Enantioselective disposition of clenbuterol in rats

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Abstract

Clenbuterol is a long-acting β2-adrenoceptor agonist and bronchodilator that is used for treatment of asthma, but the desired activities reside almost exclusively in the (-)-R-enantiomer. Here, we examined enantioselectivity in the disposition of clenbuterol following administration of clenbuterol racemate to rats.

Concentrations of clenbuterol enantiomers in plasma, urine, and bile were determined by LC-MS/MS assay with a Chirobiotic T column. This method was confirmed to show high sensitivity, specificity, and precision, and clenbuterol enantiomers in 0.1-mL volumes of plasma were precisely quantified at concentrations as low as 0.25 ng/mL. The pharmacokinetic profiles of clenbuterol enantiomers following intravenous and intraduodenal administration of clenbuterol racemate (2 mg/kg) in rats were significantly different. The distribution volume of (-)-*R*-clenbuterol (9.17 L/kg) was significantly higher than that of (+)-*S*-clenbuterol (4.14 L/kg). Total body clearance of (-)-*R*-clenbuterol (13.5 mL/min/kg) was significantly higher than that of the (+)-*S*-enantiomer (11.5 mL/min/kg). An *in-situ* absorption study in jejunal loops showed no difference in residual amount between the (-)-*R*- and (+)-*S*-enantiomers. Urinary clearance was the same for the two enantiomers, but biliary excretion of (-)-*R*-clenbuterol was higher than that of the (+)-*S*-enantiomer. The fractions of free (non-protein-bound) (-)-*R*- and (+)-*S*-clenbuterol in rat plasma were 48.8, and 33.1%, respectively. These results indicated that there are differences in distribution and excretion of the clenbuterol enantiomers, and these may be predominantly due to enantioselective protein binding.

Keywords: clenbuterol, enantiomer, pharmacokinetics, excretion, protein binding

Introduction

Many drugs are still formulated as racemic mixtures, though enantiomers may differ in their pharmacokinetic and pharmacodynamic properties. For example, *S*-(-)-ofloxacin, a new quinolone antibacterial agent, is 8-128 times more active than *R*-(+)-ofloxacin against both gram-positive and gram-negative bacteria [1,2]. In addition, conversion of *S*-(-)-ofloxacin to the glucuronide in rat liver microsomes is 7-fold greater than that of *R*-(+)-ofloxacin [3]. Recently, the histamine H_1 antagonist levocetirizine and the sleep inducer eszopiclone have been approved by the FDA for use as optically active preparations [4]. On the other hand, only the *S*-enantiomer of thalidomide is teratogenic, but chiral inversion occurs readily in aqueous media and human serum, so that this finding does not imply the *R*-enantiomer is clinically safe [5,6]. Thus, information about enantiomeric conversion is important for drug product formulation.

Clenbuterol (4-amino-α-[(tert-butylamino)methyl]-3,5-dichlorobenzyl alcohol) is a long-acting β2-adrenoceptor agonist that is used as a bronchodilator for treatment of asthma [7]. It also possesses physiological properties, promoting the growth of muscle tissue and reduction of body fat [8-10]. Clenbuterol is commercially available as a racemic mixture of (-)-*R*- and (+)-*S*-enantiomers. Beta2-stimulant activities such as bronchodilation and uterine relaxing activity are almost exclusively possessed by (-)-*R*-clenbuterol [11-14]. On the other hand, (+)-*S*-clenbuterol has been reported to cause adverse effects such as reduction of blood pressure, increase of blood glucose levels, and elevation of glucocorticoid levels [15].

The pharmacokinetics, tissue distribution and metabolism of clenbuterol have been studied in a number of species, including human [16-19]. Residual clenbuterol in meat and muscle tissue is also a matter of concern in the food industry and in connection with drug abuse [20-24]. However, there is no available information on stereoselectivity in the absorption, distribution, and excretion of clenbuterol. Therefore, we investigated the

enantioselective disposition of clenbuterol in rats. We also examined the interconvertibility of clenbuterol enantiomers *in vitro* and *in vivo*.

Materials and Methods

Chemicals and Reagents

(rac)-Clenbuterol hydrochloride, ammonium formate, LC-MS grade water and LC-MS grade methanol were obtained from Sigma-Aldrich, Co. (St. Louis, MO, USA). Carvedilol used as an internal standard (IS) and acetonitrile were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). (-)-*R*- and (+)-*S*-clenbuterol enantiomers were obtained from Fujiyakuhin Co., Ltd (Saitama, Japan). The purities of these compounds were 100%, and 93.6%, respectively. The (-)-*R*-clenbuterol was free from (+)-*S*-clenbuterol, while the (+)-*S*-clenbuterol consisted of 93.0% (+)-*S*-clenbuterol and 7.0% (-)-*R*-clenbuterol. All other solvents and reagents used were of reagent grade.

Sample preparation for LC-MS/MS analysis

Plasma. A 10-µL volume of methanol containing carvedilol (100 ng/mL) as an IS and 900 µL of acetonitrile were added to 100 µL of plasma in a 1.5-mL microcentrifuge tube. This solution was mixed for 1 min on a vortex mixer, followed by centrifugation at 18,000 g for 5 min. The supernatant (900 µL) was applied to a solid-phase extraction column, SUPELCO HybridSPE®-Phospholipid (Supelco, PA, USA), and allowed to stand for 20 min. The eluate was transferred into an autosampler vial, and 10 µL was injected into the LC-MS/MS system.

Urine and Bile. A 10-µL volume of methanol containing carvedilol (100 ng/mL) as an IS and 900 µL of acetonitrile were added to 100 µL of urine or bile diluted 20-fold in distilled water. This solution was mixed for 1 min on a vortex-mixer, followed by centrifugation at 18,000 g for 5 min. The supernatant (900 µL) was applied to a solid-phase extraction column, SUPELCO HybridSPE®-Phospholipid (Supelco, PA,

USA), and allowed to stand for 20 min. The eluate was transferred into an autosampler vial, and 10 µL was injected into the LC-MS/MS system.

Chromatographic conditions

The HPLC system (Shimadzu Co., Kyoto, Japan) consisted of the following components: DGC-20A5 degasser, LC-20AD solvent delivery pump, SIL-20AC automatic sample injector, and CTO-20AC column oven. The analytical column for enantioseparation was a 5-µm Chirobiotic T column, 100 x 2.1-mm i.d. (ASTEC, Whippany, NJ). This column has a macrocyclic antibiotic chiral stationary phase. The mobile phase was a mixture of methanol and water (95:5, v/v) containing 2.5 mM ammonium formate, and the flow rate was set at 0.2 mL/min. The column was maintained at 40°C.

Mass spectrometry conditions

Mass spectrometric (MS) analysis was conducted using an API 4000 triple quadrupole instrument (AB Sciex Inc., MA, USA), equipped with an electrospray ionization source (ESI) operating in the positive ion mode. Analysis was performed in multiple reaction monitoring (MRM) mode by monitoring the ion transition from m/z 277.0 $[M+H]^+ \rightarrow$ 203.0 for clenbuterol enantiomers and m/z 407.1 → 100.0 for the IS. The analytical parameters were as follows: ion spray, 5,500 V; declustering potential, 56 V; entrance potential, 10 V; collision energy, 23 V; collision cell exit potential, 12 V; temperature 600°C. Nitrogen was used as the collision gas. The total run time was 20 min. The data were acquired and processed with Analyst 1.4™ software (AB Sciex, USA). No endogenous peak interfering with determination of these compounds was observed in the chromatogram of blank plasma. The retention times of (-)-*R*-clenbuterol, (+)-*S*-clenbuterol, and the IS were 12.8, 14.5, and 13.1 min, respectively. The two

enantiomers were well separated.

Calibration curves

The linear concentration range was determined by adding 10 µL of working standard solutions containing racemic clenbuterol to 100 µL of rat blank plasma. The plasma samples were then assayed using the method described above. The peak area ratios were calculated by dividing the peak area of the (-)-*R*- or (+)-*S*-clenbuterol signals by that of the IS and were plotted against the concentration of each compound.

The calibration graphs for the plasma assay were linear in the range of 0.25–50 ng/mL for clenbuterol enantiomers when 1 ng of the IS was used (regression equations; $y = 0.2637x + 0.0207$, $r^2 = 0.9998$ for (-)-*R*-clenbuterol, and $y = 0.2662x + 0.0275$, $r^2 = 0.9999$ for (+)-*S*-clenbuterol.) Similarly, the calibration graphs were linear in the range of 50–250 ng/mL for clenbuterol enantiomers when 1 ng of the IS was used (regression equations; $y = 0.23391x + 0.00612$, $r^2 = 0.9998$ for (-)-*R*-clenbuterol, and $y = 0.23604x + 0.0172$, $r^2 = 0.9998$ for (+)-*S*-clenbuterol). The lower quantitation limit was 0.25 ng/mL.

Accuracy and precision

To examine the accuracy and precision of the present method, three different racemic clenbuterol concentrations (2, 10, and 50 ng/mL; 1.0, 5.0, and 25 ng/mL of each enantiomer, respectively) were prepared in rat blank plasma. For the evaluation of inter-assay precision, 0.1-mL aliquots of plasma were analyzed. For intra-assay precision measurements, aliquots of the plasma samples were analyzed in duplicate on six consecutive days.

Reproducibility of the clenbuterol enantiomer analysis in rat plasma is summarized in Table 1. For intra-assay precision, the mean coefficients of variation for (-)-*R*-clenbuterol and (+)-*S*-clenbuterol were 2.7 and 3.0%, respectively. For inter-assay

precision, the mean coefficients of variation for (-)-*R*-clenbuterol and (+)-*S*-clenbuterol were 2.4% and 2.5%, respectively.

Animals

Four male Wistar rats (200 - 250 g) were provided by Kumagai-shigeyasu Co., Ltd (Japan, Miyagi), and housed in cages with unlimited food and water, except that food was not provided for 12 hours before the experiment. The animals were maintained on a 12-hours light/dark cycle (light on from 7:00 to 19:00) at ambient temperature (22-24°C) and at 60% relative humidity. All rats were deprived of food but given free access to water for 12 hours before the experiment.

The animal study was approved by the Ethics Committee for the Use of Animals of the International University of Health and Welfare (IUHW), in accordance with The Rules of Animal Experiments of the IUHW.

Pharmacokinetic study of clenbuterol in rats

Rats were randomly divided into six groups. The rats in the first and second groups were given *(rac)*-clenbuterol at a dose of 2 mg/kg via the intravenous (i.v.) or intraduodenal (i.d.) route, respectively. A third group of rats received (-)-*R*-clenbuterol i.v. at a dose of 1 mg/kg. The femoral artery and vein were cannulated under anesthesia with diethylether, and the abdominal cavity of the second group of rats was opened for cannulation into the duodenum. Animals were given *(rac)* or (-)-*R*-clenbuterol dissolved in phosphate-buffered saline (pH 7.4) after they had recovered from anesthesia. An appropriate volume of the dosing solution (ca. 2 mL/kg) was given to each of the animals through the femoral vein cannula, and the cannula was flushed with 0.5 mL of normal saline. Blood samples (250 µL) were collected through the arterial cannula at 0.5, 1, 3, 6, 9, 12, 24, 36, 48, 60 and 72 hours after administration

of the drug, and the lost fluid was replaced with an equal volume of saline via the vein cannula. The blood samples were placed in heparinized tubes. Plasma was obtained by centrifugation at 18,000 rpm for 5 min and stored at -30°C until analysis. The concentrations of clenbuterol enantiomers were determined by LC-MS/MS.

In-situ permeability studies

The permeability of rat intestinal membrane was evaluated by the *in situ* intestinal closed-loop method. In a fourth group of rats, the abdominal cavity was opened, and an intestinal loop (length: 10 cm) was made at the upper jejunum by cannulation with polypropylene tubing. Both ends of the loop were ligated under anesthesia with diethylether. The test solution containing *(rac)*-clenbuterol 100 µg/0.5 mL was introduced into the intestinal loop. At various times, the luminal solution in the loop and the washing obtained with 7 mL saline were collected. The supernatant fluid was analyzed. The intestinal loops were homogenized, extracted and analyzed. The permeability of clenbuterol enantiomers was evaluated in terms of the percentage of dose absorbed, calculated by subtracting the remaining amount of clenbuterol in intestinal loops from the administered amount.

Urinary Excretion

The femoral artery, vein and urinary bladder of a fifth group of rats were cannulated under anesthesia with diethylether. *(rac)*-Clenbuterol 2 mg/kg was administered as an i.v. bolus dose through the femoral vein. Urine was collected at 3, 6, 12, and 24 hours after dosing. The volume of the collected urine was measured, and the urine was stored at -30°C until analysis. Blood samples (250 µL) were collected through the arterial cannula at 0.5, 1, 3, 6, 9, 12 and 24 hours after dosing, and the lost fluid was replaced

with an equal volume of saline. The blood samples were collected into heparinized tubes. Plasma samples were obtained by centrifugation at 18,000 rpm for 5 min and stored at -30°C until analysis.

Biliary Excretion

The femoral artery and vein were cannulated in a sixth group of rats, and the abdominal cavity was opened for cannulation of the common bile duct under anesthesia with diethylether. *(rac)*-Clenbuterol 2 mg/kg was administered as an i.v. bolus dose through the femoral vein. Bile was collected at 3, 6, 12, and 24 hours after dosing. The volume of collected bile was measured, and the bile was stored at -30°C until analysis. Blood samples (250 µL) were collected through the arterial cannula at 0.5, 1, 3, 6, 9, 12 and 24 hours after dosing of the drug, and the lost fluid was replaced with an equal volume of saline. The blood samples were collected into heparinized tubes. Plasma samples were obtained by centrifugation at 18,000 g for 5 min and stored at -30°C until analysis.

Protein binding study

Plasma protein binding of (-)-*R*- and (+)-*S*-clenbuterol was determined using the ultrafiltration technique. Appropriate amounts of *(rac)*-clenbuterol were added to rat drug-free plasma to give final concentrations of 20, 100 and 200 ng/mL (10, 50 and 100 ng/mL, respectively, as enantiomer concentration). The plasma samples were incubated at 37°C for 30 min. One milliliter of each sample was then loaded onto a Centrifree® UF Device (Millipore Co, Billerica, MA). The tube assembly was centrifuged at 2,000 g for 10 min at room temperature. The protein-free ultrafiltrate was collected for drug analysis. Before centrifugation, another 0.1 mL of the plasma sample was saved for analysis of the total drug concentration. Protein unbound fraction was calculated

according to the following equation:

$$\%\ \text{protein unbound fraction} = \frac{\text{unbound clenbuterol concentration}}{\text{total clenbuterol concentration}} \times 100$$

Data Analysis

Plasma concentration-time profiles were subjected to non-compartmental pharmacokinetics analysis using the macro-program MOMENT (EXCEL) [25]. The area under the plasma concentration-time curve from time zero to time T, $AUC_{0\text{-}T}$, where T is the time of last measurable concentration, was calculated using the linear trapezoidal method. The area under the plasma concentration-time curve from time zero to infinity ($AUC_{0\text{-}\infty}$) and the area under the first-moment time curve (AUMC) were determined by using the combined log-linear trapezoidal rule and the extrapolated area. Mean residence time (MRT) was calculated using the equation MRT = AUMC/ $AUC_{0\text{-}\infty}$. The apparent elimination half-life ($t_{1/2}$) was calculated as $0.693/k_e$ and the k_e was estimated by linear regression of the plasma concentration in the log-linear terminal phase. Clearance (CL_{tot}) following i.v. dosing was calculated as Dose/$AUC_{0\text{-}\infty}$. The apparent volume of distribution (Vd_{ss}) was estimated as MRT x CL_{tot}.

The absolute bioavailabilty (%F) of clenbuterol enantiomers was calculated using the relationship.

%F = [$AUC_{0\text{-}\infty(\text{ i.d.})}$ x $Dose_{(\text{i.v.})}$/ $AUC_{0\text{-}\infty(\text{i.v.})}$ x $Dose_{(\text{i.d.})}$] x 100

The urinary clearance (CL_r) and biliary clearance (CL_{bile}) were obtained by dividing the amount excreted into the urine and bile by the area under the concentration-time curve from time zero to time 24 hours ($AUC_{0\rightarrow24}$).

Chiral Inversion and Degradation in Buffer and Plasma Solutions

Enantiomeric stability was evaluated by measuring changes in the concentrations

of (-)-*R*- and (+)-*S*-clenbuterol in 0.1 M phosphate buffer pH 7.4 and rat plasma. The sample solutions consisted of (-)-*R*- and (+)-*S*-clenbuterol 10 ng/mL in 0.1 M phosphate buffer pH 7.4 and rat plasma. Aliquots of 100 µL of these solutions were incubated at 37°C for 0, 24, and 72 hours, and then stored at -30°C until analysis.

Statistical Analysis

Values are expressed as means ± S.D. Statistical analysis was performed by paired *t* test analysis of variance with $p < 0.05$ as the minimal level of significance.

Results

Pharmacokinetics of clenbuterol enantiomers in rats

To clarify the pharmacokinetic disposition of clenbuterol enantiomers in the rat, we examined plasma concentrations of each enantiomer after intravenous administration of *(rac)*-clenbuterol. Plasma concentration-time profiles of clenbuterol enantiomers in rats after i.v. administration of 2 mg/kg *(rac)*-clenbuterol are shown in Figure 1. Initially, (+)-*S*-clenbuterol showed a higher plasma concentration, but at 24 hours, the plasma concentration of (-)-*R*-clenbuterol was higher than that of (+)-*S*-clenbuterol.

The pharmacokinetic parameters of (-)-*R*- and (+)-*S*-clenbuterol estimated following i.v. administration of 2 mg/kg *(rac)*-clenbuterol are summarized in Table 2. The $AUC_{0-\infty}$ values of (-)-*R*-clenbuterol and (+)-*S*-clenbuterol were 1.25 ± 0.18 μg/mL·hr and 1.51 ± 0.35 μg/mL·hr, respectively (mean ± S.D., n = 4). The CL_{tot} of (-)-*R*-clenbuterol (13.5 ± 1.8 mL/min/kg) was significantly higher than that of the *S*-enantiomer (11.5 ± 2.7 mL/min/kg, $p < 0.05$). The MRT of (-)-*R*-clenbuterol was significantly longer than that of (+)-*S*-clenbuterol. The Vd_{ss} of (-)-*R*-clenbuterol (9.17 ± 2.76 L/kg) was larger than that of the *S*-enantiomer (4.14 ± 1.00 L/kg, $p < 0.05$).

Next, intraduodenal administration experiments were performed in rats. Plasma concentration-time profiles of clenbuterol enantiomers in rat after i.d. administration of 2 mg/kg *(rac)*-clenbuterol are shown in Figure 2. Both enantiomers were absorbed rapidly, and the maximal concentrations (C_{max}) for (-)-*R*- and (+)-*S*-clenbuterol, reached within 15 min after administration, were 124.8 and 195.1 ng/mL, respectively. As observed in i.v. administration, (+)-*S*-clenbuterol showed a higher blood concentration at the early times after dosage, but after 24 hours, the plasma concentration of (-)-*R*-clenbuterol was higher than that of (+)-*S*-clenbuterol.

The pharmacokinetic parameters for (-)-*R*- and (+)-*S*-clenbuterol, estimated

following i.d. administration of 2 mg/kg *(rac)*-clenbuterol, are also summarized in Table 2. (-)-*R*- and (+)-*S*-clenbuterol were almost completely absorbed and the bioavailabilities (F) after intraduodenal administration were 92% and 101%, respectively. The mean CL_{tot}/F of (-)-*R*-clenbuterol was 15.6 ± 3.8 mL/min/kg, which is higher than that of the *S*-enantiomer (11.7 ± 3.5 mL/min/kg, $p < 0.05$). The mean Vd_{ss}/F of (-)-*R*-clenbuterol was 10.3 ± 2.9 L/kg, which is also higher than that of the *S*-enantiomer (5.0 ± 1.5 L/kg, $p < 0.05$).

In-situ permeability study

To clarify whether it is different in intestinal absorption between the enantiomers, the absorption rate of the enantiomers was determined in rat jejunum by *in situ* closed loop method. The fractions of (-)-*R*- and (+)-*S*-clenbuterol absorbed in the jejunal loop at 10 min after administration were 31.7% and 32.0%, respectively. The fractions absorbed at 30 min after administration were 72.9% and 73.3%, and those at 1 hour were 87.8% and 87.4%, respectively. There was no significant difference between the enantiomers at any time point examined.

Urinary elimination

The urinary excretion of the clenbuterol enantiomers was determined after i.v. dosing to rats. Excretions of (-)-*R*- and (+)-*S*-enantiomers in urine over 24 hours were 19.7 ± 1.2%, and 22.3 ± 1.5% of the administered dose, respectively (Figure 3). Calculated renal clearance (CL_r) values of the (-)-*R*- and (+)-*S*-enantiomers were 4.9 ± 0.7 mL/min/kg, and 4.5 ± 0.9 mL/min/kg, respectively, showing no significant difference.

Biliary excretion

Next, the clenbuterol concentrations in bile were determined after i.v. dosing to rats. About 9.3 to 14.4 mL of bile was obtained from each rat during 24 hours after i.v. dosing of clenbuterol. It was found that amounts of about 7.7 ± 1.8%, and 3.3 ± 0.4% of the administered dose were recovered cumulatively in the bile collected up to 24 hours (Figure 4) for (-)-*R*-clenbuterol and (+)-*S*-clenbuterol, respectively. Calculated biliary clearance (CL_{bile}) of (-)-*R*-clenbuterol (1.62 ± 0.88 mL/min/kg) was markedly higher than that of the (+)-S-enantiomer (0.45 ± 0.23 mL/min/kg).

Protein binding

The protein-unbound ratio has a substantial influence on drug distribution, so we examined *in vitro* protein binding of clenbuterol. The unbound fraction of clenbuterol, estimated by ultrafiltration of rat plasma spiked with *(rac)*-clenbuterol, showed no marked concentration dependence between 20 and 200 ng/mL clenbuterol. However, there was a marked difference between the two enantiomers. Unbound fractions of (-)-*R*-clenbuterol were 52.6, 46.4, and 47.6% at the concentrations of 10, 50, and 100 ng/mL (-)-*R*-clenbuterol, respectively. Unbound fractions of (+)-*S*-clenbuterol were 34.0, 31.4, and 33.9% at the concentrations of 10, 50, and 100 ng/mL (+)-*S*-clenbuterol, respectively. The mean unbound fractions of (-)-*R*-clenbuterol and (+)-*S*-clenbuterol were 48.8 ± 3.1 and 33.1 ± 1.5%, respectively.

Tissue distribution

Table 3 shows the tissue-to-plasma concentration ratios (K_p) and the tissue-to-plasma free concentration ratios ($K_{p,f}$) of (-)-*R*-clenbuterol and (+)-*S*-clenbuterol in various tissues at 36 hours after i.v. administration of *(rac)*-clenbuterol. The $K_{p,f}$ as well as K_p values of (+)-*S*-clenbuterol in the liver, kidney, lung and muscle were significantly greater than those of the (-)-*R*-enantiomer.

Chiral inversion *in vitro* and *in vivo*

Experiments were carried out to evaluate whether clenbuterol enantiomers undergo chiral inversion and degradation in buffer or plasma, or *in vivo*.

The peak area of (-)-*R*-clenbuterol enantiomer in PBS (pH 7.4) and rat plasma showed no significant change during 72 hours incubation, and no peak of the (+)-*S*-enantiomer was detected (data not shown). A similar result was obtained for the (+)-*S*-enantiomer. The (+)-*S*-clenbuterol used consisted of 93.0% (+)-*S*-clenbuterol and 7.0% (-)-*R*-clenbuterol, and the (+)-*S*-clenbuterol/(-)-*R*-clenbuterol ratio remained unchanged during 72 hours incubation. Thus, inversion or degradation did not occur up to 72 hours.

Plasma concentration-time profiles of clenbuterol enantiomers in rat after intravenous administration of 1 mg/kg (-)-*R*-clenbuterol are shown in Figure 5. No conversion to the (+)-*S*-enantiomer was detected within 72 hours.

Discussion

In this study, we found that Vd_{ss}, CL_{tot} and MRT were significantly different (2.2-fold, 1.2-fold and 1.9-fold, respectively) for the two enantiomers of clenbuterol in rats (Table 2). There was also a difference in protein binding between the enantiomers (1.5-fold).

The enantioselective protein binding of clenbuterol might account for the enantioselectivity of systemic clearance, but it was not sufficient to explain the difference in Vd_{ss} of the enantiomers. Given the biliary clearance of clenbuterol and the rapid and complete absorption after intraduodenal administration (Table 2), the existence of entero-hepatic circulation seems likely. Indeed, Manchee et al. [26] reported that the β2-adrenoceptor agonist salmeterol undergoes entero-hepatic circulation. Therefore, we think a difference in entero-hepatic circulation of clenbuterol enantiomers could contribute to the enantioselectivity of Vd_{ss} as well as MRT. Moreover, enantioselective tissue-binding should also be considered. Accordingly, enantioselectively in tissue uptake or membrane transport processes, i.e., from sinusoidal uptake to biliary excretion, and/or transport in the renal tubules might contribute to the enantioselective systemic clearance.

In our preliminary study, the tissue concentrations of (-)-*R*- and (+)-*S*-clenbuterol were evaluated at 3 hours and 24 hours after administration. The K_p values of (-)-*R*- and (+)-*S*-clenbuterol in the lung were 52.3 / 43.6 and 11.7 / 19.9 at 3 hours and 24 hours, respectively. Those in the liver were 63.7 / 49.2 and 10.7 / 19.2 at 3 hours and 24 hours, respectively. The K_p values of (-)-*R*-clenbuterol were larger than those of (+)-*S*-clenbuterol at early time points after administration. These results may reflect the larger distribution volume of (-)-*R*-clenbuterol compared to (+)-*S*-clenbuterol. However, we cannot exclude the possibility that the distribution process to tissues was incomplete. Therefore, we determined $K_{p,f}$ values at 36 hours after administration, by which time equilibration between blood and tissues should be completed. The $K_{p,f}$ values of

(+)-*S*-clenbuterol in liver and kidney were significantly greater than those of the (-)-*R*-enantiomer at 36 hours after administration (Table 3). This result is consistent with the findings of von Deutsch *et al*. [14] and Smith [27], who reported that the concentrations of (+)-*S*-clenbuterol in these organs were greater than those of the (-)-*R*-enantiomer after repeated dosing of clenbuterol, although they did not determine blood concentrations. The difference of K_p values estimated at 36 hours may reflect irreversible tissue binding of the (+)-*S*-enantiomer, which could be related to the side effects of the (+)-*S*-enantiomer.

We also found that the CL_{tot} of (-)-*R*-clenbuterol (13.5 mL/min/kg) was slightly but significantly higher than that of the (+)-*S*-enantiomer (11.5 mL/min/kg). However, no significant difference between the enantiomers was observed in urinary clearance and metabolic clearance (estimated as non-renal and non-biliary clearance). The only significant difference between (-)-*R*-clenbuterol and (+)-*S*-clenbuterol was observed in biliary clearance (1.62 ± 0.88 mL/min/kg and 0.45 ± 0.23 mL/min/kg, respectively). The difference of plasma protein binding may also affect the uptake of clenbuterol by the liver, thereby influencing CL_{bile} of (-)-*R*- and (+)-*S*-clenbuterol. Although the contributions of (-)-*R*- and (+)-*S*-clenbuterol biliary clearance to CL_{tot} are small, i.e., 12% and 4%, respectively, the difference in biliary clearance may contribute to the enantioselectivity of CL_{tot}.

The glomerular filtration rate of rats is reported to be 5.24 mL/min/kg [28]. Estimated values of f_p×glomerular filtration rate of (-)-*R* and (+)-*S*-clenbuterol based on the plasma protein binding rates were 2.6 and 1.7 mL/min/kg, respectively. The values of clearance ratio ($=CL_{r,u}/(GFR*f_p)$) for (-)-*R*- and (+)-*S*-clenbuterol (1.9, and 2.6, respectively) suggest that reabsorption occurred during the urinary excretion process.

Plasma protein binding of *(rac)*-clenbuterol was reported to be 68-70%. In this study, the average of (-)-*R*- and (+)-*S*-clenbuterol plasma protein-binding rates was 59%,

which is similar to the reported range. The unbound fraction of (-)-*R*- and (+)-*S*-clenbuterol were 0.488 and 0.331, respectively, and this may have contributed substantially to the differences of Vd_{ss} and excretion between the enantiomers observed in this study. Very recently, enantioselectivity in serum protein binding of a β1-blocker with β3 agonistic property, nevivolol, has been reported by Sanaee *et al* [29]. The unbound fraction of (-)-nebivolol (0.112) was significantly higher than that of (+)-nebibolol (0.061), which resulted in the systemic stereoselectivity. However, it has also been reported that the tissue binding of salbutamol is not enantioselective and plasma protein binding is relatively low [11]. Therefore, differences appear to exist even between medicines in the same category, so it is important to evaluate each medicine individually.

The $MRT_{0-\infty}$ of (-)-*R*-clenbuterol was about twice that of (+)-*S*-clenbuterol. Since (-)-*R*-clenbuterol exerts the desired therapeutic effect, this is favorable. On the other hand, C_{max} of (+)-*S*-clenbuterol was larger than that of the (-)-*R*-enantioner. Since (+)-*S*-clenbuterol has side effects including increased blood pressure and increased blood sugar level and glucocorticoid, it is undesirable for C_{max} to be high. Moreover, it was predicted from CL_{tot} that the steady-state plasma concentration of (+)-*S*-clenbuterol would be higher than that of (-)-*R*-clenbuterol. Beta2-stimulant activities such as bronchodilation and uterine relaxing activity are almost exclusively exhibited by (-)-*R*-clenbuterol. On the other hand, (+)-*S*-clenbuterol has been reported to cause adverse effects such as reduction of blood pressure, increase of blood glucose level, and elevation of glucocorticoid level. Clenbuterol treatment improves muscular functional capacity by increasing muscular strength. However, our unpublished data indicate that (-)-*R*-clenbuterol is responsible for muscle reinforcement, while (+)-*S*-clenbuterol acts to reduce bone density. Because (+)-*S*-clenbuterol clearance is smaller, and (+)-*S*-clenbuterol tissue distribution is larger than those of (-)-*R*-clenbuterol, the adverse effects of (+)-*S*-clenbuterol cannot be ignored. Therefore, we believe it would be

preferable for pharmaceutical products to contain only (-)-*R*-clenbuterol. We think the racemate should not be used as an aid for muscle-building.

Thus, we believe development of enantiomerically pure (-)-*R*-clenbuterol as a therapeutic agent would be desirable. Fortunately, we found that no enantiomeric conversion of clenbuterol occurred in the *in vitro* and *in vivo* experiments. This is in contrast to thalidomide, whose optical isomers are interconverted in PBS [5,6] and MK-0767 [30], a dual peroxisome proliferator-activated receptor agonist, whose enantiomers are interconverted in plasma even though they are stable in PBS. These results suggest that it may be feasible to develop enantiomerically pure (-)-*R*-clenbuterol as a therapeutic agent.

Conclusion

We examined the disposition of (-)-*R*- and (+)-*S*-clenbuterol following i.v. and i.d. administration of *(rac)*-clenbuterol in rats. Our results suggest that the distribution and excretion characteristics of (-)-*R*-clenbuterol and (+)-*S*-clenbuterol are significantly different, and these differences seem to be predominantly due to a difference in protein binding (*i.e.*, in unbound fraction) between the enantiomers.

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Figure Legends

Figure 1.

Plasma concentration versus time curves for (-)-*R*-clenbuterol (closed circles) and (+)-*S*-clenbuterol (open circles) after i.v. administration of 2 mg/kg *(rac)*-clenbuterol to rats. (n = 4, Mean ± S.D.) *$p < 0.05$, (-)-*R*-clenbuterol vs. (+)-*S*-clenbuterol.

Figure 2.

Plasma concentration versus time curves (-)-*R*-clenbuterol (closed circles) and (+)-*S*-clenbuterol (open circles) after i.d. administration of 2 mg/kg *(rac)*-clenbuterol to rats. (n = 5, Mean ± S.D.) *$p < 0.05$, (-)-*R*-clenbuterol vs. (+)-*S*-clenbuterol.

Figure 3.

Cumulative urinary excretion ratio (-)-*R*-clenbuterol (closed circles) and (+)-*S*-clenbuterol (open circles) after i.v. administration of 2 mg/kg *(rac)*-clenbuterol to rats. (n = 5, Mean ± S.D.). *$p < 0.05$, (-)-*R*-clenbuterol vs. (+)-*S*-clenbuterol.

Figure 4.

Cumulative biliary excretion ratio of (-)-*R*-clenbuterol (closed circles) and (+)-*S*-clenbuterol (open circles) after i.v. administration of 2 mg/kg *(rac)*-clenbuterol to rats. (n = 5, Mean ± S.D.). *$p < 0.05$, (-)-*R*-clenbuterol vs. (+)-S-clenbuterol.

Figure 5.

Plasma concentration versus time curves for (-)-*R*-clenbuterol (closed circles) after i.v. administration of 1 mg/kg (-)-*R*-clenbuterol to rats (n = 4, Mean ± S.D.).

Table 1

Intra- and inter-day precision and accuracy in determination of clenbuterol enantiomers in rat plasma.

	Nominal (ng/mL)	Mean (ng/mL)	± S.D.	Accuracy[a] (%)	C.V.[b] (%)
Intra-day (n = 6)					
(-)-*R*-clenbuterol	1	0.97	0.05	97.0	2.7
	5	5.03	0.19	100.6	4.3
	25	25.1	0.19	100.4	1.0
(+)-*S*-clenbuterol	1	0.97	0.04	97.0	3.2
	5	5.01	0.24	100.2	4.5
	25	24.9	0.14	99.6	1.4
Inter-day (n = 6)					
(-)-*R*-clenbuterol	1	1.04	0.04	104.0	3.6
	5	4.63	0.1	92.6	2.1
	25	25.4	0.36	101.6	1.4
(+)-*S*-clenbuterol	1	0.99	0.03	99.0	3.2
	5	4.85	0.15	97.0	3.1
	25	25.2	0.34	100.8	1.3

[a] Accuracy (%) is expressed as mean found concentration/nominal concentration × 100.

[b] Coefficient of variation.

Table 2

Pharmacokinetic parameters of clenbuterol enantiomers after i.v. and i.d. administration of 2 mg/kg (rac)-clenbuterol to rats.

		(-)-R-clenbuterol	*(+)-S-clenbuterol*
i.v.			
$AUC_{0\rightarrow\infty}$	(µg/mL·hr)	1.25 ± 0.18	1.51 ± 0.35
MRT	(hr)	11.2 ± 2.0	6.0 ± 0.7 *
$t_{1/2}$	(hr)	19.1 ± 5.5	14.1 ± 5.1 *
CL_{tot}	(mL/min/kg)	13.5 ± 1.8	11.5 ± 2.7 *
Vd_{ss}	(L/kg)	9.17 ± 2.76	4.14 ± 1.00 *
i.d.			
$AUC_{0\rightarrow\infty}$	(µg/mL·hr)	1.15 ± 0.42	1.52 ± 0.46 *
MRT	(hr)	11.1 ± 2.1	7.1 ± 0.7 *
$t_{1/2}$	(hr)	12.9 ± 4.9	5.4 ± 1.0 *
F	(%)	92.0 ± 33.6	101.0 ± 30.2
CL_{tot}/F	(mL/min/kg)	15.6 ± 3.8	11.7 ± 3.5 *
Vd_{ss}/F	(L/kg)	10.3 ± 2.9	5.0 ± 1.5 *

Upper: Moment analysis of clenbuterol from plasma concentration after i.v. administration of *(rac)*-clenbuterol (2 mg/kg) to rats (298 ± 27 g, n = 4).

Lower: Moment analysis of clenbuterol from plasma concentration after i.d. administration of *(rac)*-clenbuterol (2 mg/kg) to rats (235 ± 29 g, n = 5).

* Significant difference between (-)-*R*-clenbuterol and (+)-*S*-clenbuterol at $p < 0.05$; Student's *t* test.

$AUC_{0\rightarrow\infty}$, area under the plasma concentration-time curve from time zero to infinity; MRT, mean residence time; $t_{1/2}$, terminal half-life; CL_{tot}, total body clearance; Vd_{ss}, distribution volume at steady state.

Table 3

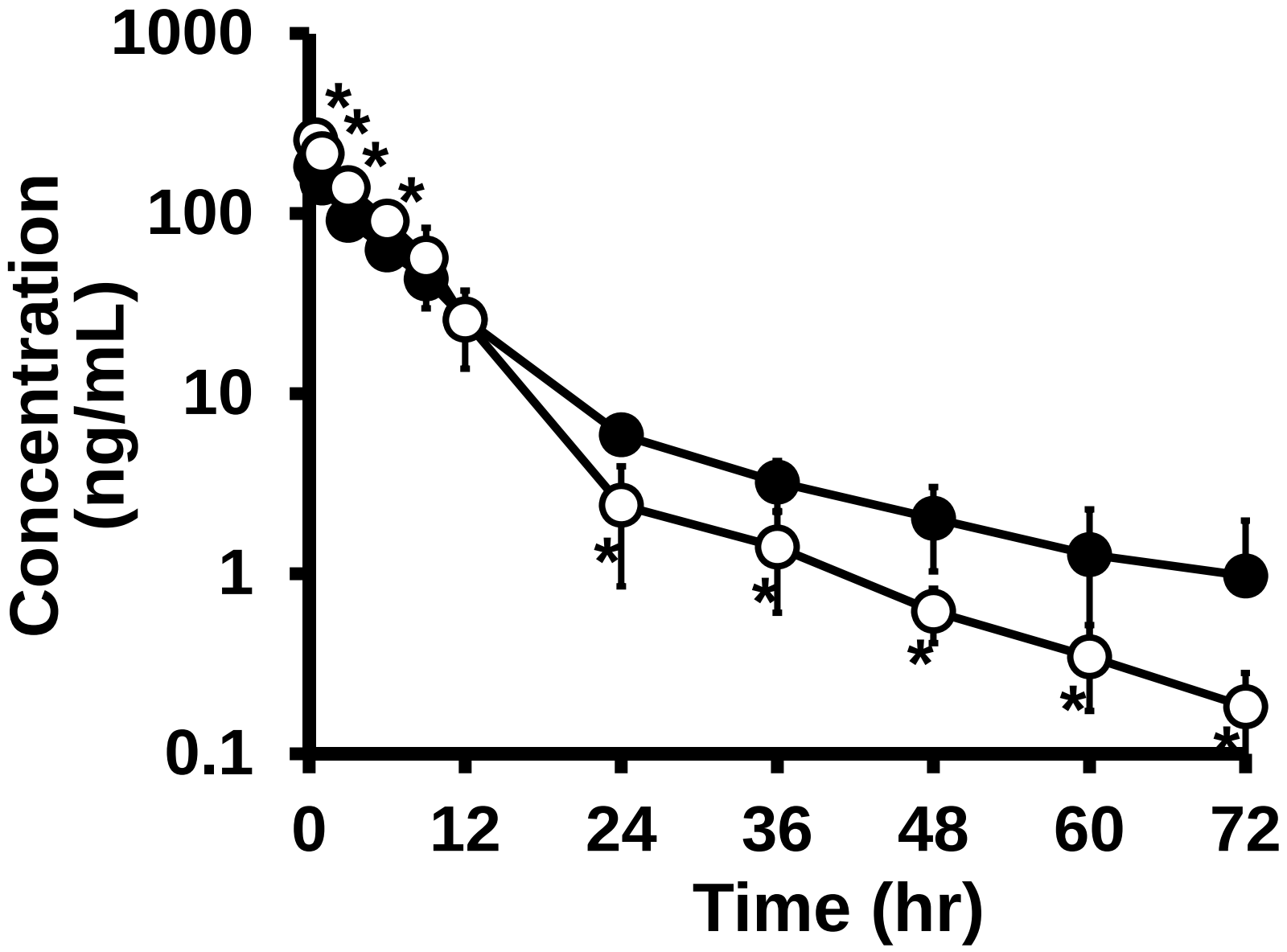
Tissue-to-plasma concentration ratio (Kp) of (-)-R-clenbuterol and (+)-S-clenbuterol at 36 hours after intravenous administration of (rac)-clenbuterol.

Tissue	***(-)-R-clenbuterol*** Kp (Kp,f)	***(+)-S-clenbuterol*** Kp (Kp,f)
Lung	2.63 ± 1.04 (5.01 ± 1.97)	9.75 ± 4.69 * (28.7 ± 13.8 *)
Liver	5.28 ± 2.19 (10.0 ± 4.2)	24.9 ± 5.8 * (73.1 ± 17.1 *)
Kidney	1.99 ± 0.65 (3.79 ± 1.24)	11.8 ± 3.0 * (34.8 ± 8.9 *)
Muscle	0.58 ± 0.28 (1.10 ± 0.54)	1.91 ± 0.60 * (5.61 ± 1.75 *)

(rac)-Clenbuterol (2 mg/kg) was administered intravenously to rats. At 36 hours after administration, rats were decapitated and the plasma and tissues were isolated and weighed. The tissue and plasma contents and plasma unbound fractions of (-)-*R*-clenbuterol and (+)-*S*-clenbuterol were determined as described in Materials and Methods. Each value represents the mean ± S.D. (n = 5).

* Significantly different between (-)-*R*-clenbuterol and (+)-*S*-clenbuterol at $p < 0.05$; Student's *t* test.

Iori Hirosawa, Figure 1.



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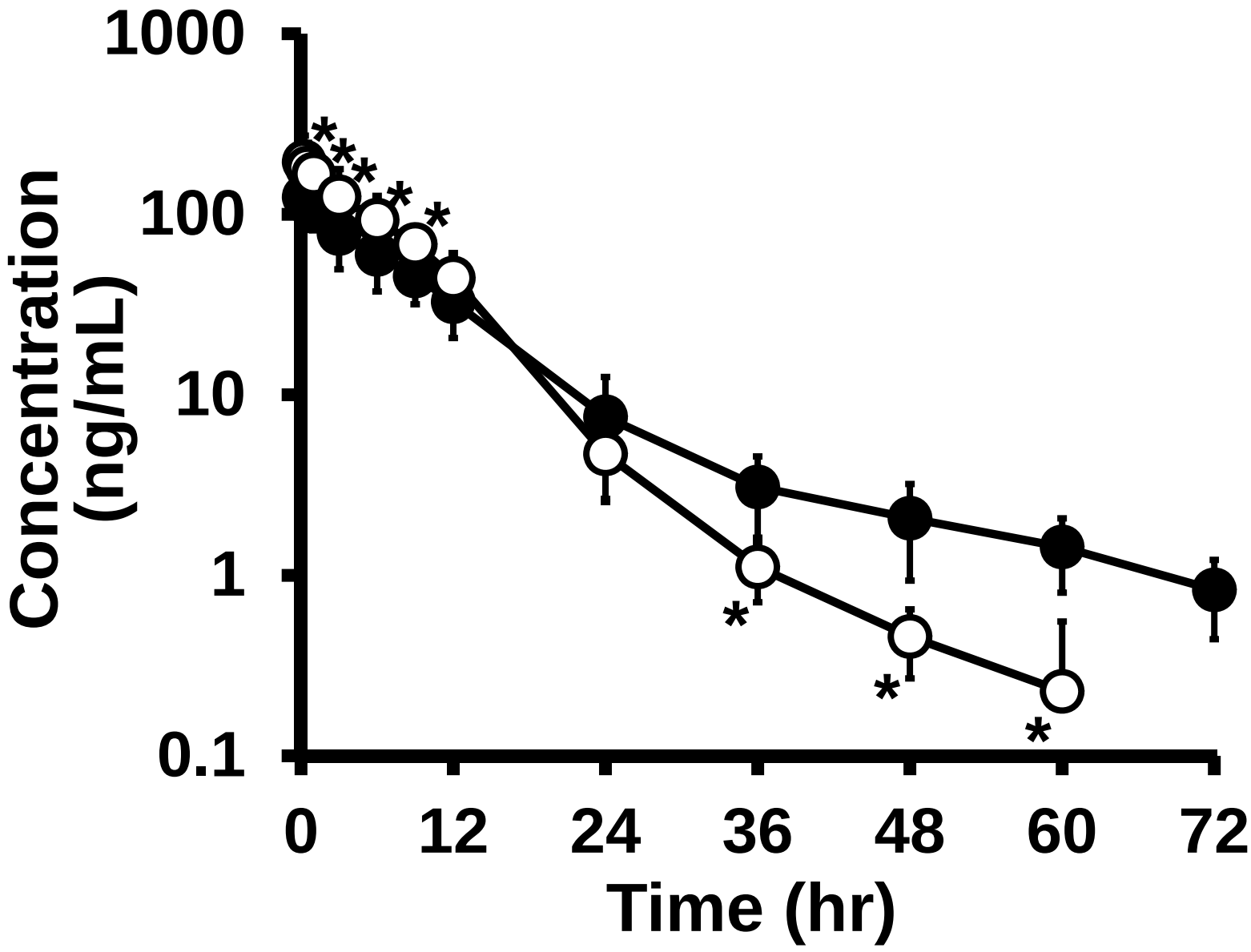

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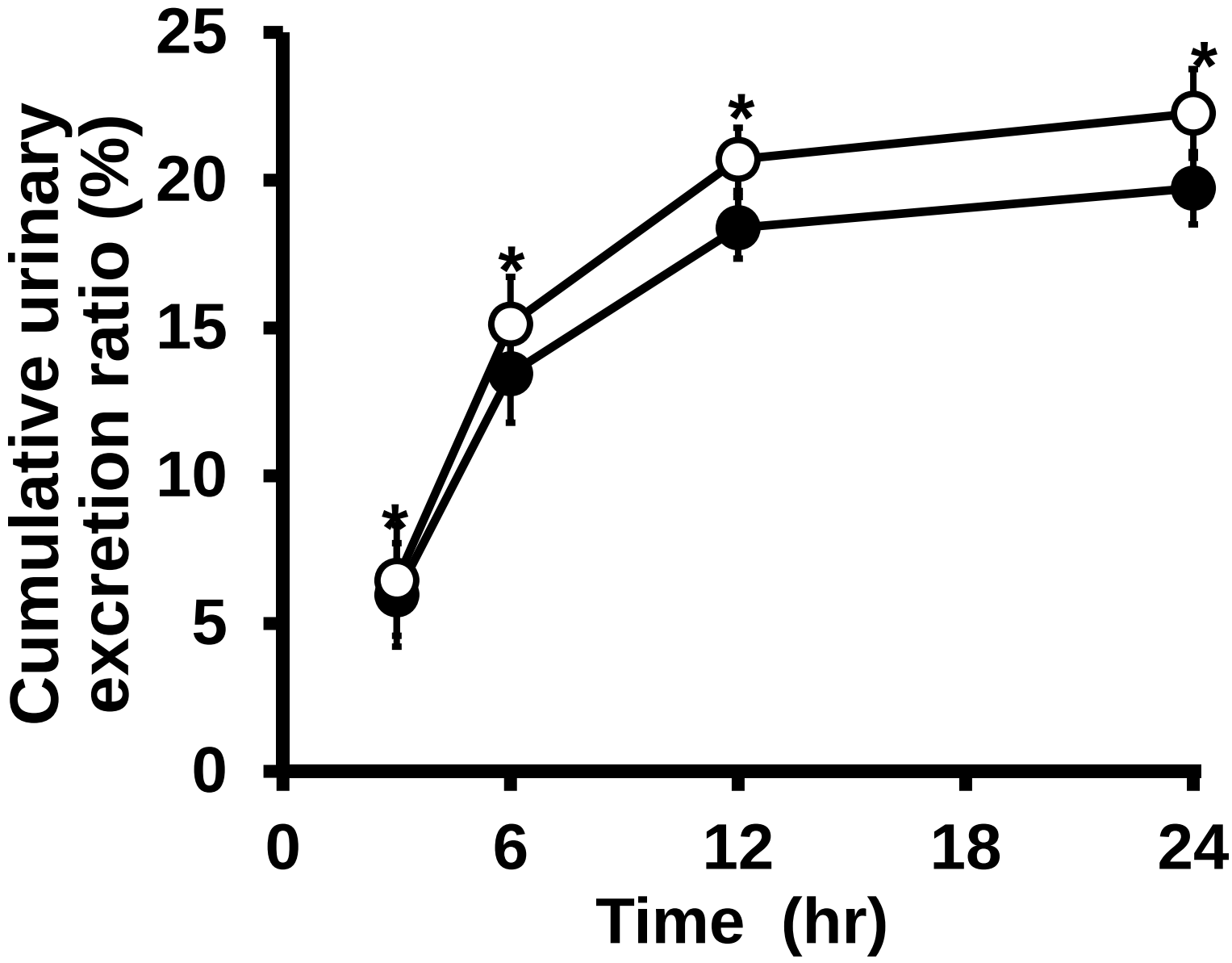

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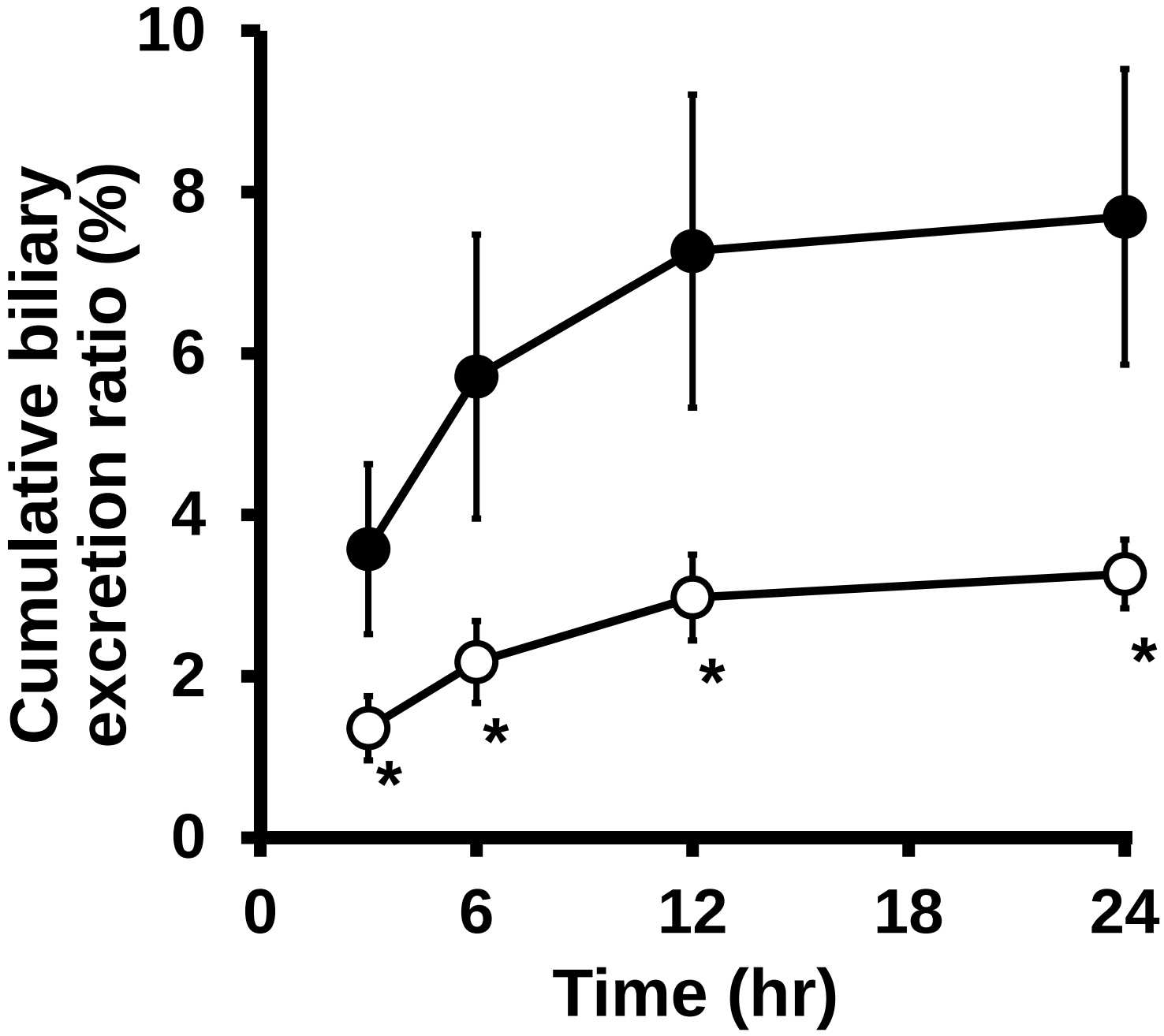

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