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Short communication

A gradient HPLC method for the quality control of chlorogenic acid, linarin and luteolin in *Flos Chrysanthemi Indici* suppository

Qunlin Zhang *, Jun Li, Chao Wang, Wei Sun, Zhongtang Zhang, Wenming Cheng

School of Pharmacy, Anhui Medical University, Hefei, Anhui 230032, PR China

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Abstract

The *Flos Chrysanthemi Indici* suppository, a famous traditional Chinese medicine, is marketed for the treatment of prostatitis and chronic pelvic inflammation. A simple, relatively rapid and accurate high-performance liquid chromatographic (HPLC) method was applied to the simultaneous determination of chlorogenic acid, linarin and luteolin in *Flos Chrysanthemi Indici* suppository. The good separation was achieved on a Hypersil ODS column by a gradient elution using the mixture of acetonitrile and aqueous 1.0% acetic acid as mobile phase within 23 min. The method was validated for three active phenolic compounds with the relative standard deviations (R.S.D.) of intra- and inter-day precision below 3.0%, and the detection limits (S/N = 3) of chlorogenic acid, linarin and luteolin were 0.32, 0.08 and 0.05 μg ml^{-1}, respectively. This assay was successfully applied to the determination of three active phenolic compounds in *Flos Chrysanthemi Indici* suppository samples with the quantitative recoveries in the range of 94.7–101.8%. The results indicate that the developed HPLC method can be readily utilized as a quality control method for *Flos Chrysanthemi Indici* suppository and its related traditional Chinese medicinal preparations.

Keywords: High-performance liquid chromatography; Chlorogenic acid; Linarin; Luteolin; *Flos Chrysanthemi Indici* suppository

1. Introduction

Traditional Chinese medicines (TCMs) have been extensively used to prevent and cure human disease for over a millennium. Because of their high pharmacological activity, low toxicity and rare side effects, TCMs have attracted more and more interests in this field. It is well known that the therapeutic effect of herbal medicines is based on the synergistic effect of their multi-constituents, which makes TCMs different from Western medicines [1–4]. An herbal medicine may consist of hundreds of phytochemicals, and their contents vary depending on climate, regions of cultivation and seasons of harvest, which make it difficult to ensure the batch-to-batch uniformity. The quality control of standardized herb extracts is essential for the therapeutic reproducibility, efficacy and safe application of extracts [5]. Conventional quality control mainly focuses on the analysis of the active constituents of herbal medicines. However, it seems necessary to simultaneously determine not only all the available active constituents, but also the marker compounds derived from the herbal materials to ensure the uniformity of their extraction procedures.

Flos Chrysanthemi Indici, anthotaxy of *Chrysanthemum indicum* L. is used as a heat-clearing and detoxication herb. It can inhibit the agglutination of blood platelet and promote myocardial blood circulation and white cell phagocytosis, which has been used to cure diseases, such as furuncle and skin nodules [6,7]. Recent study showed that the extract of *Flos Chrysanthemi Indici* had the antibacterial and dephlogisticate activity, in which three active phenolic compounds including chlorogenic acid, linarin (acacetin-7-*O*-β-D-glucopyranoside), and luteolin (Fig. 1) were identified. *Flos Chrysanthemi Indici* suppository is prepared from the extract of *Flos Chrysanthemi Indici* and marketed for the treatment of prostatitis and chronic pelvic inflammation [8]. Chlorogenic acid has antibacterial, antiphlogistic, antimutagenic, antioxidant and other biological activities [9]. Linarin and luteolin belong to flavonoids widely distributed in the plant kingdom, which are used as remedies because of their antiphlogistic, spasmolytic, good antioxidant and free radical scavenging properties [10]. In the practice of quality control, chlorogenic acid, linarin and luteolin should be simulta-

* Corresponding author. Tel.: +86 551 5161115; fax: +86 551 5167735.
E-mail address: qlzhang@ahmu.edu.cn (Q.L. Zhang).

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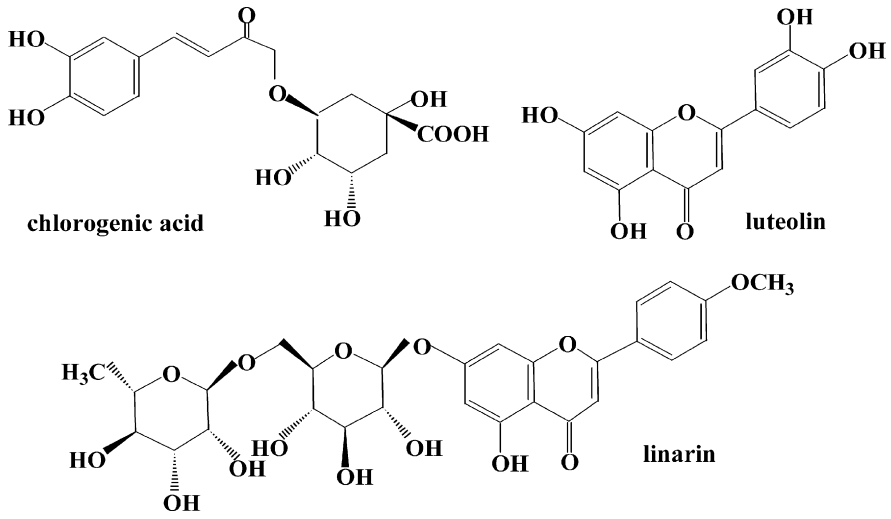

Fig. 1. Molecular structures of the main components of *Flos Chrysanthemi Indici* suppository.

neously determined in the *Flos Chrysanthemi Indici* suppository, since they possess the similar effects, such as antibacterial and antiphlogistic activity.

The analysis of volatile constituents by gas chromatography–mass spectrometry (GC–MS) [11,12] and chlorogenic acid [13] and linarin [14] by high-performance liquid chromatography (HPLC) has been accomplished for the quality assessment of *Flos Chrysanthemi Indici* samples. HPLC method with UV detection for the determination of linarin in *Flos Chrysanthemi Indici* suppository was reported in Chinese Pharmacopoeia [8]. However, no literature is available to date for the co-quantification of three active phenolic compounds (chlorogenic acid, linarin and luteolin), which is highly desirable in the QA/QC laboratories to ensure the synergistic effect of multi-components for the traditional Chinese medicinal preparations of *Flos Chrysanthemi Indici*.

In the present paper, an approach of gradient HPLC coupled with photo diode array detection was developed and validated to simultaneously identify and determine chlorogenic acid, linarin and luteolin in *Flos Chrysanthemi Indici* suppository. The proposed method can be readily utilized as a quality control tool and has been successfully employed to analyze the above three phenolic compounds in the methanolic extracts from different batches of *Flos Chrysanthemi Indici* suppository.

2. Experimental

2.1. Chemicals and solutions

HPLC grade acetonitrile was obtained from Hanbang Reagent Company (Jiangsu, China). Methanol, glacial acetic acid and petroleum benzene were of analytical-reagent grade and obtained from Shanghai Chemical Reagent Company (Shanghai, China). Ultrapure water was used to prepare all the solutions. Chlorogenic acid, linarin and luteolin were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and used without further purification.

Stock solutions of 1.0×10^{-4} g ml^{-1} phenolic compounds were prepared by dissolving appropriate amount of chlorogenic acid, linarin and luteolin in methanol, respectively. These stock solutions remained stable during 3 months if stored in refrigerator at 4 °C. Standard solutions were prepared by further dilution of stock solutions with acetonitrile–water (20:80, v/v). The HPLC mobile phase was prepared fresh daily, filtered through a 0.45 μm membrane filter, and then degassed before injecting into the column.

2.2. HPLC apparatus and operating conditions

The HPLC system (Waters model 600E system, Waters, Milford, MA, USA) consisted of a quaternary pump, a photodiode array detector (Waters 2996), an inline-degasser AF, a 20 μl-loop manual injector and Waters Empower software. A Hypersil ODS column (4.6 mm i.d. × 200 mm, 5 μm) was used for the separation of chlorogenic acid, linarin and luteolin. The tested samples were separated at 25 °C with a gradient elution program at the flow rate of 1.0 ml min^{-1}. The mobile phase was a mixture of acetonitrile (solvent A) and water–glacial acetic acid (99:1, v/v, pH 2.8) (solvent B). The gradient elution program was: 10% A (0–4 min), 10–30% A (4–15 min), 30% A (15–25 min) and 30–10% A (25–30 min). This was followed by a 10 min equilibrium period prior to the injection of next sample. The UV spectra were recorded between 210 and 400 nm for the identification of phenolic compounds and for the test of peak purity. The injection volume of each sample was 20 μl.

2.3. Compounds identification and quantification

Identification of the tested phenolic compounds was carried out by comparing their retention times and on-line UV spectra with those of available standards. Identified peaks were then confirmed by spiking samples with standard mixtures. A photo diode array detector recording at 310, 335 and 350 nm was used to detect chlorogenic acid, linarin and luteolin, respectively. Quantification was performed according to an external standard

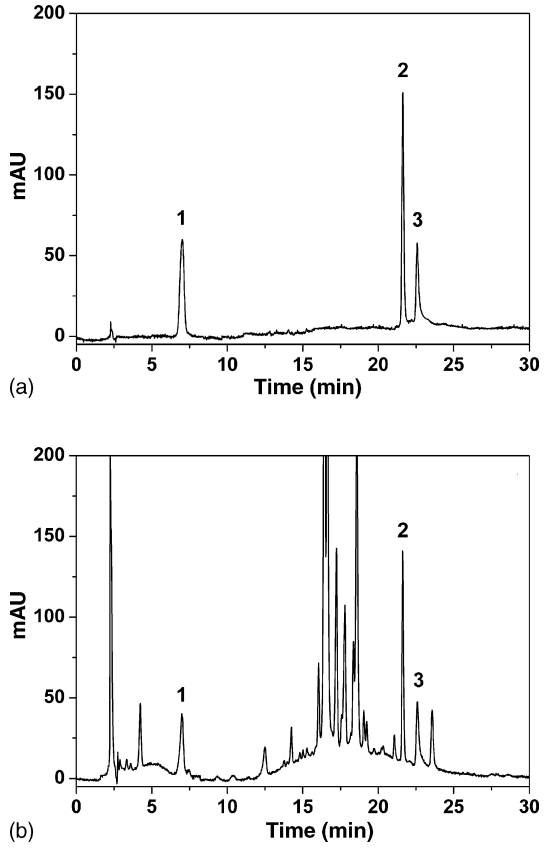

Fig. 2. HPLC chromatograms of (a) three standard phenolic compounds and (b) *Flos Chrysanthemi Indici* suppository. Peaks: (1) chlorogenic acid; (2) linarin; (3) luteolin. The mobile phase consisted of acetonitrile (A) and water–glacial acetic acid (99:1, v/v, pH 2.8) (B). The gradient elution program was: 10% A (0–4 min), 10–30% A (4–15 min), 30% A (15–25 min) and 30–10% A (25–30 min). The flow rate was 1.0 ml min^{-1} and DAD detection was performed at 310 nm.

method. The quantitative data were obtained by plotting the concentrations of standard versus peak areas.

2.4. Sample preparation

The *Flos Chrysanthemi Indici* suppository samples (batches 060118, 060215 and 060309; Table 3) were manufactured from the water extract of *Flos Chrysanthemi Indici* by Kanghua Pharmaceutical Ltd. Company (Guangxi, China). Eight suppositories were accurately weighed into a beaker and melted in a water bath at 55 °C. The melt was thoroughly mixed with a magnetic stirrer. The molten mix was placed in a refrigerator and allowed to solidify. After solidification the solidified mass was broken up, and a portion equivalent to the weight of one suppository (2.4 g) was quantitatively transferred into a beaker. The beaker was again placed into a water bath at 55 °C in order to melt the mass. Twenty-five milliliters of petroleum benzene (boiling range: 60–90 °C) was added to the melt and sonicated at room temperature in an ultrasonic bath for 20 min. The extract was centrifuged, and the supernatant was discarded. The residue was evaporated to dryness under reduced pressure. Thirty milliliters of methanol was added to the residue and then sonicated for 30 min. After cooling, the mixture was filtered through filter paper, and the filtrate was transferred to a 50 ml volumetric flask and diluted with methanol to volume. The obtained sample solution was then diluted tenfold with acetonitrile–water (20:80, v/v) and passed through a 0.22 μm Millipore membrane prior to HPLC analysis.

Table 2
Precision and accuracy of phenolic compounds

Phenolic compound	Precision (R.S.D., %)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
Chlorogenic acid	1.2	2.7	101.3	95.4
Linarin	1.8	1.6	97.0	102.7
Luteolin	2.0	2.6	99.4	101.5

3. Results and discussion

3.1. Method development and optimization

The effectiveness of HPLC separation was tested using the standard solution of three phenolic compounds including chlorogenic acid, linarin and luteolin. The gradient elution profile was optimized to obtain the highest resolution of phenolic compounds and the shortest time of analysis. Our previous results indicated that chromatographic gradient system composed of methanol and water, when adding acetic acid, could sharpen peak shapes and improve analytical sensitivity and resolution for the HPLC analysis of phenolic compounds [15]. The effects of acetic acid and methanol concentration of the mobile phase on the phenolic compound separation were studied, respectively. Unfortunately, neither isocratic elution nor gradient elution resulted in good chromatographic separation of linarin and luteolin. A mixture of acetonitrile and water–acetic acid was then used as the mobile phase in this study. It was found that a moderate addition 1.0% of acetic acid yielded the good chromatographic peak. Under the condition of 1.0% (v/v) acetic acid, the effects of acetonitrile concentration of the mobile phase

Table 1
Calibration curve of phenolic compounds ($n \geq 5$)

Phenolic compound	Calibration curve ($y = ax + b$)		r	Test range (μg ml^{-1})	LOD (μg ml^{-1})
	Slope (a)	Intercept (b)			
Chlorogenic acid	10077.5 ± 45.8	88.4 ± 11.9	0.9997	2.0–10.0	0.32
Linarin	21501.3 ± 100.1	635.8 ± 129.6	0.9999	1.0–20.0	0.08
Luteolin	48754.7 ± 175.6	1538.2 ± 491.2	0.9995	0.8–8.0	0.05

t_R, Retention time; LOD, limit of detection.

Table 3
Concentration and recovery of phenolic compounds in *Flos Chrysanthemi Indici* suppository ($n = 3$)

Batch no.	Phenolic compound	Content ($\mu g\,g^{-1}$)	Added ($\mu g\,g^{-1}$)	Recovered ($\mu g\,g^{-1}$)	Recovery (%)	R.S.D. (%)
060118	Chlorogenic acid	7.2	7.0	14.0	96.5	1.3
	Linarin	15.3	15.0	30.4	100.9	1.0
	Luteolin	5.0	5.0	9.7	94.7	0.8
060215	Chlorogenic acid	6.8	7.0	13.7	98.3	1.8
	Linarin	14.6	15.0	29.0	95.8	0.9
	Luteolin	5.1	5.0	10.2	101.8	1.7
060309	Chlorogenic acid	6.9	7.0	13.9	100.6	2.0
	Linarin	14.8	15.0	29.6	98.8	1.2
	Luteolin	4.7	5.0	9.7	99.1	1.8

on phenolic compound separation were studied. Isocratic elution at 10% (v/v) acetonitrile concentration also resulted in poor chromatographic separation of linarin and luteolin, which were eluted after 35 min. Thus, an optimal gradient elution was needed. As a result, the best resolution of all the peaks was obtained using a gradient of the mobile phase consisting of acetonitrile and 1.0% acetic acid within 23 min (the profile see Section 2.2). The retention times of chlorogenic acid, linarin, and luteolin are 7.0, 21.5 and 22.6 min, respectively. The chromatogram of the mixture of chlorogenic acid, linarin and luteolin with DAD detection at 310 nm obtained under the above conditions is shown in Fig. 2a.

3.2. Linearity, sensitivity, precision

Under the optimum conditions described above, the results obtained with the proposed method are summarized in Table 1. The calibration curves were tested over the range 0.8–20.0 $\mu g\,ml^{-1}$, and at least five samples covering the whole range were used for each phenolic compound. Each point of the calibration graph corresponded to the mean value from three independent peak measurements. The linearity relationship between peak areas and concentrations was good, and the regression coefficients (r) were greater than 0.9995 for all the curves. The detection limits for chlorogenic acid, linarin and luteolin at a signal-to-noise ratio of three were 0.32, 0.08 and 0.05 $\mu g\,ml^{-1}$, respectively.

The intra-day precision of the proposed method was tested with five repeated injections of phenolic compound solution at the concentration level of 5.0 $\mu g\,ml^{-1}$. The inter-day precision was studied by analyzing 10.0 $\mu g\,ml^{-1}$ phenolic compound solution, with seven injections randomly executed in a 20-day period. The relative standard deviations were below 3.0%. The intra-day and inter-day accuracy was also determined by the same procedure, and the accuracy data were calculated as (mean analyzed concentration)/(nominal concentration) × 100 (Table 2). The obtained data demonstrate that the proposed analytical method provide good validation.

3.3. Sample analysis

Three batches of *Flos Chrysanthemi Indici* suppository samples were prepared as described in Section 2.4. Each sample was determined in triplicate. The chromatographic profile of phenolic compounds in the methanolic extract of *Flos Chrysanthemi Indici* suppository shows that there is no interference by matrix, and the resolution between the peaks of analytes and nearby peaks is above 1.5 (Fig. 2b). The content of each phenolic compound was calculated from the corresponding calibration curve. The variation of contents of TCMs may be derived from the different quality of raw material, the difference of production procedure, storage, transportation, etc. The data in Table 3 indicate that the contents in three batches of suppository produced by the same manufacturer were relatively consistent with the variations not more than 8.5%. In order to evaluate the validity of the proposed method for the assay of phenolic compounds in *Flos Chrysanthemi Indici* suppository samples, a recovery experiment was carried out. *Flos Chrysanthemi Indici* suppository samples were spiked with the known amount of standard phenolic compounds, and the percent ratios between the recovered and expected concentrations were calculated. The satisfied recoveries of 94.7–101.8% indicate that the proposed gradient HPLC method is reliable for the quantification of chlorogenic acid, linarin and luteolin in *Flos Chrysanthemi Indici* suppository (Table 3).

4. Conclusion

A simple, relatively rapid and accurate HPLC method was successfully developed and validated. To the best of our knowledge, it is the first time that a gradient HPLC method has been applied to the simultaneous determination of three active phenolic compounds (chlorogenic acid, linarin and luteolin) in *Flos Chrysanthemi Indici* suppository. The results indicate that the proposed HPLC method can be considered as a quality control method for *Flos Chrysanthemi Indici* suppository and its related traditional Chinese medicinal preparations.

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