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Separation of basic drug enantiomers by capillary zone electrophoresis using ovoglycoprotein as a chiral selector

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Abstract

The utilization of crude ovomucoid from chicken egg whites (OMCHI) for the separation of drug enantiomers as a chiral selector in capillary zone electrophoresis (CZE) proved to be unsatisfactory. Because only about 10% ovoglycoprotein (OGCHI), which is responsible for chiral recognition ability, was included in crude OMCHI, we investigated the utilization of OGCHI as a chiral selector in CZE. To avoid protein adsorption on the wall, two kinds of modified capillaries, a vinyl capillary and a linear polyacrylamide-coated capillary, were employed. Separations of basic drug enantiomers were achieved by optimizing the concentrations of OGCHI and running buffer, running buffer pH, and the nature and content of organic modifier. The present method was successfully applied to the assay of eperisone enantiomers. © 1997 Elsevier Science B.V.

Keywords: Enantiomer separation; Chiral selectors; Ovoglycoprotein; Ovomucoid; Eperisone; Chlorpheniramine; Tolperisone; Verapamil

1. Introduction

Various capillary electrophoretic methods have been developed for the separation of drug enantiomers using chiral selectors as the additives. The chiral additives so far employed have included polysaccharides, proteins, bile salts and chiral mixed micelles [1]. Recently, proteins, albumins (such as human serum albumin [2,3] and bovine serum albumin [4–8]), and glycoproteins (such as cellulase [6,7,9], α_1-acid glycoprotein [6–8], avidin [8,10] and ovomucoid [6–8,11]), were successfully used as buffer additives for chiral resolution of basic, acidic and uncharged drugs in capillary zone electrophoresis (CZE). Also, HPLC chiral stationary phases based on these proteins have been developed for the separation of enantiomeric forms [12]. One expected similar chiral recognition abilities of a protein in both CZE and HPLC techniques, since the underlying molecular interactions should be the same. An HPLC stationary phase based on ovomucoid could separate a wide range of enantiomeric mixtures [13–15] with good chiral recognition ability. Busch et al. [6] reported that no separation of the enantiomers of the test components could be achieved by using ovomucoid in CZE, and that immobilization of ovomucoid protein might bring about a change in the structure of the protein that results in an excellent chiral HPLC stationary phase. Chiral resolution of basic and neutral racemates was attained with ovomucoid in CZE by other investigators [7,8,11]. However, those methods did not give the satisfactory results expected from the HPLC behaviors.

Recently, we found that the ovomucoid used in

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both techniques was crude, and we isolated and characterized a new protein from egg whites [16]. It was termed ovoglycoprotein (OGCHI, ovoglycoprotein from chicken egg whites), and was included in crude ovomucoid (OMCHI, ovomucoid from chicken egg whites) preparations. We made OMCHI and OGCHI columns from the isolated, pure proteins and compared chiral recognition abilities of two columns. It was found [16] that the chiral recognition ability of OMCHI reported previously [17] was due to OGCHI, that pure OMCHI had no appreciable chiral recognition ability, and that OGCHI showed far better chiral recognition properties than reported previously for OMCHI. Further, good chiral recognition ability of a chiral stationary phase based on crude OMCHI is due to the fact that OGCHI is preferentially bound to *N,N'*-disuccinimidyl carbonate-activated aminopropyl-silica gels compared with OMCHI [18].

In this study, we used isolated, pure OGCHI as a chiral selector for the separation of basic drug enantiomers in CZE. We investigated precisely the influences of concentrations of OGCHI and running buffer, running buffer pH, and the nature and content of organic modifier, on the separation of basic drug enantiomers.

2. Experimental

2.1. Reagents and materials

Eperisone and verapamil hydrochlorides were kindly donated by Eisai (Tokyo, Japan). Tolperisone hydrochloride and chlorpheniramine maleate were purchased from Nacalai Tesque (Kyoto, Japan). Sephadex G-25 (fine) and SP Sepharose FF were purchased from Pharmacia Biotech (Tokyo, Japan). Acrylamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED), ammonium peroxodisulphate (APS), thionyl chloride and tetrahydrofuran (THF) of analytical reagent grade were obtained from Wako (Osaka, Japan). Vinylmagnesium bromide (1 *M* solution in THF) was obtained from Tokyo Chemical Industries (Tokyo, Japan). Other solvents and reagents were used without further purification.

Water purified with a Nanopure II unit (Barnstead, Boston, MA, USA) was used for the preparation of the electrophoretic buffer and the sample solution.

2.2. Isolation of OGCHI from egg whites

OGCHI was isolated as reported previously [19]. Briefly, crude OMCHI was precipitated from egg whites with ethanol, according to procedures modified slightly from those of Fredericq and Deutsch [20]. The obtained crude OMCHI, which includes about 10% OGCHI by weight, was further purified by cation-exchange chromatography. Two grams of the OMCHI were applied to an SP Sepharose FF column (12×5 cm) that was equilibrated with 10 m*M* CH_3COONH_4 (pH 4.6) applying a linear gradient to 700 m*M* CH_3COONH_4 (pH 4.6) for 6 h at an average flow-rate of 100 ml/h, and then the eluent was changed to 1000 m*M* CH_3COONH_4 (pH 4.6). The eluant was monitored at 280 nm with a Model AC-500 spectrophotometric monitor (Atto, Tokyo, Japan). The separation was performed at 4°C. Two fractions, OMCHI and OGCHI, were collected and lyophilized. The lyophilized OMCHI and OGCHI were desalted with a Sephadex G-25 (fine) column (20×5 cm) using 15 m*M* NH_4HCO_3 as the buffer with an average flow-rate of 120 ml/h. The eluate was collected and lyophilized. The purity of OGCHI and OMCHI obtained was estimated to be 99 and 100%, respectively, based on the peak area by reversed-phase chromatography [18,19].

2.3. Preparation of vinyl capillary and linear polyacrylamide-coated capillary

A vinyl capillary or linear polyacrylamide-coated capillary was prepared according to the procedures reported by Nakatani et al. [21]. Briefly, the capillary was first treated with 1 *M* NaOH and 1 *M* HCl at ambient temperature for 30 min, and then washed with distilled water and dried at 100°C by flushing with nitrogen for 3 h. Thionyl chloride was passed through the dried capillary for several minutes by suction. The capillary was sealed at both ends and kept at 70°C for 6 h. After opening the seal, nitrogen was passed through the capillary for 5 min to remove excess thionyl chloride. A 0.25 *M* vinylmagnesium bromide solution in THF was introduced into the capillary. Then, the capillary was sealed again and

reacted at 70°C for 6 h. After opening the seal, the capillary was washed with THF for several minutes, and then with distilled water. The obtained capillary was a vinyl capillary. An aqueous solution containing 3% (w/v) acrylamide, 0.1% (w/v) APS and 0.1% (v/v) TEMED was added into the capillary by suction and kept at ambient temperature for polymerization for 1 h, and then washed with distilled water. The obtained capillary was a linear polyacrylamide-coated capillary.

2.4. Capillary zone electrophoresis

CZE separations were performed with a Beckman P/ACE system 5500 equipped with a photodiode array detector (Fullerton, CA, USA). A vinyl capillary or linear polyacrylamide-coated capillary (75 μm I.D., effective length 30 cm) was used for separation. All capillaries were thermostated at 23°C by using a liquid coolant.

Electrophoretic buffer (running buffer) solutions used in this study were phosphate buffers containing appropriate amounts of an organic solvent. Separation solutions were prepared by dissolving a protein as a chiral selector in the running buffer solutions. The running buffer solutions and separation solutions were filtered through a 0.45-μm membrane filter (Gelman Sciences Japan, Tokyo, Japan) and degassed with a Branson Model B-2200 ultrasonic bath (Yamato, Tokyo, Japan) prior to use. Stock solutions of samples were prepared in water or methanol. Sample solutions for injection were prepared by dilution of the stock solution with water. The capillary was rinsed with water for 1 min, 50 m*M* phosphate buffer (pH 2.5) for 3 min, water for 1 min and the running buffer for 2 min prior to the run. Then the capillary was filled with the separation solution. The sample solution was injected at 0.5 p.s.i. for 1 s (1 p.s.i.=6894.76 Pa). Both ends of the capillary were dipped into the running buffer solution, and a constant voltage of 12.0 kV was applied for the separation. Detection was performed at 254 or 214 nm.

The enantioseparation factor is calculated from the equation $\alpha=t_2/t_1$, where t_1 and t_2 correspond to the migration times of the first- and second-migrated peaks, respectively. Resolution is calculated from the equation $R_s=2(t_2-t_1)/(t_{w1}+t_{w2})$, where t_{w1} and t_{w2} are the peak widths.

3. Results and discussion

3.1. Chiral recognition ability of crude OMCHI, OMCHI and OGCHI

First, we employed an unmodified capillary for the separation of basic drug enantiomers using crude OMCHI, OMCHI and OGCHI as chiral selectors in

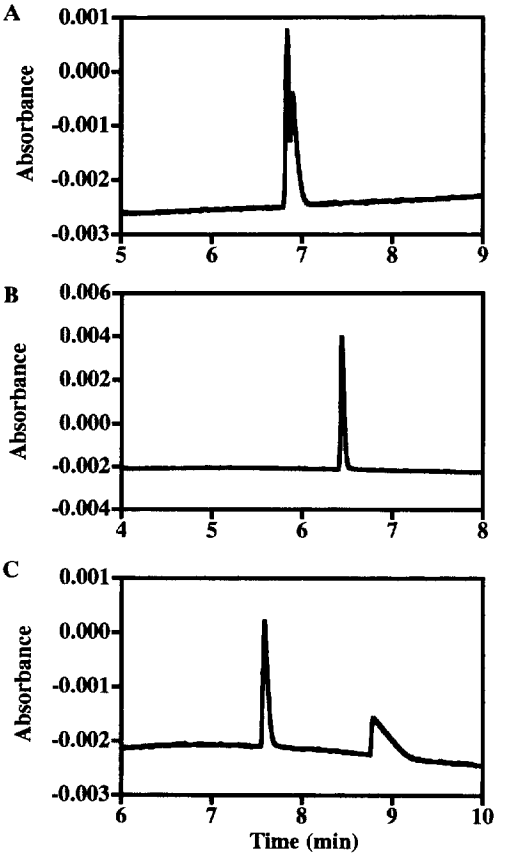

Fig. 1. Separation of tolperisone enantiomers by CZE on a vinyl capillary. Conditions: capillary, 75 μm I.D., effective length 30 cm; running buffer solution, 50 m*M* phosphate buffer (pH 5.0)–2-propanol (95:5, v/v); separation solution, 50 m*M* phosphate buffer (pH 5.0)–2-propanol (95:5, v/v) containing 50 μ*M* crude OMCHI (A), OMCHI (B) or OGCHI (C); sample, 0.1 mg/ml racemic tolperisone hydrochloride; applied voltage, 12.0 kV; detection, 254 nm.

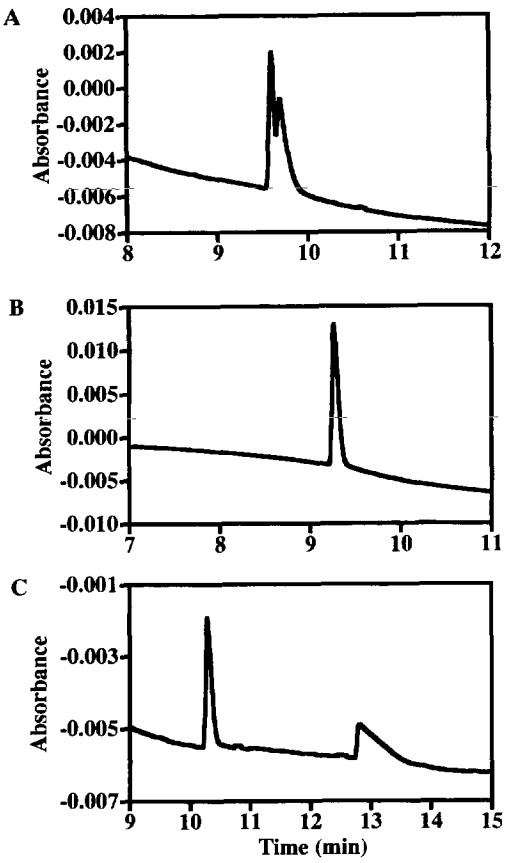

Fig. 2. Separation of tolperisone enantiomers by CZE on a linear polyacrylamide-coated capillary. Conditions as in Fig. 1.

CZE. However, the reproducible results were not obtained because of protein, especially OGCHI, adsorption on the wall. We used two kinds of modified capillaries, a vinyl capillary and a linear polyacrylamide-coated capillary, to avoid protein adsorption to the wall.

Fig. 1A–C show the separation of tolperisone enantiomers using crude OMCHI, OMCHI and OGCHI, respectively, as chiral selectors in CZE, where a vinyl capillary was employed. We showed that pure OMCHI had no appreciable chiral recognition ability, and that the chiral recognition ability of crude OMCHI originated from OGCHI [16]. In addition, crude OMCHI included about 10% (w/w) OGCHI. The obtained results were consistent with those reported previously; tolperisone enantiomers were not resolved by using OMCHI, slightly resolved by crude OMCHI, and completely resolved by OGCHI. Fig. 2A–C show the separation of tolperisone enantiomers using crude OMCHI, OMCHI and OGCHI, respectively, as chiral selectors in CZE, where a linear polyacrylamide-coated capillary was employed. In this case, the migration times of tolperisone enantiomers became longer than shown in Fig. 1, because of very low, negligible, electroosmotic mobility. These results indicate that a lot of racemates should be separated using OGCHI as a chiral selector in CZE, as expected from an HPLC chiral stationary phase based on crude OMCHI. Next, we investigated various factors af-

Table 1
Effect of OGCHI concentration on the migration time, enantioseparation factor and resolution of chlorpheniramine[a]

OGCHI concentration (μ*M*)	Capillary					
	Vinyl			Linear polyacrylamide-coated		
	t_1	α	R_s	t_1	α	R_s
12.5	7.47	1.040	0.91	8.19	1.049	1.14
25.0	7.67	1.104	1.24	8.44	1.13	1.34
50.0	8.12	1.187	2.00	8.95	1.253	2.40
75.0	9.03	—[b]	—	9.58	—	—

[a] CZE conditions: capillary, 75 μm I.D., effective length 30 cm vinyl capillary or linear polyacrylamide-coated capillary; separation solution, 50 m*M* phosphate buffer (pH 5.0)–2-propanol (95:5, v/v) containing OGCHI; sample, 0.25 mg/ml racemic chlorpheniramine maleate; applied voltage, 12.0 kV; detection, 214 nm. Other conditions as in Section 2. The t_1, α and R_s were the migration time of first-migrated enantiomer, enantioseparation factor and resolution, respectively.

[b] Not estimated because second enantiomer did not migrate.

Table 2
Effect of buffer pH on the migration time, enantioseparation factor and resolution of chlorpheniramine[a]

Buffer pH	Capillary					
	Vinyl			Linear polyacrylamide-coated		
	t_1	α	R_s	t_1	α	R_s
4.5	8.77	1.089	1.37	8.87	1.115	2.45
5.0	8.12	1.187	2.00	8.95	1.253	2.40
5.5	7.96	1.333	2.50	11.11	—	—
6.0	8.79	—[b]	—	14.53	—	—

[a] CZE conditions as in Table 1 except for the separation solution, 50 m*M* phosphate buffer–2-propanol (95:5, v/v) containing 50 μ*M* OGCHI.
[b] Not estimated because the second enantiomer did not migrate.

fecting the separations of basic drug enantiomers by CZE.

3.2. Effect of OGCHI concentration

Table 1 shows the effect of the OGCHI concentration on the migration time, enantioseparation factor and resolution of chlorpheniramine enantiomers. An increase in the OGCHI concentration resulted in an increase in the migration times and enhanced enantioseparation factor and resolution. The migration times of chlorpheniramine enantiomers increased almost linearly with an increase in OGCHI concentration, as reported by Tanaka and Terabe [8]. The differences in the intercepts of regression lines between the vinyl and linear polyacrylamide-coated capillaries are due to the differences in the electroosmotic mobility on the two capillaries. The electroosmotic mobility on the former is larger than the latter. The second-migrated enantiomer was not observed at an OGCHI concentration of 75 μ*M*. This is due to the strong binding of the second-migrated enantiomer with OGCHI and/or excessive broadening of the peak. In the following experiments, we selected 50 μ*M* as the OGCHI concentration.

The plate numbers obtained with these runs were not so high as those with the conventional CZE techniques. In addition, the second-migrated enantiomer always gave a much broader and more tailed peak than the first-migrated one. It was considered that these low efficiencies were due to the slow kinetics of interaction between chlorpheniramine enantiomers and OGCHI. The migration times of chlorpheniramine enantiomers were scattered around average values. This reveals that adsorption of OGCHI on the capillary wall is avoided by modification.

3.3. Effect of pH

The enantioseparation of chlorpheniramine was investigated in the pH range 4.5–6.0 with 50 m*M* phosphate buffer–2-propanol (95:5, v/v) including 50 μ*M* OGCHI as the separation solution. Table 2 shows the effect of running buffer pH on the migration time, enantioseparation factor and resolution of chlorpheniramine enantiomers. On a linear polyacrylamide-coated capillary, where the electroosmotic mobility is negligible over pH range 4.5–6.0, the migration time of the first-migrated enantiomer and the enantioseparation factor were increased with an increase in the buffer pH; that is, with an increase in the interaction of chlorpheniramine enantiomers with OGCHI. On a vinyl capillary, where

Table 3
Effect of buffer concentration on the migration time, enantioseparation factor and resolution of chlorpheniramine[a]

Buffer concentration (μ*M*)	Capillary					
	Vinyl			Linear polyacrylamide-coated		
	t_1	α	R_s	t_1	α	R_s
10	7.76	1.219	2.24	6.95	1.176	1.95
20	8.00	1.220	1.94	7.82	1.216	1.96
50	8.12	1.187	2.00	8.95	1.253	2.40
100	8.39	1.172	2.15	9.45	1.182	2.46

[a] CZE conditions as in Table 1 except for the separation solution, phosphate buffer (pH 5.0)–2-propanol (95:5, v/v) containing 50 μ*M* OGCHI.

the electroosmotic mobility is increased with an increase in the buffer pH, the migration time became minimum at pH 5.5. This is due to the result of compromise of the electroosmotic and electrophoretic mobilities of chlorpheniramine on the vinyl capillary. With an increase in the buffer pH, the enantioseparation factor of chlorpheniramine was increased. However, the second-migrated enantiomer was not observed at pH 6.0 for a vinyl capillary, and at pH values 5.5 and 6.0 for a linear polyacrylamide-coated capillary. In the following experiments, we selected pH 5.0 as the running buffer pH.

3.4. Effect of running buffer concentration

The effect of the running buffer concentration on the migration time, enantioseparation factor and resolution of chlorpheniramine enantiomers was examined. As shown in Table 3, with an increase in the buffer concentration, the migration times were increased, but the enantioseparation factor and resolution were not so affected as the OGCHI concentration and running buffer pH. The increase in the migration time is due to the decrease in the electroosmotic mobility and/or the change in the conformation of OGCHI with ionic strength, which might have influenced the hydrophobic, ionic and hydrogen-bonding interaction of a solute and OGCHI. We selected 50 m*M* phosphate buffer as the running buffer.

3.5. Effect of organic modifiers

For the purpose of improvement of the peak shape

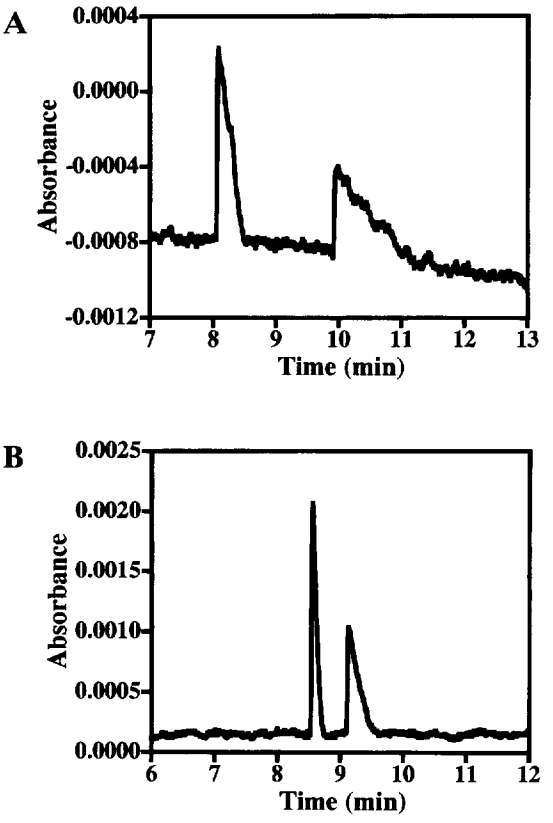

Fig. 3. Separation of chlorpheniramine enantiomers by CZE on a vinyl capillary with addition of 5 (A) and 10% (B) 2-propanol to the separation solution. Conditions: capillary, 75 μm I.D., effective length 30 cm; running buffer solution, 50 m*M* phosphate buffer (pH 5.0)–2-propanol (95:5 or 90:10, v/v); separation solution, 50 m*M* phosphate buffer (pH 5.0)–2-propanol (95:5 or 90:10, v/v) containing 50 μ*M* OGCHI; sample, 0.25 mg/ml racemic chlorpheniramine maleate; applied voltage, 12.0 kV; detection, 214 nm.

Table 4
Effect of 2-propanol concentration on the migration time and enantioseparation factor of basic drug enantiomers[a]

2-Propanol conc. (%)	Basic drug racemate							
	Eperisone		Tolperisone		Chlorpheniramine		Verapamil	
	t_1	α	t_1	α	t_1	α	t_1	α
5	7.61	1.021	7.29	1.126	8.12	1.187	9.86	1.071
10	8.58	1.008	8.05	1.031	8.62	1.063	10.35	1.019
20	11.95	1.00	11.23	1.00	12.04	1.007	14.15	1.00
30	16.57	1.00	15.01	1.00	15.95	1.00	18.98	1.00

[a] CZE conditions as in Table 1 except for the separation solution, 50 m*M* phosphate buffer–2-propanol containing 50 μ*M* OGCHI. A vinyl capillary was used. Detection was performed at 254 nm for eperisone and tolperisone, and at 214 nm for chlorpheniramine and verapamil.

Table 5
Effect of type and content of organic modifier on the migration time and enantioseparation factor of basic drug enantiomers[a]

Organic modifier	Basic drug racemate							
	Eperisone		Tolperisone		Chlorpheniramine		Verapamil	
	t_1	α	t_1	α	t_1	α	t_1	α
5% ethanol	7.31	1.044	7.25	1.232	—[b]	—	—	—
10% ethanol	7.96	1.023	7.87	1.050	9.01	1.100	10.89	1.038
10% methanol	7.79	1.045	7.88	1.273	—	—	—	—
15% methanol	8.35	1.036	8.30	1.098	10.42	1.195	—	—

[a] CZE conditions as in Table 4.
[b] Not estimated because of no migration of both enantiomers.

in CZE using a protein as a chiral selector, an organic modifier, such as methanol, ethanol and 2-propanol, was added to the separation solution [1]. Table 4 shows the effect of 2-propanol concentration on the migration times and enantioseparation factor of basic drug enantiomers. Fig. 3A,B show electropherograms of chlorpheniramine enantiomers, where 5 and 10% 2-propanol, respectively, are added to the separation solution. The peak shape was improved significantly by further addition of organic modifier, while the enantioseparation factor was diminished with an increase in 2-propanol concentration. Table 5 shows the effect of the type and content of organic modifier on the migration times and enantioseparation factor. These results are very similar to those observed in HPLC chiral stationary phases based on proteins [12]. By addition of a higher content of organic modifier, chiral recognition ability was sometimes lost, while some solutes were not eluted by addition of a lower content of organic modifier because of the strong binding with a protein. Further, addition of an organic modifier has been found to be effective in controlling retention times and enantioselectivity. The results described above suggested that by changing the type and content of organic modifier, the migration times and enantioselectivity could be controlled, and the separation of various drug enantiomers could be achieved with high efficiency.

3.6. Assay validation

Repeatability of migration times and peak areas were estimated for the eperisone enantiomers by the present method, as shown in Table 6. A calibration curve was prepared for each eperisone enantiomer in the range 5–250 μg/ml of eperisone racemate. The calibration curve was linear for each enantiomer, with a correlation coefficient of 0.999 or more for both enantiomers.

4. Conclusion

We used OGCHI for the separation of basic drug enantiomers as a chiral selector in CZE. The sepa-

Table 6
Repeatability of the assay of eperisone enantiomers by the present method[a]

	Migration time		Peak Area[b]	
	Mean (min)	R.S.D. (%)	Mean (arbitrary units)	R.S.D. (%)
First-migrated enantiomer	7.61	0.25	128 600	4.15
Second-migrated enantiomer	7.77	0.14	130 168	4.14
			(n=5)	

[a] CZE conditions: capillary, 75 μm I.D., effective length 30 cm vinyl capillary; separation solution, 50 m*M* phosphate buffer (pH 5.0)–2-propanol (95:5, v/v) containing 50 μ*M* OGCHI; sample, 0.25 mg/ml racemic eperisone hydrochloride; applied voltage, 12.0 kV; detection, 254 nm.
[b] Peak areas were normalized by the migration time.

ration of various drug enantiomers should be achieved by optimizing the concentrations of OGCHI and running buffer, running buffer pH, and type and content of organic modifier. The present method is expected to have wide applications.

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