Differences in the Pharmacokinetics of 4-Amino-3-Chlorophenyl Hydrogen Sulfate, a Metabolite of Resatorvid, in Rats and Dogs

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Abstract:

The pharmacokinetics of 4-amino-3-chlorophenyl hydrogen sulfate, M-III of resatorvid, in rats and dogs were investigated using radiolabeled M-III ([14C]M-III). The elimination half-life of 14C in the plasma of rats was approximately 1/30 of that of dogs after intravenous dosing of [14C]M-III at 0.5 mg/kg to rats and dogs. In vitro and in vivo plasma protein binding ratios of M-III were relatively high and were the same in both species. The intrinsic clearance (CLint) of M-III in rats was much higher than that in the kidney. On the contrary, in dogs, the concentration of [14C]M-III in the kidney was very much lower than that in the plasma. These results indicated that M-III was effectively taken up into the kidney and was excreted into the urine in rats; however, in dogs, ineffective renal uptake of M-III was presumed. When [14C]M-III and probenecid were simultaneously and continually infused intravenously to rats, the CLint of M-III decreased with increasing plasma concentrations of probenecid, indicating that kidney uptake of M-III in rats was inhibited by probenecid. It was also thought that uptake by the organic anion transporter(s) in the basolateral membrane is involved in the renal excretion of M-III in rats. The pharmacokinetic differences of M-III between rats and dogs are considered to be mainly caused by the difference in the urinary excretion via the renal distribution processes.

Introduction

Ethyl (6R)-6-[N-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (CAS 243984-11-4, TAK-242, resatorvid) was discovered by Takeda Pharmaceutical Company Limited (Osaka, Japan) and was studied in clinical trials conducted in Japan, in the European Union, and in the United States as a potential new antisepsis drug, which acts as a cytokine production inhibitor (Yamada et al., 2005). Sepsis is a clinical condition defined by the presence of both infection (usually bacterial) and a systemic inflammatory response (Parrillo, 1993; Levy et al., 2003). In patients with sepsis, blood pressure drops, resulting in shock. Major organs and body systems, including the kidneys, liver, lungs, and central nervous system, stop functioning properly.

TAK-242 is a suppressor of the pathogen-induced release of inflammatory cytokines and is believed to inhibit/suppress the release of these cytokines by binding to the intracellular domain of Toll-like receptor 4 (Li et al., 2006).

Nonclinical pharmacokinetic studies of TAK-242 were conducted using two types of radiolabeled TAK-242, because TAK-242 has been shown to be metabolized to a phenyl ring moiety and a cyclohexene ring moiety by the cleavage of the sulfonamide bond. The phenyl ring moiety of TAK-242 yielded M-I, 2-chloro-4-fluoroaniline, and M-I was further acetylated and conjugated to form M-II and the glucuronide (M-I-G), respectively. M-I was also converted to M-III and M-IV by hydroxylation and subsequent sulfate conjugation (Fig. 1). Meanwhile, the cyclohexene ring moiety of TAK-242 was confirmed to be metabolized to the glutathione conjugate, M-SG, followed by further metabolism of M-SG to form the cysteine conjugate (M-Cys) and the mercapturic acid conjugate (M-Mer), which were M-SG, M-Cys, and M-Mer in our previous studies (Jinno et al., 2011).

When phenyl ring-labeled TAK-242, [phenyl ring-U-14C]TAK-242, was given intravenously to rats and dogs, the concentration-time profiles in the plasma of the total 14C showed extreme species differences. The extreme species differences were characterized by the elimination half-life (t1/2) of the total 14C from the plasma between rats and dogs with the values of 5.6 and 158 h, respectively (Jinno et al., 2011). Regarding the 14C components in the plasma, M-I-G was the major component in rats; however, in dogs, M-III accounted for most of the radioactivity. From these results, we considered that the presence or absence of the metabolic pathway from TAK-242 to M-III would derive the species difference for pharmacokinetic profiles of TAK-242 between rats and dogs. However, in the 14C composition analysis of rat plasma, M-III was detected with 4.2 and 3.3% of total 14C at 0.5 and 1 h, respectively, after dosing and M-III in the rat
plasma was rapidly decreased despite the long half-life for M-III in dogs (Jinno et al., 2011). Furthermore, M-III was also detected in the urine of rats and dogs, and urinary clearance of M-III in rats was calculated to be much higher than that in dogs. These findings indicated that species differences in the elimination of M-III from plasma to urine via the renal distribution would be one of the possible factors for the extremely different 14C concentration-time profiles in the plasma between rats and dogs after intravenous injection of [phenyl ring-U-14C]TAK-242.

In this study, we focused on the disposition properties of M-III, and the pharmacokinetic studies in rats and dogs were performed using synthesized [14C]M-III. This report describes the results of the investigation of the elimination processes via the renal distribution of M-III in rats and dogs and possible factors for the species differences in the pharmacokinetic profiles of M-III.

Materials and Methods

**Materials.** TAK-242 and 4-amino-3-chlorophenyl hydrogen sulfate (M-III) were prepared by the Chemical Development Laboratories of the Production Division and the Medicinal Chemistry Research Laboratories of the Pharmaceutical Research Division, respectively, at Takeda Pharmaceutical Company Limited (Osaka, Japan). 4-Amino-3-chloro[U-14C]phenyl hydrogen sulfate ([14C]M-III) with a specific radioactivity of 5.29 MBq/mg was synthesized by GE Healthcare (Giles St. Chalfont, Buckinghamshire, UK). The radiopurity (≥95%) and chemical identity of the labeled compound were verified by thin-layer chromatography and high-performance liquid chromatography (HPLC).

**Animals.** The animals used in this study were male Cr:CD(SD)IGS rats (weight, 285–324 g, aged 8 weeks; Charles River Japan Inc., Yokohama, Japan) and male beagle dogs (weight, 7.4–10.0 kg, aged 5 or 7 months; Oriental Yeast Co., Ltd., Tokyo, Japan). They were fed laboratory chow [CR-LPF for rats (Oriental Yeast Co., Ltd.) or CD-5 for dogs (CLEA Japan Inc., Tokyo, Japan)], had free access to water, and were housed in temperature- and humidity-controlled rooms (20–26°C, 40–70%), with 12-h light/dark cycles, for more than 1 week before use. This study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) in the Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited and approved by the ethics committee for animal experiments in our division.

**Pharmacokinetics of M-III in Rats after Intravenous Dosing of [14C]M-III.** [14C]M-III was dissolved in saline (0.5 mg/ml) for intravenous injection to rats at a dose of 0.5 mg–2.6 MBq/kg. Blood samples (300 μl) were collected from the tail vein at 5, 10, 15, 30, and 45 min and 1, 2, 3, 4, 6, 8, and 24 h after dosing. Immediately after blood sampling, the heparinized blood was centrifuged at 8450 g and 4°C for 3 min, and plasma samples were obtained. The aliquots of the plasma samples (50 μl) were used to determine the radioactivity and the residual samples were kept frozen at −20°C until analysis for [14C]M-III by HPLC. Urine and feces were collected in metabolic cages for rats equipped with a separator for urine and feces. The urine and feces samples after determination of the radioactivity were kept frozen (−20°C) until analysis.

**Pharmacokinetics of M-III in Dogs after Intravenous Dosing of [14C]M-III.** [14C]M-III diluted appropriately with unlabeled compound was dissolved in saline (2.5 mg/ml) for intravenous injection of dogs at a dose of 0.5 mg–1.0 MBq/kg. Blood samples (5 ml) were collected from the cephalic vein at 5, 10, 15, and 30 min and 1, 2, 3, 4, 6, 8, 24, 48, 72, 96, 120, 144, and 168 h after dosing. Immediately after the blood sampling, the heparinized blood was centrifuged at 8450g and 4°C for 3 min, and plasma samples were obtained. The aliquots of the plasma samples (50 μl) were used to determine the radioactivity and the residual samples were kept frozen at −20°C until analysis for [14C]M-III by HPLC. Urine and feces were collected in metabolic cages for dogs equipped with a separator for urine and feces. The urine and feces samples after determination of the radioactivity were kept frozen (−20°C) until analysis.
Pharmacokinetics of \([^{14}C]M-III\) in Rats Infused a Mixture of \([^{14}C]M-III\) and Probenecid. \([^{14}C]M-III\), diluted appropriately with unlabeled compound, was dissolved in a mixture of saline and an appropriate sodium hydroxide mixture (1 mg/mL), which included probenecid (38, 76, and 149 mg/mL) for infusion at a dose of 0.125 mg–0.06 MBq \(\cdot\) rat \(^{-1}\) \(\cdot\) h \(^{-1}\). All rats were fitted with a cannula (PE50; 0.58 mm i.d.) into the jugular vein and the femoral artery (Harms and Ojeda, 1974; Ervine et al., 1996) approximately 2 h before the experiment. Each rat was placed in a Bollmann cage and was given \([^{14}C]M-III\) containing probenecid by continuous infusion into the jugular vein using an infusion pump (Harvard Pump 22; Harvard Apparatus, South Natick, MA). Blood samples (300 μl) were taken from the cannula in the femoral artery at 0.5, 1, 2, 3, and 4 h during an infusion. At 5 h after an infusion was started, the blood samples were collected from the abdominal aorta under anesthesia with diethyl ether. The plasma obtained by centrifugation was analyzed for the radioactivity and probenecid.

Distribution of Radioactivity to the Kidney of Rats and Dogs. \([^{14}C]M-III\) diluted appropriately with unlabeled compound was dissolved in saline for continuous infusion to rats (1 mg/mL). The infusion rate was 0.5 mg–0.23 MBq \(\cdot\) rat \(^{-1}\) \(\cdot\) h \(^{-1}\), rats were fitted with a cannula (PE50; 0.58 mm i.d.) into the jugular vein for drug administration before the experiment using the same procedure as described in the previous section. Each rat was placed in a Bollmann cage and was given \([^{14}C]M-III\) by continuous infusion into the jugular vein using an infusion pump (Harvard Pump 22; Harvard Apparatus). At 4 h after the infusion was started, the blood of the rats was collected from the abdominal aorta under anesthesia with 0.3 ml of 5% w/v pentobarbital solution, which was dosed from a cannula fitted into the jugular vein. Immediately after blood collection, the kidney was milled and weighed. The blood was centrifuged to obtain the plasma fraction. The removed kidneys were minced and were processed to prepare aqueous homogenates.

With regard to the dogs, \([^{14}C]M-III\) (0.5 mg–0.5 MBq/kg) was intravenously injected in the same way as described in the previous section. At 24 h after dosing, blood was collected from the cephalic vein, and then the dogs were sacrificed by bleeding under anesthesia with 2.5% w/v sodium thiopental, which was dosed from the cephalic vein, and the dosed volumes were depending on anesthetic depth (2–4 ml). The kidneys were removed and rinsed, followed by division into the cortex and the medulla. The cortex and the medulla were minced and were processed to prepare aqueous homogenates.

In Vivo Plasma Protein Binding of \([^{14}C]M-III\). \([^{14}C]M-III\) saline solution was added in vitro to the plasma of the rats and dogs at the final concentrations of 0.5, 5, and 50 μg/mL, and the protein binding was determined by the ultrafiltration method. Aliquots of the plasma were transferred into ultrafiltration devices (Amicon Centrifiuce; Nihon Millipore K.K., Tokyo, Japan) and centrifuged at 1500 g for 10 min. The concentrations of \(^{14}C\) in the plasma and filtrate were determined.

In Vivo Plasma Protein Binding of \([^{14}C]M-III\) (Plasma Protein Binding of \([^{14}C]M-III\) in Dogs). The plasma samples obtained from the distribution experiment with the dog kidney were used. The protein binding was determined with the same method as described in the previous section.

Pharmacokinetics of M-III in Rats Infused a Mixture of \([^{14}C]M-III\) and Probenecid. Aliquots of the plasma samples after a 5 h infusion were transferred into ultrafiltration devices (Ultrafree-MC, 10,000 nominal molecular weight limit; Nihon Millipore K.K.) and centrifuged at 1500g and 37°C for 10 min. The concentrations of \(^{14}C\) in the plasma and filtrate were determined.

Analytical Method for \([^{14}C]M-III\). The concentrations of \([^{14}C]M-III\) in the plasma samples were determined by the HPLC fraction collecting method and liquid scintillation counting. Acetonitrile for deproteinization was added to the plasma collected at specified time points from the rats and dogs after intravenous dosing. After centrifugation of the mixtures, the supernatants were injected into the high-performance liquid chromatograph. Urine samples from the rats and dogs were directly injected into the high-performance liquid chromatograph. HPLC was performed on an LC-10 system coupled with a SPD-10A UV-visible detector (Shimadzu) and syringe loading sample injector chromatograph. HPLC was performed on an LC-10 system coupled with a SPD-10A UV-visible detector (Shimadzu) and syringe loading sample injector chromatograph. The column eluate for every 1 min was collected into scintillation counting vials. The radioactivities in the processed vials were determined by liquid scintillation counting. The elution times of \([^{14}C]M-III\) were identified by comparison with the retention time of the reference standard detected with a UV detector. The concentrations of \([^{14}C]M-III\) were calculated from the ratio of the eluted radioactivity at M-III retention time to the total injected into the high-performance liquid chromatograph.

Analytical Method for Probenecid. The concentrations of probenecid in the plasma of rats were determined by HPLC. Acetonitrile was added to the plasma at specified time points, and the supernatants obtained by mixing and centrifugation were injected into the high-performance liquid chromatograph. The HPLC analysis was carried out on a reverse-phase column (Inertsil ODS-3, 5 μm, 250 × 4.6 mm i.d.; GL Sciences, Tokyo, Japan) at 40°C. The mobile phase, a mixture of 10 mM ammonium acetate and acetonitrile, was programmed as a linear gradient from 18 to 42% acetonitrile during a 20-min period and subsequently as a linear gradient from 42 to 90% during 5 min followed by isocratic elution with 18% acetonitrile for an additional 10 min to equilibrate the column. The flow rate was 1 ml/min, and the UV detector was set at 240 nm. The concentrations were determined by the calibration curve, which was established by probenecid-spiked plasma at final concentrations of from 0.1 to 100 μg/ml.

Data Analysis. Data are expressed as mean values or mean values ± S.D. of the results from three to five animals. The concentrations of probenecid at 5 min after the injection (\(C_{\text{sat, probenecid}}\)) were noted directly from the data. The apparent volume of distribution (\(V_{\text{d}}\)) was calculated from division of the dose by the initial concentration (\(C_{\text{initial, probenecid}}\)). \(V_{\text{d}}\) was estimated by back extrapolation from the first two or three concentrations. The AUC\(_{\text{last}}\) was calculated by summing AUC\(_{\text{last}}\) + \(C_{\text{sat, probenecid}}\) × \(k_{\text{e}}\). Where AUC\(_{\text{last}}\) is the area under the curve from 0 to last measured plasma concentration and \(C_{\text{sat, probenecid}}\) is the last measured concentration divided by the \(k_{\text{e}}\) in the terminal phase of the plasma disappearance curve calculated by linear regression. The total body clearance (CL\(_{\text{total}}\)) of M-III was calculated by dividing the dose by the AUC\(_{\text{last}}\). These parameters were computed by WinNonlin (version 5.2.1; Pharsight, Mountain View, CA). The renal clearance (CL\(_{\text{renal}}\)) of M-III was calculated by dividing the excreted amount of M-III in urine by the AUC\(_{\text{GFR}}\) of rats and AUC\(_{\text{GFR}}\) of dogs, respectively. The amount of M-III in urine was calculated from the excretion ratio of the total \(^{14}C\) into urine and the composition analysis of \([^{14}C]M-III\) in the urine. In the pharmacokinetic study in rats infused the mixture of \([^{14}C]M-III\) and probenecid solution, the pharmacokinetic parameter of CL\(_{\text{renal}}\) for M-III was calculated according to the following equation:

\[
\text{CL}_{\text{renal}} = \text{Inf} / C_{\text{M-III}}
\]

where Inf and \(C_{\text{M-III}}\) represent the infusion rate of \([^{14}C]M-III\) and the steady-state concentration of this metabolite in the plasma at 120 to 180 min after infusion, respectively. A competitive inhibition model (eq. 1) based on a general inhibition equation in which the rate of elimination was replaced by the clearance was fitted to the data by MULTI:

\[
\text{CL}_{\text{renal}} = \text{CL}_{\text{renal}}(1 + C_{\text{M-III}}/K_i) + \text{CL}_{2}
\]

where \(\text{CL}_{\text{renal}}\) is the intrinsic total body clearance of M-III in rats, and \(\text{CL}_{1}\) and \(\text{CL}_{2}\) are the components affected and are not affected by probenecid, respectively. \(\text{CL}_{2}\) was set at 300 ml · h \(^{-1}\) · kg \(^{-1}\), which is the glomerular filtration rate (GFR) value for rats. \(C_{\text{M-III}}/K_i\) is the steady-state concentration of probenecid determined in this study, and \(K_i\) is the inhibition constant for probenecid.

Results

Pharmacokinetics of M-III in Rats and Dogs. Concentration-time profiles of \(^{14}C\) in the plasma of rats and dogs given a 0.5 mg/kg bolus
intravenous injection of $[^{14}C]\text{M-III}$ are shown in Fig. 2. The rats and dogs tolerated the intravenous administration of $[^{14}C]\text{M-III}$ well. The pharmacokinetic parameters in the rats and dogs are summarized in Table 1.

**Plasma Protein Bindings of M-III in Rats and Dogs.** Table 2 shows the in vitro plasma protein binding of M-III at the concentrations of 0.5, 5, and 50 $\mu$g/ml in rats and dogs. The percentages of the plasma protein binding of M-III in rats ranged from 89.8 to 92.5% and those in dogs ranged from 84.6 to 91.3%. Furthermore, the percentages of the plasma protein binding of $[^{14}C]\text{M-III}$ in rats and dogs in vivo were 91.2 and 92.0%, respectively (Table 3).

**Excretion of M-III into the Urine in Rats and Dogs.** After a 0.5 $\text{mg/kg}$ bolus intravenous injection of $[^{14}C]\text{M-III}$ to rats and dogs, the excretion ratio of $[^{14}C]$ in urine in rats from 0 to 24 h and that in dogs from 0 to 168 h were 84.7 ± 6.7 and 44.6 ± 12.1%, respectively. The ratios in feces were 10.3 ± 8.1 and 7.7 ± 0.3%, respectively, in rats and dogs. The composition of $[^{14}C]\text{M-III}$ in the urine of rats and dogs was 76.9 ± 7.6 and 19.5 ± 9.0%, respectively, of the total dose amount. $\text{Cl}_{\text{renal}}$ and unbound $\text{Cl}_{\text{renal}}$ of M-III calculated by in vivo plasma protein binding were 568 and 5778 ml $\cdot$ h$^{-1}$ $\cdot$ kg$^{-1}$, respectively, in rats. On the other hand, the values were 0.281 ± 0.169 and 3.51 ± 2.11 ml $\cdot$ h$^{-1}$ $\cdot$ kg$^{-1}$, respectively, in dogs (Table 3).

**Distribution of M-III to the Kidney and Liver in Rats and Dogs.** The kidney/plasma $[^{14}C]$ ratios were investigated in rats and dogs after intravenous continuous infusion of $[^{14}C]\text{M-III}$ at a rate of 0.83 $\text{mg} \cdot \text{h}^{-1} \cdot \text{kg}^{-1}$ and an bolus intravenous injection at a dose of 0.5 $\text{mg/kg}$, respectively. Data in Fig. 3 are expressed as the mean values of the results for three rats and two dogs. The kidney/plasma $[^{14}C]$ ratios in rats was approximately 3.5, and those in the kidney medulla and cortex in dogs were approximately 0.2 and 0.1, respectively. The rats and dogs tolerated the intravenous administration of $[^{14}C]\text{M-III}$ well.

**Pharmacokinetics of $[^{14}C]\text{M-III}$ and Probenecid in Infused Rats.** Figure 4 shows the concentration-time curves of $[^{14}C]\text{M-III}$ and probenecid in the plasma of rats given an intravenous infusion of $[^{14}C]\text{M-III}$ and probenecid simultaneously. The rats tolerated the intravenous administration of $[^{14}C]\text{M-III}$ and probenecid well. When $[^{14}C]\text{M-III}$ was infused at the rate of 0.125 $\text{mg} \cdot \text{rat}^{-1} \cdot \text{h}^{-1}$, probenecid was concurrently infused at rates of 4.7, 9.4, and 18.6 $\text{mg} \cdot \text{rat}^{-1} \cdot \text{h}^{-1}$. In the plasma, the concentrations of $[^{14}C]\text{M-III}$ and probenecid reached steady state approximately 2 h after the infusion was started, and the concentration of $[^{14}C]\text{M-III}$ increased from approximately 0.6 to 3.6 $\mu$g/ml at 3 h after the infusion was started with ascending doses of probenecid.

Table 4 also includes the in vivo plasma protein binding of $[^{14}C]\text{M-III}$ in the rats when probenecid was infused simultaneously. The percentages of the unbound fraction increased from 8.8 to 19.7% with increasing concentrations of probenecid. The CL$_{\text{int}}$ of M-III was calculated from CL$_{\text{tot}}$ divided by the unbound fraction. Furthermore, the percent-protein binding of M-III in rats ranged from 89.8 to 92.5% and in dogs, although the $V_d$ values in the rats were larger than those in the dogs, although the $V_d$ values in the rats and dogs were smaller than the extravascular volumes. The CL$_{\text{tot}}$ and $k_d$ of M-III calculated from the terminal phase in the rats were extremely higher than those in the dogs. Consequently, the $t_{1/2}$ of $[^{14}C]$ in the plasma of the rats was considered to be approximately 1/30 of that in the dogs (Fig. 2). Because M-III is a small molecular weight compound with high polarity, this finding indicated that unbound M-III could be excreted into urine via glomerular filtration. Therefore, the differences in the percentages of plasma protein binding between rats and dogs could be attributed to species differences in the renal uptake of M-III.

**Discussion**

The pharmacokinetic profile of M-III in the rats showed biphasic pharmacokinetics and the $V_d$ values in the rats were larger than those in the dogs, although the $V_d$ values in the rats and dogs were smaller than the extravascular volumes. The CL$_{\text{tot}}$ and $k_d$ of M-III calculated from the terminal phase in the rats were extremely higher than those in the dogs. Consequently, the $t_{1/2}$ of $[^{14}C]$ in the plasma of the rats was considered to be approximately 1/30 of that in the dogs (Fig. 2). Because M-III is a small molecular weight compound with high polarity, this finding indicated that unbound M-III could be excreted into urine via glomerular filtration. Therefore, the differences in the percentages of plasma protein binding between rats and dogs could be attributed to species differences in the renal uptake of M-III.
influence the GFR of M-III. The in vitro plasma protein binding of M-III in plasma of both rats and dogs were relatively high and showed little concentration dependence in both species, suggesting that there were no difference between rats and dogs in the nature of the plasma protein binding (Table 2). The in vivo plasma protein binding ratio also showed that there was no difference in the binding of [14C]-M-III in the systemic circulation (Table 3). These results suggested the species difference in the plasma protein binding could not be a factor for the differences in the M-III pharmacokinetics between rats and dogs. Because only the unbound fraction could be filtered through the glomeruli, the Clrenal of M-III was compared with the GFR in rats. Inulin clearance is considered to be equivalent to the GFR, and the inulin clearance of rats has been reported to be approximately 300 ml · h⁻¹ · kg⁻¹ (Caron and Kramp, 1999). The pharmacokinetic analysis revealed that the Clrenal of M-III in rats was 631 ml · h⁻¹ · kg⁻¹ and the plasma protein binding of M-III in rats was 91.2% (Table 3), indicating that the Clrenal of M-III was calculated to be 7170 ml · h⁻¹ · kg⁻¹ in rats. These results showed that the Clrenal of M-III for rats was much higher than the inulin clearance (in other words, the GFR). Therefore, another mechanism besides glomerular filtration must be involved in the excretion of M-III into the urine in rats. In contrast to the rats, the reabsorption system could contribute to the much lower values for the Clrenal of M-III than the GFR in the dog kidney.

Similar interspecies differences in pharmacokinetics were reported for phenoxyacetic acid (Timchalk, 2004). Timchalk (2004) reported that the dog plasma half-lives for 2,4-dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid at 5 mg/ml dosage ranged from 92 to 106 and was 63 h, respectively. The half-lives of these compounds were longer than those in the rat with values of less than 1 and 6 h or in humans with values of 12 and 11 h, respectively. As a result, it was inferred that a lack of or ineffectiveness of the active transport process and potential reabsorption in the renal tubules collectively resulted in a slower renal clearance in dogs relative to other species including humans.

If there were any differences between rats and dogs in the renal uptake of M-III, the distribution of M-III to the kidney should be different between rats and dogs. To investigate the distribution of M-III to the kidney, 14C kidney/plasma ratios were determined in rats after intravenous continuous infusion of [14C]-M-III at a rate of 0.83 mg · h⁻¹ · kg⁻¹ and dogs after an intravenous injection at a dose of 0.5 mg/kg. At 4 h after the infusion was started, the steady-state phase of M-III concentration in the plasma, the 14C in the kidney was much higher than that in the rat plasma. On the contrary, in dogs, at 24 h after a bolus intravenous injection, almost the plateau phase of the M-III concentration in the plasma, the 14C in the kidney was much lower than that in the plasma (Fig. 3). These results indicated that M-III was taken up effectively into the rat kidney; however, in dogs, the contribution of the renal uptake to the removal of M-III from plasma appears to be minor. Furthermore, the much lower Clrenal and unbound Clrenal of M-III in dogs suggested that rapid and effective

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**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>Rat</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excretion ratio of [14C]M-III into urine, %</td>
<td>84.7 ± 6.7</td>
<td>44.6 ± 12.1</td>
</tr>
<tr>
<td>Composition of [14C]M-III in urine (% of dose)</td>
<td>76.9 ± 7.6</td>
<td>19.5 ± 9.0</td>
</tr>
<tr>
<td>Plasma protein binding (% (in vivo) (the concentration of M-III)</td>
<td>91.2 (0.6 μg/ml)</td>
<td>92.0 (2.5 μg/ml)</td>
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<tr>
<td>Clrenal, ml · h⁻¹ · kg⁻¹</td>
<td>5778</td>
<td>3.51 ± 2.11</td>
</tr>
<tr>
<td>Unbound Clrenal, ml · h⁻¹ · kg⁻¹</td>
<td>300</td>
<td>190–270</td>
</tr>
<tr>
<td>GFR, ml · h⁻¹ · kg⁻¹</td>
<td>190–270</td>
<td>190–270</td>
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**FIG. 4.** The concentration-time curves of [14C]-M-III and probenecid in plasma of rats after an intravenous infusion of [14C]-M-III and probenecid simultaneously. The concentrations of [14C]-M-III and probenecid are shown as solid lines and dashed lines, respectively. Dose of [14C]-M-III: 0.125 mg · rat⁻¹ · h⁻¹ (infusion). Infusion rate of probenecid: 16 mg · h⁻¹ · kg⁻¹ (●), 31 mg · h⁻¹ · kg⁻¹ (▲), and 62 mg · h⁻¹ · kg⁻¹ (×); and vehicle alone (♦).
reabsorption from urine to systemic circulation might be active in dogs.

Because the difference in the $^{14}$C concentrations in the kidney was considered to be caused by the difference in the renal uptake system for M-III between rats and dogs, it was thought that some transporters, which take M-III into the kidney, could be involved in the mechanism of removal of M-III from rat plasma. Most of the M-III exists as an anion at biological pH because the pK$_a$ of M-III was 2.4. Therefore, it was thought that organic anion transporters (OATs) on the renal basolateral membrane (BLM) could be involved in the uptake of M-III in the rat kidney.

Probenecid is well known as a broadband inhibitor of OATs for various species (Shitara et al., 2005). When $[^{14}]$C-M-III and probenecid were simultaneously and continuously infused into the rat jugular vein, the concentrations of M-III increased, depending on the increase in the probenecid concentrations. A decrease in the plasma protein binding, which might be caused by saturation at high concentrations and competitive binding with probenecid, was considered. Therefore, the apparent steady-state concentrations, infusion rate, and unbound ratios of M-III at each dose level of probenecid were used for calculation of the CL$_{int}$ for M-III. The calculated CL$_{int}$ of M-III decreased with an increase in the probenecid concentrations in the rat plasma. Figure 5 shows the relationship between the CL$_{int}$ of M-III and C$_{ss,probenecid}$ and the optimum parameters were calculated by a nonlinear regression method based on the competitive inhibition model. Because the CL$_{int}$ of M-III at the 62 mg·h$^{-1}·$kg$^{-1}$ infusion rate of probenecid was 563 ml·h$^{-1}·$kg$^{-1}$ (Table 4), it was suggested that OATs could be inhibited by probenecid and only excretion via glomerular filtration would function in the rats. Therefore, the CL$_2$ value was fixed at 300 ml·h$^{-1}·$kg$^{-1}$, which is the GFR in rats. The $K_i$ value (106 µM) was considered reasonable to compare with the values for Oat1 (31.0 µM) and Oat3 (20.0 µM) reported previously (Sugiyama et al., 2001). These results indicated that the renal uptake of M-III in rats was competitively inhibited by probenecid. Furthermore, the OAT transports expressed on the BLM could be involved in the uptake of M-III and the pharmacokinetics differences for M-III between rats and dogs were considered to be mainly caused by different renal uptake processes.

In conclusion, the pharmacokinetic analysis revealed that CL$_{int}$ and $K_i$ of M-III for rats were much higher than those for dogs. The kidney/plasma $^{14}$C concentration ratio for rats was much higher than that for dogs after intravenous doses of $[^{14}]$C-M-III. The relationship between the CL$_{int}$ of M-III and C$_{ss,probenecid}$ showed that the renal uptake of M-III in rats was competitively inhibited by probenecid and also suggested that the OAT transport system expressed in the BLM could be involved in the renal uptake of M-III in rats. These results indicated that the observed species differences in the plasma pharmacokinetics of M-III between rats and dogs are considered to be caused by the differences in the renal uptake system between the two species.

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Authorship Contributions

Participated in research design: Jinno, Takeuchi, and Tagawa.
Conducted experiments: Jinno and Tagawa.
Performed data analysis: Jinno, Takeuchi, and Tagawa.
Wrote or contributed to the writing of the manuscript: Jinno, Takeuchi, Tagawa, Kondo, Itoh, and Asahi.

References


### TABLE 4

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<tr>
<th>Probenecid</th>
<th>C$_{ss,probenecid}$</th>
<th>CL$_{tot}$</th>
<th>CL$_{int}$</th>
<th>Protein Binding</th>
<th>Unbound</th>
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<tr>
<td>0 mg·h$^{-1}·$kg$^{-1}$</td>
<td>0</td>
<td>687</td>
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<tr>
<td>16 mg·h$^{-1}·$kg$^{-1}$</td>
<td>63.8±9.9</td>
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<td>3.23×10$^3$</td>
<td>92.2%</td>
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<tr>
<td>31 mg·h$^{-1}·$kg$^{-1}$</td>
<td>189±44</td>
<td>141</td>
<td>7.15×10$^2$</td>
<td>80.3%</td>
<td>19.7%</td>
</tr>
<tr>
<td>62 mg·h$^{-1}·$kg$^{-1}$</td>
<td>433±92</td>
<td>110</td>
<td>5.63×10$^2$</td>
<td>81.6%</td>
<td>18.4%</td>
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### Figures

**Fig. 5.** Relationship between M-III clearance and probenecid steady-state plasma concentration in rats. Dose of $[^{14}]$C-M-III: 0.125 mg·h$^{-1}·$kg$^{-1}$ (infusion). Infusion rate of probenecid: 16 mg·h$^{-1}·$kg$^{-1}$ ( ), 31 mg·h$^{-1}·$kg$^{-1}$ ( ), and 62 mg·h$^{-1}·$kg$^{-1}$ ( ); vehicle alone ( ).


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