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Original Article

Comparative pharmacokinetics of naringin and neohesperidin after oral administration of flavonoid glycosides from *Aurantii Fructus Immaturus* in normal and gastrointestinal motility disorders mice

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ABSTRACT

Objective: To compare the pharmacokinetics of naringin and neohesperidin after oral administration of Zhishi total flavonoid glycosides (ZSTFG) extracted from *Aurantii Fructus Immaturus* in normal and gastrointestinal motility disorders (GMD) mice.

Methods: ZSTFG was orally given to normal and GMD mice induced by atropine or dopamine. The plasma samples were incubated with β-glucuronidase/sulfatase, the total (free + conjugated) naringenin and hesperitin were extracted with acetonitrile. The validated HPLC-MS/MS method was successfully applied to the pharmacokinetic study.

Results: The results showed that, compared with the normal group, $AUC_{0-\infty}$, AUC_{0-t} and C_{max} for total naringenin and hesperitin were significantly higher ($P < 0.01$ or $P < 0.05$), while CL_Z/F for total naringenin and hesperitin was significantly lower ($P < 0.01$) in the GMD group. T_{max}, $t_{1/2z}$, MRT_{0-t}, and $MRT_{0-\infty}$ for naringenin were longer ($P < 0.01$) in the GMD group than those in the normal group.

Conclusion: The results showed that there were significant differences in pharmacokinetic parameters of naringenin and hesperitin between normal and GMD groups. It was suggested that the absorption of naringenin and hesperitin was increased, and the elimination processes of naringenin and hesperitin were slower in the GMD group than the normal group. The data are of value for further pharmacological studies of ZSTFG and would be useful to provide a reference for improving the therapeutic regimen of ZSTFG in clinical trials.

1. Introduction

Aurantii Fructus Immaturus, the fruitlet of *Citrus aurantium* L. or *Citrus sinensis* (L.) Osbeck, have been mainly used to treat gastrointestinal (GI) disorders such as stagnant, fullness, diarrhea, constipation and organ prolapse in China (Committee of National Pharmacopoeia of RP China, 2015). Recently, pharmacological and clinical researches have demonstrated that *Aurantii Fructus Immaturu* exert potent prokinetic activities in functional dyspepsia (FD) animals (Zhu, Zhang & Zhang, 2005) and in humans with FD (Zhang, 2007).

Flavonoids are considered to be the major bioactive constituents of *Aurantii Fructus Immaturus*. Naringin and neohesperidin as quality control markers for Zhishi products, were found abundantly (Xu, Xu, Zhang, Zhang & Liu, 2018), which can increase the gastric emptying rate and small intestinal propulsion rate, and promote gastrointestinal motility in normal mice (Yi et al., 2015) and in rats with FD (Huang, Chi & Lu, 2012). Neohesperidin can improve the symptoms of rats with gastric ulcer induced by indomethacin, mainly through the expression of a stomach cyclooxygenase 2 (COX 2) and DNA fragments (Hamdan, Mahmoud, Wink & El-Shazly, 2014).

Zhishi total flavonoid glycosides (ZSTFG) extracted from *Aurantii Fructus Immaturus*, mainly including naringin and neohesperidin developed by the Jiangxi Qingfeng Pharmaceutical Co., Ltd., being a category of five new drugs of traditional Chinese medicine (TCM) for gastrointestinal motility disorders (GMD) treatment. The company has received the approval for clinical studies from the Chinese State Food and Drug Administration. In our previous

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in vivo study, ZSTFG increased the gastric emptying rate and small intestinal propulsion rate in normal mice and inhibited the reduction in gastric emptying and small intestinal motility in GMD mice induced by atropine or dopamine.

Several reports suggested that naringin and neohesperidin were poorly absorbed from the gastrointestinal tract in its original form (Kanaze, Bounartzi, Georgarakis & Niopas, 2007), the free form of naringin and neohesperidin were transiently present in the plasma, and the glucuronides/sulfates of naringenin and hesperitin were the main circulating metabolites (Fang et al., 2006; Felgines et al., 2000; Hsiu, Huang, Hou, Chin & Chao, 2002; Manach, Morand, Gil-Izquierdo, Bouteloup-Demange & Rémésy, 2003). In the present study, we chose naringin and neohesperidin as the representative components, and developed a highly sensitive HPLC-MS/MS method for simultaneous determination of naringenin and hesperitin by hydrolysis with β-glucuronidase/sulfatase in mouse plasma after oral administration of ZSTFG and further to apply it to the pharmacokinetics of normal and GMD mice induced by atropine or dopamine. It was expected that the results of this study would be helpful for establishing clinical project of treatment and pharmacological studies of ZSTFG. The chemical structures of naringin, naringenin, neohesperidin and hesperitin and the internal standard (IS) hexadecadrol are shown in Fig. 1.

2. Materials and methods

2.1. Chemicals and reagents

ZSTFG (total flavonoid glycosides 93.6%; naringin 29.5%, neohesperidin 32.5%) was provided from Jiangxi Qingfeng Pharmaceutical Co., Ltd. Naringin (HPLC $\geq$ 98%) and neohesperidin (HPLC $\geq$ 98%) standards were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Naringenin (HPLC $\geq$ 98%) and hesperitin (HPLC $\geq$ 98%) were purchased from Tianjin Science and Technology Co., Ltd., (Tianjin, China). Hexadecadrol was purchased from Sigma-Aldrich. β-glucuronidase (IX-A from *E. Coli*) and sulfatase (H-1 from *Helix pomatia*) were purchased from Sigma-Aldrich (Shanghai, China). Methanol and ammonium formate (HPLC-grade) were purchased from Merck (Merck, Germany). All other reagents were analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd., China. Water was purified using a Watson's water purification system (Guangzhou Watson's Food & Drinks Co., Ltd.)

2.2. Animals

Male KM mice [body weight (20 $\pm$ 2) g] were provided by the Experimental Animal Center of Fudan University (license number: SCXK 2014-0004). The animals were kept under controlled conditions: 12 h/d fluorescent light (07:00–19:00), humidity: (55 $\pm$ 5)%, (25 $\pm$ 2) °C room temperature for one week acclimation before experiment and were allowed free access to standard laboratory diet and water during the period. All animal studies were performed following Shanghai Institute of Pharmaceutical Industry Ethics Committee Guidelines for the Care and Use of Laboratory Animals.

2.3. HPLC-MS/MS conditions

Chromatography was performed on an HPLC/MS/MS system equipped with an UltiMate 3000 integrated HPLC (Dionex, USA) and a triple quadrupole mass spectrometer with trap function (API 4000 QTrap, AB SCIEX Toronto, Canada). Data acquisition was performed with an analyst 1.5.2 workstation (AB SCIEX USA). Chromatographic separation was performed on a Kromasil® C_{18} column (250 mm $\times$ 2.1 mm i.d., particle size 5 µm, Kromasil, Sweden). The mobile phase consisted of 10 mmol/L ammonium formate (B) and methanol (A) at a flow rate of 0.3 mL/min. The gradient elution program was as follows: 0 min, 30% B; 0.1 min, 90% B; 2.2 min, 10% B; 2.3 min, 30% B; 5 min, 30% B. The injection volume was 5 µL and the column temperature was 30 °C.

2.4. Preparation of calibration standards and quality control (QC) samples

Naringenin and hesperitin standards were accurately weighed and dissolved in methanol respectively, then mixed together. The concentrations of mix stock solution of naringenin and hesperitin were 0.5 mg/mL respectively, IS (0.2 mg/mL) was prepared in methanol, working solutions were prepared in methanol by appropriate dilution of the stock solution. The calibration standards were prepared by spiking 50 µL of blank plasma, 20 µL of β-glucuronidase, 20 µL of sulfatase (250 U dissolved with phosphate buffer), and 2.5 µL of standard working solution. The effective concentrations in plasma samples were prepared at concentrations of 0, 2, 10, 50, 100, 200, and 500 ng/mL. The quality control (QC) solutions were prepared at concentrations of 4, 80, and 400 ng/mL in a similar way to the calibration standards. These samples were

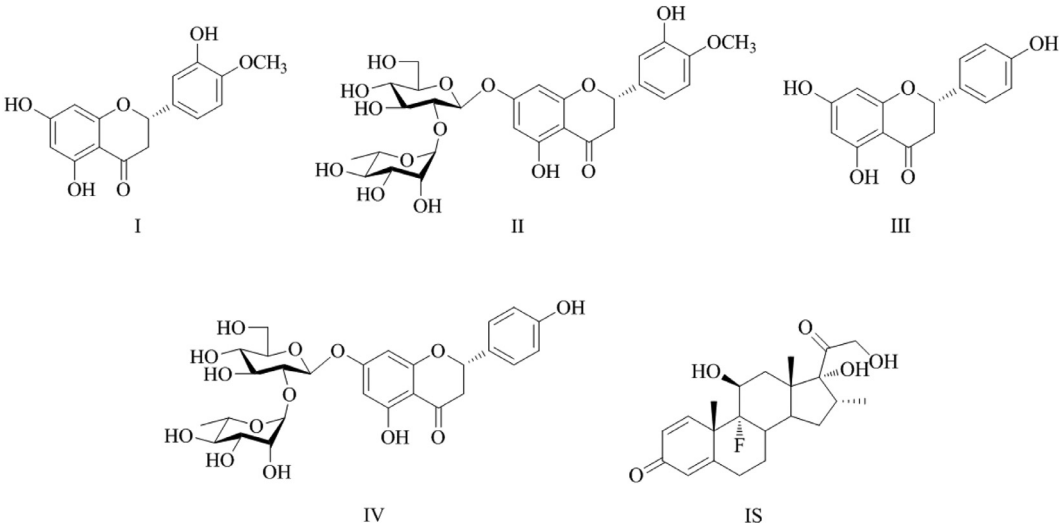

Fig. 1. Chemical structures of hesperitin (I); neohesperidin (II), naringenin (III), naringin (IV), and internal standard (IS) hexadecadrol.

stored at −80 °C until use. QC samples were used to determine the precision, recovery, matrix effect and stability.

2.5. Sample preparation

The 20 μL of β-glucuronidase and 20 μL of sulfatase were added into 50 μL of plasma sample, after vortex for 30 s, the mixture was incubated at 37 °C for 1 h, 5 μL of IS solution (500 ng/mL) were added into the mixture, and then stopped the incubation with 270 μL ice-cold acetonitrile by vortex mixing for 1 min. The mixture was centrifuged at 13 000 rpm for 10 min, and 5 μL of supernatant was injected into the HPLC/MS/MS system. All procedure for sample preparation was performed at room temperature.

2.6. Method validation

2.6.1. Linearity of calibration curve and lower limit of quantification

Each calibration curve of naringenin and hesperitin was constructed in six different concentrations by using weighed linear regression of the peak area ratios of the two flavonoids versus the IS (Y) against the concentrations of the calibration standards (X). The lower limit of quantification (LLOQ) of the assay was defined as the lowest concentration on the standard curve that can be quantitated with accuracy within 20% bias of the nominal concentration and precision not exceeding 20% (US Department of Health & Human Services, FDA, 2001).

2.6.2. Specificity

Specificity was assessed by analysis of six different samples of blank matrix with and without spiking with naringenin, hesperitin, and IS.

2.6.3. Accuracy and precision

The precision and accuracy were evaluated by assaying six replicates of QC samples at low, medium, and high concentrations on the same day and over 3 d consecutively. The accuracy was expressed by the relative error (RE), which should be within 15%, except at low concentration, where it should not deviate by more than 20%. The precision was evaluated by the relative standard deviation (RSD), which should not exceed 15%, except at low concentration, where it should not exceed 20% according to FDA (US Department of Health & Human Services, FDA, 2001).

2.6.4. Extraction recovery and matrix effect

The extraction recoveries of naringenin and hesperitin were evaluated at the three QC concentrations with five replicates by comparing the mean peak areas from extracted samples with those from post-extracted blank plasma samples spiked with the standard solutions at equivalent concentrations. The absolute matrix effects of the two components were assessed by comparing the mean peak areas of the post-extracted blank plasma samples spiked with the standard solutions with those of equivalent standards prepared with mobile phase. The extraction recovery and absolute matrix effect of IS was assessed at a single concentration of 500 ng/mL as the same procedure.

2.6.5. Stability

The stabilities of QC samples were determined by analyzing samples stored at room temperature for 4 h, at −80 °C for 4 weeks and three successive freeze (−80 °C) and thaw (room temperature) cycles. The QC samples were stored at −80 °C for 24 h and thawed unassisted at room temperature.

2.7. Atropine, dopamine induced GMD mouse model

Mice were randomly divided into normal, atropine and dopamine group. Mice were fasted overnight. Atropine solution (2.5 mg/kg, ip), or dopamine solution (2 mg/kg, ip) was injected. Normal group were intraperitoneally administered the same volume of saline and Evans blue solution (0.1%, *w*/*v*, in DW) was administered orally 30 min after the atropine or dopamine injection. Each mouse was sacrificed by decapitation 20 min after receiving the Evans blue solution. The length from the pylorus to the front of the Evans blue (A) and the total length of the small intestine (B) were measured 30 min after injection. Intestinal transit rate (ITR) (%) was expressed as a percentage: ITR (%) = $A/B \times 100$.

2.8. Collection of plasma samples

Mice were randomly divided into normal, atropine and dopamine group, with 36 mice in each. After fasted for 12 h with free access to water, the mice were injected saline, atropine solution, or dopamine solution as described above, 30 min after injection, each mouse was given ZSTFG at a dose of 40 mg/kg (equivalent to 11.8 mg/kg of naringin, and 13.0 mg/kg of neohesperidin) by oral administration. Blood samples were collected in heparinized tubes via the postorbital venous plexus veins from each six mice before and at 0.08, 0.25, 0.3, 1, 2, 3, 4, 6, 8, 10, 12, and 24 h after dosing. The blood was centrifuged at 8000 rpm for 10 min. Plasma was transferred into clean tubes and stored at −80 °C until analysis.

2.9. Statistical analysis

Non-compartmental pharmacokinetic parameters were calculated by DAS 2.1 pharmacokinetic program (Chinese Pharmacological Society). Significant difference between normal and GMD groups was determined using SPSS 16.0 (Statistical Package for the Social Science) by independent sample *t*-tests after natural logarithmic transformation for AUC_{0-t}, $AUC_{0-\infty}$, C_{max} and by the nonparametric Mann-Whitney test for t_{max}, $t_{1/2z}$, MRT_{0-t} and CLz/F. *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Atropine, dopamine induced GMD mouse model

Intestinal transit rate (ITR) were significantly ($P < 0.05$) decreased in either atropine or dopamine treated group compared with normal group (Table 1), which proved the success of the model.

3.2. Specificity

The MRM mode provided high selectivity and sensitivity for the quantification assay (Fig. 2). The representative chromatograms of naringenin, hesperitin and IS under the optimized conditions and the results showed that no interferences existed under the present analytical conditions.

Table 1
Intestinal transit rate of Evans blue in normal and gastrointestinal motility disorder mice (mean ± SD, $n = 10$).

Groups	Dose/(mg·kg^{-1})	Intestinal transit rate (ITR)/%
Normal	/	65.3 ± 5.6
Atropine	2.5	39.5 ± 5.9**
Dopamine	2	46.0 ± 6.6**

** $P < 0.01$ *vs* normal group.

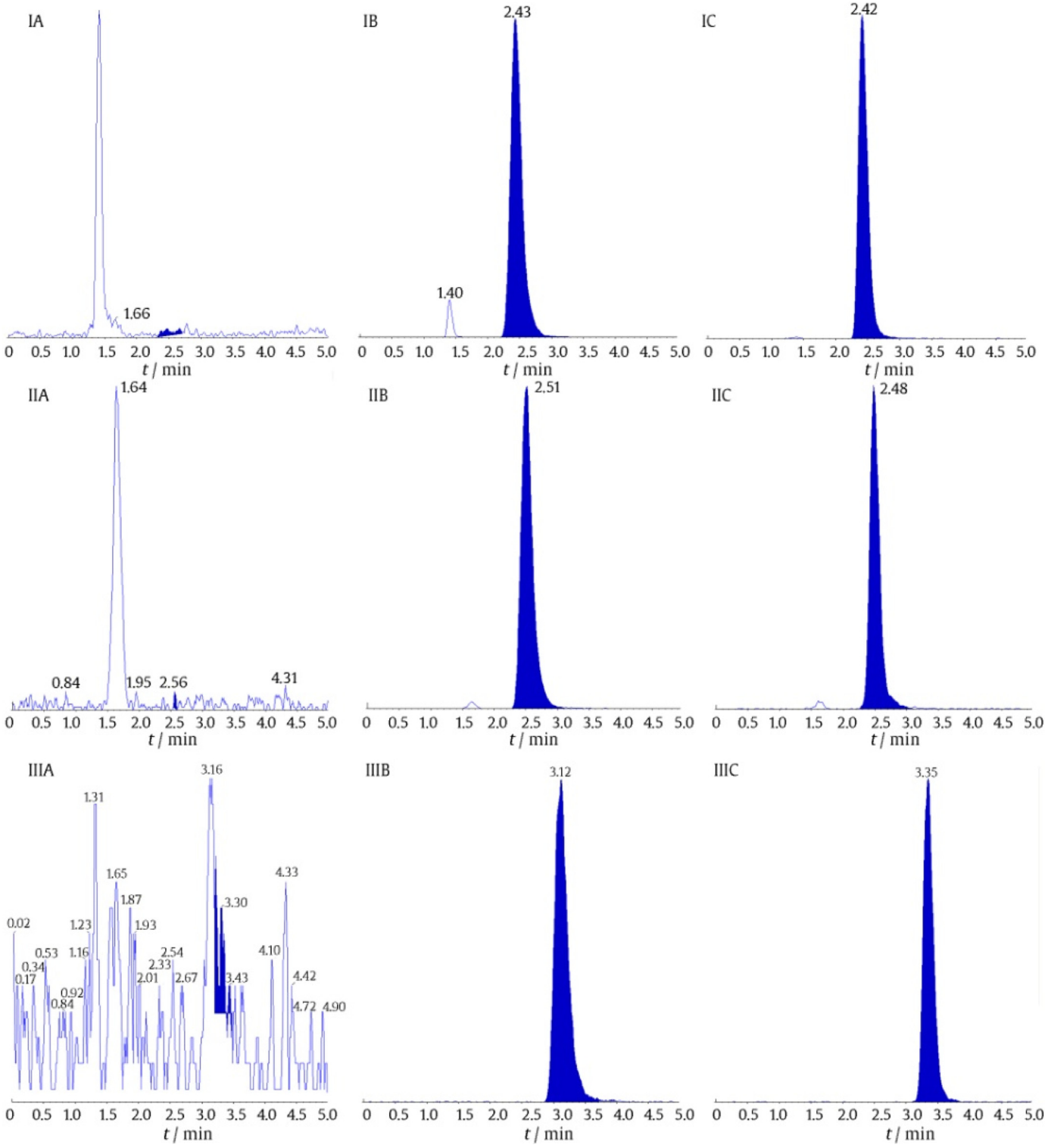

Fig. 2. Representative chromatograms of naringenin (I), hesperitin (II) and IS (III) in blank plasma (A), plasma sample spiked with standards (B) and plasma samples (C) after oral administration of ZSTFG.

3.3. Linearity of calibration curve and LLOQ

Linear calibration curves were obtained within the concentration range of 0.0 – 500 ng/mL for the two components in the plasma using weighed ($1/x^2$) least-squares linear regression analysis. Representative regression equations and LLOQ of the compounds were calculated as follows: Naringenin, $Y = 0.0313X + 0.00725$ ($r = 0.9989$); Hesperitin, $Y = 0.0120X + 0.00278$ ($r = 0.9999$). The coefficients of variation were 9.5% and 9.6%, respectively. The LLOQs for naringenin and hesperitin in plasma were both 2.0 ng/mL.

3.4. Precision and accuracy

Intra-day precision was less than 10.9% for both naringenin and hesperetin. Intra-day accuracy was between −7.65% to 2.15% for both flavanone aglycones (Table 2). Inter-day precision was less than 10.7% for both naringenin and hesperetin. Inter-day accuracy

for naringenin was between −7.15% to −1.4%, for hesperetin was between −3.76% to −2.28% (Table 2). Since both relative error and relative standard deviation values did not exceed ± 15%, this indicated acceptable degree of accuracy and precision of the proposed method.

3.5. Extraction recovery and matrix effect

The extraction recovery and matrix effect for naringenin and hesperitin were summarized in Table 3. The extraction recoveries were in the range of 74.9% to 83.7% and the extraction recovery of IS was 77.3%, which indicated that recoveries were consistent and reproducible. The matrix effects of naringenin and hesperitin were in the range of 86.5%–90.8%, and the matrix effect of IS was 97.9%. The results showed that there was no obvious matrix effect for naringenin, hesperitin, and IS.

3.6. Stability

The two analytes in plasma exhibited good stability during storage at room temperature for 4 h (RSD: 4.31%–7.11% for naringenin, 4.13%–9.01% for hesperitin), three freeze-thaw cycles (RSD: 6.46%–13.46% for naringenin, 5.47%–12.24% for hesperitin) and at −80 °C for 4 weeks (RSD: 5.11%–6.69% for naringenin, 7.89%–10.47% for hesperitin).

3.7. Pharmacokinetics

The validated analytical method was applied to a comparative pharmacokinetic study of naringenin and hesperitin in normal and GMD mice after oral administration of ZSTFG.

The plasma concentration-time profiles were shown in Fig. 3 and the corresponding pharmacokinetic parameters were shown in Tables 4 and 5. The results demonstrated that there were significant differences in pharmacokinetic parameters, including AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, t_{max}, $t_{1/2z}$, and CL_Z/F for naringenin and hesperitin between normal and GMD groups. Remarkable increases ($P < 0.01$ or $P < 0.05$) in the values of $AUC_{0-\infty}$, AUC_{0-t} and C_{max} for naringenin and hesperitin, significant decrease ($P < 0.01$) of CL_Z/F for naringenin and hesperitin, and longer t_{max}, $t_{1/2z}$, MRT_{0-t} and $MRT_{0-\infty}$ ($P < 0.01$) for naringenin were observed in the GMD group compared with the normal group.

Table 2
Intra-day and Inter-day precision and accuracy of naringenin and hesperritin in mouse plasma (mean ± SD, $n = 6$).

Analytes	Spiked/(ng·mL^{-1})	Intra-day			Inter-day		
		Measured/(ng·mL^{-1})	RSD/%	RE/%	Measured/(ng·mL^{-1})	RSD/%	RE/%
Narringenin	4	4.009 ± 0.345	8.61	0.23	3.944 ± 0.361	9.16	−1.40
	80	73.88 ± 4.001	5.42	−7.65	74.28 ± 3.768	5.07	−7.15
	400	400.6 ± 23.19	5.79	0.15	380.4 ± 30.53	8.03	−4.90
Hesperitin	4	3.790 ± 0.415	10.9	−5.25	3.880 ± 0.414	10.7	−3.00
	80	75.94 ± 4.234	5.57	−5.08	76.99 ± 4.083	5.30	−3.76
	400	408.6 ± 22.43	5.49	2.15	390.9 ± 29.37	7.51	−2.28

Table 3
Matrix effect and recovery of naringenin and hesperitin in mouse plasma.

Analytes	Spiked/(ng·mL^{-1})	Matrix effect/%	Recovery/%
Naringenin	4	90.3	74.9
	80	86.5	80.0
	800	87.7	83.7
Hesperitin	4	90.8	80.2
	80	86.7	83.7
	800	89.6	82.1
IS	500	97.9	77.3

4. Discussion

Recently, the pharmacokinetic studies of flavanone aglycones naringenin, hesperitin and their glycosides naringin, neohesperidin have been previously published in several articles and different pharmacokinetic characters have been observed in rabbits (Hsiu et al., 2002), rats (Chen et al., 2014; Fang et al., 2006; Li, Xiao, Liang, Shi & Liu, 2004; Silberberg et al., 2006; Tong et al., 2012), beagles (Mata-Bilbao Mde et al., 2007) and in human

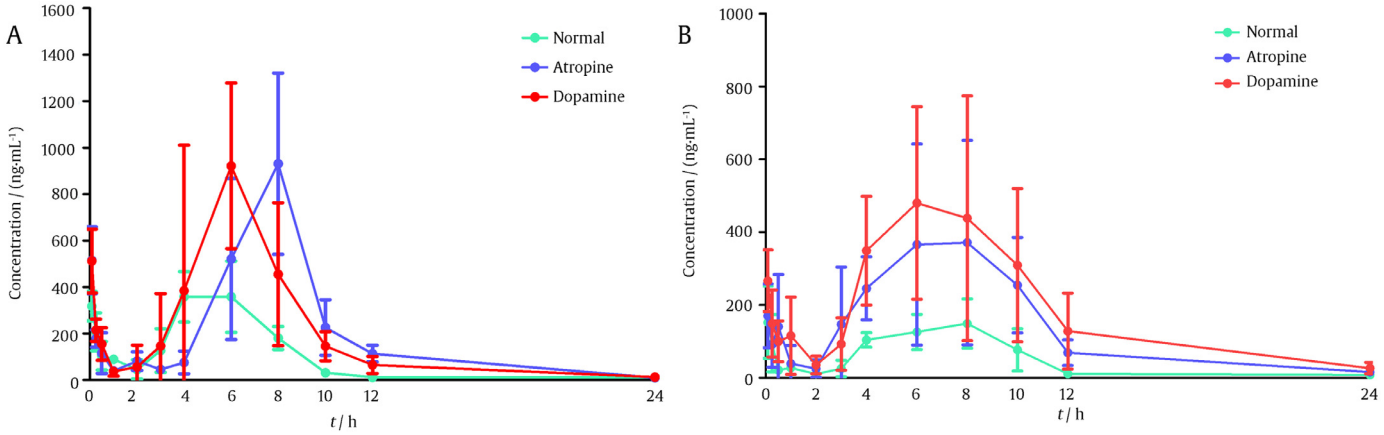

Fig. 3. Plasma concentration-time curves of naringenin (A) and hesperitin (B) after oral administration of ZSTFG (40 mg/kg (11.8 mg/kg of naringin, and 13.0 mg/kg of neohesperidin)) to normal and GMD mice.

Table 4
Pharmacokinetic parameters of naringenin in normal and GMD mouse plasma after oral administration of ZSTFG (mean ± SD, $n = 6$).

Parameters	Units	Naringenin		
		Normal	Atropine	Dopamine
AUC_{0-t}	$ng \cdot h^{-1} \cdot mL^{-1}$	2591.10 ± 484.69	4837.35 ±1256.01**	4179.54 ±1623.69*
$AUC_{0-\infty}$	$ng \cdot h^{-1} \cdot mL^{-1}$	2591.12 ± 484.68	4870.51 ±1253.10**	4230.98 ±1639.26*
MRT_{0-t}	h	5.77 ± 0.43	8.16 ± 0.66**	7.37 ± 0.74**
$MRT_{0-\infty}$	h	5.95 ± 0.44	8.45 ± 0.76**	7.66 ± 0.87**
$t_{1/2z}$	h	1.19 ± 0.15	2.71 ± 1.10**	2.98 ± 1.05**
t_{max}	h	4.67 ± 1.03	7.33 ± 1.03**	6.67 ± 1.03**
C_{max}	$ng \cdot mL^{-1}$	651.33 ± 230.72	1008.67 ± 320.36*	982.22 ± 304.29*

* $P < 0.05$, ** $P < 0.01$ *vs* normal group.

Table 5
Pharmacokinetic parameters of hesperitin in normal and GMD mouse plasma after oral administration of ZSTFG (mean ± SD, $n = 6$).

Parameters	Units	Hesperitin		
		Normal	Atropine	Dopamine
AUC_{0-t}	$ng \cdot h^{-1} \cdot mL^{-1}$	1501.22 ± 249.86	2577.85 ± 931.08*	4078.04 ± 1451.55**
$AUC_{0-\infty}$	$ng \cdot h^{-1} \cdot mL^{-1}$	1525.01 ± 241.85	2670.46 ± 956.39*	4195.33 ± 1515.59**
MRT_{0-t}	h	7.72 ± 0.68	8.19 ± 0.49	8.29 ± 0.76
$MRT_{0-\infty}$	h	8.30 ± 0.99	8.97 ± 0.50	8.97 ± 1.04
$t_{1/2z}$	h	2.95 ± 1.85	4.03 ± 0.85	3.39 ± 0.94
t_{max}	h	7.00 ± 1.10	5.35 ± 2.98	6.01 ± 3.55
C_{max}	$ng \cdot mL^{-1}$	298.15 ± 96.25	412.43 ± 192.87*	589.83 ± 317.94*
CLz/F	$L \cdot kg^{-1} \cdot h^{-1}$	26.71 ± 3.69	16.42 ± 5.04**	10.8 ± 4.4**

* $P < 0.05$, ** $P < 0.01$ *vs* normal group.

(Kanaze et al., 2007; Zhang & Brodbeh, 2004). However, comparative studies on the pharmacokinetics of these active compounds between normal and gastrointestinal motility disorder mice in plasma after oral administration of ZSTFG have not been reported. The findings of this study indicated for the first time that hesperitin and naringenin in ZSTFG have different pharmacokinetic characters in normal and GMD mice.

Gastrointestinal motility is regulated by various neurotransmitters such as 5-hydroxytryptamine (5-HT) (Mine et al., 1997; Takeda et al., 2008; Tominaga et al., 2011), dopamine (Cooper & McRitchie, 1985; Iwanaga et al., 1991; Li, Schmauss, Cuenca, Ratcliffe & Gershon, 2006) and acetylcholine (Decktor, Pendleton, Elinistsky, Jenkins & McDowell, 1988; Fujii et al., 2002). Atropine is an acetylcholinesterase inhibitor. Hence, atropine and dopamine are regularly used for establishing GMD animal model.

Naringin and hesperidin are hydrolyzed in the gastrointestinal tract by the enzymes of intestinal bacteria followed by absorption and conjugation of their aglycones, the main present forms are naringenin and hesperitin glucuronide/sulfatase conjugations and a small amount of the free aglycones in plasma (Ameer, Weintraub, Johnson, Yost & Rouseff, 1996; Choudhury, Chowrimootoo, Srai, Debnan & Rice-Evans, 1999; Erlund, Meririnne, Alfthan & Aro, 2001). Hence, the concentration of naringin and neohesperidin in mouseplasma can be expressed respectively by the total (free + conjugated) concentration of naringenin and hesperitin which were determined by HPLC-MS/MS after hydrolysis with β-glucuronidase and sulfatase.

Our data indicated that the absorption processes and metabolic rate of naringin and neohesperidin became slow in GMD mice. It might be attributed to the reduced gastric emptying rate, slowed small intestine movement and reduced the metabolic ability induced by atropine or dopamine. It was also found that the C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ of two flavonoids significantly increased in GMD group by comparison with normal group. It might be a consequence of decreased gastrointestinal motility affected by atropine and dopamine, result in extended retention time, delayed absorption, decreased excretion rate of two flavonoids in the intestine and finally lead to the total absorption increased and the accumulation in blood. On the other hand, the possible reasons may be due to the disease status of gastrointestinal motility disorders in mice, enteric flora disturbance leading to the increased naringin and hesperidin decomposition, while naringenin and hesperitin are easier to be absorbed. Further exploration is needed to confirm the findings.

5. Conclusion

In conclusion, this work shows that there were significant differences in pharmacokinetic parameters, including AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, t_{max}, $t_{1/2z}$, and CL_Z/F for naringenin and hesperitin between normal and GMD mice. The absorption of naringenin and hesperitin was increased, and the elimination processes of naringenin and hesperitin were slower in the GMD mice than the normal mice. The data are of value for further pharmacological studies of ZSTFG and would be useful to provide a reference for improving the therapeutic regimen of ZSTFG in clinical trials.

Conflict of interest

The authors have declared that there is no conflict of interest.

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