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Partial separation zone technique for the separation of enantiomers by affinity electrokinetic chromatography with proteins as chiral pseudo-stationary phases

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Abstract

A previously reported technique which employed a discontinuous separation zone built in a part of a capillary was applied to the separation of enantiomers by affinity electrokinetic chromatography using proteins as chiral pseudo-stationary phases to avoid a detection problem caused by the presence of UV-absorbing materials in the separation solution. The technique was named "partial separation zone technique", where the capillary was partially filled with a solution containing a protein and the protein was not in the detector cell when analytes reached that cell. It was shown that the technique was successfully performed automatically using a commercial capillary electrophoresis instrument. The migration velocity of the protein was practically reduced to zero by using a coated capillary. The protein concentration could be increased in this technique to enhance resolution without the deterioration of the detector sensitivity. The detection sensitivity was remarkably improved using short-wavelength light in comparison with the conventional technique where the protein was added to the whole separation solution. The reproducibilities of the migration time and the peak area were evaluated, giving results comparable to those with the conventional method. Successful separations of basic racemates were achieved by the technique using bovine serum albumin, α_1-acid glycoprotein, ovomucoid and conalbumin.

1. Introduction

The separation of enantiomers is critical in the development of new medicines, because enantiomers have not only different pharmacological activities but also different pharmacokinetic and pharmacodynamic effects. High-performance liquid chromatography (HPLC) is the most popular tool for the separation of enantiomers at present. Various kinds of chiral stationary phases have been developed and are commercially available for the separation of various racemic compounds. In particular, stationary phases with bonded proteins such as bovine serum albumin (BSA), α_1-acid glycoprotein (α_1-AGP) and ovomucoid have been widely accepted in the pharmaceutical field because of an extremely wide range of applications under reversed-phase conditions [1–4]. The drawbacks of these stationary phases are that: (1) the columns are relatively expensive; (2) many different types of columns are required for successful separations; (3) lifetimes of the columns are short; and that (4) the efficiency of the columns is rather low.

Affinity electrokinetic chromatography (EKC), where a charged biopolymer such as a

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protein or polysaccharide is employed as a pseudo-stationary phase, is a new separation technique having high resolving power, short separation time, simple operating procedure, and minimal sample and solvent requirements similarly to the capillary electrophoretic (CE) techniques for enantiomer separation [5–8]. Many papers have been published on successful separations of enantiomers using BSA [9–11], human serum albumin [12], α_1-AGP [11], ovomucoid [13] and avidin [14] as chiral selectors. A major drawback of affinity EKC with the protein as a pseudo-stationary phase is in low detection sensitivity caused by the UV absorption by the proteins in the shorter-wavelength region, in addition to an inherent weak point of low concentration sensitivity of CE detectors. In order to overcome this drawback, Valtcheva et al. [15] developed an approach in which the protein did not pass through the detection cell and did not interfere with the detection of analytes. However, in the technique, a 2–3 mm long agarose gel must be plugged at the injection end of the capillary prior to each run in order to prevent hydrodynamic flow. We modified the technique to run automatically using a commercial CE instrument. The technique is named "partial separation zone technique", which keeps the same advantage in detection sensitivity as the original method mentioned above. In this paper, the operating principle and the procedure of the technique are described, and the method validations for a quantitative analysis are discussed. Separations of various basic racemic drugs by this method using BSA, α_1-AGP, ovomucoid and conalbumin as chiral selectors are presented.

2. Experimental

2.1. Apparatus

Affinity EKC was performed with a BioFocus 3000 automated CE system (Bio-Rad, Hercules, CA, USA). A 50 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was coated with linear polyacrylamide by the method reported by Nakatani et al. [16]. A coated capillary of 36 cm in total length (31.5 cm to the detector) was incorporated into a user-assembled capillary cartridge. The instrument control and data collections were performed with a Bravo LC 4/33 personal computer (AST Research, Irvine, CA, USA).

2.2. Reagents

Ovomucoid and conalbumin were gifts from Eisai (Tokyo, Japan). BSA as fraction V powder and bovine α_1-AGP as Cohn fraction VI were purchased from Sigma (St. Louis, MO, USA). These proteins were used as received. Bunitrolol and epinastine were pharmaceutical products of Nippon Boehringer Ingelheim (Hyogo, Japan). Tolperisone and clorprenaline were obtained from Eisai. Chlorpheniramine was purchased from Tokyo Kasei Kogyo (Tokyo, Japan), oxyphencyclimine was from Sigma, and pindolol was from Wako (Osaka, Japan). Primaquine, propranolol, trimebutine and trimetoquinol were donated by Tanabe Seiyaku (Osaka, Japan). Homochlorcyclizine, arotinolol and verapamil were obtained from Shinwa Chemical (Kyoto, Japan). All other reagents were of analytical grade. Water was purified with a Milli-Q Labo system (Nihon Millipore, Yonezawa, Japan)

2.3. Procedure

Electrophoretic buffer (running buffer) solutions used in this study were 50 m*M* phosphate buffers containing appropriate amounts of an organic solvent or an amphoteric surfactant, when necessary. The running buffer solution was placed in multiple buffer vials and the running buffer was exchanged before every run in order to obtain reproducible results. Separation solutions were freshly prepared by dissolving a protein as a chiral selector in the running buffer, and filtered through a 0.45-μm syringe-type membrane filter prior to use. Stock solutions of samples (ca. 500 μg/ml) were prepared in water or methanol. Sample solutions for injection were prepared by tenfold dilution of the stock solution with water.

The capillary was thermostated at 25°C with a liquid coolant. The temperature of vial holders for the separation solution, running buffer solu-

tion and sample solutions were maintained at 15°C. The capillary was rinsed with water followed by the running buffer for 30 s each prior to the run, and was partially filled with the separation solution at 1 p.s.i. (6.9 kPa) for 190 s. The sample solution was injected at 1 p.s.i. for 2 s. Both ends of the capillary were dipped into the running buffer, and a constant voltage of 12.0 kV was applied for the separation. Migrating analytes were detected by on-column measurement of UV absorption at 210 or 220 nm.

3. Results and discussion

3.1. Principle of the partial separation zone technique

The operating principle of the technique is illustrated schematically in Fig. 1. The capillary is rinsed with water and the running buffer at 100 p.s.i. for 30 s each. The capillary is then partially filled with the separation solution containing a protein as a chiral selector at 1 p.s.i. for 190 s (Fig. 1a). The separation zone thus built in the capillary is about 27 cm in length. Because the distance from the injection end of the capillary to the detector cell is 31.5 cm, the separation zone does not reach the detection cell. A sample solution is introduced at the end of the capillary filled with the separation solution (Fig. 1b), and the injection end is dipped into the running buffer followed by the application of a high voltage. Basic analytes which are positively charged in the running buffer (pH 4.0–7.0) migrate toward the cathode. On the other hand, α_1-AGP (p*I* 2.9–3.2), ovomucoid (p*I* 3.9–4.3), BSA (p*I* 4.7–4.9) and conalbumin (p*I* 6.1–6.6) are slightly negatively charged in the running buffers (pH 4.0 for α_1-AGP, pH 5.0 for ovo-

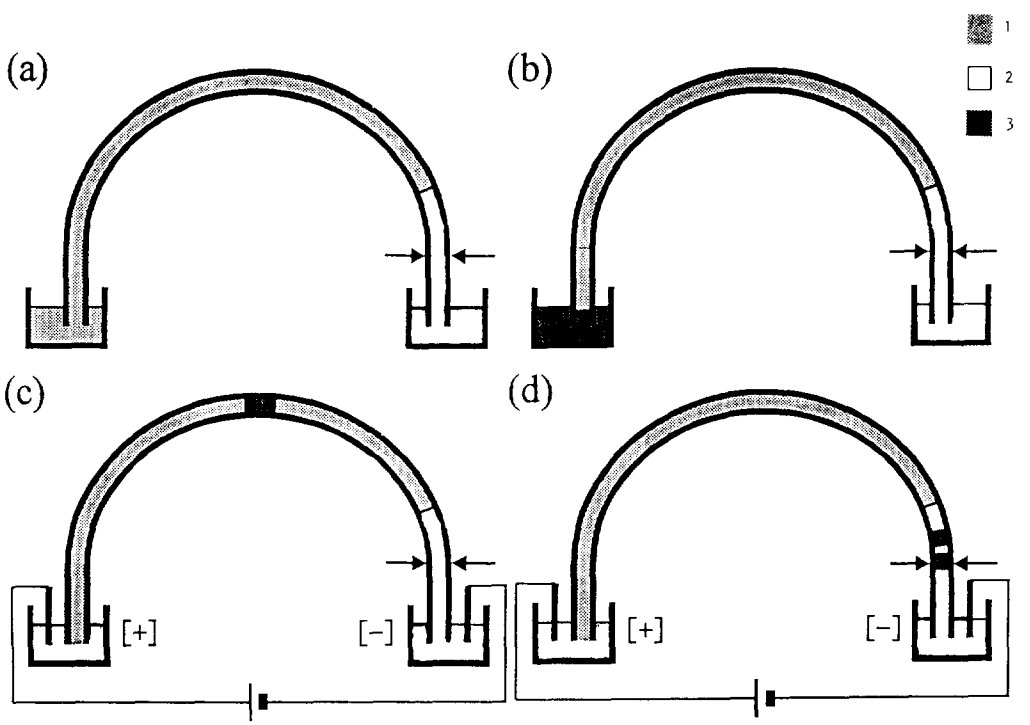

Fig. 1. Schematic illustration of the partial separation zone technique: 1 = separation zone; 2 = running buffer; 3 = sample solution; arrows indicate detection window. (a) The separation zone is introduced from the injection end to a point short of the detector cell after the rinse of the capillary with water followed by the running buffer; (b) the sample solution is introduced into the capillary; (c) a high voltage is applied between both ends of the capillary after both ends are dipped into the running buffer and the analytes migrate toward the detector; (d) a separated zone reaches the detector cell but the separation zone does not reach this cell.

mucoid, pH 6.0 for BSA and pH 7.0 for conalbumin) and migrate in the opposite direction. However, the electrophoretic velocities of these proteins are very low. In this study, a linear polyacrylamide-coated capillary was used to suppress the electroosmotic flow as well as to reduce the protein adsorption on the capillary wall. Therefore, the separation zone or the protein did not migrate significantly during the run. The separation of enantiomers is achieved only while the solute is migrating in the separation zone (Fig. 1c). The enantiomers migrate at identical velocities outside the separation zone, reaching the detection cell. The protein is absent in the detector cell when the enantiomers pass through that cell (Fig. 1d). In this method, the detection sensitivity can be remarkably improved by measuring absorption at a shorter wavelength. Although the separation solution must be replaced before each run, the consumption of the separation solution is only about 0.5 μl for each run. Therefore, the total volume of the separation solution required for a series of analysis is less than that required for the conventional method, where the protein must be dissolved into the whole running solution. The whole running solution containing the protein must often be replaced in the conventional method to obtain good reproducibility, whereas the total protein solution can be used for the separation in the partial separation zone technique. For only one or two runs, the technique may not be advantageous, but for several runs with a protein, the advantage is clear.

In a previous paper [14], we proposed a simple model based on the model developed for cyclodextrin-modified capillary zone electrophoresis [17] for the separation of enantiomers by affinity EKC using a protein as a chiral selector. If we assume that the analyte interacts with a single site of the protein, the apparent mobility of the analyte in the separation zone, $\mu_{app,s}$, is written as

$$\mu_{app,s} = \mu_{eo} + \frac{1}{1 + K[P]} \cdot \mu_a + \frac{K[P]}{1 + K[P]} \cdot \mu_p \quad (1)$$

where μ_{eo} is the electroosmotic mobility, μ_a the electrophoretic mobility of the free analyte, μ_p the electrophoretic mobility of the protein, which is assumed to be equal to the mobility of the protein–analyte complex, K the binding constant and [P] the protein concentration. Since μ_{eo} and μ_p are negligible in this technique as described above, Eq. 1 is simplified to

$$\mu_{app,s} = \frac{1}{1 + K[P]} \cdot \mu_a \quad (2)$$

Actually, μ_{eo} and μ_p could not be measured because the velocities were too low and the estimated values were less than $1.1 \cdot 10^{-4}$ mm^2 s^{-1} V^{-1} for μ_{eo} and less than $4.2 \cdot 10^{-4}$ mm^2 s^{-1} V^{-1} for $(\mu_{eo} + \mu_p)$. Enantiomers 1 and 2 have different binding constants, K_1 and K_2, and therefore apparent mobilities of the enantiomers are different. However, when the analytes migrate in the buffer zone out of the separation zone, K_1 and K_2 become zero and both enantiomers have the same mobility, $\mu_{app,b}$,

$$\mu_{app,b} = \mu_{eo} + \mu_a \approx \mu_a \quad (3)$$

Therefore, only the separation zone is effective for the separation of enantiomers and the rest of the zone to the detector, which does not contain the protein, serves to transport the separated zones to the detector. Band broadening outside the separation zone can be expected to be minimum, because the same running buffer is employed in both separation and transportation zones. A representative electropherogram observed at 210 nm is shown in Fig. 2 for the separation of epinastine racemates with 750 μM BSA at pH 6.0. Baseline drift was not observed in spite of the short-wavelength detection at 210 nm in Fig. 2.

3.2. Concentration of chiral selector

For the separation of enantiomers, the difference in apparent mobilities, $\Delta\mu_{app} (= \mu_{app,1} - \mu_{app,2})$, between the two enantiomers must be maximized. The difference in mobilities in affinity EKC with a protein is given as a function of the concentration of the protein as [14],

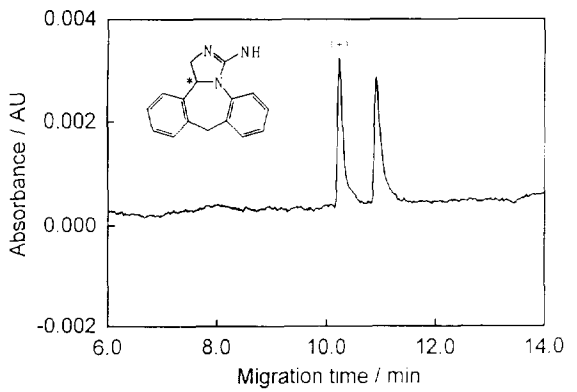

Fig. 2. A representative electropherogram obtained by the partial separation zone technique for the separation of (±)-epinastine with BSA as the chiral selector. Conditions: capillary, 36 cm (31.5 cm to the detector) × 50 μm I.D., separation zone length, 27 cm; separation solution, 750 μ*M* BSA in the running buffer; running buffer, 50 m*M* phosphate buffer (pH 6.0); applied voltage, 12.0 kV; detection wavelength, 210 nm.

$$\Delta\mu_{app} = \frac{(\mu_s - \mu_p)(K_2 - K_1)[P]}{(1 + K_1[P])(1 + K_2[P])} \quad (4)$$

where subscripts 1 and 2 refer to enantiomers 1 and 2. The optimum concentration of the protein, which gives the maximum resolution, can be calculated as [14],

$$[P]_{opt} = (K_1 K_2)^{-1/2} \quad (5)$$

Although the binding constants of most racemates are not known, if we assume the binding constants are in the order of from $1 \cdot 10^3$ to $1 \cdot 10^5$ M^{-1}, $[P]_{opt}$ will be in the range 10–1000 μ*M*. Thus, the optimum concentration of the protein can be very high. Actually, the concentrations of the proteins used for the separation of enantiomers were in the range of 20–100 μ*M* [9–13], except for two cases: ca. 600 μ*M* cellulase [15] and 250 μ*M* ovomucoid [13]. The use of a high concentration of cellulase was allowed by a similar technique [15], whereas 250 μ*M* ovomucoid caused noisy and unstable baselines [13]. We observed that increasing the protein (avidin) concentration higher than 50 μ*M* caused a serious baseline drift even with detection at 240 nm, although the resolution was improved for most enantiomers [14]. Therefore, the maximum avidin concentration in the running solution was usually limited lower than 50 μ*M* in the conventional method when the analytes were to be detected at a wavelength below 240 nm. On the other hand, the partial separation zone technique permits the use of a high protein concentration without the detection problems in order to enhance resolution.

The relationship between the migration time, t_a, and [P] can be given by assuming Eq. 2,

$$t_a = \frac{l_s}{\mu_{app,s}E} + \frac{l_b}{\mu_{app,b}E} = \frac{l_s(1 + K[P])}{\mu_a E} + \frac{l_b}{\mu_a E} \quad (6)$$

where l_s is the length of the separation zone, l_b the rest of the effective length filled with the running buffer and E the electric field strength. To obtain reproducible results, it is essential to build separation zones of identical length in the capillary. The length of the separation zone can be determined experimentally from the time the separation solution is delivered into the capillary at a constant pressure and the time required for the separation solution to reach the detector cell at the same pressure. In this experiments, the separation solution was introduced at 1 p.s.i. The effect of the protein concentration on the length of the separation zone built during a constant time at a constant pressure was examined by measuring the filling time required for the separation solution to reach the detector cell (31.5 cm) when it was introduced into the capillary at 1 p.s.i. The results are given in Table 1, from which data the times to fill a constant length of the capillary (27 cm) with BSA solutions of different concentrations can be easily calculated. When the BSA concentration was higher than 1 m*M*, significant increases in viscosity were observed, as shown in Table 1.

The effect of the protein concentration on the migration time was examined for the separation of epinastine racemate with BSA as a chiral selector. The complete resolution was achieved with the concentration higher than 750 μ*M* BSA. The resolution increased with an increase

Table 1
Effect of BSA concentration on the time required to fill the capillary with the BSA solution by pressurization at a constant pressure

Concentration of BSA/μM	Detection time[a] of BSA/s
100	220
250	225
500	225
750	225
1000	230
1500	240
2000	268

Conditions: capillary, 36 cm (31.5 cm to the detector) × 50 μm I.D.; BSA solution, BSA in 50 mM phosphate buffer (pH 6.0); pressure, 1 p.s.i.
[a] The time required to observe a stepwise change of the baseline.

in the protein concentration as shown in Fig. 3. As expected from Eq. 6, the migration times of epinastine enantiomers increased almost linearly with increasing BSA concentration. Above 1 mM BSA the plots slightly deviated from linearity because of the significant increase in viscosity, as given in Table 1. The linear plots below 1 mM BSA given in Fig. 3 permitted the calculation of the binding constants of epinastine enantiomers with BSA, giving 830 M^{-1} for (+)-epinastine and 910 M^{-1} for the (−)-enantiomer. It should be mentioned that the binding constants obtained here are rough estimates, because [P] in Eq. 2 is the concentration of the non-binding protein but the total protein concentration is used instead here. However, it is not the purpose of this paper to discuss the binding constant and therefore, the detailed discussion will be given elsewhere.

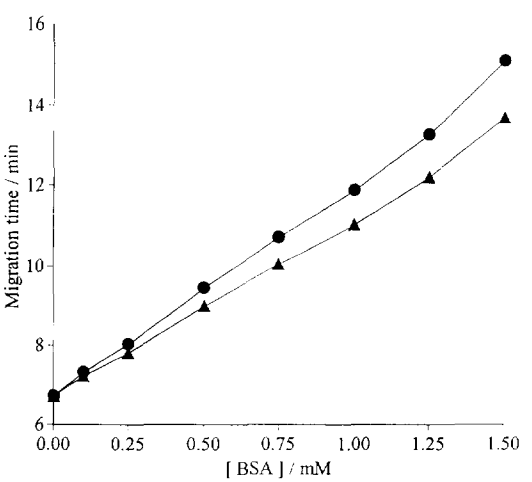

Fig. 3. Dependence of the migration times of (+)- and (−)-epinastine on the BSA concentration. Conditions as in Fig. 2 except for the BSA concentration.

3.3. Assay validation

Reproducibilities of migration times and peak areas were measured for the separation of enantiomers of epinastine with BSA as the pseudo-stationary phase by the partial separation zone technique. The results in Table 2 are comparable to those obtained with conventional capillary zone electrophoresis and suggest that if the automated CE instrument is employed, high reproducibility can be obtained even for the partial separation zone technique.

A calibration curve was prepared for each enantiomer of epinastine separated with 750 μM BSA in the range 10–500 μg ml^{-1} of epinastine racemate hydrochloride. The peak areas divided by the migration times were plotted against the concentration of each enantiomer. The calibration curve was linear below 100 μg ml^{-1} for each enantiomer [$r = 0.9997$ for (+)- and 0.9996 for (−)-enantiomer], but above 100 to 200 μg the plotted line for (+)-epinastine, which migrated faster than the (−)-enantiomer, slightly deviated from linearity, probably due to the incomplete resolution of enantiomers at the high concentrations.

3.4. Separations of some enantiomers by the partial separation zone technique

The partial separation zone technique was applied to the separation of various basic drug racemates using BSA, α_1-AGP, ovomucoid and conalbumin as chiral selectors, and the results are summarized in Table 3. In the case of ovomucoid, most analytes were not detected using 500 μM ovomucoid in 50 mM phosphate buffer (pH 5.0) without any additives to the

Table 2
Repeatability of migration time and peak area by the partial separation zone technique ($n = 5$)

	Migration time		Peak area	
	Average (min)	R.S.D. (%)[a]	Average (arbitrary units)	R.S.D. (%)[a]
(+)-Epinastine	10.20	0.19	123 784	2.95
(−)-Epinastine	10.86	0.21	121 707	3.12

Conditions as in Fig. 2.
[a] Relative standard deviation.

separation solution. The additives were often required not only for ovomucoid [13] but also for the other proteins [12,14]. In most cases, organic solvents or amphoteric surfactants were used as effective additives. In this study, an alcohol or an amphoteric surfactant was added both in the separation solution and the running buffer solution at an identical concentration. A combination of a high concentration (500 μM) of ovomucoid and a relatively higher concentration of alcohol that reduce the interaction between the analyte and protein seemed unreasonable, because the choice of a low concentration of ovomucoid without additive could be natural judging from the separation mechanism. The additive was essential even for the use of a low concentration of ovomucoid [13,18] and the use of the high concentration of ovomucoid with the

Table 3
Separation of basic racemates with proteins by affinity electrokinetic chromatography employing the partial separation zone technique

Protein	Concentration (μM)	pH	Additive	Compound	Migration times/min	R_s
BSA	500	6.0	None	Homochlorcyclizine	12.29, 13.42	1.8
				Oxyphencyclimine	8.93, 9.22	1.1
				Propranolol	11.44, 12.04	1.7
				Trimebutine	13.04, 13.48	0.8
	750	6.0	None	Epinastine	10.23, 10.91	3.1
OVM	500	5.0	CHAPS (10 mM)	Bunitrolol	8.98, 9.15	0.9
			Ethanol (8%)	Pindolol	10.93, 11.36	1.5
			2-Propanol (6%)	Arotinolol	12.97, 13.42	1.3
			2-Propanol (8%)	Oxyphencyclimine	12.64, 12.97	1.1
			2-Propanol (10%)	Tolperisone	11.51, 12.00	2.0
			2-Propanol (10%)	Verapamil	17.65, 18.02	0.9
			1-Propanol (8%)	Chlorpheniramine	11.49, 11.83	1.5
			1-Propanol (8%)	Primaquine	11.83, 12.24	1.5
			1-Propanol (8%)	Trimebutine	13.92, 14.35	1.6
α_1-AGP	500	4.0	None	Clorprenaline	9.58, 9.92	1.3
Conalbumin	500	7.0	None	Trimetoquinol	12.40, 13.17	1.5

BSA = Bovine serum albumin; OVM = ovomucoid; α_1-AGP = α_1-acid glycoprotein; CHAPS = 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate.

additive gave better resolution than the use of the low concentration, although the reason is not clear. The other proteins, BSA, α_1-AGP and conalbumin, did not require the additive. With these proteins relatively high concentrations were necessary to achieve successful enantiomer separations, and the partial separation zone technique was extremely effective especially for these conditions.

4. Conclusions

For the separation of enantiomers by affinity EKC, we described the partial separation zone technique previously developed by Valtcheva et al. [15] in which UV absorption by a protein used as a chiral selector did not disturb the detection. The resolution of enantiomers was enhanced by increasing the protein concentration without losing the detection sensitivity. The technique could be performed automatically using commercial CE instruments. Separations of some basic drug racemates were successful by this method employing relatively high concentrations of BSA, α_1-AGP, ovomucoid and conalbumin as pseudo-stationary phases. An additional advantage of the technique is a low consumption of the protein solution in comparison with the conventional method. The method validations in terms of the linearity of the calibration curve, reproducibility of peak areas, and that of the migration times make it clear that the technique can be used for quantitative analysis.

In conclusion, the partial separation zone technique is very powerful for the separation of enantiomers by affinity EKC using high concentrations of proteins. Furthermore, it is remarkably useful for a routine analysis with commercial instruments because of the automated procedures and good reproducibilities. We are convinced that many other enantiomeric separations can be achieved by the technique. Furthermore, the technique can be easily extended to the use of other UV-absorbing pseudo-stationary phases or additives in capillary zone electrophoresis. However, when the pseudo-stationary phase migrates in the same direction as the analytes, this method cannot be used because of migration of the pseudo-stationary phase through the detection cell.

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References

[1] K.M. Kirkland, K.L. Neilson and D.A. McCombs, *J. Chromatogr.*, 545 (1991) 43–58.
[2] S.R. Narayanan, *J. Pharm. Biomed. Anal.*, 10 (1992) 251–262.
[3] L. Siret, N.B. Leyder, A. Tambuté and M. Caude, *Analusis*, 20 (1992) 427–435.
[4] Y. Oda, N. Mano, N. Asakawa, Y. Yoshida, T. Sato and T. Nakagawa, *Anal. Sci.*, 9 (1993) 221–228.
[5] S. Terabe, K. Otsuka and H. Nishi, *J. Chromatogr.*, 666 (1994) 295–319.
[6] M.M. Rogan, K.D. Altria and D.M. Goodall, *Chirality*, 6 (1994) 25–40.
[7] T.J. Ward, *Anal. Chem.*, 66 (1994) 632A–640A.
[8] M. Novotny, H. Soini and M. Stefansson, *Anal. Chem.*, 66 (1994) 646A–655A.
[9] G.E. Barker, P. Russo and R.A. Hartwick, *Anal. Chem.*, 64 (1992) 3024–3028.
[10] P. Sun, N. Wu, G. Barker and R.A. Hartwick, *J. Chromatogr.*, 648 (1993) 475–480.
[11] S. Busch, J.C. Kraak and H. Poppe, *J. Chromatogr.*, 635 (1993) 119–126.
[12] R. Vespalec, V. Šustáček and P. Boček, *J. Chromatogr.*, 638 (1993) 255–261.
[13] Y. Ishihama, Y. Oda, N. Asakawa, Y. Yoshida and T. Sato, *J. Chromatogr. A*, 666 (1994) 193–201.
[14] Y. Tanaka, N. Matsubara and S. Terabe, *Electrophoresis*, 15 (1994) 848–853.
[15] L. Valtcheva, J. Mohammad, G. Petterson and S. Hjertén, *J. Chromatogr.*, 638 (1993) 263–267.
[16] M. Nakatani, A. Shibukawa and T. Nakagawa, *Biol. Pharm. Bull.*, 16 (1993) 1185–1188.
[17] S.A.C. Wren and R.C. Rowe, *J. Chromatogr.*, 603 (1992) 235–241.
[18] S. Terabe, H. Ozaki and Y. Tanaka, *J. Chin. Chem. Soc.*, 41 (1994) 251–257.