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Title Page

In vivo kinetics of indoxyl sulfate in humans and its renal interaction with angiotensin-converting enzyme inhibitor quinapril in rats

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Running title page

Running title: In vivo kinetics of indoxyl sulfate and drug interaction

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Abbreviations: IS, Indoxyl sulfate; CKD, chronic kidney disease; AUC, area under the curve; Fu, fraction of unbound; GFR, glomerular filtration rate; CL_{cr}, creatinine clearance; Thio, Sodium thiosulfate; PAH, sodium para-aminohippurate; RPF, renal plasma flow; CL_R, renal clearance; CL_{TS}, renal tubular secretion; $E_{TS,}$ extraction rate by the renal tubules; OAT, organic anion transporter; ACEI, angiotensin-converting enzyme inhibitor; HPLC, high performance liquid chromatography

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Abstract

Indoxyl sulfate (IS) is an organic anion uremic toxin which accumulates in chronic kidney disease (CKD) patients. The aims of this study were to examine the kinetic profiles of IS in humans at a steady state after multiple doses of L-tryptophan (Trp), a precursor of IS, and the in vivo interaction of IS with the angiotensin-converting enzyme inhibitor (ACEI) quinapril, whose active metabolite is a substrate of organic anion transporter 3 (OAT3), in rats. First, 12-h kinetics after the single doses of Trp (2, 4 and 8 g) were examined in two healthy volunteers. Second, 24-h kinetics after the single dose of 2 g Trp was studied in 6 volunteers. Third, 35-h kinetics after the single and multiple doses of 2 g Trp were examined in 5 volunteers. In anesthetized rats, quinapril or probenecid, an inhibitor of OATs, following IS was intravenously given and blood and urine were taken until 90 min. Trp and IS concentrations were determined by high performance liquid chromatography (HPLC). Ultrafiltration was used to measure serum unbound IS concentrations. Renal tubular secretion of IS accounted for more than 90% of its renal clearance in the steady state of serum IS levels after multiple doses in humans. In animals, the serum area under the curve (AUC) of IS increased in conjunction with a decrease in renal clearances after co-administration of IS with quinapril or probenecid. It is concluded that quinapril may inhibit the urine excretion of IS *via* OAT3-mediated renal tubular transport in CKD patients.

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Introduction

Many metabolic products accumulate in patients with CKD and those that are harmful to organs are called uremic toxins. Uremic toxins are classified into 3 groups; free water-soluble low-molecular-weight solutes, middle molecules, and protein-bound solutes (Vanholder et al., 2003). IS, one of the major uremic toxins, is characterized as a protein-bound solute and is a substrate of OAT 1, 3 and 4 (Enomoto et al., 2003). IS has been found to inhibit the albumin binding of anionic drugs *in vitro* (Bowmer and Lindup, 1982; Mabuchi and Nakahashi, 1988) and its intracellular uptake was mostly inhibited by organic anion drugs in the rat kidney (Deguchi et al., 2004). Therefore, concomitant use of drugs which have high protein binding and are substrates or inhibitors of OATs may cause the accumulation of IS in CKD patients. It has been demonstrated that the plasma protein binding of organic anion drugs was decreased, increasing the fraction unbound (Fu) of organic anion drugs, in chronic renal failure where GFR is less than 15 mL/min/1.73m^2 (Dreisbach and Lertora, 2003).

The aim of this study was to investigate the pharmacokinetic interactions between IS and drugs with respect to protein binding and urine excretion *in vivo*. First, the kinetic profiles of IS were examined in healthy people to clarify the contribution of renal active transport to urine excretion. As serum levels of IS in people with normal renal function are low, L-Trp, a precursor of IS, was administered to healthy volunteers in order to increase the serum IS levels (Niwa and Ise, 1994). IS is produced primarily through degradation of Trp to indole by enterobacteria and indole is subsequently oxidized and then conjugated with sulfate in the liver (Smith and Macfarlane, 1996). In this study, we examined the kinetics of IS under a steady state after multiple doses of L-Trp. Second, pharmacokinetic interactions were investigated in rats by intravenous administration of organic anion drugs and IS. In these experiments, the serum IS levels were set to be similar to those in the above human study. Quinapril, an ACEI, whose active metabolite quinaprilat is a substrate of OAT3 and protein binding rate in plasma is high along with quinaprilat (about 97% in human), was used as a probe drug (Yuan et al., 2009).

Probenecid, an inhibitor of intracellular IS uptake via OAT1, OAT3 and OAT4, was chosen as a positive control (Enomoto et al., 2003).

METHODS

Human study

Subjects

The subjects were 6 healthy male volunteers. Two, 6 and 5 volunteers participated in the first, second and third protocol, respectively. Each volunteer is identified by a unique number in the text. The age, body weight, and body mass index (BMI) of the subjects are presented in Table 1. Only subjects whose serum creatinine was within normal ranges were included in the study. Subjects were excluded for any of the following reasons: suffering from an active disease requiring treatment, clinically abnormal laboratory tests, a history of allergy, a psychiatric disease, drug abuse or digestive tract surgery, positive results for human immunodeficiency virus antigen or antibody, hepatitis B surface antigen, hepatitis C antibody or serological test for syphilis, a blood donation of 200 mL within 4 weeks or 400 mL within 12 weeks before the study, and participation in other clinical studies within 3 months before the present study.

All subjects gave written informed consent before the study began. The studies were approved by the Institutional Review Board of Kitasato University East Hospital and were conducted at the Clinical Trial Center of Kitasato University East Hospital.

Treatment and Formulation

Trp was provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo) as a powder of L-Trp, which is used as a food additive and is listed in the Japanese Pharmacopoeia, Fifteenth Edition. Enteric coated capsules filled with Trp (333.3 mg L-Trp/capsule) were obtained from Sunsho Pharmaceutical Co., Ltd. (Shizuoka, Japan).

Sodium thiosulfate (Thio) and sodium para-aminohippurate (PAH) were purchased from Banyu Pharmaceutical Co., Ltd. (Tokyo) and Daiichi

Sankyo Company, Ltd. (Tokyo), respectively.

Study Design

The study consisted of 3 separate protocols (Table 2). First, a single oral dose of L-Trp was administered to two fasting volunteers at 8 AM on Day 1 at escalating doses of 2, 4 and 8 g with a 2-week washout period between each dose. The second protocol, a single oral dose of L-Trp was administered to 6 volunteers at 8 AM 30 min after breakfast on Day 1 at a dose of 2 g. The third protocol, a sequential 2-phase study, consisted of single and multiple doses with a 11-days washout period. In the single-dose study, L-Trp at a dose of 2 g was administered at 10 PM on Day 1. In the multiple-dose study, L-Trp at a dose of 2 g was administered at 10 PM daily from Day 1 to Day 8. A renal function test was conducted during the washout period.

Low-Trp diets were consumed on Day -1 and Day 1 in the first protocol, on Day 1 and Day 2 in the second protocol and on Day 1, Day 2, Day 8 and Day 9 in the third protocol (Table 3).

Blood and urine sampling were performed at 0, 2, 3, 4, 6, 8 and 12 h after administration of L-Trp and every 2 h for 12 h, respectively, in the first protocol. Those were performed at 0, 4, 6, 8, 10, 12, 14, 16, 24 h after administration of L-Trp and every 2 h for 24 h, respectively, in the second protocol. In the single dose study of the third protocol, blood was taken at 0, 8, 11, 14, 15, 20, 23, 26, 32 and 35 h after administration of L-Trp and urine was collected for 0-8, 8-14, 14-20, 20-26 and 26-35 h. In the multiple-dose study, blood was taken in the evening on Day 1, 4 and 6 before each administration of L-Trp and at 0, 8, 11, 14, 15, 20, 23, 26, 32 and 35 h after the last administration of L-Trp. Urine was collected in a manner similar to that as the single dose study. Blood was kept at room temperature and serum was separated from blood by centrifugation at 4,000 *g* for 15 min. Serum and urine samples were stored at -20°C until the analysis.

Renal function tests

Renal function tests were performed 8 days after the single dose of

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L-Trp in the second protocol where the washout period was thought to be long enough for the elimination of IS. In brief, 80 mL of 10% Thio solution and 12 mL of 10% PAH were given for 10 min by an intravenous drip injection 30 min after intake of 500 mL of water. The subjects then urinated 25 min after the injections and this urine was discarded. Then, urine was collected for 30 min. Blood was collected at 10 and 20 min after urination. The clearances of Thio and PAH were calculated as eq. 1:

$$C_{Thio\ or\ PAH} = U_{Thio\ or\ PAH} / P_{Thio\ or\ PAH} \times V \times 1.48 / A \quad (1)$$

Where $C_{Thio\ or\ PAH}$ (mL/min) is the clearance of Thio or PAH, $U_{Thio\ or\ PAH}$ (mg/dL) is the urine concentration of Thio or PAH, $P_{Thio\ or\ PAH}$ (mg/dL) is the calculated serum concentration of Thio or PAH at the middle time of two measured points, V (mL/min) is urine volume per min, 1.48 is a standard body surface area for Japanese (m^2), and A is body surface area. A is calculated using the eq. 2:

$$A = \text{body weight}^{0.425} \times \text{height}^{0.725} \times 0.007271 \quad (2)$$

$C_{Thio\ and}$ C_{PAH} refer to glomerular filtration rate (GFR) and renal plasma flow (RPF), respectively.

Kinetic parameters

Mean C_{max}, T_{max}, and $AUC_{0\text{-}35}$ were obtained from the serum concentration-time curves of IS for individuals in the single- and multiple-dose studies in the second protocol. Urinary excretion rates of Trp and IS were calculated as a percent of urine excretion to the administered dose of L-Trp. Percent of Fu of IS in the serum was calculated as the ratio of $AUC_{0\text{-}35}$ for unbound IS to that for total IS. Renal clearance of IS (CL_R) was obtained from the urine excretion of IS for 35 h divided by $AUC_{0\text{-}35}$ of IS. The clearances of renal tubular secretion (CL_{TS}) and extraction rate of IS by the renal tubules (E_{TS}) were calculated as eqs. 3 and 4:

$$CL_{TS} = CL_R - GFR*Fu \quad (3)$$

$$E_{TS} = CL_{TS} / RPF \quad (4)$$

The ratio of CL_R to Fu*GFR, a measure expressing the renal elimination pattern of a drug, was also calculated. When the value is more than 1, it is inferred that active secretion is apparent in the renal elimination of a drug.

Animal study

Animals

Seven week-old male LEW/CrlCrlj rats (Oriental Yeast Co., Ltd., Tokyo) were housed in our animal care facility under the constant humidity and temperature and 12 h light-dark cycle. The animals were maintained on a certified diet of MF pellets (Oriental Yeast) and tap water *ad libitum*. The experiments were approved by the Animal Experimentation and Ethics Committee of Kitasato University School of Medicine.

Reagents

IS potassium salt, quinapril, and probenecid were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). IS was dissolved in saline at concentrations of 0.5% and 0.05%. Quinapril was dissolved in saline at 0.2%. Probenecid was first dissolved in 1M NaOH solution and then diluted with saline and titrated at pH 7.9 to pH 8.1 by a diluted HCL solution at a concentration of 5%. Saline was also prepared at a pH similar to the above range as the vehicle control for probenecid treatment.

Study design

Rats were initially anesthetized with pentobarbital sodium (Nembutal: Abbott Laboratories, North Chicago, IL, USA) at a dose of 65 mg kg^{-1} by i.p.. For blood sampling with time, an intramedic polyethylene tube (PE50, Nippon Becton Dickinson Co., Japan) was inserted into the left femoral vein toward the inferior vena cava. A tube inserted into the blood vessels was filled with saline solution containing heparin. Blood was taken via a tube before administration of the co-administered drugs and at 5, 15, 30,

45, 60 and 90 min after administration of IS. Total blood sampling volume was about 2 mL. A co-administered drug such as quinapril or probenecid was given 2 min before administration of IS. All drugs were given intravenously. The urinary bladder was cannulated through a small abdominal skin incision using a polytetrafluoroethylene tube (Feeding needles, Fuchigami Ltd., Kyoto, Japan). Urine was collected for 90 min. Doses of quinapril following IS were set at 2 mg/kg and 0.5 mg/kg, respectively, molar doses of which were comparable (4.2 and 2.3 μmol/kg, respectively). Those of probenecid following IS were set at 50 mg/kg and 5 mg/kg, respectively, by referring to the previous study where rats were co-administrated with IS and probenecid at 10 mg/kg (46.9 μmol/kg) and 50 mg/kg (175 μmol/kg), respectively, to investigate the pharmacokinetic interaction (Deguchi et al., 2003).

There were 14 rats in the administration of IS groups with and without quinapril, respectively, and 4 and 5 rats in the administration of IS groups with and without probenecid, respectively.

Kinetic parameters

Mean $AUC_{0\text{-}90}$ values were obtained from the serum concentration-time curves of IS and unbound IS. Renal clearance of IS (CL_R) was obtained from the total IS amount in urine for 90 min divided by the $AUC_{0\text{-}90}$ of IS.

High-performance liquid chromatography (HPLC)

Reagents

Acetonitrile and methanol were purchased from Kanto Chemical Co., Inc. (Tokyo), and indoxyl sulfate potassium salt was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Sample preparation

Aliquots of serum were divided into two portions, one of which was used to determine the total concentrations of Trp and IS, and the other to measure Fu of IS. The samples were prepared as previously described (Lagana et al., 1986). For determination of the former, 200 μL of methanol was added to 100 μL of serum. After vortex-mixing, the mixture was kept

for 1 h at 4°C followed by centrifugation at 6000 *g* for 20 min. The resulting supernatants were filtered through a 0.45 µm cellulose acetate filter (ADVANTEC DISMIC-13cp, Advantec Toyo Kaisha, Ltd., Tokyo) to remove large insoluble particles. For the latter, 500 µL of serum was ultrafiltered through a MICROCON YM-10 membrane (Millipore Corp., MA, USA) and left for 30 min at room temperature followed by centrifugation at 6000 *g* for 20 min. In both cases, 20 and 10 µL samples were injected. Urine was filtered through a 0.45 µm filter to remove large insoluble particles and a 5 µL sample was injected.

Trp and IS assay

Trp and IS concentrations in serum and urine were quantified by HPLC. The chromatograph assembly (Waters™ 600 controller) consisted of a Waters™ 600 pump, Waters™ 717 plus autosampler, JASCO FP-920 Intelligent Fluorescence Detector, and a reverse-phase column (Develosil Column, Nomura Chemical Co., Ltd, Japan) with a guard column. Mobile phase consisting of solution A (distilled water/acetic acid, 100:0.08, v/v) and solution B (acetonitrile/acetic acid, 100:0.08, v/v) was delivered at a flow rate of 1 mL/min at ambient temperature. The mobile phase was linearly programmed from 100% of solution A to 100% of solution B within 20 min, and then to 100% of solution A within 1 min and being maintained for 9 min before the next injection. The eluate was monitored by detection of fluorescence at an excitation wavelength of 295 nm and an emission wavelength of 390 nm. Calibration was obtained using standards of 10.2 and 10.7 µg/mL of Trp and IS, respectively. The intra-assay and inter-assay coefficients of variation were 0.9 and 4.3% for Trp at a concentration of 3.4 µg/mL, 0.7 and 1.9% for IS at a concentration of 3.6 µg/mL, and 8.9 and 9.8% for IS at a concentration of 0.036 µg/mL, respectively. Regarding the unbound fraction of IS, the intra-assay and inter-assay coefficients of variation were 0.8 and 2.1% for IS at a concentration of 11 µg/mL and 4.3 and 8.3% for IS at a concentration of 0.11 µg/mL IS. Linearity of the standard curves was obtained at ranges from 0.16 to 10 µg/mL for Trp and 0.0083 to 11 µg/mL for IS.

Data Analysis

The results are expressed as the mean ± SD in the human study and as the mean ± SE in the animal study. Statistical comparisons of the kinetic variables were performed using the paired-*t* test in the human study and the unpaired-*t* test in the animal study. Differences in the serum concentrations of IS with time due to administration of IS alone and with co-administration of quinapril or probenecid were assessed using repeated-measures ANOVA with the post-hoc Fisher PLSD test or Scheffé test, respectively. The level of statistical significance was $P < 0.05$ (2-sided).

RESULTS

Changes in total concentrations of Trp and IS in serum and excretion rates of Trp and IS in urine after single, ascending doses of L-Trp after low-Trp diets.

Figure 1 presents individual data for the changes in total concentrations of Trp (A, B, C) and IS (D, E, F) in serum for 12 h after L-Trp administration at doses of 2, 4, and 8 g. Trp increased in the blood early after administration. At the lower 2 doses, Trp reached a maximum 2 h after administration and the increases were dose-dependent in both subjects. Trp levels had decreased to baseline by 12 h. The increases in Trp were biphasic for the highest dose of L-Trp. IS levels increased gradually in both subjects after administration of 2 and 4 g L-Trp. No increases in IS were observed with 8 g L-Trp in either subject. Figure 2 shows the urinary excretion rate of Trp (A) and IS (B) for 12 h after administration of L-Trp. The urinary excretion rate of Trp increased with doses up to 4 g L-Trp. Those of IS at doses of 2 and 4 g did not show dose-dependent increases within the observation period.

Changes in total concentrations of IS in serum and excretion rates of Trp and IS in urine after single dose of L-Trp after uncontrolled diets.

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Figure 3A and 3B show the individual data for changes in serum concentrations of IS and those for urinary excretion rates of Trp and IS for 24 h after single dose of L-Trp, respectively. The baseline concentrations of total IS in serum were higher in all subjects that those in the first protocol (Figure 1D, 1E and 1F). An apparent increase in IS concentrations in serum were not observed in the subjects except for subject 2. As shown in Figure 3B, a large inter-individual differences in urinary excretion rates of IS was demonstrated after administration of L-Trp. The urinary excretion rates of Trp and IS ranged from 1.1 to 2.5% and 1.8 to 8.1%, respectively.

Comparison of changes in total concentrations of IS in serum and urinary excretion rates of Trp and IS between single and multiple doses of L-Trp after low-Trp diets.

Figure 4 shows the individual data for changes in total serum concentrations of IS in the single- and multiple-dose studies. Subject 8 was withdrawn from the multiple-dose study because of treatment for a moderate headache accompanying nausea during the single dose study. Therefore, only data from the single dose study were displayed for this subject (Figure 4E). Serum concentrations of IS increased in 4 subjects after multiple doses compared with a single dose, while the increase was small in subject 4 (Figure 4D). In both the single- and multiple-dose studies, the elevation of serum IS concentrations gradually decreased toward the basal levels within each observation period. Large intra-individual differences in serum IS concentrations were displayed in subject 3 and 7 during the multiple doses (Figure 4C and 4D). As it was shown in Figure 3B, a large inter-individual differences in urinary excretion rates of IS was also demonstrated after the single (2.1 to 6.6%) and multiple doses (2.8 to 8.4%), respectively (Figure 5).

Kinetic parameters of IS after single and multiple doses of L-Trp.

Table 4 summarizes the kinetic parameters of IS after single and

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multiple doses of L-Trp and the results of renal function tests. Data for subject 5 who was withdrawn after the single dose study were not included in the analysis. Both mean C_{max} and AUC_{0-35} increased by 1.4-fold after multiple doses compared with those after a single dose, although the changes were not significant. The Fu of IS in serum after single and multiple doses were both small. CL_R significantly decreased after the multiple doses compared with after the single dose. More than 90% of the total renal clearance undergoes tubular secretion as indicated by the rates of CL_{TS} to CL_R for IS. The net renal tubular secretion of IS was shown to be about half of the GFR. The ratios of CL_R to Fu*GFR greatly exceeded 1 and the E_{TS} values were low.

Safety of L-Trp in the human study.

No adverse events were observed with a single dose of 2 g L-Trp in the first and second protocols. Mild fuzzy headache and sleepiness developed in subject 1 from 2 h to 10 h after administration of 4 g L-Trp. After administration of 8 g L-Trp in subject 1, dull headache and sleepiness, diarrhea, and nausea occurred at 20 min, 1 h, and 2.5 h after administration, respectively, and all of these symptoms except the diarrhea disappeared by 5 h after administration. The diarrhea improved the next morning. These adverse events were mild. In subject 2, muddle, sleepiness, and nausea occurred 20 min after administration. Postural discomfort and pale occurred from 4 h to 6 h after administration. The subject had been rested at a supine position due to these adverse events. Muddle, sleepiness, and nausea disappeared 7, 6, and 8 h after administration, respectively. Diarrhea appeared 4 h and 1 day after administration and resolved by the morning 2 days after administration. The events of nausea and postural discomfort and pale were moderate and others were mild. In the single and multiple-dose study a headache accompanying nausea was reported in subject 5 after the single dose and the subject took ibuprofen at 21.5 h. Since the subject experienced a headache and nausea before participation in the study, it is unlikely they were related to study drug administration.

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Pharmacokinetic interactions of IS with quinapril in rats.

As shown in Figure 6, the serum concentrations of IS gradually decreased with time after intravenous administration of IS. The concentrations at time -2 min were baseline values of endogenous IS before administration of quinapril or its vehicle. The decrease was suppressed by co-administration of quinapril. With respect to the kinetic parameters of IS, the $AUC_{0\text{-}90}$ increased and CL_R decreased in the quinapril co-administered group compared with the IS alone group (Table 5). The Fu of IS was also low in rats, but the protein binding rates did not differ between the IS alone group and quinapril co-administered group as represented by the $AUC_{0\text{-}90}$ of free IS and Fu rates.

Pharmacokinetic interactions of IS with probenecid in rats.

Figure 7 shows the changes in serum concentrations of IS with time after administration of IS with and without probenecid. The decreases in serum IS concentrations were suppressed by probenecid. Similar to the above results, $AUC_{0\text{-}90}$ increased and CL_R decreased after co-administration of probenecid with IS (Table 6). The $AUC_{0\text{-}90}$ of free IS increased in the probenecid co-administered group compared with the IS alone group, while Fu of IS was not significantly different.

DISCUSSION

The aims of the present study were to examine the kinetics of IS, focusing on the protein binding rate and renal elimination pathway, in a steady state of serum concentrations of IS after multiple doses of L-Trp in humans, and the pharmacokinetic interactions between IS and organic anion drugs in rats. The percentage of conversion of L-Trp to IS could be less than 10% as indicated by the 35-h urinary excretion rate of IS after multiple doses of L-Trp (Figure 5B), when assuming indole was absorbed into the blood by 100%. A higher conversion rate was demonstrated in

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healthy volunteers when L-Trp was administered by injection into the distal ileum and cecum through intestinal intubation where the 24-h urinary excretion rate of IS was nearly 14% (Bryan, 1966).

The biphasic increases in Trp at the highest single dose may be due to suppression of intestinal absorption, as nausea appeared in both subjects during the times which corresponded to the phase of the decreased concentrations of Trp (Figure 1C). The serum IS concentrations gradually increased, followed by an increase in Trp up to the single administration of 4 g L-Trp, however dose-proportionality was not described because of an insufficient observation period. Thus, it was extended to 24 h in the second protocol. However, the concentration-time curves of IS in serum were not reproduced as it was demonstrated in the first protocol (Figure 3A). A reason for this failure was probably due to an insufficient dietary control of Trp intake. Indeed, the baseline concentrations of IS in serum were higher in the second protocol than the first one (Figure 1D to 1F and Figure 3A). The subjects started the low-Trp diet 24 h before and were fasted for 12.5 h before administration of L-Trp in the first protocol, while the subjects started the low-Trp diet just before administration of L-Trp and were not fasted before administration of L-Trp in the second protocol. We also measured the concentrations of indole in serum and urine to investigate possible mechanisms for the delay in the appearance of IS in serum and no peaks were detected at the retention time for indole (16.71 min, data not shown). These results suggest indole is rapidly conjugated with sulfuric acid in the liver and a delay in the appearance of IS in serum may reflect the time required to reach L-Trp at the end of the intestine. Taking into consideration that a dietary Trp affects on the baseline concentrations of IS and serum IS appeared late after administration of L-Trp, designs of the third protocol were modified. The low-Trp diet was started 14.5 h before administration of L-Trp, the time for administration of L-Trp was moved forward from in the morning to at night, and the observation period was extended from 24 h to 35 h.

Serum IS increased in each subject after multiple doses of L-Trp compared with those after single dose, however the increases in mean C_{max} and AUC_{0-35} were not significantly different. This was probably due to

a small number of the subjects and still higher baseline concentrations of IS compared with those in the first protocol. Thus, a desirable wash out treatment before administration of L-Trp may be the 24-h low-Trp diet and 12.5-h fasting which were adopted in the first protocol.

A result of the decrease in CL_R after multiple doses of L-Trp suggests that the renal active transport of IS was saturated at a higher concentration of IS in the blood. When comparing the starting points of the increase in serum IS levels focusing on subject 1 and 2, they were delayed after administration at night compared with those in the morning (Figure 4 and Figure 1). These results may be due to a circadian rhythm of intestinal motility, which decreases at night and increases while awake (Rao et al., 2001). Mild increases in serum IS concentrations at 35 h may have been due to dietary Trp.

As it was represented by the high values of CL_R/Fu*GFR, a major pathway of renal elimination of IS was tubular secretion (Table 4). The elimination rates of IS by renal tubules were found to be low as expressed by the low values of ETS. According to the concept of drug clearance, the clearance of a drug with a low extraction rate is influenced by the protein binding rate and intrinsic clearance (Rowland and Tozer, 1995). The intrinsic clearance of a drug is generally defined by metabolism and transport activities in the organ of elimination. These results suggest that IS may interact with organic anion drugs, which are highly protein bound and undergo renal tubular transport in vivo.

Animal experiments were conducted to examine the above hypotheses. The results indicate that the kinetic interaction of IS with quinapril resulted from the inhibition of renal elimination of IS, not from the inhibition of protein binding (Figure 6, Table 5). The reason why probenecid interacted with IS at the protein binding may be due to relative differences in doses of the combined drugs, *i.e.* IS and probenecid were 5 mg/kg and 50 mg/kg, and IS and quinapril were 0.5 mg/kg and 2 mg/kg, respectively, so that probenecid might easily displace the protein binding of IS. Based on the Table 6 and 7, the inhibitory effect of probenecid on CLr of IS seems to be more potent (about 85%) than that of quinapril (about 50%). Given that probenecid is a general inhibitor not only for

OAT1, OAT3, and OAT4; but also for other organic anion transporters, while the active metabolite of quinapril is an inhibitor for OAT3, the results indicate that the clearance of IS may involve such organic anion transporters. It was suggested that OAT3 may contribute to the overall urine excretion of IS by nearly 50/85*100=59% in rats.

In both the human and animal studies, the serum IS concentrations were comparable to those in patients with low CL_{cr} (1.2±1.1 μg/mL, mean ± SE; 30 to 80 mL/min, n=27) (Niwa and Ise, 1994). Such patients are usually referred to as mild and moderate CKD where the decreases in GFR are defined as 60-89 and 30-59 mL/min/1.73m^2, respectively.

Finally, regarding the safety of L-Trp, sleepiness, headache, nausea, postural discomfort, and pale and diarrhea were observed in the subjects administered L-Trp at doses over 4 g accompanying the increases in Trp levels in serum. Similar adverse reactions to L-Trp have been also reported (Lieberman et al., 1984; Yuwiler et al., 1981; Greenwood et al., 1975). It was concluded that a single administration of L-Trp was tolerated up to 4 g.

In summary, the results of this study have demonstrated that IS was highly bound to serum protein and the major renal elimination route for IS was renal tubular secretion in humans. Kinetic interaction was demonstrated between IS and quinapril in rats where serum IS levels were comparable to those in the above human study.

It is concluded that urine excretion of IS, a major organic anion uremic toxin, may be inhibited by co-administered organic anion drug quinapril *via* renal tubular transport in CKD patients with a less progressive stage.

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Authorship Contributions

Participated in research design: Fujita and Majima.

Conducted human studies: Fujita, Nakamura, Maeda, Kobayashi, Sahashi, Ikeda.

Conducted animal studies: Fujita and Yasuda.

Contributed assay of drug concentrations: Ishihara.

Contributed kinetic analysis: Fujita and Kumagai.

Conducted data analysis: Fujita.

Wrote or contributed to the writing of the manuscript: Fujita.

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Tables

Table 1. Age, body weight, and BMI of the human subjects.

Protocol number	Subject number	Age (years)	Body weight (kg)	Body mass index (kg/m^2)
1	1	25	70.4,	23.8
	2	31	65.1	24.4
2	1	25	75.4	25.5
	2	31	68.2	25.7
	3	24	82.3	24.1
	4	27	63	20.8
	5	32	57.6	20.1
	6	24	67.1	22.5
3	1	26	71.9	24.1
	2	32	65.7	24.6
	3	24	85.4	25.0
	7	29	62.1	22.2
	8	38	63.1	22.8

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Table 2. Schedules for protocol 3.

	Single dose			Washout period	Multiple doses									
Days after Trp dose	1	2	3		1	2	3	4	5	6	7	8	9	10
Low-Trp diet	●	●										●	●	
Doses of Trp	●				●	●	●	●	●	●	●	●		
Kinetics (Blood and urine)	●	●	●		●			●		●		●	●	●
Renal function test				●										

Closed circles indicate the day on which an item listed at the left was performed.

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Table 3. Energy and amounts of Trp in low-Trp diets.

Protocol	Single or multiple doses of L-Trp	Days after dose of L-Trp	Energy (kcal)	Trp (mg)
1	Single dose	Day -1	2400	484
		Day 1	1590	354
2	Single dose	Day 1	1705	296
		Day 2	898.0	178
3	Single dose	Day 1	2402	460
		Day 2	1652	399
	Multiple doses	Day 8	2310	460
		Day 9	1669	367

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Table 4. Kinetic parameters of IS and renal function test results in human studies.

	After single dose	Washout period	After multiple doses	*P*
C_{max} (μg/mL)	0.9 ± 0.3		1.2 ± 0.4	0.06
T_{max} (h)	19 ± 6		17 ± 3	0.35
AUC_{0-35} (μg*h/mL)	21 ± 8		31 ± 13	0.06
Fu (%)	6.3 ± 3.7		4.0 ± 1.3	0.20
Urine excretion rate (%)	4.9 ± 2.2		5.4 ± 2.9	0.52
CL_R (mL/min)	74 ± 9		57 ± 8	0.04
CL_{cr} (mL/min)	158 ± 17		156 ± 21	0.57
GFR (mL/min)		111 ± 13		
RPF (mL/min)		418 ± 100		
CL_{TS} (mL/min)	68 ± 12		52 ± 9	0.08
E_{TS}	0.17 ± 0.06		0.13 ± 0.03	0.11
CL_R / Fu*GFR	13 ± 6		14 ± 5	0.68

Fu, fraction of unbound of serum IS; $CL_{TS,}$ clearances of renal tubular secretion, CL_R-GFR*Fu; E_{TS}, extraction rate of IS by the renal tubules, CL_{TS}/RPF. Data were expressed as the mean ± SD. Paired-*t* tests were performed. *P<0.05* was significantly different.

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Table 5. Kinetic parameters of IS co-administered with or without quinapril in animal studies.

	IS	Quinapril + IS	*P*
AUC_{0-90} of IS (μg*min/mL)	236 ± 18	374 ± 59	<0.05
AUC_{0-90} of free IS (μg*min/mL)	28.7 ± 4.7	34.3 ± 5.2	0.43
Fu (%)	13.3 ± 2.9	11.3 ± 2.2	0.58
CL_R (mL/min/kg)	1.2 ± 0.2	0.6 ± 0.2	<0.05

Data were expressed as the mean ± SE. Unpaired-*t* tests were performed. *P<0.05* was significantly different.

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Table 6. Kinetic parameters of IS co-administered with or without probenecid in animal studies.

	IS	Probenecid + IS	*P*
AUC_{0-90} of IS (μg*min/mL)	1630 ± 135	3553 ± 197	<0.01
AUC_{0-90} of free IS (μg*min/mL)	225 ± 28	577 ± 60	<0.01
Fu (%)	14.0 ± 1.8	16.5 ± 2.3	0.41
CL_R (mL/min/kg)	1.4 ± 0.1	0.2 ± 0.1	<0.01

Data were expressed as the mean ± SE. Unpaired-*t* tests were performed. *P<0.05* was significantly different.

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Legends for figures

Figure 1. Concentration-time curves of total concentrations of Trp and IS in serum after single escalating doses of L-Trp.

Two subjects were administered L-Trp at doses of 2, 4, and 8 g (A and D, B and E, C and F) in a fasting state in the morning. Blood sampling was conducted until 12 h after administration. Total concentrations of Trp (A, B and C) and IS (D, E and F) in serum were determined by HPLC. The subjects started to consume a low-Trp diet on the day before administration.

Figure 2. Urinary excretion rates of Trp (A) and IS (B) after single escalating doses of L-Trp.

Two subjects were administered L-Trp at doses of 2, 4, and 8 g in a fasting state in the morning. Urine sampling was conducted until 12h after administration. The amounts of Trp (A) and IS (B) in 12-h urine samples were determined by HPLC. The urinary excretion rates of Trp and IS were calculated as a percentage of the amounts of Trp and IS in the collected urine relative to L-Trp administered.

Figure 3. Concentration-time curves of total concentrations of IS in serum and urinary excretion rates of Trp and IS after single administration of 2 g L-Trp.

Six subjects were administered L-Trp at a dose of 2 g in a non-fasting

state in the morning. Blood and urine sampling were conducted until 24h after administration. Total concentrations of IS in serum (A) and the amounts of Trp and IS in 12-h urine samples (B) were determined by HPLC. The subjects started to consume a low-Trp diet just before administration of L-Trp. The urinary excretion rates of Trp and IS were calculated as a percentage of the amounts of Trp and IS in the collected urine relative to L-Trp administered.

Figure 4. Concentration-time changes in total concentrations of IS in serum after single (open circles) and multiple (closed circles) doses of L-Trp.

Subjects were administered L-Trp at a single dose of 2 g following multiple doses of 2 g daily for 8 days, respectively, in a non-fasting state at night, with an 11-day interval between the single and multiple doses. Subject 8 was withdrawn after the single-dose study due to an accidental headache, therefore, only the single-dose data is shown for subject 8 (E). Blood sampling was conducted until 35 h after the single dose (open circles) and after the last dose in the multiple-dose study (closed circles). It was also conducted on Day 1, 4 and 6 before each dose in the multiple-dose study (closed circles). The total concentrations of IS in serum were determined by HPLC. The subjects started to consume a low-Trp diet on the day of single administration of L-Trp and on the last administration of the multiple doses.

Figure 5. Urinary excretion rates of Trp and IS after single and multiple doses of L-Trp.

Five and four subjects were administered L-Trp at doses of 2 g as single and multiple doses of L-Trp, respectively. Urine sampling was conducted until 35 h after single (A) and multiple doses (B) of L-Trp. The amounts of Trp and IS in 35-h urine samples were determined by HPLC. The urinary excretion rates of Trp and IS were calculated as a percentage of the amounts of Trp and IS in the collected urine relative to L-Trp administered.

Figure 6. Concentration-time curves of total concentrations of IS in serum after intravenous administration of IS followed by quinapril (closed circles) or vehicle (open circles) in anesthetized rats.

Blood was taken before quinapril (at -2 min) and at 5, 15, 30, 45, 60 and 90 min after administration of IS. Quinapril was administered intravenously at a dose of 2 mg/kg 2 min before administration of IS. IS was administered at a dose of 0.5 mg/kg. There were 14 rats in the groups with and without quinapril, respectively. Data are expressed as the mean ± SE.

Figure 7. Concentration-time curves of total concentrations of IS in serum after intravenous administration of IS followed by probenecid (closed circles) or vehicle (open circles) in anesthetized rats.

Blood was taken before quinapril (at -2 min) and at 5, 15, 30, 45, 60 and 90 min after administration of IS. Probenecid was administered intravenously at a dose of 50 mg/kg 2 min before administration of IS. IS was administered at a dose of 5 mg/kg. There were 4 and 5 rats in the groups with and without probenecid, respectively. Data are expressed as the mean ± SE.

Figure 1

（A） （D）

（B） （E）

（C） （F）

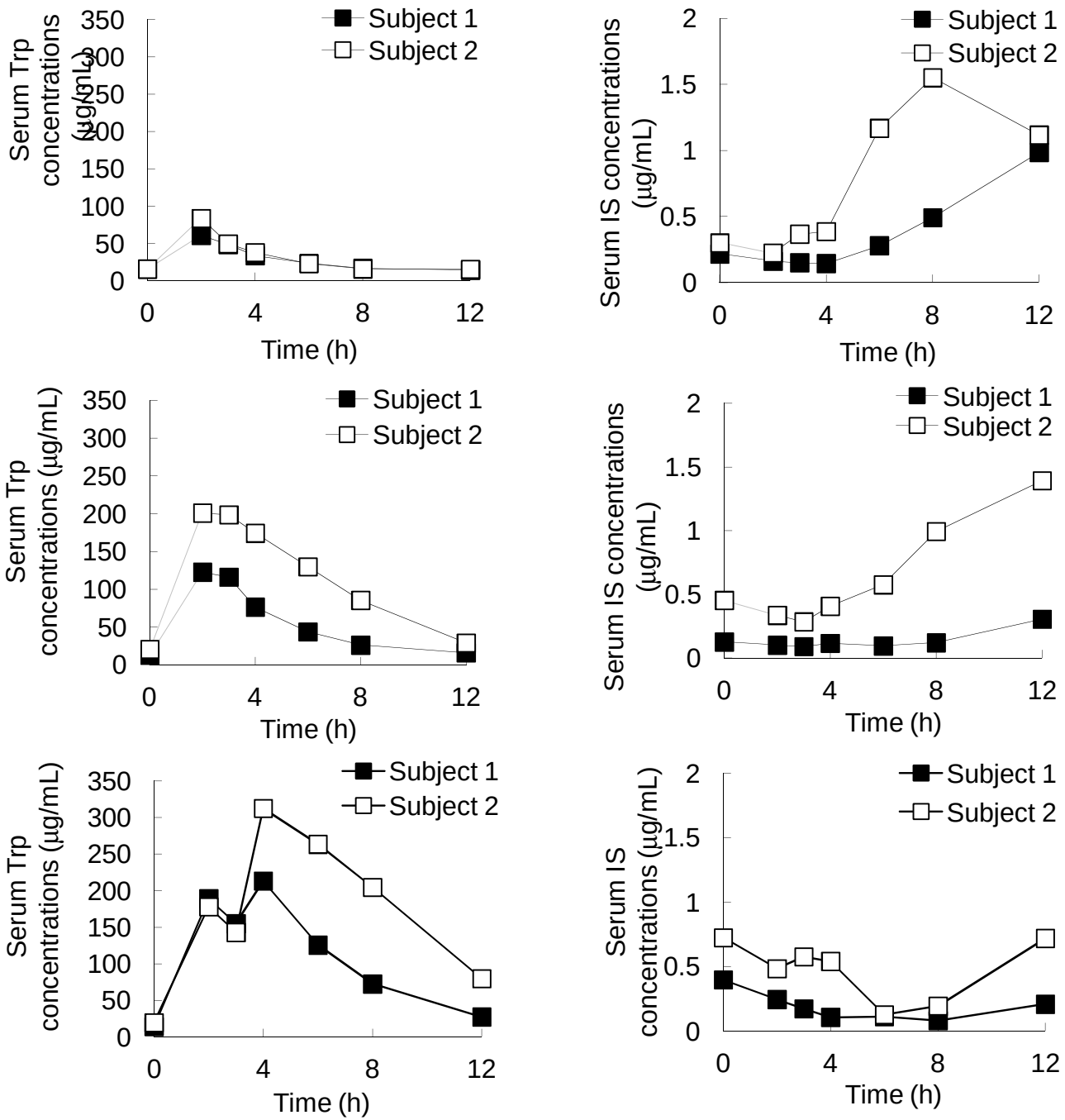
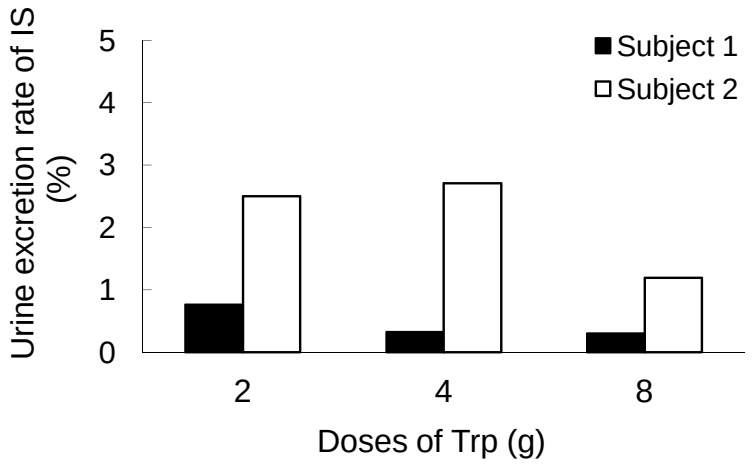


Figure 2

(A)

(B)



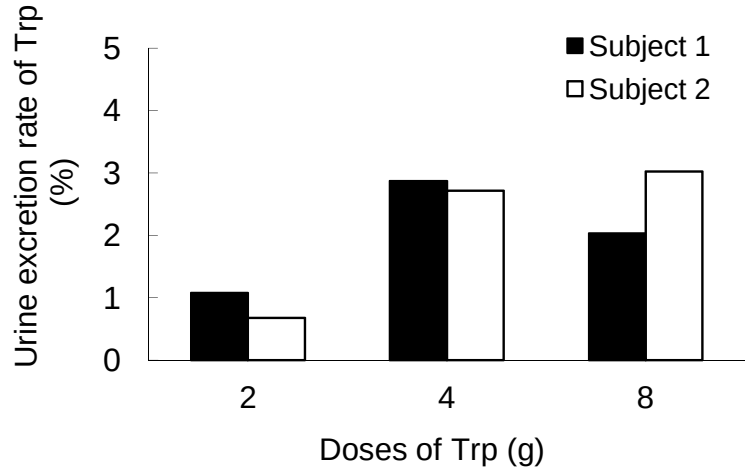
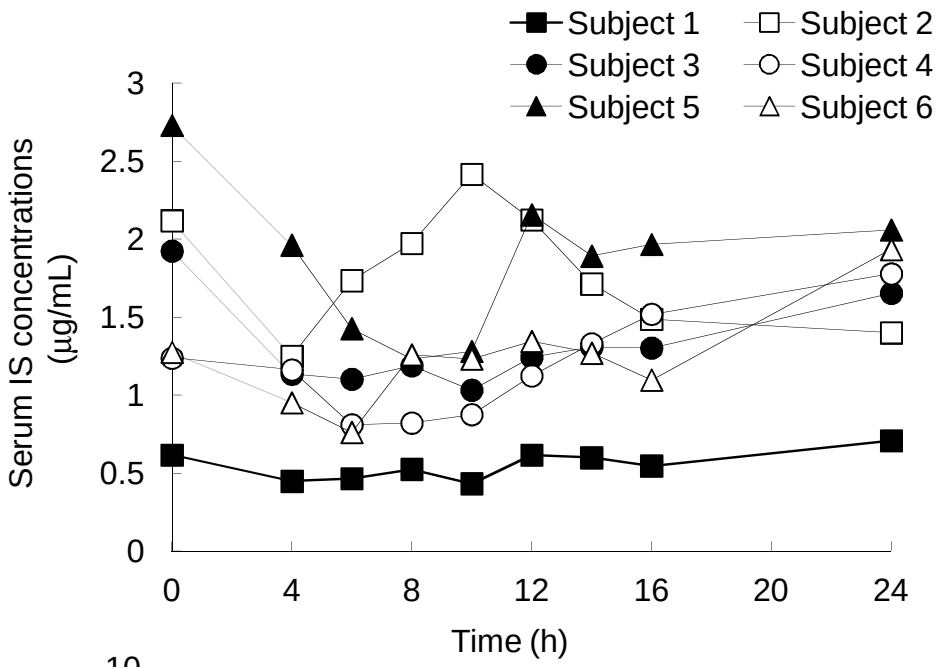


Figure 3

(A)

(B)



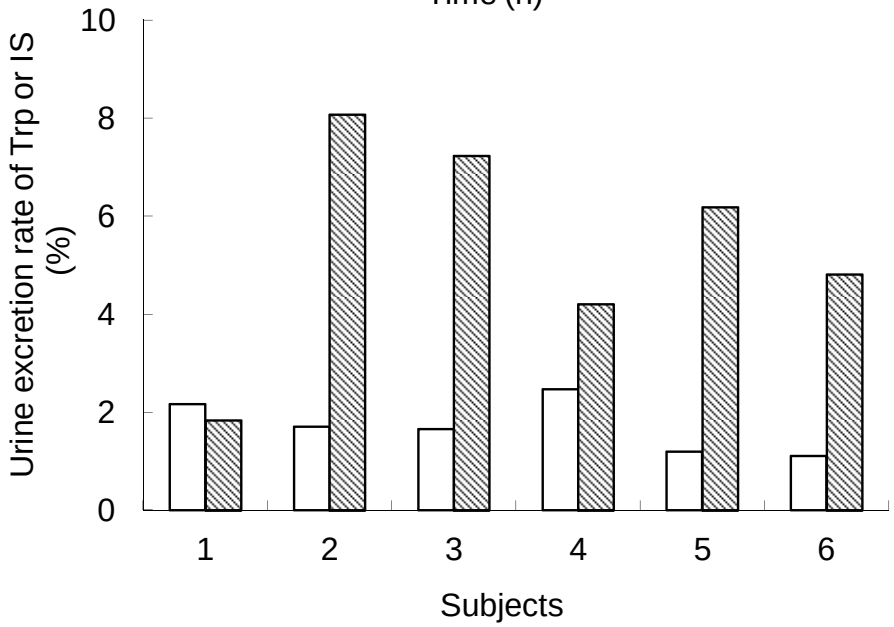


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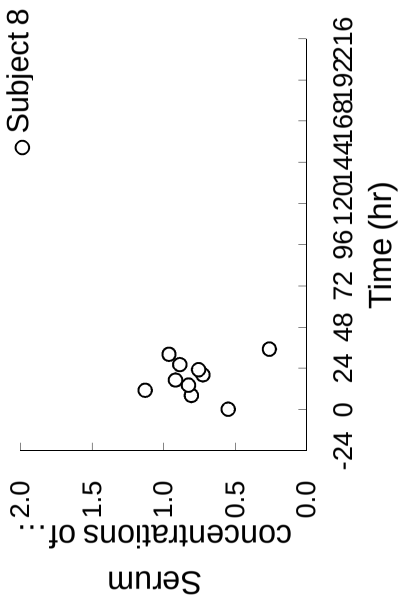
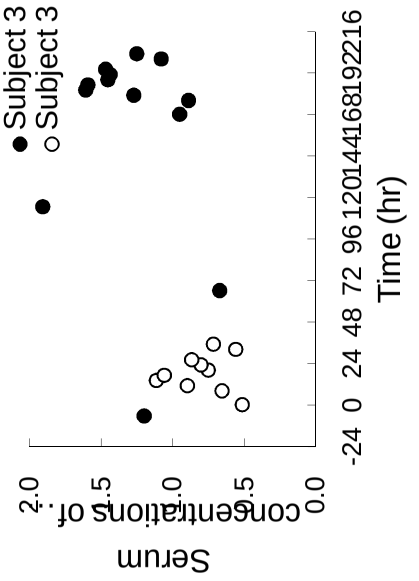
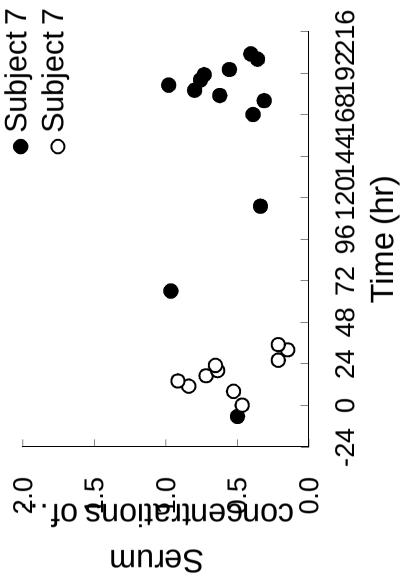
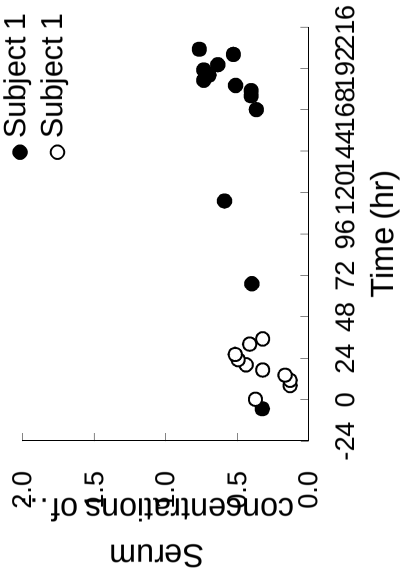
Figure 4 (A) (C) (E)
(B) (D)









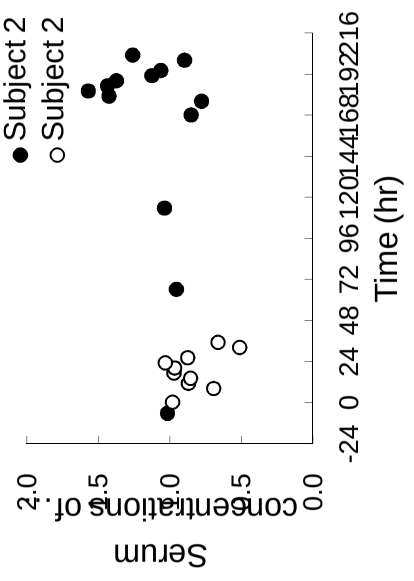


Figure 5

(A)

(B)

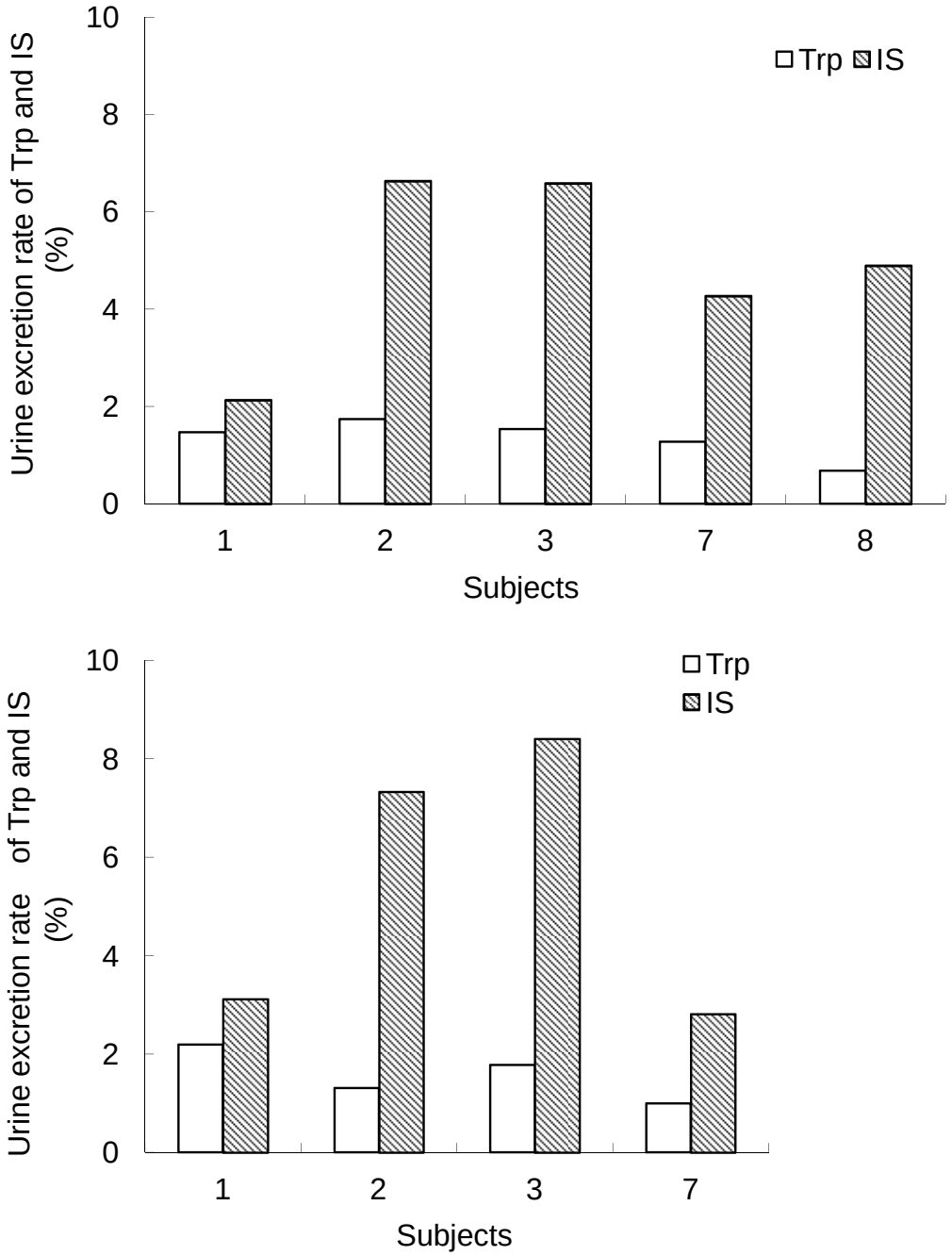


Figure 6

Figure 6

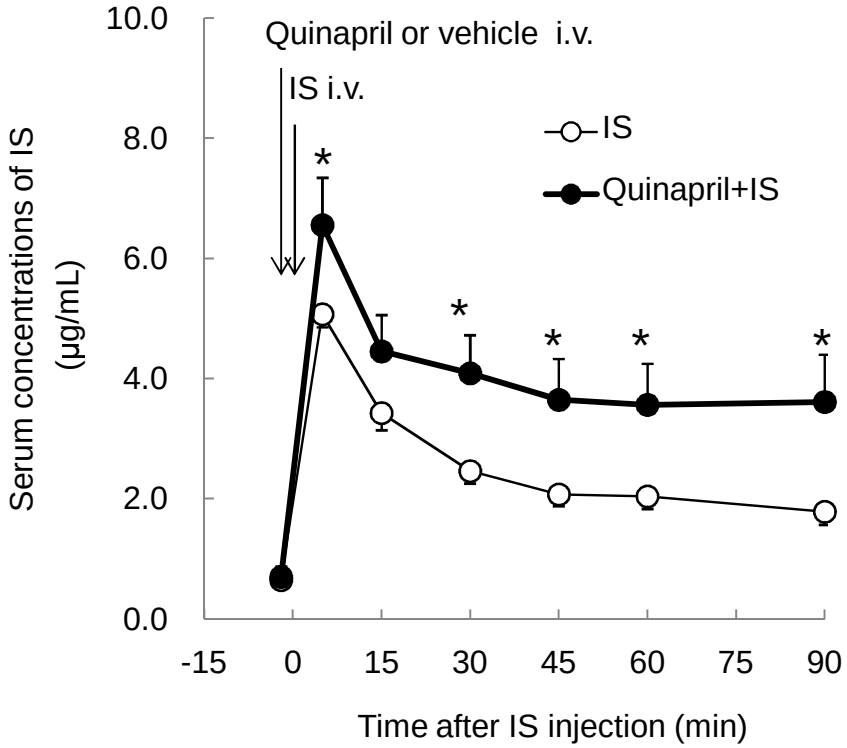


Figure 7

Figure 7