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Validation of a capillary electrophoresis method for determination of 5-aminolevulinic acid and degradation products

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Abstract

A capillary electrophoresis method was developed for simultaneous quantification of 5-aminolevulinic acid (ALA) and its degradation products 2,5-dicarboxyethyl-3,6-dihydropyrazine and 2,5-dicarboxyethylpyrazine in aqueous solution within a total analysis time of 9 min. The optimized method was validated with respect to specificity, precision, linearity, limits of detection and quantitation, and robustness. The degradation products were quantified with respect to the ALA peak. A related micellar electrokinetic chromatography method, involving the addition of sodium dodecylsulfate to the running buffer solution, was applied for direct injection of an oil–in–water emulsion containing ALA, i.e. without sample pretreatment.

Keywords: Validation; Pharmaceutical analysis; Aminolevulinic acid; Dicarboxyethylpyrazines

1. Introduction

The precursor 5-aminolevulinic (ALA) of the photosensitizer protoporphyrin IX in the heme biosynthetic pathway has been proposed for photodynamic diagnosis and therapy of epithelial cancer. The development of stable ALA-containing pharmaceutical formulations requires an appropriate analytical method to evaluate the chemical stability of the compound. ALA undergoes dimerization to an intermediate dihydropyrazine derivative, which is further oxidized to 2,5-dicarboxyethylpyrazine (PY) (Fig. 1). The structure of the degradation products has been verified by ^{1}H- and ^{13}C-NMR spectrometry [1]. In the past, ALA has been quantified by HPLC methods using common derivatization reactions, typical for amino acid analysis [2–4]. Besides, only a few direct determination methods have been reported. In reversed-phase chromatography, no sufficient retention is achievable for nonderivatized ALA, because of its high polarity. For that reason, ion-exchange chromatography [5,6] or ion-pairing chromatography [7] may be used. A very useful method for the determination of charged substances is capillary electrophoresis (CE). Its use for the simultaneous determination of ALA and porphobilinogen in biological samples by micellar electrokinetic chromatography (MEKC) has already been reported by Luo et al. [8]. In comparison to liquid chromatographic methods, capillary electrophoresis offers

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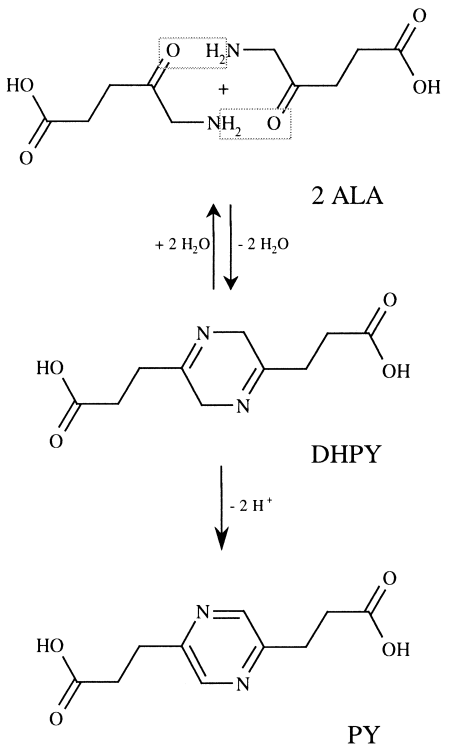

Fig. 1. Main degradation pathway of ALA.

several advantages, e.g. high resolution and efficiency, short analysis time as a requirement for routine analysis, small sample volumes, small volumes of running buffers, inexpensive column, direct injection without sample pretreatment. Therefore, CE has become a versatile alternative to HPLC for drug analysis. CE of drug-related impurities has been reviewed by Altria [9].

An objective of this work was to develop an analytical CE method, which permits the simultaneous quantification of ALA and its two degradation products. Quantification of active moiety and impurities in drug formulations is of great importance for drug registration. The International Conference on Harmonisation (ICH) provided guidelines for quality control of drug formulations applicable in the European Union (EU), USA and Japan. ICH-guidelines also describe the validation of analytical methods [10]. Validation of CE has been the topic of a growing number of publications showing the comparable performance of CE and HPLC [11–16]. In the present study, we report on the validation of a CE method to demonstrate its suitability for ALA assay and impurity testing.

2. Experimental

2.1. Chemicals

ALA was purchased from Sochinaz (Vionnaz, Switzerland). $Na_2B_4O_7$, NaH_2PO_4, Na_2HPO_4, NaOH and Sudan III were from Fluka (Buchs, Switzerland). Acetone was from Scharlau (Barcelona, Spain). Water was double-distilled. The degradation products of ALA, i.e. 2,5-dicarboxyethyl-3,6-dihydropyrazine (DHPY) and PY, were obtained as described previously [1].

ALA and its main degradation products were analyzed in two formulations, i.e. an aqueous solution and an oil–in–water emulsion. Initial ALA concentration of aqueous solution was 100 mg ml^{-1}, and its pH was adjusted to 5.5 by addition of 0.6 *M* NaOH. For the emulsion, a commercial parenteral fat emulsion (Intralipid from Pharmacia and Upjohn, Dübendorf, Switzerland) was used, wherein ALA was dissolved at 100 mg. The pH value of the water phase was approximately 2 and not further adjusted.

2.2. Instrumentation

CE was performed using a P/ACE 5010 CE, instrument (Beckman, Fullerton, CE, USA) equipped with an on-column UV detector and a diode array detector. 'Gold-Nouveau'-software (Beckman) was used for data acquisition. Fused-silica capillaries (eCAP Capillary Tubings) of the following dimensions were used: 37 cm (effective length 30 cm)×50 μm I.D×375 μm O.D., and 57 cm (effective length 50 cm)×75 μm I.D.×375 pm O.D.L (Beckman).

2.3. Methods

Samples were injected hydrodynamically (3447 Pa, 3 s). The voltage during the analysis was 20 kV, and the temperature 30°C. Analytes were detected by UV absorbance at 200 nm, unless indicated otherwise. The capillary was rinsed with 0.1 *M* NaOH for 2 min prior to each analysis.

The running buffer solution was a 50 m*M* borate

buffer of pH 9.4. The pH of the borate buffer was not adjusted. The buffer solution was filtered through a 0.45-μm pore size filter (Schleicher/Schuell, Dassel, Germany) and degassed by ultrasonication.

The electrophoretic mobility of the micelles during MEKC was measured by injecting sudan III as marker substance. Acetone was used to measure the electroosmotic flow (EOF).

ALA and its degradation products were quantified relative to external standards. The intermediate degradation product DHPY, however, was not available as reference substance, so that it was quantified by another method. Based on the observation that ALA degraded completely to DHPY in 5 *M* potassium hydroxide purged with nitrogen, the DHPY content of the solution was calculated stoichiometrically from the initial ALA concentration. Electropherogram peak analysis at 200 nm revealed a high DHPY purity of approximately 95%, and a good linearity at different concentrations.

Separation parameters were calculated according to the European Pharmacopoeia. Calculations of detection limit (DL) and quantitation limit (QL) were carried out according to the ICH guidelines [10].

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

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

where σ is the standard deviation of the *y*-intercept and *S* the slope of the calibration curve.

3. Results and discussion

3.1. Method optimization

CE conditions for assaying ALA and its two degradation products were optimized by using a solution containing all three substances, i.e. ALA, DHPY and PY (Fig. 1). As the intermediate degradation product of ALA, DHPY, could not be isolated, it was not possible to spike an ALA solution with a defined amount of this component. Hence, a partially degraded ALA solution was used instead. For this purpose, ALA was dissolved in a 200 m*M* phosphate buffer at pH 7.4, where ALA is known to degrade readily [1], and stored at 50°C overnight. According to the amphoteric structure of the compounds (Fig. 1), separation was expected to be feasible at low and high pH values. Indeed, good separation of all three substances was achieved in the borate buffer of pH 9.4, whereas only two peaks with less resolution were obtained in a phosphate buffer of pH 2.0. We further aimed at optimizing the separation of the analytes as anions in the basic running buffer. The first peak was identified as ALA, the second as the intermediate DHPY and the third as PY, as described previously [1]. In a phosphate buffer of pH 8, ALA did not dissociate sufficiently and was detected near the electroosmotic flow, as expected from the pK_a value of 8.2 [17]. Separation of ALA from the EOF peak was appropriate with the pH 9.4 borate buffer.

To achieve a baseline separation of ALA and its degradation products within a short analysis time, further separation conditions were optimized. Increasing the buffer concentration up to 50 m*M* borate improved the resolution of DHPY and PY. In order to avoid joule heating effects in the initially used 75-μm capillary, a capillary with a smaller inner diameter of 50 μm and a length of 37 cm (30 cm effective length to the detection window) was chosen. Applying a voltage of 20 kV, the separation was achieved within 7 min (Fig. 2).

Because ALA lacks aromatic or other favorable UV-absorbing structural moieties, the detection was carried out at a wavelength of 200 nm to avoid derivatization.

The selective separation of ALA and its degradation products was demonstrated here. High efficiency and resolution were achieved under optimized conditions, as outlined above (Table 1).

3.2. Method validation

3.2.1. Specificity

Thanks to the migration-time reproducibility achieved, the CE method allowed the unambiguous discrimination of both degradation products. Best results were obtained for relative migration times related to the ALA peak. The reproducibility of migration time was determined by injecting twice each of six individual samples containing ALA, DHPY and PY. The confidence interval of relative

Table 1
Electrophoretical parameters of separation of ALA and its degradation products[a]

Parameters	ALA	ALA/DHPY	DHPY	DHPY/PY	PY
Effective mobility ($cm^2\ V^{-1}\ s^{-1}$)	$-2.60\cdot10^{-4}$		$-3.81\cdot10^{-4}$		$-3.87\cdot10^{-4}$
Separation factor α		1.764		1.038	
Resolution R_s		38.39		2.83	
Symmetry factor	1.51		1.55		1.11

[a] Separation conditions: 50 m*M* borate buffer of pH 9.4; 200 nm; uncoated capillary 37 cm (effective length 30 cm)×50 μm; 30°C; 20 kV; electroosmotic mobility=$5.40\cdot10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$; efficiency N=113 434 theoretical plates.

migration times for double injections amounted to ±0.49% for DHPY and ±0.55% for PY.

As analyzed by peak purity, co-migration of further unknown impurities with one of the three detected compounds was excluded.

3.2.2. Precision

The precision of the analytical method was evaluated by determining the repeatability of the corrected peak area ($A_{corr}=A/t_M$) of six test samples, containing ALA, DHPY and PY. The obtained data shows sufficient repeatability for assay and purity testing of ALA (Table 2).

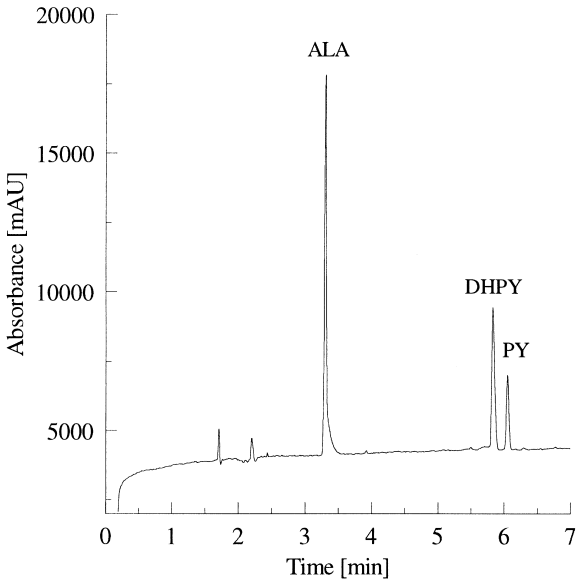

Fig. 2. Electropherogram of ALA and its degradation products DHPY and PY. For conditions see Table 1. The volume of the injected solution (1 mg ml^{-1}) was 4.3 nl.

3.2.3. Linearity and range of ALA and its degradation products

ALA and its degradation products were quantified relative to external standards. For a target concentration of 10 mg ml^{-1} of ALA, good correlations were determined between corrected peak areas of ALA, DHPY and PY and their respective concentrations (Table 3). The correlation coefficients were calculated from six sample concentrations within ranges of 72–119% of ALA and 1–20% of impurities related to ALA.

From the slope of the regression lines, relative response factors were calculated, allowing the quantification of the degradation products DHPY and PY, relative to the ALA peak (Table 3). Such

Table 2
Precision of analytical procedure, as determined by corrected peak area (repeated injections of six samples containing ALA, DHPY and PY)

	ALA	DHPY	PY
RSD (%) for $n=6$	0.75	3.18	1.50
C.I. (%) for $n=6$	±0.78	±3.34	±1.57
C.I. (%) calculated for $n=2$	±1.36	±5.78	±2.72

Table 3
Linearity determination of ALA, DHPY and PY from six respective concentrations. Response factors for quantification of DHPY and PY related to main peak of ALA

	ALA	DHPY	PY
Range (mg ml^{-1})	0.7–1.2	0.01–0.15	0.01–0.14
Correlation coefficient	0.9976	0.9972	0.9987
Response factor f_r		0.5032	0.1805

response factors allow the use of the ALA peak as internal standard for the determination of the degradation products by Eq. (3).

$$m_{\mathrm{DHPY/PY}} = \frac{m_{\mathrm{ALA}} A_{\mathrm{DHPY}} f_{\mathrm{r}}}{A_{\mathrm{ALA}}} \quad (3)$$

where m_{ALA} is the mass of ALA in the sample solution, estimated during assay determination by external standard, $m_{\mathrm{DHPY/PY}}$ the mass of DHPY or PY in the sample solution, A the peak area and f_{r} the response factor.

3.2.4. Limits of detection and quantitation

From the calibration curve of five sample dilutions, containing DHPY or PY in the range of detection limit, the detection and quantitation limits were calculated according to equations indicated in the ICH guidelines [6] (Table 4). The values of these limits demonstrated that the developed CE method is suitable for impurity testing of ALA solutions containing low concentrations of degradation products.

3.2.5. Robustness

The electrophoretic resolution of the closely adjoining DHPY and PY peaks, were used to evaluate the method under modified conditions. Sufficient resolution for DHPY and PY was obtained under all separation conditions tested (Table 5), demonstrating sufficient robustness.

Table 4
Detection and quantitation limits of DHPY and PY

	DHPY	PY
Range of determination (ng ml^{-1})	7.6–153.5	0.7–3.4
Correlation coefficient of calibration curve	0.9944	0.9734
Detection limit (ng ml^{-1})	0.12	0.32
Quantitation limit (ng m^{-1})	0.38	0.96

3.3. Application of the CE-method to a pharmaceutical ALA formulation

The CE method developed was not directly applicable to oil–in–water-type emulsions, which we considered as potentially useful pharmaceutical formulations for ALA. Baseline disturbance occurred, which was caused by the oily ingredients of the emulsion (Fig. 3). To improve the separation, we modified the CE running buffer by addition of SDS. Using the MEKC-mode, a suitable electropherogram was obtained (Fig. 3). In this method, the lipophilic compounds were incorporated in negatively charged micelles, which were moving in opposite direction to the analytes and did not reach the detection window (Fig. 3). A similar separation

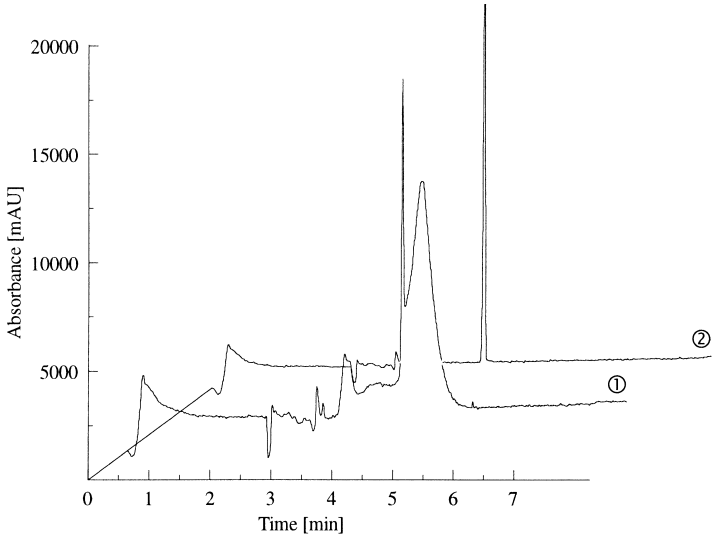

Fig. 3. Electropherograms of ALA in oil–in–water-type emulsion. Separation conditions: uncoated capillary 37 cm (effective length 30 cm)×50 μm; 30°C; 20 kV; 200 nm; running buffer 50 m*M* borate buffer of pH 9.4 (①), MEKC mode with addition of 10 m*M* SDS (②).

Table 5
Robustness of the analytical method upon variation of CE separation conditions[a]

Parameter	R_s
Voltage (kV)	
15	2.51
20	2.83
25	2.66
Temperature (°C)	
25	2.73
30	2.83
35	2.63
Buffer concentration (m*M*)	
30	1.58
50	2.83
75	3.61

[a] The resolution R_s of the DHPY and PY peaks was used to test the suitability of the method. Standard separation conditions are summarized in Table 1.

principle is described for analysis of biological fluids containing proteins that complicate separation in CZE [18]. MEKC yields an 'analytical window', wherein the analyte is detected before elution of protein [19]. A number of methods for direct injections of complex matrices without sample pretreatment in CE has been reviewed by Watzig et al. [20].

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