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Enantioseparation of vesamicol in human serum by capillary electrophoresis with solid phase extraction and sulfated-β-cyclodextrin

Meng Zhou, James T. Stewart *

Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA 30602, USA

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Abstract

An enantioseparation of racemic vesamicol in human serum by capillary electrophoresis with solid phase extraction and sulfated B-cyclodextrin (S-B-CD) is presented The separation was achieved on an uncoated 72 cm × 50 μm id fused silica capillary maintained at 30 °C and + 15 kV applied voltage using a run buffer of 128 μM S-B-CD in 50 mM phosphate buffer at pH 5. The detection wavelength was 260 nm. Bond Elut C18 solid phase extraction cartridges were used in the sample preparation of the vesamicol samples from serum. Among the CDs studied, the migration order of the enantiomers was reversed in CM-B-CD compared to S-B-CD. Increases in migration time and differences in time between enantiomers was observed with increasing concentrations of S-B-CD. Baseline separation was achieved in the 2–20 μg/ml range of enantiomer concentration ($r > 0.996$). A sample stacking technique was used to improve peak shape and LOD. LODs were 0.5 μg/ml for each enantiomer. Studies of various factors and CE conditions showed the effect of CD type, CD concentration, buffer type, buffer concentration and pH on stability and resolution.

Keywords: Enantioseparation; Vesamicol; Capillary electrophoresis; Cyclodextrin

1. Introduction

Vesamicol [*trans*-(±)-2-(4-phenyl-1-piperidinyl)-cyclohexanol] is a potent synthetic inhibitor of vesicular acetylcholine storage. Vesamicol inhibits the transport of acetylcholine into synaptic vesicles in cholinergic nerve terminals [1]. Two vesamicol enantiomers in which the amino alcohol substituents are in the *trans* relationship are readily obtained in a high yield by addition of secondary amines to epoxides under S_N2 conditions [2]. Pharmacological studies showed that the inhibition of acetylcholine transport is stereospecific and concentration-dependent between the two enantiomers. L (−)-Vesamicol is 25-fold more potent than D (+)-vesamicol. A chiral HPLC separation was reported using a Chiracel OD column [3], but there were no quan-

* Corresponding author. Tel.: +1-706-5425770; fax: +1-706-5425358
E-mail address: jstewart@rx.uga.edu (J.T. Stewart).

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titative methods studied. As a complementary technique, CE has some advantages over chiral HPLC, such as low consumption of run buffer, high efficiency and small sample size.

In chiral CE, enantiomeric compounds are separated due to the complexation with chiral selectors and different mobility between complexes and uncomplexed analytes [4]. Neutral and charged CDs are the most commonly used chiral selectors, due to low cost, commercial availability, and UV transparency. Native CDs are cyclic, chiral oligosaccharides with a hydrophobic cavity and hydroxyl groups on the rim. The intermolecular forces including hydrophobic, hydrogen bonding, dipole–dipole and van der Waals interactions are present in the formation of inclusion complexes between CDs and chiral compounds. In derivatized CDs, hydrogen bonding, electrostatic interactions or ion pairing between the substituents and chiral compounds may further enhance the chiral recognition. In particular, negatively-charged cyclodextrins such as carboxymethyl-β-cyclodextrin (CM-β-CD) or sulfated-β-cyclodextrin (S-β-CD), which move opposite to electroosmotic flow (EOF) in CE, may show a higher enantioselectivity. Since charged cyclodextrin derivatives can be used in extremely low concentrations, they are advantageous in achieving higher resolution with a short analysis time and at low cost.

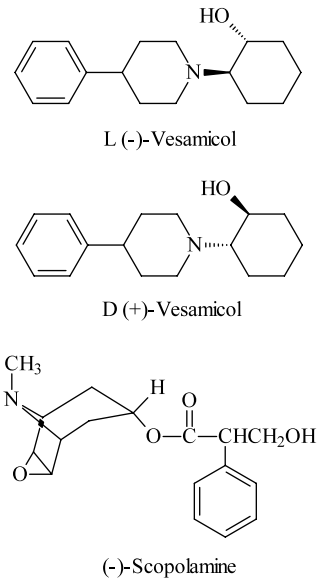

Fig. 1. Structures of L (−)- and D (+)-vesamicol and (−)-scopolamine (IS).

In our study, enantioseparation of L (−) and D (+)-vesamicol in serum was obtained using S-β-CD as the chiral selector. SPE was used for sample preparation. Various CDs and CE conditions were explored to gain an understanding of chiral recognition.

2. Experimental

2.1. Instrumentation

An Applied Biosystems 270A capillary electrophoresis system (Applied Biosystems, Foster City, CA) equipped with a HP 3395 integrator (Hewlett-Packard, Avondale, PA) was used with an uncoated 72 cm (effective length 50 cm) × 50 μm ID fused-silica capillary (Polymicro Technologies, Phoenix, AZ). The capillary temperature was maintained at 30 °C with air coolant. The applied voltage was +15 kV for the enantioseparation with S-β-CD and the detection wavelength was 210 nm. The detection window was created by stripping the polyimide coating of the capillary in the length of 5 mm. The samples were injected by applying a 5 in. Hg vacuum to the outlet of the capillary for 1 s. The capillary was conditioned with 1 N NaOH for 1 h followed by 30 min of run buffer before each day's run. Before each run, the capillary was rinsed with 0.1 N NaOH for 2 min and run buffer for 3 min.

2.2. Chemicals and reagents

(±)-Vesamicol, D (−)-vesamicol, L (+)-vesamicol and internal standard (−)-scopolamine were purchased from Aldrich RBI (Natick, MA). The chemical structures are shown in Fig. 1.

Sulfated-β-cyclodextrin (S-β-CD), sulfated-α-cyclodextrin (S-α-CD), β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD), heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CD), heptakis(2,3,6-tri-*O*-

methyl)-β-cyclodextrin (TM-β-CD) and hydroxypropyl-β-cyclodextrin (HP-β-CD) were obtained from Aldrich Chemical Co. (Milwaukee, WI). α-Cyclodextrin (α-CD), methyl-β-cyclodextrin (Me-β-CD) and carboxymethyl-β-cyclodextrin (CM-β-CD) were gifts from Cerestar (Hammond, IN). 2-[*N*-morpholino]ethanesulfonic acid (MES buf-fer), citric acid, sodium citrate, sodium acetate, and acetic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium phosphate, concentrated phosphoric acid and sodium hydroxide were purchased from J.T.Baker (Phillipsburg, NJ). All chemicals were of analytical grade or HPLC grade. Deionized water was purified using a cartridge system (Picotech Water System, RTP, NC). All solutions were filtered through a 0.22 μm nylon filter (Alltech Associates, Deerfield, IL).

Drug free human serum was obtained from Biological Specialty (Colmar, PA). Varian Bond Elut C18 and Abselut Nexus cartridges (1 cc/100 mg) were obtained from Varian Sample Preparation Products (Harbor City, CA). Waters Oasis HLB cartridges were purchased from Waters Corp (Milford, MA).

2.3. Buffer and sample preparation

Four types of buffers used in the method development—sodium phosphate, sodium citrate, sodium acetate and MES buffer—were prepared at different concentrations in deionized water. The pH was adjusted with appropriate acids or bases. The run buffers were prepared accordingly after the addition of appropriate amounts of the chiral selectors. The run buffer of 128 μM S-β-CD in 50 mM phosphate buffer (pH 5.0) was used in the CE assay of the vesamicol serum samples. All buffer solutions were filtered and degassed by sonication before use.

Stock solutions of each enantiomer and (−)-scopolamine (internal standard) were prepared in deionized water to give concentrations of 1 mg/ml respectively. Samples were stored at 4 °C. To make the solutions for calibration curves and spiked samples, appropriate volumes of individual analyte solutions were mixed with 20 μl internal standard solution, followed by drug free serum to 1 ml.

2.4. Solid phase extraction

Varian Bond Elut C18, Waters Oasis HLB and Varian Abselut Nexus SPE cartridges were studied. Bond Elut C18 and Oasis cartridges were conditioned with 2 × 1 ml methanol and 1 ml deionized water before applying the serum samples. Serum samples were directly applied on the Abselut Nexus cartridges without conditioning. The Oasis cartridges were washed after sample application with 1 ml water–methanol (95:5, v/v) and the other two cartridges were washed with 1 ml deionized water. All SPE cartridges were eluted with 1 ml methanol containing 3% TEA. Both Bond Elut C18 and Oasis HLB cartridges showed absolute recoveries in excess of 87% for the enantiomers and internal standard. Abselut Nexus cartridge gave recoveries of 60–80% for enantiomers and internal standard. Thus the Bond Elut C18 cartridges were selected for use in the assays of vesamicol serum samples. The eluting solvent was evaporated and reconstituted in 1 ml deionized water. One-second hydrodynamic injections of samples were made in the anodic end of the capillary.

2.5. CE assay procedure

Calibration curves were constructed using drug/internal standard (D/IS) peak area ratios versus analyte concentrations at 2, 5, 10, 15, and 20 μg/ml for both L (−)-vesamicol and D (+)-vesamicol. Triplicate injections were run on each sample. The method accuracy was obtained via two spiked samples (14 and 6 μg/ml) by comparing the concentrations calculated from calibration curves versus concentrations added. The absolute recoveries of sample clean-up procedure were obtained by comparing the extracted serum samples to unextracted stock solutions.

3. Results and discussion

3.1. Effect of the cyclodextrin type on enantioseparation

Recent studies on NMR and molecular dynamics simulations showed that aromatic groups of

Table 1
Enantioseparation of (±)-vesamicol (0.1 mM) using CM-β-CD and S-β-CD

Selector	Concentration (mM)	t_1 (min)	t_2 (min)	α	Rs	First eluted enantiomer
None	–	8.65	–	–	–	–
CM-β-CD	0.1	8.67	–	–	–	–
	0.5	10.18	10.26	1.01	0.16	D (−)
S-β-CD	0.1	9.67	9.99	1.03	1.23	L (+)
	0.5	14.78	16.44	1.11	3.18	L (+)

50 mM phosphate buffer (pH 5.0), applied voltage of 15 kV.

chiral analytes formed stronger complexes with CDs than alkyl groups based on association constants [5]. Chiral analytes containing less bulky substituents on the aromatic ring tended to be resolved better with α- and β-CDs than γ-CD [6]. Limited interactions of hydroxyl groups on the native CD rim provide resolving ability for a small number of chiral drugs.

Although possessing phenyl and hydroxyl groups (Fig. 1), vesamicol enantiomers were not resolved with native and neutral derivatised CDs, including α-CD, β-CD, γ-CD, Me-β-CD, DM-β-CD, TM-β-CD and HP-β-CD. The increased migration time using these CDs indicated a weak interaction with vesamicol. With charged CDs, S-α-CD showed similar electropherograms to native and neutral CDs except there was no chiral separation. CM-β-CD exhibited a slight chiral recognition at a higher concentration, but only S-β-CD provided a base line separation at both low and high concentrations (Table 1). Interestingly, the migration order of the vesamicol enantiomers was reversed in CM-β-CD compared to S-β-CD.

3.2. Effect of the sulfated-β-cyclodextrin concentration on enantioseparation

In our studies, an increase in the migration times and migration time differences between enantiomers was observed with increasing concentration of S-β-CD. This dependency between migration time and CD concentration was also reported in the cases of other enantiomer–CDs complexes [7,8]. We studied the effects of S-β-CD concentration on the resolution Rs and separation selectivity α in a 50 mM phosphate buffer at pH 5.0. An increase of both resolution and separation selectivity was observed with an increasing S-β-CD concentration. When the S-β-CD concentration was lower than 25.6 μM, no resolution was observed. The migration times were too long and peak dispersion was severe when the S-β-CD concentration was higher than 1.0 mM.

Strong binding constants, 1099 and 1181/M for D (+)- and L (−)-vesamicol, respectively, were calculated from a mobility ratio method, which was developed for zone electrophoresis by Alberty and King [9], and later rewritten to simplify [10]. The strong binding allowed enantioseparation of vesamicol enantiomers in the presence of a small amount of S-β-CD in the run buffer. This is one of the remarkable advantages of charged CDs over native and neutral CDs.

3.3. Effect of the buffer type and buffer concentration on enantioseparation

The physicochemical properties such as buffering range, electric conductivity, running current, and background absorbance need to be considered when choosing a CE run buffer. In our experiments, we studied sodium phosphate, sodium acetate and sodium citrate buffers at pH 5.0, and sodium phosphate and MES buffers at pH 6.0 (Fig. 2). Sodium phosphate and sodium citrate buffers exhibited a high running current, which consequently resulted in severe peak dispersion due to Joule heating. The running currents for MES and sodium acetate buffers were

lower than inorganic buffers. This allowed a higher concentration of organic buffer to be used, such as 200 mM for MES buffer. All organic buffers possess higher UV cut-offs than phosphate buffer, and in particular, greater baseline noise was observed for citrate buffer. In Fig. 2, all run buffers showed similar enantioselectivity except for sodium acetate, which had a higher selectivity with the same S-β-CD concentration. However, the peak efficiency was reduced using acetate buffer.

Besides running current, buffer concentrations affect buffering capacity and EOF. Generally, the higher the buffer concentration, the better the buffering capacity. This will prevent buffer depletion and improve assay reproducibility. Also, higher buffer concentration will decrease the effective charges on the capillary wall, and consequently reduce the EOF [11]. In our experiments, we found that concentrations of phosphate buffer had more impact on peak efficiency than selectivity. Shorter migration times and sharper peaks for the enantiomers were obtained with higher buffer concentrations, and significant changes were found with changes in MES concentrations.

3.4. Effect of buffer pH on enantioseparation

The effects of buffer pH on enantioseparation of L (−) and D (+)-vesamicol were studied in a 50 mM sodium phosphate solution at pH 3–8. A constant S-β-CD concentration of 128 μM (0.025% w/v) was used in the run buffers. The S-β-CD is negatively-charged over the entire pH range and moves opposite to EOF. Increasing migration times were observed in buffers containing S-β-CD compared to free solutions. In addition to ionized acidic or basic solutes, the buffer pH can change the EOF in CE. At pH <5, the EOF is relatively small and peak efficiency is high. EOF increases with increasing pH, and migration times decrease but with an adverse impact on peak efficiency. Separation selectivity and resolution were depicted over the pH range 3–8, and the highest selectivity and resolution were achieved at a pH of 5.0.

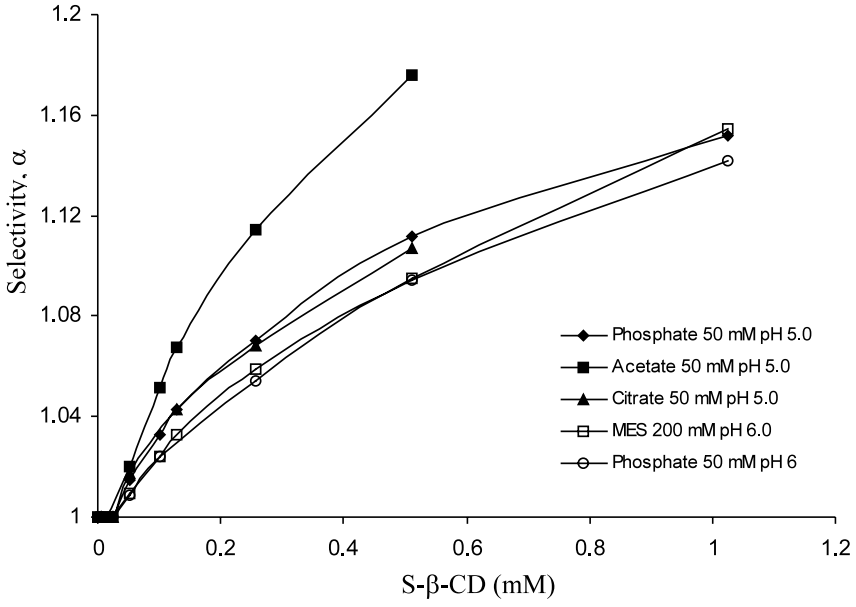

Fig. 2. Effect of S-β-CD concentration on separation selectivity α in different run buffers.

Table 2
Accuracy and precision of serum samples spiked with D (+)- and L (−)-vesamicol

Analytes	Concentration added (μg/ml)	Concentration found (μg/ml)	Percent error (%)	RSD (%)
Inter-day[a]				
D (+)-vesamicol	14	13.70 ± 0.82	2.11	5.23
	6	6.19 ± 0.14	3.15	2.00
L (−)-vesamicol	14	13.99 ± 0.60	0.11	4.02
	6	6.28 ± 0.02	4.60	0.39
Inter-day[b]				
D (+)-vesamicol	14	13.86 ± 0.38	1.00	2.72
	6	5.94 ± 0.15	1.05	2.58
L (−)-vesamicol	14	13.69 ± 0.67	2.18	4.93
	6	6.15 ± 0.25	2.45	4.12

[a] Based on $n = 6$.
[b] Based on $n = 9$.

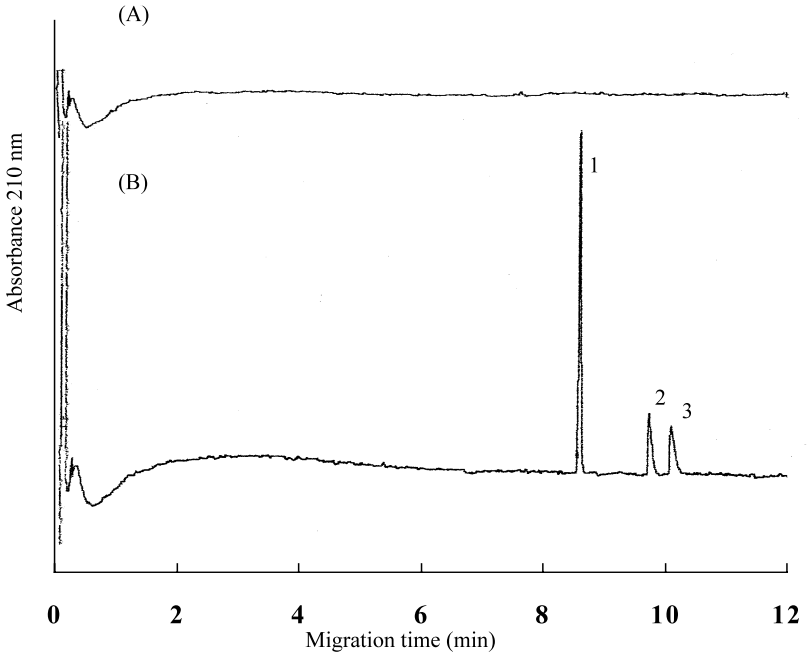

Fig. 3. Representative electropherograms of (−)-scopolamine (1), L (−)-vesamicol (2), and D (+)-vesamicol (3) using 50 mM phosphate run buffer (pH 5.0) containing 128 μM S-β-CD as chiral additive; (A) blank serum; (B) spiked vesamicol samples at concentrations of 5 μg/ml.

3.5. Quantitative analysis

An optimized run buffer of 128 μM S-β-CD in 50 mM phosphate buffer at pH 5.0 was used for CE assays of vesamicol enantiomers in serum samples. Base line separation was achieved in the 2–20 μg/ml range of sample concentrations.

A sample stacking technique was used to improve peak shape and limits of detection. Deionized water was used as solvent in sample

solutions. Thus, the electrical conductance of sample zone was lower than that of the capillary. An overall applied voltage of 15 kV was held constant, and a higher field was developed across the sample zone. The samples migrated faster until they reached the boundary of the sample zone, where they stacked at the interface with the running buffer.

The limits of detection were 0.5 μg/ml for both L (−) and D (+)-vesamicol enantiomers at 210 nm (molar absorptivity of 10750). Calibration curves were obtained over the range of 2–20 μg/ml with regression coefficients greater than 0.996 ($n = 15$). The quantitative aspects of the method were examined and results are shown in Table 2. Representative electropherograms of blank serum and spiked samples are shown in Fig. 3. No interferences were observed from endogenous compounds in the serum.

4. Conclusion

The enantioseparation of L (−) and D (+)-vesamicol enantiomers was achieved using 128 μM S-β-CD in a 50 mM phosphate run buffer (pH 5.0). The studies of various factors and CE conditions showed the effects of CD type, CD concentration, buffer type, buffer concentration, and pH on selectivity and resolution. One of the advantages of S-β-CD over native or neutral CDs is that only a small concentration of S-β-CD was required due to the strong binding of the vesamicol-S-β-CD complexes.

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References

[1] C. Prior, I.G. Marshall, S.M. Parsons, Gen. Pharmacol. 23 (6) (1992) 1017–1022.
[2] G.A. Rogers, S.M. Parsons, D.C. Anderson, L.M. Nilsson, B.A. Bahr, W.D. Kornreich, R. Kaufman, R.S. Jacobs, B. Kirtman, J. Med. Chem. 32 (1989) 1217–1230.
[3] D.L. Gildersleeve, Y.W. Jung, D.M. Wieland, J. Chromatogr. A 667 (1994) 183–189.
[4] B. Chankvetadze, Capillary Electrophoresis in Chiral Analysis, Wiley, England, 1997, pp. 141–228.
[5] M. Zubiaur, C. Jaime, J. Org. Chem. 65 (2000) 8139–8145.
[6] D. Belder, G. Schomburg, J. Chromatogr. A 666 (1994) 351–365.
[7] S. Fanali, J. Chromatogr. 474 (1989) 441–446.
[8] S. Fanali, J. Chromatogr. 545 (1991) 437–444.
[9] R.A. Alberty, E.L. King, J. Am. Chem. Soc. 73 (1951) 517–523.
[10] K.L. Rundlett, D.W. Armstrong, J. Chromatogr. A 721 (1996) 173–186.
[11] K.D. Altria, C.F. Simpson, Anal. Proc. 23 (1986) 453–454.