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Pharmacokinetics and Conjugation Metabolism of Naringin and Naringenin in Rats after Single Dose and Multiple Dose Administrations

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ABSTRACT

Naringin and naringenin are major flavonoids in citrus fruits. This study investigated the pharmacokinetics of naringin and naringenin in rats. Single dose of naringenin was administered to rats *via* intravenous bolus and oral route, whereas naringin was given orally as single dose and multiple doses (twice daily for 9 days). The serum concentrations of naringenin were assayed by HPLC method prior to and after hydrolysis with β-glucuronidase and sulfatase, respectively. After intravenous bolus of naringenin, naringenin sulfates and glucuronides emerged instantaneously, the profile of naringenin parent form declined rapidly. In contrast, when naringenin and naringin was orally administered, naringenin sulfates and glucuronides were found almost exclusively circulating in the bloodstream, whereas no naringin and naringenin was detected in most serum samples. After multiple-dose administration of naringin, naringenin sulfates and glucuronides gradually accumulated in the circulation and reached steady state on day 5. The profile of naringenin sulfates was much higher than that of naringenin glucuronides. In conclusion, the sulfates and glucuronides of naringenin were exclusively present in the circulation whether naringenin or naringin was orally given to rats. Therefore, pharmacologists should focus more on the bioactivities of conjugated metabolites of naringenin, in particular naringenin sulfates.

Key words: naringenin, naringin, pharmacokinetics, sulfates, glucuronides

INTRODUCTION

Polyphenols are widely distributed and abundant in herbs, plant foods and beverages. Accumulation of epidemiological and biochemical studies suggested a wide range of beneficial biological activities of polyphenols, including antioxidation, anti-inflammation, anticancer and antivirus[1-3]. Many studies suggested that polyphenols may be involved in cardioprotection, neuroprotection and chemoprevention. Among them, flavonoids are the largest group and have attracted increasing attention in recent years.

Naringin and naringenin (structures shown in Figure 1) are the major flavonoids in grapefruit (*Citrus paradisi* M_{ACF}.) and citrus herbs including fruits of *C. aurantium*, *C. grandis* var. *tomentosa* and *C. maxima*, which are widely used in clinical Chinese medicine. Naringin and naringenin were reported to possess various pharmacological activities including anti-ulcer[4], anti-microbial[5], superoxide scavenging[6], anti- oxidation[7] and anti-cancer effects[8-9]. However, many of them were *in vitro* results.

Till now, detailed information concerning the pharmacokinetics of naringin and naringenin is limited. Our previous study reported the pharmacokinetics of naringin and naringenin after single-dose administration to rabbits,

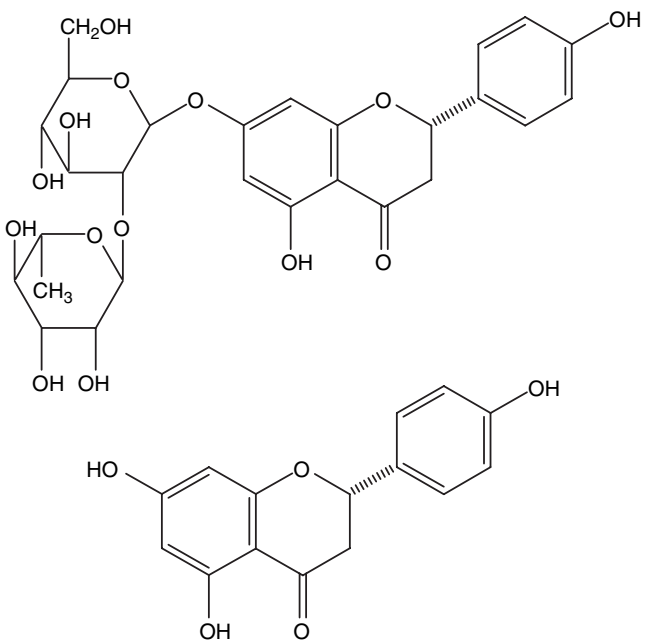

Figure 1. Chemical structures of naringin and naringenin.

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but only the mixed concentration of naringenin sulfates and glucuronides in serum had been determined. Our results revealed very poor oral bioavailbility of naringin[10]. This study attempted to investigate the pharmacokinetics of naringenin and naringin after single-dose and multiple-dose administrations to rats. Moreover, the detailed pharmacokinetics of naringenin sulfates and glucuronides were measured individually.

MATERIALS AND METHODS

I. *Chemicals*

Naringin (purity 95%), (±)-naringenin (purity 95%), β-glucuronidase (Type B-1, 666,400 units/g, from bovine liver) and sulfatase (Type H-1, 20,000 units/g, from *Helix pomotia*) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 5,7-Dimethoxycoumarin (99%) was purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile, methanol and ethyl acetate were of HPLC grade and were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). L(+)-Ascorbic acid was obtained from RdH Laborchemikalien GmbH & Co. KG (Seelze, Germany). Other reagents were of analytical grade. Milli-Q plus water (Millipore, Bedford, MA, USA) was used throughout this study.

II. *Instrumentation and HPLC Conditions*

The HPLC apparatus was equipped with a pump (LC-10AT, Shimadzu, Japan), a diode array detector (SPD-M10AVP, Shimadzu, Japan), an automatic injector (SIL-10A, Shimadzu, Japan) and a Cosmosil C18 column (5 μm, 4.6×150 mm, Nacalai tesque, Kyoto, Japan). The detection wavelength was set at 288 nm. The mobile phase consisted of acetonitrile – 0.1% phosphoric acid (36:64 v/v) and the flow rate was 1.0 mL/min for naringenin assay in serum.

III. *Drug administration and Blood Collection*

Rats were purchased from the National Science Council, ROC, and accommodated in the animal center of China Medical University. Before drug administration, male Sprague-Dawley rats weighing 300-350 g were fasted for 15 h and continued for another 3 h after dosing. For intravenous administration, naringenin was dissolved in a solvent mixture containing dimethylacetamide: PEG 400: H_2O (1:5:4) and filtered through a 0.22 μm membrane. The iv bolus was given to eight rats *via* the tail vein at a dose of 37 μmol/1 mL/kg (10 mg/kg) and blood samples (0.5 mL) were withdrawn *via* cardiac puncture prior to dosing and at 5, 15, 30, 60, 90, 120, 180, 240, 360, 480, 720 and 1440 min postdosing. The rats were anesthetized with ether before iv administration and cardiopuncture.

For oral administration, either naringin or naringenin was dissolved in the above-mentioned solvent mixture and administered *via* gastric gavage. Naringenin was given at a dose of 184 μmol/kg (50 mg/kg) and blood samples were withdrawn via cardiac puncture at 5, 15, 30, 60, 90, 120, 180, 240, 360, 480, 720 and 1440 min post dosing. Naringin was administered at two doses of 184 μmol/kg (107 mg/kg) and 367 μmol/kg (213 mg/kg) and blood collected at 5, 15, 30, 60, 90, 120, 180, 240, 360, 480, 720, 1440, 2880, 4320 and 5760 min.

In another study, six rats were orally given 367 μmol/kg (213 mg/kg) naringin twice daily for 9 days. Blood samples were withdrawn once daily before the morning dose and at 5, 15, 30, 60, 90, 120, 180, 240, 360, 480 and 720 min post the morning dose on day 9. All blood samples were centrifuged at 9860 g for 15 min, and then stored at –30°C for later analysis. The animal study adhered to "The Guidebook for the Care and Use of Laboratory Animals (2002)" (Published by The Chinese Society for the Laboratory Animal Science, Taiwan, R.O.C.).

IV. *Quantitation of Naringenin and Its Sulfates and Glucuronides in Serum*

The concentrations of sulfates and glucuronides of naringenin in serum were determined after β-glucuronidase and sulfatase treatments individually. For glucuronide quantitation, 100 μL of serum sample was mixed with 100 μL of β-glucuronidase (1000 units/mL in pH 5 buffer) and 100 μL of ascorbic acid (150 mg/mL) and incubated at 37°C for 2 h. As for sulfate quantitation, 100 μL of serum sample was mixed with 100 μL of sulfatase (1000 units/mL in pH 5 buffer) and 100 μL of ascorbic acid (150 mg/mL) and incubated at 37°C for 1 h. After hydrolysis, the serum sample was acidified with 100 μL of 0.1 N HCl and partitioned with 500 μL of ethyl acetate (containing 2.0 μg/mL of 5,7-dimethoxycoumarin as the internal standard). The ethyl acetate layer was evaporated under N_2 gas to dryness and reconstituted with an appropriate volume of acetonitrile, then 20 μL was subjected to HPLC analysis. For the assay of naringenin free form, 100 μL of serum sample was subjected to the process described above except for the addition of 100 μL of pH 5 buffer without β-glucuronidase or sulfatase.

For calibrator preparation, 100 μL of serum was spiked with various concentrations of naringenin, and then added with 100 μL of pH 5 buffer. The later procedure was the same as that described above. The calibration graph was plotted by linear regression of the peak area ratios (naringenin to the internal standard) against concentrations of naringenin.

V. *Validation of Assay Method of Naringenin in Serum*

The system suitability was evaluated through intra-day and inter-day analysis of precision and accuracy. The accuracy of this method was further assessed with

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recovery studies by spiking naringenin into blank serum and water in triplicates to afford 0.8, 6.3 and 50.0 μg/mL. In addition, the concentrations obtained in blank serum to the corresponding ones in water were compared. The LLOQ (lower limit of quantitation) represents the lowest concentration in a sample that can be determined with acceptable precision and accuracy, whereas LOD (limit of detection) represents the lowest concentration in a sample that can be detected (with S/N>3).

VI. *Data Analysis*

The concentrations of naringenin sulfates and glucuronides in serum were calculated by subtracting the free form concentration from the total naringenin concentrations obtained after hydrolysis with respective enzyme. The peak serum concentration (C_{max}) and the time to peak concentration (T_{max}) were obtained from experimental observation. The pharmacokinetic parameters were analyzed by noncompartmental model for intravenous bolus and oral administrations, with the aid of the program WINNONLIN (version 1.1 SCI software, Statistical Consulting, Inc., Apex, NC). The AUC_{0-t} was calculated using trapezoidal rule to the last point. The paired Student's *t*-test was used for statistical comparison of pharmacokinetic parameters, taking $P < 0.05$ as significant.

Table 1. Pharmacokinetic parameters of naringenin, its glucuronides and sulfates of eight rats after intravenous administration of naringenin (37 μmol/kg).

Parameters	Naringenin	Glucuronides	Sulfates
AUC_{0-720} ($nmol \cdot min \cdot mL^{-1}$)	333.7 ± 29.5^a	454.9 ± 130.6^a	12024.2 ± 1908.8^b
$T_{1/2}$ (min)	39.5 ± 29.3^a	71.6 ± 19.0^a	255.3 ± 48.7^b
MRT (min)	8.6 ± 1.2^a	38.0 ± 10.4^a	173.4 ± 19.0^b
Cl ($mL \cdot min^{-1}$)	31.9 ± 4.4	38.3 ± 15.7	2.7 ± 1.8
V (mL)	806.8 ± 286.2^a	2291.0 ± 357.7^b	855.3 ± 479.1^a

Data expressed as mean ± S.E. Means in a row without a common superscript differ, $P < 0.05$.

RESULTS

Good linear relationship was obtained in the concentration range of 0.8–50.0 μg/mL (y = 0.78x + 0.08, r = 0.9996) of naringenin in serum. The precision and accuracy of the method indicated that all coefficients of variation (CVs) and the relative errors were below 7.1% and 12.7%, respectively. The LLOQ and LOD for naringenin in serum were 0.8 and 0.02 μg/mL, respectively. The recoveries of naringenin from serum were 97.2, 98.0 and 99.0% for the concentrations of 0.8, 6.3 and 50.0 μg/mL, respectively.

Figure 2(a) depicts the mean serum concentration-time profiles of naringenin, its sulfates and glucuronides after intravenous bolus (iv) of naringenin, revealing that the profile of naringenin sulfates was much higher than that of naringenin parent form and glucuronides. The pharmacokinetic parameters are listed in Table 1, showing that the parent form, glucuronides and sulfates accounted for 2.6%, 3.6% and 93.8%, respectively, of the total exposure of naringenin derivatives. The mean elimination half-lives of naringenin parent form, naringenin glucuronides and sulfates were 39.5, 71.6 and 255.3 min, respectively.

After oral administration of naringenin, no free form naringenin was detected except for a few samples with very low level at early phase. The serum profiles of naringenin sulfates and glucuronides are shown in Figure 2(b). The pharmacokinetic parameters are listed in Table 2, showing that the glucuronides and sulfates accounted for 25.4% and 74.6% of the total naringenin absorbed, respectively. The C_{max} of naringenin sulfates was 1.6 times of naringenin glucuronides.

After oral administration of naringin, naringenin glucuronides and sulfates were detectable up to 24 and

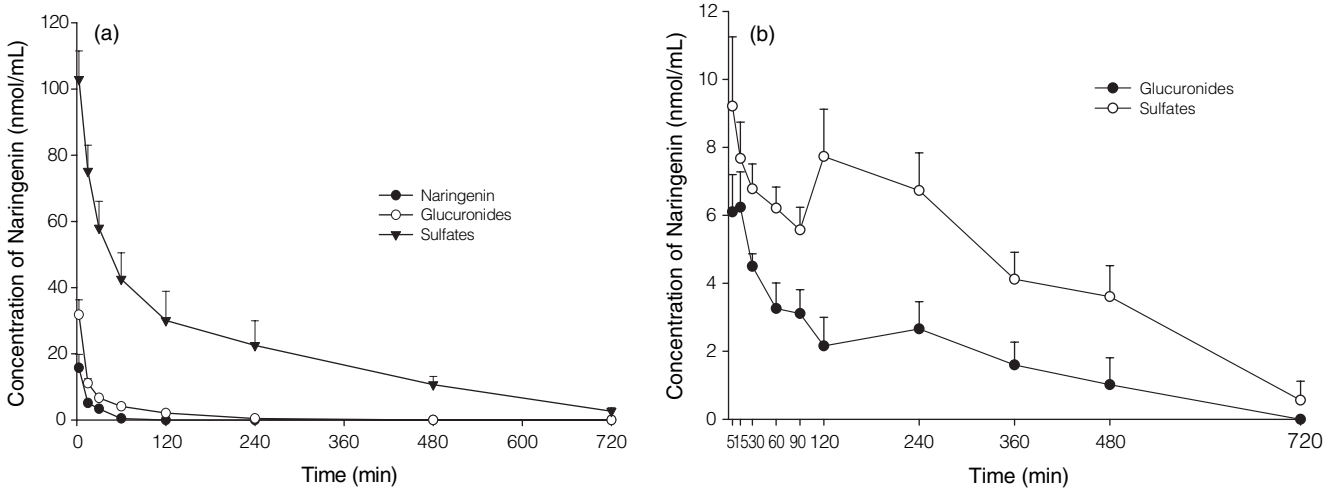

Figure 2. Mean (± S.E.) serum concentration-time profiles of naringenin, naringenin glucuronides and sulfates of eight rats after intravenous bolus of 37 μmol/kg naringenin (a) and oral administration of 184 μmol/kg naringenin (b).

96 h, respectively, whereas no naringin and naringenin was detected in most samples. After administration of 184 μmol/kg naringin, the serum profiles of naringenin sulfates and glucuronides are shown in Figure 3(a), and the pharmacokinetic parameters are listed in Table 3. The $AUC_{0\text{-}5760}$ and C_{max} of naringenin sulfates were 16.5 times and 6.4 times of those of naringenin glucuronides. After administration of 367 μmol/kg naringin, the serum profiles of naringenin sulfates and glucuronides are shown in Figure 3(b), and the pharmacokinetic parameters are listed in Table 3. The $AUC_{0\text{-}5760}$ and C_{max} of naringenin sulfates were 21.2 times and 3.9 times of naringenin glucuronides. The systemic exposure of naringenin sulfates after the higher dose was 1.3 times of the lower dose, whereas those of naringenin glucuronides were comparable between two doses.

The free form of naringenin was largely undetectable after multiple-dose oral administration of naringin (twice daily for 9 days). The profiles of naringenin sulfates and glucuronides in the serum sample before each morning dose indicated that naringenin sulfates and glucuronides were gradually accumulating in the circulation and reached steady state by day 5 (Figure 4(a)). Figure 4(b) depicts the serum concentration profiles of naringenin sulfates and glucuronides post the morning dose on day 9 and the pharmacokinetic parameters are listed in Table 4. The profile of naringenin sulfates was much higher than that of naringenin glucuronides. The $AUC_{0\text{-}720}$ and C_{max} of naringenin sulfates were 5.4 times and 2.3 times of naringenin glucuronides, respectively.

Table 2. Pharmacokinetic parameters of naringenin glucuronides and sulfates of eight rats after oral administration of naringenin (184 μmol/kg).

Parameters	Glucuronides	Sulfates
$AUC_{0\text{-}1440}$ (nmol·min·mL^{-1})	1009.3 ± 279.8	2958.2 ± 416.5**
C_{max} (nmol·mL^{-1})	7.4 ± 1.3	11.7 ± 1.6
T_{max} (min)	39.4 ± 17.8	94.4 ± 48.3
MRT (min)	128.4 ± 26.4	218.9 ± 20.6**

Data expressed as mean ± S.E. ** $P < 0.01$

DISCUSSION

Since authentic standards of naringenin glucuronides and naringenin sulfates are unavailable, their concentrations in serum cannot be determined directly. Serum samples were thus assayed prior to and after hydrolysis with β-glucuronidase and sulfatase in order to calculate the concentrations of naringenin sulfates and glucuronides. The conditions for enzymatic hydrolysis were optimized in terms of reaction time and the addition of

Table 3. Pharmacokinetic parameters of naringenin glucuronides and sulfates after single dose administration of 184μmol/kg and 367μmol/kg naringin.

Parameters \ Dose	184 μmol/kg (n = 8)		367 μmol/kg (n = 7)	
	Glucuronides	Sulfates	Glucuronides	Sulfates
$AUC_{0\text{-}5760}$ (nmol·min·mL^{-1})	302.3 ± 33.1^{a}	4985.7 ± 370.4^{b}	298.2 ± 74.4^{a}	6375.8 ± 651.8^{c}
C_{max} (nmol·mL^{-1})	0.6 ± 0.1^{a}	3.6 ± 0.2^{b}	1.5 ± 0.4^{a}	5.8 ± 0.9^{c}
T_{max} (min)	129.4 ± 46.3^{a}	135 ± 33.1^{a}	280 ± 72.5^{ab}	415 ± 65.0^{b}
MRT (min)	703.9 ± 114.5^{ab}	973.9 ± 79.6^{b}	385.8 ± 37.9^{a}	833.6 ± 159.7^{b}

Data expressed as mean ± S.E. Means in a row without a common superscript differ, $P < 0.05$.

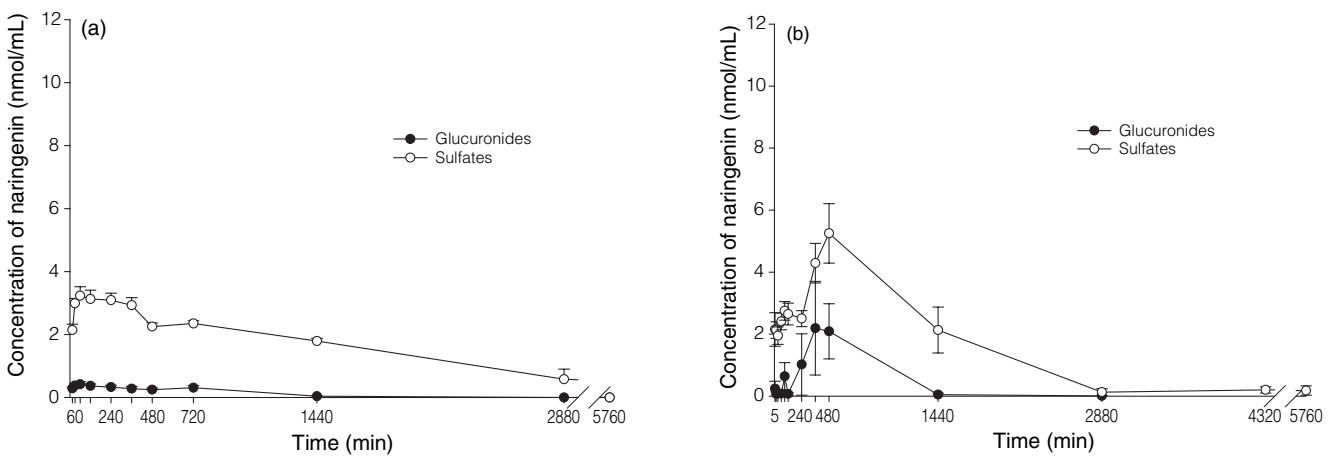

Figure 3. Mean (± S.E.) serum concentration-time profiles of naringenin glucuronides and sulfates of rats after 184μmol/kg (n = 8) (a) and 367μmol/kg (n = 7) (b) of naringin.

antioxidant. The results indicated that hydrolysis of naringenin glucuronides (2h) take longer time than sulfates (1h) as other flavonoids[11].

After intravenous bolus of naringenin (37 μmol/kg), the serum profiles of naringenin, its sulfates and glucuronides indicated very rapid and extensive conjugation metabolism of naringenin to yield sulfates as the major metabolites in the bloodstream. The AUC_{0-t} of naringenin sulfates constituted 93.8% of the total exposure of naringenin with its conjugates. In contrast to our previous study on rabbits, in which 60% of naringenin was metabolized to its sulfates/glucuronides[10], this rat study exhibited a more extensive sulfation metabolism of naringenin. The systemic exposure of naringenin sulfates in the previous rabbit study was probably underestimated due to the use of mixed glucuronidase/sulfatase (1104/42 unit/mL) which could result in incomplete hydrolysis of sulfates. The mean elimination half-life of naringenin sulfates was four times longer and clearance (Cl) was 14 times lower than that of naringenin glucuronides. The volume distribution of naringenin sulfates was 2.7 times lower than that of naringenin glucuronides. It suggested that the longer half-life and lower volume distribution of naringenin sulfates resulted in much lower clearance and higher systemic exposure. In a previous report, the bile clearance (Cl_b) of glucuronide metabolite of 6-hydroxy-5,7-dimethyl-2-methylamino-4- (3-pyridylmethyl) benzothiazole calculated based on the partition to hepatocytes was almost the same as the hepatic blood flow rate in rats. On the other hand, the Cl_b for the sulfate metabolite was only 30% of that[12]. In another study using isolated rat liver, the net efflux clearances (Cl_{eff}) of sulfates of 4-methylumbelliferone and p-nitrophenol calculated based on the unbound concentration from the sinusoidal membrane were much higher than that of glucuronides[13]. Likewise, the lower uptake or higher efflux of sulfates from the liver might explain the much higher concentration of naringenin sulfates than glucuronides in bloodstream.

When naringenin (184 μmol/kg) was administered orally, the sulfates and glucuronides of naringenin were found almost exclusively circulating in the bloodstream, whereas naringenin free form was negligible. The systemic exposure of naringenin sulfates was 3 times higher than naringenin glucuronides. The first peaks of naringenin sulfates and glucuronides occurred at 5 min, indicating very rapid absorption of naringenin and simultaneous sulfation/glucuronidation. The second peak of naringenin sulfates was observed at 120 min, implying enterohepatic circulation of these conjugated metabolites. Comparison of the systemic exposure of naringenin free form between iv and oral administrations of naringenin indicated almost null bioavailability of naringenin. Therefore, an extensive conjugation metabolism occurred in naringenin during the first pass through gut and liver, which was in good agreement with previous studies reporting that conjugation was central to flavonoid metabolism[14].

After naringin was administered orally, only little naringenin was detected, predominantly naringenin sulfates and less glucuronides were found in the bloodstream. The systemic exposure of naringenin sulfates was 16.5 times and 21.4 times higher than glucuronides at two doses of 184 μmol/kg and 367 μmol/kg. The greater difference than oral naringenin indicated a different sulfation/glucuronidation ratio between oral intake of naringenin and naringin. This might be accounted for by the different absorption site between glycoside and

Table 4. Pharmacokinetic parameters of naringenin glucuronides and sulfates of six rats after the morning dose on day 9.

Parameters	Glucuronides	Sulfates
AUC_{0-720} (nmol·min·mL^{-1})	1259.9 ± 356.8	6841.7 ± 325.9***
C_{max} (nmol·mL^{-1})	2.8 ± 0.5	6.4 ± 1.2
T_{max} (min)	130.0 ± 72.8	207.5 ± 116.2
MRT (min)	342.2 ± 15.3	374.6 ± 15.8

Data expressed as mean ± S.E. *** $P < 0.001$.

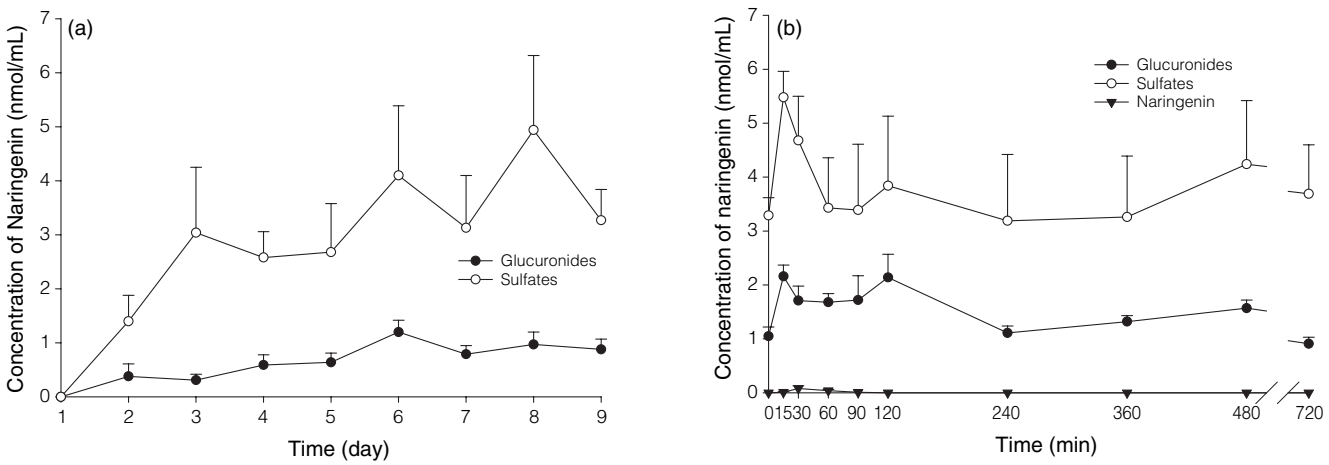

Figure 4. Mean (± S.E.) serum concentration-time profiles of naringenin, naringenin glucuronides and sulfates of six rats after oral administration of 367μmol/kg naringin twice daily for 9 days (a) and after the morning dose on day 9 (b).

aglycone. Moreover, oral naringin resulted in lower C_{max}, latter T_{max} and longer MRT of naringenin sulfates and glucuronides than those of oral naringin after dose correction. This could be explained by the gradual hydrolysis of naringin to naringenin before absorption. The comparison of the exposure of naringin metabolites between two doses of naringin revealed that sulfation and glucuronidation were saturated at the higher dose, indicating a nonlinear pharmacokinetics of naringin.

After multiple-dose oral administration of naringin, the serum concentration-time profiles showed that the conjugated metabolites gradually accumulated in the circulation and reached steady state by day 5. A pharmacokinetic study on day 9 showed that the AUC_{0-t} of naringenin sulfates and glucuronides was 1.1 and 4.2 times of those after single dose treatment, respectively, indicating that glucuronidation of naringenin might be induced after multiple-dose administration. On the contrary, sulfation was comparable between single-dose and multiple-dose administration. The profile of naringenin sulfate showed a rather stable mean blood levels above 3 μM during the dosing interval (12 h) and the system exposure was 5.4 times of naringenin glucuronides.

Previous *in vitro* studies on bioactivities of naringenin all focused on the naringenin parent form[8-9], but failed to report activities of the sulfates or glucuronides of naringenin. Based on the biological fates of naringenin and naringin after oral administration, the sulfates and glucuronides of naringenin are most likely to exert bioactivity and express beneficial effects in humans and animals. A recent study on morin glucuronides/sulfates demonstrated that the conjugated metabolites of morin were 1000 times more potent than morin aglycone in anti-inflammation[15]. On the other hand, human tissues including small intestine, liver and neutrophils are capable of effectively cleaving glucuronides to the aglycone[16]. Moreover, inflammation enables the release of aglycone from glucuronides metabolites because of the elevation of β-glucuronidase activity[17]. It is therefore of much interest to understand the tissue distribution and diverse *in vivo* effects of naringenin conjugated metabolites in their intact forms and as precursors of naringenin. The tissue distribution study is underwent in our laboratory.

In conclusion, naringenin were rapidly transformed into the sulfates and glucuronides of naringenin *in vivo* whether naringenin was given intravenously or orally. When naringin was given orally, naringenin sulfates and glucuronides were predominantly circulating in the bloodstream. Therefore, the bioactivities of naringenin sulfates and glucuronides in particular sulfates are worthy for investigation because they may determine the *in vivo* efficacy of naringenin and naringin.

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