Short Papers in Pharmaceutical Analysis

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Effects of space-charge formation on Thermally Stimulated Current (TSC) spectroscopy results

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TSC is a relatively novel and powerful technique for studying molecular mobility in the pharmaceutical field. For example, we have previously shown that TSC may be used to assess the glass transition of amorphous materials (Antonijević & Barker 2002) and solid-state polymorphic transitions (Antonijević & Barker 2003). One issue with the interpretation of TSC spectra is the presence of post-main transition peaks, which may not always be reproducible. The TSC response is manifested in the form of a current generated by dipolar relaxations in a sample over the temperature range of interest. In addition to the dipolar (heterocharge) responses, ionic (homocharge) responses may also be observed. These latter are due to space-charge formation. Injection of free electrons (space-charge) could occur from the electrode to the sample due to the differences in band structure of the two materials or from the air gap between them, with the extent of such injection varying with the applied energy (ie electric field). Injected free electrons are deposited in cathodic (CDR) and/or anodic (ADR) depletion regions (Thielen et al 1996). Here we have assessed the ability of TSC to monitor and characterise space-charge formation and investigated the possibility that space-charge formation is responsible for at least some of the irreproducible post-main transition peaks observed in our studies on amorphous indometacin. TSC experiments were carried out using a TSC/RMA 9000 spectrometer (Setaram, France) in the TSDC mode over the temperature range 0–80°C (Tg of amorphous indometacin is approximately 42°C). Experimental results indicated a reproducible main peak corresponding to the published Tg, with variable moderate post transition peaks between 40 and 80°C. The characteristics of these peaks varied with experimental conditions, in particular temperatures of stabilisation and polarisation. The results can be rationalised by considering the contact between the sample and the electrode and how this varies with experimental conditions. As the temperature is raised, the sample expands, reducing any air gap between the sample and electrode. Subsequently, the shape of the CDR is changed, which then causes a diffusion and drift of space-charge. As a result, a small positive shoulder ρ_{RD} peak appears on the higher-temperature side of the main transition peak. After the ρ_{RD} current vanishes, the system is not in a steady-state but rather in quasi-steady state. A secondary relaxation stage of the system, from quasi-steady state to the true steady state, involves electronic injection from the electrodes. The subsequent space charge peak is referred to as an injection peak, ρ_I, and is dependent on the characteristics of the interfacial potential barrier and amount of charge accumulated in the corresponding space charge regions. Given the thermal and electrical pre-treatment of the sample inherent in running in the TSDC mode, it is logical to consider that the experimental conditions chosen will affect the extent of generation of space-charge and hence the post-main peak results. Our results are consistent with the model that space-charge formation occurs and is responsible for the non-regular peaks seen in the post-main transition temperature region.

Antonijević, M. D., Barker, S. A. (2002) Proceedings of the American Association of Pharmaceutical Scientists Pharmaceutics and Drug Delivery Conference, 3013

Antonijević, M. D., Barker, S. A. (2003) Proceedings of the British Pharmaceutical Conference, 063

Thielen, A., Niezette, J., Feyder, G. et al (1996) *J. Phys. Chem. Solids* **57**: 1567–1580

002
The application of near-infrared spectroscopy for the off-line and on-line analysis of intact Lipitor tablets

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Near-infrared (NIR) spectroscopy has found widespread application as one of the enabling tools for the recent FDA process analytical technology (PAT) initiative (Fountain et al 2003). NIR methods provide a quick and accurate alternative to the reference analytical method (e.g. HPLC), which allows for increased testing frequency, greater process knowledge and understanding, and facilitates the move of quality systems towards continuous verification. This study aimed to demonstrate the feasibility of a global off-line single tablet NIR assay for the active in Lipitor (atorvastatin) 10 mg tablets, which represented a significant challenge due to the low concentration of atorvastatin present (nominal 6.6% m/m). A secondary objective was transfer of the assay with minimal re-validation to a second, equivalent, instrument (e.g. at another testing site), and to an on-line process instrument, thereby considerably expanding the applicability of the method. Currently, the transfer of NIR calibration models is relatively uncommon, with the main obstacle being differences in the response of instruments and a lack of guidelines for evaluating transfer. Nine batches (from three manufacturing sites) of uncoated Lipitor (atorvastatin) 10 mg tablets were used in this study, with the actual active content determined by UV analysis (4.80–8.21% m/m active content). Transmittance NIR spectra were measured at 7000–12 000 cm^{-1} using two equivalent Bruker MPA Fourier transform (FT) spectrophotometers (laboratory systems) configured with InGaAs detectors. Spectra were also collected using a Bruker Martix-T FT spectrophotometer, an optically similar process instrument configured for on-line tablet analysis. The optimal NIR assay, developed using data from a single laboratory instrument (A), was used to analyse samples measured on the second laboratory instrument (B) and the process instrument (C) without any form of correction (i.e. direct transfer). The prediction errors (root mean standard error of prediction (RMSEP)) were poor compared with instrument A, with significant bias between the NIR predicted and the UV reference values (Table 1). Applying the mean sample residual spectrum correction to instruments B and C, a method previously utilised for calibration transfer (Smith et al 2002), however, produced comparable results. Statistical evaluation proved the assay to be accurate on all instruments. An accurate NIR assay for atorvastatin in intact tablets, developed at a single site using an off-line instrument, was successfully transferred to a second equivalent instrument as well as a process instrument configured for on-line analysis. The potential for transferring the method to further manufacturing sites was also demonstrated as samples from three locations were included in the development procedure. NIR spectroscopy as a tool for the process control and testing of Lipitor tablets was established during this study.

Table 1 Calibration transfer statistics

Inst	Cal set	Direct transfer		Corrected transfer	
	RMSEC (% m/m)	RMSEP (% m/m)	Bias (% m/m)	RMSEP (% m/m)	Bias (% m/m)
A	0.039	0.054	0.007	0.054	0.007
B	N/A	0.158	−0.134	0.083	0.007
C	N/A	0.334	−0.327	0.069	0.007

Fountain, W., et al (2003) *J. Pharm. Biomed. Anal.* **33**: 181–189

Smith, M. R., et al (2002) *Analyst* **127**: 1682–1692

003
The novel use of near-infrared spectroscopy to determine the limits of detection of drugs in powders

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Near-infrared spectroscopy (NIRS) has demonstrated its effectiveness within the pharmaceutical industry as a quantitative tool for the assay of the active component within a sample matrix such as tablets (Eustaquio et al 1998). Recent work has described the ability to calculate the limit of detection (L.O.D.) by a direct measurement NIRS method for the polymorphic forms of sulphathiazole (Patel et al 2001), but there have been no published methods to measure the L.O.D of a drug in powders. This study investigated different methods of measuring the L.O.D for caffeine in a sucrose substrate using NIRS. Three particle size fractions of sucrose at 710–250 μm, 500–180 μm and 38 μm–180 μm in triplicate were used as substrates. One sample of each particle size range was selected to which caffeine (180–90 μM) 1–6% w/w in 1% increments was added (making a total of 18 samples), mixed and scanned to obtain six replicates per increment per particle size fraction. A linear relationship was derived between the average spectral intensity values of six replicate spectral scans of the 2nd derivative caffeine peak at 1672 nm and the concentration of the caffeine over the range 1.0–6.0% w/w for each of the particle size fractions. In addition, pure sucrose of each particle size fraction before caffeine was added shaken and scanned. The L.O.D was defined as any mean intensity

value outside ±3 standard deviations of the six replicate scans at 1672 nm, for either the original samples (3 × six scans) or the three particle size fractions combined (18 replicate scans) of the pure sucrose. The equation generated from each linear relationship was then subsequently confirmed in a separate experiment by concentrations of approximately 0.2, 0.4, 0.6 and 0.8% w/w caffeine. The L.O.D of caffeine obtained by this direct measurement method in each sucrose sample was 0.8% w/w in the 38–180 μm sucrose particle size fraction, 0.9% w/w caffeine in the 500–180 μm particle size fraction and 0.5% w/w in the 710–250 μm particle size fraction. Differences in variability for each pure sucrose fraction were obtained when defining the population based on six replicate scans against defining it based on the combination of three particle size fractions (18 replicate scans). These differences were minimised with the 38–180 μm fraction. A Wavelength Distance model was also created with the data. This enabled the Wavelength Distance model to detect the caffeine at a level of 0.2% in the 38–180 μm particle size and 0.3% for the 500–180 μm particle size fractions. The results indicate that the L.O.D is dependent on the variability of particle size fractions of the substrate and strategies to improve this can enable lower L.O.D values to be obtained. It also demonstrates that the Wavelength Distance model offers a better L.O.D than the direct measurement method for drugs such as caffeine. Preliminary experiments with other drugs confirm the applicability of the methods outlined.

Eustaquio, A., et al (1998) *Analyst* **123**: 2303–2306
Patel, et al (2001) *J. Pharm. Sci.* **90**: 360–369

004
Determination of pyrazine 2,5-dipropionic acid formed in formulations containing 5-aminolevulinic acid

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Aqueous solutions and topical dosage forms containing 5-aminolevulinic acid (ALA) are commonly used in the fluorescence diagnosis of bladder tumours (Waidelich et al 2003) and photodynamic therapy of neoplastic lesions, respectively (McCarron et al 2003). ALA is chemically unstable when not in solutions of low pH. Pyrazine-2,5-dipropionic acid (PY) is the main degradation product formed in aerated aqueous solutions. The toxicity of PY is currently unknown. As a result, concentrations in pharmaceutical formulations and human cavities should be kept to a minimum. Solutions for fluorescence diagnosis of bladder tumours are, therefore, prepared immediately before use, while topical dosage forms are kept at −20°C before use. The aim of this work was to quantify the amounts of PY formed in aqueous solutions, of different pH values and ALA concentrations, over time at room temperature. The formation of PY in various aqueous and organo gel and bioadhesive patch formulations, stored under different conditions was also followed over a period of 6 months. Since significant amounts of PY may be formed in the urine within the bladder after instillation, the stability of ALA in artificial urine was also studied. A high performance liquid chromatography assay was developed that allowed quantification of PY in solution. This method was used to study PY formation in solutions buffered to various pH values, and artificial urine as well as the range of topical dosage forms. All formulations contained defined initial loadings of ALA. Samples of each of the topical dosage forms were dissolved in borate buffer pH 5.0 before assay. To ensure that PY was the only degradation product formed under the conditions investigated, ALA loss was followed using the method of Oishi et al (1996). The method developed was found to be specific for PY. PY was detectable down to low levels using HPLC with UV detection at 265 nm. The limits of detection and quantification were 0.01 μg mL^{-1} and 0.04 μg mL^{-1}, respectively. The method was reproducible, with inter-day and intra-day variation in peak areas within acceptable limits. PY was stable under the autoinjector conditions used, meaning multiple samples could be sequentially injected without fear of analyte degradation. Significant PY formation was detected in all ALA solutions studied, as well as in artificial urine. PY was found to be the only degradation product formed under the conditions studied, since the amounts of PY detected were in close agreement with the theoretical yields of PY calculated based on ALA loss. No other peaks were detected in the chromatographs. As a result of the significant amounts of PY formed, steps should be taken to increase ALA stability in solutions intended for instillation into the bladder for fluorescence diagnosis of bladder tumours. In addition, since PY was also formed in artificial urine, the toxicity of PY, at concentrations found in this study, should be investigated using cell culture techniques. PY was not the major degradation product in the topical dosage forms, presumably due to their multi-component natures and the inherent reactivity of ALA.

McCarron, P. A., Donnelly, R. F., Woolfson, A. D., et al (2003) *Drug Deliv. Sys. Sci.* **3**: 59–64
Oishi, H., Nomiyama, H., Nomiyama, K., et al (1996) *J. Anal. Toxicol.* **20**: 106–110
Waidelich, R., Beyer, W., Knuchel, R., et al (2003) *Urology* **61**: 332–337

005
Applications of Raman microscopy: fast scanning chemical mapping of drug delivery systems

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Raman spectroscopy is a versatile and information rich spectroscopic technique. As a tool for pharmaceutical analysis it has many applications, ranging from the polymorphic characterisation of drug and excipients to process control. Coupling a Raman spectrometer to a microscope produces a technique capable of giving detailed chemical and physicochemical information with spatial resolution at the micrometer scale. However, the technique is not without problems. The Raman signal can be small relative to intrinsic fluorescence. In some cases Raman scattered light is not observable at all in the presence of strong fluorescence, a problem often encountered with natural carbohydrates and their derivates, a set encompassing many pharmaceutical excipients. Additionally, Raman microscopy is often regarded as a slow technique, and indeed acquisition of Raman maps can, in some cases, extend to several days. Here we report on the performance of a high sensitivity Raman microscope (Witec Confocal Raman Microscope CRM 200) giving rapid scanning and high spatial resolution. Use of a cooled Charge Coupled Device (CCD) with efficient coupling of excitation laser (high flux point source) and collection optics allow the acquisition of Raman spectra from $< 1\ \mu m^3$ voxel (true confocal conditions) with a dwell time in the millisecond range. This in turn allows the acquisition of Raman maps with micrometer spatial resolution, where spectra are acquired for each individual pixel, in hours rather than days. Here two examples where Raman microscopy complements other high-resolution spatial imaging techniques are shown. In the first example Raman maps of the distribution of a micronised drug within a tablet formulation give the drug particle size distribution. This data is subsequently correlated with Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS) images. In the second example Raman mapping was performed on a formulation comprised of a drug substance compounded with hydroxypropylmethyl cellulose (HPMC). Data were acquired using a 785 nm excitation source, which reduces the fluorescence problem frequently encountered with HPMC. The maps show that the drug can exist as a molecularly dispersed state or as a phase separated state. In the latter, detailed examination of the spectra from regions of interest show that the drug is detected in a crystalline state. This is reflected in the compromised bioavailability. These results were verified using Scanning Thermal Microscopy (SThM) and Localised Thermal Analysis (LTA). In summary one of the major disadvantages of Raman microscopy, namely slow acquisition rates, has been addressed by a new generation of Raman microscope design. High sensitivity allows collection of true confocal spectra in millisecond time periods, and consequently detailed chemical maps with a resolution approaching the diffraction limit.

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Stability of flucytosine in polypropylene syringes

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Flucytosine, 4 amino-5-fluoro-2 (1H)-pyrimidinone is a fluorinated pyrimidine antifungal drug used to treat severe *Candida* and *Cryptococcus* infections. Parenteral aseptic preparations of such medicines are used frequently in hospitals, but little stability data is available. Data that is available is usually incomplete, unreliable and often confusing. The purpose of this study was to obtain the ultimate shelf life for flucytosine in prefilled syringes so that they may be prepared in bulk in appropriately licensed facilities. The stability of flucytosine 10 mg mL^{-1} was determined in 2-mL polypropylene syringes at 25°C/60% relative humidity (RH) and at room temperature in the light (RTL) for up to 546 days. Flucytosine and fluorouracil, 5-fluoro-2, 4 (1H, 3H) pyrimidine-dione (a cytotoxic decomposition product) concentrations were determined at appropriate time intervals by stability-indicating high-performance liquid chromatography (HPLC). The chromatographic separation was performed at ambient temperature on a reversed phase Supelcosil LC-18-DB column, 150 mm × 4.6 mm i.d., 5 μm particle size. Elution was established (Vermes et al 1999) with a mobile phase composition of ammonium dihydrogen phosphate (pH 3.5, 0.03 M) and heptane sulphonic acid (0.005 M)

at a flow rate of 1.0 mL min^{-1}. The chromatographic signal was monitored at 266 nm. The samples were also monitored for pH and the appearance of the solution and container. Results showed that flucytosine in polypropylene syringes remains very stable at both storage conditions for the duration of the study. No measurable loss in flucytosine assay values was noted, the solution remained clear and colourless and no visual change was observed in the syringe components. The pH did not alter throughout the study period. The concentration of fluorouracil, however, doubled from 0.24% to 0.47% relative to flucytosine. In consideration of the increase in the toxic decomposition product and the possible microbiological growth associated with storage of syringes at room temperature, a shelf life of 3 months is recommended.

Vermes, A., Van Der Sijs, H., Guchelaar, H. (1999) *Pharm. World Sci.* **21**: 35–39

007

Determination of protein-ligand binding affinity by NMR: observations from serum albumin model systems

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NMR is becoming an increasingly popular drug discovery tool in the study of small molecule–protein interactions. We have used the protein bovine serum albumin (BSA) as a model to investigate the use of NMR methods to determine K_ds for drug–protein interactions. Using ligands such as L-and D-tryptophan, naproxen, ibuprofen, salicylate and warfarin, we have attempted to test and validate this technique. We have also employed isothermal titration calorimetry (ITC) to determine K_ds for these systems to allow comparison of results obtained by these two methods. Serum albumin is known to have affinity for a wide range of small molecules. It has two major binding sites, one of which appears to bind ligands, such as L-tryptophan, naproxen and ibuprofen, tightly and specifically, and one that is less well defined and whose ligand binding is less specific. Salicylate and warfarin are known to bind at this second site. As well as these two binding sites, serum albumin is also known to bind with weak affinity many molecules of ligand at other poorly defined binding sites. Using ITC, a method that determines K_d from the heat evolved or absorbed during titration of a ligand solution into a protein solution, we were able to accurately determine the K_d and stoichiometry of the interaction between BSA and three drug molecules — L-tryptophan, naproxen and ibuprofen. These all appear to form specific complexes with BSA, with a stoichiometry of 1:1. It was reported in the literature that D-tryptophan has no affinity for BSA but using ITC we were able to show that this molecule does indeed bind to BSA but with a K_d at least 10 times less than L-tryptophan. However, in the case of warfarin and salicylate, the ITC experiments were less reproducible and the stoichiometry for these interactions appeared greater than 1:1. These data could not be fitted satisfactorily using the ITC data analysis software. We studied the same systems using the NMR titration method, where the ligand is the observable species. The ligand concentration was increased while the protein concentration remained constant and the results interpreted using using two data interpretation methods — changes in chemical shift and changes in linewidth. In the case of L-tryptophan, a K_d of $160 \pm 130\,\mu$M was determined with good reproducibility. However, in the case of D-tryptophan, no line broadening or chemical shift changes were observed. Naproxen was shown by ITC have an extremely high affinity for BSA ($K_d \sim 0.035\,\mu$M) and as such is not suitable for NMR analysis as this value is outwith the range of this technique. At present we are studying BSA complexes with ibuprofen, warfarin, and salicylate and hope to be able to report our results in due course.

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The use of near-infrared microscopy for the identification of counterfeit Viagra tablets

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This work examines the capabilities of Near Infrared (NIR) microscopy for the identification of counterfeit medicines. Counterfeit medicines may be seized by the Medicines and Healthcare products Regulatory Agency (MHRA) or other regulatory agencies abroad. Existing techniques used to identify counterfeit products such as High Performance Liquid Chromatography (HPLC) are time consuming and require skilled personnel. An additional problem is that counterfeit products are often sophisticated, containing the active ingredient in similar amounts to those of the authentic, which means that simply testing for the presence or absence of the active ingredient(s) using existing methods may be insufficient. The use of NIR microscopy would allow for not only the identification of individual components in a product, but also the determination of their relative distribution and percentage composition, all of which can be compared with the properties of the authentic product. A Perkin Elmer FT-NIR IdentiCheck spectrophotometer coupled with an AutoIMAGE microscope was used to map several authentic and counterfeit Viagra tablets supplied by Pfizer Ltd and the MHRA. In addition to this, several different generic versions of Viagra (not licensed in the UK but available over the internet) were imaged to demonstrate the sophistication of the technique. A 30 μm^2 area of a flat cross-section of the tablet was used to collect a single NIR spectrum (known as a pixel), each tablet image consisting of 100×100 pixels. These tablet image spectra were then compared with databases holding spectra of pure sildenafil citrate, the active ingredient and commonly used tablet excipients, including those known to be present in authentic Viagra tablets. Chemometric methods used to compare mathematically pre-treated spectra included Principal Components Analysis and Partial Least Squares analysis. Visual differentiation between the authentic, generic and counterfeit tablets was possible. A semi-quantitative method was developed, which showed that the sildenafil citrate content was similar for the authentic and generic tablets, while the majority of the counterfeit tablets contained less of the active ingredient. Particle size analysis of sildenafil showed agglomeration of particles was more evident with the generic and the counterfeit tablets than with the authentic Viagra. In addition components such as lactose were identified as being present in the generic and counterfeit samples, which are not present in Viagra, while components such as microcrystalline cellulose and magnesium stearate present in Viagra were not identified in the other two sets of tablets. NIR microscopy would play a useful role in supplementing existing techniques used in the combat against counterfeit pharmaceuticals as it is able to not only identify counterfeit products but also to compare the spatial distribution of components within a tablet with other seized counterfeit products, which can help in tracing their source.

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