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ORAL PRESENTATIONS

Short Papers in Pharmaceutics

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Use of disintegrants in the crystallization of a poorly water-soluble drug

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Objectives Spherical crystallization is a particle-design technique by which crystallization and agglomeration can be carried out simultaneously in one step. It has been successfully utilized for improvement of flowability and compactibility of crystalline drugs (Paradkar et al 1994, Nokhodchi et al 2007). The aim of the current study was to improve the flow, compaction and dissolution properties of a poorly water-soluble drug with poor compactibility, naproxen, by incorporating a disintegrating agent in the drug agglomerates by spherical crystallization technique.

Methods Naproxen crystals were prepared in the presence of different ratios of disintegrants. Hydroxypropyl cellulose was dissolved in distilled water (500 mL), and one-third of the total disintegrant was uniformly dispersed in the solution at room temperature by stirring for 20 minutes. Acetone containing naproxen and the other two-thirds of the disintegrant was also separately prepared. The latter dispersion was added immediately to the dispersion containing dissolved polymer under constant stirring conditions. The resulting agglomerates were then filtered and dried overnight. The agglomerates were compressed at different pressures and dissolution studies were carried out for the tablets produced at the lowest compression force. The dried agglomerates were also characterized in terms of shape, size, flow and tensile strength, and the solid state of the agglomerates was studied using X-ray powder diffraction (XRPD) and differential scanning calorimetry (DSC).

Results The results showed that the tablets prepared using the untreated (original) naproxen particles were prone to capping at compression pressures above 40 MPa, whereas the agglomerated crystals were successfully tableted without capping at any of the compression pressures applied. The improved compactibility of the agglomerates could be attributed to their structural characteristics. The agglomerates comprised small adherent crystals and this particular structure was responsible for the large relative volume change, which occurred during the early stage of the compression process as a consequence of fragmentation. It is apparent that when the disintegrant was incorporated via the crystallo-*co*-agglomeration techniques the disintegration of the resultant tablets was faster than if the tablets were produced by physical mixing of the disintegrant with naproxen. For example, when starch was added to the crystallization medium the disintegration time was 4.76 minutes, whereas the corresponding disintegration time for the naproxen tablets when an equivalent amount of starch was added physically to the obtained recrystallized naproxen was 12.2 minutes. The former tablets containing these agglomerates dissolved at a faster rate (90% dissolved in 10 minutes) than the tablets containing crystallized naproxen with the same amount of disintegrant incorporated only extragranularly by physical mixing (55% dissolved in 10 minutes). Similar results were obtained when starch was replaced with sodium starch glycolate. DSC and XRPD studies showed that naproxen particles, crystallized in the presence of hydroxypropyl cellulose and disintegrant, did not undergo structural modifications.

Conclusions In conclusion, the properties of agglomerated crystals, such as flowability, compactibility and dissolution rate, were improved profoundly using the developed technique. This resulted in successful direct tableting without need for the additional process of physically blending the agglomerates and disintegrants.

Nokhodchi, A. et al (2007) *Powder Technol*. **175**: 73–81
Paradkar, A. R. et al (1994) *Indian Drugs* **31**: 229–233

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Determining particle size distribution of non-spheres from chord length measurements

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Objectives Determination of particle size distribution (PSD) is vital in many processes such as polymerization, precipitation and crystallization in which product quality depends on control of PSD. Laser diffraction (LD) is a reliable and well-validated measurement technique but it can only be applied directly in low-concentration systems. Focused-beam reflectance measurement (FBRM) is a measurement technique that works in high-concentration systems and is easy to use, but it returns chord length distribution (CLD) rather than the more easily understood PSD. Compared with CLD, PSD is more useful because it relates directly to a well-defined and clearly understood product property which facilitates use of automatic control. However, it has not been easy to convert a measured CLD into its corresponding PSD accurately due to the lack of a theoretical analysis, especially for non-spherical particles. The aim of the study is to develop mathematical methods to convert a measured CLD into its corresponding PSD.

Methods Two distinct issues were addressed to transform a CLD into its corresponding PSD. The simpler is the PSD-to-CLD translation model, which calculates the CLD corresponding to a known PSD of particles of a given shape. The PSD–CLD model facilitates the CLD-to-PSD inversion in which a measured CLD is compared with CLDs generated by known PSDs. Here, a general model was generated to translate PSDs into their corresponding CLDs for particles of arbitrary shapes. Then an inversion method was developed to obtain PSDs from measured CLDs. The inversion has been used to convert CLDs of particles of several different shapes measured by a Lasentec model D600L (Mettler Toledo) FBRM instrument. The retrieved PSD is an equivalent-diameter distribution of particles having the projected area of the measured particles.

Results CLDs of three different inorganic materials – spherical ceramic beads and non-spherical plasma aluminium and zinc dust particles – were measured using the Lasentec FBRM instrument. The particle shape and PSD of these materials were also investigated by image analysis. Comparison of PSDs retrieved from FBRM data with PSDs measured using image analysis showed that the PSD can be retrieved from a measured CLD successfully using the iterative NNLS method based on the PSD-to-CLD model. CLD measurements were made during crystallization of L-glutamic acid in both its prismatic α-form and the acicular β-form. These CLD measurements have been transformed into PSDs of the crystals so that crystal growth data may be obtained.

Conclusions From this study it can be seen that a measured CLD can be translated successfully into its PSD. The accuracy of this translation depends substantially on particle shape, which determines the optimal aspect ratio in the model. For a dynamic system such as a crystallization process, the PSD changes during the process. If the shape information is constant during the process, then, similar to an instrument calibration, the optimal aspect ratio can be first determined for a sample with known PSD using the optimal searching algorithm. Then unknown PSDs can be recovered from a series of CLD measurements using the NNLS method based on the PSD–CLD model at the same aspect ratio.

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Interactive effects of drugs and diluents on early gel-layer formation in hydroxypropyl methylcellulose hydrophilic matrices

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Objectives The aim of this study was to investigate the interactive effects of drugs (diclofenac and meclofenamate sodium) and diluents (lactose and microcrystalline cellulose (MCC)) on hydroxypropyl methylcellulose (HPMC) hydrophilic matrix gel-layer morphology, swelling and functionality. An earlier study had confirmed the differing effects of two structurally similar non-steroidal anti-inflammatory drugs on gel-layer formation and functionality in binary mixtures (Pygall et al 2006). Subsequently, ternary formulations containing drug, diluent and HPMC were manufactured and investigated for the effects of drugs and diluents on gel-layer development.

Methods Tablets (8 mm, 200 mg) containing 20–60% w/w HPMC (E4M) drug (diclofenac sodium and meclofenamate sodium) (20–60% w/w) and a diluent (MCC or lactose) (either 19 or 59% w/w) were manufactured to a constant compression pressure (180 MPa) on an instrumented tablet press. The tablets were subsequently held between two clear Perspex discs, allowing imaging from above using a Bio-Rad MRC 600 confocal microscope. Tablets were hydrated in aqueous 0.008% Congo red, maintained at 37°C. Congo red is known to fluoresce

when bound to hydrated cellulose, thus allowing a fluorescent image to be obtained of the tablets as hydration proceeds (Bajwa et al 2006). Disintegration of these matrices was determined using a standard US Pharmacopoeia (USP) methodology. Tests were carried out maximally for 120 minutes.

Results The gel-layer formation of these matrices appeared to be affected by both the drug and the diluent. At low levels of diluent incorporation (19% w/w), the effect of drugs appeared to be related to the ratio of drug to HPMC. Meclofenamate appeared to 'salt-in' the HPMC and enhance swelling, whereas the opposite was seen with diclofenac. MCC appeared to confer a resistance to these drug effects, necessitating higher ratios of drug to HPMC for the drug effects to be observed. With higher levels of diluent incorporation (59% w/w), the disruptive effects of the diluents were exacerbated by the presence of drugs on the HPMC, becoming more significant with higher drug loadings. Lactose appeared to modify the effect of drugs by suppressing polymer hydration. This resulted in a synergistic 'salting-out' effect with diclofenac and antagonism of the salting-in effect of meclofenamate. From the images, MCC appeared to increase tortuosity within the gel layer and promoted modulation of HPMC properties by the drug through a possible retardation of diffusion and an increase in concentration within the gel layer.

Conclusions This study illustrates how individual components in a hydrophilic matrix formulation can interact with one another. It shows that diluents can affect the manifestation of drug effects on HPMC gel-layer functionality through effects on polymer hydration and swelling. The choice of diluent can resist or exacerbate the effect of drugs, which may be related to their solution properties. The possible interplay between diluents and drug effects should be considered during formulation-development processes.

Bajwa, G. S. et al (2006) *J. Pharm. Sci.* **95**: 2145–2157
Pygall, S. R. et al (2007) *J. Pharm. Pharmacol.* **59** (Suppl.): A40

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Dynamic gastric model (DGM): a novel *in vitro* apparatus to assess the impact of gastric digestion on the droplet size of self-emulsifying drug-delivery systems

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Objectives Self-emulsifying drug-delivery systems (SEDDSs) can improve drug bioavailability and pharmacokinetics by avoiding erratic oral absorption. However, assessment of formulation performance within the gut is often unpredictable, due to a lack of adequate *in vitro* digestion models. The dynamic gastric model (DGM) was developed at the Institute of Food Research (Norwich, UK) and can accurately simulate human stomach physiology. It was used here to evaluate the impact of gastric digestion on SEDDS droplet size.

Methods Empty Licaps® capsules (n = 4; Capsugel) were filled with 500 mg of SEDDS (Span 80®/Tween 80®/soybean oil). Ibuprofen (BASF) was used as a model drug (6% w/w). DGM was primed with 20 mL of acid solution (HCl (19.7 mL/L), NaCl (4.675 g/L), $CaCl_2$ (0.03 g/L) and NaH_2PO_4 (0.105 g/L) to simulate the residual fasted gastric volume, then fed 250 mL of ultrapure water. After pH equilibration the capsule was introduced. Simulated gastric secretions containing NaCl (4.675 g/L), $CaCl_2$ (0.03 g/L), NaH_2PO_4 (0.105 g/L), phosphatidylcholine (from egg yolk; 0.127 mM, 1.96 mL/L), pepsin (9 kU/mL, 2.72 g/L) and gastric lipase (60 U/mL, 0.4 g/L) were added during processing, at a flow rate modified during gastric emptying to mimic real-time *in vivo* dynamic addition, within physiological limits. The rate of gastric acid addition was controlled by a pH-monitoring/-feedback loop to simulate physiological flow rates and to produce a gastric pH profile as seen *in vivo*. All simulated gastric digestions were carried out at 37°C and had a total residence time of approximately 25 min. At predetermined time intervals, digested samples were collected for droplet size analysis. A total of eight fractions for each capsule were collected. Size measurements were performed on samples diluted in ultrapure water using a Coulter LS-230 laser light-scattering apparatus (Beckman-Coulter) equipped with a polarization intensity differential scattering (PIDS) unit and small-volume module.

Results Simulated gastric digestion had a greater effect on placebo droplet size than the ibuprofen formulation. For the placebo, the emulsion's droplet size changed during gastric digestion: the first emptied samples (at 3 min 39 s and 6 min 26 s) contained very small droplets ($D_{50} \leq 0.9963 \pm 0.6666$ μm); then between 9 min 5 s and 19 min 58 s the droplet size showed only a moderate increase ($2.316 \pm 1.060 < D_{50} < 4.127 \pm 2.691$ μm). However, at the end of digestion (25 min 22 s) the formed emulsion showed a significant size increase ($D_{50} = 13.68 \pm 4.451$ μm). Formulation containing ibuprofen also showed a slight increase in droplet size, as digestion proceeded, but not the large increase at the end of digestion ($D_{50} = 5.285 \pm 2.404$ μm).

Conclusions Usually, SEDDS droplet size is evaluated using volumetric flasks or dissolution apparatus II as dispersive systems; however, these models show great limitations and approximations in modelling gastric digestion. As an example, artificial gastric juice (British Pharmacopoeia 2008) does not contain gastric lipase, and the pepsin content is higher than the physiological concentration; also, stomach motility has an impact in determining the emulsion's droplet size, once emptied through the pyloric sphincter (Dressman et al 2007). Using DGM, we demonstrated that the physiology of the stomach can be accurately simulated, and the impact of the physical and biochemical processes on the formulation droplet size estimated, with satisfactory reproducibility within different samples.

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Dressman, J. et al (2007) *J. Pharm. Sci*. **96**: 522–531

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Can dietary sugars influence the extended-release performance of hydroxypropyl methylcellulose matrices?

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Objectives Despite the widespread use of hydroxypropyl methylcellulose (HPMC) in extended-release applications, there have been isolated reports describing HPMC matrix failure in fed individuals (Abrahamsson et al 1998). The purpose of this study was to establish whether the presence of dietary sugars can influence the performance of HPMC matrices.

Methods The effect of D-fructose, D-glucose, D-galactose, maltose, lactose and sucrose on the sol–gel transition temperature of 1% w/v HPMC solutions (Methocel™ K4M-CR) was investigated turbidimetrically (cloud-point apparatus; QMC, Nottingham, UK). Extended-release matrices (8 mm diameter, 250 mg) containing 30% w/w HPMC, 10% caffeine anhydrous and a 2:1 mixture of lactose and microcrystalline cellulose were manufactured using an F3 Manesty tablet press (Manesty, Liverpool, UK) at 230 MPa. Drug release in aqueous sugar solutions was investigated using US Pharmacopoeia (USP) apparatus I at 100 rpm with 900 mL deaerated medium maintained at 37 ± 0.5°C. Early gel-layer development during matrix hydration in sugar solutions at 37 ± 0.5°C was imaged using a Bio-Rad MRC-600 confocal microscope (Bio-Rad, Hemel Hempstead, UK) by a method described previously (Bajwa et al 2006).

Results All dietary sugars were found to progressively depress the HPMC sol–gel transition temperature as sugar concentration increased. The potency of different sugars varied considerably from –6.7°C M^{-1} (D-fructose) to –31.1°C M^{-1} (lactose). Increases in sugar concentration in the dissolution medium at first slowed drug release, from $T_{80\%}$ = 5.5 h to $T_{80\%}$ = ≈7.5 h, but at a critical sugar concentration (C_{CRIT}) there was a dramatic change in dissolution kinetics. At C_{CRIT}, matrices at first showed the development of extended-release properties, but then rapidly disintegrated, liberating 100% drug within 4 hours. Values of C_{CRIT} were found to vary from 0.5 M (lactose) to 1.15 M (D-fructose) and correlated directly with the ability of the sugar to depress the HPMC sol–gel transition temperature ($r^2 = 0.9091$). Images of early gel-layer formation showed that matrices below C_{CRIT} formed normal coherent gel layers, but at sugar concentrations immediately above C_{CRIT} images showed highly extended gels and evidence of incomplete polymer hydration. Further increases in sugar concentration suppressed polymer swelling completely, and prevented the formation of a gel layer.

Conclusions The presence of dietary sugars in the dissolution medium above a theshold concentration C_{CRIT} was shown to promote burst release from HPMC matrices. Images of early gel-layer formation suggested that matrix failure was in response to the formation of diluted and fragile gel layers, following the suppression of polymer particle swelling and coalescence processes. Since sugars were also shown to lower HPMC sol–gel transition temperatures, the observed matrix failures in sugar-rich environments may arise from the ability of sugars to disrupt stable hydration sheaths around the hydrophobic areas of HPMC through their solvent-ordering properties and therefore lower the aqueous solubility of the polymer.

Abrahamsson, B. et al (1998) *J. Control. Rel*. **52**: 301–310
Bajwa, G. S. et al (2006) *J. Pharm. Sci.* **95**: 2145–2157

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Calcium-enriched titanium surfaces via a hydrothermal process

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Objectives Titanium is currently the favoured biomaterial for many load-bearing applications. A variety of methods have been applied for increasing the osseo-integration capabilities of titanium surfaces. Among these methods hydrothermal

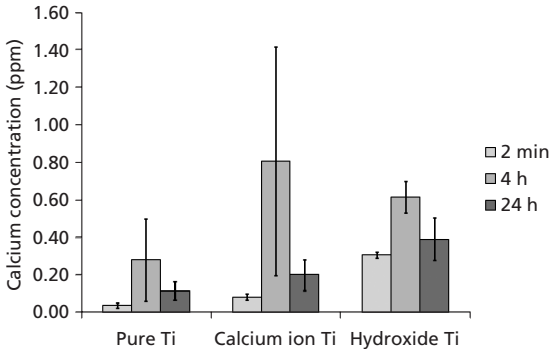

Figure 1 Calcium ion concentrations found in test solutions at the indicated time. Samples: Pure Ti, polished, grade 1 Ti; Calcium ion Ti, calcium ion-implanted Ti; Hydroxide Ti, hydrothermally treated Ti.

treatments (Kim et al 1996) and calcium ion implantation (Nayab et al 2005, Armitage et al 2007) have been shown to offer improvements. As calcium ion implantation is an involved and expensive process, the objective of this study is to evaluate an alternative method for loading titanium surfaces with calcium.

Methods Titanium discs (grade 1, commercial purity) were ground with silicon carbide paper and polished to a mirror finish using colloidal silica and hydrogen peroxide. Hydrothermal treatment was carried out using NaOH and CaOH solutions. Following rinsing in pure water, the samples were heat treated in an air furnace at 600°C for 24 hours. Dissolution testing was performed using ion chromatography on pure water with the appropriate samples immersed for 2 minutes, 4 hours and 24 hours.

Results The results of the dissolution test (Figure 1) indicate that there is a significant sustained calcium release from the hydrothermally treated sample which is similar in magnitude to that observed for calcium ion-implanted surfaces. Preliminary experiments indicate that only a small proportion of the calcium in the near-surface region is released, offering much potential for sustained calcium ion release from these surfaces.

Conclusions Modified titanium surfaces were produced with a calcium-release profile broadly similar to that of calcium ion-implanted titanium. This treatment process is far more commercially viable than ion-implantation techniques and warrants further investigation.

Armitage, D. A. et al (2007) *App. Surf. Sci.* **253**: 4085–4093
Kim, H. M. et al (1996) *J. Biomed. Mater. Res.* **32**: 409–417
Nayab, S. N. et al (2005) *Biomater.* **26**: 4717–4727

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Kafirin proteins and their pharmaceutical applications as a novel excipient for solid oral dosage forms

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Objectives To explore the potential applications of kafirin storage proteins from sorghum as a novel tablet excipient. Our previous work has established that other cereal proteins, zeins, could be utilized for controlled release of oral drugs. In particular, this study investigated the dissolution of a model drug from the kafirin tablets and any changes in the secondary structure of the kafirin macromolecules upon compression and dissolution.

Methods Kafirin was extracted from a Sudanese sorghum cultivar and was tested by a range of physical techniques. Direct compression tablets containing kafirin (65.1% w/w), calcium hydrogen phosphate (27.9% w/w) and magnesium stearate (1% w/w) as excipients and caffeine (6%) as a model drug were prepared using a Manesty E press fitted with 12.7 mm normal concave punches. The effect of compression and dissolution on the secondary structure of kafirin was studied using a Bruker Fourier-transform infrared spectrometer. Dissolution studies were conducted in 0.1 M HCl, purified water and pH 6.8 buffer using a British Pharmacopoeia model II apparatus.

Results Fourier-transform infrared data on kafirin showed that α-helices and β-sheets predominated, with their relative contents being slightly affected during processing. After dissolution at pH 1 there was an increase in β-sheets. Dissolution profiles showed that the amount of caffeine released after 5 hours was the greatest at pH 1, suggesting that deamidation of the kafirin might be occurring at this pH, which is consistent with the Fourier-transform infrared results. Tablets remained intact throughout the dissolution study, irrespective of the pH, suggesting that kafirin has potential as a vehicle for oral controlled drug release. Figure 1 illustrates the appearance of the kafirin tablets after the dissolution studies at various pH values. A pale pink coloration was observed at pH 1, possibly due to the tannins present in the kafirin extract, which underwent a colour shift in acidic conditions.

Conclusions Kafirin shows promise as a pharmaceutical excipient, particularly when its hypo-allergenicity is considered. Work is currently ongoing to fully characterize its potential.

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Bacille Calmette–Guérin (BCG)-loaded alginate beads as a potential oral tuberculosis vaccine for wildlife

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Objectives The aim of this work was to evaluate the protective effect of encapsulating live Bacille Calmette–Guérin (BCG) in alginate beads for oral delivery. To investigate this, *in vitro* studies have been performed to assess the release behaviour and protective effects of alginate beads on BCG in simulated gastric fluid (SGF), phosphate-buffered saline (PBS) and simulated intestinal fluid (SIF).

Methods Alginate solution (2% w/w; Protanal SF200 69%G 31%M) was mixed with a suspension of BCG in an equal ratio. The mixture was then added dropwise into a solution of 0.5 M $CaCl_2$ using a syringe (30 gauge needle), producing beads approximately 1.8 mm in diameter. After 15 minutes the beads were removed from the $CaCl_2$ solution and washed with double-distilled water. BCG-loaded beads were then incubated in SGF (0.1 M HCl pH 1.2 without pepsin) for 2 hours. The BCG was subsequently extracted from the beads by dissolving them in sodium citrate (2% w/v) and then cultured on Middlebrook 711 agar containing OADC supplement at 37°C for 4 weeks. Following incubation colony counts were taken to calculate viability. BCG-loaded beads were also incubated in SGF for 2 hours followed by incubation in PBS at pH 7.5, for up to 2 hours. Finally this study was also repeated using SIF (containing monobasic potassium phosphate 0.05 M, pancreatine 10 g/L, co-lipase 20 μg/L, pancreatic lipase 300 μg/L and sodium cholate 2.7 g/L, adjusted to pH 7.5 with 1 M NaOH) rather than PBS to provide results using conditions that are more representative of the small intestine (Chambers 2007). Parallel experiments using 'naked' BCG were also performed. Statistical analysis of results was performed using one-way analysis of variance with *post hoc* Tukey test to identify significant differences between datasets.

Results Initial results show that alginate beads were able to offer significantly enhanced ($P < 0.05$) protection to BCG during 2 hours' exposure to SGF, with 65 ± 5% viability being retained (no BCG colonies were recovered from the remaining volume of SGF) compared with 5 ± 0.9% of naked BCG. Total release into PBS pH 7.5 following 2 hours of SGF exposure occurs within 45 min and following release BCG appears to be stable for up to 2 hours in PBS, retaining 90% viability. In contrast, when BCG was released in SIF, although dissolution and release from the beads occurred within 45 minutes viability was reduced by 70 ± 7% following 1 hour of exposure. When exposure to SIF was repeated with naked BCG a similar reduction in viability occurred (78 ± 4%), which was not statistically different ($P < 0.05$) from the viability of BCG released from alginate

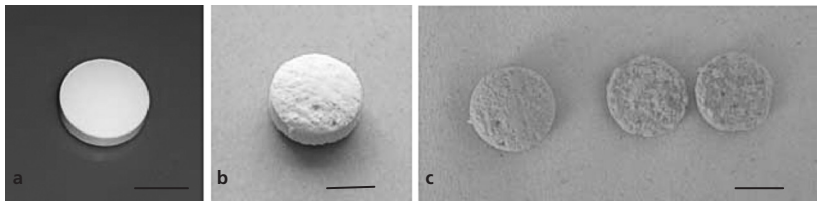

Figure 1 Kafirin tablets: (a) untreated, (b) after dissolution in water and (c) after dissolution at pH 6.8 (left) and at pH 1 (centre and right). Scale bars, 10 mm.

beads. These results highlight the need to develop dissolution media that are more representative of the gastrointestinal tract, especially where physiologically sensitive biopharmaceuticals are concerned.

Conclusions BCG entrapped in alginate enhances viability in SGF for up to 2 hours compared with naked BCG. However, incubation in SIF resulted in large losses in viable BCG, possibly due to the cocktail of enzymes in the SIF. Ongoing *in vivo* studies will investigate the suitability of this approach to oral tuberculosis vaccination of wildlife and the efficacy of the vaccine.

Chambers, M. (2007) *Interim project report SE3223*. DEFRA

Short Papers in Pharmacognosy and Pharmaceutical Chemistry

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Analysis of linear polyamine natural product conjugates

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Objectives The applications of linear polyamines, for example spermidine and spermine and their conjugates, in drug delivery are continuing to be investigated. Our studies of organic nano-bioparticles as vehicles for non-viral gene delivery have afforded a library of semi-synthetic polyamine conjugates, which are efficient at DNA condensation and delivery. However, their quantitative analysis is challenging as they lack any chromophore. In order to analyse these polyamine conjugates, fluorescence derivatization has been applied.

Methods We have developed methods for poly-derivatization with a panel of extrinsic fluorophores (e.g. dansyl chloride (Minocha and Long 2004), *o*-phthalaldehyde (OPA), 9-fluorenyl-methoxycarbonyl chloride (FMOC; Koros et al 2007) and fluorescamine) followed by high-performance liquid chromatography (HPLC) with both fluorescence and UV absorption detection. These methods enable the analytical optimization of synthetic routes. Reaction and chromatographic conditions were optimized for each fluorophore using a series of model mono- and diamines and finally applied to natural and semi-synthetic polyamines. The structures of the resulting derivatives were confirmed by off-line high-resolution electrospray ionization mass spectrometry (H ESI-MS). Linear responses were obtained over the concentration range 0.01–1.00 mM. The relative quantum yields of the polyamine-fluorophore derivatives were examined to measure any intramolecular fluorescence quenching. We have also developed an alternative methodology for distinguishing the similarly functionalized polyamines spermidine and spermine (tri- and tetra-amines) by preparing hexahydropyrimidines (Chantrapromma et al 1980) before derivatizing with a chromophoric reagent and HPLC separation.

Results Our results show that synthesis of polyamine derivatives in quantitative yield depends on the reaction conditions: time, temperature and the molar ratio of derivatization reagent to substrate amine. Off-line mass spectrometry analysis of the products demonstrated complete derivatization of both primary and secondary amino groups with dansyl and FMOC fluorescent derivatives and of primary amine groups for OPA and fluorescamine derivatives. Under the H ESI-MS ionization conditions used, the dansyl derivatives often showed double protonation as the cations $[(M+2H)/2]^{2+}$ in addition to the expected monovalent ions $[M+H]^+$. Presumably this is because this chromophore contains basic amino groups that can be protonated easily, whereas FMOC derivatives gave predominantly $[M+Na]^+$ ions. Dansyl derivatization of polyamines showed no apparent steric hindrance. The OPA reaction with polyamines is rapid, but the products have poor stability. Derivatization with fluorescamine gave multiple products (HPLC analysis). The chromatographic separation of poly-dansyl derivatives of spermidine (three) and spermine (four) as mono- and di-hexahydropyrimidines (as di-dansyl derivatives) showed retention times for spermidine, spermine and their hexahydropyrimidines of 11.60, 17.80, 7.50 and 10.00 minutes respectively (HPLC, Luna C8, 5 μm, 150 × 4 mm, isocratic 70:30 acetonitrile/water, λ_{ex} = 310 nm, λ_{em} = 550 nm).

Conclusions This methodology provides a useful way to analyse these important natural products and their semi-synthetic analogues which lack any chromophores. These compounds are being developed as efficient vehicles for gene delivery and as anti-cancer lead compounds where quantitative analysis is important.

Acknowledgement We thank the CRN for financial support (studentship to SB).

Chantrapromma, K. et al (1980) *Tetrahedron Lett.* **21**: 2475–2476
Koros, A. et al (2007) *J. Chromatogr. A* **1149**: 46–55
Minocha, R., Long S. (2004) *J. Chromatogr. A* **1035**: 63–73

10
Investigation of scopoletin biosynthesis during post-harvest physiological deterioration in cassava roots using stable isotopic labelling

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Objectives Coumarins are pharmacologically active and have roles in plant defence. Despite their importance, key aspects of the biosynthesis of these secondary metabolites remain unresolved. Here we exploit the observation that the accumulation of scopoletin and its glucoside scopolin increases in cassava roots (*Manihot esculenta* Crantz) during post-harvest physiological deterioration to test alternative pathways for the biosynthesis of these hydroxycoumarins.

Methods Cassava roots (cv. Mcol22 and NGA 19), within 2 hours of harvesting, were fed with different labelled intermediates on the postulated biosynthetic pathway of scopoletin (e.g. *trans*-cinnamic-d_7, *trans*-cinnamic-3,2′,3′,4′,5′,6′-d_6, *trans*-cinnamic-2′,3′,4′,5′,6′-d_5, *trans*-cinnamic-2-d, *trans*-cinnamic-$^{18}O_2$, *p*-coumaric-2-^{13}C, caffeic-2-^{13}C and ferulic-2-^{13}C acids). Also, competition feeding experiments were carried out with a mixture of *trans*-cinnamic-2′,3′,4′,5′,6′-d_5 acid with each of 2′,4′-dihydroxycinnamic, caffeic and ferulic acids. Post-harvest physiological deterioration was allowed to occur. Ethanolic extracts of the deteriorated roots were separated (high-performance liquid chromatography) and analysed (high-resolution electrospray ionization mass spectrometry).

Results Deuteriated cinnamic acids were incorporated, and typically 29% of the scopoletin was deuteriated. Incorporation (in both scopoletin and scopolin) of only three deuterons when fed with *trans*-cinnamic-d_7 and *trans*-cinnamic-d_6, and non-deuteriated scopoletin when cinnamic-2-d acid was fed, indicates that the pathway involves exchange of the 2-hydrogen atom in cinnamic acid, and strongly supports our thesis that the *trans–cis* isomerization step is enzymic, and not photochemical, as found *in vitro* and in other plants where four deuterons would have been found in the labelled product. Incorporation of *p*-coumaric-2-^{13}C, caffeic-2-^{13}C and ferulic-2-^{13}C acids in the biosynthesis of scopoletin gave an average increase of 14% ^{13}C-labelled scopoletin and ^{13}C-labelled scopolin. There was no reduction in label incorporation when either caffeic or ferulic acids were fed in competition to deuterium-labelled cinnamic acid whereas competition with unlabelled *trans*-2′,4′-dihydroxycinnamate caused a decrease from 4.6 to 3.4% (compared with the labelled scopoletin isolated when the roots were fed with labelled cinnamic acid only). *Trans*-cinnamic-$^{18}O_2$ was incorporated (5%) in the biosynthesis of scopoletin with only one ^{18}O-labelled oxygen atom in the product.

Conclusions The ready accumulation of scopoletin and scopolin in cassava roots during post-harvest physiological deterioration makes it a good model to investigate their biosynthesis. We have shown that the *E–Z* isomerization step (of the cinnamic acid derivative) during the biosynthesis is enzymic. Furthermore, all three proposed pathways (as found in different plants) are operating in cassava, but the pathway via 2′,4′-dihydroxycinnamate is likely to be the predominant one. A pathway via a spirolactone-dienone (quinol) intermediate has been previously established in *Streptomyces niveus* for novobiocin biosynthesis in elegant work by Kenner and co-workers (Bunton et al 1963), and also proposed from UV studies in cultures of the plant *Ammi majus* L. (Apiaceae, Bishop's flower, large bullwort) (Matern 1991) following work by Grisebach and Ollis (1961). The absence of doubly enriched ^{18}O-scopoletin means that the lactonization step is through *ortho*-hydroxylation not via a spirolactone-dienone intermediate, where both ^{18}O-atoms would be incorporated in the final product.

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An evaluation of the chemotaxonomy of *Lignosus rhinocerus*

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Objectives To identify a chemotaxonomic marker compound that may be suitable for evaluating the quality and safety of traditional herbal medicinal products (THMPs) containing *Lignosus rhinocerus*. The use of THMPs has increased over the last 10 years in Malaysia. However, the quality and safety of these products are still questionable since they usually contain a range of herbal constituents which are complex and may also contain adulterants. Hence, it is essential to identify a chemotaxonomic marker compound for a herbal plant that could give an indication