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Determination of enantiomers of 1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]methylpiperidine hydrochloride (E2020), a centrally acting acetylcholine esterase inhibitor, in plasma by liquid chromatography with fluorometric detection

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ABSTRACT

Two high-performance liquid chromatographic methods for the determination of enantiomers of E2020, 1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]methylpiperidine (I) hydrochloride, in rat plasma have been developed. The first method involves chiral separation of I on an ovomucoid-bonded column, and native fluorescence detection of I with excitation at 318 nm and emission at 390 nm. The fluorometric detection is without interference from background components and is about five times more sensitive than ultraviolet detection at 271 nm. The method was successfully applied to monitoring the racemization of each enantiomer of I in buffer solutions and in rat plasma. The second method involves separation of I from background components of rat plasma on an achiral column, collection of the I fraction into a sample loop, concentration of I to a trap column, transfer of I to a chiral column, resolution of the enantiomers of I on the chiral column and fluorometric detection of the enantiomers of I with excitation at 318 nm and emission at 390 nm. The detection limits of I and each enantiomer of I were 1 and 1 ng/ml, respectively, with a 200-μl injection of deproteinized plasma samples.

INTRODUCTION

E2020, 1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]methylpiperidine (I) hydrochloride, is a centrally acting acetylcholine esterase inhibitor that is currently in Phase II clinical trials to test its effectiveness in treating Alzheimer's disease [1]. As shown in Fig. 1, I is a chiral compound that may easily racemize because the chiral centre is adjacent to a carbonyl group. In order to determine the enantiomeric composition of I in standard samples or pharmaceuticals, and to quantitate enantiomers of I in body fluids, it was necessary to develop a sensitive, selective high-performance liquid chromatographic (HPLC) method. We tried to achieve the direct resolution of I using a chiral stationary phase and an aqueous mobile phase. Among the chiral stationary phases used with aqueous mobile phases, protein-bonded columns are thought to have wide appli-

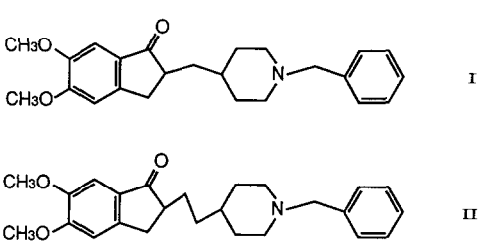

Fig. 1. Structures of I and the internal standard (II).

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cability for various solutes, especially drugs. In preliminary experiments, we examined commercially available columns bonded with bovine serum albumin, α_1-acid glycoprotein and ovomucoid (OVM) [2–4]. Only an OVM-bonded column (Ultron ES-OVM) could resolve enantiomers of I. Further, we found that I had native fluorescence with excitation at 318 nm and emission at 390 nm.

This paper describes selective, sensitive HPLC methods for the monitoring of the racemization of each enantiomer of I in rat plasma and for the determination of the enantiomers of I in the presence of plausible metabolites of I in plasma. The former was carried out by direct chiral resolution of each enantiomer of I on an OVM-bonded column and fluorometric detection. On the other hand, preliminary studies revealed that I and a plausible metabolite of I could not be resolved on the OVM-bonded column, so we developed a coupled achiral–chiral chromatographic method involving a trap column and a dilution tube, and fluorometric detection.

EXPERIMENTAL

Reagents and materials

The racemate and both enantiomers of I and racemic 1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]ethylpiperidine (II), which is used as an internal standard (I.S.), were sythesized by Eisai Research Laboratory (Tsukuba, Ibaraki, Japan) as were the plausible metabolites of I. These structures are illustrated in Figs. 1 and 2. Acetonitrile of HPLC grade was obtained from Wako (Osaka, Japan). Other reagents of analytical grade were used without further purification.

Water purified by a Nanopure II unit (Barnstead, Boston, MA, USA) was used for the preparation of the eluent and the sample solution.

Chromatography

To monitor the racemization of I in buffer solutions and in rat plasma, the HPLC system consisted of an LC-5A pump, an RF-535 fluorospectrophotometer (both from Shimadzu, Kyoto, Japan) and a Rheodyne 7125 injector (Cotati, CA, USA). An OVM-bonded silica column (Ultron ES-OVM, particle diameter 5 μm, 150 mm × 4.6 mm I.D.) (Shinwa Chemical Industries, Kyoto, Japan) was used with a guard column (10 mm × 4.0 mm I.D., Ultron ES-OVM · G, Shinwa Chemical Industries) packed with the same materials. The eluent was 20 m*M* phosphate buffer (pH 7.2)–acetonitrile (87.5:12.5, v/v). Chromatograms were recorded and integrated by a C-R6A integrator (Shimadzu). The flow-rate was maintained at 0.6 ml/min. Detection was performed with excitation at 318 nm and emission at 390 nm. All separations were performed at 20°C by using a CO-1093C column oven (Uniflows, Tokyo, Japan).

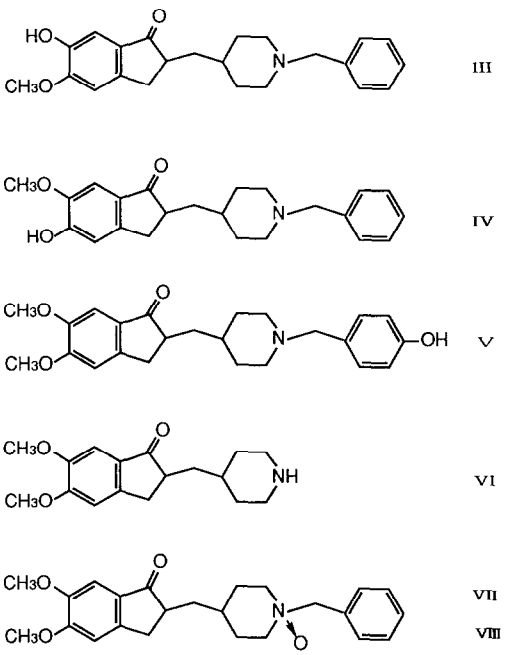

Fig. 2. Structures of plausible metabolites of I. Compounds VII and VIII are axial and equatorial conformations, respectively.

To determine the enantiomers of I in rat plasma, the HPLC system consisted of three HPLC pumps (Model 880-PU pump from Japan Spectroscopic, Tokyo, Japan), two fluorospectrophotometers (a Model RF-535 and a Model 821-FP from Japan Spectroscopic), two six-port switching valves (Model PT-8000 from Tosoh, Tokyo, Japan), a Rheodyne 7125 injector equipped with 500-μl loop, and a Model C-R6A integrator.

Three columns were used: a Capcell Pak C1 (150 mm × 4.6 mm I.D., Shiseido, Tokyo, Japan) with a 10 mm × 4.0 mm I.D. guard column packed with the same packing materials for separating I from the background components of serum and from plausible metabolites of I; an OVM-bonded column (150 mm × 4.6 mm I.D.) with a 10 mm × 4.6 mm I.D. guard column packed with the same packing materials for separating enantiomers of I; and an OVM-bonded guard column (10 mm × 4.0 mm I.D.) for trapping I. The eluents used were as follows: 20 m*M* phosphate buffer (pH 7.5)–acetonitrile (70:30, v/v) at a flow-rate of 1.0 ml/min for separating I; 5 m*M* phosphate buffer (pH 7.5) at a flow-rate of 2.3 ml/min for trapping I after dilution; and 20 m*M* phosphate buffer (pH 7.2)–acetonitrile (87.5:12.5, v/v) at a flow-rate of 0.8 ml/min for separating enantiomers of I. All procedures were performed at ambient temperature, except that the chiral separation of the enantiomers of I is performed at 20°C, using a CO-1093C column oven. Detection was performed fluorometrically with excitation at 318 nm and emission at 390 nm. The procedures were based on the modified method of Oda *et al.* [9]: I and II were separated on an achiral column (Capcell Pak C1), the fractions containing I were collected in a 2-ml loop, I was concentrated on a trap column (Ultron ES-OVM · G) after dilution, and the enantiomers of I were transferred to a chiral column (Ultron ES-OVM) and resolved, then detected fluorometrically.

Monitoring of racemization of each enantiomer of I

A 1-ml aliquot of rat plasma or phosphate buffer (pH 7.4, $\mu = 0.2$) containing 500 ng/ml (*R*)- or (*S*)-I was incubated at 37°C. At predetermined times, 100-μl aliquots were withdrawn, deproteinized with 100 μl of acetonitrile and centrifuged at 10 000 *g* for 5 min. To a 100-μl aliquot of the supernatant, 200 μl of 20 m*M* phosphate buffer (pH 6.9) were added. Aliquots of 50 μl of the resulting solution were loaded onto the OVM-bonded column.

Determination of I and each enantiomer of I in rat plasma

A known amount of racemate or each enantiomer of I was dissolved in water or methanol, and the solution was diluted with rat plasma to the desired concentration. A 500-μl aliquot of plasma sample was deproteinized with 500-μl of acetonitrile and centrifuged at 10 000 *g* for 5 min. A 200-μl aliquot of the deproteinized plasma sample was loaded onto the column.

RESULTS AND DISCUSSION

Loadability of I on an OVM-bonded column

It is well known that the loading capacity of a protein-bonded column is fairly low [5]. Thus, the loadability of I on an OVM-bonded column was

TABLE I

DEPENDENCE OF CAPACITY FACTOR (k'), ENANTIOSELECTIVITY (α), RESOLUTION (R_s) AND NUMBER OF THEORETICAL PLATES (N) OF ENANTIOMERS OF I ON THE LOADING AMOUNTS ON AN OVM-BONDED COLUMN

The eluent was 20 m*M* phosphate buffer (pH 7.2)–acetonitrile (85:15, v/v) at a flow-rate of 1.0 ml/min. The k'_1 and k'_2 values correspond to the capacity factors of the first and second eluted peaks, respectively, (*R*)-I and (*S*)-I. The capacity factor is $k' = (t_R - t_0)/t_0$, where t_R and t_0 are the elution times of retarded and unretarded solutes, respectively. The N_1 values, corresponding to the number of theoretical plates for the first eluted peak, were calculated according to the equation $N_1 = 2\pi(t_{R1}H/A)^2$, where t_{R1} is the retention time of the first eluted peak, H is the peak height and A is the peak area. The N_2 values, corresponding to the number of theoretical plates for the second eluted peak, were calculated by the same procedure as N_1. R_s values were calculated according to the equation $R_s = 2t_0(k'_2 - k'_1)/(t_{w1} + t_{w2})$, where t_{w1} and t_{w2} are peak widths of the first and second eluted peaks, respectively.

Loaded amount (μg)	k'_1	N_1	k'_2	N_2	α	R_s
0.02	3.38	2260	4.53	2120	1.34	2.52
0.08	3.39	1950	4.52	2180	1.33	2.40
0.2	3.33	2020	4.50	2180	1.35	2.52
0.4	3.24	2030	4.35	2100	1.34	2.50
1.0	3.28	2040	4.28	1880	1.33	2.33
2.0	3.25	2140	4.28	1630	1.32	2.14
4.0	3.20	2150	4.16	1270	1.30	1.85
10	3.08	1950	3.89	780	1.27	1.22

examined. Table I shows the dependency of the capacity factor (k'), column efficiency (N), enantioselectivity (α) and resolution (R_s) on the load of racemic I. The change of α values was small over the load range 0.02–10 μg. Although an R_s was constant over the load range 0.02–0.4 μg, it decreased with an increase of the load and drastically decreased with loads of more than 4.0 μg. This is ascribable to a drastic decrease in the N_2 value: the number of theoretical plates of the first eluted peak corresponding to (*R*)-I was slightly decreased by a 500-fold increase in load (from 0.02 to 10 μg), whereas that of the second eluted peak corresponding to (*S*)-I was decreased to one third. These results suggest that (*S*)-I might bind to a specific site(s) of the OVM, whose capacity is low, and (*R*)-I might bind to a non-specific site(s), whose capacity is high.

Effects of the eluent pH, the type and content of organic modifier, and the buffer concentration on retention, enantioselectivity and enantiomeric elution order of I

In a previous paper [6], we reported that for chiral resolution of propranolol and its ester derivatives it is necessary to select the most suitable organic modifier as well as the eluent pH to give the maximum enantioselectivity. We examined methanol, ethanol, 2-propanol and acetonitrile as organic modifiers, and found that acetonitrile gave the highest enantioselectivity. Other factors affecting the retention, enantioselectivity and enantiomeric elution order were also examined, including the eluent pH, the acetonitrile content and the buffer concentration. Table II shows the effect of the eluent pH on the resolution of I. Although the highest enantioselectivity was obtained at an eluent pH of 5.2, severe peak tailing was observed. Greater resolution of racemic I was obtained at an eluent pH of above 7. The enantiomeric elution order was reversed around an eluent pH of 5–6. However, by altering the acetonitrile content and buffer concentration, the enantiomeric elution order could be kept unchanged. The data on the effects of acetonitrile content and buffer concentration reveal that a buffer concentration of 20 m*M* and above and an organic modifier content of 12.5–15% are suitable for the chiral resolution of I. Thus, we selected the optimum eluent conditions described in Experimental.

TABLE II

EFFECT OF pH ON RETENTION, ENANTIOSELECTIVITY AND ENANTIOMERIC ELUTION ORDER OF I

Eluent pH	k'_1	α	R_s	Elution order
7.7[a]	7.13	1.34	2.30	(*R*)/(*S*)
7.3[a]	4.92	1.35	2.18	(*R*)/(*S*)
6.4[a]	1.58	1.25	0.51	(*R*)/(*S*)
5.4[a]	0.57	1.00	–	–
5.2[b]	5.04	1.41	<0.5	(*S*)/(*R*)
4.0[b]	2.48	1.17	<0.5	(*S*)/(*R*)

[a] The eluent was 20 m*M* phosphate buffer–acetonitrile (87.5:12.5, v/v).

[b] The eluent was 20 m*M* phosphate buffer–acetonitrile (99.5:0.5, v/v).

Fluorometric detection of I in plasma

Fig. 3A and B show chromatograms of standard and rat plasma samples, respectively, con-

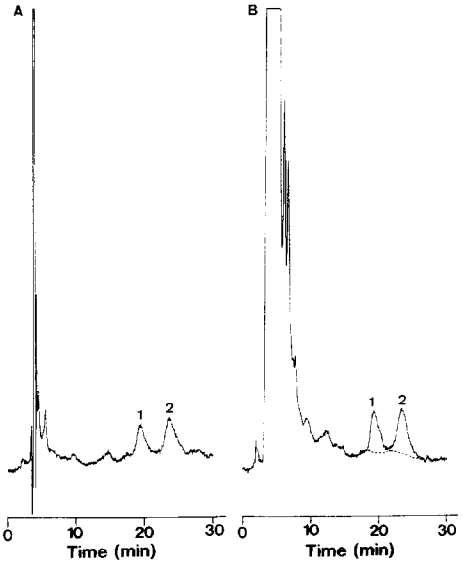

Fig. 3. Chromatograms of (A) standard and (B) rat plasma samples containing racemic I, with UV detection at 271 nm. Eluent, 20 m*M* phosphate buffer (pH 7.2)–acetonitrile (87.5:12.5, v/v); flow-rate, 0.6 ml/min. Other conditions as in Experimental. Peaks: 1 = (*R*)-I; 2 = (*S*)-I. Dashed line indicates plasma blank.

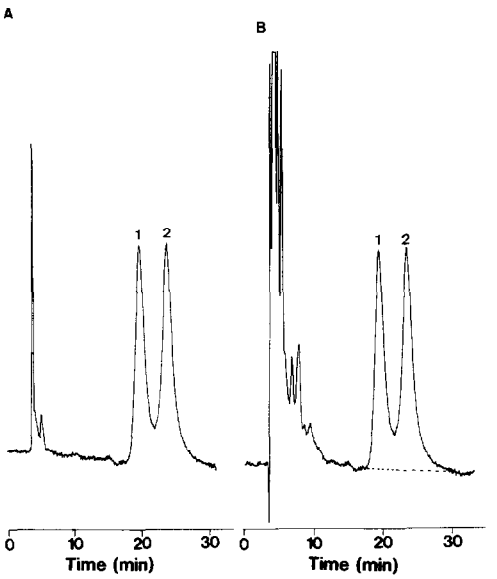

Fig. 4. Chromatograms of (A) standard and (B) rat plasma samples containing racemic I, with fluorometric detection with excitation at 318 nm and emission at 390 nm. HPLC conditions were the same as in Fig. 3. Peaks: 1 = (*R*)-I; 2 = (*S*)-I. Dashed line indicates plasma blank.

taining racemic I, with UV detection at 271 nm. Although the separation of racemic I was good, the high background and poor signal-to-noise ratio prevented sensitive detection of I. Thus, we investigated fluorometric detection of I. Fig. 4A and B show chromatograms of the same samples as in Fig. 3A and B, except that detection was performed fluorometrically with excitation at 318 nm and emission at 390 nm. The advantages of fluorometric detection for I in rat plasma samples compared with UV detection are as follows: (1) fluorometric detection is about five times more sensitive than UV detection; (2) it is easy to avoid interference from background components; and (3) repetitive injection is possible without interference due to the preceding injection. Therefore, we used fluorometric detection to monitor the racemization of each enantiomer of I, as described below.

Monitoring of racemization of each enantiomer of I in buffer and rat plasma

Before using our new method to monitor the racemization of each enantiomer of I in rat plasma, reproducibility and recovery data were collected. No racemization was observed during pretreatment procedures. The reliability of the procedure (recovery, 103–99.7%; precision 0.56–0.72%; $n = 5$) makes it suitable to monitor the racemization of each enantiomer of I. Table III illustrates racemization of (*R*)- and (*S*)-I in phosphate buffer and rat plasma. These results reveal that, in phosphate buffer, only *ca.* 10 and 20% racemization occurs 6 and 12 h after incubation, whereas in rat plasma (*R*)- and (*S*)-I racemize completely during 6 h of incubation.

Retention and enantioselectivity of I and its plausible metabolites on achiral and chiral columns

Table IV illustrates the capacity factors of I, II and plausible metabolites of I on an achiral column (Capcell Pak Cl), and the capacity factors and enantioseparation factors for them on a chiral column (Ultron ES-OVM). On the achiral

TABLE III

TIME-DEPENDENT CONVERSION OF (*R*)- AND (*S*)-I IN PHOSPHATE BUFFER SOLUTION AND IN RAT PLASMA AFTER ADDITION OF (*R*)- OR (*S*)-FORM

Phosphate buffer solutions (pH 7.4, $\mu = 0.2$) and rat plasma samples spiked with (*R*) or (*S*)-I (500 ng/ml) were treated as described in Experimental.

Added		Found (mean ± S.D., $n = 3$) (%)						
		0 h	1 h	2 h	4 h	6 h	8 h	12 h
In phosphate buffer solution								
R-form	*R*-form	98.6		95.4	92.6	89.6	86.9	81.7
	S-form	1.4		4.6	7.4	10.4	13.1	18.3
		(−)		(1.4)	(2.3)	(2.4)	(1.1)	(0.3)
S-form	*R*-form	3.2		6.6	9.6	12.9	14.3	20.8
	S-form	96.8		93.4	90.4	87.1	85.7	79.2
		(−)		(0.9)	(1.3)	(0.9)	(1.3)	(1.5)
In rat plasma								
R-form	*R*-form	98.6	88.4	77.8	59.2	56.5		
	S-form	1.4	11.6	22.2	40.8	43.5		
		(−)	(2.3)	(2.7)	(3.1)	(0.9)		
S-form	*R*-form	3.2	11.9	23.6	39.3	46.1		
	S-form	96.8	88.1	76.4	60.7	53.9		
		(−)	(1.5)	(1.1)	(2.5)	(3.0)		

TABLE IV

CAPACITY FACTORS OF I, II AND PLAUSIBLE METABOLITES OF I ON ACHIRAL AND CHIRAL COLUMNS

k' is the capacity factor for a solute; k'_1 is the capacity factor for the first eluted enantiomer; α is the enantioseparation factor.

Compound	Achiral column[a]	Chiral column[b]	
	k'	k'_1	α
I	8.58	7.33	1.21
II	11.9	8.52	1.13
III	4.64	7.22	1.17
IV	2.99	5.33	1.00
V	3.04	5.84	1.00
VI	1.10	3.78	1.00
VII	1.58	2.41	1.09
VIII	1.54	2.07	1.00

[a] HPLC conditions: column, Capcell Pak Cl (150 mm × 4.6 mm I.D.); eluent, 20 m*M* phosphate buffer (pH 7.5)–acetonitrile (7:3, v/v); flow-rate, 1.0 ml/min; temperature, ambient.

[b] HPLC conditions: column, Ultron ES-OVM (150 mm × 4.6 mm I.D.); eluent, 20 m*M* phosphate buffer (pH 7.2)–acetonitrile (85:15, v/v); flow-rate, 0.8 ml/min; temperature, 20°C.

column, I, II and III were separated, whereas IV and V, VII and VIII gave almost the same capacity factors. On the chiral column, racemic I and racemic III were resolved under the HPLC conditions used, but the peaks corresponding to the separate enantiomers overlapped when racemic I and racemic III were cochromatographed. Although the eluent composition was changed in several experiments, the four peaks corresponding to enantiomers of I and III were not completely resolved. Also, the separate enantiomers of II, in which a methylpiperidine ring of I is converted into an ethylpiperidine ring, overlapped with the enantiomers of I and III. These results reveal that the chiral column is superior to the achiral one for resolving a racemic solute, but is inferior for separating related compounds, such as a desmethyl derivative or a derivative different in alkyl side-chain length. Similar results have been reported by Wainer and co-workers [7,8] on the resolution of verapamil and norverapamil, and leucovorin and 5-methyltetrahydrofolate on reversed-phase and protein-bonded columns. Racemic VII was resolved on the OVM-bonded column, but other plausible metabolites (IV, V, VI and VIII) were not resolved into their enantiomers. Their capacity factors were different from those of the enantiomers of I and III and from one another.

It is interesting to compare the chiral recognition properties of I, II and plausible metabolites of I on the OVM-bonded column. Enantiomers of the I.S. were resolved on the OVM-bonded column despite a lower enantioseparation factor than for I. Racemic IV, a desmethyl derivative of the methoxy group at the 6-position, was not resolved on the OVM-bonded column, but racemic III, a desmethyl derivative of the methoxy group at the 5-position, was resolved. Also, V (a hydroxy derivative of a benzyl group of I) and a dimethoxy derivative of a benzyl group of I (structure not shown) were not resolved. These results reveal that a methoxy group at the 6-position and an unsubstituted benzyl group are indispensable to chiral resolution of I derivatives on the OVM-bonded column.

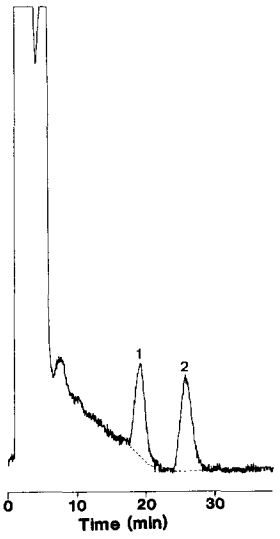

Fig. 5. Separation of I (1) and II (2) from the background components of rat plasma on an achiral column with fluorometric detection. Concentrations: I = 20 ng/ml; II = 40 ng/ml. The plasma sample was treated as described in Experimental, and 200 μl of deproteinized plasma sample were loaded onto the column. Eluent, 20 m*M* phosphate buffer (pH 7.5)–acetonitrile (70:30, v/v); flow-rate, 1.0 ml/min; excitation, 318 nm; emission, 390 nm. Other conditions as in Experimental. Dashed line indicates serum blank.

Determination of I and enantiomers of I in rat plasma

The above results suggest that coupled achiral–chiral column chromatography should be tried to attain the determination of the enantiomers of I in rat plasma. In initial experiments, the I fraction that eluted from an achiral column was collected with a 500-μl sample loop and transferred to a chiral column using a column-switching technique. However, adequate detection of the enantiomers of I was not attained, because of low enantioselectivity and high detection limits. Next, we examined a column-switching method followed by concentration of I by a trap column. Recently, Oda *et al.* [9] reported an elegant HPLC method for the determination of verapamil enantiomers. The system involves coupled achiral–chiral columns, a dilution tube and a trap column. In the present study, a modified method of Oda *et al.* [9] was used to determine the enantiomers of I in rat plasma. Fig. 5. shows the separation of I and II in rat plasma on an achiral column with fluorometric detection. I and II were separated from background components of rat plasma samples and from each other, and were sensitively detected. Fig. 6 shows the chiral resolution of I in rat plasma on the OVM-bonded column after column switching.

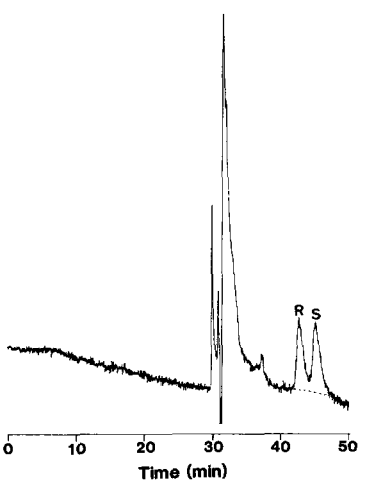

Fig. 6. Resolution of (*R*)-I (R) and (*S*)-I (S) on a chiral column after column switching. Concentration of I, 20 ng/ml in rat plasma; eluent, 20 m*M* phosphate buffer (pH 7.2)–acetonitrile (87.5:12.5, v/v); flow-rate, 0.6 ml/min; excitation, 318 nm; emission, 390 nm. The I fraction was collected for 2 min (at retention time of *ca.* 18-20 min), I was concentrated on a trap column after dilution for 8 min, and the enantiomers of I were transferred to a chiral column for 2 min and resolved. Other conditions as in Experimental. Dashed line indicates serum blank.

TABLE V

ASSAY PRECISION OF I

Racemic I (20 ng/ml) was spiked in plasma; $n = 5$.

	Achiral column	Chiral column
Accuracy (ng/ml)	20.2	–
(*R*)-/(*S*)-I peak-area ratio	–	50.4:49.6
R.S.D. (%)	1.39	4.36

Based on the chromatograms obtained with Figs. 5 and 6, assay validation of the proposed method was examined. Table V shows the accuracy and assay precision for I on the achiral column, the peak-area ratio (*R*)-/(*S*)-I and assay precision for the enantiomers of I on a chiral column. The calibration graphs for the assay of racemic I constructed with peak-area ratio ranging from 5 to 50 ng/ml and 50 to 500 ng/ml I in rat plasma were linear through the origin, with a correlation coefficient of 0.999 or more. The concentration of racemic I was determined using the achiral column, and the percentages of (*R*)-I and (*S*)-I were determined using the coupled achiral–chiral columns. The detection limits of I and the enantiomers of I were 1 and 1 ng/ml, respectively, with a 200-μl injection of deproteinized plasma samples.

The present method will be applicable to the determination of concentrations of I and the enantiomers of I in plasma after the administration of I.

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