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Short communication

Quantitative determination of 5-aminolaevulinic acid and its esters in cell lysates by HPLC-fluorescence

Francesca Giuntini[a], Ludovic Bourré[b], Alexander J. MacRobert[b], Michael Wilson[c], Ian M. Eggleston[a,*]

[a] Wolfson Laboratory of Medicinal Chemistry, Department of Pharmacy and Pharmacology, University of Bath, Bath, UK
[b] National Medical Laser Centre, Division of Surgical and Interventional Sciences, University College Medical School, University College London, London, UK
[c] Division of Microbial Diseases, Eastman Dental Institute, University College London, London, UK

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ABSTRACT

The development of a reliable sensitive method for the HPLC determination of 5-aminolaevulinic acid (ALA) and ALA esters in cell lysates is described. The method relies on the quantification of a fluorescent derivative of ALA following its derivatisation with acetylacetone and formaldehyde. Following this procedure it is possible to quantify ALA in cell lysates with no need for pre-purification of the sample. The method has been validated in two ranges of concentration (0.6–65 μM, 0.1–10 μg/mL, and 30–600 μM, 5–100 μg/mL), and has also been extended and validated for the determination of ALA released from ALA prodrugs after acidic hydrolysis.

1. Introduction

Photodynamic therapy (PDT) is a binary therapeutic modality, which is currently under investigation for the treatment of several kinds of malignancies [1,2]. It relies on the interaction of two individually harmless components: a photosensitiser and an external source of radiation. The interaction of a photosensitiser with light of the appropriate wavelength and molecular oxygen results in the generation of cytotoxic species, namely singlet oxygen and/or radicals, and localised destruction of tumours or infected tissue, relative to normal adjacent tissue.

In ALA photodynamic therapy (ALA-PDT), exogenous administration of ALA is employed to generate elevated intracellular levels of a natural photosensitiser, protoporphyrin IX (PpIX), via metabolism through the haem biosynthetic pathway [3,4]. ALA-PDT has proved to be a successful and safe tool for the treatment of a number of neoplastic lesions or pre-malignant disorders, including basal cell carcinoma, actinic keratosis, Bowen's disease, as well as non-neoplastic diseases [5–7].

The detection and quantification of ALA in cells and biological fluids is of considerable significance, not only in PDT, but also in many other fields. In clinical chemistry, for example, an increase in the concentration of ALA in serum and urine is associated with disorders such as acute porphyrias, hereditary tyrosinemia, and lead poisoning [8–10]. Similarly, in green plants, the biosynthesis of ALA may be affected by environmental conditions [11], and elevated levels of ALA here represent an important biomarker for increased tolerance of the plant to stress conditions [12,13].

Despite this, and the great interest in improving ALA-PDT, there is a lack of simple and reliable procedures for the detection and quantification of ALA in solution and/or in biological samples. This is particularly relevant to studies with esters of ALA and other prodrugs, which have been developed to overcome difficulties that are associated with the hydrophilic character of ALA that severely impair its ability to cross cell membranes, and thus lower its bioavailability, especially in topical applications [14–16]. An increase in intracellular PpIX concentration after administration of an ALA prodrug is often used as the sole indicator of accumulation of exogenous ALA. While such an approach gives a useful estimate of the efficiency of ALA delivery, the level of PpIX production is also subject to other factors, such as the efficiency of ALA release from a given prodrug by cytoplasmic esterases or peptidases. An accurate absolute measure of intracellular ALA is therefore essential to properly judge accumulation.

ALA is a small aliphatic molecule whose only weak chromophore is not useful for conventional UV detection. Other methods based on gas–liquid chromatography with electron-capture detection [17] or capillary electrophoresis coupled to UV detection [18] have been proposed, but the instruments they rely on are not readily available in most laboratories. Thus the most common methods to quantify ALA involve its detection after chemical derivatisation. The most popular method for the quantification of ALA is the Ehrlich method,

* Corresponding author. Tel.: +44 1225383101; fax: +44 1225386114.
E-mail address: ie203@bath.ac.uk (I.M. Eggleston).

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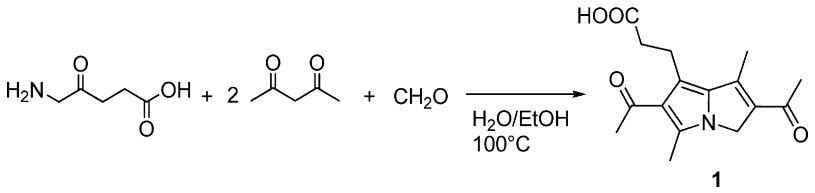

Scheme 1. Derivatisation of ALA.

originally described by Mauzerall and Granick [19]. The procedure relies on the condensation of ALA with acetylacetone, and on the subsequent reaction of the pyrrole obtained with an aromatic aldehyde (generally 4-dimethylaminobenzaldehyde). The derivative thus obtained can be quantified by colorimetry (λ_{max} = 555 nm). One major disadvantage of the Ehrlich method is the lack of stability of the dipyrromethane derivative, which dictates that the analysis be run immediately after the derivatisation [20]. Another important drawback is that several substances present in biological media are likely to interfere with the derivatisation reaction [21]. As a consequence of this and the instability of the Ehrlich derivative, ALA must be separated from the matrix prior to the reaction [19,22]. Several other detection methods involving the formation of fluorescent derivatives of ALA have been developed [23–25], which should be intrinsically more sensitive than UV-based approaches.

Although such derivatives are usually stable enough to be isolated by HPLC, which allows for better specificity even in the presence of interfering substances, they are often generated by the reaction of a given fluorescent label with the amino group of ALA, hence their applicability in biological media is still severely hampered by the presence of a variety of substances (e.g. amino acids) which are reactive towards the derivatisation reagent.

In addition, they tend to require harsh reaction conditions or cumbersome procedures to remove the unreacted derivatisation reagent [24]. A few methods have been reported which are specific for α-aminoketones and lead to the formation of fluorescent derivatives [26–29], two of which in particular have been the subject of optimization and validation studies. One of these methods, originally presented by Meisch [26,27], relies on the reaction of ALA with *o*-phthalaldehyde in the presence of mercaptoethanol to yield a stable fluorescent isoindolic derivative. The other method was first reported by Tomokuni [29] and later optimised by Oishi [28]: it relies on the reaction of ALA with two molecules of acetylacetone and one molecule of formaldehyde, to give 2,6-diacetyl-1,5-dimethyl-7-(2-carboxyethyl)-3-*H*-pyrrolizine **1**, a stable fluorescent derivative that can be isolated by HPLC (Scheme 1).

As part of our ongoing interest in the development of ALA prodrugs [30–32], we wished to establish a method to detect and quantify ALA in cell lysates and other biological media over a broad range of concentrations. For our purposes, we needed the method to be sensitive enough to detect potentially small amounts of ALA in the target cells, specific enough to be performed directly in the biological samples without pre-purification, and efficient when used in conjunction with an *in vitro* procedure to release ALA from its prodrugs. We therefore undertook a study to assess the applicability of the Oishi method to the determination of ALA and its esters directly from cell lysates and to adapt it to our requirements.

2. Materials and methods

2.1. Chemicals

Aminolaevulinic acid hydrochloride and hexyl-5-aminolaevulinate hydrochloride were purchased from Organix (Colchester, UK). Methyl and ethyl-5-aminolaevulinate hydrochloride were obtained by acid-catalysed esterification of ALA, by analogy with published procedures [16,33] Acetylacetone and formaldehyde (37%, v/v in water) were purchased from Sigma–Aldrich (Gillingham, UK). Methanol HPLC grade was purchased from Fisher Scientific (Loughborough, UK). Glacial acetic acid was purchased from Fisher Scientific. HPLC grade water was obtained in house, using a Milli-Q Plus PF system (Millipore, UK).

2.2. Cell culture

The spontaneously transformed murine keratinocyte cell line, PAM212 (obtained from Prof. R. Groves, Imperial College, London) was cultured in RPMI-1640 medium (Gibco BRL, Life Technologies Ltd., Paisley, UK) containing L-glutamine (2 mM) and phenol red (PR), supplemented with 10% foetal calf serum (FCS, Sigma–Aldrich Ltd.,) and penicillin and streptomycin (500 units/mL and 0.5 mg/mL, Gibco BRL). The cells were routinely grown as monolayers in 75 cm^2 culture flasks (TPP, Helena Bioscience, Gateshead, UK), at 37 °C, 5% CO_2, atmosphere until confluent.

2.3. Preparation of lysates

PAM212 cells were seeded into 100 mm Petri-dishes at a density of 5×10^5 cells/mL for 48 h. After removing the culture medium and washing with phosphate-buffered saline (PBS), CelLytic™ (Sigma–Aldrich Ltd.) (1 mL) was added and incubated for 15 min at room temperature, then the cells were mechanically scraped. Cell lysates were centrifuged at 2279 rpm for 10 min to remove the cell debris, and the supernatant was collected. The lysates were stored at −20 °C and thawed prior to use.

2.4. Solutions

Stock solutions of ALA and ALA esters in deionised water were prepared daily from powders stored at 4 °C. Working solutions were obtained after dilution of the stock solutions using class A glassware. Calibration samples were prepared by spiking blank cell lysate with the appropriate working solution. Acetylacetone reagent was prepared by mixing water, absolute ethanol, and acetylacetone in a 55/30/15 volumetric ratio. 10% formaldehyde was obtained by dilution of the commercially available 37% (v/v) aqueous solution in water. The solutions were stored at 4 °C.

2.5. Instruments

The derivatisation reactions were performed in a Greenhouse Plus® Parallel Synthesiser (Radley, UK) equipped with a 24-position reaction block. HPLC analyses were performed on a Dionex Ultimate 3000 system (Dionex, UK). The system consisted of a LPG-3400 pump fitted with an internal vacuum degasser, a WPS-300SL semi-preparative autosampler equipped with a 130 μL loop, a TCC-3000 column compartment, a VWD-3400 variable wavelength detector, and a RF-2000 fluorescence detector. The separations were performed on a Gemini 5μ C18 110A column,

150 mm × 4.6 mm (Phenomenex, UK), equipped with a Security-Guard C18 (ODS) 4 mm × 3.0 mm ID guard column (Phenomenex, UK), at 35 ± 0.1 °C. Linear regression analysis and one-way analysis of variance (ANOVA) were performed using the software package Origin® 8 (OriginLab Corporation).

2.6. Methods

2.6.1. Sample preparation

For the determination of ALA, the calibration samples were prepared by spiking 90 μL of cell lysate with 10 μL of the appropriate working solution.

For the determination of ALA esters, 370 μL of cell lysate was spiked with 30 μL of the appropriate working solution. 200 μL of such a mixture was diluted to 2 mL with 10^{-3} M aqueous HCl in a 16 mm ∅ Greenhouse reaction tube. The tubes were transferred into the Greenhouse reactor preheated at 100 °C, refluxed for 3 h under stirring, then cooled in an ice-bath.

2.6.2. Derivatisation procedure

In a Greenhouse reaction tube equipped with a magnetic stirrer, 50 μL of calibration sample was added to 3500 μL of acetylacetone reagent and 450 μL of 10% formaldehyde solution. The tubes were placed in the Greenhouse reactor preheated at 100 °C, and stirred for 10 min. The reactor chamber was wrapped with foil in order to protect the tubes with the reaction mixture from light. The samples were then cooled in an ice-bath in the dark for 2 h, transferred into HPLC vials and kept in the autosampler at room temperature until the analysis was performed.

2.6.3. HPLC-fluorescence method

The mobile phase consisted of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in methanol (solvent B). The composition of the mobile phase was as follows: 5.0–0.0 min at 60% solvent B, 0.0–6.0 min at 60% solvent B, 6.0–6.1 min 60–95% solvent B, 6.1–12.0 min at 95% solvent B, 12.0–12.1 min 95–60% solvent B. The flow rate was 0.7 mL/min. $\lambda_{exc.}$ = 370 nm and $\lambda_{em.}$ = 460 nm were used for the detection. For the low concentration range, 20 μL of sample were injected and the fluorescence detector was set on high sensitivity. For the high concentration range, 2 μL were injected and the detector was set on medium sensitivity. The peak corresponding to **1** eluted at 4.72 min. The total time required for the analysis was 12.1 min.

2.6.4. Method validation

The methods developed were validated for linearity, accuracy and precision, limits of detection and quantification. For the determination of ALA the two ranges of concentrations investigated were 0–5 μg/mL (0–30 μM) and 5–100 μg/mL (30–603 μM). For the determination of ALA esters after hydrolysis, the method was validated over the range 30–603 μM. For every curve, nine calibration points (in duplicate) were generated on 6 consecutive days, and analysed by HPLC (in duplicate) as reported above. The detector response (peak area) was plotted against the concentration of the analyte. Linear regression analysis was applied to calculate the slope, the intercept and the linear correlation (R^2). The intra-day precision was evaluated by analysing three different samples within the concentration range, for six times in the same day. Inter-day precision was determined at every level of calibration, over three days. The limit of detection (LOD) and the limit of quantification (LOQ) were determined mathematically from the standard curve equations by multiplying the standard deviation (SD) of the intercepts by 3.3 and 10, respectively, and dividing by the slope [34].

The recovery of ALA was determined by analysing nine independent samples over the linear range, obtained by spiking both cell lysate and water. For the determination of ALA after hydrolysis of ALA esters, the recovery from refluxed cell lysate samples was evaluated against non-refluxed samples both in water and in cell lysates.

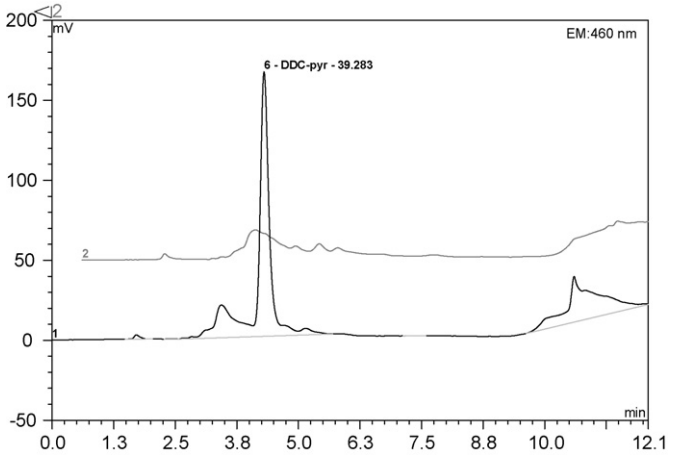

Fig. 1. Chromatogram obtained upon injecting 20 μL of ALA 10 μM in cell lysate (Line 1), of unspiked matrix (Line 2) after derivatisation.

3. Results and discussion

3.1. Derivatisation procedure and HPLC-fluorescence analysis

The derivatisation and the HPLC analysis were performed according to the indications reported [28], with some important modifications. Firstly, the composition of the acetylacetone reagent originally reported in Oishi's work (acetylacetone/ethanol/water = 15/10/75 by volume) yielded, in our hands, an emulsion, which eventually becomes a homogeneous solution upon standing overnight. Increasing the percentage of ethanol in the mixture (acetylacetone/ethanol/water = 15/30/55) afforded a homogeneous solution that could be used immediately for the derivatisation reaction without affecting its performance. The use of an inexpensive multiposition parallel synthesis unit (Greenhouse®) equipped with magnetic stirring bars and a reflux condenser allowed a fast and reliable equalisation of the temperature in every reaction tube and excluded fluctuations in the volume of the reaction mixture caused by evaporation of the solvents. The use of methanol/water (0.1% acetic acid) 60/40 (v/v) as the mobile phase, at a flow rate of 0.7 mL/min affords high values of peak purity and a good peak shape throughout the range of concentrations for the peak of the fluorescent derivative of ALA (t_r = 4.72). 20 μL injection volume and high sensitivity mode of detection were suitable for the detection and quantification in the range of concentration 0.6–65 μM. 2 μL injection and medium sensitivity detection were used for the higher range of concentration to avoid saturation of the signal. Fig. 1 shows the typical chromatograms obtained from unspiked and spiked (10 μM ALA) samples of cell lysates after derivatisation. This demonstrates that besides the peak of endogenous ALA, there is no other interference from the matrix, confirming that this derivatisation procedure is suitable for the analysis of ALA directly in biological media.

3.2. Method validation

For the detection of ALA in cell lysates, the linearity was verified over the range 0.6–65 μM and over the range 60–600 μM. In the range of concentrations 0–6–65 μM, the linear regression equation was $y = (2.83 \pm 6.4E{-}3)x + (0.22 \pm 0.007)$ (R^2 = 0.994). With the

Table 1
Recovery of ALA from the matrix.

	Nominal concentration (μM)								
	0.6	3.2	6.3	13.2	20.3	31.7	46.3	51.4	64.7
0.6–65 μM									
Found (water)	0.7	3.5	6.1	12.9	20.6	31.4	46.0	51.7	65.1
Recovery from cell lysates (%)[a]	102.7	98.6	96.1	97.8	96.9	97.4	98.0	98.5	97.5
	Nominal concentration (μM)								
	29.8	59.7	119.4	179.0	298.4	358.1	417.6	477.1	597.0
60–600 μM									
Found (water)	31.1	61.2	120.6	176.9	294.4	351.8	419.1	472.3	592.0
Recovery from cell lysates (%)[a]	97.1	96.4	98.0	97.8	98.2	98.6	101.3	97.0	96.8
	Nominal concentration (μM)								
	29.8	59.7	119.4	179.0	298.4	358.1	417.6	477.1	597.0
60–600 μM after hydrolysis									
Found (water)	30.4	58.0	116.6	185.8	295.9	350.4	420.8	490.3	591.1
Recovery from cell lysates (%)[a]	98.3[b]	98.0[b]	96.6[b]	98.4[b]	97.1[b]	97.8[b]	98.5[b]	103.0[b]	101.2[b]
	97.5[c]	97.4[c]	95.8[c]	97.1[c]	95.9[c]	94.9[c]	95.4[c]	96.0[c]	96.9[c]

The values reported are the mean of 5 independent experiments.
[a] Recovery % compared to the value found in water.
[b] Samples not submitted to the hydrolysis step.
[c] Samples submitted to the hydrolysis step.

Table 2
comparison between the recoveries of ALA esters after the hydrolysis step under optimised conditions.

Nominal concentration (μM)	Found			
	ALA	ALAMe	ALAEt	ALAHex
119.4	118.6 ± 0.93	119.2 ± 1.11	119.4 ± 0.78	119.8 ± 0.94
298.4	297.9 ± 1.26	298.5 ± 1.05	298.2 ± 2.05	298.7 ± 3.85
477.1	477.4 ± 0.62	477.9 ± 0.80	478.2 ± 1.00	477.1 ± 1.42

The data are expressed as means ± SEM and were analysed by one-way analysis of variance (ANOVA) ($P < 0.01$).

exception of the lower concentration examined (0.6 μM), the accuracy varied within ±2%. Inter- and intra-day precisions (RSD) never exceeded 6.4% and 2.61%, respectively. The LOD and LOQ were found to be 0.04 and 0.12 μM, respectively. For the higher range of concentrations (30–600 μM) the regression line obtained was $y = (0.22 \pm 8.3\text{E}{-4})x + (-2.05 \pm 0.27)$ ($R^2 = 0.998$). The accuracy was 97.9–108.8%, exceeding +2.3% in only one point of calibration. Inter-day precision was never higher than 8.1% within the whole range, and intra-day precision never exceeded 1.4%. LOQ and LOD were 4.0 and 13.2 μM, respectively. Samples analysed on the day of preparation and on subsequent days gave curves with similar slopes and intercepts (data not shown). Table 1 shows the recovery of ALA from the matrix over both concentration ranges.

3.3. Quantification of ALA after release from its esters

The method described above can be successfully applied to the detection of ALA contained in widely used ester prodrugs [16] after a simple acidic hydrolysis step. Preliminary experiments showed that pH 3 is the lowest pH tolerated by the derivatisation reaction (data not shown) that is consistent with efficient ester hydrolysis and maximum production of **1**. ALAMe, ALAEt, and ALAHex (600 μM) were thus all completely converted to free ALA when refluxed in 10^{-3} M aqueous HCl for 3 h (the reactions were followed by TLC, dichloromethane/methanol/33% aqueous ammonia = 18/5/1). Table 2 shows the results of a set of experiments in which equimolar samples of ALA and its esters were exposed to hydrolysis and the reaction solutions submitted directly to the derivatisation protocol. ANOVA showed that the difference in mean values obtained from equimolar samples of different prodrugs for each level of concentrations was not statistically significant.

The regression line obtained over the range 30–600 μM after hydrolysis is described by the equation $y = (0.20 \pm 8.95\text{E}{-4})x + (-1.79 \pm 0.30)$ ($R^2 = 0.996$). The hydrolysis step did not affect the intra- or inter-day precisions, which never exceeded 3.14% and 7.4%, respectively. The accuracy was within 10% over the whole range. LOD and LOQ were 4.9 and 15 μM, respectively. As shown in Table 1, the hydrolysis step does not impact on the recovery.

4. Conclusions

We hereby report the development of a HPLC-fluorescence based method for the detection of ALA in cell lysates. The method has been validated over two different ranges of concentration: 0.6–65 μM (corresponding to 0.1–10 μg/mL) and 30–600 μM (corresponding to 5–100 μg/mL). The derivatisation procedure and the subsequent HPLC analysis are straightforward, do not require specialised apparatus, and the intensity of fluorescence of the derivative is stable for several days in the dark at room temperature. The method can be successfully applied to the quantification of ALA esters, directly in the biological matrix following a simple hydrolysis procedure. The overall efficiency and simplicity of the method, plus the absence of interference from the matrix itself should encourage the application of this method as a powerful tool for evaluating intracellular levels of ALA or its prodrugs in biomedical science.

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