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Stereoselective analysis of benazepril and its stereoisomers by reversed-phase high-performance liquid chromatography on a chiral AGP column

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Abstract

A direct HPLC method was developed for the determination of benazepril, its stereoisomers and its active metabolite benazeprilat. The separation was achieved, without any derivatization, on an α_1-acid glycoprotein-based column (chiral AGP). The influence of pH, temperature, buffer ionic strength and organic modifiers of the mobile phase on retention and enantioselectivity was evaluated. The optimised method permits baseline separation of benazepril, its stereoisomers and its metabolite in less than 35 min, without the use of charged organic modifiers or tandem coupled chromatography.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Benazepril

1. Introduction

There is great interest in determining the chiral purity of pharmaceuticals because of the pharmacological differences, which may exist between the optical isomers. Several examples of these differences are available; the (*S*)-enantiomer of penicillamine is an antiarthrytic while the (*R*)-enantiomer is extremely toxic [1]; the (*S*)-enantiomer of propanolol, a β-adrenergic blocking agent, widely used for the treatment of hypertension and angina pectoris, is 100-times more active than its enantiomer [2].

At present there are many chiral stationary phase (CSP) high-performance liquid chromatography (HPLC) columns available that can directly separate enantiomers [3–9].

Protein-based CSPs have become widely used for the direct separation of drug enantiomers because of their broad applicability and the use of aqueous buffered mobile phases that are compatible with many polar compounds.

Examples of such kind of CSPs are the bovine serum albumin (BSA) and α_1-acid glycoprotein (AGP) as a result of the pioneering work of Allenmark [10] and Hermansson and Eriksson [11], respectively.

Chiral AGP stationary phase has been obtained by immobilising α_1-acid glycoprotein on silica [12]. This CSP has been used for the determination of a series of drugs in bulk form or in biological fluids [13–17].

Based on our interest in the chiral recognition of

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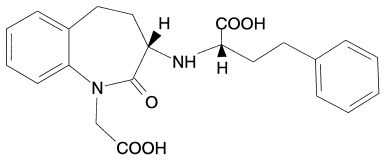
drugs, we have focused our attention on benazepril hydrochloride, (3-[(1-ethoxycarbonyl-3-phenyl-(1*S*)-propyl)-amino]-2,3,4,5-tetrahydro-2-oxo-1-(3*S*)-benzazepine-1-acetic acid hydrochloride) (Fig. 1), a prodrug, which, following oral administration, is hydrolysed in vivo to its active dicarboxylic acid metabolite, benazeprilat (3-[(1-carboxyl-3-phenyl-(1*S*)-propyl)-amino]-2,3,4,5-tetrahydro-2-oxo-1-(3*S*)-benazepine-1-acetic acid) (Fig. 1), a nonsulfhydryl angiotensin converting enzyme (ACE) inhibitor [18].

Benazepril contains two stereogenic centres, but is currently available as single enantiomer (*S*,*S* configuration) for the treatment of hypertension. Its enantiomer (compound **3**, Fig. 1) and the diastereoisomeric pair of enantiomers (compounds **4**, **5**, Fig. 1) can be regarded as impurities.

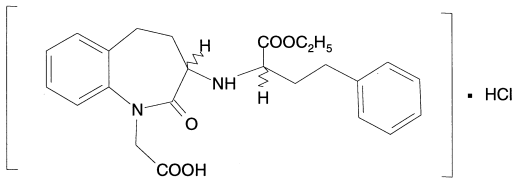
Literature surveys have revealed very little regarding the chiral resolution of this compound; therefore, a selective (chemo-, diastereo- and enantioselective) analytical method to apply in pharmaceutical and pharmacological studies is needed.

Using a reversed-phase column and enalapril, as internal standard, benazepril has been determined in pharmaceutical dosage forms [19].

The present work reports the separation of benazepril stereoisomers and its active dicarboxylic acid metabolite, benazeprilat, by a rapid and simple HPLC procedure. The method is suitable for the estimation of the optical purity of this compound in pharmaceutical formulations and for investigations of stereoselective pharmacokinetics and biotransformations.



1: Benazeprilat (S,S configuration)



2 : Benazepril hydrochloride (S,S configuration)

3 : CGP 42456A hydrochloride (R,R configuration)

4 : CGP 42454A hydrochloride (absolute configuration not noted)

4 : CGS 14829 hydrochloride (absolute configuration not noted)

Fig. 1. Structures of the solutes investigated.

2. Experimental

2.1. Chemicals

Stainless steel chiral AGP (100×4 mm I.D.) (J.T. Baker, Phillipsburg, NJ, USA) and Kromasil C_8 (250×4.6 mm I.D.) (Eka Nobel, Bohus, Sweden) columns were used.

HPLC-grade solvents were obtained from Carlo Erba (Milan, Italy).

Benazepril, benazeprilat and stereoisomers were obtained from Novartis Farma (Origgio, VA, Italy).

2.2. Apparatus

Analytical chromatography was performed on a Waters (Milford, MA, USA) chromatograph equipped with a Model U6K injector and a Waters Model 966 programmable multi-wavelength diode array detector.

Thermodynamic data were obtained from variable-temperature chromatography using an HPLC Waters oven (range 25°C–65°C, $\Delta T \pm 0.5$°C).

pH was measured with a 686 Titroprocessor Series 04 pH meter (Metrohm, Herisau, Switzerland).

The circular dichroism (CD) spectra of samples, dissolved in ethanolic solution (0.2 m*M*) in a quartz cell (1-cm path length) at room temperature, were measured using a Jasco Model J-700 spectropolarimeter (Jasco, Ishikawa-cho, Hachioji City, Tokyo, Japan).

2.3. Chromatographic system

Phosphate buffers of pH 3–7 were prepared using potassium dihydrogen phosphate solutions (0.01 *M*–

0.06 *M*), which were adjusted by addition of potassium hydroxide or phosphoric acid to the desired pH.

The eluents for the separations were mixtures of appropriate percentages of different organic modifiers (ethanol, 2-propanol, acetonitrile) and phosphate buffer. The mobile phases were filtered and degassed by sonication immediately before use. A flow-rate of 0.9 ml min^{-1} was used.

The wavelength of detection was 240 nm.

All analytical separations were performed at 25°C, except those used for the study of the thermodynamic data.

For the studies on the influence of the mobile phase pH, buffer concentration, organic modifiers type and concentration, a standard solution of compounds **1**–**5** was prepared by dissolving 3 mg of each analyte with 25 ml of HPLC-grade ethanol. This solution was further diluted to give a final concentration of 24 μg ml^{-1} for each compound. The injection volume was 10 μl.

3. Results and discussion

3.1. Influence of mobile phase pH on retention and selectivity

The influence of mobile phase pH on retention and selectivity of solutes **1**–**5** on chiral AGP phase was studied by changing the pH of the aqueous buffer (0.06 *M*) of the mobile phase from 3.0 to 6.0, using 2-propanol as organic modifier.

Since the isoelectric point (p*I*) of α_1-glycoprotein is 2.7 in phosphate buffer [12], an increase in the mobile phase pH causes an increment in negatively charged groups on the immobilised protein; moreover the pH changes affect the ratio of unionised to ionised solute.

Fig. 2 shows the dependence of the retention of compounds **1**–**5** on pH. A pronounced maximum in retention for the four stereoisomers **2**–**5** was observed at pH close to 4.5. Conversely the retention of compound **1** decreased progressively as pH increased.

An attempt to rationalise the retention mechanisms by comparing the changes of the capacity factors due to a pH increase on chiral AGP and achiral reversed-phase Kromasil C_8 columns was performed. The *k′* values of diastereomers **2** and **4** increase progressively with increasing pH (Fig. 3), which is probably due to a reduced ionisation causing increased hydrophobic interaction.

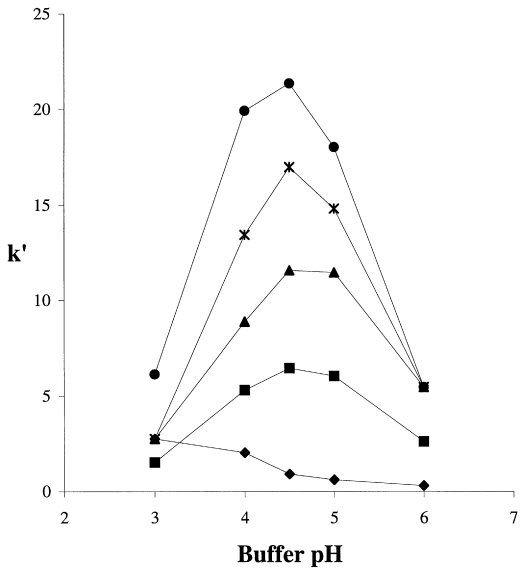


Fig. 2. Effect of mobile phase pH on the capacity factors (*k′*) of compounds **1**–**5**. Column, chiral AGP (100×4.0 mm I.D.); eluent, 0.06 *M* phosphate buffer–2-propanol (92.5:7.5, v/v); flow-rate, 0.9 ml min^{-1}; column temperature, 25°C; detection wavelength, 240 nm. ♦=**1**; ■=**2**; ▲=**3**; ★=**4**; ●=**5**.

The fact that, at pH values greater than 4.5, the elution order of the stereoisomers **2** and **4** on the chiral AGP column is inverted, might indicate the prevalence of others phenomena, such as conformational changes in the protein and/or repulsive electrostatic interactions, over hydrophobic interactions.

The effects of pH on enantioselectivity were studied keeping 2-propanol concentration (7.5%) and molarity buffer (0.06 *M*) constant.

Data obtained are summarised in Fig. 4. The separation factor of the enantiomers **4** and **5** ($\alpha_{5,4}$) decreased from 2.24 to 1.00 with a pH change from 3.0 to 6.0; whereas the influence on $\alpha_{3,2}$ was relatively modest. $\alpha_{4,3}$ shows a pH dependence similar to that observed in Fig. 2.

On the basis of the aforementioned data, the pH of the mobile phase should be a critical parameter to provide separation of compounds being tested; in fact the optimum pH value for the simultaneous

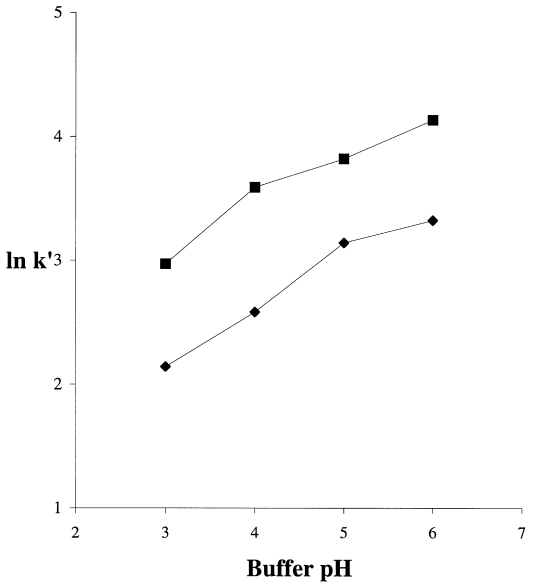


Fig. 3. Effect of mobile phase pH on the capacity factors (k') of diastereomers **2** and **4**. Column, Kromasil C_8 100 Å, 5 μm (250×4.6 I.D.); eluent, 0.06 *M* phosphate buffer–2-propanol (80:20, v/v); flow-rate, 1.0 ml min^{-1}; column temperature, 25°C; detection wavelength, 240 nm. ♦=**2**; ■=**4**.

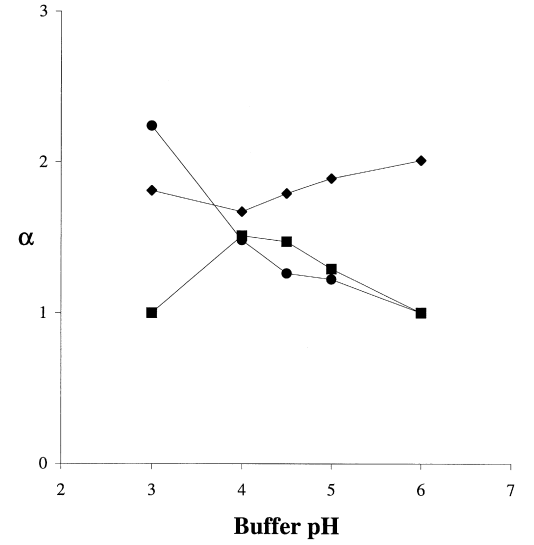


Fig. 4. Effect of mobile phase pH on the separation factors (α). Column, chiral AGP (100×4.0 mm I.D.); eluent, 0.06 *M* phosphate buffer–2-propanol (92.5:7.5, v/v); flow-rate, 0.9 ml min^{-1}; column temperature, 25°C; detection wavelength, 240 nm. ♦=$\alpha_{(3,2)}$; ■=$\alpha_{(4,3)}$; ●=$\alpha_{(5,4)}$.

baseline separation of tested solutes is 4.0, whereas outside the 4.0–5.0 range, too short retention times or partial overlapping of peaks were observed.

3.2. Effect of the buffer molarity

Little effect on retention times of compounds **1**–**5** by varying the concentration of buffer phosphate from 0.01 *M* to 0.06 *M*, at different concentrations of 2-propanol, was observed.

The dependence of both enantioselective and resolution factors of two critical pairs **2**, **3** and **4**, **5** are given in Fig. 5. As can be seen from our data, stereoselective interactions are slightly affected by ionic strength of mobile phase; however, higher buffer concentration resulted in increased resolution of the enantiomers **4** and **5**.

3.3. Influence of the nature and concentration of uncharged organic modifiers

The influence of the nature and concentration of uncharged organic modifiers such as 2-propanol,

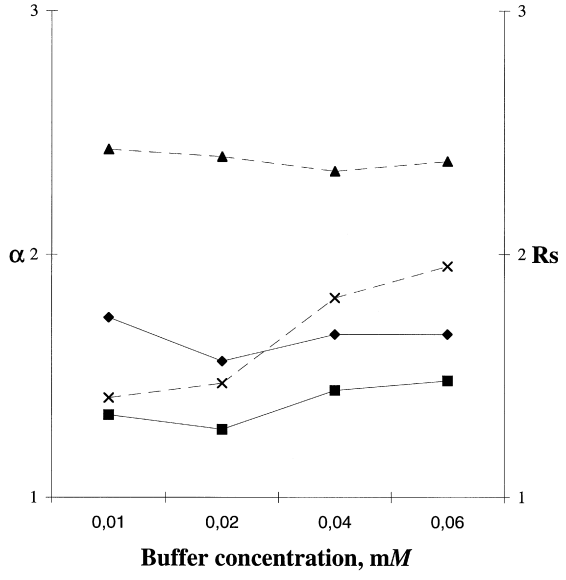


Fig. 5. Effect of buffer molarity on the enantioselectivity factors (α) and resolution factors (R_s). Column, chiral AGP (100×4.0 mm I.D.); eluent, phosphate buffer (pH 4.0)–2-propanol (92.5:7.5, v/v); flow-rate, 0.9 ml min^{-1}; column temperature, 25°C; detection wavelength, 240 nm. ♦=$\alpha_{(3,2)}$; ■=$\alpha_{(5,4)}$; ▲=$R_{s(3,2)}$; ×=$R_{s(5,4)}$.

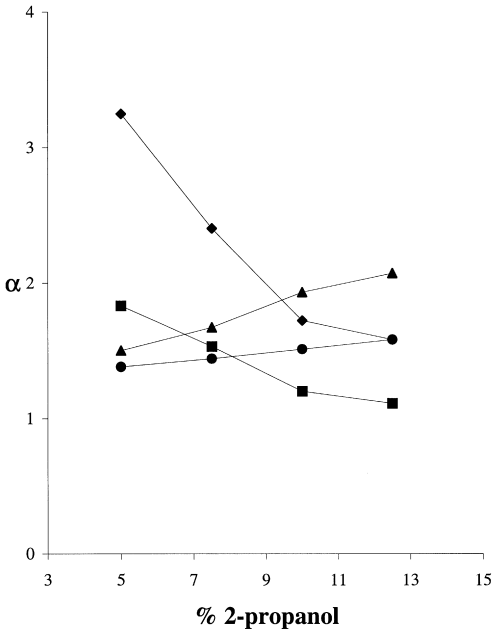


Fig. 6. Effect of 2-propanol concentration on stereoselectivity. Column, chiral AGP (100×4.0 mm I.D.); eluent, 0.04 *M* phosphate buffer (pH 4.0)–2-propanol; flow-rate, 0.9 ml min^{-1}; column temperature, 25°C; detection wavelength, 240 nm. ♦= $\alpha_{(2,1)}$; ▲= $\alpha_{(3,2)}$; ■= $\alpha_{(4,3)}$; ●= $\alpha_{(5,4)}$.

ethanol and acetonitrile was studied using a 0.06 *M* phosphate buffer (pH 4.0), as mobile phase, at a column temperature of 25°C. Preliminary trials were carried out to evaluate the effect of different percentages of ethanol and 2-propanol on retention and selectivity.

An increase in the amount of alcoholic modifier in the mobile phase gave a decrease of k' values for all compounds; moreover, by changing concentration of organic modifier, it was possible to obtain differences in enantioselectivity and diastereoselectivity.

Indeed the separation factors of both pairs of enantiomers **2**, **3** and **4**, **5** ($\alpha_{3,2}$ and $\alpha_{5,4}$, respectively) increased with the concentration of alcohol present in the mobile phase, while the separation factors of diastereomers **3** and **4** ($\alpha_{4,3}$) and compounds **1** and **2** ($\alpha_{2,1}$) showed a different trend. The effect of 2-propanol concentration on selectivity is shown in Fig. 6.

After the preliminary studies, three different organic modifiers (2-propanol, ethanol and acetonitrile) were tested; the concentrations in the mobile phases were suitably chosen to obtain approximately the same retention times for benazepril.

Table 1 summarises the experimental data ob-

Table 1
Effect of uncharged organic modifiers

Organic modifier (%)	k'[a]					Critical pair[b]	α[c]	R_s[d]
	1	2	3	4	5			
2-Propanol (7.5)	2.20	5.31	8.88	13.43	19.92	3,2	1.67	2.38
						4,3	1.51	2.00
						5,4	1.48	1.95
Ethanol (15)	1.40	4.41	15.44	9.32	24.63	3,2	2.11	3.83
						3,4	1.66	2.06
						5,4	1.60	3.79
Acetonitrile (12.5)	2.20	6.93	11.06	14.94	29.81	3,2	1.60	1.93
						4,3	1.35	1.27
						5,4	1.99	2.87

Column, chiral AGP (100×4.0 mm I.D.); eluent, 0.06 *M* phosphate buffer (pH 4.0)–organic modifier; flow-rate, 0.9 ml min^{-1}; column temperature, 25°C; detection wavelength, 240 nm.

[a] The capacity factor of compounds **1**–**5**.

[b] The closest eluting pair of stereoisomers; the first number of the critical pair indicates the most retained stereoisomer.

[c] The separation factor of critical pair.

[d] The resolution factor of critical pair.

tained; in all cases simultaneous separation of benazepril (compound **2**), its enantiomer (compound **3**), the diastereomers (compounds **4** and **5**) and the chiral active metabolite benazeprilat (compound **1**) was achieved.

The best results in terms of resolution were obtained when ethanol was employed as organic modifier. The use of acetonitrile in the mobile phase gave rise to a partial overlapping of third and fourth eluted peaks, corresponding to diastereomers **3** and **4**, respectively.

Fig. 7 shows the chromatograms obtained under identical chromatographic conditions using ethanol and 2-propanol as organic modifiers. Substituting ethanol with 2-propanol in the mobile phase, caused inversion of the elution order of diastereomers **3** and **4**.

The CD spectra of benazepril and its enantiomer were recorded in ethanolic solution (0.2 m*M*). Benazepril (compound 2) exhibits intense negative first Cotton effect at 243 nm and a weak positive second Cotton effect at 217 nm.

The limits of detection (LODs) of compounds **1**–**5** were determined by using a mobile phase containing 2-propanol as organic modifier. The LODs, obtained at a signal-to-noise ratio of 3:1, were approximately of 0.6 ng for benazeprilat, 1.5 ng for benazepril and 5 ng for its stereoisomers. Benazeprilat and the stereoisomers of benazepril can be determined at the levels of 0.2% and 0.5%, respectively.

3.4. Effect of temperature on enantioselectivity

The enantioselectivity factor, α, is related to the difference between the free energies of association to the CSP for the enantiomers of a racemic solute ($\Delta_{j,i}\Delta G$) by:

$$-\Delta_{j,i}\Delta G = RT\ln k'_j/k'_i = RT\ln\alpha \quad (1)$$

where the subscripts j and i refer to the more and less retained enantiomer, respectively, R is the gas constant and T the temperature in K.

The combination of Eq. (1) with the Gibbs–Helmholz relationship:

$$\Delta_{j,i}\Delta G = \Delta_{j,i}\Delta H - T\Delta_{j,i}\Delta S \quad (2)$$

gives:

$$\ln\alpha = -\Delta_{j,i}\Delta H/RT + \Delta_{j,i}\Delta S/R \quad (3)$$

where $\Delta_{j,i}\Delta H$ and $\Delta_{j,i}\Delta S$ are the differences between the two enantiomers in enthalpy and entropy of adsorption, respectively. These thermodynamic parameters can be obtained by plotting ln α vs. $1/T$ (slope $=-\Delta_{j,i}\Delta H/R$; intercept $=\Delta_{j,i}\Delta S/R$). If there is no change in the enantioseparation mechanism over the temperature range studied, the plot of the ln α vs. $1/T$ yields a straight line.

The thermodynamic data relative to the resolution of two pairs of enantiomers **2**, **3** and **4**, **5** were calculated from Eq. (3), using three different mobile phases (Table 2). The data were collected in 10°C increments from 25°C to 65°C. As showed, both enthalpic and entropic terms were negative, for both enantiomer pairs and under all experimental conditions examined. According to these results, enthalpy-driven separations occurred. The good chiral recognition ability of the CSP observed at 25°C (see Table 1) can be explained in terms of larger $-\Delta\Delta H$ values upon the unfavourable $-T\Delta\Delta S$ contribution. As a general trend, ethanol gave the largest $-\Delta\Delta H$ and $-\Delta\Delta S$ quantities, when it was used as organic modifier in aqueous buffer. The large negative entropic contribution implies a strong dependence of enantioseparation factor on temperature. In these cases the isoenantioselective temperature (T_{iso}), defined as the temperature at which enthalpy and entropy terms are balanced and $\alpha=1$, can be achieved at lower value than those normally used in gas chromatography and supercritical fluid chromatography.

Van't Hoff plots depicted in Fig. 8 show how the capacity factor for compound **3** declines more rapidly with respect to its enantiomer **2**, which results in a practically complete peak coalescence at 65°C ($-\Delta\Delta S=7.69$ cal mol$^{-1}\cdot$K; $T_{iso}=67$°C).

By substituting 2-propanol with acetonitrile, the identical isoenantioselective temperature value for enantiomers **2** and **3** was obtained (Table 2). Above the isoenantioselective temperature, enantiomer separation is entropy-controlled and a reversal of the elution order could be observed. However, higher temperatures than 65°C were not used to avoid damage to the column.

The correlation coefficient values ($r^2>0.98$) show that, for all the separations carried out, a good

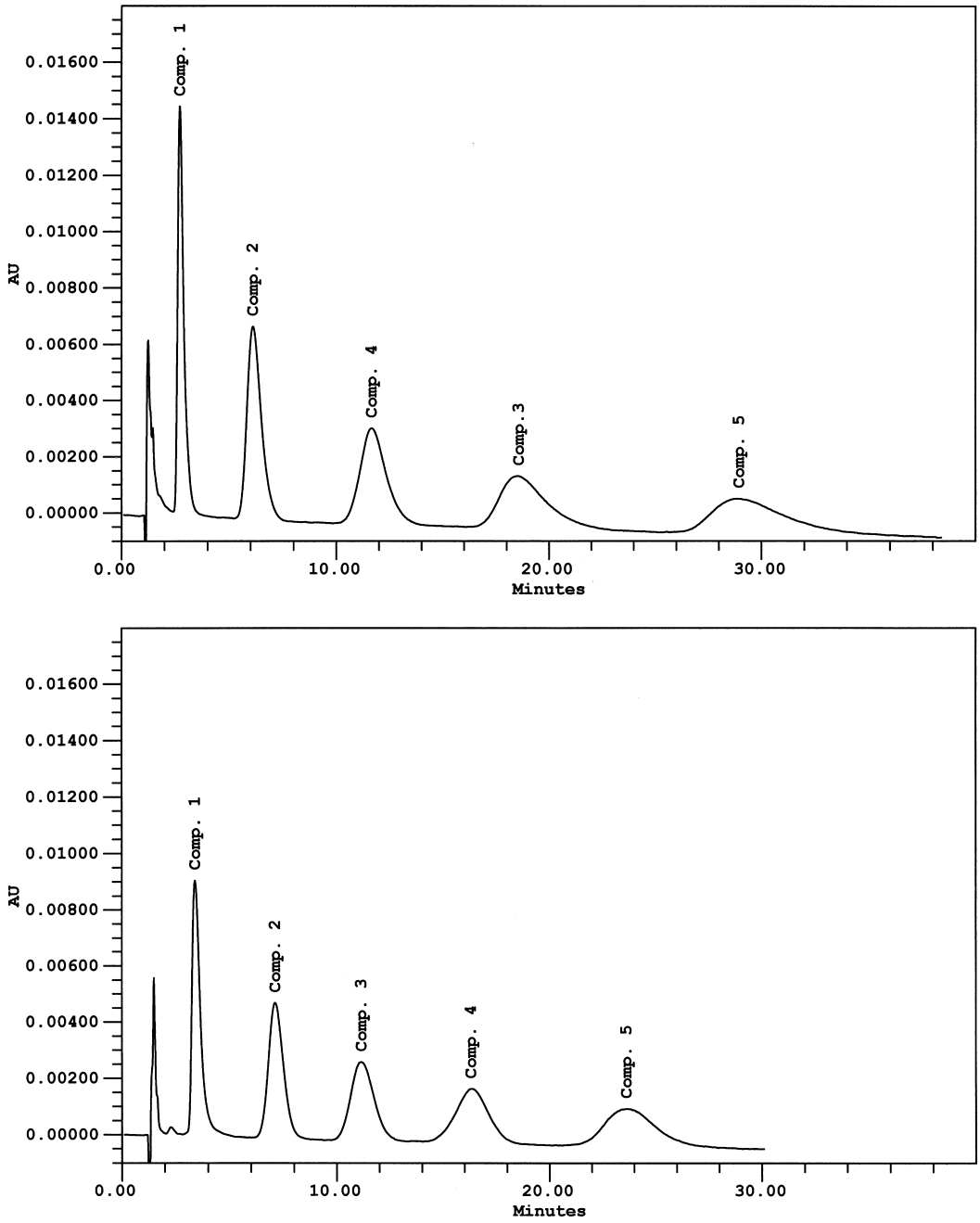


Fig. 7. HPLC of compounds **1**–**5**. Column, chiral AGP (100×4.0 mm I.D.); (top): eluent, 0.06 *M* phosphate buffer (pH 4.0)–ethanol (85.0:15.0, v/v); (bottom): eluent, 0.06 *M* phosphate buffer (pH 4.0)–2-propanol (92.5:7.5, v/v); flow-rate, 0.9 ml min^{-1}; column temperature, 25°C; detection wavelength, 240 nm.

Table 2
Thermodynamic data

Organic modifier (%)	Critical pair	$-\Delta\Delta H$ (kcal mol^{-1})	$-\Delta\Delta S$ (cal mol^{-1}·K)	r^2	T^{a}_{iso} (K)
2-Propanol (7.5)	3, 2	2.66	7.8	0.994	340
	5, 4	1.13	2.9	0.984	396
Ethanol (15)	3, 2	4.80	13.6	0.985	352
	5, 4	3.24	8.9	0.998	363
Acetonitrile (12.5)	3, 2	2.61	7.7	0.992	340
	5, 4	2.32	6.2	0.995	372

Column, chiral AGP (100×4.0 mm I.D.); eluent, 0.06 *M* phosphate buffer (pH 4.0)–organic modifier; flow-rate, 0.9 ml min^{-1}; detection wavelength, 240 nm.
[a] Calculated from $T_{iso} = \Delta\Delta H/\Delta\Delta S$.

linearity was observed. This is consistent with the previous observation that chiral discrimination mechanism remains essentially constant within the temperature interval studied.

Acknowledgements

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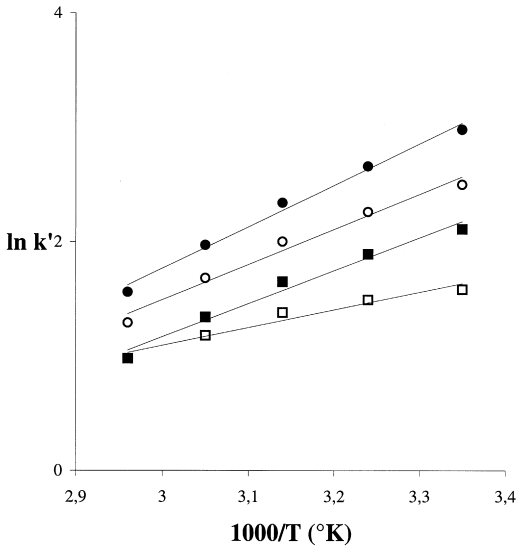

Fig. 8. Linear inverse relationship between ln k' and temperature for compounds **2**–**4**. Column, chiral AGP (100×4.0 mm I.D.); eluent, 0.06 *M* phosphate buffer (pH 4.0)–2-propanol (92.5:7.5, v/v); flow-rate, 0.9 ml min^{-1}; detection wavelength, 240 nm. □=**2**; ■=**3**; ○=**4**; ●=**5**.

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