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Capillary electrophoresis method development for determination of impurities in sodium cysteamine phosphate samples

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Abstract

A capillary electrophoresis method for determination of impurities in sodium cysteamine phosphate—an alternative drug to use in place of cysteamine (Cystagon®, Mylan Laboratories Inc.) in the treatment of cystinosis—was developed. The administration of cysteamine, divided in four doses due to the short half-life of this drug, is a helpful treatment, but several patients show intolerance, due to the very unpleasant odor and taste of cysteamine. Sodium cysteamine phosphate is less organoleptic aversive and also has a larger active time, allowing the compression of the doses to 2 per day, increasing the acceptance of the drug. In the developed method the two main decomposition products of sodium cysteamine phosphate, cystamine and cysteamine, can be determined with LOQs of 30 μg/ml (0.2%) and 16 μg/ml (0.1%), respectively. The background electrolyte is 15 mM ammonium acetate (pH 8.85) with 10% methanol and the separation takes less than 4 min. UV detection is performed at 195 nm. This volatile method was developed with the purpose of further hyphenation to a mass spectrometer.

Keywords: Capillary electrophoresis; Sodium cysteamine phosphate; Cystinosis; Quality control; Impurities analysis

1. Introduction

Cystinosis is an autosomal recessive disease. The incidence is about 1 case per 200,000 live births, but can vary significantly depending on the subpopulation. Higher incidence is observed in Caucasians. The male-to-female ratio has been reported to be 1.4:1 [1].

Ingested protein enters the lysosome and is hydrolysed into amino acids, including cysteine, which is quickly oxidized to cystine. In normal individuals both cysteine and cystine can be transported through the lysosomal membrane and enter the cytoplasm, where cystine is reconverted to cysteine via glutathione. Then cysteine can be or incorporated into a new protein or suffer degradation to inorganic sulphate for excretion. The disease is caused by an inability to remove the molecules of cystine from the lysosomal compartment due to the lack of the transport protein named cystinosine. The increase of the concentration of cystine combined with its low solubility leads to the formation of crystals that cause injuries in the cells, probably by impairment of cellular energy metabolism [1,2].

Two different phenotypes can be observed: the nephropathic and the non-nephropathic cystinosis. The non-nephropathic cystinosis is considered a benign variant, and diagnosis is usually done in middle age. Nephropathic cystinosis is subdivided in infantile (the most severe form) and late-onset, dependent on age at manifestation.

The symptoms of infantile nephropathic cystinosis appear in the first year of life and in few months severe dehydration, electrolyte imbalance, acidosis and heat intolerance (due to impairment of sweat production) can be observed. Lack of treatment leads to rickets, impairment of growing, renal Fanconi syndrome [1,3] and finally renal failure by the age of 7–10 years.

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Patients with nephropathic cystinosis can postpone the need of hemodialysis and/or kidney transplantation taking cysteamine (dose gradually increased till 1.3–1.95 g/m^2/day), divided in four doses due to the short half-life of this drug [1]. This is a helpful treatment, but several patients show intolerance, due to the very unpleasant odor and taste of cysteamine. This is one of the biggest drawbacks for a good compliance of the treatment, especially because the majority of the patients are children and adolescents. Sodium cysteamine phosphate (SCP) is less organoleptic aversive [4] and also has a larger active time, allowing the compression of the doses to 2 per day, increasing in this way the acceptance of the drug.

After ingestion SCP is converted to cysteamine in the intestine and can freely enter the lysosomes where it takes part in a thiol–disulfide interchange reaction converting cystine into cysteine and cysteine–cysteamine molecules that can be transported out of the lysosome by the cysteine and lysine transporters [1,2].

The two main decomposition products of SCP are cysteamine (CTE) and cystamine (CTA), products of the phosphate hydrolysis and thiol oxidation, respectively (Fig. 1). There are some studies describing the separation of thiols, including CTA and CTE, in physiological samples [5–7], yet no method has been described for the separation of SCP and its impurities for quality control purposes. Thin layer chromatography can be used for the quality control of SCP (unpublished data). In order to enhance the sensitivity and resolution of impurity determination, a capillary electrophoresis (CE) separation method with UV detection was developed. This method was developed employing a volatile system with the purpose of further hyphenation to a mass spectrometer (CE–MS).

Previous attempts of our laboratory to develop a liquid chromatography (LC) method for the quality control of SCP were not successful. Indeed, the retention of SCP on reversed phase columns appeared to be too low, due to the highly polar and hydrophilic character of SCP. It was possible to separate it from its two impurities, but SCP co-eluted with the dead volume. Even the use of stationary phases especially designed for polar compounds could not improve the situation. Ion pair chromatography was also tried out, with cationic as well as anionic surfactants because of the bipolar character of SCP, and with mixtures of both, but this could not sufficiently improve the retention characteristics of SCP on reversed phase columns.

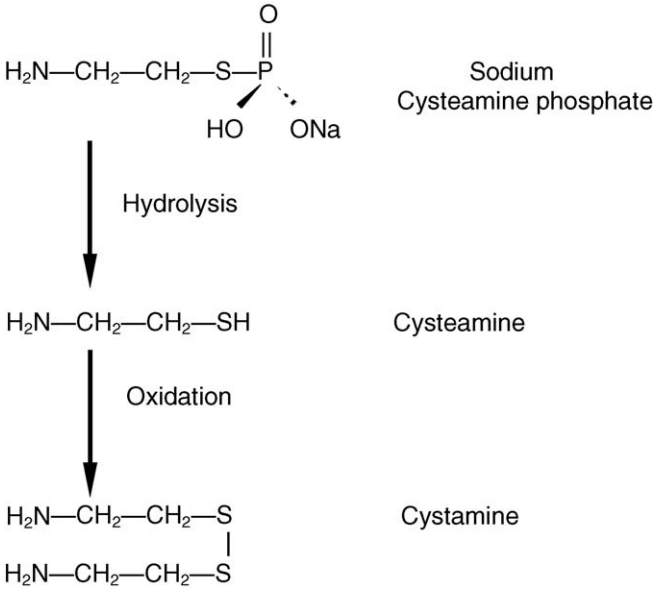

Fig. 1. Sodium cysteamine phosphate and its hydrolysis and oxidation.

Subsequently, an ion exchange chromatography was explored with ammonium acetate solution as the mobile phase. No satisfactory separation could be obtained. The analysis of SCP on a cation exchange column at low pH seemed to provide better results within a short analysis time. With a gradient system from pH 3 to 1 it was possible to obtain a separation between all the peaks, but the sensitivity and the efficiency of this system were rather low.

Therefore further investigations on LC of SCP were abandoned and efforts were put into the development of a CE method.

2. Material and methods

2.1. Chemicals

The chemicals used were of analytical grade: ammonium hydroxide 25% (Riedel-de-Haën, Seelze, Germany), ammonium acetate, sodium acetate, glacial acetic acid, sodium hydroxide pellets (BDH Analar, Poole, UK), benzoic acid 98% (Sigma–Aldrich, Gillingham, UK) or HPLC grade: methanol (Fisher Chemicals, Loughborough, UK). The water was purified (18 MΩ/cm) in a Milli-Q® system (Millipore, Milford, MA, USA).

The samples of sodium cysteamine phosphate tetrahydrate were home synthesized by a two-step procedure. In the first step thiophosphoryl trichloride is added to a sodium hydroxide solution under constant stirring to produce sodium thiophosphate. The latter is crystallized and subsequently reacted with aminoethylbromide to obtain SCP. The compound is crystallized and repeatedly washed with ethanol. Cysteamine hydrochloride, min. 98%, and cystamine dihydrochloride, min. 97%, were purchased from Avocado Research Chemicals, Heysham, UK. Due to stability reasons the solutions were prepared immediately before and CTE·HCl as well as SCP were stored under refrigeration (4 and −20 °C, respectively).

2.2. Buffer preparation

The buffers utilized were at most 2 days old. The amount corresponding to the indicated molarity was weighed and dissolved in water, and the pH was adjusted with diluted solutions of acetic acid or ammonium hydroxide, depending on the required pH. For buffers containing methanol the reported pH corresponds to the aqueous solution before the addition of the alcohol. The pHs were measured and adjusted with the aid of a pH-meter Metrohm 691 (Herisau, Switzerland). In order to ascertain the accuracy of these measurements, it was calibrated before each measurement with reference buffer solutions, as described in the European Pharmacopoeia [8].

2.3. Software

The design of optimization experiments was made with the aid of the software MODDE 5.0 (Umetrics, Umeå, Sweden).

2.4. Capillary electrophoresis

Experiments were performed on a P/ACE™ MDQ equipment with diode array detector and the data acquisition was done by means of 32 Karat™ 4.0 software (both Beckman–Coulter, Fullerton, CA, USA). The capillaries used were purchased from Polymicro Technologies (Phoenix, AZ, USA). All of the capillaries used were uncoated fused silica, 75 μm i.d., 40 cm long, with the detection window burnt at 10 cm from the opposite end. New capillaries were conditioned by rinsing with water for 5 min, then with NaOH (1 M) for 5 min and keeping them filled with NaOH (1 M) for 120 min. Finally they were rinsed with NaOH (0.2 M) followed by water, 5 min each. Daily, prior to analysis, the capillary was conditioned by the following washing sequence: water (5 min), 0.2 M NaOH (10 min), running electrolyte (5 min), application of 15 kV (5 min) and once more running electrolyte (5 min). Between each run one single flush of 2 min was performed with running electrolyte. During the optimization experiments, when the composition of the electrolyte had to be changed, a flush of 5 min with the new running electrolyte was applied. All the washing procedures were performed by applying a pressure of 138 kPa. The capillary was thermostated at 25 °C during all of the experiments, including washing procedures. The inlet/outlet vials were replaced every three runs.

3. Results and discussion

SCP, CTA and CTE are very soluble substances and are present as an ion in a wide range of pH, making the separation by capillary zone electrophoresis (CZE) an obvious choice. SCP ionizes in water, and the pK_a values of cysteamine phosphate (CP) are 2.2, 5.0 and 10.3, values corresponding to the phosphate and the amino group, respectively [9]. CTE has one amino group and CTA has two amino groups with no pK_a data available in the literature, but expected to be close to the observed value for the CP amino group, due to the similarity between structures.

The development of a CE–MS method restricts significantly the choice of buffers to volatile systems (formate, acetate, hydrogen carbonate, carbonate and ammonium). An initial screening was made, using at this moment also non-volatile systems. The following buffers were tested, all at 15 mM: sodium acetate (pHs 3.8; 4.8; 5.8) and ammonium acetate (pHs 8.3; 9.3; 10.3). In order to overcome the instability of the hydrogen carbonate system, 15 mM sodium phosphate pH 7.0 was used. Also sodium phosphate buffers (15 mM, pHs 3.0 and 12.0) were used to extend the range. In the most alkaline condition instability of the compounds was observed.

Best separation was obtained with the buffers sodium acetate pH 4.8 and ammonium acetate pH 8.3. In both systems CP is in the anionic form while CTE and CTA are in the cationic form.

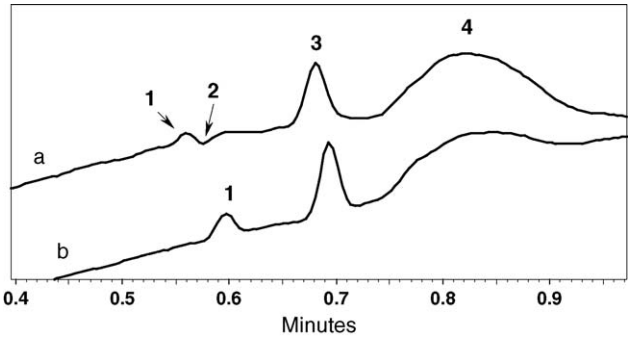

Fig. 2. Electropherograms showing the presence of a negative system peak when ammonium acetate (a) is used in place of sodium acetate (b). Conditions: buffer concentration 15 mM, pH 4.2, separation potential +28 kV, and positive 0.7 kPa/3 s for hydrodynamic injection. Capillary 75 μm i.d., 40 cm long (30 cm till detection window). Peaks: (1) cystamine (25 μg/ml); (2) system peak; (3) cysteamine (25 μg/ml); (4) disturbance in the baseline after cysteamine peak.

Since better peak shape was observed in the acid system, the first efforts to optimize the method were done between pH 4.0 and 5.0.

Further experiments showed that when sodium is replaced by ammonium, in order to increase the volatility, a negative system peak arises around the CTA migration time. Independent of the acetate counter-ion, another disturbance in the baseline just after the CTE peak is present, especially with higher SCP concentration (above 5 mg/ml) (Fig. 2). In this pH range significant variation in the migration time was observed, especially intra-day, and when a new capillary was used, tending to achieve equilibrium after several injections. This behavior can be attributed to changes in the capillary wall, since the anionic specimens, such as CP and benzoate, show much higher shifts when compared with the cationic substances, such as CTA and CTE.

The migration time repeatability improved at higher pHs (between 8 and 9). The resolution between CTA and the negative system peak was pH-dependent, and improved at high pH.

The use of methanol was helpful to increase the resolution. Also, the presence of organic modifiers increases the volatility of the buffer and is thus appropriate. For fine optimization of pH, buffer concentration and methanol percentage, a factorial design experiment was carried out, with use of the software MODDE 5.0 (Umetrics, Umeå, Sweden). The separation potential used is the highest possible, without Joule heating (easily determined by plotting potential × current), so as to reach the highest efficiency possible.

To correct for injection errors, sodium benzoate (30 μg/ml) is used as internal standard. The factors were tested at two levels: pH 8.3 and 9.0 as low and high level, buffer concentration 10 and 20 mM as low and high level, no methanol and 20% methanol as low and high level. In order to calculate the error, a triplicate experiment of the central point (arithmetic average of low and high level of all parameters) was done, totalizing 11 experiments (factorial design $2^3 + 3$). In order to use the central point for determination of the precision, all of the experiments were performed in randomized order. The responses investigated were the time distance between the negative system peak

Table 1
Analytical parameters of the developed method

	Linearity				
CTA	$y = 2.4662x - 0.0016$; R^2 0.9984; range 0.038–0.306 mg/ml; $n = 8$				
CTE	$y = 5.7783x - 0.0864$; R^2 0.9993; range 0.019–0.611 mg/ml; $n = 16$				
CP	$y = 0.6939x + 0.0015$; R^2 0.9990; range 0.082–0.656 mg/ml; $n = 8$				
	Repeatability (R.S.D., %)			Sensitivity	
	Migration time (min)	Area	Area/area IS	LOQ	LOD (μg/ml)
CTA	1.25	53.6	5.2 (0.06 mg/ml; $n = 16$)	30	9
CTE	1.18	29.7	4.8 (0.06 mg/ml; $n = 16$)	16	5
CP	0.33	40.5	3.2 (0.40 mg/ml; $n = 9$)	40	12
Benzoate	0.27	40.4	–(0.03 mg/ml; $n = 9$)		

and CTA ($DIS_{SP/CTA}$), the resolutions between CTA and CTE ($RES_{CTA/CTE}$), CP and benzoate ($RES_{CP/BENZ}$), the time distance between CTE and the beginning of the EOF ($DIS_{CTE/EOF}$) and analysis time, measured as the benzoate migration time.

The individual effects of the factors (pH, buffer concentration, methanol concentration) and the interaction of combined effects (pH and buffer concentration, pH and methanol concentration, and so on) were evaluated. Modde 5.0 was used to develop a model using partial least squares (PLS), calculated using the PLS2 non-linear iterative partial least squares (NIPALS) algorithm. With this approach an R^2 value is obtained, representing the fraction of the variation of the response explained by the model and a Q^2 value, representing the fraction of the variation of the response that can be predicted by the model. Fig. 3 presents the coefficient plots. In these plots, a coefficient bar denotes the effect of a factor and an error bar represents the 95% confidence interval. When the error bar is bigger than the coefficient bar, there is no significant effect. The R^2/Q^2 values obtained were 0.919/0.644, 0.961/0.691, 0.943/0.266, 0.837/0.106 and 0.974/0.588 for $DIS_{SP/CTA}$, $RES_{CTA/CTE}$, $RES_{CP/BENZ}$, $DIS_{CTE/EOF}$ and analysis time, respectively.

Although some models show low Q^2 values, it is possible to clearly see an improvement of the resolutions with the use of methanol and with the increment of the buffer concentration, which can simply be attributed to the reduction of the EOF mobility. The pH should play a role in the separation just by the difference in the ionization constants of the substances, since above pH 8 no significant changes in the EOF are expected. No significant interaction effects were observed.

The final composition of the running electrolyte, 15 mM ammonium acetate (pH 8.85) with 10% methanol, was established in order to ensure good resolutions with a minimal increase of the analysis time (less than 4 min). Although pH 9.0 leads to the best resolution values, the use of pH 8.85 avoids some possible interference of the EOF in the integration of the CTE peak. The separation potential is +28 kV, yielding a typical 47 μA running current. A positive 3.5 kPa pressure was applied during 5 s for hydrodynamic injection. Fig. 4 shows a typical electropherogram. Despite the fronting peak shape of CP, good linearity and repeatability are achieved for this substance. In CE the peak shape is dependent on the differences between the mobility of the analyte and the co-ions of the running electrolyte, an intrinsic phenomenon of the technique called electrodispersion that is described since the early development of CE [10]. Table 1 displays the repeatability (of migration time and area), linearity, LOQ and LOD. The LOQ and LOD, corresponding to a signal to noise ratio (S/N) = 10 and S/N = 3, respectively, were calculated using the lowest level of the linearity curve.

The LOQ and LOD can also be expressed with respect to $SCP{\cdot}4H_2O$, as percentage of the highest amount of $SCP{\cdot}4H_2O$ that can be injected without lack of resolution between CP and benzoate peaks (about 14 mg/ml). The calculated LOQ and LOD for CTA and CTE are 0.2 and 0.06% and 0.1 and 0.03%, respectively.

The robustness of the method was evaluated by plotting the response surface for the most significant pairs of factors for each evaluated response, according to Fig. 3. $DIS_{CTE/EOF}$ was not included in the evaluation since no significant effects were observed for this item. Table 2 shows the data obtained through the response surface plots, and demonstrates the good robustness of the method.

Differences in the response factors (RF) between the substances were observed. The relative RF was obtained making a calibration curve of CTE and CTA and injecting a sample of SCP in the range with response (area) covered by the calibration curves. The RFs were determined with the corrected areas (area/migration time) with internal standard correction. The following values were found: $RF_{CTE/CP} = 9.6$ and $RF_{CTA/CP} = 4.0$. Table 3 shows the results of the analysis of four different batches of SCP. The results were calculated comparing the areas of CTE and CTA with the area of CP in a 1% dilution of the sample. Triplicate analysis was carried out on a sample solution containing

Table 2
Robustness evaluation

Response	Factor 1	Factor 2	Variation
$DIS_{SP/CTA}$	MeOH 9–11 %	pH 8.8–9.0	0.16–0.2 min
$RES_{CTA/CTE}$	pH 8.8–9.0	b.con 14–16 mM	3.1–3.9
$RES_{CP/BENZ}$	MeOH 9–11%	b.con 14–16 mM	11–13.5
Analysis time	MeOH 9–11%	b.con 14–16 mM	3.5–4.0 min

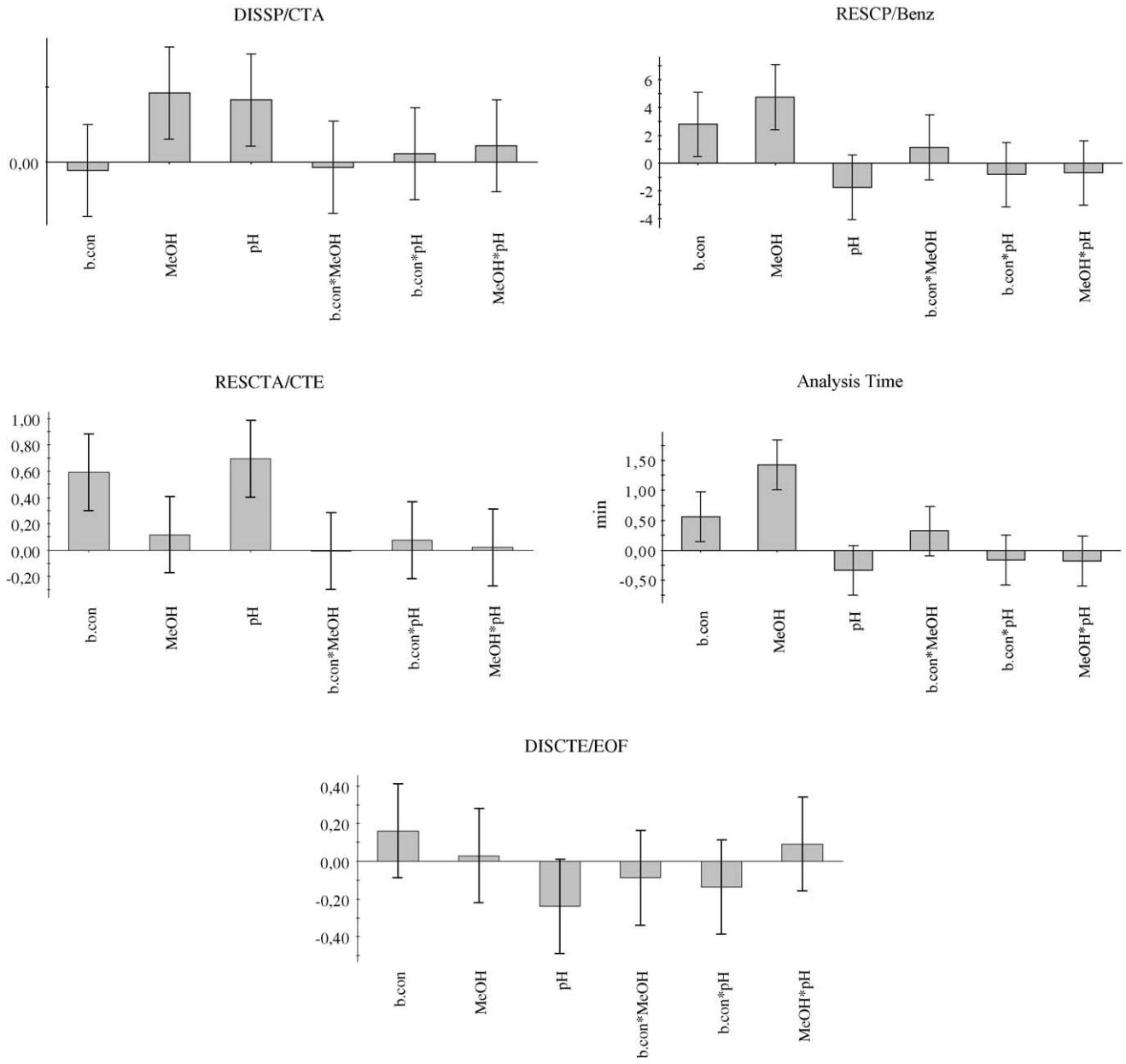

Fig. 3. Regression coefficient plots for the response variables: time distance between the negative system peak and CTA ($DIS_{SP/CTA}$); resolution between CTA and CTE ($RES_{CTA/CTE}$); the time distance between CTE and the beginning of the EOF ($DIS_{CTE/EOF}$); resolution between CP and benzoate ($RES_{CP/BENZ}$); and analysis time. The solid bar denotes the effect of a factor and an error bar represents the experimental error. An error bar bigger than the coefficient bar means that the respective effect is not significant. b.con = buffer concentration.

about 14 mg/ml of SCP. Fig. 5 shows typical electropherograms. Since CTE and CTA can be purchased, the determination of the impurities can also be done by the construction of calibration curves, but the analyst should be careful with the standards because CTE is gradually oxidized to CTA, leading to underestimation of CTA and overestimation of CTE.

Since SCP is not very stable it is practically not possible to use it as a reference substance for quantitative determination of the sample content by CE.

As shown in Fig. 1 CP can be oxidized (after hydrolysis to CTE) to CTA. This oxidation can be performed quantitatively using excess iodine, and titration of the excess iodine by thiosulfate is the assay method actually performed for SCP (unpublished data). In an analogous way, iodine oxidation may be used to transform CP quantitatively into CTA, which is determined by CE, using CTA as a reference standard. In contradistinction with SCP, CTA is stable.

Table 3
Analysis of SCP batches

Batch	CTA	CTE (%) (R.S.D., %)
290304	<LOD	0.1 (10)
300304	<LOD	<LOQ
200404	<LOD	0.2 (6)
260404	<LOD	0.1 (10)

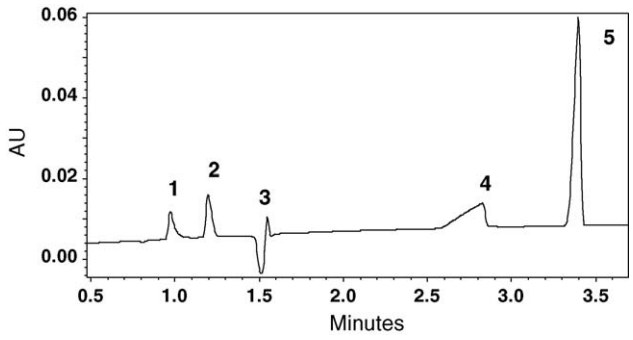

Fig. 4. Electropherogram showing the separation of cysteamine phosphate and its impurities in a reference mixture. Conditions: 15 mM ammonium acetate, pH 8.85 with 10% methanol, separation potential +28 kV, and positive 3.5 kPa/5 s for hydrodynamic injection. Capillary 75 μm i.d., 40 cm long (30 cm till detection window). Peaks: (1) cystamine (0.15 mg/ml); (2) cysteamine (0.075 mg/ml); (3) EOF; (4) cysteamine phosphate (0.66 mg/ml); (5) benzoate (0.03 mg/ml).

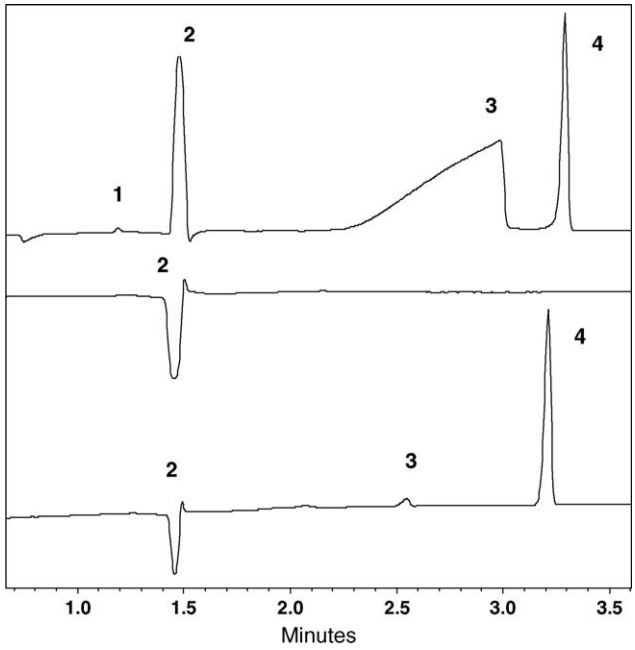

Fig. 5. Series of electropherograms obtained for impurity analysis in a sample of SCP. Conditions: as described in Fig. 4. From top to bottom: concentrated SCP sample (approx. 14 mg/ml), blank and diluted SCP sample (approx. 0.14 mg/ml). Peaks: (1) cysteamine; (2) EOF region; (3) cysteamine phosphate; (4) benzoate (30 μg/ml).

4. Conclusions

A fast method for determination of impurities in samples of SCP was developed. Good repeatability of migration time and satisfactory sensitivity and repeatability were achieved for the separation and determination of CTA and CTE.

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