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Stereoselective determination of the active metabolites of a new anti-inflammatory agent (CS-670) in human and rat plasma using antibody-mediated extraction and high-performance liquid chromatography

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ABSTRACT

The main metabolites of (±)-2-[4-(2-oxocyclohexylidenemethyl)phenyl]propionic acid (CS-670), a new pro-drug anti-inflammatory agent of the 2-arylpropionic acid type, have one or two chiral centres arising from reduction of the oxocyclohexylidene moiety in addition to an original chiral centre in the propionic acid moiety. To determine these metabolites stereoselectively, antibody-mediated extraction was investigated as a stereoselective clean-up method prior to chiral HPLC. Immunoglobulin G, which recognizes each stereoisomeric cyclohexanol moiety, was coupled to cyanogen bromide-activated Sepharose 4B to prepare re-usable immobilized antibody, and its specificity was improved by examination of a washing process after charging of samples. Plasma extracted with the immobilized antibody column was derivatized with a chiral reagent to separate the enantiomers of the propionic acid moiety by HPLC. This newly developed analytical method clarified the stereoselective biotransformation of the pro-drug to pharmacologically active forms in humans and rats related to reduction of the oxocyclohexylidene moiety and chiral inversion in the propionic acid moiety.

INTRODUCTION

Many analytical methods, *e.g.* high-performance liquid chromatography (HPLC), gas chromatography and immunoassay [1], have been used for stereoselective determination of chiral drugs and their metabolites, and the implications of stereoselectivity in the pharmacokinetics of these drugs are now expected to be understood. (±)-2-[4-(2-Oxocyclohexylidenemethyl)phenyl]-propionic acid (CS-670, I) is a 2-arylpropionic acid anti-inflammatory agent belonging to the chiral drug category [2] and, further, it is known to be a pro-drug [3,4] like loxoprofen [5,6]. That is to say, **I**, with a chiral centre in its molecule, is in humans mainly reduced to unsaturated alcohol with two chiral centres, and *trans*- and *cis*-alcohols with three chiral centres, as shown in Fig. 1 [3]. Both (2*S*,2′*S*)-unsaturated- and (2*S*,1′*R*,2′*S*)-*trans*-alcohols have been found to

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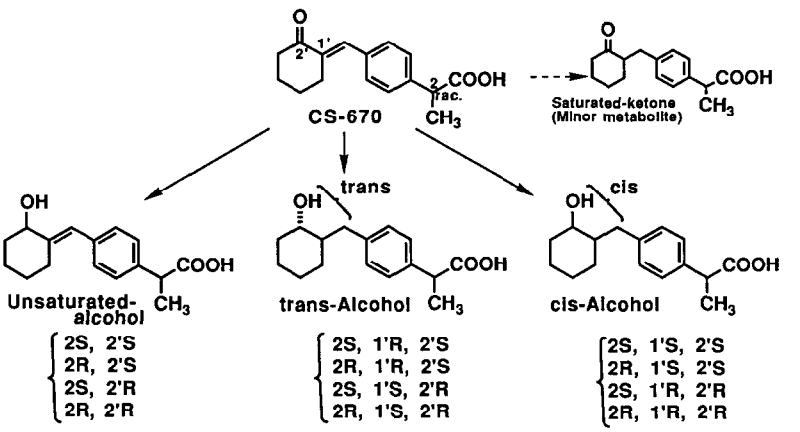

Fig. 1. Structures of I and its main plasma metabolites.

be active metabolites from an *in vitro* study of prostaglandin synthesis [4]. Thus, each stereoisomer of mono-alcohol metabolites should be stereoselectively determined to evaluate the pharmacological effect of I.

Antibody-mediated extraction is now a common technique for biologically active materials. This technique has been used as a selective clean-up method to determine hapten compounds, and we have applied it to protaglandins [7–9] and loxoprofen, which is the prototype of I [10]. However, application of this method to hapten compounds has not been studied as precisely as for high-molecular-mass compounds, such as enzymes or plasma proteins. In this work, specificity and preparation methods, *e.g.* cyanogen bromide-activated [11] and hydrazido-derivatized [12] methods, of immobilized antibody were investigated using immunoaffinity chromatography against the metabolites of I. The immunoaffinity chromatographic method for stereoselective extraction of each stereoisomer concerning the cyclohexanol moiety was followed by chiral HPLC, which enabled the enantiomeric separation of the propionic acid moiety (Fig. 2). The established analytical method was applied to stereoselective determination of the main metabolites of I in plasma of humans and rats.

EXPERIMENTAL

Materials

Bovine serum albumin, human serum albumin

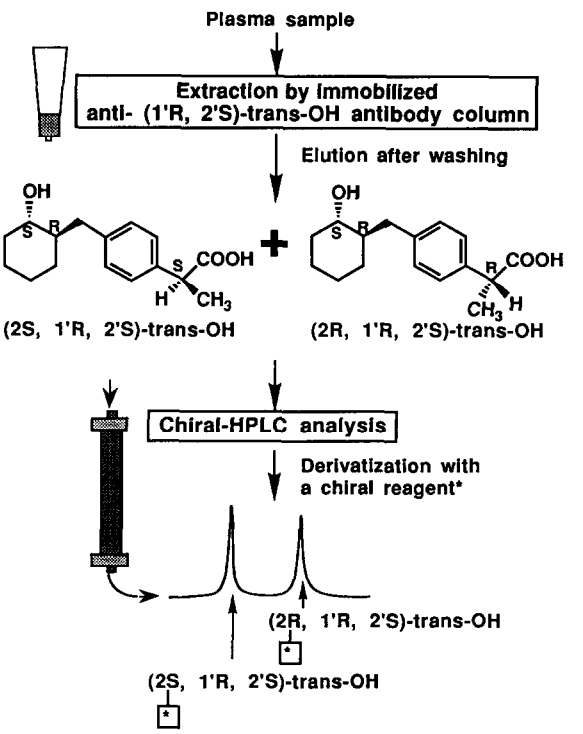

Fig. 2. Strategy for determination of (1'*R*,2'*S*)-*trans*-alcohol diastereomers.

and Freund's complete adjuvant were purchased from Sigma (St. Louis, MO, USA). Goat immunoglobulin G against rabbit immunoglobulin G was obtained from Cappel (West Chester, PA, USA), and microtitre plates for enzyme-linked immunosorbent assay (ELISA) were from Sumitomo Bakelite (Tokyo, Japan). Horseradish peroxidase (EC 1.11.1.7, grade I-C, 260 units/mg) was purchased from Toyobo (Osaka, Japan), and β-glucuronidase (type A-I) was from Tokyo Zoki Kagaku (Tokyo, Japan). 3,3′,5,5′-Tetramethylbenzidine, (+)-*R*-1-(1-naphthyl)ethylamine, 1-hydroxybenzotriazole and N,N′-dicyclohexylcarbodiimide were from Tokyo Kasei Kogyo (Tokyo, Japan). Affigel protein A MAPS-II and Affi-Gel Hz Hydrazide Gel kits were obtained from Bio-Rad (Richmond, CA, USA), and Sepharose 4B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Standard samples of **I** and its metabolites were synthesized in the Medicinal Chemistry Research Laboratories of Sankyo. Other solvents and chemicals, purchased from Wako (Osaka, Japan), were of analytical grade and were used without further purification.

Preparation of antisera against each mono-alcohol metabolite

(2*S*,1′*R*,2′*S*)-*trans*-, (2*S*,1′*R*,2′*R*)-*cis*- and (2*S*,2′*S*)-unsaturated alcohols were conjugated with bovine serum albumin by the N-succinimidyl ester method. Each diastereomer (0.08 mmol) dissolved in acetonitrile (0.6 ml) was mixed with N,N′-disuccinimidyl carbonate (0.1 mmol) and triethylamine (0.1 mmol) and gently stirred at room temperature for 15 min. Each mixture was diluted with ethyl acetate followed by washing with saturated sodium hydrogencarbonate and water. After evaporating the solvent, the N-succinimidyl ester of each diastereomer was obtained as a powder.

Each active ester ($6 \cdot 10^{-5}$ mol) was reacted with bovine serum albumin (10^{-6} mol) in 0.05 *M* phosphate buffer (pH 7.3)–pyridine (1:1, v/v, 3 ml) at room temperature for 24 h, and was dialysed against dimethylformamide (DMF)–water (50:50, v/v, and 20:80, v/v) and saline for two days. The antigen obtained was diluted to 1 mg of protein per ml with saline and emulsified with an equal volume of Freund's complete adjuvant. Three rabbits for each antigen were immunized with 1 ml of immunogen by several intradermal injections every two weeks. Four months after the first immunization, blood was taken by cardiac puncture from each rabbit and the antisera were prepared by the conventional method.

Enzyme-linked immunosorbent assay

Preparation of labelled antigens. Peroxidase-labelled antigens of (2*S*,1′*R*,2′*S*)-*trans*-, (2*S*,1′*R*,2′*R*)-*cis*- and (2*S*,2′*S*)-unsaturated alcohols were prepared according to the method described by Hosoda *et al.* [13]. N-Succinimidyl ester ($1.5 \cdot 10^{-7}$ mol) dissolved in dioxane (0.2 ml) was added to a solution of horseradish peroxidase (2 mg) in 0.05 *M* phosphate buffer (pH 7.3, 0.4 ml) at 0°C. The mixture was gently stirred at 4°C for 4 h, followed by dialysis against phosphate-buffered saline (PBS, pH 7.4)–DMF(50:50, v/v, and 80:20, v/v) and PBS at 4°C. The dialysate was diluted with PBS containing 0.1% gelatin to a concentration of 500 μg of peroxidase per ml, and refrigerated.

Procedure of assay. Microtitre plates, which were coated with a second antibody and post-coated with human serum albumin, were washed three times with PBS containing 0.05% (w/v) Tween 20 (buffer A) just before use. Antiserum (50 μl) diluted appropriately with PBS containing 0.1% human serum albumin (buffer B) was placed in each well of the plates. Then peroxidase-labelled antigen (50 μl), diluted appropriately with buffer B, and the standard solution or a sample (50 μl) were added to the wells. The plates were incubated for 20–24 h at 4°C and were washed with buffer A (3 × 350 μl). The peroxidase activity retained on the plates was measured according to a method described previously with slight modification [14]. Briefly, a solution (200 μl) of 0.01% tetramethylbenzidine in 0.05 *M* acetate–citric acid buffer (pH 5.5) containing 3% (v/v) dimethyl sulphoxide (DMSO) and 0.002% hydrogen peroxide was added to the wells, and the plates were incubated at room temperature for 30 min. The reaction was terminated

by adding 0.5 *M* sulphuric acid (50 μl), and the absorbance of the sample was measured at 450 nm using an MTP-100 microplate reader (Corona-denki, Ibaragi, Japan).

Preparation of immobilized antibody column

Each antiserum was affinity-purified by a protein A–Sepharose column, according to the method of the Bio-Rad kit. The immunoglobulin G obtained was coupled to cyanogen bromide-activated Sepharose 4B [11], as random immobilization, and to hydrazido-derivatized agarose [12], as oriented immobilization. Cyanogen bromide-activated Sepharose 4B (150 ml settled volume) was suspended in 80 ml of 0.1 *M* carbonate buffer (pH 9.0), and antibody (600 mg of immunoglobulin G in 40 ml of the same buffer) was added quickly. The mixture was rotated gently on a rotary evaporator at 4°C for 16 h. The remaining active groups were inactivated with 0.1 *M* Tris–HCl buffer (pH 8.0), and the antibody-coupled Sepharose 4B was stored in PBS at 4°C. Immobilization by the oriented method was carried out according to the Affi-Gel Hz Hydrazide Gel kit (Bio-Rad). Immunoglobulin G (80 mg), whose oligosaccharide moieties had been oxidized with sodium periodate, was mixed with hydrazido-derivatized gel (10 ml) in coupling buffer (20 ml) of pH 5.5 at room temperature for 20 h, and the antibody-immobilized gel was washed with the coupling buffer and stored in PBS at 4°C.

Immunoaffinity chromatography

The immobilized antibody gel (1 ml) was packed into a polypropylene column (Sepacol-mini-pp, Seikagaku-kogyo, Tokyo, Japan) and handled at room temperature during the clean-up. A sample was charged to an immobilized antibody column and washed with water (3 × 5 ml). Further, to improve the specificity of each immobilized antibody, 60% (v/v) methanol, 10% (v/v) methanol, and a solution containing the methyl ester of *trans*-alcohol (5 μg) were used as washing solvents for, respectively, (1′*R*,2′*S*)-*trans*-, (1′*R*,2′*R*)-*cis*- and 2′*S*-unsaturated alcohols. Then, each mono-alcohol retained on the column was eluted with 95% (v/v) methanol (5 ml), and the solvent was evaporated and subjected to derivatization for chiral HPLC analysis.

The capacity and specificity for each mono-alcohol in the immobilized antibody column were examined by HPLC, which resulted in the separation of **I** and its possible metabolites. After extraction of each metabolite using the immobilized antibody column, the residue obtained was redissolved in ethanol and subjected to HPLC, which was performed with acetonitrile–0.05 *M* phosphoric acid (46:54, v/v) as a mobile phase at a flow-rate of 1.0 ml/min with ultraviolet detection (203 nm).

Chiral HPLC using derivatization of the propionic acid moiety

First, each internal standard corresponding to the analytical objective was added to the residue obtained from immunoaffinity chromatography (see the legend to Fig. 4). Next, dichloromethane–pyridine (98:2, v/v) solutions of (+)-*R*-1-(1-naphthyl)ethylamine (20 μg, 0.1 ml), 1-hydroxybenzotriazole (40 μg, 20 μl) and N,N′-dicyclohexylcarbodiimide (40 μg, 0.1 ml) were added and the mixture was allowed to stand at room temperature for 1 h. After evaporation of the solvent, the residue was mixed with 0.5 ml of ethyl acetate and 0.5 ml of hydrochloric acid and stirred for 1 min using a vortex mixer. The organic phase was separated, dried over anhydrous sodium sulphate, and a 50-μl aliquot was injected into the HPLC system. HPLC was performed with *n*-hexane–ethanol–dichloromethane (95:5:10, v/v) as mobile phase at a flow-rate of 2.0 ml/min with fluorimetric detection (excitation at 283 nm and emission at 330 nm). A 655 liquid chromatograph (Hitachi, Tokyo, Japan) equipped with ERC-Silica-1282 (250 × 6 mm I.D., ERMA CR., Saitama, Japan) and an F-1000 spectrofluorimeter (Hitachi) was used.

*Administration of **I** to humans and rats*

Six fasted (for 10 h) male volunteers received a single oral dose of 120 mg of **I**. Blood was collected from a peripheral vein at an appropriate time after the oral administration and was centrifuged

to obtain plasma. A 1-ml aliquot of plasma was analysed by HPLC to determine the total *trans-*, *cis-* and unsaturated alcohols according to the method described by Matsuki *et al.* [3]. The HPLC was performed with a Nucleosil $5C_{18}$ (150 × 4.6 mm I.D., Nihon Chromato Works, Tokyo, Japan) column and acetonitrile–water–phosphoric acid (25:45:0.1, v/v) as mobile phase at a flow-rate of 1.0 ml/min with ultraviolet detection (203 nm). Additionally, 0.1–0.6 ml of plasma was applied to three immobilized antibody columns, to determine each diastereomer of mono-alcohols. A urine sample appropriately diluted was hydrolysed with β-glucuronidase in 0.1 *M* acetate buffer (pH 5.2) and subjected to HPLC analysis or antibody-mediated extraction in a similar manner as described above.

A dose of 2 mg/kg **I** dissolved in PBS was given orally to male Wistar–Imamichi rats that had been fasted for 16 h. Whole blood was obtained from a carotid artery of each rat at regular intervals under ether anaesthesia, and plasma samples were prepared and analysed in the same manner as described previously.

RESULTS

Comparison of random and oriented immobilization of antibodies in hapten immunoaffinity chromatography

The relationship between antibody ligand density and capacity for antigen was examined with two immobilization methods: cyanogen bromide-activated and hydrazido-derivatized. The result for anti-(1′*R*,2′*R*)-*cis*-alcohol antibody is shown in Table I, when 2 μg of (2*S*,1′*R*,2′*R*)-*cis*-alcohol were charged. Both matrices showed similar capacity, *ca.* 500 ng of hapten per mg of immunoglobulin G, at various antibody-matrix densities, without showing a tendency for capacity to decrease as density increased, though the hydrazido-derivatized matrix has been reported to be a high-capacity immunosorbent for high-molecular-mass compounds. In Fig. 3, the two methods are compared in terms of re-usability. The capacity of immobilized antibody coupled to matrix by the cyanogen bromide-activated method was more than 80% of the capacity of the first time, even after three uses. On the other hand, the capacity of the hydrazido-derivatized method was decreased to 20% of the first-time capacity after exposure to 95% (v/v) methanol three times.

TABLE I

INFLUENCE OF ANTIBODY LIGAND DENSITY ON CAPACITY FOR ANTIGEN IN AN IMMOBILIZED ANTIBODY COLUMN

Antibody[a] coupled (mg/ml gel)	Capacity for antigen	
	ng/ml gel	ng/mg IgG
Cyanogen bromide-activated Sepharose 4B		
1	400 ± 9	400 ± 9
4	1708 ± 33	427 ± 8
12	5481 ± 143	457 ± 12
Affi-Gel Hz Hydrazide Gel		
1	588 ± 8	588 ± 8
4	1907 ± 13	477 ± 3
12	5787 ± 105	482 ± 9

[a] Anti-(1′*R*,2′*R*)-*cis*-alcohol antibody was coupled to each matrix.

Specificity of immobilized antibody

Cross-reactivities with other metabolities in ELISA were examined for each antiserum, and the results of representative antisera, which had satisfactory specificity and titres, are shown in Table II. Because almost the same affinity was

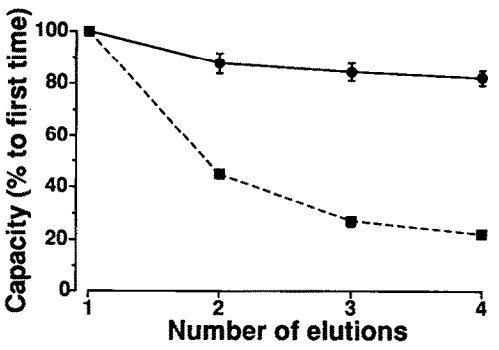

Fig. 3. Decrease in capacity by re-using immobilized antibody column. The immobilized antibody column prepared by cyanogen bromide-activated (●) or hydrazido-derivatized method (■) was exposed to 95% (v/v) methanol and re-used after adequate washing with water and PBS.

TABLE II

CROSS-REACTIVITIES (%) OF ANTIBODY IN ELISA OR IMMOBILIZED COLUMN BY SEVERAL WASHING METHODS

Figures in parentheses indicate recovery (%) in 95% methanol fraction compared with water washing.

Compound	(A) Anti-(1′*R*,2′*S*)-*trans*-alcohol			(B) Anti-(1′*R*,2′*R*)-*cis*-alcohol			(C) Anti-2′*S*-unsaturated alcohol		
	ELISA	Immobilized antibody		ELISA	Immobilized antibody		ELISA	Immobilized antibody	
		Water	60% Methanol		Water	10% Methanol		Water	Cross-reacting compound
trans-Alcohol									
(2*S*,1′*R*,2′*S*)	100	100	100(98.1)	<0.1	2.9	<0.1	3.6	28.5	15.2
(2*R*,1′*R*,2′*S*)	100	98.0	74.1	<0.1	2.7	<0.1	6.1	34.3	18.0
(2*S*,1′*S*.2′*R*)	1.4	97.2	9.1	0.5	11.5	3.1	0.1	7.9	1.8
(2*R*,1′*S*,2′*R*)	0.5	51.7	14.3	0.9	14.4	2.0	0.2	10.2	1.1
cis-Alcohol									
(2*S*,1′*S*,2′*S*)	0.3	33.3	0.6	0.4	8.6	1.8	1.5	49.5	11.7
(2*R*,1′*S*,2′*S*)	0.3	60.6	0.5	0.4	8.5	2.3	0.9	41.3	11.1
(2*S*,1′*R*,2′*R*)	0.4	61.6	0.1	100	100	100(76.3)	<0.1	11.9	1.4
(2*R*,1′*R*,2′*R*)	0.4	82.4	1.4	79.3	98.7	83.9	0.1	18.9	1.4
Unsaturated alcohol									
(2*S*,2′*S*)	5.8	86.5	11.7	<0.1	4.1	1.8	100	100	100(78.2)
(2*R*,2′*S*)	7.0	88.7	12.0	0.2	5.6	2.8	93.8	100	95.0
(2*S*,2′*R*)	0.4	39.9	5.7	3.4	25.3	8.2	2.6	33.1	7.0
(2*R*,2′*R*)	0.4	32.2	4.4	2.2	28.8	8.3	3.3	29.6	10.4
Saturated ketone	1.5	69.1	11.3	0.7	14.6	6.0	0.2	10.8	0.5
CS-670 (I)	<0.1	49.7	6.5	<0.1	0.1	<0.1	<0.1	34.2	1.5

observed between 2*S* and 2*R* forms of the analytical objective in every case, each antiserum prepared by immunization with (2*S*,1′*R*,2′*S*)-*trans*-, (2*S*,1′*R*,2′*R*)-*cis*- and (2*S*,2′*S*)-unsaturated alcohol–bovine serum albumin conjugate was against (1′*R*,2′*S*)-*trans*–, (1′*R*,2′*R*)-*cis*- and 2′*S*-unsaturated alcohol, respectively. Next, to examine the specificity of the immobilized antibody, other metabolites were applied in the amount of 2 μg. The specificity after washing with water and elution with 95% (v/v) methanol was not as high as the result obtained by ELISA in every case. When methanol diluted to 60% (v/v) was used as a washing solvent in the case of anti-(1′*R*,2′*S*)-*trans*-alcohol, cross-reactivity against (2*S*,1′*S*, 2′*R*)-*trans*-alcohol was reduced to 9.1% from 97.2%, and the capacity for the (2*S*,1′*R*,2′*S*) form remained 98.1%. A similar effect on the specificity of immobilized anti-(1′*R*,2′*R*)-*cis*-alcohol antibody was observed by using 10% (v/v) methanol; cross-reactivities against (1′*S*,2′*S*)-*cis*-alcohols were improved to *ca.* 2% from more than 8%. To improve the specificity of immobilized anti-2′*S*-unsaturated alcohol antibody, 5 μg of the methyl ester of racemic *trans*-alcohol in PBS, which was a cross-reacting compound and could not be determined by chiral HPLC using derivatization with (+)-*R*-1-(1-naphthyl)ethylamine, were used as washing solvent. This washing method with a solution containing the cross-reacting compound reduced the cross-reactivity against (2*S*,2′*R*)-unsaturated alcohol to 7.0% from 33.1%, retaining more than 78% of the analytical objectives.

Chiral HPLC analysis combined with antibody-mediated extraction

Metabolites of **I** derivatized with (+)-*R*-1-(1-naphthyl)ethylamine were stable after extraction with ethyl acetate for injection into the HPLC system. A chromatogram of a standard mixture under optimal chiral HPLC conditions is shown in Fig. 4A. Three compounds, *i.e. cis*-, *trans*- and unsaturated alcohols, were separated with different retention times, and each enantiomeric pair was resolved completely. Human plasma samples purified by each immobilized antibody column

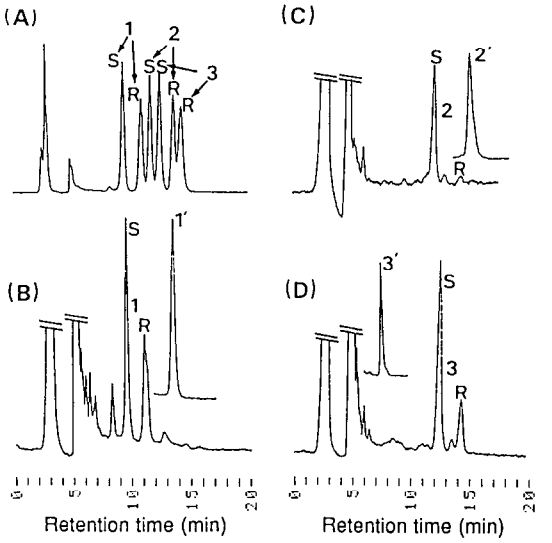

Fig. 4. High-performance liquid chromatograms of diastereomeric amides. (A) A mixture of *cis*-, *trans*- and unsaturated alcohols was derivatized with the chiral reagent. Human plasma collected 1 h after oral administration of **I** was passed through the immobilized antibody column for the stereoselective extraction of (B) (1′*R*, 2′*R*)-*cis*-, (C) (1′*R*,2′*S*)-*trans*- and (D) 2′*S*-unsaturated alcohols, and was derivatized with the chiral reagent. Peaks 1, 2 and 3 correspond to *cis*-, *trans*- and unsaturated alcohol, respectively. Each internal standard is identified by a number with a superscript prime: 1′ = 2*S*-*trans*-alcohol form of loxoprofen; 2′ = 2*R*-*trans*-alcohol form of loxoprofen; 3′ = 2*R*-loxoprofen. *S* or *R* represents the absolute configuration of position 2.

were derivatized with the amine and subjected to HPLC (Fig. 4B–D). Peaks, except for each objective alcohol, could not be observed on chromatograms, because they were almost completely removed by the antibody-mediated extraction. Therefore, the retention time of the internal standard was appropriate for the determination of each alcohol.

Standard samples of 25–350 ng of (1′*R*,2′*S*)-*trans*-alcohol and 25–200 ng of (1′*R*,2′*R*)-*cis*- and 2′*S*-unsaturated alcohols in 0.2 ml of human control plasma were extracted by each immobilized antibody column and determined by chiral HPLC. Calibration curves of the HPLC combined with antibody-mediated extraction showed good linearities between the amounts of metabolite and the peak-height ratios to internal standard (r = 0.997–0.998), and passed close to the origin.

Plasma levels of each alcohol diastereomer in rats

The plasma concentrations of *trans-*, *cis-* and unsaturated alcohol diastereomers in rats after oral administration of **I** at a dose of 2 mg/kg are shown in Fig. 5, and pharmacokinetic parameters of each metabolite in rat plasma are summarized in Table III. The area under the curve (AUC, 0–8 h) of (1′*R*,2′*S*)-*trans*-alcohol accounted for 82% of the AUC (0–8 h) of total *trans*-alcohol, suggesting highly stereoselective reduction of the oxocyclohexylidene moiety to the (1′*R*,2′*S*) form. Further, almost all of the (1′*R*,2′*S*)-*trans*-alcohol was in the 2*S* form (80% of total *trans*-alcohol), and peak plasma time (t_{max}) and half-life ($t_{1/2}$) of (2*S*,1′*R*,2′*S*)-*trans*-alcohol were later and longer, respectively, than the t_{max} and $t_{1/2}$ of (2*R*,1′*R*,2′*S*)-*trans*-alcohol. These results suggest that chiral inversion of *trans*-alcohol from 2*R* to 2*S* occurs in rats. However, in the cases of *cis-* and unsaturated alcohols, the stereoselective phenomena were not so clearly observed. That is to say, reduction of the oxocyclohexylidene moiety was selective to a certain extent, but the ratios of 2*S*/2*R* were 1.7 (48/29) and 1.1 (37/33) in *cis-* and unsaturated alcohol, re-

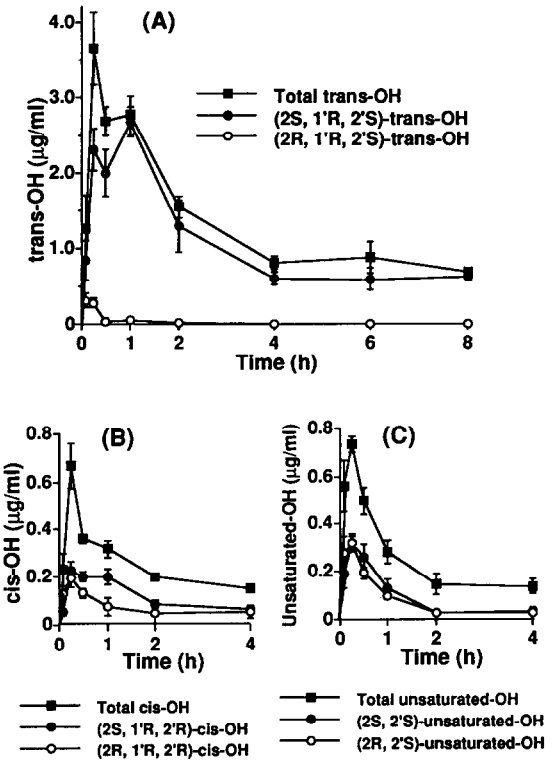

Fig. 5. Plasma levels of the main metabolite diastereomers after oral administation of **I** to rats. **I** was given to rats at a dose of 2 mg/kg, and the mean plasma concentration of three rats was plotted for (a) *trans-*, (B) *cis-* and (C) unsaturated alcohol.

TABLE III

PHARMACOKINETIC PARAMETERS OF DIASTEREOMERS OF MAIN METABOLITES IN RAT PLASMA AFTER ORAL ADMINISTRATION OF **I** (2 mg/kg, $n = 3$)

Compound	C_{max}[a] (μg/ml)	t_{max} (h)	$t_{1/2}$ (h)	AUC[b] (μg · h/ml)	
Total *trans*-alcohol	3.66	0.25	1.73	10.37 (100)[c]	
(2*S*,1′*R*,2′*S*)-*trans*-alcohol	2.67	1.0	1.83	8.29 (79.9)	(81.7)
(2*R*,1′*R*,2′*S*)-*trans*-alcohol	0.32	0.08	0.13	0.19 (1.8)	
Total *cis*-alcohol	0.67	0.25	1.63	1.00 (100)	
(2*S*,1′*R*,2′*R*)-*cis*-alcohol	0.23	0.25–1.0	1.95	0.48 (48.0)	(77.0)
(2*R*,1′*R*,2′*R*)-*cis*-alcohol	0.20	0.25	0.55	0.29 (29.0)	
Total unsaturated alcohol	0.73	0.25	0.50	0.98 (100)	
(2*S*,2′*S*)-unsaturated alcohol	0.32	0.25	0.50	0.36 (36.7)	(69.4)
(2*R*,2′*S*)-unsaturated alcohol	0.32	0.25	0.45	0.32 (32.7)	
Saturated ketone	0.18	0.25	–[d]	0.16	
CS-670 (I)	0.11	0.08–0.25	–	0.10	

[a] Peak plasma concentration.
[b] AUC was calculated from the data of 0–8 h for *trans*-alcohol, and the data of 0–4 h were used for the other metabolites.
[c] Figures in parentheses indicate the percentage of the total for each alcohol.
[d] $t_{1/2}$ could not be calculated.

spectively. The degree of stereoselective metabolism, including reduction of the oxocyclohexylidene moiety and chiral inversion in the propionic acid moiety, was observed to be in the order of *trans-* > *cis-* > unsaturated alcohol in rats.

Plasma levels of each alcohol diastereomer in humans

The plasma concentrations of each alcohol diasteromer in humans after oral administration of I at a single 120-mg dose are shown in Fig. 6.

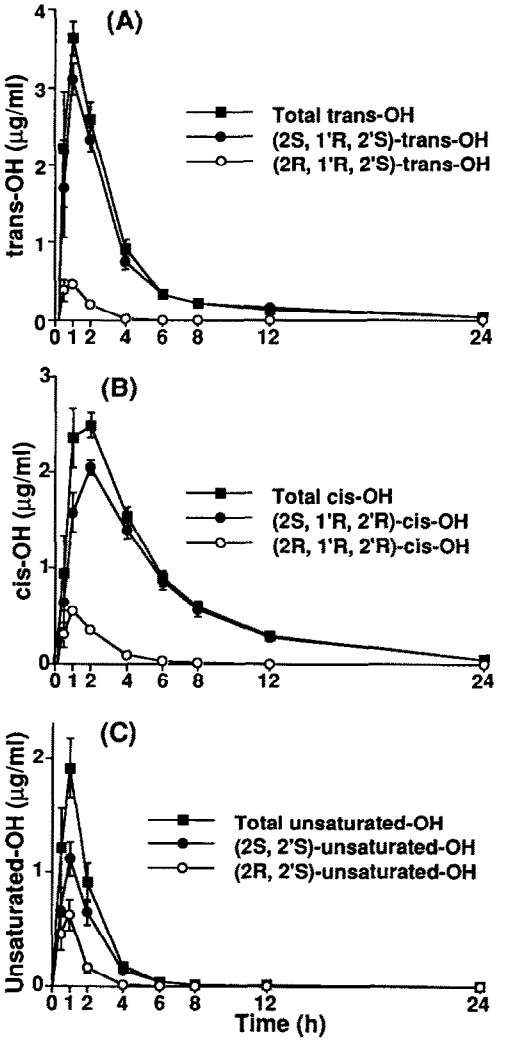

Fig. 6. Plasma levels of main metabolite diastereomers after oral administration of I to human volunteers. I was given to humans in a single 120-mg dose, and the mean plasma concentration of six volunteers was plotted for (A) *trans-*, (B) *cis-* and (C) unsaturated alcohol.

The pharmacokinetic parameters of each metabolite in human plasma are summarized in Table IV. The AUC (0–24 h) of (1′*R*,2′*S*)-*trans*-alcohol in humans calculated as a percentage of total *trans*-alcohol AUC (0–24 h) was higher than in rats, and 91% of the total of four *trans*-alcohol stereoisomers was in the (2*S*,1′*R*,2′*S*) form. A stereoselective metabolism, including a chiral inversion phenomenon, was clearly observed with *cis-* and unsaturated alcohols, and it was different from that seen in rats. When reduction of the cyclohexylidene moiety was noticed, the AUC (0–24 h) of (1′*R*,2′*R*)-*cis*-alcohol accounted for 96% of the AUC (0–24 h) of total *cis*-alcohol, and similarly 2′*S*-unsaturated alcohol accounted for 89% of total unsaturated alcohol. As chiral inversion occurs from the 2*R* to the 2*S* enantiomer, some extension of t_{max} and $t_{1/2}$ of the main metabolite (2*S*) compared with the minor metabolite (2*R*) was more clearly observed with *trans-* and *cis*-alcohol than with unsaturated alcohol.

These stereoselective phenomena were supported by measurement of metabolites in urine after treatment with β-glucuronidase. The representative forms of *trans-*, *cis-* and unsaturated alcohol accounted for 82, 80 and 50% of each total alcohol, and the order of stereoselectivity in metabolism was the same as in plasma.

DISCUSSION

The analytical strategy described in this paper, which is antibody-mediated extraction combined with chiral HPLC, resulted in stereoselective measurement of three haptens that are the main metabolites of I in plasma and have complicated structures. In a similar manner, we applied the immobilized antibody as a chiral reagent to determine the active metabolite of loxoprofen [10]. But the general character of immobilized antibody against hapten is not, as yet, understood in detail. In the present work, (1) a comparison of immobilization methods and (2) an improvement of specificity on immobilized antibody were studied in the field of immunoaffinity chromatography against hapten.

TABLE IV

PHARMACOKINETIC PARAMETERS OF DIASTEREOMERS OF MAIN METABOLITES IN HUMAN PLASMA AFTER ORAL ADMINISTRATION OF I (120 mg per person, $n = 6$)

Compound	C_{max} (μg/ml)	t_{max} (h)	$t_{1/2}$ (h)	AUC[a] (μg · h/ml)	
Total *trans*-alcohol	3.72 ± 0.31	1.08 ± 0.20	1.50 ± 0.09	12.15 ± 0.82 (100)[b]	
(2*S*,1′*R*,2′*S*)-*trans*-alcohol	3.20 ± 0.27	1.08 ± 0.20	1.73 ± 0.34	11.07 ± 0.82 (91.1)	(98.2)
(2*R*,1′*R*,2′*S*)-*trans*-alcohol	0.61 ± 0.06	0.75 ± 0.11	0.93 ± 0.17	0.87 ± 0.06 (7.1)	
Total *cis*-alcohol	2.79 ± 0.20	1.50 ± 0.22	3.00 ± 0.22	15.24 ± 1.19(100)	
(2*S*,1′*R*,2′*R*)-*cis*-alcohol	2.07 ± 0.10	1.83 ± 0.17	3.15 ± 0.22	13.21 ± 1.13 (86.7)	(95.8)
(2*R*,1′*R*,2′*R*)-*cis*-alcohol	0.59 ± 0.07	1.08 ± 0.20	1.17 ± 0.18	1.39 ± 0.06 (9.1)	
Total unsaturated alcohol	2.13 ± 0.23	0.75 ± 0.11	0.87 ± 0.06	3.94 ± 0.43 (100)	
(2*S*,2′*S*)-unsaturated alcohol	1.23 ± 0.14	0.75 ± 0.11	0.93 ± 0.03	2.53 ± 0.30 (64.2)	(88.9)
(2*R*,2′*S*)-unsaturated alcohol	0.84 ± 0.09	0.75 ± 0.11	0.58 ± 0.07	0.98 ± 0.13 (24.7)	

[a] AUC was calculated from the data of 0–24 h for all metabolites.
[b] Figures in parentheses indicate the percentage of the total for each alcohol.

At first, cyanogen bromide-activated Sepharose [11] and hydrazido-derivatized agarose [12] were compared as immunoaffinity matrices. Antibody immobilized on hydrazido-derivatized agarose, which has been reported to be a high-capacity immunosorbent for high-molecular-mass compounds, such as enzymes and plasma proteins, owing to its oriented immobilization [15–18], was not re-usable when 95% methanol was used as an eluting solvent. However, the cyanogen bromide-activated method, namely random immobilization, resulted in re-usable immobilized antibody having the same capacity as the hydrazido-derivatized method. From these results, it might be speculated that hapten can more easily bind to the randomly immobilized antibody than high-molecular-mass antigen, and its random and hidden manner of immobilization causes protection of immobilized antibody against organic solvent, which is necessary for hapten elution.

In addition, the specificity of immobilized antibody in hapten immunoaffinity chromatography was investigated. When only water was used as a washing solvent prior to elution of an objective hapten from immobilized antibody column, the specificity of the antibody was not as high as the result obtained by ELISA in all cases of three antisera against different antigens. This contradiction may be explained by the great discrepancy in the amount of antibody used and the difference in antibody situation. Furthermore, washing with water containing some organic solvent or a cross-reacting compound improved the unexpected specificity of these immobilized antibodies, and this procedure can be generally applied to the selective clean-up method for hapten using immunoaffinity chromatography.

The ability of immunoaffinity chromatography to recognize the cyclohexanol moiety was combined with chiral HPLC, which enables separation of the enantiomers of the propionic acid moiety, and this newly developed analytical method clarified the stereoselective metabolism of **I**. In rats, the active metabolite with the 2*S*,1′*R*,2′*S* configurations accounted for 80% of the total amount of four *trans*-alcohol stereoisomers. In humans, stereoselectivity was more clearly observed regarding the formation of not only the (2*S*,1′*R*,2′*S*) form of *trans*-alcohol but also the (2*S*,1′*R*,2′*R*) form of *cis*-alcohol and the (2*S*,2′*S*) form, which is another active metabolite,

of unsaturated alcohol. The stereoselective ketone reduction and chiral inversion of 2-arylpropionic acid in **I** resulted in more effective biotransformation to the pharmacologically active forms than in the case of loxoprofen [10]; this means that **I** is more attractive as a pro-drug than loxoprofen.

In this work, the usefulness of immobilized antibody for stereoselective extraction of hapten was confirmed in combination with HPLC analysis. We can also apply the immobilized antibody as a clean-up material to other methods of analysing haptens. Additionally, further investigations of monoclonal antibody and physically stronger matrices than agarose will be necessary to develop the field of immunoaffinity chromatography against hapten more extensively.

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