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Short communication

Quantitative determination of apigenin and its metabolism in rat plasma after intravenous bolus administration by HPLC coupled with tandem mass spectrometry

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Abstract

Apigenin is a flavone and is being developed for treatment of cardiovascular disease. A sensitive and accurate quantitative detection method using liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) for the measurement of apigenin and luteolin levels in rat plasma is described. Analytes were separated on a separation by a Luna C_{18} (5 μm, 100 mm × 2.0 mm) column with acetonitrile:methanol:water (35:40:60, v/v/v) as a mobile phase. The eluted compounds were detected by tandem mass spectrometry. Good linearity ($R^2 > 0.9997$) was observed for both analytes over the range of 2.5–5000 ng/mL in 0.1 mL of rat plasma. The overall accuracy of this method was 93–105% for apigenin and 95–112% for luteolin in rat plasma. Intra-assay and inter-assay variabilities were less than 11% in plasma. The lowest quantitation limit for both apigenin and luteolin was 2.5 ng/mL in 0.1 mL of rat plasma. Practical utility of this new LC/MS/MS method was demonstrated in a pilot pharmacokinetic study in rats following intravenous administration of apigenin. Metabolism of apigenin to luteolin in vivo was established.

Keywords: Apigenin; Metabolism; Luteolin; LC/MS/MS; Pharmacokinetics

1. Introduction

Apigenin (5,7,4′-trihydroxyflavone) belongs to a subclass of flavones and is a commonly used dietary component of some vegetables, fruits and traditional Chinese medical herbs [1–4] (for chemical structure see Fig. 1). It has been reported to be anticarcinogenic, anti-inflammatory, and antimutagenic [5–9].

Extensive studies of its pharmacology and pharmacokinetics have been conducted in which ex vivo isolated perfused liver techniques have deduced that the major hepatic metabolite of apigenin is luteolin (3′,4′,5,7-tetrahydroxyflavone, for chemical structure see Fig. 1) [10], following isolation of phase II conjugates by HPLC with UV detection [11]. The pharmacokinetics of apigenin and luteolin in dogs, following oral administration of traditional Chinese medicine containing these two components, has been reported. Unfortunately, the concentration of apigenin was too low to be quantified accurately [12]. In other studies, the interactions of flavonoids and P-glycoprotein mediated transport is growing in popularity [13–16] and such interest might reasonably include apigenin and luteolin. In order to study the pharmacokinetics of the compounds, a robust and accurate analytical method needs to be developed.

In this work, we developed an LC/MS/MS method and applied it to the determination of apigenin and luteolin plasma levels in rat. We attained a limit of quantitation of 2.5 ng/mL in rat plasma. The speed of sample preparation and analysis, selectivity and sensitivity proved to be satisfactory. In addition, using the method described above, the pharmacokinetic profile of apigenin in rats has been determined following intravenous bolus administration and it has been shown that apigenin metabolizes to luteolin in vivo.

2. Materials and methods

2.1. Chemicals and materials

Apigenin (lot no. 060305) was extracted from parsley with a purity of 98.0%. Luteolin (lot no.060205) was extracted from

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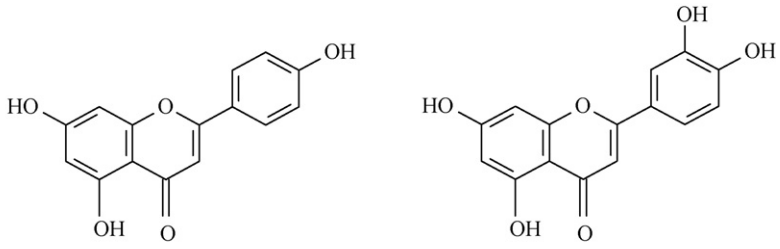

Fig. 1. Chemical structure of apigenin and luteolin.

the *Arachis hypogaea* nutshell with a purity of 98.0%. Apigenin and luteolin were supplied by Shanghai Healthjoy Chemical Co., LTD. Both of them were analyzed for purity and stability using HPLC method by the supplier. Internal standard lovastatin (lot no. QR-93121) was purchased from the Shanghai Food and Drug Administration (Shanghai, China). Acetonitrile and methanol (Tedia Inc., CA, USA) were HPLC grade. Distilled, deionized water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA). All other solvents and chemicals were analytical grade or better.

2.2. Standard solutions

Stock solutions of apigenin and luteolin were prepared in methanol at a concentration of 10 mg/mL. Further stock solutions were made by diluting the limited stock standard solution with methanol. Internal standard was prepared in methanol at a concentration of 0.2 mg/mL then diluted to 50 ng/mL before use. Both stock solutions were stored at 4 °C until use.

2.3. Apparatus and chromatographic conditions

The LC system comprised an isocratic pump (1100 series), and autosampler (1100 series) and a degasser (1100 series) (Agilent Technologies Inc. Palo Alto, CA, USA). Mass spectrometric analysis was performed using an API3000 (triple-quadrupole) instrument with an ESI interface with TurboIon spray (Applied Biosastems, Foster City, CA, USA). The data acquisition and control systems used Analyst 1.4 software from ABI Inc. The analytical column used was a Luna C18 (5 μm, 100 mm × 2.0 mm, Phenomenex, CA, USA). The mobile phase was a mixture of acetonitrile, methanol, and water (35:40:60, v/v/v). The mobile phase was degassed automatically using the electronic degasser system. The column was equilibrated and eluted under isocratic conditions utilizing a flow rate of 0.2 mL/min at ambient temperature (25 °C).

High-purity nitrogen was provided by a liquid nitrogen tank. Negative ion mass spectrometry was performed using the following conditions: nebulize gas: 8 L/min; curtain gas: 8 L/min; collision gas: 4 L/min; ionspray voltage: −4500 V; temperature: 500 °C. Other analyte specific parameters are shown in Table 1.

2.4. Sample preparation

The plasma samples were processed by the protein precipitation method [17]. A 0.1 mL of plasma sample was combined with 300 μL internal standard lovastatin (50 ng/mL), then vortexed for 1 min. After centrifugation at 15,000 rpm (15,000 × *g*) for 5 min, 100 μL of the supernatant liquid was transferred to a sample vial and 5 μL was injected into the LC/MS/MS for quantitative analysis.

2.5. Assay validation

A calibration curve was generated to confirm the linear relationship between the peak area ratio and the concentration of apigenin and luteolin in the test samples. Apigenin and luteolin were added to the plasma blank to yield final concentrations of 2.5, 5, 10, 50, 100, 500, 1000 and 5000 ng/mL in 0.2 mL of rat plasma. To determine the within-day precision of the method, three samples of plasma with the concentrations of 3.5, 650 and 3000 ng/mL were analyzed five times on the same day. To determine the between-day precision and the accuracy, a further three samples of plasma were run on each of five different days.

In short-term stability tests, samples were analyzed after 4 h at room temperature and 24 h stored in a refrigerator (4 °C). The freeze–thaw stability was evaluated by analyzing samples after undergoing two freeze–thaw cycles. Long-term stability was studied by assaying samples after storage for 1 week at −70 °C.

Table 1
MS data for apigenin and luteolin identified

Drug name	Q1	Q3	Dell time (ms)	DP (v)	FP (v)	EP (v)	CE (v)	CXP (v)
Apigenin	269.0	150.9	200	−90.0	−200	−10	−35.23	−15
Luteolin	284.9	133.0	200	−90.0	−200	−10	−35.88	−15
Lovastatin	421.0	319.1	200	−90.0	−200	−10	−47.03	−15

2.6. Drug administration and sampling

Four male Sprague–Dawley rats (200 ± 20 g) were provided by Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed in Shanghai Medicilon Inc. animal facility (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 ca. 60% relative humidity. Animal studies were approved by IACUC of Shanghai Medicilon Inc. and were carried out in accordance with the SOPs of the facility.

Apigenin was dissolved in 0.9% saline and the pH adjusted to 9 using 2 M NaOH. Upon preparation, the dosing solution was administered intravenously to rats at a dose of 20 mg apigenin/kg body weight. The blood samples were collected via carotid cannulation at 0 min (pre-dose), 5 min, 15 min, 30 min, 60 min, 2 h, 4 h, 6 h, 8 h, 24 h, and 48 h following administration. The blood samples were placed in heparinized tubes and the plasma separated by centrifugation at 8000 rpm (4400 × *g*) for 6 min. All plasma samples were stored at −20 °C until analysis.

2.7. Pharmacokinetic analysis

Pharmacokinetic parameter for apigenin and luteolin were calculated using PK analysis software DAS 2.0 (Gaosi Data Analysis Inc., Wuhu, China) and the non-compartmental model was applied. The area under the curve (AUC) was calculated by the trapezoidal rule between first (0 h) and last sampling time plus C_n/λ_n, where C_n is the concentration of last sampling and λ_n is the elimination rate constant. $AUC_{0\to t} = \Sigma(C_i + C_{i-1}) \times (t_i - t_{i-1})/2$; $AUC_{0\to\infty} = AUC_{0\to t} + C_n/\lambda_n$. The elimination half-life was estimated by linear regression analysis of the terminal phase of the plasma concentration-time profile.

3. Results and discussions

3.1. 3.1Chromatographic separations

Several combinations of acetonitrile, methanol and water were evaluated as possible mobile phases. It was determined that the combination of acetonitrile, methanol and water (35:40:60, v/v/v) described herein was found to be the most suitable for separating either apigenin and internal standard lovastatin or luteolin and lovastatin. Under the described chromatographic conditions, the retention time was about 1.7 min, 1.5 min, and 2.0 min for apigenin, luteolin, and lovastatin, respectively, which all were eluted without any endogenous interferences from the blank rat plasma.

3.2. Assay validation

The standard curves for rat plasma, containing known amounts of apigenin and luteolin, were linear over the range 2.5–5000 ng/mL in 0.1 mL of plasma and could be described by the regression equations: $Y = 0.000669X + 0.00167$ ($R^2 > 0.9998$) for apigenin, and $Y = 0.00193X - 0.00067$ ($R^2 > 0.9997$) for luteolin, in which Y was the peak area ratio of apigenin or luteolin to the internal standard, and X was the analyte concentration in ng/mL in the plasma.

Table 2
Accuracy and precision of the determination of apigenin and luteolin in plasma

	Apigenin (ng/mL)			Luteolin (ng/mL)		
	3.5	650	3000	3.5	650	3000
Intra-day accuracy and precision						
Mean	3.29	664.81	3048.49	3.35	630.67	3343.92
SD	0.30	27.42	59.54	0.12	37.75	24.51
RSD (%)	9.12	4.12	1.95	3.58	5.99	0.73
Accuracy (%)	94.00	102.28	101.62	95.71	97.03	111.46
Inter-day accuracy and precision						
Mean	3.59	677.84	3077.87	3.40	653.51	3219.05
SD	0.39	50.33	229.33	0.35	38.78	156.82
RSD (%)	10.78	7.43	7.45	10.29	5.93	4.87
Accuracy (%)	102.61	104.28	102.60	97.14	100.54	107.30

The lowest quantitation limit for both apigenin and luteolin was 2.5 ng/mL in 0.1 mL of rat plasma. The method was found to be sufficiently sensitive for the determination of pharmacokinetic analysis of both apigenin and luteolin in rats treated with apigenin.

The inter-assay and intra-assay precision and accuracy, displayed in Table 2 demonstrate that the assay method is reliable and reproducible.

The results of stability test demonstrate that the tests compounds, apigenin and luteolin, were stable in rat plasma when either stored at −70 °C, during freezing and thawing, or at room temperature for 4 h (RSD ≤ 9.43).

3.3. Pharmacokinetics of apigenin following intravenous administration of apigenin

The plasma concentration of apigenin following intravenous administration is presented in Fig. 2. Selected pharmacokinetic parameters are shown in Table 3.

Following an IV bolus injection of apigenin at 20 mg/kg, the mean ± SD value of systemic clearance was 6.12 ± 0.79 L/h/kg,

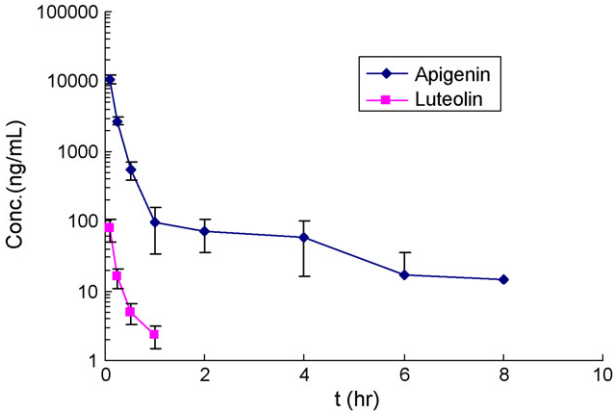

Fig. 2. Concentration–time curve in male rats following intravenous administration of apigenin at a dose of 20 mg/kg ($n = 4$).

Table 3
Selected pharmacokinetics parameters of apigenin and luteolin in male SD rats following intravenous administration of apigenin at 20 mg/kg ($n = 4$)

Parameters	Apigenin	Luteolin
$AUC_{(0-t)}$ (μg/L h)	3211.54 ± 554.88	24.70 ± 8.99
$AUC_{(0-\infty)}$ (μg/L h)	3312.10 ± 473.30	28.73 ± 11.33
$MRT_{(0-\infty)}$ (h)	0.65 ± 0.50	0.75 ± 0.86
$t_{1/2_z}$ (h)	1.75 ± 1.18	0.97 ± 1.25
V_z (L/kg)	15.75 ± 11.73	NA
CL_z (L/h/kg)	6.12 ± 0.79	NA
C_{max} (μg/L)	10933.88 ± 1730.11	78.16 ± 26.63

which corresponded to 1.85-fold of rat hepatic blood flow (3.31 L/h/kg).

The volume of distribution at terminal phase was 15.75 ± 11.73 L/kg, which was greater than the total body water (0.67 L/kg) in the rats. Apigenin distributes well into the tissues.

3.4. Pharmacokinetics of luteolin following intravenous administration of apigenin

A finding in this experiment was that luteolin, determined absent in the dosing solution, was detected in the plasma of treated rats and was showing an elimination tendency too.

Following an IV bolus injection of apigenin at 20 mg/kg, luteolin was detected in the plasma of treated rats at 5 min, 15 min, 30 min and 1 h. The mean ± SD values of C_{max} and T_{max} for luteolin following IV administration of apigenin at a nominal dose of 20 mg/kg were 78.16 ± 26.63 μg/L and 0.08 ± 0.00 h.

In summary, we have developed a highly sensitive and accurate analytical LC/MS/MS method for quantitative detection of apigenin and luteolin in plasma. By applying this method we have evaluated the pharmacokinetic profile of both apigenin and luteolin following intravenous administration of apigenin to the rat. The previously described in vitro metabolism of apigenin to lutolin was confirmed to occur in vivo. Our study focused on method development and the concentration level of luteolin as metabolism of apigenin according to the concentration of apigenin. Because there may be other metabolism route and to what degree the parent compound would degrade in this way is unknown yet, further study needs to be initiated. The availability of this assay will now permit further detailed pharmacokinetic studies of apigenin and luteolin in the future.

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