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Short communication

Simultaneous determination of luteolin and apigenin in dog plasma by RP-HPLC

Liping Li, Huidi Jiang*, Haohao Wu, Su Zeng

Department of Pharmaceutical Analysis and Drug Metabolism, College of Pharmaceutical Sciences, Zhejiang University, 353 Yan'an Road, Hangzhou 310031, China

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Abstract

A specific and accurate high-performance liquid chromatographic method has been developed and validated for the simultaneous determination of luteolin and apigenin in the plasma of dog. The sample was treated with 6.0% perchloric acid to precipitate the protein. Luteolin and apigenin were extracted with ethyl acetate. The organic layer separated was dried and reconstituted in the mobile phase. The HPLC separation was performed on C_{18} column and the UV detector was set at 350 nm. The standard curve for luteolin and apigenin in plasma were linear over the range of 38.5–4350 and 16.5–1860 ng/ml, with the correlation coefficients 0.9996 and 0.9999, respectively. The assay recoveries for luteolin and apigenin ranged from 102.7 to 104.5% and 93.8–101.8%, respectively. The intra- and inter-day precisions (R.S.D.) for luteolin and apigenin were all less than 7.9%. The sample was stable within 24 h at 4 °C storage, 30 days at −20 °C storage, and undergoing four freeze–thaw-assay cycles. The limits of detection (LOD) of luteolin and apigenin were 1.82 and 1.94 ng/ml, while the limits of quantification (LOQ) were 7.84 and 6.29 ng/ml, respectively. The method developed was applied successfully to study pharmacokinetics of the effective composition (luteolin) of *Chrysanthemum morifolium* extract in dogs after single dose of oral administration.

Keywords: *Chrysanthemum morifolium* extract; Luteolin; Apigenin; HPLC analysis

1. Introduction

The flower of *Chrysanthemum morifolium* Ramat. (CM) has been widely used as a healthy food and folk medicine in China for a long time. Its antioxidation, vasoactive effects and anti-ischemia/reperfusion injury action have been proved in animal models and clinic [1,2]. It is found that the flavonoids are abundant in CM. The main flavonoids in CM were luteolin-7-O-β-D-glucoside and apigenin-7-O-β-D-glucoside [3] which could be biotransformed to their aglycone forms—luteolin (3′,4′,5,7-tetrahydroxyflavone), and apigenin (4′,5,7-trihydroxyflavone) (Fig. 1), respectively, in intestine by microorganism when the extract of CM was orally administrated [4,5]. Our previous studies also showed luteolin-7-O-β-D-glucoside and apigenin-7-O-β-D-glucoside were not be detected in plasma of dog even if 15 g/kg of CM was administrated orally. Luteolin and apigenin possess diverse pharmacological activities which could be related with most actions of CM in clinic [6,7]. We suggested that luteolin and apigenin might be the bioactive components of CM when it was orally administrated.

Flavonoids absorption and metabolism have been studied by in vitro methods for instance in human intestinal Caco-2 cells [8] and rat liver microsome [9]. However, to the best of our knowledge, the pharmacokinetics of flavonoids or flavonoids contained Chinese traditional medicine and other natural drugs has not been reported broadly [10]. Therefore, our present study aims to develop a HPLC method for simultaneous determination of luteolin and apigenin in plasma for further studies on the pharmacokinetics and action

* Corresponding author. Tel.: +86 571 87 217226; fax: +86 571 87 964190.
E-mail address: hdjiang@zju.edu.cn (H. Jiang).

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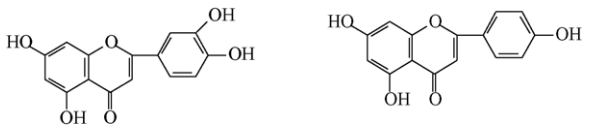

Luteolin ($C_{15}H_{10}O_6$) Apigenin ($C_{15}H_{10}O_5$)

Fig. 1. The chemical structures of luteolin and apigenin.

mechanism of bioactive compositions of CM extract (CME) orally administered by dog.

2. Experimental

2.1. Materials

Luteolin and apigenin (purity > 99%) were obtained from J & K-ACIoS (serial number: 62696) and Lot 111K1520 SIGMA-ALDRICH CO., respectively. Methanol and acetonitrile, HPLC grade, were purchased from Merck Co. Ltd. CME was provided by the Institute of Medicine, Zhejiang University, China, containing 7.60 and 5.19% of luteolin and apigenin determined by HPLC after being hydrolyzed with hydrochloric acid. The other reagents were of analytical purity.

Beagle dogs, 13.0–15.0 kg, were purchased from the Experimental Animal Center of Zhaoqin, Guangdong Province, China. Animal welfare and experimental procedures were strictly in accordance with the guide for the care and use of laboratory animals and the related ethical regulations of our university.

2.2. Instrumentation

Agilent 1100 HPLC system, equipped with G1314A isocratic pump, a thermostatted column compartment, a variable-wavelength UV detector (VWD) and Agilent Chemstation software.

2.3. Sample collection

Dogs were orally administrated with CME at the dosage of 102 mg/kg after being fasted for 12 h. The blood samples (1.0 ml) were collected from saphenous vein in a 5 ml tube containing heparin at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 24.0 h after administration. The plasma was isolated by centrifugation for 10 min at 3000 rpm. Blank plasma was collected by the same method before dogs were orally dosed with CME.

2.4. Chromatographic condition

The analysis was performed on Diamonsil ODS C_{18} column (250 mm × 4.6 mm, 5 μm). The optimum separation of HPLC was carried out with a mobile phase composed of methanol and 0.2% phosphoric acid aqueous solution (58:42, v/v) at a flow-rate of 1.0 ml/min. The volume of sample injected was 50 μl, the detective wavelength and the column temperature were set at 350 nm and 30 °C, respectively.

2.5. Preparation of reagents and solutions

A stock solution containing 232.0 μg/ml luteolin and 99.2 μg/ml apigenin was prepared in methanol. This solution was diluted with the mobile phase to prepare the working solutions containing 1.544, 4.632, 23.16, 46.31, 77.33, 116.0 and 174.0 μg/ml of luteolin and 0.660, 1.980, 9.900, 19.80, 33.07, 49.60 and 74.40 μg/ml of apigenin, respectively. The analytical standards were used to assess the linearity, recovery, accuracy, precision and stability studies.

Plasma calibration standards, containing 38.50, 115.8, 578.8, 1158, 1933, 2900 and 4350 ng/ml of luteolin and 16.50, 49.50, 247.5, 495.0, 826.8, 1240 and1860 ng/ml of apigenin, respectively, were prepared individually by diluting 5 μl of the working standards to 0.2 ml plasma. This method was used to prepare appropriately calibration standards in plasma in replicates. The calibration standards were prepared freshly on each day of analysis.

2.6. Assay sample preparation

0.2 ml 6.0% perchloric acid was added slowly into 0.2 ml blank, control or sample plasma to precipitate protein completely. Luteolin and apigenin were extracted with 3.0 ml of ethyl acetate by vortexing for 5 min. After centrifugation for 10 min at 3500 rpm, 2.0 ml of the supernatant organic phase were carefully transferred to another tube and evaporated to dryness in the vacuum desiccator under room temperature. The residue was reconstituted in 250 μl of mobile phase, the mixture was centrifuged for 10 min at 12,000 rpm and the supernatant was injected into the HPLC apparatus.

3. Results

3.1. Validation

3.1.1. Separation and selectivity

Individual blank plasma sample, control sample and plasma sample following an oral administration of CME were assayed. Fig. 2 showed the chromatograms of blank sample, blank plasma sample spiked with 1158 ng/ml of luteolin and 495.0 ng/ml of apigenin. Luteolin and apigenin were well separated under the present chromatographic condition. Retention time was approximately 11.0 min for luteolin and 17.0 min for apigenin. No interference peaks of endogenous constituents from plasma and reagents were observed at the retention time of luteolin and apigenin.

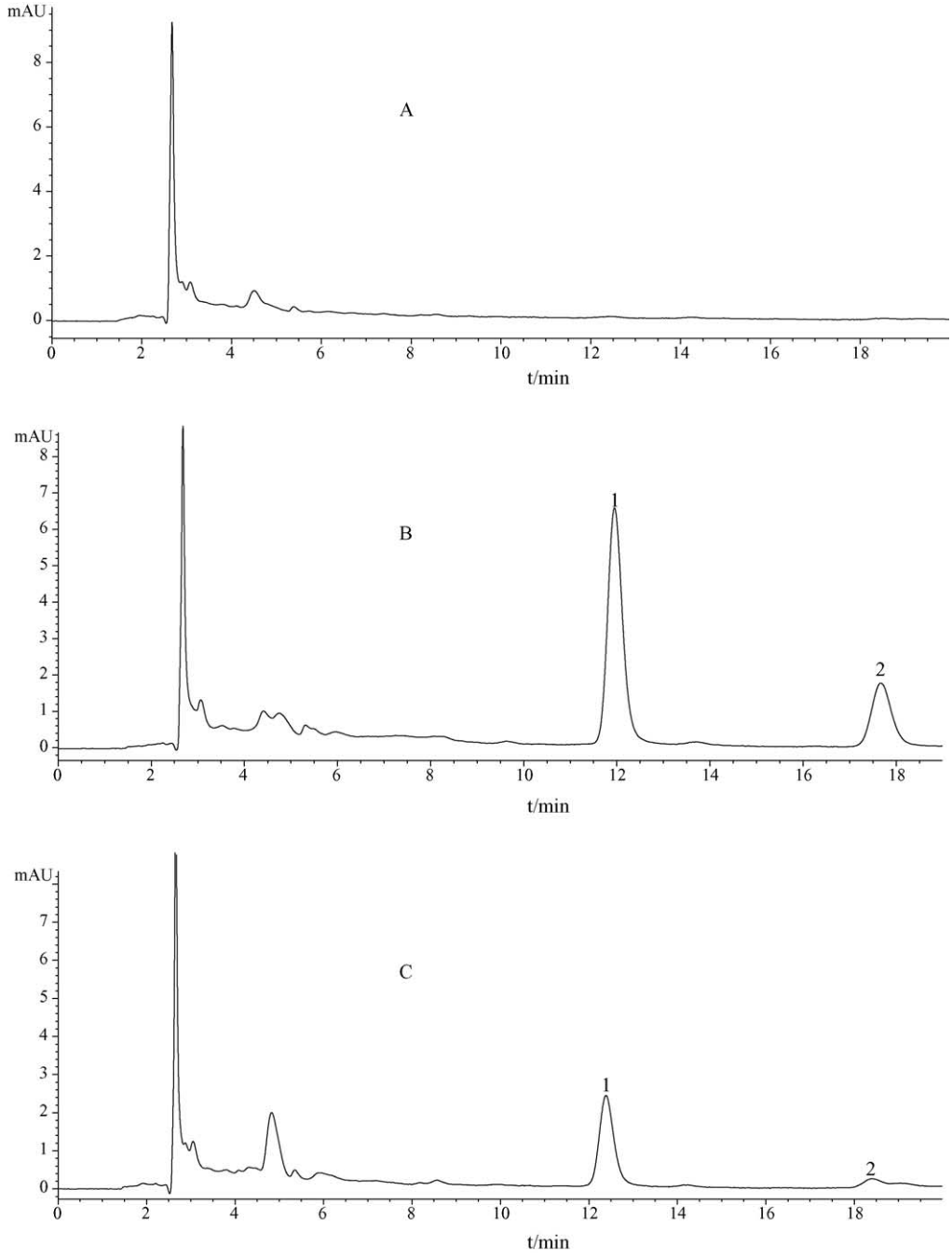

Fig. 2. Chromatograms of blank plasma (A); plasma spiked with 1158 ng/ml luteolin and 495.0 ng/ml apigenin (B); plasma sample after an oral administration of CME (102 mg/kg) for 2.0 h (C). (1) Luteolin; and (2) apigenin.

3.1.2. Calibration curve

The peak area (y) of the luteolin and apigenin was measured and plotted against the concentration (x) of luteolin and apigenin after HPLC analysis. The standard curve of luteolin and apigenin in dog plasma were linear over the range of 38.50–4350 and 16.50–1860 ng/ml, respectively. The regression equations were $y = 0.1337x + 0.5419$ ($r = 0.9996$, $n = 7$) for luteolin and $y = 0.1103x + 0.5125$ ($r = 0.9999$, $n = 7$) for apigenin, respectively.

3.1.3. Recovery

Blank plasma of dog was spiked with luteolin and apigenin at low, medium and high concentration, respectively (see Table 1). Five samples for each concentration were processed as described previously. The assay recovery was obtained by comparing the peak area of luteolin and apigenin of the extracted samples with which calculated from the calibration curve. While, absolute recovery were determined by comparing the peak area of luteolin and apigenin of the extracted

Table 1
Recovery for assay of luteolin and apigenin ($n = 5$)

Theoretical concentration (ng/ml)	Absolute recovery		Assay recovery	
	Mean (%)	R.S.D. (%)	Mean (%)	R.S.D. (%)
Luteolin				
38.50	91.08	7.9	104.5	8.7
1933	88.35	0.70	102.7	0.70
4350	90.69	0.83	103.2	1.0
Apigenin				
16.50	96.52	8.1	93.82	11
826.8	89.92	0.52	101.6	0.53
1860	91.73	1.5	101.8	1.5

samples with the peak area obtained from direct injections of a standard solution containing the same concentration of luteolin and apigenin. As the results shown in Table 1, the lowest absolute recovery was 88.35% while the lowest assay recovery was 93.82%, which revealed that the accuracy of the method was satisfied.

3.1.4. Precision

Blank plasma spiked with three different concentrations of luteolin and apigenin (Table 2) were used for precision study. The intra- and inter-day precision (R.S.D.) of the entire analytical procedure were evaluated by analysis homogeneous sample in five replicates in 1 or 5 days. All values of R.S.D. of intra-assay were less than 7.9%, while the values of R.S.D. for inter-assay precision were less than 6.0% (Table 2).

3.1.5. Stability

The plasma samples were assayed under three different conditions to assess the stability of luteolin and apigenin in plasma samples. All stabilities were assayed at low and high concentrations (38.50 and 4350 ng/ml for luteolin, 16.50 and 1860 ng/ml for apigenin) in triplicate. In short-term stability test, samples were stored in refrigerator (4 °C) for 0–24 h, the assay was performed at 0, 2, 4, 8, 12, 24 h, the results showed 94.0–105.1% and 91.3–104.5% of initial content of luteolin and apigenin were recovered. Long-term stability was studied by assaying samples after 1, 3, 7, 15, and 30 days of storage (−20 °C), 97.6–107.5% and 95.2–103.2% of initial content of luteolin and apigenin were recovered. The stability of freeze–thaw cycles was done over four cycles at −20 °C, 97.5–104.1% and 94.1–105.9% of initial content of luteolin and apigenin were detected. The previous stability tests demonstrated that the analytes were stable under these conditions.

Table 2
Precision of assay for luteolin and apigenin

Theoretical concentration (ng/ml)	Precision (% R.S.D.)	
	Intra-assay	Inter-assay
Luteolin		
38.50	7.9	6.0
1933	2.3	3.4
4350	0.86	1.6
Apigenin		
16.50	7.5	3.9
826.8	2.2	3.9
1860	1.3	1.7

3.1.6. Sensitivity

The limit of detection (LOD) of luteolin and apigenin was 1.82 and 1.94 ng/ml in dog plasma, respectively, which was measured based on signal/noise (S/N) = 3 with dilution step by step.

The limit of quantification (LOQ) was defined as the lowest nominal concentration of the control sample in five replicate with acceptable precision and accuracy (±20% R.S.D.). The LOQ of assaying luteolin and apigenin in dog plasma was 7.84 and 6.29 ng/ml, with R.S.D. = 6.2 and 5.9%, respectively.

3.2. Pharmacokinetic study of CME in dogs

The developed HPLC method was applied to determine luteolin and apigenin plasma concentrations in the dog following single oral dosing of CME (102 mg/kg). Unexpectedly, the concentration of apigenin was below the LOQ, the mean plasma concentration–time profile of luteolin best-fitted two-compartment model with 0.333 h lag time was shown in Fig. 3.

Software 3P87 was applied to the various pharmacokinetic parameters for luteolin, the results were listed in Table 3. Apparent maximal concentration (C_{max}) and the corresponding time (t_{max}) of luteolin were about 450 ng/ml and 3.0 h determined visually from the concentration–time profile. The parameters of luteolin after oral administration of CME including: the half-life for first exponential term ($t_{1/2\alpha}$), and half-life for second exponential term ($t_{1/2\beta}$) which were calculated by $\ln(AUC_{0-24\,h})/\alpha$ and $\ln(AUC_{0-24\,h})/\beta$, where α and β are the rate constants for the first and second exponential terms, respectively, the total area under the plasma concentration versus time curve ($AUC_{0-24\,h}$) which was determined

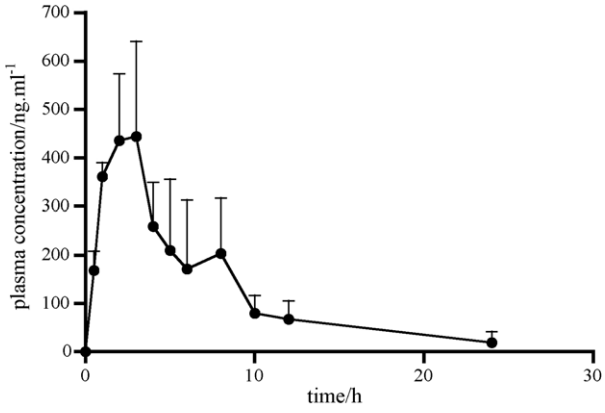

Fig. 3. Mean (±S.D.) plasma concentration–time profile of luteolin after oral administration of CME (102 mg/kg) to dogs ($n = 3$).

Table 3
Pharmacokinetic parameters of luteolin after oral administration of CME (102 mg/kg) to three dogs

Parameters	Mean ± S.D.
$t_{1/2\alpha}$ (h)[a]	1.799 ± 0.37
$t_{1/2\beta}$ (h)[b]	6.968 ± 3.0
V_d/F (c) (l/kg)[c]	149.6 ± 2.9
$AUC_{0-24\,h}$ (ng/ml h)[d]	3095 ± 742
CL (l/kg h)[e]	33.14 ± 8.7
C_{max} (ng/ml)[f]	463.9 ± 87
T_{max} (h)[g]	1.540 ± 0.30

[a] The half-life of luteolin for the first exponential.
[b] The half-life of luteolin for the second exponential.
[c] Apparent volume of the distribution of central compartment.
[d] Area under the plasma concentration–time curve to time infinity.
[e] Plasma clearance.
[f] The maximum plasma concentration.
[g] The time to reach C_{max}.

by the linear trapezoidal rule from time zero to last sampling point equal to or above the lower limit of quantitation AUC_t, added as the residual area as estimated by log-linear extrapolation to infinity, and plasma clearance (CL) as well as apparent volume of distribution (V_d/F) were calculated from CL = Dose/AUC and $V_d/F = CL/\beta$, respectively, where F is the fraction of the administered dose absorbed.

4. Discussion

4.1. Extraction solvent

According to Ref. [11], the solvent of acetone and diethyl ether with different ratio used to extract luteolin and apigenin, the results showed that the extraction recoveries of luteolin and apigenin were about 55 and 40%, respectively. It was deduced that due to multiple hydroxies present in the structure of luteolin and apigenin (Fig. 1), both of them had strong polarity which resulted in low solubility in low polarity solvent. Therefore, the higher polarity of solvent, ethyl acetate was employed, and the satisfactory extraction recoveries were obtained. The extraction recoveries obtained with 3.0 ml of ethyl acetate were more than 88 and 89% for luteolin and apigenin, respectively. Moreover, the endogenous components of the dog plasma did not interfere with the elution of any of the analytes (Fig. 2).

4.2. Reagent for protein precipitation

To get clear biological sample and protect the HPLC column, it is necessary to precipitate the plasma protein. The organic precipitating agents, acetonitrile and methanol, were tested. However, both of them failed due to poor recoveries and incomplete precipitation. Finally, 6% perchloric acid was adopted, as a result, the recovery was increased markedly.

4.3. Internal standard (IS)

The study was initially tried with β-naphthol and quercetin as IS. It was regretted that β-naphthol was oxidized so easily that it was undetectable after sample preparation. According to the retention time in the present chromatographic condition, quercetin was suitable to be an internal standard, however, the absolute recovery of the quercetin was only about 10% because of its extensive binding to plasma proteins [12]. The IS was given up ultimately. The variations in the observed analyte concentrations for absolute- and assay-recovery (% mean) (Table 1) and intra- and inter-assay precision (% R.S.D.) (Table 2) were within the acceptable limits of ≤20% at lowest concentration and ≤15% at all other concentrations without IS [13]. Therefore, the method without IS was also feasible.

4.4. Application of the method

CME contains luteolin glucoside and apigenin glucoside which could be hydrolyzed to their aglycones by the action of intestinal microorganism or intestinal hydrolytic enzyme when it was administrated orally. The present study showed that the concentration of apigenin was too low to be measured accurately after the dog was orally administered with single dose of CME, while the plasma concentration of luteolin could be determined without any problem. The same circumstance occurred in rabbit. Other study in our lab showed that the concentration of apigenin was very low in plasma of dogs even 920 mg/kg of CME was administrated orally. However, the content of apigenin in CME used in this study was 5.19%. The concentration of apigenin was also lower than LOQ even apigenin was orally administrated to rabbit. Therefore, we suggested that apigenin may be transformed to luteolin in vivo [14,15]. However, the pharmacokinetic process was best-fitted to two-compartment model by comparing the AIC values between some possible models whose AIC was smallest.

Though, the method was unable to determine the apigenin when apigenin and its glucoside were orally administrated, it can be applied to assay apigenin injected. The study is still underway in our lab.

5. Conclusion

A reversed-phase HPLC assay for simultaneous determination of luteolin and apigenin in dog plasma has been developed and validated. It has been proved simple, rapid, and reliable method for quantitative determination of luteolin in plasma samples from three dogs after single oral administration of 102 mg/kg CME. Its applications to the determination of luteolin in plasma of experimental animals after administration of Chinese traditional medicine or natural drug containing luteolin or luteolin glucoside are in progress in our lab.

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