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Stereospecific analysis of lorazepam in plasma by chiral column chromatography with a circular dichroism-based detector

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Abstract

The chiral separation of lorazepam was achieved on a chiral column with UV and circular dichroism (CD) detection. The good resolution of lorazepam enantiomers was obtained on the column of β-cyclodextrin derivative immobilized silica gel under reversed-phase conditions. The enantiomeric separation and identification of lorazepam were succeeded by CD detector. The method described allows the quantitation of the stereoisomers of lorazepam in human plasma following the administration of a therapeutic dose of the racemic drug. Chiroptical detection is useful for the pharmacokinetic study of chiral drugs.

Keywords: Enantiomer separation; Circular dichroism detection; Detection, LC; Lorazepam; Benzodiazepines

1. Introduction

Lorazepam, 3-hydroxybenzodiazepine with a chiral center at the C3 position, is marketed as a racemate. Benzodiazepines may exert antianxiety effects through the potentiation of the inhibitory neurotransmitter, GABA (γ-aminobutyric acid). Lorazepam is used clinically as an antianxietic or a sedative with central nervous system depressant activity.

Though there are several reports for enantiomeric separation of benzodiazepines, the stereochemical compositions of the drug in biological fluids have been scarcely studied. We have investigated the analysis of sedatives and anesthetics in biological fluids by using liquid chromatography–mass spectrometry (LC–MS) [1,2] and have been interested in determining the enantiomeric composition of the drug in plasma after racemate administration. Recently, we reported the chiral separation of lorazepam on chiral column by LC–MS [3].

Chiral drugs continue to be a significant force in the pharmaceutical market [4]. Enantioselective metabolism and excretion are known in various chiral drugs. Sometimes specific pharmaceutical activity is present in only one enantiomer and inactive in the other, or the other enantiomer has a different kind of activity from the first approved as a racemate. Pharmacological studies such as chiral-form stability, interconversion, pharmacokinetic properties, and dosage equivalence of chiral therapeutics are required.

Recent advances in methods to resolve racemic mixture is to exploit the subtle three-dimensional

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differences of the enantiomers [5–8]. Depending on the chirality of a molecule, it will differ in its ability to rotate plane-polarized light in either of two opposite directions. The optical rotation (OR) detectors have commonly been used for detection of chiral compounds in high-performance liquid chromatography (HPLC). Circular dichroism (CD) detection based on the absorption difference between left and right circularly polarized light is also effective in separating chiral mixtures [5–7,9,10]. In this study, we investigated stereospecific analysis of lorazepam in plasma by chromatography–CD detection.

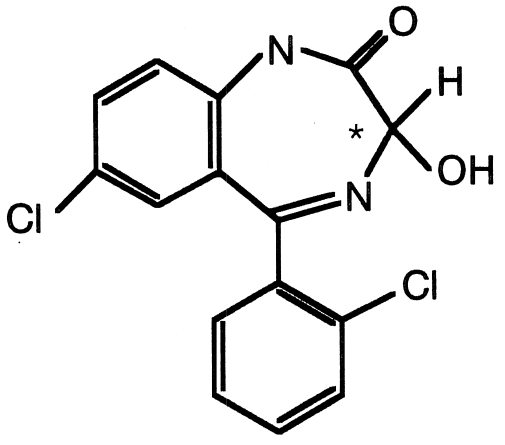
2. Experimental

2.1. Material and reagents

Lorazepam was purchased from Sigma (St. Louis, MO, USA). The structures of the drug are shown in Fig. 1. Water was distilled and passed through a Milli-Q purification system (Millipore, Bedford, MA, USA). All other chemicals and solvents were of analytical-reagent grade.

2.2. Apparatus

HPLC was performed using an L-6200 pump, a D-2500 integrator (Hitachi, Tokyo, Japan), a Shodex AO-30 column oven (Showa Denko, Tokyo, Japan), and a CD-1595 circular dichroism detector (Jasco, Tokyo, Japan).



Lorazepam

Fig. 1. The structures of lorazepam.

2.3. Chromatographic conditions

Chiralcel OD (250×4.6 mm I.D., Daicel, Tokyo, Japan), a column packed with cellulose tris(3,5-dimethylphenylcarbamate) was used under normal-phase conditions. The mobile phase for chiral separation was *n*-hexane–2-propanol–ethanol (5:5:1) and the eluate was monitored at 254 nm. The flow-rate was 1 ml/min and the column temperature was 25°C.

Shodex ORpak CDBS-453 (150×4.6 mm I.D., Showa Denko), a column packed with silica gel chemically bonded by a derivative of β-cyclodextrin was used under reversed-phase conditions. The mobile phase was a mixture of acetonitrile–0.2 *M* NaCl (13:87, v/v) containing acetic acid (1%, v/v), and its flow-rate was 0.5 ml/min. The UV detector was set at 254 nm and the column temperature was 15°C.

2.4. Sample preparation

Plasma sample was a 200-μl volume of human plasma spiked with 2 μg of lorazepam. To remove proteins prior to injection, the plasma sample was pretreated with solid-phase extraction as follows; a 200-μl volume of the plasma was loaded onto an Oasis HLB cartridge (Waters, Milford, MA, USA) after conditioning the cartridge with methanol, water and 0.2 *M* NaCl containing 1% acetic acid. A 3-ml volume of 0.2 *M* NaCl containing 1% acetic acid and 5 ml volume of water as washing solvents were passed through the cartridge. The sample fraction was obtained by elution with 5 ml of methanol. After evaporation, the residue was dissolved in 100 μl of the eluent.

A series of calibration standards were prepared by dissolving known amounts of lorazepam in drug-free human plasma. Calibration curves were constructed by plotting peak area against concentration of the lorazepam and the data were subjected to linear regression analysis.

Plasma samples were collected from a patient who was being treated with lorazepam (Wypaks; Yamanouchi Pharmaceuticals, Tokyo, Japan). The

samples were stored frozen at −20°C until required for analysis. A 1-ml volume of the plasma was loaded onto an Oasis HLB cartridge. After the same treatments as described above, the residue was dissolved in 100 μl of the eluent.

3. Results and discussion

Fig. 2 shows UV and CD chromatograms of lorazepam on the Chiralcel OD column under the conditions described in Experimental. The first and second peak on the UV chromatogram with retention times of 5.49 and 6.20 min are assigned to (+)- and (−)-lorazepam, respectively, from the CD chromatograms. The (+)-enantiomers of 3-substituted 1,4-benzodiazepines have been found to correspond to the *S*-enantiomers [11,12].

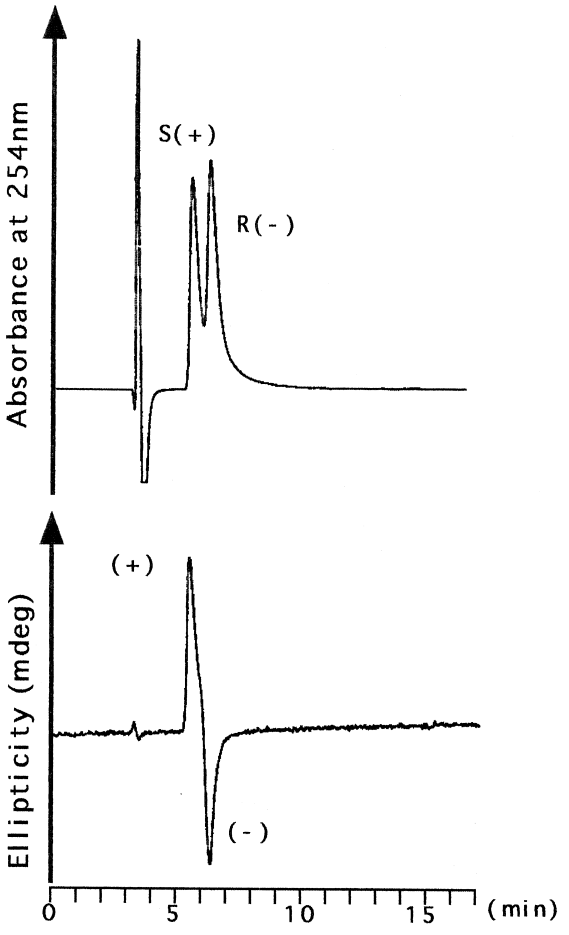


Fig. 2. UV (upper) and CD (lower) chromatograms of lorazepam on the Chiralcel OD column. Chromatographic conditions are described in Experimental. Injection volume is 10 μl containing 1 μg of the racemic lorazepam.

UV and CD chromatograms of lorazepam under the reversed-phase conditions are shown in Fig. 3. The column used for chiral separation was the Shodex ORpak CDBS-453 (150×4.6 mm) using acetonitrile–0.2 *M* NaCl (13:87, v/v) containing acetic acid (1%, v/v) as an eluent. The flow-rate was 0.5 ml/min and the column temperature was 15°C. The CDBS-453 column is β-cyclodextrin derivative immobilized silica gel. The good resolution of lorazepam enantiomers was obtained on the column under the conditions. The enantiomeric elution order was *R*- and *S*-lorazepam with retention times of 29.52 and 43.20 min, respectively.

The elution order of lorazepam enantiomers was reversed on the CDBS-453 column under the reversed-phase conditions. UV detection does not distinguish between a pair of enantiomers but CD detection does by responding in the opposite direction. Table 1 shows the selectivity factors (α) and resolutions (R_s) of lorazepam enantiomers on the chiral columns. R_s on the CDBS-453 column was not less than 1 and higher than on the Chiralcel OD column.

3.1. Plasma sample

Fig. 4 shows UV and CD chromatograms of an extract of human plasma spiked with lorazepam and blank plasma. The well resolved chromatogram with chiral separation was obtained without any influence of endogenous compounds in plasma.

The (−)-enantiomer of lorazepam is eluted faster than (+)-enantiomer on the column. Absolute con-

Table 1
Chromatographic parameters for the separation of lorazepam[a]

Column	k_1	k_2	α	R_s
CDBS-453	5.2	8.1	1.6	22.8
Chiralcel OD	0.7	0.9	1.3	1.8

[a] Retention factor: $k=t_R/t_0$, k_1=retention factor for the first-eluted enantiomer, selectivity factor: $\alpha=k_1/k_2$, resolution: $R_s=2(t_{R2}-t_{R1})/(w_1+w_2)$ where w is the baseline peak width.

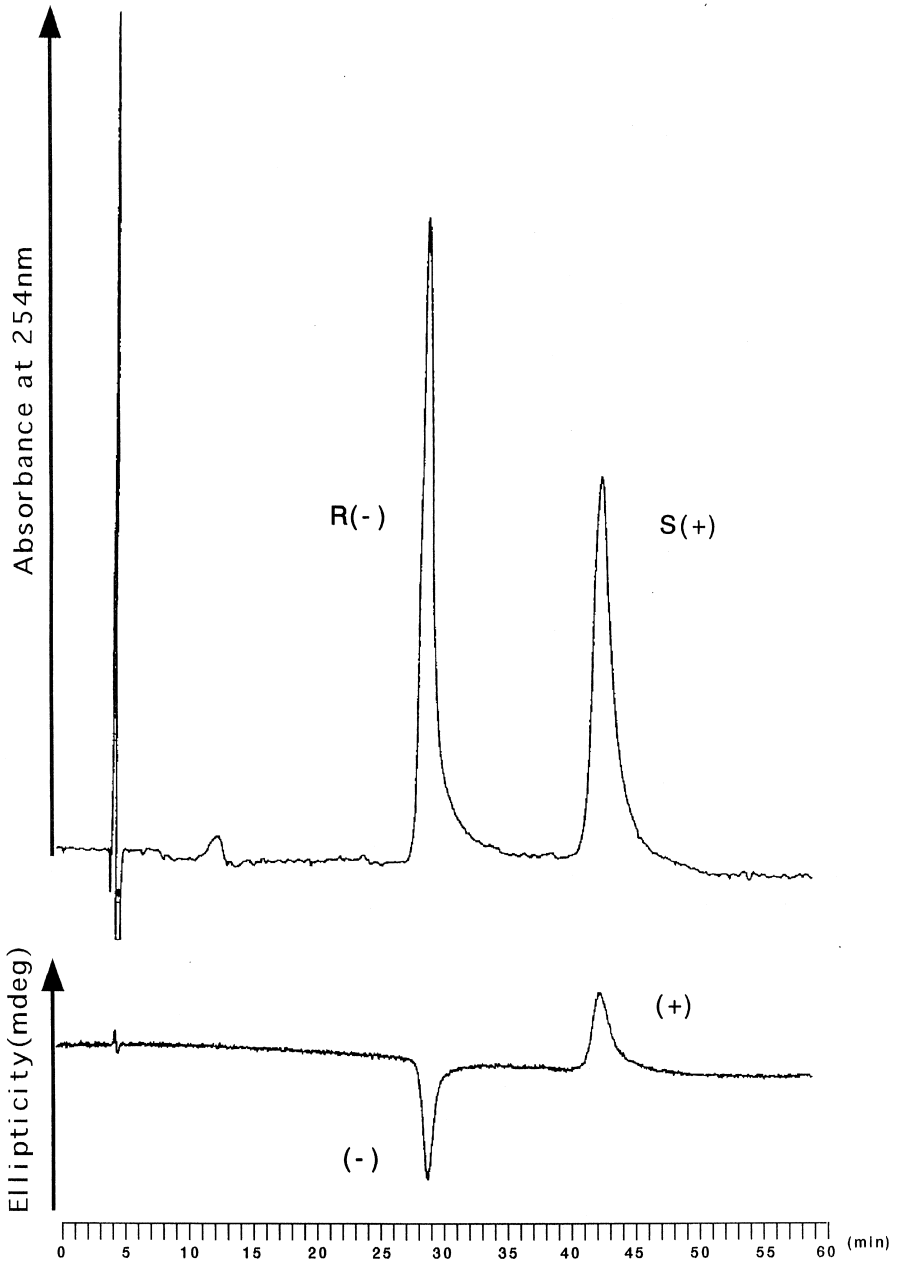


Fig. 3. UV (upper) and CD (lower) chromatograms of lorazepam on the CDBS-453 column. Chromatographic conditions are described in Experimental. Injection volume is 10 μl containing 1 μg of the racemic lorazepam.

figurations of the enantiomer can be assigned according to their CD spectral properties. Recent studies indicated that the pharmacologically more active *S*-enantiomer of benzodiazepines was metabolized at a slower rate than the pharmacologically less active *R*-isomer [13]. The clinical implications of these enantioselective metabolic pathways remain to be established.

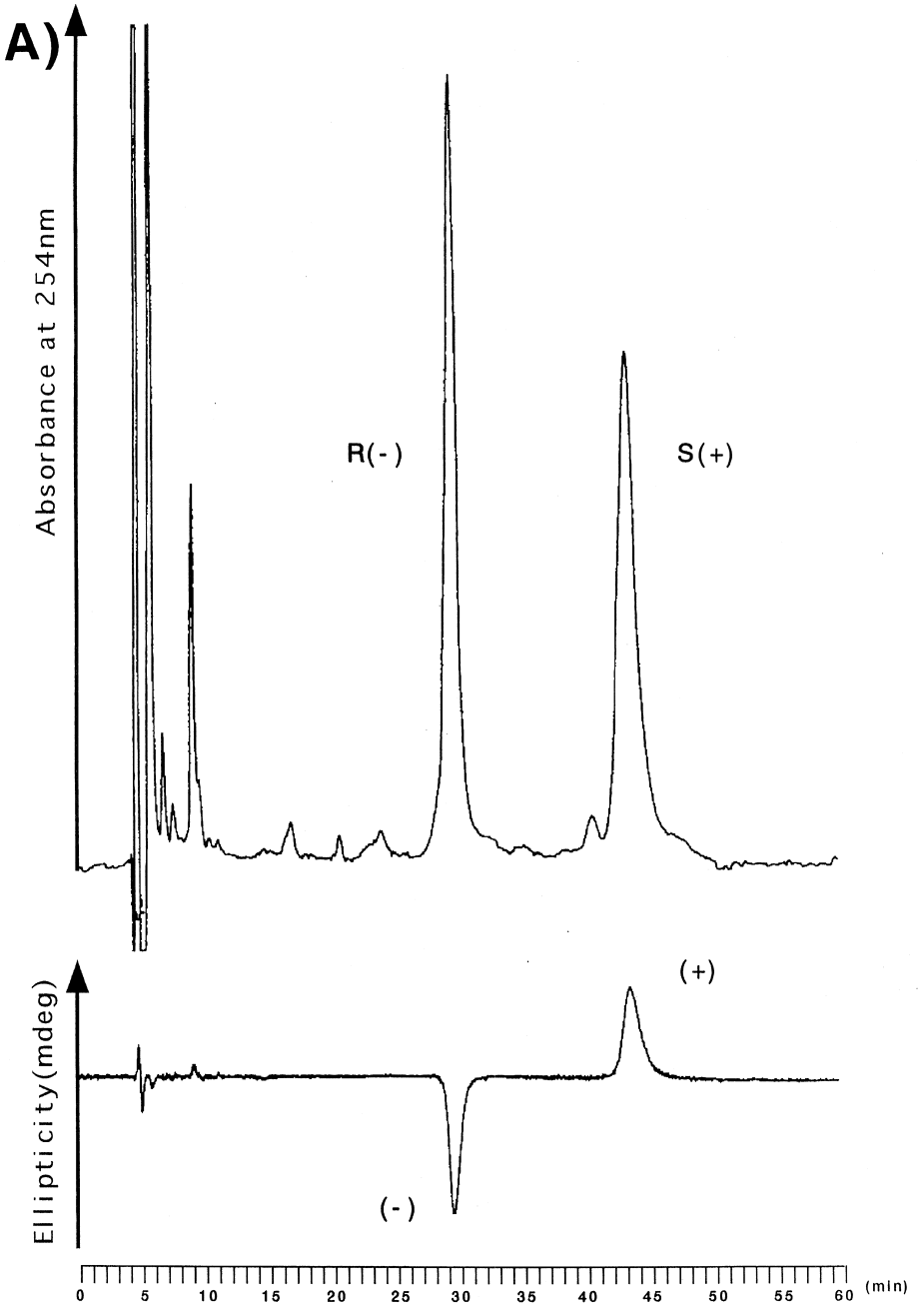


Fig. 4. UV (upper) and CD (lower) chromatograms of an extract of human plasma spiked with lorazepam (A) and blank plasma (B). Chromatographic conditions are described in Experimental. Injection volume is 50 μl.

3.2. Calibration curves and precision

The linearities between the amount of racemic lorazepam and the peak areas of each enantiomer in the CD chromatogram were obtained up to 0.1 μg. The relationship calculated between the peak area

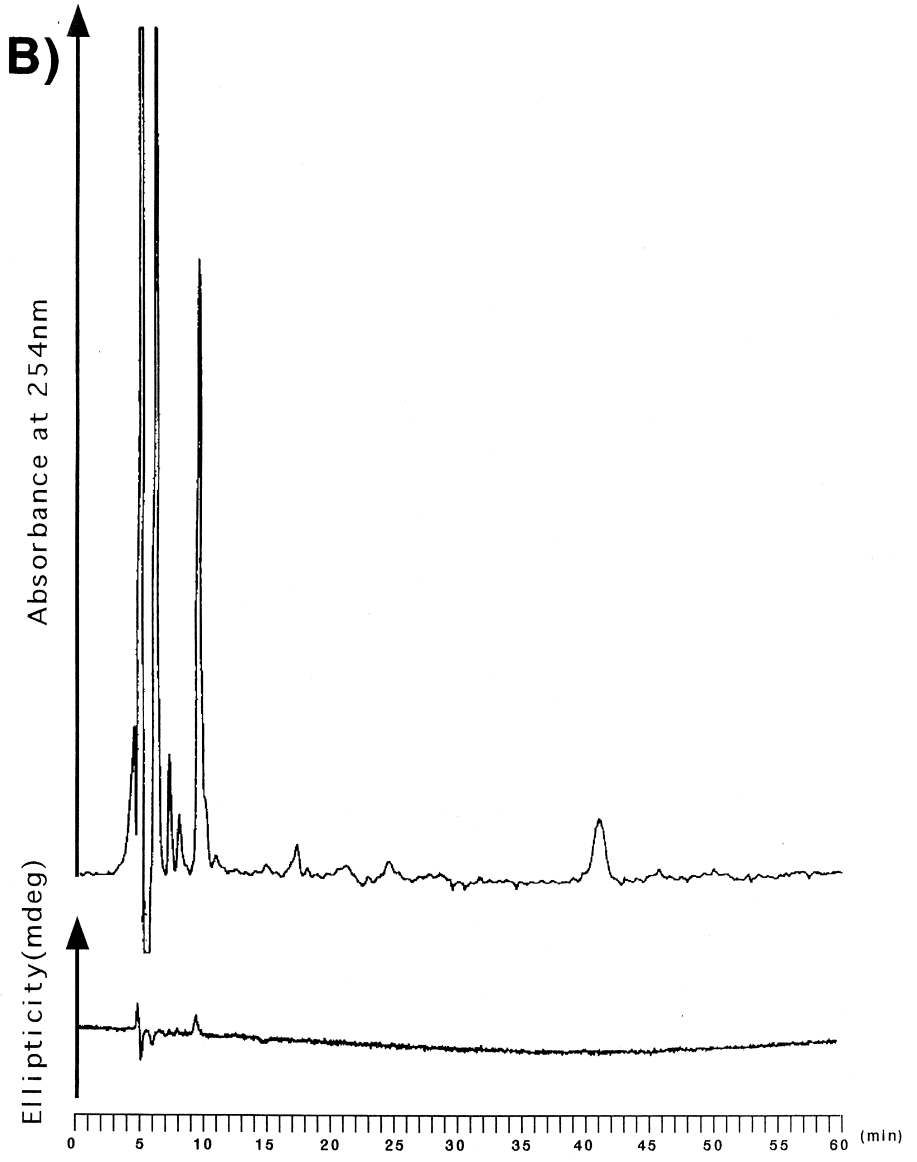


Fig. 4 (*continued*).

(y) and the μg amounts of lorazepam (x) and the correlation coefficients (r) were as follows: (−)-lorazepam; $y/10^4=1.81x-0.01$ ($r^2=0.996$) and (+)-lorazepam; $y/10^4=1.92x-0.03$ ($r^2=0.984$).

The lower limit of quantification for lorazepam from plasma sample was 3 ng/ml, at a signal-to-noise ratio of 3. In our previous report, the calibration graphs were obtained using a standard dissolved in mobile phase [7]. Therefore the method needed to be improved in order to monitor therapeutic drug concentration in plasma after administration. We improved mobile phase conditions and pretreatment procedure. The precision of the enantiomeric composition of racemic lorazepam was established from five assays and the mean values and relative standard deviations (RSDs). The RSD values of each enantiomer in the UV chromatograms were less than 5%.

3.3. Application to the quantitation of the enantiomers of lorazepam in human plasma

Fig. 5 shows UV and CD chromatograms of an extract of plasma sample obtained from the patient orally administered racemate. The well resolved chromatogram with chiral separation was obtained without any influence of endogenous compounds in plasma. The peak of *S*-enantiomer of lorazepam was identified with retention time of 42.38 min, but that

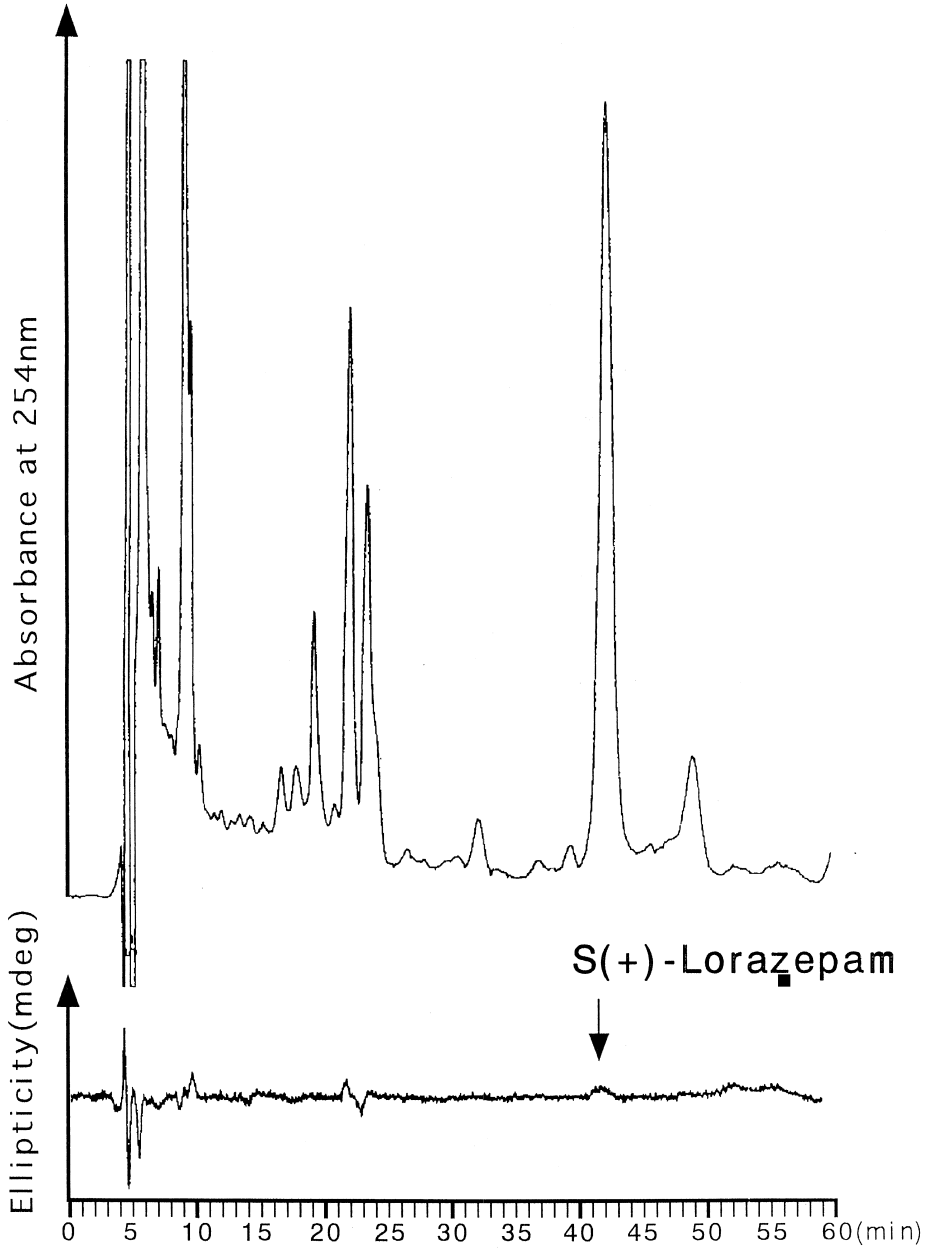


Fig. 5. UV (upper) and CD (lower) chromatograms of an extract of human plasma obtained from the patient orally administered lorazepam. Chromatographic conditions are described in Experimental. Injection volume is 50 μl.

of *R*-enantiomer was not observed in the plasma. The concentration of *S*-enantiomer as determined by the present method, was 32 ng/ml.

Lorazepam has short-to-intermediate half-life of elimination. The usual oral dosage of the drug for treatment of anxiety may range from 1–10 mg daily in divided doses. The present method showed acceptable precision and sensitivity for the pharmacokinetic studies of lorazepam enantiomers in biological samples.

In our previous report, the sensitivity by CD detection was more than 50-times of that by OR detection for enantioseparation of lorazepam [7]. The lorazepam enantiomers were not detected by OR detection under the present conditions and the same lorazepam concentration.

4. Conclusion

In present study, the chiral separation of lorazepam was achieved on a chiral column with UV and CD detection. We were successful in enantiomeric separation and identification of lorazepam in plasma by CD detection. Knowledge of the pharmacokinetics of drugs should be necessary in clinical practice. Chiroptical detection is useful for the pharmacokinetic studies of chiral drugs.

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