HIV microbicides. The life cycle of HIV has several steps that could be targeted to prevent infection, and there is a strong rationale for the use of HIV microbicide combinations having different mechanisms of action to increase efficacy. Vaginal rings are currently being developed for sustained delivery of single HIV microbicide compounds. In this study, feasibility of providing simultaneous sustained release of maraviroc (an entry inhibitor) and dapivirine (a nonnucleoside reverse transcriptase inhibitor) from matrix-type vaginal rings was evaluated for the first time.

## Method

Matrix-type silicone elastomer vaginal rings (dimensions: 54 mm diameter, 7.6-mm cross-sectional diameter, $n = 4$) containing 25 mg of dapivirine and various loadings of maraviroc (25, 50, 100, 200 and 400 mg) were manufactured by reaction injection moulding at elevated temperature. Release *in vitro* over 28 days was evaluated for each ring formulation using a sink condition model and ultra performance liquid chromatography analysis for quantitation of release of each microbicide. The saturation solubilities of dapivirine and maraviroc in thin films of silicone elastomer were measured, based on a method reported previously.[1] DSC analyses of silicone + dapivirine + maraviroc combinations were also evaluated.

## Results and Discussion

The release of each microbicide compound from the vaginal rings was characterised by matrix-type $t^{1/2}$ kinetics, wherein the daily release rate decreased with time. For the 25-mg dapivirine/100-mg maraviroc combination vaginal rings, the day 1 dapivirine release was 3135 $\mu$g (±249 $\mu$g) declining to 116 $\mu$g (±15 $\mu$g) on day 25, while day 1 maraviroc release was measured at 3232 $\mu$g (±3 $\mu$g) declining to 157 $\mu$g (±6 $\mu$g) on day 25. Over 28 days, similar amounts of each microbicide compound were released from the 25/100 mg vaginal rings (11.2 mg maraviroc and 10.3 mg dapivirine) despite a 4-fold difference in initial loadings, and indicative of maraviroc having a lower solubility in the silicone elastomer matrix, as confirmed by the results of solubility studies.

## Conclusion

The study demonstrates for the first time that microbicide combinations may be effectively incorporated within a single matrix-type vaginal ring device to provide sustained release of HIV microbicides at rates independently determined by their initial loading. Such combination vaginal rings may ultimately be useful in providing broad protection against sexually transmitted HIV infection.

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# Short Papers in Pharmaceutical Analysis

## 36
## A study of vial headspace moisture in an entire freeze-dried batch and the factors affecting moisture content variability

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## Introduction and Objectives

Freeze drying (lyophilisation) is used for production of pharmaceuticals, vaccines, diagnostics and other materials to prevent loss of activity and increase product shelf life. Regulatory authorities require proof that lyophilisation cycles are developed logically and demonstrate uniformity. One measure of uniformity can be residual water content throughout a batch, which will be influenced by vial location, degree of shelf contact, radiative heating, packing density, product formulation and the cycle conditions themselves. In this study, vial headspace moisture was mapped using frequency-modulated spectroscopy (FMS) for 100% of vials after different lyophilisation cycles in a laboratory freeze dryer.

## Methods

Sucrose and mannitol solutions (480 vials/cycle) were freeze-dried using a range of temperatures and pressures. The FMS is a high-sensitivity laser absorption technique and can be used to measure partial pressure of water within the headspace of each vial. Coulometric Karl Fischer (KF) titration was used to measure total water content in a limited sample set covering a range of headspace moisture results. A calibration curve was constructed by analysing a subset of the same vials by KF against FMS to yield a best fit line. The FMS was then used to map headspace vapour pressure against location for all vials.

## Results and Discussion

For sucrose, a 17-point curve of KF % w/w moisture against vial headspace moisture yielded a best fit line with $r^2 = 0.9225$. Similar curves were constructed for batches lyophilised using a range of cycles. Earlier reports have suggested that drying times are affected by the presence of solid trays and that evenness of the contact surface is an important factor. However, in much of primary drying, heat transfer is primarily controlled by gaseous convection and conduction. Our data show that based on headspace moisture, the relative significance of each of these parameters varies with cycle and formulation. For example, where vials of sucrose were freeze-dried in a solid tray, FMS values ranged from 0.5 to 3.2 torr (mean = 1.05; SD = 0.5; $n = 240$) while

for vials in direct contact with the shelf, FMS values ranged from 0.2 to 1.0 torr (mean = 0.50; SD = 0.17; $n$ = 240) demonstrating that for this particular cycle, format and solution, the presence of a solid tray led to significant intrabatch variation in headspace moisture, this can be related to shelf location, degree of shelf contact and radiative heating. For mannitol, a higher shelf temperature and pressure were used and the results show a much smaller range in headspace moisture.

## Conclusion

For the excipients studied here, the effect of varying product and process parameters on headspace moisture could be assessed using FMS, and these values correlated with total residual moisture as measured by KF. Therefore, FMS may enable 100% batch inspection due to the speed of the analysis and its nondestructive nature and shows the potential to be fitted into a quality-assurance framework. Studies are now being extended to encompass more realistic pharmaceutical and biopharmaceutical formulations in a wide range of drying formats and a more comprehensive set of freezing and drying conditions.

# 37
# Selection of a suitable analytical method for evaluating bovine serum albumin concentrations in controlled release polymeric formulations

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## Introduction and Objectives

Bovine serum albumin (BSA) is a commonly used model protein in formulation development. To assay its release, either the bicinchoninic acid (BCA) assay[1] or a more specific size-exclusion high-performance liquid chromatography (SEC-HPLC) assay is commonly used.[2] However, these can give erroneous results in the presence of some polymers. We therefore investigated the ability of these methods compared with an reversed phase HPLC (RP-HPLC) technique to determine BSA concentrations in standard solutions containing Noveon AA, Gantrez, hydroxyethyl cellulose (HEC), hydroxypropyl methylcellulose (HPMC), polyvinylpyrrolidone (PVP), sodium carboxymethyl cellulose (Na-CMC) or chitosan before selecting one method with which to perform drug release studies from freeze-dried polymer-based formulations.

## Methods

BCA assay kit (Pierce, USA), SEC-HPLC and RP-HPLC (Perkin-Elmer system, Buckinghamshire, UK) methods were evaluated for BSA analysis. The SEC-HPLC method: BioSep-SEC-S 3000 column (Phenomenex, Cheshire, UK); 0.05M ammonium acetate buffer mobile phase; UV detection at 280 nm; RP-HPLC method: Jupiter 300 Å column (Phenomenex, UK); gradient flow (0.1% triflouro acetic acid (TFA) in water(A), 0.1% TFA in acetonitrile(B); A/B from 95 : 5 to 35 : 65) mobile phase; UV detection at 220 nm. BSA-polymer solutions were analysed using these techniques to evaluate BSA polymer interactions. An application to an in-vitro BSA release study from novel freeze-dried gel formulations was studied by the selected method.

## Results and Discussion

Noveon AA, Gantrez and PVP showed interaction with BCA working reagent and interfered with BSA analysis with the BCA kit method. Noveon AA and Gantrez eluted at the same retention time as BSA (8.86 min) with the SEC-HPLC method at the flow rate of 1 ml/min and 100-$\mu$l injection volume. There was no interference of any polymers found with BSA (retention time 17.08 min) by the RP-HPLC method at the same flow rate and injection volume. The RP-HPLC was the most suitable technique and further validated. The performance characteristics were also judged in terms of linearity, accuracy, precision, limit of detection and quantification. The plot of the area of BSA versus concentration of BSA in microgram/millilitre was found linear in the range of 0.5–100 $\mu$g/ml with high correlation coefficient of 0.9999. The limit of detection and quantification were 0.110 and 0.335 $\mu$g/ml, respectively. The performed '$t$'-test for the estimated and theoretical concentration indicated no significant difference providing the accuracy, and low %RSD values (0.8–1.39%) indicate the precision of the method. Further, in-vitro BSA release studies from polymeric freeze-dried formulations showed that the BSA release could be quantified without interference of the polymers.

## Conclusion

BCA kit, HPLC-SEC and RP-HPLC techniques were compared for analysis of BSA, and RP-HPLC was selected on the basis on no interference of polymers, selectivity and sensitivity. The method was shown to be highly reproducible and was not affected by the presence of polymers. This method may be used as a substitute for other methods of determining BSA levels which are complex, time consuming and need specialised kits.

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## 38
## A physicochemical and biological assessment of UV filters as substances and formulated products

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### Introduction and Objectives

Sunscreen materials contain UV filters, some of which have been implicated as sources of free radicals within the skin. There have also been some reports about their potential as endocrine disrupting chemicals (EDCs). 'An EDC is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, subsequent to changes in endocrine function' (WHO, 2002). Worldwide, there is increasing focus on UV radiation and skin cancer. In addition to growing usage, these (mostly) aromatic molecules are highly lipophilic and can bioaccumulate. An oestrogenic, mutagenic and cytotoxic screen for these agents and associated finished formulations is indicated.

### Method

The yeast estrogen screen (YES) assay is a reporter gene assay and used here to indicate potential oestrogenic activity of selected UV filters, as active sunscreen ingredients (ASIs) and as formulated sunscreen products. The AMES assay is the hallmark of mutagenic activity and is used here to assess the mutagenic potential of each filter, individually and synergistically. The cytotoxic response of each active ingredient was carried out at various concentrations using the MTT reduction assay on HepG2 (liver) and XB2 (skin, keratinocyte) cell lines.

### Results and Discussion

A number of biological in-vitro assessments have been conducted on selected UV filters, namely 4-methylbenzylidene camphor (4-MBC), benzophenone-3 (Bp-3), homosalate (HMS), octyl dimethyl para-aminobenzoic acid, (OD-PABA), butyl methoxydibenzoylmethane (BMDM) and octyl methoxycinnamate (OMC). Figure 1 indicates the oestrogenic profile for OMC and Bp-3. The latter was also noncytotoxic using our HepG2 cell line and has shown minimal instability on exposure to UV light. Thus far, all parent compounds in the absence of metabolising enzymes are not mutagenic. The mutagenic potential of metabolites has been assessed as have combinations of ASIs. A series of emulsified house formulations have been tested for parameters based on the nature and concentration of key vehicle ingredients including paraben-based preservatives. Physicochemical determinations include a stability-indicating, reverse-phase high-performance liquid chromatographic assay and spectrophotometric monitoring of degradation and radiation scattering.

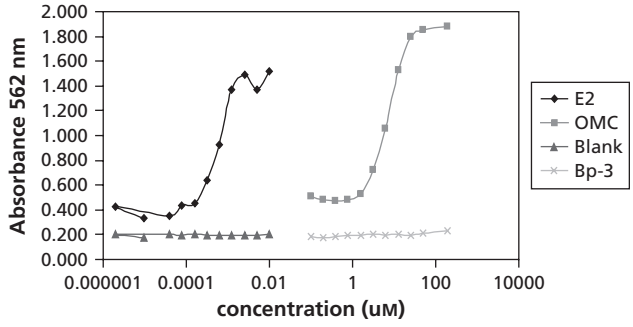


**Figure 1** Dose response curves for the oestrogenic activity of octyl methoxycinnamate and benzophenone-3 in the yeast oestrogen screen, including the positive control 17$\beta$-oestradiol (E2).

### Conclusion

This work will present a ranking for the oestrogenic potential of the selected sunscreens and will discriminate between those formulation types prone to enhanced synergistic-based effects. The results have been correlated with analytical indicators of photointegrity.

## 39
## Quantitative determination of mebeverine HCl by $^1$H nuclear magnetic resonance chemical-shift migration

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### Introduction and Objectives

A novel quantitative $^1$H nuclear magnetic resonance (NMR) method is proposed for the analysis of mebeverine HCl in aqueous solutions based on concentration-dependent chemical-shift variations. The chemical shifts of different nonexchangeable aromatic protons of mebeverine HCl migrate to different extents as the concentration of the analyte is varied in aqueous solution.[1] This concentration-dependent chemical-shift variation is linear across a useful range, and it has, therefore, been applied in the quantitative analysis of mebeverine HCl in its authentic form and in its pharmaceutical tablets (Colofac IBS, Solvay Healthcare Limited, UK).

### Method

The $^1$H NMR spectra of different concentrations of mebeverine HCl (0.01–100 mg/ml) in $D_2O$ were recorded and proton

chemical shifts were plotted against concentration. The regression equation for each proton chemical shift was derived over the linear range (5–50 mg/ml). The Stokes–Einstein equation was used to determine the apparent hydrodynamic volume, $V_H$ for mebeverine HCl in $D_2O$, $CDCl_3$ and $CD_3OD$.

## Results and Discussion

Comparison of the $^1H$ NMR spectra obtained at different concentrations showed that the chemical shift of the mebeverine HCl aromatic protons changed with changing concentration in $D_2O$ solution, but not in $CDCl_3$, $CD_3CN$, dimethyl sulfoxide (DMSO-$d_6$) or $CD_3OD$. In $D_2O$, the protons of mebeverine HCl move upfield when the concentration increases and can even result in coalescence of signals, thus significantly changing the appearance of the spectrum and its associated integration. The proton with the best regression coefficient (H3", 5", $r^2 = 0.9998$) was used in a quantitative analysis of mebeverine HCl. The apparent hydrodynamic volume, $V_H$ for mebeverine HCl in $D_2O$ solution is 10-fold greater than that seen in either $CDCl_3$ or $CD_3OD$, 1320, 217 and 133, respectively, and this presumably reflects the aggregation of several (six to seven) molecules. The method was evaluated, it is not affected by magnetic field strength, and it showed good accuracy and precision as indicated by statistical comparisons of the results obtained to those of the British Pharmacopoeia method (nonaqueous titration) using the student's *t*-test and the variance ratio test (*F*-test); there was no significant difference between them. The chemical shift variation observed can be a result of molecular aggregation, perhaps, due to intermolecular hydrogen bonds, p–p aromatic stacking and electrostatic interactions, leading to 'head to head' and/or 'head to tail' dimers. The number of molecules, their orientations, their interactions and 'tightness' of association in the aggregate may vary as a function of concentration, which leads to the altered chemical shifts.[2,3]

## Conclusion

This quantitative NMR assay of mebeverine HCl in $D_2O$ shows acceptable characteristics regarding accuracy, precision and robustness.

## Acknowledgement

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# 40
# LC-MS determination of Streptococcal M protein fragments in hyaluronic acid injectables by enzymatic digestion

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## Introduction and Objectives

Hyaluronic acid (HA) is produced commercially by bacterial fermentation using *Streptococcus pyogenes*. The injection of HA preparations (viscosupplementation) has been used in the relief of pain associated with osteoarthritis by mimicking the synovial fluid between joints. An increased level of discomfort has been reported from specific batches of some injectable HA products, and it has been proposed that this may be due to the presence of traces of bacterial M protein fragments, membrane proteins remaining after HA purification.[1] The development of an analytical method was required to identify and confirm the presence of M protein fragments in HA preparations.

## Method

Proteomic fragments were identified by the enzymatic digestion of HA solutions using trypsin protease, and liquid chromatography-mass spectrometry (LC-MS) analysis was carried out to compare the digested and undigested samples. Digested samples were prepared in triplicate using a buffered solution, and the samples were reduced using dithiothreitol and treated with iodoacetamide to ensure all proteins remained reduced before overnight tryptic digestion. Undigested samples (single preparation) were treated in the same manner and incubated overnight in the absence of trypsin. LC-MS analysis was carried out in positive electrospray ionisation mode over a mass range of 100–20,000 Da, and chromatographic separation was achieved using a gradient method.

## Results and Discussion

LC-MS analysis was carried out on the digested samples and the data collated from a single injection of each. The data for undigested control samples were collated from the triplicate injection of a singular preparation of each sample. Mass spectral interpretation software was used to identify compounds from the raw data, and the observed differences between compounds present in digested and undigested samples were attributed to the presence of proteomic fragments as a consequence of the trypsin digest. To ensure a clear definition of the presence or absence of proteomic fragments, a criterion was set that the compounds identified from the total ion chromatogram relating to a particular mass ion must be present in all three replicate injections within a data set. The masses for compounds identified as proteomic fragments were inputted into the Mascot online proteomic

database, and potential protein matches were evaluated based on the predicted trypsin digest map using published amino acid sequences. streptococcal M protein homologous to *S. pyogenes* was identified, and a significant proportion of its sequence was assigned to the proteomic fragments identified from the trypsin digest of the HA samples.

## Conclusions

Compounds present in the digested samples and absent in undigested samples were identified from the LC-MS raw data; these compounds were attributed to proteomic fragments generated from the trypsin digest and were identified as streptococcal M protein fragments. The M protein fragments were detected in samples of HA preparations where an inflammatory response had been previously reported, and subsequent analysis of samples where no inflammatory response was reported yielded no detectable M protein fragments. This work supports the hypothesis of streptococcal M protein contamination of HA preparations being responsible for the inflammatory response reported.

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# 41
# Identification of tablets through their blister packaging by near-infrared spectroscopy

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## Introduction and Objectives

The aim of this study was to develop a near-infrared spectroscopic (NIRS) method for the identification and discrimination of blister-packed tablets.

## Methods

A total of 27 batches of various products in colourless transparent (CT) blister packaging were purchased from the world market. The drugs were atorvastatin, azithromycin, carbamazepine, clarithromycin, clopidogrel, esomeprazole, metronidazole, montelukast, norfloxacin, ofloxacin, olanzapine, pantoprazole, quetiapine, raloxifene, risperidone, valsartan, valsartan with hydrochlorothiazide and venlafaxine. Ciproxin, which was available in multiple batches, was used to standardise the system. The spectra of these tablets were measured with a FOSS 6500 NIR spectrometer using a Smart Probe and processed as standard normal variate second-derivative spectra (SNV-D2) excluding the regions 1666–1796 nm and 2200–2500 nm. Comparison of the spectrum of a standard Ciproxin tablet with a variety of other tablets in their blisters was made using correlation in wavelength space (CWS) and principal component analysis (PCA) to provide a numerical and graphical profile of the tablets.

## Results and Discussion

To set up a threshold for discrimination, a simple ceramic reference was used, which gave a correlation value of 0.94 between an intact Ciproxin tablet and the tablet in its blister. To improve the identification method, we examined a set of tablets in CT top blisters using a reference of an opened empty CT blister above a National Institute of Standards and Technology (NIST) USA federal agency 20% photometric reference standard. A correlation value of 0.98 between the Ciproxin tablet and the tablet in its blister was now obtained. The variation in individual blisters had little effect on the identification. A single Ciproxin tablet examined in 10 similar CT blisters gave a minimum correlation of 0.97. Individual Ciproxin tablets within a batch also showed good correlation: 10 Ciproxin tablets of the same batch measured within their own blisters gave a minimum correlation value of 0.98. In addition, the measurement of eight different batches of Ciproxin tablets gave correlation values higher than 0.94, which was then used as the threshold for identification. This identification method was applied to compare a set of 19 products other than Ciproxin all in CT blister packaging. Of the 20 products, only 3 mismatches of a correlation higher than 0.94 were observed between Lipitor (atorvastatin), Risperidal (risperidone) and Singulair (montelukast). This may be due to the similar composition of excipients in these products. When PCA was applied to these products, no better discrimination was observed. The precise position of the probe in relation to the blister and the tablet was investigated and found to have little effect. However, NIRS could not be applied to coloured opaque and aluminium-foil-packaged tablets.

## Conclusion

The use of NIRS on opened CT blister packaging above 20% NIST standard could identify the tablets in the CT blisters and discriminate them from the substitutes. The method could identify 80% of the 20 products examined with a threshold value of 0.94.