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Practical Considerations in the Pharmaceutical Analysis of Methyl and Hexyl Ester Derivatives of 5-Aminolevulinic Acid

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Abstract: Photodynamic therapy (PDT) using topical 5-aminolevulinic acid (ALA), a water-soluble precursor of the potent endogenous photosensitiser, protoporphyrin IX (PpIX), is a treatment and diagnostic tool for premalignant and malignant skin cancers. However, to improve drug delivery to deeper skin lesions, more lipophilic ALA esters have been investigated. Owing to the necessity in drug delivery research for efficient and validated assays for ALA esters in solution, this paper aims to describe optimised protocols to quantify the methyl and hexyl esters of ALA.

ALA esters were derivatised using acetyl acetone and formaldehyde reagents and analysed using reversed phase HPLC. For the first time, the significance of ALA-impurities in ester samples has been highlighted. Furthermore, it was shown that for a given concentration, peak areas obtained for ALA-esters were significantly smaller than those obtained for ALA ($p < 0.05$). This may be due to parent drug undergoing the derivatisation reaction more efficiently or because the ALA-derivate is inherently more fluorescent than the ester derivatives. The method was optimised to give acceptable intra- and inter-day variability (CV values < 5%) and the limits of detection and quantification were determined for both drugs.

The validated methods were used to determine the release profiles of ALA, m-ALA and h-ALA from an o/w cream formulation. The percentage of drug loading released after five hours across a model membrane was in the order of ALA (45.2%) > m-ALA (38.3%) > h-ALA (33.9%). These findings may explain why, historically, some of the benefits seen with ALA-esters using cell culture models have not been demonstrated *in vivo*.

Keywords: 5-Aminolevulinic acid, Drug delivery, Esters, HPLC, Assay validation.

1. INTRODUCTION

Photodynamic therapy (PDT) is a medical treatment by which a combination of visible light and a sensitising drug causes the destruction of selected cells [1]. A drug without dark toxicity is introduced into the body and accumulates in rapidly dividing cells. A measured light dose of appropriate wavelength is then used to irradiate the target tissue [2]. This activates the drug and elicits the toxic reaction in the presence of oxygen [1]. The most widely used agent in topical PDT is 5-aminolevulinic acid (ALA). Topical application of ALA induces an overproduction of the endogenous photosensitiser, protoporphyrin IX (PpIX), which can subsequently be activated by light of the appropriate wavelength.

ALA-PDT is largely limited to the treatment of superficial lesions, such as actinic ketatosis (AK) and superficial nonmelanoma skin cancers (NMSC) [3]. Indeed, the clearance rates associated with ALA-PDT for superficial lesions are often high and the treatment promises significantly better cosmetic outcomes than surgery and radiotherapy [3]. However, clearance rates for deeper lesions, such as nodular basal cell carcinoma (BCC) are less impressive [4]. The poor penetration of ALA into nodular BCCs is not surprising considering the compounds physicochemical properties. Hydrophilic charged molecules, such as ALA generally penetrate the *stratum corneum* poorly [5]. Consequently, there has been a concerted effort to develop innovative strategies to enhance the penetration of ALA into skin. Techniques that have been studied include the use of ion pairing agents [6], laser ablation [7], iontophoresis [8] and microneedle arrays [9]. However, to date, the most widely used enhancement technique has been the incorporation of more lipophilic ALA esters into conventional dosage forms, such as creams, ointments, gels and solutions [10, 11].

ALA (Fig. **1a**) is a straight chain, primary amino acid with a weak chromophore (isolated carbonyl group). Thus, ALA is rarely quantified by conventional ultraviolet (UV) absorption spectroscopy [12]. Instead, chemical derivatisation procedures are employed to produce compounds detectable by simple spectroscopic analysis [13]. For more complex samples, whereby ALA needs to be separated from interfering substances, high performance liquid chromatographic (HPLC) methods based on chemical derivatisation of ALA have been employed. Meisch *et al.* [14] reported a method for derivatisation of ALA with 2-amino-3-hydroxynaphthalene (AHN) to yield a cyclic compound, which may be determined by HPLC with fluorescence detection. Similarly, HPLC with fluorescence detection was used for quantification of ALA derivatised with dansyl chloride [15], *o*-phthaldialdehyde [16], 9-fluorenylmethyloxycarbonylchloride (FMOC) [17] and

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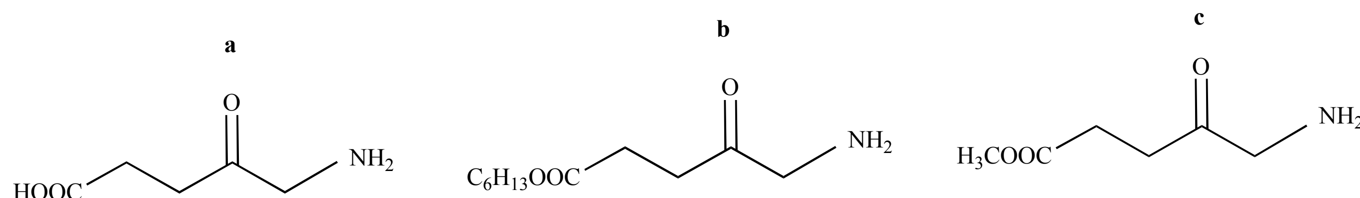

Fig. (1). Chemical structure of ALA (**a**), h-ALA (**b**) and m-ALA (**c**).

fluorescamine [4-phenylspiro(furan-2[3H],1-phthlan)-3,3-dione] [18]. At present, however, the most commonly employed methods for determination of ALA involves derivatisation with acetyl acetone and formaldehyde [19] followed by chromatographic separation and, usually, fluorescence detection. Indeed, recent work by Donnelly *et al.* [16] reported an optimised and validated method employing these derivatisation reagents. The authors reported a LOD and LOQ of 0.05 µg ml^{-1} and 0.14 µg ml^{-1}, respectively.

Fig. (**1b**, **c**) illustrate that, as with the parent molecule, straight chain ALA esters do not have any significant chromophores. Consequently, it is necessary to perform a derivatisation procedure to enable quantification of ALA esters. The procedure used by Mauzerall and Granick for ALA quantification has been adapted to allow determination of ALA esters in solution by UV spectroscopy [20]. In contrast, Turchiello *et al.* [21] derivatised ALA esters with acetyl acetone and formaldehyde and quantified the derivative using spectrofluorimetry (excitation 378 nm, emission 466 nm). However, to differentiate between ALA and its esters, an appropriate separation technique is required. HPLC methods employing a pre-column derivatisation using a mixture of *o*-phthaldialdehyde and 2-mercaptoethanol [22] or acetyl acetone and formaldehyde [23, 24], have also been used to quantify ALA esters.

As discussed above, the quantification of ALA esters has been reported in the literature before. However, LOD and LOQ values are rarely reported and, to date, an optimised and validated quantitative procedure that can simultaneously quantify ALA esters and the parent drug is yet to be published. This paper aims to discuss the importance of appropriate and efficient ALA-ester analysis in solution and highlight the practical considerations of such work. Importantly, methods for the analysis of m-ALA and h-ALA should be validated to International Conference on Harmonisation (ICH) standards. The overall objective of this work, therefore, was to develop optimised and validated procedures to facilitate accurate and convenient analysis of ALA esters in solution as a means of quantifying the amount of drug released from semi-solid preparations intended for use in clinical PDT.

2. MATERIALS AND METHODS

2.1. Chemicals

5-aminolevulinic acid, hydrochloride salt (ALA) was obtained from Crawford Pharmaceuticals, Milton Keynes, UK. Acetyl acetone, hexanol, methyl 5-aminolevulinate hydrochloride salt (m-ALA), thionyl chloride, formaldehyde 37% w/w solution in water were obtained from Sigma Aldrich, Dorset, UK. Unguentum Merck® was supplied by Merck, Darmstadt, Germany. All other chemicals were of analytical reagent grade.

2.2. Synthesis of ALA Hexylester Hydrochloride Salt

ALA hexylester was synthesised by reacting ALA with hexanol based on the method described by Kloek *et al.* [25]. A defined volume of hexanol (3.5 ml) was cooled in an ice bath containing iced methanol to -10°C, to which 0.5 ml thionyl chloride was added at such a rate that the temperature did not rise above 10°C. ALA hydrochloride salt (0.5 g) was added whilst stirring.

The solution was heated to 70°C for 2-3 hours until the ALA was completely dissolved, leaving a slightly cloudy solution. The solution was then cooled to room temperature, 30 ml diethyl ether added and the solution stored at -70°C for two hours to ensure the ester had precipitated out. The precipitate was then filtered under vacuum, and dried. The ester was recrystallised by dissolving it in 1.5 ml of methanol, to which 8.5 ml of diethyl ether was then added. After cooling for two hours at -70°C, the ester was filtered off, washed with ether and dried at 40°C.

2.3. Identification of ALA Hexylester Hydrochloride Salt

Samples for infrared (IR) analysis were prepared as follows. Approximately 25 mg of the h-ALA derivative was ground in a mortar. Then 10 mg of sample was thoroughly mixed with 300 mg of dried potassium bromide. The powder was poured into a 13 mm die and compressed under a load of 10 tonnes for three minutes. Resultant discs were analysed using a Nicolet Protégé 460 E.S.P.™ spectrometer, Madison, USA.

Solution state ^{1}H nuclear magnetic resonance spectroscopy (NMR) was carried out at the School of Chemistry, Queens University Belfast. Approximately 20 mg of h-ALA was dissolved in deuterated DMSO (containing tetramethylsilane (TMS) as an internal standard) and analysed using a Bruker Avance DRX 500 NMR spectrometer, Bruker, Rheinstetten, Germany. Spectra were recorded with a variable number of scans. Chemical shift of the internal standard was set at 0 for TMS.

2.4. Determination of ALA and ALA Esters with Acetyl Acetone and Formaldehyde

Previously our group has presented a validated protocol for the determination of ALA [16] based on the Hantzch reaction [19]. In the present study, ALA esters were also derivatised by reaction with acetyl acetone (AA) and formaldehyde. AA reagent was prepared by mixing 15 ml acetyl acetone, 10 ml ethanol and 75 ml distilled water. Formaldehyde solution (10% w/w) was made by a 3.7-fold dilution of the chemical reagent (37% w/w) with distilled water and stored in the dark.

To a HPLC vial, 3.5 ml AA reagent, 50 µl of sample and 0.45 ml 10% w/w formaldehyde solution were added and mixed on a vortex mixer for approximately 5 s. This mixture was heated for 20 minutes at 100°C. The vial was then cooled in an ice bath.

The solution (20 µl) containing ALA or ALA-ester AA/formaldehyde reagent derivative was injected (Agilent 1200 series autoinjector, Agilent technologies, Stockport, UK) onto a Spherisorb® column (250 mm × 4.6 mm, C_{18} ODS2 with 5 µm packing, Waters associates, Harrow, UK). The mobile phase consisted of 1% v/v acetic acid in water (solvent A) and 1% v/v acetic acid in methanol (solvent B). The m-ALA derivative was eluted using an isocratic mobile phase containing 40% A and 60% B. The following gradient elution, employing the same solvents, was performed for the hexyl ester, 0-10 minutes: 50-100% B, 10-10.5 minutes: 100-50% B, 10.5-20 minutes: 50% B.

For both analytes, the flow rate was set at 1.0 ml min^{-1} (Agilent 1200 series binary pump, Agilent technologies, Stockport, UK). Detection was by fluorescence with excitation at 370 nm and emission at 460 nm (Agilent 1200 series fluorescence detector, Agilent technologies, Stockport, UK). The chromatographs obtained were analysed using proprietary Agilent ChemStation software.

Calibration plots were performed for m-ALA and h-ALA on three separate days. Each plot was based on at least five separate calibration standards of different concentrations. On each day of the analysis, three standard solutions were prepared separately for each drug concentration. These calibration plots were used to produce a representative calibration curve for each derivatisation method. Least squares linear regression analysis and correlation analysis were performed on the curve produced, enabling determination of the equation of the line, its coefficient of determination and the residual sum of squares (R*SS*), as recommended by Shabir [26]. This representative calibration curve was used to determine the limits of detection (*LoD*) and quantification (*LoQ)* of the method as described in the International Conference on Harmonisation's Topic Q2(R1) (2005) [27]. Equations 1 and 2 may be used to calculate the *LoD* and LoQ, respectively;

$$LoD = \frac{3.3\sigma}{S} \quad (1)$$

$$LoQ = \frac{10\sigma}{S} \quad (2)$$

where σ is the standard deviation of the response (peak area) of the data used to construct the regression line and S is the slope of that line.

The inter-day variation of the analysis was investigated by separately injecting samples of high and low drug concentrations onto the column on five different days. The intra-day variation of the analysis was investigated by separately injecting samples of high and low drug concentrations onto the column on five different occasions on the same day. The mean concentration determined (± S.D.), the coefficient of variation (C.V.) and the percentage accuracy of the method were reported in all cases.

2.5. Preparation of Ester-Loaded Formulations

A lipid-rich hydrophilic base o/w cream (Unguentum Merck®) was employed as the drug vehicle. The appropriate amount of ALA was added to the3 required amount of cream to give drug loadings of 20% w/w. For ALA esters, the appropriate amount of drug was added to Unguentum Merck® so that the preparations contained equivalent molar loadings to the ALA cream (226 µm cm^{-2}).

2.6. Evaluation of Drug Permeation Across a Model Membrane

The release of ALA and ALA-esters from cream formulations was investigated using the modified Franz cell apparatus (Fig. **2**). The orifice diameter in both donor and

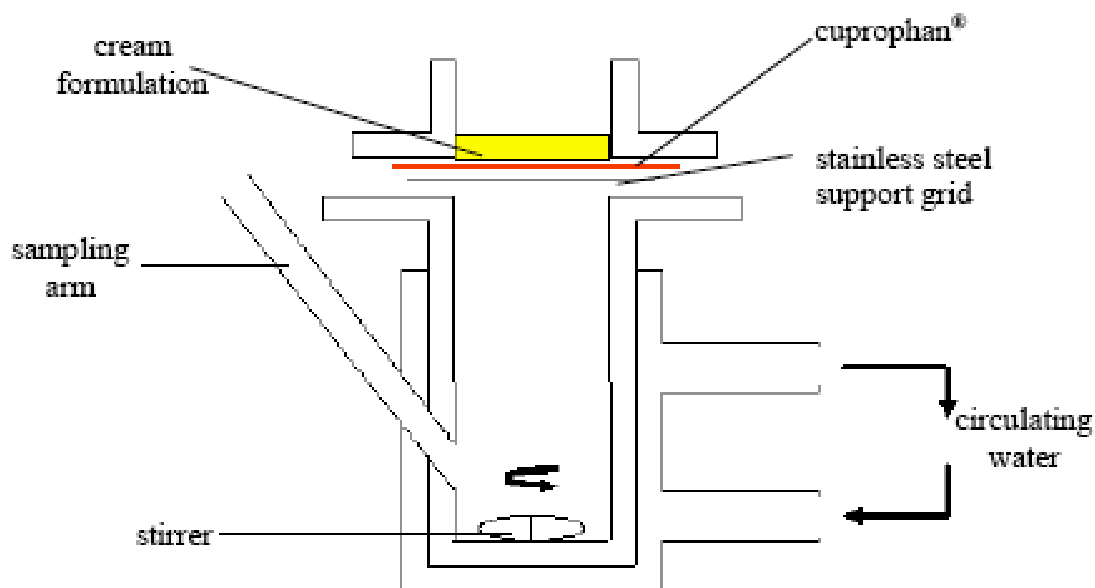

Fig. (2). Diagram showing the modified Franz cell apparatus used to investigate the release of ALA and ALA-esters from cream formulations.

receptor compartments was 15 mm. Receptor compartment volumes, approximately 10 ml, were exactly determined by triplicate measurements of the weights of water they could accommodate. Account was taken for the volumes occupied by magnetic stirring bars. Compartment temperatures were kept constant at 37°C by recirculating water from a thermostatically controlled bath. The receptor phase was 0.1 M borate buffer pH 5 (*Pharmacopoeia Helvetica*). The buffer was degassed prior to use by sonication. Continuous stirring was provided by Teflon-coated stirring bars, rotating at 600 rpm. Stainless steel filter support grids were used to support Cuprophan® membranes. The membranes and support grids were sandwiched between the donor and receptor compartments. High vacuum grease and spring clips were used to hold the entire assembly together. The donor compartments were covered with laboratory film (Parafilm®).

Release from ALA and ALA-ester-loaded creams were investigated in the thickness used clinically. Previously it has been shown that a Dermatologist would typically apply 0.19g of ALA cream per cm^2 of skin area for use in clinical PDT [28]. Therefore, this was the mass of cream that was applied in each experiment. At defined time intervals (5, 10, 15, 30, 60, 120, 180, 240 and 300 minutes), 0.1 ml samples were removed from the receptor compartment, derivatised and analysed by HPLC. This volume was immediately replaced using blank, pre-warmed buffer. Results were reported as the means (± S.D.) of three replicates.

2.7. Statistical Analysis

Where appropriate, data were analysed using a one-way Analysis of Variance (ANOVA) with post-hoc comparisons made using Fisher's PLSD test. Mathematical characterisation of the representative calibration plots was performed using least squares linear regression, following analysis of residuals. Confirmation of the validity of the linear regression was performed using ANOVA and correlation analysis. Comparison of cream formulations, in terms of the amount of drug released, was made using the Mann-Whitney U test. In all cases, $p < 0.05$ denoted significance.

3. RESULTS

The IR spectrum of h-ALA was measured over the region of 650 - 4000 cm^{-1}. The information given in Table **1** is confined to the more easily recognisable bands of the IR spectra (absorptions > 1500 cm^{-1}). This is said to be the functional group region, where the primary functional groups absorb [29]. The IR spectrum of the compound synthesised is in agreement with IR spectrum for h-ALA reported by Chen *et al.* [30].

Table 1. Assignment of Observed IR Peaks of h-ALA.

Wavenumber (cm^{-1})	Assignment
3436	NH_2 stretch (sym)
3416	NH_2 stretch (asym)
2932	CH_2 stretch (asym)
1728	C=O stretch
1588	NH_2 (def)

Abbreviations: sym, symmetric; asym, asymmetrical; def, deformation.

The ^1H NMR spectrum confirmed the structure of h-ALA. A summary of the assignments made to various chemical shifts is presented in Table **2**. Alkyl protons not attached to electronegative groups resonate between 0.2 – 2 ppm. Protons on CH_2 groups attached to the C=0 groups, oxygen atoms or NH_3 groups resonate between 2 and 4 ppm, whilst protons attached to the electronegative nitrogen are positioned at much higher frequencies (further downfield). The prominent shift observed at 3.3 ppm corresponds to the chemical shift of water in DMSO [31].

Table 2. Summary of ^1H NMR Spectra of h-ALA. The Data Refers to the Chemical Shifts, in Parts Per Million (ppm) Associated with Each Bond on the Molecule

Bond	Number of Hydrogens	^1H Shift (ppm)
CH_3	3	0.88
$3CH_2$	6	1.26
CH_2	2	1.54
CH_2	2	2.53
CH_2	2	2.78
NH_3^+-CH_2-C=O	2	3.94
OCH_2	2	4.00
NH_3^+	3	8.30

Fig. (**3a**) illustrates a typical chromatograph following injection of a 20 µl aliquot of a 400 µg ml^{-1} sample of m-ALA standard derivatised with the AA reagent. A large fluorescent signal was observed at approximately 6 minutes, which was followed by a smaller peak at approximately 11 minutes. When a sample of ALA derivative (5 µg ml^{-1}) was injected onto the column, a single peak was observed at approximately 6 minutes (Fig. **3b**). To quantify how much ALA was present in m-ALA samples, a dilution was performed so that the ALA-derivative peak was within the range of the detector. A ratio approach, using the peak area of a known ALA standard, was then used to calculate the amount of ALA present in m-ALA samples. It was found that the ALA peaks observed following injection of derivatised m-ALA samples (50 µg ml^{-1}) represent solutions containing approximately 2.18 µg ml^{-1} ALA. Clearly, the peak areas observed for the ALA-derivative were much greater than those seen for ALA-esters of the same concentration. For example, although the peak corresponding to the ALA derivative in Fig. (**3a**) is large, it represents a relatively low concentration of ALA in the sample.

When the m-ALA mobile phase was used to analyse h-ALA, only a single peak, corresponding to ALA, was observed (Fig. **3c**). However, by employing a gradient elution, whereby the fraction of methanol was increased to 100% over 10 minutes, two distinct peaks were observed (Fig. **4**). By injecting a derivatised ALA standard solution and using the h-ALA HPLC conditions, the peak with the earlier retention time (7.3 minutes) was shown to correspond to the ALA-derivative. The percentage of ALA present following the derivatisation of h-ALA samples was

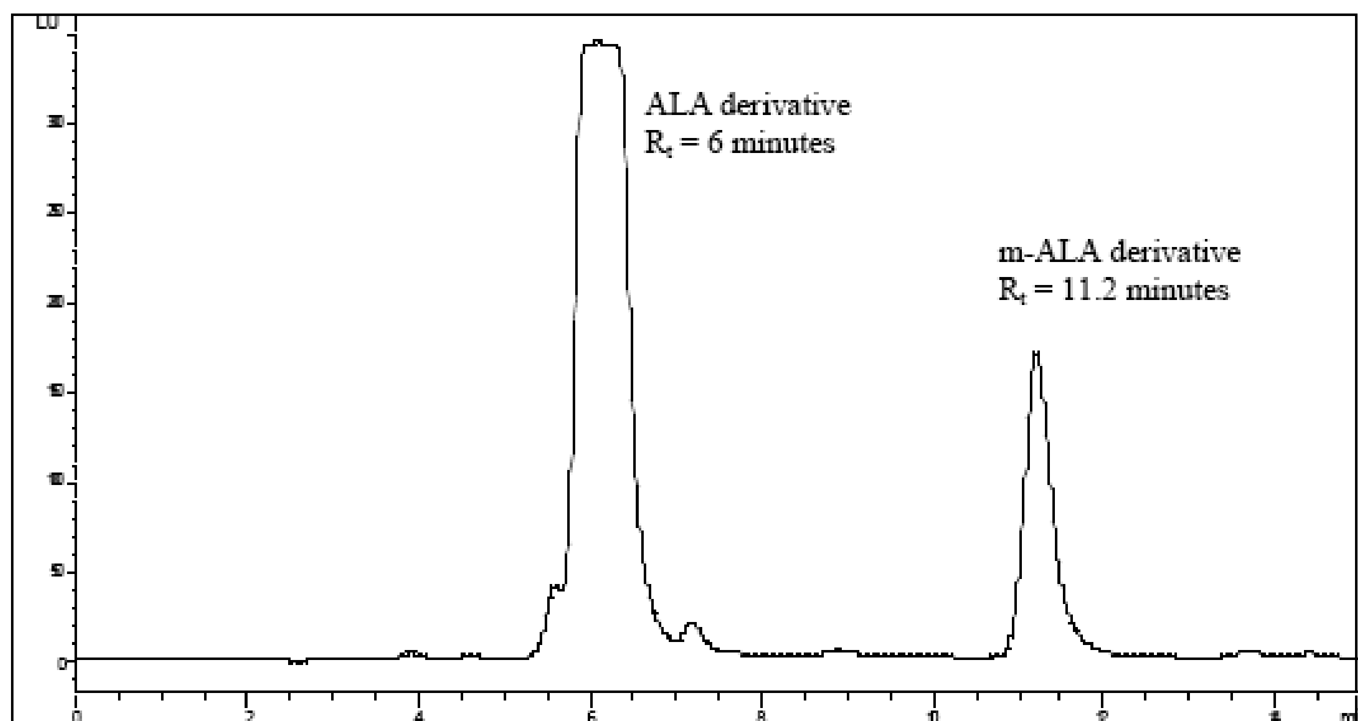

Fig. (3a). Chromatograph obtained by injection of a 20 µl aliquot of a 400 µg ml^{-1} sample of m-ALA derivatised with the AA reagent.

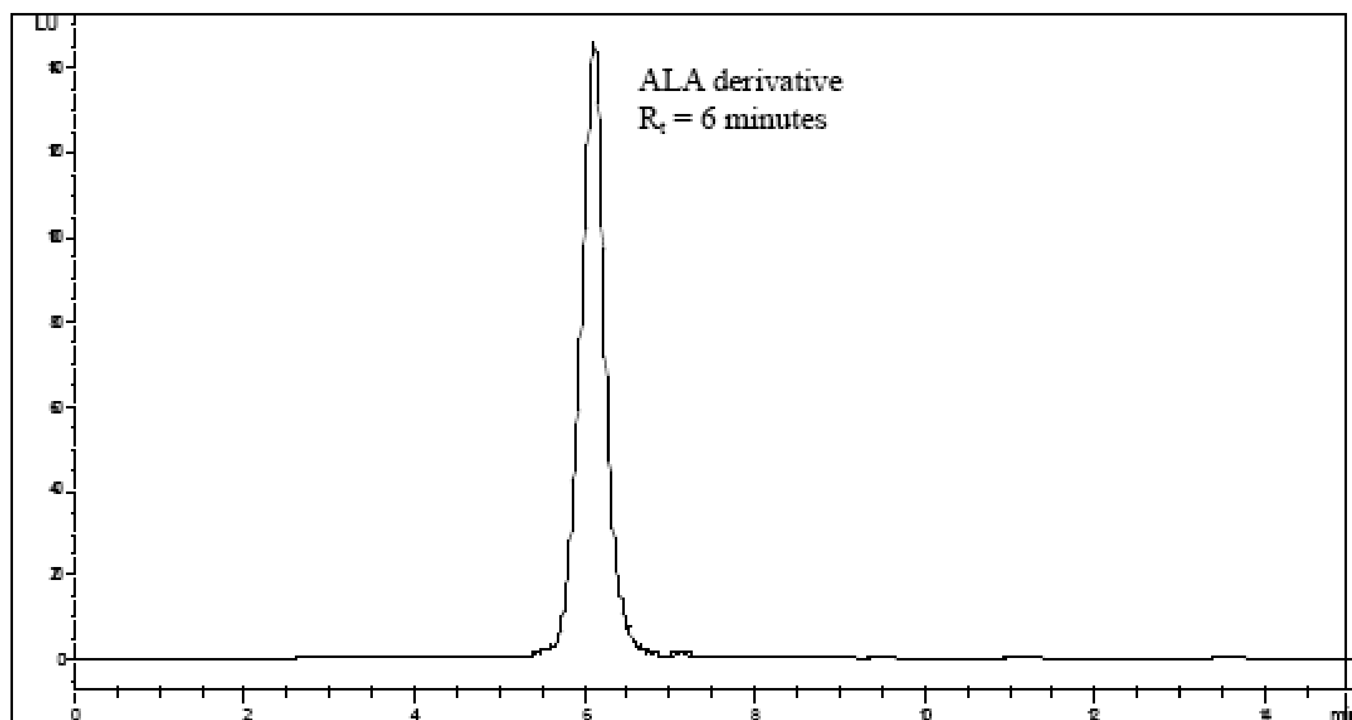

Fig. (3b). Chromatograph obtained by injection of a 20 µl aliquot of a 5 µg ml^{-1} sample of ALA derivatised with the AA reagent.

determined to be 1.39%. Table **3** shows the influence of acetic acid concentration in the mobile phase on the peak areas determined for each ester. Statistical analysis revealed that the peak area ratio of ALA-AA : m-ALA-AA was not significantly altered by increasing the acetic acid concentration from 0.1 – 2.0% v/v (p values > 0.6513). Similarly, there was no significant change in the ratio of ALA-AA : h-ALA-AA upon increasing the acetic acid concentration (p values > 0.2493).

Table **4** shows the slope, y-intercept and coefficient of determination obtained from linear regression analysis followed by correlation analysis of the representative calibration curve for the two derivatives. The residual sum of squares (R*SS*) of the regression lines is also reported. The limits of detection (*LoD*) and quantification (*LoQ*) of the two

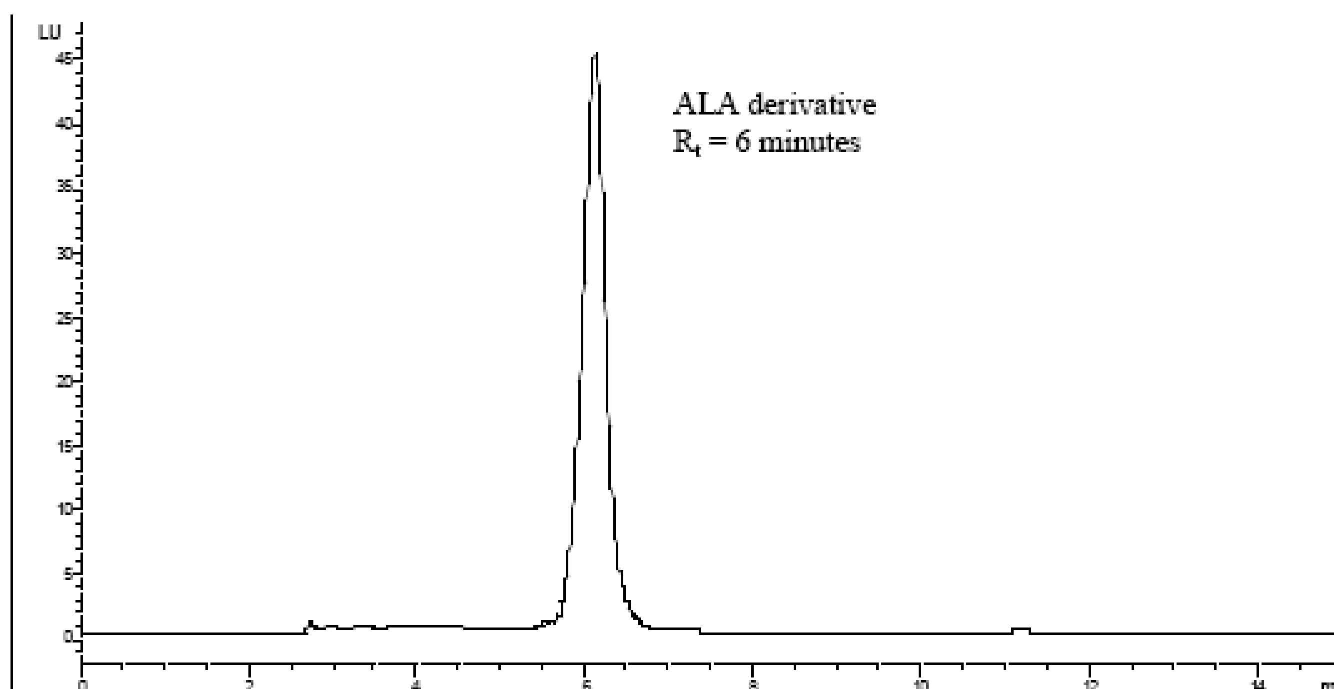

Fig. (3c). Chromatograph obtained by injection of a 20 µl aliquot of a 100 µg ml^{-1} sample of h-ALA derivatised with the AA reagent.

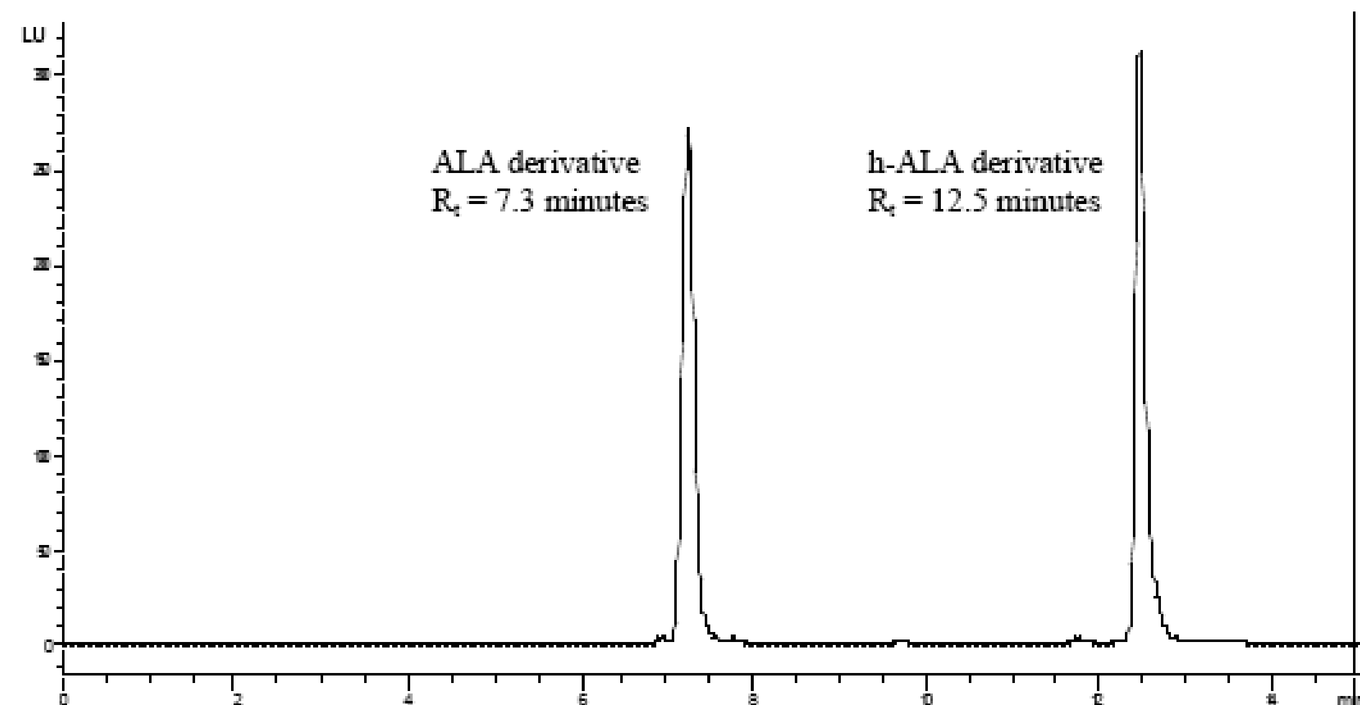

Fig. (4). Chromatograph obtained by injection of a 20 µl aliquot of a 300 µg ml^{-1} sample of h-ALA derivatised with the AA reagent.

esters were determined from the representative calibration curves, as described above. Table **5** details the results obtained from investigations into the inter-day and intra-day variation of the AA derivatisation method.

Fig. (**5**) illustrates the release of ALA, m-ALA and h-ALA from cream preparations across a semi-permeable membrane. Statistical analysis revealed that there was no significant difference in release from the m-ALA and h-ALA creams (p value > 0.0833). However, the ALA cream released significantly more of its drug loading after five hours than the formulations containing m-ALA (p value < 0.05) or h-ALA (p value < 0.05).

4. DISCUSSION

The analysis of aminolevulinic acid (ALA) in biological samples has been an important diagnostic tool for many years. For example, serum ALA levels are elevated in lead poisoning, hereditary tyrosinema and the acute porphyrias. Consequently, a number of groups have reported methods for ALA determination in biological samples [32,33]. More

recently, ALA has been applied topically for the diagnosis and treatment of premalignant and malignant skin lesions. Typically, ALA is formulated as a liquid or semi-solid preparation and applied to the skin for 3-6 hours. Quantitative methods employing HPLC with fluorescence detection have been successfully used to determine the stability of ALA in such topical preparations [21]. In addition, ALA quantification allows the determination of partitioning coefficients, drug solubility, drug release from a given formulation and drug permeation across skin membranes [16].

Table 3. Influence of the Acetic Acid Concentration in the Mobile Phase on the Peak Area Ratio of ALA-AA:ALA-Ester-AA Derivatives

Acetic Acid Concentration in Mobile Phase (% v/v)	Mean Peak Area Ratio ALA-AA:m-ALA-AA	Mean Peak Area Ratio ALA-AA:h-ALA-AA
0.1	4.26 ± 0.20	1.50 ± 0.01
1.0	4.23 ± 0.17	1.48 ± 0.01
2.0	4.20 ± 0.19	1.51 ± 0.04

Table 4. Calibration Curve Properties for the AA Derivatisation of m-ALA and h-ALA as Determined by Linear Regression and Correlation Analyses

m-ALA					
Slope	y-Intercept	r^2	RSS	LoD (µg ml^{-1})	LoQ (µg ml^{-1})
10.10	11.80	0.9997	200290.3	9.72	29.45
h-ALA					
Slope	y-Intercept	r^2	RSS	LoD (µg ml^{-1})	LoQ (µg ml^{-1})
9.27	-3.92	0.9998	39475.65	4.91	14.88

In an attempt to improve topical PDT, intense efforts have been made to develop more lipophilic ALA prodrugs. Currently, two ALA esters have been approved for human use, namely methyl ALA (m-ALA) [34] and hexyl ALA (h-ALA) [35]. Over the past 20 years numerous studies have examined the potential benefits of using ALA esters compared to the parent drug. Many investigations employ cell culture methods, whereby, ester-induced PpIX levels are compared to those induced by the parent molecule. However, relatively few studies have examined the permeability of ALA esters into and across the skin. Such a procedure requires an analytical method which is selective for the prodrug and is sensitive enough to quantify the low levels of drug likely to penetrate the skin barrier.

The m-ALA ester is relatively inexpensive to purchase and is readily available in the UK. The h-ALA ester is more difficult to obtain and considerably more costly. Consequently, h-ALA had to be synthesised. The structure of h-ALA was confirmed by IR and NMR.

In the present study, the Hantzch reaction was used to derivatise both the methyl and hexyl prodrugs of ALA. Although several groups have used this approach to quantify ALA esters, only one study has validated the procedure [23]. However, rather than directly assaying ALA esters, the authors performed an acid hydrolysis step to convert the esters to the parent drug, which were subsequently derivatised and quantified. Clearly, such a procedure is incapable of distinguishing between the ester and the parent drug, which is of principal importance in a number of scenarios. For example, enzymatic hydrolysis of ALA esters to the parent drug is likely to occur in cell culture experiments and during skin penetration studies. In addition, ester hydrolysis may occur in drug delivery systems, before the formulation is even administered. Clearly, the ability to accurately determine both analytes is essential to provide a greater understanding of complex mechanisms, such as

Table 5. Assay Variability for Fluorimetric Determination of m-ALA and h-ALA Following Derivatisation with the AA Reagent (Means ± S.D., n=5).

Selected Concentration (µg ml^{-1})	Mean Concentration Found (µg ml^{-1})	S.D.	C.V. (%)	Accuracy (%)
Inter-day m-ALA				**n = 5**
50	49.99	1.51	3.01	99.99
500	502.96	14.51	2.88	100.59
Intra-day m-ALA				**n = 5**
50	48.60	1.39	2.86	97.19
500	494.55	12.56	2.54	98.90
Inter-day h-ALA				**n = 5**
50	51.87	0.78	1.51	103.75
300	297.83	7.98	2.68	99.28
Intra-day h-ALA				**n = 5**
50	51.55	0.88	1.70	103.11
300	299.88	13.74	4.58	99.96

cellular uptake, permeation pathways across skin and drug stability.

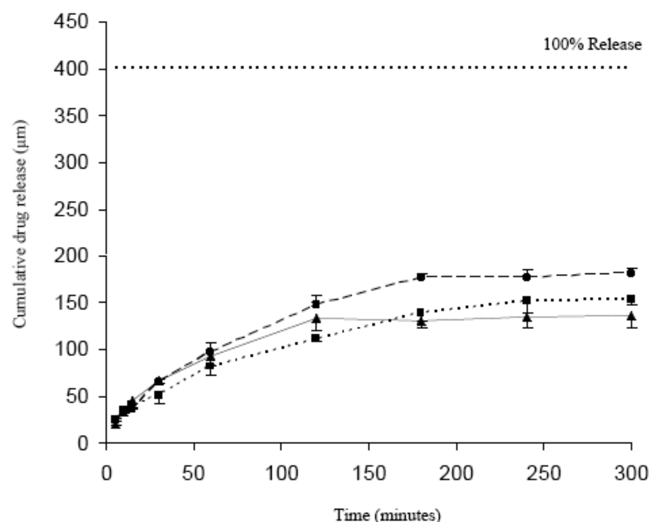

Fig. (5). Release of ALA (-●-), m-ALA (--■--) and h-ALA (-▲-) across Cuprophan® membranes from cream preparations tailored to deliver 226 µm cm^{-2}. Results represent means ± S.D., n=3.

Chromatograms obtained following injection of derivatised samples of m-ALA and h-ALA displayed two peaks (Fig. **3a**, **4**). By running samples containing only the ALA-AA derivative, the early peaks were shown to represent the ALA-AA derivative. Kajiwara *et al.* [36] reported the structure of the methyl ester derivative as 2,6-diacetyl-1,5-dimethyl-7-(2-methoxyethyl)-3-*H*-pyrrolizine (Fig. **6b**). The structure is more lipophilic than the ALA-AA derivative (Fig. **6a**), due to the hydroxyl group being replaced by a methoxy group. Consequently, ALA-esters have a greater retention time than the parent drug. Adequate peak separation and an efficient run-time were achieved for the h-ALA-AA derivative by employing a gradient elution method (Fig. **4**).

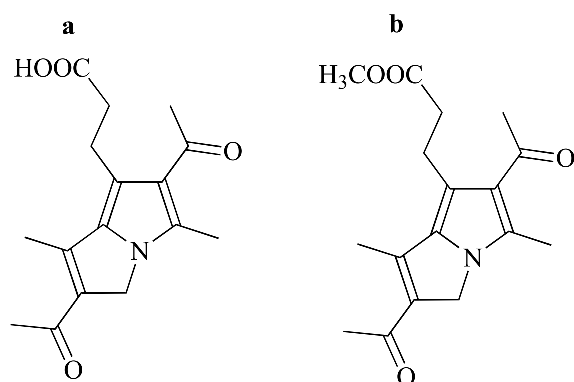

Fig. (6). The fluorescent derivatives 2,6-diacetyl-1,5-dimethyl-7-(2-carboxyethyl)-3-*H*-pyrrolizine (**a**) and 2,6-diacetyl-1,5-dimethyl-7-(2-methoxyethyl)-3-*H*-pyrrolizine (**b**) prepared by reacting ALA and m-ALA, respectively with acetyl acetone and formaldehyde. Reproduced from Kajiwara *et al.* [36].

ALA standards of known concentration were analysed using the m-ALA and h-ALA mobile phases. Using the peak areas obtained for these standards, it was possible to calculate how much ALA was being detected, in the m-ALA and h-ALA samples. It was found that ALA constituted approximately 4.38% and 1.39% of the m-ALA and h-ALA samples, respectively. The m-ALA ester was purchased at ≥ 98% purity, indicating that small amounts of the ester may have hydrolysed prior or during the derivatisation procedure. The peak area ratio of ALA-AA : ALA-ester-AA did not significantly change when the acetic acid concentration was increased in the mobile phase (Table **3**), therefore, indicating that the hydrolysis was not due to the acid component of the mobile phase.

It is important to highlight that, to date, there has been no report in the literature of ALA-AA derivatives being observed during the analysis of ALA esters. Clearly, ALA-derivatives are intensely fluorescent compounds. Therefore, when present in even very low concentrations they should be detectable during analysis. Merclin *et al.* [37] adjusted their mobile phase composition in order to separate ALA-AA and m-ALA-AA peaks. However, the authors did not clarify whether their m-ALA samples had been purposely spiked with ALA, had ALA present as an impurity or if the ALA peaks were a result of ester hydrolysis during the derivatisation procedure. A chromatogram produced by Winkler *et al.* [22] showed multiple peaks, following the derivatisation of butyl-ALA with OPA reagent. It should be noted that the chromatograph was taken during *in vitro* permeation studies through excised *stratum corneum*. The authors suggested that these fluorescent peaks were due, either to amino acids present in the biological tissue reacting with the OPA reagent or components of the derivatisation reagent itself. Interestingly, they did not examine whether any of the peaks could have been a result of the ALA-OPA fluorescent derivative.

From Figs. (**3a**, **4**) it is apparent that the derivatisation procedure allows for much greater sensitivity for the detection of ALA compared to the two esters studied. Turchiello *et al.* [21] demonstrated that the fluorescence intensities of 4 mM solutions of ALA esters were approximately one order of magnitude less than that of a 4 mM solution of ALA. Merclin *et al.* [37] reported linear calibration ranges for ALA and m-ALA detection, following derivatisation with AA reagent. The authors reported that the lower limit of the m-ALA calibration profile was approximately 35 times greater than that seen for ALA. It is possible that the ALA esters do not perform the derivatisation procedure as readily as the ALA molecule. Potentially, the additional alkyl chain may stearically hinder the reaction and prevent all the ester from being derivatised. Alternatively, the ester-AA derivatives may not be as fluorescent as the ALA-AA derivative. Indeed, it is known that small changes in chemical structure can have a significant impact on a molecule's fluorescence properties [38]. For example, adrenaline and nordrenaline, differ in their structures by only a single methyl group, but noradrenaline exhibits fluorescence intensities nearly 20 times higher than those of adrenaline for the same concentrations [29]. Although methyl groups do not have an electron capable of participating in π-bonding, they do have weak electron donor properties which can exert a considerable influence on the fluorescence properties of the molecule [39].

Rosa *et al.* [24] reported a linear correlation between concentration and response down to approximately 0.054 µg

ml^{-1} and 0.076 μg ml^{-1} for m-ALA and h-ALA, respectively. Similarly, Merclin *et al.* [37] reported that the calibration plot for m-ALA was linear to approximately 5.6 μg ml^{-1}. However, in both these studies, no specific details of r^2, LOD or LOQ values were given. In the current study, representative calibration curves were linear in the range of 30 to 500 μg mL^{-1} for m-ALA ($r^2 = 0.9997$) and 30 to 300 μg ml^{-1} for h-ALA ($r^2 = 0.9998$). Limits of detection were calculated as 9.72 μg ml^{-1} for the methyl ester and 4.91 μg ml^{-1} for the hexyl ester. Quantification limits were determined as 29.45 μg ml^{-1} and 14.88 μg ml^{-1} for the m-ALA and h-ALA esters, respectively. Importantly, the derivatisation was also shown to be reproducible in terms of both inter- and intra-day variation.

Fig. (**5**) shows how the validated methods were employed to evaluate the release of ALA-esters from a typical cream formulation across Cuprophan® membrane. Cuprophan® is a dialysis membrane with a molecular cut-off of 10,500 Da and has been previously used as a model for human *stratum corneum* [40]. Owing to the high drug loadings used in PDT, the amounts of drug released after only five minutes could be accurately determined. Interestingly, the ALA cream was shown to release significantly more of its drug loading than the two ester-containing preparations (p values < 0.05). The more lipophilic prodrugs will have enhanced solubility in the internal phase of the o/w cream. In contrast, ALA is likely to be predominantly located in the external aqueous phase. Consequently, ALA esters have additional partitioning and diffusion steps to reach the Cuprophan® membrane. Indeed, these results may explain some of the findings found elsewhere, whereby, ALA-esters have been shown to be much more efficient at inducing PpIX formation in cultured cells [10,25,41]. However, *in vivo* studies have generally not shown a significant benefit of these prodrugs over the parent ALA molecule [11,42,43]. This work indicates that many of the formulations typically used in these studies may not be the most appropriate and efficient for ester delivery. Rather, formulations that maximise the thermodynamic activity and facilitate rapid partitioning from the vehicle should be sought. For example, Donnelly *et al.* [44] prepared pressure sensitive adhesive patches loaded with m-ALA. Inspection of the patches using light microscopy revealed that the drug was present in the crystalline state. Clearly, these systems were saturated with drug and consequently, the thermodyanamic activity of the drug is likely to remain close to one during the duration of the experiment. Indeed, the work showed that these patches could induce high levels of PpIX *in vivo* at relatively low drug loadings (4 mg cm^{-2}).

5. CONCLUSION

In summary, sensitive and reliable assays have been developed for quantification of ALA esters in aqueous solution. For the first time, the potential of ALA fluorescent derivatives to interfere with the routine analysis of ALA esters has been highlighted. Hence, if the HPLC conditions are not optimised, ester release could be greatly over-estimated, when in fact it has been hydrolysed to ALA. Importantly, the two methods have been validated to ICH guidelines and could be used for a wide range of drug delivery applications. To date, many studies have employed simple o/w creams to deliver ALA esters, however, to maximise the delivery of these agents, alternative formulations may offer improved release profiles and consequently, lead to better therapeutic outcomes. This work has shown that by using fully characterised and validated methods, such formulations can be evaluated.

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