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On-column enantiomerization of 3-hydroxybenzodiazepines during chiral liquid chromatography with optical rotation detection

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Abstract

Oxazepam and lorazepam, 3-hydroxybenzodiazepines that undergo reversible enantiomerization at ambient temperature, were eluted on a chiral HPLC column at various temperatures and various flow-rates. The profiles obtained by a UV detector were various, such as two peaks, a plateau sandwiched between two peaks, and one fused peak. The profiles obtained by an optical rotation detector were also various, such as two sharp peaks with opposite chirality (−, +), two peaks (−, +) with a great tailing or leading, two small peaks (−, +) next to each other. These peculiar profiles could be explained by on-column enantiomerization. Unexpectedly, the elution order of the (−)- and (+)-enantiomers was reversed for oxazepam and lorazepam. The simultaneous detection by UV and chiroptical measurements allowed us to plot HPLC profiles of the individual enantiomers.

Keywords: Enantiomer separation; Optical rotation detection; Enantiomeric ratio; Lorazepam; Oxazepam; Benzodiazepines

1. Introduction

Many compounds exist in multiple reversible forms reacting on the time order of minutes at ambient temperature. When a reversible reaction (usually isomerization) occurs during chromatography, a peculiar chromatogram is exhibited such as split peaks, a plateau sandwiched between two peaks, a tailed peak and a broad peak.

We had analyzed HPLC profiles of some convertible isomers such as saccharides [1], proline dipeptides [2] and immunosuppressive drugs (cyclosporin derivatives, FK506 and rapamycin) with UV or refractive index detectors [3–5]. These detectors do not distinguish between a pair of enantiomers but an optical rotation detector does by responding in opposite directions. We then analyzed chiroptical profiles of two compounds, oxazepam and lorazepam.

Both compounds are 3-hydroxy-1,4-benzodiazepines with a chiral center at the C3 position undergoing reversible enantiomerization (Fig. 1) [6–11].

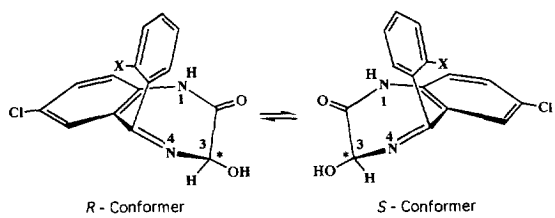

Fig. 1. Reversible enantiomerization of oxazepam (X=H) and lorazepam (X=Cl), shown in their dominant conformation.

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Both constitute a class of benzodiazepines clinically used as antianxietics or sedatives with central nervous system depressant activity.

2. Experimental

Liquid chromatography was performed using a PU-980 pump (Jasco, Tokyo, Japan), a Rheodyne 7125 injector equipped with a 20 μl loop, a 556C column oven (GL Sciences, Tokyo, Japan) with a heater and cooler and a D-2500 integrator (Hitachi, Tokyo, Japan) for each detector. The column effluent was first introduced to a UV-970 UV detector (Jasco) set at 285 nm, then directly to an OR-990 optical rotation detector (Jasco) with a Hg–Xe lamp operating at 350 to 900 nm.

A chiral stationary phase of Shodex ORpak CDB-453 HQ (Showa Denko, Tokyo, Japan) in a 150 mm×4.6 mm I.D. column was used. It consisted of a β-cyclodextrin derivative chemically bonded to a polymer gel. The mobile phase was a mixture of acetonitrile–50 m*M* pH 7.4 phosphate buffer (3:7).

The peak area corresponding to non-converted or converted molecules is defined as unreacted molecule zone or reacted molecule zone, respectively. The area of the unreacted molecule zone, shown in Fig. 3, was measured by cutting the profile copy and weighing the paper.

Oxazepam and lorazepam were kindly supplied by pharmaceutical companies. A 25 μl solution of oxazepam (1.0 μg/ml) or lorazepam (3.0 μg/ml) was injected into the column.

3. Results

3.1. Resolution of oxazepam and lorazepam enantiomers

Elution profiles of oxazepam and lorazepam were various, depending on column temperature and eluent flow-rate as described below. Elution at 17 to 32°C gave a peak of each unreacted enantiomer in the UV profile as shown in Table 1. The elevation of the column temperature shortened their retention time and decreased both separation factor (α) and resolution (R_s) of the enantiomer pairs. The low

Table 1
Resolution of enantiomers of oxazepam and lorazepam

Temperature	Oxazepam				Lorazepam			
Flow-rate	Retention time of peak		[a]α	[b]R_s	Retention time of peak		[a]α	[b]R_s
	1st (min)	2nd (min)			1st (min)	2nd (min)		
17°C								
0.6 ml/min	5.78	11.07	2.64	2.70	6.41	8.76	1.61	1.31
0.3 ml/min	11.39	21.79	2.61	3.34	12.59	17.2	1.60	1.62
27°C								
0.6 ml/min	5.23	9.00	2.41	2.79	5.64	7.20	1.50	1.16
0.3 ml/min	10.24	17.55	2.38	3.25	11.07	14.07	1.49	1.38
32°C								
0.6 ml/min	5.08	8.4	2.31	2.52	5.42	6.74	1.46	1.08
0.3 ml/min	10.06	16.43	2.25	3.17	10.71	13.19	1.43	1.31
37°C								
0.6 ml/min	4.94	7.72	2.16	2.36	5.36	6.42	1.38	[c]ND
0.3 ml/min	9.88	15.44	2.13	ND	ND	ND	ND	ND

[a] α: Separation factor ($=k'_2/k'_1$).
[b] Resolution $=2(t_2-t_1)/(W_1+W_2)$, where t_1, t_2 are the retention times, and W_1, W_2 the peak widths (for the first and second peak).
[c] ND: Not detectable.

flow-rate of 0.3 ml/min gave a slightly lower α but a higher R_s than the high flow-rate of 0.6 ml/min.

3.2. Profiles of oxazepam eluted at a low temperature

Fig. 2 shows both UV and chiroptical profiles of oxazepam eluted at 17, 27 or 32°C. The first and second peak on the UV profile could be assigned to (−)- and (+)-oxazepam, respectively, from the chiroptical profile. The areas of the two UV peaks were equal to each other with elution at 17°C, so was the chiroptical-peak area with opposite signs. These results indicated that no molecules underwent conversion within 20 min at this low temperature.

With elution at a higher temperature (27 or 32°C), a certain number of molecules should have undergone conversion, exhibiting a plateau on the UV profile as a reacted molecule zone between the two peaks. The chiroptical profile showed a (−) peak with a tailing that gradually approached optical rotation zero and crossed over the baseline; a (+) peak with a leading that was gradually rising from the crossing point on the baseline.

3.3. Profiles of oxazepam eluted at various flow-rates: estimation of the reaction velocity in the column

Elution at 32°C at a flow-rate between 0.6 and 0.3 ml/min exhibited a UV profile with a sandwiched plateau (Fig. 3). Elution at a lower flow-rate should give a longer retention time, therefore, should give the increased reacted molecule zone and the decreased unreacted molecule zone on the UV profiles. For the chiroptical profiles with a lower flow-rate, the valley of the unreacted (−)-enantiomer was

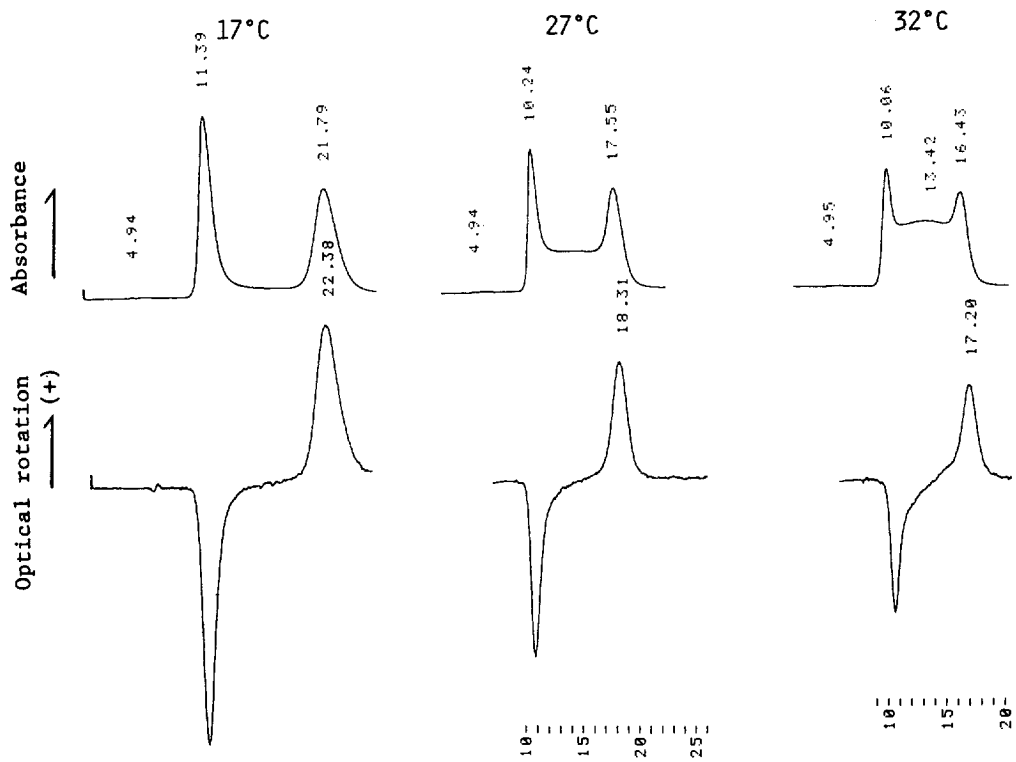

Fig. 2. HPLC profiles of oxazepam eluted at 17, 27 or 32°C at a 0.3 ml/min flow-rate. Detection: UV absorbance measurement at 285 nm at attenuation 10 of the integrator (upper profiles) and optical rotation measurement at attenuation 3 (lower profiles).

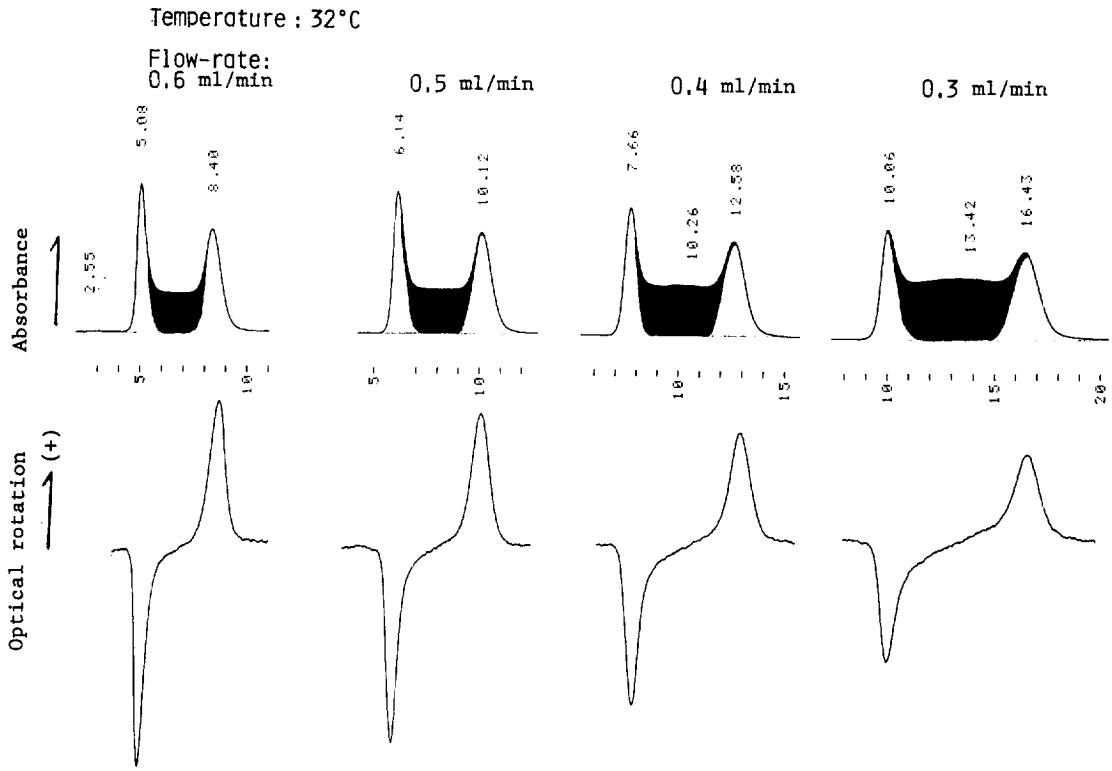

Fig. 3. HPLC profiles of oxazepam eluted at 32°C at a 0.6 to 0.3 ml/min flow-rate. The shaded area is considered to be the reacted molecule zone. Other HPLC conditions as in Fig. 2.

shallower with a greater tailing, and the peak of the unreacted (+)-enantiomer was lower with a greater leading.

The unreacted molecule zone on the UV profile was fused with the reacted molecule zone but could be separated on the assumption that it adopts a symmetrical peak (Fig. 3). The velocity of this first-order reaction could be estimated from the slope of the line obtained by plotting log area (%) of the unreacted molecule zone against the retention time (Fig. 4). For example, the half-lifes were about 9 and 18 min at 32°C for the first and second peaks, respectively, with the rate constants (=0.693/half-life) 0.077 and 0.0385 min^{-1}. The reaction velocity was higher at the higher temperature, as expected. Both areas coalesced to 50% of the total area by extrapolation of the lines to time zero, supporting the idea that the injected solute was a racemic mixture in an interconversion equilibrium.

3.4. Profiles of oxazepam at higher temperatures

The UV profile with elution at 37°C showed the sandwiched zone adopting a broad peak rather than a flat plateau (Fig. 5). On the chiroptical profile, the valley that can be attributed to the unreacted (−)-enantiomer was shallow, and the summit that can be attributed to the unreacted (+)-enantiomer was low.

The elution at a higher temperature of 47 or 57°C exhibited only one fused peak on the UV profile, indicating that all the injected molecules had undergone conversion in the column. The corresponding chiroptical profile showed one recess with a conical bottom and one eminence with a conical summit, indicating that the (−)-enantiomer was dominant in the early half and the (+)-enantiomer in the late half of the UV peak. With elution at a further elevated temperature, any fraction of the eluate would show an optical rotation of almost zero.

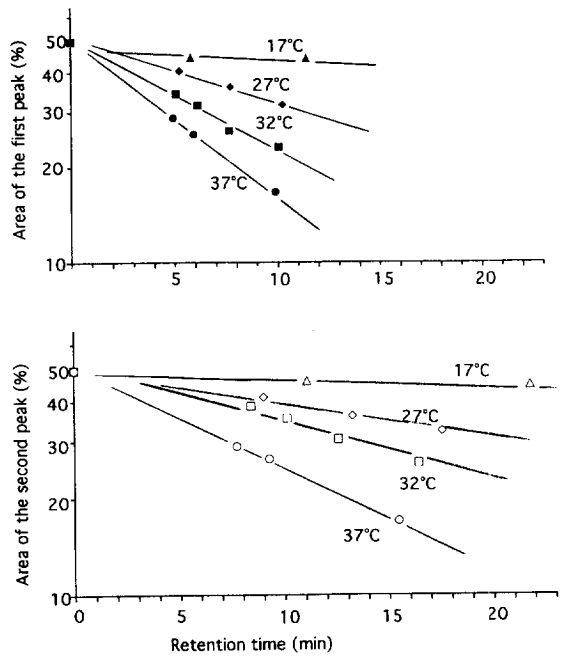

Fig. 4. Plots of area (%) against retention time of the first and second peak (upper and lower graph, respectively) of unreacted oxazepam enantiomer on the UV profile. The half-lifes for the first and second peak were estimated as: 17 and 30 min at 27°C; 9 and 18 min at 32°C; 6 and 10 min at 37°C.

3.5. Calculated profiles of each oxazepam enantiomer

The following equations hold true if the molar response of the two detectors is corrected to equality:

$$UV = P + N$$

$$OR = P - N$$

where UV stands for UV absorbance, OR for net optical rotation angle, P for (+)-enantiomer concentration and N for (−)-enantiomer concentration. Addition or subtraction of these equations leads to:

$$P = 1/2(UV + OR)$$

$$N = 1/2(UV - OR)$$

According to these equations the profiles of P and N could be plotted (Fig. 6). The optical purity was over 95% in the split UV peaks with elution at 17°C. The optical purity at the summit fraction of the split UV peaks became lower with elution at higher temperatures (27 to 37°C). One symmetrical UV peak with elution at 47 or 57°C (the enantiomer

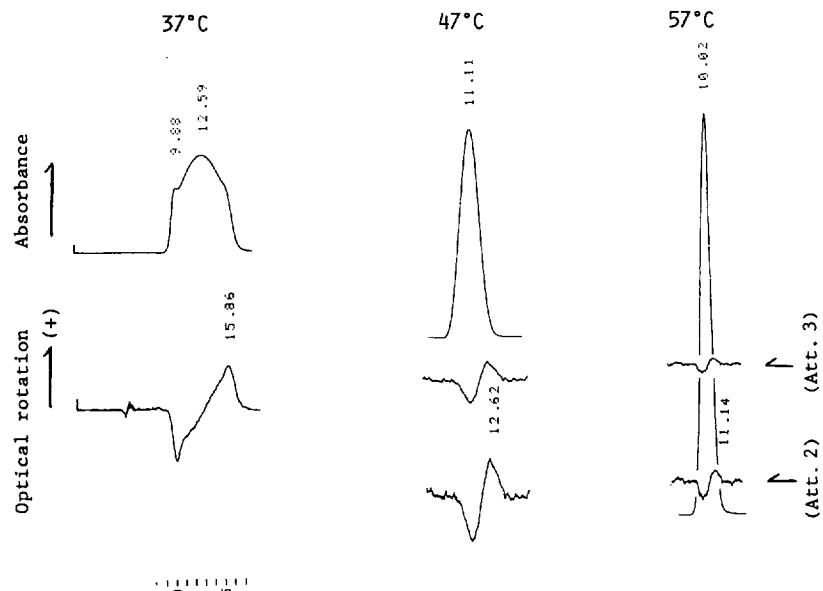

Fig. 5. HPLC profiles of oxazepam eluted at 37, 47 or 57°C at a 0.3 ml/min. Others are same as in Fig. 2, but chiroptical profiles at attenuation 2 are shown in addition.

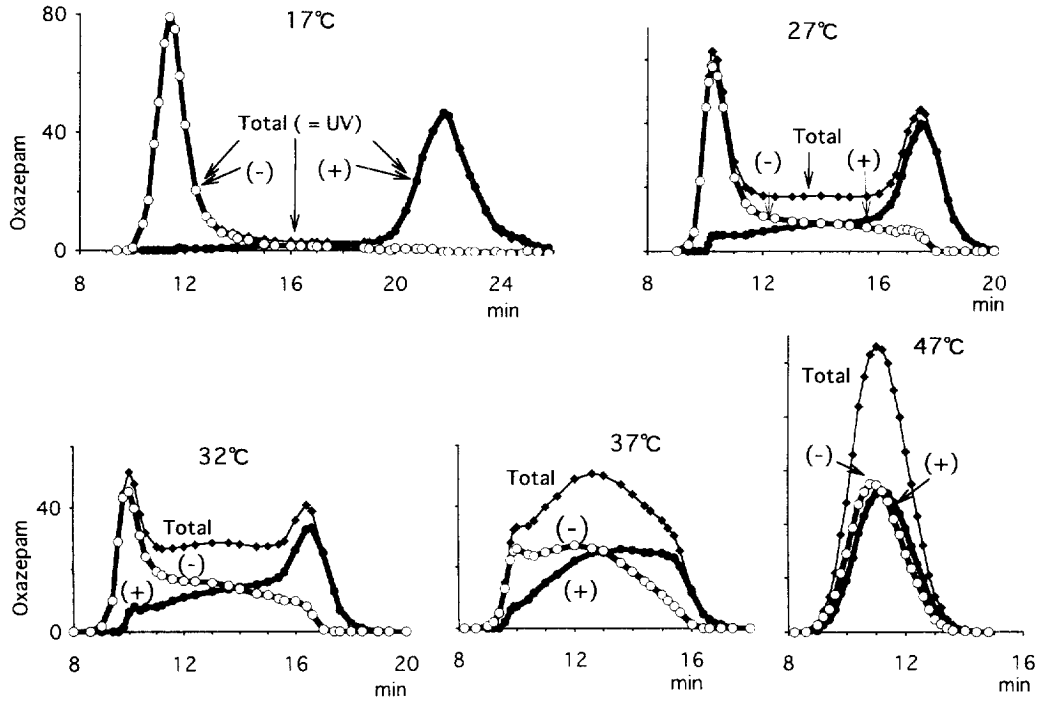

Fig. 6. HPLC profiles of total, (−)- and (+)-oxazepam eluted at a 0.3 ml/min flow-rate. The total profile was equivalent to the UV profile in Figs. 2 and 5. The profile of each enantiomer was obtained from the UV and chiroptical profiles (see Section 3).

profile at 57°C not shown) was found to be composed of the two symmetrical peaks of the enantiomers that overlapped with a slight lag. All these symmetrical peaks were apparently in shape of a Gaussian distribution.

3.6. Profiles of lorazepam

Fig. 7 shows the profiles of lorazepam, an oxazepam derivative substituted with 2′-Cl. Resolution of lorazepam enantiomers was rather poor compared to that of oxazepam (Table 1). Contrary to our expectation, the elution order of (+)- and (−)-lorazepam enantiomers was reverse to that of oxazepam enantiomers, i.e., (+)-lorazepam enantiomer eluted earlier. Though such differences existed, both UV and chiroptical profiles were essentially the same as those of oxazepam. Elution at 47 or 57°C at a 0.3 ml/min flow-rate exhibited a higher fused peak than that at the higher flow-rate of 0.6 ml/min on the UV profile, while elution at the low flow-rate exhibited a lower eminence and a shallower recess on the chiroptical profile. This effect of flow-rate was seen also for oxazepam, though not shown in Fig. 5.

4. Discussion

There are many enantiomeric drugs having different biological activities for each enantiomer. It is difficult to measure the bioactivity of 3-hydroxybenzodiazepines because of the fast conversion, however, their O-derivatives are so stable that the bioactivity measurement is feasible. In the literature 3-O-acyl- and 3-O-methylbenzodiazepine derivatives in the *S*-configuration possess much higher pharmacological activity and higher affinity for the benzodiazepine receptor than those in the *R*-configuration [12–14], suggesting that *S*-conformers of oxazepam and lorazepam are active. The *S*-conformers of oxazepam and lorazepam were suggested to have a (+) optical rotation from the chiroptical data of the

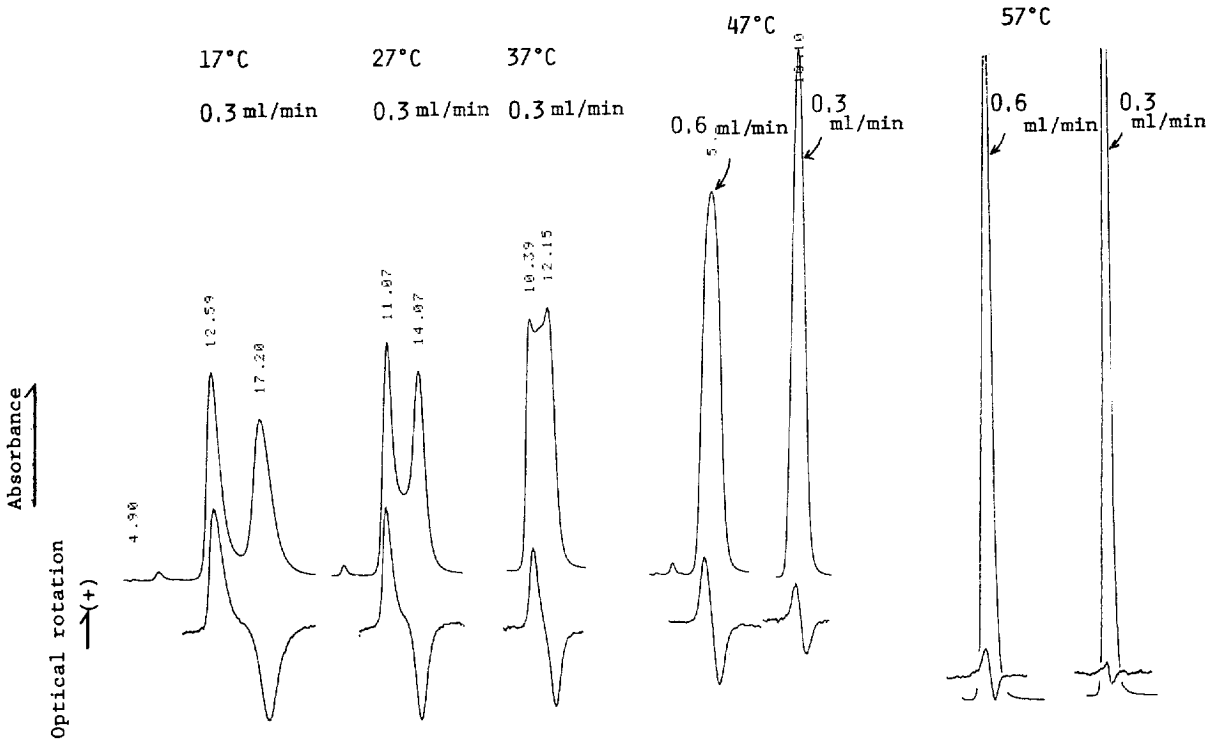

Fig. 7. HPLC profiles of lorazepam eluted at 17 to 57°C at a 0.3 or 0.6 ml/min flow-rate. Other conditions as in Fig. 2.

stable 3-O-derivatives measured with a sodium D light (589 nm) [6–8,10,12,13]. Though the optical rotation at each wavelength was not determined, another optical rotation detector (Shodex OR-1, Showa Denko) with a 780 nm light emitting diode gave essentially the same results.

The velocity of the lorazepam enantiomerization was reported to be largely dependent on the solvent; either enantiomer had a half-life of 5.0 min in pH 7.5 aqueous buffer, 9.2 min in methanol, 14.3 min in ethanol and over 5000 min in acetonitrile or dichloroethane at 23°C [8]. Though not directly comparable with these reported values, the half-life estimated from the UV profiles was plausible. The estimated velocities of the forward and reverse reaction of oxazepam in the column were different from each other (Fig. 4), though the velocity in both directions should be equal in solution. It is probable that the interaction with the chiral stationary phase affected the stability of each enantiomer, i.e., (+)-oxazepam is more stable, being retained on the chiral cyclodextrin derivative more strongly through hydrophobic binding, because the stability is dependent on the hydrophobicity of the environment as described before.

Chromatographers are sometimes confronted with peculiar profiles that make them suspicious of apparatus malfunction, sample contamination or on-column conversion. Analysis of the profile with a change in temperature or flow-rate can be useful and easy for differentiation between on-column conversion and others [1–5]. Chiroptical detection besides conventional detection will help in the comprehensive understanding of the chromatographic behavior.

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