PHARMACOKINETIC STUDY OF 4-METHYLUMBELLIFERONE IN RATS: INFLUENCE OF DOSE ON ITS FIRST-PASS HEPATIC ELIMINATION

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The dose-dependent first-pass hepatic metabolism and pharmacokinetics of 4-methylumbelliferone (4-MU) were studied at four dose levels (17.6 μg –5.29 mg) in rats. 4-MU was given intravenously and intraportally to determine the availability of 4-MU. The availability increased from 0.18 to 1.31 when the dose was increased from 17.6 μg to 5.29 mg/rat. The total body plasma clearance of 4-MU was accounted for mostly by the hepatic conjugative metabolism. The contribution of renal clearance to total plasma clearance was 11–35%, depending on the dose. The marked dose-dependency of availability may thus be explained by the saturable conjugative metabolism of 4-MU.

Keywords — 4-methylumbelliferone; clearance; pharmacokinetics; conjugation

INTRODUCTION

The quantitative assessment of bioavailability has been made by comparing the area under the blood (or plasma) concentration-time curve (*AUC*) after oral administration of a drug with the *AUC* after intravenous administration of an equal dose, provided that the proportionality between *AUC* and dose is valid, irrespective of the route of administration.¹⁾ Administration of a drug into the portal vein is equivalent to its oral administration when the drug is absorbed completely and there is no first-pass metabolism by the gastrointestinal mucosa. Clearance concepts have made clear that the hepatic extraction ratio of a drug which is eliminated solely in the liver is a function of both hepatic blood flow and the intrinsic ability (intrinsic clearance) of the liver to eliminate the drug.^{2,3)} Most drugs such as propranolol, lidocaine, phenytoin and hexobarbital which have been known to undergo a first-pass hepatic elimination are metabolized by a microsomal oxidation system (phase I metabolism). In addition, the degree of the first-pass hepatic elimination has been known to depend on dose or infusion rate in the case of propranolol.⁴⁾ Drug conjugative metabolism (phase II metabolism), especially glucuronidation and sulfation, is also one of the important detoxication reactions in the body. However, contrary to the stud-

ies on phase I metabolism, little is known about the first-pass hepatic elimination of drugs which undergo phase II metabolism except for a few drugs.^{5–9)} Although the saturation of phase II metabolism is well known, few systematic studies have been performed on the dose-dependency of the first-pass hepatic conjugative metabolism.⁹⁾ 4-Methylumbelliferone (4-MU) is well known to be conjugated to glucuronide (4-MG) and sulfate (4-MS), and has often been used as a model compound in conjugation studies.^{10–12)} The purpose of the present study is to show the dose-dependent first-pass hepatic metabolism of 4-MU by comparing the *AUC* after intravenous administration with that after intraportal administration.

MATERIALS AND METHODS

Chemicals and Reagents — 4-Methylumbelliferyl- β -D-glucuronide, 3.5 hydrate (4-MG), and 4-methylumbelliferyl sulfate, potassium salt (4-MS) were purchased from Sigma Chemical Co. (St. Louis, MO). 4-Methylumbelliferone, sodium salt (4-MU) was synthesized in Kissei Pharmaceutical Co., Ltd. (purity determined by titration method: 99.5%). All other chemicals and reagents were commercially available and of analytical grade.

Animal Experiments — Male Wistar rats

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weighing 200–240 g (Shizuoka Jikkendobutsu Co., Hamamatsu, Japan) were cannulated under ether anesthesia as described below. For intravenous administration, cannula of PE-50 polyethylene tubings in the left femoral vein was used. Administration *via* the portal vein was through a PE-50 cannula with the needle (24G) inserted with surgical glue in the portal vein. The rat was kept in a Bollman cage. Blood samples were collected from the femoral artery through a polyethylene cannula at appropriate times. Urine samples were collected from a cannula of polyethylene tubing in the urinary bladder. The experiments were started after the rats recovered from ether anesthesia. One ml of isotonic saline solution (pH 9.5) of 4-MU was administered intravenously or intraportally at four doses (17.6, 176 μ g, 1.76 and 5.29 mg/rat). To determine the effect of enterohepatic circulation, the time courses of 4-MU concentrations in the plasma were also determined at two doses (176 μ g and 5.29 mg/rat) in bile fistula rats in the same manner as described above.

Determination of Blood-to-Plasma Concentration Ratio (R_B) — Fresh blood was collected in heparinized tubes *via* the descending aorta. Two pools of blood, each pool containing blood from 7–15 rats, were used. Aliquots (2 ml) of the blood were transferred into open flasks, preincubated for 3 min at 37 °C and the uptake was initiated by adding small aliquots of stock 4-MU solution. Preliminary experiments showed that the uptake of 4-MU into blood cell was so rapid that the constant blood-to-plasma concentration ratio was obtained within 1 min. After incubation for 15 min at 37 °C, aliquots of the blood were centrifuged and the plasma concentrations of 4-MU were determined fluorometrically as described later. Simultaneous experiments were carried out at 8 different initial 4-MU concentrations in the blood which ranged from 5 to 150 μ g/ml. The concentration of 4-MU in the blood was calculated from the added known amount of 4-MU. R_B value was calculated by dividing the blood concentration by the plasma concentration.

Plasma Protein Binding — Plasma was separated from the blood which was obtained through the descending aorta. Plasma pooled from 15 rats was used and an equilibrium dialysis method was employed by using 0.5 ml of plasma. Plasma spiked with various concentra-

tions of 4-MU (5–150 μ g/ml) was dialyzed against 0.5 ml of Sørensen's buffer (pH 7.4) for 6 h at 37 °C. Semipermeable membrane (Sanko Junyaku Co., 36/32 cm) was used. Preliminary dialysis studies indicated that 4-MU reached equilibrium after a 4–5 h incubation. The concentrations of 4-MU in both the plasma and the buffer sides were determined fluorometrically as described later. The binding data were corrected for the volume shift according to the methods of Tozer *et al.*¹³⁾

Assay Methods for 4-MU, 4-MG and 4-MS — To 50 μ l of each plasma sample, or 200 μ l of each urine sample, 1 ml of methanol was added and mixed and the mixture centrifuged for 3 min in a tabletop microcentrifuge cone at 3000 rpm. All of the supernatant fluid was then applied to a precoated thin-layer chromatography (TLC) plate (Silicagel 60, without fluorescent indicator; 0.25 mm in thickness; E. Merck Darmstadt, West Germany). The TLC plate was developed in a mixture of acetone, chloroform, methanol and glacial acetic acid (12:12:1:1). 4-MU, 4-MS and 4-MG were visualized by the irradiation of the ultraviolet rays at wavelength of 365 nm. The R_f values of 4-MU, 4-MS and 4-MG were about 0.62, 0.16, and 0.05, respectively. The areas attributable to 4-MU, 4-MS and 4-MG were scraped and extracted with pH 10.5 Sørensen buffer (2 ml) for 20 min. 2 N HCl (2 ml) was then added to hydrolyze 4-MS and 4-MG in a boiling water bath for 30 min and 2 h, respectively. These solutions were neutralized with 2 N NaOH (2 ml) and added pH 10.5 Sørensen buffer (2 ml). The fluorescence of 4-MU was measured with a fluorometer (Hitachi, Fluorescence Spectrophotometer, type 650-10S). The wavelength for excitation was 365 nm and that for emission was 445 nm. The recoveries of 4-MU from plasma and urine were $97.8 \pm 1.6\%$ (mean $\pm$ S.D.) and $97.3 \pm 2.0\%$, respectively.

Data Analysis — The plasma concentration-time curve from individual rats was fitted to a two-exponential equation by the non-linear least squares method using a weight value of 1. Model-independent estimates of areas under plasma concentration-time curves from zero to infinite time (AUC) were calculated using a trapezoidal method. Areas for the portions of plasma curves following the last measurable data point were estimated by dividing the drug

concentration at that sampling time by the over-all elimination rate constant, β .

Renal clearance (CL_R) was estimated by

$$CL_R = \frac{A_{\infty}}{AUC} \quad (1)$$

where the amount of 4-MU recovered in urine up to 8 h is represented by A_{∞} . Mean values and standard errors (S.E.) of pharmacokinetic parameters obtained from an individual rat were calculated.

RESULTS

Dose Dependency of First-Pass Hepatic Elimination

of 4-MU

Figure 1 shows the time courses of 4-MU concentrations in the plasma of rats after intravenous and intraportal injection of various doses of the drug (17.6 μg –5.29 mg/rat). For all doses, the distribution phase occupied the initial 15–20 min, and the disposition of 4-MU was well described by a two-compartment model. The pharmacokinetic parameters were calculated and are listed in Table I. The volume of distribution (either V_1 or V_{ss}) increased as dose increased. For instance, V_{ss} at a dose of 5.29 mg/rat was approximately 2.6 fold greater than that at a dose of 17.6 μg /rat. The values of dose/ AUC , which represent the total body plasma clearances in the

FIG. 1. Mean Plasma Concentration-Time Curves of 4-MU after an Intraportal (○) or an Intravenous (●) Administration

Dose used are (a) 17.6 μg /rat, (b) 176 μg /rat, (c) 1.76 mg/rat and (d) 5.29 mg/rat. The lines represent the least squares fit of the data to a two exponential equation. Mean $\pm$ S.E., $n=3$.

i.v. administration, were plotted against doses (Fig. 2(a)). The availabilities which changed with doses were also plotted and are shown in Fig. 2(b). The value of dose/*AUC* after *i.v.* administration was not dependent on dose. On the other hand, the value of dose/*AUC* was decreased with increasing dose after intraportal administration. Consequently, the apparent availabilities (F_{app}) calculated from the ratio of *AUC* after intraportal administration ($AUC_{p.o.}$) to that after intravenous administration ($AUC_{i.v.}$) were increased with increasing dose. That is, the value of F_{app} (0.18) at a low dose (17.6 μ g/rat) was approximately one-seventh of that (1.31) at a high dose (5.29 mg/rat). To estimate the effect of enterohepatic circulation, the time courses of 4-MU concentrations in the plasma were determined at two doses (176 μ g and 5.29 mg/rat) also in bile fistula rats (Table II). The values of dose/*AUC* did not show significant differences between normal and bile fistula rats either after intravenous or intraportal injection. Consequently, a similar dose-dependent change in the apparent availability was seen also in bile fistula rats. The steady state distribution volume has a tendency to decrease in bile fistula rats, although

the decrease is not statistically significant ($p > 0.05$). These data suggested that enterohepatic circulation did not affect the disposition kinetics of 4-MU.

Urinary Excretion of 4-MU and Its Metabolites

The urinary metabolic pattern during 8 h after *i.v.* injection of 4-MU is shown in Table III. More than 95% of the administered 4-MU was recovered as 4-MU and its conjugates in the urine at all dosage levels. The excreted amount of 4-MU glucuronide (4-MG) as a fraction of the dose decreased, the fraction of 4-MU sulfate (4-MS) did not change appreciably and the fraction of 4-MU itself increased with increasing dose of 4-MU from 17.6 μ g to 5.29 mg/rat. Renal clearances of 4-MU increased with increasing dose of 4-MU.

Plasma Protein Binding and R_B

Plasma protein binding of 4-MU was determined by equilibrium dialysis over a wide concentration range of 4-MU (5–150 μ g/ml). As shown in Fig. 3, plasma unbound fraction of 4-MU increased slightly with increased concentrations of 4-MU. The unbound fraction (0.248 ± 0.005) at a higher concentration range ($> 50 \mu$ g/ml) of 4-MU was approximately 1.25 fold

TABLE I. *Pharmacokinetic Parameters of 4-MU in Rats*

Route	Dose (mg)	Parameters ^{a)}						Dose/ <i>AUC</i> ^{e)} (ml/min/kg)	Availability ^{d)} (<i>F</i>)
		A/dose $\times 10^3$ (ml ⁻¹)	B/dose $\times 10^3$ (ml ⁻¹)	α (min ⁻¹)	β (min ⁻¹)	$V_1^{b,e)$ (ml/kg)	$V_{ss}^{c,e)$ (ml/kg)		
Femoral vein	0.0176	46.1	4.18	0.252	0.0274	104	306	14.9	0.184
		± 3.3	± 1.55	± 0.059	± 0.0089	± 13	± 67	± 2.5	
	0.176	39.2	3.18	0.248	0.0185	124	510	16.0	
		± 2.9	± 1.59	± 0.061	± 0.0069	± 15	± 104	± 3.5	
	1.76	32.5	1.85	0.177	0.00951	152	792	14.0	
		± 2.0	± 0.35	± 0.004	± 0.00107	± 12	± 79	± 1.5	
	5.29	30.5	2.14	0.192	0.0136	158	805	15.8	
		± 0.3	± 0.65	± 0.027	± 0.0060	± 4	± 211	± 1.5	
Portal vein	0.0176	3.91	0.660	0.162	0.0152			80.8	0.313
		± 0.09	± 0.198	± 0.008	± 0.0006			± 27.3	
	0.176	4.46	1.03	0.144	0.0139			51.2	
		± 0.18	± 0.35	± 0.006	± 0.0025			± 14.3	
	1.76	8.90	2.46	0.133	0.0129			16.0	
		± 0.36	± 0.41	± 0.008	± 0.0004			± 1.2	
	5.29	12.0	3.92	0.148	0.0152			12.1	
		± 0.58	± 0.41	± 0.023	± 0.0042			± 2.4	

a) Values represent mean $\pm$ S.E. of three rats. Plasma disappearance curve was fitted to the biexponential equation, $C_p = Ae^{-\alpha t} + Be^{-\beta t}$ by an iterative least squares method. b) Distribution volume of the central compartment (V_1) was calculated by dose/($A + B$). c) Steady state distribution volume (V_{ss}) was calculated by dose $\times$ ($A/\alpha^2 + B/\beta^2$) / ($A/\alpha + B/\beta$)². d) Calculated by $AUC_{p.o.}/AUC_{i.v.}$. e) The parameters obtained from individual rat were normalized by individual body weight.

larger than that (0.197 ± 0.005) at a lower concentration range ($<30 \mu\text{g/ml}$) of 4-MU. R_B of 4-MU was essentially constant over the plasma concentration range from 5 to $150 \mu\text{g/ml}$. The mean value of 7 determinations each in two

blood pools (1 blood pool is obtained from 7–15 rats) was 0.880 ± 0.035 .

DISCUSSION

Extrahepatic conjugative metabolism has been known for some drugs, such as isoprenaline, harmol¹⁴⁾ and *p*-nitrophenol.⁸⁾ The possibility of extrahepatic metabolism of 4-MU in rats was examined as follows. Hepatic clearance (CL_H) is given by

$$CL_H = Q_H \cdot E_H \quad (2)$$

where Q_H is the hepatic blood flow and E_H is the hepatic extraction ratio. E_H can be expressed by

$$E_H = 1 - AUC_{p.o.}/AUC_{i.v.} \quad (3)$$

where $AUC_{p.o.}$ and $AUC_{i.v.}$ are the areas under the blood concentration curve following intraportal and intravenous administration, respectively. Eqs. 2 and 3 can apply in principle to drugs that show extrahepatic metabolism, if they can be described by linear pharmacokinetics.¹⁾ On the other hand, non-renal clearance (CL_{nr}) can be given by Eq. 4.

$$\begin{aligned} CL_{nr} &= \frac{1}{R_B} (CL_{tot} - CL_R) \\ &= \frac{1}{R_B} (\text{dose}/AUC_{i.v.} - CL_R) \end{aligned} \quad (4)$$

where CL_{tot} is the total body clearance and CL_R is the renal clearance. The other metabolism of

FIG. 2. Mean Values of Dose/AUC and Availability after an Intravenous or an Intraportal Administration of Various Doses of 4-MU

(a) Dose/AUC vs. dose: key (○), intraportal administration; (●), intravenous administration. Vertical bars represent standard errors. (b) Availability vs. dose: availability was calculated by dividing the AUC after intraportal administration by that after intravenous administration.

TABLE II. Pharmacokinetic Parameters of 4-MU in Bile Fistula Rats

Route	Dose (mg)	Parameters ^{a)}						Dose/AUC (ml/min/kg)	Availability (F)
		A/dose $\times 10^3(\text{ml}^{-1})$	B/dose $\times 10^3(\text{ml}^{-1})$	α (min^{-1})	β (min^{-1})	V_1 (ml/kg)	V_{ss} (ml/kg)		
Femoral vein	0.176	35.0 ± 2.4	3.49 ± 0.60	0.202 ± 0.023	0.0290 ± 0.0014	132 ± 6	290 ± 21	17.0 ± 2.5	
	5.29	32.7 ± 1.5	3.64 ± 0.38	0.213 ± 0.003	0.0280 ± 0.0007	142 ± 8	344 ± 26	17.2 ± 1.6	
Portal vein	0.176	3.68 ± 0.31	1.26 ± 0.21	0.141 ± 0.006	0.0286 ^{c)} ± 0.0014			71.6 ± 11.8	0.237
	5.29	11.6 ± 0.5	4.56 ± 0.28	0.162 ± 0.008	0.0239 ^{b)} ± 0.0016			15.9 ^{c)} ± 1.3	1.08

a) Values represent mean $\pm$ S.E. of three rats. Plasma disappearance curve was fitted to the biexponential equation, $C_p = Ae^{-\alpha t} + Be^{-\beta t}$ by an iterative least squares method. b, c) Significantly different from the normal rats (shown in Table I) at the level of $p < 0.05$ and $p < 0.01$, respectively.

TABLE III. *Urinary Excretion and Renal Clearance after Intravenous Administration of 4-MU*

Dose (mg)	Urinary excretion (% of dose) ^{a)}			Renal clearance of 4-MU (CL_R) (ml/min/kg)
	4-MU	4-MG	4-MS	
0.0176	12.9 ± 0.5	53.8 ± 0.8	29.4 ± 0.5	1.94 ± 0.38
0.176	19.3 ± 0.2 ^{c)}	48.5 ± 0.6 ^{b)}	28.0 ± 0.3	3.08 ± 0.65
1.76	33.6 ± 0.7 ^{c)}	36.7 ± 0.8 ^{c)}	26.0 ± 0.4 ^{c)}	4.72 ± 0.58 ^{b)}
5.29	34.8 ± 0.7 ^{c)}	34.6 ± 0.4 ^{c)}	26.1 ± 0.5 ^{b)}	5.50 ± 0.61 ^{c)}

a) Values represent mean ± S.E. of three rats. Urinary excretions (% of dose) of 4-MU and its metabolites during 8 h after intravenous injection of 4-MU were obtained, respectively. b,c) Significantly different from the dose of 0.0176 mg at the levels of $p < 0.05$ and $p < 0.01$, respectively.

FIG. 3. *Binding of 4-MU to Pooled Plasma*

Plasma unbound fraction is plotted against the plasma concentration of 4-MU. Binding was measured by an equilibrium dialysis method at 37°C. Plasma pooled from 15 rats was used.

4-MU, other than the conjugations to sulfate and glucuronide, might be neglected since more than 95% of the administered 4-MU was recovered as 4-MU and its conjugates in the urine (Table III). Therefore, the possibility of extrahepatic conjugative metabolism can be examined by comparing the value of CL_H obtained by Eq. 2 and that of CL_{nr} obtained by Eq. 4. That is, if the value of CL_{nr} is larger than that of CL_H , the extrahepatic conjugative metabolism should be considered. However, one has to keep in mind that the above described theory is applicable only when the pharmacokinetics of a drug is linear. Along these lines, calculations were made using the data at the lowest dose (17.6 µg/rat), where the pharmacokinetics may be close to linear. Thus, substituting 40–80 ml/min/kg,^{8,15,16)} 0.184, 14.9 ml/min/kg, 1.9 ml/min/kg and 0.88 for Q_H , $AUC_{p.o.}/AUC_{i.v.}$, dose/ $AUC_{i.v.}$, CL_R and R_B value, respectively, the values of CL_H and CL_{nr} were calculated to be 33–65 and 15 ml/min/kg, respectively. The value of CL_{nr} is somewhat lower than that of CL_H . The reason for the lower value of CL_{nr}

than that of CL_H is not known, but the hepatic blood flow might have been smaller in the groups of rats used in the present experiments. Alternatively, the assumption of linear pharmacokinetics may still not hold even at the lowest dose. Thus, we cannot tell whether the extrahepatic metabolism of 4-MU exist or not from the present analysis.

The finding that there existed a marked dose dependency in the first-pass hepatic metabolism of 4-MU (Fig. 2) may suggest the saturation of conjugative metabolism of 4-MU in the liver. In fact, the saturation of sulfation and glucuronidation of 4-MU was previously reported by others¹¹⁾ using isolated rat liver cells. Co-substrate depletion has been thought to be a primary cause of the dose-dependent metabolism of phenolic compounds to sulfate such as salicylamide.^{9,17,18)} Most recently, it was found that both adenosine 3'-phosphate 5'-phosphosulfate (PAPS) and uridine diphosphate (UDP)-glucuronic acid concentrations in the liver were decreased after administration of acetaminophen. It was suggested that capacity-limited sulfation and glucuronidation of acetaminophen are due to a decrease in co-substrate availability.¹⁹⁾ In the present studies with 4-MU, we did not measure either the plasma concentrations of inorganic sulfate or the concentrations of PAPS and UDP-glucuronic acid in the liver which can possibly be altered with increasing dose of 4-MU. Therefore the mechanism of saturation of the 4-MU conjugation pathway cannot be clarified clear at the present time.

Mostly recently, Mulder *et al.*²⁰⁾ have found that the rat liver almost completely (97%) extracted 4-MU during steady-state infusion (4-MU concentration in the portal vein blood was 86 µM) and that the total body clearance of

4-MU (approximately 50–60 ml/min/kg), determined by either single *i.v.* injection or by steady-state infusion method, approximated liver blood flow. In addition, they found that the liver and the gastrointestinal region are so efficient that their conjugation activity can account for total body clearance of 4-MU. Taking their finding into consideration, the lower total body clearance (approximately 20 ml/min/kg) obtained in the present study may be attributed to the smaller blood flow rate in the present groups of rats, although the liver blood flow rates were not determined. A high hepatic extraction ratio (0.84) of 4-MU obtained at a low dose (Table I) confirmed the finding by Mulder *et al.*²⁰⁾ Most recently, we have confirmed this dose-dependent first-pass hepatic extraction of 4-MU by a liver perfusion method called multiple indicator dilution method.²¹⁾ In this experiment, 4-MU (50 or 3000 μ g) and ¹²⁵I-human serum albumin as an extracellular reference were injected as a bolus into the portal vein. Subsequently, the total hepatic vein effluent was collected and the recovery ratios (availability) were obtained. The hepatic extraction ratios at a low dose (50 μ g) and a high dose (3000 μ g) were 0.89 and 0.27, respectively, supporting the present finding obtained from *in vivo* experiment.

Renal clearance (CL_R) can be described by the following equation when the reabsorption can be assumed negligible¹⁾

$$CL_R = CL_{RF} + CL_{RS} = f_u \cdot CL_{cr} + CL_{RS} \quad (5)$$

where CL_{RF} is renal filtration clearance, CL_{RS} is renal secretion clearance, f_u is the unbound fraction of drug in plasma and CL_{cr} is the creatinine clearance. In the present studies, CL_R ranged from 1.9 (low dose) to 5.5 ml/min/kg (high dose), f_u ranged from 0.2 (low concentration) to 0.25 (high concentration). CL_{cr} in rats has been reported to be 3.3 ml/min/kg.¹⁸⁾ Using these values, CL_{RS} was estimated to range from 1.1 ml/min/kg (low dose) to 4.0 ml/min/kg (high dose), suggesting the contribution of the secretion process to the renal clearance of 4-MU. However, dose-dependent change in the CL_R cannot be explained by the secretion process, because the CL_R value should be decreased with increased dose. This dose-dependency may rather be accounted for by the saturable reab-

sorption process. In fact, 4-MU is a very lipophilic compound with an octanol-buffer (pH 7.4) partition coefficient of 33, suggesting the existence of a reabsorption process. Under this circumstance, the CL_{RS} value calculated by Eq. 5 underestimates the true value.

The volume of distribution (V_{ss}) of 4-MU has a tendency to increase as dose increases, as shown in Table I. Saturation of plasma protein binding of 4-MU might be the possible mechanism to account for this dose-dependent change in V_{ss} . In fact, the unbound fraction of 4-MU at a higher concentration range was approximately 1.25 fold larger than that at a lower concentration range (Fig. 3). However, at two higher doses (1.76 and 5.29 mg/rat) where the increases in V_{ss} compared to lower doses were observed, the plasma concentration of 4-MU exceeded 30 μ g/ml only for short periods of time. In addition, V_{ss} at a high dose (5.29 mg/rat) was approximately 2.5 fold larger than that at a low dose (17.6 μ g/rat), and the dose-dependent change in V_{ss} may not be quantitatively explained only by the saturation of plasma protein binding of 4-MU. It has been shown with some drugs that sulfate conjugates bind more avidly to plasma proteins than the parent compounds.^{20,22,23} In the present study, the plasma protein binding of 4-MU was determined using rat plasma to which only 4-MU was spiked. Therefore, it is possible that the unbound fraction of 4-MU *in vivo*, where 4-MU sulfate exists in plasma, may be different from that determined *in vitro* due to the competition of plasma binding between 4-MU and its sulfates. The actual plasma concentration range of 4-MU sulfate after the *i.v.* administration of the highest dose of 4-MU (5.29 mg/rat) were 3.7–12.5 μ g/ml (up to 90 min). Quantitative binding studies to check this possibility are now in progress.

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