Letter

Human plasma and urinary metabolic profiles of trimethylamine and trimethylamine *N*-oxide extrapolated using a simple physiologically based pharmacokinetic model

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ABSTRACT — Trimethylamine, a dietary- and medicinal carnitine-derived amine, is extensively metabolized by liver to non-malodorous trimethylamine *N*-oxide. Although trimethylamine and trimethylamine *N*-oxide under daily dietary consumption or carnitine treatment are generally regarded as nontoxic, they have been, and remain, of toxicological and clinical interest because of their potential association with atherosclerosis. The aim of the current study was to model the pharmacokinetics of trimethylamine after oral administration of trimethylamine in humans and compare the results with reported measured values. Adjusted biomonitoring equivalents from rat studies based on reported plasma concentrations were scaled to human equivalents using known species allometric scaling factors. *In vitro* metabolic clearance data were obtained using rat and human liver microsomal preparations. Renal clearances in humans for trimethylamine and trimethylamine *N*-oxide were calculated with a clearance concept approach using reported 24-hr urinary excretion rates and assumed areas under plasma concentration curves. The resulting modeled plasma and urinary concentration curves by simple physiologically based pharmacokinetic models (or semi-physiological pharmacokinetic models) were consistent with reported concentrations. This study provides important information to help simulate human plasma levels of trimethylamine and trimethylamine *N*-oxide in trimethylamine loading tests and during treatment with prescribed medicinal l-carnitine, showing the similar range as that resulting from daily dietary foodstuff consumption along with little toxicological impacts. The present models could estimate relationship between plasma and urine concentrations of trimethylamine or trimethylamine *N*-oxide and the daily oral doses by both forward and reverse dosimetry from viewpoint of human risk assessment.

Key words: PBPK modeling, Allometric scaling, TMA, TMAO, Rat, Human

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INTRODUCTION

Trimethylamine and trimethylamine *N*-oxide are generally regarded to be nontoxic (WHO, 2006) in daily dietary consumption (Fennema *et al.*, 2016) and prescribed medicine L-carnitine treatment (Bain *et al.*, 2006b). The no-observable-adverse-effect-level of trimethylamine for general toxicity was estimated to be 160 mg/kg/day in rats, implying little risk in humans from normal daily dietary intake (Amoore *et al.*, 1978). The majority (95%) of the administered ¹⁴C-labeled trimethylamine (100 mg administered orally) in three male volunteers was excret-

ed as trimethylamine *N*-oxide in urine during the first 24 hr (Al Waiz *et al.*, 1987). We reported the metabolic fate of trimethylamine after normal daily dietary consumption in healthy Japanese volunteers (Shimizu *et al.*, 2009) and also trimethylamine levels in Japanese dialysis patients treated with prescribed carnitine (Ozasa *et al.*, 2014; Fukami *et al.*, 2015). Human plasma and urinary levels of trimethylamine and trimethylamine *N*-oxide in loading tests have been investigated in five male subjects before and after single oral doses of 300 and 600 mg trimethylamine (Lundh *et al.*, 1995), however, these human plasma levels in 300 mg trimethylamine loading tests

have been considered be within the range of interindividual variability resulting from daily dietary foodstuff consumption. However, there is continued clinical interest in their safety because of their potential to form carcinogenic *N*-nitrosodimethylamine (Bain *et al.*, 2005), their contribution to neurological toxicity (Bain *et al.*, 2006a), and their association with atherosclerosis (Wang *et al.*, 2011; Koeth *et al.*, 2013).

Although a few pharmacokinetic models for carnitine have been reported (Evans and Fornasini, 2003; Fornasini *et al.*, 2007), modeling of the detailed metabolic fates of trimethylamine and trimethylamine *N*-oxide have yet to be investigated. Limited data on the metabolic fates of trimethylamine in rats have been reported after a single oral administration of 20 mg/kg (Nnane and Damani, 2001); however, accounting for species differences in drug metabolism and disposition between rats and humans is a complex issue currently being addressed in the field of preclinical research. Consequently, we focused on combining the *in vitro* drug clearance rates of trimethylamine in rats and humans with simplified physiologically based pharmacokinetic (PBPK) models (or semi-physiological pharmacokinetic models) (Takano *et al.*, 2010; Yamashita *et al.*, 2014; Yamazaki *et al.*, 2016) using reported bioequivalence findings (Lundh *et al.*, 1995; Nnane and Damani, 2001). We report herein *in silico* human plasma and urine concentrations of trimethylamine and trimethylamine *N*-oxide estimated after virtual oral administration of trimethylamine by forward dosimetry. Biomonitoring trimethylamine and trimethylamine *N*-oxide concentrations in human biofluid would also yield daily intake of trimethylamine by reverse dosimetry in toxicological risk aspects.

MATERIALS AND METHODS

Liver microsomes from 7-week-old male Sprague-Dawley rats were prepared as described previously (Yamashita *et al.*, 2014); the use of animals and humans in this study was approved by the Ethics Committees of Showa Pharmaceutical University. *In vitro* elimination rates of trimethylamine (Wako Pure Chemicals, Osaka, Japan) mediated by pooled liver microsomes from rats and pooled liver microsomes from humans (H150, Corning, Woburn, MA, USA) were determined using our previously established gas chromatography methods (Yamazaki *et al.*, 2007) with slight modifications. Briefly, a typical incubation mixture consisted of 100 mM potassium phosphate buffer (pH 8.4), an NADPH-generating system, liver microsomes (0.50 mg protein/mL), and trimethylamine (300 μ M) in a final volume of

0.50 mL. Incubations were carried out at 37°C for 30 min to evaluate the elimination of trimethylamine within a linear range. Hepatic intrinsic clearances were calculated from the elimination rates and the liver microsomal unbound fraction ($f_{u,mic}$) for trimethylamine (0.992) determined by SimCYP software. $CL_{h,int,in vitro}$ values were assumed from trimethylamine elimination rates and extrapolated using the following values: 40 mg liver microsomal protein per 1 g liver and 10 g liver weight per 0.25 kg of rat bodyweight or 1.5 kg liver weight per 70 kg of human bodyweight (Yamashita *et al.*, 2014; Takano *et al.*, 2010).

Simplified PBPK models for trimethylamine (59.11 molecular weight) and trimethylamine *N*-oxide (75.11) consisted of a chemical receptor (gut) compartment, a metabolizing (liver) compartment, and a central compartment and were set up as described previously (Yamashita *et al.*, 2014; Yamazaki *et al.*, 2016). Values of the octanol-water partition coefficient ($\log P$; 0.240 and 0.179) and the plasma unbound fraction ($f_{u,p}$; 0.900 and 0.714) of trimethylamine and trimethylamine *N*-oxide, respectively, were obtained by *in silico* estimation using Chemdraw and Simcyp software (Emoto *et al.*, 2009). The blood-to-plasma concentration ratio (R_b ; 0.719 and 0.845) and liver-to-plasma concentration ratio ($K_{p,h}$; 0.752 and 0.675) of trimethylamine and trimethylamine *N*-oxide, respectively, were estimated from $f_{u,p}$ and $\log P$ (Poulin and Theil, 2002). Parameters that represent physiological properties such as hepatic blood flow rates (Q_h) in rats (0.853 L/hr) and humans (96.6 L/hr) were taken from the literature (Kato *et al.*, 2008).

Plasma concentrations of trimethylamine after oral administration in rats were taken from the literature (Nnane and Damani, 2001). Values of the absorption rate constant (k_a), the volume of the systemic circulation (V_1), and the hepatic intrinsic clearance ($CL_{h,int}$) were calculated by fitting with non-linear regression analyses as described previously (Yamashita *et al.*, 2014). The final parameter values for rat PBPK models are shown in Table 1. Finally, the following system of differential equations was solved to conduct the modeling for trimethylamine (Takano *et al.*, 2010):

$$\begin{aligned}\frac{dX_g(t)}{dt} &= -k_a \cdot X_g(t), \text{ when } t=0, X_g(0) = dose \\ V_h \frac{dC_h}{dt} &= Q_h \cdot C_b - \frac{Q_h \cdot C_h \cdot R_b}{K_{p,h}} + k_a \cdot X_g - CL_{h,int} \cdot \frac{C_h}{K_{p,h}} \cdot f_{u,p} \\ V_1 \frac{dC_b}{dt} &= -Q_h \cdot C_b + \frac{Q_h \cdot C_h \cdot R_b}{K_{p,h}} - CL_r \cdot C_b \\ V_u \frac{dC_u}{dt} &= CL_r \cdot C_b\end{aligned}$$

Table 1. Physiological, experimental, and final calculated parameters for rat and human PBPK models for trimethylamine and trimethylamine *N*-oxide.

Parameter	Abbrev. (unit)	Rat	Human
Absorption rate constant	k_a (1/hr)	1.17 ± 0.33	0.87
Volume of systemic circulation for trimethylamine	$V_{1\_substrate}$ (L)	0.394 ± 0.084	112
Hepatic intrinsic clearance for trimethylamine	$CL_{h,int\_substrate}$ (L/hr)	0.157 ± 0.012	27.7
Hepatic clearance for trimethylamine	$CL_{h\_substrate}$ (L/hr)	0.121	25.5
Volume of systemic circulation for trimethylamine <i>N</i> -oxide	$V_{1\_N\text{-oxide}}$ (L)	Not available	21.2
Hepatic intrinsic clearance for trimethylamine <i>N</i> -oxide	$CL_{h,int\_N\text{-oxide}}$ (L/hr)	Not available	0.000001

Data in the first three rows for the rat are means $\pm$ standard deviations. The fraction absorption $\times$ intestinal availability ($F_a \cdot F_g$) and hepatic intrinsic clearance for *N*-oxide were estimated to be 1 and almost 0 (0.000001), respectively. Renal clearances in humans for trimethylamine and trimethylamine *N*-oxide (1.55 and 2.53 L/hr, respectively) were calculated using reported 24-hr urinary excretion values in humans (22.8 and 585 mg, respectively, (Lundh *et al.*, 1995)) and assumed areas under the concentration curve by trapezoid methods (AUC_{0-24} , 14.7 and 231 mg hr/L, respectively). Parameters such as $K_{p,h}$, R_b , and $f_{u,p}$ were assumed to be the same for humans and for rats.

where X_g is the amount of compound in the gut, V_h and the volumes of liver and urine, respectively, C_h is the hepatic substrate concentration, and C_b is the blood substrate concentration. The system of differential equations to model concentrations of the trimethylamine *N*-oxide metabolite (indicated with subscript *m*) (Yamashita *et al.*, 2014) is as follows:

$$V_h \frac{dC_{h,m}}{dt} = Q_h \cdot C_{b,m} - \frac{Q_h \cdot C_{h,m} \cdot R_{b,m}}{K_{p,h,m}} + CL_{h,int} \cdot \frac{C_h}{K_{p,h}} \cdot f_{u,p} - CL_{h,int,m} \cdot \frac{C_{h,m}}{K_{p,h,m}} \cdot f_{u,p,m}$$

$$V_{1,m} \frac{dC_{b,m}}{dt} = -Q_h \cdot C_{b,m} + \frac{Q_h \cdot C_{h,m} \cdot R_{b,m}}{K_{p,h,m}} - CL_{r,m} \cdot C_{b,m}$$

$$V_u \frac{dC_{u,m}}{dt} = CL_{r,m} \cdot C_{b,m}$$

To evaluate the parameters for simplified human PBPK models of trimethylamine and its *N*-oxide metabolite based on the parameters of the rat PBPK models, relevant data from liver microsomal experiments and physiological parameters derived from the literature were used. The values of k_a , V_1 , and $CL_{h,i}$ for trimethylamine in humans were estimated using a scale-up strategy from rats to humans as described previously (Takano *et al.*, 2010; Yamashita *et al.*, 2014; Yamazaki *et al.*, 2016). Briefly, rat absorption rate constant (k_a) was multiplied by 0.744 to give the human k_a value; the human systemic circulation volume ($V_{1,human}$) was estimated using V_h and the volume of blood (V_b), i.e., $V_{h,human}$, $V_{b,rat}$, and $V_{b,human}$ values of 1.50 L, 0.0160 L, and 4.90 L, respectively (Yamashita *et al.*, 2014):

$$V_{1,human} = V_{b,human} + (V_{d,rat} - V_{b,rat}) \times \frac{R_{b,rat}}{f_{u,p,rat}} \cdot \frac{f_{u,p,human}}{R_{b,human}} - V_{h,human} \times \frac{K_{p,h} \times F_h}{R_b}$$

where V_d is the distribution volume per body weight and

F_h is the fraction unmetabolized in the liver. The *in vivo* hepatic intrinsic clearance ($CL_{h,int}$) in humans was estimated by multiplying the calculated initial parameters for *in vitro* hepatic intrinsic clearance values in humans (1.5 L/hr) by the ratio of *in vivo* (0.157 L/hr) to *in vitro* (0.0085 L/hr) hepatic intrinsic clearance for trimethylamine in rats according to the methods (Yamashita *et al.*, 2014; Takano *et al.*, 2010). The final parameter values are shown in Table 1. Sensitivity analysis for some final parameter values were performed. The above-described system of differential equations was also solved to determine the concentrations in each compartment and the levels of urinary excretion in humans. The modeled plasma and urinary concentration curves of trimethylamine and trimethylamine *N*-oxide for humans were verified by comparison with reported median concentrations (and ranges) for five men after single oral doses of 600 mg per subject (Lundh *et al.*, 1995).

RESULTS AND DISCUSSION

The circles in Fig. 1A show the reported levels of trimethylamine in plasma from six male rats after oral administration of 20 mg/kg (Nnane and Damani, 2001). From these concentrations, the final kinetic parameters such as the absorption rate constant (k_a), the volume of the systemic circulation (V_1), and the hepatic intrinsic clearance ($CL_{h,int}$) for the rat PBPK models were calculated by fitting and are shown in Table 1. By solving the equations that make up the PBPK models, a virtual plasma concentration curve was created for rats. The resulting estimated *in silico* concentration curve is shown in Fig. 1A and agreed with the reported *in vivo* data points (Nnane and Damani, 2001). Nnane and Damani (2001) also studied

Fig. 1. Estimated plasma (A, B) and urine (C) concentrations of trimethylamine and trimethylamine *N*-oxide in rats (A) and humans (B, C). The reported values for trimethylamine (circles) in rats after single oral doses of 20 mg/kg (Nnane and Damani, 2001) and for trimethylamine (circles) and trimethylamine *N*-oxide (triangles) in humans after administration of 600 mg trimethylamine were taken from the literature (Lundh *et al.*, 1995). Lines show the rat and human PBPK model results for trimethylamine (solid lines) and trimethylamine *N*-oxide (broken lines) after virtual single oral doses. The original units of urinary excretion ($\mu\text{mol/hr}$) in the literature were converted to the current $\mu\text{mol/L}$ units using the detection threshold description ($0.04 \mu\text{mol/L}$ in plasma and $0.005 \mu\text{mol/hr}$ in urine) per subject (Lundh *et al.*, 1995).

Fig. 2. Effects of deviation of the k_a ($\circ$), $V_{1\_substrate}$ (Δ), $CL_{h, int\_substrate}$ ($\square$), $V_{1\_N\text{-oxide}}$ ($\blacktriangle$) and $CL_{r\_N\text{-oxide}}$ ($\blacksquare$) values on the simulated plasma C_{max} values for trimethylamine (lower) and trimethylamine *N*-oxide (upper) in humans by the PBPK modeling after oral administration of 600 mg dose.

the disposition of trimethylamine *N*-oxide in rats at similar plasma levels to those of trimethylamine. However, it has been pointed out that predicting results in humans from data generated in rats may be difficult because of species differences in flavin-containing monooxygenases expression and function (Bain *et al.*, 2005). In this study,

our simulation in rats focused on trimethylamine disposition only.

The plots in Figs. 1B, 1C show the reported median values (and range) of trimethylamine and trimethylamine *N*-oxide in plasma and urine in five male volunteers after oral administration of 600 mg per subject (Lundh *et al.*, 1995). To establish PBPK models for trimethylamine in humans, the *in vitro* substrate elimination of trimethylamine was investigated using liver microsomes from rats and humans. Rates of trimethylamine elimination by human liver microsomes ($0.13 \text{ nmol/min/mg protein}$) were twice those of the rat ($0.065 \text{ nmol/min/mg protein}$) under the present conditions. In the present study, renal clearances in humans for trimethylamine and trimethylamine *N*-oxide (1.55 and 2.53 L/hr , respectively) were calculated using reported 24-hr urinary excretion values in humans (22.8 and 585 mg , respectively, (Lundh *et al.*, 1995) and assumed areas under the concentration curve by trapezoid methods (AUC_{0-24} , 14.7 and 231 mg hr/L , respectively). Parameters for the human PBPK model for trimethylamine (Table 1) were set up based on the parameters in the rat PBPK model. By solving the equations for the simplified human PBPK model, *in silico* plasma and urinary concentration curves after the virtual administration of trimethylamine were created using these kinetic parameters, as shown in Figs. 1B, 1C. The results of a sensitivity analysis are shown in Fig. 2.

It should be mentioned that reported studies concern-

ing trimethylamine and trimethylamine *N*-oxide elimination in humans involved the administration of unlabeled chemicals. Trimethylamine and trimethylamine *N*-oxide will have undoubtedly undergone dilutions to an unknown extent as a result of normal dietary intake of trimethylamine (Al Waiz *et al.*, 1987). The loading test carried out by Lundh *et al.* (1995) involved the administration of 300 and 600 mg trimethylamine. For the present modeling, we adopted the concentrations resulting from the administration of 600 mg trimethylamine (Lundh *et al.*, 1995), because 1 hr after the administration of 300 mg trimethylamine, plasma trimethylamine levels had reportedly increased in only one of the five subjects tested. We previously reported data showing that plasma trimethylamine and trimethylamine *N*-oxide concentrations in a cohort of 18 male and 6 female hemodialysis patients treated with 1,000 mg of L-carnitine (L-Cartin injection, Otsuka Pharmaceutical, Tokyo, Japan) intravenously on 3 days over 1 week or with 300 mg of oral L-carnitine (L-Cartin tablets, Otsuka Pharmaceutical) three times per day for 7 days are likely in the same range as those of healthy subjects consuming a typical Japanese diet (Ozasa *et al.*, 2014). Under the regime of three doses of 354 mg trimethylamine (to correspond to 1,000 mg L-carnitine, 6.2 mmol) per week, trimethylamine and trimethylamine *N*-oxide were extensively excreted in the urine (results not shown).

The present PBPK models were able to estimate human plasma and urine concentrations of trimethylamine and trimethylamine *N*-oxide after ingestion doses of trimethylamine by forward dosimetry, but were also capable of reverse dosimetry from the concentrations to trimethylamine doses for toxicological risk assessments. The trimethylamine ($26 \pm 17 \mu\text{M}$) and trimethylamine *N*-oxide ($197 \pm 100 \mu\text{M}$) concentrations measured in plasma from 10 healthy Japanese volunteers (Ozasa *et al.*, 2014) may imply exposure to $936 \pm 612 \mu\text{g}$ trimethylamine/day and $811 \pm 412 \mu\text{g}$ trimethylamine/day, respectively, by reverse dosimetry analysis with the current human PBPK model, assuming that the reported concentrations had reached steady state values (calculated as the area under the concentration curve divided by 24 hr under multiple administrations). As shown in the sensitivity test (Fig. 2), the current PBPK model could also assess the influences of genetic flavin-containing monooxygenase variations (Yamazaki and Shimizu, 2007) on the $CL_{\text{h, int}}$ in *in vivo* the trimethylamine pharmacokinetics in humans for the toxicological assessment.

In conclusion, in the present study, *in silico* human plasma and urine concentrations of trimethylamine and trimethylamine *N*-oxide estimated using a simplified PBPK

modeling method after virtual oral doses of trimethylamine were consistent with reported concentrations after non-radiolabeled trimethylamine treatments in several subjects (Lundh *et al.*, 1995). Using the simplified PBPK modeling, clinical plasma trimethylamine and trimethylamine *N*-oxide concentrations after receiving the medicine L-carnitine yielded to the similar dose ranges corresponding to virtual ingestion of a typical Japanese diet, implying little risk in humans. The human plasma or urine levels of trimethylamine and trimethylamine *N*-oxide in trimethylamine loading tests were considered to be within the range of interindividual variability resulting from daily dietary foodstuff consumption (Shimizu *et al.*, 2009; Ozasa *et al.*, 2014; Fukami *et al.*, 2015) using the simplified PBPK modeling method. The present results suggested that the forward and reverse dosimetry of trimethylamine or trimethylamine *N*-oxide using the current simplified PBPK model would contribute human risk assessment of dietary- and medicinal carnitine-derived trimethylamine.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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