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Identification of the UDP-glucuronosyltransferase responsible for bucolome N-glucuronide formation in rats

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Bucolome N-glucuronide (BCP-NG), a major metabolite of bucolome (BCP), is the first unique N-glucuronide of barbituric acid derivatives to be reported. The purpose of the present study was to identify the UGT isoform(s) responsible for BCP-NG formation in rats. A pharmacokinetic study of BCP and the biliary excretion of BCP-NG was carried out in Wistar rats pretreated with phenobarbital (PB) (PB-pretreated rats), and the results were compared with those of Wistar rats not pretreated with PB (untreated rats). BCP N-glucuronidation activities were studied using hepatic microsomes prepared from Wistar rats pretreated with PB (primarily induces UGT1A1, 1A6 and 2B1) or with clofibric acid (CF, primarily induces UGT1A1 and 1A6), and from Gunn rats (deficiency of UGT1A family), and the results were compared with those of untreated rat microsomes.The plasma elimination clearance value of BCP in PB-pretreated rats was approximately 1.4 times greater than that of untreated rats. The cumulative amount (20.4 ± 5.9 % of dose) of BCP-NG excreted in PB-pretreated rat bile was approximately 1.5-fold higher than that (13.4 ± 2.5 % of dose) in untreated rat bile, and BCP-NG (5.9 ± 3.0%) and BCP (3.0 ± 2.6%) excreted in PB-pretreated rat urine were approximately 3.0- and 1.8-fold higher than those in untreated rat urine (BCP-NG: 2.0 ± 1.4%; BCP: 1.7 ± 1.3%), respectively.BCP N-glucuronidation activities in PB- and CF-pretreated microsomes were approximately 1.5- and 1.6-fold higher than in untreated microsomes, respectively. BCP N-glucuronidation activity in the microsomes of Gunn rats was markedly reduced by approximately 8.5% in untreated rat microsomes.The results suggest that UGT 1A1 is primarily responsible for BCP N-glucuronide formation in rats.

1. Introduction

Bucolome (5-n-butyl-1-cyclohexyl-2,4,6-trioxoperhydropyrim idine, BCP; Fig. 1) is a nonsteroidal anti-inflammatory drug for treatment of chronic articular rheumatism (Senda et al. 1967). In recent years, BCP has been coadministered to help maintain long-term plasma levels of warfarin (Takahashi et al. 1999). BCP is also known to be a potent choleretic, and to increase canalicular bile flow in dogs (Kitani et al. 1977). Two hydroxylated compounds, 5-(3-hydroxybutyl) and 1-(4-hydroxycyclohexyl) derivatives of BCP, were identified as BCP metabolites in human urine (Yashiki et al. 1971 a, b, c). We have previously reported that a major metabolite of BCP in rat bile was the novel N-glucuronide, BCP-NG (Fig. 1) (Mohri et al. 1985), which was the first reported N-glucuronide of barbituric acid derivatives. Although the metabolic fate and pharmacokinetics of BCP have been studied in humans (Yashiki et al. 1971 d,e), rabbits (Yashiki et al. 1971e) and rats (Mohri et al. 2001a), the UDP-glucuronosyltransferase (UGT) responsible for the formation of this unique BCP-NG has not been studied.

UGTs are broadly classified into two distinct families, UGT1 and UGT2, based on similarities in their amino acid sequences (King et al. 2000; Mackenzie et al. 1997). The majority of UGTs display broad and overlapping substrate specificities; however, a few specific characteristics have been described for certain UGT isoforms, e.g., phenobarbital primarily induces UGT1A1, 1A6 and UGT2B1 (Ishii et al. 1997; Hanioka et al. 2001), and clofibric acid (CF) primarily induces UGT1A1 and 1A6 (Osabe et al. 2008), while it is known that Gunn rats are deficient in UGT1A family proteins (Chowdhury et al. 1993; Watanabe et al. 2000). In order to identify the UGT isoform(s) involved in BCP N-glucuronidation, we administered BCP to rats pre-administered phenobarbital (PB) (PB-pretreated rats). The pharmacokinetic parameters of BCP and the amounts of BCP-NG excreted in bile were compared with those of rats not pre-administered PB (PB-untreated rats) following identical doses of BCP.

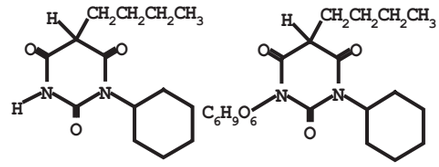

Fig. 1: Chemical structure of bucolome and bucolome N-glucuronide

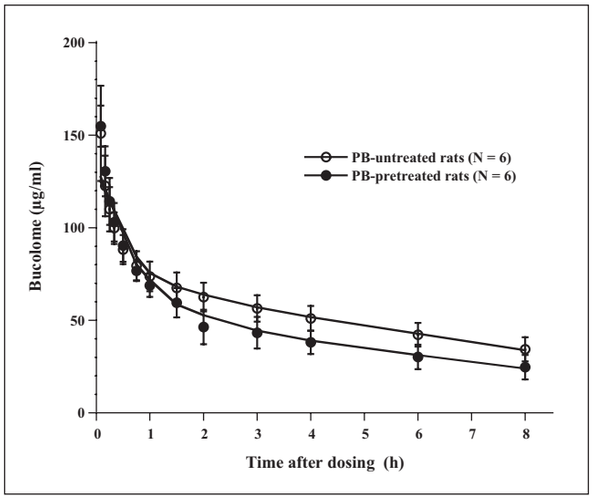

Fig. 2: BCP levels in plasma after a single i.v. dose of BCP (10 mg/kg body weight) in rats pretreated with phenobarbital, and untreated rats (n = 6)

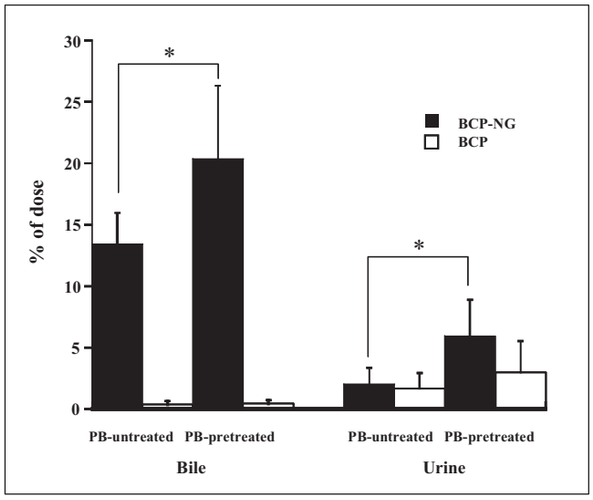

Fig. 3: Cumulative excretion (% of dose) of BCP-NG and BCP in bile and urine of untreated and PB-pretreated rats. *Significantly different from PB-untreated rats ($p < 0.05$)

In addition, BCP N-glucuronide formation activities were investigated *in vitro* using hepatic microsomes prepared from PB-untreated, PB-pretreated, and CF-pretreated rats, and Gunn rats, respectively.

In this study, the UGT isoform involved in BCP N-glucuronidation was assessed *in vivo* using PB-pretreated rats, and *in vitro* using hepatic microsomes prepared from PB-pretreated rats, CF-pretreated rats, and Gunn rats, respectively.

2. Investigations and results

2.1. Pharmacokinetics of BCP in rats

In both PB-pretreated and PB-untreated rats, plasma BCP concentration-time curves indicated a transition profile with typical distribution and elimination phases (Fig. 2). The plasma elimination of BCP in PB-pretreated rats was more rapid than in PB-untreated rats. Preliminary AUC, MRT, CL_{tot} and apparent Vd values obtained from the plasma concentration of BCP versus time data (for 8 h after i.v. drug administration) are summarized in Table 1.

2.2. Biliary and urinary excretion of BCP and BCP-NG in rats

The percentage of the cumulative amount of BCP and BCP-NG excreted into the bile in PB-pretreated and PB-untreated rats over the 8-h period after i.v. administration of BCP are depicted in Fig. 3. In PB-pretreated rats, the cumulative amounts of BCP-NG and BCP excreted in bile were 20.4 ± 5.9 % and less than 1 % of the dose, respectively. Total urinary excretion of BCP-NG and BCP was 5.9 ± 3.0 % and 3.0 ± 2.6 % of the dose, respectively. On the other hand, the cumulative amounts of BCP-NG and BCP excreted in the bile of PB-untreated rats were 13.4 ± 2.5 % and less than 1 % of the dose, respectively. The total urinary excretion of BCP-NG and BCP was 2.0 ± 1.4 % and 1.7 ± 1.3 %, respectively.

Table 1: Pharmacokinetic parameters of BCP in PB-pretreated and PB-untreated rats

	PB-pretreated rats Mean ± S.D.	PB-untreated rats Mean ± S.D.
*k*el (h^{-1})	0.11 ± 0.03	0.10 ± 0.03
$t_{1/2}$ (h)	6.67 ± 2.08	7.32 ± 1.97
CL_{tot} (l/h/kg)	$17.20 \pm 4.35^*$	12.68 ± 3.06
Vd (l/kg)	0.14 ± 0.02	0.12 ± 0.02
AUC (0−8 h) (μg/ml/h^{-1})	$367.5 \pm 47.10^*$	450.14 ± 54.37
MRT (0−8 h) (h)	$2.96 \pm 0.17^*$	3.24 ± 0.14

PB, phenobarbital; *k*el, apparent elimination rate constant; $t_{1/2}$, half-life; CL_{tot}, plasma total clearance; Vd, volume of distribution; AUC, area under the concentration-time curve; MRT, mean residence time.
* Significantly different from PB-untreated rats ($p < 0.05$).

2.3. Optimal conditions for maximal enzymatic activity in BCP N-glucuronidation

The optimal incubation conditions established with the microsomes were as follows: microsomal protein (2 mg/ml) solubilized with lubrol WX (0.2 mg/ml) on ice for 30 min. $MgCl_2$ (8 mM), saccharo-1,4-lactone (4 mM), BCP (3 mM), and UDPGa (26 mM) in 0.2 M Tris-HCl (pH 7.4). The enzymatic reaction rate was linear for the first 25 min. therefore, the time for the standardized enzymatic reaction was set to 20 min. The relationship between the amount of BCP-NG produced *in vitro* and the amount of microsomal protein for the above conditions was linear up to 10 mg/ml microsomal protein in the incubation mixture. BCP N-glucuronidation activities in untreated, CF-pretreated, PB-pretreated and Gunn rat microsomes were measured under the above conditions.

2.4. BCP N-glucuronidation activities in the microsomes of untreated, CF-pretreated and PB-pretreated Wistar rats and in Gunn rats

BCP N-glucuronidation activities in the microsomes of untreated, CF-pretreated and PB-pretreated Wistar rats and Gunn rats are shown in Fig. 4. BCP N-glucuronidation activities (pmol/min/mg protein) were as follows: untreated rats, 647.7 ± 20.8; PB-treated rats, 975.5 ± 35.0; CF-treated rats, 1004.1 ± 30.9; Gunn rats, 54.8 ± 5.4.

2.5. K_m, V_{max}, and V_{max}/K_m values for BCP and UDPGa in untreated, PB-pretreated and CF-pretreated Wistar rat microsomes and in Gunn rat microsomes

K_m, V_{max} and V_{max}/K_m values towards BCP and UDPGa in four kinds of microsomes were obtained under the conditions

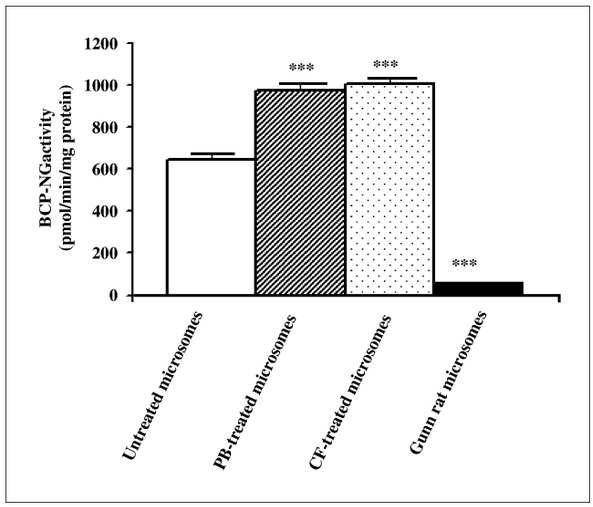

Fig. 4: BCP N-glucuronidation activities (pmol/min/mg protein) in the microsomes of untreated, PB-pretreated and CF-pretreated rats and in Gunn rats. ***Significantly different from untreated microsomes ($p < 0.001$)

showing the maximal BCP-NG formation activities, respectively. The results are summarized in Table 2.

3. Discussion

BCP-NG is the first N-glucuronide of barbituric acid derivatives isolated from rat bile as a BCP metabolite (Mohri et al. 1985). In 1995, Neighbors and Soine reported phenobarbital N-glucuronide in mouse urine; however, the enzymatic characteristics of the UGT responsible for both BCP N-glucuronide formation in rats and phenobarbital N-glucuronide formation in mice have not been studied. Therefore, we investigated *in vivo* and *in vitro* studies using rats and rat hepatic microsomes to estimate the UGT isoform(s) responsible for BCP-NG formation, as a preliminary study. First, we investigated the pharmacokinetic profiles of BCP using PB-pretreated rats in order to clarify whether PB has an influence on the BCP metabolic fate *in vivo*. As shown in Fig. 2, the plasma elimination of BCP in PB-pretreated rats was more rapid than that in PB-untreated rats. Since BCP has a long half-life (over 6 h), preliminary pharmacokinetic parameters were evaluated from the 8-h data after dosing in both PB-pretreated and untreated rats (Table 1).

In PB-pretreated rats, the mean CL_{tot} value was approximately 1.4-fold higher than in PB-untreated rats, and the mean AUC and MRT values were 0.82- and 0.91-fold, respectively. PB pre-administration thus increased the plasma elimination rate of BCP. The amounts of BCP-NG excreted in the bile and urine of PB-pretreated rats were greater than in PB-untreated rats (Fig. 3). The cumulative amount of BCP-NG excreted in the bile of PB-pretreated rats was approximately 20.5 % of the dose, and about 1.5-fold higher than in PB-untreated rats. BCP excreted in bile was less than 1% of the dose in both groups. Total urinary BCP-NG and BCP were about 2.9- and 1.8-fold higher than in PB-untreated rats. These *in vivo* comparative results using PB-pretreated and untreated rats strongly suggested that the UGT responsible for BCP N-glucuronidation was induced by PB.

Furthermore, since it is known that PB primarily induces UGT1A1, 1A6 and 2B1 (Ishii et al. 1997; Hanioka et al. 2001), and that CF mainly induces UGT1A1 and 1A6 (Osabe et al. 2008), we investigated BCP-NG formation activity in rat hepatic microsomes pretreated with PB or CF, and compared them with those in untreated microsomes (Fig. 4). BCP-NG formation activity in PB- and CF-pretreated microsomes was 1.5- and 1.6-fold higher, respectively, than in untreated microsomes. These *in vitro* findings also suggest that the UGT isoform(s) catalyzing BCP N-glucuronide formation is induced by not only PB but also CF.

Next, we investigated BCP-NG activity in the microsomes of Gunn rats that are deficient in UGT1A family proteins (Chowdhury et al. 1993; Watanabe et al. 2000). BCP-NG formation activity in Gunn rat microsomes was reduced markedly, less than 8.5% that of untreated microsomes (Fig. 4). This finding also supports that UGT1A1 is the key enzyme responsible for BCP-NG formation.

We obtained K_m, V_{max} and V_{max}/K_m values for BCP and UDPGa in untreated, PB-pretreated and CF-pretreated microsomes and Gunn rat microsomes (Table 2).

Although PB and CF treatments did not change the K_m values of BCP and UDPGa, PB and CF increased V_{max} values of BCP 1.6- and 1.8-fold, and of UDPGa both 1.4-fold higher than in untreated microsomes, respectively. Significant differences were not observed in V_{max}/K_m values of BCP between untreated and PB-pretreated microsomes, statistically; however, PB increased the K_m value slightly. It is considered that since PB increased both K_m and V_{max} values in parallel by enzymatic induction, significant differences were not observed in V_{max}/K_m values. On the other hand, significant differences were observed in all K_m, V_{max}, and V_{max}/K_m values of BCP and UDPGa between untreated microsomes and Gunn rat microsomes. The results of *in vitro* studies using enzymatic-induced microsomes by PB or CF suggested that UGT1A1, 1A6 and/or 2B1 might be responsible for BCP-NG formation in rats. The *in vitro* findings using Gunn rat microsomes also strongly suggest that UGT1A1 is the key enzyme responsible for BCP N-glucuronide formation in rats. We have already investigated BCP N-glucuronidation activity using recombinant UGT microsomes expressing rat UGT isoforms in yeast. Although recombinant UGT 1A1 showed strong BCP N-glucuronidation

Table 2: K_m V_{max} and V_{max}/K_m values of BCP or UDPGa in BCP N-glucuronidation

For BCP	Untreated	PB-treated	CF-treated	Gunn rats
K_m (mM)	0.72 ± 0.11	1.00 ± 0.21	0.95 ± 0.16	0.12 ± 0.04**
V_{max} (nmol/min/mg)	0.85 ± 0.06	1.37 ± 0.14***	1.52 ± 0.14***	0.09 ± 0.003***
V_{max}/K_m (μL/min/mg)	1.20 ± 0.10	1.40 ± 0.17	1.61 ± 0.13*	0.80 ± 0.23*
For UDPGa				
K_m (mM)	4.62 ± 0.49	4.56 ± 0.96	3.48 ± 0.60	2.14 ± 0.29**
V_{max} (nmol/min/mg)	0.78 ± 0.13	1.13 ± 0.01**	1.12 ± 0.06**	0.08 ± 0.002***
V_{max}/K_m (μL/min/mg)	0.18 ± 0.01	0.26 ± 0.05*	0.33 ± 0.03***	0.04 ± 0.005**

Untreated, untreated microsomes; PB-treated, phenobarbital-pretreated microsomes; CF-treated, clofibric acid-pretreated microsomes.
$p < 0.05$,
* $p < 0.01$,
** $p < 0.001$: significantly different from untreated microsomes.

activity; however, recombinant UGT 1A6 and 2B1 showed little BCP N-glucuronidation activity (unpublished data) (Ikushiro et al. 2004); therefore, we determined that UGT1A1 is the main UGT isoform responsible for the formation of this unique BCP N-glucuronide in rats, based on the effects of enzymatic inducers (PB and CF), and BCP-NG formation activities in Gunn rats on BCP-NG formation activity

In recent years, metabolic inhibition by BCP has been used to maintain long-term plasma levels of warfarin (Takigawa et al. 1998; Takahashi et al. 1999; Osawa et al. 2005). The present study showed that PB and CF accelerate BCP metabolism; therefore, the results of this study suggest that in patients with prothrombin levels controlled by the co-administration of warfarin and BCP, the co-administration of PB and/or clofibrate should be conducted with caution.

In conclusion, we showed here for the first time the N-glucuronidation of barbituric acid derivatives using rat hepatic microsomes. BCP N-glucuronidation in rats is enhanced by pretreatment with not only PB but also CF; in addition, Gunn rats produce little BCP-NG. We have determined that UGT1A1 is the primary UGT isoform responsible for BCP-NG formation in rats.

4. Experimental

4.1. Chemicals and reagents

BCP was synthesized from cyclohexylurea and n-butylmalonate by the method of Senda et al. (1967). BCP-NG was obtained biosynthetically as previously reported (Mohri et al. 1985). UDP-glucuronic acid trisodium salt (UDPGa), anhydrous magnesium chloride ($MgCl_2$), sodium phenobarbital (PB), tris (hydroxymethyl) aminomethane hydrochloric acid, and dimethylsulfoxide (DMSO) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka). Phenylbutazone (PBZ, I.S.) and D-saccharic acid 1,4-lactone monohydrate were purchased from Nacalai Tesque, Inc. (Kyoto). Clofibric acid (CF) was purchased from MP Biomedicals, LLC (Morgan, Irvine, CA). Both methanol (MeOH) and tetrahydrofuran (THF) were of HPLC grade (Nacalai Tesque). Lubrol WX was purchased from Sigma-Aldrich Co. (St. Louis, MO). Water was double distilled in a glass still. All other chemicals were of analytical grade.

4.2. Drug standard solutions

A stock solution containing BCP (1 mg/ml in DMSO) or PBZ (1 mg/ml in DMSO) was stored at −30 °C, and diluted with distilled water to the desired concentrations when used. All stock solutions showed insignificant degradation over a 3-month period, and were used within 3 months.

4.3. HPLC Apparatus and chromatographic conditions

BCP, PBZ (I.S.), and BCP-NG were analyzed by the previously reported HPLC method modified slightly (Mohri et al. 2001a). HPLC data were analyzed using a Borwin computer program (JASCO Corp., Tokyo). The detector was set at 268 nm using a sensitivity of 0.005 absorbance units full scale (a.u.f.s.). BCP, I.S. and BCP-NG were separated on a reverse-phase Capcell Pak C18 octadecylsilane (ODS) column (SG 120) [6.0 mm (inside diameter, i.d.) × 150 mm; particle size, 5 μm] (Shiseido Co. Ltd., Tokyo) equipped with a guard column packed with the same resin [4.6 mm i.d. × 10 mm] at room temperature (23 °C). The mobile phase (0.05 M phosphate buffer (pH 5.7):MeOH:THF, 50:40:4, v/v/v) was pumped through the column at 1.5 ml/min. Retention times of BCP, I.S., and BCP-NG were 7.2, 9.9, and 12.9 min. respectively.

4.4. Calibration curves

Calibration curves for BCP were obtained from the analyte/I.S. peak area ratio versus various concentrations of BCP using linear least square regression analyses. BCP-NG and BCP concentrations in the biological fluids were determined from the calibration curve (0–250 μg/ml) of BCP based on equivalents of the molar absorption for BCP-NG (Mohri et al. 2001a). The amount of BCP-NG formed in *in vitro* studies was calculated from the BCP calibration curve (0–0.02 mM), which was prepared by the addition of known amounts of BCP to the incubation assay mixture (i.e., containing all the reaction components except UDPGA). All analyses were performed in triplicate.

4.5. Animals and drug administration

All animal procedures were approved by Meiji Pharmaceutical University Committee for Ethics of Experimentation and Animal Care. Twelve male Wistar rats (Sankyo Labo Service Co., Tokyo, Japan) weighing 235–285 g were used. Six rats received i.p. injection of sodium phenobarbital dissolved in purified water at a dose of 80 mg/kg body weight (as phenobarbital) at 12:00 once a day for 4 successive days prior to 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) for 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 the experimental procedures, rat body temperatures were maintained at 38 ± 0.5 °C with a heat lamp to prevent hypothermic alterations of bile flow. The solution of BCP for injection was prepared by dissolving 100 mg BCP in 1 M sodium hydroxide and 0.5 M NaH_2PO_4 (final concentration, 100 mg BCP/10 ml total volume). BCP was administered at a dose of 10 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 units/ml heparin in saline. Blood samples (each approximately 0.2 ml) were collected from the femoral artery at 0, 5, 10, 15, 20, 30, 45 min. and 1.0, 1.5, 2.0, 3.0, 4.0, 6.0 and 8.0 h after i.v. administration of BCP. The collected blood was immediately centrifuged at 18,000 × g for 15 min. at 4 °C, and the plasma was separated. Bile was collected into 2-ml plastic tubes at successive 30-min. intervals. Urine samples were collected continuously for 8 h through a PE-50 tubing bladder cannula into 5-ml glass tubes containing 50 mg solid $NaHCO_3$. Saline supplements were administered to the rats through the femoral cannula to supplement body fluids, at volumes equivalent to the 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.

4.6. Assay procedures for the biological samples

Plasma, bile or urine sample (20 μl) was added to a mixture of 0.5 ml of 0.5 M phosphate buffer (pH 8.5), 200 μl EtOH, 50 μl I.S. (50 μg/ml PBZ in H_2O), 50 μl distilled water, and 0.5 g solid ammonium sulfate in a 2-ml plastic tube. The mixture was mixed vigorously for 1 min. and centrifuged at 18,000 × g for 10 min. at 4 °C. The supernatant (10 μl) was injected directly onto the HPLC. The assays were performed in triplicate.

4.7. Pharmacokinetic analysis

The individual plasma concentration versus time data (0–8 h) were analyzed by the model-independent method using the MULTI computer program (Yamaoka et al.1983). The area under the concentration-time curve (AUC) was calculated by the trapezoidal rule from the observed values (0–8 h). The mean residence time (MRT) was calculated by the means of moment analysis (Yamaoka et al. 1978). The volume of distribution (Vd) was calculated from dose × MRT/AUC. The apparent plasma total clearance (CL_{tot}) was calculated as dose divided by AUC. The half-life ($t_{1/2}$) was obtained from ln2 divided by kel, where kel is the apparent elimination rate constant, as obtained from the elimination phase gradient.

4.8. Preparation of rat hepatic microsomes

Fifteen male Wistar rats (Sankyo) and three Gunn rats (Sankyo) were used for the preparation of hepatic microsomes. Five Wistar rats received an i.p. injection of sodium phenobarbital dissolved in purified water at a dose of 80 mg (as PB)/kg body weight. Another five Wistar rats received a p.o. administration of CF dissolved in polyethylene glycol #400 at a dose of 280 mg/kg body weight. PB or CF was administered to the rats at 12:00 once a day for 4 successive days prior to the experiments. Untreated, PB-pretreated, CF-pretreated rats and Gunn rats were given no food overnight before the experiments, respectively. The microsomes of each animal were prepared by the previously reported method (Mohri et al. 2001b), and stored at -80 °C until use. All procedures were conducted in a cold room (4 °C). Protein concentrations were measured by the method of (Lowry et al. 1951).

4.9. Determination of optimal reaction conditions for enzymes catalyzing BCP N-glucuronidation

In order to investigate BCP N-glucuronidation *in vitro*, optimal conditions for enzymatic reactions were determined using untreated rat hepatic microsomes. Microsomal protein was solubilized with lubrol WX (final concentration of 0–1 mg/ml). The protein suspension with added lubrol WX was incubated on ice for 30 min. with mixing, and the solubilized microsomal protein was then used directly as the enzyme preparation. Tested incubation mixtures contained the following components over the concentration ranges indicated: BCP (0–4 mM), $MgCl_2$ (0–15 mM), saccharo-1,4-lactone

(0–8 mM), UDPGa (0–30 mM), and each microsomal protein preparation (0–3 mg/ml protein) in 0.25 ml of 0.2 M Tris-HCl buffer (pH 7.4). After 5 min. pre-incubation, the enzymatic reaction was started by the addition of BCP or UDPGa, followed by mixing for 0–40 min. The reaction was terminated by the addition of EtOH (200 μl), IS (15 μl of 100 μg/ml PBZ) and solid ammonium sulfate (0.5 g) to the reaction mixture, which was then mixed vigorously for 1 min. and centrifuged at 18,000 × g for 10 min. at 4 °C. An aliquot (10 μl) of the EtOH layer was injected directly onto the HPLC. The optimal conditions for enzymatic reactions using PB-pretreated and CF-pretreated microsomes were determined in the same manner.

4.10. K_m, V_{max}, and V_{max}/K_m values for BCP and UDPGa in untreated, PB-pretreated and CF-pretreated Wistar rat microsomes and in Gunn rat microsomes

K_m and V_{max} values were obtained from Eadie-Hofstee plots against BCP or UDPGa under the conditions showing maximal enzymatic activity in BCP N-glucuronidation, by varying BCP and UDPGa concentrations, respectively.

4.11. Statistical analysis

Data are shown as the means ± standard deviation (SD), and analyzed by unpaired Student's *t* test when there were only two treated groups. Dunnett's post-hoc test was used when there were three or more treated groups (JMP® Statistical Discovery Software; SAS Institute Japan Co., Tokyo). A *p* value of less than 0.05 was considered significant (*).

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