Short Papers in Pharmaceutical Analysis

001
Raman mapping of modified release beads

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Increasingly pharmaceutical dosage forms are being developed in which the rate of release of the active ingredient has in some way been modified compared with conventional formulations. The European Pharmacopoeia defines modified release as a modification of the rate or place at which the active substance is released. The release may be delayed for a finite lag time after which release is unhindered or prolonged by reducing the release of the active after administration. The objective of the modification may be to maintain therapeutic activity without frequency dosing, to reduce toxic effects or for some other therapeutic purpose.
Drug products under development are routinely analysed by methods such as dissolution testing to assess their performance. These blunt tools will identify variations in the products, which may for example be due to changes occurring in the formulation when stored at increased temperature or humidity. The cause of these variations cannot always be determined, in which case Raman mapping can be used as a sharp tool to provide molecular information on a microscopic scale about the product.
Here we report the use of Raman mapping to help in the design and assessment of two modified-release formulations, X and Y, in development. Raman line mapping was carried out using a high-power 785 nm diode laser. The spectral ranges collected depended upon the formulation being investigated and were chosen to best discriminate between all the materials present in the formulations. A typical spectral range would span 500 cm^{-1}. The hyper-spectral data cubes (that is, spectral and spatial data from the sample) were then processed using principal component analysis to produce chemical images to visualise the differences between samples.
The cause of variations in dissolution profiles between batches of formulation X was identified as drug migration into the control release polymer layer. A new formulation was developed as a result of this, which showed the expected dissolution profile. Beads from formulation X were found to have a complex drug layer composition. To better understand the drug layer, Raman maps were collected at different stages of the manufacturing process. These maps show the stages of formation of the complex drug layer. Beads on stability trials for each of the formulations X and Y were analysed to determine whether any changes had arisen on storage.

002
In-vitro cytotoxic activities of the major bromophenols present in the British alga, *Polysiphonia lanosa*

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The red alga *Polysiphonia lanosa* is rich in brominated phenolic compounds. The chloroform fraction of *P. lanosa* containing the brominated phenols was reported to have cytotoxic activity against DLD-1 cells. This fraction was further fractionated (Shoeib *et al* 2002) and three of the fractions showed higher activity than the parent fraction. The phenolic compounds of the most active fraction were identified by GLC-MS analysis and synthesis was employed to prepare the major compounds. In this study, five compounds were synthesised and their cytotoxic activity was evaluated.
The major compounds identified by GLC-MS were lanosol (**1**; 2,3-dibromo-4,5-dihydroxybenzyl alcohol), the ethyl ether of lanosol (**2**; 2,3-dibromo-4,5-dihydroxybenzyl ethyl ether), the aldehyde of lanosol (**3**; 5,6-dibromoprotocatechualdehyde), the methyl ether of lanosol (**4**]; 2,3-dibromo-4,5-dihydroxybenzyl methyl ether) and the propyl ether of lanosol (**5**; 2,3-dibromo-4,5-dihydroxybenzyl propyl ether).
The first attempt to synthesise lanosol using the published methodology (Lundgren *et al* 1979) was unsuccessful as 5-bromo, and 2,5 dibromo analogues of catechualdehyde were obtained instead. Another route starting from vanillin was successful.
Vanillin was brominated using bromine and acetic acid under reflux (Kubo *et al* 1990). The 2,3 dibromo analogue of vanillin was obtained and this was demethylated by boron tribromide to give the aldehyde of lanosol. Lanosol was prepared by the reduction of the latter with potassium borohydride. The ether derivatives of lanosol were prepared by refluxing lanosol with the corresponding alcohol.
DLD-1 and HCT-116 cells (human colon cancer) were cultured according to a standard procedure. Cells were incubated for 96 h in the presence of compounds and cytotoxicity was then determined by a modification of the MTT colorimetric method. The cytotoxic activities are represented in Table 1

Table 1 Cytotoxic activity of the synthetic compounds (n = 3)

Compound	IC50 (μM) against DLD-1 cells	against HCT-116 cells
1	18.27 ± 0.94	20.39 ± 2.90
2	13.46 ± 2.27	2.51 ± 0.95
3	30.94 ± 2.70	NT
4	14.56 ± 3.07	14.14 ± 2.50
5	12.35 ± 1.14	1.32 ± 0.30

Means ± s.d.

The propyl ether of lanosol (**5**) was the most active compound against both cell lines. Interestingly, its activity against HCT-116 cells was nearly 10-fold higher than against DLD-1 cells.

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003
Stability of dobutamine 500 mg in 50 mL CIVAS pre-filled syringes

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A risk assessment of the Central Intravenous Additive Service (CIVAS) products prepared at Guy's and St Thomas' Hospitals NHS Trust has identified dobutamine 500 mg in 50 mL prefilled syringes as being the highest risk (Taki *et al* 2003). The preparation of CIVAS dose units as individual items (rather than manufacture involving a homogenous bulk intermediate) means that quality control testing for drug content has limited value as no unit is representative of the batch. However, the Medicines Control Agency has advocated periodic evaluation of final product for drug content as a quality assurance (QA) exercise. Such testing requires a specific quantitative assay, the availability of which would also allow stability testing and expiry date verification. The aim of this study was to develop an HPLC method for dobutamine for use in regular QA activity and to verify the expiry date of dobutamine prefilled syringes.
An HPLC assay was developed using a Merck-Hitachi system incorporating a L-6200 Intelligent pump, L-400 UV detector and D-2500 chromo-integrator. The system was fitted with a 20-μL injection loop and separations were carried out on a HiChrom Excel Range column 100 mm × 4.5 mm i.d. octadecylsilane, 10% carbon loading with hypersil 5 μm packing. The mobile phase comprised acetonitrile/methanol/KH_2PO_4 0.05 M (82:12:6 v/v/v) plus triethylamine 0.3% v/v, at pH 4.0. The flow rate was 1.0 mL min^{-1} with detection at

280 nm. Using dobutamine hydrochloride powder (USP, Rockville, USA) as a standard, an assay was developed and established as fit-for-purpose according to standard requirements. Specificity was assessed by reference to acid/alkali, temperature and photo- degraded dobutamine solutions. Linearity precision and accuracy were determined.

For stability testing, a batch of dobutamine pre-filled syringes was prepared. Syringes were stored at 4°C protected from light (recommended storage conditions), 40°C protected from light, and room temperature in the light. Dobutamine content was measured in triplicate at t = 0, 3, 7, 14, 21, 28, 35 and 42 days.

Using the HPLC method described above, dobutamine had a retention time of approximately 7 min. The assay was specific for dobutamine and linear ($r^2 = 0.9999$). Intra-day and inter-day accuracy and precision were acceptable (intra-day n = 6, deviation < 0.76%, relative standard deviation < 0.86%; inter-day n = 8, deviation < 1.18%, relative standard deviation < 0.90%). Syringes assayed immediately post preparation were all within specification with a uniform dobutamine content (n = 4). Throughout the study the dobutamine content of syringes stored under all conditions was within ± 5% of 10 mg mL^{-1}. The maximum deviations were recorded at the end of the study (t = 42 days) when the loss of dobutamine in the syringes stored at 40°C protected from light (−3.2%) was greater than that in the syringes stored at 4°C (−1.7%) or room temperature (−1.9%).

The HPLC method will permit regular QA testing to verify dose accuracy in accordance with the product specification. The stability data indicates that the product is stable beyond the 28-day expiry date currently assigned.

Taki, M., *et al.* (2003) A novel approach to risk assessment of CIVAS products. *BPC:* submitted

004
Isolation and hormonal properties of phenolphthalein from the seeds of ripe bitter melon (*Momordica charantia*)

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Many medicinal plants used traditionally by ancient Indian physicians have recently been tested for anti-fertility activity (Hiremath *et al* 1999). In a continuation of this search for suitable contraceptive molecules from the natural sources, extracts from the seeds of *Momordica charantia* (Cucurbitaceae) were screened for anti-fertility activity using proven fertile albino rats. As the ethanol extract of the seeds had exhibited both estrogenic and androgenic properties (Naseem *et al* 1998), it was decided to proceed to attempt to isolate and identify active constituents from the extract. As in the original study, the shade dried seeds of *M. charantia* were powdered and subjected to Sohxlet extraction with various solvents with increasing polarity (i.e., petroleum ether, chloroform and ethanol) for 48 h each. The extracts were concentrated to dryness under reduced pressure and controlled temperature at 50°C. The ethanol extract was then subjected to column chromatography with silica gel G (60–120 mesh) as the adsorbent using the mobile phase (benzene–methanol 80:20 v/v). Five major fractions were collected and evaporated to dryness. HPLC analysis of these five semi-purified fractions was conducted on a Spherisorb ODS 1 column (25 × 4.6 mm i.d.) at ambient temperature, using mobile phase (methanol–water 50:50 v/v) at a flow rate of 2 mL min^{-1} with injection volume of 20 μL. UV detection was at 215 nm. Three of the fractions exhibited very similar profiles showing a prominent early-eluting peak (k 0.9) and accordingly these were combined for the subsequent preparative HPLC carried out with a view to isolating a pure sample of the compound giving rise to this peak. The preparative HPLC of the combined semi-purified fractions was conducted under broadly equivalent conditions to the analytical work using a 25 × 22 mm i.d Spherisorb ODS 1 column at ambient temperature with the same (methanol–water 50:50 v/v) mobile phase but at a flow rate of 8 mL min^{-1} to allow for the scale-up and an injection volume of 2 mL to allow for scale-up and increase sample loading and thereby throughput. Sample concentrations were 7 mg mL^{-1} and UV detection was again at 215 nm. Structural elucidation using NMR (^{1}H, ^{13}C, HMQC, HMBC), LC-MS and infrared data and then comparing with data from an authentic sample revealed that the component isolated from the crude extract of *M. charantia* was, surprisingly, phenolphthalein. This obviously led to a review of the literature but there was no evidence of this well known analytical laboratory reagent having been previously found in natural sources. The isolation and identification work was followed up by anti-fertility testing with pure phenolphthalein to confirm that it had both androgenic properties as well as its known estrogenic properties (Nieto *et al* 1990).

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005
The compatibility and stability of five drugs frequently used in syringe drivers in the palliative care of cancer patients

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The management of symptoms of cancer patients in palliative care often requires subcutaneous infusions of multiple drug mixtures. The most common drugs premixed prior to administration are analgesics, antiemetics, sedatives and anticholinergics (Back 2001). A single syringe driver is desirable for ease of refilling, monitoring, patient comfort, mobility, and for the efficient use of available resources (Hughes *et al* 1999). The use of multiple drugs in a single syringe can result in potential problems with drug–drug interactions including increased degradation of drugs in the mixture, resulting in reduced efficacy and symptom control. Precipitation of the drugs and the formation of new and potentially toxic by-products can also occur. The aim of this investigation is therefore; to provide evidence for the compatibility and stability of five commonly used drugs used in the palliative care of cancer patients.

The following drugs were chosen for their importance and frequent use in clinical practice: diamorphine, metoclopramide, levomepromazine, midazolam and hyoscine hydrobromide. The drugs were investigated individually, and in three mixes; diamorphine and metoclopramide, levomepromazine and midazolam and a mix containing all four drugs. The drugs were diluted to the appropriate concentrations used in clinical practice, and stored in 20-mL polypropylene syringes in the dark at 0–4°C, 20–24°C and 37°C. Samples were taken daily for five days (96 h) and run on a calibrated HPLC system (column: Phenosphere 5μ C18 (260 × 4.8 mm); mobile: acetonitrile/phosphate buffer 1.0 mL min^{-1}; detection: UV 254nm). The results were displayed graphically and predicted time for 10% degradation ($T_{90\%}$) calculated using first order kinetics.

The results showed that all drug combinations were stable for at least 48 h at all storage temperatures (Table 1).

Table 1 Drugs and drug mixes classified unstable for 96-h time period

Drug mix	Temp.	< 48 h	72 h	96 h	T90% (h)
Hyoscine hydrobromide	20–24°C	Y	Y	N	83
	37°C	Y	Y	N	76
Diamorphine and Metoclopramide	37°C	Y	N	N	69
Levomepromazine and Midazolam	20–24°C	Y	Y	N	85
	37°C	Y	Y	N	75

Y = stable, N = not stable

It can be concluded that the drug mixes investigated are safe to use at any temperature likely to be experienced during the course of a standard 24-h syringe driver infusion. If a longer storage or administration period is to be considered, care needs to be taken with the drugs and drug mixtures identified in Table 1, due to their relative lack of stability in storage in 20-mL polypropylene syringes.

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006
Use of Fourier Transform (FT-) Raman spectroscopy to detect protein denaturation in solid state after thermal stress and correlation with biological activity

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Proteins require their native structure to be biologically active and their conformation is important in developing protein pharmaceuticals. Proteins may denature with structural changes under stresses such as heat and the molecule loses its activity. Thus, in an attempt to identify the influence of the protein's initial solid-state form on its propensity to thermal denaturation, changes in the secondary structure due to thermal stress were studied for two model proteins, lactate dehydrogenase (LDH) and trypsin, in commercial and spray dried forms.

More specifically, our aim was to assess the use of FT-Raman spectroscopy to study changes in conformation of heated solid protein samples from a differential scanning calorimeter (DSC). The spectra of thermally treated samples were compared with those of unheated proteins, to determine if these changes affect the biological activity, and to probe differences between commercial and spray dried proteins upon heating.

DSC (solid state) was employed to heat protein samples to two temperatures, one before the apparent denaturation temperature, T_m, and the other after the T_m. Accordingly, LDH was heated from 25 to 150°C or to 250°C at 20°C min^{-1}. Trypsin samples were raised from 25 to 125°C or to 225°C at 10°C min^{-1}. Treated samples were analysed using FT-Raman, along with untreated materials. Untreated and thermally treated LDH and trypsin were assayed enzymatically.

DSC analysis indicated that T_m of LDH was ~153°C and that of trypsin was ~220°C. It is known that proteins are more stable in dry state than in aqueous state, so T_m of solid protein is substantially higher than that of aqueous state due to increased flexibility in presence of water. FT-Raman spectra showed that commercial and spray dried LDH heated to 150°C exhibited a small shift in a band at 1554–1552 cm^{-1}, and trypsin heated to 125°C showed a shift of a band at 1341–1339 cm^{-1}. There were larger changes in the spectra of LDH heated to 250°C as all characteristic bands disappeared. Spectra of trypsin samples heated to 225°C revealed perturbations to the fresh sample (splitting and shifting of amide I band from 1669 to 1665 cm^{-1}, appearance of a new band at ~1785 cm^{-1} and decrease intensities of all bands). Biological analysis supported FT-Raman data as samples heated below their T_m showed some activity (approximately 50%), while materials heated to the higher temperature were insoluble, with complete loss of activity.

In conclusion, heating of dry proteins above their T_m perturbed the secondary structure as indicated by FT-Raman and complete loss of activity in biological assay. Thermal stress affected the commercial and spray dried LDH and trypsin to the same extent. FT-Raman is a useful method to detect protein structural changes due to thermal stress and its results correlate with enzymatic activity data.

007
Antibacterial and antifungal medicinal plants of Botswana

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Given the alarming incidence of antibiotic resistance in bacteria of medical importance (Monroe & Polk 2000), there is a constant need for new and effective therapeutic agents. Plants define constant interest as a source of antimicrobial agents (Chariandy *et al* 2000) particularly as plant-derived medicines have been part of the traditional healthcare in most parts of the world for thousands of years. In many developing countries where western medicine is inaccessible, too expensive or not accepted by the people, the majority of the population still relies on traditional herbal remedies (Van Staden *et al* 2000). Indigenous medicines used in Botswana have not yet been studied extensively, thus the antimicrobial activity of selected medicinal plants sourced from herbalists in Botswana is reported.

Chloroform and ethanol extracts from different parts of nineteen traditional Botswana medicinal plants were evaluated for their therapeutic potential (i.e. antimicrobial compounds), against a selection of Gram-positive (*Staphylococcus aureus* NCIMB 9518, *Bacillus subtilis* NCTC 10073) and Gram-negative (*Escherichia coli* NCTC 9002 and *Pseudomonas aerogenosa* NCIMB 10421) bacteria and *Candida albicans* NCPF 3179, employing the agar-well diffusion and micro dilution assays.

Of the nineteen-screened species, the following were active: chloroform extracts (6 mg mL^{-1}) of *Clerodendrum uninatum* (Verbenaceae), *Commiphora glandulosa* (Burseraceae), *Commiphora marlothii* (Burseraceae), *Cassine transvaalensis* (Celastraceae) showed significant activity against Gram-positive microorganisms. Active crude extracts exhibited minimum inhibitory concentrations of 125–500 μg mL^{-1} against Gram-positive bacteria with the micro dilution assay.

Generally, both chloroform and ethanol extracts inhibited *Bacillus subtilis* more than *Staphylococcus aureus*. None of the extracts showed activity against the Gram-negative microorganisms. Chloroform extract (10 mg mL^{-1}) of the *C. uninatum* root showed activity against *Candida albicans*. The results indicate that these medicinal plants represent an untapped source of potentially useful antimicrobials. Further work relating to the isolation of the active constituents is in progress in our laboratories.

Funding for this work is provided by Botswana College of Agriculture, Botswana. The traditional healers are thanked for guiding in the selection of plants.

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008
Serum triglycerides determination with lipase, glycerol kinase, glycerol-3 phosphate oxidase and peroxidase co-immobilized onto alkylamine glass beads

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Triglyceride level in serum is considered to be important as a risk factor for coronary artery disease, important in terms of diagnostic value and for determination of low-density lipoprotein cholesterol (Klotzsch & McNamara 1990). Most of the methods, like chemical (Van Handel & Zilversmit 1957), enzymic fluorimetric (Voysey & Wilton 1994), bioluminescent (Bjorkhem *et al* 1982; Kukis *et al* 1983) and chromatographic methods available for triglyceride estimation in serum, have not become commercially popular, because of their poor precision, expensive instrumentation, pretreatment and derivatization of the analyte. On the other hand, ease of operation, good accuracy and precision offered by the enzymic colorimetric method (Fossati & Prencipe 1982) makes it the most commercially viable method for triglyceride estimation, for which enzo kits are also available. Bulk quantity of expensive free enzymes required by the kits and other quantitative systems make them expensive for routine estimation of large clinical samples. Immobilization of enzymes on a support serves the primary purpose of facilitating their recovery from reaction mixture and also often increases their stability and thus reduces the cost of the procedure.

A simple method for discrete analysis of serum triglycerides has been developed to economize available commercial enzokit methods. Lipase, glycerol kinase, glycerol-3-phosphate oxidase and peroxidase were co-immobilized onto alkylamine glass beads (pore diameter 55 nm) through glutaraldehyde coupling.

In the method, H_2O_2 generated from triglyceride by a series of reactions catalysed by the immobilized enzymes is measured at 510 nm by a colour reaction consisting of 4-aminophenazone and 3,5-dichloro-2-hydroxy benzene sulfonate (DHBS) and immobilized peroxidase as chromogenic system. An interference study of various metabolites at their physiological concentration was conducted. Storage stability and reusability of the co-immobilized enzymes was also studied. The method was validated with respect to linearity, precision, accuracy and correlation. The minimum detection limit of the method was 0.54 mmol L^{-1}. The analytical recovery of added triolein in serum was 97.55 ± 1.5% (mean ± s.d). The within and between batch coefficient of variation (CV) were < 1.5% and < 1.35%, respectively. Mean value of serum triglycerides as determined in clinical samples, showed a good correlation (0.9840) with those by the Sigma kit method.
Besides increasing the stability of the enzymes on co-immobilization, the method gave linearity, precision and accuracy comparable to the available methods and was found to be cost effective.

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009
Antimicrobial and antioxidant properties of two Ghanaian plants used traditionally for wound healing

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Wound healing is an underestimated aspect of healthcare but important in many diseases, especially in injuries in developing countries. It is a complex process and a variety of different in-vitro bioassays can be used to indicate properties which might aid the process (Mensah *et al* 2001). Protection against infection and damage to tissues caused by oxygen free radicals are two of these properties which can be tested for by the standard antimicrobial zone of inhibition assay and the DPPH (2,2-diphenyl-1-picrylhydrazyl) test (Cuendet *et al* 1997), respectively. These tests also lend themselves to bioassay-guided fractionation. A literature search and interviews with traditional healers in Ghana revealed that the stembark of *Spathodea campanulata* (Bignoniaceae) (SC) and the shoot of *Secamone afzelii* (SA) are commonly used as wound-healing agents, being applied as a paste to the wound.
Chloroform and methanol extracts were made of the two plants, dried and the extract dissolved 1% w/v in DMSO. One-hundred microlitres of each methanolic extract were tested against 4 bacterial species and the yeast *Candida albicans*, as shown in Table 1, using wells cut into the agar. The diameter of zones of inhibition were measured after 48 h and results are shown in Table 1. Both extracts showed considerable activity against *C. albicans* and SC also showed a wide spectrum antibacterial activity. Chlorampenicol 1% w/v (C) and clotrimazole 1% w/v (CL) were used as controls.

Table 1 Zones of inhibition given by methanol extracts against microorganisms

Plant	Microorganism				
	StA	BS	PA	EC	CA
SC	5.5 ± 0.5	4.5 ± 0.5	4.5 ± 0.5	5.0 ± 0.5	14.5 ± 0.5
SA	0	0	0	0	14 ± 0.5

Diameter is given in mm (mean ± s.e.m., n = 3).
StA, *Staphylococcus aureus*; BS, *Bacillus subtilis*; PA, *Pseudomonas aeruginosa*; EC, *Escherichia coli*; CA, *Candida albicans* (all from KNUST culture collection)

Antioxidant activity in the extracts was demonstrated by examining 20 μL of each extract by TLC (silica gel GF_{254}/chloroform and silica gel GF_{254}/ethyl acetate:methanol 5:1) and visualising (UV light 254nm followed by spraying with 0.2% DPPH, which shows free-radical-quenching compounds as pale spots against a purple background). All extracts showed several intense antioxidant zones. Fractionation of the chloroform extract of SA, using column chromatography (silica gel/hexane:chloroform:methanol gradient), monitored by the DPPH TLC procedure, resulted in the isolation of the major compound which was shown to be vitamin E by mass spectrometry, NMR spectroscopy and chromatographic comparison.
This activity supports the traditional reputation of these two species as wound healing agents.

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010
Determination of colchicine in plant cell suspension culture of *Colchicum autumnale* and *Gloriosa superba* by reverse-phase HPLC

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Colchicine is a traditional drug used to relive the symptoms of gout, and has been recognized for some time as an anti-tumour agent (Davis & Klein 1980). In this study we have refined existing methods to produce a simple, sensitive and rapid HPLC method that is particularly suitable for detection and analysis of colchicine in plant cell suspension culture media. The mobile phase was phosphate buffer (50 mM, pH 6.2)–acetonitrile–methanol (50:25:25 v/v), pumped at a flow rate of 1.0 mL min^{-1}, using LiChroCART RP 18 analytical column (5μm, 250 × 4.6 mm i.d.). Acetosyringone was used as internal standard. Absorbance was monitored at 350nm and the total running time was 8 min.
The method was linear over the range 25 ng mL^{-1} to 180 mg mL^{-1} (r^2 = 0.998). To complement the HPLC method we have developed an ELISA assay for colchicine which is sensitive down to 10^{-10} g mL^{-1} and shows good linearity between 10^{-9} g mL^{-1} and 10^{-6} g mL^{-1} (r^2 = 0.989).

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011
Nanoelectrospray ionisation tandem mass spectrometric identification of cytochrome P450s in human liver and hepatocytes

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The cytochrome P450s (CYPs) have a central role in oxidative metabolism of xenobiotics (carcinogens and drugs) and steroid hormones. Current research approaches to the identification of individual CYP forms include specific CYP inhibitor or substrates, antibody-based identification, and mRNA-based expression profiling. All of these approaches suffer from one common disadvantage — they all are indirect methods. Western blots, whilst being very sensitive, rely on the availability of isoform-specific antibodies; it is necessary to pre-select which CYPs are expected to be found and identify each isoform in turn. Activity assays invariably require multiple analysis techniques, and even so may not be totally isoform-specific. Measurement at the expression level is fraught with uncertainty since the presence and abundance of a particular type of mRNA does not infer similar presence and abundance of the corresponding protein. On the other hand, current developments in mass spectrometry provide a direct approach to protein identification with sensitivity in the femtomole range.

We have applied nanoelectrospray ionisation-tandem mass spectrometry (NSI-MS/MS) as a method of choice for the identification of CYPs from complex protein mixtures. A method has been developed and optimised using recombinant CYP1A2, 3A4 and 2E1 (Lane *et al* 2002). NSI-MS/MS coupled with nano-liquid chromatography (nanoLC/MS/MS) provided the sensitivity, specificity and resolution required to identify CYPs from biological media. The method has now been extended to the identification of CYPs from six human livers and one hepatocyte microsomal preparation. The region between 45 and 68 kDa on SDS-PAGE was cut into five bands, digested with trypsin and resultant peptides were mass analysed. In order to obtain the peptide sequence information, peptide ions from the full MS spectrum were subjected to MS/MS. This generates fragment ions from which Sequest information could be obtained using Sequest software. Peptides from each digest have been identified with high confidence using TurboSequest Xcorr values (Ducret *et al* 1998). Results from human liver microsomes identified that CYPs 1A2, 3A4, 2C9, 2C10 and 2E1 were present in abundance, while 2A6, 3A5, 4A11, 4A13, 2C17, 2C19, 2D6, 4F3, were identified with lower sequence coverage. The hepatocytes preparation contained CYP2C9, 3A4, 4A11 and 2E1.

It can be concluded that nanoLC/MS/MS is a reliable method for the simultaneous identification of multiple CYP isoforms at the level of protein expression found in human liver and hepatocytes. The apparently lower CYP complement of human hepatocytes may be due to prolonged time required for hepatocytes isolation prior to CYP analysis.

Ducret, A., *et al*. (1998) *Protein Science* **7**: 706–719
Lane, C. S., *et al*. (2002) *AAPS Pharm. Sci.* **4**: Abstract R6013

012
The use of near-infrared spectroscopy and orthogonal signal correction to determine the assay of a low-level active (0.3% w/w) gel

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The use of near-infrared spectroscopy as a technique to assay for actives in pharmaceutical products is well established as it offers the ability to measure a sample without the need for any prior sample preparation. However, it is noted that these successes have been often limited to products containing high levels of actives, with majority of products containing more that 50% of active content (Blanco *et al* 2001). This work aims to explore the feasibility of constructing NIR calibration for a gel formulation containing only 0.3% w/w active, with the application of a novel data-pretreatment method called Orthogonal Signal Correction, along with other more conventional methods such as Multiple Scatter Correction (MSC), Standard Normal Variate (SNV), etc. The criteria recommended by the EMEA guidelines (2003) of accuracy, linearity, range, precision were applied to assess for method validity.

Samples from 5 different batches, with active content ranging from 0 to 0.6% w/w active (0–200% nominal content), were available. The gel was measured by reflectance using the FOSS 6500 Spectrophotometer attached to the Direct Content Analyzer. The wavelength range of 1100–2500 nm was used. Partial Least Square calibrations based on MSC, SNV, 1st derivative, 2nd derivative and OSC spectra were then constructed. HPLC analysis was used as the reference method.

From Table 1, it is evident that the OSC-based calibration gave the lowest standard error at 0.011% w/w. This corresponds to a relative standard deviation (RSD) of ~ 3% w/w, which is acceptable. The Standard Error of Calibration (SEC) values for the other pre-treatments (> 0.028% w/w), however, are too high to give sufficiently accurate calibrations with RSD values of ~ 10% w/w. The OSC-based calibration also meets the requirements for a linearity test given that the 95% confidence intervals for slope and intercept included 1 and 0, respectively. Further validation of the OSC calibration with an independent prediction set gave a comparable standard error of 0.0112% w/w. A paired two tailed *t*-test, comparing the NIR values for the prediction set and the HPLC values, confirmed that there were no significant difference ($P = 0.05$).

Table 1 Basic summary of statistics for PLS calibrations based on the different data-pretreatments

Pretreatment	No. of factors	SEC	Slope (95% confidence interval)	Intercept (95% confidence interval)
MSC	2	0.033	0.94 (± 0.03)	0.015 (± 0.011)
SNV	4	0.028	0.98 (± 0.05)	0.004 (± 0.015)
1st derivative	2	0.031	0.95 (± 0.03)	0.013 (± 0.012)
2nd derivative	2	0.031	0.95 (± 0.03)	0.013 (± 0.010)
OSC	1	0.011	0.99 (± 0.01)	0.002 (± 0.004)

In conclusion, a NIR method for assaying a 0.3% w/w active level can be achieved with the use of OSC and PLS. Also, this work has demonstrated that OSC pre-treatment also improves the accuracy and linearity properties of a calibration, when compared to conventional methods.

Blanco, M., *et al*. (2001) *Analyst* **126**: 1129–1134
EMEA/CVMP/961/01 (2003) Note for Guidance on the Use of Near Infrared Spectroscopy by the Pharmaceutical Industry and the Data Requirements for New Submissions and Variations.

013
A novel algorithm for the optimisation of robust partial least squares regression calibration models in near-infrared spectroscopy

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The use of near-infrared (NIR) spectroscopy in the pharmaceutical industry has grown rapidly in the last decade (Blanco *et al* 1998). A stage has now been reached, with the recent publication of guidelines, where NIR methods for product release can be more readily accepted. However, a major regulatory concern remains over the development of multivariate regression calibration models (Moffat *et al* 1997). The overall procedure and optimisation of parameters is still perceived as an art rather than a science.

This study aimed to address the issue through the development of the optimal partial least squares regression (PLSR) model for the NIR assay of moisture within a pharmaceutical product. This encompassed the steps necessary for sample selection, choice of spectral pre-treatments and PLSR parameters. In addition, employing a novel approach, the influence of spectral regions utilised by the model was assessed with regards to overall robustness. This range optimisation algorithm would provide end-users with an invaluable tool for method development and also for troubleshooting existing PLSR models.

Thirty-two production batches of Feldene 20 mg Zydis units were used in the study with the actual moisture content determined by Karl Fischer analysis (1.74–5.32% m/m moisture content). Transmittance NIR spectra were measured from 600 to 1900 nm using a Foss NIRSystems 6500 spectrophotometer configured with an InTact tablet transmission analyser.

The optimal PLSR calibration was developed for moisture content using the full spectral range that did not contribute noise; 2 factor PLSR utilising standard normal variate (SNV) pre-treated spectra over the range 830–1700 nm. Validation statistics were determined (A in Table 1) with acceptable results obtained for accuracy (root mean standard error of prediction (RMSEP)) and bias. However, when the model was used to predict the moisture content of an external sample set (test set) a high bias result was observed between the NIR results and the corresponding Karl Fischer values.

Table 1 Calibration model statistics

	Cal Set SEC (% m/m)	Val Set RMSEP (% m/m)	Bias (% m/m)	Test Set RMSEP (% m/m)	Bias (% m/m)
A	0.21	0.24	−0.02	0.27	−0.26
B	0.22	0.27	0.05	0.10	−0.05

The range optimisation algorithm identified a continuous region of approximately 40 nm as contributing bias to the prediction and essentially corrupting the model. The region, which was not associated with the main moisture peak, was removed with no significant detriment to the validation statistics but with a major improvement to the accuracy and bias of the test set (B in Table 1). The algorithm was applied successfully, improving the robustness of the PLSR calibration model by excluding a variable spectral region not correlated to the analyte of interest.

Blanco, M., Coello, J., Iturriaga, H., *et al.* (1998) *Analyst* **123**: 135–150
Moffat, A. C., Jee, R. D., Watt, R. (1997) *Eur. Pharm. Rev.* **2**: 37–41

014
Measuring and mapping particle–particle interactions in formulations by atomic force microscopy

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An example of the importance of interactions between particulates is found in the manufacture of dry powder inhalers where active ingredients are micronised to allow delivery to the lungs. Unfortunately powders with these dimensions are difficult to aerosolise due to agglomeration. Consequently a common approach in dry powder inhalers is to add larger lactose particles, to promote the formation of an ordered mixture. In such a mixture, the micronised active ingredient sticks to active sites on the lactose, effectively increasing the particle size.
We demonstrate the use of atomic force microscopy (AFM) to measure and map the adhesive interaction forces and electrostatic forces in a typical inhaler system (active ingredient/carrier/canister coating). In this study the interactions between micronised lactose monohydrate, micronised salbutamol sulphate, and polytetrafluoroethylene (PTFE) and a single salbutamol particle have been characterised
If the spring constant (stiffness) of the AFM cantilever is determined, then the force-distance curves can be used to calculate the magnitude of the adhesion force between the tip and the substrate. It is apparent that while a consistent adhesion ranking is obtained (lactose > salbutamol > uncharged PTFE), there are variations in the magnitude of the adhesion between each tip. This is attributed to variations in geometry of the attached salbutamol particles.
This work was extended by recording an array of force-distance curves, to produce an image of spatial variations in adhesion between a salbutamol particle functionalised AFM tip, and a lactose carrier particle. The force-volume image reveals differences in salbutamol adhesion across the lactose carrier surface. To confirm this, force distance curves are extracted from two regions giving different image contrast.
Electric Force Microscopy (EFM) allows static charge distributions to be spatially imaged with micron resolution. In addition, by varying the EFM tip voltage during imaging, charge polarity and magnitude can be determined. Here, we demonstrate the use of this technique to look at the static charge distribution on inhaler component surfaces before and after exposure to micronised active ingredient particles. This information provides an insight to the role of electrostatics in determining device performance and the mechanism of attachment of active particles to charged regions.
AFM can directly measure the adhesion of single pharmaceutical particles to other formulation components. Here, the adhesion of salbutamol to the substrates is ranked lactose > salbutamol > PTFE. This order of interactions will favour the dispersal of aggregates of salbutamol onto the surface of the lactose carrier. In addition, using force-volume imaging it is possible to spatially map variations in interaction force between particles of interest. This has large potential for the ability to engineer inhalation formulations to provide advanced drug delivery systems.

015
Solid form investigation of the zwitterion histidine and its salts

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Zwitterionic drug molecules have been developed for a number of pharmaceutical medications, for example the anti-convulsant agent Gabapentin (Neurontin) (Taylor 1993). However, zwitterionic compounds and their salts often show a high propensity towards the formation of multiple hydrates, more so than non-zwitterionic compounds. This often makes the selection of the optimum solid form of these compounds problematic.
We present here our study of the solid form characteristics of histidine and its salts, which has been undertaken to gain a molecular perspective of crystalline zwitterionic compounds. Histidine is a particularly suitable probe molecule for this kind of investigation as it is known to form salts with both anionic and cationic counterions, many of which have hydrated crystalline forms (Allen 2002). Furthermore, histidine is chiral and so stereochemical effects on salt formation and hydration can be studied by comparing the single enantiomer and racemic crystalline forms.
We have used two complementary approaches within this study. Firstly we have applied an array of analytical techniques to classify the solid form characteristics of histidine and its salts. These have included ambient and non-ambient powder X-ray diffraction, Raman spectroscopy, TGA, DSC, dynamic vapour sorption, isothermal microcalorimetry, light microscopy and SEM. Secondly we have undertaken an in-depth study of the salt crystal structures to understand how the different packing arrangements relate to these solid form characteristics. This has been extended to also encompass data mined from the Cambridge Structural Database (CSD) (Allen 2002) focusing on the packing arrangements and intermolecular interactions for zwitterionic compounds in general.

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016
Establishment of inherent stability of secnidazole and development of a validated stability-indicating HPLC assay method

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The revised parent drug stability test guideline Q1AR issued by International Conference on Harmonisation (ICH, 2000) requires that analysis of stability samples should be done through the use of validated stability-indicating testing methods. It is also recommended that stress testing on the drug substance should be carried out to establish its inherent stability characteristics and for supporting the suitability of the proposed analytical procedures. It is suggested that stress testing should include the effect of temperature, humidity, light, oxidizing agents as well as susceptibility across a wide range of pH values.
The objective of the present study, therefore, was to study degradation of secnidazole under different ICH recommended stress conditions, and establishment of a validated stability-indicating HPLC method that separates drug from chromophoric degradation products formed under various conditions. Secnidazole, is chemically, (1-(2-hydroxypropyl)-2-methyl-5-nitroimidazole) and is used in the management of protozoal infections and bacterial vaginosis. There are a few reports in literature on the stability of secnidazole. Pfoertner & Daly (1987) and Moustafa & Bebawy (1999), have reported the behaviour of degradation of

secnidazole in photolytic conditions. There is only one report on stability-indicating methods for secnidazole by Moustafa and Bebawy (1999) wherein separation of the drug from only photolytic degradation products was shown.

Secnidazole was subjected to forced decomposition studies under hydrolytic, oxidative, photolytic and thermal conditions. Analyses of the stressed samples were performed using HPLC with photodiode array (PDA) detector. Formation of non-chromophoric degradation products was also checked by LC-MS studies. The chromatographic separations were carried out on C-18 column (250 mm × 4.6 mm i.d. with particle size of 5 μm). Mobile phase that was employed for initial studies involved water:acetonitrile in the ratio 86:14.

The drug was found to degrade significantly in alkaline conditions, oxidative stress, and also in the presence of light (especially in acidic environment). Mild degradation of the drug occurred in acidic and neutral conditions. The drug was stable to dry heat. LC-MS studies revealed formation of additional non-chromophoric products on photolytic degradation of the drug.

Method optimisation studies were performed as satisfactory resolution of the drug and the chromophoric degradation products was not achieved by using the above-mentioned mobile phase. Separation of drug and all the degradation products formed on forced decomposition studies was successfully achieved on a C-18 column utilizing water-methanol in the ratio of 85:15 and at the detection wavelength of 310 nm. The method was validated with respect to linearity, precision, accuracy, specificity and ruggedness. The response for the drug was strictly linear ($r^2 = 0.9994$) in the concentration range between 50–500 μg mL^{-1}. The RSD values for intra-day and inter-day precision studies were $< 0.5\%$ and $< 1.5\%$, respectively. Good recoveries (98.45–100.22%) of the drug spiked in to the mixture of stressed samples were obtained. Specificity of the method towards the drug was proved through PDA peak purity studies. Ruggedness of the method was checked on different chromatographic systems on different days.

The developed stability-indicating HPLC method is simple and can be used to analyse the drug in stability samples.

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