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Journal of Chromatography B, xxx (2007) xxx–xxx
JOURNAL OF CHROMATOGRAPHY B
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Liquid chromatography assay for 5-aminolevulinic acid: Application to in vitro assessment of skin penetration via Dermaportation

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Received 18 September 2006; accepted 29 December 2006

Abstract

The purpose of the present study was to develop a reverse-phase high performance liquid chromatographic (HPLC) assay for quantifying 5-aminolevulinic acid (ALA). The assay was applied to study the skin permeation of ALA and the influence of a novel skin penetration enhancement technology. Separation was achieved utilizing a Phenomenex Jupiter C_{18} column following fluorescence derivatization with fluorescamine. The assay was linear ($r^2 > 0.99$) with a minimum limit of quantitation of 400 ng/mL. The inter- and intraday variation was 1.7 and 0.9% at the lower end of the linear range and 1.9 and 1.1% at the upper end, respectively. The HPLC assay and fluorescence derivatization procedure is sensitive, simple, rapid, accurate and reproducible and offers advantages with regard to stability of ALA in comparison to other fluorescence derivatization methods. Results from the preliminary skin permeation study demonstrated substantial skin penetration of ALA only when applied with Dermaportation as a skin penetration enhancement device.
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Keywords: HPLC; Penetration enhancement; Transdermal drug delivery; Electroporation

1. Introduction

ALA is a small, water soluble, prodrug (Fig. 1) that is a naturally occurring precursor in the biosynthetic pathway of porphyrins, especially protoporphyrin IX (Pp IX) and heme [1]. Administration of excessive amounts of exogenous ALA surpasses the negative feedback control that heme exerts over its biosynthetic pathway leading to an accumulation of protoporphyrin IX in the cells due to the limited capacity of ferrochelastase to convert PpIX into heme. Fluorescent and photosensitizing properties of protoporphyrin accumulated after the exogenous administration of ALA, can be used to visualize and destroy malignant cells in photodynamic diagnosis and therapy [2]. Subsequent activation by light at a wavelength matching one of its absorption wavelengths leads to the formation of highly reactive singlet oxygen that causes the destruction of target cells by a complex cascade of chemical, biological and physiological reactions. The accumulation of PpIX is more pronounced in malignant cells as compared to normal cells, thus providing some therapeutic discrimination in cancer chemotherapy.

Photodynamic therapy with topical application of ALA has been used successfully in the treatment of basal cell carcinoma, actinic keratosis, Bowen's disease, vulval intraepithelial neoplasia and vulval Paget's disease. However, ALA penetration to the site of action is slow [3], requiring a 3–4 h wait before administration of light. A number of approaches have been investigated to increase the skin permeation of ALA, for example, use of iontophoresis [4,5], chemical penetration enhancers [6], encapsulation into liposomes [7] and increasing lipophilicity by use of ester derivatives or counter-ions [6]. Of these, iontophoresis has been the most promising, achieving approximately a six-fold enhancement of skin penetration compared to passive administration [5].

Dermaportation is an electrically generated energy technology to enhance skin penetration of drugs which has been developed by the Perth based biotechnology company, OBJ Ltd. The Dermaportation device generates electromagnetic fields in target tissues. Biological and therapeutic effects of electromagnetic fields and inductive effects on biological tissues have

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1570-0232/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2006.12.040

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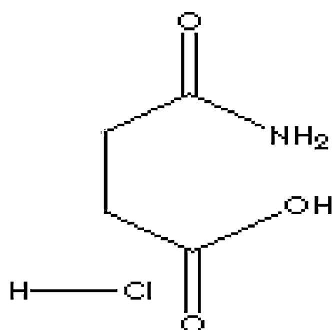

Fig. 1. Chemical structure of ALA hydrochloride.

been widely reported, for example, enhancement of healing of venous ulcers and bone fractures and effects on a range of cellular functions [8,9]. The Dermaportation technology applies these principles to the field of skin penetration enhancement. The system utilizes low energy so that the effect is not sensed by human individuals, a potential advantage over other electrical skin penetration enhancement technologies such as iontophoresis and electroporation [10,11]. It has the potential to increase therapeutic effectiveness and hasten the onset of action of concomitantly applied drugs. It is proposed that Dermaportation energy influences both the molecular movement of drug molecules in the epidermis and the ordered structure of the stratum corneum lipid bilayers, and/or increases follicular transport to provide an enhancement of penetration of topically applied drugs. The precise mechanism of enhancement is an area of continuing investigation. Dermaportation may provide a suitable skin penetration enhancement of ALA to provide a more convenient and effective treatment of skin cancers.

In order to investigate the skin penetration of ALA a suitable analytical assay is required. ALA belongs to the class of alpha amino ketones with a weak chromophoric carbonyl group and consequently is unsuitable for quantification by conventional ultraviolet (UV) absorption spectroscopy. A number of methods have been used for ALA quantification including ion exchange chromatography [12] and capillary electrophoresis [13]. HPLC is one of the most routine tools for pharmaceutical analysis in both industrial and university laboratories. Consequently, analytical methods using chromatography with chemical derivatization are most commonly utilized in the quantification of ALA.

Chemical derivatization of ALA was first described by Mauzerall and Granick, who used acetyl acetone to convert ALA into a cyclic pyrrole [14]. This was then reacted with a modified Ehlich's reagent, which consists of *p*-dimethyl-aminobenzaldehyde (DMAB) dissolved in a mixture of perchloric acid and glacial acetic acid. The fluorescent derivative was determined by colorimetry [14,15]. Oishi et al. modified this method to permit determination of ALA in plasma and urine [16]. Their method was further modified and used for ALA analysis by other researchers [17,18].

Apart from acetyl acetone and formaldehyde, the suitability of derivatizing ALA with a number of other fluorescent probes has been reported. Ho et al. reported the derivatization of ALA, porphobilinogen and porphyrins with *o*-phthaldehyde [19]. A drawback of this method is the instability of the *o*-phthaldehyde ALA-complex resulting in decreasing fluorescence intensity with time. A method involving fluorenylmethyloxycarbonylchloride (FMOC) for ALA derivatization was reported to be more robust than the standard *o*-phthaldehyde method. The FMOC/ALA-complex was stable with no change in its fluorescence chromatography after 1 week storage at room temperature in daylight. The calibration curve of the FMOC/ALA-complex was linear down to <1 ng ALA per sample [20]. These authors also evaluated 2-amino-3-hydroxyl naphthalene to derivatize ALA in biological samples. The method reported appeared more sensitive with a detection limit of 40 pmol ALA compared to a detection limit of about 4 nmol ALA for the colour reaction with Ehlrich's reagent [21]. Costa et al. [22] utilized HPLC with electrochemical detection following derivatization of ALA with *o*-phthaldehyde at room temperature. Validation data for the reported ALA assays is summarized in Table 1.

The aim of this work was to assess the potential enhancement effect of Dermaportation on penetration of ALA through human epidermis using an in vitro skin diffusion model. In order to facilitate this study, a suitable HPLC assay was developed that provides advantages over previously reported HPLC methods with regard to stability of ALA and convenience.

2. Experimental

2.1. Materials and methods

The following chemicals were used as supplied: 5-aminolevulinic hydrochloride salt (purity approximately ≥97%) and fluorescamine (>99% pure) were obtained from Sigma–Aldrich–Fluka Chemie (Australia); methanol HPLC solvent, JT Baker (USA); potassium chloride, analytical reagent, May and Baker Australia Pty Ltd.; sodium chloride, analytical reagent; Asia Pacific Speciality Chemicals Limited (Australia); boric acid, Ajax Chemical Ltd. (Australia); sodium hydroxide, analytical grade, Merck Pty Ltd. (Australia). Phosphate buffered saline solution was prepared according to the United States Pharmacopoeia.

2.2. HPLC instrumentation and conditions

The HPLC system (Agilent 1100) consisted of a quaternary pump (G1311A), autosampler (G1313A), degasser (G1312A) equipped with a fluorescence detector (G1321A). Separation was achieved on a Phenomenex Jupiter C_{18} 300 Å column (5 μm, 150 mm × 4.6 mm) with a guard column wide pore C_{18}. Integration was undertaken using Chemstation software.

The elution was performed at ambient temperature at a flow rate of 1 mL/min and the excitation/emission wavelengths were 395/480nm. The mobile phase used was 30% acetonitrile (0.1% TFA): 70% water (0.1% TFA) which gave a retention time of 7.25 min for fluorescamine derivatives of ALA. All

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Table 1
Assay validation parameters for ALA in published reports and this study

Validation parameters	Fluoremetric detection after acetyl acetone derivatization [15]	Electrochemical detection after OPA derivatization [21]	Fluoremetric detection after derivatization with OPA and 2 mercaptoethanol at 100 °C for 80 min [14]	Capillary electrophoresis [12]	Fluorescamine assay
Linearity R^2 (1.25–50 μg/mL)	0.99	0.99	0.9952	0.9976	0.9993
Precision (CV) at 0.3, 5, 25 100 μg/mL (six injections)	–	2 and 15% for replicate reaction mixtures (n = 5)	–	R.S.D. = 0.75% with a test sample of ALA (six injections)	1.7% at 5 μg/mL 1.4% at 0.3 μg/mL 0.9% at 25 μg/mL 0.41% at 100 μg/mL
Intraday repeatability (CV) at 5 and 25 μg/mL	–	–	1.68% at 3 μg/mL 0.16% at 10 μg/mL	–	0.89 at 5 μg/mL 1.9% at 25 μg/mL
Interday repeatability (CV) at 5 and 25 μg/mL	–	–	1.67% at 3 μg/mL 0.05% at 10 μg/mL	–	1.56% at 5 μg/mL 1.49% at 25 μg/mL
Limit of detection (LOD)	0.003 μg/mL ALA in plasma and urine	0.003 pg/0.02 mL injection in water	0.05 μg/mL	LOD and LOQ was calculated only for the degradation products of ALA	120 ng/mL in buffer
Limit of quantitation (LOQ)	–	–	0.17 μg/mL		400 ng/mL
Accuracy	–	Recovery = 100 ± 1.8%	Accuracy (interday) 96.92% at 3 μg/mL 103.64 at 10 μg/mL Accuracy (intraday) 97.19 at 3 μg/mL 103.50 at 10 μg/mL	–	At 25 μg/mL = 100.6% At 5 μg/mL = 104.3%

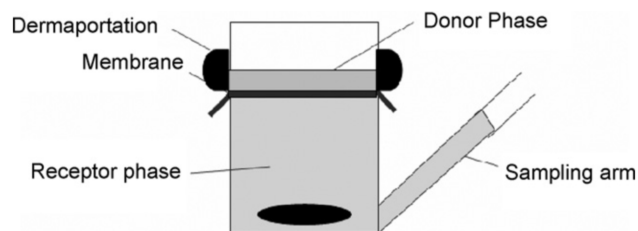

Fig. 2. Franz cell with Dermaportation coil in position.

samples were analysed by HPLC using injection volumes of 10 μL.

2.3. Derivatization of ALA using acetyl acetone and formaldehyde

ALA hydrochloride was derivatized according to the method used by Oishi et al. for the HPLC determination of ALA in the plasma and urine of lead workers [16]. The derivatization mixture was prepared by addition of 3.5 mL acetyl acetone reagent and 450 μL of 10% formaldehyde solution to a 50 μL standard solution of ALA hydrochloride in phosphate buffered saline (PBS) with mixing for 5 s. The mixture was heated at 100 °C for 10 min, cooled in an ice bath and was allowed to stand in the dark at room temperature until analysis.

2.4. Derivatization of ALA using fluorescamine

ALA hydrochloride solution in PBS was reacted with 0.1% fluorescamine solution and 0.1 M borate buffer for 10 min at room temperature (Fig. 2). A 200 μL unknown or reference sample containing drug in PBS was derivatized with 200 μL fluorescamine solution and 600 μL of 0.1 M borate buffer. ALA hydrochloride was assayed after conversion to its fluorescent derivative by reacting with fluorescamine for 10 min at room temperature.

Calibration curves were obtained using 1.25, 2.5, 5, 10, 25 and 50 μg/mL of ALA hydrochloride standard solutions in phosphate buffered saline at pH 7.4 (PBS). Linearity (quoted as R^2) was evaluated by linear regression analysis, which was calculated by the least square regression method. The precision of the assay was determined by injecting four standard concentrations (0.3, 5, 25 and 100 μg/mL ALA hydrochloride) six times on the HPLC. The intraday repeatability was assessed by injecting 5 and 25 μg/mL ALA hydrochloride standards six times at different times in a day. The interday repeatability was determined by injecting 5 and 25 μg/mL ALA hydrochloride standards six times on 3 different days. The intra- and interday repeatabilities were quoted as the coefficient of variance. The minimum detectable and quantifiable limits (LOD and LOQ) were measured by diluting ALA hydrochloride in PBS to give a concentration range from 1.25 to 50 μg/mL and then injected on the HPLC. ALA hydrochloride was also spiked into PBS which had been in contact with human epidermis for 4 h and this was assayed to ensure that there was no interference with substances leaching from the skin. Accuracy of the analyti-

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cal method was determined for in vitro skin diffusion studies as follows. Two separate samples of PBS which had been in contact with human epidermis for 4 h were spiked with ALA hydrochloride standards to give final concentrations of 5 and 25 μg/mL ALA hydrochloride, respectively. Each spiked sample and standard was injected six times on the HPLC and the percentage difference between each standard (5 and 25 μg/mL ALA hydrochloride) and the corresponding spiked sample was calculated.

Stability of the fluorescent derivative obtained by the reaction of ALA hydrochloride with fluorescamine was verified at room temperature and at 4 °C over 4 days. The stability of the derivative was also assessed for the auto injection cycle performed by the HPLC.

2.5. Skin penetration of ALA hydrochloride

In vitro permeation studies across human epidermis were performed in Pyrex glass Franz-type diffusion cells (enabling permeation across skin sections of cross sectional area 1.18 cm^2; receptor volume approximately 3.5 mL). Ethical approval for using human skin was obtained from the Health Research Ethics Committee of Curtin University. Epidermal membranes were obtained by the heat separation method [23,24]. Briefly, the subcutaneous tissue was removed by dissection from skin samples (abdominal region following abdominoplasty surgery at Perth hospitals). The resultant full thickness skin was immersed in water at 60 °C for 2 min. The epidermal membrane was teased off the dermis; placed onto aluminium foil, air dried then stored at −20 °C until required. Epidermal membrane was placed between the donor and receptor compartments and allowed to equilibrate for 30 min with the receptor solution (PBS) which was stirred continuously with a magnetic flea. The receptor compartment of the cell was immersed in a water bath at 37 ± 0.5 °C. PBS (1 mL) was placed in the donor compartment, allowed to equilibrate for 30 min and the membrane integrity determined using electrical resistance (>20 kΩ) using a digital multimeter. The PBS solutions were then removed from the donor and receptor compartments and the receptor refilled with fresh prewarmed PBS. The donor solution, which consisted of 1 mL of 2% ALA hydrochloride in PBS, was then added. Samples of the receptor phase were withdrawn and replaced with fresh pre-warmed (37 °C) PBS over a 4 h period. The ALA hydrochloride content in the samples was determined using HPLC. Dermaportation coils were added to the exterior of the donor compartment (Fig. 3) and energy applied for 4 h (Dermaportation cells), whilst other cells had no external Dermaportation energy applied (passive cells). Experiments were repeated four times for both Dermaportation and passive.

The cumulative amount of drug permeated through the epidermis versus time was plotted. The flux of ALA hydrochloride through the human epidermis for both passive and Dermaportation cells was determined from the slope of the plot of cumulative amount versus time and expressed as μg/cm^2 h. Permeability coefficients were calculated for ALA hydrochloride for both passive and Dermaportation.

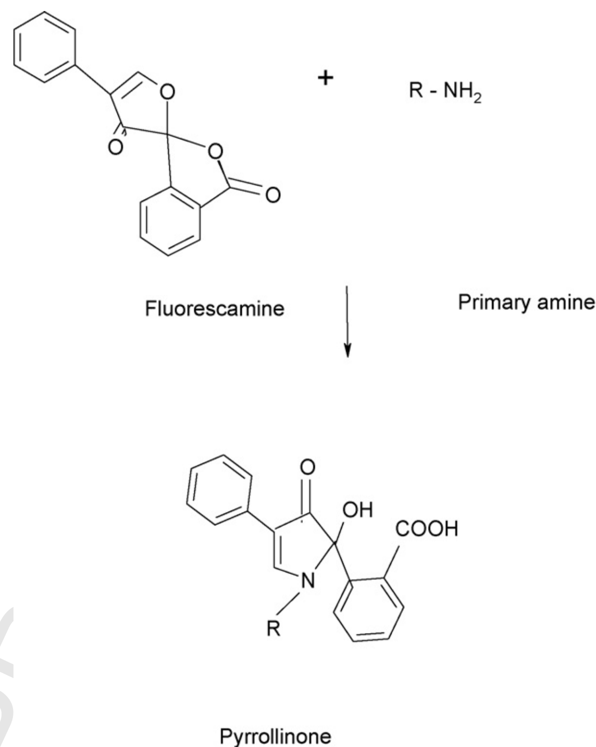

Fig. 3. Reaction scheme for fluorescamine derivatization [27].

3. Results and discussion

3.1. Analytical method development for HPLC analysis of ALA hydrochloride

Initially, we followed the derivatization technique outlined by Oishi et al. [16] utilizing acetyl acetone and formaldehyde to form the fluorescent ALA derivative which was recently identified to be 2,6 diacetyl-1,5-dimethyl-7-(2-carboxyethyl)-3H-pyrrolizine [25]. The excitation and emission wavelengths were confirmed in our laboratory using a Cary Eclipse Spectrofluorometer and were found to be 395 and 480 nm, respectively. In preparing a calibration curve across a concentration range of ALA hydrochloride standards with acetyl acetone and formaldehyde, it was found that the relationship was not linear (correlation coefficient was poor with a R^2-value of 0.9475; Fig. 3a). The sensitivity was lost at a concentration of 0.3 μg/mL ALA hydrochloride standard solution compared to the previously reported 0.03 μg/mL (Table 1, [16]).

It is generally acknowledged that derivatization reactions must be versatile, rapid and preferably should not require forcing conditions. Heating at 100 °C necessary for the derivatization reaction with acetyl acetone and formaldehyde can lead to the degradation of ALA since ALA is not stable at such high temperatures [26]. Hence these experiments were suspended and instead the use of a reagent which can be reacted at a lower temperature, and which will improve the linearity and sensitivity of detection was pursued.

A suitable fluorescent probe that would attach to the amine group on ALA hydrochloride was sought. There are numerous fluorescent probes used for the determination of primary

aliphatic amines [27]. Fluorescamine [4-phenylspiro (furan-2[3H],1-phthlan)-3,3-dione] is one such probe that reacts almost instantaneously with primary amines, at room temperature, to yield blue-green fluorescent pyrrolinones (Fig. 2). It directly reacts with amines, whether aliphatic or aromatic, to form fluorophores of high intrinsic fluorescence and the excess reagent is rapidly converted by water to non-fluorescent products [28]. It is used in the pharmaceutical analyses of a wide variety of compounds containing amino groups [27].

The completion of the reaction and the time required to achieve maximum fluorescence was determined by reacting ALA hydrochloride with the derivatizing agent and allowing it to react for 10 min. Samples were taken at 0, 5, 15, 30, 45, 60, 90 and 120 min and analysed by HPLC connected to a Cary Eclipse Spectrofluorometer. This allowed the confirmation of the excitation and emission wavelengths to be 395 and 480 nm, respectively. Experiments were conducted to assess the stability of the fluorescent derivative in order to confirm that degradation did not occur during analysis. It was found that 25 μg/mL ALA hydrochloride standard solution was stable for 4 days at 4 °C, while at room temperature some degradation was observed. Consequently, all experimental samples were refrigerated at 4 °C and were analysed within 4 days. The sample stability allowed auto injection cycles to be performed. The samples derivatized by fluorescamine remained stable for 4 days after derivatization, while samples derivatized by the acetyl acetone method had to be used within 24 h.

3.2. Chromatography and resolution

ALA hydrochloride fluorescent derivatives were analysed by fluorescence detection using HPLC. ALA fluorescamine derivatives eluted without any interfering peaks with a retention time of 7.25 min (Fig. 4). The linearity obtained by this new method was

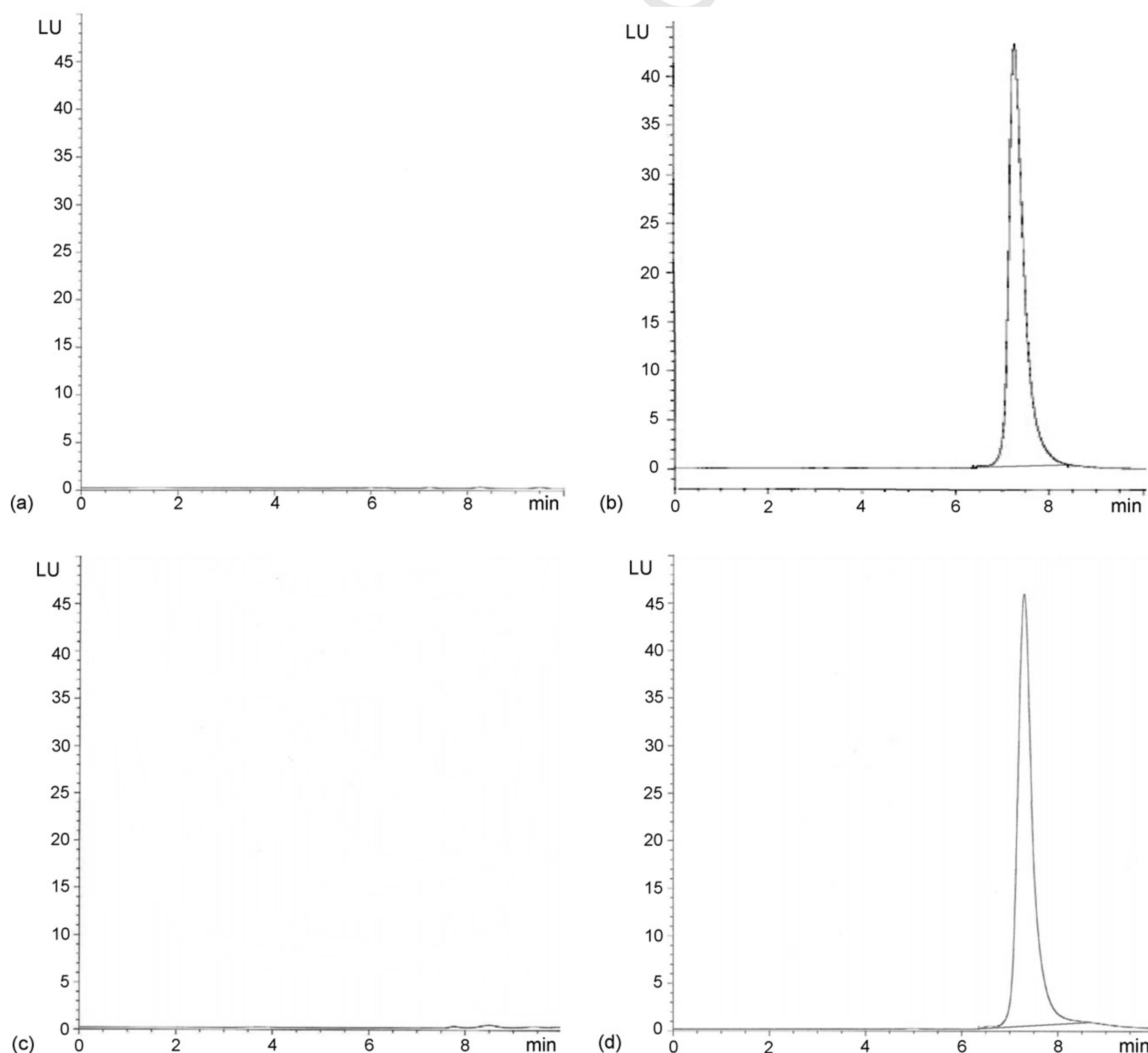

Fig. 4. Sample chromatogram analysed from receptor solutions of Franz cells after 4 h: (a) passive and (b) Dermaportation; (c) blank receptor solution in contact with human epidermis for 4 h; (d) ALA hydrochloride standard solution in water.

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0.9993 over the range of the calibration curve (1.25–50 μg/mL) for ALA fluorescamine derivatives (Fig. 3b).

3.3. Assay precision

A calibration curve was obtained by plotting the peak area versus concentration of standards injected. The CV for precision, determined from the relative standard deviation ($n=6$), was 1.7% for 5 μg/mL and 1.4% for 0.3 μg/mL ALA hydrochloride standard solutions in PBS. The intraday variation was 1.9 and 0.89% and the interday variation was 1.49 and 1.56% at 25 and 5 μg/mL ALA hydrochloride standard solutions in PBS, respectively. These are within the acceptable criteria for intra- and interday repeatability of R.S.D. < 5%.

3.4. Minimum LOD and LOQ for ALA fluorescamine derivative

The minimum LOD, calculated as greater than three times the baseline noise level in the assay, was 120 ng/mL. The lower LOQ, calculated as greater than 10 times the baseline noise level in the assay, was 400 ng/mL. The suitability of the assay with PBS which had been in contact with skin for 4 h to simulate a diffusion cell study, was checked to ensure no interference was present (Fig. 4c).

3.5. Accuracy

This method permitted the detection of 5 μg/mL of ALA hydrochloride sample with 104.3% accuracy and 25 μg/mL of ALA hydrochloride sample with 100.6% accuracy.

3.6. Dermaportation of ALA hydrochloride through human epidermis

Epidermal penetration of ALA hydrochloride over a 4 h period was determined using an in vitro experimental protocol. Fig. 4 is a typical HPLC chromatogram of ALA hydrochloride analysed 4 h after (a) passive and (b) Dermaportation showing that Dermaportation does not alter the ALA structure. A comparison of the cumulative amount of ALA hydrochloride penetrating the epidermis to the receptor solution versus time was plotted for Dermaportation and passive application (Fig. 5). The results indicated an increase in the flux of ALA hydrochloride over 4 h in cells where Dermaportation was applied, as compared to cells where the solution was applied without Dermaportation. Over the initial 2 h period of application, 8 mg (that is around 40% of the applied dose of ALA hydrochloride) penetrated the epidermis to the receptor solution. Over the 4 h application period 9 mg ALA (that is around 45% of the applied dose of ALA) penetrated the epidermis to the receptor. Due to this high rate of epidermal permeation, donor depletion of ALA hydrochloride in the Dermaportation applied cells occurred during the time course of the experiment. Consequently, an estimated flux value was calculated as the slope of the cumulative amount permeated in the first 2 h as linearity was not maintained beyond this time (Fig. 5). As this measure of flux is based on a small number of data points it is deemed as an estimate only. Over this period the estimated flux of ALA hydrochloride was 50.79 $\mu g/cm^2$ h as compared to passive flux of 0.12 $\mu g/cm^2$ h. The estimated permeability coefficient for ALA hydrochloride was 6.16×10^{-6} cm/h with passive diffusion and 2.5×10^{-3} cm/h with Dermaportation. Thus, Dermaportation substantially increased the transdermal permeation of ALA hydrochloride in this preliminary experiment and performed well in comparison to other penetration enhancement approaches. For example, Merclin et al. demonstrated that the amount of ALA delivered after 15 h of iontophoresis was increased six-fold over passive experiments that showed no detectable amounts of ALA [5]. Smaller increases in ALA skin penetration were achieved by other researchers using lipophilic counter-ions and chemical penetration enhancers. For example, De Rosa et al. showed that ALA induced PpIX accumulation and penetration across epidermis was increased 2.5-fold by dimethyl sulfoxide and ethylenediamine-tetraacetic acid disodium salt [6]. Less than two-fold increase in skin penetration of ALA was achieved with lipophilic counter ions or incorporation into liposomes [6,7].

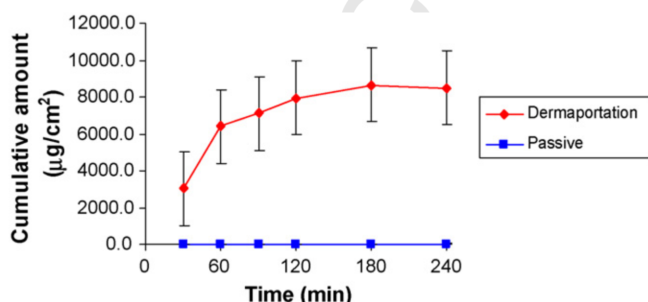

Fig. 5. Cumulative penetration (μg in receptor: mean ± S.E.M.; $n=4$) of ALA.

In conclusion, this assay provides an efficient means of quantifying ALA hydrochloride by fluorescence derivatization. Although a derivatization step is required this is simple to perform and does not require forcing conditions that can result in instability of the active drug. Comparison with previously reported assay methods is favourable although many of these assays have not been fully validated (Table 1). The applicability of the assay to quantification of ALA hydrochloride following skin penetration has been demonstrated.

Acknowledgements

The authors wish to acknowledge the financial support of OBJ Ltd. The work could not be conducted without the generous donation of skin tissue from hospitals in the Perth area. Technical support from Dr. Yan Chen and Mr. Mike Boddy is gratefully acknowledged.

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