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Use of negatively charged cyclodextrins for the simultaneous enantioseparation of selected anesthetic drugs by capillary electrophoresis–mass spectrometry

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Dedicated to Professor Dr Gottfried Blaschke on the occasion of his 65th birthday

Abstract

The simultaneous enantioseparation of selected anesthetic drugs was studied by capillary electrophoresis (CE) in presence of three different negatively charged cyclodextrins (CDs). Among the chiral selectors tested, namely carboxymethyl, sulfobutyl ether and sulfated-β-CD, the latter appeared to be the most effective to achieve the enantiomeric resolution of the investigated compounds. Beside CD type, resolution was greatly influenced by the buffer pH, the molecular structure of the anesthetic compounds, CD concentration and temperature. The optimum electrophoretic conditions for the stereoselective analysis of the studied anesthetics were obtained with a poly(vinyl alcohol) coated capillary (48.5 cm total length × 50 μm I.D.), a 50 mM Tris–phosphate buffer at pH 2.5 containing 6 mg ml^{-1} of sulfated-β-CD, an applied voltage of 30 kV and a temperature of 30 °C. Under these optimized conditions, four drugs, namely bupivacaine, mepivacaine, ketamine and prilocaine, were simultaneously enantioresolved in less than 12 min. Furthermore, the method was applied to the stereoselective analysis of mepivacaine in a pharmaceutical preparation. Finally, the method was on-line coupled to electrospray ionization mass spectrometry using the counter current partial-filling technique.

Keywords: Enantioseparation; Capillary electrophoresis; Mass spectrometry; Partial-filling; Cyclodextrins; Anesthetic drugs

1. Introduction

Enantioselective analytical methods are nowadays necessary to meet the regulatory guidelines for the development and manufacture of chiral drugs [1,2]. In this context, high performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC) and gas chromatography (GC) are often used to conduct enantioseparation studies [3]. Besides these conventional chromatographic techniques, capillary electrophoresis (CE) and more recently capillary electrochromatography (CEC) have been

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found complementary alternatives to resolve drug enantiomers [4–7]. The high separation efficiency, the short analysis time, the ease of manipulation, the cost effectiveness and the use of extremely small volumes of sample and chiral selector are the main reasons for this success.

Enantioseparations by CE are generally performed in a direct separation mode by adding a chiral selector to the background electrolyte (BGE). The common chiral selectors used in CE are cyclodextrins (CDs), chiral crown ethers, proteins, antibiotics, bile salts, chiral micelles and ergot alkaloids [8–10]. Nevertheless, CDs are by far the most widely used selectors in CE [11,12]. Neutral CD derivatives presenting various functional groups have been developed to induce different stereoselective interactions and enhance separation selectivity. Recently, anionic substituted CDs, such as sulfated, sulfobutyl ether, phosphated and carboxymethylated CDs, as well as cationic CDs, such as quaternary ammonium β-CD, have become commercially available and have been applied as chiral selectors [13]. A negatively charged chiral selector, migrating in the opposite direction to the electroosmotic flow (EOF) and the basic drug substances, effectively increases the time window in the separation of free and fully complexed enantiomers. Moreover, anionic CDs proved to be effective for the enantioseparation of neutral [14] and even acidic [15] species. Dual CD systems [16], involving charged and electrically neutral CDs, have also been reported to significantly increase enantiomeric resolution.

Local anesthetic agents play an important role in modern anesthesiology. They are widely used for local anesthesia and for local management of major pain, either via central administration (spinal and epidural) or via peripheral administration [17,18]. These compounds possess a chiral center and are administered as a racemic mixture. Several studies showed that both enantiomers exhibit different pharmacological properties. In the case of bupivacaine, one of the most frequently used local anesthetic, both enantiomers are active as nerve blockers but the *R*-(+) is more toxic than the *S*-(−) form [19]. Therefore, selective and robust analytical methods for the enantioseparation of the anesthetic drug may prove to be useful in understanding pharmacokinetics and toxicity of anesthetic drugs following their administration. Chromatographic [20,21] and, more recently, electrophoretic [22–27] methods have been reported for the enantioseparation of anesthetics. In this context, various native and derivatized neutral CDs have been applied and it was found that methyl-β-CD was the most appropriate selector for the resolution of these enantiomers [22,23]. However, acceptable resolution was only achieved at a high CD concentration.

In the present study, capillary zone electrophoresis with three negatively charged CDs, namely sulfated (S-β-CD), sulfobutyl ether (SBE-β-CD) and carboxymethyl (CM-β-CD), was extensively investigated for the chiral separation of anesthetics including bupivacaine, mepivacaine, prilocaine and ketamine. Parameters such as buffer pH, chiral selectors type and concentration, as well as separation temperature, were studied in order to achieve the simultaneous enantioseparation of the investigated drugs. The optimized method was successfully applied to the enantioseparation of mepivacaine in injectable solutions. Finally, the potential of negatively charged CDs is also highlighted for the on-line coupling with ESI–MS, using the counter current partial-filling technique.

2. Experimental

2.1. Chemicals and reagents

All local anesthetic drugs, including bupivacaine, mepivacaine and prilocaine, were obtained in their hydrochloride form and were supplied by Sintetica (Mendrisio, Switzerland). Ketamine was purchased from Fluka (Buchs, Switzerland). Carboxymethyl-β-CD (DS 3.5), sulfated-β-CD (DS 13) and Sulfobutylether (DS 4.5) were purchased from Cyclolab (Budapest, Hungary) Aldrich (Buchs, Switzerland) and CyDex Inc. (Overland Park, KS, USA), respectively. All chemicals such as phosphoric acid, tris(hydroxymethyl)-aminomethane (Tris), acetic acid and sodium acetate were obtained from Fluka (Buchs,

Switzerland). The injectable solution was donated by Sintetica (Mendrisio, Switzerland). Ultra-pure water obtained by Milli-Q RG purification unit from Millipore (Bedford, MA, USA) was used for standard and sample preparation. Before use, electrolyte solutions were filtered through a 0.45-μm pore size filter (Millipore Corp., Milford, MA, USA) and degassed in an ultrasonic bath for 10 min.

2.2. Instrumentation

2.2.1. Capillary electrophoresis

CE data was generated by a HP ^{3D}CE system (Hewlett–Packard, Waldbronn, Germany) equipped with an on-column diode-array detector, an autosampler and a power supply able to deliver up to 30 kV. A CE CHEMSTATION software package (Hewlett–Packard) was used for CE and mass spectrometry instrument control, data acquisition and data handling. An Agilent Technologies (Waldbronn, Germany) polyvinyl alcohol (PVA) coated capillary of 48.5 cm (40 cm to the detector window) × 50 μm I.D. was used for all CE-UV experiments. This coated capillary was selected to reduce the EOF as well as the interactions between analytes or negatively charged selectors and silica capillary wall. An alignment interface, containing an optical slit matched to the internal diameter, was chosen and the detection wavelength was set at 200 nm with a bandwidth of 10 nm.

All experiments were carried out in cationic mode (anode at the inlet and cathode at the outlet). A constant voltage of 30 kV, with an initial ramping of 500 V s^{-1}, was applied during analysis. Sample injections (approximately 8 nl injection volume) were achieved with pressure mode for 5 s at 50 mbar.

The carrier buffer was obtained by dissolving a suitable amount of chiral selector in the BGE. Before use, the capillary was washed with 0.1 M phosphoric acid for 10 min, followed by water for 10 min. To achieve high migration time reproducibility, the capillary was washed between analyses with 10 mM phosphoric acid for 2 min, followed by water for 2 min and the buffer without chiral selector for 2 min, then equilibrated with the running buffer with chiral selector for 2 min.

2.2.2. Mass spectrometry

Electrospray mass spectrometry measurements were carried out in positive ionization mode and performed on a single quadrupole HP Series 1100 MSD (Hewlett–Packard, Palo Alto, CA, USA), which has an upper mass limit of 3000 amu. On-line coupling of the CE instrument to the mass spectrometer detector was achieved with a commercial co-axial sheath liquid interface (Hewlett–Packard), which is orthogonaly positioned at the MS entrance. CE–MS experiments were performed in a PVA coated capillary with 50 μm I.D. and 75 cm total length. To maintain a stable electrospray, a 20 mm portion of polyimide coating was removed from the outlet capillary edge. This procedure was found effective in providing better electrospray characteristics at the probe tip. The drying and nebulizing gases were both nitrogen. ESI capillary was set at $+4.0$ kV. Nebulizing pressure and drying gas flow rate were set at 4 psi and 4 l min^{-1}, respectively. Gas temperature was set at 150 °C and the fragmentor voltage at 50 V. A coaxial sheath liquid, consisting of isopropanol–water (50/50, v/v) in presence of 0.5% formic acid, was delivered by a Harvard Model 22 syringe pump (South Natick, MA, USA) at 3 μl min^{-1}. MS detection was carried out in the selected ion monitoring (SIM) mode for each positive molecular ion. Other ESI–MS conditions are given in the figure captions.

2.3. Buffer and sample preparation

2.3.1. Standard solutions

Stock standard solutions were prepared by dissolving each compound in methanol (1 mg ml^{-1}) and were suitably diluted in water to obtain the desired standard.

The tested injectable solution was constituted of a mepivacaine racemate and sodium chloride as isotonic agent. Before analysis, this solution was only diluted with water to the desired concentration (ca. 50 μg ml^{-1}).

2.3.2. Buffer solutions

Buffer solutions at different pHs (2.5–7.0) and fixed ionic strength (50 mM) were prepared with the help of the PHOEBUS software 1.0 (Centre Analyse, Orleans, France). Buffer solutions were always freshly prepared and filtered immediately before use.

2.4. Calculation

Resolution was calculated as:

$$R_s = 2\left(\frac{t_2 - t_1}{W_1 + W_2}\right) \quad (1)$$

where t_2 and t_1 are the migration times of second and first enantiomers, respectively, and W_1 and W_2 the peak width at the base line.

The electrophoretic mobility difference of a given analyte was calculated from the observed migration time:

$$\Delta\mu = \mu_1 - \mu_2 = \frac{L_t L_d}{V}\left(\frac{1}{t_1} - \frac{1}{t_2}\right) \quad (2)$$

Where μ_1 and μ_2 are the electrophoretic mobilities of first and second enantiomers, respectively, L_t is the total length of the capillary, L_d is the length from the injection end to the detector, V is the applied voltage.

3. Results and discussion

According to previous studies using negatively charged β-CD selector [28], a coated capillary was chosen for the enantioseparation of the selected basic compounds (Table 1) to prevent adsorption of the analytes on the fused silica capillary wall. The studied negatively charged CDs included sulfated, sulfobutyl ether and carboxymethyl-β-CD (Table 2). Some of these chiral selectors have already been reported for the enantioseparation of anesthetics, but to the best of our knowledge no systematic investigation has yet been conducted [29–31]. Since pure anesthetics enantiomers were not available, peaks assignation was not carried out.

3.1. Chiral CE-UV

3.1.1. Effect of pH and CD type on chiral resolution

Both buffer pH and concentration should be considered during the optimization of enantiomeric separations. In particular, the buffer pH significantly affects enantioseparation due to the concomitant changes of solute ionization state as well as EOF velocity. To investigate the effect of buffer pH on anesthetic drug enantioseparation, three different electrophoretic media, ranging from acidic to neutral pH, were selected, namely phosphoric acid, acetate and Tris. The selected BGE were used at 50 mM ionic strength. The buffer at a basic pH was not considered, since the investigated compounds are partially uncharged and that, upon complexation with CDs, their electrophoretic migration is dictated by the negatively charged resolving agents. Thus, they are not detected within acceptable time in positive separation mode.

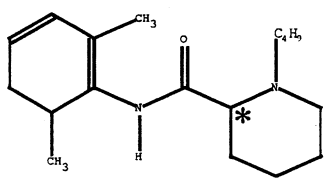
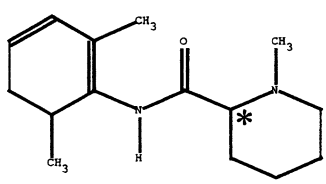
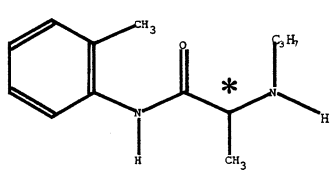
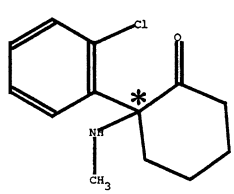
Since separation voltage was not considered as critical for enantioseparation, this parameter was held at 30 kV throughout the study, while the concentration of the chiral selector was maintained at 5 mg ml^{-1}.

As illustrated in Table 3, strong influences on enantioresolution were observed over the studied pH range (3.0–7.0). From these results, S-β-CD seems to be the most effective to achieve baseline resolution of the investigated anesthetics, which confirms the high potential of this chiral selector for the enantioseparation of basic compounds. Optimum enantioresolution of anesthetic drug substances was conducted at pH 3 (Table 3). At this pH, both selector and selectand were completely charged. Moreover, the EOF is negligible, resulting in high resolution for all investigated compounds. At higher pH, the selector is still fully charged while at pH 7, the analytes are partially ionized. Furthermore, residual EOF increased leading to lower resolution. Under the studied electrophoretic conditions, migration times and enantioresolution followed the same order (i.e. ketamine, prilocaine, mepivacaine and bupivacaine). It has to be noted that prilocaine

and ketamine, with secondary amine groups closer to the chiral center, exhibit higher resolution than mepivacaine and bupivacaine with tertiary amine groups. At pH 7, around the pK value of ketamine (7.5), the latter was mainly carried by the negatively charged CD, thus it migrated in the opposite direction and was not detected within reasonable time.

Due to the high substitution degree of the sulfated-β-CD (DS = 13 according to the manufacturer), high Rs values were obtained; this can be partially attributed to the strong electrostatic interactions between the positively charged drugs and negatively charged selector. Further hydrogen bonding and steric effects may also explain the high Rs values and wide enantioselectivity.

Table 1
Structure and pK_a values of the investigated anesthetics

Compound (Identification)	Structure	Molecular weight	pK_a
Bupivacaine (1)		288.4	8.1
Mepivacaine (2)		246.4	7.7
Prilocaine (3)		220.3	7.9
Ketamine (4)		237.7	7.5

*, Asymmetric carbon.

Table 2
Some characteristics of the investigated CDs

Type of CD	Manufacturer	Degree of substitution	Average mass (g mol^{-1})
Carboxymethyl-β-CD	Cyclolab	3.5	1309
Sulfobutylether-β-CD	Cydex	4.5	1976
Sulfated β-CD	Aldrich	13	2563

The same behavior was observed for the studied compounds in presence of sulfobutyl ether-β-CD (i.e. an increase in resolution with a decrease of buffer pH). However, if bupivacaine and mepivacaine enantiomers are well separated at pH 3, a loss of resolution was beheld in the case of ketamine and prilocaine. This behavior might be explained by the presence of the sulfobutyl group in the β-CD. Furthermore, at pH 3, migration times and enantioresolutions were different to those observed for S-β-CD. It has to be noted that above pH 4 no resolution was recorded for bupivacaine due probably to a strong interaction with SB-β-CD.

In comparison to sulfated and sulfobutyl ether-β-CDs which are negatively charged over the entire pH range, carboxymethyl-β-CD exhibits a pH dependent mobility [13,32]. In fact, due to the presence of a carboxylic group, this selector is mainly negatively charged above pH 5 while it behaves as an electrically neutral CD below pH 3. Therefore, in contrast to other CDs, increasing the pH from 3 to 7 has a favorable effect on the resolution of chiral anesthetics, as reported in Table 3. As expected, the improvement of enantioselectivity is partly attributed to the deprotonation of the carboxy groups of CM-β-CD. Thus, electrostatic interactions in the host–guest complexation seem to be critical.

From these results, it appears that enantioselectivity cannot be easily predicted from physicochemical properties of selector and selectand. Several interactions can be involved in the recognition mechanism such as inclusion complexation, ionic interactions, hydrogen bonding and hydrophobic interactions. Therefore, on the basis of resolutions achieved with the three investigated CDs, S-β-CD and acidic pH were chosen for subsequent investigations.

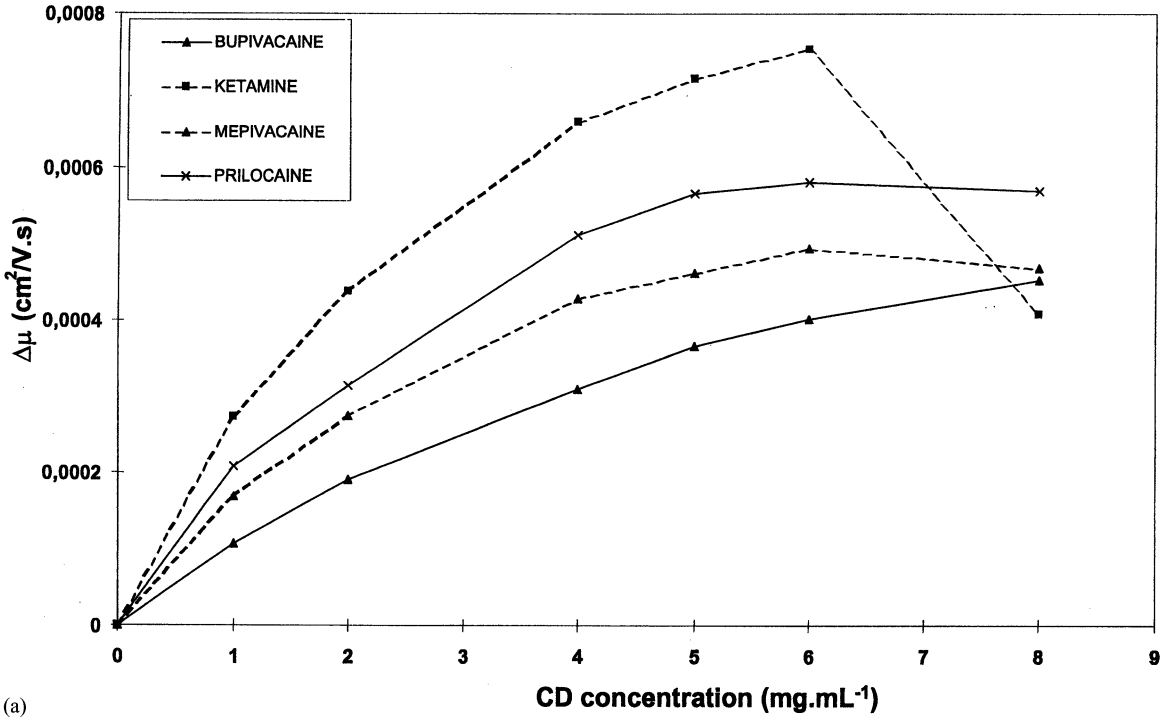
3.1.2. Effect of CD concentration on chiral resolution

Since CD concentration is an important parameter to optimize, S-β-CD concentration was further tested in 50 mM Tris–phosphate buffer set at pH 2.5. The chiral selector concentration was increased from 0 to 8 mg ml^{-1} since a more elevated CD concentration leads to excessive generated current with detrimental effects on both efficiency and enantiomeric resolution.

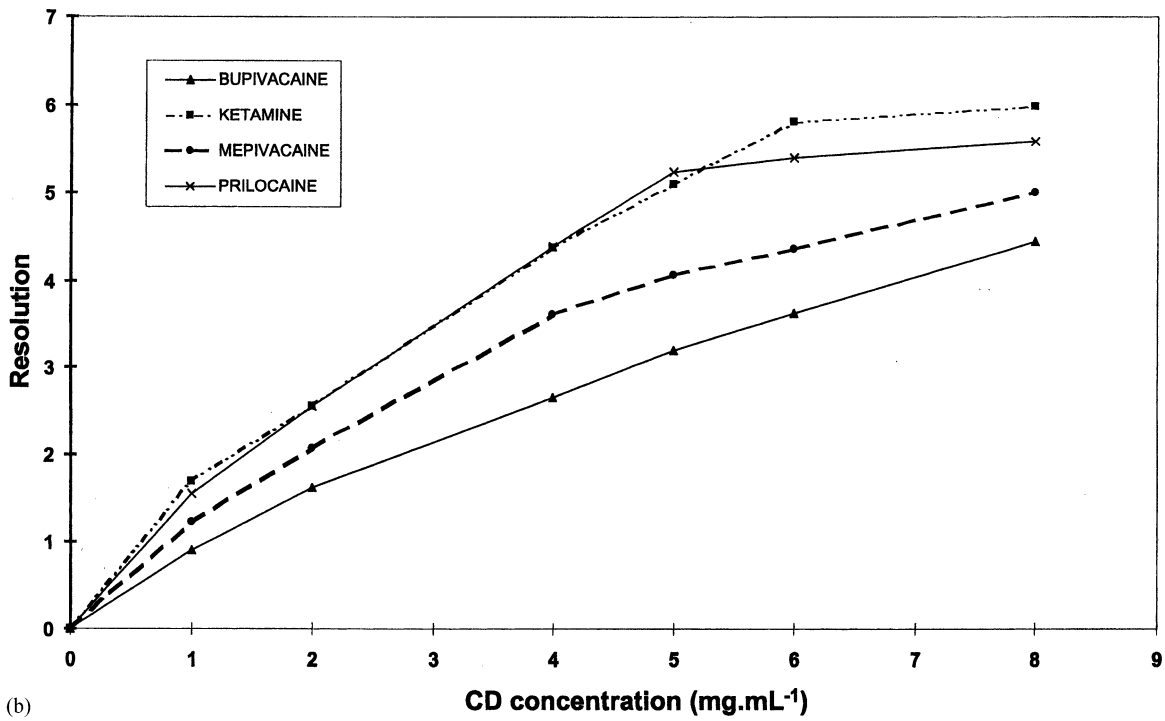
To explain CD concentration effect on enantioseparation, it is appropriate to report the apparent mobility difference between enantiomers, $\Delta\mu$, as a function of chiral selector concentration. According to Wren and Rowe model [33,34], $\Delta\mu$ often presents a maximum value which is dependent on enantiomer affinity for the chiral selector. As expected, migration times increased as a function of S-β-CD concentration (data not shown). It has to be noted that, within the studied concentration range, all investigated anesthetic drugs present an optimum value around 6 mg ml^{-1}, except bupivacaine for which no optimum was reached (Fig. 1a). These results indicate that bupivacaine enantiomers have a lower affinity for S-β-CD than other studied drugs.

As shown in Fig. 1b, resolution was improved by increasing CD concentration. However, except for bupivacaine and mepivacaine, resolution, according to Wren and Rowe model, was impeded at higher CD concentration. It is noteworthy that an optimum value of $\Delta\mu$ does not give a maximum resolution because the latter is not only dependent on apparent mobility difference.

Due to the limited studied concentration range of the negatively charged selector, optimum resolution of bupivacaine and mepivacaine enantiomers could not be achieved. Therefore, a concentration of 6 mg ml^{-1} was selected as a



(a)



(b)

Fig. 1. Effect of sulfated-β-CD concentration on, (A) apparent electrophoretic mobility difference; (B) resolution. Running buffer, 50 mM Tris–phosphate at pH 2.5 containing a suitable amount of sulfated-β-CD. PVA coated capillary (48.5/40 cm) × 50 μm I.D. Voltage, 30 kV; temperature, 20 °C. Pressure injection, 50 mbar for 5 s.

good compromise for good resolution, high efficiency and short migration time.

3.1.3. Effect of temperature on simultaneous enantioseparation

Under the above conditions, simultaneous enantioseparation of the studied anesthetic drugs was not possible, due to interference of prilocaine enantiomers with those of mepivacaine. However, numerous parameters, such as buffer viscosity, pK_a and complexation constant of the solute, are directly affected by temperature [35]. Therefore, enantioseparation can be tuned by modifying this parameter. As illustrated in Fig. 2, temperature considerably affects the chiral separation. In particular, migration time was reduced at higher temperature, while enantioselectivity decreased. Nevertheless, working at 30 °C resulted in the simultaneous separation of the eight enantiomers.

3.1.4. Application

Mepivacaine chloride is an amide type local anesthetic agent, commonly used for all types of infiltration and regional nerve block anesthesia. It is marketed as a racemic mixture of R(+) and S(−) mepivacaine. Both enantiomers differ in their biological activity with the S(−) form being biologically more active than the R(+) mepivacaine [27]. The optimized method was applied to the stereoselective analysis of mepivacaine enantiomers in a pharmaceutical preparation containing mepivacaine racemate and sodium chloride as isotonic agent. Both enantiomers were clearly separated without interference from excipients (data not shown).

Repeatability (within-day precision) of the method was determined by performing replicate injections ($n = 6$) of a 50 μg ml^{-1} solution containing racemic mepivacaine. In Table 4, relative standard deviation (R.S.D.) values are given for migration times and normalized peak area of mepivacaine enantiomers. In all cases, R.S.D. was lower than 0.5% for migration time and 3% for normalized peak area. Therefore, this method is appropriate for the quality control of pharmaceutical solutions containing mepivacaine enantiomers.

4. Chiral CE–ESI–MS

To overcome the low sensitivity of CE coupled to UV detection, due to the short optical pathlength of the capillary, the on-line coupling of CE–ESI–MS has evolved as an attractive combination in terms of high sensitivity and selectivity as well as molecular structure elucidation [36–38]. However, the presence of CDs in the separation buffer can contaminate the MS source and negatively affect electrospray performances. To circumvent these limitations, the use of the partial-filling technique has been established as a powerful and efficient alternative [39–46]. This technique was first introduced by Valtcheva et al. [47] to improve the sensitivity of systems involving chiral selectors that elicit a strong detector re-

Table 3
Effect of buffer pH and CD type on chiral resolution

CD type	Buffer (pH)	Bupivacaine (Rs)	Mepivacaine (Rs)	Prilocaine (Rs)	Ketamine (Rs)
Sulfated β-CD	3	3.68	4.91	6.26	7.01
	5	2.21	2.83	3.03	5.24
	7	2.22	2.95	3.7	–
Sulfobutyl ether-β-CD	3	3.52	5.2	0.82	0.62
	5	–	2.94	0.61	0.59
	7	–	2.44	–	–
Carboxymethyl-β-CD	3	0.7	–	0.61	0.81
	5	1.55	0.65	0.97	1.95
	7	1.79	0.88	1.01	–

–, No resolution.

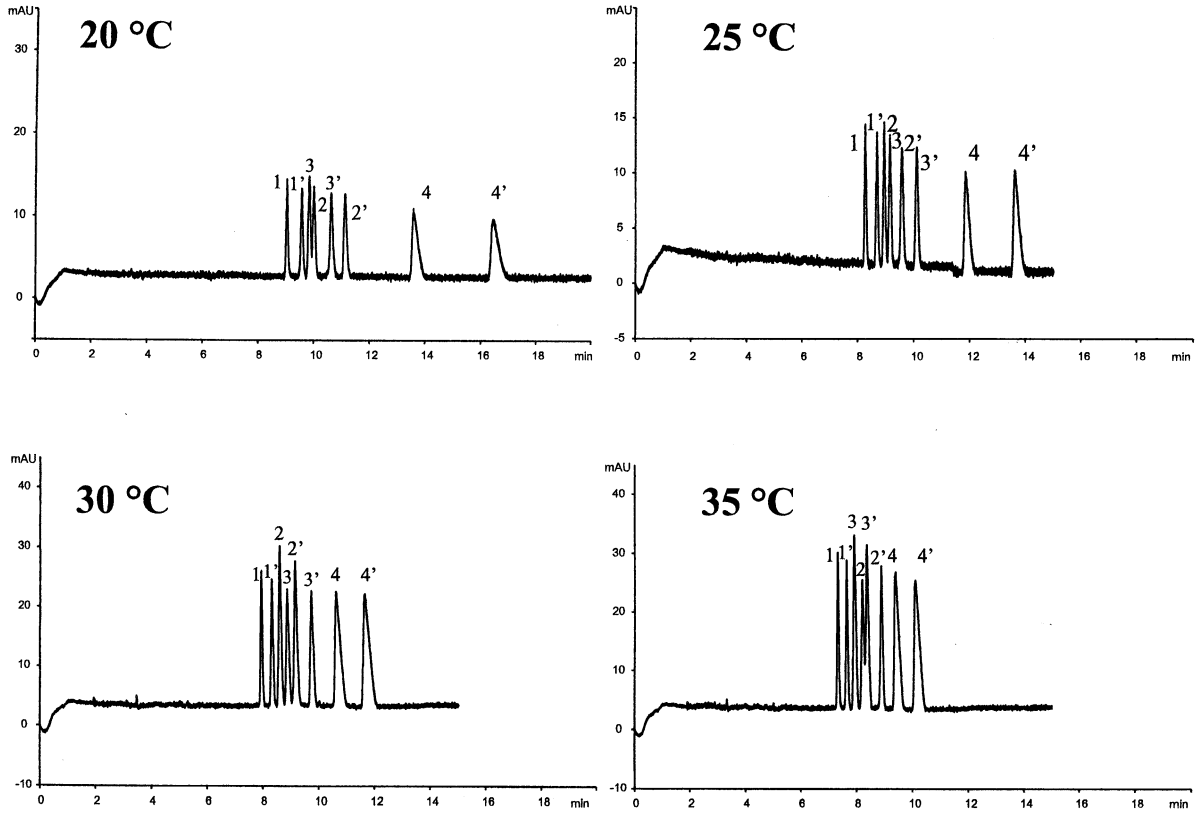


Fig. 2. Effect of temperature on the simultaneous separation and enantioseparation of anesthetic drug substances. Running buffer, 50 mM Tris–phosphate at pH 2.5, 6 mg ml^{-1} of sulfated-β-CD. PVA coated capillary (48.5/40 cm) × 50 μm I.D. Voltage, 30 kV. Pressure injection, 50 mbar for 5 s.

sponse, such as proteins or macrocyclic antibiotics. It requires filling the capillary with a BGE solution without a chiral selector, then partially introducing a solution containing a suitable amount of chiral selector to achieve enantioseparation, and finally injecting a sample. In the case of anesthetics drugs, which are positively charged at acidic pH, negatively charged selectors are generally appropriate since applying the electric field results in a counter-current process in which the chiral selector and the enantiomers migrate in opposite directions. In particular, it has been demonstrated that negatively charged CDs migrating opposite to the analyte in a counter current process decreased the negative effect of the nebulization pressure on the enantiomeric resolution [46].

Thus, for a successful interfacing of CE with ESI–MS, Tris–phosphate was replaced by a volatile buffer, namely ammonium formate at pH 3. Moreover, the partial-filling technique was applied by filling 90% of the PVA coated capillary with the appropriate S-β-CD concentration. For optimum resolution, enantioseparation of ketamine and other anesthetics were conducted at different S-β-CD concentrations (Fig. 3). Ketamine enantiomers were baseline resolved at a

Table 4
Method repeatability given as R.S.D. values in %

	Enantiomer 1	Enantiomer 2
Migration time	0.47	0.49
Normalized peak area	1.57	2.90

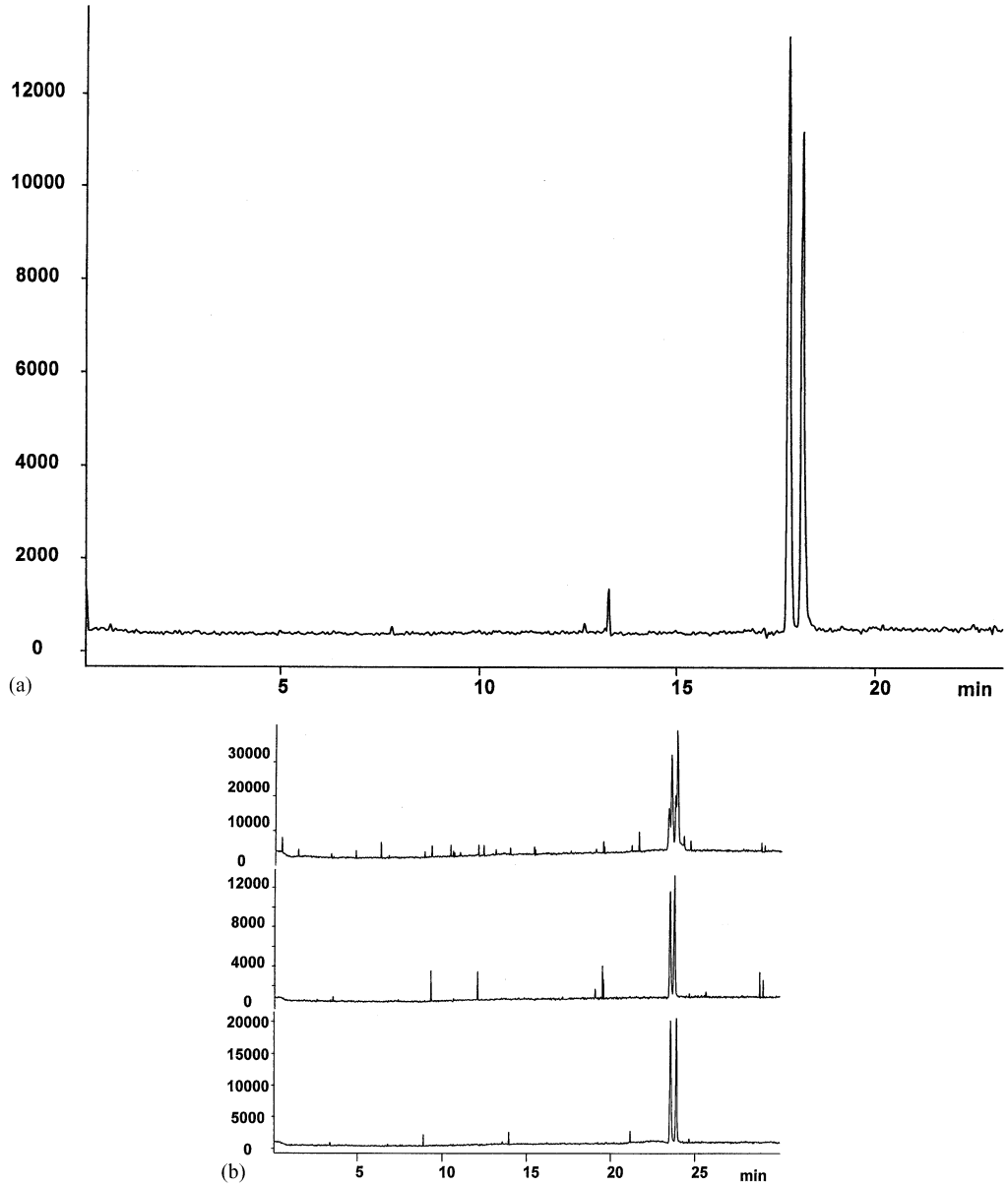


Fig. 3. CE–ESI–MS enantioseparation of investigated anesthetics. CE conditions, running buffer, 20 mM ammonium formate at pH 3 in the presence of S-β-CD (A) 2.5 mg ml^{-1} in the case of ketamine and (B): top: reconstructed ion electropherogram, middle: ion 221, bottom: ion 247, 10 mg ml^{-1} for prilocaine and mepivacaine; PVA-coated capillary dimensions, 70 cm total length × 50 μm I.D.; partial-filling of the capillary 90%; pressure injection, 25 mbar for 5 s; applied voltage, 20 kV; temperature, 20 °C. ESI–MS conditions, SIM positive ion mode; capillary voltage, 4 kV; fragmentor, 50 V; drying gas N_2 flow rate and temperature, 4 l min^{-1} and 150 °C; nebuliser pressure 4 psi; sheath liquid, 0.5% formic acid in water–*iso*-propanol (50/50, v/v); sheath liquid flow rate, 3 μl min^{-1}.

concentration of S-β-CD inferior to 6 mg ml^{-1}. For other studied anesthetics, higher S-β-CD concentration (i.e. 10 mg ml^{-1}) was necessary to achieve baseline enantioresolution. Under the optimized electrophoretic conditions, the baseline enantioseparation of investigated compounds was

achieved. Furthermore, as illustrated in Fig. 3B, despite peak overlapping in the reconstructed ion electropherogram (RIE), the determination of anesthetic enantiomers can be achieved unambiguously with the help of the high MS selectivity.

5. Conclusion

This paper presents the potential of negatively charged CDs for the chiral separation of anesthetic drug substances. Among the three investigated CDs, sulfated-β-CD was found to be the most effective for the simultaneous enantioseparation of bupivacaine, mepivacaine, prilocaine and ketamine chosen as model substances. Resolution was greatly affected by compound structure, CD type and concentration, buffer pH and temperature. Using a PVA coated capillary and a tris-phosphate buffer set at pH 2.5 containing 6 mg ml^{-1} of S-β-CD and a separation temperature of 30 °C, the simultaneous enantioseparation of the investigated drugs was achieved in less than 12 min. This optimized method was found appropriate for the enantioselective analysis of mepivacaine in pharmaceutical solutions.

Negatively charged CDs have a distinct advantage over neutral CDs derivatives because of their self-electrophoretic mobility, which often results in resolution improvement. Moreover, in contrast to neutral CDs, very low concentration of sulfated-β-CD are required to achieve high resolution although charged CDs do usually have a higher solubility. Finally, negatively charged CDs derivatives were found to be more appropriate for the counter current partial filling technique, allowing a successful on-line coupling of CE with electrospray ionization mass spectrometry.

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