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

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Abstract

The chiral separation of loxoprofen was achieved on a chiral column with UV and circular dichroism (CD) detection. The good resolution of four loxoprofen stereoisomers was obtained. The column used for the chiral separation was Chiralcel OJ column (250×4.6 mm) using hexane–2-propanol–trifluoroacetic acid (95:5:0.1), as an eluent. The flow-rate was 1.0 ml/min and the detection was at 225 nm. In addition, CD and UV spectra were obtained by stopped flow scanning. The method allows the determination of the stereoisomers of loxoprofen in human plasma after the administration of therapeutic dose of the racemic drug, thus HPLC with CD detector is useful for the stereospecific determination of loxoprofen products in biological samples.

Keywords: Enantiomer separation; Circular dichroism detection; Detection, LC; Loxoprofen; Profens; Nonsteroidal antiinflammatrory drugs

1. Introduction

Loxoprofen, 2-[4-(2-oxocyclopentylmethyl) phenyl]-propionate with two chiral centers, is marketed as an equal parts mixture of four stereoisomers. Loxoprofen sodium is an important non-steroidal antiinflammatory drug (NSAID) of the 2-arylpropionic acid group used for the treatment of rheumatoid arthritis and osteoarthritis. Loxoprofen is a prodrug which produces effects after being absorbed from the gastrointestinal tract followed by conversion to an active metabolite. The mechanism of action of loxoprofen is due to inhibition of prostaglandin biosynthesis by its action on cyclooxygenase. After oral administration, loxoprofen sodium is absorbed as the free acid from the gastrointestinal tract rather than the sodium salt, which causes just weak irritation of the gastric mucosa, and is then converted to an active metabolite by reduction of the ketone carbonyl to the *trans*-OH form. The active isomer has the 2*S*,1*R*′,2′*S* configuration, which potently inhibits prostaglandin biosynthesis [1–3]. Though there are several reports for enantiometric separation of 2-arylpropionic acids, the stereochemical composition of loxoprofen in biological fluids has been rarely studied. We have been interested in determining the stereochemical composition of the drug in plasma after racemate administration [4–8]. Recently, we reported the stereospecific analysis of chiral drugs such as benzodiazepines and 2-arylpropionic

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acids on a chiral stationary phase by LC–MS and circular dichroic (CD) detection [6,9,10].

The analysis of drug enantiomers in biological samples is very important, since the distinct chiral forms of a drug have different reactivities and pharmacokinetic properties. Biological molecules such as enzymes and receptors recognize strictly the chirality of optically active drugs. Enantioselective metabolism and excretion are known in various chiral drugs. Sometimes specific pharmacological activity is present in only one enantiomer the other being inactive, or has a different activity.

Depending on the chirality of the stereoisomer, they will differ in their ability to rotate the plane of polarized light in either of two opposite directions. CD is based on absorption difference between right and left circularly polarized light. This principal of the detection gives high-sensitive detection of optically active compounds with UV absorption (200–400 nm) [11,12]. The coupling of HPLC to a chiroptical detector, namely, a CD detector is one of the most powerful hyphenated techniques for stereochemical investigation [13].

In this study, we investigated the stereospecific analysis of loxoprofen in plasma by chiral-phase chromatography with CD detection.

2. Experimental

2.1. Material and reagents

Loxoprofen sodium and four its isomers were kindly provided by Sankyo (Tokyo, Japan). The structure of loxoprofen is 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.

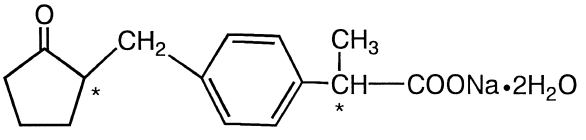

Fig. 1. Structure of loxoprofen sodium dihydrate.

2.2. Apparatus

High-performance liquid chromatography (HPLC) was performed using an L-6200 pump (Hitachi, Tokyo, Japan), a 655-A-52 column oven (Hitachi) and a Browin-NT/HSS-1500 data processor (Jasco, Tokyo, Japan). The column effluent was introduced to a CD-1595 circular dichroism detector (Jasco) and the detector used for monitoring enantiomers based on CD and UV principles. UV and CD spectra were recorded using a CD-1595 detector. Measurement of difference in absorption was performed within 20 μs in CD detection.

2.3. Chromatographic conditions

The chiral stationary phase used for separation of loxoprofen was Chiralcel OJ (250×4.6 mm I.D., Daicel, Tokyo, Japan). The mobile phase was a mixture of 2-propanol–hexane (5:95, v/v) containing trifluoroacetic acid (0.1%, v/v) as a modifier. The flow-rate was 1.0 ml/min at 25°C. UV and CD detector were set at 225 nm.

2.4. Sample preparation

For precision examination, a 200-μl volume of human plasma spiked with 20 μg of loxoprofen sodium was loaded to a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) after conditioning the cartridge with methanol, water and 0.1 *M* ammonium acetate. A 5-ml volume of 0.1 *M* ammonium acetate as washing solvents was passed through the cartridge. The sample fraction was obtained by elution with 5 ml of methanol. After evaporation, the residue was dissolved in 200 μl of the eluent and 10 μl of the sample was injected into the HPLC system.

2.5. Stereochemical composition of loxoprofen in tablet

A loxoprofen sodium tablet (Loxonin®, Sankyo) was pulverized and 20 μg of pulverized the drug was added to 200 μl of human plasma. After the same treatments as described above, the residue was dissolved in 200 μl of the eluent.

2.6. Calibration curve

Standard sample for calibration were prepared as follows: A known quantity of loxoprofen in the concentration range 5–50 μg was added to 200 μl of blank plasma samples. Calibration curves were constructed by plotting the peak-area ratios between loxoprofen and the internal standard versus the amount of loxoprofen in the spiked in drug-free human plasma. The data were subjected to linear regression analysis.

The stereochemical composition of loxoprofen was determined as follows:

$$\text{Stereochemical composition} = \frac{\text{Peak area of stereoisomer}}{\text{Total peak area of four stereoisomers}}$$

2.7. Administration of loxoprofen

Plasma samples were collected from a healthy adult 2 h after a single oral dose of 60 mg loxoprofen sodium (Loxonin®). Venous blood sample was collected into EDTA tubes and separated by centrifugation for 10 min at 1500 rpm. The samples were stored frozen at −20°C until required for analysis. A 500-μl volume of the plasma was loaded to a Sep-pak C_{18} cartridge. After the same treatments as described above, the residue was dissolved in 50 μl of the eluent and 10 μl of the sample was injected into the HPLC system.

3. Results and discussion

3.1. Chiral separation of loxoprofen

The commercially available loxoprofen sodium (Loxonin®) is an equal parts mixture of four optical isomers. UV and CD chromatograms of loxoprofen following extraction from plasma spiked with loxoprofen sodium tablet under the conditions described in the Experimental section are shown in Fig. 2a. The (1′*R*, 2*R*)-loxoprofen, (1′*S*,2*R*)-loxoprofen, (1′*R*,2*S*)-loxoprofen, (1′*S*,2*S*)-loxoprofen were observed at retention times of 22.60 (t_1), 25.57 (t_2), 30.09 (t_3) and 35.49 (t_4) min, respectively, on the UV and the CD chromatograms. The good resolutions of each of four stereoisomers of loxoprofen were obtained on the column. The selectivity factors (α; $\alpha_1 = k_2/k_1$, $\alpha_2 = k_3/k_2$, $\alpha_3 = k_4/k_3$) of loxoprofen stereoisomers under the conditions were 1.14 (α_1), 1.21 (α_2) and 1.20 (α_3), respectively, where k_1–k_4 are the retention factors calculated from t_1–t_4.

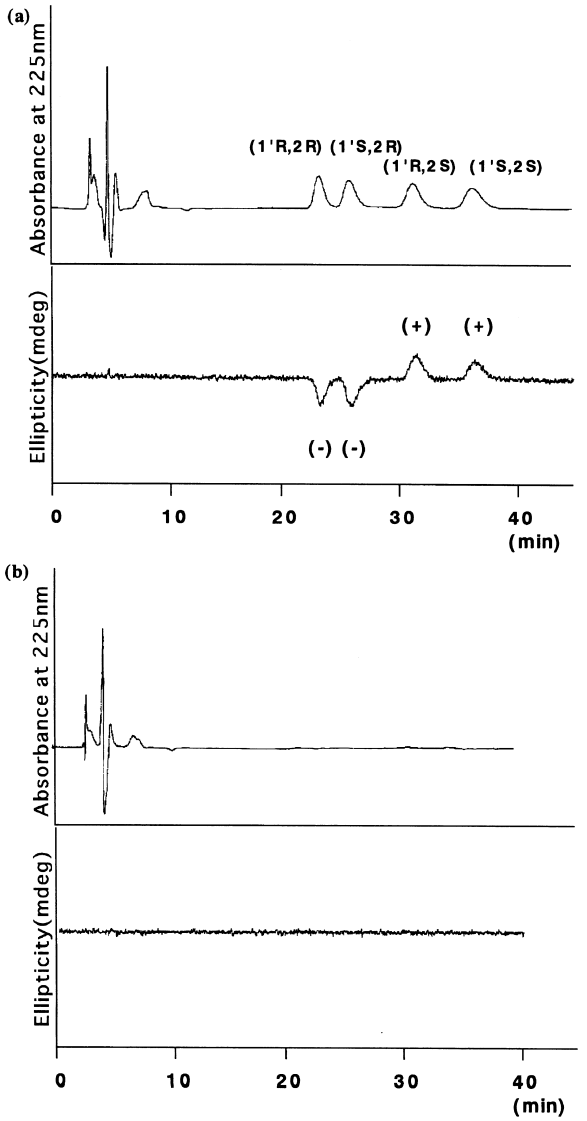

Fig. 2. UV (upper) and CD (lower) chromatograms of loxoprofen on Chiralcel OJ. (a) Loxoprofen following extraction from plasma spiked with loxoprofen sodium tablet. (b) An extract of blank plasma. Eluent: hexane–2-propanol–trifluoracetic acid (95:5:0.1); wavelength: 225 nm; column temperature: 25°C; injection volume: 10 μl.

Methanol was used for determining the hold up time (t_0). The resolution values [R_s, $R_s = 2(t_2 - t_1)/(w_1 + w_2)$, $2(t_3 - t_2)/(w_2 + w_1)$, $2(t_4 - t_3)/(w_3 + w_4)$] were 1.22, 1.79 and 1.82, respectively, where w_1–w_4 are the baseline peak widths. The stereochemical composition of (1′*R*,2*R*)-, (1′*S*,2*R*)-, (1′*R*,2*S*)-, (1′*S*,2*S*)-loxoprofen in the Loxonin® tablet were 24.75, 25.67, 25.11, 24.47%, respectively. Fig. 2b shows chromatograms of an extract of blank plasma. The well resolved chromatogram of chiral separation was obtained without any interference of endogenous compounds in plasma.

3.2. Calibration curves

The linearities between the amount of loxoprofen and the peak areas of each stereoisomer in the UV and CD chromatogram were obtained between 5 and 50 μg. The linear relationship calculated between the peak-area (y) on CD chromatogram and the concentration (x, μg/ml) of loxoprofen in plasma up to 250 μg/ml and the correlation coefficients (r) were as follows:

(1′*R*,2*R*)-loxoprofen; $y = 5.40x + 0.63$ ($r^2 = 0.987$)
(1′*S*,2*R*)-loxoprofen; $y = 6.10x + 0.22$ ($r^2 = 0.985$)
(1′*R*,2*S*)-loxoprofen; $y = 6.16x + 0.10$ ($r^2 = 0.987$)
(1′*S*,2*S*)-loxoprofen; $y = 6.86x + 0.57$ ($r^2 = 0.985$)

The linearity between the peak area on the UV chromatogram and the concentration of loxoprofen in plasma was also obtained (data not shown). The lower limit of quantification for loxoprofen was 0.35 μg by UV detection and 0.85 μg by CD detection, respectively, at a signal-to-noise ratio of 3. The recoveries of the analytical procedure loxoprofen stereoisomers from plasma were 99.7–100.4%. The precision of the method was established from five assays. The mean values and relative standard deviations (RSDs.) are shown in Table 1. The RSD values of the retention times were <1% and those of the peak areas on CD chromatograms were <4%. The present method is sufficiently sensitive and accurate to analysis of loxoprofen stereoisomers in biological samples.

3.3. Application to the quantitation of the stereoisomers of loxoprofen in human plasma

Loxonin was administered orally to a healthy adult at single dose of 60 mg. Fig. 3 shows UV and CD chromatograms of an extract of plasma sample obtained from the subject following oral administration of the mixture. The peaks of numbers 3 and 4 on the CD chromatogram should be (1′*R*,2*S*)-and (1′*S*,2*S*)-stereoisomers of loxoprofen, respectively, but that of (1′*R*,2*R*)-loxoprofen was not observed in the plasma. The peak of (1′*S*, 2*R*)-loxoprofen in the plasma was observed (peak 2), but could not be determined. These results may be due to stereospecific metabolism of loxoprofen. The concentrations of (1′*R*,2*S*)- and (1′*S*,2*S*)-loxoprofen stereoisomers as determined by the present method, were 0.79±0.02 and 1.26±0.07 μg/ml, respectively. Usually for adults, a single dose of 60 mg of loxoprofen sodium is administered orally. From the assay using achiral method, the blood level of loxoprofen reached a peak about 30 min (C_{max} 5.04 μg/ml) after administration and the elimination half-life was about 1 h 15 min [3]. Its main metabolite, which was produced by stereospecific reduction of the cyclopentanone moiety to hydroxycyclopentane, exhibited a potent inhibitory activity to the enzyme. The stereospecific configuration (*trans*-OH, 2*S*,1*R*′,2′*S*) was

Table 1
Precision of the assay by CD detection

	Retention time (min)	RSD (%)	Peak area ($\times 10^6$)	RSD (%)
(1′*R*,2*R*)-loxoprofen	22.19±0.15	0.68	23.22±0.28	1.24
(1′*S*,2*R*)-loxoprofen	24.63±0.16	0.67	27.87±0.67	2.42
(1′*R*,2*S*)-loxoprofen	29.50±0.29	0.99	26.90±0.83	3.12
(1′*S*,2*S*)-loxoprofen	34.31±0.33	0.98	28.03±0.88	3.13

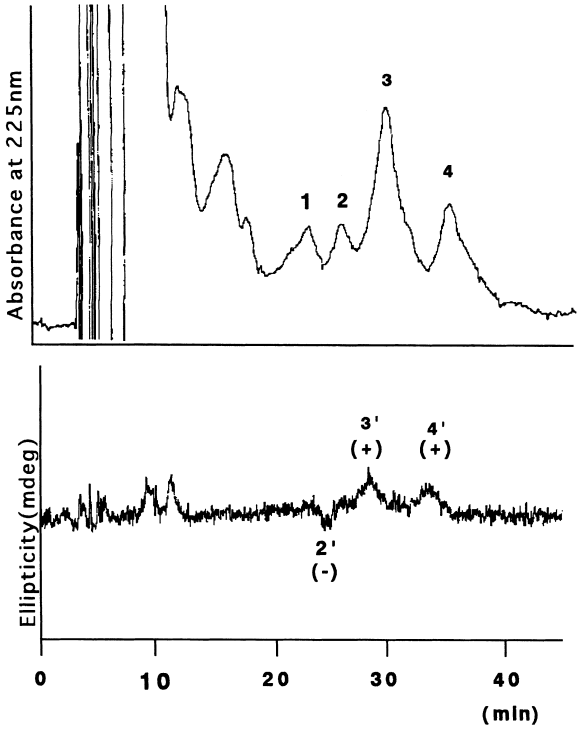

Fig. 3. UV (upper) and CD (lower) chromatograms of an extract of plasma sample obtained from the subject orally administered loxoprofen sodium as racemate. Chromatographic conditions are the same as Fig. 2. Injection volume is 10 μl.

essential for inhibitory activity [1–3]. However, the metabolites of loxoprofen were not observed in this condition.

In contrast to the four peaks being observed around the retention time of loxoprofen stereoisomers on UV chromatogram, peak 1 was not found on the CD chromatogram. CD detection is inherently sensitive only to chiral nonracemic molecules. Therefore, it operates as a filter, which cuts off signals arising from achiral components. Comparison between CD and UV detection provides relevant analytical information. Although the sensitivity for loxoprofen using CD detection is less than that of the method using UV detection, we determined the plasma concentration of loxoprofen from CD chromatogram.

The present method may be applicable to the pharmacokinetic studies of loxoprofen stereoisomers in biological samples. The clinical implications of the enantioselective metabolic pathway remain to be established.

3.4. UV and CD spectrum

Absolute configurations of the enantiomer can be assigned according to their CD spectral properties. CD spectra were obtained by stopped-flow scanning at chromatographic peak tops in UV detection (Fig. 4). UV and CD spectra of the stereoisomers of loxoprofen exhibit bands at approximately 226 nm. In the case of related *S*-α-substituted phenylacetic acid derivatives reported in the literature, the band at 226 nm was assigned to the $n \rightarrow \pi^*$ transitions of the carbonyl group [14]. Examination of the spectrum of (*S*)-ibuprofen is consistent with this report (data not

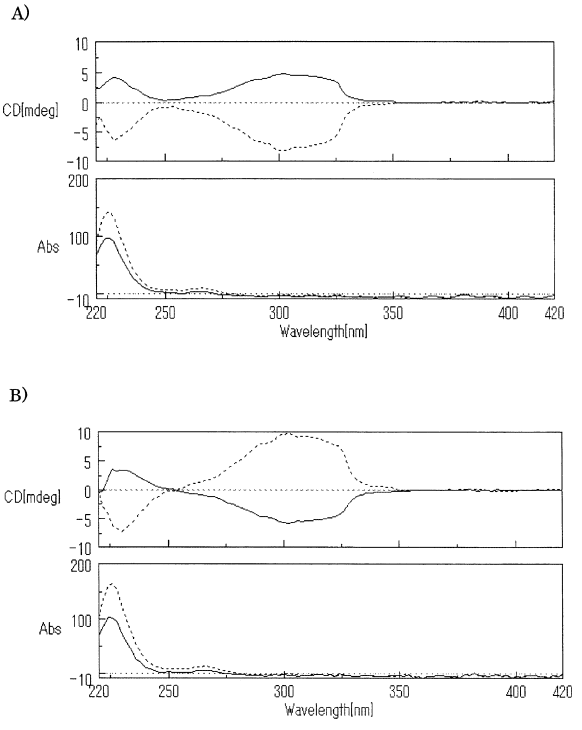

Fig. 4. CD (upper) and UV (lower) spectra of the stereoisomers of loxoprofen. (A) Solid line: (1′*R*,2*S*)-loxoprofen sodium, dotted line: (1′*S*,2*R*)-loxoprofen sodium. (B) Solid line: (1′*S*,2*S*)-loxoprofen sodium, dotted line: (1′*R*,2*R*)-loxoprofen. Column: Chiralcel OJ, Eluent: hexane–2-propanol–trifluoroacetic acid (95:5:0.1); wavelength: 220–420 nm, column temperature: 25°C.

shown). As ibuprofen is structurally similar to the phenylpropionic acid moiety of loxoprofen, examination of its CD spectrum should provide information on the relative contribution of the chiral center at the 2-position in loxoprofen. In our previous report [9], (*S*)-ibuprofen exhibits the positive CD band at 226 nm and the band of (*R*)-ibuprofen at this wavelength is negative. These data indicated that the configuration of propionic acid moiety contribute to the CD band at 226 nm. The CD spectra of (1′*R*,2*S*)-and (1′*S*,2*R*)-loxoprofen sodium were opposite along the wavelength axis each other. (1′*S*,2*S*)- and (1′*R*,2*R*)-loxoprofen also showed opposite spectra along the wavelength axis each other. The CD spectrum of (1′*R*,2*S*)-loxoprofen exhibits the positive bands at approximately 226 and 300 nm. The CD bands of (1′*S*,2*R*)-diastereoisomer at these wavelengths were negative. With (1′*S*,2*S*)- and (1′*R*,2*R*)-loxoprofen, the two centers are the same configuration, but in these cases the CD bands at 226 and 300 nm were opposite. These results suggest the contribution of configuration of the 1′ center to the CD bands at 300 nm.

The signs of CD at all wavelengths for two enantiomers are opposite. CD can be used to determine the elution order of an antipode pair. The chromatographic resolution has enabled the characterization of the stereoisomers by CD spectroscopy.

4. Conclusion

In the present study, the chiral separation of loxoprofen was achieved on a chiral stationary phase with UV and CD detection. We were successful in the stereochemical separation and identification of the loxoprofen stereoisomers in plasma by CD detection. Knowledge of the pharmacokinetics of the enantiomers of chiral drugs should be necessary in clinical practice. Chiroptical detection is useful for analysis of stereoisomers of chiral drugs such as loxoprofen in biological samples and the adaptation of the method described above for the pharmacokinetic studies of the chiral drugs is currently in progress.

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