and micelle-associated AmB was determined using UV spectroscopy. The antifungal activities of formulations were assessed against *Candida albicans* by microdilution susceptibility testing in 96-well microtitre plates. Haemolysis method was used to establish the safety of these carriers for AmB delivery. Micelles (5 ml) were placed in a Pari LC Star (Pari GmbH, Starnberg, Germany) nebuliser attached to a TurboBoy N compressor (Pari GmbH) and nebulisation undertaken to 'dryness' into a twin impinger (TI). The AmB output and deposition in the fine particle fraction (FPF) (i.e. lower stage of the TI) were determined by UV analysis.

Results and Discussion

Results indicate that amphiphilic HYA-PLA can solubilise AmB during self assembly of the graft copolymer (Figure 1). Up to 50% of drug loading efficiency was obtained with 25% w/w theoretical AmB loading. The surface charge of drug-loaded polymeric carriers was negative. Polymeric carrier-loaded AmB exhibited antifungal activity against *C. albicans* comparable to Fungizone (AmB desoxycholate; Sigma Aldrich, UK). Haemolytic studies showed that encapsulation of AmB in HYA-PLA carriers reduced drug toxicity up to 20 μg/ml, whereas AmB in Fungizone was haemolytic at less than 10 μg/ml. Following nebulisation, the FPF of AmB was 58% AmB. Further in-vitro studies are ongoing with regard to stability and toxicity of these micellar systems.

Conclusion

These studies show that novel HYA-PLA polymer can be useful for solubilisation and delivery of hydrophobic drugs to the lung.

Short Papers in Drug Delivery

30
Preparation of polymeric microspheres as an ophthalmic drug delivery system for brimonidine tartrate

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Introduction and Objectives

A dramatic increase in the incidence of eye diseases, such as age-related macular degeneration, and inadequate therapeutic control has led to the search for advanced methods of sustained and targeted drug delivery. The development of polymeric devices, matrix implants and microspheres has facilitated the prolonged controlled release of therapies to the target sites. The aim of this study is to prepare brimonidine tartrate–loaded microspheres, which possess suitable characteristics for development into an implantable device that will later exploit the binding properties of ocular melanin to influence the release properties.

Method

Microspheres were prepared using a solvent evaporation technique for encapsulation developed from those methods described in the literature.[1–3] Resomer RG502 (Boehringer Ingelheim, Ingelheim, Germany) (PLGA; ~50 : 50 D,L-lactide : glycolide) was dissolved in various nonhalogenated solvents after which a known quantity of brimonidine tartrate was added. In each case, this solution was added to the stirred aqueous continuous phase containing polyvinyl alcohol or Pluronic F-127. The emulsion was stirred for 3 h at room temperature after which the microspheres were collected by filtration or centrifugation and dried under vacuum. The collected microspheres were assessed for size and shape by optical light microscopy, and the size distribution was determined using a zeta-sizer. Percentage yield was calculated, and the encapsulation efficiency was determined by dissolving a known quantity of the microspheres in dichloromethane and extracting the brimonidine in 0.001 M tartaric acid. The aqueous layer of extraction was analysed for drug content using UV spectroscopy. Determination of release profiles was performed using an established method.

Results and Discussion

A range of microspheres were successfully prepared, which possessed different physical properties. An example of one of the batches of microspheres prepared from ethyl formate and a 0.5% w/w Pluronic F-147 aqueous phase is shown in Figure 1. A uniform size range was found where the average diameter of the particles was 7 μm, and each possessed a smooth spherical shape, although a more detailed investigation into their surface characteristics needs to be performed. This was true for all the microspheres prepared although problems with particle aggregation were encountered with the acetone solvent system due to their submicron size. It was found that as the average particle size of the microsphere decreased, the encapsulation efficiency decreased, especially in the case of those prepared from acetone where it was believed that their small size inhibited efficient encapsulation. Reduction in particle size also increased the rate of release. Further optimisation is ongoing to find the appropriate balance between particle size, encapsulation efficiency and release profile.

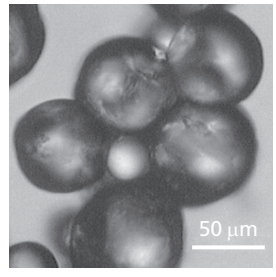

Figure 1 Optical light microscopy image of microspheres prepared from ethyl acetate.

Conclusion

Initial studies have shown that microspheres suitable for ophthalmic drug delivery can be prepared from solvents of minimal toxicity. The physical properties of the microspheres can be manipulated by varying the conditions of encapsulation which in turn affects the release properties. Investigations into the formation of a biopolymeric drug delivery system encompassing melanin, a naturally occurring substrate present in the eye, are continuing and may provide another dimension in controlling this type of sustained release.

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31
Effects of surfactants on integrity and biological activity of crystallised and spray-dried proteins

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Introduction and Objectives

The formulations of stable and biologically active protein therapeutics are challenging due to the chemical and physical instabilities of protein. Accordingly, crystallization and spray-drying of lysozyme, a model protein, in the presence of different concentrations of surfactants (at and above their critical micelle concentrations (CMC)) were evaluated to determine their effects on protein integrity, stability and biological activity.

Method

Unprocessed lysozyme was crystallised (batch crystallisation) and spray-dried (Büchi Spray Dryer, BÜCHI Labortechnik AG, Meirseggstrasse 40 Postfach, Switzerland) in the absence and presence of two different surfactants (Caprol PGE860, Ohio, USA; CremophorRH40, BSAF, Ludwigshafen, Germany) at and above CMC. To the best of our knowledge, those surfactants have not been used before to stabilise proteins. Preparations were characterised using biological activity assay, polarised microscopy, differential scanning calorimetry (DSC) and spectroscopic analysis with Fourier transform infrared (FTIR). Storage stability study was conducted for protein solutions for 8 weeks at room temperature (20 ± 2°C) and at high temperature (44 ± 3°C); then biological activity results for samples after storage were compared with those before storage.

Results and Discussion

Comparing activities of all preparations with that of unprocessed lysozyme, the activity of pure protein crystals (95.6%) was statistically higher ($P < 0.05$) than pure spray-dried formula (50%); this is in agreement with the study by Elkordy *et al.*[1] However, the biological activity of spray-dried protein with surfactants was greatly enhanced to be similar ($P > 0.05$) to that of crystallised protein, for example, activity of lysozyme crystals with CremophorRH40 above CMC was 102.3 versus 96.9% for spray-dried formulation with same surfactant. The FTIR spectra of protein samples with surfactants were identical to that of unprocessed protein, suggesting that surfactants retained the secondary structure of lysozyme. However, DSC thermograms showed decreased denaturation temperatures (T_m) in the presence of surfactants, e.g. T_ms were 192.8, 199.3, 189.2 and 198.3°C for crystallised, spray-dried, crystallised-CremophorRH40 and spray-dried-CremophorRH40 (at CMC) lysozyme, respectively. Polarised microscopy showed that the morphology of crystals was different from that of spray-dried form (amorphous). Regarding storage stability at 44°C, enzymatic activity exhibited significantly increased ($P < 0.05$) activity retention for protein crystals with surfactants (such as 41.1 versus 18.6% for crystallised and spray-dried protein (with CremophorRH40, CMC), respectively). Nevertheless, there was no significant difference in activity of samples stored at room temperature for 8 weeks (activity was ~43%).

Conclusion

The stability and biological activity of spray-dried lysozyme were improved by the addition of surfactants. The best biological activity of lysozyme was observed with CaprolPGE860 in crystallised and spray-dried forms. Accordingly, these results show promise for preparation and delivery of stable and effective protein pharmaceuticals.

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32
A comparative biodistribution study investigating the choice of cationic liposome for the delivery of a subunit tuberculosis antigen

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Introduction and Objectives

The use of cationic liposomes for the delivery of the *Mycobacterium tuberculosis* subunit protein 'Ag85B-ESAT-6'

has shown a strong immunogenic profile with marked Th1 cellular response.[1] The immunological method of action of liposomal adjuvants remains unclear; however, antigen retention in the tissue is considered favourable for maximum uptake by circulating immune cells. Our biodistribution studies quantitatively investigate the dissemination of both adjuvant and antigen after intramuscular injection.

Methods

The lipid-film method was used to form liposomes composed of an H^3 radiolabel tracker, trehalose 6,6′-dibehenate (TDB) and the cationic lipid dimethyldioctadecylammonium bromide (DDA). Comparative liposomes were chosen based upon their physical relation to DDA and their favourable immunological profile in immunisation studies. Ag85B-ESAT-6 (radiolabeled with I^{125}) was added to the liposomes prior to intramuscular injection of triplicate groups of mice: each 50 μl dose contained 0.4 μmol lipid, 0.05 μmol TDB and 2 μg protein. At time-points 1, 4 and 14 days p.i. mice were culled, and various tissues analysed for the presence of both radiolabeled components.

Results and Discussion

The delivery of Ag85B-ESAT-6 by liposomes based on cationic lipids such as DDA, [N-(N′,N′-dimethylaminoethane)-carbonyl] cholesterol (DC-Chol), N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride (DOTAP) and N,N-dioleyl-N,N-dimethylammonium chloride (DODA) results in the retention of antigen at the site of injection (SOI) to levels still detectable 14 days p.i. The injection of Ag85B-ESAT-6 alone leads to rapid dissemination with only 1% of the original dose being present four days p.i. Substitution of the cationic charge using the neutral lipid distearoyl-glycero-phosphatidylcholine (DSPC) results in a significantly lower level of Ag85B-ESAT-6 at the SOI at all time points studied and by day 14 p.i. the levels are almost undetectable. The importance of bilayer fluidity (determined by the lipid transition temperature) as opposed to vesicle size in controlling liposome retention at the SOI is apparent in that DOTAP and DODA vesicles, both approximately 600 nm with fluid bilayers, are not held at the SOI to the high levels seen with DC-Chol liposomes (rigid structure of approximately 200 nm). However, liposome retention at the SOI is not a determining factor for antigen retention: Ag85B-ESAT-6 delivery by DODA liposomes results in the highest antigen presence at the SOI over the studied time-points.

Conclusion

The antigen depot effect noted when cationic liposomes are used for the delivery of Ag85B-ESAT-6 is particularly important for activation of cognate immune cells and the trafficking of antigen to draining lymphoid tissue. Prolonged liposome deposition in the tissues is also observed when liposomes of a rigid structure, such as DDA and DC-Chol, are used in the delivery of Ag85B-ESAT-6. Whilst their deposition does not appear to significantly affect the antigen depot effect, the prolonged presence of immunostimulatory molecules such as DDA and TDB may be of benefit in the production of a strong and successful adaptive immune response.

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33
The role of thiol groups in redox-sensitive disulphide-crosslinked polymeric gene delivery vectors

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Introduction and Objectives

Disulphide-crosslinked polymers are redox-sensitive vectors, which are stable in the extracellular medium but undergo dissociation in the reductive environment of the cell to facilitate plasmid release.[1] Since crosslinking is believed to increase the molecular weight and size of the polymer, it has not been applied to high molecular weight polymers and there are always residual thiol groups on the polymer as not all thiols are crosslinked. The objective of this study is to investigate the nature of disulphide crosslinks, role of thiols and degree of crosslinking on transfection efficiency by comparing thiolated and crosslinked derivatives of polyethylenimine.

Method

Polyethylenimine (25 kDa) was thiolated by a two-step process, using 3-(2-pyridyldithio) propionic acid *N*-hydroxysuccinimide ester, followed by reduction with dithiothreitol. The crosslinked polymers were synthesised by oxidation of thiolated polymers using air. The two sets of polymers were characterised by Ellman's assay to determine their thiol content; the sizes of polymers and polyplexes were measured using dynamic light scattering and their membrane interactive nature was studied using haemolysis assay. The cytotoxicity of the polymers was investigated by MTT assay, whilst the strength of polymer–DNA interactions was measured by ethidium bromide exclusion assay. Transfection efficiencies of the polyplexes were evaluated in HEK293 cells.

Results and Discussion

Crosslinked derivatives showed lower buffering capacities in solution than thiolated derivates. They were also smaller

(between 11 and 13 nm), suggesting that the crosslinks were intramolecular and did not increase the molecular weight of the polymer. The haemolysis study showed that thiol groups, when present in sufficiently large concentrations, can increase membrane interaction. They may interact with thiol groups on membrane proteins and increase cellular uptake by adsorptive endocytosis.[2] This was supported by the transfection and cytotoxicity experiments, where the polymers with high concentrations of thiol groups showed significantly higher transfection efficiencies, but did not increase cytotoxicity compared to polyethylenimine. The crosslinked polymers condensed DNA effectively, improved the polyplex stability in the extracellular medium and polyplex dissociation in the cell, which enhanced transfection. However, polymers with an increased degree of crosslinking overstabilised the polyplexes and did not effectively release the plasmid within the cell, which consequently decreased transfection efficiency.

Conclusion

Disulphide-crosslinking in crosslinked polymers are intramolecular and hence do not increase the molecular weight of the polymer. Therefore, disulphide crosslinking can be applied to relatively high molecular weight polymers without detrimental effects on cytotoxicity. Optimising the degree of crosslinking, and hence the concentration of residual thiol groups, can provide optimal polyplex stabilisation and increase cellular uptake, thereby increasing transfection efficiency. Thus, these vectors can overcome key blocking steps of nonviral gene delivery by improving polyplex stability in the extracellular medium, enhancing cellular uptake of polyplexes and facilitating release of plasmid selectively in the cell cytoplasm.

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34
Giant unilamellar vesicles as models to study the interaction of gene delivery vectors with endosomal membranes

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Introduction and Objectives

Nonviral gene delivery vectors hold great promise for treating a wide range of diseases; however, their clinical applicability is limited due to numerous biological barriers to transfection. Because 'endosomal escape' is one of the main barriers to successful gene delivery, it is essential to understand the interactions between gene delivery vectors and endosomal membranes. While conventional studies often use small unilamellar vesicles (SUVs) as model membranes to study such interactions, it is proposed that giant unilamellar vesicles (GUVs) present more realistic endosomal models due to their larger size, superior membrane packing due to reduced surface curvature and the ability to visualise GUVs using light or confocal microscopy.[1]

Method

GUVs composed of a mixture of neutral or negatively charged lipids, representing early or late endosomal membranes, were prepared by electroformation in 10 μM calcein followed by addition of cobalt chloride to quench background fluorescence (Figure 1a).[2] GUVs were then observed using a confocal fluorescence microscope upon the addition of Triton-X (positive control) or various lipid or polymer gene delivery vectors over time. Any photobleaching or osmotic effects were accounted for by exposing the GUVs to similar light intensities or control buffer for the same period of time.

Results and Discussion

Calcein-encapsulating GUVs in the size range 30–50 μm were selected for observation using a Leica confocal microscope, and the encapsulated fluorescence leakage was quantified and plotted as a function of time. It was evident that the addition of Triton-X caused abrupt membrane rupture and deformation in the neutral early and negatively charged late endosomal GUV models (Figure 1b and d) within 15 min of Triton-X addition. The introduction of a lipid gene delivery vector comprising dimethyldioctadecylammonium bromide and dioleoylphosphatidylethanolamine (DDAB/DOPE : DNA) at 4 : 1 charge ratio caused the encapsulated calcein to leak at an accelerated rate compared to controls in the neutral GUV model (Figure 1c); however, no such effect was observed in the negatively charged GUV model. Finally, the addition of the polymeric vector polyethyleneimine (PEI : DNA) 4 : 1 ratio did not cause any evident membrane destabilisation in either GUV model. The results obtained using GUV models were similar to those obtained using SUV models prepared using the same lipids encapsulating carboxyfluorescein, whereby 95 and 20% dye release compared to Triton-X was measured upon the addition of DDAB/DOPE : DNA and PEI : DNA, respectively, in the neutral SUV model compared to 20 and 5% release in the negatively charged SUV model.

Conclusion

In conclusion, it is proposed that lipid gene delivery vectors incorporating DOPE can efficiently destabilise early neutral endosomal membranes, probably due to lipid fusion; however, this process becomes less efficient at later endosomal or lysosomal stages in the presence of negatively charged membrane components. Furthermore, it is unlikely

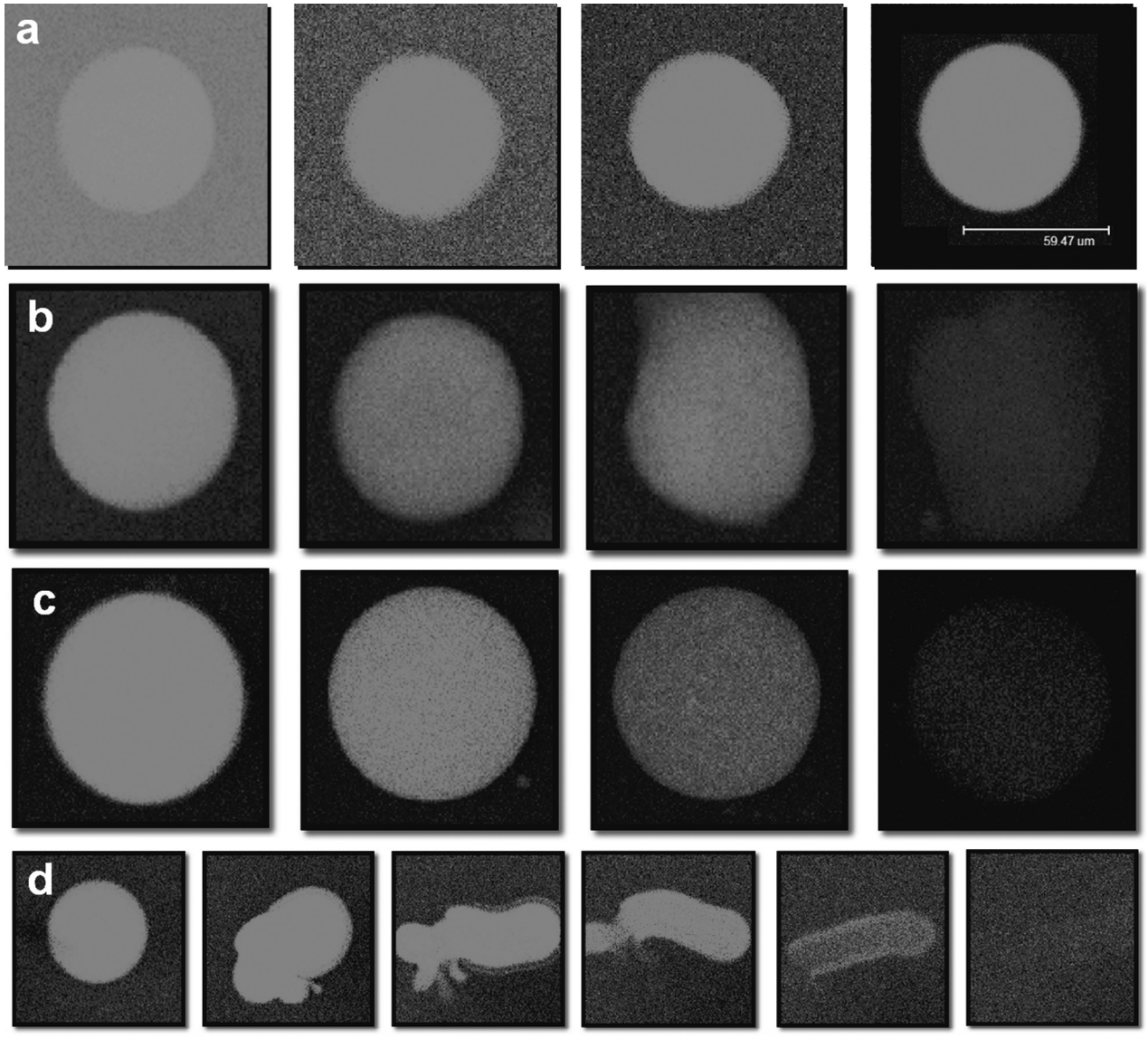

Figure 1 (a) Calcein background quenching upon the addition of increasing concentrations of cobalt chloride to GUVs. Destabilisation of a neutral early endosomal GUV model upon the addition of (b) Triton-X at 0, 5, 10 and 12 minutes and (c) DDAB/DOPE:DNA vector at 0, 10, 20 and 35 minutes. (d) Deformation and subsequent destabilisation of a negatively charged late endosomal GUV model upon the addition of Triton-X at 0, 15, 20, 22, 25 and 30 minutes.

that polymeric gene delivery vectors such as PEI cause endosomal release by membrane destabilisation in the absence of other specialised mechanisms such as the proton sponge effect. Finally, this study shows that GUVs are plausible model endosomal membranes, although future work should seek to apply more relevant physiological conditions such as changes in temperature and pH.

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35
Sustained corelease of HIV microbicides from silicone elastomer vaginal rings

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Introduction and Objectives

Over 17.5 million women worldwide are living with HIV-AIDS, and there is a need for female-initiated

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 μg (±249 μg) declining to 116 μg (±15 μg) on day 25, while day 1 maraviroc release was measured at 3232 μg (±3 μg) declining to 157 μg (±6 μg) 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