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Liquid chromatographic resolution of the isomers of tipredane and phenylthioproline using urea-solubilized β-cyclodextrin in the mobile phase

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Abstract: Two LC assays were developed using urea-solubilized β-cyclodextrin (β-CD) as a mobile phase additive in combination with reversed-phase columns. A methylsilane column gave optimal resolution of the steroid tipredane from its epimer. An investigation of the effects of β-CD concentration, column temperature, column type, and addition of ethanol on the chromatographic separation is detailed. The enantiomer and diastereoisomers of L-*cis*-phenylthioproline were best resolved by urea-solubilized β-cyclodextrin and a trimethylsilane column. The elution order of L-*cis*-phenylthioproline relative to its stereoisomers was reversed after adding ethanol to the β-CD containing mobile phase or by changing from a β-CD to an acetylated β-CD column. The resolution factors for these separations obtained using the β-CD mobile phase were larger than those obtained using β-CD columns. Mobile phases containing up to 0.15 M β-CD and 8 M urea were investigated. The separation of these isomers are dramatically affected by column polarity.

Keywords: *Chiral HPLC; β-cyclodextrin mobile phase additives; HPLC isomers.*

Introduction

β-cyclodextrin (β-CD) is a cyclic oligosaccharide composed of *d*-(+)-glucopyranose units bonded through α-(1,4)-linkages [1]. The unique property of cyclodextrins to form inclusion complexes with analytes has been utilized in a variety of analytical applications [1]. Among these are their use as chiral or stereoselective phases in HPLC [2–7], TLC [8, 9], GC [10], and capillary zone electrophoresis [11, 12]. They also find uses as matrices which increase spectroscopic response [13, 14], induce room temperature phosphorescence [14], and enhance photochemical reactions [15]. Recently, it has been shown that the aqueous solubility of β-CD is markedly increased by urea [8, 9]. The maximum solubility of β-CD is 0.0168 M in water at 25°C [1] while greater than 0.2 M can be solubilized upon addition of 8 M urea [8, 9]. The solubilization of higher concentrations of β-CD with urea is important to HPLC method development, since higher concentrations in HPLC mobile phases increase chromatographic resolution and are often required to elute the analyte from the column.

This paper describes the effect of column type, β-CD concentration, ethanol concentration and temperature on the resolution of tipredane and its 17-thio epimer (structures also are depicted in Figs 1 and 2) and of some of these parameters on the resolution of L-*cis*-phenylthioproline, a synthetic precursor of an antihypertensive agent, and its enantiomer and diastereoisomers. These parameters were investigated in order to optimize chromatographic resolution, selectivity, assay time, sensitivity, and ruggedness for purposes of practical assay development of chiral drugs The results indicate that high concentrations of β-CD in the mobile phase can provide greater resolution than β-CD columns for these compounds, and that the reversal in elution order obtained (relative to the β-CD column) can be advantageous. In addition, ruggedness is enhanced because a variety of conventional columns can be used to attain the separations.

Experimental

Chemicals

Tipredane (9-fluoro-11β-hydroxyandrosta-1,

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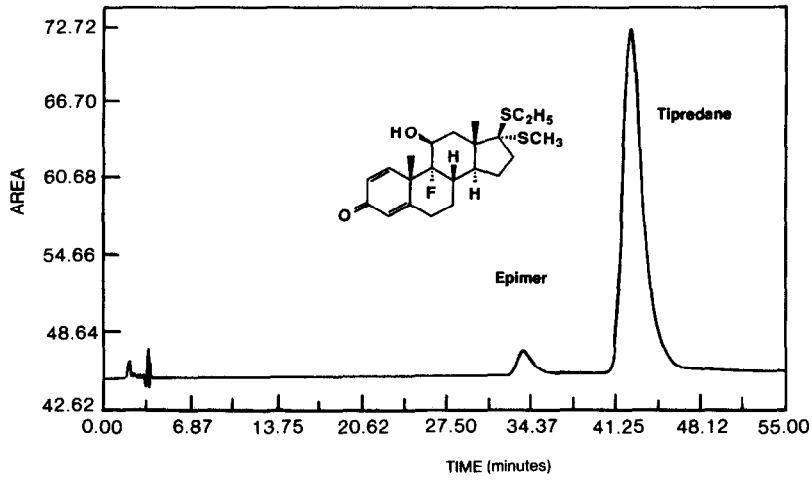

Figure 1
Chromatogram of tipredane and added epimer. Conditions: Chromegabond C-2 column, 25% ethanol, 75% aqueous 0.04 M β-CD and 1 M urea flowing at 1 ml min^{-1}, 40°C and absorption at 239 nm.

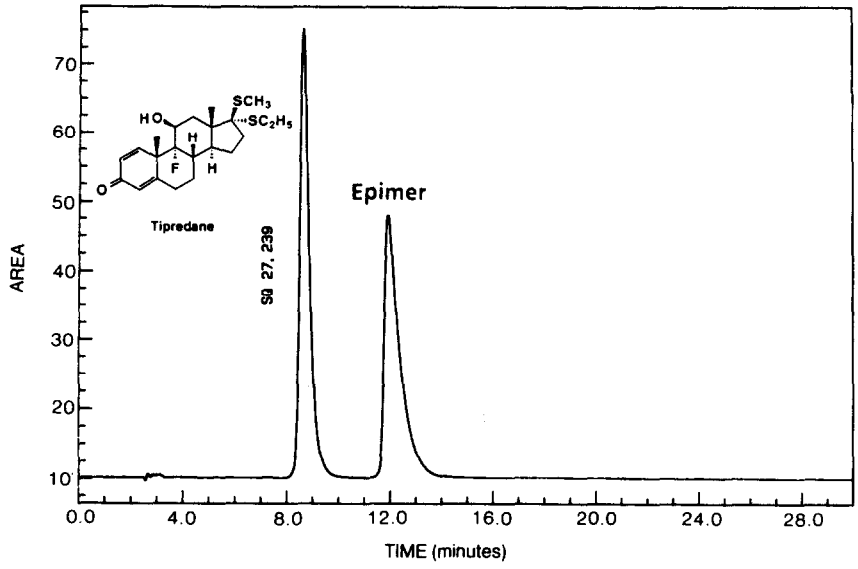

Figure 2
Chromatogram of tipredane and added epimer. Conditions: *p*-toluoyl-β-cyclodextrin column, 30% water–70% acetonitrile flowing at 1 ml min^{-1}, ambient temperature and absorption at 239 nm.

4-diene-3,17-dione (17*R*)-17-(ethylmethylmercaptole)), its epimer at the 17-thio-position (epimer), L-*cis*-phenylthioproline (*S*,*S*), L-*trans*-phenylthioproline (*S*,*R*), D-*cis*-phenylthioproline (*R*,*R*), and D-*trans*-phenylthioproline (*R*,*S*) were obtained from the Bristol–Myers Squibb Pharmaceutical Research Institute. Absolute configurations of the first three compounds were directly verified by single-crystal x-ray crystallography (M. Peters, personal communication, 1992). β-cyclodextrin was purchased from Astec (Whippany, NJ, USA). The HPLC mobile phases were prepared with ethanol (100%, Quantum Chemical Corp., Newark, NJ, USA), water that was doubly-distilled and stored in glass, urea (Fisher Scientific, Fair Lawn, NJ, USA), and *o*-phosphoric acid (Fisher Scientific).

Chromatographic methods

The aqueous portion of the mobile phases were prepared by initially adding urea (to the desired concentration) to water. β-cyclodextrin was then added while heating (50°C) and simultaneously stirring. The mobile phase was then vacuum-filtered through a 0.45 μm Nylon

filter (Micron Separations, Inc., Westboro, MA, USA). Organic modifiers, if desired, were than added. The resulting solutions were water-white. (Impure urea and/or β-CD can lead to hazy mobile phases.) Chromegabond C-1 and C-2 columns were obtained from ES Industries (Marlton, NJ, USA). Deltabond C-1, C-8 and cyano came from Keystone Scientific (Bellefonte, PA, USA). Astec (Whippany) supplied β-cyclodextrin, acetylated β-cyclodextrin and *p*-toluoyl-β-cyclodextrin columns, and Merck (Darmstadt, Germany) was the source of 5-μm C-18 columns. The chromatographic system consisted of a block-type column heater (Jones Chromatography, Boulder, CO, USA), a Perkin–Elmer ISS-100 autoinjector (Elmwood Park, NJ, USA) equipped with a 20-μl sample loop, a Beckman 110B constant-volume pump (Somerset, NJ, USA), and either a Model 783A or 785 UV absorption detector (Applied Biosystems, Foster City, CA, USA) set at 239 nm for tipredane and its epimer, and 232 nm for the phenylthioprolines. A saturator column packed with silica was placed between the pump and the injector; a necessity due to the relatively high pH of 7.5 for the urea-containing mobile phases. A rinse consisting of a burette containing distilled water was set to drip over the pump's exposed piston (about 1 drop min^{-1}) in order to remove deposited β-cyclodextrin and urea. Peak responses were determined with the aid of either a Hewlett–Packard Model 3357 (Avondale, PA, USA) or a VG/FISONS (Danvers, MA, USA) laboratory computer.

Results and Discussion

Selected assay conditions for tipredane and its epimer

After completing the investigation of the chromatographic parameters, which are described subsequently, the optimized assay conditions were chosen for the analysis of epimer in tipredane (see chromatogram, Fig. 1). The resolution factor (R_s) between tipredane and epimer was 3.3 and the possible impurity (the epimer), eluted first. The lengthy retention times were necessary to resolve the epimers from system peaks. These lengthy retention times led to broader and lower peaks, as expected due to increased diffusion, but the singlet nature of these peaks indicated one mode of separation. β-CD in the mobile phase was an improvement over the original β-CD column method [16] where the R_s was 1.5, and the epimer eluted after tipredane. Linearity of response vs concentration for tipredane (0.05–0.24 mg ml^{-1}) and epimer (0.1–5.0% w/w relative to tipredane) were excellent yielding coefficients of correlation of 0.9999 and 0.9998, respectively. The limit of detection of epimer was 0.05% (w/w, relative to tipredane at a concentration of 0.1 mg ml^{-1}). Typically, batches of tipredane contained 0.05–0.1% epimer. Reproducibility of area response and retention time of tipredane were excellent (RSDs of 1.7 and 0.3%, respectively, for five injections). A new *p*-toluoyl-β-cyclodextrin column gave the separation of tipredane containing added epimer shown in Fig. 2. The R_s factor was 3.1. Linearity of response vs concentration for epimer (0.06–0.18 mg ml^{-1}) in the presence of tipredane gave a coefficient of correlation of 0.9999. Reproducibility of area responses of tipredane and epimer (six injections of a synthetic mixture) were <0.8%. However, the epimer elutes after tipredane and all HPLC columns were more or less unstable with time. For these reasons, β-CD in the mobile phase was investigated.

Effect of column type

Table 1 contains a list of the separation factors obtained using a variety of stationary phases and a constant β-CD mobile phase composition. It is evident that the stationary phase contributed significantly to the resolution and elution times of epimer and tipredane. As seen in Table 1, the least polar columns gave the largest separation factors. For example, a Deltabond C-1 column showed a larger separation factor than a Chromegabond C-1 column. The Deltabond column was less polar because it was cross-linked. The C-18 column (silica-based, Merck) gave the longest retention times, whereas, the C-1 column (silica-based, Chromegabond) gave the shortest retention times and the smallest separation factor. Similar trends were noted for the separation of L-*cis*-phenylthioproline from its enantiomer (Table 2). Thus, choice of column was essential to the development of LC methods using CD mobile phases. Shorter columns were preferred due to lower back-pressure. Columns used with mobile phases containing urea lasted at least 3 months.

Because the column polarity affected the separation factor or resolution, it was likely

Table 1
Effect of column type on the separation factor (α) of tipredane and epimer*

Column type	α†	Column brand
C-1	1.1	Chromegabond
	6.9	Deltabond
C-2	4.0	Chromegabond
C-8	8.7	Deltabond
Cyano	4.1	Deltabond
C-18	Retention excessive (about 45 min for the epimer)	Merck

*For all studies, the β-CD concentration was 0.1 M, the urea concentration was 4 M, and the column temperature was 45°C. All columns were 25 cm.

†$\alpha = k'_2/k'_1$, where k'_2 and k'_1 are the capacity factors of tipredane and epimer, respectively.

Table 2
Effect of column type on the separation factor (α) for phenylthioproline and its enantiomer*

Column type	α†	Column brand
Trimethylsilane	2.6	Chromegabond
C-1	2.5	Chromegabond
C-8	33.1	Keystone

*For all studies, the α-CD concentration was 0.1 M, the urea concentration was 4 M, and the column temperature was 45°C. All columns were 25 cm × 4.6 mm.

†$\alpha = k'_2/k'_1$, where k'_2 and k'_1 are the capacity factors of tipredane and epimer, respectively.

Table 3
Effect of β-CD concentration on the separation factor and retention times of tipredane and epimer*

		Retention time (min)	
[β-CD]†	α‡	Epimer	Tipredane
0.15	14.1	3.3	17.8
0.10	12.0	5.0	36.9
0.05	9.6	12.3	99.6

*Studies done using a Deltabond C-8 column, 4 M urea, a column temperature of 25°C, and a flow rate of 1.5 ml min^{-1}.

†Concentration, mol l^{-1}.

‡$\alpha = k'_2/k'_1$, where k'_2 and k'_1 are the capacity factors of tipredane and epimer, respectively.

that partitioning of the CD–analyte complex itself with the stationary phase was a component of the separation mechanism. If this mechanism was not contributory, resolution would not be affected by column polarity and would solely be a function of the stability constant of the complex.

Effect of β-cyclodextrin concentration

As shown in Table 3, increasing the β-CD concentration reduced the retention times of tipredane and epimer, and increases resolution. Separation factors were significantly increased at the higher β-CD concentrations. The lowest concentration of CD required to obtain a suitable separation was chosen because lower concentrations produced less detector noise, thus improving sensitivity, and the mobile phase is less viscous, which reduced back-pressure and permitted the option of using higher flow rates.

Using data from a concentration optimization study, the following equation, derived by Uekama [17] and applied by Sybilska and Zuckowski [3, 18], was utilized to evaluate the mechanism:

$$k' = \frac{k'_G - k'}{K_G[CD]} + k'_{G\cdot CD}, \qquad (1)$$

where: k' is the capacity factor of the analyte (epimer or tipredane) measured at various concentrations of β-CD, k'_G is the capacity factor of the uncomplexed analyte, measured when the CD concentration is zero, K_G is the stability constant of the CD–analyte complex, [CD] is the concentration of cyclodextrin and $k'_{G\cdot CD}$ is the capacity factor of the cyclodextrin inclusion complex.

The equation is based on the assumptions that inclusion complexation is 1:1 and that the CD does not adsorb to the stationary phase [3]. Also, the effects of the ethanol additive are not considered. If these assumptions are correct, a plot of $(k'_G - k')/(CD)$ vs k' will yield a straight line. The intercept will then be the capacity factor for the complex and the slope will be equal to $1/K_G$. Data were taken from a concentration study performed using 25% ethanol, 4 M urea, 0.01–0.1 M β-cyclodextrin and a Deltabond, methyl column maintained at

40°C. Plots derived from tipredane and epimer retention data were linear (Figs 3 and 4), respectively, with coefficients of correlation of 0.995 for both sets of data. K_G values were obtained from the slopes (epimer: 42.3, tipredane: 30.6). Thus, the epimer eluted first due to a larger analyte–CD stability constant. The existence of a larger stability constant was supported by the observed greater solubility of the epimer in the mobile phase relative to tipredane. The intercepts were negative (epimer: −13.4, tipredane: −15.7). This indicates that the assumptions of the equation may not be entirely correct. The results imply that one or more of the following exist; either the presence of a 1:2 steroid–CD complex, that the stationary phase is modified by the cyclodextrin, or that ethanol interactions require inclusion in the separation mechanism. The different intercept values infer different $k'_{G\cdot CD}$ values and, thus, possibly, a contribution to the separation mechanism of the CD–analyte complex interacting with the stationary phase.

Effect of column temperature

Tables 4 and 5 show decreases in separation factors with increases in column temperature for tipredane and phenylthioproline, and their respective isomers. For tipredane and its epimer, elution times were increased by raising the column temperature. The opposite was observed for the isomers of phenylthioproline

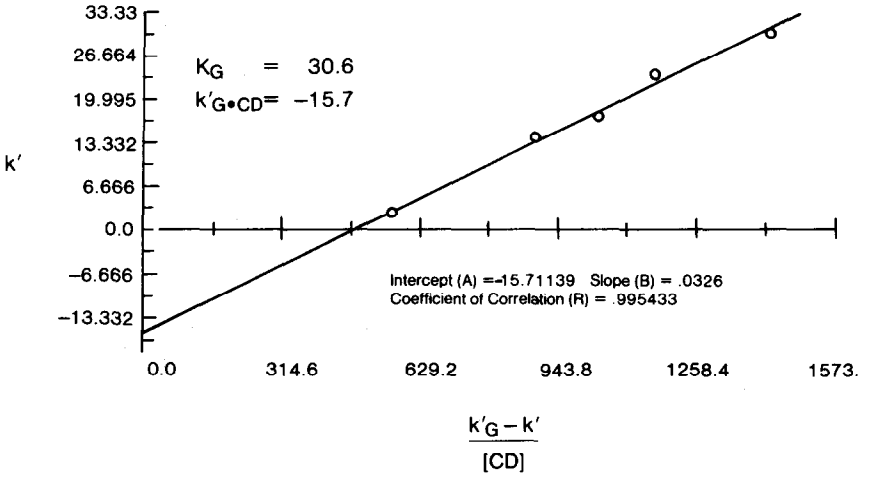

Figure 3
Plot of k' vs $(k'_G - k')$/[CD] for tipredane.

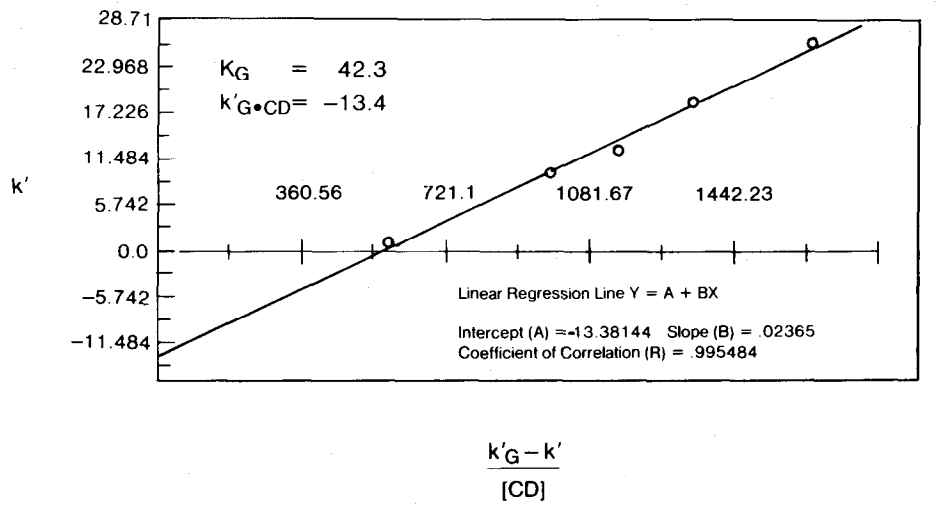

Figure 4
Plot of k' vs $(k'_G - k')$/[CD] for epimer.

Table 4
Effect of column temperature on the separation factor for tipredane and epimer*

		Retention time (min)	
T(°C)	α†	Epimer	Tipredane
25	12.0	5.0	35.9
35	10.5	5.5	37.3
45	8.7	6.6	40.0

*Studies done using a Deltabond C-8 column, 0.1 M β-CD, 4 M urea, and a flow rate of 1.5 ml min^{-1}.
†$\alpha = k'_2/k'_1$, where k'_2 and k'_1 are the capacity factors of tipredane and epimer, respectively.

identical methyl column, and β-CD and urea concentrations. Ethanol, which is known to bind with cyclodextrin [1, 4, 5], can be used to optimize the assay run time by reducing the large separation factor. Other effects of other modifiers were not investigated.

For phenylthioproline and its enantiomer and diastereoisomers, the elution order was reversed upon addition of ethanol [Fig. 5(A) and (B)]. Elution order was also reversed when an acetylated β-cyclodextrin column was used [Fig. 6(A) and (B)]. Generally, the mechanism

Table 5
Effect of column temperature on the separation factor of phenylthioproline and its enantiomer*

		Retention time (min)	
T(°C)	α†	L-*cis*-phenylthioproline	D-*cis*-phenylthioproline
25	2.9	5.28	11.61
35	2.8	4.76	9.59
45	2.6	4.53	8.63

*Studies done using a Chromegabond trimethylsilane column, 0.1 M β-CD, 4 M urea, and a flow rate of 1.0 ml min^{-1}.
†$\alpha = k'_2/k'_1$, where k'_2 and k'_1 are the capacity factors of phenylthioproline and its enantiomer, respectively.

where the elution times decreased. One possible explanation was the contribution to the separation mechanism of $k'_{G\text{-}CD}$. As the temperature increased, K_G decreased and there was more uncomplexed analyte in the mobile phase. Uncomplexed tipredane and epimer are more hydrophobic than their CD complexes, and are retained longer on the hydrophobic stationary phase. Similarly, uncomplexed phenylthioproline is more polar than its CD complex and elutes faster. (The minor differences between Tables 3 and 4 showing slightly different retentions under identical conditions are due to the data being obtained on different days.) Column temperatures of about 40°C were preferred, since the viscosity of the mobile phase is less than at room temperature and because the solubility of β-cyclodextrin was further increased.

Effect of additives

It has been shown previously that ethanol reduces the magnitude of the stability constants of the analyte–CD [2], thus decreasing resolution, and resulting in more closely eluting peaks. Table 6 shows the effect of ethanol content on the separation factor and retention times of tipredane and epimer, using the

of chiral recognition by β-CD involves interaction of substituents near the chiral centre with hydroxyl groups at the mouth of the CD cavity [4, 5], in addition to inclusion complexation. The binding of ethanol to β-CD and derivatization by acetylation may alter the chiral recognition mechanism. Elution order reversal had been previously noted when CD derivatives are used in GC [10].

Several separations performed with mixtures of tipredane and its epimer, 0.1 M β-cyclodextrin and either 8 or 4 M urea showed no

Table 6
Effect of ethanol on the separation factor of tripredane and epimer*

		Retention time (min)	
% Ethanol	α†	Epimer	Tipredane
0	7.4	32.4	238.5
5	4.2	29.3	122.7
15	2.0	32.7	64.7
25	1.3	23.9	30.9
35	1.0	—	—

*The concentration of β-CD and urea were maintained constant at 0.04 and 1 M, respectively. A Deltabond methyl column at 40°C and 1.0 ml min^{-1} was used.
†$\alpha = k'_2/k'_1$, where k'_2 and k'_1 are the capacity factors of tipredane and epimer, respectively.

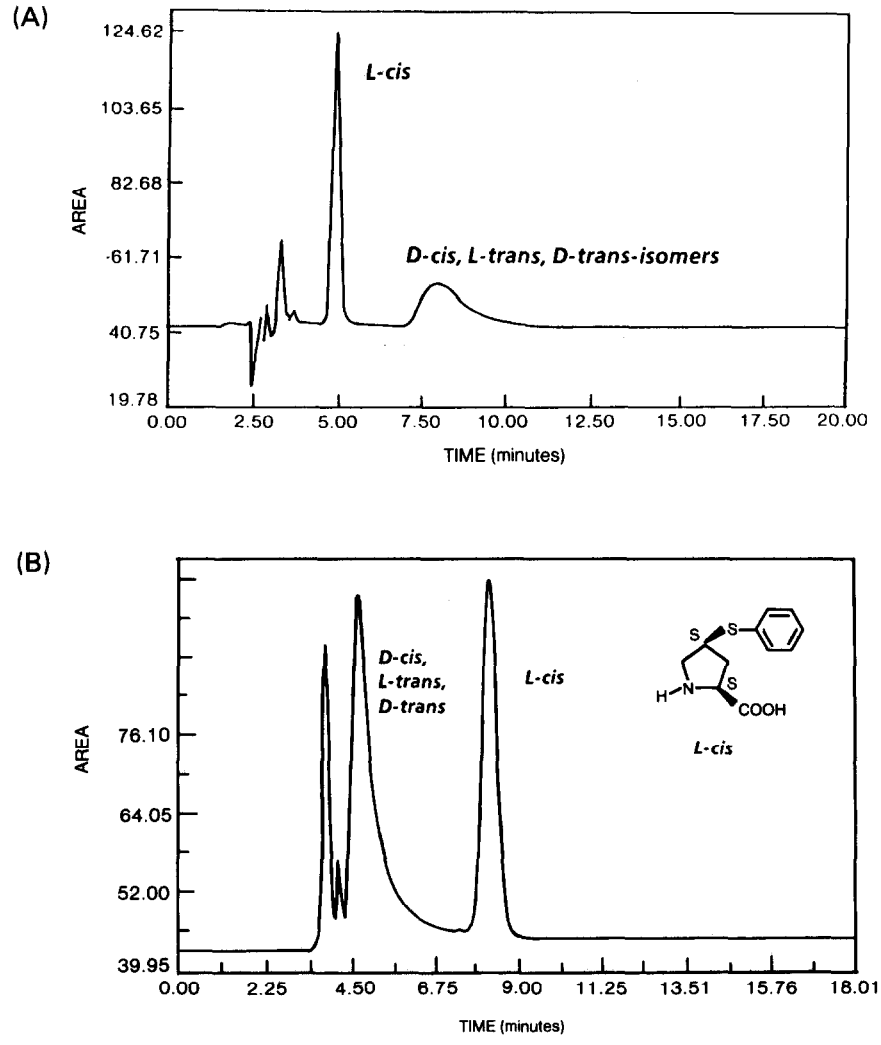

Figure 5
(A) Chromatogram of a synthetic mixture of L-*cis*-phenylthioproline and its isomers. Conditions: trimethylsilane column, 0.1 M β-CD in 8 M aqueous urea (pH 6.7), 45°C, flowing at 0.7 ml min^{-1} and absorption at 232 nm. (B) Reversal of elution order occurs upon addition of ethanol. Conditions: trimethylsilane column, 20% ethanol, 80% aqueous 0.04 M β-CD and 4 M urea (apparent pH 6.5), 45°C, flowing at 1.0 ml min^{-1} and absorption at 210 nm.

apparent differences in retention times or separation factors. This indicated that at these concentrations, urea had little or no effect on the chromatographic behaviour of tipredane and epimer. Urea is not believed to complex with β-cyclodextrin [8, 9]; however, further investigation at low urea concentrations should be performed. The failure to obtain useful resolution of terbutaline from its unwanted enantiomer [19] using 2 M urea to solubilize β-cyclodextrin may be due to either the choice of a phenylpropyl column, too low a concentration of β-cyclodextrin in the mobile phase, the lack of organic modifier or the intrinsic properties of the compounds to be resolved.

Conclusions

LC assays using urea-solubilized β-cyclodextrin in the mobile phase are feasible, rugged, provide enhanced selectivity and, in some instances, produce improved resolution over the β-CD column methods. Urea-solubilized CD also provides a means of investigating analyte–CD interactions at CD concentrations greater than are available without a solubilizing agent. Of special interest is the reversal in the elution order of L-*cis*-phenylthioproline and its isomers by the addition of ethanol to the urea-solubilized β-CD mobile phase or by the use of an acetylated β-CD column. In

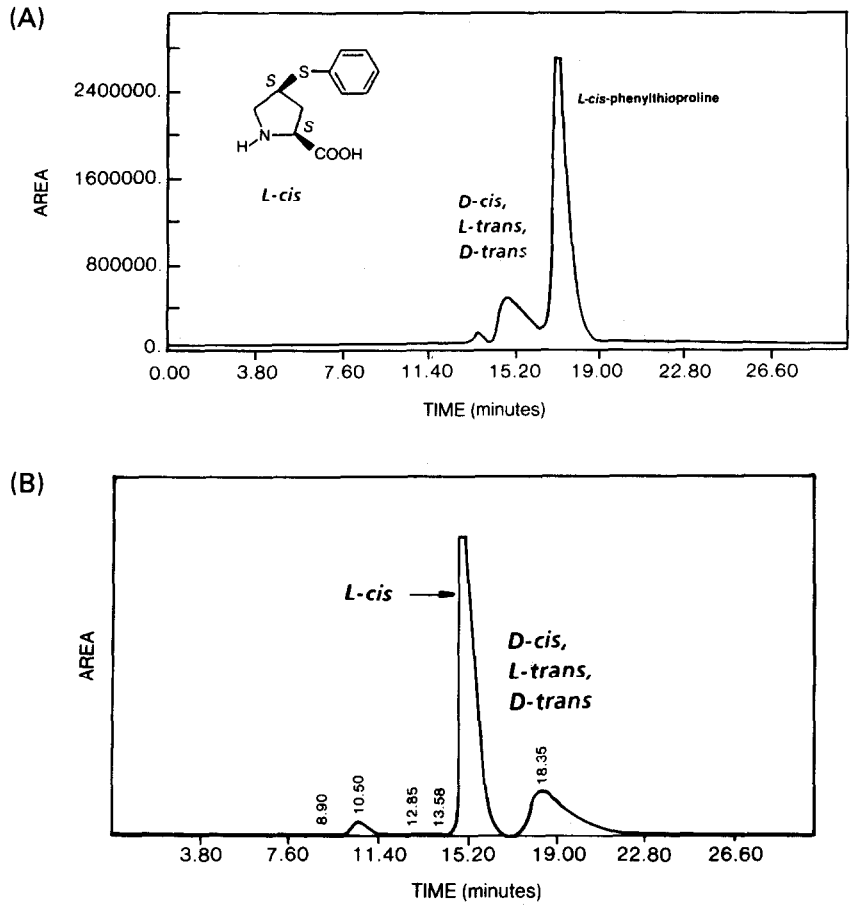

Figure 6
(A) Chromatogram of a synthetic mixture of L-*cis*-phenylthioproline and its isomers using a β-CD column. Conditions: 40% methanol–60% water (apparent pH = 6.7), flowing at 0.3 ml min^{-1}, ambient temperature and absorption at 210 nm. (B) A reversal of elution order occurs by using an acetylated, β-CD column. Conditions are identical to those described in Fig. 5(A).

addition, resolution can be greatly affected by column polarity. This is likely due to the interaction of the CD–analyte complex with the stationary phase.

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