Journal of Chromatography A, 1217 (2010) 6545–6554

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Characterization of warfarin unusual peak profiles on oligoproline chiral high performance liquid chromatography columns

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ARTICLE INFO

Article history:
Received 28 March 2010
Received in revised form 25 June 2010
Accepted 24 August 2010

Keywords:
Warfarin
Oligoproline chiral stationary phase
System peak
Peak broadening
Peak compression
Effect of temperature
HPLC

ABSTRACT

Unusual peak profiles of warfarin were characterized on two oligoproline chiral stationary phases (CSPs). The pattern of 1st peak (*S*(−)) broadening and the 2nd peak (*R*(+)) compression was observed under mobile phase of hexane (0.1% TFA)/2-propanol (IPA) on a triproline CSP 1, and with other alcohol modifier such as ethanol, 1-propanol, 1-butanol, 2-butanol, and *tert*-butanol as well. Through analyzing system peak of additives, the unusual peak profile was interpreted by perturbation of TFA additive system peak. The unusual peak profile was also found in enantioseparation of coumachlor and on a covalently bonded doubly tethered diproline CSP 2. The pattern of 1st peak (*S*(−)) broadening and the 2nd peak (*R*(+)) compression can change to pattern of 1st peak compression and the 2nd peak broadening from 15 to 50 °C. Chiral separation of warfarin created nonlinear van't Hoff plots on CSP. No peak broadening/compression were observed with methyl tertiary butyl ether or ethyl acetate as the modifier. The peak shapes of the two warfarin enantiomers can thus be tuned by varying alcohol concentration and column temperature. High separation factor and resolution may be carried out to tune the peak profiles into Langmuir/anti-Langmuir band-shape composition. Using none hydrogen donor modifier may avoid interference of the TFA system peak.

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1. Introduction

Direct HPLC enantioseparation with chiral stationary phase (CSP) is nowadays majority choice that spurs activities of development, modification and characterization of CSP [1–4]. Chiral compound as probe is essential in evaluation of CSP. Warfarin, a prescription medicine and rodenticide, is one of those frequently used compounds. It has successively enantioseparated on various CSPs [5] such as polysaccharide derivatives, proteins, brush-types, cyclodextrin, cinchona alkaloid, cellulase (CBH I), and α-chymotrypsin [6–19]. Several chiral analysis methods were developed and validated for determination of warfarin enantiomers in plasma [20–24]. Binding characteristics of warfarin enantiomer on human serum albumin (HSA) has been extensively studied for pharmacokinetic and pharmacodynamic purpose, because warfarin is a specific probe of binding site I on HAS [25–27]. Therefore, warfarin is a very important probe compound in evaluating CSP.

Many unusual behaviors of enantiomer in enantioseparation were reported previously [28,29]. For example, Pirkle [30] observed that the van't Hoff plots of enantiomers for a conformationally rigid spirolactam on (*R*)-N-(3,5-dinitrobenzoyl) phenylglycine varied with both the temperature and the concentration of isopropanol (IPA) in the hexane mobile phase, and interpreted this unusual behavior was caused by the temperature-dependent interaction between IPA and either chiral selector phase (CSP) and/or analyte [30]. Fornstedt et al., using nonlinear chromatography and thermodynamic method, explained the unusual chiral separation of propranolol on a cellobiohydrolase I bonded silica column [31]. The retention was entropy driven for *R*-propranolol, while enthalpy driven for *S*-propranolol. A bi-Langmuir adsorption isotherm model was fitted with the equilibrium data, showing different contributions from the non-enantioselective and enantioselective sites. Another type of unusual phenomena in chiral separation is peak broadening for the 1st eluted enantiomer. For instance, 1-phenylethanol and camphor showed peak broadening on a Chirasil-Nickel CSP used in supercritical fluid chromatography [32]. Slow kinetics of diastereometric equilibration was suggested to cause the peak broadening. In HPLC, though less pronounced, the 1st eluted enantiomer of a fungicidal triazolyl alcohol showed remarkable tailing on a Chiralpak AD column [33].

When solvent of sample solution is different from the mobile phase, injection perturbs equilibrium of stationary and mobile phases because analyte compete for interaction site with mobile phase component. This result in desorption of some additive molecule migrating along the column to generate system peak. System peak also termed as pseudo peak, ghost peak, eigenpeak, vacancy peak, induced peak, dip peak, or perturbation peak has

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0021-9673/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.chroma.2010.08.066

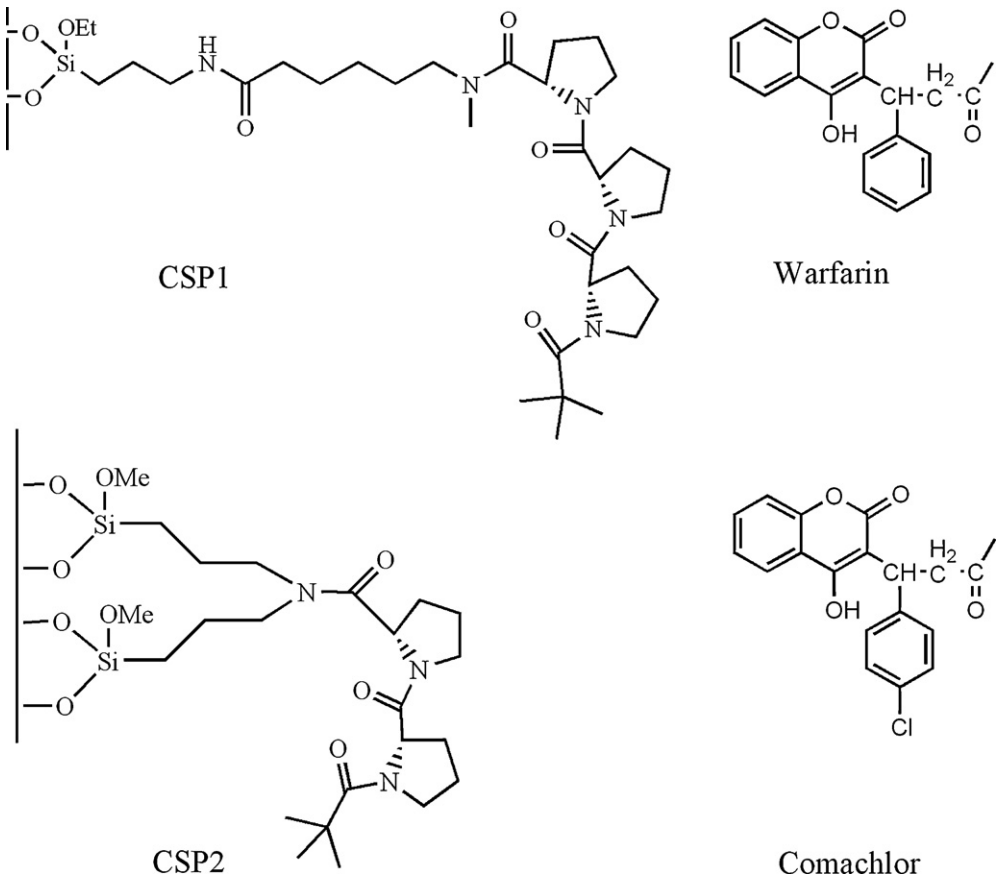

Fig. 1. Structure of CSP1 and CSP2, warfarin and coumachlor.

been studied since 1970s [34–44]. Levin and Grushka realized the system peaks were produced in relaxation process to a new state of equilibrium [41]. They further linked system peaks to the calculation of adsorption isotherm, capacity ratio and column void volume [45–47]. Golshan-Shirazi and Guiochon theoretically and experimentally studied system peak in linear or nonlinear chromatography [48–50]. System peaks have been used in calculation of retention factors of the mobile phase components and column void volume, measurement of the adsorption isotherm, indirect detection, and studying of drug–protein interactions [41,44,51].

System peak influences analyte peak shape but offers valuable information of separation process. A HPLC peak deviating from classical Gaussian, Langmuirian and anti-Langmuirian profiles has been defined as deformed peak by Samuelsson et al. [52]. Peak

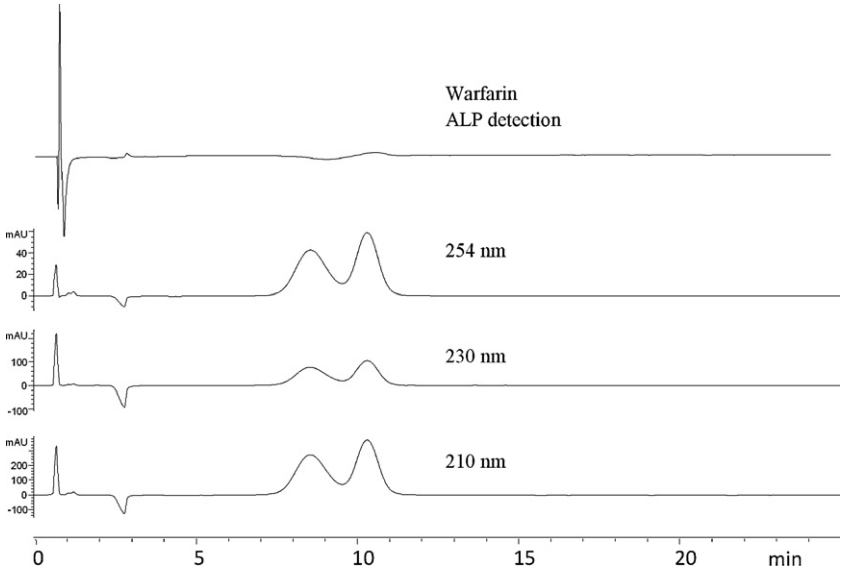

Fig. 2. Chromatograms of warfarin on CSP1. Chromatographic conditions: UV and ALP detectors; injection volume, 20 μL; sample concentration: 0.6 mg/mL; flow rate, 1.0 mL/min; column temperature, 20 °C; mobile phase: 90/10 (v/v) hexane (0.1% TFA)/IPA.

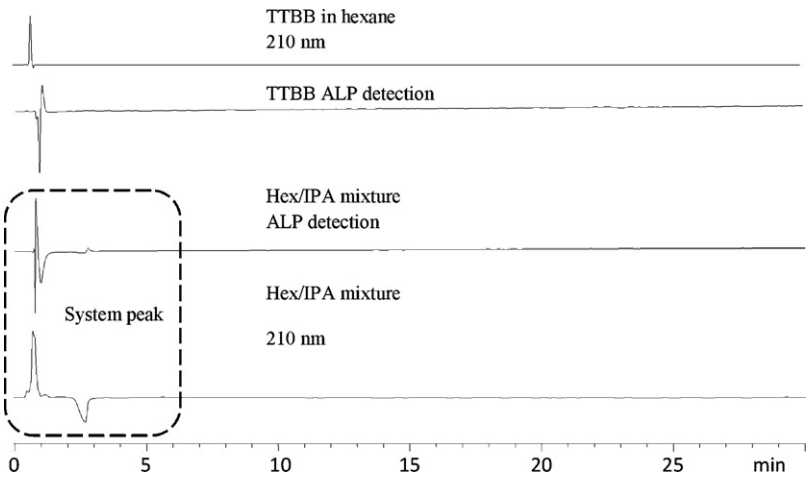

Fig. 3. Chromatograms of TTBB and hexane/IPA mixture by on CSP1. Chromatographic conditions: column temperature, 15 °C; mobile phase: 88/12 (v/v) hexane (0.1% TFA)/IPA. Other chromatographic conditions are same as in Fig. 2.

deformation including compressed, broadened, distorted and split peaks from a normal isocratic peak can be induced by system peak, whereas gradient elution usually causes peak compression [53–56]. In practical use, term of peak compression only indicates the peak shows apparently compressed profile without great other distortion, while peak deformation indicates the peak shape has obviously distortion and deviates from classical Gaussian, Langmuirian or anti-Langmuirian profile [53,57,58]. In principle, peak compression, broadening and deformation are created by local change in mobile phase concentration in comparison with the equilibrium composition, resulted from either system peak or gradient elution on the analyte migration. Fornstedt and Guiochon et al. developed a rule of thumb for judging peak profile by comparing the ratio of the initial slopes of the additive and analyte isotherms at infinite dilution in the weak solvent such as hexane, and the ratio of the retention factors of the primary additive system peak and the solute peak in the mobile phase [50,59,60].

Peak deformation effects were studied in ion-pair reversed-phase HPLC [57,58,61–64]. It has been revealed that the compressed and distorted peaks are caused by co-elution of the analyte peak with a system peak originating from a mobile phase additive or modifier. Fornstedt showed the compression and broadening effects on co-eluting analyte peaks of large system peak in study of peak distortion effects of suramin using ion-pair adsorption chromatography [53]. He concluded that the front and rear parts of the large system peak consist of either increasing or decreasing concentration gradients of mobile phase components which may make analyte peak experience continuously increasing or decreasing migration velocity to cause compression or broadening peak [64]. A tracer-pulse method using mass spectrometric detection has been implemented in characterization of invisible system peak and determination of competitive adsorption isotherms [65].

In chiral separation, Levin et al. exhibited competition between two 2,2,2-trifuoro-1-(9-anthryl) ethanol (TFAE) enantiomers from their system peaks on three chiral columns [66]. Lindholm and Fornstedt observed peak deformations of 2-phenylbutyric acid enantiomers due to a co-eluting system peak of formic acid on a tartardiamide-based network-polymeric CSP [67]. Arnell et al. from the same group identified the system peak of the strongly adsorbed additive and carried out varying mobile phase composition to tune the peak shapes of the two β-blocker enantiomers on a teicoplanin CSP (Chirobiotic T) [68]. In chiral preparative chromatography, Forssen et al. further applied the properties of the strong additive to possible tune the enantiomers peak profiles into any combination of the apparent band shapes: anti-Langmuir/anti-Langmuir, anti-Langmuir/Langmuir, Langmuir/Langmuir and Langmuir/anti-Langmuir using methanol/acetonitrile as the mobile phase and acetic acid/triethylamine as the additives [69]. Later, a numerical investigation ascertained the most favorable band-shape composition in preparative chromatography for a particular enantiomer [70]. These previous studies demonstrated adsorbing additives, in particular strongly bound, can have extreme impact on retention times and peak shapes [71,72].

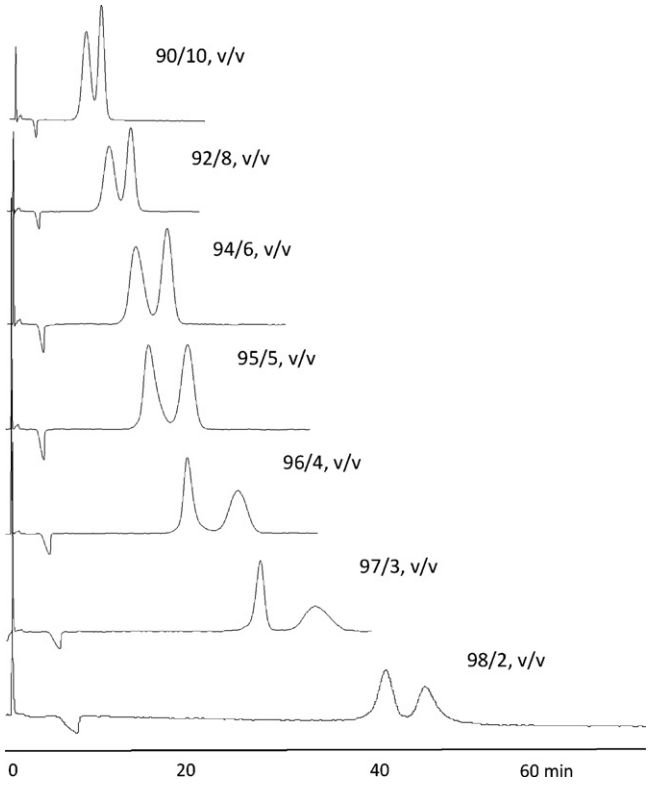

Fig. 4. Effect of mobile phase composition on chiral separation of warfarin on CSP1. Chromatographic conditions: temperature, 25 °C; detection, 254 nm; mobile phase: hexane (0.1% TFA)/IPA (v/v). Other chromatographic conditions are same as in Fig. 2.

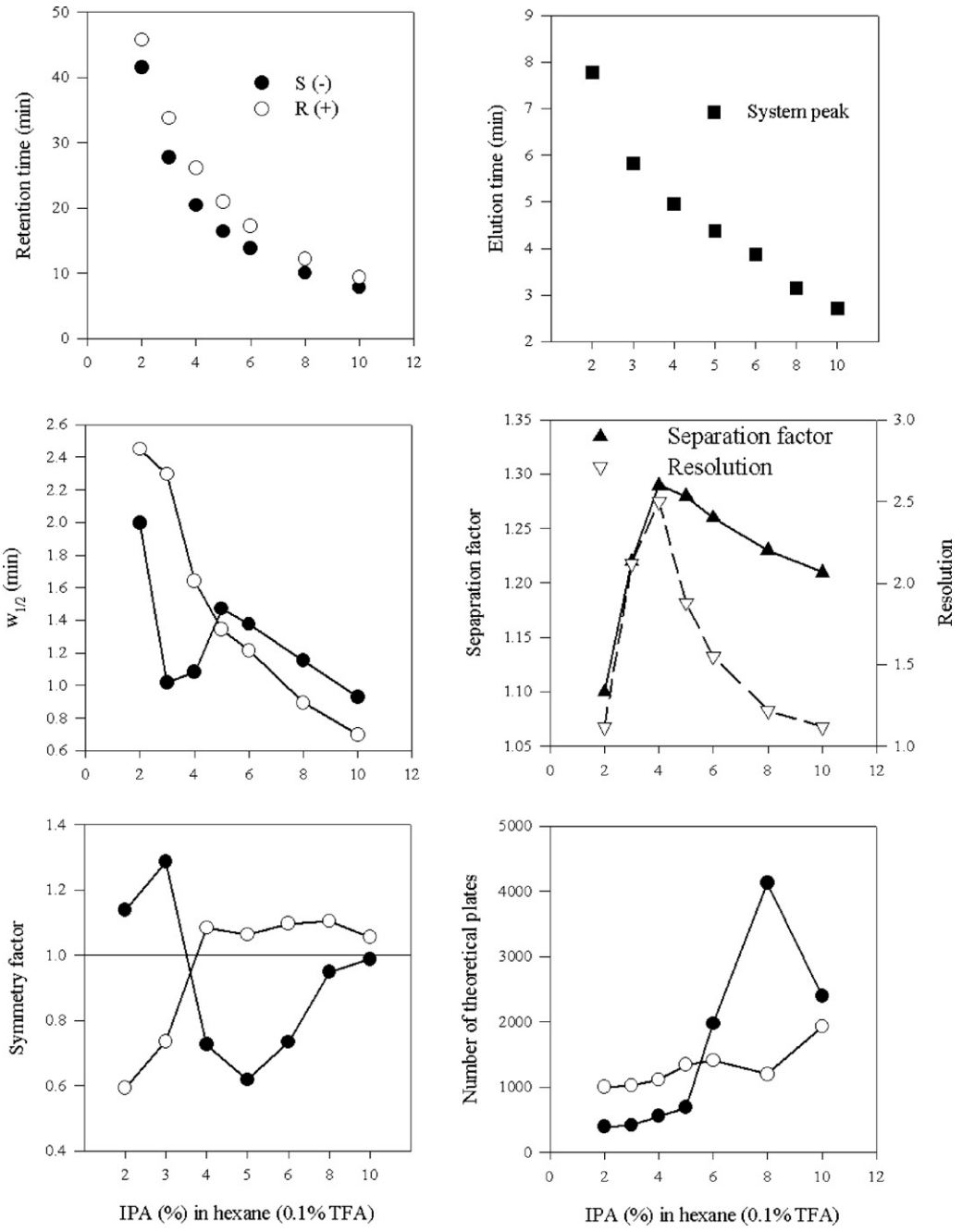

Fig. 5. Variability of warfarin chromatographic parameters along with composition of mobile phase on CSP1.

We have previously prepared and evaluated two novel oligoproline CSPs (Fig. 1) [19,73]. In this study chromatography behavior of warfarin on the two oligoproline CSPs was characterized with trifluoroacetic acid (TFA) as mobile phase additive. Effect of mobile phase modifier and column temperature was evaluated. Behavior of system peak of mobile phase additive was employed to interpret unusual peak profiles of warfarin enantiomers in enantioseparation.

2. Experimental

2.1. Chemicals

Racemic warfarin (≥98%) and coumachlor (98%), trifluoroacetic acid (TFA, 99%) and 1,3,5-tri-*tert*-butylbenzene (TTBB, 97%) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). All HPLC-grade solvents were purchased from Fisher (Pittsburgh, PA, USA).

2.2. Apparatus

An Agilent 1100 HPLC system (Agilent, Wilmington, DE, USA) equipped with a vacuum degasser, a quaternary pump, an autosampler, a thermostatic column compartment, a multiple wavelength detector (UV detector) and Chemstation software was used. An advanced laser polarimeter (ALP detector, PDR-Chiral, Lake Park, FL) was connected after the UV detector to determine sign of rotation of the resolved enantiomer at 675 nm. ALP is a non-absorbance based detector and does not require chromophore or wavelength tuning to match an absorbance band. It may response not only optical activity of analyte but also component change of mobile phase.

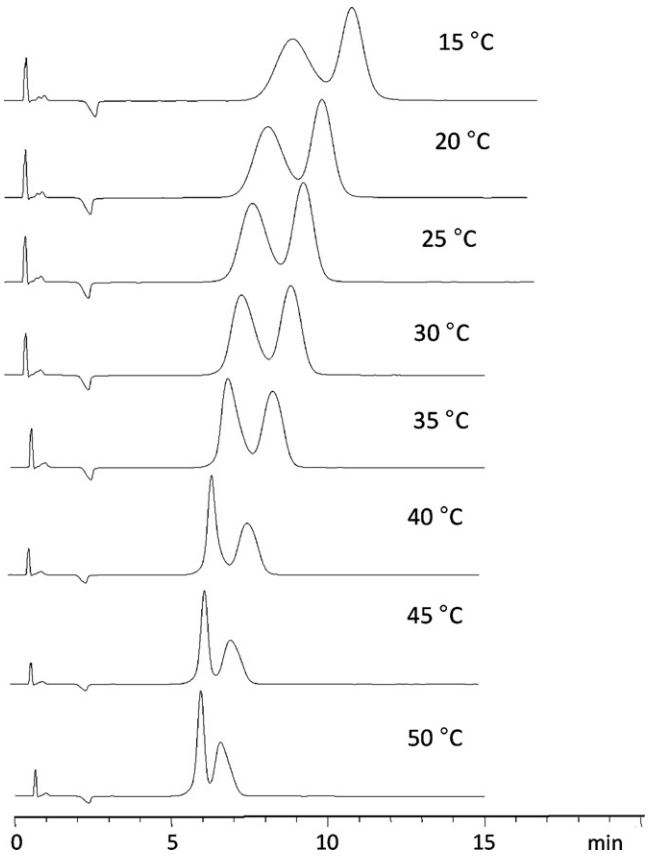

Fig. 6. Effect of column temperature on chiral separation of warfarin on CSP1. Mobile phase, 90/10 (v/v) hexane (0.1% TFA)/IPA. Other chromatographic conditions are same as in Fig. 2.

Two previously reported columns (4.6 mm × 50 mm), i.e., a triproline and a covalently bonded doubly tethered diproline CSPs, were utilized (Fig. 1) [19,73]. The column dimension is 4.6 mm internal diameter × 5.0 cm length (Isolation Technologies, Hopedale, MA, USA).

2.3. Chromatographic measurements

Concentration of warfarin or coumachlor dissolving in IPA was 0.6 mg/mL (0.002 mol/L). The flow rate was 1.0 mL/min, and the detection wavelength was set at 210, 230 and/or 254 nm with a 4 nm bandwidth. The injection volume was 20 μL unless it is specified. The hold-up time (t_0) was measured with TTBB. For the thermodynamic experiment, the column temperature was changed step-wisely over the range of 15–50 °C. The column was equilibrated at each temperature (15, 20, 25, 30, 35, 40, 45 and 50 °C) with the mobile phase for 1 h before sample injection.

The extra column time (t_{ext}) of the HPLC system is 0.07 min. The retention factor (k) is calculated using the equation $k = (t_r - t_0)/(t_0 - t_{ext})$ where t_r is the retention time, and t_0 is the hold-up time. The separation factor (α) is calculated as the ratio $\alpha = k_2/k_1$, where k_1 is retention factor of the 1st eluted enantiomer and k_2 is retention factor of the 2nd eluted enantiomer. The resolution factor (R_s) is calculated using the equation $R_s = 1.18 \times (t_{r2} - t_{r1})/((w_{1/2})1 + (w_{1/2})_2)$, where $(w_{1/2})_1$ and $(w_{1/2})_2$ are the widths at half peak height. Number of theoretical plates (N) is calculated with $N = L/(5.54 \times (t_r/w_{1/2})^2)$, where L (mm) is the length of the column packing.

3. Results and discussion

3.1. Enantiomer peak profiles of warfarin CSP1

Enantiomers of warfarin were resolved on various CSPs in previous studies [74,75]. Given that the asymmetric carbon is attached with four different substituent varying greatly in polarity and size, it was not surprising for the resolution of warfarin enantiomers on CSP1 with hexane (0.1% TFA)/2-propanol (IPA) (90/10, v/v) as mobile phase [19]. However, as illustrated in Fig. 2, chromatograms of simultaneous UV recordings at 210, 230 and 254 nm all show that shape of the first (1st) peak of enantiomer (*S*(−)) clearly is broader than that of the second (2nd) enantiomer (*R*(+)). Moreover, the shape of 1st or 2nd peaks has no apparent deformation in the three UV chromatograms besides compression of the 2nd peak. This phenomenon is unusual in HPLC chiral separation because the normal peak shapes should be the 1st peak higher but narrower than the 2nd peak. A simultaneous recording from an optical rotation ALP detector exhibits an injection shock and signs of optical rotation of warfarin enantiomers as well as the sample purity. The unusual peak profiles prompted there was possible effect of system peak on the peak shape, which could be produced by mobile phase additive [58,61,63,67,68]. In the present mobile phase, IPA and TFA are the two additives (or strong solvents) that can be adsorbed on the stationary phase by intermolecular interaction such as hydrogen bonding, while hexane is the pure weak solvent. To evaluate warfarin enantioseparation on CSP1and further characterize this unique chromatographic pattern, we followed two experimental approaches by varying content of alcohol additive and column temperature. The concentration of warfarin was kept 2 mM that was in the range of linear chromatography. TFA concentration (0.1%, v/v) in hexane was kept constant through all the study.

3.2. Identify system peaks and the strongly adsorbing component on CSP1

With mobile phase of hexane (0.1% TFA)/2-propanol (IPA) (90/10, v/v), additional injections of two solutions lacking warfarin were made to assign system peak. One of the solutions with TTBB dissolving in hexane only created one t_0 peak of UV signal at 210 nm and one injection pulse of ALP signal indicating equilibrium of additives between the mobile and stationary phase was not perturbed (Fig. 3). It implied there was no competition between hexane/TTBB and IPA or TFA, and therefore adsorption of hexane and TTBB on the stationary phase could be negligible [68]. Moreover, it also displayed no retention of TTBB on CSP 1. Another injection was made using a solution of hexane/IPA of that concentration was different from the mobile phase. This injection apparently resulted in one positive and one negative UV peaks (Fig. 3). Besides the injection pulse, the ALP detector recorded additional one negative and one positive peak that were corresponding to the two system peaks of UV signal (Fig. 3). Comparing with the APL signal, the positive peak at 210 nm was identified a co-eluting signal of the t_0 peak and a system peak. Theoretically, introduction of one of two additives perturbs the equilibrium of the two additives at the column inlet so that four system peaks will be produced if each additive is involved in interaction with stationary phase and compete with each other [59]. In order to assign original of the two system peaks, 10 and 50 μL of IPA were injected, respectively. The corresponding areas of the negative system peak (at about 2.7 min) were 147 and 730 of which proportion is same as the injection amounts, suggesting this pair of system peaks was introduced by IPA. If the equilibrium of TFA was disturbed, its system peak at least should be recorded at 210 nm as TFA has absorbance at this wavelength [76]. No system peak of TFA meant no perturbation of its equilibrium between the stationary and mobile phases by IPA. It is concluded that TFA, a

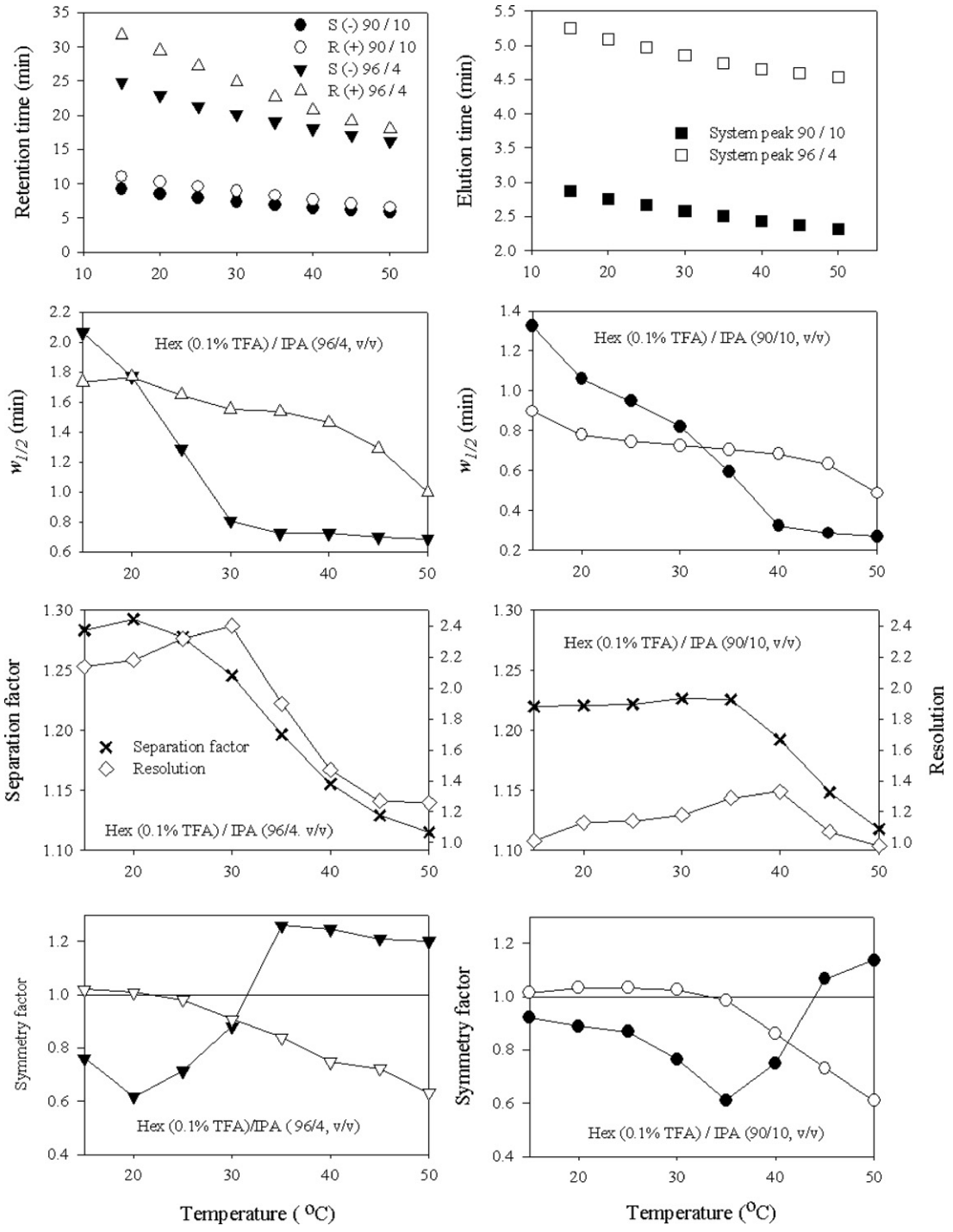

Fig. 7. Temperature effect on warfarin chromatographic parameters.

strong carboxylic acid with pK_a of 0.23 [77], is the strongly adsorbing component on the stationary phase [78].

3.3. Tunable peak profiles with IPA concentration on CSP1

Without TFA additive in hexane/2-propanol (IPA) (90/10, v/v) mobile phase, on peak of warfarin was observed out of the column because warfarin, a weak acid with pK_a of 5.1 [79], was also strongly adsorbed. Adding TFA in mobile phase, interaction between warfarin and stationary phase was reduced to allow it elution out. The chromatograms using different IPA concentration in hexane (0.1% TFA) mobile phase at room temperature (≈22 °C) are shown in Fig. 4. It can be seen from 98/2 to 97/3 mobile phase, the 1st peak was compressed, while the 2nd peak was broadened. Apparent peak compression of the 2nd enantiomer occurred from 97/3 to 90/10 mobile phase along with deceased retention. However, the 1st peak continuously was broadened from 97/3 to 95/5 mobile phase, and was narrowed since 94/6 mobile phase, which is more clearly shown by peak width ($w_{1/2}$) values in Fig. 5. The elution time of IPA negative system peak (at 2–3 min) and retention times of the two enantiomers reduced along with the increase of mobile phase strength. A transition point of value for separation factor and resolution takes place with 96/4 mobile phase. The number of theoretical plate for the 1st peak dramatically increased from

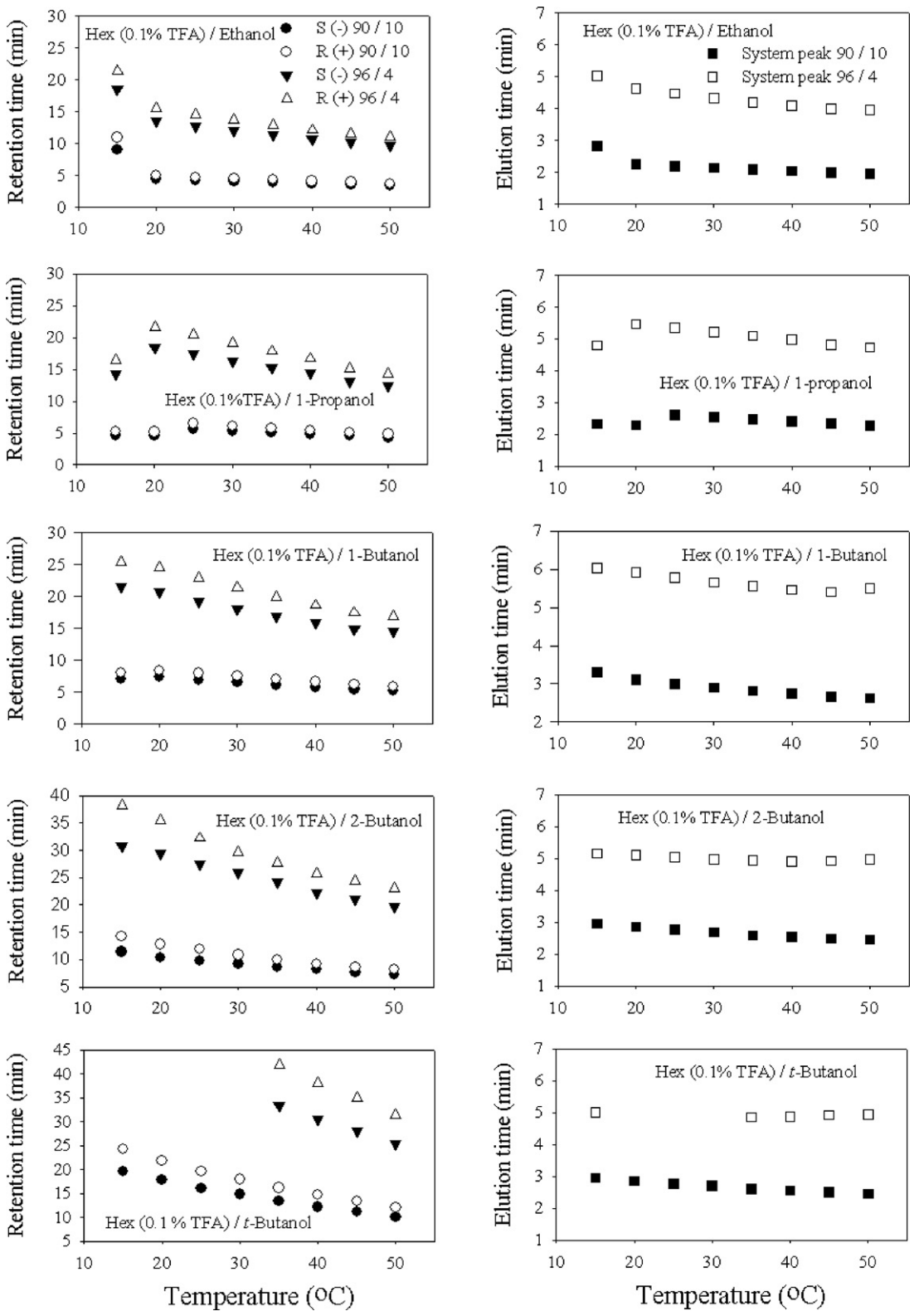

Fig. 8. Temperature effect on warfarin chromatographic parameters with different alcohol modifiers.

94/6 mobile phase, whereas it only gradually enhanced for the 2nd peak from 98/2 to 90/10 mobile phases. The symmetry factors measured by the Agilent Chemstation software are also plotted in Fig. 5. Note that being just opposite to standard concept, this symmetry factor is below unity for peak with compressed front and extended rear (Langmuirian peak), and exceeding unity for peak with diffuse front and steep rear (anti-Langmuirian peak) [17]. At IPA level below ~3%, the 1st peak of warfarin is anti-Langmuirian, whereas the 2nd peak is Langmuirian. The symmetry inversion of the two peaks occurs with 96/4 mobile phase to give Langmuir/anti-Langmuir band-shape composition, which is consistent with the change profiles of peak width, separation factor and resolution. It

Table 1
Variation of peak width ratio of 2nd/1st eluted warfarin enantiomers with temperature in mobile phase of 90/10 (v/v) hexane (0.1% TFA)/alcohol.

Temperature (°C)	Ethanol 1.89[a] (mol/L)	1-PA 1.49 (mol/L)	IPA 1.44 (mol/L)	1-Bu 1.21 (mol/L)	2-Bu 1.21 (mol/L)	*t*-Bu 1.16 (mol/L)
15	0.68	0.56	0.68	0.47	0.87	0.79
20	0.62	0.59	0.74	0.48	0.97	0.9
25	0.68	0.65	0.79	0.58	1.32	1.01
30	0.72	0.71	0.88	0.69	1.51	0.98
35	0.78	0.79	1.19	0.78	1.45	1.02
40	0.83	0.84	2.11	0.86	1.06	1
45	0.86	0.91	2.21	0.91	0.95	1.03
50	0.9	0.95	1.81	0.95	0.92	1.05

[a] Molar concentration of alcohol in hexane.

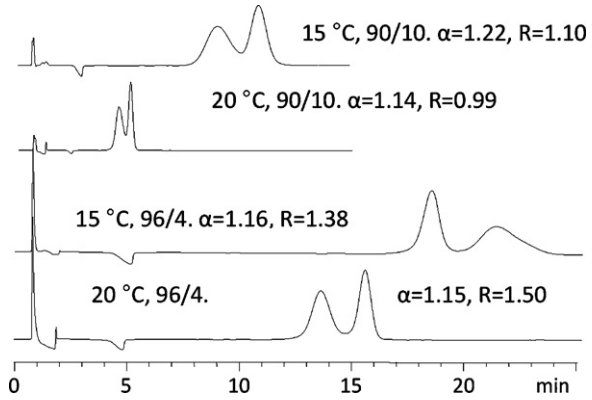

Fig. 9. Comparison of effects of column temperature on enantioseparation of warfarin with ethanol as modifier on CSP1.

shows the chiral separation of warfarin on CSP1 is favorable with this band-shape composition [70]. In fact, increasing IPA concentration reduced the retention time so that partially cancelled the favorable effect of band-shape composition. These observations illustrated IPA played an important role in the changing peak profiles, and consequently the peak profile can be tuned by changing IPA concentration.

Because of weak adsorption of IPA, injection of warfarin solution produced same of IPA system peaks in Figs. 3 and 4 [80]. TFA molecule must be adsorbed on the oligoproline chiral selector besides on silanol because proline residue is a featured hydrogen bonding acceptor. The interaction sites of CSP should be also associated by IPA molecules because of its higher concentration in mobile phase than TFA and property of hydrogen bond acceptor/donor. When the sample was introduced into the column, warfarin molecule expelled IPA and/or TFA adsorbed on the CSP. However, no TFA system peak was observed before the 1st peak of warfarin implying TFA was more retained than warfarin enantiomers, which is understandable by comparing their acidities and structures. When warfarin concentration declined on the stationary phase, the same amount of TFA/IPA in the mobile phase was re-adsorbed to restore the local equilibrium [59]. As warfarin migrating along the column, chiral selector and each enantiomer form transient diastereomeric complex with different energy and association constant that make enantiomers gradually be separated. Fornstedt and Westerlund schematically illustrated of broadening and compression effects on analyte peaks of elution with a large co-ion negative fronting system peak [64]. The evolution of the band-shape profiles in present study implies the warfarin enantiomers co-elute with a negative TFA fronting system peak, because competition of TFA and warfarin can be considered as co-ion and analyte in ion-pair adsorption chromatography. According to the description of Fornstedt [53,64], the 1st peak of warfarin should be at the front of the negative TFA system peak where has deceasing concentration gradient of mobile phase, while the 2nd peak must be at the rear of the TFA system peak to suffer an accelerating mass transfer to present a compressed peak eventually [61,64,66]. In fact, broadening effect on the 1st peak was effective from 97/3 to 90/10 mobile phases, but it was apparently cancelled by increase of strength from 94/6 to 90/10 mobile phase. This view is supported by the symmetry factor profiles (Fig. 5). On contrast, compression effect for the 2nd peak overlapped with the effect of increase of mobile phase strength.

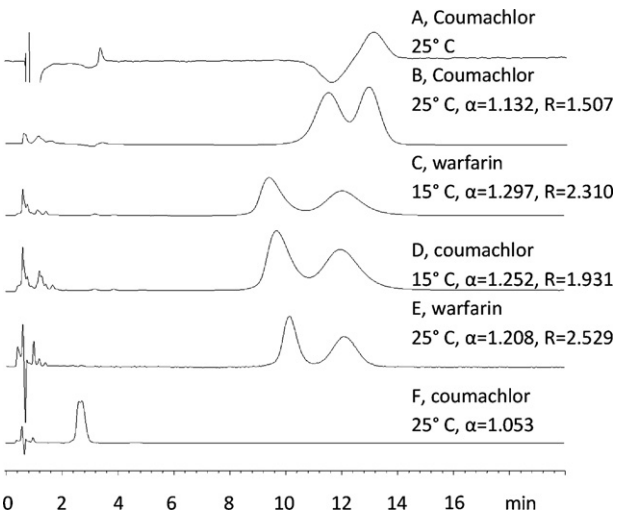

Fig. 10. Chromatograms of coumachlor, and warfarin on CSP1. Chromatographic conditions: detection, A, APL; B-F, UV at 254 nm. Mobile phase: A and B, 92/8 (v/v) hexane (0.1% TFA)/IPA; C and D, 30/70 (v/v) hexane (0.1% TFA)/MTBE; E and F, 80/20 (v/v) hexane (0.1% TFA)/EA. Other chromatographic conditions are same as in Fig. 2.

3.4. Effect of temperature and other alcohol modifiers on peak profiles

Two mobile phase systems, i.e., 90/10 and 96/4 (v/v) hexane (0.1% TFA)/IPA were selected for a temperature test. In 90/10 mobile phase, the 1st peak broadening and the 2nd peak compression are depicted in the temperature range of 15–25 °C (Fig. 6). At 30 °C, the two peaks displayed approximately the same width, suggesting that a transition state existed in course of temperature response. From 35 to 50 °C, the 1st peak was compressed, while the 2nd peak was broadened. This view is supported by the width change of the 2nd peak and symmetry factor profiles on which transition points locate at 35 °C (Fig. 7). The change of peak profile was also evident from the width ratio of 2nd/1st enantiomer (Table 1). The ratios are <1 in the range of 15–30 °C indicating 2nd peak compression, and vice versa in the 35–50 °C. Retention times for the enantiomers and elution time for the IPA system peak reduced along with increase of temperature. Separation factor is almost constant at 15–35 °C, and then rapidly declines from 35 to 50 °C with highest resolu-

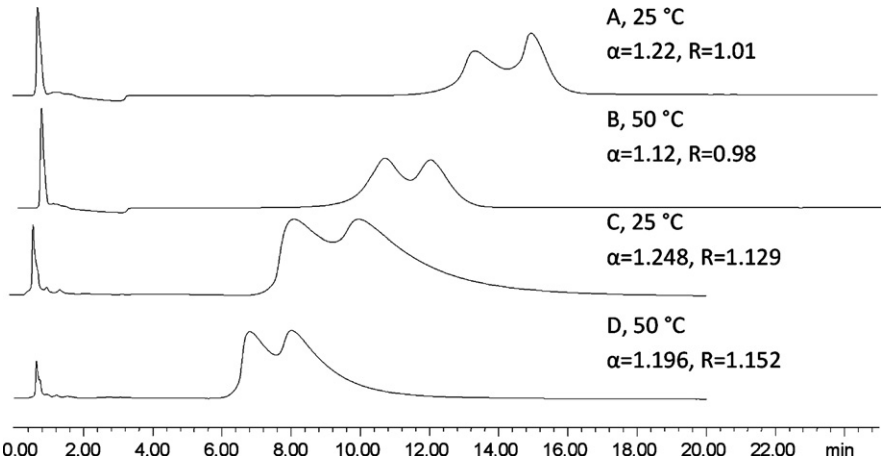

Fig. 11. Chromatograms of warfarin on CSP 2. Mobile phase: A and B, 90/10 (v/v) hexane (0.1% TFA)/IPA; C and D, 30/70 (v/v) hexane (0.1% TFA)/MTBE; detection, 254 nm. Other chromatographic conditions are same as in Fig. 2.

tion at 40 °C. This can be explained by the change of band-shape composition from favorable Langmuir/anti-Langmuir to adverse anti-Langmuir/Langmuir at 35 °C (Fig. 7).

The highest values for separation factor and resolution are at 20 and 30 °C, respectively, using the 96/4 (v/v) hexane (0.1% TFA)/IPA mobile phase (Fig. 7). Comparatively, separation factors and resolution with 96/4 mobile phase are larger than with 90/10 mobile phase at 15–30 °C. They all rapidly reduced at elevated temperature. The pattern of the 1st peak broadening and the 2nd peak compression likely just occurred at 15 °C with 96/4 mobile phase, and it gradually changed to reverse one (Table 2). The band-shape composition also reflects this change (Fig. 7). These observations exhibit the 1st peak broadening and 2nd peak compression tends to occur at low temperature, and imply the band-shape of the TFA system peak possible change from fronting to tailing. This possibility should be verified in the consecutive study. Consequently, the peak shape can be tuned by varying column temperature.

Five commonly used alcohol modifiers were also evaluated using the same temperature program and solvent volume ratios (Tables 1 and 2). Retention and elution time profiles of enantiomers and the system peak are shown in Fig. 8. Retention times decreased along with increase of temperature with ethanol, 2-butanol (2-Bu), and *tert*-butanol (*t*-Bu) as modifiers. Retention times increased from 15 to 20 °C and then decreased from 20 to 50 °C with 1-propanol (1-PA) as modifier in 96/4 mobile phase. The elution time of the system peak showed the similar trend. The unusual peak profiles the two enantiomers were found with all of these alcohols. Specifically, the pattern of 1st peak broadening/2nd peak compression persisted over the whole experimental temperature range in 90/10 mobile phase with ethanol, 1-PA, or 1-butanol (1-Bu) as the modifier. This pattern was recorded only at 15 and 20 °C for *t*-Bu. Interestingly, this pattern was observed for 2-Bu at low (15 and 20 °C) and high (45 and 50 °C) temperatures. Moreover, the 2-Bu modifier also yielded a unique observation that pattern of 1st peak broadening/2nd peak compression occurred only at high (35–50 °C) temperature in the 96/4 mobile phase. Nevertheless, with 4% level of ethanol in mobile phase, obvious changes of peak width for the two enantiomers were found from 15 to 20 °C (Fig. 9). These observations indicate the peak profiles can be impacted by the structure of alcohol additive. As a result, there will be more options of additive to tune peak shape in warfarin enantioseparation.

In additional, van't Hoff plots of separation factor for the hexane/IPA mobile phases (90/10 and 96/4, v/v) were nonlinear in the range of 15–50 °C. The two nonlinear plots are dissimilar in their shapes, and apparently have different transition temperatures that were 20 and 35 °C for the 96/4 and 90/10 mobile phase, respectively. van't Hoff plot for 1-PA, 1-Bu and 2-Bu are all nonlinear, whereas only for ethanol at 4% IPA level and *t*-BU at 10% IPA level are close to a linear line.

3.5. Peak profile of coumachlor and on CSP2

The above unusual chromatographic behavior was not limited to warfarin and CSP1 column, and had been reproduced by another analyte and oligoproline CSP. Firstly, coumachlor, structurally relating to warfarin, was chromatographed on CSP1with the same of above the mobile phase. As shown in Fig. 10(A) and (B), similar peak profile of warfarin was appeared, which confirmed the observations of peak shapes on CSP1. When methyl tertiary butyl ether (MTBE) or ethyl acetate (EA) was the modifier, however, the enantioseparation peak profiles became normal (Fig. 9(C)–(E)). Note that coumachlor only gained initial optical resolution with mobile phase of hexane (0.1% TFA)/EA (80/20, v/v) (Fig. 9(F)). MTBE or EA being only hydrogen bond acceptor was not adsorbed by the chiral selector so that the enantiomers were easily to access chiral selector as well as other interaction sites. Hence, the enantiomers gained res-

Table 2
Variation of peak width ratio of 2nd/1st eluted warfarin enantiomers with temperature in mobile phase of 96/4 (v/v) hexane (0.1% TFA)/alcohol.

Temperature (°C)	Ethanol 1.78 (mol/L)	1-PA 1.39 (mol/L)	IPA 1.35 (mol/L)	1-Bu 1.14 (mol/L)	2-Bu 1.14 (mol/L)	*t*-Bu 1.09 (mol/L)
15	1.94	0.56	0.84	0.65	1.53	
20	0.66	0.77	1.1	0.76	1.51	
25	0.74	0.78	1.52	0.86	1.36	
30	0.81	0.85	1.92	0.9	1.13	
35	0.87	0.94	2.12	0.98	0.89	1.57
40	0.93	1.0	2.02	1.0	0.89	1.15
45	0.95	1.03	1.85	1.07	0.94	1.61
50	0.99	1.06	1.45	1.09	0.99	1.27

olution on the CSP without peak deformation probably due to no perturbing adsorption of TFA.

On CSP2 the pattern of 1st peak broadening/2nd peak compression for warfarin was also observed at 25 °C with hexane (0.1% TFA)/IPA (90/10, v/v) as mobile phase (Fig. 11(A)). This pattern was disappeared at 50 °C (Fig. 11(B)). When MTBE was used as additive, both enantiomers showed tailing peaks instead of any pattern of broadening\ compression (Fig. 11(C) and (D)). Combining above observations suggested the unusual peak profile can take place on oligoproline CSPs, and is impacted by type and concentration of modifier as well as column temperature.

Browsing previous chromatograms of warfarin enantioseparation on many CSPs and with various additives including TFA, formic acid, and acetic acid has not found any unusual peak profile [6–19,24]. Anti-Langmuirian peaks of warfarin enantiomers were observed on a β-cyclodextrin CSP with triethylamine as additive in acetonitrile/methanol mobile phase [81]. In the same study, mass transfer rate of the 2nd enantiomer was found faster than the 1st enantiomer while no apparent peak compression or broadening was created. It is thus believed the unusual peak profile is unique for warfarin chiral separation on the oligoproline CSPs. As a pilot study, many other variable factors such as TFA concentration, type of acidic additive, and injection amount of warfarin were excluded. Learning from effects of these factors and from result of nonlinear chromatographic method as well as employing a refractive index detector should allow to further understanding the chromatographic behavior of warfarin on oligoproline CSPs. In practical application, the tunable peak via changing concentration of modifier and temperature is particularly useful in displaying and identifying enantioselectivity in real matrix [62,63].

4. Conclusions

Unusual peak profiles of warfarin enantiomers were observed during chiral separation of warfarin in hexane (0.1% TFA)/alcohol mobile phase systems on a novel triproline CSP. This phenomenon had been reemerged by coumachlor and on a diproline CSP. Through identifying system peak of additive, perturbation of TFA is believed to cause the peak broadening and compression. The perturbation of TFA system peak cannot be eliminated by changing modifier type of alcohol, but it has been illustrated varying alcohol concentration and column temperature can tune the peak shapes. Selection of Langmuir/anti-Langmuir band-shape composition, e.g., with hexane (0.1% TFA)/IPA 96/4 (v/v) mobile phase or at low temperature, may achieve high separation factor and resolution. However, using none hydrogen donor modifier (e.g., MTBE) may avoid interference of system peak. Going to show somewhere else, the tunable peak profile has been utilized in quantitative determination of warfarin enantioselective degradation in soils.

Acknowledgments

This study was supported by a USDA-National Research Initiatives grant no. 2005-35107-16189. The authors would like to thank Dr. Zhiwei Wang and Dr. Dong Yan for their informational assistances. We are grateful to the anonym reviewer for valuable comments and suggestions.

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