Liquid chromatographic retention behaviour and separation of promethazine and isopromethazine on a β-cyclodextrin bonded-phase column

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ABSTRACT

A liquid chromatographic method for the separation of promethazine (PR) and its positional isomer isopromethazine (IPR) is described. PR is an N-substituted phenothiazine with the actions and the uses of the antihistamines (H1-receptor antagonists). IPR is an impurity in the pharmaceutical preparations of PR and must be controlled at a level below 1%. The liquid chromatographic behaviour of PR and IPR on a hydrolytically stable β-cyclodextrin (β-CD) column with respect to mobile phase composition, pH, ionic strength and the nature of the organic modifier was also investigated. Based on the results, conditions were chosen for the isocratic separation of the two isomers. The proposed separation method is simple and rapid and permits the simultaneous determination of PR and IPR. The separation selectivity of a cyclodextrin bonded-phase column was examined. Special attention was devoted to modelling the inclusion complexes of PR and IPR with β-CD in order to predict their optimum orientation within the β-CD cavity.

INTRODUCTION

Even though much progress has been achieved in the separation of structural [1] and positional isomers [2] of a number of compounds, most of the effort has been directed at developing complex mobile phases that can provide the required selectivity. In the last few years, however, some attention has been directed towards the development of tailor-made column packing materials specifically designed to provide multi-point or specific chemical interactions with analytes in order to achieve more selective separations. Among such column packings are a number of stable cyclodextrin (CD)-bonded phases, which were first described in 1984 [3]. This allows cyclodextrin columns to effect numerous chemical separations by selectively including, in solution, a wide variety of organic and inorganic guest molecules in the cyclodextrin cavity [4].

Cyclodextrins are toroidally shaped oligosaccharides formed by the action of *Bacillus macerans* amylase on starch. These supramolecules contain 6–12 D-glycopyranose units bonded through α-(1,4)-glycosidic linkages. The physical shape of the molecule is that of a truncated cone, with an internal hydrophobic

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cavity whose dimensions are determined by the number of glycose units and hydrophylic exterior faces [5,6].

The ease with which CD packings discriminate between molecules of different chemistry, size, shape and spatial geometry is obviously due to its ability to form intimate inclusion complexes of different strength. In the mechanism of separation a variety of interactions are involved, including hydrophobic interactions with the interior of the cavity, hydrogen bonding with the hydroxyl groups at the periphery of the cavity, the release of high-energy water or modifier during complex formation or a combination of these factors [7,8].

Promethazine (PR) hydrochloride is an N-substituted phenothiazine (Fig. 1) with the actions and uses of the antihistamines (H1-receptor antagonists). It also has some antimuscarinic, antiseretoninergic and marked local anaesthetic properties [9]. It is commonly used in pharmaceutical preparations such as tablets, injections and elixirs. Thus it is a molecule of great analytical interest and it has been determined by colorimetric [10], spectrophotometric [11], spec-
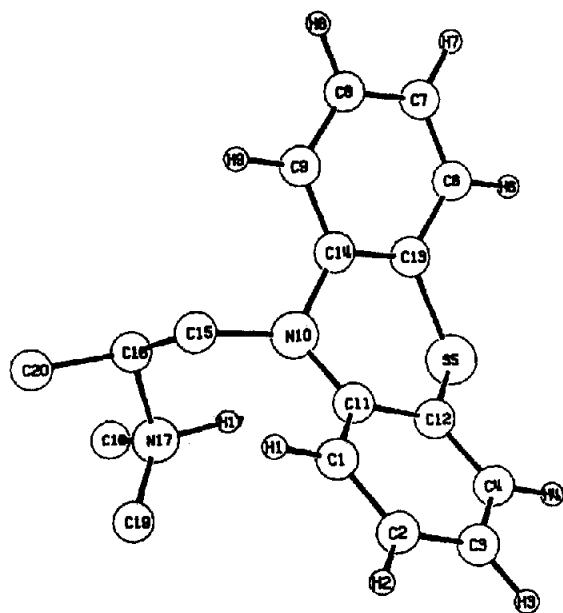
trofluorimetric [12], flow injection [13], GC [14], GC–MS [15] and LC [16–26] methods.

On the other hand, isopromethazine (IPR) is an impurity in PR pharmaceutical preparations and its level must be controlled below 1%. Their simultaneous determination has been performed by high-speed LC [27].

Our initial motivation in undertaking this work was the direct LC separation of PR and its positional isomer IPR. To the best of our knowledge, this is the only separation of these two isomers by HPLC using a β-CD column. Further, we investigated the LC behaviour of PR and IPR on a hydrolytically stable β-CD column with respect to mobile phase composition, pH, ionic strength and the nature of the organic modifier. Based on the results, chromatographic conditions for the isocratic separation of the two isomers were chosen. Special attention was devoted to modelling the inclusion complexes of PR and IPR with β-CD, in order to predict their optimum orientation within the β-CD cavity.

EXPERIMENTAL

Apparatus

The LC system consisted of a Waters Model 501 pump, a Rheodyne Model 7125 injector with a 20-μl loop and a Waters Model 486 spectrophotometer with an 8-μl flow cell. The chromatograms were obtained by using an HP-3394A integrator (Hewlett-Packard).

A Cyclobond I column (250 × 4.6 mm I.D.) was obtained from Advanced Separation Technologies. It has β-CD molecules chemically bonded to a spherical silica support through a five-atom, non-nitrogen bonded-containing spacer arm [1]. When not in use the column was stored in 100% methanol.

pH readings were obtained using a Metrohm Model 654 pH meter.

Fig. 1. Crystallographic structure of promethazine. In isopromethazine the methyl group at C-20 is attached to C-15.

Chromatographic conditions

The effect of pH on the retention of PR and IPR was investigated by changing the pH of the aqueous phase of mobile phase from 3.5 to 7.0 with triethylamine acetate buffer (2.0%, w/v). The effect of ionic strength on retention was examined by varying the concentration of tri-

ethylamine buffer (pH 4.5) from 0.1 to 2.5 (w/v).

The effect of the organic modifier on the resolution of two isomers was examined by preparing binary aqueous–organic mobile phases with the same polarity index (*ca.* 8.64). The polarity of each mobile phase was calculated according to Snyder based on the following equation:

$$P_m = \varphi_1 P_1 + \varphi_2 P_2$$

where P_m is the polarity of the mixture, P_1 and P_2 are the polarities of the pure solvents and φ_1 and φ_2 are the volume fractions of the two solvents. The organic modifiers used were methanol (MeOH), ethanol (EtOH), 2-propanol (i-PrOH), *tert.*-butanol (t-BuOH), acetonitrile (ACN) and tetrahydrofuran (THF).

All experiments were performed at room temperature (about 25°C). The compounds were detected at a wavelength of 254 nm. The mobile phases, consisting of triethylammonium acetate buffer and the appropriate amount of the organic modifier, were freshly prepared, filtered and degassed under vacuum using a Millipore system.

Solutions

Stock standard solutions of all drugs (1.00 mg/ml) were accurately prepared by dissolving an appropriate amount of the compound in HPLC-grade water and were kept in an amber-coloured bottle in a refrigerator and renewed every week. Working standard solutions (for promethazine and isopromethazine) were prepared every day in the mobile phase. Typically a volume of 20 μl of each solution was injected.

To obtain comparable results, experiments were performed under identical chromatographic conditions, *i.e.*, a flow-rate of 0.8 ml/min and an integrator attenuation of 3. The void volume of the column was determined by injecting 20 μl of pure methanol each time.

To evaluate the reproducibility of the retention times, each run was performed three times.

Materials

Methanol, acetonitrile, ethanol, 2-propanol, *tert.*-butanol and tetrahydrofuran were of HPLC-grade and purchased from Tech-Line. Triethylamine and glacial acetic acid were of analytical-reagent grade and purchased from Aldrich. Pure promethazine and isopromethazine, in form of hydrochloride salts, were kindly provided by Rhone-Poulenc Rorer and used as received.

RESULTS AND DISCUSSION

Effect of mobile phase composition

The effect of the mobile phase composition on the retention times and the resolution of the two positional isomers was investigated by changing the methanol-to-buffer ratio in the mobile phase from 10:90 to 60:40. It was observed that the dependence of log k' of each compound on the methanol content in mobile phase was mostly linear, suggesting a reversed-phase mechanism for the interaction between the solute and stationary phase [28].

After these preliminary studies, buffer–methanol (85:15) was chosen as the mobile phase composition providing the optimum chromatographic conditions for the studies described below. The flow-rate was 0.8 ml/min.

Effect of ionic strength

The effect of ionic strength on the separation of PR and IPR was investigated in the concentration range 0.1–2.5% (w/v) triethylamine acetate (TEAA) at pH 4.5, where both PR and IPR are expected to be mostly in their ionic forms (pK_a of PR = 9.1 [29]).

As shown in Table I, the retention times of PR and IPR decrease as the electrolyte concentration increases. This type of behaviour can be rationalized in terms of the TEAA molecules included in the β-CD cavity, thus competing with the solute molecules. This mechanism may be possible through the formation of a TEAA ion pair within the hydrophobic environment of the cavity. The binding constant for such a complex is likely to be enhanced by the hydrophobic interactions between the cavity and the hydrophobic shell provided by the ethyl moieties of the triethylamine cation, which surround and shield its central, positively charged nitrogen atom. Owing to the comparable sizes of the

TABLE I

EFFECT OF TEAA CONCENTRATION ON RETEN-
TION, RESOLUTION AND SELECTIVITY OF
PROMETHAZINE AND ISOPROMETHAZINE USING
A β-CYCLODEXTRIN-BONDED COLUMN

TEAA concentration ($\%$, w/v)	t_1 (min)	t_2 (min)	R	α	Log α
0.10	19.61	19.61	0.000	1.000	0.000
0.50	19.45	21.15	0.324	1.087	0.036
0.75	18.54	21.02	0.558	1.135	0.055
1.00	17.94	20.64	0.675	1.150	0.061
1.25	17.47	20.23	0.736	1.158	0.064
1.50	16.95	19.87	0.973	1.172	0.069
1.75	16.30	19.21	1.058	1.179	0.072
2.00	16.19	19.05	1.185	1.201	0.080
2.25	14.86	18.00	1.256	1.211	0.083
2.50	14.44	17.51	1.505	1.213	0.084

β-CD cavity and the triethylamine cation, these interactions are likely to be of considerable magnitude [28].

An alternative mechanism may be that the TEAA molecules interact with the hydrophilic secondary hydroxyls at the opening of the β-CD cavity, thus blocking the entry of the analytes [28].

This decrease in the retention time of the analytes as the concentration of TEAA increases was described by eqns. 1 and 2 for IPR and PR, respectively, which express the linear relationship between the retention time (t) of the solute and the TEAA concentration ($\%$ w/v).

$$t = 20.57 \ (\pm 0.22) - 2.47 \ (\pm 0.13) \ [\text{TEAA}]$$

$$n = 9, r = 0.990 \quad (1)$$

$$t = 22.41 \ (\pm 0.21) - 1.85 \ (\pm 0.13) \ [\text{TEAA}]$$

$$n = 9, r = 0.983 \quad (2)$$

The negative slope values are in agreement with the above theoretical concept. It is important to note that the slope in eqn. 1 is much greater than that in eqn. 2. Hence the influence of the variation of electrolyte concentration on the retention time of IPR (first-eluted component) is much greater than that on the retention of PR.

Resolution is also affected by ionic strength. It has been reported [30] that peak shapes become sharper with increasing buffer concentration and the resolution is slightly affected. However, the separation of PR and IPR is greatly affected by ionic strength. As is shown in Fig. 2, an increase in salt concentration in the range 0.1–2.5% (w/v) results in a variation of the resolution (R) between 0 and 1.5, i.e., complete resolution.

There is a linear relationship between R and TEAA concentration ($\%$, w/v) and eqn. 3 describes the fit of all the experimental data:

$$R = 0.038 \ (\pm 0.042) + 0.580 \ (\pm 0.027) \ [\text{TEAA}]$$

$$n = 10, r = 0.991 \quad (3)$$

The selectivity of the separation is affected very slightly by the ionic strength, as evidenced by the absence of any changes in the elution order of the solutes; in all instances IPR is eluted before PR. In the molecule of PR, one of the three methyl groups is in an α-position with respect to the nitrogen of the ethanamine group. In contrast, in the molecule of IPR this methyl group is in a β-position and gives rise to steric effects in the formation of the inclusion complex with β-CD. As the ionic strength of the mobile phase increases, the above selectivity is expressed more by the following possible mechanism.

Chromatographic separations using CD-bonded phases are mainly the result of variations in the stability of the inclusion complexes of the analytes with the CD [7], which reflects the respective variations in the stability constants of the complexes (K_f). The molecule of PR and also that of IPR have a hydrophobic three-ring system (phenothiazine structure) and a hydrophilic chain carrying an ionizable nitrogen group which at pH 4.5 is mostly positively charged. It has been assumed that the hydrophobic part is buried inside the hydrophobic CD cavity and the hydrophilic part can participate in hydrogen-bonding interactions with the hydroxyl groups located outside the cavity.

On the other hand, as the ionic strength increases, the polarity of the mobile phase also increases. Consequently, the hydrophobic part of the PR molecule is forced to be "hidden" in the
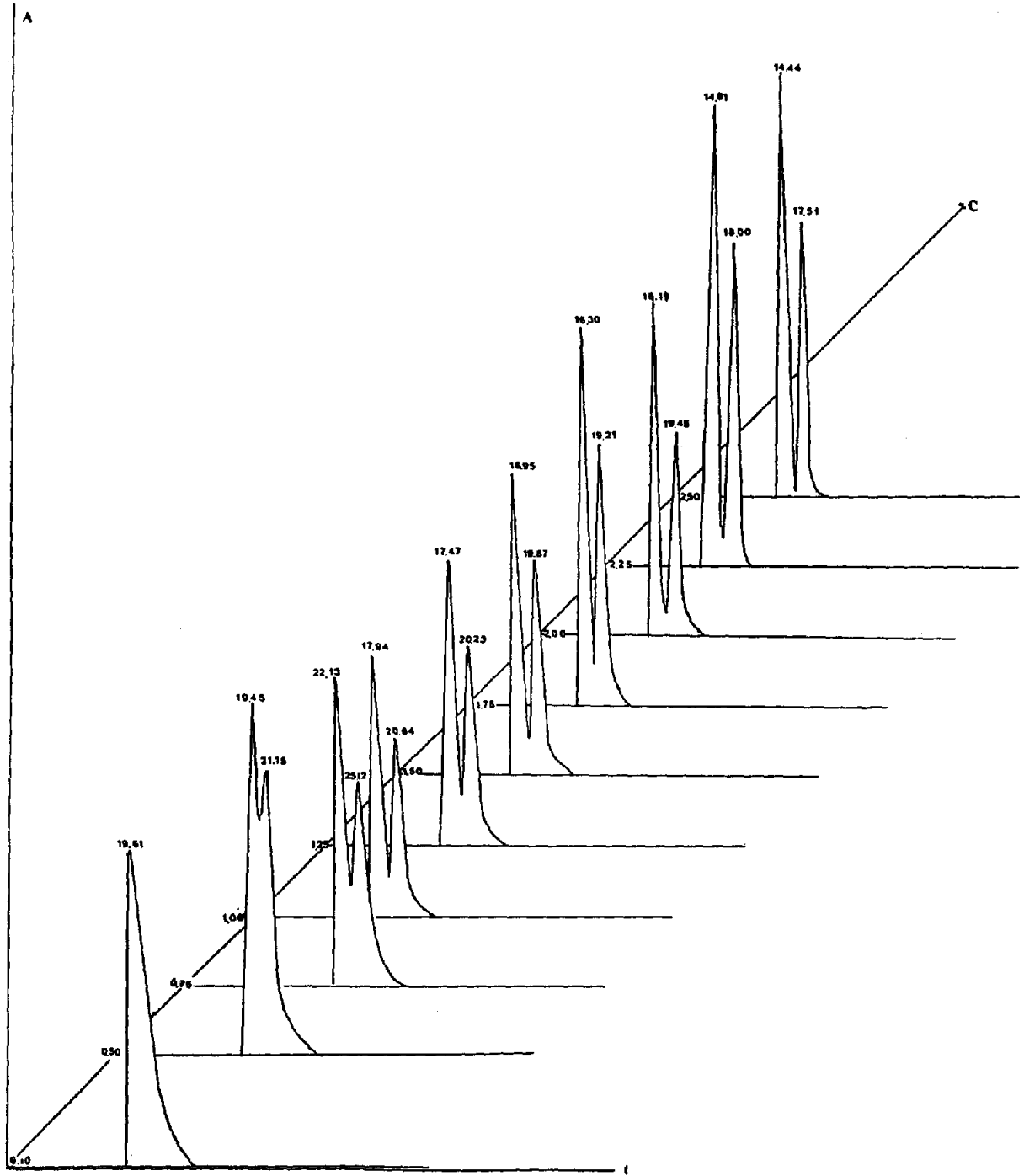
Fig. 2. Effect of ionic strength on the chromatographic separation of IPR and PR. C = Salt concentration in %C (w/v) and t = retention time in min.

CD cavity in order to avoid contact with the hydrophillic mobile phase environment. Hence the stability constant of the inclusion complex which is formed between PR and β-CD increases as the ionic strength of the mobile phase increases. In contrast, the inclusion complex formation between IPR and β-CD is characterized, as mentioned above, by a steric effect. Thus, as the TEAA concentration increases the above variation in the stability constants of the two complexes increases.

The chromatographic selectivity factor (α) is an important experimental probe in studies of the solute retention process. It reflects the difference between two solutes in the Gibbs free energy of transfer from the mobile phase to the stationary phase:

$$\alpha = k_2'/k_1' \tag{4}$$

$$\ln \alpha = -\Delta(\Delta G)/RT \text{ or } \log \alpha$$

$$= -\Delta(\Delta G)/2.303RT \tag{5}$$

where k_1' and k_2' are the capacity factors of the two solutes, ΔG is the Gibbs free energy, R is the gas constant and T is the absolute temperature [31].

As shown, the regression analysis plot represent a multiplicative model described by the equation

$$\alpha = a \, [\text{TEAA}]^b \quad n = 10, \, r = 0.995 \tag{6}$$

where $a = 0.1368(\pm 0.0020)$ (the intercept is equal to $\log a$) and $b = 0.0609(\pm 0.0021)$ represent the intercept and the slope, respectively. Eqn. 6 can be rearranged to eqn. 7, which express a linear relationship between $\log a$ and TEAA concentration (%, w/v):

$$\log \alpha = \log a + b(\log [\text{TEAA}])$$

$$n = 10, \, r = 0.995 \tag{7}$$

where $a = 0.05954(\pm 0.00085)$ and $b = 0.0611(\pm 0.0022)$. From eqns. 5 and 7, it is evident that the effect of the ionic strength on the selectivity factor (α) reflects an effect on the difference between the two solutes in the Gibbs free energy of transfer from the mobile phase to the stationary phase.

Effect of pH

The effect of pH on the retention of PR and IPR was investigated by changing the Ph of the aqueous component of the mobile phase from 4.00 to 7.00 (respecting the column limitations) using triethylamine buffer (2.5%, w/v). In order to obtain comparable results, experiments were performed under chromatographic conditions identical with those mentioned under Experimental.

Table II shows the effect of pH on retention, resolution and selectivity of PR and IPR using a β-CD-bonded stationary phase.

As discussed earlier, inclusion has been suggested as the predominant mode for their retention. The existence of a well defined hydrophobic part in the molecules of PR and IPR provides opportunities for hydrophobic interactions with the interior of the β-CD cavity. On the other hand, the charged hydrophilic nitrogen group provides opportunities for hydrogen bonding interactions with the OH groups on the circumference of β-CD molecule. These two kinds of interactions are slightly affected by pH, owing to the higher pK_a values of PR and IPR than the maximum pH value examined.

However, an increase in retention time with increasing pH is observed (Fig. 3). A plausible explanation may be that the charged hydrophilic nitrogen group in the molecules of PR and IPR and the acetate anion form an ion pair within the CD cavity.

TABLE II

EFFECT OF pH OF THE AQUEOUS COMPONENT OF THE MOBILE PHASE ON RETENTION, RESOLUTION AND SELECTIVITY OF PROMETHAZINE AND ISOPROMETHAZINE USING A β-CYCLODEXTRIN-BONDED COLUMN

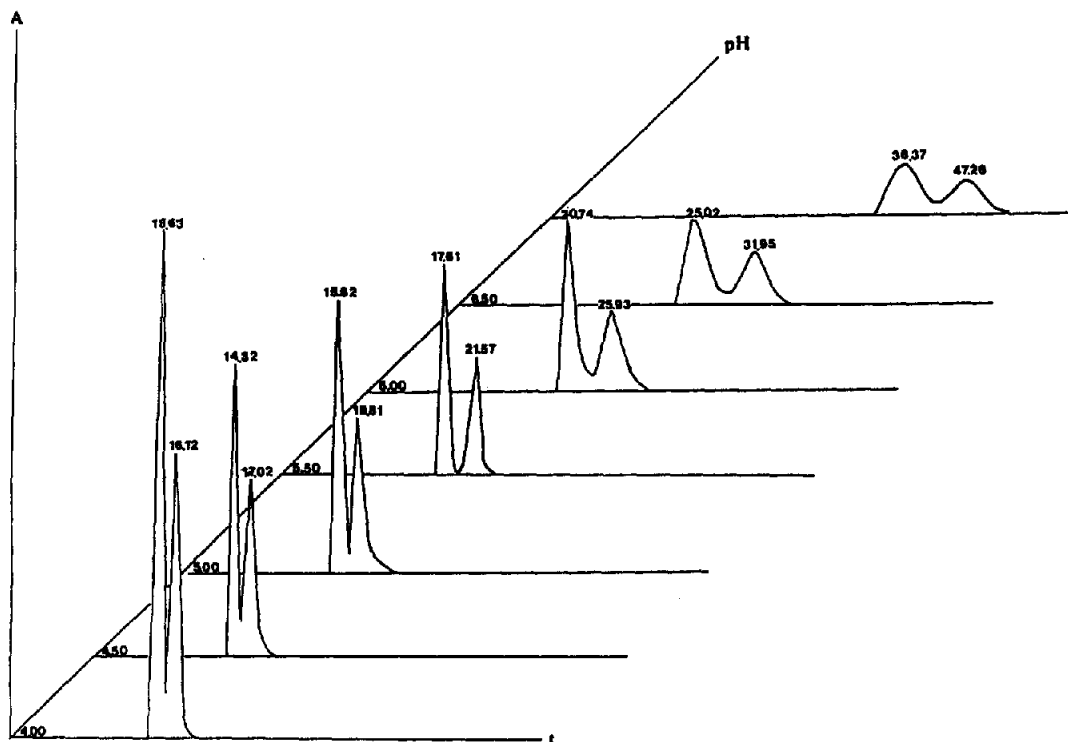| pH	t_1 (min)	t_2 (min)	R	α
4.00	13.63	16.12	1.072	1.182
4.50	14.32	17.02	1.160	1.188
5.00	15.62	18.81	1.245	1.204
5.50	17.61	21.57	1.320	1.225
6.00	20.74	25.93	1.260	1.250
6.50	25.02	31.95	1.153	1.277
7.00	36.30	47.26	1.089	1.288

Fig. 3. Effect of pH on the separation of IPR and PR.

The dependence of the retention time of IPR and PR on the pH of the aqueous component of the mobile phase is described by a reciprocal model (eqns. 8 and 9, respectively):

$$1/t = 0.1380(\pm 0.0062) - 0.0152(\pm 0.0011) \text{ pH}$$

$$n = 7, r = 0.987 \quad (8)$$

$$1/t = 0.1200(\pm 0.0047) - 0.01372(\pm 0.00084) \text{ pH}$$

$$n = 7, r = 0.991 \quad (9)$$

By increasing the pH the solutes become more retained and hence the column's inclusion selectivity also increases. The dependence of the selectivity factor (α) on pH is described by the equation

$$\alpha = 1.018(\pm 0.015) + 0.0388(\pm 0.0026) \text{ pH}$$

$$n = 7, r = 0.990 \quad (10)$$

It could be expected that the resolution R would also increase with increase in pH owing to the increase in column selectivity. However, in the pH range 4.0–5.5 the resolution increases whereas it decreases at pH >5.5. This dependence follows a linear model described by eqns. 11 and 12, respectively:

$$R = 0.412(\pm 0.020) + 0.1658(\pm 0.0042) \text{ (pH)}_{aq}$$

$$n = 4, r = 0.9994 \quad (11)$$

$$R = 2.206(\pm 0.080) - 0.160(\pm 0.013) \text{ (pH)}_{aq}$$

$$n = 4, r = 0.994 \quad (12)$$

This behaviour can be rationalized by considering that as the pH increases both retention and selectivity increase, whereas concurrently at pH >5.5 the peaks become broader in such a way that the resolution decreases.

Effect of solvent selectivity

"Selectivity" primarily refers to the ability of a solvent to exhibit specific solute interactions which another solvent of approximately similar strength or polarity do not undergo. Polarity describes the gross solvent strength. Selectivity

346

S. Piperaki et al. / J. Chromatogr. A 660 (1994) 339–350

describes the fine structure of strength, the profile of polarity sub-parameters [32].

Selectivity describes the degree to which a solvent is chromatographically stronger for a particular solute by virtue of its ability to enter into specific intermolecular interactions to a greater extent than for other solutes.

With CD-bonded phases, retention is predominantly due to inclusion complex formation. Because molecules of different chemistry, size, shape and spatial geometry form inclusion complexes of different strengths with CDs, separation is readily achieved. The organic mobile phase modifier tends to compete with all solutes. There are a definite number of adsorption sites on the stationary phase on which the solute and the mobile phase components can bind. It is assumed that the organic modifier has a greater affinity for the adsorption site than water but lower than solutes [3].

It was of interest to estimate the solvent selectivity using different organic modifiers in the mobile phase which had the same polarity index (ca. 8.64). Thus, all the differences observed in separation and resolution reflect differences in the inclusion process.

As shown in Fig. 4, when methanol, ethanol or acetonitrile was used, a very good resolution between IPR and PR was achieved. The use of 2-propanol provided partial resolution of the two isomers but using THF or tert.-butanol no resolution was obtained.

It may be useful to investigate the relationship between resolution and the logarithm of the partition coefficient (octanol–water) of each solvent (log P) (Table III). There is a linear relationship between R and log P, described by the equation

$$R = 0.82(\pm 0.15) - 2.2(\pm 0.33) \log P$$

$$n = 6, r = 0.956 \quad (13)$$

This good relationship ($r > 0.94$) suggests that the hydrophobicity of the organic modifier is one of the dominant factors with respect to solvent selectivity in inclusion process phenomena. The competition between solutes and the organic modifier for the same retention site (β-CD cavity) controls the retention, which in this

instance depends on the relative hydrophobicity of the solvent and solute. Obviously, as the hydrophobicity of the organic modifier increases its affinity for the adsorption site (β-CD cavity) also increases.

For log P values greater than 0.35 no resolution was observed for the two isomers. It is important to note that other workers have suggested the use of log P as a solvent "strength" parameter in RPLC [33]. According to the present observations, this concept can be extended to inclusion complex chromatography.

It should be noted that the relationship between R and log P shows excellent linearity ($r > 0.999$) when the point for acetonitrile is omitted:

$$R = 0.9060(\pm 0.0029) - 2.428(\pm 0.061) \log P$$

$$n = 5, r = 0.9994 \quad (14)$$

The interaction potential of acetonitrile is different to those of the other solvents used [34]. Using acetonitrile as the organic modifier, selective dipole–dipole interactions between solutes and the solvent may occur owing to the high dipole moment of acetonitrile. These dipole–dipole interactions usually occur between individual polar functional groups of solvent and solute molecules. Both PR, and IPR possess a polar amine group which can participate in such interactions. On the other hand, it has been reported that acetonitrile will bind more strongly to CD than other solvents [3]. For these two reasons, solutes are less retained and consequently would tend to elute more quickly using acetonitrile as organic modifier.

The selectivity factor (α) in the chromatographic separation of PR and IPR is also affected by solvent selectivity. A reciprocal model relationship exists between α and log P, described by the equation

$$1/\alpha = 0.9014(\pm 0.0080) + 0.222(\pm 0.018) \log P$$

$$n = 6, r = 0.990 \quad (15)$$

Isocratic separation of PR and IPR

After studying the LC retention behaviour of PR and IPR on a β-CD column with respect to
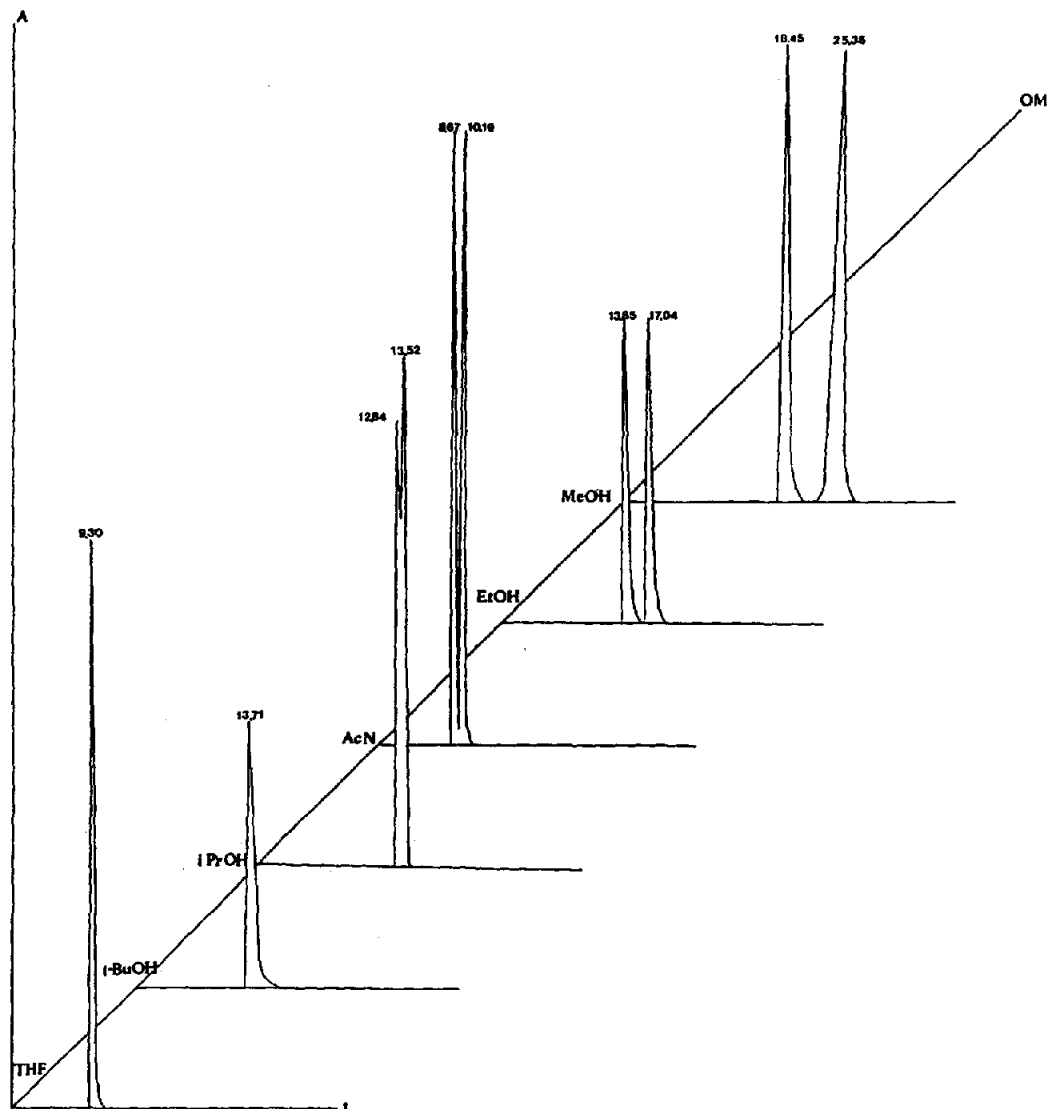
Fig. 4. Effect of solvent selectivity on the separation of IPR and PR.

mobile phase composition, pH, ionic strength and solvent selectivity, a set of chromatographic conditions were chosen for the simultaneous separation of the two positional isomers. These conditions are as follows: methanol–buffer (83:17); buffer, 2.5% (w/v) TEAA; pH, 5.50; and flow-rate, 0.8 ml/min. These conditions permit the determination of 1% of IPR in PR solution using a UV–Vis detector at 254 nm (Fig. 5).

Molecular modelling for the PR- and IPR–β-CD complexes

In order to understand the basis of the separation of these positional isomers using a β-CD column, the interactions of the crystal structures of PR and IPR [35,36] with β-CD were examined using computer graphic modelling. Computer modelling using the program CHEM-X revealed interesting docking arrangements. The computer program positioned the two posi-

348

S. Piperaki et al. / J. Chromatogr. A 660 (1994) 339–350

TABLE III

EFFECT OF SOLVENT TYPE AND HYDROPHOBICITY ON RETENTION, RESOLUTION AND SELECTIVITY OF PROMETHAZINE AND ISOPROMETHAZINE USING A β-CYCLODEXTRIN-BONDED COLUMN

Solvent	t_1 (min)	t_2 (min)	R	α	Log P
MeOH	18.45	25.35	2.760	1.374	−0.77
EtOH	13.85	17.04	1.707	1.230	−0.32
ACN	8.67	10.19	0.933	1.181	−0.34
i-PrOH	12.84	13.52	0.227	1.053	0.30
t-BuOH	13.71	13.71	0.000	1.000	0.35
THF	9.30	9.30	0.000	1.000	0.46

Fig. 6. Stick model of the inclusion complex of PR with β-CD.

tional isomers minimizing steric and electronic hindrances.

A model of the binding of the two isomers to β-CD is proposed. In both instances it is assumed that the hydrophobic part (three-ring system) of the two molecules is buried inside the cavity while the other part is pointing towards the outside. It is apparent that PR is able to form a better inclusion complex than IPR. The three-ring system of PR was found to penetrate deeper in the β-CD cavity compared with IPR, as can be seen in Figs. 6 and 7. Further, Fig. 8 shows

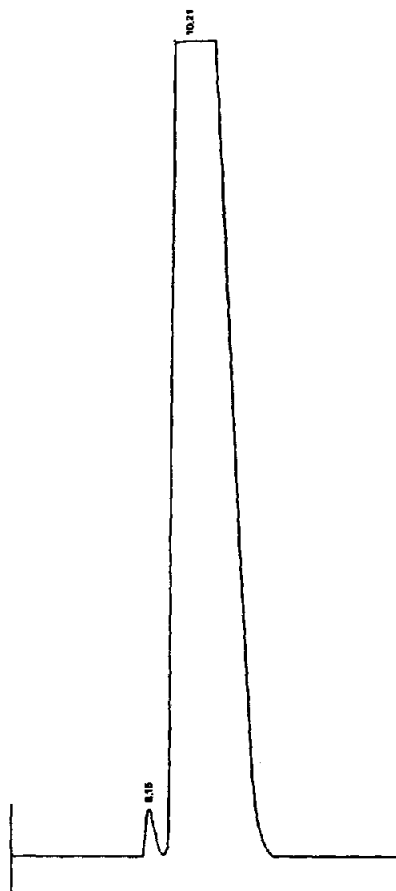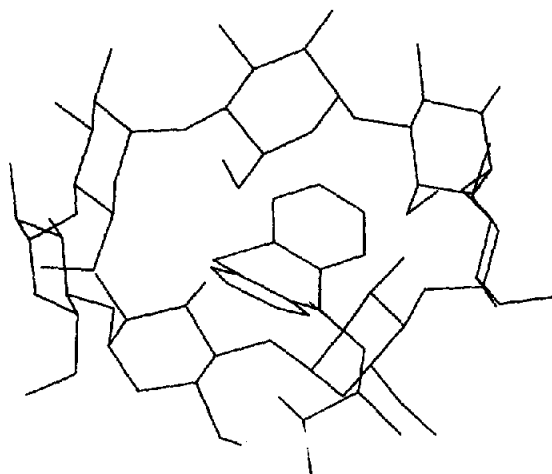
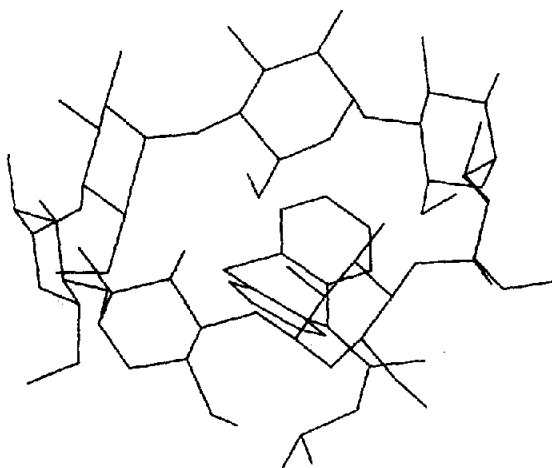Fig. 5. Chromatographic separation of IPR and PR (1% IPR in PR solution).

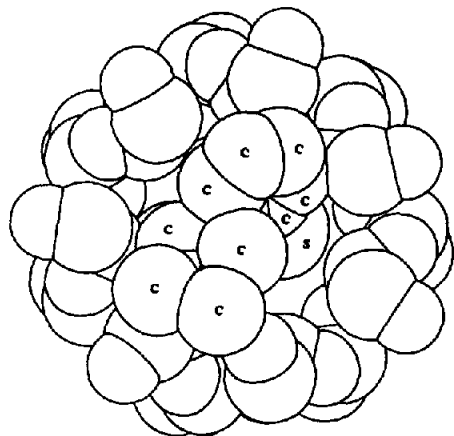Fig. 7. Stick model of the inclusion complex of IPR with β-CD.

Fig. 8. Space-filling model of the inclusion complex of PR with β-CD.

the space-filling model of the PR inclusion complex with the β-CD cavity.

This difference was qualified by measuring the distance from the mouth of the β-CD cavity. For the molecule of PR (crystallographic numbering system, Fig. 1) the rings are modelled to enter as deeply as possible inside the β-CD cavity without having any bad contacts, in such a way that a possible hydrogen bond is formed between N-17 and one OH group located at the ring of β-CD cavity (N–O distance 2.71 Å). Supposing that the orientation of the rings remains the same for IPR when bonded to β-CD, the three-ring system cannot enter as deeply as in the first case because the methyl group at C-20, now in a β-position, closer to the rings, has bad contacts with the hydrophobic cavity. In order to remove these contacts, IPR must be dragged towards the outside. This procedure also weakens the hydrogen bond mentioned above (N–O distance 2.93 Å) and minimizes the hydrophobic interaction between the β-CD cavity and the three-ring system of the molecule of IPR.

The difference in penetration allows PR to be more tightly complexed in the hydrophobic cavity of β-CD. This result corresponds with the chromatographic data.

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