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Metabolism of bucolome in rats
Stability and biliary excretion of bucolome *N*-glucuronide

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Abstract

Bucolome (BCP) is a non-steroidal anti-inflammatory drug, which is used in the treatment of chronic articular rheumatism. Bucolome *N*-glucuronide (BCP-NG), a metabolite of BCP, is the first unique *N*-glucuronide of barbituric acid derivatives. First, the stability of BCP-NG in various pH aqueous solutions was studied. BCP-NG was quite unstable under neutral and acidic conditions, and is easily hydrolyzed to BCP. Based on these characteristics of BCP-NG, a simple, rapid and highly sensitive method for the simultaneous determination of BCP and BCP-NG with phenylbutazone (I.S.) in biological fluids was developed using high-performance liquid chromatography (HPLC). A reversed-phase ODS column was used for the separation of BCP, BCP-NG and I.S. A pharmacokinetic study for BCP and BCP-NG was carried out in male Wistar/ST rats following i.v. administration of BCP at a dose of 10 mg/kg body weight. The slow plasma elimination of BCP with time was shown. A major metabolite of BCP in bile was *N*-glucuronide. The cumulative amounts of BCP and BCP-NG in the bile over 8 h were approximately 2.4±1.4% and 12.6±2.3% of the dose, respectively. BCP and BCP-NG in the urine were 2.7±0.7% and 3.2±0.3% of the dose. Although BCP had a long half-life (over 8.5 h), the preliminary pharmacokinetic parameters (0–8 h) were determined: $t_{1/2}$, 8.52±1.96 h; AUC, 419.9±45.2 μg·h/ml; MRT, 3.29±0.11 h; CL_{tot}, 5.93±0.54 ml/h; and Vdss, 19.5±1.3 l. These observations are the first pharmacokinetic findings for the *N*-glucuronide of the barbituric acid derivatives.

Keywords: Bucolome; Bucolome *N*-glucuronide

1. Introduction

Bucolome (5-*n*-butyl-1-cyclohexyl-2,4,6-trioxoperhydropyrimidine, BCP) is a nonsteroidal anti-inflammatory drug that is used in the treatment of chronic articular rheumatism [1]. BCP is also known to be a potent choleretic and increases the canalicular bile flow in rats [2] and dogs [3]. Previously, we reported that a major metabolite of BCP in rat bile was a novel *N*-glucuronide (BCP-NG) (Fig. 1) [4], which is the first *N*-glucuronide of the barbituric acid derivatives. Although the metabolic fate and pharmacokinetics of BCP were studied in man [5–9] and rabbit [9] in 1968, the pharmacokinetics of BCP in rats have not yet been established.

Some methods have been reported to determine the concentrations of BCP and its metabolites in human plasma and urine using the ultraviolet absorp-

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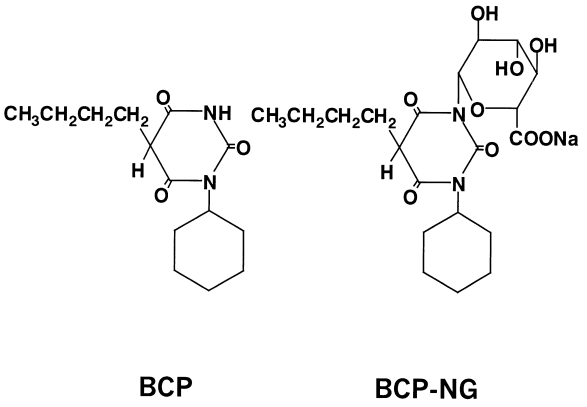

Fig. 1. Chemical structures of bucolome (BCP) and bucolome *N*-glucuronide (BCP-NG).

tion method [6] and the gas–liquid chromatographic method [7]. Not only did these previous methods not measure BCP-NG, but also these methods may be overestimating the free BCP concentrations, because BCP-NG is totally unstable even in neutral aqueous solution.

First, we studied the stability of BCP-NG in various pH aqueous solutions. BCP-NG was quite unstable under neutral and acidic conditions, and easily hydrolyzed to BCP. Based on these characteristics of BCP-NG, a simple, rapid and highly sensitive method for the simultaneous determination of BCP and BCP-NG with phenylbutazone (internal standard, I.S.) in biological fluids was developed using high-performance liquid chromatography (HPLC). A reversed-phase octadecylsilane (ODS) column was used for the separation of BCP, BCP-NG and I.S.

A pharmacokinetic study for BCP and BCP-NG was carried out in male Wistar/ST rats following i.v. administration of BCP at a dose of 10 mg/kg body weight, including the biliary excretion of BCP and BCP-NG.

2. Experimental

2.1. Chemicals

BCP was synthesized from cyclohexylurea and *n*-butylmalonate by the method of Senda et al. [1]. BCP-NG was biosynthetically obtained as previously reported [4]. Dimethylsulfoxide (DMSO) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka). Phenylbutazone (PBZ) was purchased from Nacalai Tesque, Inc. (Kyoto). Both methanol (MeOH) and acetonitrile (MeCN) were of HPLC grade (Nacalai). The water used was double distilled in a glass still. All other chemicals were analytical grade reagents from commercial sources and used without purification.

2.2. Drug standard solutions

A stock solution containing BCP (1 mg/ml in DMSO) or phenylbutazone (I.S., 1 mg/ml in DMSO) was used after being further diluted with distilled water to the desired concentrations when used. All stock solutions were stored at −30°C until used, and showed insignificant degradation over a period of 3 months. BCP-NG (100 μg/ml in 0.1 *M* phosphate buffer, pH 9.0) was prepared immediately before use.

2.3. HPLC apparatus and chromatographic conditions

The HPLC system consisted of a JASCO Intelligent HPLC pump Model PU-1580 (JASCO Co. Ltd., Tokyo) equipped with a JASCO Intelligent UV detector Model UV-1570, a JASCO Intelligent Sampler Model 851-AS and Shimadzu C-R4A Chromatopac integrator (Shimadzu Co., Kyoto). The detector was set at 268 nm, and a sensitivity of 0.005 absorbance units full scale (a.u.f.s.). The separation of BCP, BCP-NG and I.S. was performed on a reversed-phase Capcell Pak ODS column (SG 120) [6.0 mm (inside diameter, I.D.)×15 cm; particle size, 5 μm] (Shiseido Co. Ltd., Tokyo) equipped with a guard column packed with the same resin [4.6 mm I.D.×1 cm] at room temperature. The mobile phase (0.05 *M* phosphate buffer (pH 5.7): MeOH, 100: 45, v/v) was pumped through the column at a rate of 1.5 ml/min. The mobile phase was passed through a 0.45 μm filter (Millipore, Bedford, MA) prior to use, and degassed with an ERC-3322 degasser (Erma Co. Ltd., Saitama). The solvent was degassed by reduced pressure.

2.4. Calibration curves

For the stability study of BCP-NG, a calibration curve was prepared as follows: standard BCP-NG was arranged in drug free 0.1 *M* sodium phosphate buffer (pH 8.0) by adding known amounts of BCP-NG to give final concentrations ranging from 0.1 to 50 μg/ml, to which I.S. was added for a final concentration of 0.3 μg/ml. The prepared standard solution was directly injected onto the HPLC. In the in vivo study, the BCP-NG concentrations in the biological fluids were determined from the calibration curves (0–500 μg/ml) of BCP as equivalent to molar absorption index of BCP-NG. The calibration curves for BCP and BCP-NG were obtained from the analyte/I.S. peak area ratio versus the various concentrations of BCP and BCP-NG, respectively. All samples were performed in triplicate. The concentrations of BCP and BCP-NG in the buffer or biological samples were calculated from the calibration curves using linear least square regression analyses.

2.5. Reproducibility, linearity, and sensitivity

Reproducibility studies were performed by analyzing three different pooled concentrations (each 0.2, 2 and 20 μg/ml) of the BCP and BCP-NG samples every day for 6 days. The BCP and BCP-NG concentrations in the samples were calculated each time from the calibration curves for BCP and BCP-NG. The linearity was checked from the calibration curves (0.1–50 μg/ml) for BCP and BCP-NG. The sensitivity of the method was determined by preparing dilutions of the BCP and BCP-NG samples containing known concentrations, respectively.

2.6. Assay procedures for the stability study of BCP-NG

The 0.1 *M* phosphate buffers at various pHs (2.0, 5.0, 7.4, 9.0 and 11.0) were prepared by adding 0.1 *M* phosphoric acid or 0.1 *M* sodium hydroxide to a mixture of 0.1 *M* sodium dihydrogenphosphate and 0.1 *M* disodium hydrogenphosphate. A mixture of 1 ml of 0.1 *M* phosphate buffer (pH 2.0) and 1 ml of MeOH was also prepared as an incubation mixture. An aliquot (100 μl) of standard BCP-NG (100 μg/ml in 0.1 *M* phosphate buffer, pH 9.0) was added to 2.0 ml of incubation buffer at various pHs in a 5-ml glass centrifuge tube with a screw cap. After vigorously mixing, the solution was incubated immediately at 37°C for 6 h. The sample (100 μl) was collected at before and 0.5, 1.0, 2.5, 3.0, 4.0 and 6.0 h after start of the incubation. Since we already know from our pilot experiments that BCP-NG is quite unstable under acidic and neutral conditions, the incubation samples were immediately treated as follows: the collected incubation samples at pHs 2.0, 5.0, 7.4, 9.0 and 11.0 were added to each 100 μl of 1 *M* sodium hydroxide aqueous solution, 0.1 *M* sodium hydroxide aqueous solution, 0.1 *M* phosphate buffer (pH 11.0), mobile phase and 0.1 *M* phosphate buffer (pH 7.4). Each 100 μl of phosphate buffer (pH 2.0)–MeOH or phosphate buffer (pH 2.0)–MeCN mixture was added to 100 μl of 1 *M* sodium hydroxide. The samples were stored at −80°C until analysis. All samples became slightly alkaline (around pH 8.0–9.0). A 60-μl aliquot of I.S. (5 μg/ml in mobile phase) was added to 200 μl of the collected sample, mixed vigorously, and 20 μl of the mixture was directly injected onto the HPLC. The experiments were performed in triplicate.

2.7. Animals and drug administration

Five male Wistar rats (Sankyo Labo Service Co., Tokyo), weighting 230–250 g, were used throughout the study. The rats were housed in stainless steel cages in groups of five, in a temperature-controlled (20–28°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) for instillation of the 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 the bile samples, and closed with surgical clips. During the experimental procedures, body temperatures were maintained at 38±0.5°C with a heating lamp to prevent hypothermic alterations of the bile flow. The solution of BCP for injection was prepared by dissolving 100 mg of BCP

in a 10-ml mixture of 1 *M* sodium hydroxide, 0.5 *M* NaH_2PO_4 and purified water. Saline was injected into the rats through the cannula to supplement body fluids. 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 before and 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. The collected blood was immediately centrifuged at 15,000 g for 15 min at 4°C, and the plasma was then separated. Bile was collected in 2-ml plastic tubes at intervals of 30 min. Urine samples were continuously collected for 8 h through a PE-50 tubing bladder cannula into 5-ml glass tubes containing 50 mg of solid $NaHCO_3$. Saline supplements were administered to the rats through the femoral cannula 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 collected plasma, bile and urine samples were stored at −80°C until analysis.

2.8. Assay procedures for the biological samples

Twenty μl of plasma, bile or urine sample was added to a mixture of 0.5 ml of 0.5 *M* phosphate buffer (pH 8.5), 300 μl of ethanol, 25 μl of I.S. (50 μg/ml PBZ in ethanol) and 0.5 g of ammonium sulfate in a 2-ml plastic tube. The mixture was vigorously vortexed for 30 s and centrifuged at 15 000 *g* for 5 min. The supernatant (5 μl) was injected onto the HPLC.

2.9. Kinetic analysis for BCP-NG degradation

The BCP-NG concentration versus time data were analyzed by the apparent first-order kinetics.

2.10. Pharmacokinetic analysis

The individual plasma concentration versus time data (0–8 h) were analyzed by the model-independent method using the computer program MULTI [10]. 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 using the moment analysis [11]. The steady state volume of distribution (Vdss) was calculated from dose×MRT/AUC. The apparent plasma total clearance (CL_{tot}) was calculated as the 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 elimination phase gradient. The data are shown as mean±SD. Significant differences were estimated by the analysis of variance (ANOVA in STAX Institute, Tokyo).

3. Results

3.1. Chromatography

Fig. 2 shows a typical chromatogram of BCP, I.S. and BCP-NG. The retention times of BCP, I.S. and BCP-NG were 8.0, 11.7 and 14.1 min, respectively. Fig. 2(A1–3) shows typical HPLC chromatograms for the analysis of the plasma samples. Fig. 2(A1) is a control plasma sample with added I.S., Fig. 2(A2) shows a calibration curve sample (BCP 100 μg/ml), and Fig. 2(A3) is the 30-min plasma sample after dosing (BCP 83.1 μg/ml). Fig. 2(B1–3) shows typical HPLC chromatograms for the analysis of the bile samples. Fig. 2(B1) is a control bile sample added I.S., Fig. 2(B2) is a calibration curve sample (BCP 100 μg/ml), and Fig. 2(B3) is a 0.5-h bile sample after dosing (BCP: 5.03 μg/ml and BCP-NG: 110.1 μg/ml as equivalent to BCP).

3.2. Sensitivity

Under the experimental conditions used, the lower detection limit of BCP and BCP-NG were approximately 0.025 and 0.006 μg/ml, respectively, at a signal-to-noise ratio of 3:1.

3.3. Linearity

For the linearity, a regression analysis gave the following equations: $y=5.08x+0.010$ (for BCP, $n=3$, $r=0.999$, SD of slope=0.0055) and $y=2.76x-0.012$ (for BCP-NG, $n=3$, $r=0.999$, SD of slope=0.036).

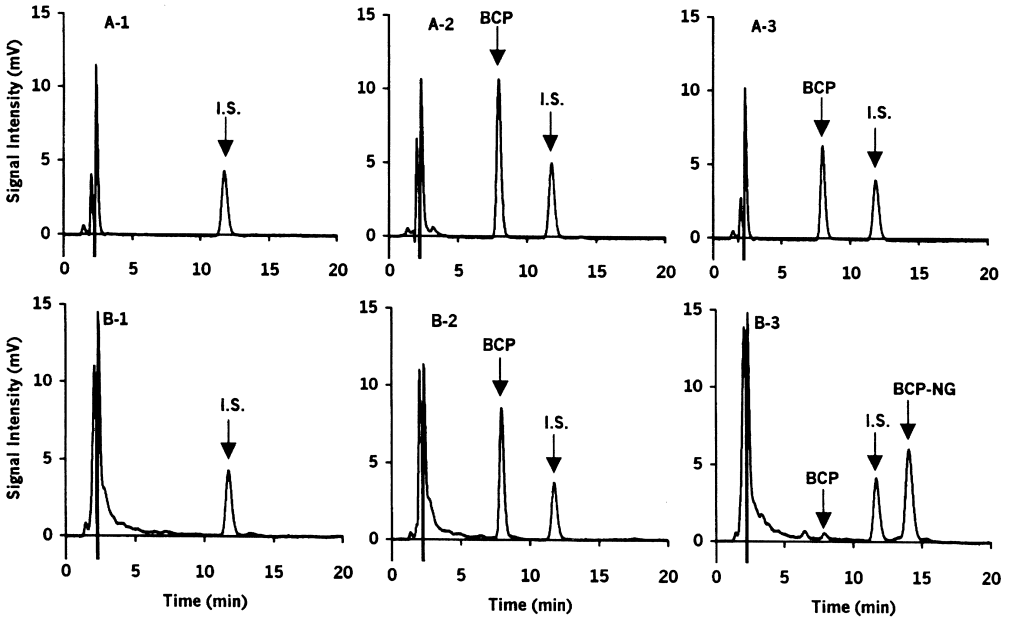

Fig. 2. HPLC chromatograms of BCP, BCP-NG and I.S. in plasma and bile. A-1 is a chromatogram of blank plasma sample with added I.S., A-2 is a calibration curve sample with added I.S., and A-3 is a 30-min plasma sample with added I.S. after dosing. B-1 is a chromatogram of blank bile sample with added I.S., B-2 is a calibration curve sample with added I.S., and B-3 is a 30-min bile sample with added I.S. after dosing.

3.4. Accuracy and precision

As shown in Table 1, the within-day CVs (coefficient of variation) were below 3.9% for BCP and 4.3% for BCP-NG at all the analyzed concentrations. The accuracy of the method (within-day), expressed by the bias, varied between −1.5 and 0.2% for BCP, and between −0.1 and 19.9% for BCP-NG, respectively. The between-day CVs (Table 1) were below 8.1% for BCP and 8.9% for BCP-NG at all the analyzed concentrations. The accuracy of the method varied between −0.2 and 1.6% for BCP, and between 0 and 7.8% for BCP-NG.

3.5. Stability of BCP-NG

Degradation profiles of BCP-NG versus incubation time were expressed by % of remaining BCP-NG over incubation (Fig. 3, Table 2). The results showed that BCP-NG was quite unstable under the acidic and neutral conditions.

3.6. Pharmacokinetics of BCP in rats

As shown in Fig. 4, the plasma BCP concentration–time curve indicated a transition profile with typical distribution and elimination phases. The preliminary AUC, MRT, CL_{tot} and apparent Vdss values obtained from the plasma BCP concentration versus time data until 8 h after i.v. administration of the drug were 419.9±45.2 μg·h/ml, 3.29±0.11 h, 5.93±0.54 ml/h and 19.5±1.3 ml, respectively. The half-life ($t_{1/2}$) of BCP obtained from the elimination phase was approximately 8.52±1.96 h. BCP-NG could not be detected in the plasma.

3.7. Biliary and urinary excretion of BCP and BCP-NG

The cumulative BCP and BCP-NG excreted into the bile over 8 h after i.v. administration of the BCP are depicted in Fig. 4. The cumulative BCP and BCP-NG excreted in the bile were 2.4±1.4% and 11.3±3.2% of the dose, respectively. The total

Table 1

BCP concentration (μg/ml)	Measured BCP concentration (μg/ml) mean±SD	*n*	CV (%)	Bias
(a) Within and between day precision (CV) and accuracy (bias) in the HPLC assay for BCP				
Within-day				
0.2	0.197±0.008	6	3.92	−1.5
2	2.003±0.008	6	0.42	0.2
20	20.000±0.001	6	0.00	0.0
Between-day				
0.2	0.203±0.016	6	8.10	1.6
2	1.997±0.018	6	0.91	−0.2
20	20.000±0.002	6	0.01	0.0
BCP-NG concentration (μg/ml)	Measured BCP-NG concentration (μg/ml) mean±SD	*n*	CV (%)	Bias
(b) Within and between day precision (CV) and accuracy (bias) in the HPLC assay for BCP-NG				
Within-day				
0.2	0.240±0.006	6	2.31	19.9
2	2.162±0.094	6	4.33	8.1
20	19.983±0.009	6	0.05	−0.1
Between-day				
0.2	0.216±0.019	6	8.94	7.8
2	2.018±0.112	6	5.54	0.9
20	19.998±0.011	6	0.06	0.0

urinary BCP and BCP-NG were 2.7±0.7% of the dose for BCP and 3.2±0.3% for BCP-NG, respectively.

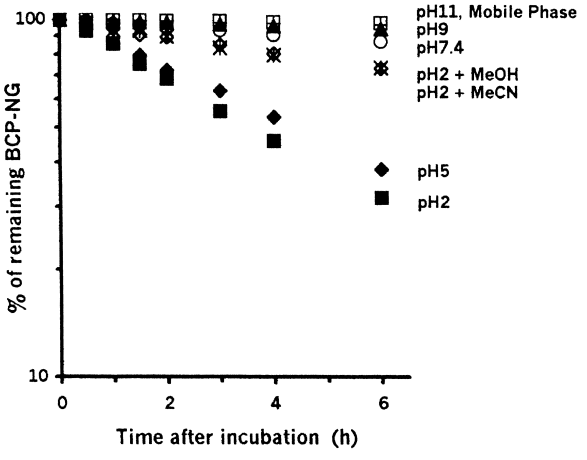

Fig. 3. Stability profiles of BCP-NG in various pH phosphate buffers.

4. Discussion

BCP-NG is the first reported *N*-glucuronide of the barbituric acid derivatives isolated from rat bile as a BCP metabolite [4]. However, the pharmacokinetic profiles of BCP-NG have not yet been clarified. To investigate the pharmacokinetics of BCP and BCP-NG in rats, the assay method for the simultaneous determination of BCP and BCP-NG in biological fluids was established using HPLC. The method was rapid, sensitive and specific. First, the effect of pH on the stability of BCP-NG was studied (Fig. 3). The result showed that BCP-NG was stable over pH 8.0, however, it was rapidly hydrolyzed from BCP-NG to BCP under acidic and neutral conditions. Therefore, the previous reports [5–9] might be overestimating the concentration of BCP in the biological fluids, because not only these previous methods did not measure BCP-NG, but also they did not pay any attention to the treatment of BCP-NG. Since it was found that BCP-NG was stable under alkaline conditions from the pilot experiments for the stability of

Table 2
Stability of bucolome *N*-glucuronide in various pH buffer solutions

Buffer Solution	First-order equation	Apparent K_{deg}	Apparent $T_{1/2}$ (h)
pH 2	$y=-0.1948x+4.6203$	0.1948	3.6
pH 5	$y=-0.1599x+4.6173$	0.1599	4.3
pH 2+MeCN	$y=-0.0518x+4.6018$	0.0518	13.4
pH 2+MeOH	$y=-0.0503x+4.5985$	0.0503	13.8
pH 7.4	$y=-0.0224x+4.6044$	0.0224	30.9
pH 9	$y=-0.0088x+4.6051$	0.0088	78.8
pH 11	$y=-0.0043x+4.6052$	0.0043	161.2
Mobile phase	$y=-0.0021x+4.6086$	0.0021	330.0

K_{deg}: degradation constant for BCP-NG; $T_{1/2}$: half-life.

BCP-NG, rat urine was collected into a glass tube containing 50 mg of sodium bicarbonate to prevent the spontaneous hydrolysis of BCP-NG. As the pH of rat bile was 8.5, we did not add sodium bicarbonate to the plastic container used for bile collection. The cumulative BCP-NG excreted in bile was approximately 13% of the dose (as equivalent to BCP). The results suggested that *N*-glucuronidation is the major metabolic pathway of BCP in rats, and the biliary excretion of BCP-NG was the main elimination route of BCP. Both rabbits and guinea pigs also had the abilities to metabolize BCP to BCP-NG and to excrete them into the bile (unpublished data). This report showed the first pharmacokinetic findings with respect to the *N*-glucuronide of the barbituric acid derivatives.

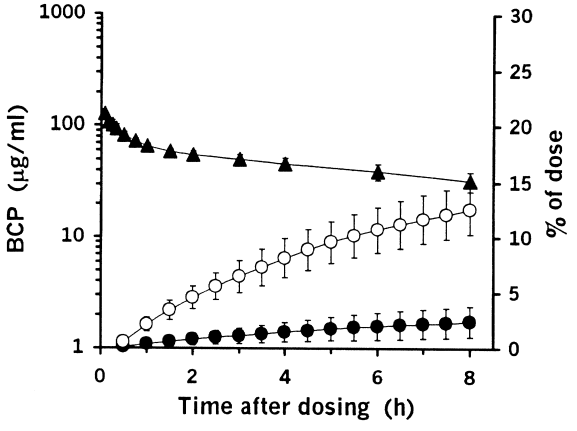

Fig. 4. BCP (▲) levels in plasma (left ordinate) and cumulative BCP (●) and BCP-NG (○) in bile (right ordinate) after a single i.v. administration of BCP (10 mg/kg body weight). Each point and vertical bar represent the mean and SD ($n=5$), respectively.

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