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Study of competitive binding of enantiomers to protein by affinity capillary electrochromatography

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Dedicated to Professor Dr Gottfried Blaschke on the occasion of his 65th birthday

Abstract

Affinity capillary electrochromatography (CEC) with zonal elution method was used to probe the competitive interactions of enantiomers with protein. In this approach, a known concentration of a competing agent is continuously applied to a CEC column with bovine serum albumin (BSA) physically adsorbed on SAX packing while injections of a small amount of analyte are made. The binding sites of solutes on the BSA molecule were determined by the changes in the retention factors of the solutes resulted from the addition of competitive agent. By using D- or L-tryptophan as competitive agents and D-, L-tryptophan and benzoin enantiomers as injected analytes showed that BSA molecule has a primary site to strongly bind L-tryptophan, but D-tryptophan dose not bind at this site; D- and L-tryptophan share a weak binding site on the BSA molecule. Benzoin enantiomers do not share any binding sites with either D- or L-tryptophan. Non-chiral compounds of trichloroacetic acid and *n*-hexanoic acid were applied as the competitive agents to study the binding of warfarin enantiomers to BSA, it was observed that trichloroacetic acid and *n*-hexanoic acid had a same binding site for warfarin enantiomers binding to BSA molecule.

Keywords: Affinity capillary electrochromatography; Enantiomers; Drug–protein interaction; Bovine serum albumin

1. Introduction

Most drugs undergo some degree of reversible binding to plasma proteins, a process which may have significant effects on the overall activity profile of the compounds [1,2]. High-performance affinity chromatography and zonal elution are typically used to probe the drug–protein interactions [3–7]. In this approach, one of the drugs is used as a solute to be injected onto the protein-based stationary phase, and the other drug is used as a competitive agent added into the mobile phase. The magnitude and direction of the changes in the retention factor (k') of the solute resulted from the addition of competitive agent can be used to determine the magnitude of the

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interactions and the sites on the protein at which they occur [3]. Large amount of mobile phase components including additive and competitive agent is necessary to perform experiments in conventional HPLC, which makes this method expensive to be performed for probing the interaction of some species in biochemistry field where only trace sample can be available in some cases. Therefore, a miniaturized separation technique is valuable.

Capillary electrochromatography (CEC) has experienced explosive growth in recent years since Pretorius et al. first proposed in 1974 [8]. The advance of CEC is reviewed by several authors [9–12]. The capillary columns with inner diameter of 50–100 μm packed with 1.5–10 μm conventional HPLC packing are typically used in CEC. The mobile phase in CEC is driven by electroosmotic flow (EOF), neutral solutes are separated based on the different interactions of them with the stationary phase, electrophoretic mechanism also contributes to the migration of the ionic solutes besides chromatographic mechanism. Use of EOF in CEC results in two important advantages over conventional HPLC. First, the nearly flat profile of EOF reduces the band broadening caused by transchannel diffusion and eddy diffusion, thereby higher column efficiency can be obtained in CEC than in HPLC. Second, the EOF is independent of particle size and dose not generate back pressure, thereby smaller particle and longer column can be used in CEC than in HPLC, and the plate numbers can be further improved. All of these features have made CEC an attractive separation technique. On the current stage, CEC is only used as a tool for separation. In fact, CEC can also be used to probe the intermolecular interactions as that in HPLC. However, no much effort has been made in this field yet.

Silica particles with immobilized α_1-acid glycoprotein and human serum albumin (HSA) were packed into CEC columns for chiral separation [13,14]. One limitation for the using of these conventional HPLC packings in CEC is the difficulty for separation of acidic enantiomers due to the counter directional migration of the EOF and the electrophoretic mobility of the analytes. It was observed that some acidic enantiomers, like fenoprofen, ibuprofen, ketoprofen and naproxen, cannot be loaded in CEC column with α_1-acid glycoprotein immobilized silica gel as stationary phase [14]. In order to resolve acidic enantiomers, the packing with positive net surface charge is desirable because anodic EOF can be generated. To synthesize this kind of packing material may be complex and time consuming. An alternative way is to prepare such packing material by adsorption of protein on SAX material. It was found that BSA pre-adsorbed onto SAX could still display enantioselectivity in CEC [15]. In this system the EOF direction is from cathode to anode due to the positively charged surface, which is preferable to separate acidic enantiomers. The anionic enantiomers, tryptophan, ketoprofen, fenoprofen and warfarin, were successfully resolved [15]. In this work, the zonal elution approach in CEC was performed to study the competitive binding of enantiomers to protein with the SAX column containing the pre-adsorbed BSA.

2. Materials and methods

2.1. Instrumentation and material

All the CEC experiments were performed on a P/ACE system MDQ (Beckman, Fullerton, CA, USA), a Spectra-Physics pump (Spectra-Physics Inc., San Jose, CA, USA) was used to pack capillary columns. Fused silica capillary (50 μm I.D., 365 μm O.D.) was obtained from Yongnian Optic Fiber Plant (Hebei, China). Spherisorb-SAX (5 μm, 120 Å) was purchased from the Waters Phase Separation (Milford, MA, USA).

2.2. Reagents and buffers

The racemic benzoin and warfarin were purchased from Sigma (St. Louis, MO, USA). DL-, D-, and L-tryptophan were from Shanghai Institute of Biologic Chemistry, Academia Sinica (Shanghai, China). BSA was purchased from Sino-American Biotechnology Company (Beijing, China). All other chemicals used were of analytical or chromatographic grade. The mobile phases

with D- or L-tryptophan as competitive agent were prepared by adding 0–250 μM of the desired competing agent to 5 mM sodium phosphate buffer (pH 6.5) containing 5% acetonitrile. The mobile phases with *n*-hexanoic acid or trichloroacetic acid as competitive agent were prepared by adding 0–10 mM of the desired competing agent to 5 mM sodium phosphate buffer (pH 6.5) containing 15% acetonitrile. The mobile phase was degassed in an ultrasonic bath for 30 min. The concentrations of the enantiomers were 100–500 μg/ml. The stock solution of BSA with concentration of 2 mg/ml was prepared in 10 mM phosphate buffer at pH 6.5.

2.3. Column preparation and separation conditions

CEC columns were prepared by slurry packing technique as reported in literature [15]. All columns were 31 cm long with effective length of 10 cm (packed length). The procedure for the adsorption of BSA to SAX packing was the same as in our previous report [15]. The column was first flushed with 10 mM phosphate buffer (pH 6.5) for 30 min by a syringe, whose needle was connected to the capillary column with a PTFE tube. Then the column was flushed with 2 mg/ml BSA in 10 mM phosphate buffer (pH 6.5) for 4 h. The residual BSA was flushed out by 10 mM phosphate buffer (pH 6.5). Finally, the column was rinsed with mobile phase for 30 min, and conditioned on electrophoresis instrument by both applied voltages of -5 and -10 kV (the minus sign means that placing the cathode at the inlet side and the anode at the outlet side of the column) for 2 h. The temperature was kept at 25 °C. On column detection was used in this work, and the wavelength was set at 214 nm. The separation voltage was at -10 kV. Sample was injected by applying voltage of -3 kV for 10 s.

3. Results and discussion

Theory for zonal elution experiments is well documented in the literatures [3–7]. In general, a known concentration of a competing agent (I) is continuously applied to a column packed with the ligand (L) immobilized stationary phases while injections of a small amount of analyte (A) are made. If I and A compete at a single type of site on L, and A binds to no other sites, a plot of reciprocal of the retention factor of A (k'_A) versus the concentration of I ([I]) should be linear. The competing agent and the injected analyte can be the same solute, if $1/k'$ versus the concentration of the solute in the mobile phase is linear, that also means single-site competition takes place [4]. However, the determination of retention factor (k') for ionic solute in CEC, which is resulted from only chromatographic mechanism, is very difficult because of the involvement of electrophoretic mechanism. The retention factors for ionic solutes in micellar electrokinetic capillary chromatography can be calculated by considering both the effects of micellar solubilization and electrophoresis of the solutes [16], the determination of retention factor (k') for ionic solute in CEC was not reported until now. In order to describe the migration process, apparent retention factor or electrochromatographic retention factor (k^*) was defined similar to that in HPLC [9]:

$$k^* = (t_r - t_0)/t_0, \quad (1)$$

where t_r is the migration time of the solute, and t_0 is the migration time of a neutral and chromatographically unretained solute. It was reported that the influence of electrophoretic mobility on the retention behavior of ionic solutes was insignificant if they are strongly retained on the ion-exchange stationary phase [17]. A good linear relationship between $\log k^*$ and $\log(c)$ as in ion-exchange HPLC was observed in ion exchange CEC for separation of small peptides [17]. Because the difficulty in determination of k' in CEC for ionic solutes, the k' was substituted by k^* in this work for ionic solutes. The separation selectivity (α) was calculated by:

$$\alpha = (t_2 - t_0)/(t_1 - t_0), \quad (2)$$

where t_0 is the void time, t_1, t_2 are the migration time of the first and later eluted enantiomer, respectively. Chiral separation in CEC with pre-adsorbed BSA on SAX material was achieved, and it was observed that the relative standard deviations (RSD) for void time and retention

times 21 by consecutive runs were less than 1.0% [15]. This means that the repeatability of this system is acceptable, thus the average retention times of solutes by three injections with relative deviation (RD) less than 1.5% were applied for probing the interaction of enantiomers with the pre-adsorbed BSA in this work.

Some of the protein molecules will be denatured after the adsorption or immobilization of them on the support surface, but most of them will still keep the biological behavior as the free molecule in the solution. The enantiomers of tryptophan, ketoprofen, fenoprofen and warfarin were successfully resolved in CEC with the pre-adsorbed BSA stationary phase [15], which indicated that most of the binding sites on the BSA molecules are not damaged after the adsorption. The retention of tryptophan enantiomers in this system is contributed from at least three different factors as: (i) electrophoretic mobility of the negatively charged tryptophan; (ii) the electrostatic interaction of anionic tryptophan with positive groups on the SAX packing material; and (iii) the binding of tryptophan to the protein pre-adsorbed on SAX surface. The retention of tryptophan may be changed based on above three factors by addition of tryptophan enantiomer into the mobile phase as a competitive agent. Therefore, it is necessary to determine if the change in the retention of a solute is originated from the displacing effect for the binding of competitive agent to the pre-adsorbed protein. If CEC column packed with SAX is adopted, the migration of tryptophan is contributed only by the first two mechanisms, and their effect on the retention of the analyte can be investigated with different concentration of D- or L-tryptophan as the competitive agents in the mobile phase. The retention values of both D- and L-tryptophan on SAX packed column with mobile phases containing different concentration of tryptophan enantiomer and 5% acetonitrile in 5 mM phosphate buffer (pH 6.5) were measured. The obtained results for the plots of $1/k^*$ of D, L-tryptophan versus the concentration of L- and D-tryptophan presented in the mobile phase are shown in Fig. 1(a) and (b). It can be seen from Fig. 1 that the change in the k^* values of D, L-tryptophan is not significant with RSD less than 2.2% when the L- or D-tryptophan concentration in the mobile phases varied from 0 to 250 μM. Benzoin is a neutral compound, its retention on SAX is mainly contributed from the molecular interaction. Similarly, the retention of benzoin on SAX column at different D, L-tryptophan concentration was measured, and the obtained results are also shown in Fig. 1. As can be seen from Fig. 1, the $1/k^*$ value of benzoin almost does not change with addition of tryptophan. These results indicate that the addition of low concentration of tryptophan has almost no influence on the retention of solutes on SAX column contributed from the electrophoretic and chromatographic mechanisms. When the BSA was pre-adsorbed onto the SAX material, the binding of the solute to the protein will also make a contribution to the retention of solute. Under this condition, any signifi-

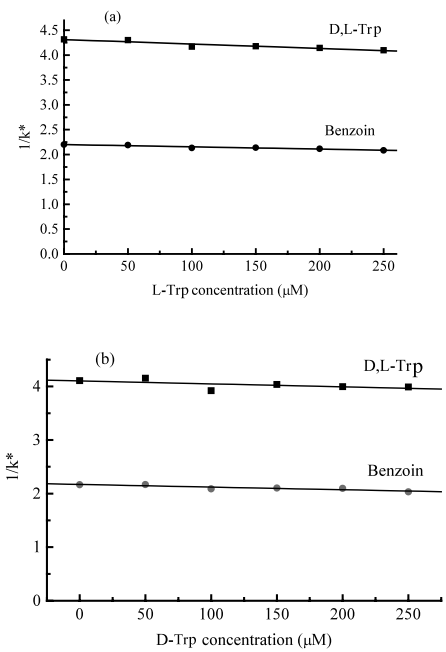

Fig. 1. Effect of: (a) L-tryptophan; and (b) D-tryptophan concentration in mobile phase on the retention of D,L-tryptophan and benzoin in CEC with SAX column without pre-adsorption of BSA. Experimental conditions: column, 50 μm i.d. × 375 μm O.D., packed/total length = 10 cm/31 cm, the column was packed with 5 μm SAX; applied voltage, − 10 kV; injection, − 3 kV × 10 s; detection wavelength, 214 nm; mobile phase, 5% acetonitrile, 5 mM phosphate buffer (pH 6.5) and 0–250 μM D- or L-tryptophan.

cant variations in the retention of the tryptophan and benzoin enantiomers are mainly caused by their binding to the protein pre-adsorbed on SAX when some amount of tryptophan enantiomer is added, which makes it possible to probe the interaction between the enantiomers and protein pre-adsorbed.

Several studies on binding of tryptophan molecules to BSA have been reported, but different conclusions have been obtained. King and Spencer [18] reported that D-tryptophan competitively displaced L-tryptophan from BSA, suggesting that both ligands bind at the same site. Garnier et al. [19] also reported competitive binding of D- and L-tryptophan on a single site to BSA. McMenamy and Oncley [20] reported that a small amount of tryptopan is bound to a secondary site with a very low order of magnitude. Gilpin et al. [21] reported that the D-tryptophan does not have a specific binding site, whereas the L-tryptopan exhibits a high specific binding site. In order to determine whether or not D- and L-tryptophan share a common binding site on the pre-adsorbed BSA, D- or L-tryptophan was added in the mobile phase as the competitive agent and both enantiomers of tryptophan were injected as the analyte. Initial study was carried out by injection of small amount of D- and L-tryptophan onto the BSA pre-adsorbed column with L-tryptophan as a competing agent. For L-tryptophan, a serious decrease in retention was observed as increasing amounts of L-tryptophan in the mobile phase. While the effect of L-tryptophan concentration on the retention of D-tryptophan was insignificant. As addressed previously, the retention of solutes in CEC with SAX column by chromatographic and electrophoretic mechanisms was almost not affected by the addition of tryptophan in the mobile phase. Accordingly, the serious decrease in retention of L-tryptophan was mainly caused from the competitive displacement on the binding sites of pre-adsorbed BSA by the L-tryptophan presented in the mobile phase. The plot of $1/k^*$ of D- and L-tryptophan versus the L-tryptophan concentration added in the mobile phase was shown in Fig. 2(a). As can be seen from Fig. 2(a) that the plot with L-tryptophan as the analyte was linear when the concentration of L-tryptophan from 50 to 250 μM, and the linear correlation coefficient was 0.9965. Above result means that the chromatographic capacity factor k' can be substituted by the electrochromatographic retention factor k^* in affinity CEC for probing enantiomer–protein interaction approximately. The linear relationship indicates that single site competitive binding was occurred, therefore L-tryptophan had one primary class of binding sites on the BSA. The deviation of the plot from linear relationship at low L-tryptophan concentration in the mobile phase may be resulted from two reasons. Firstly, the initial binding of L-tryptophan to BSA may induce a change in the protein conformation, which result in the alteration of the binding ability of BSA. Secondly, a secondary site with a very low order of magnitude was involved in the binding of L-tryptophan to the pre-adsorbed BSA. As can be seen from Fig. 2(a), the increase of L-tryptophan concentration in the mobile phase from 50 to 250 μM had almost no influence on the retention of D-tryptophan. This result means that D-tryp-

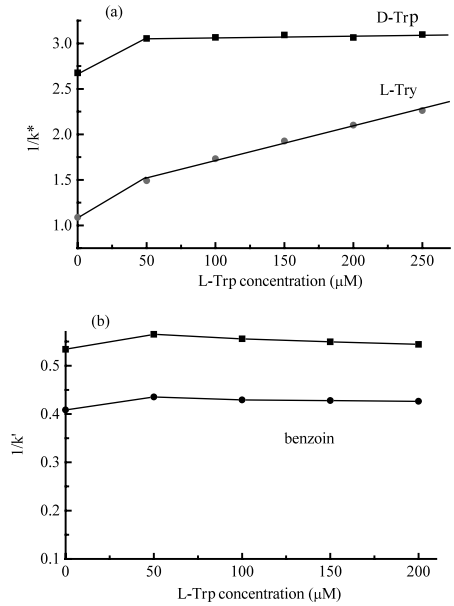

Fig. 2. Effect of L-tryptophan concentration on the retention of: (a) tryptophan; and (b) benzoin enantiomers in CEC with SAX column containing pre-adsorbed BSA. Experimental conditions: mobile phase, 5% acetonitrile, 5 mM phosphate buffer (pH 6.5) containing different concentration of L-tryptophan, other conditions as in Fig. 1.

tophan does not bind to the primary L-tryptophan binding site on the BSA molecule. However, the decrease of the retention of D-tryptophan was found by addition of 50 μM L-tryptophan into the mobile phase, which may also be resulted from the conformation change of BSA induced by the initial binding of L-tryptophan and sharing of a binding site with D-tryptophan and L-tryptophan.

L-tryptophan has stronger affinity to bind to the adsorbed BSA, thereby it eluted in longer time than D-tryptophan. The retention of L-tryptophan decreased quickly with increasing L-tryptophan concentration in the mobile phase, while no significant change in the retention of D-tryptophan was observed with increase of L-tryptophan concentration. This resulted in dramatic decrease in separation selectivity (α) for the separation of the tryptophan enantiomers as shown in Fig. 3. Fig. 4 shows the chromatograms for the separation of tryptophan enantiomers at different L-tryptophan concentration presented in the mobile phase. The tryptophan enantiomers can be separated with resolution as high as 2.46 when the mobile phase does not contain L-tryptophan. When 100 μM L-tryptophan was added in the mobile phase, the resolution decreased to 1.77, but two enantiomers were still baseline separated under these conditions. When the L-tryptophan concentration increased to 250 μM, only partial enantioseparation was observed.

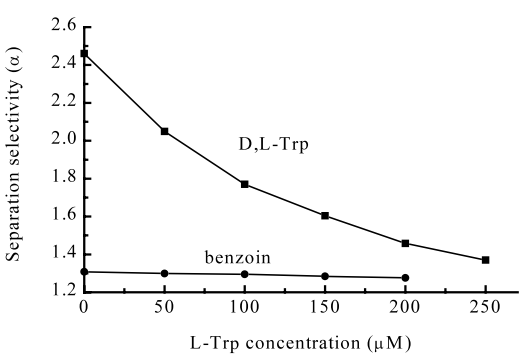

Fig. 3. Effect of L-tryptophan concentration on separation selectivity of tryptophan and benzoin enantiomers in CEC with SAX column containing pre-adsorbed BSA. Experimental conditions as in Fig. 2.

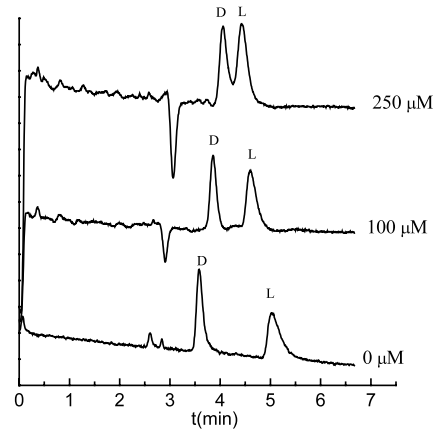

Fig. 4. Chromatograms for separation of tryptophan enantiomers at different L-tryptophan concentration in mobile phase. Experimental conditions as in Fig. 2.

Competitive binding study was also performed with using benzoin enantiomers as the injected solutes and L-tryptophan as a mobile phase additive. The resulting plot of $1/k'$ versus L-tryptophan concentration is shown in Fig. 2(b). The effect of L-tryptophan concentration on the retention of benzoin enantiomers was not significant. The k' values decreased about 6% by addition of 50 μM L-tryptophan and slightly increased with further addition of L-tryptophan. This result means that L-tryptophan does not compete with benzoin enantiomers at the same binding sites. The reason for the initial decrease of the retention of benzoin enantiomers may be caused from allosteric interactions: L-tryptophan that binds to BSA causes a change in the protein conformation, which further affects a remote binding site in such a way that its ability to bind benzoin enantiomers is reduced. Unlike to that of tryptophan enantiomers, the addition of L-tryptophan in the mobile phase has almost no influence on the separation selectivity of benzoin enantiomers.

Similarly, the competitive studies were performed with D-tryptophan as a competitive agent. Retention values of tryptophan enantiomers were measured with mobile phases containing different concentration of D-tryptophan, and the obtained results are shown in Fig. 5(a). Both the k^* of the tryptophan enantiomers decreased when the D-tryptophan concentration increased from 0 to100

μM. Obviously competitive displacement of L-tryptophan by D-tryptophan was observed, this confirms that the tryptophan enantiomers share a common binding site on the BSA molecule. The k^* values for the two enantiomers almost maintain constant with further increasing D-tryptophan concentration. The reason may be that the binding of tryptophan enantiomers at this site is very weak, and its binding capacity was saturated at the high concentration of D-tryptophan. The result observed in this work is in consistence to as reported by McMenamy et al. [20], that a small amount of tryptophan is weakly bound to a secondary site.

The interactions of benzoin enantiomers with BSA were also studied by using D-tryptophan as the mobile phase additive. The k' values of benzoin enantiomers were almost kept at constant with increasing concentration of D-tryptophan from 0 to 250 μM. The plot of $1/k'$ versus the concentration of D-tryptophan is shown in Fig. 5(b). No competitive displacement was observed between the benzoin enantiomers and D-tryptophan. This behavior means that benzoin enantiomers and D-tryptophan do not share the same binding site. Separation selectivity of tryptophan and benzoin enantiomers at different concentration of D-tryptophan is shown in Table 1. Separation selectivity of benzoin enantiomers almost did not change with the increasing D-tryptophan concentration. While the variation for separation selectivity of the tryptophan enantiomers was obvious at low D-tryptophan concentration, the highest selectivity can be obtained at 100 μM of D-tryptophan. The reason is that the D-tryptophan concentration has influence on the retention of the tryptophan enantiomers.

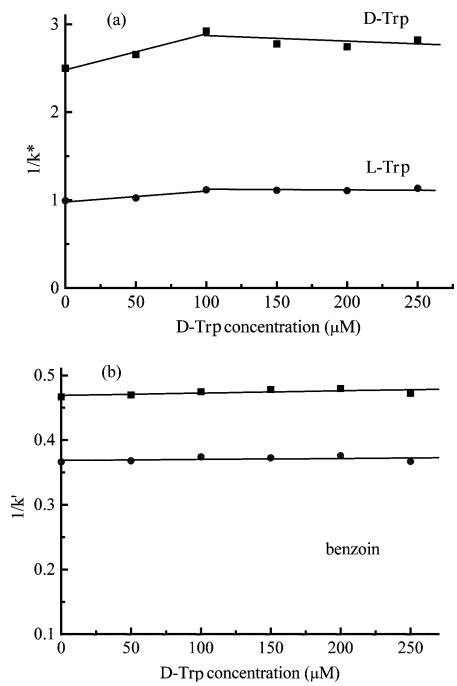

Fig. 5. Effect of D-tryptophan concentration on the retention of: (a) tryptophan; and (b) benzoin enantiomers in CEC with SAX column containing pre-adsorbed BSA. Experimental conditions: mobile phase, 5% acetonitrile, 5 mM phosphate buffer (pH 6.5) different concentration of D-tryptophan; other conditions as in Fig. 1.

Table 1
Separation selectivity of tryptophan and benzoin enantiomers at different D-tryptophan concentration in mobile phase

Enantiomers	D-tryptophan concentration (μM)					
	0	50	100	150	200	250
Tryptophan	2.52	2.60	2.62	2.50	2.48	2.49
Benzoin	1.27	1.28	1.27	1.28	1.28	1.29

Experimental conditions as in Fig. 5.

The retention of warfarin enantiomers on the immobilized BSA stationary phase could be reduced dramatically by addition of trichloroacetic acid (TCA) in the mobile phase in HPLC [3,22]. When TCA was added to the mobile phase, up to a concentration of 5 mM, there was approximately a 50% reduction in the k' values of both warfarin enantiomers, while α was reduced by only 3% [3]. A plot of $1/k'$ versus TCA concentration yielded straight lines for both warfarin enantiomers. It was concluded that the warfarin enantiomers and TCA bind at the same site on the BSA molecule. A similar behavior was also observed in CEC with pre-adsorbed BSA stationary phase. The chromatograms for separation of warfarin with different TCA concentration are shown in Fig. 6. R- and S-warfarin were eluted at 107 and 84 min without addition of TCA in the mobile phase, but the retention times for the two

enantiomers were reduced to 46 and 39 min, respectively, when 2 mM TCA was added in the mobile phase. The retention times of warfarin enantiomers at different TCA concentrations are shown in Table 2. The retention times decrease significantly with increasing the TCA concentration. When the concentration of TCA increased to 10 mM, the warfarin enantiomers can be eluted in less than 29 min. The k^* values for both warfarin enantiomers were reduced by about 80%, while the separation selectivity was only reduced by about 9%. It was reported that the strong retention of warfarin in this system was contributed from its strong binding to the pre-adsorbed protein [15]. Therefore, the significant reduction in the retention of the warfarin enantiomers is mainly caused from the competitive displacement on the binding site of pre-adsorbed BSA by TCA. As shown in Fig. 7 a plot of $1/k^*$ versus TCA concentration from 2 to 10 mM yielded straight lines for both R- and S-warfarin with correlation coefficient of 0.9964 and 0.9959, respectively. The results are very similar to that observed in HPLC [3]. This linear relationship means that single site competitive displacement was occurred, which means that warfarin enantiomers and TCA bind at the same site on pre-adsorbed BSA molecule. The first point without addition of TCA deviated from the linear relationship, which could also be found in the plot of the same relationship in HPLC [3]. The reason may be that the initial binding of TCA on the pre-adsorbed BSA molecule induces a change in the protein conformation, which results in the alteration of the ability of BSA to bind warfarin enantiomers.

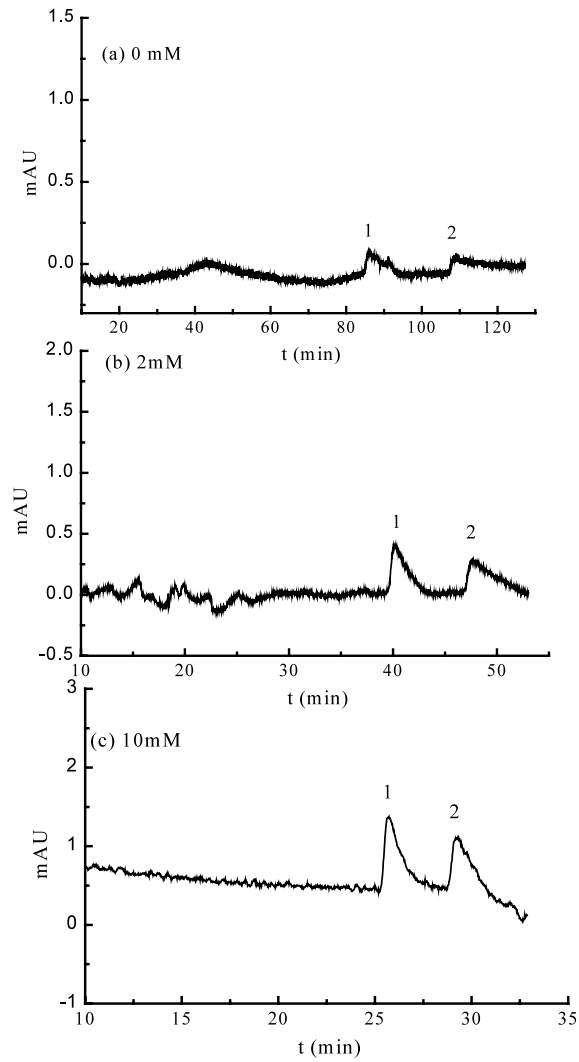

Fig. 6. Chromatograms for separation of warfarin enantiomers at different trichloroacetic acid concentration in mobile phase with SAX column containing pre-adsorbed BSA. Experimental conditions: mobile phase, 15% acetonitrile in 5 mM phosphate buffer (pH 6.5) containing: (a) 0 mM; (b) 2 mM; and (c) 10 mM trichloroacetic acid; other conditions as in Fig. 1.

The retention of warfarin in this system could also be reduced significantly by addition of *n*-hexanoic acid in the mobile phase [15]. The k^* values of the R- and S-warfarin were decreased by 80.3 and 77.1%, respectively, and the separation selectivity decreased by 14% by changing the *n*-hexanoic acid concentration from 0 to 10 mM. The relationship of $1/k^*$ of the enantiomers and the *n*-hexanoic acid concentration is shown in Fig. 8, which was very similar to that in CEC with addition of TCA as shown in Fig. 7. The linear relations for R- and S-warfarin were obtained with correlation coefficient of 0.9986 and 0.9992, respectively, when the *n*-hexanoic acid concentration varied from 2 to10 mM. These results also demonstrated that the warfarin enantiomers and *n*-hexanoic acid bind at the same site on the BSA molecule. Deviation of the data without *n*-hexanoic acid in the mobile phase from the linear relationship may be caused from the same reason as that by addition of TCA.

Table 2
Retention times of warfarin enantiomers at different trichloroacetic acid concentration in mobile phase

Retention time (min)	Trichloroacetic acid concentration (mM)						
	0	2	3	4	6	8	10
t_1	84.5	39.1	35.5	33.5	29.5	27.4	25.6
t_2	106.8	46.0	41.5	38.9	33.8	31.2	29.0

Experimental conditions as in Fig. 6.

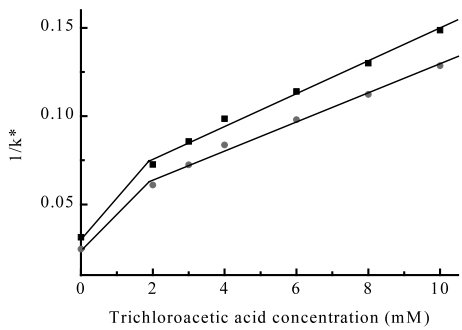

Fig. 7. Effect of trichloroacetic acid concentration on the retention of warfarin enantiomers in CEC with SAX column containing pre-adsorbed BSA. Experimental conditions: mobile phase, 15% acetonitrile, 5 mM phosphate buffer (pH 6.5) containing different concentration of trichloroacetic acid; other conditions as in Fig. 1.

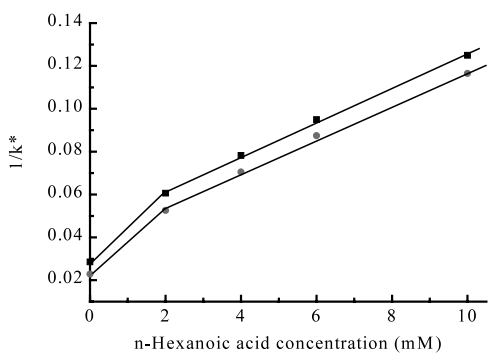

Fig. 8. Effect of *n*-hexioc acid concentration in mobile phase on the retention of warfarin enantiomers in CEC with SAX column containing pre-adsorbed BSA. Experimental conditions: mobile phase, 15% acetonitrile, 5 mM phosphate buffer (pH 6.5) containing different concentration of *n*-hexanoic acid; other conditions as in Fig. 1.

4. Concluding remarks

Affinity capillary electrochromatography has been developed to study the competitive binding of enantiomers to pre-adsorbed protein. By using D- or L-tryptophan as the mobile phase additives, it was observed that BSA molecule has a primary site to which L-tryptophan binds strongly, but D-tryptophan does not bind to this site. However, L- and D-tryptophan share a weak binding site on the BSA molecule. Benzoin enantiomers were resolved on CEC with BSA pre-adsorbed stationary phase, but their binding site on BSA molecule is different from to which either D- or L-tryptophan binds. Trichloroacetic acid and *n*-hexanoic acid were applied as the mobile phase additives to study the binding of warfarin enantiomers to BSA molecule pre-adsorbed, it was observed that warfarin enantiomers, trichloroacetic acid and *n*-hexanoic bind to BSA at the same binding site.

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