Short Papers in Pharmaceutical Analysis

001

Analysis of human cytochrome P450 isoforms using nanoelectrospray ionisation tandem mass spectrometry

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Cytochrome P450 (CYP) isoforms are known to be expressed in human cancers, including colon, breast, lung, liver, kidney and prostate. This raises the possibility of the use of CYP isoforms in tumour-specific prodrug activation (Patterson et al 1999). As a prerequisite for this work, protocols must be established for the analysis of CYPs. Nanoelectrospray ionisation tandem mass spectrometry (NSI-MS/MS) has been adopted as the method of choice because of the large amount of protein sequence information that can be obtained from very small volumes of sample.

Recombinant human CYP isoforms 1A2, 2E1 and 3A4 were analysed intact, digested with trypsin in solution or subjected to 1D SDS-PAGE followed by in-gel tryptic digestion and extraction. Approximately 50 pmol of each protein was loaded onto the gels; solution digests contained protein concentrations of 1.7 pmol μL^{-1}. MS/MS experiments were performed on a ThermoFinnigan LCQ^{duo} with a nanospray ion source. Flow rates were less than 50 nL min^{-1}. Samples were analysed as mixtures with no prior chromatographic separation. Protein digest identifications were made using ThermoFinnigan TurboSequest software.

Peptides from each digest have been identified with high confidence using TurboSequest Xcorr and DelCn values (Ducret et al 1998). Table 1 shows the percentage sequence coverages obtained.

Table 1 Sequence coverage of CYP isoforms by amino acid count

CYP	Sequence coverage (single MS analysis)	Total sequence coverage (4–5 MS analyses)
3A4	26%	42%
2E1	36%	54%
1A2	21%	42%

Single-MS-analysis data refer to in-gel tryptic digestions

MALDI-TOF MS, performed by C. Lenz (Applied Biosystems) provided higher sequence coverage per single analysis, at 40–60% by amino acid count. However, data shown in Table 1 is from analysis of the total digest mixture for each protein, with no prior HPLC separation. It is probable that differential ionisation and matrix effects have caused reduced sequence coverage for the NSI-MS/MS analyses, with not all of the tryptic peptides present being observed. The introduction of online nano-HPLC prior to NSI-MS/MS analysis should improve sequence coverage and provide better sensitivity.

Nanoelectrospray mass spectra have been obtained of intact CYP2E1 and 1A2, with average masses of 54446 Daltons and 57410 Daltons, respectively.

It has been shown that human proteins from the cytochrome P450 family can be isolated from polyacrylamide gels and, following tryptic digestion, successfully identified by NSI-MS/MS.

Ducret, A., Van Oostveen, I., Eng, J. K., et al (1998) *Protein Science* **7**: 706–719

Patterson, L. H., McKeown, S. R., Robson, T., et al (1999) *Anti-Cancer Drug Design* **14**: 473–486

002

Sensitive high-performance thin-layer chromatographic method for monitoring plasma levels of rifampicin and its major active metabolite 25-desacetylrifampicin

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Rifampicin (RIF) is a key drug used, in the form of single component formulation or as a fixed dose combination (FDC) formulation with isoniazid, pyrazinamide or ethambutol, for the treatment of tuberculosis. FDC of two, three or four drugs is a preferred formulation for better patient compliance, efficient reduction in viable bacterial population and minimizing duration of the treatment as well as development of resistance to anti-tuberculosis drugs. However, poor bioavailability of RIF from its FDCs has been a subject of much concern and has been critically reviewed (Shishoo et al 2001; Singh et al 2001).

WHO and IUATLD have cautioned that FDCs only with convincingly proven bioavailability of RIF should be used. Most of the methods employed for estimation of RIF in biological fluids are microbiological, spectrophotometric or HPLC methods (Ellard & Fourie 1999). Microbiological and spectrophotometric methods are nonspecific.

The objective of this study was to develop a simple, sensitive and specific HPTLC method for estimation of RIF and its major active metabolite, 25-desacetylrifampicin (25-DAR), in plasma which can be utilized for assessment of bioavailability/bioequivalence of RIF formulations or FDCs.

In the proposed HPTLC method, RIF and 25-DAR were extracted from human plasma (0.5 mL) with ethyl acetate. Ethyl acetate extract was evaporated to dryness and the residue was reconstituted in methanol and spotted on to the precoated TLC plates (silica gel 60 F_{254}, layer thickness, 0.2 mm). The chromatographic separation was performed using a mixture of chloroform–methanol–isopropyl alcohol (9:1:1 v/v) as mobile phase, under following conditions: chamber saturation time, 45 min; temperature, 25 $\pm$ 2°C; migration distance, 35 mm; slit dimension, 4 $\times$ 0.1 mm; wavelength of detection, 333 nm; spraying rate, 10 s μL^{-1}.

The peaks for RIF and 25-DAR (Rf = 0.77 $\pm$ 0.02 and 0.55 $\pm$ 0.02, respectively) were well resolved without any interference from plasma components or related substances (rifampin quinone and 3-formyl rifamycin SV) or co-administered drug (isoniazid). The method was validated and the validation parameters are summarized in Table 1.

Table 1 Validation parameters for the proposed HPTLC method for estimation of RIF and 25-DAR in human plasma

Parameter	RIF	25-DAR
Linearity range (ng/spot)	100–1200	100–1200
Precision (%CV)		
Intra-day	1.78–16.88	0.54–10.69
Inter-day	4.53–15.14	2.95–16.70
Repeatability of measurement	0.31	0.79
Repeatability of sample application	2.20	2.30
% Accuracy	89.02–107.16	103.16–109.44
Limit of detection (ng/spot)	20	40
Limit of quantification (ng/spot)	100	100
Specificity	Specific	Specific
Specific		

Thus, the method was found to be simple, sensitive, precise, accurate and specific for estimation of RIF and 25-DAR in human plasma and can be successfully employed for bioavailability/bioequivalence/pharmacokinetic studies of RIF.

One of the authors (S.S.S.) is thankful to CSIR, New Delhi, for financial assistancc

Ellard, G. A., Fourie, P. B. (1999) *Int. J. Tuberc. Lung Dis.* **3**: S301–S308

Shishoo, C. J., et al (2001) *Indian J. Pharm. Sci.* **63**: 443–449

Singh, S., et al (2001) *Int. J. Pharmaceutics* **228**: 5–17

003

Factors affecting a medicine's erosive potential

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Dental erosion is recognised as a common dental problem and its treatment can be complex. Acidic foods and drinks in the diet are a prime cause of dental erosion. In addition, some medicines with prolonged oral clearance (liquids, syrups, dispersible, effervescent and chewable tablets) may require the addition of weak acids to optimise their palatability and formulation properties. Prolonged oral clearance medicines, often used regularly and long-term in children and the elderly are described by Maguire & Rugg-Gunn (1994) and Baqir & Maguire (2000), and certain formulations and their patterns of use may put some patients more at risk from dental erosion. However, information regarding the erosive potential of these medicines is limited and the effect of sugar content, dose form, dose strength, and branding is unclear.

Our aims were to determine the mean endogenous pH and titratable acidity of 126 formulations with prolonged oral clearance and to assess the effect of sugars content, dose form, dose strength and brand of medicine on the erosive potential of these medicines. Each medicine was prepared according to its dose form. Two 2-mL samples, each diluted with 40 mL of distilled water, were tested at room temperature for endogenous pH using a Pye Unicam pH meter. The sample was then titrated to pH 5.5 and pH 7.0 with 0.1 M sodium hydroxide solution, using a Mettlor Automated Memo Titrator. The titratable acidity was calculated as a standard 5-mL dose for liquids and a single tablet dispersed in 50 mL of distilled water for solids. The data were analysed descriptively and analytically using independent and paired *t* tests and linear regression methods.

Of the 126 formulations tested, 99 were liquids or syrups, 12 were chewable tablets and 15 dispersible or effervescent tablets. Of the 52 generic medicines, 65% were sugar-free, while 62% of the 74 branded formulations were sugar-free. For all medicines, the differences in titratable acidity or mean pH between sugar-free and sugar-containing medicines was not statistically significant. Paired *t* tests showed no difference in mean pH or titratable acidity for sugar content, dose form or brand. However, the difference in mean pH between paired medicines with differing dose strengths was statistically significant ($P = 0.018$). Linear regression showed respiratory medicines ($P = 0.018$) as significant predictors of a lower mean pH and effervescent tablets and nutrition/blood preparations as significant predictors of higher titratable acidity ($P < 0.001$). Conversely, gastrointestinal medicines ($P < 0.001$), dispersible tablets ($P = 0.002$), and proprietary medicines ($P = 0.029$) were significant predictors of a higher mean pH.

It was concluded that the sugar content of a medicine does not predict its erosive potential; more significant predictors are dose form, therapeutic group and brand of medicine.

This project was supported by a SmithKline Beecham/British Dental Association Toothwear/Dental Erosion Research Grant.

Baqir, W., Maguire, A. (2000) *Br. Dent. J.* **189**: 267–272

Maguire, A., Rugg-Gunn, A. J. (1994) *Commun. Dent. Health* **11**: 91–96

004

Validation of cleaning practices and associated analytical chemistry

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The relationships between purposely contaminated surfaces and the extraction or recovery of drug or excipient, from stainless-steel coupons was investigated. The test stainless-steel coupons (10 × 10 × 0.3 cm) were prepared specifically for the trial studies from 316L pharmaceutical-grade manufacturing sheeting having the appropriate highly polished finish (roughness ~1 μm). This approach was taken because of the direct relevance of planar surface and dry residue to pharmaceutical manufacture.

Four criteria, in terms of the fouling of surfaces, were reviewed experimentally. These were deposition, recovery, measurement and interpretation of the consequences of carry-over contamination. The deposition process was investigated with a view to consistently contaminating the coupon surface, which was then studied in further detail. Removal of drug or excipient substrate from the test surface was facilitated by a direct contact, swab procedure and additionally by a combination of both passive and more active rinse techniques.

The data were compared in terms of the effective recovery of three different probe analytes from the contaminated plates. The purpose of the recovery study was to consider the scope or margin of error in terms of an on-going cleaning validation study. Three substances were evaluated, paracetemol, sodium dodecyl sulphate (SDS, as the cleaning agent) and zinc sulphate which was found to act as a dual excipient salt and as the active component in numerous pharmaco-cosmetic preparations. The components were assayed and identified using an array of methods, principally involving conventional ultra-violet spectroscopy and a combination of atomic flame emission and atomic absorption spectroscopy. A regime of varied polarity and acidity-basicity solvents were used to optimise dissolution of the analyte and also act as simple diluents.

The effective removal of the zinc sulphate, 4 ppm/plate, followed a linear decrease when plotted against the logarithm of sequential rinse number. After four rinses no zinc was detected (< 0.05 ppm). Notably, approximately 99.4% of the SDS, deposited from the cleaning solution (0.1% w/v) and used for primary cleaning, was removed from the coupon after the first rinse. The effectiveness in securing the surface contaminant and re-dissolution (recovery) were assessed. With the paracetamol-doped steel coupons, direct surface sampling (swab) proved far less effective that an indirect surface sampling (rinse jet) technique (60–100% recovery). Alginate-based swabs proved most effective (15–40% increase) of the varieties used. The direct surface sampling made use of two different types of spun fibre. The recovery by the swab technique (10–20%) was far less than the 70% recovery, widely considered to be the target level.

The investigation showed both rinsing and swabbing to be effective means of analyte recovery from contaminated surfaces. Monitoring analyte concentration via a re-solubilisation procedure linked to spectroscopy, proved a simple, cheap and effective and therefore, accessible means of monitoring surface contamination. Further work looking at optimum and acceptable re-solubilisation solvents and extraction procedures would prove useful. These data provide important information in an area that greatly concerns the increasingly highly regulated pharmaceutical industry.

005

The influence of relative humidity on the surface energy of a partially amorphous drug substance

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Inverse gas chromatography at infinite dilution was used to determine the surface energies of the crystalline and partially amorphous forms of a drug developed for inhalation delivery using a dry powder inhaler . The surface energy of a partially amorphous drug is shown to vary according to the relative humidity (RH) and this affects the fine particle fraction (FPF) of the dry powder inhaler product (DPI). Levels as low as 3% amorphous are shown to influence FPF.

FPFs were determined for DPIs manufactured using crystalline and partially amorphous batches of the drug (Table 1). As the amorphous content decreases the FPF decreases. The effect is not linked to the particle size of the drug (Table 1). Differences in FPF are shown to be related to surface energy differences between amorphous and crystalline material.

Table 1 γ_D Results for partially amorphous compound 1

Batch	Amorphous (%)	% FPF	Mean particle size (μm)
A	10	46	2.15
Recryst. A	1–3	34	2.16
B	4.5	44.5	2.02
C	1–3	35.5	2.04
D	1–3	31	3.34
Recryst. D	< 1	31	3.18
E	< 1	34	2.34

Table 2 summarises the dispersive surface energy (γ_D) obtained with our drug substance. However, crystalline and partially amorphous batches exhibit the same γ_D at 0% RH. For partially amorphous batches γ_D increases significantly at 35% RH, consistent with the onset of molecular motion above the glass transition temperature. At 45% RH γ_D decreases, corresponding to recrystallisation of the amorphous fraction.

In contrast, the γ_D values for the crystalline batches are unaffected by RH.

Table 2 Dispersive surface γ_D energies at 0, 35 and 45% RH for the partially amorphous drug substance

% Amorphous	γ_D (mJ m^{-2})		
	0% RH	35% RH	45% RH
< 1%	52	49	48
3%	51	56	50
10%	51	64	47
17%	50	62	57

We conclude that differences in FPF between the crystalline and partially amorphous drug are related to their differences in surface energy under the ambient environmental conditions used in product manufacture. These differences affect the interaction of the drug with the lactose carrier and yield different FPF.

006
Chromatography in a different dimension: studies on the 2nd order differentiation of chromatographic traces

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AQ4N (1,4-bis[2-(dimethylamino)ethyl]amino-5,8-dihydroxyanthracene-9,10-dione-bis-*N*-oxide) is currently in Phase I clinical trials with Cancer Research UK. During the initial analytical and formulation development, two AQ4N samples were submitted to the Cancer Research UK Formulation Unit.

An HPLC method was developed and validated which allowed sufficiently accurate assay of the main drug with detection and quantification of related impurities. However the HPLC method demonstrated two potential problems; firstly, low resolution between the main drug and a small impurity (nominally labelled Impurity X) and, secondly, poor linearity of an additional impurity (nominally labelled Impurity Y). Further method development did not improve these problems without compromising other aspects of the assay.

The differentiation of experimental data is used in a wide variety of analytical techniques to improve resolution and reveal information otherwise obscured.

Both the resolution and linearity issues with the AQ4N HPLC assay were resolved by using the mathematical second order derivative of the experimental chromatographic trace. The second-order derivatives were generated by using a Savitzky-Golay series within an Excel spreadsheet.

The poor resolution of the main peak from Impurity X significantly affected the quantification of the latter. A comparison of the relative quantities of Impurity X in AQ4N sample B (with respect to AQ4N sample A), as determined by peak area and second derivative are shown in Table 1.

Table 1 Impurity X contents of samples A and B as determined by peak area and second derivative (data normalised to Sample A result)

Sample	Content determined by peak area	Content determined by 2nd derivative
A	1	1
B	> 0.01	0.17

The quantification of Impurity Y suffered from poor linearity of peak area against impurity content and the use of peak height produced similar results. A comparison of the response factors determined by peak area and second derivative amplitude for Impurity Y at different concentrations are shown in Table 2.

Table 2 Response factors at different concentrations of Impurity Y as determined by peak area and second derivative amplitude (all data normalised to the results from highest Impurity Y standard concentration)

Relative Impurity Y concn	Relative RF peak area	Relative RF 2nd derivative
0.03	0.63	0.86
0.33	0.64	0.90
1	1	1

Although the use of differentiation techniques cannot replace sufficiently developed and validated HPLC methods, the use of this mathematical approach has allowed the development work on this compound to progress without excessive hindrance from the HPLC method.

The work in this laboratory is funded by Cancer Research UK. AQ4N is being developed in partnership with BTG.

007
A stability study of mechlorethamine hydrochloride ointment using direct HPLC measurement of mechlorethamine

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Mechlorethamine hydrochloride (mustine hydrochloride) was originally used as an anticancer drug but newer and more stable bifunctional alkylating agents, such as melphalan and cyclophosphamide have surpassed it in combination chemotherapy. However, another role emerged for the chemotheraputic properties of mustine hydrochloride in the treatment of the skin conditions mycosis fungoides and psoriasis. The treatment involves topical application of an ointment such as 0.01%w/w mechlorethamine in 50/50 liquid paraffin/white soft paraffin.

Because of its high chemical reactivity as an alkylating agent few selective analytical methods have been reported. High-performance liquid chromatography (HPLC) procedures have involved derivatisation with diethyldithiocarbamic acid (DDTC), (Cummings et al 1991, 1993). Colorimetric procedures (e.g. using 4-(4-nitrobenzyl)pyridine; NBP) (Kirk 1985), have been reported but lack selectivity.

We report a selective method for the analysis of mechlorethamine preparations including a topical ointment, by direct HPLC, which is rapid and convenient. Its use in a limited stability investigation of the ointment is also presented.

Validation experiments demonstrated the importance of using anhydrous solvents. Over a period of 42 h in industrial methylated spirit (IMS) mustine hydrochloride lost 60% of its initial peak area and showed the presence of a single decomposition peak. No significant decomposition was detected in absolute ethanol or acetonitrile. In the analysis of the ointment the determined accuracy was 93.1% (RSD = 4.72%, n = 6), with a mean recovery of 92.1% (RSD = 5.89%, n = 3). The precision (repeatability) was 4.93% (RSD, n = 4). A major limitation of the method is the need for an absolute assay of the Mustine Hydrochloride for Injection BP as standard.

Stability samples of mustine hydrochloride (0.01%w/w in 50/50 liquid paraffin/white soft paraffin) were stored at 25°C/60% relative humidity and 7°C over a

period of 18 months. Samples were analysed using the reported method at various time points over this period. Shelf-lives were calculated by determining the upper confidence limit of the rate of decomposition (i.e. the slope of the linear regression of the analytical data). Using this maximum rate method shelf lives of 217 and 218 days, respectively, were allocated.

The results showed no significant difference between the rate of decomposition at 25°C/60% relative humidity and 7°C. The appearance of an oily layer, dispersed with mixing, at 25°C/60% relative humidity was the only physical change in the stability samples.

Cummings, J., MacLellan, A., Smyth, J. F. (1991). *Anal. Chem.* **63:** 1514–1519

Cummings, J., MacLellan, A., Langdon, et al (1993) *J. Pharm. Pharmacol.* **45**: 6–9

Kirk, B. (1985) *Proc. Guild Hosp. Pharm.* **23:** 47–52

008

Selenium plasma concentration differences between age-related macular degeneration patients in comparison to a control group by differential pulse cathodic stripping voltammetry

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Age-related macular degeneration (AMD) can cause irreversible blindness in elderly patients, for which there is no therapy. Antioxidants such as vitamins (A, C and E) selenium (Se) and zinc have possible roles in the prevention or inhibition of this disease. Se (a nonmetallic chemical element) has received much attention from biologists because of its dual role as both an essential trace nutrient and a toxic compound. This interest has created a need for reliable analytical methods for its measurement in biological fluids such as plasma. To date, only a few methods have provided the reproducibility and sensitivity required in biological materials at trace and ultra trace levels. The high sensitivity of neutron activation methods has made them appealing, but the special skill, time and cost of these methods are major drawbacks. A few studies have employed atomic absorption spectrometry, spectrophotometric and fluorometric approaches, but these techniques often require prior chemical separation of Se to eliminate interferences. Electrochemical techniques, especially differential pulse voltammetry, can provide a simple, less expensive and highly sensitive approach for the determination of Se in most matrices.

In this investigation, the determination of Se based on differential pulse cathodic stripping voltammetry was accomplished in hydrochloric acid (0.2 M) with a scan rate of 60 mv s^{-1} and a pulse height of 100 mV by a hanging mercury drop electrode (HMDE). The solution was stirred during pre-electrolysis at –350 mV (vs SCE) for 30 s and the potential was scanned from –350 mV to –800 mV.

Under these conditions, the quantification limit of the method was 5 $\mu g\,L^{-1}$. The calibration curves were linear over the range 0–30 $\mu g\,L^{-1}$ ($R^2 = 0.996$, $P < 0.001$). Repeatability of the method, expressed as the relative standard deviation for Se, was 2.5% and 10.5% at concentrations of 30 and 0.5 $\mu g\,L^{-1}$, respectively. The plasma concentrations of Se in AMD patients were also compared with those in a control group. Although the average Se concentration in the blood of AMD patients (156.6 $\mu g\,L^{-1}$, n = 26) was lower than those of the control group (158.4 $\mu g\,L^{-1}$, n = 16), this difference is not statistically significant ($P = 0.927$). Other scientists have reported different results, such as Mayer et al (1998) who found a significant difference ($P = 0.018$) between age-related maculopathy (ARM) patients (186.6 $\mu g\,L^{-1}$, n = 9) and a control group (207.0 $\mu g\,L^{-1}$, n = 10).

There is therefore a need for a prospective study involving a large number of patients to obtain conclusive data.

Mayer, M. J., van Kuijk, F. J. G. M., Ward, B., et al (1998) *Acta Ophthalmol. Scand.* **76**: 62–67

009

Stability of hydrocortisone sodium succinate in PVC and non-PVC bags and in polypropylene syringes

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Parenteral aseptic preparations of hydrocortisone sodium succinate (HSS) are used frequently in hospitals, but little definitive stability data is available. The purpose of this study was to obtain the ultimate shelf life for the formulations so that they may be prepared in bulk in appropriately licensed facilities.

In the first study, the stability of HSS, 1 mg mL^{-1}, was determined in polyvinyl chloride (PVC) bags and non-PVC bags, in 0.9% sodium chloride at 7°C, at 25°C/60% relative humidity (RH) and at room temperature in the light (RTL) with storage for up to 135 days. In the second study, the stability of HSS, 50 mg mL^{-1} was determined in polypropylene syringes at 5°C and at 25°C/60% RH with storage for up to 120 days.

Samples from each admixture were analysed for HSS concentration by stability-indicating high-performance liquid chromatography. The HPLC system consisted of a 150 mm × 4.6 mm Lichrospher C_{18} column with UV detection at 254 nm and a mixture of phosphate buffer pH 7.0, 0.1M mixed B.P and methanol (50:50 v/v) as the mobile phase. The flow rate was 1.2 mL min^{-1}. The samples were also monitored for pH, appearance of solution and container, and the rate of appearance of decomposition products.

Shelf lives were calculated after derivation of the upper 95% confidence limit of the rate of decomposition (i.e. the slope of the linear regression of the analytical data). This corresponds to the maximum rate of decomposition represented by the analytical data. The shelf lives were assigned on the basis of the time required for the active agent (HSS) concentration to fall by up to 10% of its initial value.

The shelf lives were assigned as shown in Tables 1 and 2.

Table 1 PVC and non-PVC bags

Temperature condition	PVC (days)	Non-PVC (days)
7°C	41	48
25°C/60% RH	8	8
RTL	7	6

Table 2 Polypropylene syringes

Temperature condition	Polypropylene syringes (days)
5°C	81
25°C/60% RH	6

010

Development of a solid phase extraction (SPE) method using HLB cartridges for rapid, simultaneous HPLC determination of prednisolone and cortisol in plasma and urine

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One of the main side effects of prednisolone (P) and high-dose inhaled steroids is the suppression of plasma and urinary cortisol (C). Only three articles have been published concerning the simultaneous determination of cortisol and prednisolone in blood and urine. However, they are limited by either using liquid-liquid extraction (LL) which is time consuming and has several disadvantages or by using conventional silica-based (SPE) cartridges which can run dry during the extraction process, resulting in variable recovery of the analyte. A specific HPLC method used for determination of P and C in plasma (Adair et al 1992) has been modified and validated for use in both plasma and urine. This abstract describes the

development and validation of a simple SPE method for extraction of prednisolone and cortisol from plasma and urine using one washing step and utilising Oasis hydrophilic lipophilic balanced cartridges (HLB) (1 mL/30 mg, 30 μm). The cartridges were first conditioned with 1 mL of 100% methanol and 1 mL of water. The plasma or urine samples (1 mL) were mixed with 0.05 mL of internal standard (beclometasone) solution and loaded onto the cartridges. They were then washed using 2% NH_4OH in 40 % aqueous methanol for plasma samples or 2% NH_4OH in 50% aqueous methanol for urine samples. P and C were then eluted using 100% methanol. These percentages, used in washing and elution, were chosen after repeated analysis using different percentages of aqueous methanol and examination of the corresponding chromatograms. After elution, collected fractions were evaporated to dryness under nitrogen and then reconstituted in 350 μL of the mobile phase and 120 μL was injected. Recoveries of P and C from plasma and urine were more than 82% (CV was less than 10%). The limit of detection and limit of quantification in both plasma and urine was 1 and 5 $ng\,mL^{-1}$ for C, respectively, and 5 and 10 $ng\,mL^{-1}$ for P, respectively. The intraday and inter-day precision (measured by CV%) for both P and C in both plasma and urine was always less than 7%. The accuracy (measured by relative error %) for both P and C in both plasma and urine was always less than 8%. The advantages of the developed method are the use of a one-step washing SPE utilizing HLB cartridges which do not suffer the drying out problems of conventional SPE cartridges and the time saving when compared with LL extraction, in addition to the simultaneous determination of P and C in both plasma and urine. This method has been used successfully in measuring plasma and urinary cortisol suppression and prednisolone concentrations in 36 patients with asthma.

Adair, C., et al (1992) *Br. J. Clin. Pharmacol.* **33**: 495–499

011
Atomic force microscopy investigation of the effect of DNase I on DNA-PAMAM dendrimer complexes

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Polyelectrolyte complexes between polyamidoamine (PAMAM) dendrimers and DNA have emerged as potential non-viral vectors for DNA delivery. Methods for analysing the ability of PAMAM dendrimers to protect the DNA from degradation enzymes such as DNase I are essential (Bezanilla et al 1994). The atomic force microscope (AFM) can image individual DNA molecules and DNA–enzyme interactions in aqueous solutions through a unique combination of nanometer scale spatial resolution, millisecond temporal resolution and Pico Newton force sensitivity without the need for staining, shadowing or labelling. Here, we have applied AFM in liquid to follow, in real time, the effect of DNase I on generation 4 PAMAM dendrimers complexes with DNA. DNA–dendrimer complexes were formed by incubating the two components together in deionized water at different dendrimer–DNA ratios. The DNase I was then either added to the complex on mica surface (on-surface), or incubated with the complex in solution before adsorption onto mica surface (in-solution). Our studies show that, on-surface, complete degradation of bare DNA was observed within 20 min. At a dendrimer–DNA ratio of 0.5:1, fragmentation of the complex was observed after 60 min, while for 1:1 ratio, the complex relaxed to a maximum diameter consistent with the relaxed DNA after 127 min without fragmentation. For in-solution samples, it was impossible to follow the degradation of bare DNA. While the cleavage rates of complexes were more rapid than on-surface, the effect of DNase I on the complexes was delayed according to the DNA–dendrimers ratios used. Furthermore, differences in the cleavage rates of the DNase I on-surface and in-solution suggest the restricted accessibility of the surface confined DNA. The approach developed here allows the direct correlation of DNA–polymer complex structure to resistance to degradation by Dnase I, a potentially key step in developing successful gene delivery vectors.

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Bezanilla, M., Drake, B., Nudler, E., et al (1994) *Biophys. J.* **67**: 2454–2459

012
Chemical images for analysis of pharmaceutical formulations

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Chemical maps and images can provide information on particle size, adjacencies, etc., of the different components within a pharmaceutical formulation. Raman maps from dispersive systems and near infrared spectroscopic images have been collected from the same area of a sample and the data combined in a process called Chemical Image Fusion (Clarke et al 2001). The applicability of this technique to pharmaceutical formulations is illustrated through characterisation of standard formulations of excipients.

The time required for data acquisition has been a drawback, especially in the collection of Raman maps. We have now moved from Raman point mapping to a line focus system. The result is a great improvement in data acquisition time but with a compromise being made between spectral and spatial resolution. The practical problems with using a line focus system are discussed along with the chemometric methods required for data processing.

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013
Evaluation of sandalwood essential oil quality

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The use of essential oils in complementary medicine, particularly aromatherapy, and also in the cosmetic and perfumery industries is becoming increasingly popular. Sandalwood (*Santalum album*), which is indigenous to peninsula India, is the subject of international concern regarding its sustainability and quality. *S. album* is associated with numerous problems that contribute to declining resources, in particular a high incidence of smuggling, which prevents the occurrence of natural regeneration and establishment of artificial regeneration. In view of the current issues associated with the conservation status of plant material, an investigation was conducted to assess the quality of sandalwood essential oil being used in the trade.

High quality *S. album* wood may be substituted with inferior quality wood or the oil may be adulterated with synthetic substitutes, which can influence the chemical composition and physical properties of the oil. It is recognised that the essential oil from *S. album* should not contain less than 90% w/w of free alcohols, calculated as santalols (Martindale 1972; Food Chemicals Codex 1981). It is apparent, however, that the specifications for the santalol content of sandalwood essential oil generally lack specificity (e.g. specification of the isomeric form of the santalols). More recently it has been suggested that sandalwood oil should be evaluated by quantitation of the α- and β-santalol content, with a proposed range of 40–55% for α-santalol and 17–27% for β-santalol (Verghese et al 1990).

In this study, twenty-six samples of essential oils claimed to be *S. album*, were assessed using gas chromatography-mass spectroscopy (GC-MS) and further analysis was conducted using GC with flame-ionisation detection (FID). Eight samples of *S. album* essential oil that were prepared between 1880 and 1890 were also assessed, and sandalwood oils from Indonesia, Fiji, Brazil (*S. album*) and Australia (*Santalum spicatum*) were also investigated for their santalol content. The results showed that none of the oils assessed complied with the specified 90% santalol content. The majority of oils that were claimed to be *S. album* (including those prepared between 1880 and 1890) were composed of 40–70% santalols (α- and β-). In addition, one sample contained $<1\%$ α- and β-santalols. The total santalol content (α- and β-) of the Australian essential oils was in the range of 0.3–43%.

In conclusion, it is evident that sandalwood oil composition may vary depending on its origin, which may reflect current international demand and declining resources, perhaps as a result of collection methods and adulteration. It is also apparent that current specifications (90% santalol content) require re-evaluation, in the light of the results obtained from this study, and with consideration of the more efficient analysis methods available.

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014
Investigation of cytotoxic compounds of the British alga, *Polysiphonia lanosa*

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Polysiphonia lanosa is a marine red alga that usually grows epiphytically on the brown alga *Ascophyllum nodosum*. *P. lanosa* is rich in brominated phenolic compounds.

In a previous study (Shoeib et al 2001), the chloroform fraction of *P. lanosa* was found to have cytotoxic activity against DLD-1 cells (human colon cancer), and the chloroform fraction was further fractionated and three of the fractions showed higher activity than the parent fraction. In this study the phenolic compounds of the most active fraction were identified by GLC-MS examination followed by trials to synthesize the major compounds in this active fraction and investigate their cytotoxic activity.

The marine alga was collected from the South coast of England and quickly dried at 50°C. Tthe dried alga was extracted with methanol and the extract was concentrated under reduced pressure. The chloroform fraction was prepared by partitioning the methanol extract between chloroform and water. This fraction was further fractionated by silica-gel column chromatography.

DLD-1 cells were cultured according to a standard procedure. Cells were incubated for 96 h in the presence of extracts and cytotoxicity was then determined by a modification of the MTT colorimetric method.

The chloroform fraction and the three active fractions were found to have IC50 values of 4.58, 4.19, 2.77 and 2.35 $\mu g\,mL^{-1}$, respectively. The most active column fraction was subjected to GLC-MS analysis. Identification of the bromophenols was made from the mass spectra of the trimethylsilyl-derivatives after GLC (Pedersen et al 1974). Three major compounds were identified as lanosol (2,3-dibromo-4,5-dihydroxybenzyl alcohol), the aldehyde of lanosol (5,6-dibromoprotocatechualdehyde) and an ether of lanosol (3,4-dibromo-5-(ethoxymethyl) pyrocatechol). As the amount of material available was insufficient to enable isolation of pure compounds, the synthesis of these compounds was attempted.

Attempts to use published methodology (Lundgren et al 1979) for the synthesis of lanosol were unsuccessful as 5-bromo, and 2,5 dibromo analogues of catechualdehyde were obtained instead. The alcohol of the 2,5- dibromo derivative was prepared by the reduction of the latter with potassium borohydride. The former compounds were found to have IC50 values against DLD-1 cells of 6.02, 9.19 and 4.47 $\mu g\,mL^{-1}$, respectively.

Currently, another route of synthesis starting from vanillin to prepare lanosol and related compounds present in the active fraction and the evaluation of their cytotoxic activities are in progress.

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015
Isolation of a keratinocyte proliferation inhibitor from *Vernonia anthelmintica* (willd.) seeds traditionally used for psoriasis

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The seeds of *Vernonia anthelmintica* (Willd.) (Asteraceae) (VA) have been used traditionally in Indian medicine to treat skin disease psoriasis (Kirtikar et al 1935). A major pathological characteristic of this common skin disorder is the hyperproliferation of the epidermis, which in psoriatic plaques is 12 times the normal rate (Herfindal et al 1992).

In this study, various organic extracts of VA seeds were assayed for inhibitory activity on the growth of the fast-growing human keratinocyte cell line, SVK-14, which was used as an in-vitro model for the hyperproliferative epidermis. Experiments were carried out in 96-well plates containing 5000 cells/well. Plates were incubated for 24 h after which extracts (0.78–200 $\mu g\,mL^{-1}$) were added and incubation continued for 3 days. Cell number at the end of this period was assessed using sulphorhodamine B dye (Skehan et al 1990). A methanol extract of the VA seeds (MET) was identified to display good inhibitory activity of cell division with an IC50 value of 21.6 $\mu g\,mL^{-1}$.

Preliminary bioassay guided fractionation (by silica gel column chromatography) of MET extract highlighted the concentration of inhibitory activity in one main fraction (F1) (85mg) which had an IC50 value of 4.9 $\mu g\,mL^{-1}$. The major active, inhibitory compound in F1 was isolated using further silica gel columns and preparative reversed phase HPLC, and was found to have an IC50 value of 21 $\mu g\,mL^{-1}$ in the bioassay. It was identified from ^{1}H, ^{13}C and 2D NMR spectra recorded in $CDCl_3$ using tetramethylsilane (TMS) as the internal standard, as well as LCMS, as vernodalol, a sesquiterpene elemanolide lactone.

These studies suggest that vernodalol may be the main active anti-psoriatic compound in VA. The results are in agreement with Jisaka et al (1993) who reported similar cytotoxic activity with this compound, which was isolated from *Vernonia amygdalina*. The cytotoxic activity of *Vernonia anthelmintica* has not previously been reported. Our spectroscopic studies have enabled, for the first time, the unambiguous assignment of 3-carbonyl signals and six olefinic carbon signals in this molecule.

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016

New methods for the isolation of the principal cannabinoids in *Cannabis sativa*

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The principal cannabinoids found in herbal cannabis are either Δ^9 tetrahydrocannabinolic acid (Δ^9THCA) or cannabidiolic acid (CBDA), together with small quantities of the corresponding neutral cannabinoid, Δ^9 tetrahydrocannabinol (Δ^9THC) or cannabidiol (CBD), respectively. Extraction of neutral cannabinoids from the botanical raw material (BRM) using liquid CO_2 involves an initial heating step which consequently decarboxylates the cannabinoid acids. The liquid CO_2 extract therefore contains Δ^9THC or CBD as the principal cannabinoid.

To provide quantities of neutral cannabinoids and precursor acids for laboratory analytical reference purposes and for pharmacological studies, there is a need to isolate and characterise these compounds in a pure state. Methods are described for the reproducible and reliable isolation of the principal cannabinoids found in chemovars of *Cannabis sativa*, viz. THCA, CBDA, THC and CBD.

Low pressure column chromatography separations were carried out and qualitative compositions of the collected fractions were monitored by thin-layer chromatography. The composition of the isolated products was determined by HPLC and capillary GC. Further purification of these isolates produced high-purity cannabinoids, which were frequently crystalline.

The use of column chromatography on a non-polar extract of decarboxylated herb produces Δ^9THC as a colourless low melting point solid with a chromatographic purity of > 98%. The yield of purified Δ^9THC from crude extract with a typical content of 60% w/w, is 43% (i.e. 3.5 g of extract yields 1.5 g of Δ^9THC). The identity of the product was confirmed by comparison of FT-IR, GC and HPLC retention-time data of an authenticated standard.

CBD is obtained as a white crystalline solid (> 98% chromatographic purity). The yield of crystalline CBD from 3 g of crude extract, with a typical CBD content of 60% w/w, is 1.0 g (i.e. 33% yield on a mass basis). Identity of product was confirmed by NMR data and from comparison of FT-IR spectra and chromatographic retention times to that of an authenticated standard. Melting point was 64.66°C (lit. value 66–67°C).

Crystalline Δ^9THCA and CBDA of high chromatographic purity have also been obtained from column chromatography of a non-polar extract of BRM. Both occur as pale yellow crystalline solids and have been extensively characterised. The extract from 100 g of BRM yielded approximately 5 g of purified cannabinoid acid. Melting points were: CBDA, 45–48°C and THCA, 70°C.