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LC/MS/MS quantitation assay for pharmacokinetics of naringenin and double peaks phenomenon in rats plasma

Yan Ma [a], Peibo Li [b], Dawei Chen [a], Tiezheng Fang [b], Haitian Li [b], Weiwei Su [b,*]

[a] *School of Pharmacy, Shenyang Pharmaceutical University, Liaoning 110016, China*
[b] *School of Life Science, Sun Yat-sen University, Guangzhou 510275, China*

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Abstract

A highly sensitive and specific electrospray ionization (ESI) liquid chromatography–tandem mass spectrometry (LC/MS/MS) method for quantitation of naringenin (NAR) and an explanation for the double peaks phenomenon was developed and validated. NAR was extracted from rat plasma and tissues along with the internal standard (IS), hesperidin, with ethyl acetate. The analytes were analyzed in the multiple-reaction-monitoring (MRM) mode as the precursor/product ion pair of *m/z* 273.4/151.3 for NAR and *m/z* 611.5/303.3 for the IS. The assay was linear over the concentration range of 5–2500 ng/mL. The lower limit quantification was 5 ng/mL, available for plasma pharmacokinetics of NAR in rats. Accuracy in within- and between-run precisions showed good reproducibility. When NAR was administered orally, only little and predominantly its glucuronidation were into circulation in the plasma. There existed double peaks phenomenon in plasma concentration–time curve leading to the relatively slow elimination of NAR in plasma. The results showed that there was a linear relationship between the AUC of total NAR and dosages. And the double peaks are mainly due to enterohepatic circulation.

Keywords: LC/MS/MS; Pharmacokinetics; Double peaks; Naringenin; Enterohepatic circulation

1. Introduction

Flavonoids are a group of naturally occurring compounds ubiquitous in the plant kingdom. These flavonoids have shown many biological and pharmacological activities, such as the inhibition of enzymes (Havsteen, 2002; Keung and Vallee, 1993), free radical scavenging (Bors et al., 1990), anti-inflammation (Perri and Auteri, 1988), anti-estrogen (Tang and Adams, 1980) and the inhibition of tumor promotion (Nishino et al., 1983). Naringenin (4′,5,7-trihydroxyflavanone) and the aglycone of naringin have been demonstrated to inhibit the human cytochrome P-450 isoform, CYP1A2, in vivo (Fukr et al., 1993). In vitro NAR inhibits CYP3A4 activity in human liver microsomes (Guengerich and Kim, 1990). Moreover, NAR was found to exhibit aorta dilatory (Scanchez de Rojas et al., 1996), antioxidant effects (Kroyer, 1986), and anti-ulcer effects (Parmar, 1983) as well as inhibiting the proliferation of breast cancer and delaying mammary tumorigenesis (So et al., 1996). Limited plasma data or pharmacokinetic parameters of NAR are available in the literature. Also, there are no studies reporting the enterohepatic circulation of NAR and multiple peaks in plasma concentration–time profile. Hepatic metabolism and enterohepatic circulation are among some of the factors that may affect the pharmacokinetics of certain drugs. Therefore, the primary aim of the study was to characterize pharmacokinetics of NAR and illustrate the contribution of enterohepatic circulation to double peaks in plasma concentration–time profiles.

In recent years, various methods have been reported for quantification of narigenin in biological samples using HPLC with UV, fluorescence or mass spectrometer detection (LC/MS) (Ishii et al., 1996, 1997; Hsiu et al., 2002) to support preclinical and clinical studies, which typically required 15–30 min on-column separation, and had either a relatively higher quantitation limit or a large sample size requirement. In order to fully evaluate the pharmacokinetics of NAR in plasma, it was necessary to develop and validate an assay with appropriate sensitivity, selectivity, accuracy and precision. Most reported approaches involve

* Corresponding author. Tel.: +86 20 84110808; fax: +86 20 84112398.
E-mail addresses: lssww@zsu.edu.cn, puiarc16@zsu.edu.cn, puiarc09@zsu.edu.cn (W. Su).

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long and tedious sample detection and preparation, either solid phase extraction or protein precipitation involved multiple steps (Ishii et al., 1996, 1997; Hsiu et al., 2002). Here, we describe a sensitive LC/MS/MS method to quantify NAR in rat plasma and tissue homogenates. The sample preparation is simple and requires only a two-step liquid–liquid extraction. In the present study, the method was validated and used to quantify NAR in plasma and tissue samples to support a pharmacokinetic study in rats.

2. Materials and methods

2.1. Chemicals and reagents

NAR (purity >95%) and β-glucuronidase (25,000 unit) were purchased from Sigma Chemical (St. Louis, MO, USA). Hesperidin (HPLC grade) was obtained from The National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol (HPLC grade) was purchased from Fisher Scientific (Pittsburgh, PA) and other chemicals and solvents were of analytical-reagent grade and were used without further purification.

2.2. Instrumentation

The LC/MS/MS system that was used consisted of an Applied Biosystems MDS Sciex API3000 Triple Quadrapole mass spectrometer (Thornhill, Ontario, Canada) coupled to a Shimadzu HPLC system (Shimadzu, Columbia, MD). The HPLC system was equipped with an SCL-10A system controller, a LC-10AD HPLC pump, a DGU 12A-degasser and an SIL-10A autosampler (Shimadzu, Columbia, MD).

2.3. HPLC and mass spectrometric conditions

NAR and IS hesperidin were separated on a 150 mm × 2.0 mm Beta Basic 5 μm C_{18} ODS column (Keystone, Bellefonte, PA). The mobile phase composition was a mixture of water/methanol (30:70, v/v) at a flow rate of 0.4 mL/min. The run time was 3 min.

The mass spectrometer was operated using electrospray ionization (ESI) with an ionspray voltage of +4500 V. The positive ion multiple-reaction-monitoring (MRM) mode analysis was performed using nitrogen as the collision gas. The curtain gas (nitrogen) flow and the ionspray flow were set at 0.8 and 0.9 L/min, respectively. Precursor/product ion pairs for NAR and hesperidin were *m/z* 273.4/151.3 and *m/z* 611.5/303.3 as shown in Fig. 1. Standard solution of NAR and hesperidin was applied to optimize the detecting condition in the presence of LC mobile phase. The optimized parameters are shown in Table 1. Data acquisition and processing were performed by Sciex Analyst 1.1 software package (SCIEX).

2.4. Sample preparation

A 0.1 mL aliquot of each plasma sample was transferred to 1.5 mL polypropylene tubes with a fixed amount of internal standard (10 μL); then, 400 μL ethyl acetate (LC grade) was added to the tube and vortexed for 2 min. After centrifugation (10 min at 6000 × *g*) the supernatant was transferred to a fresh

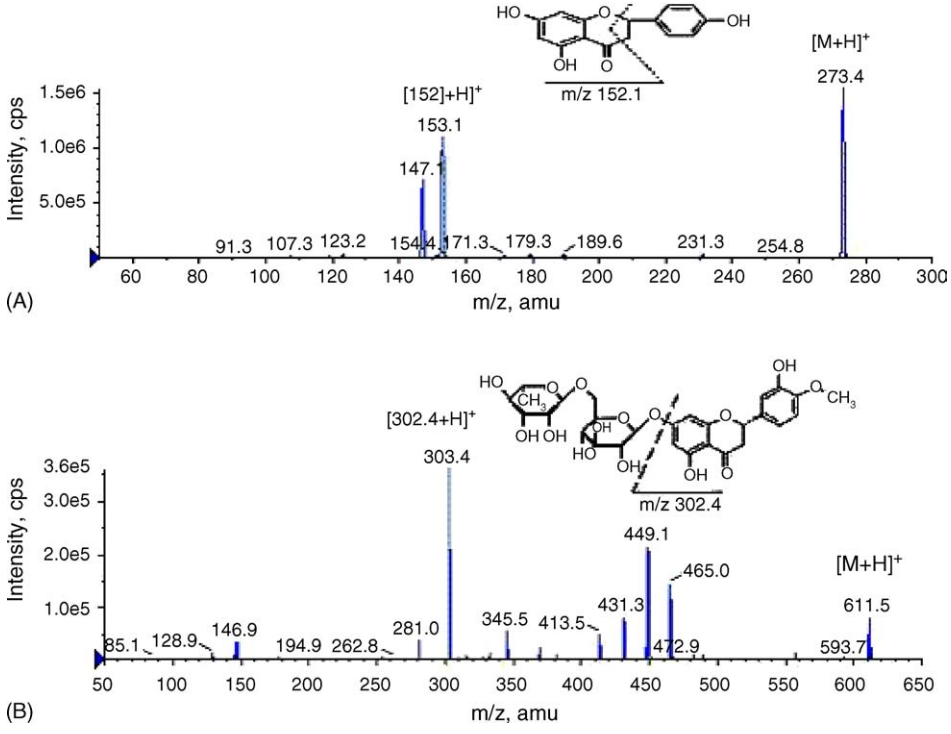

Fig. 1. Product-ion mass spectra of: NAR (A) and hesperidin (B).

Table 1
Mass-spactrometer main working parameters

Parameter	Value
Source temperature (°C)	370
Dwell time per transition (ms)	100
Ion source gas (gas 1) (psi)	3
Ion source gas (gas 2) (L/min)	6
Curtain gas (nitrogen) (psi)	10
Collision gas (psi)	7
Ion spray voltage (V)	4500
Entrance potential (V)	11
Declustering potential (V)	62
Focusing potential (V)	350
Collision energy (V)	29
Collision cell exit potential (V)	23

tube. The deposition was then added to 200 μL ethyl acetate and operated in the same way. Next the supernatant was combined and evaporated to dryness at room temperature in vacuo. The final residue was dissolved in 100 μL mobile phase with vortex-mixing for 30 s. An aliquot of 20 μL was subjected to the LC/MS/MS.

Tissues were thawed and homogenized with physiological saline (1 g:10 mL) in the tissumizer. Bile samples were diluted to 10-folds. Then the 0.1 mL aliquot of each sample (tissue, bile and tissue contents) was mixed with 10 μL IS. The preparation steps were the same for plasma.

The analysis of NAR glucuronides was reported (Ishii et al., 1996, 1997; Hsiu et al., 2002). For determination of total NAR (including NAR and its glucuronides), 0.1 mL plasma was incubated with glucuronidase 500 unit/mL at 37 °C for 1 h and then prepared as described above.

Dilutions samples were prepared by using the blank plasma, tissue and bile to dilute samples into the working calibration curve. All prepared biological samples were stored at −20 °C and all prepared stock solutions were stored at 4 °C.

2.5. Preparations of calibration standard (CS) and quality controls (QS)

CS samples were prepared by adding known amounts of NAR (0.5, 1, 5, 10, 50, 100, 250 ng) to 0.1 mL of blank plasma, homogenized tissues, tissue contents and diluted bile and then added with 5 ng of heperidin as an internal standard. The CS samples were prepared in triplicate and the standard curves were obtained by a linear fitting of the peak-area ratios versus the concentration of naringenin. The QC samples for both low and high range calibration curves in six replicates were prepared in the same manner as the CS samples to determine the precision and accuracy of the assay and to evaluate the stability of samples under various conditions.

2.6. Validation

The linearity was evaluated in the concentration range of 5–2500 ng/mL. The within-run precision values were determined in six replicates at the concentration of 5, 50, 500, 2500 ng/mL; these replicates were processed independently. The between-run precision was determined across four concentrations at five different days, and the mean concentrations and the coefficient of variation were calculated. The accuracy of the assay was determined by comparing the nominal concentrations with the corresponding calculated concentrations via linear regression. The recovery of NAR was estimated by comparing the peak area of the extracted NAR to that of the untreated NAR at concentrations of 5, 50, 500 and 2500 ng/mL. Moreover, the stability of NAR in biological samples at different conditions was estimated across four concentrations.

2.7. Pharmacokinetics of three doses in vivo

Wistar rats (College of Military Medicine Animal Research Center, Guangzhou, China), weighing between 250 and 300 g, male and female were used half-in-half. The animals had free access to water and standard laboratory chow under a controlled 12 h light–dark cycle. After at least one week of an acclimation period, the animals were divided into 10 groups (10 animals in each group).

The rats were fasted overnight and had access to water ad libitum. For oral administration, the solution was prepared by dissolving NAR in a solvent mixture (PEG 400:water = 1:1). Rats were administered with NAR (30, 90, 270 mg/kg) via gastric gavage. Blood samples were withdrawn from eyes at 0, 5, 15, 30, 45 min, 1, 2, 4, 6, 8, 12, 24 and 48 h after administration. Blood samples were collected with polythene tubes deal with anticoagulant heparin sodium 100 unit/mL and then centrifuged at 6000 × *g* for 10 min to obtain plasma stored at −20 °C until analysis.

2.8. Enterohepatic circulation

After an overnight fast, the operation on 70 rats was started. Under ether anesthesia, a laparotomy was performed through a midline abdominal skin incision. The common bile duct was exposed and ligated, and a polyethylene cannula (PE-10) was inserted into the proximal common bile duct and fixed. Then the other end of the PE cannula was penetrated through the celiac muscle and fixed on the back to collect bile at scheduled intervals. Finally, the rat was sewn up before administration.

Following a 2–3 day period, the rats were given 30 mg/kg NAR via gastric gavage and collected bile fluid at 0–2, 2–4, 4–6, 6–8, 8–12, 12–24, 24–48 h, meanwhile, blood samples were withdrawn at 5, 15, 30, 45 min, 1, 2, 4, 6, 8 h.

2.9. Gastroenteric circulation

Forty-two rats underwent enterohepatic circulation and tail intravenous administration NAR 30 mg/kg. At 5, 15, 30, 45, 60, 120, 180 min, blood, stomach and small intestine were collected. Furthermore, the stomach and small intestine were rinsed with 2 mL phosphate buffered saline to obtain the contents. All samples were stored at −20 °C until analysis.

2.10. Pharmacokinetic analysis

Plasma concentration–time profile is analyzed by the WinNonlin computer software, version 4.0.1 (Pharsight Corporation, Mountain View, CA), using the non-compartmental model.

3. Results

3.1. Chromatography and specificity

Under optimized LC/MS/MS conditions, NAR and the IS were baseline separated with the retention times of 1.08 and 0.72 min, respectively (Fig. 2C and D). The total run time was 3 min and much shorter than previously published methods (Ishii et al., 1996, 1997; Hsiu et al., 2002). Blank rat plasma showed no significant interfering peaks at the retention time of NAR and the IS (Fig. 2A and B). The addition of acetic or formic acid to the mobile phase system will improve the separation, but the system without the acid were well separated and also successful in a number of studies, as shown in the case of isoflavonoids (Farmakalidis and Murphy, 1984, 1985).

3.2. Assay validation

The calibration curves evaluated by the weighed ($1/x^2$) linear regression were linear over the concentration range of 5–2500 ng/mL of NAR in rat plasma, bile, tissues and the contents with the correlation coefficient $r^2 > 0.99$. Therefore, the LLOQ of NAR assay in rat plasma was established at 5 ng/mL. Tables 2–5 shows the within- and between-run accuracy and precision data. The method was found to be accurate with <5% deviation from the nominal values and highly precise with between-run precision, <7.85%, and within-run precision, <7.65%, at each concentration of QC samples tested. The average extraction recoveries for NAR at the four QC levels in different samples ranged from 81.24 to 88.50% as shown in Table 6. No significant loss of NAR (<4.2%) was observed after storage of plasma at

Table 2
Precision and accuracy for NAR QC samples in plasma ($n = 6$)

	Nominal concentration (ng/mL)	Observed concentration (ng/mL)	CV (%)	Accuracy (%)
Between-run	5	4.88	5.95	97.60
	50	51.23	5.21	102.46
	500	498.19	7.18	99.64
	2500	2497.54	5.07	99.90
Within-run	5	4.97	4.56	99.40
	50	50.79	3.89	101.58
	500	500.02	5.42	100.00
	2500	2490.75	7.65	99.63

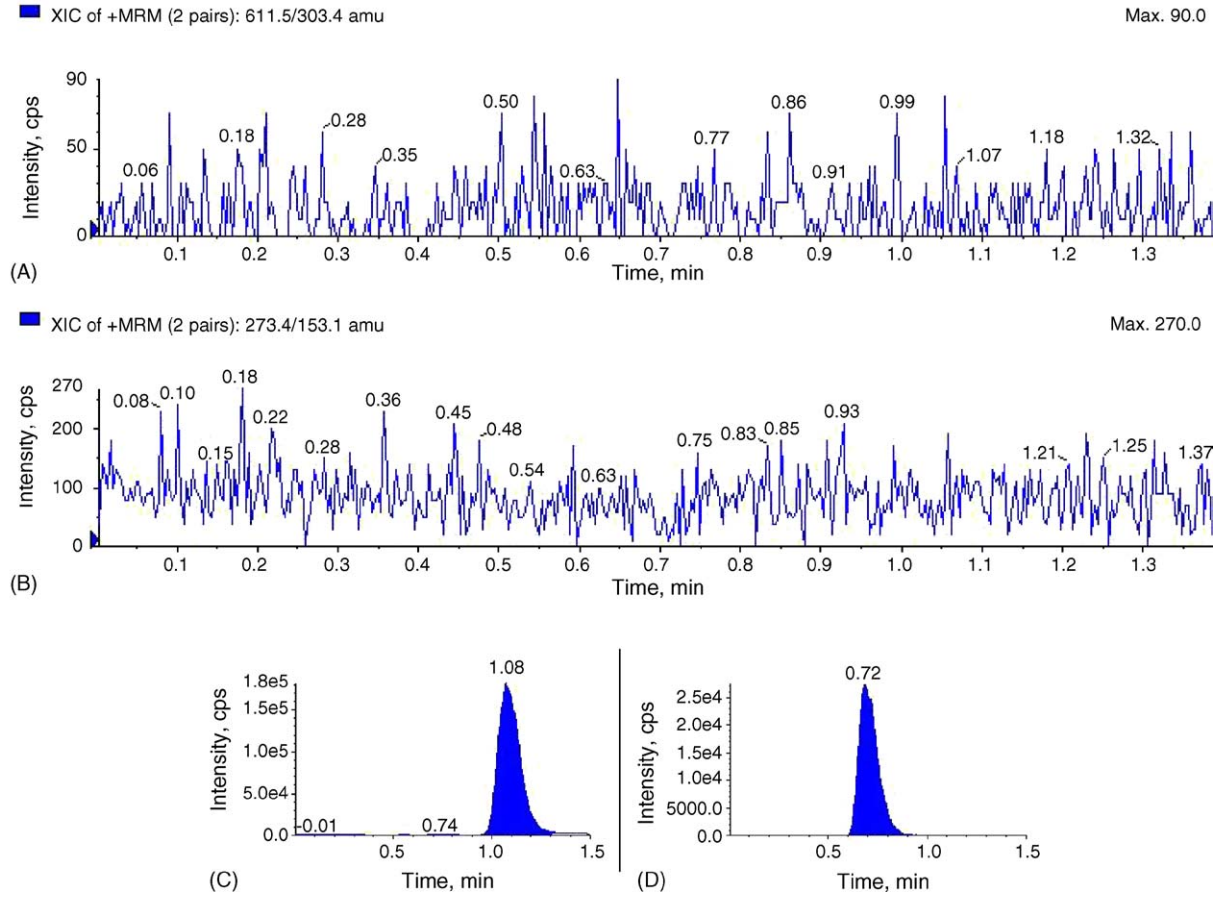

Fig. 2. Representative MRM chromatograms of: (A) blank rats plasma monitored for 611.5/303.4; (B) blank rats plasma monitored for 273.4/153.1; (C) blank rats plasma with NAR; and (D) blank rats plasma with IS hesperidin.

Table 3
Precision and accuracy for NAR QC samples in bile ($n = 6$)

	Nominal concentration (ng/mL)	Observed concentration (ng/mL)	CV (%)	Accuracy (%)
Between-run	5	4.88	6.47	97.60
	50	50.17	5.89	100.34
	500	493.19	6.98	98.64
	2500	2486.54	7.01	99.46
Within-run	5	4.94	5.18	98.80
	50	49.16	3.88	98.32
	500	499.13	4.24	99.83
	2500	2497.61	6.73	99.91

Table 4
Precision and accuracy for NAR QC samples in tissues ($n = 6$)

Nominal concentration (ng/mL)	5	50	500	2500
Stomach				
Between-run				
Observed concentration (ng/mL)	4.91	48.99	497.56	2494.75
Coefficient variation (CV%)	3.64	4.25	5.19	6.33
Accuracy (%)	98.20	97.98	99.51	99.79
Within-run				
Observed concentration (ng/mL)	5.09	49.14	501.24	2502.17
Coefficient variation (CV%)	2.38	3.29	4.15	5.11
Accuracy (%)	101.80	98.28	100.25	100.01
Intestine				
Between-run				
Observed concentration (ng/mL)	4.89	47.94	499.97	2481.17
Coefficient variation (CV%)	5.17	4.87	4.38	7.09
Accuracy (%)	97.80	95.88	99.99	99.25
Within-run				
Observed concentration (ng/mL)	4.96	48.99	500.01	2479.57
Coefficient variation (CV%)	3.13	4.29	4.19	5.55
Accuracy (%)	99.20	97.98	100.00	99.18

Table 5
Precision and accuracy for NAR QC samples in tissue contents ($n = 6$)

Nominal concentration (ng/mL)	5	50	500	2500
Stomach content				
Between-run				
Observed concentration (ng/mL)	4.87	48.27	498.11	2468.46
Coefficient variation (CV%)	5.28	4.97	4.93	7.85
Accuracy (%)	97.40	96.54	99.62	98.74
Within-run				
Observed concentration (ng/mL)	4.92	50.62	497.16	2472.21
Coefficient variation (CV%)	4.00	4.81	4.89	6.51
Accuracy (%)	98.40	101.24	99.43	98.89
Intestine content				
Between-run				
Observed concentration (ng/mL)	4.90	51.12	488.22	2476.23
Coefficient variation (CV%)	4.56	5.11	7.18	7.47
Accuracy (%)	98.00	102.24	97.64	99.05
Within-run				
Observed concentration (ng/mL)	5.03	48.98	490.54	2489.79
Coefficient variation (CV%)	3.48	3.19	5.04	6.98
Accuracy (%)	100.60	97.96	98.11	99.59

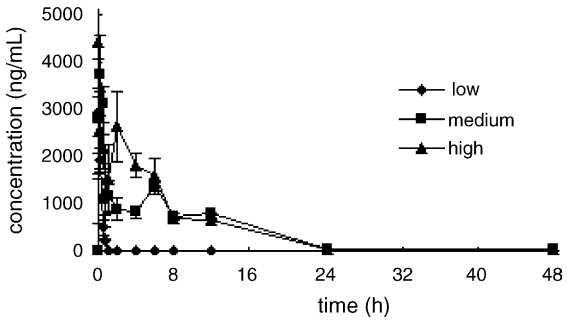

Fig. 3. Plasma concentration vs. time curve of free NAR after oral administration (low: 30 mg/kg; medium: 90 mg/kg; high: 270 mg/kg; $n = 10$).

room temperature for at least 12 h (Table 7). Processed samples were stable up to 24 h in the autosampler tray (Table 7). Plasma samples were stable at −20 °C for at least 3 weeks with no significant loss (<6.5%, Table 7) and over at least three freeze/thaw cycles (Table 7).

3.3. Pharmacokinetic analysis

The pharmacokinetic parameters of total and free NAR were obtained by WinNonlin computer software using non-compartmental model in virtue of the phenomenon of double peaks and summarized in Table 8. The plasma level of NAR conjugates was higher than that of free NAR. The mean concentration versus time profiles is shown in Figs. 3 and 4. Following oral administration 30, 90, 270 mg/kg NAR, plasma levels of free NAR rapidly reached C_{max} within 15 min, while the total NAR concentration reached C_{max} at 0.5, 2 and 2 h, respectively. The total NAR $AUC_{0–48}$ of three dosages was 30990.94, 132992.70 and 463107.43 ng/mL h, which was linearly correlated to the doses with correlation coefficient >0.999. Because of the double peaks phenomenon, the elimination half-time of the total amount of NAR is relatively long at the doses of 90 and 270 mg/kg, which is 7.58 and 10.51 h, respectively, and the respective MRT was 7.90 and 8.60 h.

It is interesting to note that there exists a dose-dependent relationship after orally administering three doses in rats; The AUC of total NAR is linearly increasing as the dosages increase. The formation of glucuronide conjugates are not

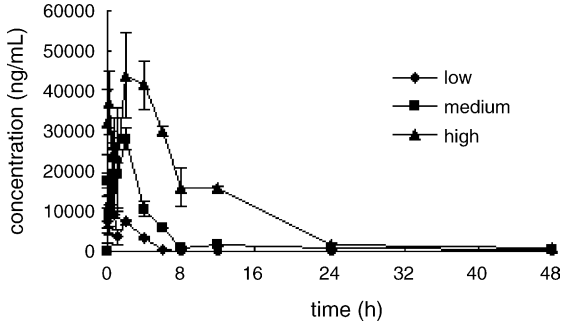

Fig. 4. Plasma concentration vs. time curve of total NAR after oral administration (low: 30 mg/kg; medium: 90 mg/kg; high: 270 mg/kg; $n = 10$).

Table 6
Absolute recovery of NAR in QC samples determined by LC/MS/MS ($n = 6$)

Absolute recovery (%, mean ± S.D.)	5	50	500	2500
Plasma	86.99 ± 4.17	87.02 ± 7.91	84.61 ± 6.78	87.27 ± 4.03
Bile	84.60 ± 2.85	85.66 ± 7.89	85.02 ± 4.08	87.76 ± 3.18
Stomach	82.38 ± 6.27	84.15 ± 5.87	83.63 ± 6.12	88.50 ± 7.45
Intestine	81.24 ± 4.46	81.98 ± 3.89	82.64 ± 5.49	84.73 ± 6.46
Stomach content	85.42 ± 4.13	84.63 ± 5.09	84.82 ± 6.41	86.60 ± 5.42
Intestine content	81.43 ± 4.32	82.25 ± 6.13	83.40 ± 4.87	83.74 ± 6.17

Table 7
Stability of NAR in rats plasma ($n = 6$)

Sample condition	Nominal concentration (ng/mL)			
	50		500	
	Observed (ng/mL)	CV (%)	Observed (ng/mL)	CV (%)
Freshly prepared	49.21	3.49	497.29	4.57
12 h at room temperature	48.59	4.12	495.31	3.91
Autosampler 24 h stability	48.14	5.04	485.42	5.24
3 weeks at −20 °C	47.72	6.49	492.89	5.22
Free/thaw cycle no.1	47.86	5.89	491.25	4.73
Free/thaw cycle no.2	47.51	6.47	489.68	5.93
Free/thaw cycle no.3	46.87	6.98	479.14	7.38

saturated in the testing dosages calculated from pharmacological experiments. The relatively slow elimination rate of NAR may be due to glucuronidation and enterohepatic circulation.

3.4. Enterohepatic circulation

Following oral administration 30 mg/kg NAR in the bile duct ligated rats, most of NAR excreted from bile exists in the form of glucuronide conjugates showed in Fig. 5, just as reported (Karen et al., 2001). NAR administered orally excreted twice as much from bile (12%) than from urine (6.25%, data unpublished in our laboratory). The total plasma concentration of NAR in ligated rats versus time profile exhibits no double peaks and relatively lower drug concentration compared with that in normal rats plasma (Fig. 6).

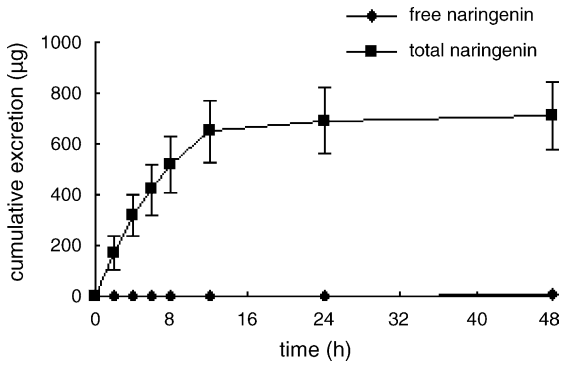

Fig. 5. Cumulative excretion of NAR in bile vs. time curve in the bile duct ligated rats ($n = 10$).

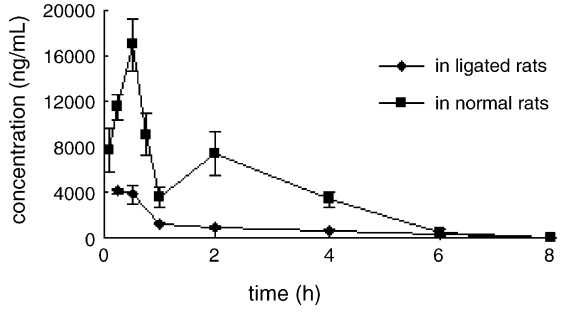

Fig. 6. Total plasma drug concentration vs. time curve of NAR in normal and ligated rats ($n = 10$).

3.5. Gastroenteric circulation

After intravenous administering 30 mg/kg of NAR in ligated rats, it was detected that the stomach and intestine as well as their

Table 8
Non-compartmental pharmacokinetic parameters of NAR after oral administration different doses (mean ± S.D., $n = 10$; low: 30 mg/kg; medium: 90 mg/kg; high: 270 mg/kg)

Parameter	Free NAR			Total NAR		
	Low	Medium	High	Low	Medium	High
Lambda_z (1/h)	4.62	0.11	0.084	1.15	0.091	0.065
HL_Lambda_z (h)	0.15	6.54	7.94	0.60	7.58	10.51
T_{max} (h)	0.083	0.25	0.083	0.5	2.0	2.0
C_{max} (ng/mL)	2925	3717.5	4420	16977.78	28000	43862.5
AUC_{0-48} (ng/mL h)	952.99	18038.58	21893.46	30990.94	132992.70	463107.43
AUC_{all} (ng/mL h)	953.64	18038.58	21893.46	30993.30	132992.70	453107.43
MRT (h)	0.25	6.97	8.52	2.01	7.90	8.60

Lambda_z: first order rate constant with the terminal (log-linear) portion of the curve; HL_Lambda_z: terminal half-life; T_{max}: time of maxium observed concentration; C_{max}: maximum observed concentration, AUC_{0-48}/AUC_{all}: area under the curve from the time of dosing to the last measurable concentration or the time of the last observation; MRT: mean residence time.

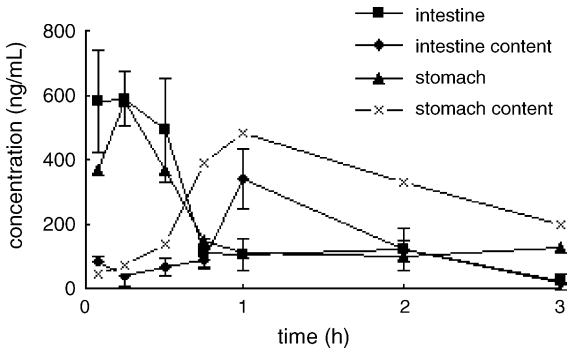

Fig. 7. Total plasma drug concentration of NAR in intestine and stomach after i.v. 30 mg/kg in normal rats ($n = 6$).

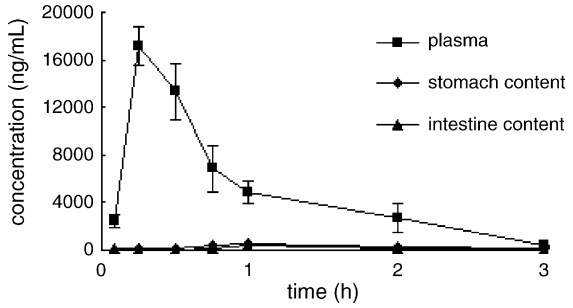

Fig. 8. Total drug concentration vs. time curve of NAR after i.v. 30 mg/kg in ligated rats ($n = 6$).

contents also observed NAR. Furthermore, the drug concentration in the contents of stomach and intestine apparently increased as NAR in both stomach and intestine sharply decreased (Fig. 7). As NAR is a polyphenolic compound bearing weakly acidity, the concentration in the stomach appeared a little higher than intestine, which was fit to the pH partition theory a certain extent. However, by comparison the drug in intestine, stomach and their contents was much less than NAR in plasma (Fig. 8).

4. Conclusion and discussion

In the study, the LC/MS/MS method to qualify NAR in plasma, bile, tissues and their contents was validated and has proven to be sensitive, specific, precise and suitable for the quantitation of drugs in biological samples. The liquid–liquid extraction method gave good and consistent recoveries for NAR and IS from rat plasma, bile, tissues and their contents with no detected interference. Consequently, the present method can be applied to pharmacokinetic study after administering NAR to rats.

Conjugation is a common detoxification reaction leading to increased solubility of a compound, which is important for excretion (Karen et al., 2001). Drugs mainly excreted in the bile have molecular weight in excess of 500. Flavonoid glucuronides are likely to be mainly excreted in bile due to their increased molecular weight and polarity. In this study, the formation of NAR conjugate (Mt > 500) results in an increase in bile excretion. Drugs excreted as conjugate empties into the duodenum through bile ducts and will be hydrolyzed to the parent drug for re-absorption known as enterohepatic circulation by the action of β-glucuronidase enzyme present in the intestine.

NAR and its conjugates were shown to rapidly appear in plasma after NAR ingestion, indicating conjugation might firstly occur within the intestinal epithelium with subsequent appearance of conjugate within the systemic circulation. And perfusion studies performed with rat intestine have indicated that at least a part of the formation of flavonoid glucuronides occurs in the intestine wall (Crespy et al., 2002; Spencer et al., 1999). Besides, from the results we can conclude that the NAR glucuronide also occurs in liver abundant in UDP–glucuronosyltransferase, the key enzyme to glucuronidation. Subsequently, the NAR glucuronides in liver undergo the enterohepatic circulation, which leads to the secondary peak in the plasma drug concentration curve (Peter et al., 2000).

After intravenous administration, some drugs can pass through the gastrointestinal membrane into GI tract, which also might lead to the drug reabsorbed and double peaks (Chen et al., 2001). Although NAR was detected within GI tract, the concentration was very little compared to the concentration in bile and plasma. So, we might conclude that enterohepatic circulation pay more contribution to the double peaks in the plasma concentration–time curve and drug re-absorption.

In summary, we have studied the three dosages pharmacokinetics of NAR in rats but then would further investigate the glucuronide conjugates and specify the position of the glucurinidation for the future. We report and testify for the first time the enterohepatic circulation. This investigation contributes to information on NAR pharmacokinetics, which is essential for understanding the safety and efficacy of NAR.

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