ELSEVIER

Journal of Chromatography B, 694 (1997) 153–161

JOURNAL OF CHROMATOGRAPHY B

Stereoselective determination of a new antidepressant, E2011, and its diastereoisomer as a metabolite by high-performance liquid chromatography

T. Naitoh[a,*], M. Kakiki[b], S. Kawaguchi[b], Y. Kagei[b], T. Horie[b]

[a]*Department of Pharmaceutical Regulatory Affairs, Eisai Co., Ltd., Koishikawa 4-6-10, Bunkyo-ku, Tokyo 112-88, Japan*
[b]*Tsukuba Research Laboratories, Eisai Co., Ltd., Tokodai 5-1-3, Tsukuba-shi, Ibaraki 300-26, Japan*

Received 3 December 1996; revised 6 February 1997; accepted 6 February 1997

Abstract

A stereoselective HPLC method has been developed for the determination of E2011 (compound I) and one of its metabolites and diastereoisomers, ER-20593 (compound II), in plasma. The two substances and the internal standard were extracted from plasma, followed by two purification steps. In the first step, a minicolumn, Bond Elut C_{18}, was used and in the second step, another minicolumn, Bond Elut Si, was used for purification of the compounds. After the purification, the compounds were analyzed by HPLC with two Chiralpak AD columns. In this procedure, compounds I and II were stable and there was no chiral inversion. The within-day and the between-day assays were performed in rat plasma, where compounds I and II existed simultaneously. The within-day and the between-day precisions of compound I were 2.0~10.1% and 1.3~7.1%, and the within-day and the between-day accuracies were −8.2~+3.0% and −6.6~+4.0% in the concentration range 0.003–10 μg ml^{-1}. The within-day and the between-day precisions of compound II were 1.7~16.9% and 0.9~4.5% and the within-day and the between-day accuracies were −9.0~+2.4% and −5.6~+3.8% in the concentration range of 0.005-0.5 μg ml^{-1}. QC samples for compound I and II were stable for at least 3 months. The method was applied to measure the levels of compound I and II in the rat plasma after oral administration of compound I.

Keywords: Enantiomer separation; E2011; ER-2059

1. Introduction

E2011 (compound I, (5*R*)-3-[2-((1*S*)-3-cyano-1-hydroxypropyl)benzothiazol-6-yl]-5-methoxymethyl-2-oxazolidinone, Fig. 1) was discovered to be a metabolite of a candidate [1]. Compound I was developed as a new selective monoamine oxidase-A inhibitor and antidepressant.

Compound I has 2 chiral centers in its structure. One is in the oxazolidinone ring and the other center is at the α position of the aromatic ring and has a hydroxy group. (*R*)-Enantiomers at the chiral center in the oxazolidinone ring are compound I and its diastereoisomer, ER-20593 (compound II, (5*R*)-3-[2-((1*R*)-3- cyano-1-hydroxypropyl)-benzothiazol-6-yl]-5-methoxymethyl-2-oxazolidinone, Fig. 1). Compounds I and II have the same potency to inhibit MAO-A and are more potent than (*S*)-enantiomers at the chiral center in the oxazolidinone ring [2].

(*S*)-Mandelic acid, which has a hydroxy group at

*Corresponding author.

PII S0378-4347(97)00111-4

the α position of the aromatic ring, was reported to invert to (*R*)-mandelic acid in vivo [3]. Compound I also has a hydroxy group at the α position of the aromatic ring. Thus, the possibility of chiral inversion of compound I to the diastereoisomer, compound II, was considered.

Compound II has the characteristics of an enantiomer of compound I because the 2 chiral centers of these compounds are far from each other. The 2 diastereoisomers of compounds I and II cannot be separated by HPLC with an ODS column. In this paper, a method for stereoselectively measuring compounds I and II was developed to study the pharmacokinetics of compound I in vivo.

2. Experimental

2.1. Chemicals

Compound I (HPLC purity with an ODS column, 99.8%; HPLC purity with a chiral column, 99.9%), compound II (HPLC purity with an ODS column, 97.8%; HPLC purity with a chiral column, 97.0%) and the internal standard (compound III, ((5*R*)-3-[2-(3-cyano-propyl)benzothiazol-6-yl]-5-methoxy-methyl-2-oxazolidinone, Fig. 1); HPLC purity, 99.4%) were synthesized at Tsukuba Research Laboratories of Eisai. Compound I was stable in the solid phase, and the inversion from compound I to compound II was not recognized after 4 weeks at 40°C or 40°C and 75% relative humidity. The HPLC chiral column, Chiralpak AD (250×4.6 mm), was purchased from Daicel (Tokyo, Japan) and was used to separate the diastereoisomers. HPLC solvents were HPLC grade. Britton–Robinson buffer was used as a buffer covering a wide pH range. The buffer was made by mixing 1/25 *M* acid solution (2.71 ml of 85% phosphoric acid, 2.36 ml of acetic acid, 2.47 g of boric acid l^{-1}) and 0.2 *M* NaOH. Chemicals were of commercially available, special grade.

2.2. Chromatographic system and conditions

The HPLC system consisted of a Waters 600E pump (Millipore, Milford, MA, USA), a Waters WISP 710B autosampler with a cooling system

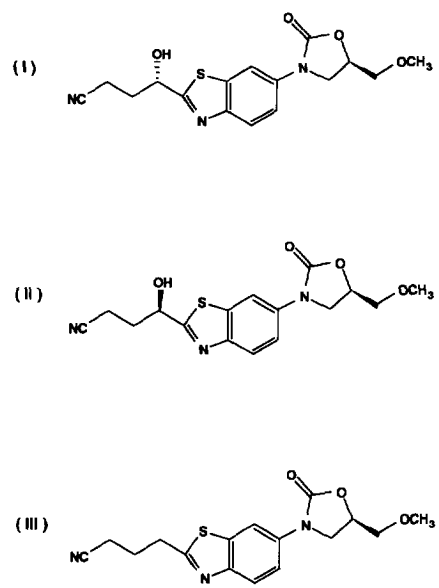

Fig. 1. Structures of compound I (I), compound II (II) and the internal standard (III).

(Millipore), a UV detector SPD-10A (Shimadzu, Kyoto, Japan) and two Unicorder U-228 recorders (Nippon Denshi Kagaku, Tokyo, Japan). The HPLC columns were two Chiralpak ADs. The mobile phase was *n*-hexane–isopropanol–ethanol (65:20:15, v/v/v) and the flow-rate was 0.9 ml min^{-1}. The wavelength of the detector was 275 nm.

2.3. Stock solutions

Stock solutions were prepared by dissolving compounds I and II in methanol. The concentrations of the stock solutions of compound I were 100, 30, 10, 3, 1, 0.3, 0.1 and 0.03 μg ml^{-1}. The concentrations of the stock solutions of compound II were 5, 1.5, 0.5, 0.15, 0.05, 0.015, 0.005 and 0.0015 μg ml^{-1}. The internal standard solution was prepared by dissolving the compound in methanol at a final concentration of 5 μg ml^{-1}. These solutions were stored at −20°C.

2.4. Sample preparation

To 1 ml of rat plasma, 30 μl of the internal standard solution, 0.2 ml of methanol, 1 ml of saline

and 10 ml of dichloromethane–diethyl ether (1:2 v/v) were added and shaken with a KM-shaker (Iwaki, Tokyo, Japan) at a speed of 250 min^{-1} for 10 min. After centrifugation at 1500 *g* for 10 min, the upper layer was separated and evaporated to dryness under nitrogen at 40°C. The resultant residue was dissolved in 1 ml of water and the solution was applied to a Bond Elut C_{18} (2.8 ml, Varian, Harbor, CA, USA) cartridge, which was conditioned with 3 ml of methanol and 3 ml of water beforehand. The Bond Elut C_{18} cartridge was washed with 3 ml of 15% methanol–water (v/v) and the isolates were eluted with 3 ml of methanol. The eluate was evaporated to dryness under nitrogen at 60°C and the resultant residue was dissolved in 2 ml of dichloromethane–diethyl ether (1:2, v/v). To purify it further, the solution of the resultant residue was applied to a Bond Elut Si (2.8 ml) cartridge, which was conditioned with 2 ml of dichloromethane–diethyl ether (1:2, v/v) beforehand. The Bond Elut Si cartridge was washed with 2 ml of isopropyl ether–ethyl acetate (1:1, v/v). The isolates were eluted with 2 ml of ethyl acetate–methanol (4:1, v/v). The eluate was also evaporated to dryness under nitrogen at 40°C, and the resultant residue was dissolved in 0.15 ml of isopropanol, followed by HPLC analysis of 0.05 ml of the solution.

2.5. Accuracy and precision of the assay

To make standard samples for calibration, each 100 μl of stock solutions of compound I and II, 30 μl of the internal standard solution and 1 ml of saline were added to 1 ml of rat plasma. The spiked concentrations of compound I and II were 100:5, that is to say, 10:0.5 μg (compound I:compound II), 3:0.15 μg, 1:0.05 μg, 0.3:0.015 μg, 0.1: 0.005 μg, 0.03:0.0015 μg, 0.01:0.0005 μg and 0.003:0.00015 μg. The samples were then processed as described in Section 2.4. In order to evaluate the within-day validity, replicate samples ($n=4$) were determined for each concentration on the same day. The between-day validity was evaluated for 6 days. The accuracy was evaluated as percentage error [(found concentration − spiked concentration)/spiked concentration] × 100(%) and the precision was evaluated by the coefficient of variation (C.V., %).

2.6. Procedure for animal experiments

Male Sprague–Dawley rats (7 weeks old, special pyrogen free) were purchased from Japan SLC (Hamamatsu, Japan). The animals were starved overnight before dosing and for 8 h after dosing.

Compound I was suspended in 0.5% methylcellulose (w/v), adjusted to a suitable concentration and administered orally to rats at a dose of 3 mg/5 ml kg^{-1}.

Rats were anesthetized with ether and whole blood was collected from the abdominal aorta with a heparinized syringe at each time point. Plasma was separated immediately by centrifugation at 4°C and stored at −20°C until analyzed.

2.7. Calculation and data analysis

Calibration curves were obtained from spiked plasma standards covering the expected concentration ranges of the substances. A non-linear least-squares regression of the peak-height ratios of the substance/internal standard versus substance concentration was calculated, using a weight factor of $1/y^2$. With this equation, the unknown concentrations of the samples were calculated from the measured peak height of each substance divided by the peak height of the internal standard.

Pharmacokinetic parameters were calculated by model-independent techniques, based on the area under the plasma concentration versus time curve [4]. The area under the plasma concentration versus time curve (AUC) from time zero to the last detection time was calculated from the observed values by the trapezoidal method.

3. Results and discussion

3.1. Selection of the HPLC column and the mobile phase

Compounds I and II are a pair of diastereoisomers, which have 2 chiral centers. The chiral carbons in these compounds are at a distance from each other. Because of this distance, these compounds cannot be separated by HPLC with an ODS column. To separate the compounds, many commercial chiral

columns were selected according to the application data of the manufactures and tested with HPLC in our laboratory. Only Chiralpak AD could separate compounds I and II, and the others could not separate these compounds under any conditions.

The column, Chiralpak AD, allows the use of a mixture of *n*-hexane–ethanol or *n*-hexane–isopropanol as the solvent for the mobile phase, as stated in the manual. In the case of compounds I and II, a mixture of *n*-hexane–ethanol–isopropanol (65:20:15, v/v/v) was the best solvent to separate these compounds.

3.2. Determination of solvent and pH for the extraction

To 1 ml of plasma, compound I, the internal standard and saline were added and extraction ratios were examined with HPLC and various organic solvents. In the case of ethyl acetate, the absolute extraction ratio was 96% and the relative extraction ratio was 100%, though the background baseline of HPLC was not suitable for analysis of very small amounts of these compounds. For diethyl ether, the absolute extraction ratio was low, 56%. For dichloromethane–diethyl ether (1:2, v/v), the absolute and relative extraction ratios were high, 74% and 100%. Using compound II instead of compound I, the absolute and relative extraction ratios were high, 71% and 97% for dichloromethane–diethyl ether (1:2, v/v). The background baseline of HPLC after extraction with dichloromethane–diethyl ether (1:2, v/v) was clearer than the baseline with ethyl acetate.

The effect of pH on the extraction ratio was examined by diluting plasma with solvents of various pH values. For the pH solvents, a 0.1 *M* hydrochloride solution, Britton–Robinson buffers of pH 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 and saline were selected. When the extraction ratio with saline was taken as 100%, the extraction ratios of a 0.1 *M* hydrochloride solution, or Britton–Robinson buffers of pH 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 were 100.9%, 106.6%, 105.5%, 103.7%, 103.5%, 102.6%, 101.5%, 100.1%, 100.2%, 95.8% and 95.1%, respectively. The extraction ratios with the buffers of pH 2 and 3 were a little higher than that of saline. To make the procedure easier, saline was selected because it is commercially available.

3.3. Clarification of background baseline

The background baseline after extraction only was not sufficiently clear for the analysis of very small amounts of compounds. For this reason, purification with minicolumns was examined. Minicolumns with normal phase and reversed-phase of silica gel were selected because they have been used extensively.

The minicolumn with normal phase of silica gel, Bond Elut Si, could clear the background peaks which were eluted far from the unchanged drug and interrupted the next analysis (data not shown). The minicolumn with reversed-phase of silica gel, Bond Elut C_{18}, could clear the background peaks which were eluted near the unchanged drug (data not shown). From this result, it was decided to use both minicolumns to clear the baseline.

3.4. pH stability of compounds I and II

The pH stability of compounds I and II after incubation for 3 h at 37°C was examined with a 0.1 *M* hydrochloride solution or Britton–Robinson buffers of pH 3, 5, 7, 8, 9 and 11 as the solvent. For the standard, solutions of these compounds in saline were kept at 0°C.

The residual percentages of compound I after incubation in a 0.1 *M* hydrochloride solution or Britton–Robinson buffers of pH 3, 5, 7, 8, 9 and 11, were 99.7%, 98.7%, 101.2%, 100.7%, 97.4%, 93.5% and 92.7%, respectively. Chiral inversion of compound I to compound II was not detected. For compound II, the residual percentages after incubation in a 0.1 *M* hydrochloride solution or Britton–Robinson buffers of pH 3, 5, 7, 8, 9 and 11 were 101.2%, 100.7%, 100.8%, 101.7%, 98.7%, 93.5% and 90.5%, respectively. Chiral inversion of compound II to compound I was not detected.

The compounds were not stable in alkaline conditions above pH 8. Otherwise they were as stable as in saline. To avoid stability problems saline was selected as the diluent solvent.

3.5. Stability in the blood

Compound I was added to blood, followed by incubation at 0°C, 25°C or 37°C and centrifugation to obtain plasma. The plasma was analyzed to examine the stability of compound I in the blood.

The residual percentage of compound I after incubation at 37°C for 15 min was 88.0% and that at 25°C for 1 h was 92.2%. Chiral inversion of compound I to compound II was not observed in either case. At 0°C, residual percentages of compound I after incubation for 0.5, 1 and 3 h were 99.5%, 101.0% and 97.6%, respectively. This result showed that compound I was stable in the blood at 0°C for at least 3 h. Blood collected from animals after administration of compound I should be centrifuged as soon as possible at 0°C and the plasma should be kept at 0°C or below.

3.6. Influence of compound I on the determination of compound II

The influence of compound I on the determination of compound II was evaluated because it was feared that the measurement of compound II would not be quantitative for the following two reasons; (1) in the HPLC chromatogram, peaks of compounds I and II were close and the peak of compound II was former; (2) a preliminary study in rats showed that the concentration of compound I was much larger than that of compound II. On the other hand, the measurement of compound I should be quantitative for these reasons. So, the influence of compound II on the determination of compound I was not evaluated.

To 1 ml of plasma in which compound I was present at a concentration of 0.1 or 1 $\mu g\,ml^{-1}$, compound II was added and the ratio of compound I/compound II was adjusted to 100:5, 100:10, 100:20, 100:40 or 100:100. These plasma samples were processed as described in Section 2.4.

The peak-height ratio (PHR) was calculated from the peak height of compound I or II divided by the peak height of the internal standard. The calibration curves were constructed by plotting (PHR of compound II)/(PHR of compound I) versus the spiked concentration of compound II. The calibration curves of compound II were linear with both concentrations of 0.1 and 1 $\mu g\,ml^{-1}$ of compound I. The equations for the calibration curve of compound II were $y=11.871x+0.002$ ($r^2=0.996$) with 0.1 $\mu g\,ml^{-1}$ of compound I and $y=1.251x-0.026$ ($r^2=0.995$) with 1 $\mu g\,ml^{-1}$ of compound I. These results showed that the concentration of compound II could be measured without interference from compound I and that chiral inversion of compounds I and II did not occur during the determination.

The within-day and between-day assay variance of compounds I and II should be evaluated in plasma which contained both compounds, because they would both be present after administration of compound I. For the ratio of compound I/compound II to be nearer to a numerical value 1 would make the value of the accuracy and precision smaller. So, 100:5 was selected for the plasma concentration ratio of compounds I and II in the within-day and between-day assays.

3.7. Within-day assay variance

A calibration curve was prepared by adding 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3 or 10 μg of compound I and 5% of compound II to each concentration of compound I in 1 ml of plasma. Four plasma samples were prepared for each concentration and analyzed to ascertain the within-day assay variance. Compound II was not detected at the three lowest concentrations, 0.00015, 0.0005 and 0.0015 $\mu g\,ml^{-1}$.

Typical chromatograms obtained in this experiment are shown in Fig. 2. Chromatogram (A) shows that no interfering peak was observed in the baseline of the background sample. Chromatogram (B) shows that the peaks of compound I, compound II and the internal standard were well separated.

Calibration curves of compounds I and II were linear over the added concentrations in the plasma, where both compounds were present. Table 1 shows the within-day assay results of compounds I and II determined by back calculation from the calibration curve. In the case of compound I, the precision was 2.0~10.1% and the accuracy was −8.2~+3.0% in the concentration range 0.003–10 $\mu g\,ml^{-1}$. In the case of compound II, the precision was 1.7~16.9% and the accuracy was −9.0~+2.4% in the concentration range 0.005–0.5 $\mu g\,ml^{-1}$. These results showed that compounds I and II could be measured by this assay without the problem of within-day variance.

3.8. Between-day assay variance

Five more calibration curves for compounds I and II were prepared at the same concentrations to study the between-day assay variance. For quality control

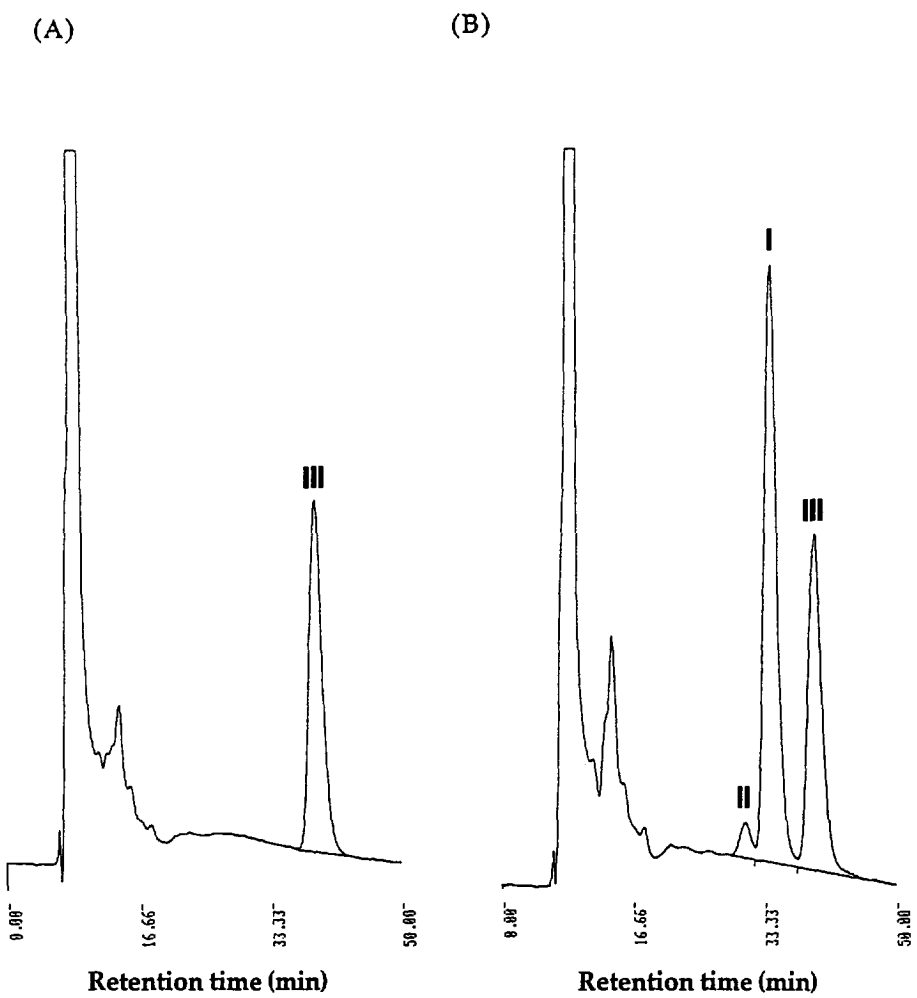

Fig. 2. Chromatograms of (A) blank plasma sample and (B) plasma sample spiked with 0.3 μg compound I and 0.015 μg compound II. The positions of compound I (I), compound II (II) and the internal standard (III) are shown.

(QC), high-, intermediate- and low-concentration samples of compounds I and II were prepared with rat plasma and stored at −20°C.

Table 2 shows the between-day assay results of compounds I and II determined by back calculation from the calibration curves. In the case of compound I, the precision was 1.3~7.1% and the accuracy was −6.6~+4.0% in the concentration range 0.003–10 μg ml^{-1}. In the case of compound II, the precision was 0.9~4.5% and the accuracy was −5.6~+3.8% in the concentration range 0.005–0.5 μg ml^{-1}. This result showed that compounds I and II could be measured in this assay without the problem of between-day variance. QC samples of compounds I and II were stable for at least 3 months.

3.9. Application

Compound I was administered orally to rats at 3 mg kg^{-1}. Whole blood was collected from these rats at each time point and centrifuged to obtain the plasma. A 1-ml sample of the plasma was analyzed to measure the levels of compounds I and II. One

Table 1
Accuracy and precision of the within-day assay (n=4)

Compound	Spiked concentration ($\mu g\ ml^{-1}$)	Found concentration ($\mu g\ ml^{-1}$)	Precision (C.V.) (%)	Accuracy (%)
Compound I	0.003	0.00288	8.8	−4.0
	0.01	0.00918	4.9	−8.2
	0.03	0.0295	4.3	−1.7
	0.1	0.103	10.1	3.0
	0.3	0.296	2.5	−1.3
	1	0.999	2.3	−0.1
	3	2.92	2.3	−2.7
	10	9.42	2.0	−5.8
Compound II	0.005	0.00455	16.9	−9.0
	0.015	0.0146	1.7	−2.7
	0.05	0.0512	2.3	2.4
	0.15	0.150	4.4	0.0
	0.5	0.473	2.0	−5.4

Compounds I and II were both present in each plasma at the ratio of 100:5.
Compound II was not detected at the 3 concentrations lower than 0.005 $\mu g\ ml^{-1}$.

example of the HPLC chromatograms is shown in Fig. 3. Fig. 4 shows the plasma concentration versus time curves for compounds I and II.

Time at the maximum concentration in plasma (T_{max}) and the maximum concentration in plasma (C_{max}) of compound I were 0.5 h and 1.043 $\mu g\ ml^{-1}$, respectively. The T_{max} and C_{max} of compound II were 1 h and 0.110 $\mu g\ ml^{-1}$, respectively. C_{max} of compound I was 9.5 times greater than that of compound II. AUC values of compounds I and II were 1.51 and 0.169 $\mu g \cdot h\ ml^{-1}$, respectively. The existence ratio (%) of compound I in the plasma was calculated from the concentration of compound I divided by the sum of the concentrations of compounds I and II. From 0.25–3 h, when both the concentrations of compounds I and II could be

Table 2
Accuracy and precision of the between day assay (n=6)

Compound	Spiked concentration ($\mu g\ ml^{-1}$)	Found concentration ($\mu g\ ml^{-1}$)	Precision (C.V.) (%)	Accuracy (%)
Compound I	0.003	0.00312	5.3	4.0
	0.01	0.00934	7.1	−6.6
	0.03	0.0299	2.5	−0.3
	0.1	0.104	5.0	4.0
	0.3	0.306	2.4	2.0
	1	1.03	3.1	3.0
	3	3.07	1.3	2.3
	10	9.61	4.1	−3.9
Compound II	0.005	0.00493	0.9	−1.4
	0.015	0.0155	2.6	3.3
	0.05	0.0519	4.5	3.8
	0.15	0.153	1.9	2.0
	0.5	0.472	3.4	−5.6

Compounds I and II were present in each plasma, at the ratio of 100:5.
Compound II was not detected at the 3 concentrations lower than 0.005 $\mu g\ ml^{-1}$.

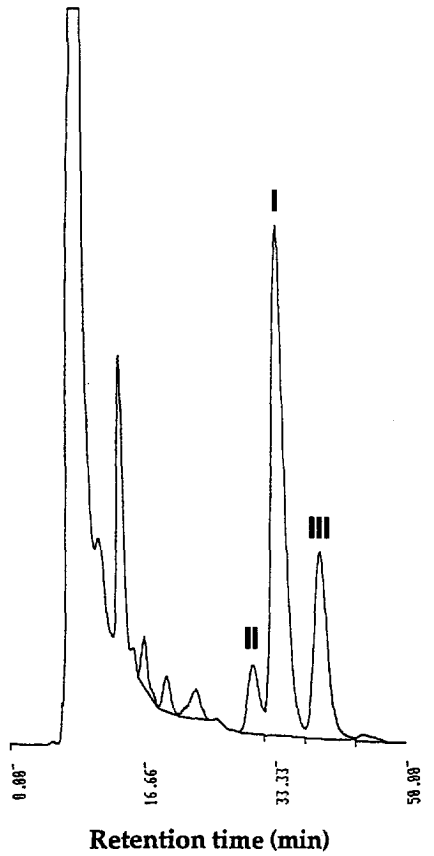

Fig. 3. Chromatogram of a plasma sample after the oral administration of compound I (3 mg kg^{-1}) to rats. The plasma was collected 2 h after oral administration. The positions of compound I (I), compound II (II) and the internal standard (III) are shown.

measured after the administration, the existence ratio of compound I was consistently 84.8~90.8%.

The chiral inversion of compounds I and II did not occur in aqueous solution, the solution of organic solvents, plasma and blood from the results in Sections 3.4–3.6 but occurred in the rat in vivo only. These results show that the free energy difference between the two diastereoisomers is not small and the chiral inversion only occurred enzymatically. The consistency of the existence ratio in vivo would result from the following presumptions: the velocity of the enzyme reaction of the chiral inversion from compound I to compound II, v_A and that from compound II to compound I, v_B, would be constant; v_B would be greater than v_A; v_A and v_B would be greater than the velocity of metabolism (except for

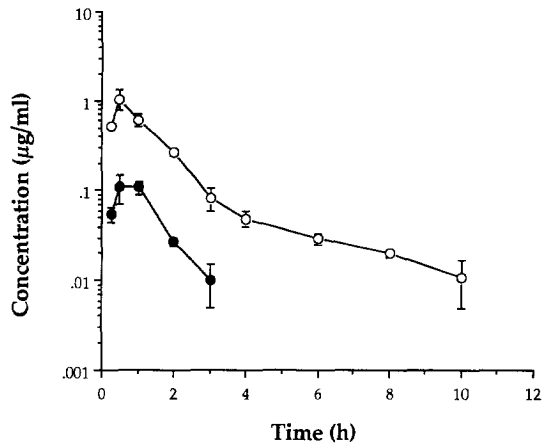

Fig. 4. Plasma concentration versus time curves of compound I (○) and compound II (●) after the oral administration of compound I (3 mg kg^{-1}) to rats (n=3, mean±S.D.).

the chiral inversion) and excretion of the two diastereoisomers in rat in vivo; the concentrations of compounds I and II in vivo would be much lower than the concentrations in which the two enzyme reactions of chiral inversion showed the maximum velocities.

From these presumptions, the existence ratio of compound I would be continuously constant also from 4 h after administration, and the concentration of compound I in vivo would be much greater than that of compound II. Accordingly, pharmacological effects after administration of compound I to rats would be mainly derived from compound I and not from compound II, though compound II is as effective as compound I in vitro pharmacologically.

Compound II has been found in rats in vivo. Compound II is produced by the chiral inversion of compound I at the α-carbon of the benzothiazole. This type of chiral inversion is rarely reported. The only example is in the case of (*S*)-mandelic acid [3], but the mechanism of its chiral inversion has not been reported. The mechanism of the chiral inversion of compound I is a further issue to be studied.

By this method, we measured the concentrations of compounds I and II, which are (*R*)-enantiomers at the chiral center in the oxazolidinone ring. The concentrations of the (*S*)-enantiomers at the chiral center of the oxazolidinone should be measured with this method, because the (*S*)-enantiomers were sepa-

rated from the (*R*)-enantiomers with these HPLC conditions (data not shown). The (*S*)-enantiomers were not detected in the experiment with rats. So, the chiral inversion at the chiral center in the oxazolidinone ring would not occur in vivo because breaking and connection of the aliphatic C–C bond would not occur in normal metabolism.

References

[1] T. Naitoh, T. Horie, S. Nagato, T. Kagaya, A. Kubota, K. Akasaka, Xenobiotica 24 (1994) 819–826.

[2] S. Nagato, A. Kajiwara, I. Yoshida, A. Sasaki, Y. Iimura, N. Karibe, M. Mizuno, A. Kubota, T. Kagaya, M. Komatsu, T. Naitoh, H. Itoh, T. Kaneko and K. Akasaka, Abstracts of American Chemical Society National Meeting 206th, Division of Medicinal Chemistry, 1993, p. 177

[3] L. Drummond, J. Caldwell, H.K. Wilson, Xenobiotica 20 (1990) 159–168.

[4] K. Yamaoka, T. Nakagawa, T. Uno, J. Pharmacokinet. Biopharm. 6 (1978) 547–558.