Pharmaceutical Research, Vol. 22, No. 1, January 2005 (© 2005)
DOI: 10.1007/s11095-004-9012-4

Research Paper

Stereoselective Taurine Conjugation of (*R*)-Benoxaprofen Enantiomer in Rats: *In Vivo* and *in Vitro* Studies Using Rat Hepatic Mitochondria and Microsomes

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Received April 3, 2004; accepted September 23, 2004

Purpose. Identify (*R*)-BOP-T in rat bile after administration of (*R*)-BOT over a 12 h period.
Methods. Each benoxaprofen (BOP) enantiomer was administered i.v. to bile duct-cannulated rats at a dose of 5 mg/kg. The optical isomers of BOP and its metabolites in plasma, urine, and bile were quantified using a chiral HPLC column. The amounts of BOP glucuronide (BOP-G), BOP taurine conjugate (BOP-T), and BOP enantiomers excreted into the bile over 12 h after administration of (*R*)-BOP were as follows: (*R*)-BOP-G and (*S*)-BOP-G, 2.1 ± 0.5 and 6.2 ± 1.4% of the dose; (*R*)-BOP-T and (*S*)-BOP-T, 5.6 ± 1.8 and 0.7 ± 0.3% of the dose; (*R*)-BOP and (*S*)-BOP, 0.7 ± 0.1 and 1.7 ± 0.2% of the dose, respectively, whereas after (*S*)-BOP administration, (*S*)-BOP-G and (*S*)-BOP were mainly excreted into the bile (14.3 ± 1.8 and 3.0 ± 0.4% of the dose, respectively). Only after (*R*)-BOP administration was the taurine conjugate of BOP found in the bile, and the configuration was *R*. BOP-T could not be found in the bile after (*S*)-BOP administration. To investigate the stereoselectivity of the conjugation enzymes responsible for BOP-T formation, *in vitro* studies were performed using rat hepatic organelles.
Results. When (*R*)-BOP was used as a substrate, rat hepatic mitochondrial and microsomal fractions exhibited stereoselective BOP-T formation activity, with microsomal activity approximately 3.0 times greater than that of the mitochondria. That of (*S*)-BOP was approximately 2.1. Mean (*R*)/(*S*) ratios of BOP enantiomer for BOP-T formation in the mitochondrial and microsomal incubations were approximately 1.7 and 2.4, respectively.
Conclusion. Although in the *in vivo* studies, only (*R*)-BOP-T originated from (*R*)-BOP was found in the bile, the configuration of BOP-T formed by the incubations of (*R*)-BOP or (*S*)-BOP with rat hepatic mitochondria or microsomes was *S* for both.

KEY WORDS: benoxaprofen; glucuronide; microsomes; stereoselective; taurine conjugation.

INTRODUCTION

Benoxaprofen [2-(4-chlorophenyl)-α-methyl-5-benzoxazole acetic acid, BOP] (Fig. 1) is a 2-arylpropionic acid derivative that exhibits anti-inflammatory effects (1–3) and that was withdrawn from the market because of unexplained fatal liver toxicity (4,5). The metabolic fate of BOP has been studied in mice, rats, rabbits, dogs, rhesus monkeys, and humans (6–9). Previously, we reported that 12-h cumulative amounts of BOP glucuronide (BOP-G) and BOP taurine conjugate (BOP-T) excreted in bile were approximately 13.2% and 2.5% of the dose, respectively, after i.v. administration to bile duct–cannulated rats at a dose of 10 mg/kg racemic BOP (10). We also reported that BOP-T was mainly in the *R* configuration and BOP-G was mainly *S*.

Here, we report the stereoselective taurine and glucuronic acid conjugation of each BOP enantiomer excreted in bile over 12 h, after i.v. administration of each BOP enantiomer at a dose of 5 mg/kg. Furthermore, we also describe the stereoselective BOP-T formation in rat hepatic subcellular fractions (mitochondria and microsomes).

MATERIALS AND METHODS

Measurements

High-resolution mass spectra were obtained with a JEOL JMS-DX302 instrument (JEOL, Tokyo, Japan), using a direct-inlet system. Operating conditions were as follows: The accelerating voltage and ionization current were 3 kV and 300 μA, respectively. Ionization was by electron impact with 70-eV electrons, with a source temperature of 150°C. Perfluorokerosene was used as a standard compound. Positive-ion FAB mass spectra were obtained with an Auto Spec mass spectrometer (Micromass UK Ltd., Manchester, England) equipped with a FAB source using cesium atoms. The instrument was operated with an accelerating voltage of 8 kV, and glycerol was used as the FAB matrix for the acquisition of positive-ion spectra.

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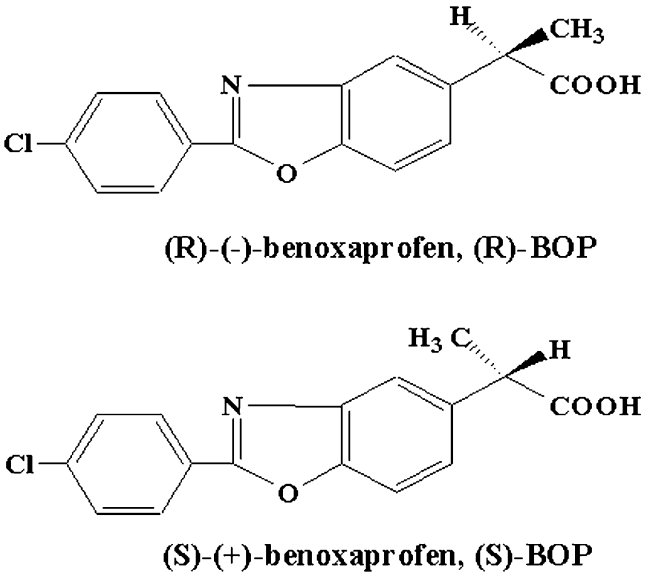

Fig. 1. Structures of benoxaprofen enantiomers.

Chemicals

Tetrabutylammonium hydrogen sulfate (TBA) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Naproxen was purchased from Sigma Chemical Co. (St. Louis, MO, USA). BOP was kindly donated by Eli Lilly Co. (Indianapolis, IN, USA) before withdrawal of the drug from the market. Triton X-100 was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Tris (hydroxymethyl) aminomethane (Tris) and ATP·2Na were purchased from Nakalai Tesque Inc. (Kyoto, Japan). Sodium ethylenediaminetetraacetic acid (EDTA·2Na), sucrose, CoASH, magnesium chloride ($MgCl_2$), and dithiothreitol (DTT) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Taurine was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Methanol (MeOH), acetonitrile (MeCN) and tetrahydrofuran (THF) were HPLC grade (Wako). All other chemicals were reagent grade. The water used had been double-distilled in a glass still. Naproxen methylester (NAP-Me, IS) was synthesized in our laboratory, as reported previously (10). Reference (*R*)-BOP and (*S*)-BOP were obtained by preparative HPLC using a chiral column described previously (10). (*R*)-BOP-T and (*S*)-BOP-T were synthesized from (*R*)- and (*S*)-BOP enantiomers respectively, as reported previously (10). Standard (*R*)-BOP-G and (*S*)-BOP-G were obtained from (*R*)-BOP and (*S*)-BOP enantiomers biosynthetically, as reported previously (11).

Animals and Drug Administration

Groups of five male Sprague-Dawley rats (Sankyo Labo Service Co., Tokyo, Japan), weighing 250–300 g, were used throughout the study. Each group of five rats was housed together in a stainless steel cage, in a temperature-controlled (23–25°C) room with a 12-h light/dark cycle. The rats were allowed free access to standard rat chow (Sankyo) and water for 1 week before the experiments. Each animal was anesthetized with 20% (w/v) urethane (1 g/kg body weight, i.p.). The femoral vein was cannulated with PE-10 tubing (Clay Adams, Parsippany, NJ, USA) for instillation of saline solution and drug administration. An abdominal incision was made and the common bile duct was cannulated with PE-10 tubing (Clay Adams), for collection of bile samples, and closed with surgical clips. During experimental procedures, body temperatures were maintained at 38 ± 0.5°C with a heating lamp, to prevent hypothermic alterations of bile flow. The solution of BOP for injection was prepared by dissolving 50 mg of BOP in a 2-ml mixture of 1 M sodium hydroxide, ethanol, and saline (8:8:4, v/v). Saline was injected into the rats through the cannula to supplement body fluids. Each BOP enantiomer was administered at a dose of 5 mg/kg body weight through the femoral vein. The femoral artery was cannulated with PE-50 tubing (Clay Adams) and a heparin-lock was established, using 100 U/ml heparin in saline. Blood samples (each approximately 0.2 ml) were collected from the femoral artery at 0, 5, 10, 15, 20, 30, and 45 min and 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, and 12 h after i.v. administration. The collected blood was immediately centrifuged at 15,000 × *g* for 15 min at 4°C, and plasma was separated. Bile was collected into 2-ml plastic tubes containing 100 μl of 10% metaphosphoric acid to prevent hydrolytic cleavage of acyl glucuronides, in successive 30-min periods. Urine samples were collected continuously for 12-h through a PE-50 tubing bladder cannula into 5-ml glass tubes containing 100 μl of 10% metaphosphoric acid. Saline supplements were administered to the rats through the femoral cannula, at volumes equivalent to blood and bile collection volumes (each approximately 0.2–0.5 ml). Bile and urine outputs were measured by weight. The plasma, bile, and urine samples collected were stored at −80°C until analysis.

HPLC Conditions

The HPLC system consisted of a model PU-1580 Intelligent pump (JASCO Corp., Ltd., Tokyo, Japan) equipped with a model FP-1520 Intelligent fluorescence detector (JASCO) and a model AS-1555 Intelligent sampler (JASCO). HPLC data were treated by a Bowin computer program (JASCO). The excitation and emission wavelengths (xenon lamp) were set at 315 and 365 nm, respectively. For the separation of optical isomers of BOP and its metabolites, a chiral HPLC column (SUMICHIRAL OA 3300, 4.6 mm i.d. × 25 cm; Sumika Chemical Analysis Service, Osaka, Japan) equipped with a guard column packed with the same resin (4.6 mm i.d. × 1 cm; Sumika) was used. The mobile phases used for the separation of the optical isomers were 0.04 M ammonium acetate in MeOH for BOP enantiomers in plasma, 0.06 M ammonium acetate in MeOH for BOP-G enantiomers in bile, and 0.01 M ammonium acetate in MeOH/MeCN/H_2O (85:15:5, v/v) for BOP and BOP-T enantiomers in bile. The analysis for BOP-T formed enzymatically was performed using a mobile phase consisted of 0.006 M ammonium acetate in MeOH. For the determination of optimal conditions for the conjugation enzymes of BOP-T formation, a reversed-phase HPLC column (CAPCELL PAK C18 UG120 ODS, 4.6 mm i.d. × 250 mm; Shiseido) equipped with a guard column packed with NUCLEOSIL 10 C18 (4.6 mm i.d. × 10 mm; Senshu Scientific Co. Ltd., Tokyo, Japan) was used with 10 mM TBA/MeCN/THF (100:35:35, v/v). The flow rate was 1.0 ml/min.

Pretreatment of Biological Samples Prior to HPLC Analysis

To 10 μl of plasma, bile, or urine samples in 2-ml plastic tubes, 180 μl of MeCN, 30 μl of IS (100 μg/ml in dimethylsulfoxide), and 30 μl of distilled water were added, with vigorous mixing. After centrifugation of the sample for 10 min at 15,000 × *g* at 4°C, 10 μl of the supernatant was injected directly into the HPLC system.

Analytical Methods

Calibration curves (1–100 μg/ml) were established, using linear least-squares regression analyses, from BOP/IS peak area ratios vs. various concentrations of BOP in drug-free plasma, bile, or urine samples to which aliquots of standard BOP had been added. For the (*R*)- and (*S*)-BOP enantiomers, the calibration curves ranged from 0.5 to 50 μg/ml. The BOP-G and BOP-T concentrations in bile and urine were calculated from the BOP calibration curve as BOP equivalents. However, because the molar absorptivities for BOP-G and BOP-T in the mobile phase were different from that of BOP, the BOP-T concentrations obtained from the BOP calibration curve were corrected using the following equation: BOP-T concentration = (1 mM BOP peak area/1 mM BOP-T peak area) × peak concentration of BOP-T calculated from the BOP calibration curve. BOP-G was hydrolyzed by incubation in 1 M NaOH for 30 min at 30°C, and the peak areas measured before and after hydrolysis were compared. The concentration was calculated as follows: BOP-G concentration = (BOP peak area after alkaline hydrolysis/BOP-G peak area before alkaline hydrolysis) × peak concentration of BOP-G calculated from the BOP calibration curve. All samples were analyzed in duplicate.

Preparation of Rat Liver Mitochondria

Preparations of rat liver mitochondrial and microsomal fractions were performed separately. Male Sprague-Dawley rats weighing 250–350 g were anesthetized with ether, and the livers removed immediately. Each liver was perfused with ice-cold 1.15% KCl, and a 20% homogenate in 0.25 M sucrose-10 mM Tris HCl (pH 7.4) containing 0.1 mM EDTA buffer (sucrose-Tris HCl buffer) was prepared. After centrifugation at 600 × *g* for 10 min, the supernatant was separated and centrifuged at 5,000 × *g* for 10 min. The precipitate was reconstituted as a 50% homogenate in Sucrose buffer, centrifuged at 2,000 × *g* for 2 min, and further at 5,000 × *g* for 8 min. The precipitate was resuspended in sucrose buffer (2 ml) and stored at −80°C as mitochondrial fractions until use. Protein was determined by the method of Lowry *et al.* (12). All preparation procedures were carried out in a cold room (4°C).

Preparation of Rat Liver Microsomes

Briefly, the 20% homogenate described in the previous section was centrifuged at 700 × *g* for 10 min, and then at 10,000 × *g* for 10 min. The supernatant was centrifuged at 105,000 × *g* for 60 min. The pellet was resuspended in sucrose-Tris HCl buffer in ten times volume of the pellet. This suspension was centrifuged at 105,000 × *g* for 60 min again. The precipitate was resuspended in 1 ml of 0.1 M Tris HCl (pH 7.4) buffer containing 10 mM EDTA and 20% glycerol, and stored at −80°C as microsomal fractions until use.

Determination of Optimal Conditions for the Conjugation Enzymes of BOP-T Formation

In order to investigate stereoselectivity of the conjugation enzymes responsible for BOP-T formation, *in vitro* optimal conditions for the enzymatic reactions were determined using rat hepatic mitochondria and microsomes, respectively. Solubilization of mitochondrial and microsomal protein was achieved with Triton X-100 (final concentrations of 0.20–0.25%). The protein suspension added Triton X-100 was allowed to stand on ice for 30 min with mixing. The mitochondrial protein solubilized was centrifuged for 10 min at 17,500 × *g* at 4°C, and the supernatant was used as an enzymatic source. The microsomal protein solubilized with Triton X-100 was used as an enzymatic source directly. Tested incubation mixtures contained the following components over the ranges indicated: each BOP enantiomer (0–1 mM), CoASH (0-4 mM), taurine (0–40 mM), ATP (0–20 mM), $MgCl_2$ (0–5 mM), DTT (0–10 mM), and mitochondrial or microsomal protein (0–4 mg protein/incubation mixture) in 0.5 ml of 0.2 M Tris HCl buffer (pH 7.5–9.5). After 3 min pre-incubation, the enzymatic reaction was started by the addition of BOP, and incubated with mixing for 30 min. The reaction was terminated by the addition of ethanol (300 μl), solid ammonium sulfate (0.3 g), and IS (20 μl of 50 μg/ml in 50% ethanol) to the reaction mixture, which was mixed vigorously, and centrifuged at 17,500 × *g* for 10 min at 4°C. The ethanol layer (300 μl) was separated, and dried by the addition of anhydrous sodium sulfate (0.3 g). An aliquot (5 μl) of the ethanol layer was injected onto the HPLC directly. Calibration curves were prepared from 0 to 0.5 μM BOP-T for the *in vitro* studies.

Statistics

The data are given as mean ± SD. Significant difference was estimated by unpaired Student's *t* test.

RESULTS

HPLC

Under the HPLC conditions described above, BOP-T and IS exhibited symmetrical peaks with base line resolution, with no interfering peaks observed for endogeneous components of rat bile. Figure 2A shows a typical chromatogram of a drug-free bile sample to which (*S*)-BOP-T, (*R*)-BOP-T, and IS had been added at concentrations of 25, 25, and 300 μg/ml, respectively. Figure 2B illustrates the analysis of a bile sample obtained 60 min after (*R*)-BOP administration (5 mg/kg, i.v.). Figure 2C shows a chromatogram of enzymatic reaction of (*R*)-BOP (0.25 mM) with microsomes (1.25 mg) after 30-min incubation.

Biliary Excretion of BOP and Its Metabolites

The enantiomers of BOP and its metabolites in plasma and bile were measured by HPLC analysis with a chiral column, as described in the section of Materials and Methods. The plasma concentration-time curves for (*R*)-BOP and (*S*)-

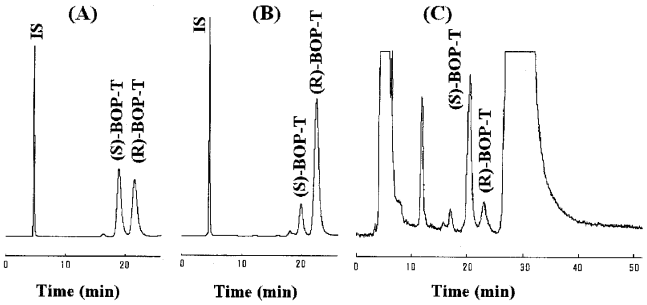

Fig. 2. HPLC chromatograms of (*S*)- and (*R*)-BOP-T enantiomers and IS. (A) Control bile supplemented with (*S*)-BOP-T (25 μg/ml), (*R*)-BOP-T (25 μg/ml), and IS (300 μg/ml). (B) Bile sample obtained 60 min after (*R*)-BOP administration (5 mg/kg, i.v.). (C) A 30-min incubation mixture of (*R*)-BOP with rat hepatic microsomes.

BOP after bolus i.v. administration of (R)-BOP, and cumulative curves for (*R*)-BOP and (*S*)-BOP, (*R*)-BOP-G and (*S*)-BOP-G, and (*R*)-BOP-T and (*S*)-BOP-T excreted into the rat bile are illustrated in Fig. 3. The plasma concentration-time curve for (*S*)-BOP after bolus i.v. administration of (*S*)-BOP, and cumulative curves for (*S*)-BOP and (*S*)-BOP-G excreted into the rat bile are illustrated in Fig. 4.

Pharmacokinetic Data

Following (*R*)-BOP i.v. administration, (*R*)-BOP exhibited rapid plasma elimination concomitant with appearance of (*S*)-BOP in plasma. The amounts of BOP, BOP-G, and BOP-T enantiomers excreted into the bile are shown in Table I. No BOP and its metabolites were found in the urine.

Optimal Conditions for Taurine Conjugation of BOP

The concentrations of each component in the reaction mixture were varied to optimize the initial velocity of the enzymatic reactions, as described in "Materials and Methods." The following incubation conditions were chosen for the *in vitro* assay of BOP-T formation: 0.25 mM BOP, 5 mM ATP, 3 mM $MgCl_2$, 5 mM DTT, 30 mM taurine, 0.4 mM

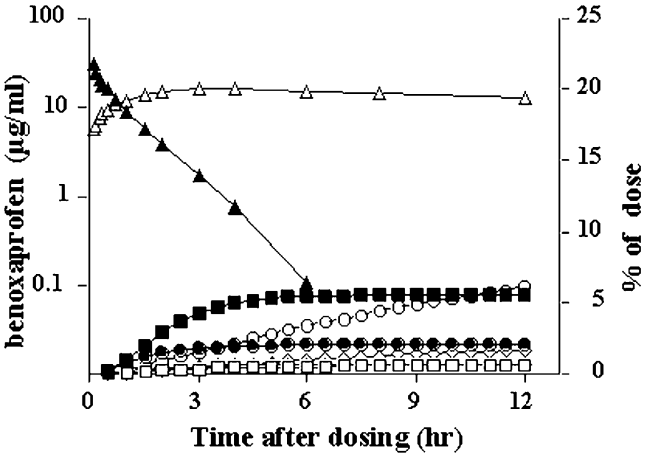

Fig. 3. BOP levels in plasma (left ordinate) and cummulative excretion of BOP and its metabolites in bile (right ordinate) after a single i.v. administration of (*R*)-BOP. The dose was 5 mg/kg (n = 5). ▲, (*R*)-BOP and △, (*S*)-BOP in plasma; ♦, (*R*)-BOP; ◇, (*S*)-BOP; ●, (*R*)-BOP-G; ○, (*S*)-BOP-G; ■, (*R*)-BOP-T; □, (*S*)-BOP-T in bile.

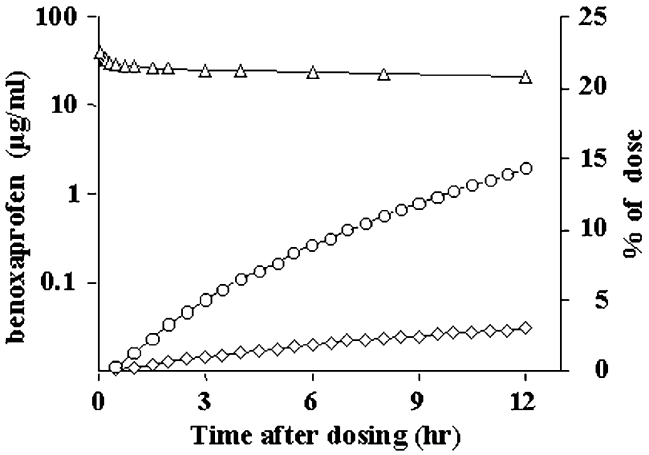

Fig. 4. BOP levels in plasma (left ordinate) and cummulative excretion of BOP and its metabolites in bile (right ordinate) after a single i.v. administration of (*S*)-BOP. The dose was 5 mg/kg (n = 5). △, (*S*)-BOP in plasma; ◇, (*S*)-BOP and ○, (*S*)-BOP-G in bile.

CoASH, and 1.25 mg mitochondrial or microsomal protein in 0.2 M Tris HCl buffer (pH 9.0) in a final volume of 0.5 ml. Mitochondrial and microsomal proteins were solubilized with 0.25% and 0.2% of Triton X-100 on ice for 30 min, respectively, prior to start of the enzymatic reaction. BOP-T formation activities (pmol mg protein^{-1} min^{-1}) were linear over 60 min incubation time. BOP-T did not formed without either mitochondria or microsomes under the same conditions.

Comparison of BOP-T Formation Activities in Rat Hepatic Mitochondria and Microsomes

The result of BOP-T formation activities in mitochondria and microsomes using (*R*)-BOP or (*S*)-BOP as each substrate are depicted in Fig. 5. In mitochondria, (*R*)-BOP-T and (*S*)-BOP-T formed from (*R*)-BOP were 0.084 ± 0.145 and 1.761 ± 0.186 pmol mg protein^{-1} min^{-1}, respectively, whereas (*R*)-BOP-T and (*S*)-BOP-T formed from (*S*)-BOP were 0.085 ± 0.109 and 1.005 ± 0.175 pmol mg protein^{-1} min^{-1}, respectively. In microsomes, (*R*)-BOP-T and (*S*)-BOP-T formed from (*R*)-BOP were 1.251 ± 0.059 and 4.367 ± 0.129 pmol mg protein^{-1} min^{-1}, respectively, whereas (*R*)-BOP-T and (*S*)-BOP-T formed from (*S*)-BOP were 0.444 ± 0.051 and 1.890 ± 0.201 pmol mg protein^{-1} min^{-1}, respectively. BOP-T formation activity in microsomes was approximately 3.0 times higher than that in mitochondria, when (*R*)-BOP was used as a substrate. That of (*S*)-BOP was approximately 2.1. BOP-T formed from (*R*)-BOP was approximately 1.7 times that from (*S*)-BOP in mitochondria, and approximately 2.4 times in microsomes. The (*S*)/(*R*) ratios of BOP-T formed from (*R*)-BOP was 21.0 in mitochondria, and 3.5 in microsomes.

DISCUSSION

Stereoselective Pharmacokinetics and Metabolism of BOP in Rats

Previously, we reported (10) that BOP-G and BOP-T were the main metabolites of BOP excreted in rat bile, following i.v. administration of a 10 mg/kg dose of racemic BOP. Furthermore, we clarified that BOP-T was of *R* configuration, whereas BOP-G was *S*. To investigate stereoselective biliary

Table I. 12-h Cumulative Excretion of BOP, BOP-G, and BOP-T in Bile

i.v. Administration	(*R*)-BOP	(*S*)-BOP	(*R*)-BOP-G	(*S*)-BOP-G	(*R*)-BOP-T	(*S*)-BOP-T
(R)-BOP	0.7 ± 0.1	1.7 ± 0.2	2.1 ± 0.5	6.2 ± 1.4	5.6 ± 1.8	0.7 ± 0.3
(S)-BOP	—	3.0 ± 0.4	—	14.3 ± 1.8	—	—

Data are expressed as mean ± SD (% of dose, n = 5). Intravenous dose of each enantiomer was 5 mg/kg body weight.

excretion of BOP and its metabolites, especially focusing upon BOP-T formation, each BOP enantiomer was administered i.v. to male Sprague-Dawley rats at a dose of 5 mg/kg.

(*R*)-BOP showed plasma elimination with appearance of (*S*)-BOP in plasma after (*R*)-BOP administration (Fig. 3), whereas following (*S*)-BOP administration, only slow plasma elimination of (*S*)-BOP was observed (Fig. 4). The plasma elimination of (*R*)-BOP was more rapid than that of (*S*)-BOP (Fig. 3 and Fig. 4). Bopp *et al.* (13) and Simmonds *et al.* (14) reported that BOP exhibited stereoselective plasma elimination, and that (*R*)-BOP was converted unidirectionally to (*S*)-BOP in the human body. Our results in rats were identical with those observed for many 2-arylpropionic acid derivatives (15).

It was found that most of BOP-G excreted into the bile was of (*S*)-configuration, when either (*R*)-BOP or (*S*)-BOP was administered. This finding is similar to the human urine results reported by Spahn *et al.* (11), where the cumulative amount of (*S*)-glucuronide in urine over 96 h after administration of 300 mg of racemic BOP was about twice that of (*R*)-glucuronide. Iwakawa *et al.* (16) reported stereoselective biliary excretion of the glucuronides of caprofen, flunoxaprofen (a BOP derivative) and naproxen in rats. They also noted that the cumulative amount of flunoxaprofen (*S*)-glucuronide in bile over 8 h was about 2.5-fold greater than that of the (*R*)-glucuronide, and naproxen (*S*)-glucuronide was 3.4-fold greater than that of the (*R*)-glucuronide.

Only after (*R*)-BOP administration, was the taurine conjugate of BOP found in the bile, and the configuration was *R*. BOP-T could not be found in the bile after (*S*)-BOP administration.

In recent years, stereoselective amino acid conjugation of 2-arylpropionic acid derivatives (profens) has been considered together with the metabolic chiral inversion mechanism, because both reactions involve drug-CoA thioester formation. The chiral inversion of BOP occurs unidirectionally from *R*-enantiomer to *S*-enantiomer in the body, as described previously (13,14). This is hypothesized to occur because only the (*R*)-BOP enantiomer can be a substrate for the enzyme "acyl-CoA ligase" during acyl-CoA thioester formation (17,18).

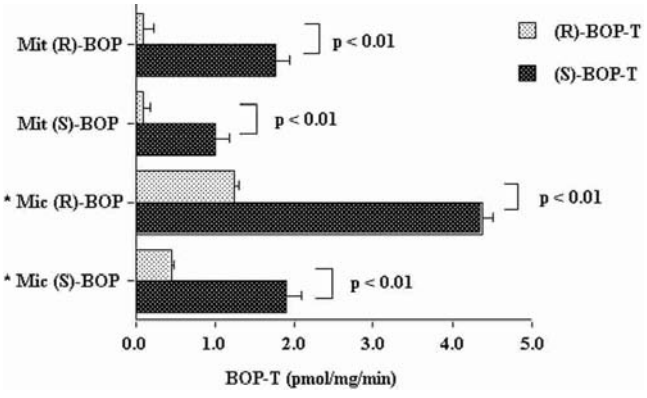

Fig. 5. Amounts of BOP-T enantiomer produced after a 30-min incubation with (*R*)-BOP or (*S*)-BOP in rat hepatic mitochondria (Mit) or microsomes (Mic). *Significant difference was observed for the total amount of BOP-T produced by the incubations of (*R*)-BOP or (*S*)-BOP with microsomes. ($p < 0.01$) Each point and vertical bar represent the mean ± SD (n = 5).

For the BOP taurine conjugation process, BOP-CoA thioester formation appears to be required as the first step, with BOP-T produced from BOP-CoA thioester by taurine *N*-acyltransferase. Because the (*S*)-BOP enantiomer cannot be converted to its CoA thioester, only when (*R*)-BOP is administered to rats, was the taurine conjugate of (*R*)-BOP excreted into rat bile. A small amount of (*S*)-BOP-T was identified in the bile after (*R*)-BOP administration. This may be the result of chiral conversion of (*R*)-BOP-CoA thioester to (*S*)-BOP-CoA thioester by epimerase, followed by the metabolism to (*S*)-BOP-T by taurine *N*-acyltransferase.

A few reports with respect to stereoselective amino acid conjugation of 2-arylpropionic acid derivatives have appeared in the literature. Shirley *et al.* (19) reported that IBP-T excreted in human urine was *S*-configuration at the propionic acid moiety (approximately 87% of IBP-T, 1.5% of the dose, after oral administration of the racemate). Asami *et al.* reported that the taurine conjugate of trans-hydroxy-CS-670 excreted in dog urine had mainly *S*-configuration, and that the *S*/*R* ratios of taurine conjugates after administration of (2*R*)-CS-670 and (2*S*)-CS-670 were 75.9:1 and 249:1, respectively (20). Kitamura *et al.* noted that the taurine conjugate of M-5011 excreted in dog urine and feces was only *S*-configuration (approximately 15.4 and 13.5% of the dose, respectively) (21). Konishi *et al.* described that the taurine conjugate of 2-[4-(3-methyl-2-thienyl)phenyl]propionic acid detected in dog urine was *S*-configuration (22). Tanaka *et al.* reported that a 2-arylpropionic acid glycine conjugate excreted in dog urine had *S*-configuration (23). Thus, most literature reports state that taurine conjugates of 2-arylpropionic acid derivatives are of S configuration, especially in the dog. However, the bile samples obtained here after administration of (*R*)-BOP enantiomer showed that the configuration at the propionic acid moiety of BOP-T was *R*.

Stereoselective BOP-T Formation in Rat Hepatic Subcellular Fractions

For the further certification of stereoselectivity of the enzymes responsible for BOP-T formation, we conducted *in vitro* studies using rat hepatic subcellular fractions, mitochondria and microsomes. Mean (*R*)/(*S*) ratios of BOP enantiomer for BOP-T formation in the mitochondrial and microsomal incubations were approximately 1.7 and 2.4, respectively. The results of the *in vitro* studies demonstrated that (*R*)-BOP rather than (*S*)-BOP was metabolized to BOP-T predomi-

nantly, consistent with that of the *in vivo* studies of the biliary excretion of (*R*)-BOP-T in rats. We have already confirmed that (*R*)-BOP-CoA is produced from only (*R*)-BOP, and (*S*)-BOP-CoA is not produced from (*S*)-BOP, by the incubations with microsomes (our unpublished data).

The *in vitro* studies also demonstrated that a large amount of (*S*)-BOP-T and a small amount of (*R*)-BOP-T were produced by the incubations of not only (*R*)-BOP but also (*S*)-BOP with rat hepatic subcellular fractions (Fig. 5). The (*S*)/(*R*)-BOP-T ratios formed by the incubations of (*R*)-BOP or (*S*)-BOP with microsomes were 3.5 and 4.3, respectively, and 21.0 and 11.8 with mitochondria. BOP-T formed in rats was *R* configuration, on the other hand was *S* configuration in the *in vitro* studies. The results of configuration of BOP-T formed *in vivo* and *in vitro* were quite opposite. In Fig. 5, (*S*)-BOP-T formation from (*R*)-BOP was speculated as follows: (*R*)-BOP-CoA formed from (*R*)-BOP was inverted to (*S*)-BOP-CoA by epimerase, and furthermore metabolized to (*S*)-BOP-T. The reason why (*S*)-BOP-T and (*R*)-BOP-T formed from (*S*)-BOP is obscure. (*S*)-BOP may be racemized to (*R*)-BOP under alkaline condition (pH 9.0) nonenzymatically. A part of (*R*)-BOP converted from (*S*)-BOP might be metabolized to (*S*)-BOP-T, via from (*R*)-BOP-CoA to (S)-BOP-CoA. Although the reasons are unexplained, it may be due to that the *in vitro* incubation conditions are quite different from the physiologic conditions in rats (e.g., concentrations of co-factors).

Shirley *et al.* reported that the incubations in freshly isolated rat hepatocyte preparations with (*R*)-IBP or (*S*)-IBP produced both IBP CoA and IBP-T (19). The results demonstrated that (*R*)-IBP rather than (*S*)-IBP was metabolized to IBP CoA and IBP-T predominantly. They also stated that, for the formation of (*S*)-IBP-T isolated from the urine of the volunteers given a pseudoracemic mixture of IBP {(*R*)-IBP/S[$^2H_3$]-IBP} was formed from original (*S*)-IBP directly, not (*S*)-IBP derived from (*R*)-IBP by chiral inversion. In spite of that acyl CoA ligase dose not catalyze IBP-CoA thioesterification reaction from (*S*)-IBP to (*S*)-IBP-CoA, they have emphasized the formation of (*S*)-IBP-T from (*S*)-IBP. Thus, stereoselective taurine conjugation of 2-arylpropionic acid derivatives is complicated and still obscure, requiring further investigations.

Proposed Stereoselective Metabolism from BOP to BOP-T

We propose here the following hypothesis depicted in Fig. 6. Our results suggest that (*R*)-BOP appears to be more favorable substrate than (*S*)-BOP for acylCoA ligase, and that the conjugation velocity from (*R*)-BOP-CoA to (*R*)-BOP-T may be more rapid than that of the chiral inversion to (*S*)-BOP-CoA *in vivo*. The reason why the formation of BOP-T stops around 5 h after (*R*)-BOP administration to the rats may be due to the depletion of (*R*)-BOP in the body by the chiral inversion from (*R*)-BOP to (*S*)-BOP, consistent with the low (*R*)-BOP concentrations in plasma (below approximately 0.1 μg/ml). As described previously, our results are quite different from previous reports with other arylpropionic acid derivatives. This may be due to species and substrate differences.

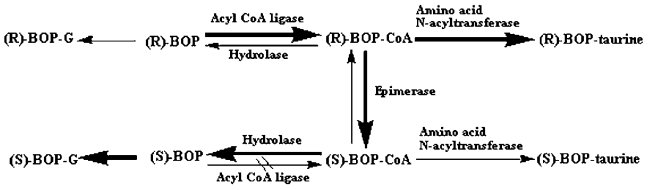

Fig. 6. Proposed mechanism of stereoselective metabolism of each BOP enantiomer.

The reason why the configuration of BOP-T formed *in vivo* and *in vitro* was opposite may be considered as follows: In the *in vitro* system, (*R*)-BOP was metabolized to (*R*)-BOP-CoA by acyl CoA ligase, which was further metabolized to (*S*)-BOP-CoA by epimerase predominantly, rather than the metabolism from (*R*)-BOP-CoA to (*R*)-BOP-T by taurine *N*-acyltransferase. It appears to be that (*S*)-BOP-CoA formed by chiral inversion may be metabolized to (*S*)-BOP-T. It may be due to that the differences of the enzymatic reaction velocities between acyl CoA epimerase and taurine N-acyltransferase against (*R*)-BOP-CoA were induced. It may be possible that some unkown factors *in vivo* stabilizing (*R*)-BOP-CoA and/or inducing the metabolism from (*R*)-BOP-CoA to (*R*)-BOP-T may be present.

James *et al.* reported that *N*-acyltransferase activity in mitochondrial fractions was the highest in rat subcellular fractions (24). However, the enzymatic activity in the microsomal fractions for the BOP-T formation was approximately 3.0 times higher than that of mitochondrial fractions, when (*R*)-BOP was used as a substrate. That of (*S*)-BOP was approximately 2.1. With respect to BOP-T formation, the enzymatic activities in the microsomes were predominant than that in mitochondria.

In conclusion, we identified (*R*)-BOP-T in rat bile only after administration of (*R*)-BOP, and it was approximately 5.6% of the (*R*)-BOP dose over 12 h. Furthermore, the enzymes in rat hepatic subcellular fractions were more stereoselective for (*R*)-BOP than (*S*)-BOP in the taurine formation. However, the configuration of BOP-T formed by the incubations of (*R*)-BOP and (*S*)-BOP with rat hepatic mitochondria and microsomes respectively was mainly both *S*. The BOP-T formation activity in the microsomal fractions was higher than that in the mitochondrial fractions.

ACKNOWLEDGMENTS

The authors are grateful to Nao Torimoto and Misato Sekine at Meiji Pharmaceutical University for skillful assistance. L.Z.B.'s participation was supported in part by NIH grant GM36633.

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