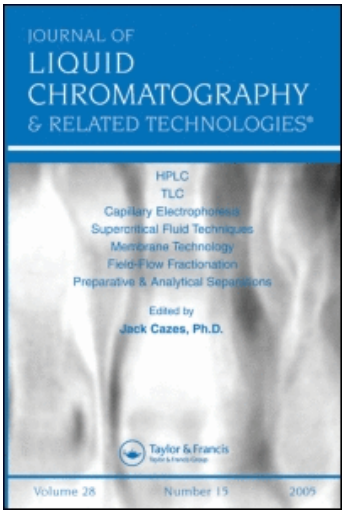

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CHIRAL SEPARATION OF LORAZEPAM ON OVOMUCOID-BONDED COLUMNS: PEAK COALESCENCE DUE TO RACEMIZATION

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ABSTRACT

Racemic lorazepam (LZ) was separated on an ovomucoid (OVM)-bonded column by changing the column temperature. Chromatographic peak coalescence, appearing as a plateau between the resolved peaks, was observed above column temperatures of 15°C. Each LZ enantiomer was isolated on another chiral column using a non-aqueous eluent. Racemization of each LZ enantiomer in two different solutions and peak coalescence on these chiral columns were investigated. These results revealed that the peak coalescence

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should be due to racemization of LZ on the OVM-bonded column. By reducing the column temperature to 7°C, the enantiomeric composition of LZ before chromatography could be determined on the OVM-bonded column.

INTRODUCTION

Chiral separation is an important and essential technology in the field of pharmaceutical analysis and drug metabolism. Generally, chiral separations have been carried out on the ordinary reversed-phase column by derivatizing enantiomers to their corresponding diastereomers. In the last decade, many chiral stationary phases (CSPs) for high performance liquid chromatography (HPLC) have been developed for optical resolution. Of the stationary phases developed, protein-bonded phases are becoming increasingly popular due to their direct optical resolution and their wide chiral recognition for the drug enantiomers in aqueous mobile phases. Moreover, they are easily applicable to resolution of drug enantiomers in biological samples. Albumins such as bovine serum albumin (BSA) (1) and human serum albumin (HSA) (2), glycoproteins such as orosomucoid (α_1-acid glycoprotein) (3) and ovomucoid (OVM) (4-6), enzymes such as α-chymotrypsin (7) and fungal cellulase (8) are reported as typical chiral protein phases. Columns based on albumins and glycoproteins described above are now commercially available.

OVM has an isoelectric point of 4.1 and a molecular weight of 28,000. The chromatographic behavior of enantiomeric drugs on

an OVM-bonded column has been reported recently by many researchers (9-13). The OVM-bonded column provides effective separation without derivatization for acidic and basic racemates having aromatic residues. In a previous study (14), we reported an optimization method for resolution of drug enantiomers on an OVM-bonded column, and reported a marked influence of column temperature on enantioselectivity as well as retention. It was found that chromatographic peak coalescence, which is seen as a plateau between the resolved peaks, was observed with the separation of racemic lorazepam (LZ) on the OVM-bonded column. The column temperature was 20 °C, which is generally used for the separation of drug enantiomers. Our aims for this study are to clarify that the peak coalescence is due to racemization of LZ on an OVM-bonded column during chromatography, and to find out the conditions for determination of the enantiomeric composition of LZ on the OVM-bonded column.

MATERIALS AND METHODS

LZ (structure, see Figure 1) used in this study was purchased from Sigma (St. Louis, MO, U.S.A.). LZ was dissolved in ethanol and a 5- to 10-μL portion of the sample was injected into the column. The sample concentration was 0.125 mg/mL. Reagents were purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan) and water was purified by a Puric-S water purifier (Organo, Tokyo, Japan). The HPLC system consisted of an LC-9A liquid chromatograph (Shimadzu, Kyoto, Japan), a Rehodyne 7125 sample

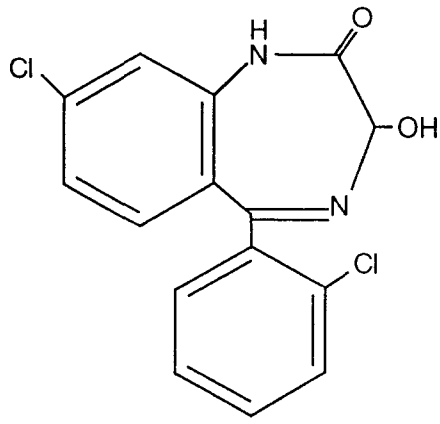

FIGURE 1. Structure of lorazepam (LZ).

injector equipped with a 20-µl loop (Cotati, CA, U.S.A.), an SPD-6AV UV-VIS spectrophotometric detector (Shimadzu) and a BX-7700 automatic column thermostatic oven (Ishido Co. Ltd., Chiba, Japan). Chromatograms were recorded and integrated on a Chromatopac C-R6A (Shimadzu) integrator. The following two columns were used: the Ultron ES-OVM column (5 µm particle diameter, 150 mm x 4.6 mm I.D.) purchased from Shinwa Chemical Industries, Ltd. (Kyoto, Japan), and the Sumichiral OA-3100 column (5 µm, 250 mm x 4.0 mm I.D.), which is bonded with N-3,5-dinitrophenylaminocarbonyl-(S)-valine, purchased from Sumika Chemical Analysis Service, Ltd. (Osaka, Japan).

RESULTS AND DISCUSSION

Figures 2A-2D show chromatograms of racemic LZ obtained with column temperatures of 7, 10, 15, and 25°C on an OVM-

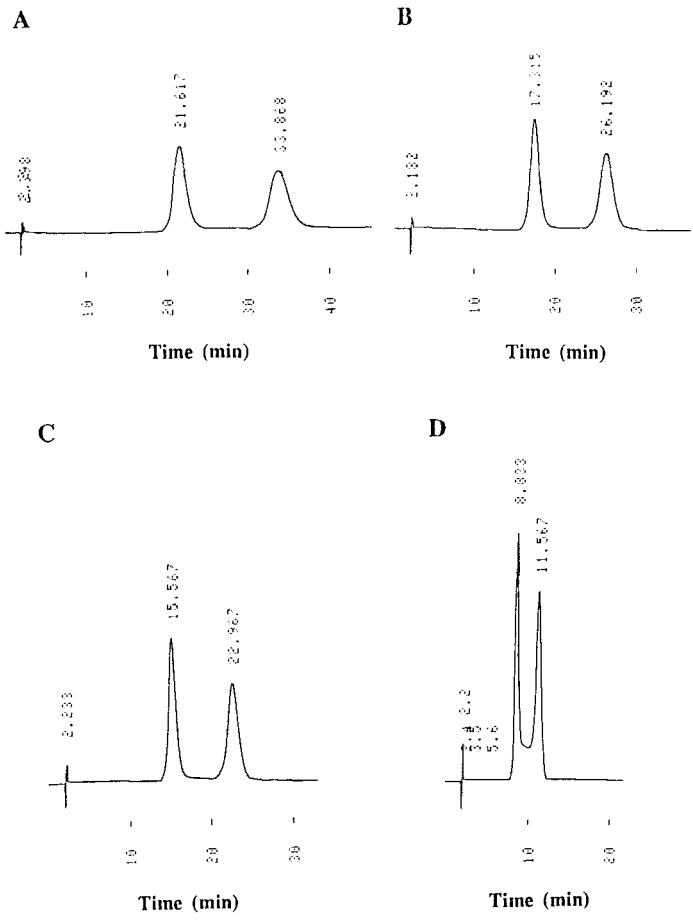

FIGURE 2. Separation of LZ obtained with an OVM-bonded column. Mobile phase: 20 mM KH_2PO_4 (pH 4.6)/2-propanol (100:10, V/V). Flow rate: 1.0 mL/min. Detection: 254 nm. Column temperatures: 7°C(A), 10°C (B) , 15°C (C) and 25°C (D).

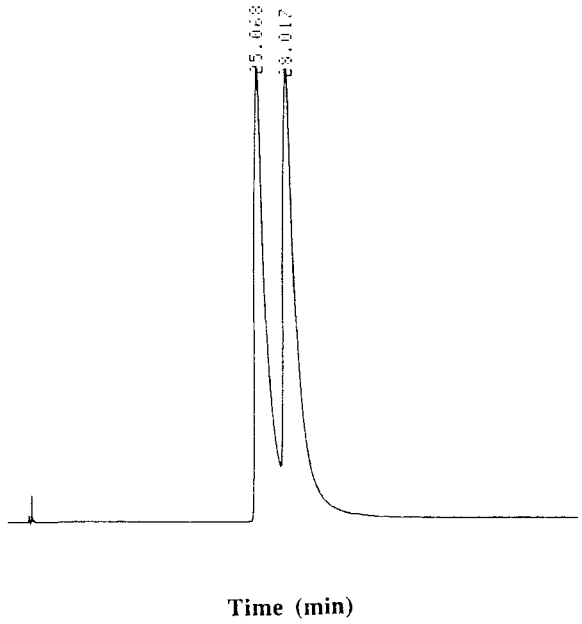

FIGURE 3. Separation of LZ on an OA-3100 column. Mobile phase: hexane/dichloroethane/ethanol (73:20:7, V/V/V). Flow rate: 2.0 mL/min. Detection: 254 nm. Column temperature: 10 °C.

bonded column. The eluent used was a mixture of 20 mM potassium dihydrogenphosphate (pH 4.6) and 2-propanol (100:10, V/V). Chromatographic peak coalescence was observed above column temperatures of 15°C. Bürkle et al. (15) reported peak coalescence due to on-column enantiomerization along with the kinetics on chiral stationary phases in gas chromatographic columns. Also, it is reported that racemization half-lives of LZ are 5 and 14.3 min in 0.1 M Tris-HCl (pH 7.5) and in ethanol, respectively, at 23°C

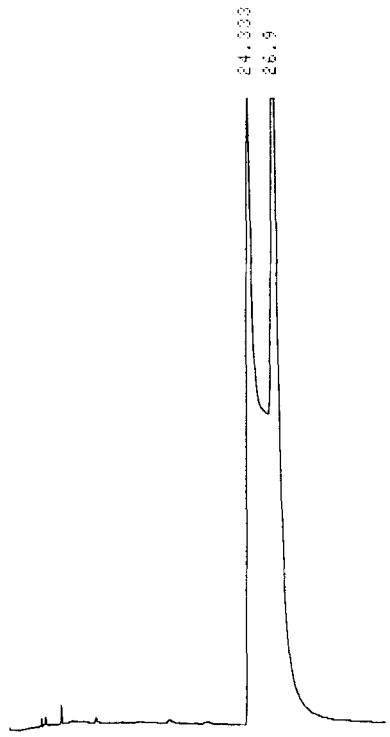

FIGURE 4. Separation of LZ on an OA-3100 column. Mobile phase: hexane/dichloroethane/ethanol (73:20:7, V/V/V). Flow rate: 1.0 mL/min. Detection: 254 nm. Column temperature: 40°C.

(16). These suggest that peak coalescence could be due to racemization of LZ on the OVM-bonded column.

First, we examined whether or not peak coalescence would occur on another column, an OA-3100 column, which is used in the non-queous mode. Figure 3 shows a chromatogram of LZ on an OA-3100 column using hexane-dichloroethane-ethanol (73:20:7,

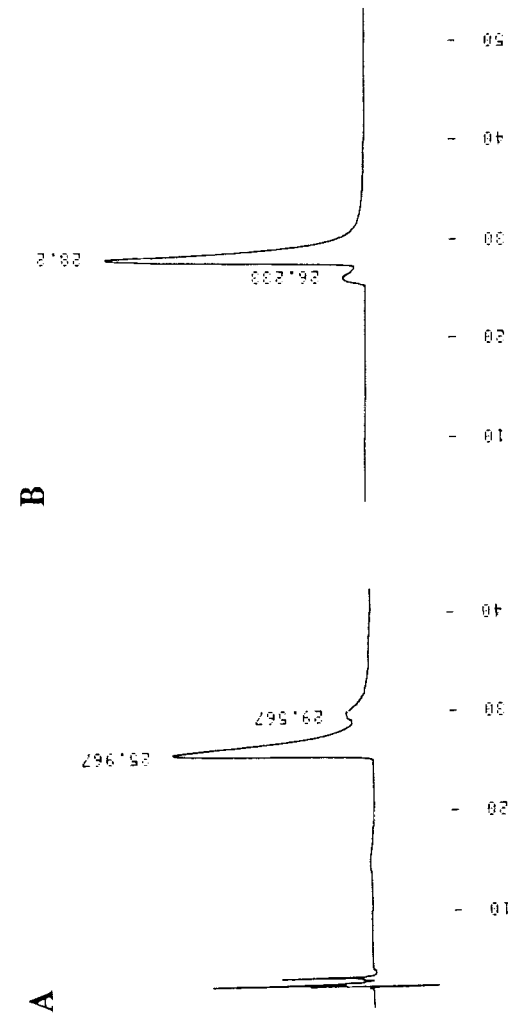

FIGURE 5. Separation of fractions of the first- (A) and second-eluted (B) LZ enantiomer in Figure 3, on an OA-3100 column.
Other chromatographic conditions are the same as in Figure 3.

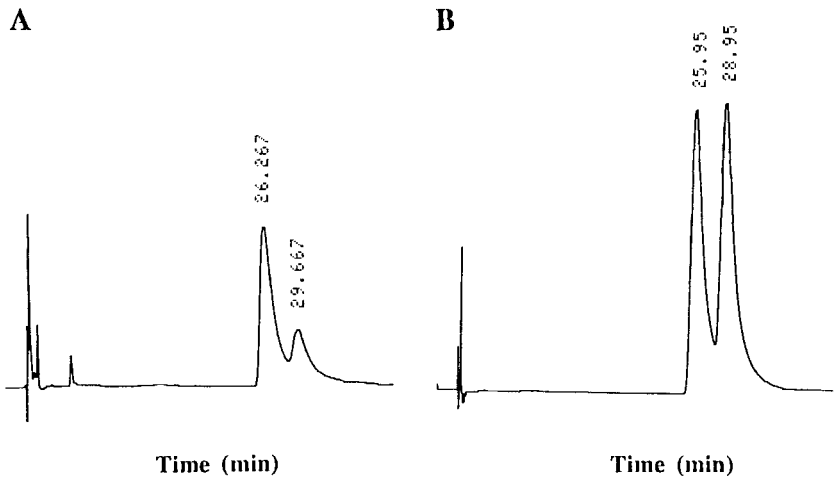

FIGURE 6. Separation of fractions of the first-eluted LZ enantiomer in Figure 3 on an OA-3100 column. Sample: dissolved in ethanol and stored for 20 min (A) and 2 h (B) at a column temperature of 10°C. Other chromatographic conditions are the same as in Figure 3.

V/V/V) as an eluent at a column temperature of 10°C. The column temperature was raised to 15, 30 and 35°C, resulting in no peak coalescence. When the column temperature was elevated to 40°C, peak coalescence appeared, as shown in Figure 4. These results suggest that peak coalescence due to racemization of LZ could occur on the OA-3100 column with a non-aqueous eluent [hexane-dichloroethane-ethanol (73:20:7, V/V/V)] by elevating column temperature.

Next, we tried to isolate each LZ enantiomer on an OA-3100 column and to examine racemization of each LZ enantiomer in two different solutions. Fractions of the two peaks in Figure 3,

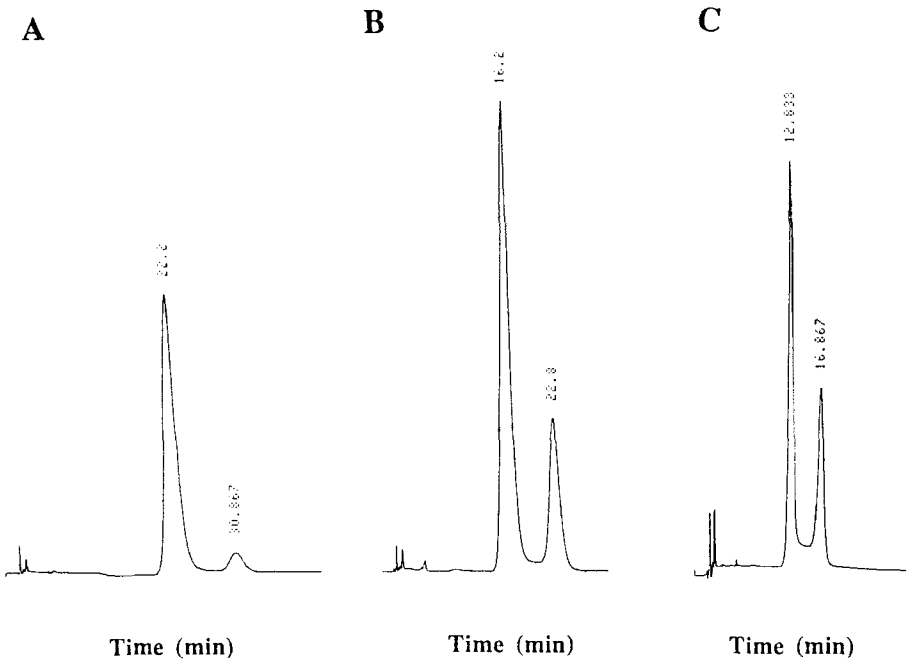

FIGURE 7. Chromatograms of fractions of the second-eluted LZ enantiomer obtained with an OVM-bonded column at various column temperatures.
Mobile phase: 20 mM KH_2PO_4 (pH 4.6)/2-propanol (100:10, V/V). Flow rate: 1.0 mL/min. Detection: 254 nm. Column temperatures: 7°C (A), 15°C (B) and

resolved on the OA-3100 column, were collected and evaporated, and the residues were again chromatographed with the same conditions as in Figure 3. As shown in Figures 5A and 5B, the fractions of first- and second-eluted LZ enantiomers were separated on the OA-3100 column at a column temperature of 10°C with peak area ratios of 95:5 and 5:95, respectively, without peak coalescence. The presence of the opposite enantiomer in the isolated fractions is mainly due to incomplete separation of each enantiomer peak.

Furthermore, after dissolving in hexane-dichloroethane-ethanol (73:20:7, V/V/V) and storing for 20 minutes at 10°C, the isolated LZ enantiomers did not racemize. On the other hand, Figures 6A and 6B show chromatograms obtained with the isolated first-eluted LZ enantiomer, after dissolving in ethanol and storing for 20 minutes and 2 hours, respectively, at 10°C. The peak area ratios were about 75:25 and 50:50, respectively. These results reveal that the LZ enantiomer does not easily racemize in hexane-dichloroethane-ethanol (73:20:7,V/V/V), but easily racemizes in ethanol. This is consistent with the fact that the racemization rate of LZ enantiomers becomes faster with an increase in solvent polarity (16).

We then examined peak coalescence on an OVM-bonded column. Figures 7A-7C show chromatograms of the isolated second-eluted LZ enantiomer in Figure 3. These were obtained with the OVM-bonded column using 20 mM potassium dihydrogenphosphate (pH 4.6) - 2-propanol (100:10,V/V) as an eluent at column temperatures of 7, 15 and 20°C. The peak area ratios were 95:5, 73:27 and 60:40, respectively, at column temperatures of 7, 15 and 20°C. These results mean that no racemization occurs at a column temperatures of 7°C, but that at column temperatures of 15 and 20°C, racemization occurs during chromatography, causing peak coalescence. Note that the elution order of LZ enantiomers should be different between the OA-3100 and OVM-bonded columns.

It is concluded that peak coalescence takes place for chiral separation of LZ on an OVM-bonded column above column

temperatures of 15°C, and that this is due to racemization of LZ on the OVM-bonded column during chromatography. Also, by reducing the column temperature to 7°C we could determine the enantiomeric composition of LZ before chromatography on the OVM-bonded column.

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