Analytical Sciences

7

Improved HPLC methodology for the determination of mercaptopurine and its metabolites in plasma and red blood cells

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Mercaptopurine (MP) is one of the most widely used medications for childhood acute lymphoblastic leukemia (ALL; Relling et al., 1999), the most common cancer in children. MP itself is inactive requiring metabolism to thioguanine nucleotides (TGN) which mediate cytotoxicity via incorporation into DNA and RNA (McLeod et al., 2000). The metabolism and bioavailability of mercaptopurine are subject to wide inter- and intra-individual variation as a result of both genetic and environmental factors and there is accumulating evidence that individualised therapy is necessary for optimisation of the therapeutic response to this drug (Shipkova et al., 2003).

In an attempt to understand the different factors that could lead to inter- or intra-patient variability in MP cytotoxic metabolites and clinical response in paediatric patients with ALL we have developed an improved analytical method that enabled the rapid quantification of MP and four of its metabolites; thiouric acid (TU), thioguanine (TG), thioxanthine (TX) and methylmercaptopurine (mMP) in both plasma and RBCs.

Erythrocytes separated from plasma by centrifugation at 1000 *g* for 10 min were washed twice with Hanks solution and then suspended at a density of 8×10^8RBCs/200 μl. Both separated plasma and the prepared erythrocytes were stored at −20°C until required for further processing. 200 μL of plasma or erythrocytes were mixed with 100 μL of dithiothreitol (75 mg/ml), 50 μL of perchloric acid (700 ml/L) and 50 μL of water for 30 sec by vortex mixing in 1.5 ml Eppendorf tubes. The tubes were then centrifuged for 15 min at 13000 g. The supernatant (300 μL) was heated for 45 min at 100°C to hydrolyze TGN to the corresponding base (TG). During hydrolysis, mMP was converted to a derivative analysed under the same chromatographic conditions. After cooling, the supernatant (30 μL) was analysed by HPLC using Waters Atlantis™ dC_{18} column (3.9 × 150 mm, 3 μm). The mobile phase consisted of ANC/MeOH/0.02 M KH_2PO_4, pH 2.25 (3:1:96, v/v) and was used at a flow rate of 1 ml/min. UV detection was employed at 322 nm (for the detection of MP and mMP derivative) and 342 nm (for TG, TU and TX).

The elution conditions used allowed good resolution between MP and its four metabolites in both plasma and RBCs. No interfering substances were present in the processed plasma or RBCs obtained from untreated healthy subjects. The total run time was 12 min with all peaks of interest being eluted within 8 min. Linear relationships were observed between the peak areas and the concentrations of all compounds ($r > 0.994$). The reproducibility of the assay was evaluated by replicate analyses ($n = 5$) of erythrocytes and plasma samples supplemented with the compounds of interest at three different concentrations. The intraday and interday precision and accuracy did not exceed 10% for the five compounds in plasma and RBCs except for MP and TU in RBCs (15% for MP and 20% for TU).

This HPLC assay allows the measurement of MP and four of its metabolites using very small volumes (200 μL) of plasma or erythrocytes which are suitable for use in paediatric patients. This method will facilitate the investigation of intra- and inter-patient variability in MP and its metabolites. Furthermore, the different metabolite concentrations measured will be used to construct pharmacokinetic models that combine a range of possible causes of intra-individual variability including patient adherence to therapy.

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8

The identification of counterfeit and sub-standard paracetamol tablets by near-infrared spectroscopy

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Although near-infrared spectroscopic (NIRS) methods have been developed to identify counterfeit proprietary medicines (Yoon et al 2001; Wilson & Moffat 2004; Shin et al 2005), little effort has been put into methods to detect substandard generic medicines. This work investigates a simple procedure for developing NIRS methods to identify counterfeit and substandard medicines by using quantitative calibration curves generated from using crushed tablets using paracetamol tablets as an example. A total of 14 batches from 8 different manufacturers of paracetamol tablets 500 mg BP were purchased from local community pharmacies. The smooth sides of intact tablets (16 from each brand) were scanned twice using a FOSS NIRSystems 6500 NIR spectrometer and the mean spectra taken using both the Rapid Scanning Analyser (RCA) and the Optical Probe (the probe touching the tablet surface). Tablets from each brand (8) were then crushed and either paracetamol or maize starch added (9 samples for each brand) to give a total of 72 samples with paracetamol concentrations from 72.37 to 93.17% m/m. All powders were measured in glass vials. The same ceramic reference standard was used for the RCA and probe, and 32 repeat scans were acquired for each spectrum. The 8 brands of paracetamol tablets 500 mg had mean weights between 556.89 and 600.81 mg (89.75–83.22% m/m paracetamol respectively, mean 87.64% m/m). The reference value for each tablet (paracetamol % m/m) was taken as the label claim of the tablet (500 mg) divided by its weight. A calibration set (45 spectra) and validation set (27 spectra)

were used in various partial linear squares regression (PLSR) models (using The Unscrambler software). Six data points were removed because they were outliers. The best model was a two component PLSR model using Multiplicative Scatter Correction (otherwise the probe values were too low) and removing the regions 1420–1560 and 1900–2000 nm (to remove the effects of water in the added maize starch which was distorting the model). This model gave calibration and validation graphs with slopes of 0.953 and 0.979, respectively, and a root mean standard error of prediction of 1.10 and 1.34% m/m, respectively. Using this model to predict the paracetamol contents (% of label claim) of the individual brands of tablets (total of 14 batches from 8 manufacturers) gave: Powders, 10 crushed tablets from each batch scanned, mean 99.9%, range 98.0–102.4%. Intact tablets using the RCA, 16 tablets from each batch scanned, mean 96.3%, range 93.7–99.2%. Of all the 224 individual tablets analysed, no individual tablet fell outside the range 93.2–102.5 % label claim. Intact tablets using the probe, 16 tablets from each batch scanned, mean 101.3%, range 98.9–103.4%. Of all the 224 individual tablets analysed, none fell outside the range 97.3–104.1 % label claim. Thus the NIRS calibration using crushed tablets and spiking with pure paracetamol or maize starch could be applied to the analysis of intact tablets using the NIRS probe giving accurate answers to within 97–105% label claim even with single tablet analysis.

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9
Assessing the structure of immobilised proteins and antigens using circular dichroism

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For certain types of administration therapeutic proteins may be advantageously immobilized as dry particles. Alternatively antigens may be bound onto insoluble adjuvant particles to improve the efficacy of vaccines. In both cases it is difficult to determine how the immobilisation process has affected the secondary and tertiary structure of the protein because the particles produced are not suitable for conventional CD analysis due to extensive scattering. This study aimed to develop a method for obtaining good quality circular dichroism (CD) spectra of proteins and antigens bound to scattering particles. A novel rotating cell was developed that ensures that particles remain homogeneously suspended during the measurement process. This was located in a conventional spectropolarimeter and positioned to maximise the collection of scattered light at the detector. CD spectra were obtained of suspensions of protein coated microcrystals in solvent and of protein bound to adjuvant in water. Model systems included lysozyme, and albumin coated onto microcrystals of valine and BSA bound onto alhydrogel. The CD spectra of solvent and aqueous suspensions were found to be similar to those obtained in aqueous solution but a reduction in the peak intensities below 240 nm was generally observed. The signal to noise could be significantly improved by optimisation of both the collection geometries and weight fraction of the suspension but the absorption flattening effect remains. It was proposed that this arises from the lower cross-sectional area of protein molecules packed onto a surface relative to when uniformly dispersed in solution. An empirical method was therefore developed to compensate for the effect based upon determining the total absorbance of the sample. Using this approach it was possible to correct for changes in spectral shape and magnitude sufficiently well that conventional secondary structural analysis could be usefully applied to the immobilised proteins. This was used to probe if differences in structure occurred during the immobilization process. These results show that changes to the structure of immobilised proteins can be probed by solid-state CD spectroscopy in both aqueous and solvent systems. This is likely to be of major benefit for improving formulations of therapeutic proteins and vaccines.

10
Transferability of near-infrared spectral libraries for the identification of pharmaceutical excipients and actives

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Near-infrared (NIR) spectroscopy is a suitable technique for the rapid and non destructive identification of pharmaceutical excipients and actives. A major limitation of the technique, however, is the difficulty in transferring a spectral library created on one instrument for use on another instrument. Instruments differ for many reasons (e.g. number of data points, wavelength accuracy, lamp age, etc.). Setting up a robust spectral library is time consuming and costly as large numbers of batches for each material are required to ensure that all expected variability is adequately modelled. The effects of differences in the wavenumber scales of the instruments on library transfer has been investigated to allow successful transfer of spectra between instruments. Reflectance spectra were measured on two different Fourier transform NIR instruments: a Bran+Luebbe (B+L) FT-NIR Infraprover II fitted with a reflectance accessory (wavenumber range 4008–9996 cm^{-1}, 500 data points at 12 cm^{-1} intervals), and a PerkinElmer System Identicheck with a reflectance accessory (wavenumber range 4000–10000 cm^{-1}, 3001 data points at 2 cm^{-1} intervals). Spectra of 53 different compounds, for which there were between eight and ten different batches for each, were measured on both instruments. A library of second-derivative spectra was created from those measured on the B+L instrument. Using Correlation in Wavenumber Space (r), and the library created using spectra from the B+L instrument, 24 of the 53 compounds were identified, $r > 0.98$ (identification criteria: $r > 0.9$ and nearest neighbour >0.1 different). The remaining compounds fell into eight groups containing between two and 16 compounds. These required the application of further more complex identification algorithms, which allowed an additional 15 compounds to be identified (Palmer et al 2005). Cubic spline interpolation was used to reduce the PerkinElmer spectra to 500 data points. 22 of the 24 compounds that were identified in the B+L library were correctly identified although r fell to 0.90–0.98. The two compounds unidentified were paracetamol and methyl hydroxybenzoate for which the r values now fell below the 0.9 limit ($r = 0.89$ and 0.85, respectively). The decrease in correlation values due to the wavenumber error was found to be more important for compounds that were unidentified in the B+L library. These compounds no longer gave maximum correlation values for their correct compound as they did in the original B+L library. The nearest neighbours ($\delta r < 0.1$) now included compounds from other unidentified groups. Closer inspection of the spectra from the two instruments clearly revealed a small wavenumber shift, particularly in the 4008–6000 cm^{-1} region. A fixed wavenumber shift of + 6 cm^{-1} was applied to the PerkinElmer spectra for compounds that were identified in the B+L library. The correlation values for all 24 compounds had now improved, ranging from 0.97 to 0.99. In conclusion, although a fixed wavenumber correction improved the situation and successful transfer was achieved, the wavenumber shift is not linear along the wavenumber scale. Future work will examine a more sophisticated wavenumber correction procedure.

Palmer, K. E. et al (2005) *J. Pharm. Pharmacol*. **57** (Suppl.): S10–S11

11
Using FTIR and dielectric spectroscopy in combination to measure protein hydration in-situ

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Although the cause of instability of therapeutic proteins following lyophilisation has not been unequivocally established, removal of part or all of the protein hydration shell is thought to play a role. Knowledge of the content and distribution of water in the formulation is therefore critical. Data (Wellner et al 1996) on wheat gluten suggests that infrared (IR) spectroscopy may be a useful method of measuring local water concentrations, since there is an indication that changes in the structure of the Amide II band (N-H bend/CN stretch) follow closely the hydration of the protein. Low frequency dielectric spectroscopy has previously been used to probe the behaviour of non-frozen water in frozen aqueous solutions of a model protein and cryo-protectant (Barker 2004). Here, we have combined these two techniques to study both the location and behaviour of water in a simple protein formulation. Gluten was used as a model protein because it shows a strong IR response and is readily available. Samples of wheat gluten (Hereward, UK) containing a range of water contents were prepared by storage over saturated salt solutions and examined by FTIR, dielectric spectroscopy and MTDSC. Peak intensity ratios in the Amide II region of the FTIR spectra showed a linear relationship with the water content of the samples up to approximately 30 g water per 100 g dry protein. In more hydrated samples, the slope of the curve decreased. In contrast, peak intensity ratios in the Amide I (C = O stretch) region show a maximum at approximately 15–20% water content. A linear relationship was apparent between the real permittivity at low frequencies and the moisture content, with a change in slope at a moisture content corresponding to that seen at the discontinuity in the Amide II data. These data suggest that the dielectric response and the FTIR response are related. Changes in the Amide I band are thought to reflect changes in conformation, rather than hydration (Dousseau & Pezolet 1990). It is postulated that the addition of water results in the hydration of the amide groups detected in the Amide II band, resulting in a change in the FTIR spectrum. Hydration also increases the motion of the amide groups and side chains, reflected in the dielectric spectrum. Once amide hydration has reached a maximum, the relationship between response and water content will be altered, as was seen here. From our data, maximum amide hydration occurs at approximately 3.3 water molecules per amino acid residue. MTDSC experiments indicate that

the non-freezing water content in wheat gluten is dependent on initial water content and ranges from 36 to 45 g water per 100 g dry protein. The differences in the MTDSC data and the spectroscopic data suggest that protein hydration and the non-freezing water are not necessarily strongly connected. This study has shown that FTIR and dielectric spectroscopy may be used in conjunction to explore protein hydration and may offer a way of probing protein hydration in-situ with no requirement for extensive sample preparation or manipulation.

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12
Use of near-infrared conformance methods for tablet in-process monitoring and quality assurance

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The attention of pharmaceutical manufacturing has recently focused on Process Analytical Technology (PAT), endorsed and highlighted by the U.S. Food and Drug Admininstration's (FDA) PAT initiative, which has directed pharmaceutical analytical chemistry to technology that can rapidly analyse key process and quality attributes in pharmaceutical product during manufacturing (FDA, CDER, 2004). As near-infrared (NIR) spectroscopy has the potential for the analysis of hundreds of tablets per batch, it lends itself to at- and on-line content uniformity testing in a PAT framework for real-time analysis and quality control of pharmaceutical tablets. This paper describes the development of an at-line conformance method that can be implemented rapidly into the process to monitor the performance of tabletting operations and assure the quality of the active ingredient in the pharmaceutical product. The conformance method assesses whether the quality attribute of interest fits within a previously established acceptable range rather than whether the attribute meets a specific specification as with conventional quantitative NIR methods (e.g. active content as % label claim). Six commercial batches of a conventional tablet solid dosage form were selected for the calibration set to establish the range covering all acceptable natural variation in the product. This calibration set was used to set thresholds for two trend charts; individual tablet active pharmaceutical ingredient (API) absorbance and variation (standard deviation,) of API absorbance for sets of 10 tablets. Four commercial batches and four development batches at 75, 85 115 and 125% of nominal active content (2.5% w/w amlodipine besylate) comprised the validation set and were used to challenge the established trend chart thresholds. Transmittance NIR spectra were measured across 600–1900 nm on a FOSS Intact Tablet Analyser. The active content of each tablet was then determined by HPLC assay and the conformance method compared with conventional assay and relative standard deviation results for each batch. Specificity for the API in the presence of the other tablet components was proven at 1122 nm with SNV, 1st derivative data treatment and the NIR analysis was shown to be precise with repeatability RSD of 0.01%. The trend charts were generated, with trend chart limits set at ± 3 standard deviations from the mean. The individual trend chart limits successfully passed tablets from the four production batches in the validation set while flagging all four development batch tablets. The SD trend chart successfully flagged three challenge sets composed of a spread of commercial and development tablets. The conformance method results for API absorbance and SD of API absorbance were found to overlay the conventional HPLC assay and RSD results for the validation set. The successfully developed conformance method was shown to perform comparably to traditional methods and required less than half the reference chemistry as conventional quantitative assay method validation. This would facilitate rapid implementation into pharmaceutical plants and would enable rapid analysis of a large volume of tablets during manufacture providing a simple system for process monitoring and quality assurance.

PAT — A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance, http://www.fda.gov/cder/OPS/PAT.htm.
United States Food and Drug Administration, Centre for Drug Evaluation and Research (2004) *Guidance for Industry*

13
In-situ process monitoring using calorimetry: assessment of powder and tablet swelling

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The ability to follow processes as they occur in-situ is the primary aim of any assay (reducing the number of assumptions required to effect interpretation) but can pose a significant analytical challenge. Typically, the more sensitive assays (chromatographic or spectroscopic) require abstraction of the principal analyte prior to analysis, as well as requiring that analyte to have some specific chemical or physical property. Heat, conversely, is a universal marker of change, which means that calorimetric techniques are ubiquitous and useable for virtually any system and thus offer significant potential for pharmaceutical systems. Here, to demonstrate this potential, it is shown how calorimetry can be used quantitatively to assess the swelling of polymers and polymeric drug delivery systems. Knowledge of the swelling characteristics of such systems, vital in order to tailor the release profile of drugs to a specific pharmacological profile, is difficult to gain by direct study; conventionally dissolution or visual/mass change data are used. The former is not a direct measurement (as it is the drug release profile that is actually recorded) and the latter is invasive and not suited to microparticulate systems. We studied formulations containing HPMC E4M and NaCMC, both as powders (using particle size fractions of 90–125 μm and 45–53 μm) and as tablets (2 mm × 1 mm). Note here that the study of swelling of powders is virtually impossible by other means. Samples were dispersed into buffered media (either pH 2.2 or 6.8 McIlvaine buffers) using the Thermometric 2265 20 mL micro solution ampoule at 25°C and the power-time data were recorded. Swelling profiles were constructed by plotting q_t/Q (where q_t is the heat released to time t and Q is the total heat release). The data could then be analysed using a conventional swelling model to yield n values (where n is a parameter that defines the mechanism of swelling). The values of n are given in Tables 1 and 2. For the powdered samples the values of n were greater than 1, which indicated that dissolution occurred immediately following hydration of the polymer. The values of n for each polymer were significantly different and, moreover, were observed to vary both as a function of particle size and dissolution medium pH. For the tablets, the values of n were ca. 0.8 at pH 2.2 and ranged from ca. 0.8 to 1.2 at pH 6.8. At both pHs the data for NaCMC tablet dissolution showed an endothermic signal after an exothermic peak. It is presently not clear what events give rise to this observation, but it is likely that for this system dissolution involves both hydration and dissociation and the calorimetric data reflect the balance of events. The work has demonstrated that (i) calorimetry can be used to monitor swelling and that there are demonstrable differences between polymer and formulation types and (ii) more generally that calorimetric methods offer an alternative suite of analytical tools for direct assays.

Table 1 n values for powdered samples

Sample	pH	n
HPMC (90–125 μm)	2.2	1.03
HPMC (45–53 μm)	2.2	1.20
NaCMC (45–53 μm)	2.2	1.31
1:1 Blend (45–53 μm)	2.2	1.23
HPMC (45–53 μm)	6.8	1.09
NaCMC (45–53 μm)	6.8	1.61
1:1 Blend (45–53 μm)	6.8	1.32

Table 2 n values for tablets

Sample	pH	n
HPMC	2.2	0.82
NaCMC	2.2	n/a
1:1 Blend	2.2	0.80
HPMC	6.8	0.81
NaCMC	6.8	1.28
1:1 Blend	6.8	1.04

14
Validated bioanalytical method development for determination of etoricoxib in human plasma and its application to pharmacokinetic studies in rats

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Etoricoxib, 5-chloro-6′-methyl-3[4-(methanesulfonyl) phenyl]-2,3′-bipyridine, is a highly selective and active cyclo-oxygenase II inhibitor. This dipyridinyl derivative was developed in order to address the safety issues associated with traditional

NSAIDs (Cochrane et al 2002). It is rapidly absorbed with a mean bioavailability of 100%. Etoricoxib reaches a maximum plasma concentration in approximately 1 h (T_{max}) after oral administration. A mean peak plasma concentration (C_{max}) of 3.6 mgL^{-1} was reported after a single oral dose of 120 mg to a human volunteer. Peak plasma levels and AUC values are approximately linear with the dose. Etoricoxib has a volume of distribution of 120 L and is 92% bound to plasma protein. To monitor intra- and inter-individual variability for the drug, it is desirable to determine plasma concentrations of the drug in clinical practice. Few HPLC methods for the estimation of etoricoxib in urine and plasma have been reported (Saha et al 2002; Brautigam et al 2003). This communication describes two simple, sensitive and highly selective, reversed-phase, isocratic, high-pressure liquid chromatographic methods for estimation of etoricoxib in plasma.

Sample preparation for the developed methods employs precipitation using acetonitrile (direct method) and liquid-liquid extraction with dichloromethane (indirect method). The technique employs a C_{18} column (5μ, 250 mm × 4.6 mm) for separation and acetonitrile: phosphate buffer (10 mM, pH 6), 50:50 v/v adjusted with 0.1 M KOH as mobile phase at a flow rate of 1.5 ml min^{-1}. Detection was accomplished at 280 nm using diode array detection. Chromatograms showed good resolution and sensitivity with no interference from plasma. The direct method was found to be linear in the range 0.1–2.5 μg ml^{-1} ($r^2 = 0.999$). Results of sample analysis gave a relative standard deviation of ± 3.97%; the limit of detection was found to be 0.019 μg ml^{-1}. The indirect method also exhibited linearity in the range of 0.1–2.5 μg ml^{-1} ($r^2 = 0.999$). The results of sample analysis gave a relative standard deviation of 3.16%; limit of detection was found to be 0.009 μg ml^{-1}. Etoricoxib was found to be stable upon storage in plasma at −20°C. All the stability samples met acceptance criteria and gave the value for accuracy better than 100 ± 10% and precision of less than 10%. Both intra-day and inter-day accuracy and precision data showed good reproducibility. The methods were applied to measure the plasma concentration following an intravenous bolus and oral administration of etoricoxib to rats and were found to be suitable for pharmacokinetic studies.

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