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Analytical study of penicillamine in pharmaceuticals by capillary zone electrophoresis

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Abstract

The ability of capillary zone electrophoresis in the development of analytical methods devoted to the quality control of the thiol drug penicillamine is shown. Using 50 m*M* phosphate running buffer (pH 2.5), good quantitations of underivatized penicillamine and its disulfide were achieved; detection at 200 nm allowed checking the presence of the disulfide impurity in pharmaceuticals. The use of 1,1′-[ethenylidenebis(sulfonyl)]bis-benzene as a thiol specific reagent resulted in an increased sensitivity for the quantitation of D-penicillamine (limit of detection at 200 nm wavelength was 1.5 μ*M*). Introducing β-cyclodextrin as chiral selector in the running buffer, enantioseparation of D,L-penicillamine was obtained; for this purpose (+)-camphor-10-sulfonic acid, a chiral ion-pairing reagent, was found to be an essential additive in obtaining a baseline separation. The resulting enantioseparative system was validated in order to evaluate the presence of the toxic L-penicillamine enantiomer in pharmaceutical samples.

Keywords: Pharmaceutical analysis; Enantiomer separation; Penicillamine

1. Introduction

Capillary electrophoresis (CE), one of the most recent separation techniques, may offer high-performance separations in very short analysis time associate to the development of simple methods and minimal sample volume requirements. It is thus suitable to solve the major problems in the analytical area of the pharmaceutical quality control [1]. In this study CE was applied to the analysis of D-penicillamine and related impurities (disulfide and L-enantiomer).

D-Penicillamine is a thiol drug used in the treatment of Wilson's disease, cystinuria, certain forms of metal intoxication and progressive systemic sclerosis; more recently it is administered in rheumatoid arthritis [2]. The pure D (or *S*) form of the drug is used, because the L (or *R*) form and the DL (*RS*) racemate are much more toxic, as shown by severe adverse reactions such as neuritis in patients treated with the DL-penicillamine [3]. The chemical processes underlying the pharmacological action of penicillamine are still unknown; however, the drug may act by reacting with sulfhydril sites on plasma proteins in the cytosol, or at the cell membrane [4]. For these reasons it seems to be of paramount importance have at one's disposal analytical methods to evaluate both the impurities penicillamine disulfide and L-penicillamine in raw materials and pharmaceutical dosage forms.

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The literature shows that the analysis of thiols and relative disulfides has been achieved by several techniques; CE proved to be able to develop methods useful for the speciation and evaluation of thiols and related disulfide also in biological samples [5–7].

Concerning the chiral separation of DL-penicillamine and other amino acid enantiomers, HPLC and CE methods based on resolution of diastereoisomers obtained after asymmetric derivatization (indirect approach) have been reported [8,9]. The direct enantioresolution (direct approach) of small size molecules in a media containing a chiral selector, suffers for the negligible differences in interactions between the optical isomers and the chiral selector (usually cyclodextrins in CE). Derivatization of aminic function with dansyl chloride is performed to improve the sensitivity and to exalt the different interactions, selector–selectand, in order to reach analytical chiral separations of amino acids [10,11]. To our knowledge, this approach wasn't applied to the determination of the penicillamine enantiomers. On the other hand, the availability of new and more rapid and selective derivatization reactions able to meet the requirements for a direct, sensitive enantioseparation of small, aliphatic thiol compounds is still a challenge.

In the present paper simple and rapid CE methods were developed for the analysis of penicillamine in a pharmaceutical dosage form (capsules), including the determination of both the impurities penicillamine disulfide (achiral separation) and L-penicillamine (chiral separation). Direct CE analysis was performed in order to determine simultaneously the analyte and its disulfide by UV detection, while derivatization of the sulfhydril function using 1,1′-[ethenylidenebis-(sulfonyl)]bis-benzene (ESB) [12] was found to increase the sensitivity of detection for penicillamine and also to provide a thiol adduct suitable for the CE enantioseparation using β-cyclodextrin (βCD) as chiral selector in the presence of (+)-camphor-10-sulfonic acid (CSA).

2. Experimental

2.1. Materials

D-Penicillamine and DL-penicillamine, penicillamine disulfide, hydroxypropyl-β-cyclodextrin (HPCD), dimethyl-β-cyclodextrin (DMCD) were purchased from Sigma (St. Louis, MO, USA); glutathione, cysteine, homocysteine, *S*-methyl-L-cysteine and CSA, γ-cyclodextrin (γCD) were from Fluka (Buchs, Switzerland); tryptophan and βCD were from Janssen (Beerse, Belgium). The derivatization reagent ESB was from Merck (Schuchardt, Germany). Methanol used for the preparation of reagent solution and as tracer of electroosmotic flow was of HPLC grade from Mallinkrodt (Germany). Potassium phosphate and phosphoric acid were from Carlo Erba (Milan, Italy). Water employed for all the preparations (running buffers and sample solutions) was deionized from a TKA ROS 300 system.

2.2. Solutions

Running buffer solutions for the direct analysis of penicillamine and its disulfide, consisted of 50 m*M* phosphate solution adjusted to pH 2.5 using phosphoric acid; for the chiral analysis, the chosen cyclodextrin and CSA are dissolved at the desired concentrations in the same electrolyte solution. A 10 m*M* phosphate buffer solution (pH 8.0) was used as media for the derivatization reaction with ESB reagent. The reagent solution (ESB) was prepared in methanol (0.15 mg/ml) and was found to be stable for at least 3 days at room temperature. Stock solutions of the thiol compounds were daily prepared in water at 1 mg/ml concentration for the direct analysis and at 0.8 mg/ml for the analysis after derivatization procedure. Internal standard solutions of tryptophan and *S*-methyl-L-cysteine were prepared in water at a concentration of 0.3 mg/ml and 0.5 mg/ml, respectively; in the final solutions (calibration graphs and sample analysis) the concentrations were as follow: 0.021 mg/ml of tryptophan in the analysis after derivatization and 0.3 mg/ml and 0.05 mg/ml of *S*-methyl-L-cysteine in the direct analysis of D-penicillamine and penicillamine disulfide, respectively.

2.3. Apparatus

All separations were carried out using a ^{3D}CE capillary electrophoresis system (Hewlett-Packard, Waldbronn, Germany), equipped with a diode array

detector. The data were collected on a HP Vectra 486 personal computer using the ^{3D}CE-ChemStation software and macro programmes based on established algorithms. Fused-silica capillaries (Supelco, Milan, Italy) 48.5 cm (40 cm effective length)×50 μm I.D. were used. The applied voltage was held constant at 25 kV (analysis after derivatization) and 30 kV (underivatized analytes); the detection wavelength was adjusted to 220 nm (analysis after derivatization) and 200 nm (underivatized analytes). All the electrophoretic runs were carried out at 15°C; the samples were introduced hydrodynamically for 10 s (injection pressure 5 kPa). The capillary was conditioned prior to each run for 3 min with the separation electrolyte. Before the injection into the CE system, each solution (running buffer and sample solutions) was subjected to filtration through a membrane 0.2 μm GyroDisc (Orange Scientific, Waterloo, Belgium),

2.4. Derivatization procedure

Stock solutions of penicillamine or other thiols were diluted with 10 m*M* phosphate buffer pH 8.0 to give final concentrations of about 0.02 mg/ml, and a 0.5 ml aliquot was reacted with 1 ml of ESB solution. The mixture was maintained at room temperature for 2 min and acidified by adding 0.5 ml of 50 m*M* buffer phosphate solution pH 2.5. The internal standard solution was added (0.15 ml of tryptophan 0.3 mg/ml). The resulting final solution was then directly introduced into the CE system.

2.5. Calibration graphs

Calibration graphs were obtained by analysing the following series of standard solutions: (1) underivatized D-penicillamine solutions (0.2–0.6 mg/ml) containing 0.3 mg/ml of internal standard (*S*-methyl-L-cysteine); (2) penicillamine disulfide ($5.5\cdot10^{-3}$–$22.0\cdot10^{-3}$ mg/ml) containing 0.05 mg/ml of internal standard (*S*-methyl-L-cysteine) in the presence of a constant amount of D-penicillamine (0.75 mg/ml); (3) derivatized D-penicillamine ($9.3\cdot10^{-3}$–$37.2\cdot10^{-3}$ mg/ml) containing 0.021 mg/ml of internal standard (tryptophan).

The peak correct area (area/migration time) ratios (Y) of the analyte to internal standard were plotted against the corresponding analyte concentration (C) to obtain the calibration graphs.

2.6. Analysis of pharmaceutical formulations

Commercially available dosage forms (capsules) containing D-penicillamine were analysed. Other components of the matrix include lactose and magnesium stearate. An aliquot of the powder contained in the capsules was dispersed in water to give a sample solution of approximate 0.4 mg/ml of D-penicillamine for penicillamine analysis and 0.75 mg/ml of D-penicillamine for disulfide analysis. When the derivatization of the thiol function was applied, the final concentration of the sample was approximately 0.02 mg/ml for the determination of the D-penicillamine content and 1.5 mg/ml for the evaluation of optical purity (ESB concentration was 4.6 mg/ml in methanol). The peak correct area ratios of analyte to internal standard were determined and the amount of the analyte in each sample was calculated by interpolating the calibration curve.

3. Results and discussion

3.1. Derivatization reaction

The reaction of ESB (I) with aliphatic thiols was studied in our previous work [12]; it proceeds quickly at room temperature yielding an adduct of the thiolate nucleophile with the activate double bond of the ESB molecule (Fig. 1). Working at pH 8.0 with a [ESB]/[thiol] ratio higher than 3 it was possible to carry out the derivatization reaction in a very short time (2 min). Keeping a methanol:water ratio of 1 to 1 in the final reaction mixture, separation of the excess reagent was not observed and the clear reaction mixture was directly injected into the CE system. The stability of the ESB adduct in the reaction mixture was evaluated by measuring the peak correct area of a sample maintained at room temperature; after replicate injections in the CE system during the day, only a negligible reduction of peak correct area was observed.

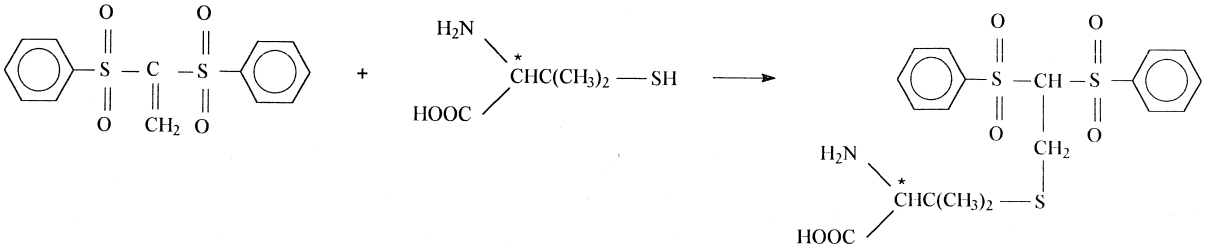

Fig. 1. Derivatization reaction scheme.

3.2. Separation conditions

The zwitterionic nature (α-amino-acidic structure) of all the analytes considered (underivatized and derivatized), provides wide opportunity in the choice of suitable running buffer to perform separation methods. Nevertheless, given the good stability displayed in acidic conditions by the thiol compounds [5] as well as the derivatization adducts with ESB [12], a pH 2.5 phosphate running buffer (50 m*M*) was chosen to carry out all the electrophoretic separations. Under the described conditions, the measured electroosmotic flow was very low (~$5\cdot 10^{-5}$ $cm^2\,V^{-1}\,s^{-1}$); therefore, applying the method based on the derivatization with ESB, the neutral reagent excess has long migration time without interferences. These electrophoretic conditions allowed us to achieve good reproducibility of the migration times for both the derivatized and underivatized penicillamine samples: RSD was 0.46% ($n=6$) for the migration time of ESB adduct and 1.39% ($n=6$) for D-penicillamine. The selectivity of the developed electrophoretic method was confirmed by the opportunity to have good separations of a mixture of derivatized thiols: ESB adduct from cysteine, glutathione, homocysteine and penicillamine were resolved in short analysis time with high resolution (Fig. 2). Moreover, the same electrophoretic conditions permit the complete resolution of the underivatized penicillamine from its disulfide (Fig. 3).

3.3. Analysis of underivatized D-penicillamine and its disulfide

Since the pharmacological effects of penicillamine and other thiol compounds are related to the presence of the sulfhydrilic group and giving the easiness of its oxidation to disulfides, the pharmacopeias establish a content limit of disulfide in pharmaceuticals containing thiols. In particular, the United States Pharmacopeia sets at 2% level the maximum content of penicillamine disulfide respect to the content in D-penicillamine, while FU IX (Italian Pharmacopeia), the British Pharmacopeia and the European Pharmacopeia more strictly fix this upper limit to 1% of the content of D-penicillamine [13–16]. Therefore, the proposed CE methods were applied to offer an analytical solution to this problem.

For quantitative applications, under the described experimental conditions, linear calibration graphs were obtained for D-penicillamine (concentration range 0.2–0.6 mg/ml) and for penicillamine disulfide (concentration range $5.5\cdot 10^{-3}$–$22.0\cdot 10^{-3}$ mg/ml) in the presence of 0.75 mg/ml of D-penicillamine, corresponding to 0.5–3% of the thiol drug content (Table 1). The detection was achieved by measuring the absorbance at 200 nm; the sensitivity expressed as LOD (limit of detection: signal-to-noise ration $[S/N]=3$), was found to be 90 μ*M* of D-penicillamine. The method was then applied to the analysis of a commercial formulation; the assay results were in good agreement with the declared content in D-penicillamine (found 100.3%, RSD 1.15%, $n=5$); moreover the analysed samples were found to be free from penicillamine disulfide (Fig. 4).

3.4. Analysis of D-penicillamine after ESB derivatization

The proposed derivatization reaction with ESB

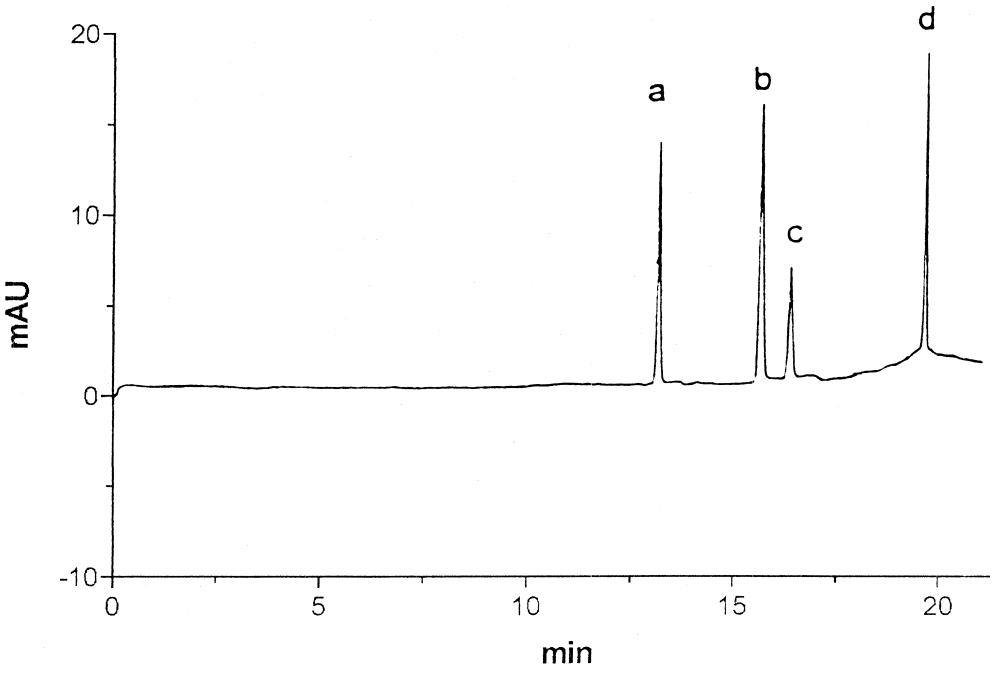

Fig. 2. Electrophoretic separation of a standard mixture of (a) homocysteine, (b) cysteine, (c) penicillamine, (d) reduced glutathione derivatized with ESB. Conditions: fused-silica capillary (48.5 cm total length); 50 m*M* potassium phosphate buffer (pH 2.5); separation voltage 25 kV; temperature 15°C; detection at 220 nm.

meets the requirements for rapid and simple analyses of aliphatic thiols without additional sample handling. Moreover, the lack of intrinsic sensitivity in the determination of underivatized thiols can be overcome with the introduction of the chromophore of ESB. Using the same background electrolyte described for the direct UV analysis of D-penicillamine and its disulfide, a linear calibration graph was obtained at 220 nm wavelength (molar absorptivity $\epsilon \sim 20\,000$ mol^{-1} cm^{-1}) in the presence of tryptophan as internal standard (Table 1). Analysis of commercial samples was performed leading to results in agreement with those obtained by direct analysis: (D-penicillamine found 100.9%, RSD 2.91%, $n=5$). The LOD, evaluated by progressive dilution of derivatized sample solution until $S/N=3$ was reached, was found to be 2.5 μ*M* at 220 nm and 1.5 μ*M* at 200 nm. In other words the increase of sensitivity after derivatization was found to be in the order of about 50-times it compared to the direct analyses.

3.4.1. Chiral analysis

The addition of the thiol drugs to the reactive double bond of the ESB, does not produce a new chiral center because of the symmetry of the reagent molecule. This resulted in the opportunity for a wide choice in the separation conditions in order to perform thiols analysis: no diastereomeric pairs are produced and even in front of high efficiency separation, peak splitting phenomena are avoided. Further, since the derivatized product keeps the chirality features of the analyte, associated to a better adaptability to the cyclodextrins cavity, enantioseparations can be performed. Thus, under the same electrophoretic conditions described for the analysis of underivatized thiol, we have tested the most common neutral cyclodextrins as chiral selector in an attempt to resolve the two enantiomers of derivatized D,L-penicillamine. While DMCD completely failed in the enantioresolution, γCD, βCD and HPCD showed ability in the enantiorecognition of the derivatized enantiomers. Nevertheless, even in the presence of

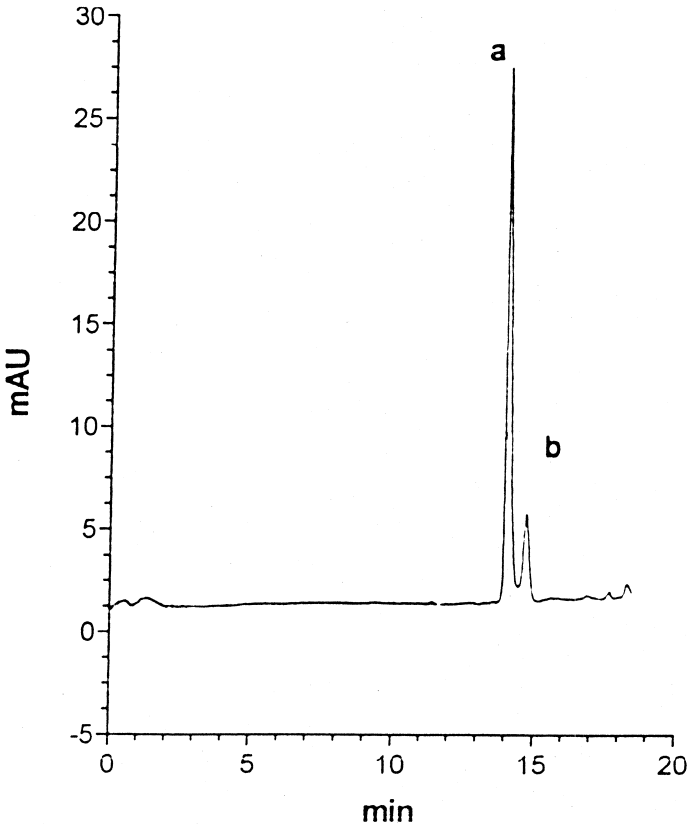

Fig. 3. CE separation of D-penicillamine (a) and penicillamine disulfide (b). Penicillamine disulfide is present at 10% level in a standard solution of D-penicillamine. Conditions: fused-silica capillary (48.5 cm total length); 50 m*M* potassium phosphate buffer (pH 2.5); separation voltage 30 kV; temperature 15°C; detection at 200 nm.

high concentrations of these chiral selectors, only partial separations were reached and the maximum resolution value (R_s=1.3) was obtained using 10 m*M* HPCD.

In order to get baseline enantioseparation of the studied compounds the combined use of a chiral ion-pairing reagent (IPR), CSA, was tried. Jira and Bunke reported the ability of such an IPR to improve chiral separations of analytes with a dialkyl-aminoethanol structure using βCD containing running buffer solution [17,18]. Although the derivatized D,L-penicillamine does not completely respond to the requirements mentioned above, we observed a definite increase in the enantioresolution when 30 m*M* of CSA was added to the running buffer. According to the proposed mechanism based on the formation of ion pairs between analyte and CSA, the electrolyte pH was kept at 2.5. In the absence of any

Table 1
Calibration graphs: $y = mx + q$; 95% confidence interval for slope (m) and intercept (q), (n=5)

Analyte	m (SD)	q (SD)	r	Conc. range (mg/ml)
D-Penicillamine	2.574 (0.172)	0.230 (0.071)	0.998	0.2–0.6
Penicillamine disulfide	5.559 (0.173)	−0.001 (0.004)	0.999	$0.55\cdot10^{-2}$–$2.2\cdot10^{-2}$
Derivatized D-penicillamine	0.065 (0.0001)	0.037 (0.014)	0.999	$0.93\cdot10^{-2}$–$3.72\cdot10^{-2}$

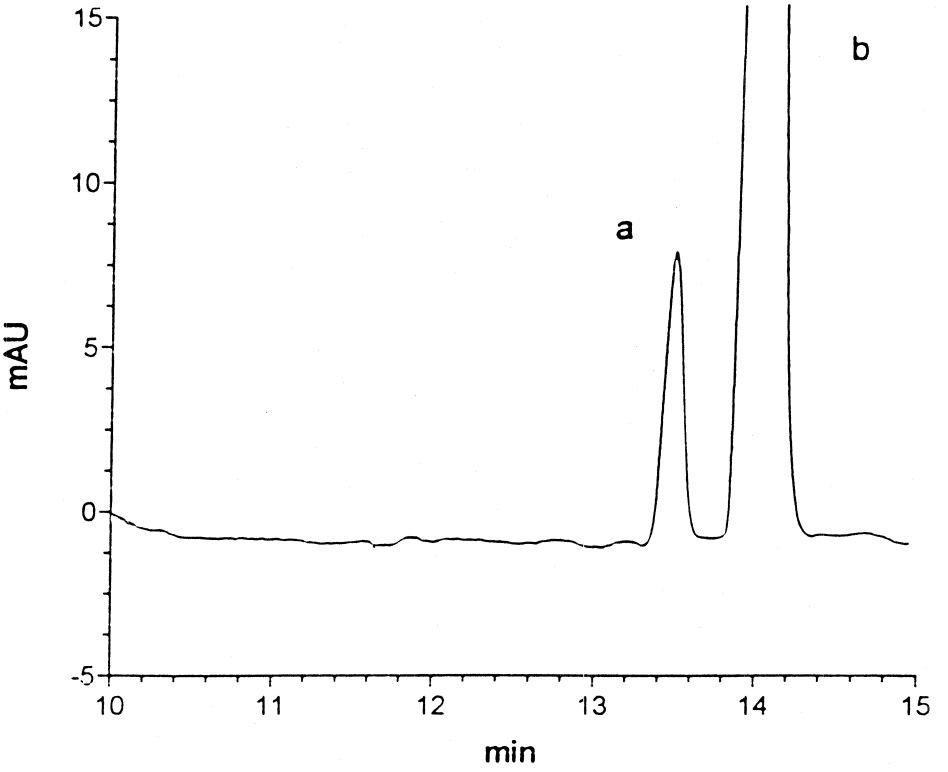

Fig. 4. Electropherogram from commercial sample: (a) methyl-*S*-cysteine (internal standard), (b) D-penicillamine. Conditions as in Fig. 3.

additive, no more than 16 m*M* (25°C) concentration in water can be reached for βCD: a suggestive increasing of its aqueous solubility in the presence of CSA (20–40 m*M*) was observed indicating the existence of interaction between IPR and cyclodextrin and subsequent improvement of the enantioresolution was obtained. For an high, fixed concentration of cyclodextrin, the increase in the CSA concentration gave a reduction in the migration time; the best compromise between resolution and analysis time, was found to be 35 m*M* βCD and 30 m*M* CSA. Under these experimental conditions a baseline separation (R_s=1.7) of the enantiomers was achieved and the migration time was reproducible (RSD 1.3%, n=5) (Fig. 5). When HPCD (50 m*M*) was used in the presence of the optimized concentration value of CSA, good but not complete enantioseparation was reached. Analyzing nonracemic mixtures of ESB-derivatized penicillamine in the optimized conditions, the migration order of enantiomers was established; the adduct with the L-penicillamine enantiomer was found to move toward the detector with slower velocity than the D-isomer. The good peak symmetry allows very low levels of this enantiomer to be detected, therefore the method resulted useful for the control of the optical purity of D-penicillamine (Fig. 6). More precisely, a linear calibration graph was constructed analyzing nonracemic mixtures constituted by a constant amount of D-penicillamine (1.5 mg/ml) and containing L-penicillamine, the toxic enantiomer, in the range of 0.5–2% level. After derivatization reaction with ESB, the reaction mixtures were subjected to enantioseparations by the developed CE method; the peak correct area values (y) were plotted against the concentration level (C) to obtain the equation $y=(0.41386\pm0.01183)x+(-0.03164\pm0.01145)$, ($r=0.999$; $n=6$). Although an overload of the main compound (D-penicillamine) was necessary to evaluate the detection limit, the achieved baseline sepa-

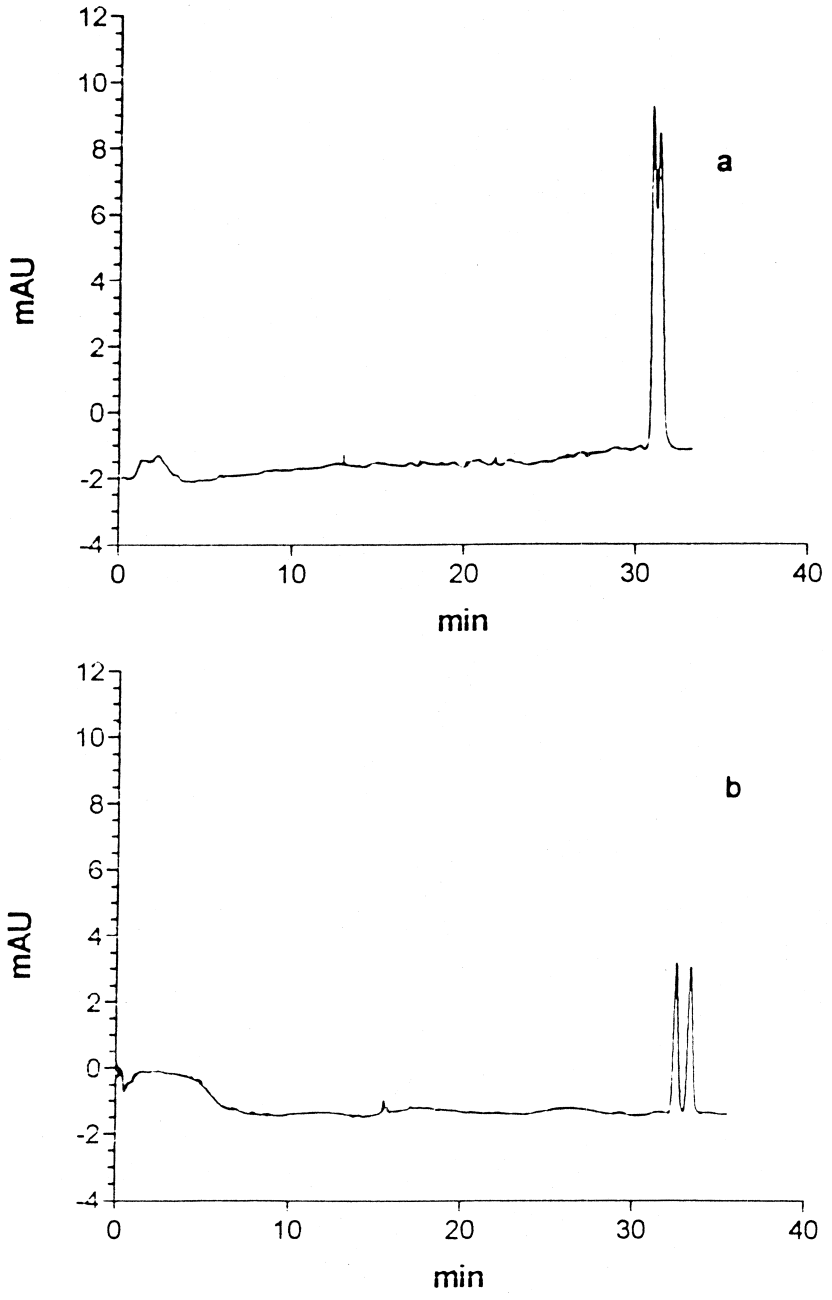

Fig. 5. Enantioresolution of D,L-penicillamine derivatized with ESB. Conditions: fused-silica capillary (48.5 cm total length); separation voltage 20 kV; temperature 15°C; detection at 220 nm; 50 m*M* potassium phosphate buffer (pH 2.5) containing (a) 5 m*M* βCD; (b) 35 m*M* βCD, 30 m*M* CSA.

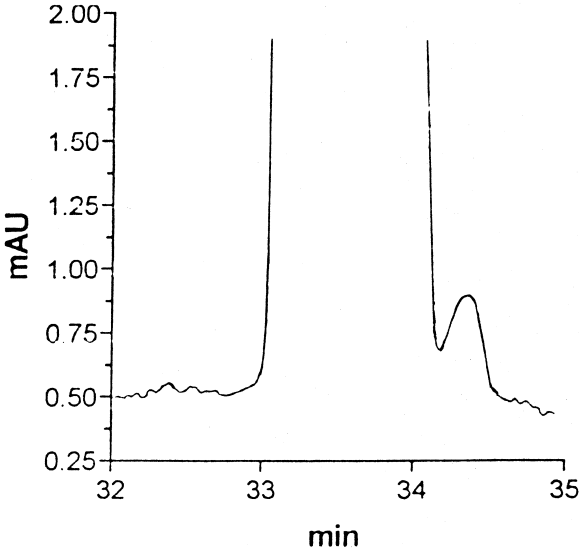

Fig. 6. Electropherogram from a derivatized (using ESB) mixture of D-penicillamine containing 0.5% of L-penicillamine. Conditions as in Fig. 5(b).

ration makes it possible to detect a ratio of 0.3:99.7 (L:D).

The method was then applied to check the optical purity of a commercial formulation and no significant levels of the toxic L-penicillamine enantiomer were detected.

4. Conclusion

Capillary zone electrophoresis was found to be suitable for the complete analysis of penicillamine in pharmaceutical samples. The simple direct determination of D-penicillamine and its disulfide is a good alternative to existing HPLC methods giving a short analysis time and permitting avoidance of the chromatographic problems due to the high polarity of such analytes.

The opportunity to apply a simple and rapid derivatization reaction to the analysis of the thiol drug is presented: besides increasing the sensitivity, the latest approach showed usefulness in the development of a method able to evaluate the optical purity of D-penicillamine. The results described here confirm that derivatization reactions could find interesting applications in CE.

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