A Stereospecific HPLC Method and Its Application in Determination of Pharmacokinetics Profile of Two Enantiomers of Naringenin in Rats

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Abstract

In this present study, a stereospecific HPLC method that could separate two enantiomers (R/S epimer) of naringenin in bottom base has been developed and validated, and then further applied it to determine stereoselective pharmacokinetics of naringenin in rats. In this method, a normal phase column (Chiralpak AD-H) was used and mobile phase consisting of *n*-hexane, isopropanol, and trifluoroacetic acid with a gradient elution program. The ultraviolet detection wavelength was at 288 nm and injection volume was 20 µL. The column temperature was at 30°C, and flow rate was 0.8 mL/min. The validation of the method showed that the calibration curves in plasma were linear ranging from 0.05 to 20 µg/mL for each enantiomer with correlation coefficients of 0.9993 and 0.9997, respectively. The inter-day assay and intra-day assay accuracies (%) for both enantiomers were between 100.89–108.33%, while the inter- and intra-day assay precisions (%RSD) were between 1.99–7.71%. Extraction recovery, elution, and stability of both enantiomers in plasma were evaluated too, and the results showed that the method was reliable. The method was applied to determine the pharmacokinetic profile of two enantiomers of naringenin in rats after intravenous and oral administration. Naringenin has a bioactive effect as antioxidant, anti-inflammatory and immune system modulator and study on its pharmacological potency is becoming popular. The variety of naringenin's bioactivity might be due to its feature of chiral structure according to some reports, the availability of a stereospecific analytical method will be of great help to interpret the findings in different areas.

Introduction

Naringenin which comprehensively exists in citrus fruits and herbal products, has multiple biological activities including antioxidant, anti-carcinogenic, anti-inflammatory, and anti-estrogenic effects (1–2). It was also reported that naringenin was able to alter pharmacokinetic profiles of a variety of clinically used drugs by inhibiting cytochrome P-450 enzymes (3) and/or ABC transporters located in the intestine (4). In natural existence, naringenin is structurally a chiral flavonone and the S/R enantiomers ratio varies remarkably, that probably lies behind its diverse biological effects. A number of methods have been reported utilizing various methods including high-performance liquid chromatography (HPLC) with UV and LC coupled with mass spectrometry to determine quantitatively naringenin in herbal products and biological fluids (5–6). Yet there is far less information concerning stereoselective determination of its two enantionmers and evaluation of its possible pharmacokinetic stereoselectivity in organism. Also, the lowest detection limit reported so far was 0.5 µg/mL (7–8), which was not sensitive enough. The purpose of this study was to develop and validate a more sensitive and simpler method for separation of S/R enantiomers of naringenin using normal-phase HPLC and further applied to characterize its stereoselective pharmacokinetic property after administration to rats.

Experimental

Chemicals and reagents

Naringenin and internal standard (IS) Apigenin with a purity of 98.0% were supplied by Shanghai Healthjoy Chemical Co., Ltd (Shanghai, China). Two enantiomers of naringenin (*R*-naringenin and *S*-naringenin) were produced using productive chromatography on a Chiralpak AD-H column in our lab. Methanol, *n*-hexane, ethanol, isopropanol, and trifluoroacetic acid provided by Tedia, Inc. (Carson City, CA, USA) were HPLC-grade. β-Glucuronidase (type IX-A) from *E. coli* and sulfatase (type H-1) from Helix pomatia were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were analytical reagent grade.

Instrumentation and chromatographic conditions

The LC was performed with an Agilent isocratic pump (1100 series), an Agilent autosampler (1100 series), a degasser (1100 series), and an UV–vis detector (Agilent Technologies, Palo Alto, CA). The whole system was controlled with an Agilent

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ChemStation software for both apparatus operation and chromatographic data acquisition and integration. A Chiralpak AD-H Column (46 mm i.d. × 250 mm) was purchased from Daicel Chemical industries, Ltd. (Fort Lee, NJ).

The mobile phase consisted of A (*n*-hexane with 0.5% trifluoroacetic acid) and B (isopropanol with 0.5% trifluoroacetic acid) and a gradient elution program was set as follows: solution A, 80–75% (0–8 min), 75–70% (8–16 min), and 80% (16–20 min). The column temperature was at 30°C, and flow rate was 0.8 mL/min. The ultraviolet detection wavelength was at 288 nm and injection volume was 20 µL.

Preparation of stock solution and standards

A stock solution of naringenin was prepared by dissolving the drug in ethanol to yield a final concentration of 1 mg/mL. An aliquot of this solution was diluted using ethanol to get a series of working solutions of 0.5,1,2.5, 5,10, 25, 50, 100, and 200 µg/mL. The stock and standard solutions were stored at 4°C. Eight calibration standard samples containing 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, and 20 µg/mL were obtained by adding 10 µL working solution prepared descvribed previously into eight Eppendorff tubes containing 100 µL blank plasma. Quality control (QC) samples were prepared by spiking 100 µL blank plasma with 10 µL working solutions of 0.5, 10, and 200 µg/mL to yield final concentration of 0.05, 1, and 20 µg/mL.

Stock solution of Apigenin (IS) was prepared by dissolving the drug in ethanol to a final concentration of 200 µg/mL. This solution was diluted with ethanol to yield a final concentration of 10 µg/mL. The stock and working IS solutions were stored at 4°C.

Plasma sample process

Plasma samples (0.1 mL) were transferred to an Eppendorff tube, 10 µL ethanol, 10 µL apigenin (IS), 100 µL ethanol were added and vortex-mixed for 15 s, then 200 µL dichlormethane was added. After vortexing for 1 min and centrifuging for 3 min at 11,250 × *g*, 200 µL of supernatant was transferred to another Eppendorff tube and evaporated at 45°C with the aid of a gentle stream of nitrogen. The residue was reconstituted with 100 µL ethanol and vortex-mixture for 15 s then centrifuged for 8 min at 11,250 × *g*. A volume of 20 µL of supernatant was injected into the chromatographic system.

Enzymatic hydrolysis was performed by addition of 25 µL β-glucuronidase (800 U/mL) and 25 µL sulfatase (800 U/mL) to 50 µL plasma, the sample was incubated in a sealed vial for 3 h at 37°C under continuous shaking. One hundred microliters of hydrolysised samples were preparated as plasma samples as mentioned previously.

Method validation

Specificity

The plasma was obtained from six different blank rats that were housed in the same environment as the treated animals. The plasma was processed by the analytical procedure to check for the absence of interfering compound(s).

Calibration curve

The analytical curves were constructed using eight nonzero standards ranging from 0.05 to 20 µg/mL. The linear regression analysis of naringenin was performed by plotting the peak area ratio of naringenin (*R/S*) over IS (*y*) against the naringenin concentration (x) in µg/mL. The linearity of the relationship between peak area ratio and concentration was demonstrated by the correlation coefficients (*R*) obtained for the linear regression of naringenin.

Intra- and inter-day precision and accuracy

Intra-day precision and accuracy were determined by repeated analysis of each QC sample in one day ($n = 5$), while the inter-day precision and accuracy by analysis of both calibration curve and QC samples in five different days. The precision was evaluated by the relative standard deviation (RSD).The accuracy was estimated based on the mean percentage error of measured concentration to the actual concentration. The mean and RSD of the QC samples at each concentration from multiple runs should be determined. The mean observed concentration should be within ± 15% of the nominal concentration, and the RSD should be less than 15%, at all concentrations. If the QC concentration is at the lower limit of quantitation (LOQ), the RE% and the RSD can be up to 20%.

Stability

The short-term stability tests were performed by analyzing spiked plasma samples after 4 h at room temperature and 24 h stored in a refrigerator (4°C). The freeze-thaw matrix stability was evaluated by analyzing spiked samples for two cycles. The long-term stability was performed by analyzing spiked samples after storage for 4 week at –80 °C. Auto-injector stability was evaluated by analyzing processed samples after kept at room temperature for up to 24 h.

Dilution

Some sample concentrations may exceed the upper LOQ, a test for sample dilution with blank matrix during validation should be performed. Prepared plasma sample (100 µg/mL) was diluted using blank plasma for 10 times and 2 times, and then underwent the process described previously. The acceptance criteria for the diluted samples are the same as described previously

Pharmacokinetic Study

Ten male Sprague–Dawley rats (250 ± 20 g) were provided by Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed in the animal facility of Shanghai Institute of Pharmaceutical Industry (Shanghai, China) with unlimited access to food and water except for 12 h of fasting before the experiment. The animals were maintained on a 12 h light–dark cycle (light on from 8:00 to 20:00 h) at ambient temperature (22 ± 4°C) and ~60% relative humidity and the carotid cannula for each animal was in place a week before dose administration. Animal studies were approved by IACUC of Shanghai Institute of Pharmaceutical Industry and were carried out in accordance with the SOPs of the facility.

Naringenin *(R/S* was 50/50) was dissolved in 0.9% saline and then 2 M NaOH solution was used to adjust the pH to 9. Upon preparation, the dosing solution was administered intravenously to rats at a dose of 20 mg/kg body weight. The blood samples (250 µL) were collected via carotid cannulation at 0 min

(predose), 2.5 min, 5 min, 10 min, 15 min, 30 min, 45 min, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, and 12 h after administration. For oral administration, naringenin (*R/S* was 50/50) was dissolved in 0.05% CMC-Na and was administered orally to rats at a dose of 20 mg/kg body weight. The blood samples (250 µL) were collected via carotid cannulation at 0 min (predose), 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, and 24 h after administration. The blood samples were placed in heparinized tubes and the plasma was separated by centrifugation at 7,500 × *g* for 6 min. All plasma samples were stored at –80°C until analysis. Pharmacokinetic parameters of naringenin were calculated using PK analysis software DAS 2.0 (Gaosi Data Analysis, Inc., Wuhu, China), and the non-compartmental model was applied.

Results

Method Development

Typical chromatograms of plasma extracts (0.5 µg/mL and blank) are presented in Figure 1. Bottom base separation was obtained with retention times at approximately 9.4 min, 11.8 min, and 14.5 min for IS, *S*-naringenin, and *R*-naringenin, respectively. The lowest limits of quantitation (LLOQ) for both enantiomers in plasma were 0.05 µg/mL, which met the requirements for the stereoselective pharmacokinetic study of naringenin in rats performed afterwards.

Method validation

Selectivity, sensitivity, and calibration curves

Potential interference from endogenous materials was investigated by analyzing rat plasma of six different sources and chromatographic conditions showed that the blank plasma has no interference to the determination of both two enantiomers of naringenin and apigenin as well (IS) determination (Figure 1). Good linearity was obtained over the plasma concentration range of 0.05–20 µg/mL for each enantiomer. The equations of the mean calibration of the enatiomers were listed in Table I.

Recovery

The recovery of both enantiomers of naringenin was determined by comparing peak areas from plasma spiked with known amounts of drug at 0.05, 1, and 20 µg/mL versus peak areas of the same concentrations prepared in ethanol and injected directly in the chromatographic system. Each sample was determined in quadruplicate. The recoveries for R-enantiomer and S-enantiomer of naringenin ranged from 88.7% to 90.0% and 86.8% to 92.6%, respectively.

Precision and accuracy

Accuracy and precision for intra- and inter-

Table I. Standard Curves of Naringenin in Plasma

Samples	Conc. ranges (µg/mL)	Regression Equation	Correlation Coefficient
S-naringenin	0.05–20	$y = 1.85x - 0.006$	0.9997
R-naringenin	0.05–20	$y = 2.01x - 0.011$	0.9993
S-naringenin hydrolysis	0.05–20	$y = 0.93x - 0.011$	0.9996
R-hesperetin hydrolysis	0.05–20	$y = 1.02x - 0.013$	0.9994

Table II. Intra- and Inter-day Assay Accuracy of the Determination of Naringenin Plasma

Analyte	Spiked conc. (µg/mL)	Found conc. (µg/mL)	Accuracy (%)	Precision (%RSD)
S-naringenin				
Intra-day	0.05	0.053 ± 0.001	105.35	4.72
Assay	1.0	1.009 ± 0.020	100.89	1.99
	20.0	20.818 ± 0.644	104.09	3.09
Inter-day	0.05	0.108 ± 0.006	108.33	5.23
Assay	1.0	1.014 ± 0.021	101.36	2.07
	20.0	20.636 ± 0.522	103.18	2.53
R-naringenin				
Intra-day	0.05	0.101 ± 0.008	100.70	7.71
Assay	1.0	1.016 ± 0.036	101.62	3.51
	20.0	20.800 ± 0.637	104.00	3.06
Inter-day	0.05	0.103 ± 0.008	103.42	7.29
Assay	1.0	1.023 ± 0.048	102.29	4.68
	20.0	20.654 ± 0.513	103.27	2.49

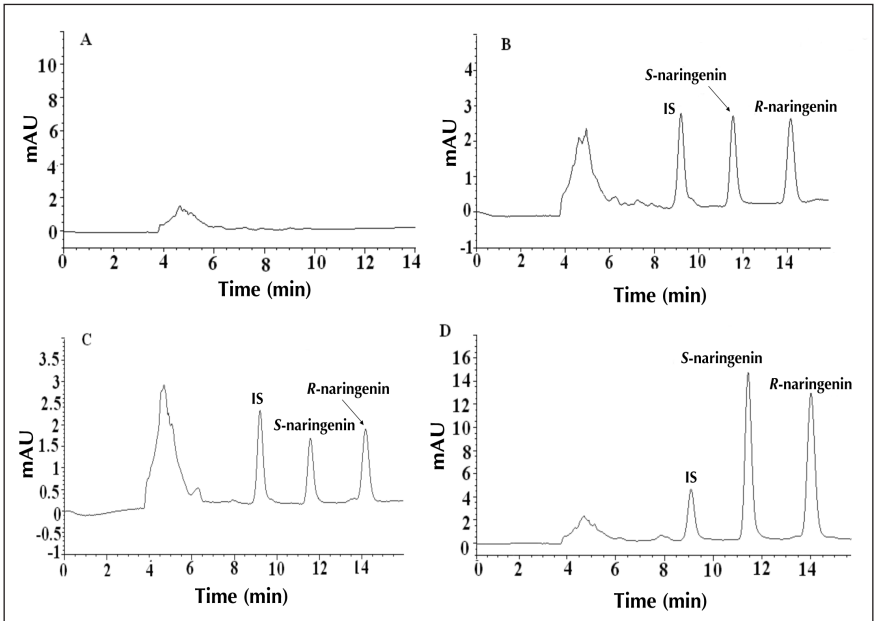

Figure 1. Representative chromatograms of naringenin in plasma. (A) Drug-free plasma, (B) naringenin and IS spiked sample, (C) plasma sample containing naringenin enantiomers 0.5 h after the administration of an iv of 20 mg/kg, without hydrolysis, and (D) plasma sample containing naringenin enantiomers 0.5 h after the administration of an IV of 20 mg/kg, enzymic hydrolysis.

day plasma samples of both enantiomers were tested at three concentration levels (0.05, 1, and 20 µg/mL) and the data are presented in Table II. It appears that the coefficients of variation are less than 7.7%, indicating that the developed HPLC method was repeatable and reproducible.

Stability and dilution test

Short-term stability and freeze/thaw cycles tests resulted in a loss less than 11.7% at three concentration levels (0.05, 1, and 20 µg/mL), and there was no perceptible loss seen for frozen at –80°C up to 4 weeks. Again, the extracts of both naringenin's enantiomers were stable at ambient temperature for at least 24 h. Besides, dilution test data resulted in a coefficient of variation of 6.8%, which was considered as satisfactory.

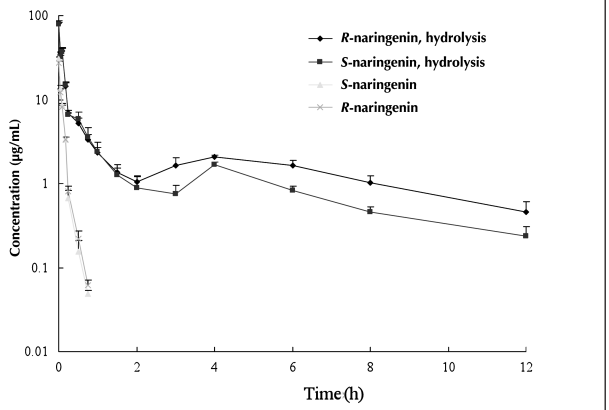

Figure 2. Plasma concentration-time profile of naringenin after IV administration of 20 mg/kg to rats (n = 5).

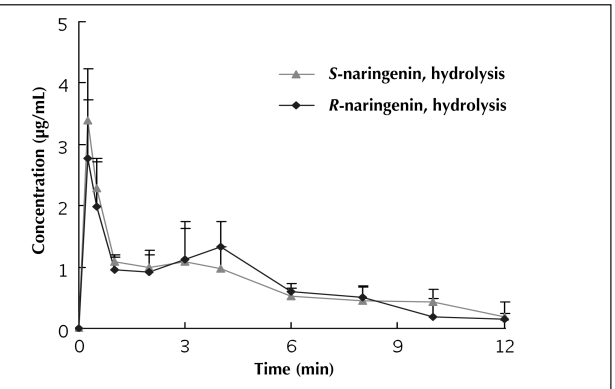

Figure 3. Plasma concentration-time profile of naringenin after oral administration of 20 mg/kg to rats (n = 5).

Stereospecific pharmacokinetics of naringenin in rats

Stereospecific pharmacokinetic study of naringenin was conducted by intravenous and oral administration of equal dose (10 mg/kg) of both enantiomers to rats simultaneously. The concentration of each enantiomer was determined using the described assay method (Figures 2 and 3) and the pharmacokinetic data analysis was performed using noncompartmental analysis modules in DAS 2.0. The bioavailability was calculated as F(%) = [Doseiv × AUCoral(0 – ∞)/[Doseoral × AUCiv(0 – ∞)] × 100% (Table III). According to the data, after intravenous and oral administration, the pharmacokinetic parameters of two enantiomers did not show significant differences except that AUCiv of R-naringenin was 7.002 µg h/mL (35%) higher than that of S form. Apparently, the data obtained in this study was still too scarce to come to any conclusion, further study is needed to collect more information about the possible stereospecific exposure of naringenin in organism.

Discussion

Traditionally, liquid–liquid extraction with ethyl acetate, solid-phase extraction (SPE) (5), and precipitating with cold acetonitrile (7,9) are used to extract flavonoids compounds, which, however, did not work as well for naringenin as expected. In this study, ethyl acetate was replaced with ethanol and dichloromethane, which proved to be excellent in improvement of extraction recovery and specificity of naringenin determination. Also, a AD-H column was used to separate naringenin in biological samples which has been not reported yet and the beautifully bottom base separation was obtained (Figure 1). The LLOQ was up to 0.05 µg/mL for both enantiomers of naringenin which was much lower than that have been reported so far using OD-RH column (0.5 µg/mL) (14).

After validation, the method was then applied to the in vivo pharmacokinetics study of individual enantiomer of naringenin in rats and no significant difference was found between two chiral ingredients in the most pharmacokinetic parameters which is consistent with previous studies. Naringenin is

Table III. Pharmacokinetic Parameters of Naringenin and Hesperitin Enantiomers After Oral Administration 20 mg/kg Racemate to Rats

	Oral administration		IV administration			
	With enzymatic hydrolysis		With enzymatic hydrolysis		Without enzymatic hydrolysis	
Parameters	*R*-naringenin	*S*-naringenin	*R*-naringenin	*S*-naringenin	*R*-naringenin	*S*-naringenin
AUC_{0-t} (µg h/mL)	9.055 ± 1.822	8.802 ± 2.767	24.643 ± 5.117	19.145 ± 3.821	2.258 ± 0.069	2.139 ± 0.072
$AUC_{0-\infty}$ (µg h/mL)	10.155 ± 1.958	10.377 ± 3.172	26.766 ± 7.521	19.764 ± 3.788	2.263 ± 0.067	2.0827 ± 2.148
C_{max} (µg/mL)	3.38 ± 0.84	3.211 ± 0.951	81.791 ± 10.794	78.097 ± 7.851	30.923 ± 1.272	27.49 ± 1.145
T1/2z (h)	3.932 ± 1.016	4.638 ± 2.561	2.924 ± 1.029	2.684 ± 0.696	0.132 ± 0.096	0.109 ± 0.032
MRT (h)	4.051 ± 0.366	4.024 ± 0.467	3.123 ± 0.346	2.306 ± 0.145	0.075 ± 0.006	0.084 ± 0.006
CL/F (L/h/kg)	2.027 ± 0.399	2.132 ± 0.653	0.785 ± 0.177	1.037 ± 0.176	8.845 ± 0.257	9.32 ± 0.317
Vz/F(L/kg)	11.57 ± 4.09	12.034 ± 3.092	3.119 ± 0.267	4.099 ± 1.368	1.231 ± 0.454	1.463 ± 0.417

appealing to researchers all around the world because of its fascinating multiple functions and chiral structural features. To make most of it for human benefits, further more studies will be conducted in future and the availability of a sensitive and reliable stereoselective assay method makes this possible and worthwhile.

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