JPAG Session: Short Papers in Pharmaceutical Analysis

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Microemulsion liquid chromatography (MELC) method validation for the quantification of terbutaline in the Bricanyl Turbohaler

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Objectives Microemulsion liquid chromatography (MELC) has received much interest and has shown great potential in separation science, with applications in both *in vitro* analysis (Marsh et al 2005, Altria et al 2006) and *in vivo* bioanalysis (Berthod et al 1992, El-Sherbiny et al 2003). This is because of its unique selectivity, high efficiency and faster analysis time compared with conventional high-performance liquid chromatography (HPLC). This report describes the development and validation of a MELC method, using salbutamol as an internal standard. Terbutaline was analysed in the Bricanyl Turbohaler while the aerodynamic characteristics of the emitted dose were examined using Pharmacopoeial methods.

Methods The mobile phase was a 95% aqueous orthophosphate buffer (adjusted to pH 3 with orthophosphoric acid), with 0.5% ethyl acetate, 1.5% Brij 35 and 3% 1-butanole (all components are expressed as w/w). The samples were injected on to a C_{18} Spherisorb (250 mm × 4.6 mm × 5 μm) column at 25°C using a constant flow rate of 1 mL/minute. The terbutaline peak was detected by fluorescence using excitation and emission wavelengths of 267 and 313 nm, respectively.

Results The method had an accuracy of more than 99%, and the calibration curve was linear (r^2 = 0.99). The intra-day and inter-day precision of the method (in terms of percentage coefficient of variation) were less than 0.1% and 0.09%, respectively. The limit of quantitation and limit of detection for terbutaline were 25 and 8 μg/L, respectively.

Conclusions The method described in this study is reliable, precise, accurate and rapid. On the other hand, it was not possible to separate terbutaline from the salbutamol peak using the conventional HPLC modes. The method was applied to determine the content of the emitted dose and the fine-particle dose of the Bricanyl Turbohaler.

Altria, K. D. et al (2006) *Chromatographia* **63**: 309
Berthod, A. et al (1992) *Anal. Chem.* **64**: 2258–2262
El-Sherbiny, D. et al (2003) *J. Sep. Sci.* **26**: 503–509
Marsh, A. et al (2005) *Chromatographia* **61**: 539–547

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An ICH-guided, validated, stability-indicating high-performance liquid chromatography assay method for estimation of carvedilol in bulk drug, tablet and nano-emulsion formulations

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Objectives The objective of the current investigation was to study the degradation behaviour of carvedilol under different International Conference on Harmonisation (ICH)-recommended stress conditions by high-performance liquid chromatography (HPLC), and to establish a validated, stability-indicating HPLC assay method.

Methods The chromatographic separation was achieved on a Supelco 516 Rp C_{18} column (250 mm × 4.6 mm, 5 μm inner diameter) using acetonitrile/0.02 M phosphate buffer/triethylamine (55:45:0.01, by vol.; pH 3.5); pH was adjusted with orthophosphoric acid as the mobile phase. The binary elution was performed at a flow rate of 1.0 mL min^{-1} with UV detection at 242 nm. The method was validated with respect to linearity, precision (including intermediate precision), accuracy, robustness and specificity. The drug was subjected to forced degradation of hydrolysis (acid and alkaline), oxidation, photolysis and thermal (dry and wet) decomposition as per ICH guidelines (ICH-Q1A 2003). The acidic and alkaline degradation kinetics of carvedilol were determined using this method at temperatures of 313, 328 and 343 K. The developed method was successfully applied to estimate the amount of carvedilol in tablets (Cardivas® and Carloc® tablets) and nano-emulsions.

Results The method was validated and response was found to be linear in the drug concentration range of 0.5–50 μg mL^{-1}. The mean values (± SD) of the slope and correlation coefficient were 120555 (± 1789.69) and 0.9991 (± 0.002), respectively. The percentage relative SD (RSD) values for intra- and inter-day precision were 0.648–1.463 and 0.892–1.540, respectively. Extensive degradation was found to occur in alkaline medium at 70°C for 3 h, and under oxidative stress (20% H_2O_2 for 3 h). Mild degradation was observed in acidic, UV (254 nm) and daylight (72 h) conditions. The drug was stable to thermal stress. All the degradation peaks were well resolved from the pure drug with significantly different retention time values. The first-order degradation kinetics of carvedilol were observed in acidic and alkaline conditions with half-lives ($t_{1/2}$) at 25°C of 6.35 and 3.14 h respectively. The utility of the method was verified by assay of drug in bulk, tablet and nano-emulsion formulations and percentage recoveries were found to be in the range of 99.95–101.13, 98.60–99.61 and 99.52–99.84%, respectively.

Conclusions A validated stability-indicating HPLC assay method was developed for carvedilol, applying the forced degradation suggested by ICH. The developed method is simple, accurate, precise and specific and could separate the drug from degradation products. It is suggested for use in analysis of samples generated during stability studies on carvedilol and its formulations.

International Conference on Harmonisation (2003) *Stability testing of new drug substances and products*. ICH-Q1A (R2). International Conference on Harmonisation

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Assay of ciprofloxacin in intact and powdered tablets by near-infrared spectroscopy

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Objectives To develop near-infrared spectroscopic (NIRS) quantitative models for the assay of ciprofloxacin in proprietary and generic ciprofloxacin 500 mg intact and powdered tablets.

Methods Ciprofloxacin 500 mg tablets were purchased from the UK and the Tanzanian markets. Tablets in both intact and powdered forms were utilized to construct calibration models. Tablets of a single proprietary brand (Bayer, 500 mg) were powdered and diluted with excipient(s) to give mixtures of different concentrations of ciprofloxacin. In addition, standard additions of ciprofloxacin were used to give samples of increasing concentrations. Spectra were measured on a FOSS NIRSystem 6500 NIR spectrometer using a rapid content analyser. Each spectrum was the sum of 32 scans over the wavelength range 1100–2500 nm at 2 nm interval. Four spectra were recorded from each tablet, two on each side at 90° to each other. Four spectra were also recorded from the powdered samples in Waters (4 mm) vials shaking between measurements. The average of the spectra was taken in both cases in standard normal variate second derivative (SNV-D2) form.

Results The ciprofloxacin tablets had a range of activity between 55 and 77% m/m estimated as the ratio of the label claim (in milligrams) to the weight of the tablet. These data were used to construct the Intact Tablet Model. The powdered models used various ranges of ciprofloxacin between 0 and 100%. In all cases, a partial least squares regression (PLSR) algorithm (Unscrambler 7.5) was utilized. The spectra were split into calibration and validation sets based on PCA data to give the greatest variability in the calibration set. The ratio of the calibration to validation sets was 75%:25%. Specific partial least squares components were chosen for each model to give the lowest root mean standard error of prediction as a percentage of the label claim (RSEP %). The PLSR loadings showed good correlation with the ciprofloxacin tablet spectrum. The predictive ability of the model was validated against known proprietary and generic intact and powdered tablets. The tablets contained ciprofloxacin or other antibiotics of different classes. A good prediction gave the label claim within ±10% of the ciprofloxacin tablets and 0% for the other tablets. In this respect, the best model that fulfilled the stated criteria was the one which utilized the standard addition of the ciprofloxacin to the crushed Bayer tablets. It gave an r^2 value of 0.9989 and RSEP% of 0.96%. In addition, it was able to predict both ciprofloxacin and blank tablets.

Conclusions The NIRS method with PLSR regression is a simple, rapid, non-destructive technique for the determination of ciprofloxacin in proprietary and generic tablets. It can be used without the need for a reference analytical technique and with an RSEP% as low as 0.96%.

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A stability-indicating high-performance thin-layer chromatography and liquid chromatography method for the determination of levetiracetam in bulk and in pharmaceutical dosage form

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Objectives Levetiracetam, known chemically as (*S*)-2-(2-oxopyrrolidin-1-yl)butan-amide, is used as an adjunct in the treatment of partial seizures in adults and children

aged 4 years and over in the UK. Adults and adolescents aged 16 years and over may also be given levetiracetam as monotherapy for this indication (Radtke et al 2001). The aim of the present work was to develop a stability-indicating chromatographic method for the determination of levetiracetam in the presence of its degradation products for assessment of purity of bulk drug and stability of its dosage forms using high-performance thin-layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC) (Rao et al 2004).

Methods Two sensitive and reproducible methods are described for the quantitative determination of levetiracetam in the presence of its degradation products. The first method was based on HPTLC followed by densitometric measurements of their spots at 225 nm. The separation was on HPTLC aluminium sheets of silica gel 60 F_{254} using toluene/methanol/acetic acid (6.0:4.5:0.05, by vol.). This system was found to give compact spots for levetiracetam after development (R_f value, 0.50 ± 0.05). The second method was based on HPLC of the drug from its degradation products on a reversed-phase Perfectsil C_{18} column (25 cm × 4.6 mm, 5 μm inner diameter) at ambient temperature using a mobile phase consisting of ammonium acetate with orthophosphoric acid/methanol (80:20, v/v) at pH 3.0 and a retention time, t_r, of 7.25 ± 0.05 minutes. Both separation methods were validated as per the International Conference on Harmonisation (ICH) guidelines.

Results No chromatographic interference from the tablet excipients was found. Levetiracetam was subjected to acid/alkali hydrolysis, oxidation, dry heat, wet heat and photodegradation. The drug was susceptible to acid/alkali hydrolysis and oxidation. The drug was found to be stable in neutral, wet heat, dry heat and photodegradation conditions. As the proposed analytical methods could effectively separate the drug from its degradation products, they can be employed as stability-indicating techniques.

Conclusions The proposed HPTLC and HPLC methods provide simple, accurate, reproducible and stability-indicating methods for quantitative determination of levetiracetam in pharmaceutical tablets, without interference from the excipients and in the presence of acidic, alkaline, oxidative and photolytic degradation products. Both the chromatographic methods were validated according to ICH guidelines. Statistical tests indicate that the proposed methods reduce the duration of analysis and appear to be equally suitable for the routine analysis in pharmaceutical formulation in quality-control laboratories, where economy and time efficiency are essential. This study separates the drug from its degradation products, and hence is a typical example of a stability-indicating assay.

Radtke R. et al (2001) *Epilepsia* **42** (suppl. 4): 24–7
Rao B. et al (2004) *J. Pharm. Biomed. Anal*. **5**: 1017–1026

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The role of high-performance liquid chromatography in a study to extend the shelf life of injection solutions of the recombinant monoclonal antibody bevacizumab (Avastin)

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Objectives Bevacizumab is a recombinant monoclonal antibody that acts as an angiogenesis inhibitor by targeting vascular endothelial growth factor. This effect prevents the formation of new blood vessels in the growing tumour. It is licensed for use in cancer therapy, but has been shown to be effective for the treatment of age-related macular eye degeneration and it is to be used in a clinical trial to compare its activity with that of ranibizumab (Lucentis), a drug licensed for this purpose. The assurance of bevicizumab's stability in pre-filled syringes is of importance for this trial. The stability of monoclonal antibodies must be assessed using a range of analytical techniques to monitor changes in the size, shape, electric charge and molecular mass of the antibody (International Conference on Harmonisation document ICH-Q5C, *Stability testing of biotechnological/biological products*). The chromatographic methods presented here were developed and validated as part of this programme.

Methods High-performance size-exclusion chromatography (SEC) was carried out using a silica-based gel-permeation column with an eluent of high salt content at a pH well separated from the isoelectric point of the antibody. The monomeric bevacizumab plus dimer and trimer were resolved using this method (Table 1). Reverse-phase high-performance liquid chromatography (RP-HPLC) utilized a wide-pore C_{18} column at a temperature of 80°C. Gradient elution was carried out with mobile phases containing trifluoroacetic acid and increasing amounts of a high-eluotropic-strength solvent. Validation of the methods' suitability for stability studies was carried out by measuring precision, linearity and ability to separate degradation products produced under accelerated stability regimes (Table 1).

Results SEC: chromatograms showed a single large peak due to monomeric bevacizumab, a small peak possibly due to dimer which was present in the bevacizumab sales pack, and after 2 weeks a further very small, broad peak that was possibly due to trimer. Validation: linearity = 0.9999, relative SD (RSD) = 0.644% (n = 6). RP-HPLC: chromatograms showed a single large peak due to bevacizumab, and two small peaks, which were also present in the bevacizumab sales pack. Validation: linearity = 0.99998, RSD = 0.124% (n = 6).

Table 1 Four-week stability data, with peak areas as percentage total peak area (internal normalization)

	RP-HPLC			SEC		
	RT = 2.6 min	RT = 12.4 min	Bevacizumab	RT = 4.5 min Trimer	RT = 6.7 min Dimer	Bevacizumab Monomer
Vial contents	0.31	1.48	98.20	N/Q	1.29	98.56
1 week	0.30	1.45	98.24	N/Q	1.39	98.53
Vial contents	0.34	1.41	98.21	1.21	1.51	96.56
2 weeks	0.37	1.41	98.22	1.33	1.44	98.23
Vial contents	0.33	1.42	98.22	N/Q	1.38	97.93
4 weeks	0.33	1.34	98.32	N/Q	1.44	98.57

N/Q, not quantifiable; RT, retention time.

Conclusions The stability study is ongoing, but the data acquired so far demonstrate that bevacizumab solutions are stable when stored under refrigeration for 1 month.

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Terahertz-pulsed imaging as an analytical technique for tablet film coating: monitoring the unit operation and process scale-up

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Objectives Terahertz-pulsed imaging (TPI) operates in the far-infrared region of the electromagnetic spectrum (2–120 cm^{-1}) (Ho et al 2007). This technique affords non-destructive extraction of coating quality parameters (coating layer thickness and density) that have direct impact on subsequent dissolution performance (Ho et al 2008). In this study, the ability of TPI to monitor a film coating-unit operation and to assess the success of the subsequent process scale-up is investigated.

Methods One hundred and ninety sustained-release (polyvinyl acetate) coated tablets were sampled in total, at 10% increments of the amount of polymer applied from a laboratory and a pilot-scale batch. These tablets were analysed with TPI, followed by dissolution testing. Process quality parameters (tablet film coating thickness and variations in coating density) were extracted using TPI. Samples with a coating layer thickness under the current axial resolution limit of the TPI set-up (around 38 μm) were discarded from the coating analysis.

Results It was shown that both coating quality parameters (coating thickness and variations in density) derived with TPI were more product-specific than the amount of polymer applied when employed to monitor the coating processes. When monitoring a film coating-unit operation (using the laboratory scale), coating layer thickness was shown to be the governing factor on the subsequent dissolution behaviour. An R^2 value of 0.89 (root mean square error (RMSE) = 0.22 hours, mean dissolution time (MDT) range = 3.15–5.48 hours) was observed for the linear regression between the coating thickness and the MDT, while an R^2 of 0.79 (RMSE = 0.31 hours) was demonstrated for the variations in coating density. The process scale-up resulted in significant changes in MDT between the laboratory and pilot scale, and this was accurately detected by monitoring variations in the film coating density (acquired from terahertz electric field peak strength, TEFPS) and confirmed with coating layer thickness measurements using TPI (Figure 1).

Conclusions As an analytical technique, TPI allows for better film coating process understanding, and can be used to improve the coating-unit operation and the scale-up procedure for sustained-release tablets.

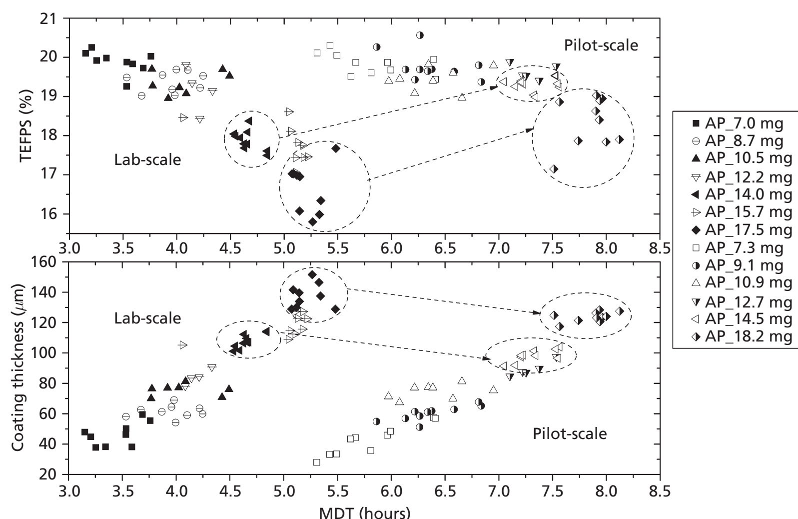

Figure 1 The MDT differences between the laboratory and pilot scales resulting from a scale-up failure can be detected using TEFPS (detecting variations in film coating density) and confirmed with coating layer thickness. The pilot scale showed higher film coating density (higher TEFPS), which was also evident in the thinner coating layer thickness for the pilot scale after process scale-up. Higher coating density for the pilot scale resulted in lower water permeability in the film coating and hence slower dissolution rate, which is shown in the longer MDT for the pilot scale. AP, amount of polymer applied.

Ho, L. et al (2007) *J. Cont. Rel.* **119**: 253–261
Ho, L. et al (2008) *J. Cont. Rel.* in press

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Liquid chromatography and liquid chromatography-mass spectrometry/time of flight (LC-MS/TOF) studies on simvastatin and its degradation products

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Objectives Simvastatin is a lipid-lowering drug used in the treatment of hypertension. The aim of the present study was development of a stability-indicating assay method and characterization of simvastatin's degradation products by using liquid chromatography-mass spectrometry/time of flight (LC-MS/TOF).

Methods Stress studies were conducted on the drug under the set of stress conditions defined by International Conference on Harmonisation (ICH): hydrolysis, oxidation, photolysis and thermal stress. Using the stressed samples, a stability-indicating method was developed on a C_8 column using a mobile phase comprising ammonium formate buffer (0.01 M, pH 3.2) and acetonitrile in a gradient manner, which was pumped at a flow rate of 1.2 mL/minute. The detection wavelength was 238 nm. The method proved to be specific to the drug and also selective to the degradation products. Nine degradation products that formed in the mixture of stressed samples were separated. The same method was transferred to LC-MS/TOF.

Results The drug was found to be highly sensitive to acidic and oxidative conditions. The molecular ion peaks of the hydrolytic degradation products were 437, 437, 419, 401, 401 and 285, respectively. The observed *m/z* values for major fragments of the drug and its degradation products demonstrated analogous mass reduction from the parent mass spectrum of simvastatin (Figure 1). On the other hand, the fragmentation pattern of oxidative products was different from that of the drug. The difference between *m/z* values of major fragments of the drug and its

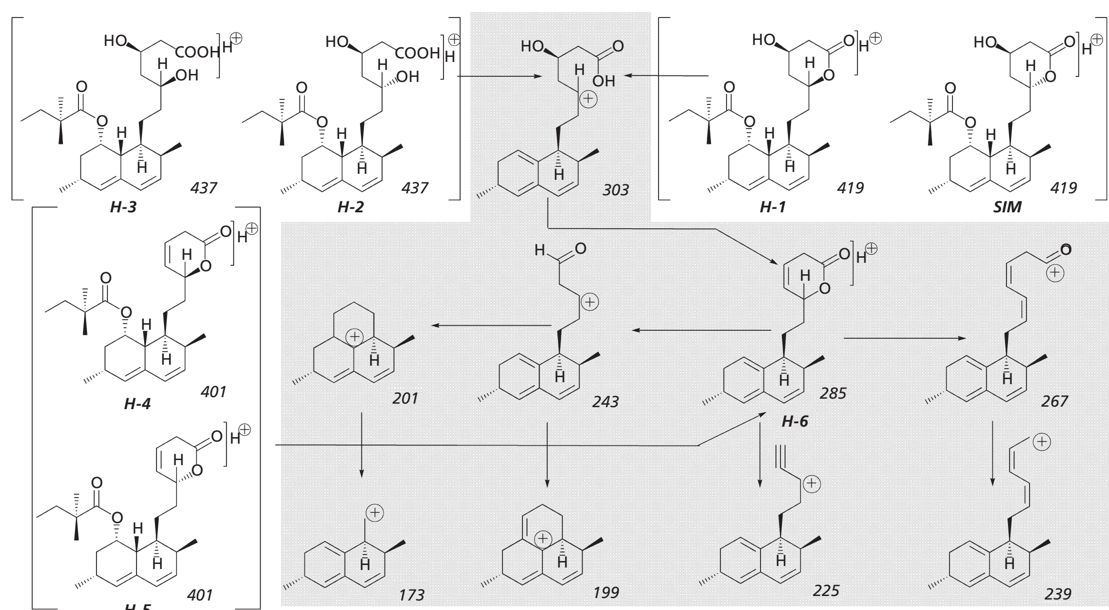

Figure 1 General fragmentation pathway and fragmentation pattern of hydrolytic degradation products (H-1 to H-6).

degradation products was 2 amu, but common losses were of 18 (corresponding to water) and 116 (corresponds to 2,2-dimethyl butanoic acid). All the major degradation products were identified and characterized on the basis of their molecular ion peak and fragmentation behaviour. The major oxidative degradation products were found to be hydroxyl ketones and dihydroxy simvastatin, whereas diastereomers of hydroxy acid, drug and dehydrated lactone were formed under acidic conditions, as in the case of atorvastatin (Shah et al 2008).

Conclusions A stability-indicating assay method was developed and in total nine degradation products of simvastatin were characterized by LC-MS/TOF.

Shah, R. P. et al (2008) *Rapid Commun. Mass Spectrom.* **22**: 613–622

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Molecular identification of St John's wort by PCR amplification of the ITS1 region: implications for medicinal plant identification

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Objectives The increase in use of medicinal plant products has highlighted the necessity for assurances of identification and quality in commercial products. We report the development of a DNA-based method for the identification and authentication of plant species, based upon the economically important St John's wort (SJW; *Hypericum perforatum* L.). Such methods can be applied to enhance the datasets (microscopic, chemical) required for the accurate identification of economically important medicinal plants. Medicinal plant products for human use in the European Union are regulated by the Traditional Herbal Medicines Directive.

Methods The ITS regions of the nuclear-encoded rRNA genes were the target for primer design. The ITS1 region is flanked on the 5′ and 3′ ends by the coding sequence of 18 and 5.8S rRNA, whereas ITS2 is flanked by 5.8 and 28S rRNA. The rRNA coding regions are highly conserved throughout plant species but the ITS sequences have an evolutionary rate which results in inter-species variation and intra-species conservation. PCR primers were designed for this variable region and specific primers, within this region, to selectively identify SJW. Nine species of vouchered DNA samples from the DNA Bank of the Royal Botanic Gardens, Kew, UK, were used as confirmed species specimens. These samples were used as a reference to ensure that the method could accurately discriminate SJW among six other *Hypericum* species grown from seed and garden-collected. Finally, three commercial medicinal products of or claiming to contain SJW were tested. PCR protocols were based upon the ITS1 region being successfully amplified in all DNA samples using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Crockett et al 2004). Further specific PCR primers were designed within the ITS region: FO2 (5′-CATAA-GAAGTGTAAGGCTCCCGG-3′), RO (5′-CGATACTTGGTGTGAATTGCA-GAA-3′) and HI-S (5′-CTCCTTCTGTTCATAACAATAACGACTCT-3′).

Results The FO2 and HI-S primers were designed to encompass the most divergent regions of the ITS1 sequence in the *Hypericum* species. This combined effect caused the primer pairing to give a specific PCR product with all three SJW samples. Of the nine *Hypericum* samples tested, a product with the primer pairing FO2 and HI-S was only found with SJW and *Hypericum delphicum*. This is expected due to the sequence similarity of this species at the FO2-binding site. However, this species is not widespread, and is unlikely to be found as a substitution or adulterant of SJW on sale commercially. Of the seven non-vouchered *Hypericum* samples tested, a product with this primer pairing also identified SJW as opposed to the other six samples. These results show that the primer combination can be used to differentiate non-vouchered samples from the economically valuable SJW. This same primer pairing also identified SJW in only two of three commercial products that listed SJW as an ingredient. DNA detection levels confirmed the PCR product from 0.00075 μg of DNA (0.1% genomic DNA).

Conclusions The method utilized in this study has enabled the design of a rapid and reliable molecular identification method for SJW. This has the potential to become a model for molecular identification design, and may be reproducible in other economically valuable plants.

Crockett, S.L. et al (2004) *Planta Medica* **70**: 929–935

Short Papers in Drug Delivery

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The use of dendriplexes as a novel vaccine-delivery system against anthrax

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Objectives Two cationic polylysine dendron complexes with DNA (so-called dendriplexes) have been studied as the basis of a potential genetic anthrax vaccine. *Bacillus anthracis* has attracted attention as an agent for bioterrorism, calling for the development of new anthrax vaccines to protect humans against their intentional use. The virulence of anthrax bacilli is due to the production of a three-component protein exotoxin. Protective antigen (PA), one of the protein components, is singularly the most important antigen required for specific immunity to anthrax. The cationic dendrons have branched asymmetrical polylysine dendritic head groups attached to a head group, one with three C_{18} chains and the other without. Dendrons interact with and condense DNA, producing a complex with a small residual negative surface charge, and enhance cellular internalization of DNA, in part protecting the DNA from degradation.

Methods The dendrons (Figure 1) were synthesized by stepwise solid-phase peptide synthesis from Boc-Lys(Boc)-OH and 2-amino octadecanoic acid for C_{18} dendron and Boc-Ala-OH for C_0 dendron (Novabiochem) on 4-methyl benzhydrylamine resin using the Boc strategy (Sakthivel et al 1998). The C_0 dendron $((C)_0(Lys)_7(NH_2)_8)$ contains seven lysine groups and eight amino groups attached to the core and the C_{18} dendron $((C_{18})_3(Lys)_7(NH_2)_8)$ contains an additional three hydrocarbon chains. Two types of plasmid DNA were developed, one encoding PA 83 cloned into the eukaryotic expression plasmid pSecTag 2B (7.3 kbp) and a control plasmid without PA 83. The plasmids were complexed with dendrons, forming particles approximately 80 nm in size depending on the lipophilicity of the dendron. A/J and Balb/c mice were vaccinated with dendriplexes containing 1 and 50 μg plasmid DNA per dose over a period of 6 weeks.

Results Immunogenicity was determined using the enzyme-linked immunosorbent assay (ELISA) method for anti-PA antibodies. Naked PA immunization with multiple dosing did not induce a sufficient antibody response even after secondary boosting after primary intramuscular immunization, whereas both dendriplexes produced a strong anti-PA antibody response. The response was dose-dependent, as depicted by the low- and high-dose dendriplex treatment groups. Throughout the *in vivo* study both dendriplexes improved immunomodulatory efficiency in comparison with the naked PA DNA. However, the elicited antibodies did not neutralize lethal toxin *in vitro* (Ribeiro et al 2008).

Conclusions To our knowledge this is the first *in vivo* study using dendriplexes in a vaccine against anthrax. Further work is required to improve these preparations to elicit functional antibodies.

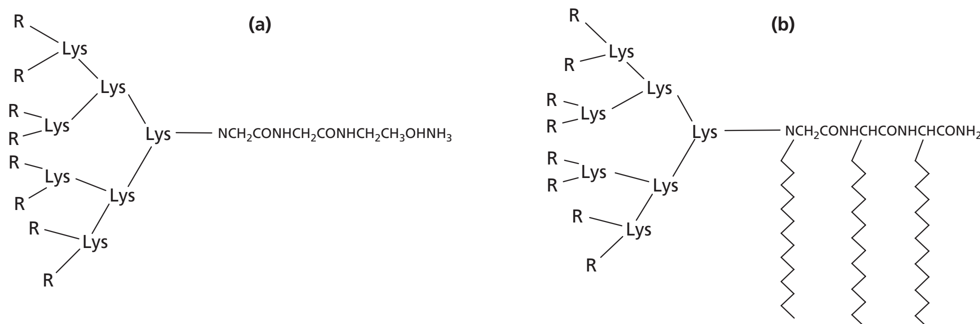

Figure 1 Simplified structure of the cationic lysine-based dendrons (a) C_0 $((C)_0(Lys)_7(NH_2)_8)$ with a molecular mass of 1174.91 Da and (b) C_{18} $((C_{18})_3(Lys)_7(NH_2)_8)$, of 1758.45 Da. Both dendrons contain 16 amino groups of which seven lysine groups (Lys) are attached to the core.