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Chiral separation by simultaneous use of vancomycin as stationary phase chiral selector and chiral mobile phase additive

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Abstract

Improved chiral selectivity was observed for numerous compounds when vancomycin was added to the mobile phase on a Chirobiotic-V column. This chiral mobile phase additive (CMPA) is the same chiral selector as that bonded to the stationary phase of this Chirobiotic-V column. A substantial increase in the difference in enthalpy of transfer, $\Delta\Delta H$, and in the difference in entropy of transfer, $\Delta\Delta S$, for two enantiomers was observed when vancomycin was used as both the mobile phase and the stationary phase chiral selector. The importance of mobile phase composition, analytical column, CMPA concentration was investigated. Also, higher resolution was observed for the separations of acidic compounds when a fluidity enhancing solvent, such as fluoroform, was added into the mobile phase. However, the most commonly used fluidity enhancement solvent, CO_2, was ineffective.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Vancomycin

1. Introduction

Life is heavily dependent on chirality. For example, the human body metabolizes only the D-enantiomer of glucose and produces only L-amino acids [1]. Chiral discrimination is frequently encountered in biological systems. Chirality is also an important issue in the pharmaceutical industry due to the potential of different activities and toxicities of drug enantiomers [2]. Meanwhile, chiral separations are often difficult. The search for a chiral selector that can discriminate specific enantiomers is often time consuming, tedious, and results in unsatisfactory performance. A specified chiral selector usually can only separate a limited range of compounds. The search for more ''universal'' chiral selectors is challenging.

A chiral selector can be employed as either a chiral stationary phase (CSP) or a chiral mobile phase additive (CMPA) in LC chiral separations. The choice can make a difference in the selectivity of the separation. For example, the retention order of hexobarbital enantiomers reversed when cyclodextrin (CD) was used as mobile phase additive compared to that observed on an achiral column where CD was strongly adsorbed to the stationary phase surface [3]. Sometimes it is possible to improve chiral enantioselectivity by combining these two techniques and obtain a ''synergistic'' effect. Kuijpers et al. demonstrated that chiral separations were improved by the use of a structurally unrelated (D)-(+)-camphorsulphonic acid as CMPA on a β-cyclodextrin CSP [4]. By using an aqueous solution of L-(+)-tartrate as the mobile phase additive, Fujita et al. increased the resolution of $[Co(en)_3]^{3+}$ enantiomers on a Sephadex

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cation exchanger that contained D-(−)-tartrate [5]. Pettersson and Gioeli's work also showed that naproxen enantiomers were better separated on an acetylquinidine–silica surface when quinidine was added into mobile phase [6]. Different models have been proposed to explain these phenomena. Duff et al. explained the synergistic effects observed in a system where CSP was used in combination with its homologous CMPA [7]. This model explained the phenomenon that chiral resolution improved when an (*S*)-CMPA was added into the mobile phase and an (*R*)-CSP was used, while the resolution degrades when an (*R*)-CMPA was used with an (*R*)-CSP.

In this study, the use of vancomycin as both the chiral stationary phase and the chiral mobile phase additive was characterized. Vancomycin is used to separate numerous types of enantiomers and is increasingly applied in HPLC separations [8]. Vancomycin is a macrocyclic antibiotic molecule. It has three fused macrocyclic rings, eighteen stereogenic centers, nine hydroxyl groups, and two amine groups [8]. This diversity in functionality opens the possibility to have different interaction mechanisms with different compounds. Vancomycin has been applied successfully as CMPA in both capillary electrophoresis (CE) [9,10] and thin-layer chromatography, TLC [11]. It often provides superior resolving power for anionic enantiomers. Previous work also includes its use as CSP in HPLC [8,12,13] and SFC [14] for a variety of compounds.

The impact of using vancomycin as a chiral mobile phase additive on enantiomer separations when surface bound vancomycin is the stationary phase will be characterized by studying the effect of vancomycin concentration in the mobile phase on the selectivity, resolution and retention factor of test solutes and by measuring the differences in enthalpy and entropy of transfer for solutes with and without vancomycin in the mobile phase.

Enhanced-fluidity liquid chromatography (EFLC) was recently shown to be useful for improving enantioselectivity on vancomycin CSP [15]. Enhanced-fluidity liquid mixtures are prepared by adding liquified gas into high solvent strength liquids. Enhanced-fluidity liquid mixtures have solvent strengths similar to those of common liquids, while providing low viscosity and high diffusivity conditions which generally results in faster mass transfer. Faster separations, higher efficiency, lower pressure drop across the column, and better selectivity have been observed for EFLC conditions. For the chiral separations studied to date [15], the variation of the retention factor and efficiency seems unpredictable using some enhanced-fluidity liquid mixtures as mobile phases. However, improved enantioselectivity was always achieved [15]. For example, in the separation of 1,1′-bi-2-naphthol enantiomers, the retention factor and reduced plate height of both enantiomers decreased initially with the addition of CO_2 into the mobile phase, but both parameters increased when CO_2 mol% exceeded 40. Meanwhile, the enantiomer resolution increased continuously as a function of increased CO_2 in the mobile phase and baseline separation was achieved for >60 mol% CO_2 in the mobile phase. Herein, the EFLC technique is applied to the combined vancomycin CSP–CMPA system and the results are compared to that obtained under common LC conditions.

2. Experimental

2.1. Materials

HPLC grade methanol and tetrahydrofuran were purchased from Fisher Scientific (Pittsburgh, PA) and used as received. Water was purified from distilled water by using a NANOpure Π filtration system (SYBRONIBarnstend, Boston, MA) and the final resistivity was 18.3 megaohms. Ammonium nitrate (99.999%) used to prepare buffer solutions was obtained from Aldrich Chemical Co. (Milwaukee, WI). Test racemates used in this investigation include: warfarin and 3-(α-acetonyl-4-chlorobenzyl)-4-hydroxy coumarin purchased from Aldrich Chemical Co.; flurbiprofen and ketoprofen purchased from Sigma Chemical Company (St. Louis, MO) and Fmoc–D(L)–Pro–OH and Fmoc–D(L)Lys(Boc)–OH obtained from AnaSpec Inc. (San Jose, CA). These compounds were dissolved in methanol with a concentration of 2 mg/ml. SFE/SFC grade CO_2 (99.999%) and CHF_3 (99.999%) were obtained from Air Products and Chemicals (Allentown, PA).

2.2. Instrumentation

The mobile phase was delivered by an Isco LC-260D syringe pump (Isco, Lincoln, NE). A Valco-W-

series high-pressure injection valve with a 200-nL internal injection loop (Valco Instruments Houston, TX) was used for sample injection. A Chirobiotic-V 150×4.6 mm HPLC column which includes 5 μm silica gel that is chemical bonded with vancomycin was used (Advanced Separation Technologies Inc., Whippany, NJ). Two other HPLC columns were used for purposes of comparison. One was a 250×2 mm Betasil Silica 60 column with 5 μm diameter particles and the other was a 250×4.6 mm ES Diphenyl Hypersil column with 5 μm diameter particles. Both were obtained from Keystone Scientific (Bellefonte, PA). A Spectra 100 variable wavelength UV–Vis absorbance detector (Thermo Separation, Fremont, CA) was used with the wavelength set to 308 nm for warfarin and 3-(α-acetonyl-4-chlorobenzyl)-4-hydroxy coumarin while 264 nm was used for the other compounds. The detection cell was created by removing the polyimide coating from a piece of 250 μm I.D. fused-silica tubing (Polymicro Technologies, Phoenix, AZ). Between the pump and the injector, a Betasil Silica pre-column (Keystone Scientific Bellefonte, PA) was installed. An inline filter with a 0.5 μm frit (Valco Instruments) was also used to protect the column from plugging. A Perkin-Elmer Sigma oven (Perkin-Elmer Corporation) was used to elevate the column temperature. Flow rate of the mobile phase was controlled by attaching a fused-silica restrictor of a certain length to the column outlet and directly monitoring the flow with the syringe pump (±0.00 1 ml/min). A Setra model 204 pressure transducer (Setra Systems, Acton, MA) was placed between the detector and the restrictor to ensure the pressure was above a minimum value that maintains the mobile phase mixture as one phase.

2.3. Mobile phase preparation and data analysis

Methanol–H_2O was applied as an initial mobile phase with a volume ratio of 80:20, which corresponds to a 64:36 mole ratio. 20 m*M* ammonium nitrate aqueous solution was always used instead of pure water to improve peak shape and maintain elution strength. Differing amounts of vancomycin were added into mobile phases. Both vancomycin and ammonium nitrate were first dissolved in water then a specific amount of methanol was added. All mixtures were degassed before use. The method used to prepare the enhanced-fluidity liquid mobile phase was the same as described previously [16]. CO_2 or CHF_3 was transferred from another Isco syringe pump into the pump that was used in the system after a certain amount of liquid was previously placed in the pump. All mixtures containing CO_2 or CHF_3 were allowed to equilibrate for at least 12 h before use and were mixed throughout the 12 h period.

The chromatographic data were collected and analyzed using EZChrom data system (Scientific software, San Ramon, CA) and Peakfit 4.0 (Peakfit Analysis Software, Jandel Scientific, San Rafael, CA). The second statistical moment was used to provide band dispersion information. Each reported data point is an average of two or three measurements.

The retention time of the injection solvent was used to determine the hold up time of the column, t_0. In the case when the solvent peak was not observed, the retention time of acetone was used instead. All experiments were done at room temperature and at an inlet pressure of 170 atm, unless otherwise specified.

3. Results and discussion

3.1. Combination of vancomycin as stationary phase and chiral mobile phase additive

For highly polar racemates we found it difficult to achieve adequate resolution using the Chirobiotic V stationary phase. For example using polar solutes such as, flurbiprofen, ketoprofen, and derivatized amino acids, with mixtures of methanol and NH_4NO_3 (aq) as the mobile phase, the enantiomers of these solutes were not separated. Several other mobile phases were tested. Tetrahydrofuran–20 m*M* NH_4NO_3 (20:80, volume ratio) was one of the most suitable mobile phase compositions found. Under this mobile phase, the enantiomers of flurbiprofen and Fmoc–Lys(Boc)–OH were separated with enantio selectivity of 1.16 and 1.10 respectively. But Fmoc–Pro–OH enantiomers remained unresolved. Furthermore, the chromatographic efficiencies of these separations were low (reduced plate height ≥30 for the first elute peak of Fmoc–Lys(Boc)–OH). Retention factors also increased significantly compared to those using 64:36 mol% CH_3OH–20

m*M* NH_4NO_3. For example, k_1 changed from 0.08 to 3.36 for flurbiprofen.

In an attempt to separate these very polar compounds more efficiently, vancomycin was studied as a chiral mobile phase additive. Several achiral HPLC columns were tested, including a 250×2 mm Betasil Silica 60 column and a 250×4.6 mm ES Diphenyl Hypersil column in combination with vancomycin as a mobile phase additive. However, all the enantiomers studied eluted as one peak. No separation was observed with up to 2 m*M* vancomycin present in the mobile phase.

Next the vancomycin was used as a chiral mobile phase additive (CMPA) when combined with the Chirobiotic-V column. These studies involved using 64:36 mol% CH_3OH–20 m*M* NH_4NO_3 as the mobile phase. Different amounts of vancomycin were dissolved in buffer solution prior to mixing the buffer with methanol. Remarkable increases in enantioselectivity were observed when vancomycin was added. Baseline separation was achieved for flurbiprofen enantiomers with as low as 1 m*M* vancomycin present in the mobile phase. Meanwhile, minimum change in the retention time was observed. For example using 64:36 mol% CH_3OH–20 m*M* NH_4NO_3 as the mobile phase at flow rate 0.5 ml/min without vancomycin added to the mobile phase, the enantiomers of flurbiprofen both elute at a retention time of 3.77 min. However, when 1 m*M* vancomycin is present in the same mobile phase the retention times of the enantiomers is 3.99 and 4.43 min and resolution, R_s of 1.73 was obtained (Fig. 1). Similar improvements in chiral resolution were also observed for Fmoc–Pro–OH (R_s from 0 to 0.46) and Fmoc–Lys(Boc)–OH (R_s from 0 to 1.08) enantiomer separations when vancomycin was added to the mobile phase.

The addition of vancomycin seems highly beneficial to enantiomer discrimination when vancomycin is also the stationary phase. Both selectivity and resolution increased for the tested molecules with the increase in vancomycin concentration. Fig. 2 illustrates the variation in enantiomer resolution with the addition of vancomycin. As shown in Table 1, there is also a slight increase in retention factor and a slight decrease in efficiency associated with the increase in CMPA concentration. As the concentration of vancomycin in the mobile phase was

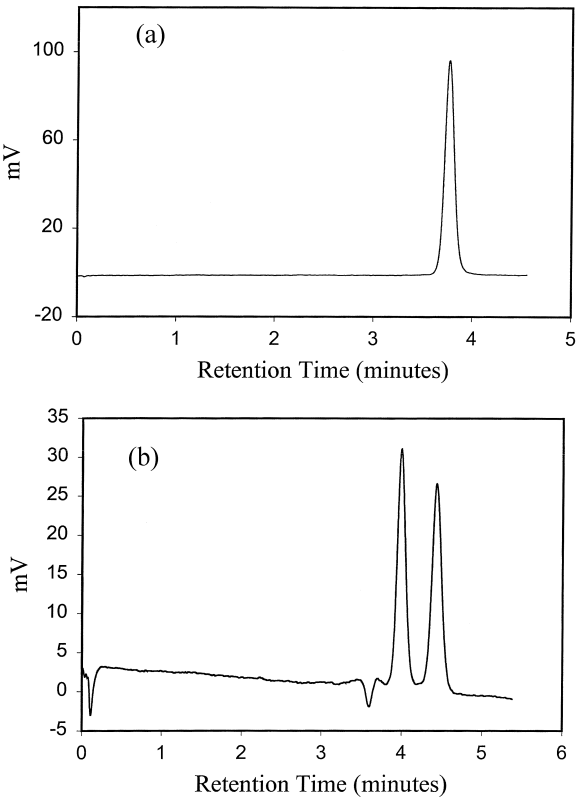

Fig. 1. Chromatograms of the separation of flurbiprofen at the same flow rate of 0.5 ml/min with: (a) 64:36 mol% CH_3OH/20 m*M* NH_4NO_3, (b) 64:36 mol% CH_3OH–20 m*M* NH_4NO_3 + 1 m*M* vancomycin as CMPA.

increased from 2 m*M* to 3 m*M*, minimal improvement in selectivity was observed.

Adsorption of vancomycin onto the support particle could contribute partially to the increase on selectivity and resolution. However, there are two reasons for not considering it the major factor. First, the amount of vancomycin adsorbed is low. For example, from breakthrough volume measurements, 30 mg of vancomycin was adsorbed to the Chirobiotic V column using the 64:36 mol% CH_3OH–20 m*M* NH_4NO_3 mobile phase and 1.5 m*M* vancomycin in the mobile phase. This is approximately 10% of the originally bonded vancomycin. Second, when the Betasil Silica 60 column was used, no enantio selectivity was observed with up to 2 m*M* vancomycin CMPA. If adsorption of vancomycin to the support was the primary cause of the increased

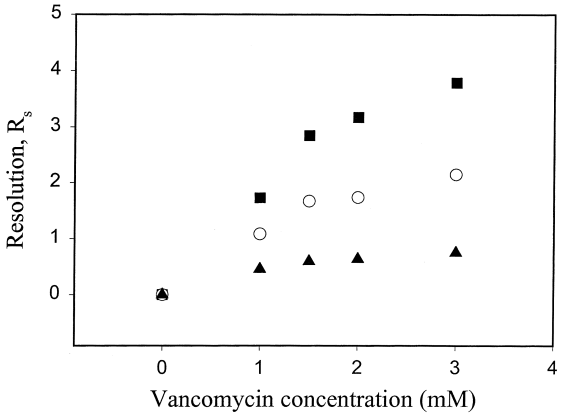

Fig. 2. Effect of mobile phase vancomycin composition on the resolution of flurbiprofen (■), Fmoc–Lys(Boc)–OH (○), and Fmoc–Pro–OH (▲). Mobile phase is 64:36 mol% CH_3OH–20 m*M* NH_4NO_3 mixture, the flow rate is 0.5 ml/min. The error bars for the data are within the size of the data points.

enantioselectivity with the applied vancomycin concentration, increased chiral selectivity would have been observed with the Betasil silica column using vancomycin as the CMPA.

3.2. Thermodynamic study

Temperature is an important factor in controlling chiral recognition processes. Chromatographic retention is related with temperature through the following equation:

$$\ln k = \frac{-\Delta H^0}{RT} + \frac{\Delta S^0}{R} + \ln \phi \quad (1)$$

where k is the retention factor, ΔH^0 and ΔS^0 are the standard state enthalpy and entropy of transfer to the stationary phase, respectively, R is the gas constant, T is absolute temperature and ϕ is ratio of the volume of mobile phase and stationary phase in the column.

By combining the equations for k_1 and k_2, chiral selectivity may be predicted as:

$$\ln \alpha = \frac{-\Delta\Delta H^0}{RT} + \frac{\Delta\Delta S^0}{R} \quad (2)$$

where $\Delta\Delta H^0$ and $\Delta\Delta S^0$ are the enthalpy and entropy differences between the two enantiomers while they

Table 1
Effect of mobile phase vancomycin concentration on enantiomeric resolution, selectivity, retention factor and band dispersion of flurbiprofen, Fmoc–Lys(Boc)–OH and Fmoc–Pro–OH. Flow rate is 0.5 ml/min. Initial mobile phase composition is 64:36 mol% CH_3OH–20 m*M* NH_4NO_3

Molarity of vancomycin	Resolution R_s	Selectivity α	Retention factor k'	Reduced plate height h
Flurbiprofen				
0	0	0	0.08	
1	1.73	1.89	0.14, 0.27	4.86, 5.12
1.5	2.85	2.14	0.21, 0.44	5.12, 5.69
2	3.17	2.21	0.23, 0.50	5.32, 5.85
3	3.79	2.34	0.26, 0.61	5.37, 6.00
Fmoc–Lys(Boc)–OH				
0	0	0	0.13	
1	1.08	1.41	0.26, 0.36	7.57, 7.93
1.5	1.67	1.52	0.36, 0.54	7.00, 8.80
2	1.74	1.55	0.39, 0.61	8.15, 10.68
3	2.15	1.60	0.46, 0.74	7.81, 10.23
Fmoc–Pro–OH				
0	0	0	0.29	
1	0.46	1.09	0.54, 0.59	5.72, 7.59
1.5	0.60	1.11	0.65, 0.73	6.63, 8.01
2	0.64	1.12	0.67, 0.75	6.97, 8.99
3	0.75	1.13	0.83, 0.94,	7.86, 9.11

interacted with the mobile and stationary phases. Assuming the enthalpy and entropy values are constant over the temperature range studied, a plot of ln α vs. $1/T$ will be linear with a slope of $\Delta\Delta H^0/R$ and a y-intercept of $\Delta\Delta S^0/R$.

Therefore, the effect of temperature variation on α was characterized using a mixture of 50:50 vol% methanol–20 m*M* NH_4NO_3 (aq) as the mobile phase with and without vancomycin CMPA. The amount of H_2O in the mobile phase was increased for this part of the study so that over the entire temperature range the two enantiomers were at least partially separated even for conditions when no vancomycin was in the mobile phase. Otherwise the thermodynamic data could not be collected on the individual enantiomers.

The temperature of the column was varied between 20 and 45°C. Higher temperatures were not considered to protect the column from degradation. The enthalpy and entropy differences between each enantiomer pair was determined from the ln α vs. $1/T$ plot; the results are listed in Table 2. The differences in the enthalpy and entropy increased significantly for flurbiprofen and Fmoc–Lys(Boc)–OH with the addition of vancomycin into the mobile phase, which indicated a possible new interaction mechanism. Meanwhile, the effect of mobile phase vancomycin additive on the differences in enthalpy and entropy of 3-(α-acetonyl-4-chlorobenzyl)-4-hydroxy coumarin enantiomers was small even though the chiral selectivity increased. The chiral recognition mechanism remained unchanged for the separation of coumarin; the slight improvement in selectivity ($\alpha = 1.67–1.81$) could be attributed to the contribution from adsorption. For the increased enthalpy and entropy of flurbiprofen and Fmoc–Lys(Boc)–OH, one possible explanation is that the dimerization of vancomycin enhanced the selectivity of the separations. Previous studies showed that vancomycin forms stable noncovalent dimers in aqueous solutions with a K_d 800 M^{-1} [17] and this dimerization plays an important role in its tight binding to ligands, such as D-Ala–D-Ala [18]. As the concentration of vancomycin in solution increases the number of dimers that are present in solution should increase. Further experiments are needed to explain the observed phenomenon.

3.3. Enhanced-fluidity LC separations

Previous studies showed that using enhanced-fluidity mobile phase improves chiral resolution [15] when using a Chirobiotic-V column. The effect of the addition of a fluidity enhancing solvent on this CSP–CMPA system was also evaluated. 10 mol% CHF_3 was added into the mixture of 64:36 mol% CH_3OH:20 m*M* NH_4NO_3 + 1.5 m*M* vancomycin CMPA to prepare EFLC mobile phase. This mixture is one phase under experimental conditions studied according to the CH_3OH:H_2O:CHF_3 phase diagram [19]. The results are shown in Table 3. Compared to

Table 2
Least-square regression analysis of ln α vs. 1/T relationship for 3-(α-acetonyl-4-chlorobenzyl)-4-hydroxy coumarin, flurbiprofen, and Fmoc–Lys(Boc)–OH. Mobile phase composition is 31:69 mol% CH_3OH–20 m*M* NH_4NO_3 with 0 or 1 m*M* vancomycin CMPA. Flow rate is 0.5 ml/min.

Compound	Slope	Intercept	$\Delta\Delta H^0_{R,S}\left(\frac{kcal}{mol}\right)$	$\Delta\Delta S^0_{R,S}\left(\times 10^{-3}\frac{kcal}{mol\cdot K}\right)$	r^2
w/o vancomycin CMPA					
3-(α-acetonyl-4-chlorobenzyl)-4-hydroxy coumarin					
	759.1	−2.08	−1.51	−4.12	0.999
Flurbiprofen	145.6	−0.34	−0.29	−0.68	0.997
Fmoc Lys	174.7	−0.45	−0.35	−0.90	0.997
w/1 mM vancomycin CMPA					
3-(α-acetonyl-4-chlorobenzyl)-4-hydroxy coumarin					
	798.6	−2.13	−1.59	−4.23	0.999
Flurbiprofen	804.7	−2.39	−1.60	−4.75	0.996
Fmoc Lys	652.4	−1.91	−1.30	−3.79	0.997

Table 3
Chromatographic performance including resolution, selectivity, retention factor and band dispersion of flurbiprofen, Fmoc–Lys(Boc)–OH and Fmoc–Pro–OH in EFLC separation mode. Flow rate is 0.5 ml/min. Mobile phase composition is 10 mol% CHF_3 been added into the 64:36 mol% CH_3OH–20 m*M* NH_4NO_3 + 1.5 m*M* vancomycin CMPA mixture

Test molecule	Resolution R_s	Selectivity α	Retention factor k'	Reduced plate height h
Flurbiprofen	3.00	1.84	0.31, 0.57	4.29, 5.79
Fmoc–Lys(Boc)–OH	1.99	1.48	0.61, 0.91	7.67, 13.66
Fmoc–Pro–OH	1.03	1.16	1.02, 1.19	7.93, 9.17

Table 1, there is an increase in both resolution and retention factor for the tested enantiomers compared to that observed when no CHF_3 was added. However, selectivity for flurbiprofen and Fmoc–Lys(Boc)–OH decreased slightly. This decrease in selectivity may be due to dilution of the vancomycin in the mobile phase when CHF_3 was added to the mobile phase. The final concentration of vancomycin would be less than 1.5 m*M*. As also shown in Table 2, efficiency increased for flurbiprofen but decreased for the derivatized amino acids in EFLC. The chromatograms for the separation of Fmoc–Pro–OH

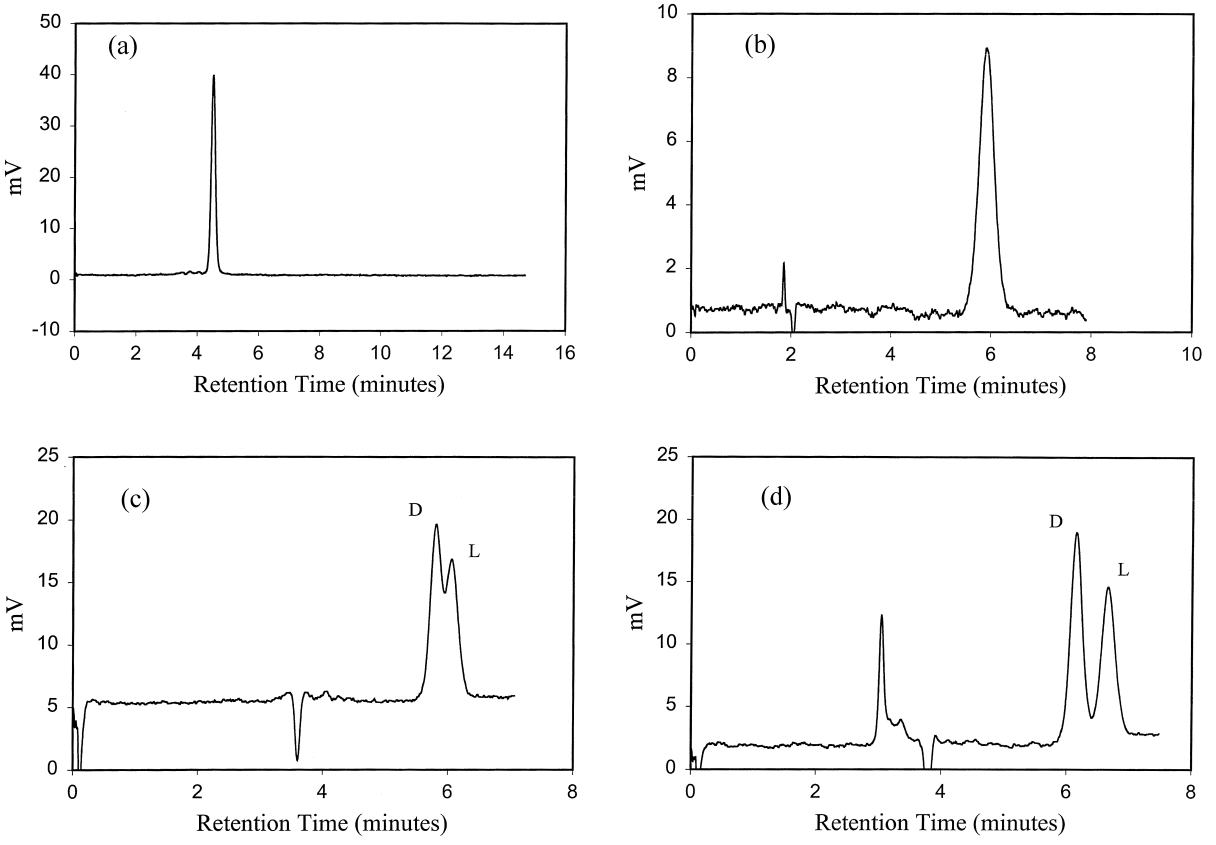

Fig. 3. Chromatograms of the separation of Fmoc–Pro–OH under different experimental conditions. Flow rate was set constant at 0.5 ml/min except that it was 1 ml/min for B. Mobile phase compositions are A) 64:36 mol% CH_3OH–20 m*M* NH_4NO_3, B) 20/80 volume ratio THF–20 m*M* NH_4NO_3, C) 64/3 6 mol% CH_3OH–20 m*M* NH_4NO_3 + 1.5 m*M* vancomycin as CMPA, and D) addition of 10 mol% CHF_3 into the 64:36 mol% CH_3OH–20 m*M* NH_4NO_3 + 1.5 m*M* vancomycin mixture.

under LC (with and without vancomycin CMPA) and EFLC conditions (with vancomycin CMPA) are shown in Fig. 3.

4. Conclusions

By combining the use of vancomycin as both a CMPA and a CSP, superior results were observed. Very polar compounds that could not be separated on the CSP alone were resolved by the addition of CMPA while using the same mobile phase. Improvements in enantiomer resolution and selectivity increased with the increase of vancomycin concentration in the mobile phase. However, increasing the concentration of vancomycin to concentrations above 2 m*M* did not improve the enantiomer separation much. A significant increase in the enthalpy and entropy difference between the two enantiomers was observed for some very polar compounds. This indicates a change of the retention mechanism for the CSP-analyte. Applying CHF_3 as mobile phase fluidity enhancing solvent was beneficial to chiral discrimination. Further studies are underway to fully understand the chiral recognition mechanism for this CSP–CMPA system.

Acknowledgements

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