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Journal of Chromatography A, 898 (2000) 53–61

JOURNAL OF CHROMATOGRAPHY A

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Effect of mobile phase composition on the separation of propranolol enantiomers using a perphenylcarbamate β-cyclodextrin bonded chiral stationary phase

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Received 9 November 1999; received in revised form 18 July 2000; accepted 10 August 2000

Abstract

An analytical column packed with a novel perphenylcarbamate β-cyclodextrin bonded chiral stationary phase was used to separate propranolol enantiomers. Good separation results were obtained using triethylammonium acetate (TEAA) buffer and methanol mixtures as the mobile phase. Effects of the methanol concentration, buffer concentration and pH value on the retention and the enantioselectivity of propranolol enantiomers were investigated on this column. The retention times and the separation factor decrease with increase of the methanol concentration as expected in reversed-phase HPLC. At trace TEAA amount, the solute eluted out with anti-Langmuirian band profiles, their retention times decreased quickly with increase of TEAA concentration and attained a minimum at a TEAA concentration of 20 ppm. Above 20 ppm, solute band profiles changed to a Langmuirian shape, the retention times of enantiomers increased with increasing buffer concentration, and eventually, they attained asymptotes at ca. 1% TEAA. A simulation considering the different interactions between the solute and the additive at above and below 20 ppm TEAA concentration as well as system peaks interference can successfully explain the anti-Langmuirian band profiles and the retention time variation trend.

Keywords: Mobile phase composition; Chiral stationary phases, LC; Enantiomer separation; Propranolol

1. Introduction

Native and derivatized β-cyclodextrin (β-CD) bonded chiral stationary phase (CSP) has been widely used for high-performance liquid chromatography (HPLC) separation of enantiomers since the beginning of the 1980s [1,2]. Several β-CD or derived β-CD bonded CSPs are commercially available now. Most of the derived CD CSPs were prepared using post-immobilization derivatization procedures so that the regioselective immobilization of CD and batch-to-batch reproducibility are not accomplished [3–5].

Recently, a novel perphenylcarbamate β-CD bonded CSP was developed by our group [3–5]. This CSP was prepared using a pre-immobilization derivatization procedure. Mono-(6-azido-6-deoxy) per-

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0021-9673/00/$ – see front matter
PII: S0021-9673(00)00859-1

functionalized β-CDs were first synthesized, purified and characterized before being chemically anchored on the surface of aminized silica gel via the hydrolytically stable urethane linkage. The new methodology afforded structurally well-defined CSPs and a facile control of batch-to-batch reproducibility. This CSP has good stability and can be used with the whole range of HPLC solvents. Since all the hydroxyl groups on the rim of the CD ring were substituted by the phenylcarbamate except for bonding to silica gel, it showed very different separation characteristics from the native β-CD CSP. This new CSP demonstrated good enantioseparation of drugs in the reversed phase where the traditional β-CD column encounters difficulties. In reversed-phase HPLC using an aqueous–organic mobile phase, small amount of additives (usually a buffer) are used to control the ionization of the solutes and alleviate the peak tailing. Accordingly, triethylammonium acetate (TEAA) is commonly used as buffer in reversed-phase HPLC. The organic solvent and the modifier concentrations significantly affect retention and separation. Usually, the retention time of the solutes was reduced with increasing concentrations of organic solvent [2,6–8]. Piperaki et al. [6] reported that the retention times of promethazine isomers decreased as the additive concentration increased.

Paleologou et al. [7] found an initial decline in retention followed by a plateau for chlorophenols on a β-CD column.

When additives are used in the mobile phase, system peaks will appear due to the disturbance of the initial adsorption equilibrium of the additives between the mobile phase and the stationary phase upon the injection of the sample [9]. These system peaks may affect solute retention times and band profiles. Simulation shows that when the isotherms of the solute and additive are both Langmuir type, the solute retention time will decline with increase of the additive concentration [10]. Sometimes anti-Langmuirian and Golshan shape band profiles will appear through the interference between the system peaks and solute peaks [11,12].

The aim of this study is an investigation of the effects of the methanol and additives concentrations on the retention and enantioselectivity of propranolol enantiomers. Optimal mobile phase compositions for analyzing these two drugs will be found through this study. The influence of additive system peaks on the solute retention time and band profile will also be studied by simulation using a dispersion–transport model. The simulation result can qualitatively explain the retention time and peak shape variation observed in the experiment.

2. Experimental

2.1. Chemicals

HPLC-grade methanol (MeOH) and glacial acetic acid were obtained from J.T. Baker (Phillipsburg, NJ, USA). Triethylamine was obtained from Merck. HPLC water was made in the laboratory using a Millipore ultra-pure water system. Propranolol hydrochloride was purchased from Sigma (St. Louis, MO, USA).

2.2. Apparatus

The experiments were carried out with a Shimadzu LC-10ATVP chromatographic system (Kyoto, Japan), which consisted of two LC-10AT$_{vp}$ pumps (A and B), a SIL-I0AD$_{vp}$ auto-injector and a SPD-10A$_{vp}$ UV–Vis detector. The software CLASS-VP was used to control the system and record the detector signal. The column used in this work was an analytical column (25 cm×4.6 mm I.D.) packed with 3.5 g Macherey-Nagel sphere silica gel (Nucleosil with 300 Å pore size, pore volume: 0.8 ml/g and 10 μm particle size. The surface area is 100 m^2/g) on which perphenylcarbamate β-CD was covalently bonded (with 0.26 $\mu mol/m^2$ surface coverage of the derivatized β-CD). This column was prepared in the Department of Chemistry at the National University of Singapore. The column temperature was thermostatted at 25°C using a water bath.

2.3. Chromatography

Solvent A contained only the desired methanol concentration in water solution. Solvent B contained the same methanol concentration as solvent A plus buffer of 1% (v/v) triethylamine (TEA) adjusted with acetic acid to the desired pH. Solvents A and B

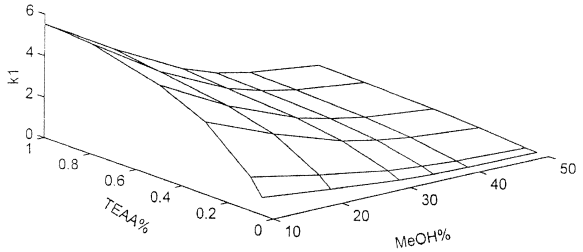

Fig. 1. Effects of MeOH and TEAA concentrations on the retention factor of *S*-(−)-propranolol.

were delivered by pumps A and B and were mixed thoroughly in a low dead-volume mixer after the pumps. The total mobile phase flow-rate was 1 ml/min. Buffer concentration was changed in the stair case mode by decreasing stepwise the proportion of solvent B, a sequence which corresponds to the decreasing retention times for the drugs used in this work. A 40-min re-equilibration time was used after each step change of the buffer concentration. All drug samples were dissolved in the mobile phase at a concentration of 1 mg/ml. Signals were detected with the UV detector at 230 nm after 10 μl of sample was injected into the column by the auto-injector.

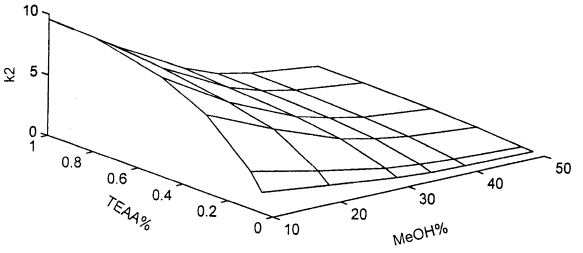

Fig. 2. Effects of MeOH and TEAA concentrations on the retention factor of *R*-(+)-propranolol.

The dead time of the column was determined by injecting pure methanol at 200 nm.

3. Results and discussion

3.1. Effect of the MeOH concentration

The retention factors at different MeOH and TEAA concentrations are shown in Figs. 1 and 2 with the *y* axis representing the volume fraction of MeOH in the mobile phase. An increase in MeOH concentration led to a decrease in retention factors for both *S*-(−)-propranolol and *R*-(+)-propranolol. This is a common rule in reversed-phased HPLC due to the increasing amount of adsorbed organic compound weakening the hydrophobic interaction between the solute and the adsorbent.

The logarithm of the retention factor *k* was found to be linearly related to the methanol volume fraction according to:

$$\ln k = a - s\varphi \quad (1)$$

where *a* is the intercept of the correlation equation, *s* the slope of the correlation equation and φ the methanol volume fraction in the mobile phase.

The slope, intercept as well as the correlation coefficient at various TEAA concentrations are listed in Table 1. The linearity was quite good at higher TEAA concentrations and fair at lower TEAA concentrations. This is due to the very short retention times for propranolol at low TEAA concentrations so that the experimental error on determination of retention times and the dead time of the column affect the retention factor much more.

The effect of MeOH concentration on the sepa-

Table 1
Slope and intercept of Eq. (1) at different TEAA concentrations

TEAA (%)	*S*-(−)-Propranolol			*R*-(+)-Propranolol		
	s	*a*	*r*	*s*	*a*	*r*
0.05	0.1024	1.0314	0.9579	0.0827	1.4868	0.9694
0.1	0.0803	1.4247	0.9799	0.0746	1.9795	0.9857
0.3	0.0693	2.0851	0.992	0.0702	2.6822	0.9925
0.5	0.0655	2.2614	0.9954	0.0674	2.854	0.9963
0.8	0.064	2.3938	0.9971	0.0671	2.9984	0.9964
1.0	0.0637	2.4468	0.9936	0.0672	3.0452	0.9927

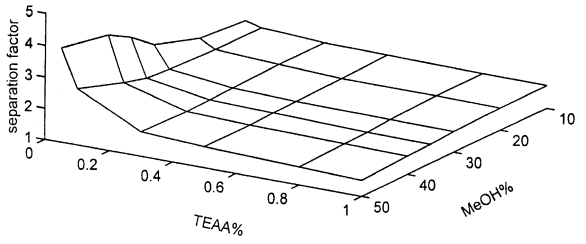

Fig. 3. Effects of MeOH and TEAA concentrations on the separation factor of propranolol enantiomers.

ration factor of propranolol is shown in Fig. 3. However, changing the MeOH concentration will not change the separation factor to the same extent as it will change the retention factor. We can see from Fig. 3 that the separation factor reaches a maximum at ca. 20% MeOH and then declined slightly from 30% MeOH to 50% MeOH. A similar effect of the MeOH concentration was found at all TEAA concentrations. The abnormally high separation factors at 0.05% and 0.1% TEAA concentrations were ascribed to the fact that the retention times of the two enantiomers were very near the column dead time, and a small experimental error would cause a large deviation of the separation factor.

3.2. Effect of TEAA concentration

3.2.1. TEAA concentration>20 ppm

At first, the TEAA concentration effect was studied in the range of 0.05% to 1% TEAA, the most commonly used buffer concentration in reversed-phase HPLC. The retention factors for both enantiomers and their separation factors are shown in Figs. 1–3. The *x* axis represents the TEAA concentration. We can see from Figs. 1 and 2 that the retention factors initially increase quickly with increasing TEAA concentration and then approach asymptotes at about 1% TEAA concentration. This variation trend of the retention factor is quite different from the common buffer effect on an organic compound in reversed-phase HPLC. This phenomenon can be attributed to the unique property of CD, i.e., forming inclusion complex. With increasing the concentration of TEAA, strong hydrogen bonding to the carbamate groups and the solute retention can be greatly reduced due to decreasing the interaction between the solute and carbamate groups.

Another plausible explanation of the abnormal trend will be given in the next section by simulation using an anti-Langmuir isotherm of the TEAA additive in this concentration range.

Fig. 3 shows the effect of the TEAA concentration on the separation factor in the *z–x* section. The separation factor declined sharply with the TEAA concentration increasing from 0.05% to 0.3% followed by a plateau in the 0.3–1% range. This means in the range of 0.3–1% TEAA, TEAA affects the retention of both enantiomers in the same manner, but did not affect the chiral recognition site very much.

We noticed from Figs. 1 and 2 that the retention times of the propranolol enantiomers were very short at 0.05% TEAA concentration, near to the column dead time. But we knew that when we used a pure water and methanol mixture as the mobile phase without any buffer, the retention times for both enantiomers were very long (longer than 60 min). In order to determine how the addition of the TEAA to the mobile phase reduced the propranolol retention, it is necessary to study the propranolol retention behavior at very dilute TEAA concentrations.

3.2.2. TEAA concentration<20 ppm

The chromatograms of propranolol separated on the perphenylcarbamate β-CD column with ppm-magnitude TEAA in the 30% MeOH aqueous solution mobile phase are shown in Fig. 4. With only 1

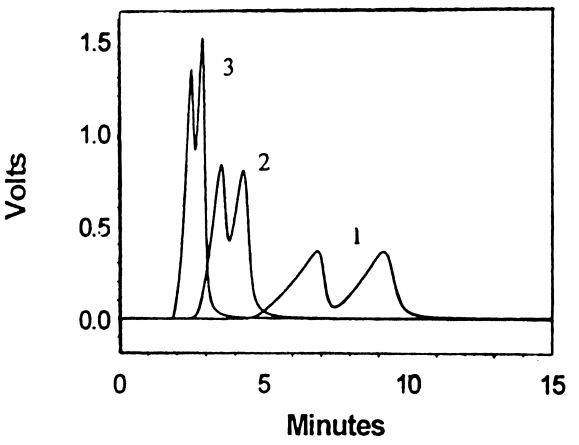

Fig. 4. Band profiles and retentions of propranolol enantiomers at trace TEAA amount in the mobile phase.

ppm TEAA in the mobile phase, propranolol enantiomers eluted out in 10 min. But the peaks were very broad. Increasing the TEAA concentration from 1 ppm to 4 ppm, decreased the retention times dramatically and the peaks were less broad. Further increasing the TEAA concentration to 20 ppm produced a minimum retention time with sharp peaks. This minimum retention time was even shorter than the column dead time determined by injection of pure MeOH (3.2 min). We still got some separation for propranolol enantiomers with such a short retention. This can be due to the fact that as the TEAA molecules are included in the cyclodextrin cavity and occupy it, TEAA strongly hydrogen bonds with the carbamate groups at the larger entrance of the cavity and make the formation of inclusion complex difficult. So the retention time was greatly reduced. When the buffer concentration is increased, solute peaks become sharper and the retention time (t_R) decreases. Without TEAA additive in the mobile phase, the basic propranolol molecules can totally go into the cavity and form stable inclusion complexes, subsequently resulting in a longer retention time and peak tailing.

The abnormal anti-Langmuirian peak shape with diffuse front and sharp rear is shown in Fig. 4. These anti-Langmuirian peaks were the result of the interference between the solute peaks and the system peaks formed by the disruption of the additive adsorption equilibrium triggered by the sample injection. This anti-Langmuirian peak shape and the retention time variation are shown clearly through a numerical simulation of a solute band profile using a mobile phase containing an additive.

3.3. Explanation of the effect of additive concentration through theoretical simulation

The different retention time variation trend for <20 ppm TEAA and for >20 ppm TEAA in the mobile phase indicated there must be a different interaction between propranolol and TEAA additive. One of the possible reasons is that the TEAA additive behavior of hydrogen bonding changes with increasing the mobile phase concentration. There is an inflection point on its adsorption isotherm. At lower concentrations, the isotherm is concave, i.e., Langmuir type; but at higher concentrations, the isotherm is convex, i.e., anti-Langmuir type. We can see from the simulation that an additive with an anti-Langmuir isotherm will produce increasing solute retention time with increasing additive concentration.

For simplicity, we consider a system consisting of one additive in the mobile phase and one solute compound in the sample. We suppose a quadratic isotherm for the additive with an inflection point as shown in Fig. 5. The solute isotherm is Langmuir type. The competitive isotherms can be represented as:

$$q_1^* = \frac{q_s b_1 c_1}{(1 + b_1 c_1 - c_1/c_{s1}) \cdot (1 - c_1/c_{s1}) + b_2 c_2}$$
$$q_2^* = \frac{q_s b_2 c_2}{(1 + b_1 c_1 - c_1/c_{s1}) \cdot (1 - c_1/c_{s1}) + b_2 c_2} \quad (2)$$

$$q_j = f \cdot (c_1, c_2) \quad (3)$$

where subscript 1 represents the additive and 2 represents the solute, b_1, b_2 are characteristic of the additive and solute in the chromatographic system, respectively, q_s, is the saturation capacity of the stationary phase and c_{s1}, is a characteristic for additive. q_j is the equilibrium concentration of species j in the stationary phase.

The parameters used in the simulation are: $b_1 = 5$, $b_2 = 10$, $q_s = 50$, $c_{s1} = 10$. Fig. 5 shows the isotherm for the pure additive adopting above parameter values.

Substituting Eq. (2) into Eq. (3) and solving the

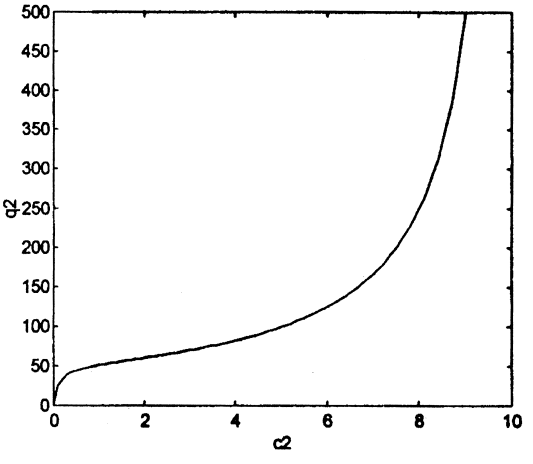

Fig. 5. Additive isotherm with inflection point.

partial equations of the dispersion–transport model using the numerical method, we obtained the band profiles of the solute and the system peaks at different additive concentration.

Fig. 6 shows two simulated chromatograms at the additive concentration corresponding to the concave (Langmuir type) part on the additive isotherm. The dotted line represents the system peaks. The scale for the additive concentration used in the plot is $c_2-c_2^0$. We should notice that the negative system peak accompanying the solute peaks does not mean the additive concentration is negative, it just means the additive concentration present is below the feed concentration. The additive system peaks did not appear in the experimental chromatograms because the additive did not have strong UV absorption at that wavelength. Two system peaks were formed for the injection of one solute. Before the injection of the sample, the stationary phase was equilibrated with additive in the mobile phase. After the sample entered the column, the equilibrium is perturbed. Some of the additive molecules formed hydrogen bonds and migrated along the column. When the injection finished, the solute concentration declined at the column inlet, the same amount of additive that was expelled had to be supplement to the stationary phase to restore the local equilibrium, thus forming a negative system peak. The system peaks formed by this perturbation interacted with the solute band, resulting in the distortion of the solute band profile.

Fig. 6a is the calculation result at the additive concentration of 0.2 m*M*, which corresponded to the strong concave isotherm part in Fig. 5. The solute band profile is strongly anti-Langmuirian, and the retention time is very long. Fig. 6b shows the calculation result at a TEAA concentration of 1 m*M*. The solute band profile is still anti-Langmuirian, but the retention time is much shorter.

Fig. 7 shows the simulated chromatograms for two additive concentrations corresponding to the concave part (anti-Langmuir) on the isotherm. The solute band profiles became Langmuirian and the retention time increased with increase of the additive concentration from 4 m*M* to 8 m*M*. The retention delay can be also understood through the theory of characteristics [13]. The migration velocity u_z of the additive at concentration c is given by:

$$u_z = \frac{u}{1 + F\dfrac{\mathrm{d}q}{\mathrm{d}c}} \tag{4}$$

The additive migration velocity at concentration c is governed by the isotherm slope at that point. A convex isotherm resulting in an increasing slope with increasing concentration, so that the migration velocity of the additive peak decreases.

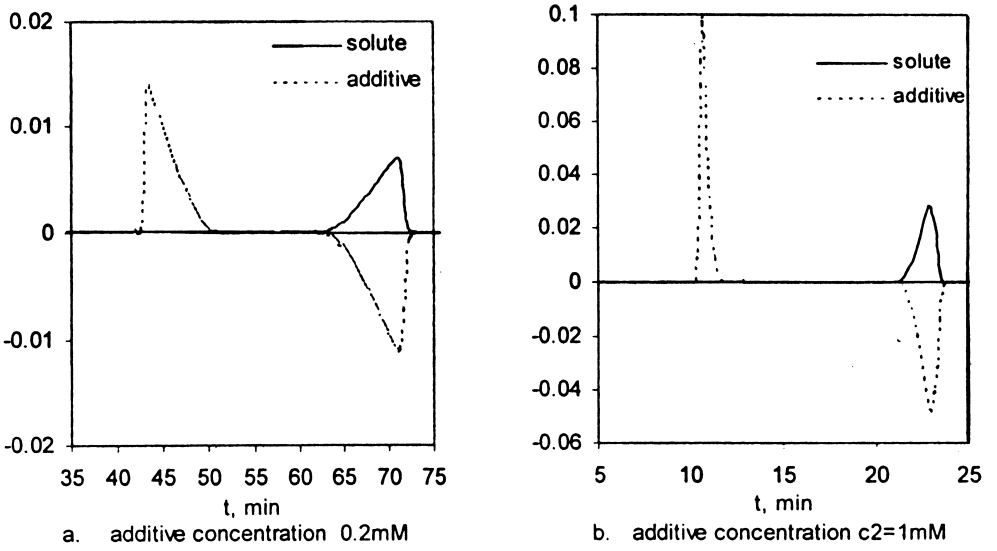

Fig. 6. Simulation band profiles at lower additive concentration.

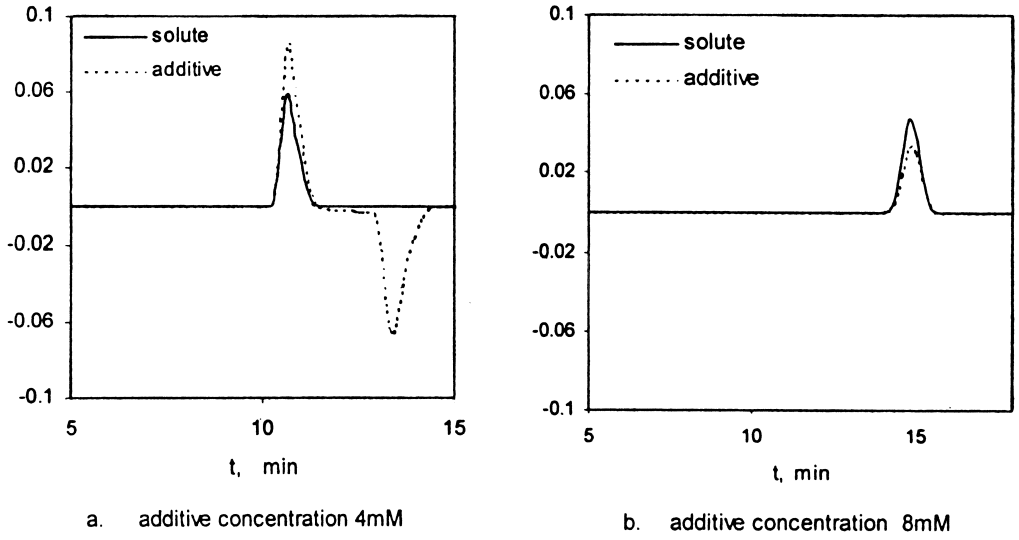

Fig. 7. Simulation band profiles at higher additive concentrations.

However, the simulation can only predict a fair degree increase of retention with increasing additive concentration in the convex isotherm period, not as significant as that observed in the experiment. There may be other factors affecting the retention at higher TEAA concentrations. One of the other possible contributions to the retention delay is that the mobile phase polarity increased with increasing TEAA concentration, which enhanced the hydrophobic interaction between propranolol and the CSP.

The simulated results using a quadratic isotherm with a reflection point for additive can successfully predict the abnormal anti-Langmuirian peaks observed at ppm-magnitude TEAA concentrations. The change of retention time variation trend was also successfully predicted.

3.4. Effect of pH

Fig. 8 shows the effect of pH value in the mobile phase. With pH increase, from 4 to 6, the retention factors of both propranolol enantiomers increased significantly. This is because the basic solute ionization degree decreased with increasing mobile phase pH. The pK_a value for propranolol is ca. 6.0. At pH 4.0, most of the propranolol molecule exists as the cation. With increasing pH, more and more propranolol molecule exists as free base in the mobile phase which definitely enhances the hydrophobic interaction between the apolar segment of the solute and the hydrophobic moiety of the CD cavity to make the inclusion complex more stable than the ionized compound. This will also increase the retention time and retention factor of solute. The separation factor of propranolol enantiomers declined slowly with the increase of pH in the experiment range. Obviously, here the hydrophobic interaction has played a more important role than hydrogen bonding between the solute and the derivatized β-CD.

3.5. Optimal method for propranolol analysis

Through the studies of the effects of the mobile phase composition, the buffer concentration and the pH on propranolol chiral separation, an optimal analysis method was obtained. The chromatograms at different sample sizes are shown in Fig. 9. Enantiomers eluted out in a relatively short time with baseline separation and symmetric peaks. The separation factor is 1.58. The resolution of a 10-μl sample size is 1.99. The propranolol concentration in the sample was 1 mg/ml. We can still get baseline separation and good peak shape at 50-μl sample size, which shows the high loading capacity of the perphenylcarbamate CSP. Considering the lower efficiency of the column packed with 10 μm silica

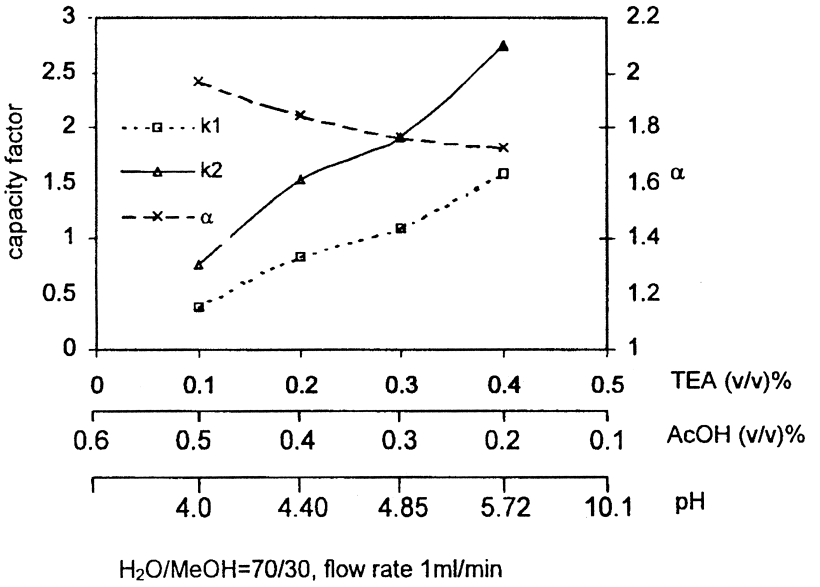

Fig. 8. Effect of pH on the retention factors and separation factor of propranolol.

gel rather than the 5 μm silica gel that usually used in most of the analytical columns, the separation result is good. However, this separation cannot be obtained in aqueous phase mode using a native β-CD CSP. This is most likely because the hydroxyl group on the β-CD rim suppressed the inclusion complexation formation of the drug molecule with the β-CD moiety. In this perphenylcarbamate β-CD, all hydroxyl groups on the top and bottom rims of the β-CD ring are substituted by phenylcarbamate, which makes the drug molecule fit the cyclodextrin cavity better than the native β-CD and increases the hydrophobicity of the ring openings. So the enantiomers can easily enter the CD cavity due to attractive interaction to form a complex with β-CD.

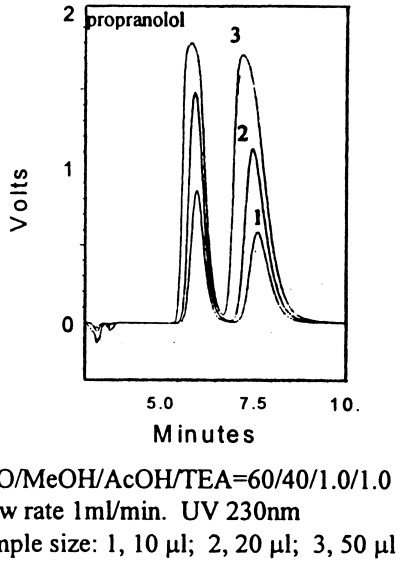

Fig. 9. Chromatograms of propranolol on the perphenylcarbamate β-CD column.

4. Conclusions

Enantiomers of propranolol were baseline separated with symmetric peaks together with short analytical times on the newly developed perphenylcarbamate β-CD column using TEAA buffer and aqueous methanol mixture as the mobile phase. The modified β-cyclodextrin looks to have a more ''tight fit'' inclusion complex in both size and shape with the propranolol molecule than native CD and enhances the hydrophobicity of the opening, which contributes to the inclusive complexation between the enantiomers and β-CD.

The retention factor and the enantioselectivity of propranolol on the perphenylcarbamate β-CD CSP

are significantly affected by the MeOH and TEAA concentration. Increasing MeOH concentration reduces the retention factors of both enantiomers and the separation factor. Above 20 ppm TEAA, retention factors increased with increasing TEAA concentration and approaches asymptotes at about 1% TEAA.

Abnormal anti-Langmuirian band profiles were observed when TEAA concentration was below 20 ppm. This is mainly caused by the interaction between the solute band and the additive system peaks. The retention times of enantiomers declined sharply to a minimum with TEAA concentration increasing from 1 to 20 ppm. Numerical simulation of the band profile using a quadratic isotherm with a reflection point for additive confirmed the anti-Langmuirian band profiles and the retention time variation trend.

Acknowledgements

We thank the National Science & Technology Board and National University of Singapore for financial support through grants GR 6450 and RP 982752.

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