

**Pressurized Capillary Electrochromatography:
Theory, Mechanism, and Application in Enantiomeric Separations**

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Abstract

A set of equations were derived to describe the retention and separation processes in pressurized capillary electrochromatography (pCEC), and to predict the effects of experimental parameters in pCEC, such as pressure and electrical field strength, on the separation parameters of capacity factor (k'), resolution (R) and selectivity (α). The enantiomers of promethazine, carteolol, celiprolol, and albuterol were used as model compounds to evaluate the validity and the applications of the theoretical modeling.

Introduction

Capillary electrochromatography (CEC) combines the best features of capillary electrophoresis (CE) and high performance liquid chromatography (HPLC): high separation efficiency of CE and the versatile selectivity and large sample capacity of HPLC. However, in practice, when CEC was used without pressure, often on a commercial CE instrument, there were problems and difficulties associated with bubbles formation and column dry-out. These problems can be solved by a pressurized CEC (pCEC) system, in which a mobile phase is driven by both a pressurized flow and an electroosmotic flow (EOF). In such a system, a pressure can be applied to the inlet end, and outlet end if necessary, of the capillary column to suppress bubble formation ^[1]. Quantitative sample introduction in pCEC can also be easily achieved through a rotary-type injector ^[2]. The EOF can either be in the same direction as, or against, the pressurized flow. Therefore, the sample elution order may be manipulated ^[2]. Most importantly, it is amenable for a solvent gradient mode, similar to that in HPLC, by programming the composition of eluents ^[3]. With pCEC, the promises of CEC can be fully exploited.

Like HPLC, the success in applications of pCEC to practical separations relies on the understanding of the separation mechanism and theoretical development, by which the controls of all experimental parameters are guided. Several groups have proposed and

derived some models to relate the chromatographic retention to experimental parameters [4-12]. In this article, we introduce a set of equations to segregate the contributions to retention by HPLC from that by CE, and analyze the effects of experimental parameters, such as pressure and electrical field strength, on resolution (R), selectivity factor (α) and capacity factor (k'). We chose enantiomers as model compounds to evaluate the equations derived and the assumptions made in the separation mechanism because enantiomers have the identical electrophoretic mobility and many other physicochemical properties. The difference between two enantiomers is their interactions with the chiral stationary phases. Practically, enantiomeric separations are also a very important field in chemical, especially pharmaceutical, analyses. The separations of enantiomers by capillary electrochromatography (CEC) have received considerable attention in recent years [13-15]. The stationary phases evaluated included Chirasil-Dex [16], α_1 -acid glycoprotein (AGP) [17], cyclodextrins [18, 19], cellulose [20, 21], "Pirkle" phase [22], macrocyclic antibiotics [23-26], anion-exchange stationary phases [27] and molecular imprinted polymers (MIPs) [28,29]. In this article, enantiomers of several drug compounds, namely, promethazine, carteolol, celiprolol and albuterol, were resolved with vancomycin, a macrocyclic antibiotic, as the CSP on a pCEC system which was developed in our laboratory. The retention data of these enantiomers were further discussed with respect to the theoretical modeling.

Experimental Section

Materials The columns, purchased from Unimicro Technologies, Inc, (Pleasanton, CA, USA), were packed with 5 μm Chirobiotic V CSP (Astec, NJ, USA) by electrokinetic packing method ^[30,31]. Promethazine, carteolol, celiprolol, and albuterol were obtained from Tianjin Institute of Drug Control (Tianjin, China). Methanol (MeOH) and acetonitrile (MeCN), both of HPLC grade, were purchased from Langfang Xingke Chemicals, Co. Ltd. (Hebei, China). Glacial acetic acid (HOAc), triethylamine (TEA) and acetone, all of analytical grade, were obtained from Tianjin Chemical Reagents Company (Tianjin, China). Fused-silica capillaries were purchased from Polymicro Technologies Inc. (Phoenix, AZ, USA). Filter, backpressure regulator, cross, tee and injector were all purchased from Upchurch Scientific, (WA, USA).

Apparatus The schematic diagram of the pCEC apparatus, a modified TriSepTM-2000GV system (Unimicro Technologies, Inc., Pleasanton, CA, USA), is shown in Fig.1. The mobile phase was driven by a solvent delivery system (HPLC pumps) (through a filter to the splitting cross. The backpressure regulator was used to maintain a constant pressure on the column. The rotary-type of injector with a small injection volume of 10 nL was described in details previously ^[2]. An UV absorbance on-column detector with a fixed wavelength of 254 nm was used. The grounded electrode was set to the splitting cross and the other electrode was set to the tee in connection with the outlet

end of the column. Note that although the instrument was capable of the gradient elution only the isocratic mode was used in this study.

Methods Analyte stock solutions were prepared in MeOH at a concentration of 1.0 mg/mL and stored at 4°C. Samples were prepared by dilution of each stock solution with MeOH. The mobile phases were prepared by adding certain volume of HOAc and TEA to MeOH. The typical mobile phase was MeOH/HOAc/TEA=100/0.1/0.1 (v/v/v). Acetone was used as the marker of a non-retained solute.

Mechanism and Theory

Capacity Factor and Its Relationship with Experimental Parameters In pCEC, if there is no electrical field, we adopt the conventional definition of the capacity factor in the same fashion as in HPLC:

$$k'_0 = \frac{t_R - t_0}{t_0} \quad (1)$$

where k'_0 is the capacity factor based upon “pure” HPLC; t_R and t_0 are the retention time of the solute and the void time without electrical field, respectively.

For pCEC systems, the linear velocity of analyte in the mobile phase is:

$$v = v_p + v_{eo} + v_{ep} \quad (2)$$

where

$$v_{eo} = \mu_{eo}E \quad (3)$$

$$v_{ep} = \mu_{ep}E \quad (4)$$

similarly, the linear velocity of the mobile phase is:

$$v_m = v_{p,m} + v_{eo,m} \quad (5)$$

where for the solute, v_p , v_{eo} , and v_{ep} are the linear velocity of pressurized, electroosmotic, and electrophoretic flow, respectively and μ_{eo} and μ_{ep} are electroosmotic and electrophoretic mobilities, respectively. For the mobile phase, v_m , $v_{p,m}$, and $v_{eo,m}$ are the overall linear velocity, linear velocity of pressurized and electroosmotic flow, respectively. Values of v_{eo} and v_{ep} or μ_{eo} and μ_{ep} can be positive or negative. While v_{eo} , $v_{eo,m}$ and v_{ep} are in the same direction as v and v_m , v_{eo} (μ_{eo}) and v_{ep} (μ_{ep}) are positive, otherwise, v_{eo} (μ_{eo}) and v_{ep} (μ_{ep}) are negative. Note that for the mobile phase, there is no electrophoretic flow.

The linear velocity of the solute (v) and the linear velocity of the mobile phase (v_m) are related to the retention time:

$$v = \frac{L_e}{t_{R'}} \quad (6)$$

$$v_m = \frac{L_e}{t_0'} \quad (7)$$

where L_e is the effective length of the column; $t_{R'}$ and t_0' are the apparent retention time of the solute and the void time, respectively.

The capacity factor defined in Eq. 1 can be expressed with the linear velocity terms,

$$k'_0 = \frac{V_{p,m} - V_p}{V_p}; \text{ or } V_{p,m} = (1 + k'_0)V_p \quad (8a)$$

$$k'_0 = \frac{V_{eo,m} - V_{eo}}{V_{eo}}; \text{ or } V_{eo,m} = (1 + k'_0)V_{eo} \quad (8b)$$

where in Eq. 8b, it is considered that under no pressure conditions, the electroosmotic flow of the mobile phase related to that of the solute due to the interactions with stationary phases, which should resemble those in regular HPLC, although details of the effect of the electrical field on the chromatographic retention process is still unknown. We will test such a hypothesis in the analysis and modeling of the retention data.

The apparent retention time of the solute and the void time under pCEC conditions can be expressed as follows (in reciprocal):

$$\frac{1}{t_{R'}} = \frac{V_p}{L_e} + \frac{(\mu_{eo} + \mu_{ep})}{L_e} E \quad (9)$$

$$\frac{1}{t_0'} = \frac{V_{m,p}}{L_e} + \frac{\mu_{eo,m}}{L_e} E \quad (10)$$

In the case of pCEC, the apparent capacity factor, k' , can be expressed in the terms of linear velocity (Eq. 11) :

$$k' = \frac{V_m - V}{V} \quad (11)$$

Combined with Eq. 2-5, the capacity factor under pCEC conditions (Eq. 11) can be expressed with experimental parameters:

$$k' = \frac{(V_{p,m} + V_{eo,m}) - (V_p + V_{eo} + V_{ep})}{V_p + V_{eo} + V_{ep}} = \frac{k'_0 V_p + k'_0 V_{eo} - V_{ep}}{V_p + V_{eo} + V_{ep}} \quad (12)$$

With solute retention parameters,

$$k' = \frac{k'_0 V_p + k'_0 \mu_{eo} E - \mu_{ep} E}{V_p + (\mu_{eo} + \mu_{ep}) E} \quad (13a)$$

With mobile retention parameters, often measurable with a non-retained solute such as acetone,

$$k' = \frac{k'_0 V_{p,m} + k'_0 \mu_{eo,m} E - (1 + k'_0) \mu_{ep} E}{V_{p,m} + [\mu_{eo} + (1 + k'_0) \mu_{ep}] E} \quad (13b)$$

Eq. 13a can degenerate into specific equations for given conditions.

A). HPLC

For HPLC, $E=0$, then k' is simplified to the conventional term, k'_0 , as described in Eq.

6.

$$k' = \frac{V_{p,m} - V_p}{V_p} = k'_0 \quad (14)$$

B). CE

For CE (no pressurized flow, no chromatographic interactions), $v_p = 0$, $k'_0 = 0$, then

Eq. 13a reduced to Eq. 15,

$$k' = -\frac{\mu_{ep}}{\mu_{eo} + \mu_{ep}} \quad (15)$$

Eq.(15) indicates that k' does not change with E in CE.

C). CEC

For “pure” CEC (no pressurized flow), $v_p = 0$, eq. 13a is degenerated to Eq. 16.

$$k' = \frac{k'_0 \mu_{eo} - \mu_{ep}}{\mu_{eo} + \mu_{ep}} \quad (16)$$

Eq.(16) indicates that k' does not change with E in CEC, which is similar to the case of CE.

The first order derivative of k' with respect to E is expressed as (Eq. 17)

$$\frac{dk'}{dE} = -\frac{v_p \mu_{ep} (1 + k'_0)}{[v_p + (\mu_{eo} + \mu_{ep})E]^2} \quad (17)$$

From Eq.(17) it can be inferred that: in pCEC, while $\mu_{ep} < 0$, i.e., the direction of electrophoresis is against the EOF, k' increases with E ; while $\mu_{ep} = 0$ (neutral analyte), k' does not change with E ; while $\mu_{ep} > 0$, i.e., the direction of electrophoresis is along the EOF, k' decreases with E . Therefore, for charged compounds, unlike CE, CEC or HPLC, the capacity factor in pCEC changes with the electrical field strength.

The Retention Mechanisms in pCEC: Separations of HPLC and CE

From Eqs. 9 and 10, we could obtain important chromatographic parameters from graphic methods. Once a series of retention time for a solute and a non-retained neutral marker under different voltage conditions are obtained, the terms of Le/t_R' and Le/t_0' can be plotted against E . The slope, A , and the intercept, B , for the solute series, can be obtained as:

$$A = \mu_{eo} + \mu_{ep} \quad (18a)$$

$$B = v_p \quad (18b)$$

for the neutral marker series, where m denotes the mobile phase,

$$A_m = \mu_{eo,m} \quad (19)$$

$$B_m = v_{m,p} \quad (19a)$$

From the slope and Eq. 8b, one could obtain the electrophoretic mobility of the mobile phase and the solute (Eqs 19, 20, 21). Note that k'_0 can be obtained from the intercept.

$$\mu_{eo,m} = A_m \quad (19)$$

$$\mu_{eo} = \frac{A_m}{(1 + k'_0)} \quad (20)$$

$$\mu_{ep} = A - \frac{A_m}{(1 + k'_0)} \quad (21)$$

Therefore, from the intercept values and Eq. 14, one could easily obtain the HPLC capacity factor, k'_0 (Eq. 22).

$$k'_0 = \frac{B_m - B}{B} \quad (22)$$

Therefore, the HPLC and CE retention mechanisms can be separated. The corresponding key parameters can be obtained from measurement of the retention time for a solute and a non-retained neutral marker under different voltage conditions.

Selectivity

The selectivity factor, α , a key parameter indicating the resolution, between the two enantiomers (with the same $v_{p,m}$, v_p , μ_{eo} and μ_{ep} values) can be derived from Eq.13a as follows:

$$\alpha = \frac{k'_2}{k'_1} = \frac{k'_{0,2}v_p + k'_{0,2}\mu_{eo}E - \mu_{ep}E}{k'_{0,1}v_p + k'_{0,1}\mu_{eo}E - \mu_{ep}E} \quad (23)$$

Based on Eq.(23), the selectivity is influenced by experimental parameters such as the field strength (E) and the linear velocity (v_p), as well as the characteristics of the solutes, the mobile phase and the stationary phase and the molecular interactions between the solutes and the stationary phase ($k'_{0,2}$ and $k'_{0,1}$).

Results and discussion

Enantiomer separations by capillary HPLC and pCEC

For antibiotic-based CSPs, some polar organic mobile phases have been proven very effective for the separations of many chiral compounds in HPLC [22]. In our study, the polar organic phase with MeOH/HOAc/TEA=100/0.1/0.1 (v/v/v) was selected for the enantiomeric separations of promethazine, carteolol, celiprolol, and albuterol by capillary HPLC and pCEC. The results were shown in Fig.2 and Table 1. The enantiomers of promethazine, carteolol and celiprolol were baseline separated by pCEC with an electrical field strength of 250 V/cm and pressure of 250 psi. It can be seen from Fig.2 that the solutes flowed out faster while the efficiency was much higher in pCEC than in capillary HPLC. Because the electroosmosis flow was superimposed to the pressurized flow in pCEC, the overall flow rate in pCEC is larger than that in capillary HPLC. Therefore, the separation time is shortened in pCEC. Since the electroosmosis has a plug-like flow profile, the overall column efficiency and resolution was increased in pCEC compared with that in capillary HPLC.

Effect of pressure The flow rate of pressurized flow was regulated by the pressure difference between the inlet end and outlet ends of the column. By changing the backpressure regulator, the linear velocity of the mobile phase, u ($u=v_p+v_{e0}$), as in the above derivations, can be changed. With promethazine as a model solute and acetone as a non-retained marker, the effects of linear velocity on efficiency, resolution and selectivity were investigated. As shown in Fig.3, the plate height (H) increased with the increase of u even at a very low linear velocity (0.34 mm/s for capillary HPLC and 0.41 mm/s for pCEC). Then we tested the relationship of $H \sim u$ under the same conditions but with thiourea as a model solute. The results were shown in Fig.3. The plate heights calculated with thiourea were much higher than that obtained with promethazine. The optimal linear velocity (u_{opt}) for thiourea was 0.50 mm/s for capillary HPLC and 0.57 mm/s for pCEC. This value was rather low, perhaps because of the existence of dead volume in the experimental apparatus. Compared with the case of thiourea, the lower H values obtained with promethazine may be due to its lower diffusion coefficient in the mobile phase. According to van Deemter equation, the lower diffusion coefficient leads to a lower molecular diffusion value and, consequently, a lower plate height. At the same time, the value of u_{opt} will be decreased with the decrease of diffusion coefficient. From Fig.4, it can be seen that the resolution decreased with the increase of linear velocity, due to the loss of the efficiency, since the selectivity (α) did not change significantly with u not only for capillary HPLC but also for pCEC. The relative independence of α on linear velocity could be explained from the effect of electrical field strength on α , discussed in the

following section.

Effect of electrical field strength The influence of electrical field strength (E) on the enantiomeric separation of promethazine by pCEC was investigated at a pressure of 250 psi. With the increase of E , the retention times of acetone (t_0) and promethazine (t_{R1} and t_{R2}) and the plate height were all decreased (Fig.5). In Fig.6 is shown that the capacity factors of promethazine (k_1' and k_2') decreased with the increase of E . However, the electrical field strength had little effect on the selectivity. It is well known that the capacity factor does not change with the flow rate in HPLC, and that it also does not change with E in CEC. In our experiment, there was evidence that capacity factor does change with E in pCEC. Therefore, a mathematical model describing the relationship of capacity factor with experimental conditions was developed as follows.

For promethazine, as described in Eq. 10, a plot of $\frac{1}{t_0}$ vs. E yielded a straight line with a slope of $\frac{\mu_{eo}}{L_e}$ and a vertical intercept of $\frac{v_{m,p}}{L_e}$ (Fig.7). Therefore, the values of μ_{eo} and $v_{m,p}$ were obtained to be 6.70×10^{-5} cm²/v/s and 2.47 cm/min. As described in Eq. 9, plotting $\frac{1}{t_{R1'}}$ and $\frac{1}{t_{R2'}}$ vs. E yielded two straight lines (Fig.7). From the intercepts, the values of v_p were obtained. With the values of $v_{m,p}$ and v_p , k_0' values of the two enantiomers of promethazine were found to be $k_{0,1}'=3.61$ and $k_{0,2}'=4.05$, respectively, using Eq. 22. From the slopes, the electrophoretic mobilities of the two enantiomers were

found to be $\mu_{ep,1}=6.25\times 10^{-5}$ cm²/v/s and $\mu_{ep,2}=6.21\times 10^{-5}$ cm²/v/s, respectively (Eqs. 19, 20 and 21). The values of $\mu_{ep,1}$ and $\mu_{ep,2}$ were nearly identical. The identical values of μ_{ep} are expected because the two enantiomers should have the same electrophoretic mobilities in the mobile phase. This validates the derivation of the above equations and the assumptions made thereof.

For a compound 1 and its enantiomer 2, substituting the values of $k_{0,1}'$, $k_{0,2}'$, v_p , $v_{m,p}$, μ_{eo} and μ_{ep} into Eq.(13a), the relationship of k_1' and k_2' vs. E can be calculated. The theoretical curve were shown in Fig. (6). It can be seen that the calculated results are in good agreement with the experimental data.

According to Eq. 23, the selectivity α is influenced by E (Fig. 6). The calculated value of α was changed from 1.12 at $E=0$ to 1.13 at $E=250$ V/cm. Therefore, apparently in the case of promethazine, the electrical field strength had little effect on α . In fact, based on Eq. 23, the effect of the electrical field strength (E) is also related to other terms such as the electroosmotic flow of the mobile phase, μ_{eo} , the HPLC capacity factor k_0' , and the electrophoretic mobility, μ_{ep} , which are the parameters often regarded as the indicators of the residence time or interaction time of the solute in the CEC column.

Clearly, in order to design the separation parameters it is very important to understand the contribution of the CE mechanism (μ_{eo} , μ_{ep} and E) on the separation, in addition to the HPLC contributions such as the choices of stationary phases, mobile phases and the

pressurized flow. Of course, the case presented here is that of μ_{ep} is identical for both enantiomeric compounds. For non-enantiomeric separations, the μ_{ep} can be different. Further investigation on the effects of the electrical field strength on non-enantiomeric model compounds under various mobile phase and stationary phase conditions is currently ongoing in our laboratory.

Conclusion

Chiral compounds, promethazine, carteolol, and celiprolol were separated by pCEC with a polar organic mobile phase and Chirobiotic V as the chiral stationary phase. The efficiencies and resolutions were found higher in pCEC than that in capillary HPLC with the same column under the same conditions. A mathematical model was developed to describe the relationship between the apparent capacity factor and the selectivity factor in pCEC and experimental parameters. These equations were used to simulate the experimental data and good agreements were found.

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Captions

Fig.1 The schematic diagram of the experimental apparatus used for enantiomer separation by pCEC

1. mobile phase vial, 2. solvent delivery system, 3. micro filter, 4. splitting cross, 5. backpressure regulator, 6. waste reservoir, 7. injector, 8. column, 9. detector, 10. tee, 11. waste reservoir, 12. high voltage supply

Fig.2 Chromatograms of enantiomer separations by pCEC (solid line) and capillary HPLC (dashed line).

Column: 150 μm id. \times 20 cm, overall length: 30cm, pack with 5 μm Chirobiotic V (vancomycin). Mobile phase: MeOH/HOAc/TEA=100/0.1/0.1 (v/v/v). Pump flow rate: 0.02 mL/min. Pressure: 250 psi. Electrical field strength for pCEC: 250 V/cm. Injection: 10 nL. Detection: 254 nm. Sample: (a) promethazine, (b) carteolol, (c) celiprolol, (d) albuterol

Fig.3 Effects of linear velocity (u) of mobile phase on the plate heights calculated by the first peak of promethazine (■, □) and thiourea (●, ○) in pCEC (□, ○) and capillary HPLC (■, ●). Conditions are the same as in Fig.2 except the pressure is changed to give different u values.

Fig.4 Effects of linear velocity of mobile phase on resolution (\bullet , \circ) and selectivity (\blacksquare , \square) of enantiomers of promethazine in pCEC (\circ , \square) and capillary HPLC (\bullet , \blacksquare). Conditions are the same as in Fig.3.

Fig.5 Effects of electrical field strength (E) on t_0 (\bullet), t_{R1} (\blacktriangle), t_{R2} (\blacksquare) and plate height calculated with the first peak of promethazine (\circ). Conditions are the same as in Fig.2 except the values of E are changed.

Fig.6 Effects of E on capacity factors of the two enantiomers of promethazine (k_1' : \blacktriangle , k_2' : \blacksquare) and selectivity (\bullet). Solid lines are calculated with model equations. Conditions are the same as in Fig. 5.

Fig.7 The relationships of $1/t_0$ (\bullet), $1/t_{R1}$ (\blacktriangle), and $1/t_{R2}$ (\blacksquare) vs. E . of promethazine (with data from Figure 5 & 6)

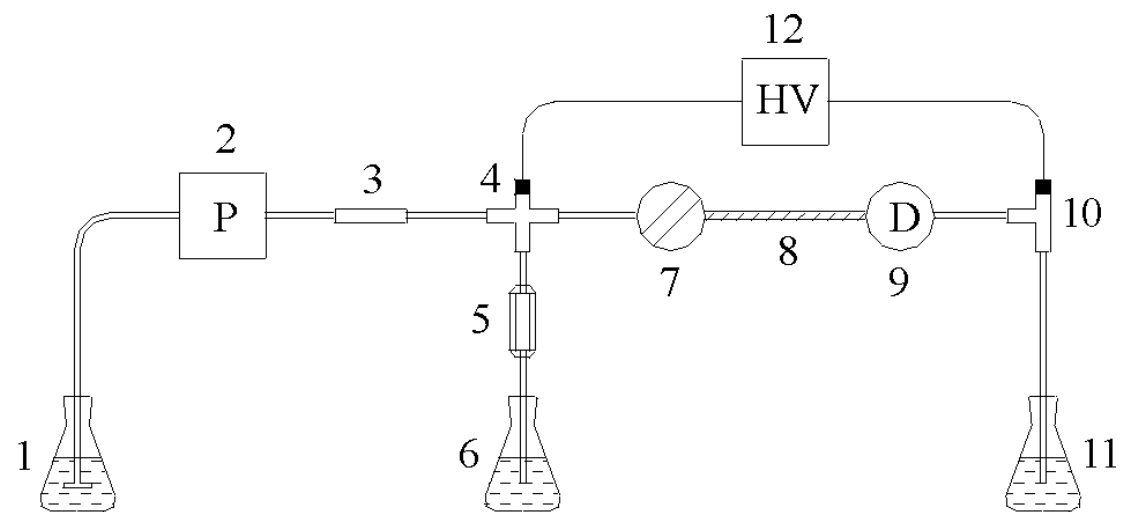


Fig.1

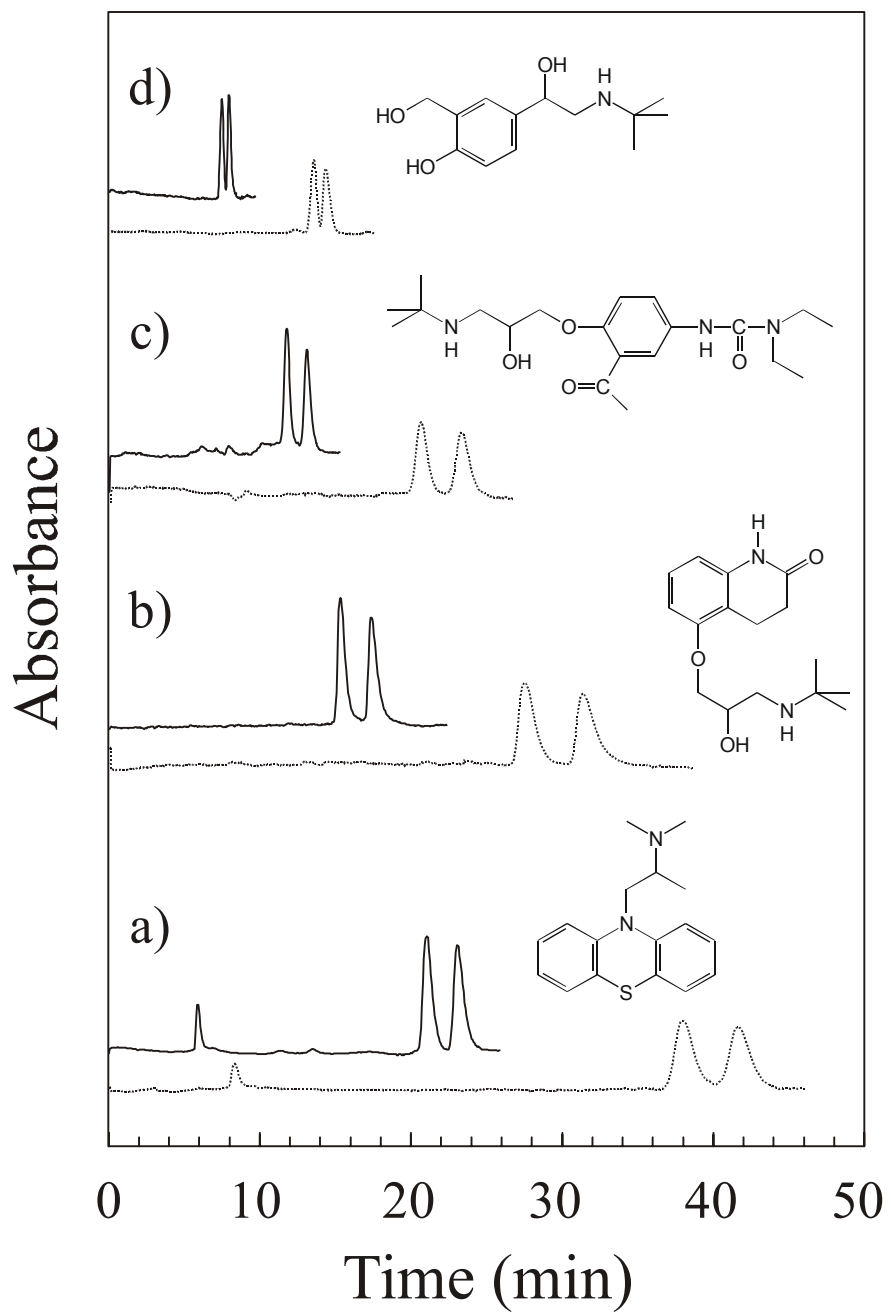


Fig.2

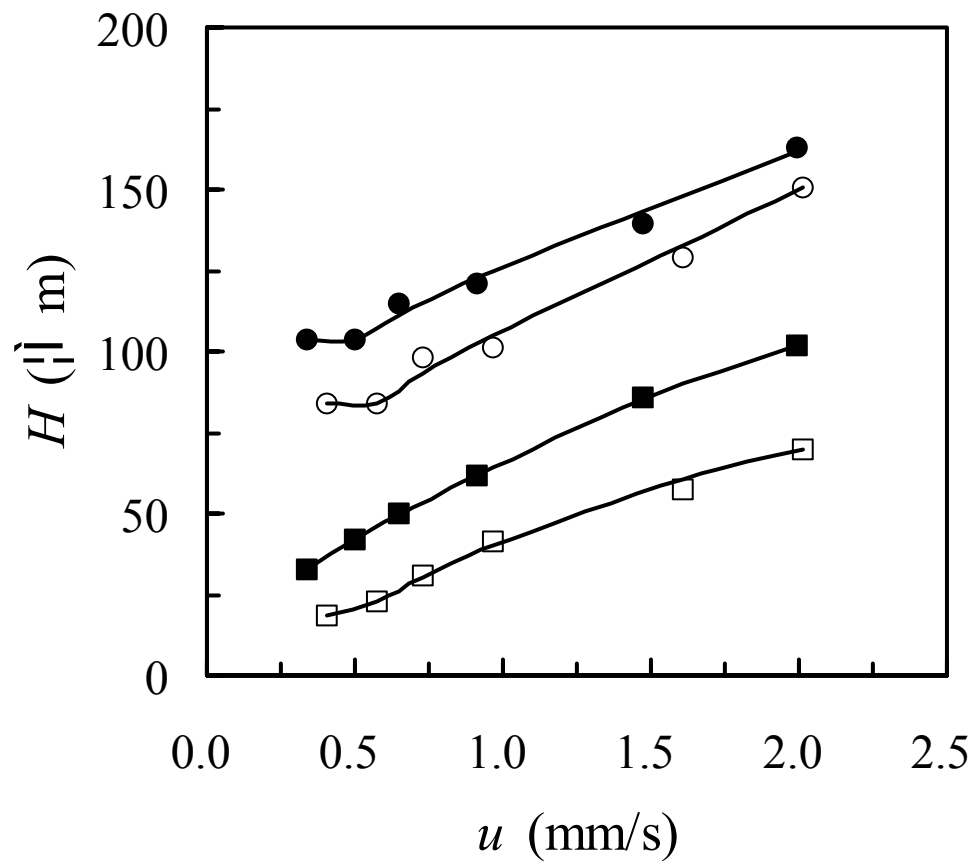


Fig.3

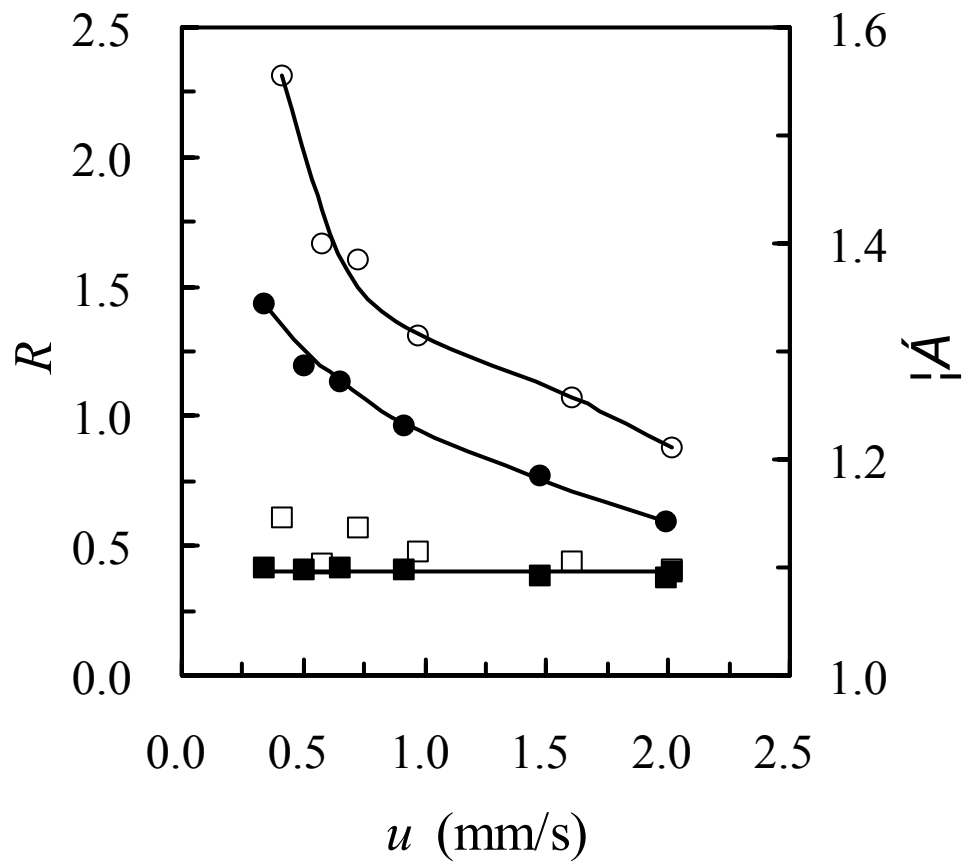


Fig.4

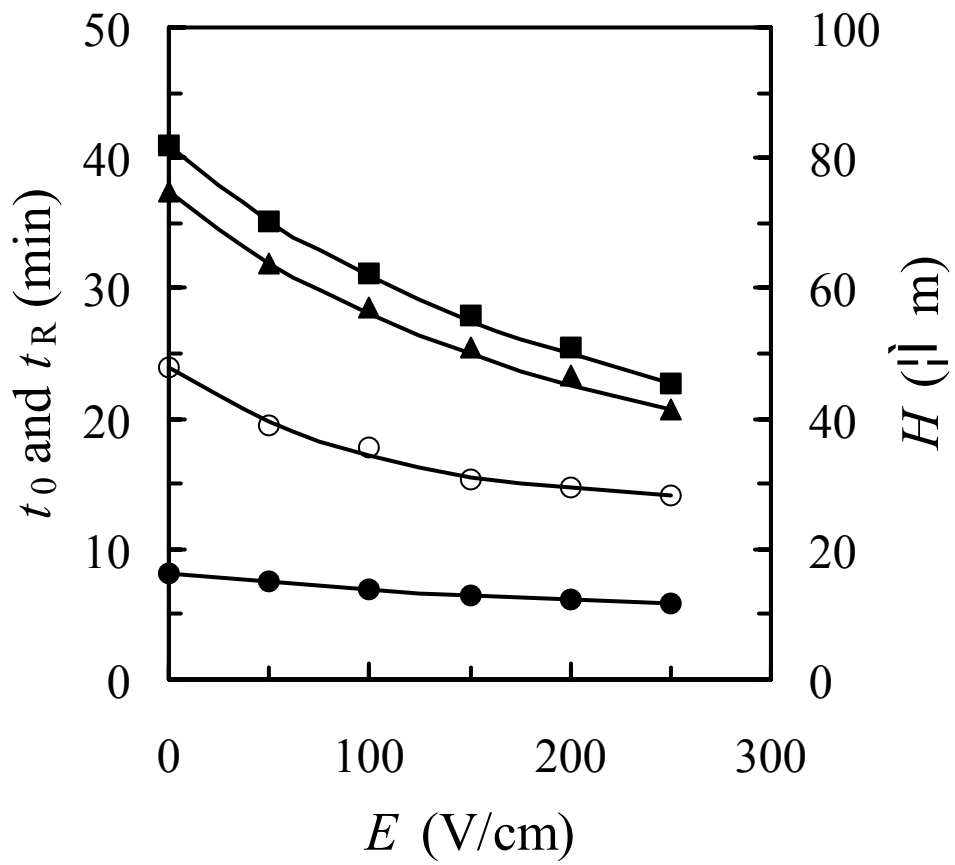


Fig.5

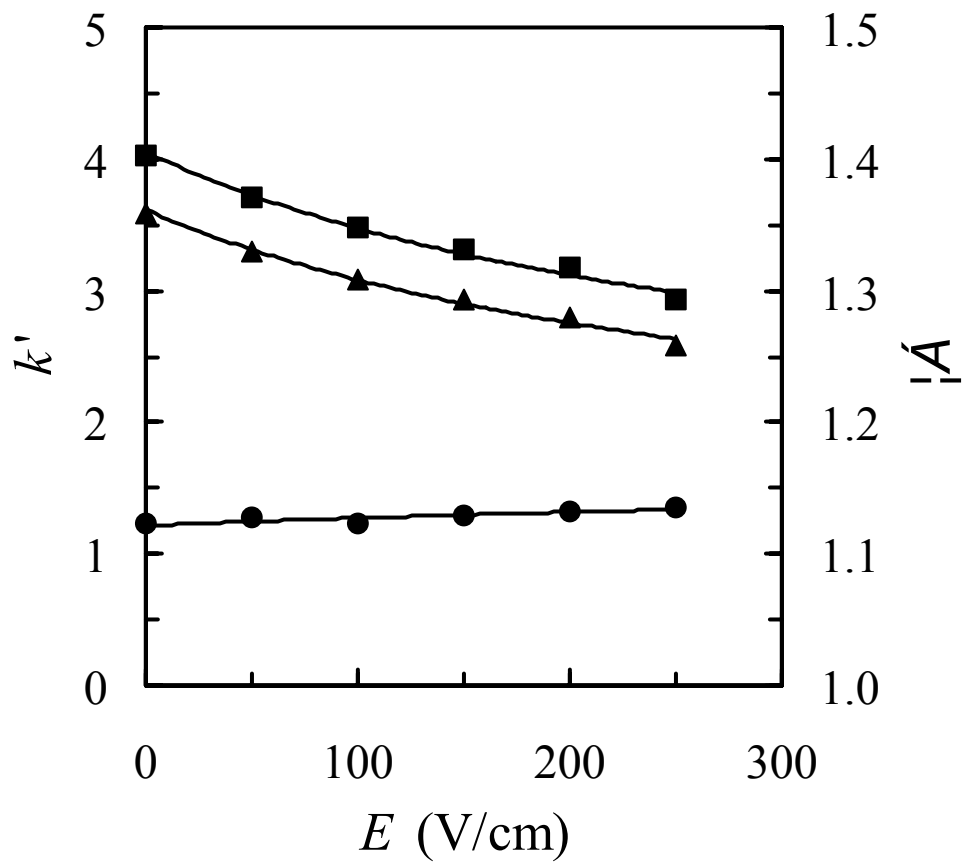


Fig.6

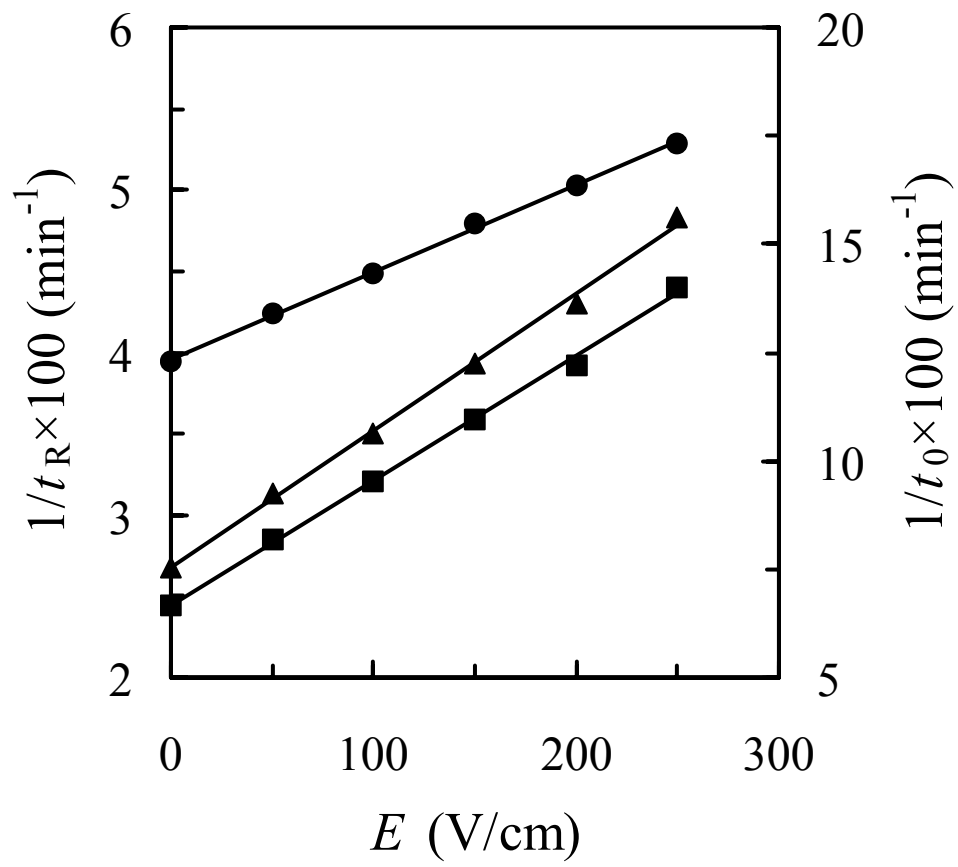


Fig.7

Table 1 Enantiomer separations by capillary HPLC and pCEC in polar organic phase with vancomycin as chiral stationary phase. Mobile phase: MeOH/HOAc/TEA=100/0.1/0.1 (v/v/v), Field strength in pCEC: 250V/cm, Pressure: 250 psi .

Compound	Separation mode	t_{R1} (min)	t_{R2} (min)	N_1 (plates/m)	N_2 (plates/m)	R_s
Promethazine	Capillary HPLC	37.4	41.0	20 894	21 028	1.48
	pCEC	20.7	22.7	35 480	32 115	1.90
Carteolol	Capillary HPLC	26.9	30.7	17 275	17 436	1.94
	pCEC	14.9	16.9	27 531	27 249	2.34
Celiprolol	Capillary HPLC	20.3	23.0	18 283	17 688	1.84
	pCEC	11.6	12.9	29 474	28 616	2.06
Albuterol	Capillary HPLC	13.1	13.9	27 407	22 149	0.98
	pCEC	7.3	7.8	28 943	32 932	1.21