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Short communication

Determination and assay validation of luteolin and apigenin in human urine after oral administration of tablet of *Chrysanthemum morifolium* extract by HPLC

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Abstract

A simple, selective, precise, and accurate RP-HPLC assay for simultaneous analysis of luteolin and apigenin in human urine was developed and validated. Prior to HPLC analysis, urine samples were incubated with β-glucuronidase/sulfatase. Separation and quantification were achieved on an Agilent C_{18} column under isocratic conditions using a mobile phase (methanol:0.2% phosphoric acid aqueous solution 55:45, v/v) maintained at 1.0 ml/min at 30 °C. The standard curves were linear over the range of 0.0975–7.800 and 0.1744–13.95 μg/ml for luteolin and apigenin, respectively ($r > 0.999$). The assay recoveries for luteolin and apigenin were above 85.7%. The intra-day and inter-day precision (R.S.D.) for luteolin were below 2.2 and 4.0%, respectively, and for apigenin were less than 2.8 and 5.4%, respectively. Stability studies showed three concentration of luteolin and apigenin in urine quality control samples were stable undergoing three freeze–thaw cycles, storage at room temperature for 4 h, and at −20 °C for 3 days. The limit of quantitation was 39.20 ng/ml ($n = 5$) for luteolin and 31.45 ng/ml ($n = 5$) for apigenin in human urine. The method developed was employed successfully to determine luteolin and apigenin in urine samples obtained from eight healthy volunteers following oral administration of tablet of *Chrysanthemum morifolium* extract (CME).

Keywords: *Chrysanthemum morifolium* extract; Luteolin; Apigenin; RP-HPLC; Urine

1. Introduction

The flower of *Chrysanthemum morifolium* Ramat. (CM) has been used as healthy food and folk medicine for sudorific, antidote, and clearing away pathogenic heat in China. The previous study of our Lab indicated CM attenuated contractile function and coronary flow reduction of isolated rat heart caused by ischemia/reperfusion [1]. Our study also showed CM induced both endothelium dependent and independent relaxation of rat thoracic aorta caused by phenylephrine or high level of K^+ [2]. Clinical study demonstrated CM had beneficial effect on coronary heart disease and could prevent of the cardiovascular diseases [3]. Studies showed flavonoids such as luteolin-7-*O*-β-D-glucoside, apigenin-7-*O*-β-D-glucoside were the main components of CM [4]. It is widely considered that flavonoid glucosides can be hydrolyzed in the gastrointestinal tract by the enzymes of intestinal bacteria [5] or the corresponding liver enzymes [6] followed by absorption and conjugation of their aglycones, which subsequently are excreted in urine mainly as sulfuric acid/glucuronic acid conjugates and to a lesser extent as free aglycones [7,8]. Our study has showed luteolin-7-*O*-β-D-glucoside and apigenin-7-*O*-β-D-glucoside in *C. morifolium* extract (CME) could be hydrolyzed when CME incubated with intestinal bacteria, which could explain why 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. As we speculated, luteolin and apigenin were detected in dog [9], rabbit, rat, and human plasma.

Since luteolin and apigenin possess the same effect as CM, such as vasodilatation [10,11] and antioxidation [12], we think they are the actual bioactive constituents of CM. The determination of luteolin and apigenin in plasma and the pharmacokinetics in dog after oral dose of CME have been reported [9]. However, the urine excretion of luteolin and apigenin after

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oral administration of CME or luteolin and apigenin or their glucosides contained drug has not been reported. Therefore, the aims of the present study were to develop and validate a simple and sensitive HPLC method for simultaneous analysis of luteolin and apigenin in human urine, and apply the developed method for determination of luteolin and apigenin in human urine of volunteers following oral administrated of CME tablet.

2. Materials and methods

2.1. Materials

Luteolin and apigenin (purity > 99%) were obtained from J & K-ACIoS (serial number: 62696) and Sigma–Aldrich Company (Lot 111K1520), respectively. *C. morifolium* extract (CME) tablet (Lot 040515), containing 7.13 mg of luteolin and 5.42 mg of apigenin in each tablet determined by HPLC after being hydrolyzed with hydrochloric acid, was provided by the Institute of Medicine, Zhejiang University, China. β-Glucuronidase (Type IX-A, from *E. coli*) and sulfatase (Type H-1, from pomatia) were purchased from Sigma Chemical Company (St. Louis, MO). Methanol, HPLC grade, was purchased from Merck Co. Ltd. The others were of analytical purity.

2.2. Chromatographic conditions

RP-HPLC analysis of urine samples was performed on the Agilent 1100 Chromatographic system, consisting of G1314A isocratic pump, a thermostatted column compartment, a variable-wavelength UV detector (VWD), and Agilent Chemstation software. Chromatographic separation was achieved on the Agilent ODS C_{18} column (250 mm × 4.6 mm, 5 μm), with a pre-column (Agilent ODS C_{18}, 10 mm × 4.6 mm, 5 μm). Determination of luteolin and apigenin was carried out with mobile phase composed of methanol and 0.2% phosphoric acid aqueous solution (55:45, v/v) at a flow-rate of 1.0 ml/min. The optimum separation of HPLC was achieved at 30 °C and monitored at 350 nm.

2.3. Reference standard solution preparation

The stock solution containing 780.0 μg/ml of luteolin and 930.0 μg/ml of apigenin was prepared in methanol. Several standard solutions of luteolin and apigenin, were prepared by diluting the stock solution with methanol according to the study demanded. All standard solutions were stable for at least 2 months when stored at −20 °C.

2.4. Urine sample collection

Four healthy women and four healthy men (body weight: 55 ± 5 kg) were enlisted in this study. The following criteria were fulfilled: no smoking, mealing without fruits and vegetables 1 day before the study.

Urine samples were collected after eight healthy volunteers administrated orally 12 tablets of CME at 0, 1, 2, 4, 6, 8, 10, and 12 h. After the volume was measured, about 5.0 ml of urine was stored in sealed containers at −20 °C.

2.5. Assay procedure

0.20 ml urine of blank, control, and sample acidified (to pH 4.9) with 20 μl of 0.58 mol/l acetic acid were incubated with β-glucuronidase (200 U/ml)/sulfatase (10 U/ml) under continuous shaking in 37 °C water bath for 2 h. After diluted with the mobile phase to 1.00 ml, the incubation solutions were centrifuged at 15,000 rpm for 10 min, and 50 μl of the supernatant was injected into the HPLC system.

3. Results

3.1. Chromatographic selectivity

This simple procedure afforded efficient separation and quantification of luteolin and apigenin in human urine, without interference of peaks of endogenous constituents from urine and reagents. Fig. 1 showed the typical chromatograms of blank urine, blank urine spiked with standard substances (0.1872 μg/ml luteolin and 0.3348 μg/ml apigenin), and urine sample after oral administration of CME tablet for 2 h. Luteolin and apigenin were eluted in approximately 10.5 and 16.0 min, respectively (Fig. 1).

3.2. Linearity and range

The calibration curves of luteolin and apigenin were constructed and the linearity of the method was estimated by regression analysis of their peak areas (y) against the concentrations (x, μg/ml) in urine, respectively. The calibration curves of luteolin and apigenin in urine were linear in the range from 0.0975 to 7.800 and 0.1744 to 13.95 μg/ml, respectively. The regression equations were $y = 51.80x + 3.547$ ($r = 0.9999$) for luteolin, and $y = 51.29x + 2.899$ ($r = 0.9997$) for apigenin.

3.3. Recovery tests

Human blank urine was spiked with the standards of luteolin and apigenin at low, medium, and high concentration, respectively (see Table 1). Five samples for each concentration were processed as described previously (Section 2.5). The assay recoveries and absolute recoveries were valuated. As the results shown in Table 1, the lowest absolute recovery was 87.1%, while the lowest assay recovery was 85.7%, which revealed that the accuracy of the method was satisfied.

3.4. Precision studies

The blank human urine, spiked with different concentrations of luteolin (0.0975, 2.340, and 7.800 μg/ml) and apigenin (0.1744, 4.185, and 13.95 μg/ml), were used for precision studies. The intra-assay and inter-assay precision (R.S.D.) were evaluated by analyzing homogeneous sample in five replicates, in 1 or 5 days. All values of the R.S.D. of intra-day precision

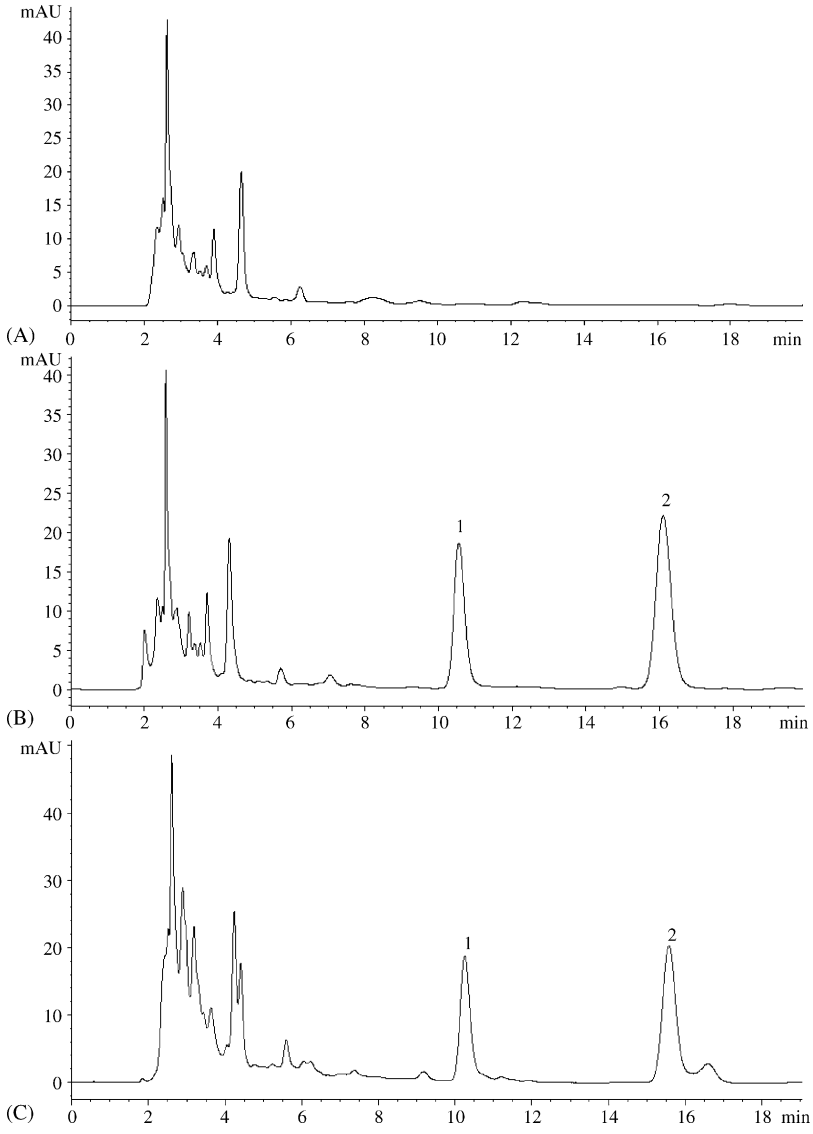

Fig. 1. Typical chromatograms of blank urine (A), blank urine spiked with 0.1872 μg/ml luteolin and 0.3348 μg/ml apigenin standards (B), and urine sample after a single oral administration of CME tablet for 2 h (C). (1) Luteolin and (2) apigenin.

were less than 2.8%, while the values of R.S.D. of inter-day precision were less than 4.0% (Table 2). The results showed that the precision of this method for analysis of luteolin and apigenin in human urine was satisfied.

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

Theoretical concentration (μg/ml)	Absolute recovery		Assay recovery	
	Mean (%)	R.S.D. (%)	Mean (%)	R.S.D. (%)
Luteolin				
0.0975	95.4	1.8	92.1	3.1
2.340	93.3	1.4	97.8	1.5
7.800	94.3	2.2	97.7	1.2
Apigenin				
0.1744	96.7	0.96	85.7	1.5
4.185	87.1	2.1	93.8	2.1
13.95	92.0	2.8	96.6	2.8

3.5. Stability studies of urine samples

The stability studies were performed by evaluating small variations in three different conditions. All stabilities were assayed at

Table 2
Presicion of assay for luteolin and apigenin ($n = 5$)

Theoretical concentration (μg/ml)	Presicion (R.S.D. %)	
	Intra-assay	Inter-assay
Luteolin		
0.0975	1.8	3.2
2.340	1.4	2.0
7.800	2.2	4.0
Apigenin		
0.1744	1.6	5.4
4.185	2.1	3.6
13.95	2.8	4.8

low and high concentration (0.0975 and 7.800 μg/ml for luteolin, 0.1744 and 13.95 μg/ml for apigenin) in triplicate. Short-term stability of luteolin and apigenin in urine samples at room temperature was determined at 1, 2, 4, 8, 12, 24 h. Freeze–thaw stability was valuated at three consecutive freeze–thaw cycles. Long-term stability was studied by assaying samples after 1, 3, 6, 12 days of storage in −20 °C. The results were expressed by the percentage of initial content of luteolin and apigenin in the freshly treated urine samples.

Except that the low concentration of apigenin in the quality control samples had significant variation after 8 h at 4 °C (less than 65.8%) and 6 days at −20 °C (less than 66.1%), all results showed more than 85.3 and 85.6% of initial content of luteolin and apigenin were remained. In the present study, all urine samples were determined within 2 days. Therefore, the stability of luteolin and apigenin in urine samples was satisfied during our study.

3.6. Sensitivity

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 urine was 39.20 and 31.45 ng/ml based on S/N = 10, with R.S.D. = 6.2 and 5.9%, respectively.

3.7. Assay of luteolin and apigenin in human urine

The described method was applied to assay luteolin and apigenin in urine samples of eight healthy volunteers. Urine samples were collected at different time after volunteers administrated orally 12 tablets of CME. The cumulative amount excreted in urine in every period was calculated. Fig. 2 showed the cumulative amount excreted in urine of total luteolin and apigenin (free + sulfuric acid/glucuronic acid conjugates) within 12 h. The mean concentrations of luteolin and apigenin in urine after 6 h administration of CME tablet were 1.239 ± 0.90 and 1.337 ± 0.91 μg/ml ($n = 8$), respectively.

4. Dicussion

4.1. Chromatographic conditions

To achieve good separation of luteolin and apigenin from interfering compounds in the human urine, methanol, and 0.2% phosphoric acid at different ratio (55:45, 52:48, 50:50, v/v) had been used as mobile phase. The results revealed that no interference peaks from endogenous constituents presented in urine and reagents were observed at the retention time of luteolin and apigenin. Due to the reasonable retention times with the symmetrical peaks and good separation, a mixture of methanol/0.2%phosphoric acid (55:45, v/v) was chosen as mobile phase. The purity of peaks for luteolin and apigenin had been detected by DAD detector (purity ≥ 0.999). Moreover, luteolin and apigenin in urine samples were identified by LC–MS (EIC ionization method, negative mode). The negative EIC–MS of peaks showed molecular ion peaks at m/z 285.2 and 269.2 $[M - H]^-$ corresponding to luteolin and apigenin, respectively. These results indicated that free luteolin and apigenin were present in hydrolyzed urine samples.

4.2. Incubation urine samples with enzymes

It is well known that major metabolites may conjugate with sulfuric acid/glucuronic acid. Our initial study also demonstrated that luteolin and apigenin could not be detected in urine samples without hydrolysis. At first, acid hydrolysis and enzyme hydrolysis were compared, but no significant difference was found. So the enzyme hydrolysis was chosen for its simple procedure and the conditions of enzyme hydrolysis, including the incubation time (1, 2, 3, 4 h) and concentration of β-glucuronidase/sulfatase (100/5, 200/10, 400/20 U/ml), were optimized by orthogonal test. Finally, enzyme incubation was carried out at 37 °C for 2 h with β-glucuronidase (200 U/ml)/sulfatase (10 U/ml) under continuous shaking.

4.3. Application of the method

The developed method was applied to determine luteolin and apigenin in urine of eight healthy volunteers following oral

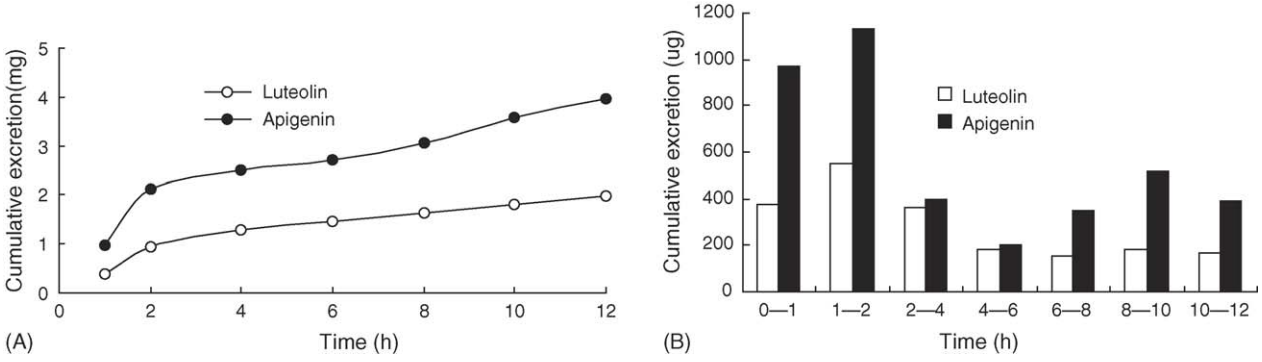

Fig. 2. Cumulative urinary excretion profile of luteolin and apigenin after a single oral administration of 12 tablets of CME. Total cumulative excretion within 12 h (A); cumulative excretion in period of time to time (B).

administration of 12 tablets of CME. The results showed that there was a quick urinary excretion after 1 h for both luteolin and apigenin, which might be attributed to their fast absorption from the gastrointestinal tract, due to rapid hydrolysis of both luteolin and apigenin glycosides by the enzymes of intestinal bacteria [5]. The fraction of the oral dose excreted in urine was only 2.30% for luteolin and 6.09% for apigenin within 12 h, respectively. The results suggested that sulfuric acid/glucuronic acid conjugates of luteolin and apigenin only represent small part of metabolites [13], other possible metabolites or potential storage tissues (blood, liver, kidney, intestines, and body residues, etc.) need to be identified. However, the experimental results showed the accumulative excretion amounts of apigenin were more than that of luteolin (Fig. 2A). Fig. 2B showed the cumulative urinary excretion of luteolin and apigenin decreased at 2 h and increased once again during 5–6 h which suggested absorption of both could occur via an enterohepatic pathway [14]. Our results were different from those observed in human volunteer after parsley ingestion: within 24 h following ingestion, only 0.58% of apigenin was recovered in urine samples [15]. This relative urinary excretion of flavones varied greatly depending on the source from which they were obtained [16]. On the other hand, large individual difference has also been reported to occur for flavones [17]. Therefore, the individual difference and the source of intake flavonoids are considerable factors in bioavailability.

5. Conclusion

In the present study, a simple, selective, precise, and accurate HPLC assay was established and employed to simultaneously analyze luteolin and apigenin in human urine of eight healthy volunteers following a single oral dose of CME tablets. It also can be applied to determine luteolin and apigenin in urine of experimental animals after administration of Chinese traditional medicine or nature drug containing luteolin, apigenin or their glucosides.

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