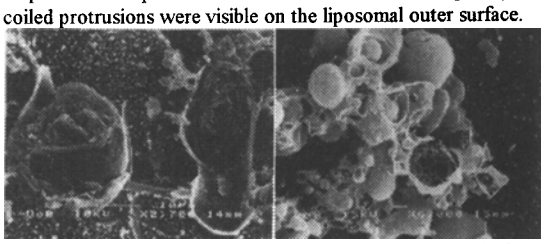

Fig. 1 Cryo-SEM micrograph of DMPC/cholesterol (2:1) liposomes

Fig. 2 Cryo-SEM micrograph of DMPC/cholesterol (2:1) liposomes containing 1% ovalbumin

The cryo-SEM method presented here provides a useful tool for the characterisation of liposomes, and a technique for the confirmation of the incorporation and location of proteins and DNA.

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144 NOVEL METHOD OF A STABLE LIPOSOME PREPARATION

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The preparation of a stable liposome product with a reasonable shelf life has been the focus of a considerable amount of work. The methods preferred are based on freezing and freeze-drying of liposomes with cryoprotectants and recently the oven drying of sugar solutions of liposomes Sun *et al*, (1996). An alternative approach by Payne et al, (1986) was the preparation of proliposomes. This work investigates the use of rotary evaporation procedures on the production of stable dry powder liposome formulations.

Egg phosphatidylcholine (EPC), 1-palmitoyl-2-oleylphosphatidylcholine (POPC), 1,2-dioleylphosphatidylserine (OOPS) and cholesterol (Chol) in various proportions were dissolved in tertiary butyl alcohol (TBA) to give a 2.5% w/v solution. 1 ml of this solution was poured into stirred 10 ml volume of phosphate buffer saline (PBS) pH 7.3 at room temperature. An appropriate volume of lactose was added to these PBS solutions to give a lactose concentration of 5 or 10% w/v lactose. The volume of TBA solutions and the rate of addition and stirring intensity were varied to determine the effect on liposome size. These solutions were then rotary evaporated at 50°C under vacuum. The dry powder was then stored under nitrogen at 4°C until required. The size of the liposomes before and after drying were measured using a Malvern 4700 PCS with a K7032 correlator and a helium neon laser. The temperature was set at 30°C and measurements made at 90°. Liposome structure was examined before and after rotary evaporation by TEM using a Joel TEM 100U and 1% w/v phosphotungstic acid as a negative stain. Fast stirring speeds produced smaller liposomes when 1 ml of TBA solutions was added to PBS (slow 159.2 nm ± 5.0 S.D., fast 107.9 nm ± 3.6 for EPC:Chol 10:1). However, in the presence of lactose solution no significant differences were seen, the values ranging from 70 to 100 nm. The greater the volume of TBA added to the PBS solution, the greater the liposome size but in the presence of lactose only a slight increases were observed (Table 1)

Table 1. Effect of TBA volume and 5% w/v lactose on liposome size

Volume of TBA added to PBS (ml)	Size nm ± S.D. n=6	Volume of TBA added to lactose 5% w/v (ml)	Size nm ± S.D. n=6
0.2	74.6 ± 3.8	0.2	82.2 ± 5.0
1.0	142.0 ± 3.2	1.0	98.1 ± 4.5
4.0	212.1 ± 5.0	4.0	115.8 ± 4.8

On rotary evaporation the size increased for the EPC liposomes prepared in PBS with or without cholesterol present. However, the increase with cholesterol was much smaller than in its absence. In the presence of lactose, the increases were insignificant (Table 2).

Table 2. Liposome size before and after rotary evaporation

Formulation	Size before nm ± S.D. n=6	Size after nm ± S.D. n=6
EPC	78.9 ± 4.7	212.0 ± 9.7
EPC:Chol 10:1	92.6 ± 3.0	146.0 ± 5.4
EPC Lactose 5% w/v	73.6 ± 3.4	82.1 ± 4.3
EPC:Chol 10:1 Lactose 5% w/v	91.8 ± 4.1	94.8 ± 4.4

Unilamellar liposomes were observed by TEM for the initial preparation and mixtures of unilamellar and multilamellar liposomes formed after rehydration of the rotary evaporated preparation. The products were free-flowing powders after 3 months. This procedure offers a simple cheap alternative to traditional freeze-drying methods for producing free flowing dry powder liposomal products for the delivery of lipohpilic drugs.

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145 THE DESIGN OF AN AQUEOUS TWO PHASE SYSTEM FOR THE PRODUCTION OF A NOVEL DRUG DELIVERY DEVICE TO TREAT TUMOURS

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The emergence of metastases in organs distant from the primary tumour is the most devastating aspect of cancer. The treatment of metastases becomes difficult as the cells in both primary and secondary neoplasms show no uniformity. The difficulty in treating such conditions is caused by the multiple cell populations within the neoplasms which exhibit differences with respect to cell surface properties, antigenicity, immunogenicity, growth rate and sensitivity to cytotoxic drugs. (Fidler, 1985)

A device capable of eradicating all neoplastic cells, whilst leaving non-neoplastic cells unharmed is required. Emphasis has been placed on antibody targeted systems, in an attempt to improve therapy and reduce toxicity. Although some success has been achieved, the altered antigenic characteristics prevent complete eradication of all tumour cells.

In the following novel approach, we describe a drug delivery device theoretically capable of accessing tumours and secondary neoplasms and destroying all tumour cells, whilst causing minimal toxicity to non-tumorigenic cells.

The work has evolved on the principle that it may be possible to exploit the natural ability of the innate immune system to recruit macrophages chemotactically to tumours, in numbers correlating with the concentration of cytokines released from in-situ lymphocytes and other tissue (Roitt, 1989). The use of macrophages as a delivery device is attractive, because once in activated state, they can recognise and destroy neoplastic cells both in-vitro and in-vivo without injuring non-tumorigenic cells. (Carazon, 1992)

The proposed exploitation will consist of the drug loading of macrophages in-vitro via the phagocytosis of liposomal drug, after which reintroduction of the modified cells into the donor may result in tumour targeting. Such a system provides several potential advantages, in that, the drug is well shielded from destructive processes prior to finding the target, there will be little uptake of the liposomal drug by the reticular endothelial system, phenotypic changes of the tumour cells will not alter the recognition by macrophages, there will be an increased success in membrane penetration, lack of provocation of hypersensitivity reactions, and, targeting time will be allowed because the drug is sequestered in the liposome and macrophage and will remain inactive until it leaks out by diffusion or destruction of the macrophage, which is a differentiated, non-dividing cell.

The formulation stage has involved designing a system in which macrophages do not adhere and can be easily removed, whilst providing, both a surface for phagocytosis and subsequent separation of any unmodified components, so as to achieve an isolated sample of liposome-loaded macrophages. For this stage, aqueous two phase polymer systems were used. It is possible to produce two immiscible phases with combinations of certain aqueous polymer solutions, which because of the high water content (80 - 90%) are compatible with cells. (Burdon, 1988) Such systems are highly variable in that changing such parameters as polymer molecular weight or concentration or by the addition of salts, the separation of any one material can be altered, thus offering the opportunity of creating the separation conditions required. A further advantage of the system is that all particulate matter, while having an affinity for one or other of the layers, can be centrifuged to the interface hence producing the surface for phagocytosis.

The polymers chosen were poloxamer F-127 and dextran 500 000. Poloxamer was chosen as it exhibits reverse thermal gelation characteristics and so it was thought possible that the phagocytosis could be conducted at a temperature of 20°C, at which the poloxamer would be in its gelled state thus providing a solid/liquid interface as opposed to a liquid/liquid interface provided by such systems as PEG/dextran. On lowering the temperature the poloxamer reverts to its liquid state and regains its potential to separate particulate matter. Studies have shown that it is indeed possible to achieve phagocytosis at the interface and separation profiles of both macrophages and liposomes in poloxamer/dextran systems containing variable phosphate and chloride ratios along with other salt additives can lead to the conclusion that although it may not be possible to achieve both phagocytosis and separation in a single system, by using several systems with differing attributes it may be possible to achieve an isolated liposome-loaded macrophage sample.

(US Patent Applic 08/481,242 Improving Effectiveness of Drugs, applies)

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146 ANTINUCLEANT SELECTION FOR SUPERSATURATED TRANSDERMAL DRUG DELIVERY

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Transdermal drug delivery has several potential advantages over the oral route of administration. However, the stratum corneum is relatively impervious to many substances including drugs. Recently supersaturated drug delivery has been shown to increase transdermal flux of several drugs without alteration of the stratum corneum structure (Davies & Hadgraft, 1991; Megrab et al. (1995). As supersaturation increases thermodynamic activity above the saturated drug level, stability is a problem. Antinucleating polymers slow down the rate of crystal growth and aid stability. To date, antinucleant selection has been largely empirical. By studying solid solutions of drugs, it is possible to investigate the potential of polymers as antinucleants for transdermal drug delivery and other areas.

Solid solutions of 17β-oestradiol, sulphisoxazole, captopril and salicylic acid were prepared by solvent evaporation before characterisation by x-ray diffraction and differential scanning calorimetry (DSC). Attenuated total reflectance Fourier Transform Infra-red (FT-IR) and FT-Raman spectroscopy were used to examine key groupings on both drug and polymer essential to antinucleant activity.

DSC scans showed reduction of the drug endothermic melts dependent on the polymer selected. This was reflected in the x-ray powder diffraction patterns which revealed an amorphous halo free from diffraction peaks seen inthe solid drug (Fig.1). The level and type of polymer were significant in this effect. Thus FT-IR illustrated changes occurring around the ketone grouping of polyvinyl pyrrolidone (PVP) molecules. Raman spectroscopy indicated that the aromatic portion of the drug molecule distorts depending on the functional groups attached to the ring. Previous work on antinucleant activity and solid solution formation has not elucidated the mechanisms of antinucleant activity.

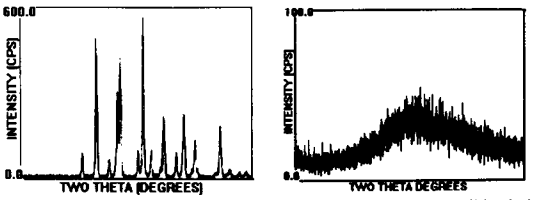

Fig1. X-ray diffractograms of crystalline 17β-oestradiol and of the solid solution of 17β-oestradiol/PVP (70:30w/w)

By studying solid solutions it was seen that precrystalline drug/polymer associations indicate that the molecules orientate between the electron deficient areas on the polymer and the electron rich aromatic rings in the drug. Thus interaction appears to be electrostatically based. Functional groups attached to the ring are important in this interaction.

Alterations in the FT-IR and Raman patterns around the ketone group of the polymer and the aromatic areas of a drug demonstrate that an affinity at the electrostatic sites of molecules is important in antinucleant activity. As formation of a solid solution depends on polymer retgardation of crystal growth, then an understanding of the nature of associations is desirable. Raman and FT-IR techniques are able to detect changes in molecular interactions and can be used to reveal the groups associated with complex formation in the solid state and may predict which polymers will stabilise a supersaturated system for transdermal delivery of medicament.

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147 EFFECT OF SODIUM LAURYL SULPHATE AND DIMETHYL SULPHOXIDE ON THE PERMEATION OF 5-FLUOROURACIL THROUGH A STRATUM CORNEUM INTERCELLULAR MODEL

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The principal barrier to transdermal delivery of most drugs is the lamellar intercellular lipid matrix of the stratum corneum (SC). Previously we reported a lamellar matrix comprising cholesterol, water and SC free fatty acids and their soaps capable of modelling the SC intercellular structure (Moghimi et al., 1996a), SC barrier performance towards permeation of 5-fluorouracil (5FU) and oestradiol (Moghimi et al., 1996b), and effects of terpenes toward permeation of these model drugs across human SC (Moghimi et al., 1996c).

Here the effects of penetration enhancers, sodium lauryl sulphate (SLS) and dimethyl sulphoxide (DMSO), on the penetration of 5FU through the matrix were investigated at 32°C. Release experiments used matrices containing 10% (w/w) enhancers and 0.1% (w/w) 5FU. Permeation studies employed matrix membranes containing 10% enhancers and saturated aqueous solution of 5FU as donor. The effect of SLS on the matrix was also studied by differential scanning calorimetry (DSC).

Release and permeation experiments revealed a diffusion coefficient of $5.97 \pm 1.75 \times 10^{-4}$ cm^2 h^{-1} and a permeability coefficient of $0.307 \pm 0.169 \times 10^{-2}$ cm h^{-1} for 5FU through the untreated matrix. SLS increased the diffusion and permeability coefficients of 5FU to $15.7 \pm 0.98 \times 10^{-4}$ cm^2 h^{-1} and $1.36 \pm 0.169 \times 10^{-2}$ cm h^{-1} respectively. Comparison of SLS-treated and untreated matrix data by t-test showed P values of 0.001 for release and 0.090 for permeation experiments. The partition coefficient of 5FU between the matrix and water was increased 1.7 times by SLS.

SLS increases the permeability coefficient of 5FU through human epidermis. As the concentration of SLS in the intercellular lipid domain of the enhancer-treated SC is not known, it is not possible to relate the present data to that of human SC quantitatively. However, the data show that our matrix models qualitatively the effect of SLS on the barrier performance of the SC toward 5FU.

In DSC studies, the matrix showed several endothermic transitions over the range of 10 to 120°C of which one covered the permeation experiment temperature. SLS decreases enthalpy of this transition significantly from 15.3 ± 1.07 to 13.1 ± 0.920 J g^{-1}. The peak temperature also decreased from 34.8 ± 0.16°C to 33.8 ± 0.12°C. The present data suggest that the mechanism of action of SLS is in part through disruption of SC intercellular structure and increased partitioning of 5FU into the SC lipids.

For DMSO, release and permeation data revealed that this molecule does not alter diffusion and permeation coefficients of 5FU through the matrix. Diffusion and permeation coefficients of 5FU through DMSO-treated matrices were $6.00 \pm 1.41 \times 10^{-4}$ and 0.307 ± 10^{-2} cm h^{-1} respectively. This might be due to the relatively low concentration of DMSO in the matrix as it has been shown that DMSO usually acts as a skin penetration enhancer at high concentrations (>60%, Williams & Barry, 1992).

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148 EFFECTS OF TERPENES ON THE SOLUBILITY OF 5-FLUOROURACIL IN LIPID BILAYERS; CORRELATION WITH SKIN PERMEATION ENHANCEMENT

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Human stratum corneum (SC) comprises of corneocytes embedded in an intercellular lamellar lipid matrix. Out studies using a lamellar model provided evidence that 5-fluorouracil (5FU) permeates the SC through to the intercellular pathway (Moghimi et al, 1996). 5FU is a hydrophilic drug and it is possible that its partitioning from the aqueous region of the lamellar structure into the hydrocarbon interior of lipid bilayers is an important step for its permeation and a target for some penetration enhancers. To investigate this hypothesis, 26 different terpenes were chosen which included chemical classes of hydrocarbons, ketones, ethers and alcohols and which have been shown to act as enhancers towards permeation of 5FU through the SC (Williams & Barry, 1991; Cornwell & Barry, 1994). Isooctane was selected as a simple model for the hydrocarbon interior of lipid bilayers and solubilities of 5FU in isooctane and isooctane:terpene mixtures were measured at 25°C. Solubility ratios (SR; isooctane:terpene/isooctane) were calculated and compared with enhancement ratios (ER; permeability coefficient through enhancer-treated epidermis divided by that of untreated samples).

All terpenes increased the solubility of 5FU in isooctane. Generally, hydrocarbons showed the least SR (3-21) and alcohols were the strongest solubilisers (SRs of up to 2620). The effects of ketones and ethers on the solubility of 5FU in isooctane lay between hydrocarbons and alcohols (SR = 63-215).

In permeation studies of 5FU through human epidermis (Williams & Barry, 1991; Cornwell & Barry, 1994), hydrocarbons also showed the least effect (ER = 1-3). But, in the case of alcohols, ethers and ketones, ER did not follow the same profile as SR; the ERs provided by alcohols (up to 23) were less than those provided by ketones and ethers (Ers of up to 94). If partitioning is the only phenomenon acting, alcohols are expected to provide the highest ER unless the rate limiting step has been changed from partitioning into, to the partitioning out of, the hydrocarbon interior of the lamellar structure in the presence of alcohols. Our determinations of systems show that such a change in the limiting step is not expected.

A possible mechanism for the lower ER of alcohols than that of ketones and ethers may lie in the bilayer disruption ability of some ethers and possibly some ketones (Moghimi et al, 1997). Analysis of data after omission of these terpenes (3 ethers and one ketone) resulted in a linear correlation with a coefficient of 0.8 between logER and logSR. These results may indicate that partitioning from the aqueous domain into the hydrocarbon interior of bilayers might be an important step in the permeation of 5FU through the SC and a target for some penetration enhancers.

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149 TRANSDERMAL DRUG DELIVERY FROM OESTRADIOL EUTECTIC FORMULATIONS

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Binary mixtures of oestradiol (OE) and polyethylene glycol (PEG) 1000 have been studied by differential scanning calorimetry (DSC), and were found to produce a simple eutectic system with a eutectic composition of 8:92 OE:PEG 1000 (% w/w) and a eutectic melting point of 29°C. This compares to a melting point of OE alone of 175°C. Kasting et al. (1987) produced a model for transdermal permeation relating permeant melting point to flux and showed an approximately exponential increase in flux as permeant melting point decreased. This model relates to individual species and was extended, empirically, to investigate the effects of melting point depression, by eutectic formation, of a binary donor system on transdermal flux.

A temperature/composition phase diagram was produced from the DSC analysis and from this a composition which was saturated at 32°C, i.e. which had excess solid OE in equilibrium with the liquid OE:PEG 1000 mixture, was selected and tested as a donor for transdermal delivery. This system (10% w/w OE) produced a flux of 0.305 (0.402, 20) $\mu g/cm^2$/hour across human epidermal membrane compared to 0.0100 (0.0029, 22) $\mu g/cm^2$/hour from a saturated aqueous control. This represents a significant ($p < 0.001$) enhancement ratio of approximately 30-fold. This effect was shown not to be specific to the skin as the eutectic donor produced a 50-fold enhancement in flux across silastic membrane.

Possible mechanisms of action of the binary eutectic systems were investigated. DSC analysis of samples stored for 2 months showed that the OE does not exist in a supersaturated state in the binary mixtures. The eutectic adjuvant, PEG 1000, does not act as a penetration enhancer and, indeed, was shown to retard permeation when applied as a pretreatment. A third possible mechanism is the formation of transient regions of supersaturation by the back diffusion of water from the receptor into the donor. This mode of action has been eliminated by repeating the permeation experiments in a non-aqueous environment. Once again the eutectic formulations produced a marked increase in flux over the control.

Therefore, it has been shown that the formation of the binary eutectic systems of OE with PEG 1000, with the corresponding reduction in the melting point of the donor systems, produces a marked increase in transdermal flux. The melting point of 17β-oestradiol is 175°C and the equilibrium liquid of the "saturated" binary eutectic system has a melting point of 32°C. This represents a melting point depression of 143°C. If one uses the Kasting model (1987) empirically to compare the flux of oestradiol with a melting point of 175°C with the predicted flux oestradiol with a melting point of 32°C, the predicted enhancement ratio is 48.8. This value is approximately 1.6 times the value obtained experimentally with human epidermal membranes.

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150 EVALUATION OF ATR-FTIR SPECTROSCOPY FOR THE MEASUREMENT OF DIFFUSION COEFFICIENTS OF PERMEANTS IN RATE-LIMITING MEMBRANES

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Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy has proven to be a useful research tool for investigating the mechanisms of action of skin penetration enhancers (Harrison et al., 1996), the morphology of human stratum corneum (Pellett et al., 1997), and the mechanisms of diffusion. However, there has been very little evaluation of the technique with more traditionally accepted methodologies, such as skin diffusion cells. The aim of this study was to compare the diffusion coefficients of 4-cyanophenol in silicone membranes as determined by ATR-FTIR spectroscopy and skin diffusion cells. The theory and application of ATR-FTIR spectroscopy has been described elsewhere (Harrison et al., 1996; Pellett et al., 1997). Silicone membranes (310μm thickness) were placed on the ATR crystal and then saturated solutions of cyanophenol in PEG 400 (with excess present) were placed in the donor compartment. FTIR spectra were collected automatically at 2 min intervals over a 2hr period, and the CN peak area (2230cm^{-1}) was determined at each time point. The donor phase was then replaced with 2ml of PEG 400 (in the absence of any solute) and the depletion of cyanophenol from the silicone membrane was monitored in a similar manner. After plotting peak areas against time, absorption and desorption curves were obtained. By applying a solution to Fick's second law of diffusion that satisfies the initial and final boundary conditions (Crank, 1975), it was possible to derive a diffusion coefficient from both the absorption and desorption curves.

Diffusion coefficients are often determined from diffusion cell data by the lag time method. This requires the attainment of steady state transport before extrapolation of the linear part of the curve. Cyanophenol diffused across a single silicone membrane very rapidly resulting in a negligible lag time. Therefore, in order to improve the accuracy in its determination, a stack of six silicone membranes were used and the transport of cyanophenol across them was monitored. Diffusion coefficients were determined from the resultant diffusion profiles using the P_1P_2 equation (Watkinson et al., 1994) and the lag time method. Table 1 shows the diffusion coefficients determined using ATR-FTIR spectroscopy and diffusion cells.

Table 1: Diffusion coefficients for cyanophenol in silicone membranes (mean±SE, n≥4)

Method	Diffusion coefficient (cm^2/hr)
ATR-FTIR spectroscopy:	
Absorption	$6.47\times10^{-4}\pm7.12\times10^{-5}$
Desorption	$4.97\times10^{-4}\pm5.94\times10^{-5}$
Diffusion cells	$3.70\times10^{-4}\pm1.18\times10^{-5}$

Using a two sample t-test, there was no difference between the diffusion coefficients generated by the absorption and desorption experiments (p=0.17), and also between the desorption and diffusion cell experiments (p=0.13). However, there was a significant difference between the absorption and diffusion cell experiments (p=0.031). This may be due to the interfacial barriers between the individual membranes in the stack, or alternatively, due to the ingress of water from the receptor phase. These experiments have shown that diffusional parameters determined using these techniques are very similar, but specific validation procedures should be addressed before they are quoted as definitive values.

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151 SUPRACHOROIDAL ROUTE FOR DRUG DELIVERY TO THE POSTERIOR SEGMENT OF THE EYE

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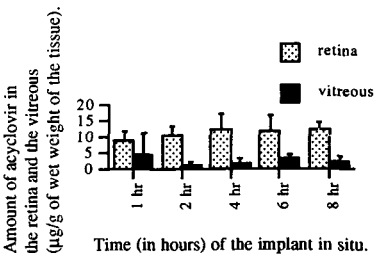
Drug delivery to the posterior tissues of the eye following topical application is complicated by the presence of several sequential barriers. In addition the blood-retinal barrier prevents intraocular delivery of the drug from the systemic circulation. Local delivery of a drug by direct intravitreal or sub-conjunctival injection are routinely used but suffer from the drawbacks of a risk of infection and poor intraocular drug levels respectively. Vitreal implants, designed to deliver drugs at a pre-determined rate and in a sustained manner, are expensive and do not avoid the possibility of endophthalmitis. A previous study by Foulds and colleagues (Foulds et al, 1988) investigated the use of a hydrophilic polymer inserted into suprachoroidal space for suprachoroidal plombage in retinal detachment surgery. It was found that over a period of time, the choroid was replaced by fibrous tissue and the retinal pigment epithelium atrophied. We have hence investigated the usefulness of the suprachoroidal space as a receptacle for a drug delivery device. Using an isolated arterially perfused sheep eye, drug levels in various tissues of the eyes were measured. Acyclovir was used as the model drug.

Polyvinyl alcohol films were prepared with a drug loading of 20% w/w. Circular slabs of 4 mm diameter were cut, containing approx. 100 μg of acyclovir. A scleral incision was made 4 mm posterior to the corneo-scleral limbus in the pars plana region of a sheep eye and the slab inserted into the suprachoroidal space. The long posterior ciliary artery was then cannulated and the eye perfused with Krebs solution. Perfusions were carried out for 1, 2, 4, 6 and 8 hours. Tissues were excised, weighed and the concentration of acyclovir determined by HPLC. Ganciclovir was used as the internal standard. The amount of acyclovir remaining in the implant was also estimated at the end of each perfusion.

Mean retinal and vitreal levels of acyclovir were found to be 10.91±1.49 μg/g and 2.42±1.40 μg/g over the 8 hour period (mean ± s.d, n=4). Retinal levels of acyclovir peaked at 12.05±5.35 μg/g at 4 hours and were maintained up to 8 hours in the sub-therapeutic range (Dorsky and Crumpacker, 1987). High drug binding to the choroid and the iris-ciliary body was observed. Scleral uptake of acyclovir was also found to be high. Very little or no drug was detected in the cornea and the aqueous humour. By 6 hours no drug was detectable at the implant site.



The suprachoroidal route provides a highly localised route for targeted delivery to the posterior space, mainly due to the proximity of the implant to this target tissue. Little lateral spread or loss to the non-target sites (cornea and aqueous humour) was seen. The study demonstrates the feasibility of dosing by the suprachoroidal route and further work is in progress to sustain the rate of delivery. Avoiding entry into the interior tissues markedly reduces the risk of endophthalmitis. Modifications to the device are in progress which will allow a higher sustained dose to be delivered.

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152 THE PRECORNEAL RESIDENCE OF A THERMALLY SENSITIVE HYDROGEL

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The precorneal residence of ophthalmic formulations is usually very short, being of the order minutes. This small contact time limits the extent of local drug absorption and can lead to significant systemic absorption from the nasolacrimal duct. There are a number of pharmaceutical approaches designed to improve drug delivery to the front of the eye by increasing corneal residence, these include the use of viscosifying agents, bioadhesive polymers and non-aqueous vehicles. This study examined the precorneal residence of a temperature sensitive hydrogel in rabbits. The hydrogel is liquid at room temperature but at 37°C exists as a solid gel which shows some bioadhesive properties.

Smart Hydrogel™, (poly(oxyethylene-b-oxypropylene-b-oxyethylene)-g-poly(acrylic acid)), is an unique interpenetrating polymer network (IPN) composed of poly(acrylic acid) and a block copolymer of poly(ethylene oxide) and poly(propylene oxide).

Gamma scintigraphy was employed as a non invasive means of monitoring the residence of the formulation on the rabbit cornea. A two-way cross over study was carried out on six New Zealand White rabbits comparing the hydrogel with a control, saline. The hydrogel formulation (1ml) was radiolabelled by the inclusion of 25 µl ^{99m}Tc-DTPA to give an activity of 3MBq per dose (20µl). The saline solution was radiolabelled in the same way. The dose (20µl) was delivered directly onto the cornea using a positive displacement pipette and the animal was immediately positioned for imaging. Scintigraphic imaging of the animal was carried out on a IGE maxicamera II using a pin-hole collimator. Imaging for the hydrogel included a dynamic acquisition for 15 minutes (60 frames at 10 seconds/frame and 10 frames at 30 seconds/frame) followed by two static images at 30 and 60 minutes post dose. The saline arm was imaged dynamically for only 10 minutes (60 frames at 10 seconds/frame). A three day washout period was allowed between the arms of the study. The data was analysed by creating a region of interest around the cornea, inner canthus, nasolacrimal area, the whole eye and by taking a sample of background counts. All data was corrected for background radiation and for decay of the isotope.

Gelation of the hydrogel on the corneal surface occurred immediately and could be visually observed. Dramatically increased retention was observed with the Smart Hydrogel™ as shown in the differences between the t_{50} values from the mean curves (n=6)of the two formulations. The t_{50} value represents the time (in seconds) for 50% of the material to be removed from the corneal surface.

Saline t_{50}	45 seconds
Smart Hydrogel™	1150 seconds

At one hour post dose (3600 seconds) there was still 23% of the administered dose on the corneal surface. The rabbits were also assessed for signs of distress after dosing with Smart Hydrogel™ but showed no symptoms of ophthalmic irritation or weight-loss. Initial studies suggest that the Smart Hydrogel™ system is a potential vehicle for the delivery of agents to the front of the eye. However it is not yet known whether these systems cause blurring or visual discomfort.

Smart Hydrogel™ is a trademark of Gelmed Inc. USA.

153 DETERMINATION OF THE NASAL RETENTION OF SMART HYDROGEL USING GAMMA SCINTIGRAPHY

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Drugs are administered via the nose for both topical and systemic action. The duration of action is limited by the rate of clearance of the drug vehicle. This study investigates the potential of a thermally sensitive hydrogel as a nasal drug delivery system when administered as a spray,

0.3 ml doses of Smart Hydrogel™, poly(oxyethylene-b-oxypropylene-b-oxyethylene)-g-poly(acrylic acid),(GelMed Inc. USA) were radiolabelled with 0.2MBq 99mTc-DTPA

The study was performed on 6 healthy males and non-pregnant females on one occasion. The radiolabelled hydrogel was administered to one nostril of each subject using the spray device. Gamma scintigraphy images of 30 s duration were taken every 30 min after the dose for 2 h. 4 h and 20 h post-dose images were also recorded. Eating and drinking was not restricted throughout the trial.

The data was analysed by creating a region of interest around the nose and corrected for background and isotopic decay before being expressed as percentage of dose remaining in the nasal cavity with time.

The Smart Hydrogel™ was deposited anteriorly in all subjects. After prolonged contact with the mucosa at the deposition site, clearance of the hydrogel followed (Figure 1).

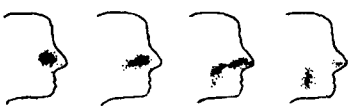

Figure 1: Clearance of Smart Hydrogel™ in one subject. Images taken every 30 minutes.

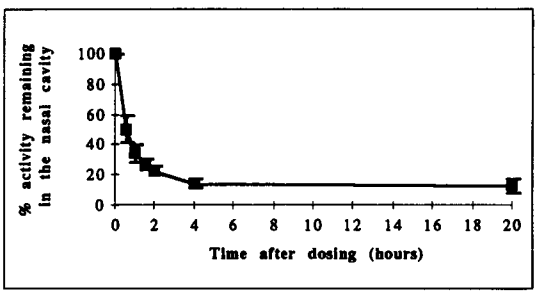

Figure 2: Mean nasal residence curve for Smart Hydrogel™ throughout the whole nasal cavity (n=6)

The temperature induced gelation of the hydrogel resulted in prolonged deposition in the nasal cavity. Approximately 10% of the formulation was present in the nasal cavity at 20 h. The half-life was 0h 31 minutes, with an AUC of 9166.91.

The clearance of the material from the nose was bi-phasic, with an initial rapid phase followed by a more prolonged clearance. This agrees with the pattern of clearance of bioadhesive starch microspheres (Ridley et al 1995).

Despite the fact that the method of dosing the hydrogel was not optimised in this preliminary study, the material appears to have potential for use as a nasal drug delivery vehicle.

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154 THE EFFECTS OF SODIUM TAURODIHYDROFUSIDATE AND PHOSPHATIDYLCHOLINE ON CILIARY BEAT FREQUENCY IN-VITRO

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Sodium taurodihydrofusidate (STDHF) has been employed previously as an intranasal peptide absorption enhancer but it has been shown to cause an increase in mucus secretion *in vitro* (Pritchard et al, 1996), a decrease in mucociliary transport rate of the frog palate (Pritchard et al, 1994) and local irritation of the mucosa (Hedin et al, 1993). The exact mechanism of STDHF absorption enhancement is not known but some of the detrimental effects can be reduced by the addition of egg PC (Pritchard et al, 1994). The aim of the present study was to investigate the effects of STDHF and PC on ciliary beat frequency (CBF) of frog palate epithelium. The epithelium was dissected into sections (2 mm^2) and placed in HEPES buffered saline, pH 7.4 (control). Cilia were observed at 25°C and CBF measured photometrically for 80 min at 10 different sites. The test solutions were; 16.6 mM (1%) STDHF and 10 mM PC alone in HEPES and STDHF combined with 5 or 10 mM PC in HEPES. STDHF (1%) halted CBF after 15 min ($p<0.05$, Table). After this time the tissue appeared to be surrounded with mucus and the cilia were no longer beating. In the presence of STDHF combined with 5 mM and 10 mM PC, the cilia continued to beat throughout the experiment. STDHF causes a cessation of ciliary activity, either due to abnormal mucus secretion or a direct effect on the cilia. This is mitigated by both 5 and 10 mM PC. We hypothesize that this is due to the formation of mixed micelles, so that less STDHF is available to interact with the epithelial cell membranes. STDHF/PC mixed micelles may be useful in nasal drug formulations containing STDHF as an absorption enhancer.

CBF (Hz, mean±S.D.)

Time (min)	STDHF (16.6mM)/ control, n=3	PC(10 mM)/ control, n=4	STDHF:PC (16.6:5mM)/ control, n=5	STDHF:PC (16.6:10mM)/ control, n=3
5	10.62±2.52 / 10.75±2.35	8.23±2.32/ 7.50±1.94	10.55±2.35*/ 8.85±2.96	6.70±2.70* / 8.58±1.86
10	7.66±5.75 / 8.33±1.82	8.19±2.27/ 7.72±2.36	8.85±2.67/ 8.49±3.21	8.67±1.53 / 8.74±1.85
15	7.66±5.77 / 9.74±1.51	7.38±2.28/ 8.08±2.86	8.73±4.81/ 8.58±2.59	7.53±2.13 / 7.37±2.10
20	0.00±0.00*/ 8.82±2.55	8.58±2.35*/ 6.35±2.04	8.79±5.32/ 9.31±3.82	7.66±3.36 */ 9.28±2.52
30	0.00±0.00*/ 10.23±1.10	6.42±2.56/ 6.64±1.69	5.17±5.76*/ 8.50±2.50	7.58±2.23 / 6.96±1.46
60	0.00±0.00*/ 10.32±2.18	8.86±2.41*/ 7.94±2.12	3.99±5.14*/ 8.84±2.55	7.25±2.04 / 6.93±1.41
80	0.00±0.00*/ 10.08±1.61	4.43±2.90*/ 8.97±2.02	4.59±5.79*/ 9.97±2.58	5.82±4.07*/ 8.27±1.86

* indicates result significantly different from control ($p<0.05$, Mann-Whitney)

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155 IMMUNE RESPONSES TO MUCOSALLY ADMINISTERED CO-ENCAPSULATED *YERSINIA PESTIS* ANTIGENS

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Simple mucosal application of antigenic proteins is usually ineffectual in terms of vaccination due to enzymatic or chemical destruction coupled with poor absorption into sub-epithelial compartments. Proteins can be protected from degradation by encapsulation within polymeric microparticulate carriers such as polylactic acid (PLA) microspheres. Such procedures may also adjuvantise by converting soluble antigenic material into particulate entities which are more efficiently taken up by microfold (M-cells) or antigen presenting cells (APCs) (Walker, 1994).

In terms of inducing immunity to plague, the fraction 1 (F1) and V subunit proteins from *Y. pestis* display an additive effect in combination. Therefore, F1 and V were co-encapsulated into PLA100Kd microspheres using a double emulsion-solvent evaporation technique. Proteins were extracted into phosphate buffered saline (PBS) using dichloromethane and assayed with a 'sandwich' type enzyme-linked immunosorbent assay (ELISA) in conjunction with a bicinchoninic acid protein assay to assess loading. 10 mice (male CBA) were immunised orally with either 11.3 µg of V and 4.4 µg of F1 on day 1 of the experiment (group A) or equivocal doses of encapsulated antigens suspended in water (group B). Both groups were boosted intra-nasally (i.n.) on day 7. Group A received free V (2.7 µg) and F1 (1.0 µg) in PBS, whilst group B was treated with an equovical dose of encapsulated vaccine. Group C consisted of 5 untreated controls. Tail vein blood was sampled periodically. On day 55 mice were terminally anathetised prior to cardiac blood sampling. Spleens were removed from dead rodents and splenic T-cell proliferation to F1 and V *in vitro*, was determined as described previously (Leary et al 1995). An indirect ELISA was employed to quantify serum IgG, IgA and IgG sub-class titres.

Serum IgG (Fig. 1) titres indicated that microencapsulated antigen facilitated the induction of superior systemic immune responses at day 28 and 55 ($P\leq0.001$) relative to free antigen. IgG_1, as opposed to IgG_{2a} or IgG_{2b}, was the predominant anti-F1 and anti-V IgG subclass in serum from both A and B. This is indicative of a Th_2 type response, and suggests that following mucosal delivery free and encapsulated sub-units are processed and presented similarly by APCs.

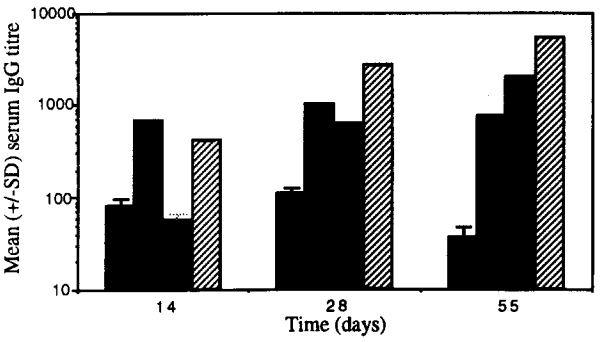

Fig 1. Groups A [■] and B [■] anti-V, and groups A[■] and B[▨] anti-F1, serum IgG titres (mean±SD; n=10).

In common with humoral responses, mucosal application of F1 induced strongest cellular activity: Stimulation indexed (SI) T-cell proliferation to F1 was 1.7±0.2 for group A and 2.0±0.2 for group B. Splenic T-cells from control mice had an SI of 1.3±0.2 upon *in vitro* exposure to F1. Group A and B T-cell recall to V was identical to that of naive animals. We conclude that mucosal application of *Y. pestis* antigens elicits both humoral and in the case of F1, cellular immune responses. Superior humoral responses result from administration of microencapsulated, as opposed to free, vaccine.

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156 ASSESSMENT OF INTRALUMINAL ENCRUSTATION AND BLOCKAGE OF URETERAL STENT BIOMATERIALS

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Ureteral stents are used to facilitate urine drainage from the renal pelvis to the bladder. Unfortunately, stents placed within the urinary tract have been shown to develop encrustation which may lead to occlusion of the lumen with subsequent loss of urine flow (Gorman et al 1993). The aim of this study was to compare the ability of ureteral stent biomaterials to resist intraluminal blockage and to determine if a relationship exists between stent inside surface roughness and rate of encrustation.

The materials chosen for comparative study were silicone, polyurethane, hydrogel-coated polyurethane (HPU), silitek and percuflex. Artificial urine (37°C, 5% CO_2, Tunney et al 1996) was pumped through 60 cm lengths of each biomaterial at a rate of 0.7 ml/min. A control experiment was also set up in which deionised water was pumped at the same rate through similar biomaterial lengths. The biomaterial lengths were checked daily to ensure that urine was still flowing. When or if urine flow through a biomaterial length became impassable through encrustation blocking the lumen, the biomaterial length and its corresponding control length were removed. Encrusted and control lengths were then dried and the total weight of encrustation determined. The inside surfaces of the biomaterials were examined by scanning electron microscopy and atomic force microscopy.

Significant differences were apparent in the number of weeks urine flow was maintained in the biomaterials prior to blockage. The polyurethane stent remained patent for the longest period of time and the HPU stent the shortest period (Table 1). Differences were also apparent in the total weight of encrustation present in the lumen of each biomaterial at the time of blockage with the greatest amount of encrustation present in the lumen of silicone and the least amount of encrustation present in the lumen of the HPU (Table 1). Atomic force microscopy revealed significant differences in the inside surface roughness of the biomaterials with root mean square values decreasing from 117 nm for silicone to 20 nm for the HPU. Increasing inside surface roughness correlated with an increase in the amount of encrustation deposited in the lumen of the biomaterial prior to blockage.

Table 1. Intraluminal patency and encrustation of ureteral stent biomaterials

Biomaterial	Duration of patency (weeks)	Weight of intraluminal encrustation (mg)
Silicone	8	382
Polyurethane	11	121
HPU	4	83
Silitek	6	213
Percuflex	10	127

This study has shown that none of the biomaterials presently used as ureteral stents are capable of resisting encrustation and intraluminal blockage. Further work is, therefore, required to develop novel biomaterials better able to resist encrustation on prolonged exposure to urine.

Gorman, S.P. et al (1993) Pharmacotherapy 73(3): 281
Tunney, M.M. et al (1996) Biomaterials 17:1025-1029

157 HYDROXYPROPYL-β-CYCLODEXTRIN IMPROVES THE FOLDING REVERSIBILITY OF AGGREGATING LYSOZYME

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Cyclodextrins (CDs) are potential stabilizing excipients for protein drugs : they may stabilize proteins against freeze-drying-induced inactivation (Hora *et al.*, 1992), heat denaturation (Izutsu *et al.*, 1993), aggregation (Katakam and Banga, 1995), precipitation (Charman *et al.*, 1993), and help protein folding (Karuppiah and Sharma, 1995). Cyclodextrins can also destabilize proteins in solution by reducing their transition temperature, T_m (Cooper, 1992). However, the effect of CDs on protein folding reversibility has not been studied directly.

We used high-sensitivity differential scanning calorimetry (HSDSC) (MCS calorimeter, MicroCal Inc., USA) to assess the effect of hydroxypropyl-β-cyclodextrin (HP-β-CD) on the stability of aggregating lysozyme (40 mg.ml^{-1}). Changes in pH and protein concentration were examined.

The addition of CD reduced T_m. An increase in pH from 3 to 6 increased T_m whereas an increase in protein concentration from 4 to 40 mg.ml^{-1} slightly decreased T_m. The addition of 30% w/w CD strongly inhibited heat-induced precipitation and greatly improved the folding reversibility (from 9.7 to 36%) of aggregating lysozyme (40 mg.ml^{-1}, pH 6). HSDSC scans are illustrated in Figure 1. Whilst the increase in protein concentration enhanced folding reversibility at pH 3, it decreased it at pH 6. The increase in pH improved folding reversibility at low protein concentration but decreased it at high protein concentration.

These results indicate that the measurement of T_m alone is not sufficient to assess protein thermal stability, since the ability of the protein to refold may be enhanced despite a decrease in T_m.

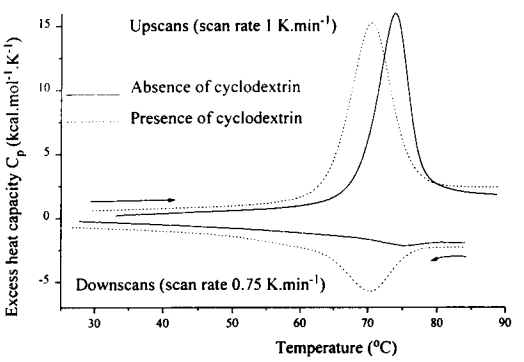

Figure 1. HSDSC upscans and downscans of lysozyme (40 mg.ml^{-1}, pH 6) in the presence and absence of HP-β-CD (30% w/w).

Charman S.A. *et al.* (1993) Pharm. Res. 10, 7: 954-962.
Cooper A. (1992) J. Am. Soc. 114: 9208-9209.
Hora M.S. *et al.* Pharm. Res. 9: 33-36.
Izutsu K.-I. *et al.* Int. J. Pharm. 90: 187-194.
Karuppiah N. and Sharma A. (1995) Biochem. Biophys. Res. Comm. 211, 1: 60-66.
Katakam M. and Banga A.K. (1995) J. Pharm. Pharmacol. 47: 103-107.

158 SYNTHESIS AND CHARACTERISATION OF POLYETHYLENE GLYCOL AND POLYPROPYLENE ESTERS OF HYDROCORTISONE 21-SUCCINATE

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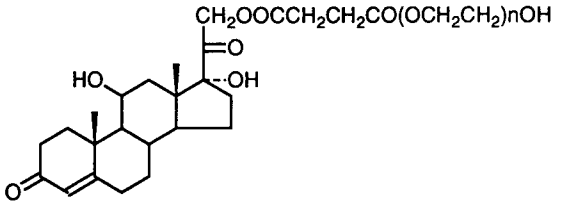
A method for the synthesis of polyethyleneglycol esters of hydrocortisone 21-hemisuccinate (H-PEGs, Figure1), potential ocular prodrugs, was developed using dicylohexylcarbodiimide to promote esterification. The products were purified by semipreparative HPLC. The steroid derivatives were, highly water soluble. The products were characterised by NMR, fast atom bombardment mass spectrometry and elemental analysis.

Figure 1 H-PEG



At constant pH and temperature pseudo first order kinetics was observed for hydrolysis of the H-PEGs. The pH of maximum stability was 4.18 ± 0.03 for H-PEG200, H-PEG400, H-PEG600, H-PEG900 and H-PEG2000. At all pH values there was no significant difference between the hydrolysis kinetics of H-PEGs with different polymer chain lengths.

The log P values for hydrocortisone and the H-PEG between n-octanol and buffer pH 7.4 were determined by the shake flask method and were also estimated from HPLC retention time (Lein, 1993). The values obtained are shown in table 1.

Table 1

Compound	No of units	Log K	Log P (obs)
Hydrocortisone	------	- 0.184	1.61
H-PEG 200	4	0.158	1.55
H-PEG 400	9	0.200	1.07
H-PEG 600	13	0.213	0.622
H-PEG 900	20	0.237	- 0.139
H-PEG2000	45	0.283	-----

The HPEGs were rapidly hydrolysed by both a commercial esterase and by homogenate of ovine cornea demonstrating their potential as prodrugs. The rate of hydrolysis decreased with increasing PEG chain length. The efficiency penetration of H-PEGs through ovine cornea and sclera was studied *in vitro* using two different diffusion cells. The HPEGs were hydrolysed upon passing though cornea and sclera. H-PEG 200 and H-PEG 400 produced better penetration of hydrocortisone through cornea and sclera than hydrocortisone at the same concentration. Hydrocortisone 21-succinate polypropylene glycol esters were also synthesised and studied with regard to their pentration through ovine cornea. Skin penetration studies are currently being conducted.

Lein E.J. Partition Coefficients In Swabrick,J. and Boylan J.C. (Ed.), *Encylopedia of Pharmaceutical Technology* , Marcel Decker, 1993 293-307.

159 ASSESSMENT OF CO-TRIMOXAZOLE PRODUCT QUALITY BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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There is growing concern about the availability of substandard pharmaceutical products to the general public in developing countries. Such products have therapeutic as well as social and economic implications. There is little data available which points to the reasons for products being substandard but the majority of literature reports contain anecdotal evidence and assume the products to be counterfeit, as highlighted by ten Ham (1993). There are, however, other reasons for products being substandard, such as poor quality control during manufacture or decomposition of the active ingredient(s). For this reason a study was established to generate quantitative information on the quality of selected pharmaceutical products (antimalarial and antibacterial agents) and obtain information which could be used to determine the cause of any compromised quality detected.

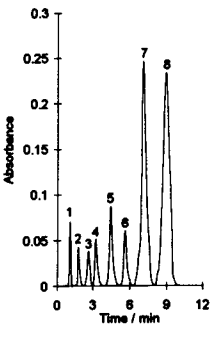
Co-trimoxazole (CTX) preparations were selected as part of this larger study since it is widely used to treat a variety of common bacterial infections in developing countries. The use of substandard CTX could lead to not only treatment failure but also the development of drug resistance.

Samples were obtained for analysis from Nigeria and Thailand. For this analysis an assay method was developed which was capable of simultaneously determining the active ingredients of CTX, trimethoprim (TMP) and sulphamethoxazole (SMX). To allow possible reasons to be assigned for any poor quality detected the method was designed to be capable of detecting the major decomposition products and the manufacturing precursors of both drugs. Such a method has not previously been reported.

The method was based on isocratic high-performance liquid chromatography (HPLC) and employed an ion pairing solvent system. The chromatographic conditions comprised a reverse phase C_{18} column (100 x 2mm ID) packed with 3µm particles with a mobile phase of methanol-10 mmol L^{-1} aqueous phosphate buffer (15-85) of pH 2.5, incorporating 80 mmol L^{-1} tetraethylammonium bromide (TEA) as the ion pairing agent. The flow rate was 0.4 mL min^{-1} and UV detection was used at 230 nm. A photodiode array detector was used for examination of peak homogeneity.

The method was validated according to current guidelines for linearity of detector response, accuracy, precision, specificity and limits of detection for the drug related impurities. The method was also used to assay pharmacopoeial standard CTX preparations.

It was found that the addition of TEA to the solvent greatly reduced the retention of TMP while having little effect on SMX retention. This effect was also observed with the other drug related compounds included in the analysis, though not to the same extent. The final concentration of TEA in the mobile phase was adjusted to provide the best possible resolution between the compounds while maintaining acceptable analysis times. A chromatogram representing the final separation achieved is shown in the figure where peaks 7 and 8 represent TMP and SMX respectively. Peaks 1 to 6 represent the possible drug related impurities included in the analysis.



The validated method was used to determine the quality of 17 CTX samples obtained from Nigeria and Thailand (these were tablet and liquid preparations). It was found that 29% of the samples failed to comply with BP (1993) limits for content of active ingredients. From the analyses it was concluded that decomposition was not the reason for low amounts of actives, however the possibility of poor control during manufacture could not be eliminated. No evidence of counterfeiting was found for the products tested.

ten Ham, M (1993), Adverse Drug React Toxicol Rev, 11 (1), 59-65.

160 METAL LABELLING OF TRANSFERRIN VACANT SITES LEADS TO IMPROVED NON-TRANSFERRIN-BOUND IRON (NTBI) QUANTIFICATION

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Human serum transferrin is an iron transporting protein which is composed of two lobes; the N- and C- lobes. In normal serum and some iron overload diseases such as haemochromatosis, transferrin is not completely saturated. In such circumstances, the quantification of non-transferrin-bound iron (NTBI) is difficult. The generally adopted method of analysis of NTBI is based on the use of a large excess of low affinity ligand (nitrilotriacetic acid; NTA). Under such conditions the conversion of NTBI to iron-NTA (FeNTA), which is normally complete, can lead to donation of a proportion of this iron pool to the vacant site on monoferric transferrin and apotransferrin, thereby generating an artificial low NTBI value. In order to avoid such complications, we have modified the method to incorporate metal ions such as gallium, indium and cobalt for blocking vacant transferrin iron binding sites. Unfortunately, indium and gallium are kinetically labile in the presence of NTA. In contrast, cobalt(III) is a kinetically inert cation and appears to be suitable for labelling the empty iron-binding sites of the protein. It binds specifically to these sites in transferrin in the presence of a synergistic anion such as bicarbonate. Bali *et al* (1991) reported that there was no cobalt loss in the presence of 100 mM NTA.

Two methods of labelling apotransferrin with cobalt have been compared, one using cobalt citrate (Aisen *et al*, 1969) and the other using sodium-tris carbonatocobaltate (III) trihydrate, $Na_3[Co(CO_3)_3].3H_2O$ (He *et al*, 1996). After adding FeNTA to 50 % saturated transferrin which was pre-labelled with cobalt (III), we found that the percent recovery of iron was 3.3 ± 2.92 % (n=3) for transferrin labelled with cobalt citrate and 93.1 ± 3.54 % (n=5) for transferrin labelled with $Na_3[Co(CO_3)_3].3H_2O$ as compared to 1.0 ± 2.18 % (n=4) for transferrin with no cobalt labelling. Similar results have been obtained with normal serum labelled with $Na_3[Co(CO_3)_3].3H_2O$ shown in Figure 1.

Clearly the nature of the cobalt complexes are critically important for the success of the method. With the cobalt citrate complex H_2O_2 was added in order to oxidise Co^{2+} to Co^{3+}. However, this reagent can also cause protein damage. In contrast, H_2O_2 is unnecessary with $Na_3[Co(CO_3)_3].3H_2O$ so the protein damage is avoided. With further development, Co(III) addition will be introduced in NTBI assays of haemochromatosis serum.

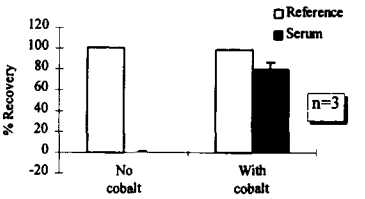

Figure 1: Percent recovery of iron after loading to normal serum compared between with and without $Na_3[Co(CO_3)_3].3H_2O$

Aisen P., Aasa R. and Redfield A.G., *J. Biol. Chem.* **1969**, 244(17), 4628-4633.

Bali P.K., Harris W.R. and Nesset-Tollefson D., *Inorg.Chem.* **1991**, 30(3), 502-508.

He Q., Mason A.B. and Woodworth R.C., *Biochem. J.* **1996**, 318, 145-148.

161 KINETICS OF THE INTERACTION OF AMINES WITH HALOGENATED HYDROCARBON SOLVENTS

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We have previously described the kinetics of the interaction between halogenated hydrocarbon solvents and amines of pharmaceutical interest and concluded that there was a pseudo first-order reaction with respect to the amine (Gaínza 1994). The order of the reaction with respect to the solvent was initially ignored; however it is important to study this aspect because it reveals unknown properties of universally used solvents.

Halogenated hydrocarbons (CCl_4, $CHCl_3$, CH_2Cl_2, and $C_2H_4Cl_2$), designated R-CH_nCl_m, react with amines of pharmaceutical interest (atropine, homatropine, tropine and quinine) forming a quaternary ammonium salt, *N*-chloroalkylammonium chloride, which was characterized using mass spectrometry and ^1H NMR spectroscopy. The reaction is a nucleophilic substitution process which may be written:

$$\text{amine} + \text{R-CH}_n\text{Cl}_m = \text{amine}^+\text{-R-CH}_n\text{Cl}_{m-1}, \text{Cl}^- \qquad (1)$$

The kinetics (order of the reaction and rate constant) with respect to the solvent were determined by using mixtures of solvents of different reactivities so as to vary the concentration of the solvent under study. The rate of the reaction (V) was determined by addition of bromocresol green to the solvent-amine mixture. This dye reveals the transformed solvent (Gaínza 1987) and hence allows the measurement of V. The data for the interaction of tropine with a number of solvents is shown in Table 1.

We found that the reaction rate could be expressed as:

$$\log V = \log k + \log [\text{solvent}] + \log [\text{amine}] \qquad (2)$$

that is, first order with respect to the solvent for any solvent and second order with respect to the whole reaction.

Inspection of the k values (h^{-1} L mol^{-1}) in Table 1 suggests that the reaction rate decreases with an increase in the number of chlorine atoms present in the methane moiety.

Table 1. Kinetics of the interaction of tropine with halogenated hydrocarbon solvents at 293 and 313 K.

Solvent	Order	k at 293 K (x 10^5)	k at 313 K (x 10^5)	r^2
CH_2Cl_2	1.01	0.58	3.72	0.972
$C_2H_4Cl_2$	1.07	0.12	0.56	0.998
$CHCl_3$	--	0.00	0.00	--
CCl_4	--	0.00	0.00	--

The activation energy is 87.05 kJ mol^{-1} for tropine-$C_2H_4Cl_2$ reaction.

Gaínza , A. H., Konyaso, R. I., Asenjo, R. A. (1994) J. Pharm. Sci. 83: 226-231

Gaínza , A. H. (1987) Can. J. Chem. 65: 1279-1291

162 SEQUENCE BASED SEPARATION OF OLIGONUCLEOTIDES IN FREE SOLUTION CAPILLARY ELECTROPHORESIS

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Separation of oligonucleotides in free solution capillary electrophoresis (FSCE) is based on differences in their charge to mass ratios (Barron & Blanch 1995). The separation of identical length oligonucleotides was therefore believed to be unachievable in FSCE especially for oligonucleotides longer than 15 bases (Paulus & Ohms 1990, Barron & Blanch 1995). However separation of two 24 mers, $pd(AT)_{12}$ and $pd(GC)_{12}$, has been achieved in this laboratory using FSCE and borate/boric acid buffer system (Hamdan et al 1997). This communication describes an extension of the previously developed method to the separation of oligonucleotides, first to non self-complementary dodecamers and then to dodecamers with identical length and base composition but with different sequences.

The non self-complementary dodecamer duplexes d[GGGCCGCGCCGC].d[GCGGCGCGGCCC] (I) and d[AAATTATATTAT]. d[AT AATATAATTT] (II) were obtained as single strands from a commercial source synthesised and separated to baseline at total borate concentration (TBC) 0.22M and pH 7.5 (Fig. 1). The separation was improved further by increasing the concentration of the buffer and/or the pH. These results are explained in terms of sequence-induced differences in the tertiary structure of DNA (Hamdan et al 1997). Two dodecamer duplexes with identical base composition but different sequences were designed to test the technique further. The duplexes d[CGCAAATTACGC].d[GCGTAATTTGCG] (III) and d[CGCTATTATCGC].d[GCGATAATAGCG] (IV) were not separable at TBC 0.22M and pH 7.5 but separation was obtained by increasing the pH and the concentration of the buffer to (TBC) 0.29 M and pH 8.0 (Fig. 2). The two duplexes III and IV have the same molecular mass and charge and differ only in the orientation of the six base pairs in the centre of the duplex. The separation in Fig. 2, although not to baseline, shows that differences in the oligonucleotide sequence can give rise to differences in the tertiary structure of the oligonucleotide which can be sufficient to bring about separation in FSCE.

It is noteworthy that the single strands of the oligonucleotides (Fig. 2: IIIa, IIIb, IVa, IVb) were also separated from each other and from the duplex forms. The peaks were identified using samples containing different ratios of the single strands, separately for each duplex.

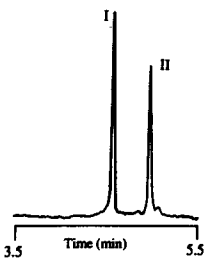

Figure 1: Electropherogram of the separation of duplexes I & II at TBC 0.22 M and pH 7.5.

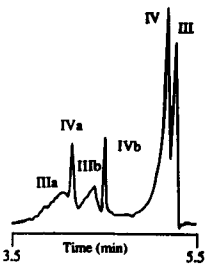

Figure 2: Electropherogram of the separation of duplexes III & IV at TBC 0.29 M and pH 8.0. Note the separation of the single strands (IIIa, IIIb, IVa, IVb).

Barron, A.E. & Blanch, H.W. (1995) Sep. Purif. Methods 24: 1-118.
Hamdan, I.I. et al (1997) submitted for publication.
Paulus, A. & Ohms, J.I. (1990) J. Chromatogr. 507: 113-123.

163 STABILITY OF OCTREOTIDE IN THE PRESENCE OF DIAMORPHINE HYDROCHLORIDE

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Octreotide (Sandostatin®), a long-acting octapeptide analogue of the hypothalamic hormone somatostatin is frequently used in palliative care for the treatment of bowel obstruction, and the control of fistula output. It is often necesary to administer octreotide in combination with diamorphine hydrochloride ((DH), Riley and Fallon, 1994). Octreotide alone, has been reported to be stable in polypropylene syringes over 24h at 25°C (Ripley et al., 1995). The effect of the presence of DH on the chemical stability of octreotide in polypropylene syringes has however not been reported. This study investigated the stability of octreotide (300µg and 600µg) in 50mg/8ml DH at 37°C in polypropylene syringes over 8h.

Samples for the stability study were prepared by aseptically aspirating appropriate volumes of Sandostatin injection (100µg/ml and 500µg/ml) into 10ml polypropylene syringes containing 0.5ml of freshly prepared 100mg/ml DH, and making up with water for injection to produce 300µg and 600µg octreotide per 8ml. Three (3) samples of each concentration were prepared and syringes subjected to a controlled temperature of 37°C for 8h. Aliquots were sampled at 0, 2, 4, 6 and 8h and analysed in duplicate by HPLC.

HPLC analysis of octreotide in samples was performed at ambient temperature using a 125 x 4.0 mm Lichrospher-60 RP-select B column (E. Merck, Germany) with a mobile phase composition of 33%v/v acetonitrile in 20mM phosphate buffer pH 7.4, at a flow rate of 1ml/min, with UV detection at 210nm. Manual injections were made via a fixed 20µl stainless-steel loop.

Octreotide, DH, and their respective degradants; des-threninol and acetyl-morphine were baseline resolved using the above chromatographic conditions with retention times of 7.5, 9.5, 2.4 and 5.0min respectively. Calibration curves were linear ($r^2 \geq 0.997$, n = 3) within the range 0µg/ml to 112.5µg/ml octreotide. Relative standard deviations of replicate injections (n ≥ 5) of standard solutions containing 300µg and 600µg octreotide per 8ml of solution were 0.10 and 0.32% respectively.

The results of the stability study (Table 1) indicate that formulations of octreotide remain stable (> 99 %) in the presence of DH at 37°C for 8h. Solutions maintained their clarity and no precipitates were observed. The pH of sample solutions remained constant at 3.8 throughout the 8h study.

Table 1 Stability of octreotide stored at 37°C for up to 8 hours.

Time (h)	Concentration* of octreotide (%)	
	300µg/8ml	600µg/8ml
2	102.50 ± 3.19	100.68 ± 0.39
4	98.38 ± 4.19	100.67 ± 1.31
6	101.28 ± 1.15	100.21 ± 1.39
8	100.15 ± 1.11	100.48 ± 2.80

*Values are mean ± S.D. (n = 6) expressed relative to the time zero control.

The study has established the stability of octreotide at 37°C in the presence of DH, in polypropylene syringes over a period of 8h. Co-administration of octreotide and DH simplifies treatment and improves the quality of life in palliative care.

This study was supported by Sandoz Pharmaceuticals (UK) and the Western General Hospitals NHS Trust, Edinburgh.

Riley, J., Fallon, M.T. (1994) Europ. J. Palliatve Care. 1: 23-25
Ripley, R.G. et al. (1995) Am. J. Health-Syst. Pharm. 52: 1910-1911

164 IMPROVED CHROMATOGRAPHY OF CYCLOSPORIN A USING A POROUS GRAPHITIC CARBON STATIONARY PHASE

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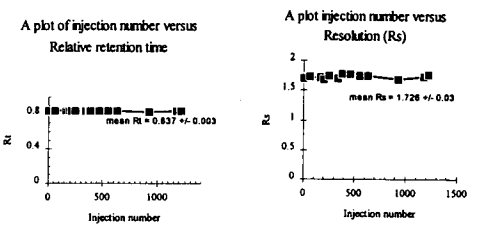
The immunosuppressant Cyclosporin A (CyA) is a neutral, hydrophobic cyclic peptide primarily used for prophylaxis and treatment of organ rejection in various transplantations (Merion et al 1984) and for the topical treatment of psoriasis (Griffiths et al 1987). RP-HPLC has been the method of analysis for monitoring CyA levels in patients after transplant surgery (Niederberger et al 1980) and quantifying levels of CyA in formulations (Laurent et al 1995). Most CyA assays use C_8, C_{18} or CN phases at temperatures of 50-75 ^{0}C which are necessary to achieve achieve the required system suitability (Bowers et al 1985). However, such elevated temperatures are thought to deteriorate these bonded phase columns. A compendial HPLC assay method exists for Cyclosporin A using EPCRS Cyclosporin U (CyU), an analogue of CyA with a difference of one CH_2 (British Pharmacopoeia Addendum 1996) but was considered inadequate due to long retention times (>30 min) and the use of a C_{18} column. Therefore a stationary phase was required which could separate CyA and CyU with shorter retention times. A relatively new stationary phase, Porous Graphitic Carbon (PGC), has shown greater selectivity, resolving solutes with differences of one CH_2 unit (Kriz et al 1994). The aim of this research was to evaluate a PGC column to resolve CyA and CyU, withstand a high sample throughput (>100 injections/day) and remain stable at elevated temperatures.

The separations were carried out on a 100 x 4.6 mm I.D. Hypercarb S 5 µm (PGC) column set at 70 ^{0}C with a mobile phase consisting of methanol:methyl-t-butyl ether (50:50 v/v) at a flow rate of 1.5 mL min^{-1}. To assess column performance 25 µL of a test mixture of CyA and CyU in methanol was injected onto the column at various intervals and the resultant peaks detected at 206 nm. From the chromatograms of CyA (t_R = 8.72 min) and CyU (t_R = 10.72 min) R_s (resolution) and R_t (relative retention) was calculated so as to assess chromatographic performance and therefore column stability. Figures show R_s and R_t (+/- std.dev) for the test mixture at various injection intervals.



In conclusion, the data show that the column is stable at elevated temperatures over a prolonged period of use and therefore the method provides an improvement on previous techniques.

Merion, R. M. et al (1984) N. Eng. J. Med., 310:148
Griffiths, C. et al (1987) Lancet, i:806
Niederberger, W. et al (1980) J. Chromatography, 182: 454-458
Laurent, T. et al (1995) Am. J. Health-Syst. Pharm., 52: 2204-2207
Bowers, L. D. et al (1985) J. Chromatography, 333: 231-238
British Pharmacopoeia (1993) Addendum (1996): 1754-1755
Kriz, J. et al (1994) J. Chromatography, 663: 151-161

165 THE OPTIMISATION OF SAMPLE PRESENTATION IN THE ANALYSIS OF PHARMACEUTICAL EXCIPIENTS USING NEAR INFRARED SPECTROSCOPY

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The continued growth in the use of near-infrared (NIR) spectroscopy in pharmaceutical analysis stems from its speed of measurement and minimal sample preparation. Only broad recommendations have been made concerning sample presentation (Osborne et al 1993, Williams 1992), however, variables such as sample cup diameter, sample cup material, sample thickness etc can affect the measured spectrum. This work aims to identify and quantify these variables and their consequences.

NIR spectra were measured over the wavelength range 1100-2500 nm using a NIRSystems Inc. 6500 spectrophotometer fitted with a Rapid Content Analyser. Each spectrum taken was the average of 32 scans. Eight excipients: Kollidon 25, A-Tab, Emcompress, Methocel E5 Premium, Avicel PH102, purified talc, Explotab and lactose monohydrate were used to investigate the effects of the various sample presentation variables.

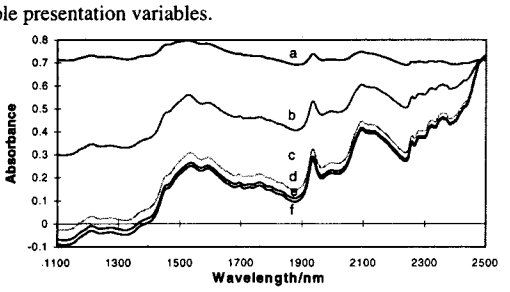

Fig 1 - NIR spectra of lactose monohydrate using a sample cup diameter of (a) 0.4 cm (b) 0.8 cm (c) 1.2 cm (d) 1.6 cm (e) 2.0 cm and (f) 4.0 cm

Increasing the diameter of the sample cup caused a multiplicative shift to spectra, resulting in peak intensity and peak order changes (see Fig 1). At an 'infinite diameter' of 2.0 cm, the multiplicative shift ceased. This value will be beam size and detector geometry dependent. The glass sample cups investigated displayed additional absorbance peaks of free SiOH overtone and silicone combinations at 1400 nm and 2200 nm respectively when compared to quartz cups, distorting poorly absorbing samples e.g. A-Tab significantly. However, correction for such effects was possible. A minimum of 1 cm sample thickness, which exceeded the penetration depth of all powders investigated, reduced spectral variation. Different methods of sample packing introduced spectral variability, the extent depending on the particle characteristics e.g. particle size distribution and extent of agglomeration. Compressing the samples up to 5000 Nm^{-2} did not affect the spectra significantly when compared to just pouring the powder into the sample cup. Tapping, which caused particle stratification and reorientation, generally produced lower absorbances and greater spectral variation. However, standard normal variate, a form of mathematical treatment, proved useful in minimising sample packing variation. Thus, sample presentation must be optimised e.g. sample cup diameter > 2.0 cm, consistent sample cup material, sample thickness > 1 cm and standardised packing to ensure spectral reproducibility.

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166 MONITORING OF HUMAN CELL GROWTH IN CULTURE: THE APPLICATION OF PIEZOELECTRIC QUARTZ CRYSTAL MICROBALANCES

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The use of in-vitro cell culture methods has expanded at an enormous rate over the past twenty years. It is now a routine technique for testing the effects of new drugs, evaluating biocompatibility and as a replacement for animal studies. Cell growth kinetics are an important research parameter in the area.

At present, measurement of cell growth over time is a slow and laborious task. Cell suspensions of known density are seeded in media onto standard polystyrene tissue culture dishes incubated at 37°C for several days and the cell number counted at two hour periods by haemocytometry.

However, many human cell strains have also been shown to bind to certain transition metals (Pouton et al 1983). We report here a novel method of evaluating the adherence and growth of cells to such surfaces using piezoelectric quartz microbalances.

Piezoelectric crystals (QCM) are high frequency, quartz resonators, excited by the application of a potential difference across a pair of transition metal electrodes. Changes in viscoelastic properties at the electrode surface are seen as proportional decreases in the resonant frequency (Suleiman and Guilbault 1994).

10MHz polished, gold electrode quartz crystals (Piezoproducts, Havant) were briefly washed in HPLC grade ethanol (Aldrich) to remove surface contamination and mounted in a cell assay chamber, designed in-house. The crystal was excited using a dual oscillator circuit developed in-house with frequency measurement being carried out by a Fluka 6685 frequency counter (Fluka Ltd , UK) connected to an Opus 486DX2 personal computer via an interface card. Fluka Timeview software was used to record data.

Human corneal fibroblasts (passage number 20-30) were trypsinised, pelleted by centrifugation (5min at 1300rpm) and re-suspended in CO^2 independent media (Gibco) to a seeding density of 2 x 10^4 cells ml^{-1}.

An aliquot of the cell suspension (30µl) was placed onto the exposed electrode of the QCM and a gas permeable membrane installed above this, the whole being placed in an incubator at 37°C and the frequency recorded at two minute intervals for a period of sixty hours.

A steady frequency was observed for approximately the first eight hours following incubation when an initial drop in frequency of 160 Hz was seen . Reasonable stability then resumed until twenty four hours of incubation was complete, when a second drop in frequency was observed (450Hz). Cells were fixed with a solution of 50/50 methanol/acetone and stained with 4,6-Diamidino-2-phenylindole (DAPI) (1mg ml^{-1}, Sigma) followed by viewing under fluorescence microscopy which confirmed the presence of nuclear DNA. A blank run with only cell medium gave a steady rise in frequency of 80Hz.

Cells usually take between six and twelve hours before depositing from suspension and adhering to solid substrates (Freshney 1987). This would account for the initial drop in frequency at approximately eight hours. With this cell line a lag phase was then expected before the start of the growth period in which the cells divide exponentially; not before twenty four hours of incubation. The size and shape of the second frequency drop would appear to coincide with the expected point of cell growth.

This work clearly demonstrates that QCM technology has the potential to provide a simple, efficient method of measuring cell growth kinetics and with suitable crystal surface modification could be used to ascertain surface biocompatibility in a range of metals as well as polymers.

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Suleiman, A. and Guilbault, G.G. (1994) Analyst. 119: 2279-80

167 PIEZOELECTRIC QUARTZ CRYSTAL AND SURFACE PLASMON RESONANCE STUDY OF BORONIC ACID SELF ASSEMBLY MONOLAYER INTERACTIONS WITH NAD

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Both piezoelectric quartz crystals sensors (QCM) and surface plasmon resonance (SPR) have been used for monitoring molecular interactions and in assay work. Generally one component involved in the interaction is bound or entrapped at the surface of the sensor. Attachment to a gold surface can be used for both sensors. This surface readily reacts with thiol or disulphide containing compounds forming a self assembly monolayer on the gold.The sensors respond to changes in the physicochemical properties of the solution at the gold surface, (mass), viscosity and density for QCM in solution and refractive index for SPR).This work is a preliminary investigation into the development of a re-usable coating for QCM and SPR sensors inorder to assay diol containing coenzymes such as nicotinamide adenine dinucleotide (NAD) and use this bound compound to study enzymes interactions. The enzyme chosen was L-Lactate dehydrogenase (LDH).

The QCM system used consisted of a 10MHz quartz crystal (Piezoproducts, Havant UK) with 4mm gold electrodes connected to an in house constructed dual crystal oscillator, Fluke PM6685 frequence counter, Opus 486 66DX2 personal computer with Fluka Timeview acquisition and control software. One face only was exposed to liquid with the crystal being sandwiched between two gaskets in a flow through cell.This was connected to a Shimadzu 9A HPLC pump via a Rheodyne 7025 valve with a 200 microlitre loop. After stabilisation of a quartz crystal in the flowing buffer a noise level of better than 1Hz was obtained. For the SPR experiments gold coated glass slides were used in an Amersham Biosensor SPR instument, equipped with a 1ml syringe pump, with data acquisition being obtained using a IBM 386sx and WIN SPR. Temperature was maintained at 30C for both procedures. Phenylboronic acid derivatives are known to reversibly bind diol containing compounds such as glucose and NAD (Schott et al 1973). For this work two new boronic acid derivatives, bis 4 borobenzyl disulphide (BBS) and 3-(5-thiolpentamide) phenylboronic acid (TPB) containing sulphur groups to bind to gold were synthesised. The compounds were subjected to elemental analysis, NMR and FTIR. The Piranaha cleaned gold coated QCM and freshly prepared SPR slide were placed in 1mM ethanolic solutions of the boronic derivatives for 20h at 30C, giving a self assembled monolayer.The deposition was followed in real time by both techniques and also by contact angle and FTIR measurements. A typical deposition profile similar to that obtained with alkanethiols involving two stage adsorption process (Pan et al 1996) was observed. The adsorption of TPB was approximately twice as fast as for BBS.

The binding of NAD to the BBS and TPB coated surfaces was achieved by passing 1 mg per ml. solutions of NAD in buffer at pH 9.0 and 7.2 respectively at flow rates of 0.01 mls per min. over the surface of the sensors. The attachment of the NAD at the surface was observed as a frequency drop of 80Hz(%rsd 9) in the case of QCM and an increase in plasmon resonance angle of 36mDa(%rsd11) for the SPR sensor. The attached NAD could be removed by flushing with pH 4.0 buffer and then re-attached at the appropriate pH. This was also followed by FTIR. A typical binding curve was obtained when response was plotted as a function of concentration.

Bovine LDH solutions were prepared in pH7.2 phosphate buffer covering a concentration range from 0.7U. to100U per ml. Again a flow rate of 0.1 mls. per min. was used. The interaction between the LDH and the bound NAD was 10 times slower than that between NAD and TPB from the SPR data. 0.7U per ml. of LDH gave a 4Hz decrease in frequency with the QCM system and a typical shaped binding curve for the interaction. This work shows that both SPR and QCM can be used for studying the interactions of the coenzyme NAD with LDH by means of the synthesised boronic acid derivatives. This system also has potential to be developed as an assay method for diol compounds.

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168 AN OPTIMIZED DERIVATIZATION METHOD FOR THE SCREENING OF ANABOLIC STEROIDS

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Anabolic steroids (AS) are a powerful group of natural/synthetic compounds similar in chemical structure to the natural hormone testosterone with both androgenic / anabolic properties. The need for a specific, rapid determination of AS has led to developments of numerous analytical methods (Sabot et al 1985). Yet there has not been a validated screening method for the analysis of all the AS. The consequences of AS abuse (supraphysiological dose) cannot be overemphasied especially in sports, psychiatry and its abuse has wide demographic spread. (Phillips 1991) Governments have legislated the control of AS (e.g Britain included them in the Misuse of Drugs Act of 1971 in 1996). Steroids present a unique challenge to chemists because of the continous structural modifications. This paper describes the setting up of criteria and the comparison of derivatives for the successful derivatisation and screening of AS using gas chromatography-mass spectrometry (GC-MS).

Criteria set were 1, detection limit; 2, precision; 3, stability; 4, reproducibility; 5, characteristic ions to monitor; 6, best for quantification; 7, best for screening; 8, large % molecular ion to monitor; 9, amenable to automation; 10, speed.

An HP 5890 GC with capillary column 12 m x 0.22 mm, temperature programmed from 45 °C to 290 °C, final time 20min was used with a mass selective detector (MSD-HP 5970) in electron impact mode. Different methods and combinations (A to F) were used in the preparation of the steroid derivatives. A, steroid + TMS for 25min; B, steroid + TMS + catalyst +antioxidant for 1hr; C, steroid + TBDMS + catalyst for 2.5hrs; D, steroid + TMS for 25min evaporated + TBDMS for 2hrs. E, steroid + PFB for 1hr; F, steroid + PFB for 30min evaporated + TMS for 40min. The performance of these 6 derivatization methods of 6 steroids: (testosterone, epitestosterone, boldenone, methandrostenolone, nandrolone, oxymetholone,) were analysed by GC-MS and measured against the criteria.

Table 1

Methods	Criteria									
	1	2	3	4	5	6	7	8	9	10
A 1TMS	x	-	-	x	x	x	x	x	x	x
B 2TMS	x	x	-	x	x	x	x	x	x	x
C..... 2TBDMS	x	x	x	x	x	x	-	x	x	-
D..1TMS -1TBDMS	x	x	x	x	x	x	x	x	x	x
E.....1PFB	-	x	x	x	x	-	x	x	x	-
F.....1PFB - 1TMS	x	x	x	x	x	-	-	x	x	x

PFB = pentafluorobenzoyl; TBDMS = tert-butyldimethysilyl; TMS = trimethylsily. x = meets the criteria; - = does not meet the criteria.

It can be seen from the results that the best performance came from TMS-TBDMS method with detection limit of 2pg (multiple ion monitoring). Trace levels of AS and their metabolites can be analysed from blood (after extraction). The choosen method demonstrated good stability with reasonable retention times. The slight limitation of the method is low abundance of ions other than the molecular ions. The 2TMS method popularly used is not as sensitive or stable as this new method. All the other methods have specific advantages. It was possible to separate all the steroids on the GC on one run.

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169 EFFECT OF GAMMA RADIATION ON MALTOSE

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Maltose can be used as an excipient in freeze-dried pharmaceutical formulations, which it may then be convenient to sterilise by ionising radiation. Irradiation (^{60}Co, γ) of solid state maltose at radiation doses of 40 to 400 kGray was carried out to investigate the physical and chemical changes to the disaccharide.

A series of analyses including assay, carbonyl group (2,4-Dinitrophenylhydrazine, DNPH), UV, IR, NMR, HPLC and gel permeation chromatography (GPC) were studied. The acidity and number of carbonyl groups were increased after irradiation. Irradiated maltose showed hyperchromic effect between 190 and 320nm, with a maxima absorbance at 260nm. This range corresponds to the increase of carbonyl group after irradiation. Little difference was detected in NMR and IR spectra between maltose and irradiated maltose.

A novel HPLC system comprising water (reversed phase) as mobile phase, a normal phase silica column (Supelcosil LC-Si, 25cm x 4.6mm i.d., 5μm), with RI and diode array detection was used in the quantification of maltose in the irradiated samples. The maltose assay after irradiation at 40 kGray is 98% (w/w). Good separation and reproducibility (c.v.<2%) were demonstrated. The results indicated that a wide range of degradants including polymeric, acidic and aldehydic/ ketonic compounds may have contributed to the total radiation degradation of less than 2% in the irradiated maltose at the radiation dosage of 40kGray. The degradation of maltose was increased from 2% to 12% at the radiation dosage of 400kGray. The G value for solid maltose monohydrate was 8.2. No glucose, maltotriose, 5-hydroxymethyl furfural, 5- methyl furfural, 2,5-furandimethanol, 2-methyl furan, 2-furaldehyde, furfuryl alcohol, D-gluconic acid, D-glucurono-6,3- lactone, δ-gluconolactone, D-glucuronic acid, maltitol and glutaric dialdehyde were found in the irradiated maltose.

Formaldehyde is one of the known toxic products beside furfuraldehyde (not detected) that is commonly seen in the carbonhydrate degradation by irradiation. The formaldehyde content in the irradiated maltose was determined through a simple formaldehyde-DNPH reaction. Formaldehyde, along with other degradants containing carbonyl functional groups, can react with DNPH to form 2,4-dinitrophenylhydrozones. The formaldehyde 2,4-dinitrophenylhydrozone was separated and identified by HPLC- PDA detector (UV detection at 350nm). The DNPH derivatised samples of irradiated maltose were eluted through a C18 column (Spheresorb ODS2, 25cm x 4.6mm i.d., 5μm) with a mobile phase of 0.05M sodium acetate : acetonitrile = 4: 6 (pH=5.02). In the irradiated maltose, less than 2.5 ppm of formaldehyde was detected in the 40kGray samples, and up to 5.5ppm in the 400kGray samples.

Under the normal radiation dosage of 40kGray, limited radiation degradation and a very low level of formaldehyde was observed in the irradiated maltose.

170 DEVELOPMENT OF THE SPECTROPHOTOMETRIC METHOD WITH PALLADIUM (II) CHLORIDE FOR DETERMINATION OF AMIKACIN IN PHARMACEUTICALS

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A number of methods have been reported for determination of aminoglucosides in pharmaceuticals, including microbiological (BP 93), high-performance liquid chromatography, McLaughlin (1992) and capillary zone electrophoresis, Ackerman (1992).

The aim of this work is to present a new spectrophotometric method for determination of amikacin in pharmaceuticals with palladium(II) chloride reagent.

The method depends on the deamination of amikacin with nitrous acid at 80 °C for 90 minutes. The reaction between the sample of deamination product of amikacin (1 mg/ml) and palladium(II) chloride reagent in alkaline medium was carried out at 80 °C for 90 minutes. The palladium(II) chloride reagent consists of: palladium(II) chloride, sodium potassium tartarate and sodium sulphite with final concentration: 1 mg/ml, 15 mg/ml and 2 mg/ml, respectively. The non-reduced palladium(II) chloride reacts with potassium iodide in acidic medium and the reaction product was determined spectrophotometrically at 318 nm. The amount of reduced palladium(II) chloride was calculated by difference between the content of palladium(II) chloride in the sample without and with amikacin, treated in the same way.

Amikacin do not possess reductive capability, but as it consists of amino-deoxy sugars (3-amino-3-deoxy-D-glucose and 6-amino-6-deoxy-D-glucose) linked via glucosidic bonds to an aglucone moiety, after deamination and hydrolysis, oxido-reductive reaction between palladium(II) chloride reagent and sugar moiety would be expected.

The deamination product of amikacin was identified by high-performance liquid chromatography using refractive index detector, as D-glucose. The proof that the reaction proceed as oxido-reduction between D-glucose and palladium(II) chloride reagent is formation of a black precipitate of elementary palladium. Under the reaction conditions described above, the oxido-reductive reaction is quantitative and could be used for the determination of amikacin in pharmaceuticals. Regression line is represented by the equation y = 0.0562 + 0.578 x, (n=5) for the concentration range 1-5 mg/ml of amikacin in the reaction with 5 ml palladium(II) chloride reagent. The correlation coefficient of the method is 0.9997. The accuracy of the proposed method was checked by the method of standard additions.

Table1. The results of the determination of amikacin by the method of standard additions

Sample	Added (mg)	Calculated (mg/amp)	Determined (mg/amp)	Recovery ϒ(%)
	-	-	498.50	-
	125	623.50	631.17	101.23
Amikacin amp.,	250	748.50	753.15	100.62
500 mg/amp.	375	873.50	871.26	99.74
	500	998.50	996.80	99.83

As it can be seen from the Table 1., the recovery values of the determination of amikacin with palladium(II) chloride reagent in dosage form, without, and after addition of amikacin standard solution 25, 50, 75 and 100% over the declared concentration, confirmed that the method is quantitative and accurate.

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2. McLaughlin, L., Henion, J., (1992) J Chromatogr., 591, 195-206
3. Ackermans, M., Everaets, F., (1992) J. Chromatogr., 606 , 229-235

171 CHARACTERISATION OF PASSIVE AND IONTOPHORETIC PERCUTANEOUS LOCAL ANAESTHESIA BY A.C. IMPEDANCE SPECTROSCOPY IN-VIVO

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Amethocaine is a highly lipophilic local anaesthetic. Additionally, it has been shown to bind to proteinaceous components of the *stratum corneum* resulting in a significant reservoir effect (Woolfson *et al.*, 1990). A.c. impedance spectroscopy has been employed successfully to characterise the effects of topically applied substances to human skin (McAdams *et al.*, 1995). This study employs the techniques of McAdams and co-workers to measure changes in the resistance (R_p) and pseudocapacitive impedance (K) during and after the passive and iontophoretic delivery of amethocaine.

Gels for passive delivery were formulated according to the amethocaine phase-change theory (McCafferty & Woolfson, 1988). Gels for iontophoresis were formulated to contain amethocaine hydrochloride 1% w/w. A novel test electrode was designed that allowed the simultaneous measurement of four adjacent skin sites. Three of these areas contained amethocaine gels. A placebo gel was employed as an internal reference at the fourth site. Passive-diffusion amethocaine gels (4% w/w) were removed after 45 minutes and replaced with placebo for the duration of the experiment. A constant direct current of 0.5 mA was used to iontophoretically deliver amethocaine for 20 minutes, thereafter the amethocaine gel was replaced with placebo. All passive drug diffusion sites demonstrated significant decreases in both R_p and K relative to placebo-treated skin sites. These changes were maintained after the active formulation was removed and replaced with placebo (Figs. 1 & 2). This suggests the existence of a substantial drug reservoir in the *stratum corneum*. Sites treated with drug-containing formulations exhibited a similar trend during iontophoresis. After iontophoresis ceased, R_p and K returned to the placebo level, suggesting that iontophoretic and passive drug delivery occur via different routes (Figs. 3 & 4). Correlation of the electrical parameters with pain scores (Irsfeld *et al.*, 1993) indicate that changes in R_p and K may be used as an accurate indicator of the effective clinical duration of anaesthesia. These results suggest that iontophoretic percutaneous local anaesthesia is a clinically limited technique as a large proportion of the potent drug bypasses the nociceptors and passes directly into the systemic circulation where it is rapidly metabolised .

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McAdams, E.T. *et al. 9th. Int. Conf. Elec. Bioimp.* Heidelberg, 1995.
McCafferty, D.F. and Woolfson, A.D. *U.K. Patent* 2,163956. 1988.
Woolfson, A.D. *et al. Br. J. Clin. Pharm.* **30** (1990) 273.

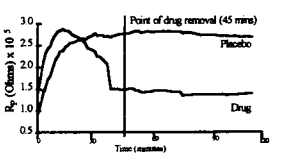

Figure 1. Typical plot of R_p against time for passive delivery of amethocaine.

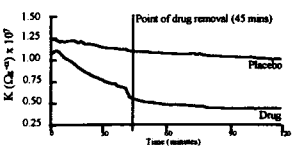

Figure 2. Typical plot of K against time for passive delivery of amethocaine.

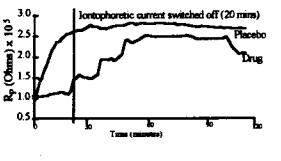

Figure 3. Typical plot of R_p against time for iontophoretic delivery of amethocaine.

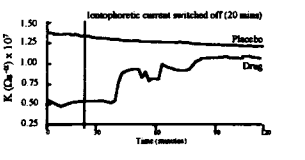

Figure 4. Typical plot of K against time for iontophoretic delivery of amethocaine.

172 A NOVEL SYNTHETIC APPROACH TO DNA-BINDING MONOMERIC AND DIMERIC PYRROLO[2,1-C][1,4] BENZODIAZEPINES AS POTENTIAL GENE-TARGETTING AGENTS

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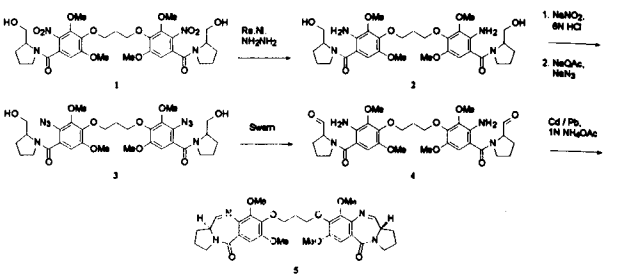
The pyrrolo[2,1-c][1,4]benzodiazepines (PBDs) are a group of antitumour antibiotics produced by Streptomyces species. Members of the PBD family include anthramycin, sibiromycin and DC-81. The antitumor activity of the PBDs is exerted through sequence-selective covalent binding to a N2 of a guanine base within the minor groove of DNA via an electrophilic imine functionality (Thurston, 1993). The preferred bonding sequence involves a 5'-Pu-G-Pu motif, however the recent synthesis of PBD dimers has successfully extended the sequence selectivity to a six base motif of 5'PuGATCPy or 5'PyGATCPu, as well as producing highly efficient DNA interstrand crosslinking agents (Bose *et al*, 1992). Compounds of this type with extended sequence selectivity also have potential use in the control of gene expression (Puvvada, 1997).

Many analogues of naturally occurring PBDs have been synthesised in the laboratory (Thurston, 1996) and the synthetic chemistry in this field has now developed to the point where there are now a large number of synthetic derivatives being produced, allowing for extensive SAR studies. There is an increasing number of published methods for the synthesis of PBDs, each of which employs mild methods for the formation of the imine functionality. A new synthesis of the novel PBD dimer, DRH-165 **(5)** has been carried out using a mild reductive method to convert an azide group to an amine in the presence of an aldehyde group thus leading to B-ring closure. The nitro-alcohol **(1)** was reduced using raney nickel and hydrazine to afford **(2)**. Diazotisation and treatment with sodium azide afforded the azido-alcohol **(3)**, which was oxidised with an excess of Swern reagent to give the aldehyde **(4)**. A mild cadmium/lead couple reduced the azide groups to afford the target dimer molecules in high yield. This approach has also been successfully applied to the synthesis of monomer PBDs, and provides a new high-yielding alternative route for the synthesis of these biologically important molecules.



The Royal Pharmaceutical Society of Great Britain and the Cancer Research Campaign are thanked for their financial support.

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173 EVALUATION OF MOLECULARLY IMPRINTED POLYMERS AS SELECTIVE SORBENTS FOR THE PRELIMINARY SCREENING OF COMBINATORIAL LIBRARIES

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Molecular imprinting is an effective method for introducing sites capable of specific or near-specific molecular recognition into an otherwise uniform matrix. Molecules can be successfully imprinted using non-covalent interactions, such as hydrogen bonding, ionic forces and hydrophobic interactions, to form a pre-polymerisation complex that is ultimately fixed within a cross-linked polymer. Subsequent removal of the imprint molecule leaves a reciprocal site capable of molecular recognition. Such molecularly imprinted polymers (MIPs) have been used as high efficiency chiral HPLC stationary phases, antibody mimics in immunoassays, chemosensory adjuncts and catalysts, Mosbach and Ramstrom (1996). This study extends imprinting technology to consider the potential role of MIPs as primary screening sorbents for chemically diverse mixtures of compounds. Such mixtures are now routinely encountered within the pharmaceutical industry as the products of combinatorial synthesis, Floyd et al. (1996).

This approach relies on either currently available drug moieties or rejected, high efficacy, drug candidates acting as template molecules to create target site mimics in an acrylic polymer matrix. Subsequently, these materials are processed and packed into columns to be used as chromatographic stationary phases.

Under HPLC conditions mixtures of compounds can then be screened. The retention time of the eluted material relative to that of the imprint molecule is used as an indication of a compound's affinity for the imprinted site. Consequently, its potential as a drug candidate, to go forward for additional biochemical screening, may be assessed.

In this study, two model systems were examined in an attempt to validate this methodology for use in more complex combinatorial library screening. Firstly, a MIP was prepared imprinted with Boc-L-phenylalanine, Kempe and Mosbach (1991). Confirmation of imprinting was achieved by successfully separating Boc-L-phenylalanine and Boc-D-phenylalanine on the column prior to the screening study. The injection of a range of Boc-protected L- and D-amino acids onto this column demonstrated a predictive variety of affinities for the Boc-L-phenylalanine imprint site. A second model used a range of commercially available beta-blockers. A propranolol MIP was prepared and the affinities of seven related compounds were compared with that of the imprint molecule. This analysis was carried out for the individual compounds and for a mixture. The mixture eluted as a single complex peak and it was necessary to fractionate the column eluent and determine, for each fraction, the relative abundance of each beta-blocker by reverse phase HPLC, Shen et al. (1991).

Results indicate that, for structurally related mixtures of compounds, this approach is capable of differentiating between compounds based on their relative affinities. Future work will extend this methodology to more complex and structurally diverse combinatorial libraries.

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174 STEROID AROMATASE INHIBITORS. NEW ROUTES TO THE SYNTHESIS OF FORMESTANE AND RELATED D-LACTONES

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Aromatase is the enzyme which catalyses the final step in the biosynthesis of estrogens. Inhibition of this enzyme is a priority goal, since it has been identified as a good endocrine therapy for the treatment of estrogen-dependent breast cancer and may be important in the treatment of other malignant diseases in aging patients.
Among the various types of aromatase inhibitors, steroids closely resembling the enzyme natural substrates androstenedione and testosterone have been shown useful for the treatment of breast cancer. The 4-hydroxyandrost-4-ene-3,17-dione (4-OHA, Formestane) has already proved very effective in the treatment of advanced breast cancer and quite recently has found clinical use (Brodie 1994).
Furthermore, ring D lactones related to testosterone proved also to be effective aromatase inhibitors (Leclercq 1992) and testolactone was one of the first steroids used in the clinical treatment of breast cancer, although it has been withdrawn before its activity as aromatase inhibitor has been established.
In order to enhance the anticancer activity and to prepare affinity labels for the elucidation of the active site of aromatase, the synthesis of new drugs related to 4-OHA, is a very active field of research. Following this idea, the synthesis of a new target molecule, the 4-hydroxy-D-homo-17a-oxaandrost-4-ene-3,17-dione with the ring A moiety of 4-OHA and the ring D moiety of testolactone, has been our goal. Moreover, the low yields reported for the synthesis of formestane drove us to approach the synthesis of this steroid with different strategies.
We report herein an appropriate synthetic strategy, that accounts for the built-in on the same structure of the ring D lactone and the ring A diosphenol, important functionalities usually present separately in steroids used to inhibit aromatase. Using the key intermediate 3α,4β-dihydroxy-5α-androstan-17-one, recently prepared in a novel approach to the synthesis of 4-OHA (Tavares da Silva et al 1996), as starting material, the desired ring D lactone was built through a Baeyer-Villiger oxidation. Further oxidation of the dihydroxylactone formed, gave a kinetic diosphenol which by base or acid-catalyzed isomerization led to the desired thermodynamic diosphenol, the 4-hydroxy-D-homo-17a-oxaandrost-4-ene-3,17-dione with an overall yield of 91%.
To circumvent the poor yields reported for the preparation of 4-OHA, we will also describe a new and straightforward synthesis using testosterone as starting material. An hydroboration/oxidation followed by DMSO-trifluoroacetic anhydride oxidation and further base-catalyzed isomerization, yielded the aforementioned steroid. This method revealed to be competitive with the already described ones.

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175 THE APPLICATION OF A POLYMERIC PHOSPHINE OXIDE RESIN AND THE DEVELOPMENT OF A NOVEL SOLID PHASE REAGENT FOR SOLID PHASE ORGANIC CHEMISTRY

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Arising from the use of solid phase methods in peptide chemistry, the use of solid phase media has burgeoned over recent years (Hermkens *et al* 1996). This development has lead to the development of combinatorial chemistry in the search for molecular diversity in drug design (Williard *et al* 1996, Doyle 1996). Conventional methodologies utilise a solid support for the combinatorial library and introduce reagents in the solution phase. We introduce here an alternative methodology where the library or a lead pharmacophore undergoing elaboration is maintained in solution. Molecular evolution is then achieved using a solid phase reagent. The advantages of such a methodology are that reactions can be readily monitored spectrophotometrically or chromatographically and samples removed for screening without the need for cleavage from the solid support.

In brief, the methodology outlined in this communication describes the development of a novel polymer supported sulphonyl chloride for the substitution of hydroxyl functionalities. Further, the use of an unusual polymeric phosphine oxide resin in solid phase chemistry is evaluated.

Polymeric phosphine oxide resin has been reported for use in enzyme immobilisation. It was synthesised was *via* the Mannich condensation of tris(hydroxymethyl)phosphine with Jeffamine T-3000 followed by peroxide mediated oxidation, following the method of Henderson *et al* (1994). The resultant amino-functionalised resin was derivatised with 2-bromoethane sulphonic acid and acetylated with acetic anhydride to remove remaining nucleophilic functionality. Subsequent conversion to the sulphonyl chloride was achieved using oxalyl chloride. The swelling capacity of the resin in organic solvents was evaluated. The most efficient swelling was achieved in DMF and dichloromethane (~4.6 cm^3/g resin). Swelling coefficients in acetonitrile, 1,4-dioxane and THF were also in the order of 3 to 4 cm^3/g resin. Times taken to achieve maximal swelling ranged from 0.5 h for dicloromethane to 4 h for DMF.

Conversion of the sulphonyl chloride resin into the benzyl sulphonate was used as a model reaction to establish conditions for alcohol activation. Treatment of the resin with benzyl alcohol in DMF at 50°C gave complete conversion to the ester after 30 min. Lower temperatures and alternative solvents do not lead to efficient conversion. Washing of the resin at this step removes undesired impurities. Generation of N,N-diethylbenzylamine and benzyl phenyl sulphide from the benzyl sulphonate by treatment with diethylamine and thiophenol respectively proceeded rapidly and cleanly with yields of at least 80%. This approach is thus readily applicable to the substitution of a free hydroxyl functionality cleanly and quantitatively, with the combined advantages of solid phase and solution phase techniques.

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176 SYNTHESIS AND CONFORMATIONAL ANALYSIS OF A SERIES OF CONFORMATIONALLY RESTRICTED CROMAKALIM ANALOGUES

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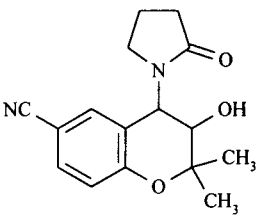
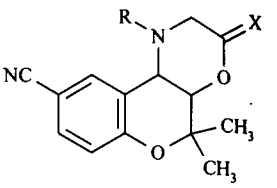
Within the past few years potassium channel activators have gained increasing attention because of their clinical potential in various diseases. One of the lead compounds within this new class of drugs is the benzopyrane derivative cromakalim (**1**). Following our ongoing studies on structure-activity relationships (Wolf et al. 1996) we synthesised a series of conformationally restricted 1-benzopyrano-[3,4-b][1,4]oxazines (**2a,b**). Synthesis of **2a** was performed via nucleophilic ring opening of the corresponding 3,4-epoxy-benzopyrane (**3**) with N-alkyl-ethanolamines, selective mesylation of the primary hydroxy group and subsequent cyclisation under basic conditions. Synthesis of **2b** was achieved via reaction of **3** with glycine-t-butyl ester, acylation of the secondary nitrogen atom and subsequent thermic cyclisation.
Conformational studies and molecular shape analysis of the new compounds were performed using semiempirical and ab initio calculations. Comparison of the compounds with cromakalim was performed by a least square fit of the coordinates of those atoms which are common in all structures.



Cromakalim (1)



2a: X = H,H
2b: X = O

Wolf, C., Weiss-Greiler, P., Wolschann, P., Ecker, G., Fleischhacker, W. (1996), Pharmazie 51: 836-839

177 DESIGN AND SYNTHESIS OF BENZOPYRANO[4,3-*c*]QUINOLINES AS TOPOISOMERASE INHIBITORS

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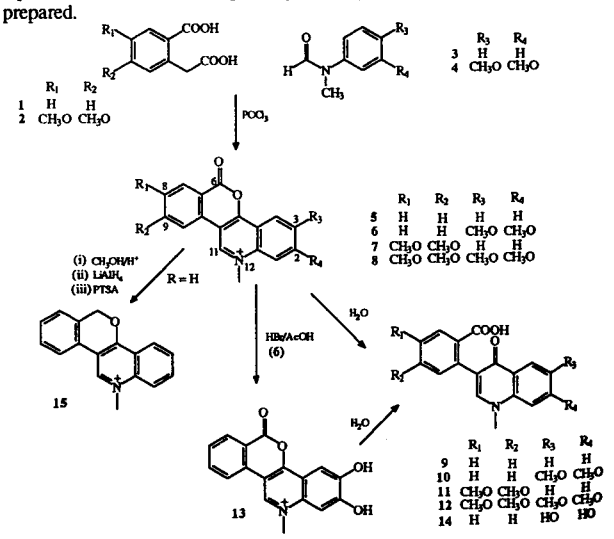
We are currently preparing new tetracyclic compounds as topoisomerase inhibitors based on the premise that topoisomerase poisoning is achieved through the formation of a labile covalent bond between a nucleophilic residue within the enzyme's active site and an electrophilic site on the drug which is bound to DNA via intercalation (Larsen *et al.*, 1993; Kerry *et al.*, 1995). Reaction between activated acetic acids **1**, **2** and *N*-methylformanilides **3**, **4** in the presence of $POCl_3$ (Meth-Cohn, 1986) yielded a series of 12-methyl-6-oxo-6*H*-[2]benzopyrano[4,3-*c*]quinolinium salts **5-8**. These compounds have a planar geometry to facilitate DNA intercalation, and possess two electrophilic sites (positions 6 and 11) which can potentially react with the enzyme's nucleophilic residue. They are acid stable, but readily undergo lactone hydrolysis in aqueous conditions to the 3-aryl quinol-4-ones **9-12**, as revealed during NMR analysis of **5** in D_2O. The influence of substituents on hydrolytic stability was determined by UV spectroscopy, and rate constants for lactone hydrolysis calculated:

5	1.34×10^6 $M^{-1}min^{-1}$
6	7.02×10^5 $M^{-1}min^{-1}$
7	2.20×10^5 $M^{-1}min^{-1}$
8	1.90×10^5 $M^{-1}min^{-1}$
13	1.74×10^4 $M^{-1}min^{-1}$

Stabilisation is achieved through the +M effect of the oxygenated substituents being greater than their destabilising -I effect. Methoxy groups at positions 2 and 3 (**6**) do not stabilise the lactone to the same extent as substituents at positions 8 and 9 (**7**), with the former substituents being more distant from the site of hydrolysis. This would explain the smaller improvement in stability with the tetramethoxy analogue (**8**) over the 8,9-substituted compound (**7**). Greater stability was achieved when compound **6** was converted to the corresponding catechol **13** using HBr in acetic acid. The instability of the lactones in aqueous solution renders these compounds unsuitable for assessment against topoisomerase. In order to generate stable compounds, the lactone **5** was converted to the corresponding reduced pyran analogue (the 6-dihydro-12-methyl-[2]benzopyrano[4,3-*c*]quinolinium salt). Hydrolysis of the lactone **5** to the methyl ester, followed by reduction with $LiAlH_4$ yielded the 3-(2'-hydroxymethylphenyl)-1-methyl-1-dihydroquinolin-4-one, which cyclised via the hemiacetal, spontaneously followed by dehydration and oxidation to yield the desired quinolinium salt **15**, which was stable in aqueous solution. The corresponding methoxy analogues are currently being prepared.



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178 DESIGN AND SYNTHESIS OF BENZO[*c*]PHENANTHRIDINE ANALOGUES AS TOPOISOMERASE INHIBITORS

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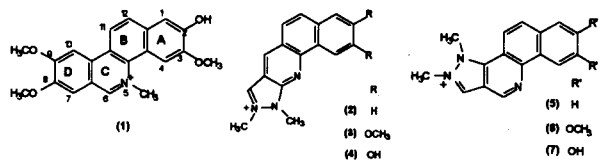
The naturally occurring benzo[*c*]phenanthridine alkaloid fagaronine (1) exhibits antileukaemic activity against P388 and L1210 tumour cell lines (Phillips & Castle, 1981). Activity is believed to be related to its ability to intercalate with DNA (Pezzuto *et al.*, 1983) and interfere with DNA replication and transcription. Fagaronine has been found to poison topoisomerase I (Wang *et al.*, 1993) via the formation of a stable cleavable complex between fagaronine, DNA and topoisomerase I which is a reaction intermediate in the enzymes natural catalytic cycle. The poisoning is reversible, and removal of fagaronine from the reaction allows topoisomerase I to continue its normal catalytic activity.



It is our belief that stabilization of the complex involves the formation of a labile covalent bond between a nucleophilic residue on the topoisomerase I enzyme and the highly electrophilic C-6 position of the iminium group of the benzo[*c*]phenanthridine nucleus. Our molecular orbital calculations for fagaronine confirm that C-6 possesses the lowest π-electron density in the molecule. Benzo[*c*]phenanthridine analogues that are devoid of an electrophilic centre at C-6 have no antitumour activity (Phillips & Castle, 1981; Mackay, 1990). Utilizing this electrophilic centre theory, we have synthesized several novel ring D benzo[*c*]phenanthridine analogues (2-7) which are able to interact with DNA via intercalation and have the potential to allow terniary complex formation. The iminium moiety of these new analogues is situated within ring D of the nucleus instead of ring C (cf. fagaronine).

We have developed novel synthetic strategies to produce these benzo[*c*]phenanthridine analogues. All previous syntheses of these alkaloids and their analogues (Mackay *et al.*, 1996) involve the final cyclization of ring B or C, requiring that all substituents are determined at the start of the synthesis. In our synthetic strategy, the final cyclization is of ring D, allowing the placement of several different rings/substituents from a single precursor.

The preparation of the quaternary pyrazolobenzo[*h*]quinoline 2 has previously been reported (Kerry *et al.*, 1995) and analogues 3 and 4 were synthesized using a similar strategy. Compounds 5 - 7 were synthesized from the corresponding 1-naphthylamine via the 3-ethyl ester of benzo[*h*]quinol-4-one. Conversion to the 4-chloro-3-ethyl ester was carried out using $POCl_3$ and then reduced to the aldehyde using DIBAL. Reaction of the ethylene glycol acetal with methyl hydrazine afforded the unquaternized species, which upon reaction with dimethylsulfate gave the final species (5 - 7).

Compounds 2 - 4 all bind to DNA with affinity constants greater that fagaronine and the UV absorption spectra on titration with DNA are characteristic of intercalation as the mode of binding.

Molecular orbital calculations for all analogues indicate that the region of lowest π-electron density is centred around the iminium moiety of ring D, and NMR and UV studies have indicated attack at this position by the hydroxide ion to form the pseudobase.

Compounds 2 - 4 are currently undergoing cell line testing to assess their antitumor activity.

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179 DESIGN OF 3-HYDROXYPYRIDIN-4-ONE CHELATORS WITH MINIMAL INHIBITORY PROPERTIES TOWARDS IRON-CONTAINING METALLOENZYMES

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3-Hydroxypyridin-4-one(HPO) chelators form a class of orally active iron chelators developed as alternatives to desferrioxamine(DFO), the only clinically available agent, for the treatment of transfusion-induced iron overload associated with thalassaemia, Hider *et al* (1992). By virtue of the low molecular weight of HPOs and favourable distribution coefficients, they rapidly penetrate most cells and hence gain access to a wide range of iron-containing metalloenzymes with the potential of effecting inhibition, Hider (1995). 5-Lipoxygenase(5-LO) and ribonucleotide reductase(RR) are two such examples. CP20(1,2-dimethyl-3-hydroxypyridin-4-one), the HPO currently in clinical trials, inhibits both 5-LO and RR more effectively than DFO. Agranulocytosis which is observed in some patients treated with CP20 may be associated with the inhibition of these critically important iron metalloenzymes. Thus the design of a chelator which lacks such inhibitory properties is highly desirable. RR is the rate limiting enzyme in DNA synthesis. The enzyme contains, within each monomer of its smaller subunit R2, an oxygen linked diferric metal centre which both generates and stabilises a tyrosyl radical essential for the enzyme activity. 5-LO in human neutrophils catalyses the first two steps in the conversion of arachidonic acid to leukotriene A4(LTA4) which is the precursor of LTB4, a potent mediator of inflammation.

The aim of this study was to design an HPO chelator of minimal inhibitory properties towards both 5-LO and RR. A set of HPO chelators with different substituents were synthesised. The inhibition of RR was monitored using an indirect method based on the measurement of tritiated-thymidine incorporation into DNA and a direct method involving the quantification of the ESR signal of the enzyme tyrosyl radical. 5-LO was monitored by measuring the rate of linoleic hydroperoxide formation. We have identified substituents which, when introduced on the HPO ring, introduce a steric factor on the chelator which interferes with the binding of the chelator with the iron centre in the enzyme active site thereby abrogating the inhibition of 5-LO(Figure 1). Some of these chelators trigger apoptosis in both thymocytes and K562 cells at a much slower rate than CP20. Thus it is anticipated that the in vivo toxicity of these chelators will much less than that the simple dialkyl chelators as typified by CP20.

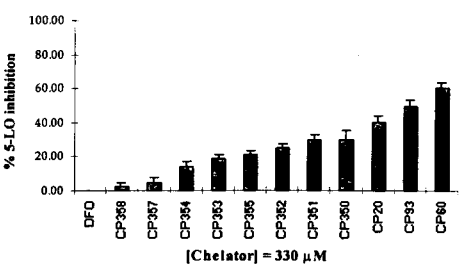

Figure 1: 5-LO inhibition by iron chelators

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180 A PRACTICAL SYNTHETIC ROUTE TO UNSYMMETRICALLY PROTECTED POLYAMINES

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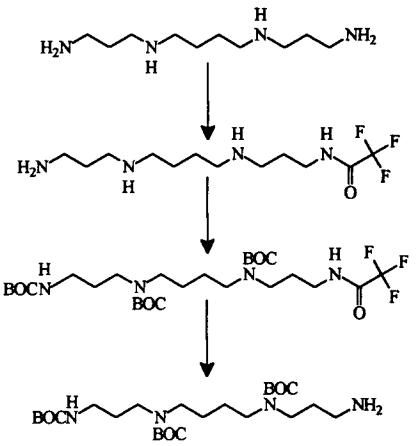
Polyamines are important naturally occurring bases which regulate the functions of certain ion channels Usherwood & Blagbrough (1991) and the folding of polynucleic acids Schmid & Behr (1995). Conjugates of 1,11-diamino-4,8-diazaundecane (norspermine, 3.3.3) and 1,12-diamino-4,9-diazadodecane (spermine, 3.4.3) occur in the spider toxins CNS 2103 and ArgTX-673 respectively. These compounds (polyamine amides) are blockers of glutamate-gated ion channels. Spermine has also found recent use in our cytotoxic 9-aminoacridine conjugates, and in steroid-conjugates to form a cationic lipid for efficient gene delivery. These and other pharmacological/medicinal applications require a practical route to unsymmetrical substituted polyamines Blagbrough et al (1997) and Hsieh et al (1995). Selective monoacylation, in high yield, of polyamines such as spermine is a synthetic challenge. Here, we report a practical, one-pot synthesis of N^1-N^2-N^3-tributoxycarbonylspermine. Spermine was selectively protected on one of the primary amines by reaction with trifluoroethyl acetate in methanol at -78 °C (1 h then to 0 °C over 1 h), to afford the mono-trifluoroacetamide (TFA) derivative. Immediately, in this solution, the remaining three amino functional groups were BOC protected with di-*tert*-butyl dicarbonate (0 °C to 25 °C over 1 h). The TFA protecting group was then cleaved by increasing the pH of the mixture to 11, with conc. aqueous ammonia, stirring (25 °C, 15 h), to afford (after flash chromatography over silica gel) N^1-N^2-N^3-tributoxycarbonylspermine in a 50% yield from a convenient, one-pot reaction. This practical experimental protocol is a significant improvement over existing procedures for the preparation of this synthetically important unsymmetrically protected polyamine.



We thank Celltech Therapeutics and EPSRC for a CASE studentship (to A.J.G.). We thank Dr. M.A.W. Eaton (Celltech Therapeutics) for his interest in these studies.

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181 SYNTHESIS AND ANTIMICROBIAL PROPERTIES OF SOME NOVEL RHODANINE DERIVATIVES

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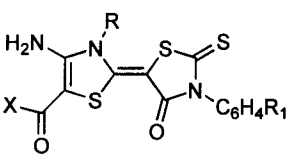
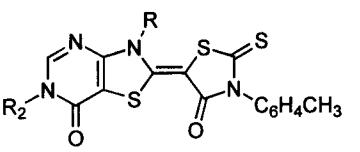
Novel compounds have been designed based on the biocidal activities of rhodanines (Das et al 1990), dithiazoles (Lakhan & Singh 1991) and 4,5-dipyrimidines (Nagarhara et al 1990). Our goal was to synthesize compounds with various rhodanine moieties, with the intention of increasing the bactericidal efficiency either by rapid cell-wall penetration or increased synergism.
Two series of compounds (I and II) were prepared by reacting the appropriate thiazole-2 (3H)-thiones or thiazolo[4,5-d]pyrimidines with dimethylsulphate in boiling acetonitrile to form the 2-methylthiazolium salt, followed by reaction with rhodanines in the presence of triethylamine (Gewald 1966; Gewald et al 1981).
Those new compounds with structures listed opposite showed significant antifungal potency (MIC against *A. niger* and *P. sp* of better than 25 μg mL^{-1}) worthy of further investigation.



Series I	R	R_1	X
	Allyl	CH_3	NH_2
	C_6H_5	Cl	NH_2
	C_6H_5	CH_3	OCH_3



Series II	R	R_2
	C_6H_5	$N{=}CHC_6H_4OCH_3$
	Allyl	H

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182 SYNTHESIS AND EVALUATION OF CYSTEAMINE ANALOGUES

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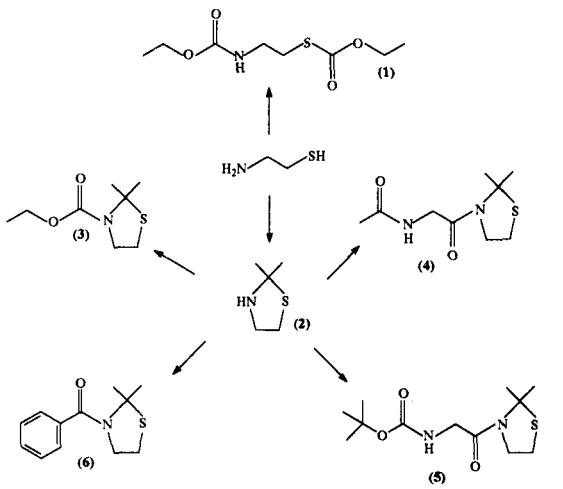
Cystinosis is a genetic condition characterised by deficiencies in the metabolism of the amino acid cysteine (Gahl *et al* 1989, Gahl 1986). This results in an accumulation of cysteine in the body, particularly in the eyes and kidneys. Patients suffering from cystinosis fail to thrive and usually do not survive beyond their teenage years (Gretz *et al* 1983). Current treatment is by adminstration of cysteamine base $HSCH_2CH_2NH_2$ or one of its salts (Yudkoff *et al* 1981). Patient compliance is very poor due to the extremely unpleasant taste and smell of cysteamine (Schneider *et al* 1995). We have synthesized a number of derivatives of cysteamine in an attempt to produce compounds with a more palatable taste and less offensive smell.
Cysteamine was reacted initially with ethylchloroformate to produce the carbamate/thiocarbonate derivative **(1)**, in good yield.
Reaction of cysteamine with acetone, under reflux, produced the dimethylthiazolidine **(2)**. This compound was further reacted with ethyl chloroformate, N-acetyl glycine and N'BOC glycine to produce compounds **(3)**, **(4)** and **(5)** respectively. Finally, compound **(2)** was reacted with benzoyl chloride to yield **(6)**. All synthesized compounds were purified and characterised by mass spectrometry, ir and nmr spectroscopy.
The release of cysteamine from the synthesized compounds was then investigated. 200 mg of each compound was added to 0.1M HCl and incubated at 37°C for up to one hour, aliquots of solution were removed at various time intervals and extracted with ethyl acetate, both phases were then chromatographed on silica gel G plates using dichloromethane 95 : methanol 5 as the developing solvent and visualized with iodine vapour. Unexpectedly, the ethylcarbamate/thiocarbonate group was relatively stable to acid hydrolysis under these conditions. The dimethylthiazolidine, however, represents an ideal cysteamine derivative as it is easily synthesized and produces cysteamine readily on contact with acidic aqueous media.



Gahl, W.A., Renlund, M., Thoene, D.I.G., (1989) Metabolic Basis of Inherited Diseases 6th edition 2619-2647
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183 SYNTHESIS AND PHARMACOLOGICAL ACTIVITY OF 3-OXYARYLRHODANINES

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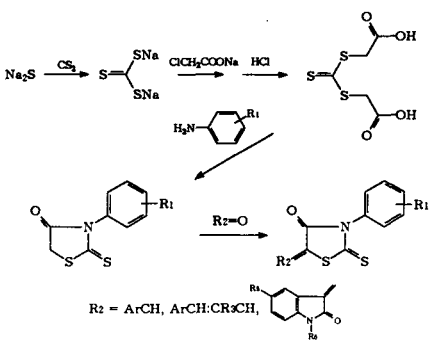
High biological activity has been reported for the derivatives of rhodanine (2-thionthiazolidone-4). Our researches with 3-(1-phenyl-2,3-dimethylpyrazolone-5-yl-4) rhodanines (Lesyk et al 1996) revealed compounds of low toxicity and high anti-inflammatory activity, confirming the utility of this approach. We have now synthesized a series of 3-oxyarylrhodanines using the Golmberg reaction for condensation of thiocarbonyl-bis-thioglycolic acid with *p*-phenetidine, and *o*- *m*- and *p*-aminophenols Scheme 1).
All structures were confirmed by UV and ^{3}H NMR spectroscopy.

Scheme 1



Anti-inflammatory activity was determined using formalin-induced paw oedema in the rat (Trinus et al 1975). Anti-exudative activity was compared with that of established anti-inflammatory compounds.
The majority of our synthesized compounds showed anti-exudative activity in the range 17-75% in comparison with diclofenac sodium (50%), phenylbutazone (40%), aspirin (40%) and acetominophen (4%). As the synthesized compounds are also structurally related to the choleretic drugs, cyclovalone and osalmidum, we also studied their anti-choleretic activity in the acute fistula-free method of Litvinchuk et al (1979). A group of substances was found with a greater choleretic activity than the etalonic drug, Flaminum. The acute toxicity (LD50) for the new compounds ranged from 330 to 3000 mg kg^{-1}.
Structure-activity studies suggested that derivatives of 3-(3-oxyphenyl) rhodanine had the highest anti-inflammatory and choleretic activity, with the arylidenic component at position 5 being important.
We suggest that the new compounds offer the opportunity to develop new compounds with favourable separation of anti-exudative, choleretic and toxic properties.

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Litvinchuk, M. D. et al (1979) Pharmakol. Toxicol. 6: 674-676 (in Russian)
Trinus et al (1975) In: Non-steroidal Anti-inflammatory Drugs, Zdorov'ya, Kyiv, 239p (in Russian)

184 SYNTHESIS OF SCHUMANNIFICINE AND DEVELOPMENT OF NOVEL ANALOGUES FOR ANTI-HIV TESTING

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Dr K. Jones, King's College London Dept of Chemistry, The Strand, London

The anti-HIV activity of *Schumanniophyton magnificum* has been attributed to the chromone alkaloids [Houghton (1994)], of which schumannificine **1** is the most potent.

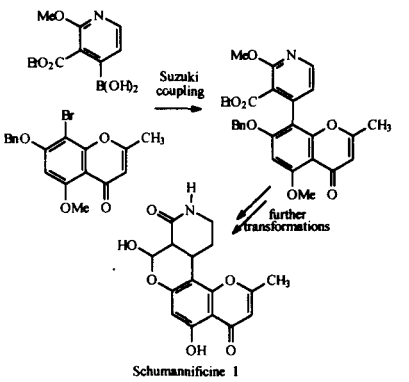
Schumannificine exhibits an activity against HIV-infected cells *in vitro* 1000 times less than that of AZT. Schumannificine is therefore not therapeutically useful but is a worthy lead compound in the development of novel anti-HIV agents.

Semi-synthetic derivatives did not have a greater anti-HIV activity than the parent compound. A total synthesis of schumannificine is thus desired which can be modified to generate a range of related compounds for testing. SARs would direct future synthesis towards targets with a greater selectivity and potency.

Retrosynthetic analysis of schumannificine reveals that it could be constructed by linking two suitably prepared fragments: a chromone and a pyridine. The fragments must first be synthesised, elaborated and protected so that they will link in the desired way.

A mild cross-coupling reaction known as the Suzuki coupling is indicated for the present case. This generic reaction uses palladium catalysis to link an aryl bromide to an aryl boronic acid. The way in which it might be used in the synthesis of schumannificine is shown below.

The coupling product would undergo further transformations to yield the target molecule.



Schumannificine 1

The bromochromone fragment has been successfully synthesised and characterised. Investigations have begun into the Suzuki reaction between the bromochromone fragment and benzene boronic acid.

Campi, E.M. *et al.* (1994); *J. Chem. Soc., Chem. Comm.*, 2395
Houghton, P.J. *et al.* (1994); *Antiviral Research*, 25, 235
Kelly, T.R. & Kim, M.H. (1992); *J. Org. Chem.*, 57, 1593

185 4-HYDROXYLATION OF RETINOIC ACID: IN-VITRO INHIBITION BY SOME CYTOCHROME P450 LIGANDS

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Retinoic acid (RA) plays a crucial role in the growth and differentiation of epithelial tissues. The biological efficacy of RA is greatly reduced by its rapid metabolism to inactive products (Napoli 1996). A major route is a P450-mediated 4-hydroxylation eventually leading to the formation of 4-keto RA and more polar metabolites (Van Wauwe et al 1992). Inhibition of C-4 hydroxylation by appropriate P450 inhibitors would be expected to slow down RA metabolism which would result in higher and more sustained tissue levels of RA. In theory, such P450 inhibitors would be capable of generating RA mimetic effects *in vivo* and this has potential therapeutic application in diseases such as cancer. In the present study, some established P450 inhibitors together with some of their analogues were screened as inhibitors of RA metabolism.

(11,12-^{3}H)-RA (10µl:3µM) was incubated with hepatic mocrosomes (10µl:10mg protein.ml^{-1}) from the male albino rat and NADPH (50µl:2mmol) in phosphate buffer (pH7.4), in the absence and presence of inhibitor (100µM in DMSO 10µl) for 15 min at 37°C. Following extraction into ethyl acetate, ^{3}H-RA and its oxidative metabolites were separated by reverse phase HPLC with radiochemical detection (Ahmad et al 1994). The column was a 10µm C18µ Bondapak (3.9x300mm, Millipore) and the mobile phase was acetonitrile:water:formic acid (75:25:0.05v/v) containing 10mM ammonium acetate. Metabolism was determined from the % conversion of RA into its metabolites based on AUC values. The results (Table 1) show that not all the compounds inhibited RA hepatic metabolism although they are all P450 inhibitors. We have previously observed that the P450 isozymes 1, 2 and 3A are involved in the metabolism of RA (Ahmad et al 1995). This difference of activity is most likely due to the isozyme specificity of the inhibition. The most potent inhibitor was N-benzyl-2-phenethylamine and this may serve as a useful lead for the development of more potent and selective inhibitors as potential anticancer agents.

Table 1: Effect of various compounds (100µM) on the *in vitro* hepatic metabolism of ^{3}H-retinoic acid.

Name of compound	% Inhibition*
4-Hydroxy coumarin	38.5
Coumarin	37.0
7-Methoxy coumarin	11.7
S-Warfarin	6.5
R-Warfarin	3.0
Aminoglutethimide	30.8
Nitroglutethimide	- 1.4
Benzphetamine	46.0
N-Benzyl-2-phenethylamine	85.0
Aspirin	5.6
Paracetamol	3.5
Diflunisal	2.0
Indomethacin	0
Ketoprofen	- 3.5

*Values are means of three determinations. Individual values differ from the mean by less than 3%. (-) denotes stimulation.

Ahmad, M. et al (1994) J.Pharm.Pharmacol. 46(Suppl); 1055
Ahmad, M. et al (1995) J.Pharm.Pharmacol. 47(Suppl); 1075
Napoli, J.L. (1996) FASEB J. 10; 993-1001
Van Wauwe, J. et al (1992) J.Pharmacol.Exp.Ther. 261; 773-79

186 INHIBITION OF AROMATASE ($P450_{AROM}$) BY 3-(4'-THIOPHENYL)PYRROLIDINE-2,5-DIONES

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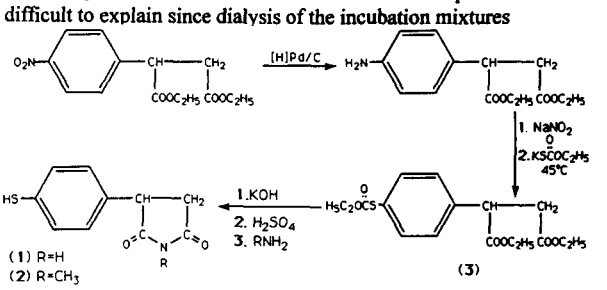
Aromatase ($P450_{AROM}$) inhibitors have been used in the treatment of hormone-dependent breast cancer[1] in post-menopausal women to prevent oestrogen biosynthesis and thus stimulus to growth of metastases. Aminoglutethimide (AG) used clinically is being replaced by more potent, specific reversible and irreversible inhibitors free of the undesirable effects of AG on cortisol production (due to CSCC inhibition) and the CNS[2]. 17β-Hydroxy-10β-mercaptoestra-4-ene-3-one and 19-mercapto-androst-4-ene-3,17-dione are mechanism-based (irreversible) inactivators of the enzyme[3]. Accordingly, we have synthesised the 4-mercapto analogues (**1**) and (**2**) (Scheme 1) of the reversible, selective inhibitor 3-(4'-aminophenyl)pyrrolidine-2,5-dione[4] as potential mechanism-based inactivators of the enzyme and evaluated them as inhibitors of $P450_{AROM}$ and CSCC ($P450_{SCC}$). $P450_{AROM}$: Incubation mixtures (0.5ml) in triplicate, containing either [1β,2β-^{3}H]-androstenedione (0.6μM) or [1β,2β-^{3}H]-testosterone (1.5μM), NADPH-generating system (50μl) and inhibitor (100μM) in ethanol (10μl) were warmed to 37°C and then placental microsomes (0.5mgml^{-1}, 25μl) added. After 6 min incubation an aliquot (300μl) was added to activated charcoal (1%, 900μl), mixed thoroughly for 15 min and then centrifuged (3000xg) for 20 min. Aliquots (500μl) of the supernatant were dispersed in scintillation fluid ('Instagel') and counted for ^{3}H. $P450_{SCC}$: [^{14}C]-cholesterol (24μM) and bovine adrenal enzyme (10,000xg fraction, 0.7mgml^{-1}) were used. After 30 min aliquots (500μl) were added to alkaline buffer pH 9.5 (1.7 ml) extracted with $CHCl_3$ and centrifuged Aliquots (500μl) of the supernatant were added to alumina, centrifuged (2000xg) and aliquots (1 ml) counted in the usual manner. Pre-incubation of the inhibitors with $P450_{AROM}$ and NADPH followed by removal of the inhibitor with charcoal and then assay revealed that the inhibition was time-dependent. This is difficult to explain since dialysis of the incubation mixtures



Scheme (1) Synthesis of the 3-(4'-thiophenyl)pyrrolidine-2,5-diones.

regenerated enzyme activity in accordance with reversible inhibition. Compounds (**1**), (**2**) and the intermediate xanthate (**3**) inhibited $P450_{AROM}$ by 49, 12 and 55% respectively (androstenedione) and 54, 50 and 63% (testosterone) indicating that **1** and **3** are comparable in potency to AG (85 and 84%). However although **1** (24%) and **3** (29%) are weaker inhibitors of $P450_{SCC}$ than AG, (66%) they would not be expected to show a greater selectivity on a dose-to-dose basis over AG towards interference with cortisol production.

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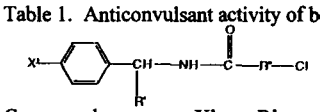
187 SYNTHESIS AND PHARMACOLOGICAL EVALUATION OF SOME ACYLATED CHLOROPROPIONAMIDES AS POTENTIAL ANTICONVULSANT AGENTS

I.P.S. Sehmbhi, M. Ahmadi, H.J. Smith and P.J. Nicholls, Welsh School of Pharmacy, University of Wales, Cardiff

Although no longer marketed as an anti-epileptic, beclamide (**I**; N-benzyl-3-chloropropionamide, Table 1) remains of interest because of its tranquillizing and anti-aggressive effects. However, the drug possesses a short $t_{0.5}$ (3h) and is of low potency. Greater than 90% of **I** is metabolically cleared, the main pathways being 4-hydroxylation of the aromatic ring and C-oxidation at the CH_2 of the benzyl and propionamide moieties (Nicholls et al 1979). The present study prepared beclamide derivatives with a view to increasing potency and improving pharmacokinetics by modifying sites of metabolism on **I**. Both aryl- and side chain-substituted derivatives of **I** were synthesised by the Schotten Baumann method, benzylamine or a substituted derivative being reacted with an appropriate ω-halo acid chloride. The ability of the compounds to protect against maximal electroshock-induced seizure (MES) was examined in male mice 0.5h following an oral dose equivalent to 400mgI/kg. Seizures were scored according to a numerical scale and results were expressed as % score of control animals receiving dosing vehicle (1% carboxymethyl cellulose) before MES.

The results presented in Table 1 show that, apart from **II** (equivalent activity to **I**) and **IX** (slightly more potent than **I**), none of the derivatives was more potent than **I**. Indeed, several compounds (**IV, V, X, XI**) were notably less active than **I**. Derivatives **II - VII** were 4-substituted on the aromatic ring, a site of oxidative metabolism accounting for ≈ 30% metabolism of **I**. It was therefore surprising that the fluoroderivative (**II**) was not more active. However, log P_c for **II** (1.435) was only marginally higher than that for **I** (1.292). Another major metabolic route for **I** is oxidation of the benzylic CH_2 resulting in benzoic acid formation. However, CH_3 substitution at this locus (**VIII**) was ineffective in increasing anticonvulsant potency. The methylene chain of the propionamide moiety is a site of oxidation but not of scission. However, modification of this side-chain in compounds **X-XII** was unable to enhance potency. A similar outcome was observed for **XIII** where modification at both X' and R" sites had been made. It was therefore of interest that the simplest compound of the series (**IX**) was the most active.

Table 1. Anticonvulsant activity of beclamide and derivatives



Compound	X'	R'	R"	% Seizure score*
I	H	H	$(CH_2)_2$	43
II	F	H	$(CH_2)_2$	40
III	Cl	H	$(CH_2)_2$	48
IV	Br	H	$(CH_2)_2$	73
V	NO_2	H	$(CH_2)_2$	62
VI	NH_2	H	$(CH_2)_2$	53
VII	CH_3O	H	$(CH_2)_2$	58
VIII	H	CH_3	$(CH_2)_2$	58
IX	H	H	CH_2	37
X	H	H	$(CH_2)_3$	82
XI	H	H	$(CH_2)_4$	68
XII	H	H	$C(CH_3)_2$	59
XIII	Cl	H	$C(CH_3)_2$	54

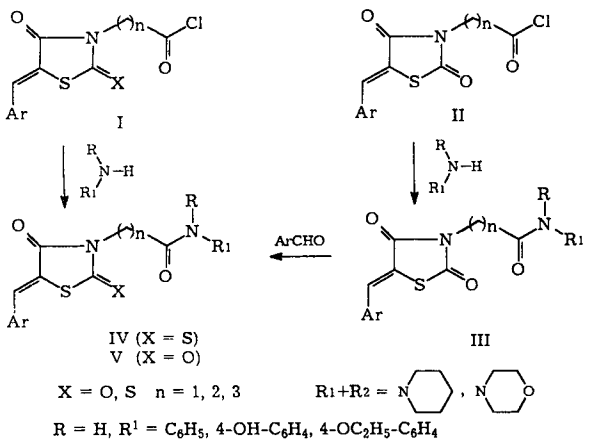
*values are means (n=6); individual values <15% from mean

Nicholls, P.J. et al (1979) Xenobiotica 9: 129-140

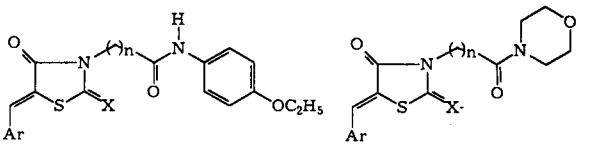
188 SYNTHESIS AND ANTI-INFLAMMATORY EFFECT OF AMIDES OF 4-THIAZOLIDONE-3-CARBOXYLIC ACIDS

V. Horishny, Department of Pharmaceutical, Organic and Bioorganic Chemistry, Medical University, Pekarska 69, 290010 Lviv-10, Ukraine

In the search for analgetic, anti-pyretic, and anti-inflammatory drugs, we have synthesized amides of 5-arylidenethiazolidine-2-thione-4-one-3-carboxylic (**4**) and 5-arylidenethiazolidine-2,4-dione-3-carboxylic (**5**) acids by reaction of acid chlorides with primary and secondary amines (Horishny et al 1990, 1997). Compound **5** was also obtained by condensation of aromatic aldehydes with the corresponding compounds unsubstituted at position 5.



Anti-inflammatory activity was determined using formalin-induced paw oedema in the rat (Horishny et al 1995).
Two series of compounds were identified which showed promise as non-steroidal anti-inflammatory compounds:



Horishny, V. Ya. et al (1990) Farm. Zhurnal 5: 40-43 (in Ukrainian)
Horishny, V. Ya. et al (1997) Sci. Pharm. 65: 91
Horishny, V. Ya. et al (1995) Farm. Zhurnal 4: 50-53 (in Ukrainian)

189 9-AMINOACRIDINE-SPERMINE CONJUGATES ARE CYTOTOXIC AGAINST MURINE MELANOMA CELLS

Simon Carrington, Mousa A. Qarawi, Stephen H. Moss, Colin W. Pouton and Ian S. Blagbrough, School of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

Recently, we reported our studies with compounds containing anthracene or acridine covalently linked, at position 9, through an amide bond to spermine. These conjugates have bifunctional modes of DNA binding (Carrington et al 1996) and are cytotoxic against B16 murine melanoma cells. We now report novel conjugates of acridine N-alkylated at position-9 by spermine (**1**) or by spermine linked to either a 5-aminovaleric acid (**2**, **3**) or an aniline (**4-6**) derived spacer. These should confer a region of some flexibility between the acridine and the spermine, allowing the two binding regions to optimise their interactions. In order to minimise side-reactions, spermine was protected on three amine functional groups with benzyloxycarbonyl groups leaving one free terminal amine. Conjugation reactions were carried out using DCC as the coupling reagent together with HOBt (cat.), and alkylations onto acridine were achieved by the displacement of phenoxide from 9-phenoxyacridine or chloride from 9-chloroacridine. Deprotection was achieved by hydrogenolysis or by action of trimethylsilyl iodide. The methoxy substituted aniline (**6**) was synthesised from spermine which had been protected with one t-butyloxycarbonyl group on a primary amine, which was then used in the conjugation reactions as before and was deprotected with trifluoroacetic acid.

The conjugates were assayed on B16 murine melanoma cells in the tetrazolium microtitre plate (MTT) assay as described by Mosmann (1983). Figure 1 shows the calculated mean EC50 values from three independent assays with six replicates in each assay. The most active conjugate was (**2**) with one 5-aminovaleric acid spacer showing an EC50 of an order of magnitude better than any of our previously reported spermine conjugates (Carrington et al 1996).

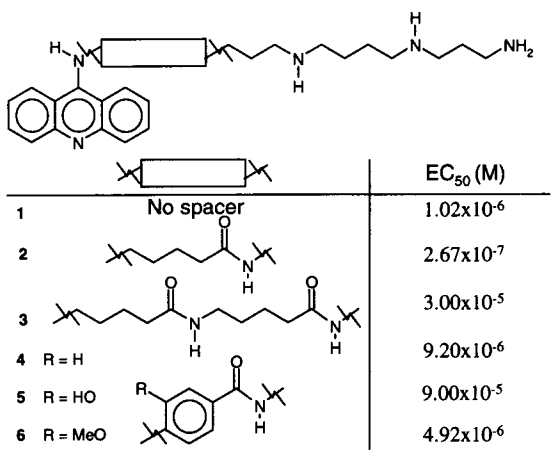


Figure 1. Structures and activities of synthetic spermine-9-acridine target compounds

We acknowledge the financial support of BTG and the School of Pharmacy and Pharmacology, University of Bath, for studentships (to SC and MAQ).

Carrington, S., Qarawi, M. A., Blagbrough, I. S., Moss, S. H., Pouton, C. W. (1996) Pharmaceutical Sciences 2: 25-27
Mossman, T. (1983) J. Immunol. Methods 65: 55-63

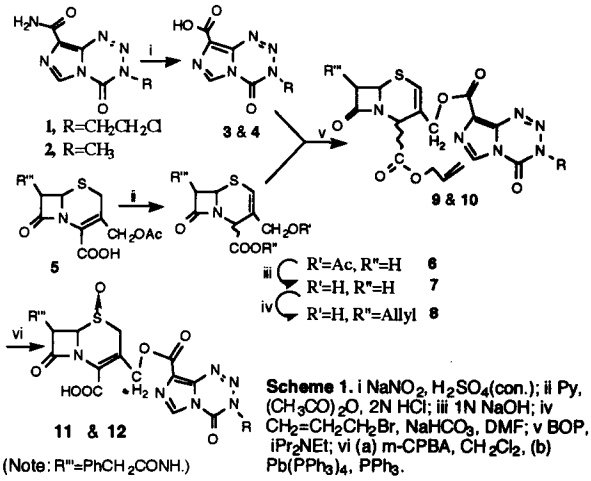
190 SYNTHESES OF CONJUGATED ESTERS OF ANTITUMOUR DRUGS MITOZOLOMIDE AND TEMOZOLOMIDE WITH CEPHALOSPORIN AS ENZYME ACTIVABLE PRODRUGS FOR ANTIBODY DIRECTED ENZYME PRODRUG THERAPY

Linxiang Zhao[a], Yongfeng Wang[a*], David C. Billington[a] & Zhizhong Ji[b]. [a]Department of Pharmaceutical and Biological Sciences, Aston University, Aston Triangle, Birmingham B4 7ET UK. [b]Pharmaceutical Institute, Shenyang Pharmaceutical University, Shenyang City, P. R. China

In recent years, antibody directed enzyme prodrug therapy (ADEPT) which aims to selectively deliver drugs to tumour sites has emerged to be a promising chemotherapeutic treatment, implying the magic bullet will have come of age (Sherwood, 1996). In on-going studies of imidazotetrazinone antitumour drugs, discovered and developed in Aston (Hickman et al, 1985), enormous efforts have been made to minimize the toxicity, to improve the delivery and to enhance the biological efficacy of the drugs, after the most promising candidate mitozolomide **1** was precluded from further clinical studies due to its concomitant side effects revealed in phase I clinical trial (Fodstad, 1985). Discovery of a good patients' tolerance to temozolomide **2**, a lower homologue of **1**, made a great impact on the development of this field (Newlands, E.S. 1985). As a result, temozolomide, one of the few compounds effective against malignant melanoma and mycosis fungoides, was licensed to Schering-Plough as a anti-brain tumour drug for world-wide exploitation. Inspired by potential of ADEPT, we designed a group of imidazotetrazinone-cephalosporin prodrugs in order to enhance the intrinsic activity of **1** and **2** against brain tumour and to achieve targeting delivery and high efficacy. We report here syntheses of the first two conjugated esters of mitozolomide **1** and temozolomide **2** with cephalosporin as enzyme activable prodrugs for ADEPT.

Conversion of 8-carboxamide group of **1** and **2** into 8-carboxylic acid derivatives **3** and **4** is accomplished with sodium nitrite (**Scheme 1**), ready for integrating with cephalosporin. Hydrolysis of 3-acetoxymethyl of **5** into 3-hydroxymethyl is completed in two steps: first the isomerization of Δ^3-cephem to Δ^2 congener promoted by pyridine, then treated with NaOH. After protection of 4-carboxylic acid of **7** with allyl bromide, the product **8** reacts with **3** and **4** in the presence of Py-BOP to afford **9** and **10**, which undergoes isomerization and the deprotection to give the targets **11** and **12**.

In studies of enzymic activation of **11** and **12** using type II penicillinase from *Bacillus cereus* in pD 7.0 buffer, a clear decomposition of the cephalosporin is observed by monitoring the change of chemical shift of its 3-methylene protons by NMR. Intensive studies of enzymic activation of **11** and **12** are now being undertaken, and *in vivo* testing is planned.



Scheme 1. i $NaNO_2$, H_2SO_4(con.); ii Py, $(CH_3CO)_2O$, 2N HCl; iii 1N NaOH; iv CH_2=CH_2CH_2Br, $NaHCO_3$, DMF; v BOP, iPr_2NEt; vi (a) m-CPBA, CH_2Cl_2, (b) $Pb(PPh_3)_4$, PPh_3.

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191 MOLECULAR MODELLING STUDY OF NON-NUCLEOTIDE INHIBITORS OF THE HIV VIRAL ENZYME – REVERSE TRANSCRIPTASE

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The reverse transcriptase enzyme in the Human Immunodeficiency Virus is a heterodimer consisting of two polypeptide chains, p66 and p51 (Kohlstaedt et al 1992). X-ray structure determinations with various inhibitors (bound within the enzyme active site) have revealed striking similarities in the geometry of the binding pocket and the inhibitors (Ding & Arnold 1995). Both appear to adopt a 'broad Y shape', and as a result, a number of interactions (both hydrophobic and polar) have been proposed for a small number of inhibitors. The effectiveness of an inhibitor has been suggested to be related to its ability to mimic the broad Y shape.

The inhibitors considered todate, have been conformationally restrained three- and/or two-ring containing structures with limited degrees of flexibility. However, a number of highly flexible non-nucleotide inhibitors exist which have not been previously considered since they were thought not to be able to take up the appropriate shape. We have therefore initiated a line of research to study the conformational analysis of these inhibitors in an effort to observe any correlation between inhibitory activity and overall inhibitor shape. Furthermore, a knowledge of the conformers adopted by these types of inhibitors would be invaluable in clarifying the nature of the active site, i.e. whether the active is flexible (and therefore able to accommodate inhibitors of any shape) or rigid (i.e. whether the inhibitors are required to adopt the broad Y shape so as to mimic the active site).

All the structures of the inhibitors required for the present study were constructed within (or visualised using) Alchemy III molecular modelling software and extensive conformational analysis of the flexible parts of the inhibitors was carried out using Powersearch. In the construction of inhibitors, atoms/fragments/groups available within the Alchemy libraries were utilised. The crystal structures of the inhibitor-bound enzymes were obtained from the Brookhaven Protein databank and simplified (involving the extraction of the ligand only) using Rasmol, version 2.6. The low energy conformers of the flexible inhibitors were all superimposed onto the crystal structure of the most rigid inhibitor bound to the polymerase active site, namely Thioazoloidine, using three or more points.

The initial results of the study suggest that the flexible inhibitors, previously presumed not to take up a broad Y shape, can indeed adopt such a shape without any large increase in energy. Furthermore, from the consideration of these flexible compounds, we hypothesise that the full Y shape need not be adopted, i.e. any part of the overall Y shape may be mimicked, e.g. the coumarin based inhibitors which are found to be of a rigid planar structure and therefore do not take up the Y shape, were found to produce good overlap with the left hand side of the Y shape. We also propose that another factor may be involved in determining the overall inhibitory activity, i.e. logP is considered to be a factor with the most potent inhibitors possessing calculated logP values of ~2.5.

Ding, J. & Arnold, E. (1995) Nat. Struct. Biol. 2:407-415
Kohlstaedt, L.A. et al (1992) Science 256:1783-1790

192 MOLECULAR MODELLING APPROACH TO THE STUDY OF THE ENZYME LANOSTEROL 14α-DEMETHYLASE – A THEORETICAL APPROACH

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Lanosterol 14α-demethylase ($P\text{-}450_{14DM}$), a cytochrome P-450 enzyme, is responsible for the first stage in the biosynthesis of cholesterol from lanosterol. Although extensively studied, no overall conclusions have been drawn on the nature of the active site of this enzyme or the attacking P-450 Fe species involved in the mechanism of C-C bond cleavage. It is postulated that a ferroxy radical (Fe^{IV}-O•), a peroxy radical (Fe-O-O) or a mixture of both may be involved within the general P-450 family of enzymes (Akhtar & Wright 1991). We have previously studied the mechanism of a related P-450 enzyme, Aromatase, and concluded that the oxidative steps in the aromatisation of the A ring of androstenedione involve only a Fe^{IV}-O• radical (Ahmed & Davis 1995).

Using the previously reported 'substrate-haem complex' approach (Ahmed & Davis 1995; Ahmed 1995) and the molecular modelling software Alchemy III, two representations of the $P\text{-}450_{14DM}$ active site (involving both the ferroxy and the peroxy attacking species) and the intermediates of the presumed mechanism (Akhtar & Wright 1991) were constructed. It is postulated that H abstraction of the C(15)-βH is an important step, thus the intermediate in a proposed mechanism was fitted onto the complexes and the OH to Fe^{IV}-O• distances determined. From a comparison of the two complexes, we postulate that the use of a mixture of two ferroxy radicals (for the two hydroxylation steps) and a peroxy radical (for the C-C bond cleavage) is unlikely as the steroid backbone would be required to move in an approximate vertical direction (2.7Å) (so as to position itself within attacking distance of the ferroxy or the peroxy attacking oxygen radical) and which therefore would require the existence of numerous hydrogen bonding sites - an observation which has not been reported for the general P-450 family of enzymes. The use of a purely peroxy attacking species is also considered unlikely as the final step in the mechanism postulates H abstraction from the C(20)-OH, and which is observed to be too far away from the appropriate oxygen (2.5Å). The use of the Fe^{IV}-O• appears to be the probable attacking species since both C(14)-CH_3 and C(15)-βH groups are positioned close to the ferroxy oxygen for H abstraction (1.3Å). The results of this and previous studies (Ahmed 1995) appear to support the hypothesis that an overall mechanism exists for the P-450 family of enzymes (Wright & Akhtar 1990).

It has been shown that inhibitors of other related cytochrome P-450 enzymes, containing hetero atoms, bond directly to the P-450 haem (Type II inhibition). In the present study, we presumed that inhibitors of $P\text{-}450_{14DM}$ undergo a similar process, and we thus bonded the inhibitors directly to the Fe so as to mimic this process (resulting in an inhibitor-substrate-haem complex). We also presume that the Fe-inhibitor hetero atom bond will undergo 'free rotation' in an effort to stabilise the enzyme-inhibitor complex (through polar-polar interaction with the remainder of the active site), as such we believe that interaction between the inhibitor's polar group and the part of the active site which would normally bind the C(3)-βOH of lanosterol is important and that the distance is an approximate indication of the strength of the interaction and therefore of the stability of the Michaelis complex. Using the inhibitor-substrate-haem complex we have studied the mode of binding of some azole based antifungal compounds such as Bifonazole, Ketoconazole and Miconazole.

Ahmed, S. (1995) Bioorg. Med. Chem. Lett. 5:2795-2800
Ahmed, S., Davis, P.J. (1995) Bioorg. Med. Chem. Lett., 5:1673-1678
Ahmed, S., Davis, P.J. (1995) Bioorg. Med. Chem. Lett. 5:2789-2794
Akhtar, M., Wright, J.N. (1991) Nat. Prod. Rep. 8:527-551
Wright, J.N., Akhtar, M. (1990) Steroids 55:142-151

193 DERIVATION OF A REPRESENTATION OF THE 5α-REDUCTASE ENZYME (5AR) ACTIVE SITE FROM THE CONSIDERATION OF THE REACTION MECHANISM

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In the treatment of benign prostatic hyperplasia (BPH), extensive research has been undertaken to produce compounds which are both potent and selective inhibitors of 5α-reductase (5AR), the enzyme responsible for the conversion of testosterone (T) to the more potent androgen dihydrotestosterone (DHT) (Rasmusson et al 1984). To date, derivatives of 4- and 6-azasteroids have proved to be potent inhibitors of 5AR, for example 17β-N,N-diethylcarbamoyl-4-methyl-4aza-5α-androstan-3-one (4-MA). Research into novel non-steroidal inhibitors is still however in its early stages, but may be assisted by clearer understanding of the active sites of both isozymes of 5AR (type I and type II), however, the specific nature of the active site of this enzyme (and more specifically the two isozymes) is not known. In an effort to obtain further information regarding 5AR, we have initiated a series of theoretical studies to produce a simplified representation of the active site involving the consideration of the proposed mechanism of 5AR.

It is proposed that testosterone and the nicotinamide part of NADPH are bound close to each other so as to allow transfer of hydride from the co-factor to the steroid. A transition state results where the steroid is present in its enolic form, protonation of which (by water) brings about ketonisation causing a conformational flip of the A ring of the steroid resulting in the de-binding of the product from the enzyme active site.

The molecular structures of T and NADPH were constructed and minimised (using the fastest minimisation routine available - cycles of 300 iterations were attempted until the gradient dropped below 10^{-3}) within the CACHE molecular modelling software. Conformational analysis was performed on flexible parts of the compounds in order to determine the lowest energy conformer. In determining the transition-state, the 'fastest procedure' was employed.

From the results of the molecular modelling study, we propose that the NADPH is bound (with respect to the steroid) such that the majority of the molecule is placed below and beyond the C(4), C(5) & C(6) positions of the steroid backbone, with the results that the hydride hydrogen is placed 2.5Å below the C(5) position of the steroid backbone. That the observed positioning of the NADPH moiety may be possible is supported by experimental data (Rasmusson et al 1984) where groups larger than methyl have been shown to reduce the inhibitory activity of 5AR inhibitors. We hypothesise that the use of the larger alkyl groups results in steric interaction between the butyl chain and the NADPH moiety resulting in the destabilisation of the enzyme-inhibitor complex, thereby reducing the potency [the C(4) H atom of T was found to be 3.4 Å away from the main body of NADPH]. Also, from a study of the possible orientations adopted by the NADPH molecule (with respect to the steroid), the difference between type I and II isozymes may be due to the different orientation (or mode of binding) of the reducing agent, i.e. we observe that two low energy orientations exist which allows the NADPH molecule to approach the C(5) from below the steroidal plane.

In conclusion, we believe that through the consideration of the mechanism, it has been possible to produce a simple representation of the 5AR active site which can be utilised in drug design discovery.

Rasmusson, G.H. et al (1984) J. Med. Chem. 27:1690-1701

194 THE MECHANISM OF THE CYTOCHROME P450 ENZYME CHOLESTEROL SIDE CHAIN CLEAVAGE (CSCC) – A MOLECULAR MODELLING APPROACH

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The P-450 enzyme Cholesterol Side Chain Cleavage (CSCC) mediates the conversion of cholesterol to pregnenolone and isocapraldehyde (Ortiz de Montellano 1986). Although extensively studied, no overall conclusions have been drawn on the nature of the attacking P-450 Fe species involved in the C-C bond cleavage. It is postulated that a ferroxy radical (Fe^{IV}-O•), a peroxy radical (Fe^{III}-O-O) or a mixture of both may be involved within the general P-450 family of enzymes. We have previously studied the mechanism of Aromatase (AR), a related P-450 enzyme, from a molecular modelling point of view and concluded that the three oxidative steps in aromatisation of the A ring of androstenedione involve only a Fe^{IV}-O• radical. In the design of inhibitors of other cytochrome P-450 enzymes, such as AR, it is important to understand the mechanism of CSCC since AR inhibitors are required which do not inhibit it, the CSCC inhibition would be that the biosynthesis of other biomolecules, such as the mineralocorticoids, would be affected.

Using the previously reported 'substrate-haem complex' approach (Ahmed & Davis 1995; Ahmed 1995) and the molecular modelling software Alchemy III, two representations of the CSCC active site (involving both the ferroxy and the peroxy attacking species) and the intermediates of the presumed mechanism (Wright & Akhtar 1990) were constructed. It is postulated that H abstraction from C(20)-OH and C(22)-OH are important steps, thus the intermediates were fitted onto the complexes and the OH to Fe^{IV}-O• distances determined.

From a comparison of the two complexes, we postulate that the use of a mixture of two ferroxy radicals (for the two hydroxylation steps) and a peroxy radical (for the C-C bond cleavage) is not likely as the steroid backbone would be required to move in an approximate vertical direction (2.7Å) (so as to position itself within attacking distance of the ferroxy or the peroxy attacking oxygen radical) and which therefore would require the existence of numerous hydrogen bonding sites - an observation which has not been reported for the general P-450 family of enzymes. The use of a purely peroxy attacking species is also considered unlikely as the final step in the mechanism postulates H abstraction from the C(20)-OH, which is observed to be too far away from the appropriate oxygen (3.5Å). The use of the Fe^{IV}-O• appears to be the probable attacking species since both C(20)-OH and C(22)-OH groups are positioned close to the ferroxy oxygen for H abstraction (1.2Å). The results of this and previous studies (Ahmed 1995) appear to support the hypothesis that an overall mechanism exists for the P-450 family of enzymes (Wright & Akhtar 1990) and that the attacking Fe species, in all steps, is probably a ferroxy radical.

Ahmed, S. (1995) Bioorg. Med. Chem. Lett. 5:2795-2800
Ahmed, S, Davis, P.J. (1995) Bioorg. Med. Chem. Lett. 5:1673-1678
Ortiz de Montellano, P. R., in Cytochrome P-450: Structure Mechanism and Biochemistry, ed. Ortiz de Montellano, P. R., Plenum Press, New York and London, 1986
Wright, J.N., Akhtar, M. (1990) Steroids 55:142-151

195 MOLECULAR MODELLING APPROACH TO THE STUDY OF THE MECHANISM OF THE P-450 ENZYME 17α-HYDROXYLASE/17,20-LYASE

Sabbir Ahmed, School of Applied Chemistry, Kingston University, Penrhyn Road, Kingston upon Thames, Surrey, KT1 2EE, UK.

The P-450 enzyme 17α-hydroxylase/17,20-lyase ($P450_{17\alpha}$) mediates the conversion of progesterone to 17α-hydroxyprogesterone which undergoes C-C lyase to androstenedione (Nakajin & Hall 1983). Although extensively studied, the mechanism of C-C bond cleavage (in particular the nature of the attacking P-450 haem species) is not clear. It is postulated that a ferroxy radical (Fe^{IV}-O•) or a peroxy radical (Fe^{III}-O-O) or a mixture of both may be involved (Robichaud et al 1994).

Using the substrate-haem complex approach previously reported for the 17α-hydroxylase component of this enzyme (Ahmed 1995), and the molecular modelling software Alchemy III, we constructed representations of the 17,20-lyase component of the $P450_{17\alpha}$ active site (both the ferroxy and the peroxy) and the postulated intermediates in the presumed mechanism (Robichaud et al 1994). In the mechanism, H abstraction from the C(17)-OH was thought to be an important step, therefore the optimised and minimised intermediates were fitted onto the substrate-haem complex and the distances between the OH and Fe-O measured.

From a comparison of the ferroxy and peroxy complexes for each component, we postulate that the use of a mixture of ferroxy and a peroxy radical is unlikely since the steroid backbone would be required to move in an approximate vertical direction (2.7Å), therefore requiring the existence of numerous hydrogen bonding sites at the active site - an observation which has not been reported for the general P-450 family of enzymes. The use of a purely peroxy attacking species has been suggested by Robichaud et al (1994), however this appears to be unlikely since in this report, the C(17)-OH group is observed to be too far away for H abstraction to take place (3.4Å). The use of a Fe^{IV}-O• appears to be the most probable as this results in distances which are considered appropriate for H abstraction from the C(17)-OH (1.2Å). Also, we postulate that the numerous products observed from the action of this enzyme on progesterone can be rationalised on the basis of loose binding, i.e. several positions of the steroid backbone being presented to the Fe^{IV}-O•. Thus, in the formation of androsta-5, 16-dien-3β-ol, we postulate that the 16α-hydrogen is positioned close to the Fe^{IV}-O• resulting in C(16)-α-H abstraction and the eventual C=C formation and acetate. The results of this and previous studies (Ahmed & Davis 1995) appear to support the hypothesis that an overall mechanism exists for the P-450 family of enzymes (Wright & Akhtar 1990) and that the attacking Fe species, in all steps, appears to be the ferroxy radical.

Ahmed, S. (1995) Bioorg. Med. Chem. Lett. 5:2795-280
Ahmed, S., Davis, P.J. (1995) Bioorg. Med. Chem. Lett. 5:2789-2794
Nakajin, Hall, P.F. (1983) J. Steroid Biochem. 19:1345-134
Robichaud, P. et al (1994) J. Chem. Comm. 12:1501-1503
Wright, J.N., Akhtar, M. (1990) Steroids 55:142-151

196 STRUCTURE-ACTIVITY RELATIONSHIP STUDIES ON PROPAFENONE-TYPE MDR-MODULATORS USING THE TOPLISS APPROACH

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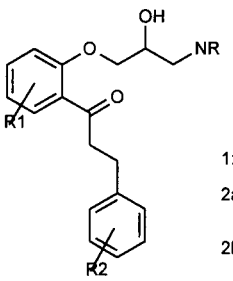
Development of unspecific mechanisms of resistance (MDR) remains a serious problem in cancer therapy. One of the predominant mechanisms responsible for MDR is overexpression of P-glycoprotein (PGP). PGP is a membrane bound, ATP-dependent transport protein which pumps a wide variety of structurally and functionally diverse cytotoxins out of tumour cells.

We recently identified propafenone analogous compounds (**1**) as beeing able to inhibit PGP-mediated drug transport. Following our approach of ligand based drug design we synthesised a series of derivatives (**2a,b**) and tested their chemosensitising potency. The compounds were varied on both aromatic rings according to the method of Topliss et al. (1976) to get insights in the physicochemical parameters influencing receptor binding in this regions of the molecules.

For substituents on the phenylpropionyl moiety the σ parameter predominantely influences pharmacological activity. Interestingly, on the central aromatic ring neither π nor σ nor combinations of both parameters seem to play a role. Nevertheless, all compounds synthesised showed EC50 values in the nanomolar range.



1: R1, R2= H, R= n-propyl

2a: R1= 3,4-dichlor, 4-chlor,4-methyl, 4-methoxy
R2= H

2b: R1= H
R2= 3,4-dichlor, 4-chlor, 4-methyl, 4-methoxy

1, 2a, 2b

Topliss, J.G. (1977), J. Med. Chem. 20: 4-??

197 RELATIONSHIP BETWEEN STRUCTURE AND BLOOD-BRAIN BARRIER PERMEABILITY FOR 3-HYDROXYPYRIDIN-4-ONE IRON CHELATORS

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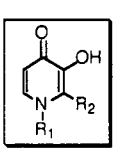
Transfusion dependent patients such as those suffering from β-thalassaemia develop a fatal secondary haemosiderosis. This iron overload is currently relieved with desferrioxamine (DFO).However, because of oral inactivity and poor pharmacokinetic behaviour, patient compliance is poor. The 3-hydroxypyridin-4-one (HPO) class of iron chelator is currently one of the main candidates for the development of orally active iron chelating alternatives to DFO [Brittenham (1992)]. The primary use of iron-chelation therapy is to reduce pathologically high levels of circulating free iron. However, if the metal chelating compounds are lipid soluble they will cross the blood-brain barrier (BBB) and will affect the level of iron and other metals in the brain, possibly leading to undesirable toxic effects. It is thus of considerable importance to investigate the relationship between chemical structure of HPOs and BBB permeability in order to identify compounds suited to particular therapeutic tasks and sites of action. The 3-hydroxypyridin-4-ones can be modified at the 1 and 2 position to give a wide variety of compounds with different physico-chemical and pharmacokinetic properties without significantly affecting the over affinity for iron (Table 1) [Dobbin et al (1993)].



Compound	R_1	R_2	Log $P_{octanol}$
CP20	CH_3	CH_3	-0.77
CP21	CH_2CH_3	CH_3	-0.31
CP94	CH_2CH_3	CH_2CH_3	0.23
CP24	$(CH_2)_3CH_3$	CH_3	0.70
CP29	$(CH_2)_4CH_3$	CH_3	1.24
CP25	$(CH_2)_5CH_3$	CH_3	1.90
CP41	$(CH_2)_5OH$	CH_3	-0.89
CP102	CH_2CH_2OH	CH_2CH_3	-0.66
CP107	$(CH_2)_4OH$	CH_2CH_3	-0.28

Table 1. Chemical structure of selected HPO ligands

A number of such HPOs have been selected to investigate the correlation between BBB permeability and Log $P_{octanol}$ of the compounds.

Preliminary studies have revealed that brain penetration is stronglydependent on lipophilicity of HPOs (Figure 1). However, replacing a terminal hydrogen with an hydroxyl group markedly reduces BBB permeability to very low levels close to the limits of detection. The apparent impermeability of N-hydroxyalkyl derivatives of HPOs into the brain suggests that lipophilicity is not the only major factor and that hydrogen bonding potential may also be a dominating factor for HPOs.

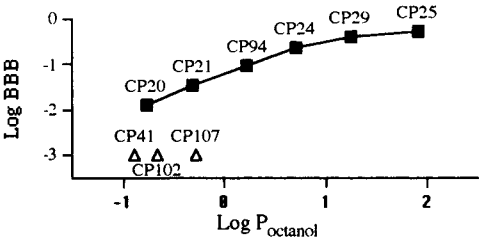


Figure 1. Correlation between brain uptake (Log BBB) of the HPO ligands and Log $P_{octanol}$ in adult Wistar rats.

In summary, there is a good relationship between BBB permeability and Log $P_{octanol}$ for N-alkyl substituted HPOs and as such this may offer a reliable predictive method for simple alkyl HPOs. Conversely, appreciable permeability of the BBB has not been observed for N-hydroxyalkyl HPOs by using the short duration vascular perfusion method adopted in this study. This is an important observation which is highly relevent to iron chelator design.

Brittenham GM (1992) Blood **80**: 569-577.
Dobbin PS, Hider RC, Hall AD, Taylor PD, Sarpong P, Porter JB, Xiao G, Van der helm D (1993) J. Med. Chem. **36**: 2448-2458.

198 AN INVESTIGATION INTO THE STRUCTURAL REQUIREMENTS OF POTENTIAL THERAPEUTIC IRON CHELATORS TO ACT AS ARTIFICIAL SIDEROPHORES

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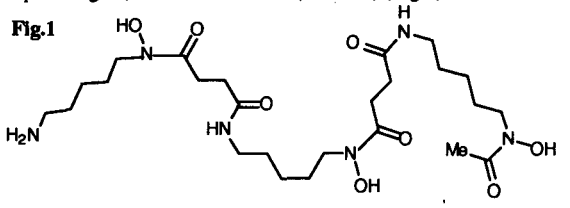
Iron is an essential element for life and plays a central role in biochemical processes by virtue of its facile redox chemistry and its high affinity of both redox states (Fe(II) and Fe(III)) for oxygen. The extreme insolubility of iron(III) under aerobic conditions and physiological pH (Ksp = 10^{-38}M) has necessitated microorganisms to develop highly elaborate systems to procure extracellular iron. To address this issue many bacteria and fungi synthesise and secrete powerful low-molecular weight (‹ 1000 Daltons) Fe(III)-selective ligands termed siderophores which facilitate the actice transport of the chelated iron into cells [Hider (1984)].
The development of orally active iron chelating agents for such conditions as transfusional iron overload (β-thalassaemia) has been driven mainly by the limitations associated with the use of the current therepeutic agent, desferrioxamine B (Desferal) (Fig.1).

Fig.1



Apart from its oral inactivity and poor pharmacokinetic behaviour, desferrioxamine B is able to donate chelated iron to pathogens thereby inducing oppurtunistic infections by such organisms such as *Yersinia enterocolitica* [Gallant et al (1986)]. Since desferrioxamine B is a natural hydroxamate-based siderophore the consequences of its therapeutic applications in inducing such infections are apparent. Since many of the receptor-mediated mechanisms for siderophore-Fe(III) chelate uptake are not specific [Winkelmann et al (1987)] it is therefore essential that novel potential therapeutic iron chelators are screened and demonstrated to be devoid of the ability to donate iron to pathogenic microorganisms. The main molecular chelating functionalities considered for incoorporation into novel structures for clinical application are catecholates (i), hydroxamates (ii), aminocarboxylates (iii), α-hydroxypyranones (iv) and α-hydroxypyridinones (v) (Fig.2).

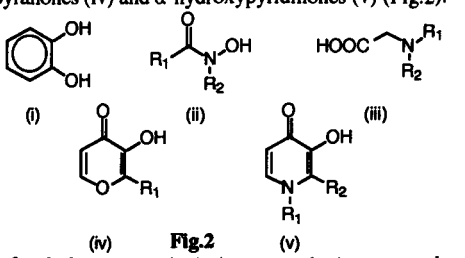


Fig.2

A range of polydentate synthetic iron-complexing agents have been synthesised and assessed for their iron-donating abilities in a growth promotion assay using mutant strains of *E. coli*. Initial findings suggest that hexadentate ligands based on the 3-hydroxy-4*(1H)*-pyranone and 3-hydroxy-4*(1H)*-pyridinone moieties are unable to supply iron utilising the receptors reserved for natural siderophores such as enterobactin, ferrichrome and ferrioxamines. However structures based on the heterocyclic unit 3-hydroxy-2*(1H)*-pyridinone retain this undesirable property and are able to utilise the enterobactin receptor. Aspects of preliminary structure-iron donation relationships will be presented

Hider RC (1984) Struct. Bond **58**: 25-87.
Gallant T, Freedman MH, Vellend H and Francomber WH (1986) N. Eng. J. Med. **314**:1643.
Winkelmann G, van der Helm D and Neilands JB (eds) (1987) in "Iron Transport in Microbes, Plants and Animals" VCH Germany.

199 PREDICTION OF CHEMICAL OXIDIZABILITIES OF ORGANIC COMPOUNDS BY QSAR TECHNIQUES

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In the generation and discovery of new and beneficial drugs, there is a need to know potential oxidizabilities of chemicals as well as many physical properties (Dearden, 1994) from the viewpoint of *in vivo* biotransformation, or metabolism. The purpose of present study is to develop a new prediction method for chemical oxygen demand (COD) from the information on molecular structures or intrinsic physical properties of chemicals.

The outline of the methodology in this study is shown in Fig. 1. The correlations between observed COD data for ca. 500 compounds of diverse structures and functionalities (Janicke, 1983) and two sets of molecular descriptors were investigated by using two tools, PLS method (Wold et al, 1984) and the neural networks (NN). For COD data, the values based on the three different oxidation methods (using permanganate (in acid solution) and dichromate/H_2SO_4 (with and without Ag^+ as a catalyst))were employed. The COD values were expressed as the percentage of theoretical COD values and used for the target or dependent variables. The NN employed for this modelling was a three-layer feed-forward network with back-propagation of errors.

For some homologous series of the compounds (e.g. hydrocarbons, halogenated hydrocarbons), the PLS approach gave statistically significant models for COD (values based on the three different measuring methods), however, this approach could not establish a single model for all studied compounds. The NN approach, which

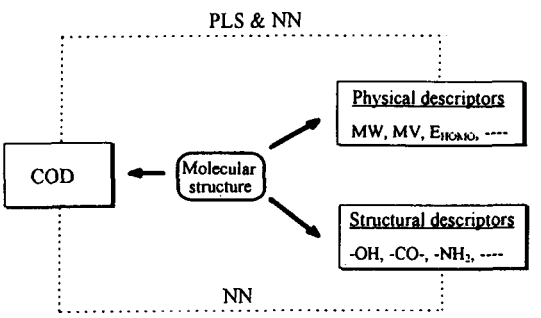

Fig. 1 Structure-property correlation scheme for COD

has an inherent ability to provide non-linear and cross product terms, gave much better results than PLS approach and could obtain global models for the three different COD data used. The predictive ability of the proposed methods were tested for the testing set compounds not included in the training data set and fairly good agreement with observed COD values was confirmed. Significant difference in adaptability of the proposed models to three different COD values was observed.

Dearden, J. C. (1994) Chemomet. Intell. Lab. Sys. 24: 77-87
Janicke, W. (1983) WaBoLu-Bericht H. 1. Dietrich Reimer Verlag, Berlin.
Wold, S. et al (1984) NATO ASI Ser. C., vol.138: 17-95

200 CLASSIFICATION ANALYSIS AND QSAR USING 2D TOPOLOGICAL DESCRIPTORS

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The two major problems with 3D QSAR/PLS are firstly the sometimes arbitrary alignment step and secondly the fact that relationships to biological activity are unlikely to be completely linear especially if one is considering efficacy or metabolic activities. With these points in mind, we have returned to our in-house 2D topologically based descriptions of molecules and used classification analysis (in the form of SIMCA) to produce QSARs with chemical/structural relevance. The SIMCA methodology published by Wold & Sjöström (1977) was described in the mid 1970s and takes a data set representing a collection of compounds from a number of classes and then derives separate principle component models to represent each class. The appropriate number of components per category is chosen using NIPALS crossvalidation and the loadings matrix indicates whether the presence/absence of a descriptor is important to a category {by the sign of the first component} and also the relative importance of a descriptor {by the magnitude}.

The 2D descriptors are those described by Kearsley et al (1996) which are in-turn derived from the atom-pair and topological-torsion descriptors of Carhart (1985) and Nilakantan (1987). These descriptors not only subdivide a molecule into its atom-type relationships but also how those relationships are modified by atomic or fragmental features such as partial charge, donor/acceptor, and hydrophobicity properties. These descriptors have been used extensively within Merck to determine a similarity metric between compounds and we believe that they do represent the major features of any compound.

SIMCA's major uses have been in process applications, or pattern recognition of analytical spectra or geological/food samples; its published applications to QSAR have been so far, very limited. These QSAR experiments all used a limited number of variables to describe their molecules and in some cases even had to go through an alignment step to generate the appropriate STERIMOL parameters (thereby including one of the problematic steps noted above).

Our work described here uses SIMCA to develop models for compound affinity in our own NMDA data set, and published sets from R.P.R. on their PDE IV inhibitors (Ashton et al (1994)) and Astra on their Calcium channel blockers (Davis et al (1994)). To illustrate the method an artificial data set of 64 halobenzenes was created and two distinct features were chosen to determine class membership. All of the compounds were correctly categorised by SIMCA and the two features chosen were easily identified by their high Discriminating and Modelling powers and their large positive loadings. Similarly the important features identified in the RPR and Astra publications (such as the presence of a 3,5-dichloro-substituted 4-pyridine in PDE and ClogP and CMR for the Ca channel data) were also identified by this procedure. In our own NMDA data set 5% of the data was kept back as a test set and the model produced from the remaining data was able to categorise the test set to a highly significant degree.

In total we believe that this combination of 2D descriptors and classification analysis is a powerful tool in identifying the important structural features within a data set. This QSAR can be used to predict the categorisation of novel compounds and hence prioritise the testing/development of compounds. This work can be applied to virtually all areas of pharmaceutical analysis and is simple enough to be applied to large data sets.

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201 QUANTITIVE STRUCTURE-PERMEABILITY RELATIONSHIPS UTILISING POLYDIMETHYL SILOXANE MEMBRANES

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An estimate of skin permeability is essential for a variety of issues including topical drug delivery and toxicity assessment. The use of *in vitro* models using excised human, or pig, skin is a potential method to provide an estimate of skin permeability, and quantitative structure-permeability relationships (QSPRs) have been developed from these (cf. Potts and Guy, 1992; Pugh et al., 1996). Recently an artificial membrane utilising polydimethylsiloxane (PDMS) has been developed and the flux through this membrane of a large number of compounds has been measured (Chen et al., 1993, 1996). In the original publications the authors developed QSPRs; however, these were for subsets of the data, and not the whole data set. This study has analysed the whole data set with the aim of using easily calculated values, and of interpreting the QSPRs mechanistically in order to justify their use as a surrogate for human skin.

Maximum steady state flux data for 269 heterogeneous compounds were taken from Chen et al (1993, 1996). For each compound 55 molecular descriptors were calculated including log P, molecular connectivities and a variety of parameters describing the hydrogen bonding ability of a compound. Statistical analysis was performed using the MINITAB statistical software.

Stepwise regression of the variables against the flux data (log J) revealed a total of 17 compounds to be outliers. Unfortunately no commonality has yet been revealed amongst these, although a number were observed to contain acid groups. After their removal the following significant QSAR was established:

$$\log J = -0.653\ HA - 0.563\ HD - 0.562\ {}^{6}\chi - 0.481$$

n=252 r=0.867 s=0.512 F=252

where n is the number of observations, r is the correlation coefficient, s is the standard error of the estimate, F is Fisher's statistic, HA and HD are the number of hydrogen bond acceptors and donors on each molecule respectively, ${}^{6}\chi$ is the sixth order path molecular connectivity (which is associated mainly with molecular size).

This equation allows for the accurate prediction of flux through a PDMS membrane and demonstrates the importance of hydrogen bonding and molecular size in passage of compounds through this membrane. However, other work has shown log P and molecular weight to be the most important physico-chemicals parameters to predict the permeability of chemicals through skin (Potts and Guy, 1992). Estimates of the skin permeability of the compounds analysed in this study were calculated using the Potts and Guy model. No correlation was found between estimated skin permeability and the flux values through the PDMS membrane. It may thus be rationalised that the mechanism of transport in PDMS is different from that in skin.

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Chen Y. et al. (1996) Int. J. Pharmac. 137:149-158
Potts R.O., Guy R.H. (1992) Pharm. Res. 9:663-669
Pugh W.J. et al. (1996) Int. J. Pharmac. 138:149-165

202 THE DEVELOPMENT OF AN AUTOMATED SYSTEM FOR THE DETERMINATION OF PHYSICOCHEMICAL CONSTANTS OF IONIZABLE SPECIES

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The physicochemical assessment of a wide range of compounds has been achieved using an adaptable fully automated titration system. This system plays a vital role in the characterisation of ligands that are in development as potentially therapeutic agents for conditions such as *thalassaemia, malaria and neurodegenerative disorders.* A fully protonated chemical species is well characterized by measuring the ionization constants (pK_a), affinity constants (β) for metals complexation and partition coefficient (*Kpart*). This has been readily achieved by the automated titration system. This system has been developed to simultaneously obtain the spectrophotometric and potentiometric titration data of compounds and the metal complexes. Moreover, the micro titration mode has been conducted using the ***HKCPD97***[1] program to deal with compounds that exhibited limited aqueous-solubility which possess (< 10^{-5}M). The data obtained by auto-titration are fed into the program to evaluate the required constants. The pK_a and β values of ligands and complexes are determined using ***TITRFIT***[2] and ***STABOPT***[2] programs consecutively. In addition, the overall affinity constant can also be obtained by competition studies with well characterized ligands (such as EDTA) and the ***HKCP95***[1] and ***COMPT1***[1] programs are used to process the data. The lipophilicity (n-octanol/H_2O partition coefficient, *Kpart*) of the ligands is assessed using a filter probe device, and the system is controlled by the ***KDHK95***[1] program. The work in the communication exemplifies the experimental procedure and the treatment of the experimental data for evaluating pK_a, *Kpart* and β values for selected heterocyclic hexadentate and bidendate ligands.

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203 MEASURING LOG P USING LIPOSOMES: A POSSIBLE TOOL TO STUDY DRUG-MEMBRANE INTERACTIONS

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The lipophilicity of a substance (measured as oil-water partition) is an important parameter which can be used to predict bioavailability of drugs. Although the use of octanol as the reference oil phase is well accepted, octanol is not very much like the physiological environment it is trying to mimic. To provide a more realistic membrane model, we have used liposomes prepared from dioleylphosphatidylcholine (DOPC) and egg-PC in experiments on the Sirius GLpKa.

Unilamellar liposomes consist of a spherical lipid bilayer with charged head groups facing into the surrounding solution, and long hydrocarbon chains forming the interior. Liposomes share many properties of biological membranes, with the advantage that they can be prepared in a pure and reproducible way and thus used as the basis for standardized analytical procedures. Partition coefficients between water and liposomes are called $\log P_{mem}$ and $\log P_{SIP}$; these terms refer to the partitioning into the membrane interior, and with the surface of the membrane as a Surface Ion-Pair.

Suspensions of unilamellar liposomes are made using the Freeze-And-Thaw Large Unilamellar Vesicles Extrusion Technique (FAT-LUVET). Lipids must be above their transition temperature between gel-like and open structure, which is the case for dioleylphosphatidylcholine (DOPC) and egg-PC at 25°C. To make the liposomes, pure lipid is dissolved in chloroform. Some of this solution is pipetted into a round-bottomed vial and the chloroform is evaporated, leaving a thin layer of lipid coating the inside of the tube. Residual chloroform is removed under vacuum. A suspension of multilamellar liposomes is made by pipetting an aqueous solution on to the lipid layer and vortexing. The liposomes are then cycled through several stages of freezing, then thawing to ambient temperature, after which they are converted into unilamellar liposomes by repeated extrusion through filters using a proprietary apparatus. Aside from the time taken to prepare the liposomes, it takes perhaps 3 to 4 hours to measure both pK_a and $\log P_{mem}$ and produce a full lipophilicity profile.

Our work to date has been to optimize techniques for preparation of liposomes, and to develop analytical protocols for the pH-metric technique. To this end we have measured lipophilicity of a number of acidic and basic drug molecules as well as some diprotic, ampholytic molecules in both water/octanol and water/liposome. The following compounds have been studied in detail in unilamellar liposomes, with excellent data quality and reproducibility.

Ibuprofen, diclofenac, warfarin; Lidocaine, Procaine, propranolol, morphine.

A noteworthy observation is that $\log P_{SIP}$ values are significantly higher than $\log P_{ion}$ values measured in water/octanol, presumably because charged molecules bind with the charged membrane surface. Also noteworthy is that ΔlogP values ($\log P_{mem}$ - $\log P_{SIP}$) are significantly smaller for bases than for acids. We will introduce the "pH-piston" theory which may account for this latter observation.

204 PREDICTION OF HYDROGEN BOND DONOR ABILITY USING NEW QUANTUM CHEMICAL PARAMETERS

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The so-called solvatochromic parameters α and β developed by Kamlet et al (1984) are undoubtedly the best quantitative measures yet devised of hydrogen bond donor and acceptor ability. Unfortunately they are available at present only from experimental measurement, which greatly restricts their applicability. We have therefore investigated how they could be calculated from theoretical considerations based only on a knowledge of chemical structure. Ghafourian (1996) obtained the following QSAR correlation for 54 phenols, amines, amides, alcohols and carboxylic acids, using the AM1 hamiltonian in the MOPAC molecular orbital calculation program (QCPE, University of Indiana):

$$\Sigma\alpha^{H}_{2} = 4.42\ Q_H - 0.0403\ E_{LUMO} - 0.384 \qquad (1)$$

$n = 54 \qquad r^2 = 0.841 \qquad s = 0.092 \qquad F = 135$

where Q_H is the charge on the hydrogen-bonding hydrogen atom and E_{LUMO} is the energy of the lowest unoccupied molecular orbital.

Recently Karabunarliev et al (1996) have modified MOPAC so as to generate additional descriptors. They kindly supplied us with a copy of their source code, which we have used to see whether we could improve on equation 1. The same 54 molecules as used by Ghafourian (1996) were constructed and minimised using NEMESIS (Oxford Molecular Ltd.), then transferred to MOPAC6. There they were further energy-minimised, and parameters were calculated using the AM1 hamiltonian. An in-house genetic algorithm program was used to select the parameters that best modelled $\Sigma\alpha^{H}_{2}$:

$$\Sigma\alpha^{H}_{2} = -11.5\ D_E - 16.8\ P_E + 3.90 \qquad (2)$$

$n = 54 \qquad r^2 = 0.946 \qquad s = 0.053 \qquad F = 447$

where D_E is the electron donor superdelocalisability of the electronegative atom bonded to the hydrogen atom involved in hydrogen bonding, and P_E is the self atom polarisability of the same atom. The correlation is outstandingly good, and indicates that sigma a values can now be calculated with a high level of accuracy. It should be noted, however, that 2-substituted phenols, water and pyrrole had to be omitted from both equations as they were outliers. The negative sign of both parameters in equation 2 is consistent with increased partial positive charge on the hydrogen atom conferring increased hydrogen bond donor ability. The parameters highlight the crucial role of the electronegative atom in controlling hydrogen bonding ability.

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205 RAPID MEASUREMENT OF PKA AND LIPOPHILICITY

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For development and formulation into a successful drug, a molecule requires suitable physicochemical properties, which should ideally be measured at an early stage after synthesis. In particular, lipophilicity (logP) and solubility both provide important information about bio-availability and permeability through membranes. The values of these parameters vary according to pK_a values for ionizable molecules, which includes the majority of drugs. Rapid, fully automated analytical techniques for pK_a and logP have not been available hitherto.

We will describe a second-generation system (Sirius GLpKa) for measurement of pK_a and logP by pH-metric titration. The system allows overnight data collection, and can measure at the rate of 20 to 30 assays (pK_a or logP) per day. The software and user interface have been designed such that relatively unskilled operators can use the instrument for data collection.

In this poster, we will report the results of several studies made with GLpKa, including:

a series of over one hundred titrations to establish pH electrode response in the presence of water mixed with three CoSolvents (acetonitrile, DMF and THF), and to establish apparent pK_a (p_sK_a) values of carbon dioxide in these CoSolvents (figure 1). The value of this work is that pK_a values of water-insoluble drugs are measured in water-CoSolvent mixtures, which requires prior characterization of pH electrode response and carbon dioxide p_sK_a values in these mixtures.

...measurements of p_sK_a values of counter-ions in CoSolvents. (acetate, phosphate, citrate, maleate, oxalate and tartrate in methanol, DMSO, dioxane, acetonitrile, DMF and THF). The value of this work is that water-insoluble drugs are often crystallised as salts with these counter-ions, and their pK_a and logP values cannot be reliably measured unless the counter-ion p_sK_a values have previously been determined.

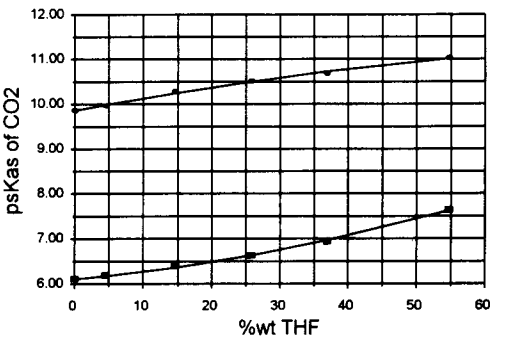

Figure 1: p_sK_a values of carbon dioxide as a function of weight percent tetrahydrofuran (THF) in THF/water mixtures made up to a concentration of 0.15M KCl

206 POLYAMINES ARE SOMETIMES, BUT NOT ALWAYS, FULLY PROTONATED AT PHYSIOLOGICAL PH

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We are continuing our studies with compounds containing spermine or an isomeric tetra-amine (e.g. thermospermine). Such polyamine conjugates have many modes of binding to biologically important proteinaceous receptors and to polynucleic acids. We now report an assessment of the pKa's of these polyamine conjugates and consider their many protonation states at physiological pH.

The structures and corresponding pKa values of some biologically important polyamines are shown in Figure 1. Spermine (**1**) is a simple endogenous polyamine, fully protonated at physiological pH, with roles in the stabilisation of chromatin as well as in cell growth and division. Unsymmetrical polyamine amide PhTX-3.4.3 (**2**) is a wasp toxin (philanthotoxin) analogue which is a potent blocker of cation channels and it is essentially fully protonated at physiological pH. The third measured pKa (9.5) accounts for the phenolic functional group, and therefore an increase in acidity from pH 10.4 to 9.5 finds both the secondary amine nearer to the tyrosine residue, and the phenoxide of tyrosine gaining protons (Jaroszewski et al 1996).

In gene therapy investigations, substituted polyamines such as DOGS (**3**), which form liposomes containing DNA, are used to effect DNA transfection. The polyamine buffers the endosome and the fact that the fourth (lowest) of the pKa values for DOGS (**3**) corresponds to the internal pH of the (acidic) endosome (Remy et al 1994) may be significant for efficient gene delivery. Other compounds of interest in the field of gene therapy include bile acids conjugates of tetraethylenepentamine (**4**), a polyamine which exhibits the co-operativity of pKa's along its polymethylene chain. Here, the fourth pKa is comparable with acetic acid (pKa 4.76), and the fifth with fluoroacetic acid (pKa 2.59).

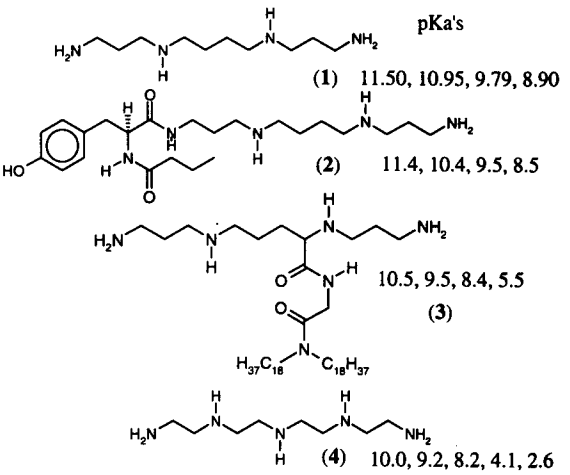

Figure 1

We acknowledge the financial support of the School of Pharmacy and Pharmacology, University of Bath, (studentship to SC), Celltech Therapeutics and EPSRC (CASE award to AJG), and BTG.

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207 A COMPARATIVE STUDY OF *AESCULUS HIPPOCASTANUM* AND *AESCULUS INDICA*

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Aesculus hippocastanum L. (Hippocastanaceae) is a common plant in Europe. Aescin, a complex mixture of triterpene glycosides, extracted from seeds of this plant exhibits anti-inflammatory and anti-edema effects in various experimental models (Bruneton, 1995). It has been widely used in the therapy of peripheral vascular disorders and the cosmetics field. *Aesculus indica* Colebr. is an indigenous plant in India and Pakistan used for the treatment of rheumatism (Chopra et al, 1956). Most studies on their phytochemistry have been carried out on *A. hippocastanum*, with only occasional studies on *A. indica* (Ikram et al, 1978).

The aim of this work is to establish a direct comparison of the two species grown under comparable conditions. The plant materials (seeds, husks and leaves) were collected from close geographic locations in order to minimise variations occurring due to geographical and climatic factors.

The constituents in methanol extracts from the two species were investigated using silica gel GF_{254} as stationary phase, butanol, acetic acid and water (4:1:3) as mobile phase and 10% sulphuric acid as detecting reagent. TLC zone profiles showed that the constituents of the leaves, seeds and husks differed within a single species. Comparing *A. hippocastanum* and *A. indica* revealed similar respective profiles for the seeds and leaves of the two species whereas the husks differed.

An investigation for the presence of the coumarin, aesculetin, and its glycoside aesculin, was carried out using TLC, followed by detection under UV and visible light. The results indicated an absence of aesculin and aesculetin from seeds, husks and leaves of both species. The absence of the compounds was not due to the unsuitability of the method, since it was effective for extracting aesculin and aesculetin from the bark of *A. hippocastanum* which has been reported to contain the two compounds (Matysik et al, 1994). *A. indica* bark was not available for comparison.

Aescin was found only in the seeds of both species but not in leaves and husks. The content of aescin in the seeds of the two species was determined using TLC-densitometry. Aescin which gave a dark brown zone occurred in dried *A. hippocastanum* seeds at 9.46±0.095 % w/w and in *A. indica* at 13.40±0.46 % w/w (n=3, separate determinations). These results suggest that *A. indica* seeds may be a viable alternative commercial source of aescin.

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208 COMPARISON OF PHYTOSOL EXTRACTION WITH CONVENTIONAL METHODS OF EXTRACTING VOLATILE OILS FROM CLOVE (*SYZYGIUM AROMATICUM* (L.) MERR. & PERRY) AND WILD OREGANO (*COLEUS AROMATICUS* BENTH.)

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Steam distillation and solvent extraction are commonly used to extract volatile oils from plants. A medium pressure method using fluorocarbons (Phytosols® A, B and D) has been proposed as an efficient alternative, and has been successfully used to extract good quality rose oil (Wilde 1993, 1994). Here, we examine the use of Phytosols for extracting volatile oils from clove and wild oregano.

Roughly ground cloves (26g) were steam distilled for 100 mins. Three further samples of the ground clove (13g) were extracted with Phytosols A, B or D (3 x 75g) using a hand held kit supplied by the company (Advanced Phytonics). The oil remained after sponataneous evaporation of the Phytosols at r.t.p.. Fresh wild oregano (WO) leaves and stems were chopped and 100-500g extracted by steam distillation (180 min), boiling with chloroform or light petroleum (5ml/g biomass; 60min) or successive extraction with Phytosols A, D and B (3 x 100g of each). Eugenol content of the clove oils was assayed spectrometrically (Sastry et al, 1985; 15 min. reaction time). Zone profiles of the WO oils were compared by silica-gel thin-layer chromatography (TLC) with hexane:ethylacetate 80:20 as mobile phase and anisaldehyde-H_2SO_4 spray reagent followed by heating as detecting method. Carvacrol content of the oils was obtained by comparing the intensity of the orange carvacrol zone in the oils with those of co-chromatographed carvacrol standards using a computer scanner and software for gel electrophoresis. Results are shown in Table 1.

Table 1. Comparison of oils obtained by steam distillation and solvent or Phytosol extraction (all values are % w/w)

Method	Clove oil		WO oil	
	Yield	[Eugenol]	Yield	[Carvacrol]
Steam distillation	12.89	31.7	0.049	37.1
Phytosol A	4.47	46.5	0.025	15.6
Phytosol B	5.05	38.6	0.014	nd
Phytosol D	3.92	37.7	0.013	nd
Chloroform	nd	nd	0.155	14.4
Light Petroleum	nd	nd	0.075	8.5

nd = not determined

Eugenol content was higher in the Phytosol extracted clove oil samples. TLC showed that the Phytosols extracted a wider range of constituents from WO than solvent extraction or steam distillation, but the carvacrol content was reduced. Thus Phytosol extraction may be a suitable method for obtaining oils from clove and WO, although these may vary in composition from the steam distilled product. The % yield from the Phytosols could possibly be increased by more exhaustive extraction.

209 COMPARISON OF THE EFFECTS OF TWO PHORBOL ESTERS ON PROTEIN KINASE C TRANSLOCATION AND MITOGENESIS IN SWISS 3T3 CELLS

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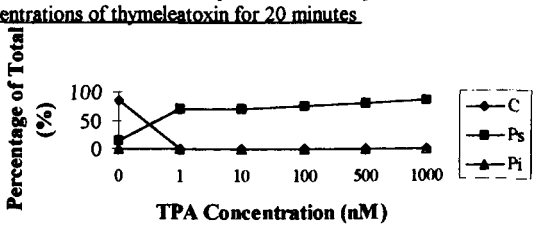
Protein kinase C (PKC) comprises of a family of at least eleven closely related isotypes (Nishizuka (1992), Hug and Sarre (1993)). We have identified by Western blotting with specific antibodies that Swiss 3T3 cells express the alpha, delta, epsilon and zeta PKC isotypes (Roberts et al (1997). In this study we have been investigating the translocation of alpha PKC with TPA and Thymeleatoxin, where PKC activation has been held to correlate with the translocation of the isotypes from the cytosol to the membrane *in vivo* (Stabel and Parker (1991)).

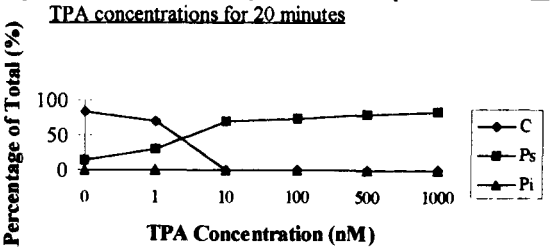
Using Western Blotting and specific antisera we have shown that in the resting state (control samples, DMSO alone) PKC alpha is found in the cytosolic fraction (C).Following stimulation with thymeleatoxin for 20 minutes the PKC band translocates to the membrane fraction (Ps). Translocation can be seen with doses greater than 1nM (Figure 1).

Figure 1: Translocation of alpha PKC after exposure to different concentrations of thymeleatoxin for 20 minutes



In comparison TPA stimulated cells translocate at doses greater than 10nM (Figure 2).

Figure 2: Translocation of alpha PKC after exposure to different TPA concentrations for 20 minutes



Stimulation of mitogenesis in Swiss 3T3 cells by phorbol esters has been attributed to their ability to bind to and activate the PKC family. In this study we have shown that both these phorbol esters stimulate mitogenesis, thymeleatoxin being more potent than TPA (ED50's 0.96nM and 8.6nM respectively).

These results suggest that PKC alpha plays an important role in the mitogenesis of Swiss 3T3 cells.

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210 ANTIPLASMODIAL ACTIVITY AND GENERAL TOXICITY OF SOME GHANAIAN PLANTS USED IN TRADITIONAL MEDICINE TO TREAT FEVERS

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The increasing drug resistance of malaria parasites has resulted in an urgent need for new agents. This paper reports on the preliminary antiplasmodial and toxicity screening of extracts of fourteen Ghanaian plants used as febrifuges in folklore medicine (Abbiw, 1990).

Antiplasmodial activities against Plasmodium falciparum (strain K1) were assessed using the parasite lactate dehydrogenase assay (Makler et al., 1993).

General toxicity of plant extracts was determined by means of the brine shrimp (Artemia salina) assay (Solis et al., 1993).

Methanolic extracts of the leaves of C. patens and the roots of P. mutica were found to have appreciable antiplasmodial activity and showed no toxicity to brine shrimps. This suggested that these plants were worthy of further study and work is being done to isolate the active principles. U. chamae showed antiplasmodial activity but also showed considerable toxicity. Investigations are being carried out to determine if the two activities are due to the same compounds.

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Solis, P.N. et al. (1993) Planta Medica, 59: 250-252.

Table 1 Antiplasmodial and toxic effects of plant extracts and standard drugs

Species/Drug	Part tested	P. falciparum IC50 (μg/ml)	Brine Shrimp LC50 (μg/ml)
Adenia rumicifolia	Leaf	>100	>250
Caesalpinia bonduc	Root	>100	>250
Cassia occidentalis	Root	>100	>250
Citrus aurantifolia	Leaf	>100	>250
Cleistopholis patens	Leaf alkaloids	8.7	>250
Cnestis ferruginea	Root	>100	>250
Momordica cissoides	Whole plant	>100	>250
Morinda morindoides	Root	>100	>250
Oncoba spinosa	Seed	>100	>250
Pleiocarpa mutica	Root	16.7	>250
Rothmania longiflora	Stem bark	>100	>250
Turraea heterophylla	Leafy twigs	>100	>250
Uvaria chamae	Root	21.6	2.5
Vernonia colorata	Stem bark	>100	>250
Chloroquine diphosphate	-	0.23	-
Emetine hydrochloride	-	-	20

211 EVALUATION OF TWO PLANT SPECIES USED IN MALAY TRADITIONAL MEDICINE FOR THE TREATMENT OF BREAST CANCER

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Phyllanthus pulcher (Baill) M.A. and Cyathostemma argenteum (Bl.) Sinclair are used in Malay traditional medicine for the treatment of breast cancer. In order to validate the use of these species in cancer treatment we have prepared crude extracts and assessed them for activity against the brine shrimp, Artemia salina, and against a murine adenocarcinoma cell line (MAC-15A).

Dried and powdered P. pulcher stem and C. argenteum roots were each extracted sequentially with petroleum ether, chloroform, ethanol and water. Extracts were evaporated to dryness under reduced pressure except for aqueous extracts which were freeze-dried. Activity against A. salina was determined using a 96-well microplate method (Solis et al., 1993). Inhibition of growth of cultured MAC-15A cells was measured using a standard MTT assay (Jabbar et al., 1989).

As shown in Table 1, the non-polar extracts from both species were highly active in both of the biological assays used. Moreover, the P. pulcher chloroform extract and the C. argenteum petroleum ether extracts were markedly more potent than the standard cytotoxic drugs emetine and 5-fluorouracil against the brine shrimps and cells respectively. These results support the reputation of the above species as anticancer plants and also demonstrate the use of A. salina for the detection of cytotoxic agents. Further work aimed at isolating the active principles is in progress.

Table 1 Activities of crude plant extracts and standard cytotoxic drugs against A. salina and MAC-15A cells

Extract/compound	Brine shrimp LC50 (μg/ml)	MAC-15A IC50 (μg/ml)
	Phyllanthus pulcher	
Pet. ether	<0.24	0.01-0.1
Chloroform	<0.24	<0.001
Methanol	1-2	0.1-1
Water	>500	-
	Cyathostemma argenteum	
Pet. ether	<1.98	<0.001
Chloroform	3.9-7.8	0.01-1
Methanol	3.9-7.8	0.01-1
Water	>500	1.10
Emetine HCl	30	-
5-Fluorouracil	-	0.2

Jabbar, S.A.B. et al. (1989) Br.J.Cancer, 16: 523-538.
Solis, P.N. et al (1993) Planta Medica, 59: 250-252.

212 PHARMACOGNOSTICAL INVESTIGATIONS ON TRADITIONAL CHINESE MEDICINAL HERBS: IDENTIFICATION OF SIX HERBS FROM THE UK MARKET

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Following our previous report on the identification of four traditional Chinese medicinal herbs(Yu et al 1995), we now report another six herbs, of which adulterants or fakes were found in the UK market. Some of those adulterants have different therapeutic functions from the authentic herbs and some of them may cause side effects not usually associated with the genuine species.

Herbal samples were collected from the UK market and standard sampling method was applied. A total of twelve samples were chosen as experimental materials and grouped into six sets with each set consisting of two specimens of the same Chinese name. After initial morphological identification, each sample was pulverised for microscopic examination with reference to the relevant authentic herb powders, the usual mountants were employed. Methanol extracts were made of each sample and the authentic herb powders for TLC comparison using silica gel pre-coated plates, different developing systems and visualising methods. TLC fingerprint chromatograms were produced for each set of materials with reference to authentic species. The authentic herbal powders of *Pulsatilla chinensis, Platycladus orientalis, Cimicifuga heracleifolia, Siegesbackia orientalis, Spatholobus suberectus* and *Pinellia ternata* were obtained from the Chinese National Institute for the Control of Pharmaceutical and Biological Products.

Two of the samples collected were supplied as Bai Tou Weng. Macroscopic, microscopic and TLC examination revealed that one complied with the Pharmacopoeial description for the authentic species *Pulsatilla chinensis,* while the other was found to be *Polycarpeae corymbosa.* Two samples were named as Cei Bai Ye but their macroscopic and microscopic characteristics were totally different. One was the Pharmacopoeial product *Platycladus orientalis* and the other was a substitute *Podocarpus macrophyllus var. maki.* A sample was supplied as Xi Xian Cao, but our examination confirmed that it was a fake herb (*Epimeredi indica*) which had different therapeutic properties from the genuine species *Siegesbackia orientali.* A sample of Ban Xia, imported from Hong Kong, was found to be a substitute *Typhonium flagelliforme* to the authentic species *Pinellia ternata.* Two samples supplied as Sheng Ma were examined. The substitute, identified as *Serratula chinensis* was more frequently encountered in the UK market than the authentic species, *Cimicifuga heracleifolia.* Ji Xue Teng (*Spatholobus suberectus*) is a commonly used herb for the blood system. However, most suppliers and practitioners in the UK often use its substitute, identified as *Sargentodoxa cuneata.*

Traditonal Chinese medicinal herbs supplied in the UK have not been properly regulated. The mis-identification, and thus the misuse of the above mentioned herbs has been a matter of concern for patient's safety. There is an urgent need for the suppliers to adopt a practice of self-regulation in order to ensure safety for patients.

Yu, H. et al (1995) Pharm. J. 255:431

213 ANTILEISHMANIAL ACTIVITY OF *KIGELIA PINNATA* ROOT BARK CONSTITUENTS

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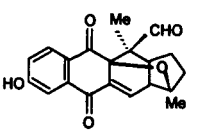
Of late there have been scant economic resources available for antiparasitic drug discovery and development (Aldhous 1994). In our continuing search for bioactive natural products with antiprotozoal activity, we consider as candidate an African plant, *Kigelia Pinnata* (Heine 1963).

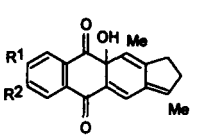
The present study is concerned with antileishmanial activity of the root bark of the plant whereby the plant extracts were tested against *Leishmania major* promastigotes JISH 118. The mixture of 100 µl of parasite at $1x10^6$/ml and 100 µl of varying concentrations of the plant extracts (from 90 to 1.3µg/ml) was incubated in a micro-titre plate at temperature of 26°C for 48 hours. Inhibition of growth of the parasite was inspected microscopically.

The result from initial experiment has shown that constituents that exhibited significant antileishmanial activity reside in the dichloromethane fraction. Subsequent experimental results led to the identification of two subfractions (from the active fraction) which gave promising activity with minimum inhibitory concentration (MIC) values of 10 and 30 µg/ml respectively.

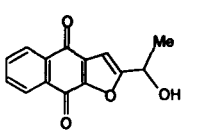
Fractionation and purification process monitored by bioassay experimental results led to the isolation of three naphthoquinoids {isopinnatal, isokigelinol and kigelinol} and one naphthoquinone {2-(1-Hydroxyethyl)naphtho[2,3-b]furan-4,9-quinone} from the fraction that gave a MIC value of 10 µg/ml. Meanwhile, from the fraction that gave a MIC value of 30 µg/ml, balanophonin, a neolignan which has not been reported to be present from the plant before, was isolated.



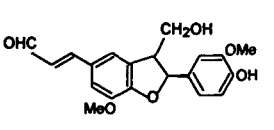
Isopinnatal



	R1	R2
Isokigelinol	OH	H
Kigelinol	H	OH



2-(1-Hydroxyethyl)naphtho[2,3-b]furan-4,9-quinone



Balanophonin

Aldhous, P. (1994). Science 264:1857-1859.

Heine, H. (1963) in Flora of West Tropical Africa (by Hutchison, J and Dalziel, J.M.) 2nd Edn (Hepper, N., ed.)Vol II, p.385.

214 ANTIPLASMODIAL ACTIVITY OF BISINDOLE ALKALOIDS FROM *ALSTONIA MACROPHYLLA*

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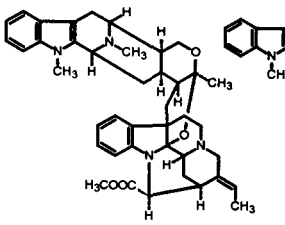
Some species of the genus *Alstonia* (Apocynaceae) have a good reputation in Southeast Asian traditional medicine for efficacy in the treatment of malaria (Perry & Metzger 1980). From the primary screening of extracts of Thai *Alstonia* species, the methanol extract of the root bark of *A. macrophylla* showed pronounced activity (in vitro) against multidrug-resistant K1 strain of *Plasmodium falciparum* with an IC_{50} value of 5.7 μg mL^{-1}.

Three novel bisindole alkaloids, alstomacroline, alstomacrophylline and O-methylmacralstonine, were isolated from this active extract along with two main bisindoles, macralstonine and villalstonine. Their structures were elucidated by spectroscopic methods.

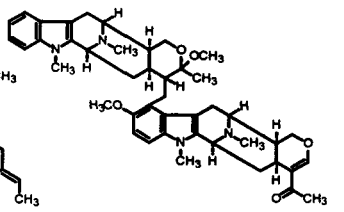
The alkaloids were tested against *P. falciparum* cultured in human blood (Desjardins et al 1979). The growth of the plasmodium was measured for various concentrations (in 4-fold serial dilutions) of each alkaloid by determination the reduction in incorporation of [G-^{3}H]-hypoxanthine into the parasites by 50 % (IC_{50}).

Villalstonine, the macroline-pleiocarpamine derivative, gave the greatest activity against K1 strain of *P. falciparum* with an IC_{50} value of 0.27 μM. This was almost as active as chloroquine diphosphate (positive control), tested in the same system. In contrast, macralstonine exhibited slight activity with an IC_{50} value of 8.91 μM. Alstomacrophylline, O-methylmacralstonine and alstomacroline were found to possess moderate antiplasmodial activity with IC_{50} values in the range of 0.65-1.60 μM.

These results lend some support to the use of *A. macrophylla* in traditional medicine for the treatment of malaria and reveal that the genus *Alstonia* is likely to yield further compounds with interesting biological activity.



Villalstonine



Alstomacrophylline

Desjardins, R.E., Canfield, C.J., Haynes, J.D., Chulay, J.D. (1979) Antimicrob. Agents Chemother. 16, 710-718.

Perry, L.M., Metzger, J. (1980) Medicinal Plants of East and Southeast Asia. MIT, Cambridge, Massachusetts, pp. 22-23.

215 STUDIES ON THE CHEMISTRY AND IN-VITRO ANTIPLASMODIAL AND ANTIBACTERIAL ACTIVITY OF *CRYPTOLEPIS SANGUINOLENTA* LEAVES

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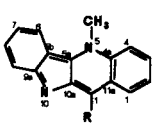
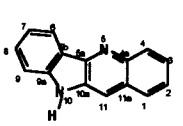
The roots of *Cryptolepis sanguinolenta* (Lindl.) Schlechtr. (Asclepiadaceae) are traditionally used in several West African countries for the treatment of malaria and bacterial infections. The "mancanha" people (Guinea Bissau) use the infusion of the leaves to treat malaria. In the past 50 years the roots have been chemically and biologically investigated but no studies on the leaves have been reported until now.

Two samples of *C. sanguinolenta* leaves, one from Nigeria and one from Guinea Bissau were investigated. The alkaloids cryptolepine [1], hydroxycryptolepine [2], and quindoline [5] were present in the ethanol extracts of both samples. From the Nigerian sample two new alkaloids were isolated and their structures elucidated by spectroscopic methods (UV, MS, and NMR) as 11-carboxycryptolepine [3] and 11-methylcarboxycryptolepine [4]. The sample infusions were analysed by HPTLC and we concluded that alkaloids [2] and [5] cannot be extracted by water. Extracts and pure compounds were tested for *in vitro* activity against the multidrug-resistant K1 strain of *Plasmodium falciparum* cultured in human erythrocytes and 6 ATCC strains of Gram - and Gram + bacteria by the twofold serial broth microdilution method. Results are shown in Table.

Our first results show that cryptolepine [1] is the major compound responsible for the antibacterial and antiplasmodial activities of the ethanol and aqueous extracts. More experiments are being carried out to determine MIC and IC_{50} values.





[1] R = H cryptolepine [5] quindoline
[2] R = OH hydroxycryptolepine
[3] R = COOH carboxycryptolepine
[4] R = $COOCH_3$ methylcarboxycryptolepine

Table: Alkaloid activities against bacteria (MIC) and *P. falciparum* (IC50) in mM

	Alkaloids			
Microorganisms	1	2	3	5
Escherichia coli	0.11	>0.40	>0.44	>0.46
Shigella dysenteriae	0.05	>0.40	>0.44	>0.46
Salmonella typhimurium	0.22	>0.40	>0.44	>0.46
Staphylococcus aureus	0.05	>0.40	>0.44	>0.46
Streptococcus faecalis	0.05	>0.40	0.22	>0.46
Vibrio cholerae	0.01	>0.40	0.11	>0.46
Plasmodium falciparum	0.0005	NT	>0.44	NT

NT = not tested

216 THE ANTIBACTERIAL ACTIVITY OF FRUITS OF SRI LANKAN *PIPER* SPECIES

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The fruits of *Piper* species (Piperaceae) have long been used as spices and in traditional medicine in southeast Asia. In Sri Lanka the fruits of *P. nigrum* (Black pepper), *P. cubeba* (Cubebs) and *P. longum* (Long pepper) are common products and used to treat infectious respiratory diseases.

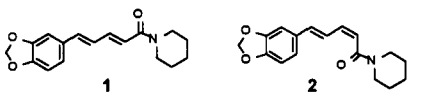
Alcoholic extracts of *P. nigrum* and *P. cubeba* have been reported to be active against *Mycobacterium tuberculosis* (Grange and Davey, 1990) but the compounds responsible were not identified.

The antimicrobial activities of the ethanolic and chloroform extracts of the three *Piper* species above were tested by applying 500 and 1000µg. of each to silica gel layers, overlaying with agar seeded with bacteria. The plates were incubated at 37° for 24 and 48h and any zones of inhibition noted. Three species of bacteria were used i.e. *E.coli*, *Staph. aureus* and *B. subtilis*. No inhibition zones were observed for any of the extracts when *E.coli* and *Staph. aureus* was used but extracts of *P. nigrum* and *P. longum* inhibited the growth of *B.subtilis*.

The extracts were separated by thin-layer chromatography (TLC) silica gel GF_{254}/toluene:ethyl acetate 7:3. Duplicate plates were run, one being visualised under UV light 254nm and then sprayed with Dragendorff's reagent and the other overlaid with agar seeded with *B. subtilis* and then incubated as above. Inhibitory zones for the active extracts were seen to correspond with the same Dragendorff-positive zones in both species. No alkaloids could be detected in *P. cubeba* extract, which had shown no antibacterial activity.

Fractionation of the extracts by solvent partition showed that the activity resided in the weakly basic alkaloid portion. Fractions arising from silica gel column chromatography of this fraction (using a solvent gradient of toluene with increasing quantities of ethyl acetate) were tested for inhibitory effect against *B. subtilis* . Two sets of fractions giving inhibition were observed for both *P. nigrum* and *P. longum*. TLC showed that each set contained one major alkaloid. These two alkaloids were isolated by prep TLC (Silica gel/toluene:ethyl acetate: hexane: butan-2-one 7:3:1:1) and their structures determined by NMR and mass spectrometry. The two compounds were shown to be piperine 1 and its E, Z isomer isochavicine 2 with piperine as the makor alkaloid.

The antibacterial properties of *Piper* fruits therefore seem to be associated with the presence of alkaloids.



1 2

Grange, J.M., Davey, R.W. (1990) J. Appl. Bacteriol. 68:587-591.

217 ALKALOID AND TANNIN DISTRIBUTION IN THE LEAVES OF *PELARGONIUM ZONALE* WITH REFERENCE TO INSECT BEHAVIOUR

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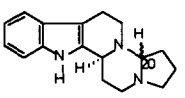
Studies of phytochemicals that influence plant-insect interaction may lead to the isolation of new bioactive substances. These are not only of relevance to the discovery of new compounds for treating parasitic insects and disease vectors but may also provide insights into mammalian CNS activity.

Pelargonium species are the most popular of the Geraniaceae family and are grown for their beautiful blooms by many amateur and professional gardeners around the world.

The presence of the indole alkaloids, elaeocarpidine **1** and epielaeocarpidine **2** have been reported from *Pelargonium* 'Zonal' cultivars but are not present in non-zonal varieties (Lis-Balchin et al, 1996).



1 20-H b Elaeocarpidine

2 20-H a Epielaeocarpidine

The leaves of many 'zonal' cultivars exhibit a distinct dark horse shoe-shaped region and it has been noted that whitefly lay their eggs on the edges of the area. Tests were carried out to see if this selective behaviour was due to differences in the chemical composition of the light and dark zones. Tests using a range of concentrations of the two alkaloids against whitefly larvae showed that **1** was toxic at 1000ppm but **2** had no activity. When **1** was applied to the centre of the leaves of non-zonal varieties of *Pelargonium* it was noted that whitefly eggs were not laid in the area. The alkaloid content was measured for each zone and also its tannin composition as it was thought that the dark area may be due to such compounds.

Fresh leaves of the zonal cultivar were cut to separate the dark and light zones. Each portion was extracted with methanol and alkaloidal content was assessed by RP-HPLC (ODS 15cm x 4.6mm i.d.; methanol:water 9:1 detection at 224nm) of the major alkaloid, elaeocarpidine **1**. This showed that the dark region contained 0.014% w/v whilst the outer light region contained only 0.001% w/v. The tannin content for each zone of the 70% ethanol extract of the leaves was measured spectrophotometrically at 300nm after the addition of 70% ethanolic iron (III) chloride solution. Tannic acid treated in the same way was used as a reference compound to construct a calibration curve. The dark zonal parts contained 1.11% w/v and the outer part 0.88% w/v equivalent of by UV-spectroscopy. TLC of the methanolic extract treated with and without showed that the alkaloids, particularly in the central dark zone of the leaves, exist in a complex, probably with the tannin, from which they are released as the free alkaloids by calcium hydroxide.

The alkaloid-tannin complex therefore seems to play some part in the deterrence of oviposition by whitefly.

Lis-Balchin, M. et al. (1996) Nat. Prod. Lett., 8:105-112.

218 SCREENING OF TUNISIAN PLANTS USED IN TRADITIONAL MEDICINE FOR IN-VITRO ANTI-INFLAMMATORY ACTIVITY

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The use of ethnobotanical information may provide a valuable short cut to new drug discoveries by indicating plants with specific folk medicinal uses which might be likely sources of biologically active compounds (Gentry, 1993).
An ethnobotanical study of the Tunisian flora has helped us identify 75 plants with reported anti-inflammatory, anti-infective and anti-rheumatic activity. In order to ensure that a wide range of potentially active compounds was covered, both hexane and methanol extracts were made of 13 of these plants. These extracts were tested in a rat neutrophil leukocyte system designed to reveal interactions, under physiological conditions, with the pro-inflammatory arachidonic acid cascade and with the secretion of preformed pro-inflammatory tissue destructive enzymes from the intracellular azurophil granules (Hoult et al, 1994; de las Heras et al, 1994).
Mixed leukocytes were obtained by peritoneal lavage from female Wistar rats pretreated 16 hours previously with 10ml 6% oyster glycogen and were resuspended at 2.5 x 10^6 cells/ml in HBSS . Cells were preincubated for 5 minutes with the plant extracts (added in 2µl of methanol or DMSO, at a final concentration of 100µg/ml) and stimulated with a calcium ionophore, A23187. Following 10 minutes incubation at 37°C, cells were pelleted and the supernatants retained for assay of generated thromboxane (RIA for TXB_2) and release of azurophil myeloperoxidase (MPO, assayed in 96 well microtitre plate format by incubating supernatant aliquots with 0.001% H_2O_2 and 0.17mg/ml o-dianisidine in pH 6.0 phosphate buffer containing 0.5% HTAB detergent).
Active extracts were defined as those producing >50% inhibition of either TXB2 generation or MPO release (or both). Of the 68 extracts screened, twelve were active against TXB_2 and eight against MPO release. None of the extracts which were active in the MPO assay had positive effects in the TXB_2 assay, indicating potential selectivity. None of these extracts were toxic to the leukocytes, either at 10 minutes (present conditions) or at 120 minutes (incubated without A23187), according to LDH and/or MTT assays.
The most promising extracts in the MPO assay were those from *Ricinus communis* L., which are undergoing more intensive evaluation.
It is interesting to note that the active extracts were made in methanol and this is in accordance with the fact that these plants are normally taken as an infusion.

Gentry, A.H. (1993)in Human Medicinal Agents from Plants. (Eds A.D. Kinghorn and A.G. Balandrin) American Chemical Society. Washington D.C. pp 13-24
Hoult, J.R.S. et al (1994) Methods in Enzymology 234 443-454
de las Heras, B. et al, (1994) Planta Med. 60 501-506

219 INVESTIGATION OF FOUR THAI MEDICINAL PLANTS FOR INHIBITION OF PRO-INFLAMMATORY EICOSANOID SYNTHESIS IN ACTIVATED LEUKOCYTES

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Four Thai medicinal plants *viz. Acanthus abracteatus* leaves (**A**), *Oroxylum indicum* roots (**B**), *Cryptolepis buchanii* stem (**C**) and *Derris scandens* stem (**D**) were selected because of their use in traditional medicine for the relief of rheumatic conditions. Aqueous and methanol extracts of 10g of each plant material were made, taken to dryness and the residue dissolved in water to give 50 mg mL^{-1} solutions.
Rat peritoneal leukocytes were obtained and were pretreated with extracts in a range of concentrations 10-500µg mL^{-1} and solvent only (negative control) (Moroney et al 1988). 10^{-6}M A23187 was added to stimulate release of the pro-inflammatory eicosanoids leukotriene B_4 (LTB_4), formed by the 5-lipoxygenase pathway, and thromboxane B_2 (TXB_2), formed by the cyclo-oxygenase pathway. The amounts of these substances released was measured by radioimmunoassay (RIA). Results for the aqueous extracts of ***A***, ***B*** and ***D*** are shown in tables 1, 2.
Significant inhibition ($p<0.05$, Student's unpaired t-test))of synthesis of LTB_4 was shown by 500µg mL^{-1} both aqueous and methanol extracts of ***A***, and by the aqueous extract of ***D*** ($p<0.001$). The aqueous extract of ***D*** also significantly inhibited TXB_2 production ($p<0.001$) at 500µg mL^{-1}. Extracts B and C caused no inhibition of release of either compound. The traditional use of two of the plants investigated, *Acanthus abracteatus* and *Derris scandens*, therefore receives some scientific validation. *D. scandens* is the more suitable candidate for further investigation as to the nature of the active constituents since it inhibits both pathways.

Table 1 TXB_2 (ng mL^{-1}) released in presence of aqueous plant extracts

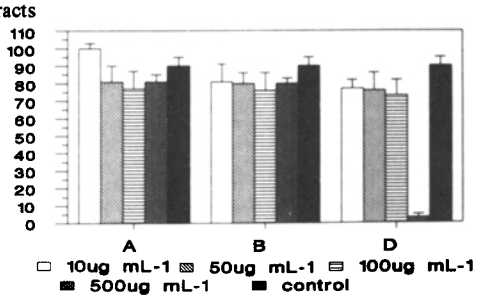
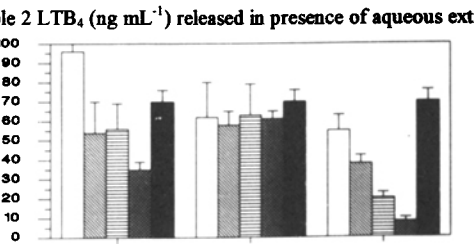


Table 2 LTB_4 (ng mL^{-1}) released in presence of aqueous extracts



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220 ANTIFUNGAL ACTIVITY OF EXTRACTS OF BIGNONIACEAE FROM MALAYSIA

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Various members of the Bignoniaceae are used in traditional medicine for the treatment of diseases associated with fungal infection. The implied antifungal activity of such plants has not been validated scientifically.

Dichloromethane extracts of members of the Bignoniaceae growing in Malaysia were examined against a range of fungal organisms. The plants used were *Oroxylum indicum* stembark (OISB), *O.indicum* roots (OIR), *Fernandao adenophylla* root (FAR), *Tabebuia chrysantha* stembark (TCB) and *Jacaranda filicifolia* stembark (JFB). 100µg of each extract were applied to paper discs which were applied to the surface of Sabouraud dextrose agar streaked with a suspension of a filamentous fungus. Fungi used were *Aspergillus niger* (AN), *Penicillium expansum* (PE) and three dermatophytes *Trichophyton mentagophytes* (TM), *Microsporum gypsum* (MG) and *Epidermophyton floccosum* (EF). Salicylic acid 5µg was used as a positive control and 0.5µg lapachol was also tested since TLC examination of the extracts showed that all except JFB contained the naphthaquinone lapachol. Plates were examined for growth after incubation for 72h at 30° and any inhibition zones were noted. Results are shown in Table 1.

The extracts were also tested against three species of wood rot fungi of importance to the tropical timber industry i.e *Coriolus versicolor* (CV), *Gloeophyllum trabeum* (GT) and *Bostryodiplodia theobromea* (BT). Tributyl tin oxide (TBTO) was used as a positive control. Malt extract agar was mixed with doses of the extract to produce standard layers of 1.66mg mL^{-1} extract in Petri dishes. Each plate was inoculated with a 4mm disc cut from the vegetative growing margin of the cultured fungus and incubated at 28° for 6 days. The colony diameters were measured against control where no extract was added to the medium. Results are shown in Table 2.

Table 1 Inhibition zones produced by 100µg extracts against filamentous fungi

Extract	Diameter of zone of inhibition (mm) n = 3 given by each organism				
	TM	MG	EF	AN	PE
OIR	10-15	16-20	16-20	0	0
OISB	<10	16-20	10-15	0	0
FAR	<10	>20	<10	0	0
TCS	16-20	16-20	16-20	0	0
JFB	0	0	0	0	0
Salicylic acid 5µg	16-20	16-20	16-20	ND	ND
Lapacho 10.5µg	10-15	10-15	10-15	0	0

ND = not done

Table 2 Diameter of colonies produced by wood rot fungi in presence of 1.66mg mL^{-1} extracts

Extract	Diameter of colony (mm) n = 3 given by each organism		
	BT	GT	CV
OIR	>20	16-20	10-15
OISB	>20	16-20	10-15
FAR	<10	16-20	10-15
TCS	>20	10-15	10-15
JFB	16-20	16-20	16-20
TBTO 5µg	<10	<10	<10
Control	>20	>20	>20

All extracts except JFB showed activity against dermatophytes and this seems to be associated with the lapachol present. Some activity against two species of wood rot fungi has also been demonstrated for all the extracts.

221 IN-VITRO INVESTIGATION OF THE WOUND-HEALING PROPERTIES OF *BUDDLEJA GLOBOSA*

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Members of the genus *Buddleja* have been employed as wound healing agents in traditional remedies in areas where they are indigenous (Houghton,1984).

B globosa leaves have traditionally been used as an aid in wound healing and for the removal of warts and callous ulcers in Chile and Bolivia (Houghton 1984). The effect of aqueous extract of the leaves on the proliferation of human dermal fibroblasts and the protective effect against hydrogen peroxide induced damage were monitored by the MTT colorimetric method of assay (Mossman, 1983). A freeze dried aqueous extract of the 10g of the leaves was prepared. Serial dilutions of the extract in Dubelco's Modified Eagle Medium (DMEM) or Hank's Buffered Saline Solution (HBSS) were used for the bioassay

In the proliferation experiment the extract at a concentration range of .05 to 1µg ml^{-1} had a slight stimulatory effect on the proliferation of fibroblasts compared to the negative control of 0.5% foetal calf serum (FCS) but on prolonged exposure this was less than that of the positive control which consisted of 2% FCS. The *B. globosa* extract was cytotoxic at 50µg ml^{-1}. Data are shown in Fig 1.

The protection against oxidant injury was observed when the cells were exposed to different concentrations of H_2O_2 alone or after 24 hour pre-incubation (Pre) with the extract. The optimum protection comparable to that of the positive control of catalase 250 IU mg/ml measured by increased absorbance reading was obtained when, in addition to the pre-incubation, the cells were treated with the extract simultaneously (Pre+S) with the H_2O_2. The extract at 5µg mL^{-1} had a statistically significant ($P<0.05$,ANOVA and Scheffe's test) antioxidant protective activity against 10^{-4} M H_2O_2-induced damage. Results for the antioxidant activity tests are shown in Fig 2.

Figure 1 MTT assay results for *B. globosa* leaf extracts on fibroblast proliferation

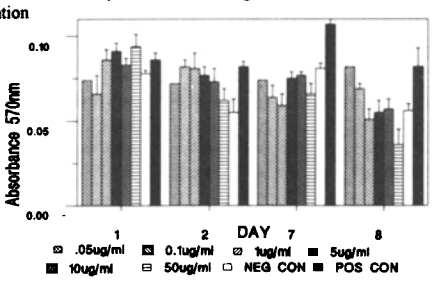
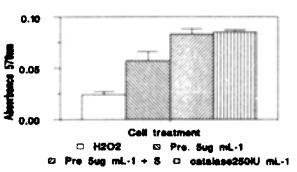


Figure 2 MTT assay results for fibroblasts treated with H_2O_2 in the presence and absence of 5µg mL-1 *B. globosa* extract



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222 THE PHYTOCHEMICAL STUDY AND CLINICAL EVALUATION OF *TAGETES ERECTA* IN THE TREATMENT OF HALLUX ABDUCTO VAGUS AND ASSOCIATED CONDITION, BUNION

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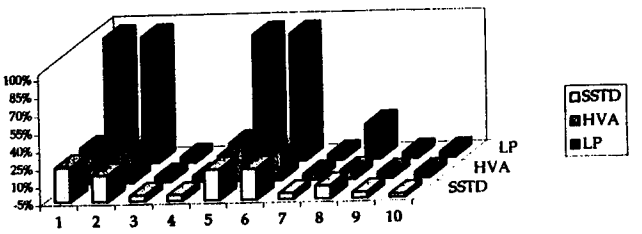
Fresh petal and leaf of *Tagetes erecta* (TeL+P) were used in a double blind placebo controlled trial (Khan, 1985, Khan *et al*, 1996, Khan and Evans, 1996). In the present study, crude extract and fractionations of TeL+P with protective pad (pp) were used in a single blind controlled study of the treatment of mild hallux abducto valgus (HAV) and its associated condition, bunion (ACB), over a period of 12 weeks to determine the active compounds and isolate and identify their mode of action.

Fifty volunteers with unilateral HAV and ACB who met the inclusion and exclusion criteria were allocated to 10 groups to evaluate the effect of crude extract and fractionations of TeL+P, reference compound and solvent with and without pp in the treatment of HAV and ACB. The crude extract which was either ethanolic or methanolic was used dried at 40mg/dose/week. The fractions were prepared using phytochemical methods, i.e. sequential extraction of the dried powder. Each patient was treated once a week for four weeks and received no treatment during the following eight weeks.

Clinical evaluation of the therapeutic effect was assessed by measurement of swollen soft tissue density (SSTD) using Venier's calipers, hallux valgus angle (HVA) using tractograph, and level of pain (LP) by Visual Analogue Pain Scale. Data pre- and post-treatment weeks 0-4 and 0-12 were analysed in terms of percentage reduction of SSTD, HVA and LP (Khan, 1996).

One of the fractions of TeL+P was found to be more effective than others in the treatment of HAV and ACB. *Fig.* The control groups showed some reduction of pain and SSTD during the treatment weeks 0-4 but no improvement at final observation week 12.

Percentage of Reduction HAV and ACB: Weeks 0 - 12



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223 SPIRITUAL HEALING AND PHYTOCHEMICAL STUDIES ON MEDICINAL PLANTS USED BY INDIGENOUS TRIBES FROM THE AMAZON REGION OF COLOMBIA

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The medicinal plants used by Amerindian tribes in Colombia, particularly the Uitoto and Muinane cultures, are being studied by us in an attempt to determine the active compounds. These indigenous groups use both preventative and curative medicine. In order to achieve a cure the "abuelos-Sabedores", wiseman such as the author (NRJ) with knowledge of medicinal plants, must treat both the spiritual and material aspects of the disease. The treatment normally begins with the spiritual cure, particularly where the illness is thought to arise through some form of witchcraft. Treatments are therefore specific to the individual and it appears that in common with many other cultures, the Uitoto and Muinane communities adopt a "holistic" approach in their medicinal practice.

The plants under investigation include several representatives of the family Burseraceae. Which is known for the production of commercial resin exudates used as incense world-wide, for example Myrrh (*Commiphora spp.*) and Frankincense (*Boswellia spp.*) found in Africa and Asia, Tucker (1986), Newall et al (1996). The plants under study are collectively known in Colombia as 'incienso'. Three individual plants have been investigated and are known to the Uitotos and Muinanes in local idiom as Uguko [*Protium* cf. *heptaphyllum* (Aubl) March], Uguna [*Dacryodes peruviana* (Loesner) Mcbr.] and Xemena [*Protium* sp.], Cuatrecasas (1957). They are burned ritually in the houses to "fill them with positive energies" and, for example Uguko resin is used as a decongestant and treatment for asthma and pulmonary infections.

Volatile oils obtained from each resin by steam destillation were subjected to GC-MS. They were analysed on an HP-1 capillary column (12m x 0.2 mm i.d.), carrier gas Helium. Injection port 250°C, and oven temp. 50°C for 5 mins., ramp 5°C min^{-1} to 300°C, isothermal 10 mins. GC-MS (Hewlett Packard 5598A) analysis operating conditions were as follows: Electron Impact 70 eV, ion source 140°C and detector at 280°C. Uguko is rich in sesquiterpenes; Uguna showed the presence of mixture of monoterpenes and sesquiterpenes while Xemena has mono-oxygenated and acetylated monoterpenes. Uguna and Xemena have some common monoterpenes such as α-thujene, *p*-cymene, Limonene, α-Terpineol, Thymol, Carvacrol; while Borneol, Bornanone, Sabinene and γ-terpinene are specific to Uguna, and O-methyleugenol, Elemicin, Eudesmol and Benzyl Benzoate are specific to Xemena. The identification of volatile oil was made comparing the mass spectra and retention times of each with authentic samples, database and/or components spectral catalogues, McLafferty (1980), Adams (1989).

The petrol, chloroform and ethyl-acetate fractions from each resin have been subjected to TLC comparison (one - two dimensional) looking the chemical components previously isolated from the petrol fraction of Uguna resin,Gray et al. (1996). Monoterpenes, penta- and tetracyclic triterpenes such as α- and β-amyrin and α-elemolic acid, were encountered in all the resins but in variable concentrations. The triterpene acids may in part be responsible for the antifungal activity against *Trichoderma viride* and *Fusarium solani* exhibited by these resins.

Acknowledgements to COLCIENCIAS, Colombia (grant to J. Robles) and J. Ratter (RBGEdinburgh), A. Garcia and W. Milliken (RBGKew) for botanical determinations.

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224 EVALUATION OF ESSENTIAL OILS AND SOME OF THEIR COMPONENT TERPENOIDS AS PEDICULICIDES FOR THE TREATMENT OF HUMAN LICE

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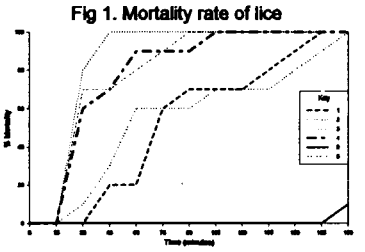
In the UK alone over 3m headlice infestations are treated annually, and recently resistance has arisen to permethrin and malathion (Burgess 1995). In addition to using accepted strategies for delaying resistance (Maunder 1991), new insecticides for use on the skin are needed.Terpenoids have been known from ancient times to be lethal and repellant to insects (Coats *et al* 1991) and are considered to be an ovicidal component of some headlice formulations (Burgess 1995). Although the succesful use of essential oils in headlice infestations is known, no scientific studies have been carried out until recently (Veal 1996). We decided therefore to look at oils with this reputation, their common constituents, and in addition to carrying out a small pilot clinical study, test some of these *in vitro* using an established test (WHO 1981). Juniper, eucalyptus, lavender, geranium, lemon and rosemary are among those oils with reputed insecticidal activity, and were used as mixtures in the pilot study. They contain terpineol, 1,8-cineole, terpenen-4-ol, α-and β-pinene, limonene, camphene, linalool and others (Williamson and Evans, 1988). The more toxic oils such as pennyroyal, bergamot and calamus were omitted for the present.

Fig 1. Mortality rate of lice



1=Cyp.oil.se, 2=terpineol, 3=Cyp.oil.sd
4=α-pinene, 5=control, 6=camphene

Leyland cypress, *Cupressus* x *leylandii*, contains an oil in high yields, and this was prepared by both steam distillation (Cyp.oil.sd) and cold solvent extraction (Cyp.oil.se) for analysis and bioassay. The major components of the *Cupressus* oil were found to be terpineol, terpenen-4-ol, 1,8-cineole and linalool. We then bioassayed the oils, together with individual terpenoids which were easily available: camphene, terpineol, and α-pinene.The pediculicide testing involved the use of the clothing louse, *Pediculus humanus*, which is more suitable for colonisation than the headlouse, *Pediculus humanus capitis*, and less likely to dehydrate during the course of the assay. Lice were kept on papers impregnated with test agent, incubated at about 37°C, and the mortality rate monitored over 3 hours. Both types of oil as well as the terpenoids tested showed high levels of activity (fig 1). The clinical pilot study, which was not controlled, was carried out by means of counter prescribing in a pharmacy, in response to requests from patients who had previously tried other preparations. In total this involved 20 children, treated with mixtures of essential oils as above, 5% in a cream/alcohol vehicle, showed full efficacy as reported by the mothers of the patients. One mild allergic reaction was observed, to a preparation containing rosemary oil which was then removed from subsequent mixtures.

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