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Short communication

Determination of phillyrin in rat plasma by high-performance liquid chromatography and its application to pharmacokinetic studies

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Abstract

A simple high-performance liquid chromatographic method was developed to study the pharmacokinetics of phillyrin in rat after intravenous administration. Plasma was extracted with ethyl acetate after addition of the internal standard, arctiin. Separation was achieved on a reversed-phase C_{18} column with UV detection at 228 nm. The calibration curves were linear ranging from 0.052 to 26.670 μg/ml. The intra- and inter-day precisions were no more than 9.83% and 12.31%, respectively. The average recovery of phillyrin was 95.44% from plasma. And the limit of quantification (LOQ) was estimated as 0.026 μg/ml with an intra-day relative standard deviation (R.S.D.)≤20%.

The analytical sensitivity and accuracy of this assay were adequate for characterization of phillyrin in rat plasma and the assay has been applied successfully to the in vivo kinetic study of phillyrin in rats.

Keywords: Phillyrin; Reversed-phase HPLC; Pharmacokinetics

1. Introduction

Fructus forsythiae (Thunb.) Vahl. has been used widely in traditional medicines to treat gonorrhea, erysipelas, inflammation, pyrexia, ulcer, etc. It has also shown anti-oxidant activity, as well as anti-bacterial, anti-viral, choleretic and anti-emetic effects [1]. Phillyrin ($C_{27}H_{34}O_{11}$), as the major effective constituent of fructus forsythiae, plays an important role in the effect of anti-inflammatory [2], anti-viral [3,4], anti-oxidant [5], anti-obesity activity [6] and inhibiting phosphodiesterases' activity [7]. Thus, phillyrin is used as the marker compound to characterize fructus forsythiae.

Pharmacokinetic studies of active ingredients in Chinese herbs are very important in order to illustrate their action mechanism for the development of traditional Chinese medicine. There were many preliminary researches about quantifying this drug in natural products and preparations [8,9], however, no study was reported about the determination of phillyrin in biological matrix. In this work, a sensitive, simple and accurate method was developed to determine phillyrin in rat plasma and it has been successfully applied to pharmacokinetic investigation of phillyrin after intravenous administration.

2. Experimental

2.1. Chemicals and reagents

Phillyrin (Fig. 1A) and arctiin (Fig. 1B) were supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade methanol was purchased from Dikma technologies (Beijing, China). Phosphoric acid, sodium dihydrogen phosphate and ethyl acetate were of analytical grade and obtained from the Chengdu Reagent Company (Chengdu, China). Water was prepared in ultra pure water system (UPA, Chongqing, China).

Male Wistar rats (180–220 g) were obtained from Animal Center at West China School of Pharmacy, Sichuan University.

2.2. Chromatographic system and conditions

The HPLC system used was a Shimadzu HPLC (Kyoto, Japan), consisting of a LC-10AT pump, a SPD-10A VP UV–vis spectrophotometric detector and a SCL-10A VP system controller. Data collection and integration were

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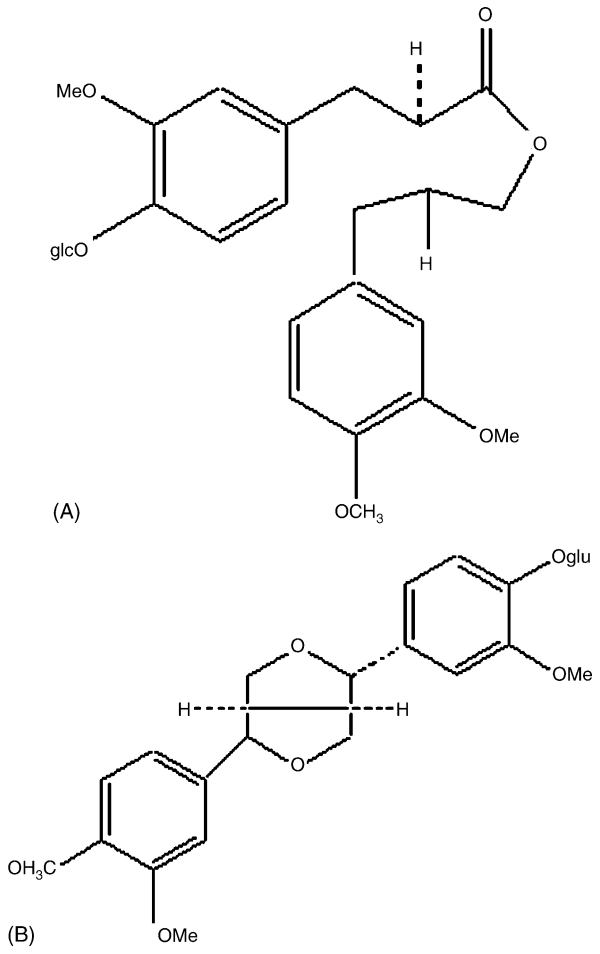

Fig. 1. (A) Chemical structure of phillyrin and (B) chemical structure of arctiin.

accomplished using Shimadzu EZ Start 7.1.1 program software.

The analytical column used was a reversed-phase Diamonsil C_{18} column (250 mm × 4.6 mm, ID, 5 μm) (Dikma technologies, Bejing, China).The mobile phase consisted of methanol and sodium dihydrogen phosphate (pH 5.75; 20 mM) (47:53, v/v), filtered and degassed under reduced pressure, prior to use. Separation was carried out isocratically at 35 °C, and a flow rate of 1.0 ml/min, with UV detection at 228 nm.

2.3. Calibration standards and quality control samples

Methanolic stock solution of phillyrin (400 μg/ml) was prepared and further diluted with methanol to give a series of standard solutions with concentrations from 0.39 to 200 μg/ml. A working internal standard (IS) solution of 35 μg/ml was made by dissolving arctiin with methanol [10]. All solutions were stored at 4 °C before use for no longer than 3 months.

Calibration standards in plasma (0.052, 0.104, 0.208, 0.417, 0.830, 1.670, 3.330, 6.670, 13.330 and 26.670 μg/ml) were prepared daily by spiking blank rat plasma with 20 μl of the standard solutions prepared above.

Quality control (QC) samples were independently prepared at concentrations of 0.104, 1.670 and 13.330 μg/ml in the same manner. The spiked samples were then treated following the sample preparation procedure as indicated in Section 2.4.

2.4. Sample preparation

Twenty-five microlitre of internal standard solution (35 μg/ml) was added to plasma standard or sample (150 μl). The mixture was vortexed for 15 s (vortex WH-3, Anting Scientific Instrument, Shanghai, China) and 1 ml of ethyl acetate was added for drug extraction. The mixture was vortexed again for 3 min and centrifuged at 1000 × *g* for 5 min (Anke TGL-16C centrifuge, Anting Scientific Instrument, Shanghai, China). The organic phase was collected and evaporated to dryness in a water bath at 37 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μl mobile phase, vortexed for 1 min, and centrifuged at 1000 × *g* for 2 min, and 80 μl of the supernatant was manually injected onto the column.

2.5. Recovery and stability of phillyrin samples

Recovery of phillyrin were assessed ($n = 6$) at 13.330, 1.670 and 0.104 μg/ml. The recoveries were determined by comparing the peak area ratio of phillyrin to arctiin in plasma with that in methanol at same concentration.

The stabilities of phillyrin in rat plasma and in processed samples were investigated using QC samples of 13.330, 1.670 and 0.104 μg/ml, in six replicates both at room temperature (25 °C) and at −20 °C.

2.6. Precision and accuracy

The intra-day precision and accuracy of the replicate assays ($n = 6$) were tested by using three different concentrations, namely 13.330, 1.670 and 0.104 μg/ml. The inter-day precision and accuracy of the assay were estimated from the results of assays of QC samples of 6 different days within 1 week.

2.7. Pharmaceutics of phillyrin in rat

A male Wistar rat (180–220 g) was anaesthetized using pentobarbital and a silastic catheter was cannulated into the right jugular vein. The animal was placed in a metabolic cage, allowed to recover overnight and fasted for 12 h before dosing. On the day of experiment, the animal was dosed intravenously with phillyrin. According to the Chinese Pharmacopeia, by mathematical conversion of doses between different species (from human to rat), three effective doses, 1.25, 2.50 and 5.00 mg/kg were selected. Serial blood samples (0.4 ml) were collected at 0, 5, 15, 25, 35, 45, 55, 65, 75, 85 and 95 min into heparinized tubes. After each sample collection, the cannula was flushed with saline. Plasma samples were obtained following centrifugation of the blood samples at 1500 × *g* for 10 min.

2.8. Data analysis

Quantification was based on calibration curves constructed using peak area ratios (PAR) of phillyrin to IS, against phillyrin

concentrations spiked in rat plasma. Concentrations of phillyrin in rat plasma after i.v. administration were determined from the peak-area ratios by using the equations calculated by SPSS (version 10.0, USA).

Pharmacokinetic data were subsequently processed by Drug And Statistics (DAS, Anhui, China).

3. Results and discussion

3.1. Selectivity

Typical chromatograms obtained from blank plasma, rat-spiked plasma and plasma sample were shown in Fig. 2. Fig. 2(C and D) demonstrated that the retention times of phillyrin and arctiin were approximately 11.9 and 9.9 min, respectively, with complete baseline resolution between peaks of interest. The selectivity of the assay was investigated by injecting blank plasma. Fig. 2(A) showed that there was an interfering peak which can not be separated from phillyrin in blank plasma obtained from the rats supplied with standard laboratory food. Then the standard laboratory food was grinded, dispersed in water, and centrifuged at 1500 × *g* for 3 min. The supernatants were injected onto HPLC system and still produced interfering peaks at the same time ($n = 3$). So it was suspected that standard laboratory food contained little phillyrin. However, no chromatographic interference derived from endogenous substance was observed in the blank plasma obtained from the rats supplied with rice (Fig. 2(B)). So the rats for experiment were fed with rice to avoid the interference from endogenous substance.

3.2. Stability and recovery

Stability investigation (Table 1) demonstrated that the concentrations of phillyrin in plasma samples (13.330, 1.670 and 0.104 μg/ml) were similar to the nominal values within 1 h. But after 8 h at room temperature or 24 h at −20 °C, the concentrations were declining with time, indicating that phillyrin was unstable in plasma samples both at room temperature (25 °C) and at −20 °C. Therefore, the plasma samples should be treated within 1 h. After being treated, the concentrations of phillyrin in processed samples (13.330, 1.670 and 0.104 μg/ml) were 99.7%, 99.2% and 98.7% of the nominal values after 24 h at room temperature (25 °C), and after storing processed plasma samples at −20 °C for 24 h, the concentrations following this storage period

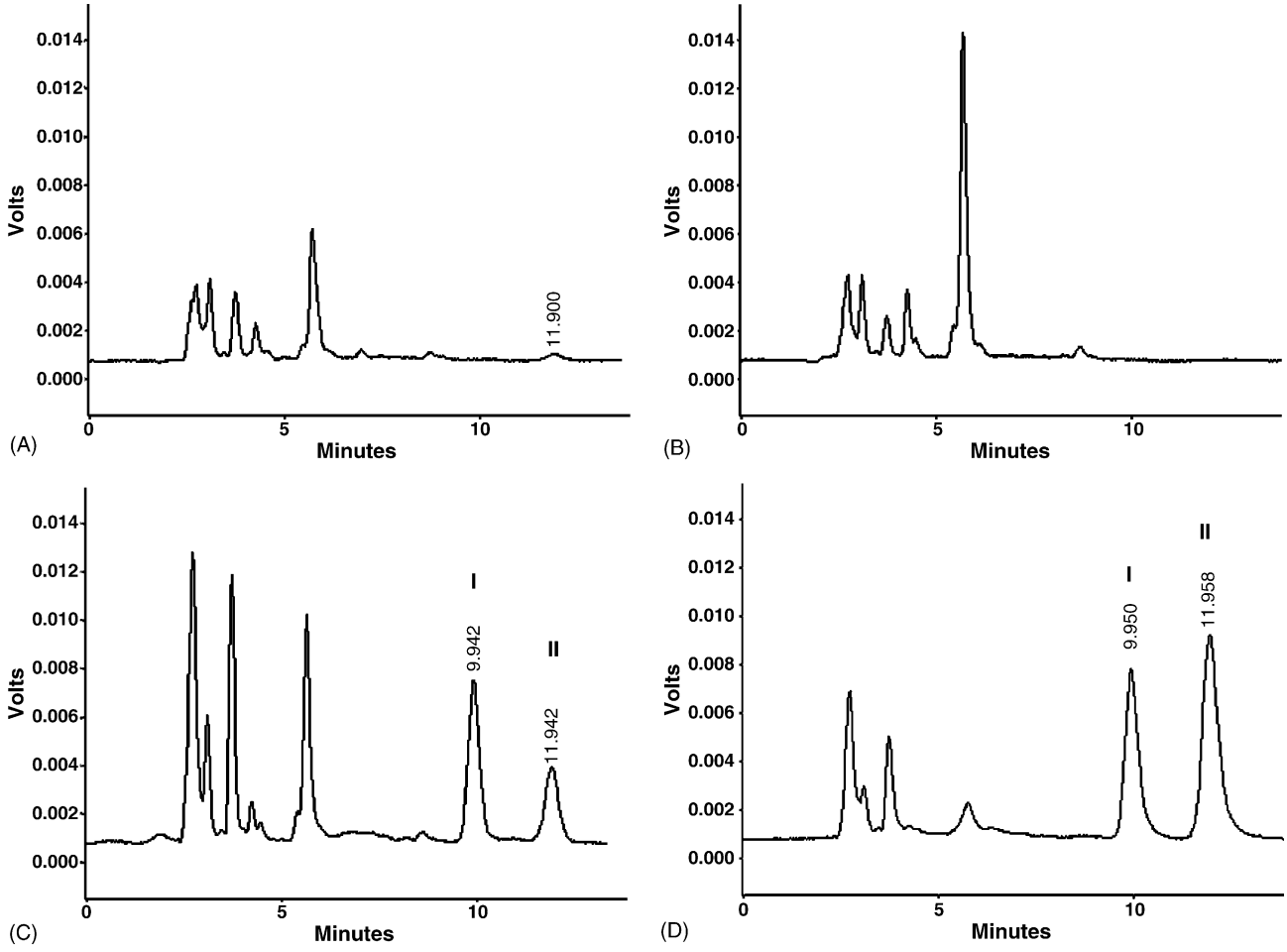

Fig. 2. Chromatograms of phillyrin in rat plasma: (A) blank plasma of rats supplied with standard laboratory food, (B) blank plasma of rats supplied with rice, (C) plasma spiked with phillyrin (1.670 μg/ml) and arctiin (internal standard) and (D) plasma sample 5 min after 2.50 mg/kg i.v. administration of phillyrin (5.098 μg/ml). I, arctiin; II, phillyrin.

Table 1
Stability of phillyrin in rat plasma ($n=6$)

Time and condition of storage	Nominal concentratio(n) (μg/ml)	Percent of nominal (%)	R.S.D. (%)
25 °C, 0.5 h	13.330	99.81	2.54
	1.670	99.57	3.78
	0.104	99.83	3.98
25 °C, 1 h	13.330	99.12	2.87
	1.670	99.21	3.45
	0.104	99.58	3.93
25 °C, 2 h	13.330	98.25	3.01
	1.670	96.71	3.54
	0.104	90.51	3.56
25 °C, 4 h	13.330	94.52	4.42
	1.670	92.14	3.87
	0.104	82.93	4.50
25 °C, 6 h	13.330	89.14	5.21
	1.670	85.21	5.34
	0.104	73.92	5.22
25 °C, 8 h	13.330	84.38	4.33
	1.670	78.38	4.89
	0.104	64.71	5.27
−20 °C, 24 h	13.330	85.98	5.21
	1.670	86.37	6.03
	0.104	66.24	5.97

Stabilities of phillyrin in rat plasma were assessed at three concentration levels (13.330, 1.670 and 0.104 μg/ml) by comparing the results obtained from the spiked plasmas after storage with the nominal values.

were 98.9%, 98.3%, 97.9% of the nominal values, respectively. No significant degradation was observed, which indicated that phillyrin in processed samples was stable enough for injection onto HPLC system.

The mean extraction efficiency was 95.44% for phillyrin from rat plasma (Table 2), and 96.83% for the internal standard ($n=6$). High recovery of phillyrin from rat plasma suggested that there was negligible loss during drug extraction.

3.3. Linearity, LOQ and LOD

The calibration curve was fitted to a 1/(concentration)/(concentration) weighted linear regression with a correlation coefficient of 0.9999 over the concentration range of 0.052–26.670 μg/ml. The slopes of the calibration graphs were 0.2762 ± 0.0148 ($n=5$) with R.S.D. of 5.368%, and the intercepts were 0.0135 ± 0.0007 ($n=5$) with R.S.D. of 5.193% throughout the study. The limit of detection (LOD) for this method defined as a signal-to-noise ratio of 3:1 was 0.013 μg/ml. The limit of quantification (LOQ) defined as the lowest drug concentration which can be determined with an intra-day relative standard deviation (R.S.D.)≤20%, was estimated as 0.026 μg/ml.

Table 2
Recovery of phillyrin from rat plasma ($n=6$)

Nominal concentration (μg/ml)	Observed concentration (μg/ml)	Recovery (%)	R.S.D. (%)
13.330	12.819	96.17	1.03
1.670	1.585	94.90	3.21
0.104	0.099	95.24	5.07

Recoveries of phillyrin were assessed at three concentration levels (13.330, 1.670 and 0.104 μg/ml) by comparing the peak area ratio of phillyrin to arctiin in plasma with that in methanol.

3.4. Precision and accuracy

The intra- and inter-day precision calculated during replicated assays of phillyrin in rat plasma were <13% over a wide range of phillyrin concentrations. The accuracy during replicate assays varied between 92% and 103%. Precision and accuracy studies indicated that the developed HPLC method is reproducible and accurate (Table 3).

3.5. Pharmacokinetic application

Kinetic analysis was accessed by a statistical non-linear regression program with DAS program. With minimum Akaike information criterion (AIC) [11] values, a two-compartment open model was proposed and validated through the program.

Table 3
Intra- and inter-day precision and accuracy of phillyrin in rat plasma ($n=6$)

Spiked concentration (μg/ml)	Observed concentration (μg/ml)	Accuracy (%)	R.S.D. (%)
Intra-day precision			
13.330	12.268 ± 0.135	92.03	1.01
1.670	1.715 ± 0.033	102.69	1.96
0.104	0.099 ± 0.010	95.19	9.83
Inter-day precision			
13.330	13.491 ± 0.336	101.21	2.52
1.670	1.717 ± 0.095	102.81	5.69
0.104	0.103 ± 0.013	99.04	12.31

The precision and accuracy of the present method were investigated by repeated analysis of phillyrin-spiked plasma samples at 13.330, 1.670 and 0.104 μg/ml.

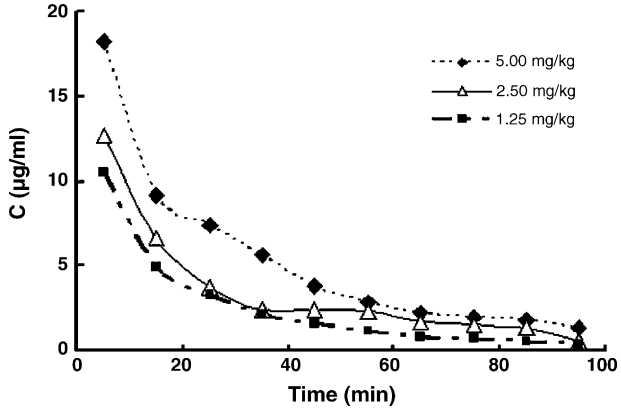

Fig. 3. Plasma concentration–time curves after i.v. administration of phillyrin to rat at doses of 1.25 (■), 2.50 (△) and 5.00 (♦) mg/kg.

Table 4
Pharmacokinetic parameters of phillyrin (1.25, 2.50 and 5.00 mg/kg, i.v., $n = 6$) in rats after intravenous administration

Parameter	Estimate (1.25 mg/kg)	Estimate (2.50 mg/kg)	Estimate (5.00 mg/kg)	One way ANOVA (p-value)
$t_{1/2\alpha}$ (min)	6.41 ± 0.21	5.07 ± 0.64	5.36 ± 0.68	>0.05
$t_{1/2\beta}$ (min)	21.38 ± 2.41	26.13 ± 3.92	23.07 ± 2.01	>0.05
$AUC_{0\rightarrow\infty}$ (μg min/ml)	84.38 ± 9.34	147.1 ± 20.97	286.2 ± 42.55	>0.05
Vol (l/kg)	5.06 ± 1.25	5.68 ± 0.51	5.42 ± 0.83	
Cl (l/min/kg)	0.15 ± 0.02	0.16 ± 0.02	0.13 ± 0.02	
MRT (min)	32.12 ± 1.45	32.36 ± 2.23	31.25 ± 3.88	

Male Wistar rats received intravenous doses (1.25, 2.50 and 5.00 mg/kg) of phillyrin. Blood samples (0.4 ml) were obtained at 0, 5, 15, 25, 35, 45, 55, 65, 75, 85 and 95 min into heparinized tubes. Plasma samples were obtained following centrifugation at 1500 × g for 10 min. Data are expressed as mean ± S.D.; $t_{1/2\alpha}$, distribution half-life; $t_{1/2\beta}$, elimination half-life; $AUC_{0\rightarrow\infty}$, area under the concentration–time curve; Vol, volume of distribution; Cl, clearance; MRT, mean residence time.

A biphasic phenomenon with a rapid distribution followed by a slower elimination phase was observed from the plasma concentration–time curves (Fig. 3). The pharmacokinetic parameters, derived from these data and calculated by the DAS program, were shown in Table 4. The short distribution half-life of phillyrin observed in this experiment indicated that phillyrin rapidly distributed in rat in about 5–7 min. The elimination was relatively slower in about 21–27 min.The values of Vol from the three groups with different doses were 5.06, 5.68, 5.42 l/kg, respectively. The AUC increase was proportional to the administrated dose. The sum of these results indicated that, in the range of the doses examined, the pharmacokinetics of phillyrin in rat was based on first order kinetics.

4. Conclusions

A sensitive, specific, accurate and reproducible reversed-phase HPLC method for the determination of philyrin in rat plasma was developed which has been successfully applied to the study of pharmacokinetics of phillyrin in rat for the first time. This method can be available for large number of biological samples very efficiently. Further studies are ongoing in our laboratory to further characterize the absorption and metabolism of phillyrin.

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