Effect of Temperature on Enantiomer Separation of Oxzepam and Lorazepam by High-Performance Liquid Chromatography on a β-Cyclodextrin Derivatized Bonded Chiral Stationary Phase

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Abstract

A reversed-phase high-performance liquid chromatography (HPLC) method with β-cyclodextrin (β-CD) derivatized as chiral stationary phase is used to directly separate oxazepam (Oxa) and lorazepam (Lor) enantiomers. The effect of temperature on the direct HPLC separation of Oxa and Lor enantiomers is studied for the commercially available β-CD derivatized bonded chiral stationary phase. Chromatographic peak coalescence, appearing as a plateau between the resolved peaks, is observed at column temperatures of above 13°C. Peak coalescence on the β-CD derivatized bonded column is attributable to racemization of the Oxa enantiomer. By reducing the column temperature to 13°C, the enantiomeric composition of Oxa and Lor could be determined on the chiral column. This method is expected to be useful for the resolution of 3-hydroxybenzodiazepines. At the same time, the separation mechanism is studied by calculating the thermodynamic parameters. The results reveal that the separation of Oxa and Lor enantiomer is a case of enthalpy-controlled separation, inclusion mechanism does not control the separation. The interaction between Oxa and β-CD is an additionally strong π–π interaction or hydrogen bonding, but that between Lor or β-CD derivatized is a weak π–π interaction or hydrogen bonding.

Introduction

Chiral separation is an important and essential technology in the field of pharmaceutical analysis and drug metabolism. There are many enantiomeric drugs having different biological activities for each enantiomer. Oxazepam (Oxa) and lorazepam (Lor), which belong to the 3-hydroxybenzodiazepines group, are used for anti-anxiety and ataractic. They have an asymmetric C_3 atom and possess a pair of enantiomers (Figure 1), but only S-conformers have pharmacological activity.

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, β-cyclodextrin (CD)-bonded phases are becoming increasingly popular because of their direct optical resolution and their wide chiral recognition for the drug enantiomers. Armstrong et al. (1,2) have demonstrated the broad enantioselectivity of chemically bonded CD phases for numerous HPLC separations of enantiomers. Normally, in gas chromatography (GC), temperature is the main parameter to be varied for optimizing separations. The effect of temperature on the resolution of enantiomers by HPLC (3–8) has not been studied greatly. It is known from binding studies that an increase in temperature diminishes the extent of complexation between the guest molecule and β-CD. Therefore, it is expected that separations on chemically bonded β-CD at different temperatures should give different results. In fact, chiral separations on such a stationary phase have been improved by decreasing the temperature (9,10).

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 [i.e., split peaks, a plateau sandwiched between two peaks, and a tailed peak (11)]. We have determined Oxa in plasma by CSPs for HPLC (12).

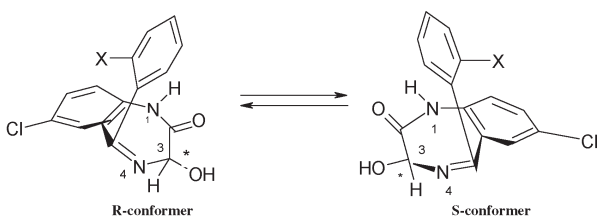

Figure 1. Reversible enantiomerization of Oxa (X = H) and Lor (X = Cl).

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This paper describes the influence of temperature on the chiral HPLC separation of Oxa and Lor using chemically bonded β-CD derivatized as the stationary phase. Our aims for this study are to clarify that the peak coalescence is caused by racemization of Oxa on a β-CD derivatized bonded column during chromatography, and to investigate into the enantioselective separation mechanism of Lor and Oxa on the β-CD derivatized bonded column by calculating the thermodynamic parameters.

Experimental

Reagents and chemicals

All of the following reagents were of HPLC grade or analytical grade. Hexanes, isopropanol, acetonitrile, triethylamine, and KH_2PO_4 were obtained from Tedia company (Fairfield, OH) and Shanghai Reagent Factory (Shanghai, China). Water was purified by a Milli-Q system (Millipore, Bedford, MA). Racemates of Oxa and Lor were kindly supplied by pharmaceutical companies. A 20-µL mobile phase solution of Oxa (10 µg/mL) or Lor (10 µg/mL) was injected into the column.

Apparatus

The HPLC system consisted of an LC-10Avp pump, Shimadzu 7725I sample injector equipped with a 50-µL loop, and SPD-10Avp UV–vis detector (Shimadzu, Kyoto, Japan). Column temperatures of 9–24°C were controlled by placing the column in a BX-7700 automatic column thermostatic oven (Ishido, Chiba, Japan). Chromatograms were recorded and integrated on a Chromatopac CDP-4S (Shimadzu) integrator.

The following two CSP columns were used in this study: (*i*) A chiral stationary phase of Cyclobond-I-2000-RSP (5 µm) column (Advanced Separation Technology, Whippany, NJ) was used. It was packed with β-CD derivatized with R,S-hydroxypropyl ether, chemically bonded to a polymer gel. (*ii*) A Chiralcel-OJ column was purchased from Daicel Chemical Industries (Tokyo, Japan), and cellulose tris(4-methylbenzoate) was coated on silica gel with a particle size of 10 µm. A guard column matching the analytical column was installed between the analytical column and injector.

Results and Discussion

Figure 2 shows the effect of temperature on the resolution of racemic Oxa and Lor obtained with column temperatures of 9°C, 13°C, 18°C, and 24°C on a β-CD derivatized bonded column, respectively. The eluent used was a mixture of acetonitrile–H_2O–1% triethylamine (17:75:8 v/v/v, pH 4.5). Chromatographic peak coalescence (the spontaneous transform from one isomer to the other) in Figure 2 was observed at column temperatures of greater than 13°C. Unit resolution was obtained by reducing the temperature to 13°C. The effect of improved resolution at lower temperatures has been reported previously for several racemic mixtures on α-acid glycoprotein- and bovine serum albumin protein-based columns (1,3). Burkle et al. (13) reported that peak coalescence was caused by oncolumn enantiomerization, along with the kinetics on chiral stationary phases in GC columns. This effect has been described by some working in GC (14) and HPLC (15). Also, enantiomers of Oxa and Lor undergo easy chiral inversion in the polar medium (16,17). This suggests that peak coalescence could be caused by racemization of Oxa.

In order to clarify that the peak coalescence in Figure 2 is caused by racemization of Oxa on a β-CD derivatized bonded column during chromatography, first, we isolated each Oxa enantiomer on a Chiralcel OJ in the nonaqueous mode at a column temperature of 9°C (Figure 3). We have not found peak coalescence below the column temperature of 24°C at the same condition. Fractions of the two peaks in Figure 3 were collected and evaporated, respectively. The residues were again chromatographed with the same conditions as in Figure 3, and the fractions of first- and second-eluted Oxa enantiomers were separated as shown in Figure 4, respectively. We found no peak coalescence. The presence of the another enantiomer in Figure 4 is attributable to incomplete separation of each enantiomer. Furthermore, after dissolving in hexane–2-propanol (9:1 v/v) and storing for 30 min at 9°C, the isolated Oxa enantiomers did not racemize. Then, we examined peak coalescence of Oxa on a β-CD derivatized bonded column. Figure 5 shows chromatograms of the isolated second-eluted Oxa enantiomer in Figure 2. These were obtained with the β-CD derivatized bonded column using acetonitrile–H_2O–1% triethylamine (17:75:8 v/v/v, pH 4.5) as an eluent at column temperatures of 9°C, 13°C, and 18°C. The peak area ratios were 96:4, 78:22, and 65:35, respectively. These results mean that no racemization occurs at column temperatures of 9°C and 13°C, but that at column temperature of 18°C, racemization occurs during chromatography, causing peak coalescence. Therefore, by reducing the column temperature to 13°C, we could determine the enantiomeric composition of Oxa and Lor on the β-CD-bonded column.

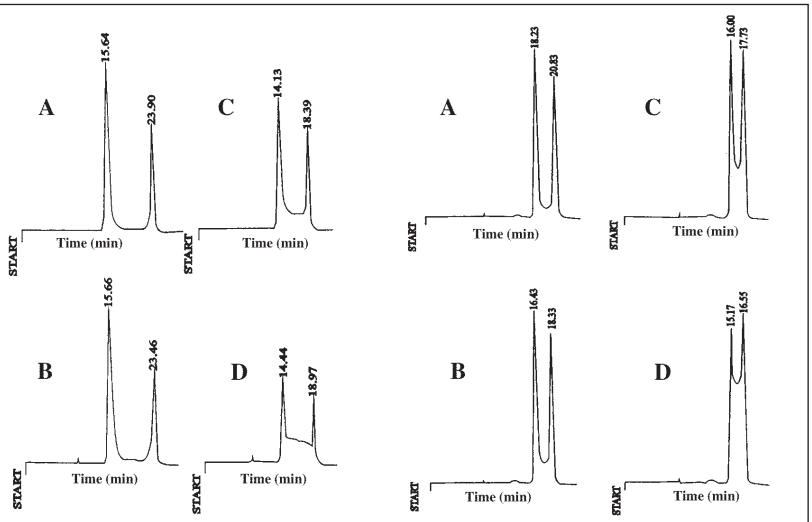

Figure 2. Chromatograms of Oxa (left) and Lor (right): separation at 9°C (A), 13°C (B), 18°C (C), and 24°C (D) as column temperatures, respectively, on a β-CD derivatized column with an acetonitrile–H_2O–1% triethylamine (17:75:8) mobile phase at pH 4.5. The flow rate was 0.5 mL/min.

To obtain more information about the chiral discrimination process, it is worth examining the Gibbs-Helmholz parameters ($D_{R,S}DH^0$ and $D_{R,S}DS^0$). The difference between the free energies can be calculated from the difference in retention via enantioselectivity α according to the following equation:

$$-\Delta_{R,S}\Delta G^0 = RT\ln\alpha,\ \alpha = k'_R/k'_S \text{ with } k'_R > k'_S \qquad \text{Eq. 1}$$

The combination of equation 1 with the Gibbs-Helmholz relationship:

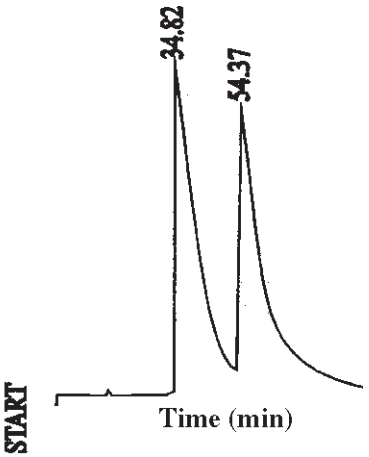

Figure 3. Separation of Oxa on a Chiralcel OJ column at a column temperature of 9°C with *n*-hexane–2-propanol (9:1) mobile phase. The flow rate was 1 mL/min.

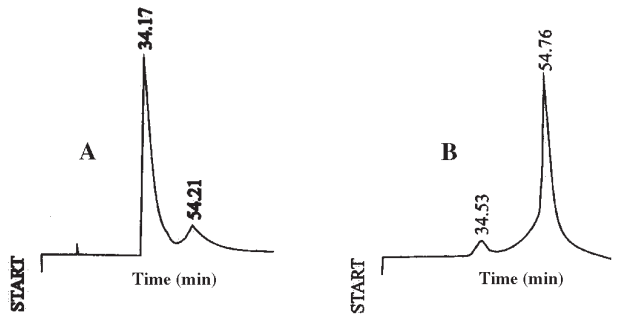

Figure 4. Separation of fractions of the first- (A) and second-eluted (B) Oxa enantiomer in Figure 3, at the same separation conditions as Figure 3.

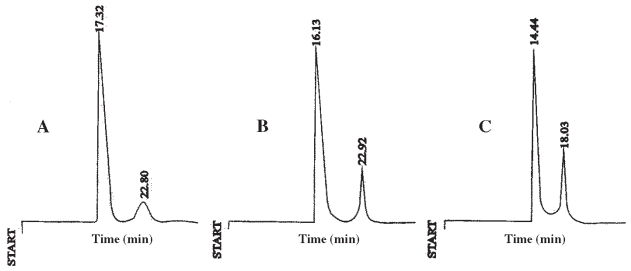

Figure 5. Chromatograms of the second-eluted Oxa enantiomer obtained with a β-CD derivatized column at temperatures of 9°C (A), 13°C (B), and 18°C (C), respectively. Other separation conditions were as shown in Figure 2.

$$-\Delta_{R,S}\Delta G^0 = -\Delta_{R,S}\Delta H^0 + T\Delta_{R,S}\Delta S^0 \qquad \text{Eq. 2}$$

gives the following:

$$\ln\alpha = -\Delta_{R,S}\Delta H^0/R\bullet 1/T + \Delta_{R,S}\Delta S^0/R \qquad \text{Eq. 3}$$

According to equation 3, the enthalpy and entropy differences for the interaction of the two enantiomers with the stationary phase can be obtained by plotting ln α versus 1/T. The slope of these plots represents $-D_{R,S}DH^0/R$, and the intercept is related to $D_{R,S}DS^0/R$. Further, from the Gibbs-Helmholz relationship (equation 3), there exists a certain temperature T_{iso} (isoenantioselective temperature) where $-D_{R,S}DG^0 = 0$, owing to enthalpy–entropy compensation:

$$T_{iso} = \Delta_{R,S}\Delta H^0/\Delta_{R,S}\Delta S^0 \qquad \text{Eq. 4}$$

At T_{iso}, no separation of enantiomers will occur.

The capacity factor (k') and separation factors ($\alpha = k_2'/k_1'$) obtained are given in Table I. As expected, the plot of ln α versus 1/T illustrates linear behavior for Oxa and Lor (Figure 6). This usually occurs when there is no change in the retention mechanism as a function of temperature. The two drugs show an entirely same pattern with temperature change and the same chiral discrimination behavior described by ln α as a function of the reciprocal of absolute temperature (l/T). Obviously the change in temperature equally influences the interaction of enan-

Table I. Resolution of Enantiomers of Oxa and Lor

Temperature	Oxa				Lor			
Flow rate	k_1'	k_2'	α	R	k_1'	k_2'	α*	R†
9°C								
0.5 mL/min	2.19	3.87	1.77	5.24	2.71	3.24	1.20	1.89
13°C								
0.4 mL/min	2.76	4.49	1.70	6.32	3.30	3.83	1.17	1.14
0.5 mL/min	2.13	3.70	1.75	4.91	2.95	3.55	1.20	1.87
0.6 mL/min	1.61	2.92	1.74	5.51	2.03	2.44	1.19	1.19
0.7 mL/min	1.23	2.36	1.75	4.92	1.59	1.95	1.19	1.15
18°C								
0.5 mL/min	1.88	3.07	1.64	NS‡	2.26	2.61	1.16	NS
24°C								
0.5 mL/min	1.79	2.86	1.60	NS	2.09	2.37	1.14	NS

* Separation factor (k_2'/k_1').
† R, resolution = $2(t_2 - t_1)/(w_2 + w_1)$.
‡ Not separated.

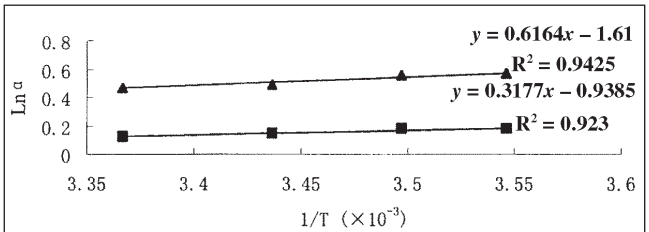

Figure 6. Ln α—1/T of Oxa (▲) and Lor (■).

Table II. Thermodynamic Data for the Enantiomeric Separation of Oxa and Lor

Enantiomer	Stationary phase	$-\Delta_{(R,S)}\Delta H$ (K Cal/mol)	$-\Delta_{(R,S)}\Delta H$ (Cal/mol • K)	Relative coefficient (*r*)	T_{iso} (°C)
Oxa	β-CD derivatized	1.23	3.20	0.9707	110°C
Lor	β-CD derivatized	0.63	1.86	0.9602	67°C

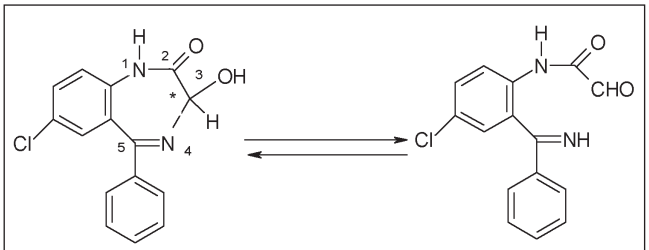

Figure 7. Mechanism of racemization of Oxa.

tiomers with the stationary phase. This may be better understood if one considers the thermodynamics involved in the separation.

Thermodynamic data for the chiral separation of Oxa and Lor are given in Table II. First, thermodynamic data revealed that the natural logarithms of the retention factors (ln α) of the investigated compounds depended linearly on the inverse of temperature (1/T). The $D_{R,S}DH^0$ values for the two pharmaceuticals have the same negative signs. From Table II, it is also found that the $D_{(R,S)}DH$ value of Oxa is more than –1.0 K cal/mol, and the $D_{(R,S)}DH$ value of Lor is between –0.5 and –1.0 K cal/mol. According to Kusters et al. (18), the separation of Oxa and Lor enantiomer is a case of enthalpy-controlled separation; the inclusion mechanism does not control the separation.

Next, if the inclusion mechanism had really controlled the separation, the enantioselectivity of Oxa and Lor would have been increased as the flow rate of mobile phase increased. But as we can see from Table I, the changes of enantioselectivity of Oxa and Lor are nearly the same with the flow rate of the mobile phase, from 0.4 to 0.7 mL/min. Those phenomena support our deduction; in a word, inclusion mechanism does not control the separation.

Finally, in our experiment, T_{iso} is 110°C and 67°C for Oxa and Lor, respectively. Koppenhoer and Bayer (19) predicted that at above T_{iso} the separation is entropy controlled. This prediction supports our conclusion. The chiral separation of Oxa and Lor is entropy controlled, specifically, it is enthalpy–entropy compensation (20,21).

Conclusion

β-CD derivatized, chemically bonded to silica, had been used as a chiral stationary phase for the HPLC separation of two chiral pharmaceuticals: Oxa and Lor. The influence of temperature on the separation of these two compounds was studied in detail. It is concluded that peak coalescence takes place for chiral separation of Oxa on a β-CD derivatized bonded column at column temperatures of greater than 13°C. The raised baseline pattern between the peaks for the two enantiomers is caused by racemization of Oxa on a β-CD derivatized bonded column during passage through the column. The chiral center of Oxa is located in a seven-ring structure that opens easily, thereby causing a chin in the configuration as shown in Figure 7.

The oncolumn enantiomerization of benzodiazopinones during chromatography has been noted by others (11). The problem of enantiomerization can, however, be avoided if the chromatography is performed at column temperatures of less than 13°C in our experiment.

The retention and selectivity factors for the enantiomers of all investigated compounds decreased with increasing temperature. The natural logarithms of the selectivity factors (ln α) of the investigated compounds depended linearly on the inverse of temperature (1/T). van 't Hoff plots afforded thermodynamic parameters, such as the apparent change in enthalpy (DH°), the apparent change in entropy (DS°) and the apparent change in Gibbs free energy (DG°) for the transfer of analyte from the mobile to the stationary phase. The thermodynamic constants DH°, DS°, and DG° were calculated in order to promote an understanding of the thermodynamic driving forces for retention in this chromatographic system. Thermodynamic data revealed that the separation mechanism of the Oxa and Lor enantiomers is a case of enthalpy-controlled separation, inclusion mechanism does not control the separation. The interaction between Oxa and β-CD is an additional strong π–π interaction or hydrogen bond, but that between Lor or β-CD is weak π–π interaction or hydrogen bond.

Acknowledgments

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