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Enantioseparation of atropine by capillary electrophoresis using sulfated β-cyclodextrin: application to a plant extract

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Abstract

A capillary zone electrophoresis (CZE) method, with sulfated β-CD as chiral selector, was optimized by means of an experimental design for the enantioseparation of atropine. In this study, a central composite design was used and the following factors were varied simultaneously: buffer concentration, buffer pH and sulfated β-CD concentration. The resolutions between littorine and its positional isomer ((−)-hyoscyamine) and between atropine enantiomers, as well as the separation time and generated current were established as responses. A model was obtained for each response by linear multiple regression of a second-degree mathematical expression. The most favorable conditions were determined by maximizing the resolution between atropine enantiomers and by setting the other responses at threshold values. Successful results were obtained with a 55 m*M* phosphate buffer at pH 7 in the presence of 2.9 m*M* sulfated-β-CD at 20°C and 20 kV. Under these optimized conditions, a baseline separation of littorine and atropine enantiomers was achieved in less than 5 min. Finally, the method allowed the enantiomeric separation of atropine in a pharmaceutical formulation and was also found to be suitable for the enantiomeric purity evaluation of (−)-hyoscyamine in plant extracts, in relation with the extraction procedure. It was demonstrated that supercritical fluid extraction induced less racemization than classical liquid–solid extraction procedures.

Keywords: Enantiomer separation; Plant materials; Alkaloids; Atropine; Cyclodextrins; Hyoscyamine; Tropane alkaloids

1. Introduction

In many drugs possessing an asymmetric center, the optical isomers exhibit a different pharmacological activity. In the case of therapeutic substances isolated from a biological source (such as alkaloids from plants), optical impurities can exist. In some cases, they are naturally present in a small amount while, in other instances, partial racemization occurs during the isolation and purification steps [1].

Atropine, a tropane alkaloid of medicinal interest, is found in plants of the Solanaceae family [2,3], such as *Atropa*, *Datura*, *Duboisia* and *Hyoscyamus*. Currently, atropine is used for its antispasmodic activity on the gastrointestinal tract, as a preanesthesic agent and in ophthalmic solutions. Atropine is a racemic mixture of optical isomers and is also referred to as (±)-hyoscyamine. However, it is well known that (−)-hyoscyamine is often more active than (+)-hyoscyamine [4].

Capillary electrophoresis (CE) has become an

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interesting alternative to classical chromatographic techniques, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC), for the separation of enantiomers [5]. Indeed, CE provides several advantages: short analysis time, high resolution power and low operational cost. Enantioseparations are generally performed by adding a chiral selector to the running buffer. Various additives acting as chiral selectors have been reported in the literature, such as cyclodextrins (CDs), crown ethers, proteins, antibiotics, bile salts and chiral micelles [6–8]. Nevertheless, CDs are the most widely used selectors in chiral CE.

Neutral CDs and derivatives presenting various functional groups have been developed to induce different stereoselective interactions and enhance selectivity. Publications have reported enantioseparation of atropine using neutral CDs but without a complete resolution [9,10]. In other cases, enantioseparation is achieved with neutral β-CD derivatives, such as HP-β-CD and TM-β-CD, but with relatively long migration times [11–13].

Charged CDs, first introduced by Terabe [14], represent an interesting alternative for CE [15–21]. The growing number of published applications demonstrates the great potential of charged CDs, such as, sulfated, sulfobutylether, phosphated and carboxymethylated CDs, for the enantiomeric separation of chiral drugs. A chiral selector with its own mobility opposite to the electroosmotic flow, shows strong resolving power, also at very low concentration. To the best of our knowledge, only two papers have reported the enantioseparation of atropine with anionic β-CDs [21,22]. However, these methods were not applied to real plant extracts and no separation of atropine and of its positional isomer, littorine, has been reported.

In relation with our investigations concerning the use of CE for the analysis of tropane alkaloids both in pharmaceutical preparations [23,24] and in plant extracts [25,26], this paper describes the optimization of atropine enantioseparation in the presence of littorine. Indeed, littorine is frequently encountered in Solanaceae plants and particularly in hairy roots. The method, optimized by a central composite design, was applied to the enantioseparation of atropine in an ophthalmic solution as well as to the evaluation of (−)-hyoscyamine racemization in hairy root extracts in relation with the extraction procedure. For this purpose, two liquid–solid extractions and a supercritical fluid extraction procedures were investigated.

2. Experimental

2.1. Chemicals and samples

Atropine sulfate and (−)-hyoscyamine free base were purchased from Sigma (St. Louis, MO, USA). Littorine was a gift of Dr K. Shimomura (Tsukuba Medicinal Plant Research Station, Japan). β-CD was obtained from Fluka (Buchs, Switzerland). α-CD, methyl-β-CD, dimethyl-β-CD, trimethyl-β-CD and carboxymethylated-β-CD were obtained from Cyclolab (Kölliken, Switzerland), whereas γ-CD was provided by Celdex (Tokyo, Japan). Hydroxypropyl-β-CD (HP-β-CD) was supplied by Roquette (Lestrem, France) and Sulfobutylether-β-CD (SBE) from CyDex (Overland Park, KS, USA). Two sulfated-β-CD, with different degree of substitution (DS) (Batch I, DS=16; Batch II, DS=13), were obtained from Aldrich (Buchs, Switzerland). All chemicals were of analytical grade: di-sodium hydrogen phosphate, sodium dihydrogen phosphate, tris(hydroxymethyl)-aminomethane (Tris), phosphoric acid, sulfuric acid, methanol (MeOH), ethanol (EtOH), chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2), diethyl ether (Et_2O) and concentrated ammonia (NH_4OH) were purchased from Fluka. Atropine (1%, w/v) ophthalmic solution was supplied by Ciba Vision (Niederwangen, Switzerland). Ultrapure water, provided by a Milli-Q RG unit from Millipore (Bedford, MA, USA), was used for standard and sample preparation. Electrolyte solutions were filtered through a 0.20-μm microfilter (Supelco, Bellefonte, PA, USA) before use.

2.2. Instrumentation and electrophoretic procedure

Electrophoresis was carried out on a Hewlett-Packard capillary electrophoresis system (Waldbronn, Germany) equipped with an on-column diode-array detector (DAD). The capillary (Composite Metal Services, Hallow, Worcestershire, UK) was 48.5 cm long (40 cm effective length) with a

50-μm internal diameter (I.D.). An alignment interface containing an optical slit matched to the internal diameter was used. Detection at 40 cm from the point of sample introduction was set at 195 nm with a bandwidth of 10 nm. A CE Chemstation (Hewlett-Packard) allowed instrument control, data acquisition and data handling.

All experiments were carried out in cationic mode (anode at the inlet and cathode at the outlet). The capillary was thermostated at 20°C and a constant voltage of 20 kV, with an initial ramp of 0.2 min, was applied during analysis. Sample injections (ca. 16 nl injection volume) were achieved with pressure mode for 10 s at 50 mbar.

The carrier buffer was obtained by dissolving a suitable amount of sulfated β-cyclodextrin in a solution prepared by mixing di-sodium hydrogen phosphate and sodium dihydrogen phosphate solutions in an appropriate ratio to give a suitable pH value between 5 and 7. All buffers were prepared using the Phoebus software 1.0 (Centre Analyse, Orleans, France). Each day, the capillary was rinsed with 0.1 *M* sodium hydroxide for 10 min followed by water for 5 min. Before each run, the capillary was equilibrated with the running buffer for 3.5 min. Before its first use, the capillary was flushed with 0.1 *M* sodium hydroxide for 30 min followed by water for 15 min.

As electrolysis can alter the running buffer and subsequently change the electroosmotic flow (EOF), a replenishment system was also applied to maintain high reproducibility. Prior to each sequence, two blank injections were performed to stabilize the capillary wall surface and allow the buffer and sample solutions to reach a constant temperature on the autosampler tray.

2.3. Standard and sample solutions

Stock standard solutions of atropine, (−)-hyoscyamine and littorine were prepared by dissolving each compound in methanol (1.0 mg/ml) and were suitably diluted in water to obtain standard solutions at a final concentration of 0.1 mg/ml. Water was used as a dissolving solvent and allowed sample stacking which was effective in enhancing sensitivity (increasing peak height) by on-column preconcentration of the sample within the capillary.

The tested ophthalmic solution was constituted of a racemate mixture of atropine, benzalkonium chloride as preservative, methylhydroxypropylcellulose as ophthalmic lubricant and sodium chloride as isotonic agent. This solution was only diluted with water to the desired concentration (ca. 0.1 mg/ml).

The culture of *Hyoscyamus albus* hairy roots was established after infection with *Agrobacterium rhizogenes,* as described elsewhere [27].

2.4. Extraction and isolation of hyoscyamine from hairy roots

Three extraction modes were tested: (1) a liquid–solid extraction with sonication in $CHCl_3$–MeOH–concentrated NH_4OH (15:5:1, v/v/v), as reported elsewhere [28]; (2) a supercritical fluid extraction (SFE) using pressurized carbon dioxide at 150 bar modified with 20% MeOH at 85°C, as already described [29]; and (3) a liquid–solid extraction according to the Swiss Pharmacopeia procedure [30] which consists in a percolation of alkaloids with alkaline solutions.

In all cases, the solvent was evaporated to dryness. The residue was suitably diluted with water in order to obtain (−)-hyoscyamine at a final concentration of about 0.1 mg ml^{-1} and was filtered before use.

2.5. Computation

Coefficients for the regression models and optimized conditions were calculated with NEMROD (LPRAI, Marseille, France) and MATLAB (version 4.2c.1) software packages. Response surfaces were drawn with Microsoft Excel (version 7.0).

3. Results and discussion

The enantioseparation of atropine as well as the separation of littorine and (−)-hyoscyamine were optimized with an experimental design. In previous CE investigations, the separation of these two positional isomers was possible by adding an organic modifier to the micellar buffer [25] or by using nonaqueous CE [26]. In our study, this separation was achieved through complexation with cyclodex-

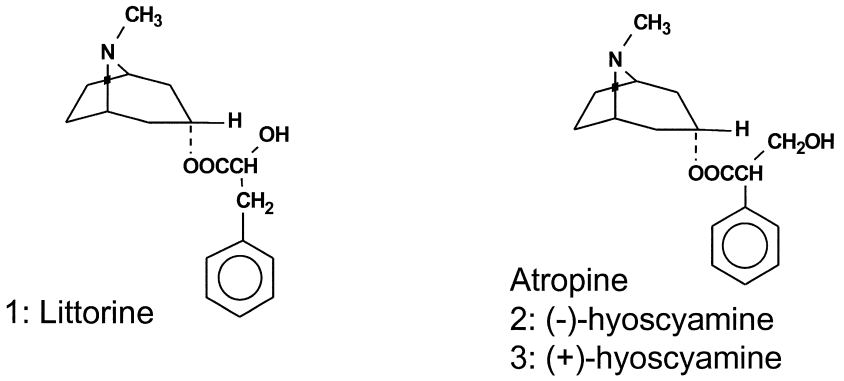

Fig. 1. Structure of investigated alkaloids.

trins. The chemical structure of the investigated compounds is given in Fig. 1.

3.1. Choice of the chiral selector

Despite the large number of applications concerning the use of CDs and their derivatives, there is no general rule for linking the stereoselectivity of these selectors to their chemical structure. Thus, for CE separation of chiral drugs, the choice of a suitable chiral selector remains of crucial importance. Therefore, various CDs and derivatives, namely α-CD, β-CD, γ-CD, HP-β-CD, methyl-β-CD, dimethyl-β-CD, trimethyl-β-CD were investigated at a concentration of 15 m*M* using a 100 m*M* Tris–phosphate buffer at pH 2.5, an applied voltage of 30 kV and a temperature of 25°C. Unfortunately, no chiral recognition was obtained under these conditions. However, as shown in Fig. 2, littorine and atropine were baseline resolved with all the investigated neutral cyclodextrins, except γ-CD. Furthermore, littorine migrated after atropine in all cases.

Prompted by the good results reported in the literature concerning the enantioseparation of some basic drugs, including atropine, in presence of anionic CDs [15,21,31–35], sulfobutylether-β-CD, carboxymethylated-β-CD and sulfated-β-CD were tested at a concentration of 5 m*M* using a 50 m*M* phosphate buffer at pH 6. Among the investigated charged CDs, sulfated-β-CD allowed the best resolution of atropine enantiomers, in agreement with recently published results [22]. Thus, this chiral agent was selected for subsequent investigations.

The degree of substitution (DS) of a cyclodextrin is of paramount importance in chiral separation by CE [36]. Two batches of sulfated β-CD from the same manufacturer but with different DS were investigated. Surprisingly, under the same electrophoretic conditions, atropine enantiomers and littorine migrated after the electroosmotic flow (EOF) with the highly substituted cyclodextrin, while they migrated in front of the EOF in the second batch (Fig. 3). The electrophoretic values for each batch are given in Table 1. In both cases, littorine and atropine enantiomers were baseline resolved. The second batch was selected for further method optimization.

3.2. Method optimization

The method was optimized using a central composite design, as already described [37,38]. Three relevant factors were simultaneously investigated: buffer concentration (X_1), buffer pH (X_2) and sulfated-β-CD concentration (X_3). Levels of the three experimental factors are listed in Table 2. In order to obtain short migration times and acceptable generated current, pH and sulfated β-CD concentration were kept below 7 and 3 m*M*, respectively.

The effect of each factor was examined by means of four responses: the resolutions (Rs_1) between littorine and (−)-hyoscyamine, and (Rs_2) between (−)-hyoscyamine and (+)-hyoscyamine, the analysis time measured as the migration time of the last migrating enantiomer ((+)-hyoscyamine in this case), as well as the generated current.

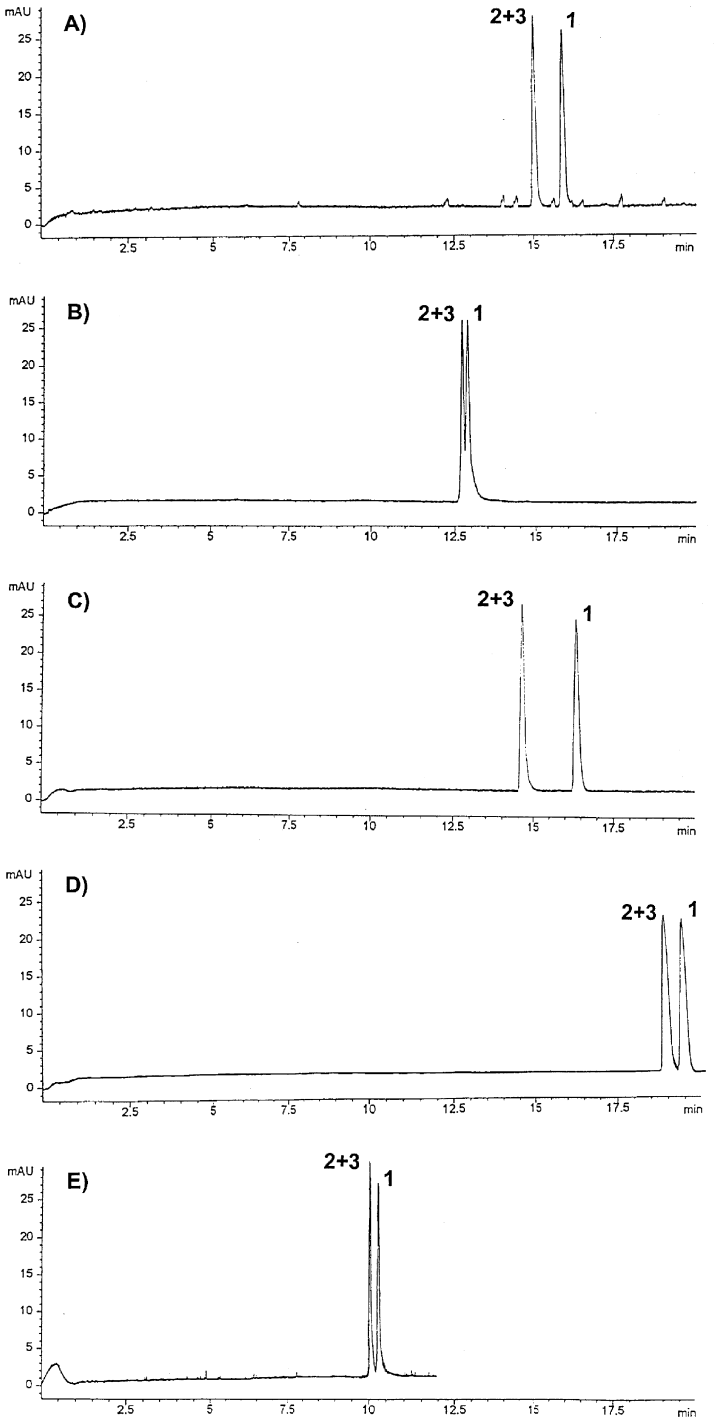

Fig. 2. Effect of cyclodextrin type on separation of two positional isomers, (−)-hyoscyamine and littorine. Buffer: 100 m*M* Tris–phosphate, pH 2.5, and 15 m*M* of (A) β-CD, (B) γ-CD, (C) HP-β-CD, (D) dimethyl-β-CD, (E) trimethyl-β-CD. Electrophoretic conditions: applied voltage, 30 kV; temperature, 25°C. Uncoated fused-silica capillary: L=48.5 cm, l=40 cm, I.D.=50 μm. Peak numbering is the same as in Fig. 1.

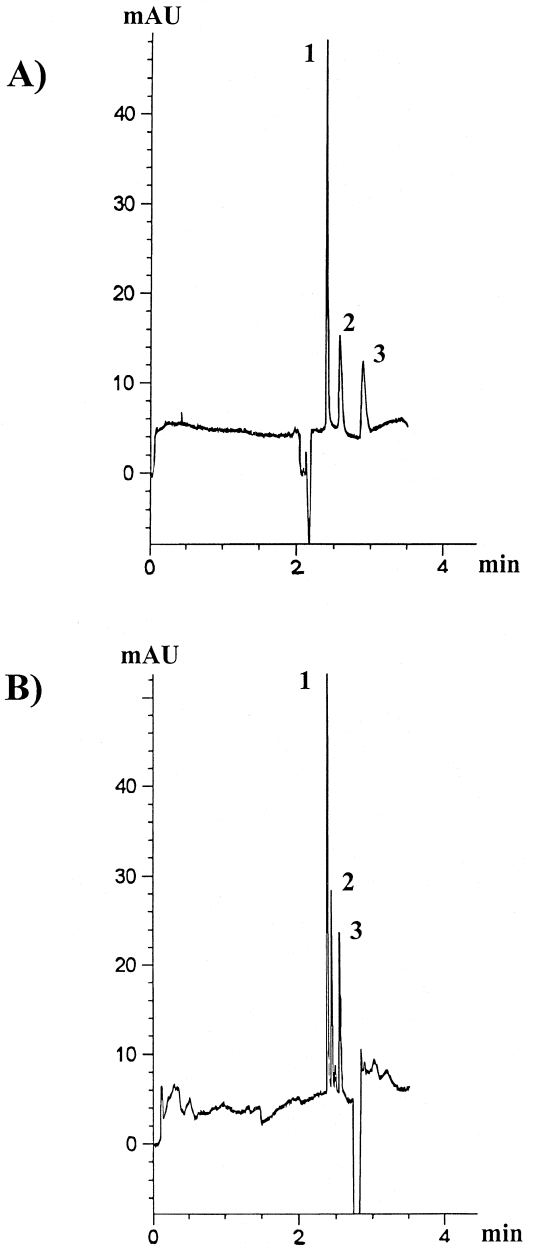

Fig. 3. Effect of sulfated β-CD degree of substitution on the electrophoretic behavior of investigated alkaloids: (A) batch I (DS=16), (B) batch II (DS=13). Buffer: 50 m*M* phosphate, pH 6, and 5 m*M* sulfated-β-CD. Electrophoretic conditions: applied voltage, 25 kV (i=77 μA for batch I and i=169 μA for batch II); temperature, 20°C. Uncoated fused-silica capillary: L=48.5 cm, l=40 cm, I.D.=50 μm. Peak numbering is the same as in Fig. 1.

Table 1
Comparison of some electrophoretic values obtained with two batches of sulfated-β-CD (conditions as in Fig. 3)

Sulfated-β-CD	Rs_1	Rs_2	Time (min)	RSD (%)
Batch I (DS=16)	3.19	4.67	2.89	3.02
Batch II (DS=13)	1.98	2.34	2.52	2.97

Rs_1, resolution between littorine and (−)-hyoscyamine; Rs_2, resolution between (−)-hyoscyamine and (+)-hyoscyamine; time, migration time of the last migrating atropine enantiomer; RSD represents the time relative standard deviation obtained by performing the injection in triplicate.

Table 2
Coded values of experimental factors

Level	X_1 Buffer (m*M*)	X_2 pH	X_3 Sulfated β-CD (m*M*)
−1	40	5	1
0	50	6	2
+1	60	7	3

A central composite design provides sufficient data for fitting a second-degree expression to each response. Coefficients of determination (R^2) and values of adjusted coefficients of determination (R^2_a) were higher than 0.97 and 0.95, respectively, indicating the good predictability of the model.

The mathematical model allowed to determine optimal conditions by maximizing the resolution between atropine enantiomers (Rs_2), and setting the other responses as threshold values. The resolution between littorine and (−)-hyoscyamine (Rs_1) was set at a value superior to 2 and the current was set at a value inferior to 70 μA to avoid excessive Joule effect. As a result, optimal conditions were reached with a 55 m*M* phosphate buffer at pH 7 and 2.9 m*M* sulfated-β-CD. All experiments were performed at 20°C and 20 kV.

The good predictability of the model was experimentally verified by the good agreement between the experimental and predicted responses under the optimized conditions (Table 3). The residual error value was contained within a range of $\pm 2\ SD_{exp}$ for each response. Under these optimal conditions, baseline separation of the three compounds, with resolutions higher than 2.9, was achieved in less than

Table 3
Comparison of predicted and measured results ($n=3$) under optimal conditions

	Time (min)	Rs_1	Rs_2	Current (μA)
2×SD	0.26	0.12	0.18	1.5
Predicted	4.32	2.97	4.53	70.0
Measured	4.33	2.91	4.54	69.5

SD represents the standard deviation obtained by performing central point in replicate ($n=6$).

5 min, as shown in Fig. 4. It is noteworthy that littorine migrated before atropine enantiomers, which allowed to quantify the three tropane alkaloids in plant extracts where littorine is generally present at low concentration.

Moreover, it is possible to draw surface responses (Rs_1 and Rs_2) as a three-dimensional plot of two factors (pH and sulfated-β-CD concentration), while keeping the buffer concentration constant at its optimal value (Fig. 5). For the sake of simplicity,

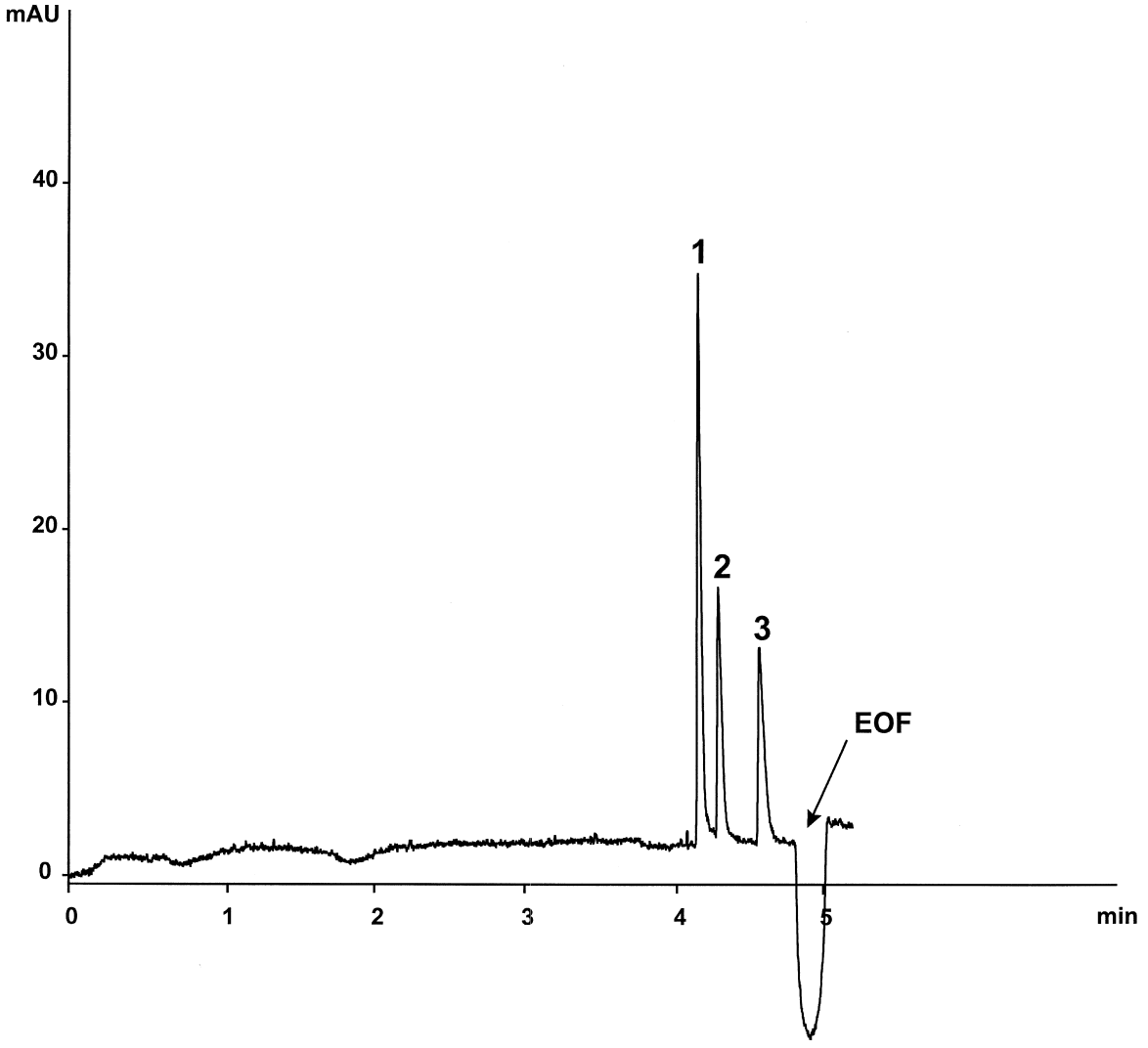

Fig. 4. Typical electropherogram of littorine, (−)-hyoscyamine and (+)-hyoscyamine (0.1 mg ml^{-1}) obtained by CZE using 55 m*M* phosphate at pH 7 and 2.9 m*M* sulfated β-CD. Applied voltage, 20 kV ($i=69.5$ μA); temperature, 20°C. Uncoated fused-silica capillary: $L=48.5$ cm, $l=40$ cm, I.D.=50 μm. Peak numbering is the same as in Fig. 1.

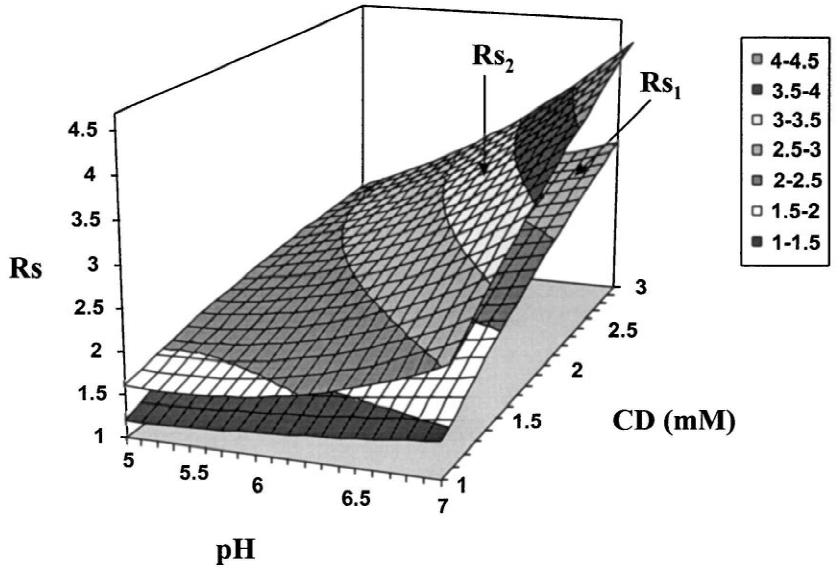

Fig. 5. Surface response plots for Rs_1 and Rs_2 as a function of the buffer pH and the sulfated β-CD concentration. The buffer concentration is set at its optimal level (55 m*M*).

other surface responses are not reported. The two response surfaces are almost similar and show an identical mechanism of interaction of sulfated β-CD with atropine and littorine. The method also proves to be robust in the tested domain.

3.3. Applications

Firstly, the optimized method was applied to the stereoselective analysis of atropine enantiomers in a commercial ophthalmic solution. The two enantiomers were clearly separated without interference from excipients and preservative (data not shown). Furthermore, as reported in Table 4, the enantiomeric ratio is not different to 1. Therefore, this method is appropriate for the quality control of ophthalmic solutions containing atropine enantiomers.

Secondly, in order to investigate the effect of the extraction procedure on (−)-hyoscyamine racemization, the optimized method was applied to the analysis of *Hyoscyamus albus* hairy root extracts. Three extraction modes were tested as described in the experimental part: (1) a liquid–solid extraction with sonication; (2) a supercritical fluid extraction; and (3) a liquid–solid extraction according to the Swiss Pharmacopeia. As reported in Table 4, it appears that SFE induces less racemization than liquid–solid extraction procedures since the calculated (+)-hyoscyamine contents are inferior to 2 and 8%, respectively. These results are in accordance with published papers which report the partial racemization of atropine during isolation and storage in solution [39,40]. Furthermore, it can be noted that besides atropine enantiomers littorine is found in this

Table 4
(+)-Hyoscyamine percentage in various solutions

	% (+)-Hyoscyamine[a]	RSD (%)
Standard	50.00	2.38
Ophthalmic solution	48.50	2.94
Extract 1	7.22	2.56
Extract 2	1.97	2.50
Extract 3	7.52	2.59

[a] The (+)-hyoscyamine percentage was calculated using normalized peak area ratio of atropine enantiomers.

Extract 1, liquid–solid extraction with sonication; Extract 2, supercritical fluid extraction; Extract 3, liquid–solid extraction according the Swiss Pharmacopeia. RSD represents the normalized peak area relative standard deviation obtained by performing the analyses in triplicate.

extract and is well separated from its positional isomer (Fig. 6). Determining this compound is very important although numerous publications dedicated to hyoscyamine dosage do not take into account this isomer and, thus, often overestimate the hyoscyamine content. Because littorine is generally the minor compound in plant extracts, its migration in front of (−)-hyoscyamine is of particular importance for quantitative purposes.

4. Conclusion

A capillary zone electrophoresis method has been developed for the enantioseparation of atropine as well as for the separation of littorine and atropine enantiomers. The electrophoretic behavior of the three alkaloids was critically affected by the substitution degree of the chiral selector.

A central composite design was applied for meth-

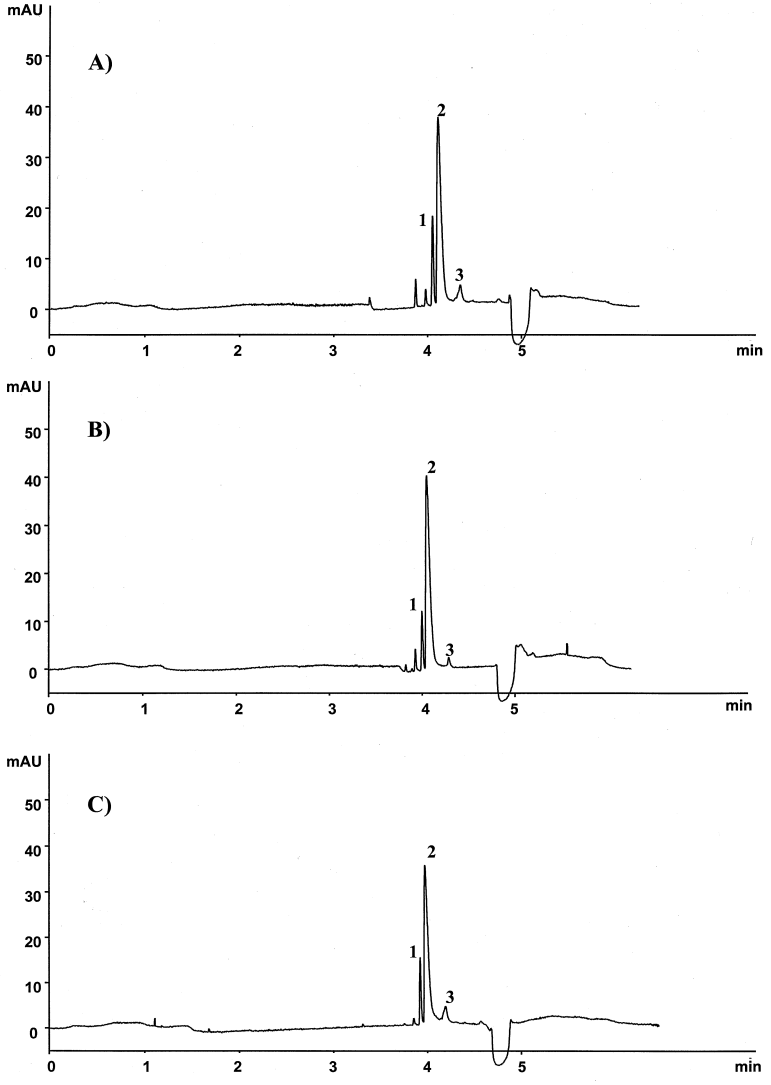

Fig. 6. Typical electropherograms of *Hyoscyamus albus* hairy root extracts analyzed under optimized conditions: 55 m*M* phosphate buffer, pH 7, 2.9 m*M* sulfated β-CD. (A) Liquid–solid extraction with sonication. (B) Supercritical fluid extraction at 150 bar, 85°C and with 20% MeOH. (C) Extraction according to the procedure described in Swiss Pharmacopeia. Other conditions are the same as in Fig. 4.

od optimization and three experimental factors were investigated: buffer concentration, buffer pH and sulfated-β-CD concentration. Four responses were evaluated: the resolutions between littorine and (−)-hyoscyamine, and between the two atropine enantiomers, the analysis time and the generated current. By using quadratic model equations, it was possible to determine the optimal conditions. Under these conditions, the separation of the three tested alkaloids was performed in less than 5 min with resolutions superior to 2.9.

Finally, the method was found suitable for a stereoselective analysis of atropine enantiomers in ophthalmic solution and for the evaluation of three extraction procedures on the racemization of (−)-hyoscyamine in hairy root extract. It was clearly demonstrated that supercritical fluid extraction induced less atropine racemization than investigated liquid–solid extraction procedures.

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