JPP Journal of Pharmacy and Pharmacology

JPP 2009, 61: 1125–1135

Received October 16, 2008
Accepted May 12, 2009
DOI 10.1211/jpp/61.09.0001
ISSN 0022-3573

Review

Aminolevulinic acid-loaded Witepsol microparticles manufactured using a spray congealing procedure: implications for topical photodynamic therapy

Rasil Al-Kassas[a], Ryan F. Donnelly[a] and Paul A. McCarron[b]

[a]School of Pharmacy, Queen's University Belfast, Medical Biology Centre, Belfast, UK and [b]Department of Pharmacy and Pharmaceutical Sciences, University of Ulster, Coleraine, Co. Londonderry, UK

Abstract

Objectives The aim was to enhance aminolevulinic acid (ALA) stability by incorporation into low-melting microparticles prepared using a spray congealing procedure and to evaluate temperature-triggered release, allowing topical bioavailability following melting at skin temperature.

Methods ALA-loaded Witepsol microparticles were prepared using a novel spray congealing technique. Entrapment efficiency was compared with conventional emulsion-based methods and modelled drug release profiles determined using a membrane separation technique. Raised receiver medium temperature was used to determine triggered release. Bioavailability and lipid-mediated enhancement of ALA penetration were determined in excised murine skin.

Key findings ALA-loaded Witepsol microparticles were spherical, with a mean diameter of 20 μm. Loading and stability studies demonstrated effective encapsulation, ranging from 91% to 100%, with no evidence of degradation to pyrazine derivatives. ALA release correlated with dissolution medium temperature, triggered at temperatures close to that of skin. Results suggested that molten Witepsol enhanced cutaneous permeation, whereas incorporation of microparticles in a semi-solid vehicle attenuated ALA penetration. Optimal use was direct application under occlusion.

Conclusions Spray congealing is superior to the emulsion-based procedures with respect to encapsulation efficiency of ALA in Witepsol matrices, providing temperature-triggered release, enhanced stability and improved penetration of ALA through keratinised skin. These features could improve ALA delivery to superficial lesions as part of photodynamic therapy.

Keywords aminolevulinic acid; microparticles; photodynamic therapy; spray congealing; Witepsol

Introduction

Aminolevulinic acid (ALA) is a photosensitising prodrug commonly used in the photodynamic therapy (PDT) of superficial abnormalities such as basal cell carcinoma.[1] Topical application causes rapid production of protoporphyrin IX (PpIX) in neoplastic lesions[2] and leads to highly specific intracellular accumulation in target cells. Clinical implementation combines the effects of visible light irradiation with subsequent biochemical events to cause selective cell death.[3] This relies on electronic excitations that lead to the generation of reactive oxygen species. Application times as short as 3 h produce clinically significant results and advantageous outcomes, such as complete clearance and lack of scarring. However, two important aspects limit the effectiveness of ALA PDT: poor skin penetration and aqueous instability.

ALA is a small, highly polar molecule with significant aqueous solubility and an octanol : water partition coefficient of 0.03.[4] Consequently, the penetration depth of ALA in intact skin is low, typically 1–2 mm.[5,6] Unsurprisingly, reports of ALA PDT for treatment of surface lesions describe long application times and formulations with high drug loadings. Times ranging from 2 to 6 h[7] and loadings of 10–30% (w/w) in a cream, ointment or gel formulation are commonplace.[8] In addition to these drawbacks, the drug is known to degrade rapidly when present in neutral or basic environments,[9] yielding

Correspondence: Paul A. McCarron, Department of Pharmacy and Pharmaceutical Sciences, University of Ulster, Coleraine, Co. Londonderry BT52 1SA, UK.
E-mail: p.mccarron@ulster.ac.uk

cyclic products that are not precursors of PpIX.[10] This degradation mechanism has been subject to much elucidation.[11] Being an α-aminoketone, ALA dimerises readily in alkaline conditions to form 3,6-dihydropyrazine 2,5-dipropionic acid (DHPY), porphobilinogen and pseudo-porphobilinogen via an open-chain dimeric ketimine. DHPY is formed initially and is then oxidised to pyrazine 2,5-dipropionic acid (PY), which is the major degradation product in aerated solutions. Neither porphobilinogen nor pseudo-porphobilinogen are formed to any great extent under such conditions.[12,13]

The presence of PY, which is deeply coloured, gives many ALA-containing formulations a non-aesthetic brown appearance. Adjusting the vehicle pH to values below neutral, where ALA stability is better, is not a simple answer to the problem of instability because cutaneous irritation then occurs. Formulations of innocuous pH typically have short shelf-lives and must be discarded 6 months after purchase.[14] Novel strategies to circumvent stability issues have included mixing ALA powder with its vehicle immediately before application (Levulan, DUSA Pharmaceuticals) and encapsulation in solid polymeric nanoparticles[15] or multilamellar liposomes.[5] Formulating vehicles that are of sufficiently low pH to allow short-term ALA stability but not so acidic that they cause irritation have also been reported.[12,16]

The aim of this work was to formulate a stable and benign formulation that could enhance ALA stability and enable topical delivery. Of particular interest was the incorporation of ALA into microparticles prepared using an aerosol-based procedure adapted from methods described for the encapsulation of indometacin[17] and 17β-estradiol.[18] A further aim was to use a low-melting lipid-like material as the matrix for the microparticles in order to prevent aqueous dissolution during application and to preserve the drug in its crystalline state. Importantly, such lipid-like materials would impart a triggered release mechanism, such as one based on elevation of temperature, so allowing topical bioavailability once the microparticles had melted at skin temperature. To achieve this, ALA-loaded Witepsol H15 microparticles were prepared using a novel spray congealing technique, as described by McCarron and colleagues.[19] Drug entrapment efficiencies produced by this novel spraying method were determined and compared with other microparticle entrapment methods, namely the oil/water (o/w) and water/oil/water (w/o/w) emulsion methods. Of particular interest in this work were the resultant drug entrapment efficiencies, size characterisation and morphological evaluation. As Witepsol is known to melt at about skin temperature, elevation of ALA-loaded microparticles to temperatures around 32°C may constitute a temperature-triggered collapse of the microparticulate structure. Thus, an additional objective of our work was to verify temperature-triggered drug release using cumulative drug-release profiles captured at various temperatures. If ALA stability is enhanced and bioavailability at skin temperature is confirmed, then it is feasible that these microparticles could be formulated into a secondary delivery vehicle such as a structured-gel base. This may overcome the difficulties associated with poor stability upon prolonged storage in aqueous media.

Materials and methods

Materials

ALA hydrochloride salt was obtained from Crawford Pharmaceuticals (Milton Keynes, UK). Witepsol H15, comprising glycerides of vegetable origin, having 12–18 carbon atoms, an ascending melting point of 33.5–35.5°C, a hydroxyl value of 5–15 mg KOH/g and an iodine value no greater than 3, was obtained from Sigma Aldrich (Poole, UK). Compritol 888 ATO (glyceryl behenate) was obtained from Gattefosse (Weil-am-Rhein, Germany). Poly(vinyl alcohol) (PVA; MW 30 000; 88% hydrolysed) was from Aldrich Chemical (Saint Quentin Fallavier, France). Acetyl acetone, formaldehyde (37% w/w) and DMSO were obtained from Sigma Aldrich. All solvents, such as 1,2-propandiol and propylene glycol (PG), were of appropriate HPLC analytical grade (if relevant) and were used without further purification.

Preparation of ALA-loaded microparticles

Microparticles loaded with ALA were prepared using two emulsification–solvent-evaporation procedures and a spray congealing method. For the o/w method, 100 mg ALA was dispersed in a solution of Witepsol (1000 mg) in dichloromethane (10 ml) by magnetic stirring for 15 min at 1500 rpm. This dispersion was poured into 250 ml PVA (0.25% w/w) prepared in phosphate buffer (pH 12) and stirred with a three-bladed propeller for 3 h at 500 rpm to form a stable o/w emulsion.

In the w/o/w method, which was based on the method of Olbrich and colleagues,[20] a solution of ALA (100 mg in 1 ml) was first emulsified by sonication (30 s) into a solution of Witepsol (1000 mg) in dichloromethane (10 ml). This primary emulsion (w/o) was poured into 750 ml PVA (0.25% w/w) prepared in phosphate buffer (pH 12). A w/o/w emulsion was formed by extensive stirring with a three-bladed propeller for 5 min at 1200 rpm. After both the o/w and w/o/w preparation methods, the formed microparticles were collected, washed twice with deionised water and allowed to dry inside a desiccator.

Microparticles with different ALA : lipid weight ratios (1 : 100, 2 : 100, 3 : 100, 5 : 100 and 1 : 10) were prepared by a spray congealing method, based on the apparatus described by McCarron and colleagues.[19] Briefly, compressed air passing through a coiled heat-exchanger device was fed to a preheated spray nozzle. The nozzle atomised a stirred ALA–lipid mixture, which was extracted from a chamber by the resulting venturi effect. Upon leaving the nozzle, the oily droplets were collected inside a cooled glass chamber, which caused the molten microparticles to solidify rapidly while avoiding aggregation. The effect of permeability enhancers was tested by addition of different concentrations of oleic acid (5% and 10% w/w) to the 1 : 10 microparticle formula. The formed microparticles were refrigerated inside the desiccator until analysis.

Particle size analysis and morphology studies

The particle size and distribution of ALA-loaded microparticles were determined using laser diffraction analysis (Helos/KF, Sympatec GmbH (Clausthal-Zellerfeld, Germany). An

appropriate amount of microparticles was placed in the cuvette to achieve an instrumental optical concentration of about 20%. ALA microparticles were suspended in a solution of Triton X-100 in deionised water (1.0% w/v) to enhance microparticle dispersion and prevent agglomeration during the sizing process. Stirring was set at 1000 rpm, with 20% sonication for the first 20 s to improve dispersion. Excessive sonication was avoided in order to protect the microparticles from fracture and to prevent localised heating, which, bearing in mind that Witepsol H15 has a low melting point of approximately 35°C, could have melted the microparticles during analysis.

In order to describe the monodispersity of microparticles quantitatively, a coefficient, SPAN, was defined as: SPAN = $(D_{90} - D_{10})/D_{50}$ where D_n (n = 10, 50, and 90) are the equivalent volume diameters at n% cumulative volume, respectively. A narrower size distribution of microparticles equates to a smaller SPAN, which indicates better monodispersity. Microparticles can be considered as monodispersed when the SPAN value is less than 0.4.[21]

An alternative sizing analysis method was used to measure particulate size immediately upon microparticle formation during the spray congealing method. This airborne procedure was performed using a modification to the instrument, comprising a glass tube designed to attach to the spray nozzle. The dimensions of this tube (internal diameter 5 cm; length 13 cm) were chosen to allow enough time for the oily droplets to harden before impinging upon the laser path. The tube was supplied with two lateral windows of appropriate diameter (5 cm) that matched the size of the laser lenses. Atmospheric compressed air was used as a blank control.

The external morphologies of microparticles were analysed by scanning electron microscopy (SEM) (Cambridge model S240, Leica Cambridge Ltd, UK) at 20 kV. Microparticles were fixed on supports with carbon glue and coated with gold–palladium under an argon atmosphere using a gold sputter module in a high-vacuum evaporator.

Differential scanning calorimetry

Thermal analyses of the microparticles and physical mixtures were investigated using differential scanning calorimetry (DSC) (2920 DSC with a refrigerated cooling system, TA Instruments, Crawley, UK). Samples (about 2.5 mg) were sealed hermetically in aluminium pans and heated twice under a nitrogen atmosphere. The resulting thermograms, covering a range of −10 to 200°C, were recorded at a heating rate of 10°C/min. The system was calibrated using an indium standard. Determinations were performed once every 2 months for 6 months after preparation, and results reported as means (± SD) of three replicates.

ALA extraction and analysis

The efficiencies of the three ALA extraction processes from intact microparticles were verified using 100 mg of the 1 : 10 ALA : Witepsol solid dispersion. In the hot-melt procedure, a sample of the solid dispersion (100 mg) was melted in 20 ml borate buffer (pH 5) at 45°C. The mixture was agitated briskly and allowed to cool before filtering through a syringe filter (0.2 μm; Minisart, Sartorius Stedim Ltd, Epsom, UK). In the second procedure, extraction using a water-miscible solvent at room temperature was used. ALA-loaded microparticles (100 mg) were dissolved in 10 ml tetrahydrofuran and the volume adjusted to 20 ml with water. The samples were centrifuged at 56 000g for 30 min to obtain a clear supernatant for analysis. In the third procedure, a water-immiscible solvent was used. Microparticles (100 mg) were weighed and dissolved in 10 ml dichloromethane. ALA was extracted into three portions of borate buffer (6.7 ml) before separation of the phases using mild centrifugation.

ALA concentrations were determined using a modification of the method described by Endo and colleagues.[22] Samples (50 μl) were derivatised by adding to 3.5 ml acetyl acetone/ethanol/water (3 : 2 : 15 v/v) and 0.45 ml 37% formaldehyde/water (27 : 73 v/v), mixed, heated at 100°C for 20 min, and cooled in an ice-cold water bath for 10 min to stop the derivatisation reaction. Derivatised samples were measured spectrofluorometrically (excitation 366 nm; emission 432 nm) once linearity of response between the fluorescence intensity of the ALA-acetyl acetone/formaldehyde derivative and ALA concentration was verified (100–500 μg/ml ALA in borate buffer; pH 5.0). Appropriate validation of derivative stability, heating and cooling times was performed during the analytical development stages of the work.

Drug loading was calculated from:

(mass of ALA in microparticles (mg)/mass of ALA-loaded microparticle sample (mg)) × 100.

Drug entrapment efficiency was calculated using the equation:

(Actual mass of ALA in microparticles (mg)/theoretical maximum mass of ALA in microparticles (mg)) × 100

Drug release and stability studies

An appropriate amount of ALA-loaded Witepsol microparticles – 132 mg, 252 mg, 412 mg, 612 mg and 1212 mg, of the 1 : 100, 2 : 100, 3 : 100, 5 : 100, 1 : 10 formulations, respectively – were placed in cellulose mesh sachets. These were immersed in release medium (40 ml borate buffer, pH 5), maintained at a controlled temperature and stirred magnetically. Samples (50 μl) were taken at defined time intervals and protected from light in amber glass vials prior to derivatisation and analysis. Extracted samples were replaced by blank release medium buffer. Samples were filtered through a 0.2 μm syringe filter to remove fine microparticulate material, and the free ALA concentration determined as described above.

The drug release experiment was modified in two ways. First, the formulation was changed by inclusion of known skin permeability enhancers in the microparticulate formulation. Thus, 5% and 10% oleic acid were added to the 1 : 10 ALA microparticle formulation and the results compared with the 1 : 10 formulation without enhancer. A second modification was to evaluate the effect of temperature of the release medium (2, 26, 30 and 37°C) on release. The 1 : 10 ALA : lipid formulation was chosen for these temperature-based studies.

Fractional drug release data from microparticulate formulations, performed at 26°C, were fitted to zero-order,

first-order and the Higuchi diffusional models and closeness of fit determined using regression analysis.[23] A limited portion of the release profile was subjected to mathematical manipulation (approximately 60%), as recommended by Costa and Sousa Lobo.[24] The Higuchi model is described by the equation: $F = k_2 t^{1/2}$; first-order kinetics by $ln(1 - F) = -k_1 t$; and zero-order kinetics by $F_t = k_o t$, where F represents the fraction of drug released in time t, and k_1, k_2 and k_o represent the first-order release constant, Higuchi dissolution constant and zero-order release constants, respectively. The diffusional exponent (n) was calculated using the equation $M_t/M_\infty = kt^n$ from the fitted regression lines of log-percent drug released (M_t/M_∞) versus log time (t) plots. The slope was taken from the linear portion of the graph and compared with values proposed by Peppas,[25] which take into account the effect of simple geometries such as a sphere as in this work. A slope of 0.43 indicates Fickian release; a slope between 0.43 and 0.85 indicates an anomalous non-Fickian transport, and a slope of 0.85 indicates Case II transport. Drug release from porous systems may lead to a slope less than 0.43 because of the combination of diffusion through the matrix and partial diffusion through water-filled pores.[25]

The stability of ALA-containing microparticles of different drug : lipid ratios (1 : 100, 2 : 100, 3 : 100, 5 : 100, 1 : 10) was monitored under controlled storage temperatures (−18, 4 and 26°C) in complete darkness. The effect of exposure to light was evaluated by storing samples in clear vials at room temperature (26°C) and ambient lighting conditions.

Skin permeation studies

The permeability of ALA through full-thickness hairless mouse skin after fixed time periods was determined using a modified Franz diffusion cell (FDC-400 flat flange, 15 mm diameter orifice) mounted in triplicate and stirred synchronously at 600 rpm. Borate buffer (pH 5) was used as the receiver medium. Each set of experiments was performed in at least four diffusion cells.

Animal experiments were conducted according to the policy of the Federation of European Laboratory Animal Science Associations and The European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, with implementation of the principle of the 3Rs (replacement, reduction, refinement). All such experiments were conducted under the observation of the animal department of the Norwegian Radium Hospital, following approval of the study by the committee of this animal department.

Twelve-week-old female nude mice (20–25 g) were euthanised by carbon dioxide aspiration. Full-thickness skin was carefully removed, and excess subcutaneous fat and connective tissue trimmed off. The skin was then washing with sterile phosphate-buffered saline (pH 7.4) and stored in aluminium foil at −70°C until further use. All skin samples were used within 2 weeks of preparation. Excised skin (1.1067 ± 0.0727 mm thickness) was cut into approximately 3 cm squares. The tissue was supported on top of a stainless-steel filter support grid that was placed across the top of the Franz cell reservoir (37°C), as described by Ahmadi and colleagues.[26] Nine formulations of two different concentrations of ALA were used (10% and 2% w/w) to study the permeability of high and low concentrations of ALA through the stratum corneum. About 265 mg or 301.4 mg ALA-loaded microparticles were applied to the stratum corneum as either a particulate bolus or suspended in a PG gel containing 3% w/w Carbopol 974P NF.[27] The three types of microparticulate formulation and two simple ALA formulations tested were 1 : 10 and 2 : 100 ALA : Witepsol microparticles; 1 : 10 and 2 : 100 ALA Witepsol microparticles suspended in PG gel; 1 : 10 ALA Witepsol microparticles suspended in PG gel containing 20% w/w DMSO; 10% and 2% w/v ALA solution; 10% and 2% ALA suspended in PG gel.

The steady-state flux and lag time were determined by plotting the cumulative permeated amounts of ALA against time for each replicate.[28] The linear section for each graph was extrapolated to estimate steady-state flux (slope) and lag time (intersection with time axis).

Statistical analysis

Data are presented as means ± SD from at least three samples. The non-parametric Kruskal–Wallis test was used to test the effect of using different microparticles preparation methods on drug entrapment efficiency, drug entrapment efficiency with different drug : lipid ratios, and the effect of different formulations on permeability of ALA through the intact skin. $P < 0.05$ was considered to be significant.

Results

The derivatisation used in this work for the determination of ALA is a complex procedure and is particularly sensitive to experimental variables such as heating and cooling. Preliminary findings confirmed that 10 min' cooling and 20 min' heating time should be used, leading to a derivative that showed no diminution of fluorescence over 4 h. Correlation between ALA concentration (100–500 μg/ml) and fluorescence was confirmed ($r^2 = 0.9955$). The limit of detection for ALA was 6.1 μg/ml and the limit of quantification was 20.4 μg/ml, as determined from calibration curves.

Images of ALA-loaded microparticles prepared by the spray congealing method are shown in Figure 1. The chosen method of preparation has a bearing on morphology, as microparticles prepared by either the o/w or w/o/w emulsion methods exhibited irregular non-spherical bodies with apparently porous surfaces, as shown in Figures 1a and b. ALA-loaded microparticles prepared by the spraying method were spherical and regular in shape, as shown in Figure 1c. In addition, the effect of using different drug : lipid ratios on the particle morphology was also studied. Microparticles with low concentrations of ALA (1 : 100, 2 : 100 and 3 : 100) and blank microparticles had a smoother surface, with a more regular and spherical shape than the other formulations with higher loadings (Figures 1c–f). It is interesting to note the presence of dark spots around the periphery of the microparticles with the highest loadings, particularly the microparticles shown in Figure 1e.

The method used to extract ALA from a candidate formulation (1 : 10 ALA–Witepsol) was found to vary in terms of extraction efficiency. Extraction-based methods performed poorly, with 78% and 87% of ALA removed from

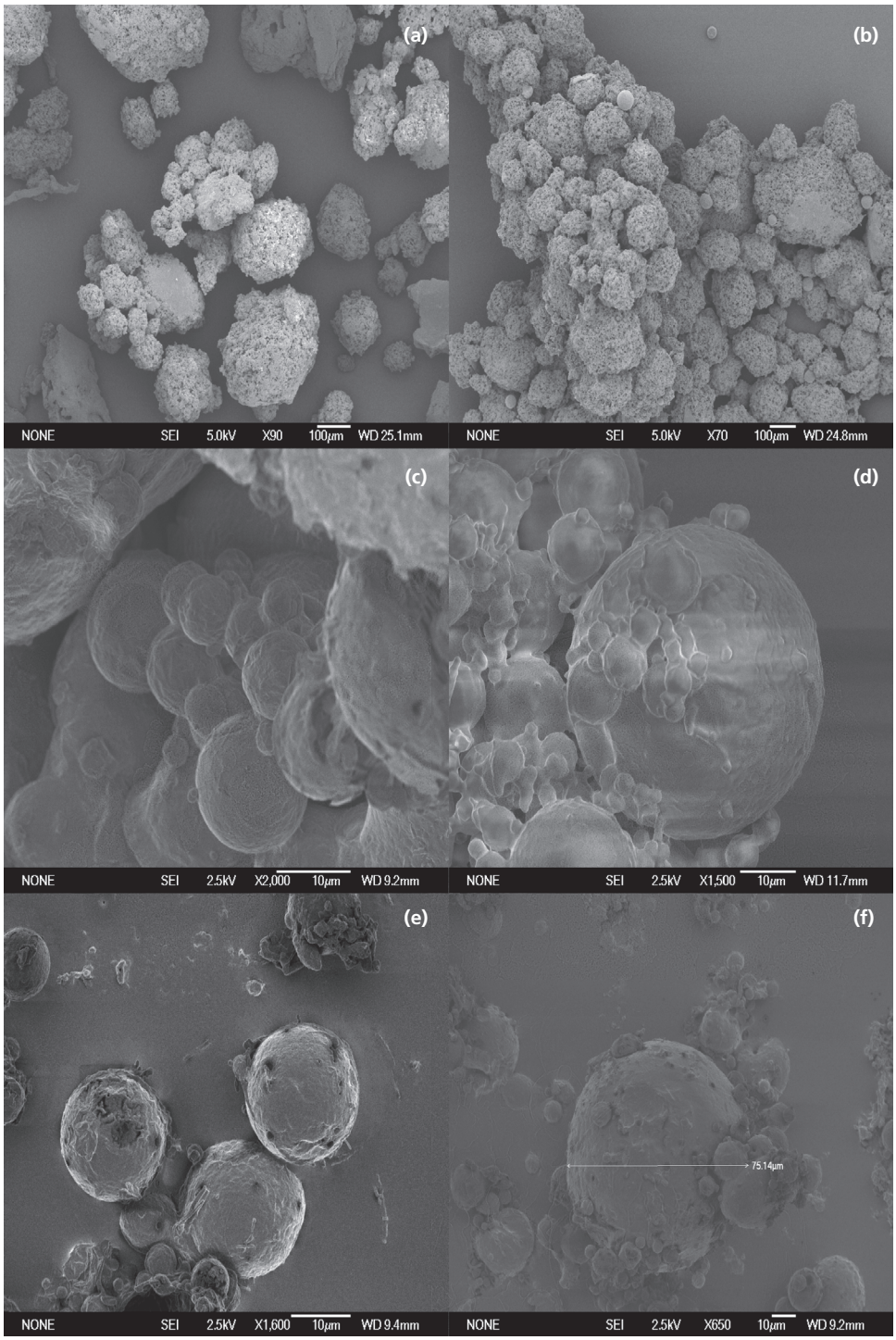

Figure 1 Scanning electron microscopy images of aminolevulinic acid. The aminolevulinic acid (ALA)-loaded microparticles were prepared by either (a) the oil/water emulsification method or (b) the double-emulsion method (water/oil/water), and Witepsol H15 microparticles were prepared using the spray congealing method and containing (c) no ALA, or ALA : Witepsol ratios of (d) 1 : 100, (e) 5 : 100 and (f) 1 : 10.

microparticles formulations using water-miscible and non-water-miscible solvents, respectively. In contrast, the amount of ALA extracted from 1 : 10 ALA-loaded microparticles by the hot melt method was significantly higher (96%; $P = 0.0273$) than with the other methods. For this reason, all further extractions were performed using the hot melt method, which showed that 0.056% ± 0.002% ALA was encapsulated by the o/w technique, and 0.76% ± 0.002% by the double-emulsion technique. The amount of ALA entrapped in the microparticles prepared by the spray

congealing method was significantly higher than in microparticles prepared by the o/w and w/o/w emulsion methods ($P = 0.0023$). Spray-congealed entrapment efficiencies ranged from about 91% to 100%, and the percentage drug loading increased in accordance with the ratios used in the initial molten blends (Table 1).

ALA-loaded microparticles were sized in both wet and dry presentations. In the wet state, the mean particle size did not increase as the amount of ALA increased, as shown in Table 2. Some variation between the different drug loadings were seen. For example, the size of the 3 : 100 ALA-loaded microparticles produced by the spray congealing method was significantly lower than the mean particle size of 1 : 10 ALA-loaded microparticles produced by the same method ($P = 0.0119$). Generally, blank microparticles were smaller than the formulations containing ALA, confirming that drug inclusion added to the bulk of the microparticles. Size determination in the dry aerosol state was also used to monitor changes in the particle size immediately after the nebulisation phase; these results are shown in Table 3. There are no clear trends between either the dry or wet methods of sizing, and both procedures estimated that the particle size was approximately 20 μm. In contrast is a comparison between the mean particle size of 1 : 10 ALA-loaded microparticles produced by the spray congealing method and those produced by either o/w or w/o/w emulsion methods, which were significantly larger ($P = 0.0273$). Additionally, the mean particle size of 1 : 10 ALA-loaded microparticles produced by the double-emulsion method (w/o/w) was significantly lower than the mean particle size produced by o/w emulsion ($P = 0.0495$). A final size method based on SEM data confirmed that no clear trend existed across the loadings, and that the blank microparticles were smaller. Interestingly, the mean size is almost twice that obtained using laser diffraction methods.

All ALA-containing Witepsol microparticles remained white in colour and additional SEM images did not reveal any discernible difference from those shown in Figure 1. The stability data showed that ALA recovery remained consistent in all formulations after 9 months' storage at −18°C. This pattern was also reproduced at the other storage temperatures investigated. The most severe storage conditions – strong daylight at room temperature – did not show any loss of ALA in any of the drug loadings. The stability of ALA-loaded microparticles with the lowest ALA concentration (1 : 100) at different temperatures, with and without light exposure,

Table 1 Loading and release data for aminolevulinic acid (ALA)-loaded microparticles prepared using different drug : lipid ratios

Parameter	ALA : lipid ratio				
	1 : 100	2 : 100	3 : 100	5 : 100	1 : 10
Loading (%)	0.91 ± 0.04	1.88 ± 0.31	2.72 ± 0.62	4.54 ± 0.12	9.09 ± 0.71
Entrapment efficiency (%)	91.5 ± 1.9	94.4 ± 1.6	93.3 ± 2.2	95.3 ± 2.5	100.3 ± 0.8
Time for complete release (min)	3300	1680	1500	720	360
Model of best fit	First-order	Higuchi	First-order	Higuchi	Higuchi
Exponent (n)	0.43 ± 0.02	0.39 ± 0.00	0.76 ± 0.02	0.31 ± 0.01	0.15 ± 0.01
Approximate mechanism	Fickian	Fickian	Non-Fickian	Non-determinable	Non-determinable

Values are means ± SD, when appropriate ($n = 3$).

Table 2 Sizing and polydispersity (SPAN) data for aminolevulinic acid (ALA)-loaded microparticles prepared using different drug : lipid ratios

Parameter and preparation method	ALA : lipid ratio					
	1 : 100	2 : 100	3 : 100	5 : 100	1 : 10	Blank
Spray congealing (wet)						
D_{50} (μm)	15.88 ± 0.14	21.64 ± 0.06	17.74 ± 0.16	20.62 ± 0.16	20.33 ± 0.15	10.72 ± 0.35
SPAN	3.38 ± 1.03	2.84 ± 0.02	4.02 ± 0.04	3.53 ± 0.02	4.12 ± 0.01	3.77 ± 0.05
Spray congealing (dry)						
D_{50} (μm)	13.43 ± 2.79	20.66 ± 7.14	48.63 ± 4.48	26.67 ± 5.30	32.21 ± 4.37	10.66 ± 0.91
SPAN	2.59 ± 1.17	3.12 ± 0.97	3.54 ± 1.02	2.48 ± 0.77	2.71 ± 0.33	3.67 ± 0.15
Oil/water						
D_{50} (μm)					223.52 ± 0.04	
SPAN					1.31 ± 0.03	
Water/oil/water						
D_{50} (μm)					188.62 ± 2.05	
SPAN					1.64 ± 1.02	
Electron microscopy						
Diameter (μm)	40–45	40–42	25–30	25–45	60–75	20–25

D_{50}, 50th percentile of the particle size distribution, as measured by volume, where 50% of the particles have a volume of this value or less. Values are means ± SD, when appropriate ($n = 3$).

Table 3 Permeation of aminolevulinic acid (ALA) through excised skin of hairless mice following application of test formulations

Formulation	Steady-state flux (μg/cm^2 per h)	Amount permeated per cm^2 per 24 h (μg)	Lag time (min)	Time for complete drug penetration (min)
1 : 10 microparticles	204.71 ± 0.11	13640.13 ± 1.91	46.0 ± 0.82	240
1 : 10 microparticles in PG gel	44.84 ± 0.02	13639.51 ± 2.19	219.5 ± 0.71	1440
2 : 100 microparticles	91.45 ± 11.0	2939.38 ± 1.71	26.0 ± 1.41	120
2 : 100 microparticles in PG gel	22.75 ± 0.01	2940.04 ± 2.01	235.0 ± 4.36	1440
10% w/w ALA solution	4.75 ± 0.19	1737.70 ± 4.06	315.0 ± 7.07	<1440
10% w/w ALA in PG gel	4.03 ± 0.04	1374.91 ± 3.25	320.0 ± 4.24	<1440
2% w/w ALA solution	1.51 ± 0.06	446.20 ± 3.47	337.5 ± 3.54	<1440
2% w/w ALA in PG gel	0.85 ± 0.05	276.20 ± 3.58	287.5 ± 17.68	<1440

Values are means ± S.D., when appropriate (n = 3). Microparticles, bolus of dry ALA-loaded microparticles; PG gel, ALA-loaded microparticles formulated in a propylene glycol gel.

showed no significant differences in recovery of ALA after 9 months (P = 0.1266). A similar lack of significant difference was observed with all other ratios tested.

DSC thermograms of pure ALA and Witepsol demonstrated sharp melting peaks at 160.52 ± 0.43 and 33.48 ± 0.49°C, respectively. Analysis of physical mixtures resulted in thermograms containing the Witepsol melting peak and the ALA melting endotherm, which diminished in proportion to the loading. Thermograms of microparticle formulations subjected to various storage conditions showed no evidence of an ALA melting peak, possibly attributable to complete homogeneity (dispersion) or solubility of the drug within the lipid matrix.

ALA release at 26°C from microparticles containing different drug : lipid ratios increased significantly as the concentration of ALA inside the microparticles increased (P = 0.0102; Figure 2). Evaluation of the time required for complete release of ALA from each formulation (Table 2) showed that 360 min was required for the 1 : 10 microparticle formulation, whereas 10-fold longer (3300 min) was needed for complete release from the 1 : 100 formulation. Most ALA-loaded microparticles with different drug : lipid ratios exhibited near Higuchi release kinetics, as judged by the model of best fit, and microparticle formulations with the low ALA concentrations (1 : 100 and 3 : 100) exhibited near zero-order drug release (Table 2). The 3 : 100 microparticles did not follow Fickian diffusion, as their release exponent was between 0.43 and 0.85, as shown in Table 2. The release exponents for the 5 : 100 and 1 : 10 microparticles were considerably below 0.43. The effect of using different concentrations of oleic acid (5% and 10% w/w) on the release rate from 1 : 10 ALA : (Witepsol–oleic acid) microparticles was similar to the equivalent loading profile shown in Figure 2, with no significant differences observed in time for complete release.

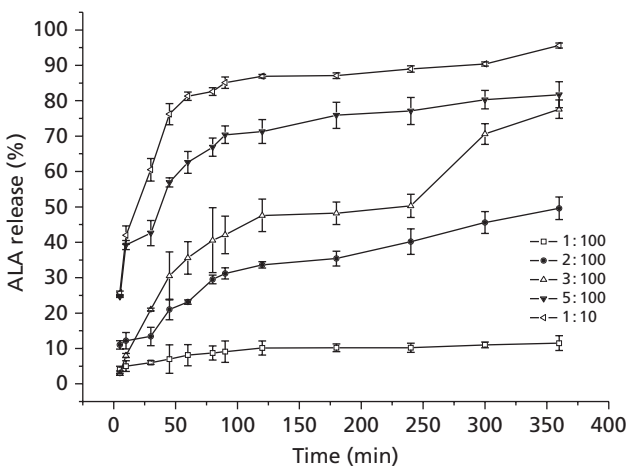

Figure 2 Effect of drug : lipid ratio on aminolevulinic acid (ALA) release from ALA-loaded microparticles at 26°C. Results are shown as means ± SD (n = 3).

The effect of temperature on the release of ALA from microparticles is shown in Figure 3. Two microparticulate formations where chosen for this study, one with a high ALA concentration (1 : 10) and one with a low concentration (2 : 100). ALA release from the 2 : 100 microparticle formulation increased significantly with increasing temperature of the release medium (P = 0.0156), and was found to fit the Higuchi drug release model at both 26 and 30°C but switched to first-order drug release kinetics at 2 and 37°C. Similarly, ALA release from 1 : 10 ALA-loaded microparticles increased significantly as the temperature of the release medium increased (P = 0.0239). The Higuchi model was prevalent in this formulation type at all temperatures. However, the complete release observed at 37°C was noticeably different between the two formulations. Figure 3a shows that complete release at this temperature is achieved in approximately 120 min with the 2 : 100, whereas almost 300 min is needed for the 1 : 10 formulation (Figure 3b).

The permeation of ALA through excised murine skin from representative high (1 : 10) and low (2 : 100) formulations is shown in Figure 4. In addition, the effect of secondary inclusion in a typical delivery vehicle, such as a topical semi-solid, is shown, along with the effect of having no microparticulate encapsulation (10% and 2% w/w ALA in aqueous solution and non-aqueous gel). Complete penetration of ALA was observed from microparticles alone (Figure 4a), followed by a more sustained release from microparticles formulated into a semi-solid vehicle. Delivery of ALA from a solution phase was poor – approximately 15% of the total ALA dose crossed the skin sample in 24 h. Steady-state fluxes obtained from this data are shown in Table 3. It is clear that flux from the microparticle-only formulation is significantly

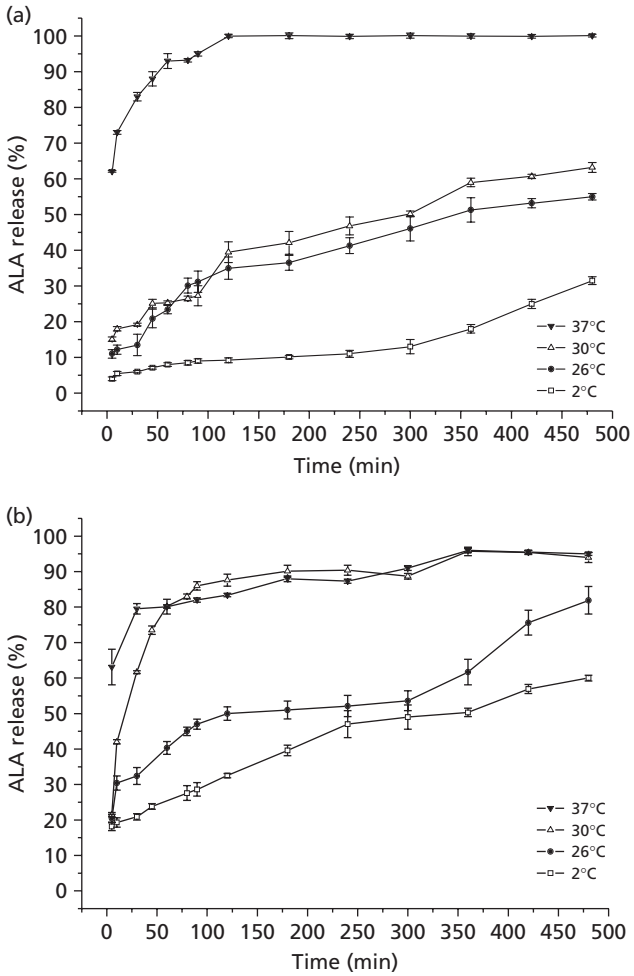

Figure 3 Effect of temperature on the release of aminolevulinic acid (ALA) from ALA-loaded microparticles containing a drug : lipid ratio of (a) 2 : 100 and (b) 1 : 10. Results are shown as means ± SD (n = 3).

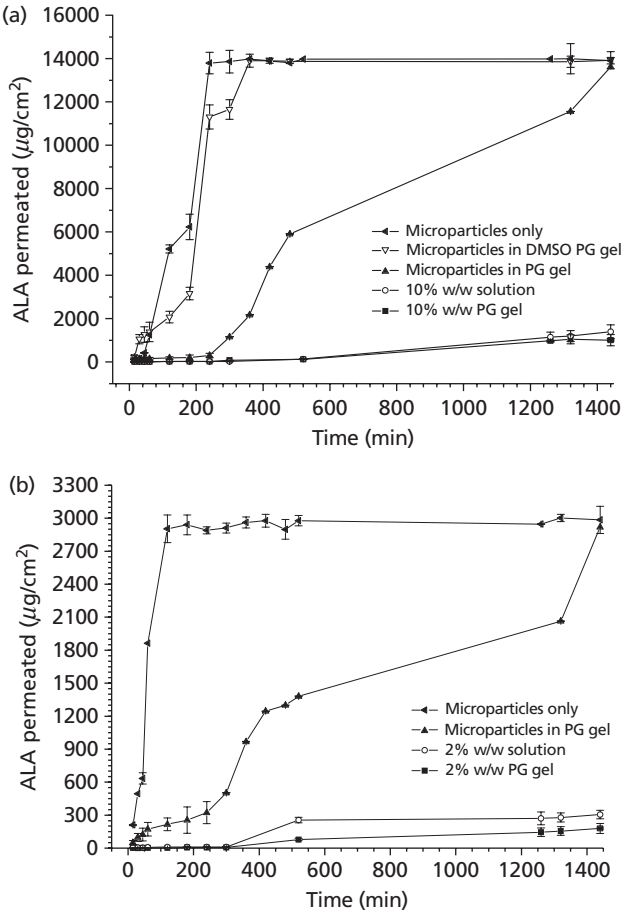

Figure 4 Percutaneous penetration of aminolevulinic acid (ALA) arising from ALA-loaded microparticles containing a drug : lipid ratio of: (a) 1 : 10 and (b) 2 : 100 applied as either a dry bolus of microparticles or suspended in different delivery vehicles. Results are shown as means ± SD (n = 5). PG, polypropylene glycol.

higher than from microparticles in gel ($P < 0.0001$). Interestingly, inclusion of DMSO enhanced flux, achieving a value close to that for microparticles only.

The results in Figure 4b for formulations based on the 2 : 100 ALA microparticles mirror those in Figure 4a. The microparticle-only formulation is clearly superior, particularly in terms of shorter lag time, steady-state flux and time to achieve complete drug permeation. The steady-state flux with 2 : 100 ALA-loaded microparticles was significantly higher than the flux obtained with 2 : 100 ALA-loaded microparticles in PG gel ($P < 0.0001$). It can also be seen that the gel and solution where 2% w/w ALA was used reflect the poor performance seen with their higher loaded counterparts. Furthermore, the lag time data in Table 3 reveal an interesting pattern: approximately 26 and 46 min were required for ALA to begin penetration through intact skin from the 2 : 100 and 1 : 10 microparticle formulations, respectively. Both 10% and 2% w/w ALA aqueous solutions and gel exhibited long lag times (287–337 min). The lag time obtained with 1 : 10 ALA-loaded microparticles was significantly lower than that induced by 1 : 10 ALA-loaded microparticles in PG gel, 10% w/w ALA solution or 10% w/w ALA gel ($P < 0.0001$). In addition, the lag time induced by 2 : 100 ALA-loaded microparticles was significantly lower than that induced by 2 : 100 ALA-loaded microparticles in PG gel, 2% w/w ALA solution and 2% w/w ALA gel ($P < 0.0001$).

Discussion

The development of topical formulations containing ALA is difficult, with major challenges related to poor stability and low bioavailability. The analytical methods used to measure ALA are also problematic and often require chemical derivatisation because of a lack of innate chromophores.[29] Validation of the analytical method is particularly important, especially when working with microencapsulation methods, as in this work, when loadings can be low. Various chemical derivatisation methods exist,[30] the most common of which involve derivatisation with either *o*-phthaldialdehyde and 2-mercaptoethanol[31] or acetyl acetone and formaldehyde,[32] followed by chromatographic separation and

fluorescence detection. In the initial stages of this current work, validation of the optimum conditions was performed because, unlike the work of Endo and colleagues,[22] analysis was by fluorometric analysis and at different excitation and emission wavelengths (366 nm and 432 nm, respectively).

The results presented in Figure 4 highlight the problem that plagues many topical delivery strategies for ALA. If the drug is in the solution phase, then flux through intact skin will be small, which was demonstrated by the fluxes arising from the gel and solution formulations used in the latter half of this work. The reason for this is well known and can be attributed to the effectiveness of the barrier function of the stratum corneum. ALA, being a small, polar molecule of low molecular weight (167.6 Da), does not penetrate skin effectively,[33] an observation confirmed in Figure 4. Simple approaches to circumvent this problem, such as increasing the concentration gradient, are not without problems as ALA is prone to rapid dimerisation in solution, which is exacerbated by higher loadings. Thus, the approach adopted in this work was to encapsulate ALA in a temperature-sensitive protective shell, keeping it in the solid state. Application to skin would induce melting of the Witepsol base and it was further hypothesised that the molten lipid would constitute some form of penetration enhancer that would fluidise stratum corneum lipids and promote ALA permeability.

Encapsulation methods generally involve an emulsification step. The results presented in Table 2 show how the efficiency of entrapment varies according to the method of manufacture. It is feasible that ALA, being water soluble, escapes to the continuous aqueous phase during emulsification, and sophisticated modifications, such as development of w/o/w systems, did little to enhance the poor loadings evident in this work. Spray congealing methods are an attractive alternative as they are characterised by high encapsulation efficiencies.[19,34,35] A comparison with equivalent o/w and w/o/w double-emulsion methods confirmed this superiority with respect to ALA (Table 1). Visualisation using SEM confirmed that spray-congealed microparticles were spherical, and the sizing data presented in Table 2 show that the emulsion procedures produced considerably bigger microparticles, by almost an order of magnitude, which concurs with the findings of Bittner and colleagues.[34] Additionally, loading of ALA caused an initial increase in microparticle size, but further increases in loading were not associated with increased diameters. It is intriguing to consider the physical form of the ALA encapsulated in the microparticles. The images in Figures 1e and 1f show what may be the presence of crystalline material either on or protruding from the surface of the microparticles. However, it should be remembered that the mean particle size of the raw ALA used in this work was approximately 25 μm, larger than the diameter of most spray-congealed microparticles (Table 3). This raises the possibility that the microparticles produced in this work were not a simple structure comprising a crystalline core and an overlying lipid shell. If this were the case, then changes in loading from 1 : 100 to 1 : 10 should see considerable increase in diameter, but this was not observed. This raises the further possibility that the ALA is present in an amorphous form as it is dispersed in a hot lipid melt close to its own melting temperature. The rapid cooling arising from impinging the cold collecting surface may promote the formation of this amorphous ALA. Indeed, results from thermal analysis showed a weak ALA signature in physical mixtures but no such peak in spray-congealed samples.

Loading drugs into microparticles and the effect on particle characteristics has not shown a consistent pattern in the literature. For example, efficiency of loading was not affected by variation in the drug : lipid ratio in some work,[36] but was found to increase in the work of others.[37] In addition, it has been reported that decreasing the core-to-wall ratio, which might be achieved by increasing the drug concentration, leads to a higher encapsulation efficiency.[38] In this current work, it was more difficult to discern a clear pattern, as ALA entrapment efficiency showed some variation but did not fall below 91.5%. The 1 : 10 formulation had the highest encapsulation efficiency (100.3%), which is in stark contrast to the emulsion techniques, presumably reflecting leaching of the hydrophilic payload (ALA) into the aqueous external phase. This loss is avoided by the spraying method, as the continuous phase is gaseous and unable to leach ALA from nascent microparticles.[34]

Stability studies will be a prominent feature of any work developing formulations of ALA in topical vehicles. Stability in aqueous solution depends on concentration, pH, temperature and the degree of oxygenation of the solution.[10,12] Degradation to PY in aerated media is rapid and resistant to the normal means of preventing oxidation such as the addition of EDTA[29] or antioxidants.[10] Sophisticated methods to address the problem, such as stabilisation in cubic phase gels[39,40] and chemical derivatisation of the ALA amino group,[41] have been tried, but with questionable success. The approach used to enhance stability in this current study was to entrap ALA inside Witepsol H15 microparticles. Stability data showed that no significant amounts of ALA were lost over a period of 9 months, even when storage conditions were made more demanding. Close observation of the microparticles did not indicate a change in colour, itself indicative of PY formation, remaining white, spherical and non-aggregated during the entire storage time. SEM studies of morphological changes during storage did not indicate any changes in appearance after 9 months and at the various conditions used in this study (images not shown).

The ALA release profile was shown to depend on the drug : lipid ratio (Figure 2). A biphasic release profile was evident in the highest loading, probably due to a burst release effect in the first hour, attributable to drug entrapped on the microparticle surface or some type of defective drug entrapment. Close observation of the images in Figures 1e and 1f show superficial black spots, which were more noticeable on the 1 : 10 and 5 : 100 formulations. It is feasible that these regions were areas of high ALA density. Also, close inspection of the microparticulate surface in Figure 1f shows that it is not entirely smooth and intact, but instead appears flaked. This effect was more noticeable in the microparticles with highest drug concentration, which could explain the initial burst effect in this formulation. It has been reported that water-soluble drugs are usually released from a matrix-type system by diffusion, whereas poorly

water-soluble drugs are released by erosion from the matrix.[24] In addition, other findings point to drug release by Fickian diffusion, as estimated by the exponent value, *n*. We produced values of approximately 0.43 from low-loaded formulations, indicative of Fickian release from spherical geometries. As the loading is increased, Fickian release appears to be lost, and the mechanism in the higher loadings becomes non-determinable. At this point, other mechanisms are contributing to the overall release pattern, possibly rapid dissolution through the disordered particulate surface observable under SEM studies.

The results shown in Figure 3 confirm the hypothesis proposed in this work, that temperature around that of normal skin can be an effective trigger for rapid drug dumping. We chose an upper threshold of 37°C because many formulations used to deliver ALA to superficial lesions use occlusion to enhance drug delivery and so localised temperatures are likely to be close to body temperature. In the 2 : 100 formulation, the triggered release process is clear as microparticles begin to melt. A similar pattern exists in the 1 : 10 formulation but release at 37°C was not as quick as expected, although it is still clearly the most rapid.

Although the microencapsulation approach was shown to overcome problems associated with stability, it is not a particularly convenient method for topical application. In PDT it is more usual to apply semi-solids such as creams and gels. Therefore, in the final part of this work, release and permeation studies were extended to include microparticles suspended in a model gel system that would not affect the integrity of the lipid coating nor have a significant impact on ALA stability. Results from in-vitro permeation studies (Figure 4) demonstrated clearly that microparticulate-only formulations were better than the others investigated. The inferior performance of semi-solid-based delivery reflects the increased diffusional path for free ALA. Delivery of ALA was poor when dissolved in either an aqueous gel or solution, with long lag times (287.5–337.5 min). This is a surprising finding as PG has been used to enhance penetration of ALA.[42] PG is known to be a good solvent for ALA and is thought to enhance penetration through skin by increasing drug partition into cutaneous layers after entering the stratum corneum.[28,43] However, beneficial effects were not demonstrated in our work.

The effectiveness of the microparticle-only formulation warrants further discussion. Witepsol H15 is a saturated triglyceride and its structure resembles typical monounsaturated fatty acids such as oleic acid. Oleic acid is known to enhance penetration of selected drug substances by disrupting packing and increasing fluidity in the stratum corneum lipid bilayer regions.[44] Lipid sponge phases, consisting of monoolein, PG and an aqueous buffer and containing 16% methyl-ALA, have demonstrated higher flux than that from Metvix cream.[45] It has also been reported that topical application of PG containing glycerol monooleate enhanced the penetration of ALA through intact mouse stratum corneum and gave greater accumulation of PpIX.[43] It is feasible that molten Witepsol performs a similar function, and the results in Figure 4 would indicate that this is happening. Nevertheless, incorporation of microparticles in a hydrophilic gel base appeared to disrupt this effect, even though the microparticles had probably melted once the applied formulation had reached 37°C.

Conclusions

The spray congealing procedure produced ALA-loaded Witepsol microparticles with similar morphology to those produced by emulsion procedures but clear superiority in terms of high loadings and close to optimal encapsulation efficiencies. Importantly, stability data showed that degradation to pyrazine derivatives, which often plagues other topical formulations, did not occur. Aerosol-based sizing procedures showed a mean diameter of about 20 μm. Calorimetry studies supported the argument that dissolution of ALA into the lipid matrix was occurring, which was itself susceptible to melting at skin temperature. This formed part of a temperature-triggered dose dumping that would be ideal for ALA delivery to superficial lesions as part of a PDT procedure. Importantly, there was evidence to suggest that molten Witepsol in direct contact with excised skin enhanced ALA permeation, overcoming poor penetration through cutaneous lesions. Incorporation of microparticles in a semi-solid vehicle for topical application was detrimental to ALA penetration and the optimal means of using these ALA-loaded microparticles was direct application under some form of occlusion.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This review received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

References

1. Fink-Puches R *et al*. Photodynamic therapy of superficial basal cell carcinoma by instillation of aminolevulinic acid and irradiation with visible light. *Arch Dermatol* 1997; 133: 1494–1495.
2. Kennedy JC *et al*. Photodynamic therapy (PDT) and photodiagnosis (PD) using endogenous photosensitization induced by 5 aminolevulinic acid (ALA): mechanisms and clinical results. *J Clin Laser Med Surg* 1996; 14: 289–304.
3. Ahmad N, Mukhtar H. Mechanism of photodynamic therapy-induced cell death. *Methods Enzymol* 2000; 319: 342–358.
4. Lieb S *et al*. Self-adhesive thin films for topical delivery of 5-aminolevulinic acid. *Eur J Pharm Biopharm* 2002; 53: 99–106.
5. Casas A *et al*. The influence of the vehicle on the synthesis of porphyrins after topical application of 5-aminolaevulinic acid: implications in cutaneous photodynamic sensitization. *Br J Dermatol* 2000; 143: 564–572.
6. Malik Z *et al*. Topical application of 5-aminolevulinic acid, DMSO and EDTA: protoporphyrin IX accumulation in skin and tumours of mice. *J Photochem Photobiol B* 1995; 28: 213–218.
7. Morton CA *et al*. Photodynamic therapy for basal cell carcinoma: effect of tumor thickness and duration of photosensitizer application on response. *Arch Dermatol* 1998; 134: 248–249.

8. De Rosa FS, Bentley MV. Photodynamic therapy of skin cancers: Sensitizers, clinical studies and future directives. *Pharm Res* 2000; 17: 1447–1455.
9. Butler AR, George S. The nonenzymatic cyclic dimerization of 5-aminolevulinic acid. *Tetrahedron* 1992; 48: 7879–7886.
10. Bunke A *et al*. Degradation mechanism and stability of 5-aminolevulinic acid. *J Pharm Sci* 2000; 89: 1335–1341.
11. Bunke A *et al*. Validation of a capillary electrophoresis method for determination of 5-aminolevulinic acid and degradation products. *J Chromatogr* 2000; A883: 285–290.
12. Novo M *et al*. Chemical instability of 5-aminolevulinic acid used in the fluorescence diagnosis of bladder tumours. *J Photochem Photobiol B* 1996; 34: 143–148.
13. de Blois AW *et al*. *In vivo* pharmacokinetics of protoporphyrin IX accumulation following intracutaneous injection of 5-aminolevulinic acid. *J Photochem Photobiol B* 2001; 61: 21–29.
14. Crawford Pharmaceuticals, Milton Keynes, UK. Porphin® Cream product information sheet, 1996.
15. Hurlimann AF *et al*. Photodynamic therapy of superficial basal cell carcinomas using topical 5-aminolevulinic acid in a nanocolloid lotion. *Dermatology* 1998; 197: 248–254.
16. Chang SC *et al*. Biodistribution of protoporphyrin IX in rat urinary bladder after intravesical instillation of 5-aminolevulinic acid. *J Urol* 1996; 155: 1744–1748.
17. Raffin Pohlmann A *et al*. Spray-dried indomethacin-loaded polyester nanocapsules and nanospheres: development, stability evaluation and nanostructure models. *Eur J Pharm Sci* 2002; 16: 305–312.
18. Mohr D *et al*. Gamma irradiation for terminal sterilization of 17beta-estradiol loaded poly-(D,L-lactide-co-glycolide) microparticles. *J Control Release* 1999; 61: 203–217.
19. McCarron PA *et al*. Comparison of a novel spray congealing procedure with emulsion-based methods for the micro-encapsulation of water-soluble drugs in low melting point triglycerides. *J Microencapsul* 2008; 25: 1–14.
20. Olbrich C *et al*. Lipase degradation of dynasan 114 and 116 solid lipid nanoparticles (SLN) – effect of surfactants, storage time and crystallinity. *Int J Pharm* 2002; 237: 119–128.
21. Xiao XC *et al*. Monodispersed thermoresponsive hydrogel microspheres with a volume phase transition driven by hydrogen bonding. *Polymer* 2005; 46: 3199–3209.
22. Endo Y *et al*. Improvement of urinary delta-aminolevulinic acid determination by HPLC and fluorescence detection using condensing reaction with acetylacetone and formaldehyde. *Sangyo Igaku* 1994; 36: 49–56.
23. Hayashi *et al.* Formulation study and drug release mechanism of a new theophylline sustained – release preparation. *Int J Pharm* 2005; 304: 91–101.
24. Costa P, Sousa Lobo JM. Modeling and comparison of dissolution profiles. *Eur J Pharm Sci* 2001; 13: 123–133.
25. Peppas NA. Analysis of Fickian and non-Fickian drug release from polymers. *Pharm Acta Helv* 1985; 60: 110–111.
26. Ahmadi S *et al*. Evaluation of the penetration of 5-aminolevulinic acid through basal cell carcinoma: a pilot study. *Exp Dermatol* 2004; 13: 445–451.
27. McCarron PA *et al*. Stability of 5-aminolevulinic acid in novel non-aqueous gel and patch-type systems intended for topical application. *J Pharm Sci* 2005; 94: 1756–1771.
28. Funke AP *et al*. In-vitro release and transdermal fluxes of a highly lipophilic drug and of enhancers from matrix TDS. *J Control Release* 2002; 82: 63–70.
29. Elfsson B *et al*. Stability of 5-aminolevulinic acid in aqueous solution. *Eur J Pharm Sci* 1999; 7: 87–91.
30. Gorchein A. Determination of delta-aminolaevulinic acid in biological fluids by gas-liquid chromatography with electron-capture detection. *Biochem J* 1984; 219: 883–889.
31. Winkler A, Muller-Goymann CC. Comparative permeation studies for delta-aminolevulinic acid and its n-butylester through stratum corneum and artificial skin constructs. *Eur J Pharm Biopharm* 2002; 53: 281–287.
32. Oishi H *et al*. Fluorometric HPLC determination of delta-aminolevulinic acid (ALA) in the plasma and urine of lead workers: biological indicators of lead exposure. *J Anal Toxicol* 1996; 20: 106–110.
33. Di Venosa G *et al*. A method for separating ALA from ALA derivatives using ionic exchange extraction. *J Photochem Photobiol B* 2004; 75: 157–163.
34. Bittner B *et al*. Recombinant human erythropoietin (rhEPO) loaded poly(lactide-co-glycolide) microspheres: influence of the encapsulation technique and polymer purity on microsphere characteristics. *Eur J Pharm Biopharm* 1998; 45: 295–305.
35. Hascicek C *et al*. Mucoadhesive microspheres containing gentamicin sulfate for nasal administration: preparation and *in vitro* characterization. *Farmaco* 2003; 58: 11–16.
36. Comoglu T. Preparation and *in vitro* evaluation of modified release ketoprofen microsponges. *Farmaco* 2003; 58: 101–106.
37. Yildiz A *et al*. Nasal administration of heparin-loaded microspheres based on poly(lactic acid). *Farmaco* 2005; 60: 919–924.
38. Wieland-Berghausen S *et al*. Comparison of microencapsulation techniques for the water-soluble drugs nitenpyram and clomipramine HCl. *J Control Release* 2002; 85: 35–43.
39. Turchiello RF *et al*. Cubic phase gel as a drug delivery system for topical application of 5-ALA, its ester derivatives and m-THPC in photodynamic therapy (PDT). *J Photochem Photobiol B* 2003; 70: 1–6.
40. Valenta C *et al*. Skin permeation and stability studies of 5-aminolevulinic acid in a new gel and patch preparation. *J Control Release* 2005; 107: 495–501.
41. Kaliszewski *et al*. The stability of 5-aminolevulinic acid and its ester derivatives. *Acta Pol Pharm* 2004; 61: 15–19.
42. Merclin N *et al*. Iontophoretic delivery of 5-aminolevulinic acid and its methyl ester using a carbopol gel as vehicle. *J Control Release* 2004; 98: 57–65.
43. Steluti R *et al*. Topical glycerol monooleate/propylene glycol formulations enhance 5-aminolevulinic acid *in vitro* skin delivery and *in vivo* protophorphyrin IX accumulation in hairless mouse skin. *Eur J Pharm Biopharm* 2005; 60: 439–444.
44. Escribano E *et al*. Assessment of diclofenac permeation with different formulations: anti-inflammatory study of a selected formula. *Eur J Pharm Sci* 2003; 19: 203–210.
45. Merclin N *et al*. Transdermal delivery from a lipid sponge phase–iontophoretic and passive transport *in vitro* of 5-aminolevulinic acid and its methyl ester. *J Control Release* 2004; 100: 191–198.