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Investigation of alkaloids from *Phyllanthus amarus* Schum. & Thonn.

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The *Phyllanthus* species has been found to exert a marked inhibitory effect on hepatitis B virus evident by its exhaustive use in Asian countries in chronic jaundice. *Phyllanthus amarus* Schum. & Thonn. has been reported to inhibit reverse transcriptase and its effect on the in vitro replication of a laboratory strain and clinical isolates of HIV has been evaluated (Notka & Meier 2004). Recently, the alkaloid extract of *P. niruri* L. was observed to have a suppressible activity on strains of HIV-1 in MT-4 cell lines (Naik 2003). The aim of this study was to investigate the alkaloid constituents is in the aerial parts of *P. amarus* Schum. & Thonn. This paper will report the isolation of its major alkaloids. The aerial part of *P. amarus* Schum. & Thonn. were collected in Ho Chi Minh City, Vietnam. A voucher specimen was deposited in the Herbarium of Department of Phamacognosy, University of Medicine and Pharmacy, Ho Chi Minh City, Vietnam. The alkaloidal fraction had a strong hepatoprotective activity. Procedures were carried out to extract these alkaloids. Procedure D, which gave a highest yield of alkaloids, was chosen for extraction on a large scale. According to this method, total alkaloids were extracted with chloroform after alkalizing the powder of *P. amarus* with 25% ammonia solution. The chloroform solution was evaporated under reduced pressure to the concentrated solution. This solution was distributed with 1% HCl solution (200 mL × 5). The combined acid layers were distributed with chloroform (100 mL, many times). The chloroform solution was concentrated to a syrupy extract. The syrupy extract was chromatographed on silicagel (0.040–0.060 μm) using increased ratios of mixtures of chloroform–methanol as mobile phase. Five following fractions, named I, II, III, IV, V, were collected. Fractions III and V were purified by column chromatography on Sephadex LH-20. Two pure alkaloids named Pa1 and Pa2 were obtained. By comparison with published MS and NMR data, Pa1 was identified to be nirurine, a known major alkaloid of *P. niruri* L., but not previously isolated from *P. amarus* Schum. & Thonn. (Houghton et al 1996). Further investigations on the structure elucidation of Pa2 and the pharmacology in vivo on cell lines of nirurine and Pa2 of this plant are in process.

Professor P. J. Houghton, King's College London; Chemical Institute of Hanoi, Vietnam for obtaining the NMR spectra.

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Poster Session 3 – Analytical Chemistry

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Comparison of near-infrared spectroscopic methods for the identification of pharmaceutical excipients and active drug ingredients

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Identification of excipients is commonly accomplished by invasive chemical methods. Near-infrared (NIR) spectroscopy is an ideal, versatile and rapid alternative that can be carried out in the warehouse or holding location of a manufacturing site so reducing the quarantine time. A key paper from Blanco & Romero (2001) proposed the use of cascading libraries for NIR spectroscopy. In this work, an NIR spectral library of excipients and active pharmaceutical ingredients (APIs) was constructed and used to compare five identification methods. Reflectance NIR spectra were collected over the range 1100–2500 nm on a FOSS 6500 Spectrophotometer fitted with a Rapid Content Analyser for 210 pharmaceutical compounds. Five pattern recognition methods were compared, based on second derivative spectra: Wavelength Correlation (WC); WC with wavelength selection (WCWS); Maximum Distance in Wavelength Space (MDWS); Peak Positioning (PP); and Soft Independent Modelling of Class Analogies (SIMCA). A reduced library of 55 compounds (containing at least eight batches of each substance) was used to optimise each method. The number of compounds correctly identified by each individual method is given in Table 1. SIMCA was the best method, but it required extensive data analysis for little advantage compared with the other methods. The next best method was PP; but it was discarded because robustness was an issue over time. MDWS came next, but multiple sample spectra are required for this procedure. While WC was the worst performing method, it was easy to update (i.e. add new compounds) and it also gave a good indication of the 'problem' compounds highlighted by the other methods. Identification was best performed by using a cascading approach, initially using WC to divide the spectral library into groups. Each group was then sub-divided sequentially using WCWS, MDWS and SIMCA. Where the group contained both chemically and physically different compounds, then WCWS was next applied. For groups containing only physically different compounds (e.g. particle size), or mixtures with different ratios of components, then MDWS was optimal. SIMCA was finally used for any groups that still remained unresolved. The procedure was initially applied to the library of 55 compounds; 36 compounds were correctly identified with the remainder grouped into starch and starch derivatives or compounds that differed only by the grade of material (Table 1). Applying the procedure to the 210 compounds, for which there were 1–91 batches for each compound, 167 compounds were correctly identified, leaving 43 that fell into 14 groups. Of these 14 groups, only one involved different chemical compounds (ceratonia, guar galactomannan and tara gum). In this study, there were insufficient batches for these compounds to allow further discrimination. In conclusion, while SIMCA is the best individual method, the cascading approach advocated is much better.

Table 1 Number of compounds identified using different pattern recognition methods

Procedure	Method				
	WC	WCWS	MDWS	SIMCA	PP
Single method (n = 55)	24	—	29	33	30
Cumulative procedure (n = 55)	24	29	33	36	—
Cumulative procedure (n = 210)	124	137	151	167	—

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Determination of anastrozole in rat plasma by liquid chromatography-tandem mass spectrometry with electrospray ionization

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A sensitive and specific liquid chromatographic-tandem mass spectrometry method was developed and validated for quantitation of anastrozole, a potent and selective aromatase inhibitor. Aromatase inhibitors are a class of compounds that act systemically to inhibit oestrogen synthesis in tissues by inhibiting aromatase, an enzyme that catalyses the conversion of androgens to oestrogen. The link between oestrogen and the growth and development of some breast cancers has long been recognised (Baum 2001) and there is substantial evidence that circulating oestrogens promote the proliferation of breast cancer. Many current therapies for breast cancer involve hormonal manipulation, with oestrogen-deprivation of the tumour being an established method of treatment. The objective of this study was to develop and validate an LC-MS/MS method for the determination of low concentrations of anastrozole in rat plasma to support formulation optimisation studies. Anastrozole and its deuterated internal standard were extracted from plasma by solid phase extraction on an automated Gilson 215 SPE workstation using 30 mg StrataX cartridges. The chromatographic separation was performed on reverse phase Aquasil C18 column with a mobile phase of acetonitrile/water (75/25, v/v). The mass spectrometer,

Sciex API 4000 was operated in the positive ion mode, employing turbo ion spray interface. Ions were detected in multiple reaction monitoring mode of mass spectrometer. The mass transitions m/z 294/225 and 298 / 229 were used to measure the analyte and the internal standard, respectively. The assay exhibited a linear dynamic range of 0.5–250 ng mL^{-1} for anastrozole in plasma. The run time was 2.5 min. The lower limit of quantitation was 0.5 ng mL^{-1} with a relative standard deviation of less than 15%. The assay required only 50 μL of plasma. Acceptable precision and accuracy were obtained for concentrations over the standard curve ranges. The average within-run and between-run relative standard deviations for quality control samples were less than 5%. The average accuracy of quality control samples was generally within ±4%. There was no evidence of instability of anastrozole in rat plasma following three complete freeze thaw cycles and samples can safely be stored for at least one month at approximately −20°C. The method was very robust and has been successfully used to analyse plasma samples originating from various in vivo studies.

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Use of an automated plate reader to measure partition coefficients

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The tumour specific expression of CYP1B1 (Murray et al 1997) provides a putative therapeutic anticancer drug target for development of non-toxic prodrugs selectively metabolized or activated only within the tumour to form highly potent tyrosine kinase inhibitors or antimitotic agents. The Cancer Drug Discovery Group at De Montfort University have utilized the pharmacophore of the only known endogenous substrate of CYP1B1, estradiol, to design a series of novel anticancer prodrugs such as DMU212 (Potter et al 1999). We are a multidisciplinary group utilizing a range of molecular biological equipment. This diversity has resulted in the novel application for an automated fluorescent plate reader in high-throughput assay of log*P* samples, which have been previously equilibrated using the traditional shake-flask method. We report here the development of this method using a prodrug in pre-clinical development DMU212 (3,4,5,4′-tetramethoxystilbene), which is a stilbene derivative, and the consequent validation of the novel application using conventional compounds (17β-estradiol and hydrocortisone) and a conventional assay (UV spectrophotometry). The log*P* of DMU212, 17β-estradiol and hydrocortisone were determined at room temperature by preparing a 50 μg mL^{-1} prodrug stock solution in octanol and mixing it with an equal volume of nanopure water. The two immiscible phases were shaken together for 1 h. After allowing the mixture to stand for 5 min, the samples were centrifuged for 30 min at 2000 rev min^{-1}, after which the two phases were separated. Samples were assayed either on a UV spectrophotometer (Unicam) or plated out into 96-well plates and measured on the fluorescence plate reader (Molecular Devices, GeminiXS). The log*P* of DMU212 determined by conventional UV spectrophotometry was 1.59 ± 0.05 (n = 3), comparable with the value determined on the fluorescent plate reader at 1.61 ± 0.19, thereby confirming the usefulness of the high-throughput method. In a further test of this method's validity, the log*P* of conventional drugs, such as 17β-estradiol and hydrocortisone, was determined utilizing both the novel and conventional methodology. The log*P* of 17β-estradiol determined by UV spectrophotometry was 2.68 ± 0.12 compared with the value of 2.70 ± 0.02 obtained with the fluorescent plate reader, correlating well with that stated in literature of 2.69 (Barratt 1995). The log*P* of hydrocortisone determined by UV spectrophotometry was 1.68 ± 0.01 and with the fluorescent plate reader was 1.58 ± 0.08, again both correlating with that stated in literature of 1.53. The results as determined on the fluorescent plate reader were more consistent compared with UV spectrophotometry; this is probably due to a higher sensitivity being achievable on the plate reader. Overall, the results indicate that the 96-well automated fluorescent plate reader can be successfully implemented for high-throughput, miniaturized, cost-effective and valid log*P* quantification.

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Investigation of a reverse-phase high performance liquid chromatography (RP-HPLC) method for a weakly basic drug

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Formulation development of combination drug products requires the availability of a rapid analytical method with low limits of detection, for the high-throughput analysis of the large number of samples generated. Salmeterol xinafoate (SX) and fluticasone propionate (FP) are co-formulated for the treatment of asthma and COPD. RP-HPLC using C-18 bonded phases of basic compounds, such as SX, can be problematic due to the occurrence of poor peak shapes. This can be overcome by decreasing the flow rate, modifying column temperature, careful selection of the column and use of methanol as organic modifier (McCalley 1999, 2000, 2002). The aim of this study was to examine the performance of SX on a second-generation silica bonded reverse-phase column, with a mobile phase containing a high content of organic modifier. The high organic solvent content should allow the rapid elution of the highly lipophilic glucocorticoid, FP, allowing future validation of a rapid assay capable of analysing both compounds. Assay development utilized an HP1050 modular HPLC system, using HP ChemStation software for analysis (Agilent Technologies UK Ltd, Wokingham, UK). The developed assay utilized an Inertsil ODS-2 base-deactivated 250 mm × 4.6 mm, 5 μm HPLC column (Capital HPLC Ltd, Broxburn, UK) with 75:25 methanol:0.6% aqueous ammonium acetate as the mobile phase. Detection was by UV absorbance at 228 nm. The system suitability data are displayed in Table 1. Salmeterol base (SB) and its counter-ion xinafoate (XA) were well resolved with low retention times at 1 mL min^{-1} and a temperature of 40°C. SB shows a low degree of tailing, even at the neutral pH employed, despite conventional recommendation that such bases should be routinely analysed using mobile phase buffered at low pH. Both SB and XA peaks displayed efficient separation, with narrow peak widths and low peak dispersion (the number of theoretical plates for either compound was never below 5000 over 85 injections). The method displayed good sensitivity and it was possible to determine the concentration of salmeterol on the basis of either the XA (LOD for SX = 0.22 μg mL^{-1}) or the SB (LOD for SX = 0.26 μg mL^{-1}) eluting peak, when a series of calibration standards of SX was analysed by replicate injection. The method also displayed good reproducibility across the calibration range of 2–50 μg mL^{-1} with a mean coefficient of variance of the peak area response of the five calibration concentrations of XA = 0.71 ± 0.18%; SB = 1.11 ± 0.64%. The assay was shown to be accurate with a % recovery of 99.58 ± 1.85% for XA and 99.49 ± 1.88 for SB (n = 11). A preliminary investigation produced a retention time of 6.94 ± 0.01 min for FP. This indicates the suitability of the developed method for full validation enabling the rapid analysis of SX and FP formulation. In conclusion, by using a base-deactivated stationary phase, it was possible to develop an assay suitable for the high throughput analysis of the SX and FP providing good chromatographic performance of the weakly basic salmeterol.

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Table 1 Retention time and peak performance parameters for the chromatographic analysis of SX by RP-HPLC

Compound	Retention time (min)	USP peak width	USP tailing factor	USP theoretical plates
XA (mean of n = 85 ± s.d.)	2.96 ± 0.02	0.19 ± 0.02	1.26 ± 0.01	5700 ± 208
SB (mean of n = 85 ± s.d.)	5.23 ± 0.11	0.33 ± 0.04	1.21 ± 0.02	5655 ± 110

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The influence of reflection and transmission geometries on powder X-ray diffraction (pXRD) patterns of an API

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Preferred orientation can be a significant problem in the analysis of APIs by powder X-ray diffraction (pXRD). Early batches of material may have undesirable habits for flat-plate sample presentation methods. In some cases, habit modification of the candidate is not possible and APIs progressed to late development may continue to have an undesirable habit for pXRD analysis. Crystals should be randomly orientated so that all lattice planes are observed in the diffraction pattern. Preferred orientation occurs when the crystals align to present only particular planes to the incident beam. Compound X is an analgesic that is currently entering phase 2 studies in the drug development process. Form 2 of this compound is the desired polymorph for progression but has an acicular habit, which presents significant handling problems for both formulators and physical properties scientists. Diffraction patterns obtained using flat-plate presentations often show significant orientation effects, which lead to interpretation difficulties. The purpose of this study was to identify all of the reflections characteristic for forms 1 and 2 of compound X, and to evaluate the influence of reflection and transmission geometries on the powder pattern of form 2 while also considering the resource demands on instrument usage and operator involvement. The powder patterns from the different geometries were assessed in terms of pattern completeness and resolution. A Phillips X'pert Pro (Phillips Analytical, Holland) with a Cu anode and an X'celerator detector was used. In reflection mode, the diffractometer was fitted with a variable divergence slit on the incident beam side. In transmission mode, a parabolic mirror or an elliptical mirror was used on the incident beam side. In reflection mode, samples were mounted on a zero background silicon wafer while in transmission mode, samples were presented in a 1-mm diameter glass capillary or between X-ray transparent foils. High purity batches of form 1 and 2 were used. The samples were analysed over the range of 3–40°2θ and experiments were performed in duplicate. Samples presented in the capillaries showed additional reflections to those obtained from samples mounted on a silicon wafer. Despite shorter data acquisition times for capillaries analysed using the elliptical mirror, data collected showed better signal-to-noise ratios and better resolution than that collected using the parabolic mirror. Data collected using the transparent foils showed additional reflections to those obtained from samples mounted in a silicon wafer. However, a few of the reflections observed in the capillary datasets were not apparent. In comparison with the transparent foils presentation system, capillary presentation was time consuming due to sample alignment requirements and filling issues: the undesirable habit and electrical properties produced poor flow. Capillary presentation of compound X with the elliptical mirror appears to produce a complete pattern while requiring less instrument time than when using the parabolic mirror. While transparent foils presentation of compound X reduces some preferred orientation, it requires considerably less resource in terms of instrument usage and sample preparation operations.

Poster Session 3 – Drug Delivery

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Enhanced release of acyclovir from intravaginal rings using common vaginal excipients

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The present scale of the HIV pandemic and the failure to develop an effective vaccine have forced scientists to evaluate alternative preventative strategies for reducing the rate of HIV transmission. Since over 90% of new infections result from heterosexual intercourse, the most viable, short-term strategy being pursued is the development of vaginal HIV microbicides. In particular, vaginal rings providing long-term, controlled-release of antiretrovirals over days/weeks/months are actively being investigated for this purpose. Several studies have demonstrated that prior infection with herpes simplex virus type 2 (HSV-2) is an important risk factor for HIV acquisition (Holmberg et al 1988), attributed to the presence of vaginal lesions and subsequent infiltration of CD4 cells to the lesion sites. Therefore, preventing and treating HSV-2 vaginal infections may be a useful strategy in reducing the incidence of female HIV infection. In this study, we evaluate the potential for controlled release of the anti-HSV-2 drug acyclovir from silicone vaginal rings. Silicone, matrix-type vaginal rings containing 10% w/w acyclovir and optionally 15% w/w of various pharmaceutically-acceptable, hydrophilic, vaginal excipients (cross-carmellose, polyacrylic acid, ascorbic acid, octyl dodocanol, povidone iodine, undecylenic acid) were manufactured according to standard methodologies (Malcolm et al 2003). In vitro release studies (37°C, 100 mL aqueous release medium, orbital incubation) were performed to assess the potential of each excipient for providing enhanced release of acyclovir from the silicone devices. Briefly, samples of the release medium were taken daily over a 14-day period, the release medium replaced to ensure sink conditions, and the samples subsequently analysed by UV-HPLC for determination of drug concentrations. For six of the seven excipients, acyclovir release was enhanced over the 14 days compared with the ring formulation containing no excipient (crosscarmellose 152%, povidone iodine 23%, ascorbic acid 18.2%, polyacrylic acid (MW 2100) 11.2%, polyacrylic acid (MW 5100) 10.8% and octyl dodecanol 3.4%). The results demonstrate that hydrophilic excipients provide greatest release enhancement and have the potential to extend the utility of the rings to the release of substances that might otherwise be difficult to release from the hydrophobic silicone elastomer. The enhanced release may be attributed to a number of factors, including water ingress and subsequent dissolution of the acyclovir. Further studies are required to determine the vaginal concentration of acyclovir required to reduce HIV transmission and to optimise the release profile from these modified ring formulations.

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Formulation of antigens in particulate delivery systems for in vitro cell stimulation assays

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Protein antigen entrapped in poly(lactide-co-glycolide) (PLGA) nanospheres has been shown to facilitate increased antigen specific in vitro cell proliferation in conjunction with increased secretion of IFN-γ and IL-6 in primary splenocyte cultures from pre-immunised mice when compared with free antigen (Eyles et al 2003). This mechanism is likely to be distinct from non-specific effects caused by components of the delivery vehicle itself. In addition to offering potential for increased and more sensitive biological readout of immunological parameters, formulation in particulate delivery systems may facilitate analysis of hydrophobic proteins or lipid antigens in aqueous environments normally problematic for such moieties. Additionally, such formulations may alter the in vitro toxicity of some antigens that can limit analysis using immunological assays (Bramwell et al 2002). To address these problems, we have designed systems that are suitable for the delivery of hydrophobic or lipid moieties for in vitro analysis of immunological cell stimulation assays. To minimise the impact of the carrier, biocompatible delivery systems based on biodegradable polymers and liposomes were thought to be promising candidates for this purpose. We used formulations based on PLGA (50:50), poly(methyl methacrylate) (PMMA), phosphatidylcholine (PC) and dioleoyl phosphatidyl ethanolamine (DOPE). Polymer carriers were prepared using a single emulsion and excipients were minimised in order to facilitate rehydration following freeze drying with the cryo/lyoprotectant sucrose. Liposome carriers were formed using thin film hydration (handshaking) in the presence of sucrose and homogenised to obtain a more desirable size range. Initial characterisation of polymer systems in 1.5% PVA is summarised in Table 1. Full characterisation and initial biological evaluation will be presented. The use of methanol (in addition to chloroform) in the polymer formulations as a co-solvent invariably led to an increase in size. The use of PVA facilitated smaller liposomes following homogenisation and did not affect the observed cell viability (in COS-7 cells) at the concentrations used for any of the formulations. Hydrophobic protein and lipid antigens are often problematic to produce and purify and are therefore generally expensive. While entrapment of such moieties in liposomes if often high, definitive analysis of entrapped material can be difficult due to the lack of specific assays, difficulties in extraction of antigen and the small amount of material available. Therefore, systems that incorporate all of the antigen, such as those outlined here, may comprise highly useful and comparatively simple tools for routine analytical purposes, especially when coupled with the versatility offered by an easily rehydrated freeze dried product that retains the characteristics of the initial formulation.