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Simultaneous determination of five marker constituents in traditional Chinese medicinal preparation Le–Mai granule by high performance liquid chromatography

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Abstract

An HPLC method for the simultaneous determination of five marker constituents was established for the quality control of traditional Chinese medicinal preparation Le–Mai granule. The marker constituents were danshensu, protocatechuic acid and protocatechualdehyde from *Salviae miltiorrhizae bunge*; paeoniflorin from *Radix paeoniae rubra* and ferulic acid from *Rhizoma chuanxiong*. Extracted samples were successfully separated on a Diamonsil C_{18} column (150 mm × 4.6 mm i.d., 5 μm) at 25 °C. The mobile phase was a mixture of methanol and 1.0% acetic acid employing gradient elution at a flow rate of 1.0 mL/min. Detection was accomplished with a diode-array detector and chromatograms were recorded at 230, 262, 280 and 322 nm. The compounds were identified by comparing their retention times and UV spectra in the 200–400 nm range with authentic standards. Regression equations revealed good linear relationship (correlation coefficients: 0.9993–0.9999) between the peak areas of the constituents and their concentrations. The average recoveries ($n = 3$) were between 96.2 and 102.5%. The proposed method has been successfully applied to the simultaneous determination of the five marker constituents in three lots of Le–Mai granule.

Keywords: Traditional Chinese medicinal preparation; Le–Mai granule; Danshensu; Protocatechuic acid; Protocatechualdehyde; Paeoniflorin; Ferulic acid; HPLC

1. Introduction

In the long history of its development, traditional Chinese medicine has demonstrated its great vitality because of its firm clinical foundation, significant therapeutic effects and specific system of theory based on clinical practice. Nowadays, concentrated Chinese medicinal preparations have become more and more popular. However, crude drugs with different origins, sources, cultural manner, harvest time, pretreatment processes, and manufacturing processes will be of different quality, which will result in significant differences in the same formula when supplied by different factories or even by the same factory. Therefore, quality control of the manufacturing process of the concentrated Chinese medicine preparations will be very critical for their future development.

In Taiwan, quality control of the production of concentrated Chinese medicinal preparations is done according to the draft issued by the Department of Health, which requires "quantification of at least two marker substances from different Chinese crude drugs in a formula" [1]. In Japan, since 1985, the Ministry of Health and Welfare has required that all concentrated Chinese medicinal preparations submitted for inspection and registration should include a content analysis with at least two chemical components as markers [2]. And since 1998, there is guidance for the marker substances which should be assayed [3]. Concerning these regulations, the goal of analytical method development is to discover more marker

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substances and simultaneously quantify them in one HPLC run [4–9]. This will promote quality control techniques for traditional Chinese medicines.

In this paper, multi-component simultaneous analysis of the preparation Le–Mai granule was discussed. Le–Mai granule, as a new generation of granule without sucrose, is refined from seven crude herbs, i.e. *Salvia miltiorrhizae bunge* (Chinese herbal name: Danshen), *Rhizoma of chuanxiong* (Chuanxiong), *Radix paeoniae rubra*(Chishao), *Flos carthami* (Honghua), *Fructus crataegi* (Shanzha), *Radix aucklandiae* (Muxiang) and *Rhizoma cyperi* (Xiangfu). It has entered the list of the protected traditional Chinese medicines and has been collected in the Chinese Pharmacopiea. Pharmacological studies reveal that Le–Mai granule has clinical effects of reducing viscosity of the whole blood and plasma, preventing the platelet from gathering, resisting the formation of thrombus element, dilating the minor artery around heart, brain and kidney. Literature search revealed that only danshensu [10] and paeoniflorin [11] in Le–Mai granule had ever been analyzed and the Chinese pharmacopiea only demands the quantification of paeoniflorin for the quality control of Le–Mai granule [11]. In order to improve the quality control of Le–Mai granule, it is necessary to develop an efficient method for the simultaneous determination of more marker constituents. In this paper, five compounds were selected as marker constituents of Le–Mai granule. They are danshensu, protocatechuic acid and protocatechualdehyde from *Salviae miltiorrhizae bunge*, paeoniflorin from *Radix paeoniae rubra* and ferulic acid from *Rhizoma chuanxiong*, respectively (their chemical structures are shown in Fig. 1).

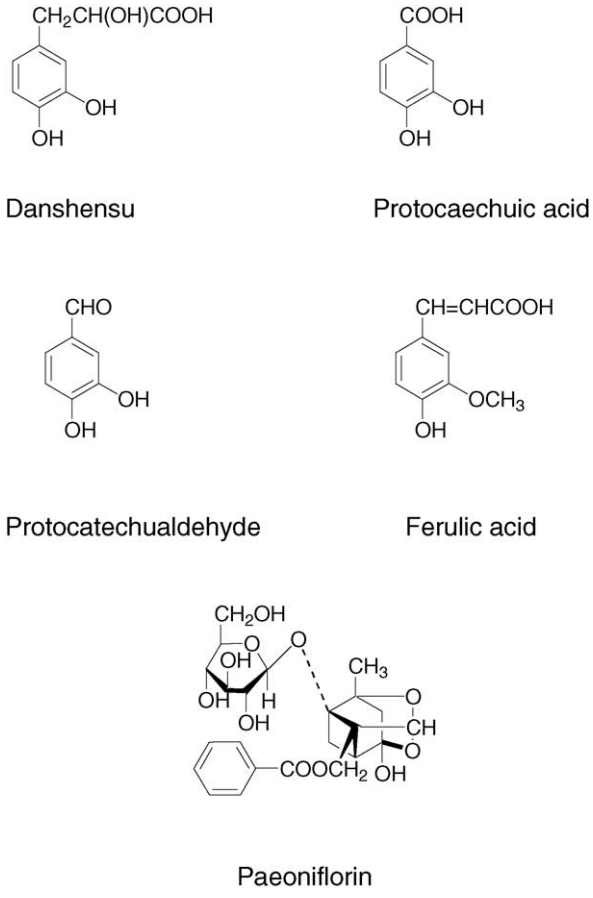

Fig. 1. Chemical structures of the marker constituents.

2. Experimental

2.1. Materials and reagents

Le–Mai granules were purchased from Ji-An-Tang Pharmaceutical Store (Beijing, China), and the products were manufactured by the Pharmaceutical Factory of West China University of Medical Sciences.

Authentic standards of danshensu, protocatechuic acid, protocatechualdehyde, paeoniflorin and ferulic acid were purchased from National Institute for Control of Pharmaceuticals and Biological Products (Beijing, China). All other reagents were of analytical grade. Double-distilled water was used to prepare all the solutions.

2.2. Apparatus and chromatographic conditions

All analyses were performed on an Hewlett Packard 1100 liquid chromatograph (Palo Alto, CA, USA) which consisted of a quaternary pump, an on-line degasser, a column thermostat, a model 0497 injection valve (sample loop 20 μL) and a photodiode-array detector. The chromatographic data were recorded and processed with an HP chemstation software. The analytical column was a Diamonsil C_{18} (150 mm × 4.6 mm i.d., 5 μm) column and the column temperature was controlled at 25 °C. The mobile phase was a mixture of methanol and 1.0% (v/v) HAc employing gradient elution as shown in Table 1. The flow rate was 1.0 mL/min. The column effluents were monitored simultaneously at 230, 262, 280 and 322 nm. Injection volume was 20 μL.

2.3. Preparation of standard solutions

Standard stock solutions of danshensu, protocatechuic acid and protocatechualdehyde were prepared in 1.0% (v/v) HAc, for these compounds are more stable [12] in acidic solution and can be stored for at least 1 month at 4 °C. Paeoniflorin was prepared in methanol and ferulic acid was prepared in methanol–1.0% HAc (1:1, v/v). Working standard

Table 1
Gradient elution programme using mobile phases A and B

Time (min)	Flow rate (mL/min)	Mobile phase A[a] (%)	Mobile phase B[b] (%)
0	1.0	12	88
10	1.0	12	88
50	1.0	50	50

[a] Methanol.
[b] 1.0% acetic acid solution.

solutions containing each of the five compounds were prepared by diluting the stock solutions with methanol–1.0% HAc (1:1, v/v).

2.4. Preparation of sample solutions

A 1.0 g of Le–Mai granule was accurately weighed and put into a 50 mL centrifuge tube. Twenty milliliters of 30% methanol was added. Then the sample was extracted at room temperature for 30 min in an ultrasonic bath. The extract was centrifuged at a speed of 11 424 g for 10 min. The supernatant was transferred to a 25 mL volumetric flask and diluted to the mark with 30% methanol. This solution was passed through a 0.45 μm syringe membrane filter, and 20 μL of the filtrate was injected into the HPLC system for analysis.

3. Results and discussion

3.1. Chromatographic conditions

A simple gradient programme was used to elute the five marker constituents in a single run within a reasonable period of time (Table 1). In order to get reproducible retention time, prior to next injection, the column was solvent conditioned by passing the initial solvent through the column until the baseline stabilized.

The on-line UV spectra of the five marker constituents were obtained by using a DAD detector. As maximally efficient detection can be obtained by selecting the wavelength where the compound has the maximum absorption, in this study, four different detection wavelengths were set according to the maximum absorption of the compounds. Protocatechuic acid, paeoniflorin and ferulic acid were detected at 262, 230 and 322 nm, respectively. Danshensu and protocatechualdehyde were detected at 280 nm.

Photodiode-array detection was used in the experiment so that UV spectra of the bioactive constituents could be compared with those of the authentic standards. Identification of the five compounds was performed by characterizing the sample peak in terms of retention time and UV spectrum. The excellent agreement between standard and sample spectra found in all analyzed samples of Le–Mai granule indicates that, under the proposed analytical conditions, determination of the five marker constituents is not subjected to the interferences from other components in the matrix. Typical chromatograms of the authentic standards and Le–Mai granule recorded at different wavelengths are depicted in Figs. 2 and 3.

3.2. Sample extraction conditions

To evaluate the extraction efficiency of different solvents, methanol, 70% methanol, 50% methanol, 30% methanol and water were employed as extraction solvents. When the sample was extracted with pure methanol, the peak shapes of the marker constituents were poor and danshensu, protocat-

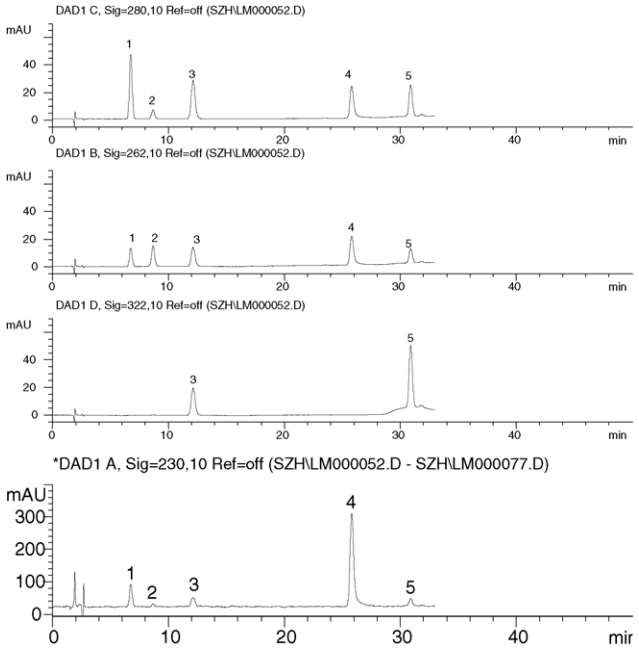

Fig. 2. Chromatograms of the authentic standards at different detection wavelengths: (1) danshensu; (2) protocatechuic acid; (3) protocatechualdehyde; (4) paeoniflorin; (5) ferulic acid. HPLC conditions: Diamonsil C_{18} column (150 mm × 4.6 mm i.d., 5 μm) controlled at 25 °C. A mixture of methanol and 1.0% (v/v) acetic acid employing gradient elution at a flow rate of 1.0 mL/min as mobile phase (see Table 1). Detection was accomplished with a diode-array detector and chromatograms were recorded at 230, 262, 280 and 322 nm. The chromatogram of 230 nm is the result of the original chromatogram substracting the blank run.

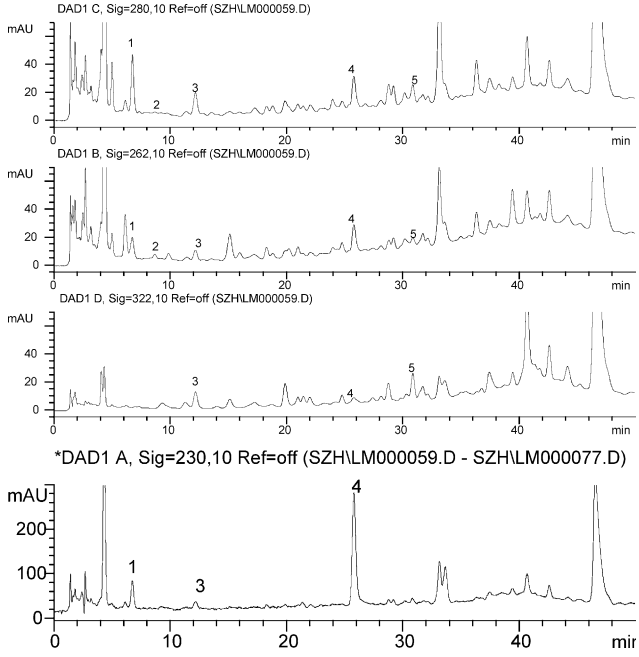

Fig. 3. Chromatograms of Le–Mai granule at different detection wavelengths: (1) danshensu; (2) protocatechuic acid; (3) protocatechualdehyde; (4) paeoniflorin; (5) ferulic acid. Chromatographic conditions as in Fig. 2.

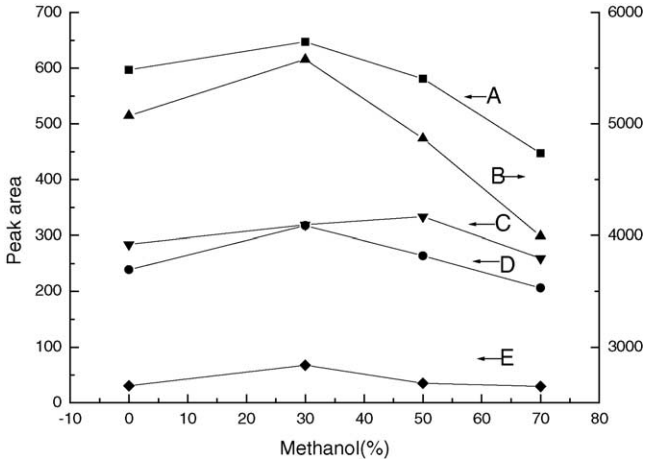

Fig. 4. Extraction efficiency of different solvents: (A) danshensu; (B) paeoniflorin; (C) protocatechualdehyde; (D) ferulic acid; (E) protocatechuic acid.

echualdehyde could not be separated with other constituents in the matrix, so the peak areas of the constituents could not be measured accurately when extracted with pure methanol.

The extraction efficiency of the different solvents are shown in Fig. 4. It could be seen that, when 30% methanol was employed, the peak areas of the five marker constituents reached the highest values. So, 30% methanol was selected as the extraction solvent.

Then the optimal extraction time was studied. Five duplicate samples were extracted with 30% methanol in ultrasonic bath for 10, 20, 30, 40 and 50 min, respectively. The peak areas of the marker constituents obtained by different extraction times are shown in Fig. 5. It could be seen from Fig. 5 that when the sample was extracted for 30 min, the peak areas of the constituents reached the highest values, so 30 min was selected as the optimal extraction time.

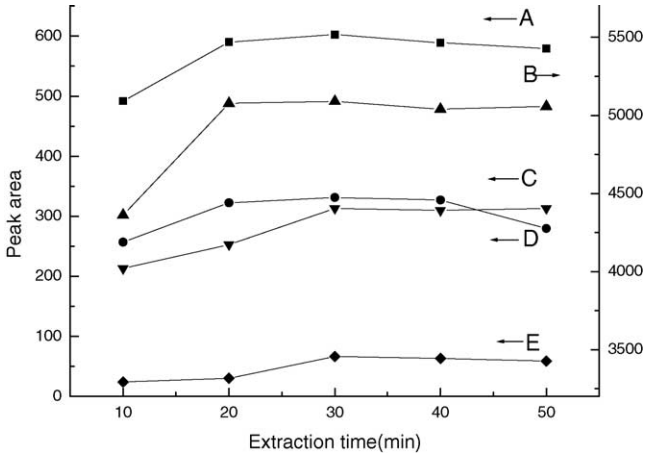

Fig. 5. Extraction efficiency of different extraction times: (A) danshensu; (B) paeonoflorin; (C) ferulic acid; (D) protocatechualdehyde; (E) protocatechuic acid.

Table 2
Regression equations, correlation coefficients, linearity ranges and limit of detection for the marker constituents of Le–Mai granule

Compound	Regression equation	Linear range (μg/mL)	*r*	LOD (μg/mL)
Danshensu	$Y = 5.14 + 11.38X$	8.0–80.0	0.9993	0.16
Protocatechuic acid	$Y = 3.86 + 66.20X$	0.55–5.50	0.9998	0.04
Protocatech-ualdehyde	$Y = -2.44 + 83.10X$	1.0–10.0	0.9997	0.03
Paeoniflorin	$Y = 3.17 + 22.18X$	40.0–400.0	0.9999	1.28
Ferulic acid	$Y = 15.31 + 89.95X$	1.25–12.50	0.9994	0.03

3.3. Calibration graphs and the limit of detection

All calibration graphs were plotted based on linear regression analysis of the integrated peak areas (Y) versus concentrations (X, μg/mL) of the five marker constituents in the standard solution at six different concentrations. The regression equations, correlation coefficients, and linear ranges for the analysis of the five marker constituents are shown in Table 2.

The limit of detection value (LOD) was calculated as the amount of the injected sample which gave a signal-to-noise ratio of 3. The LOD values of the method for the five components are also listed in Table 2.

3.4. Robustness of the method

Five duplicate samples from the same lot of Le–Mai granules were treated according to the sample preparation procedure and analyzed with the established HPLC method. The relative standard deviations (R.S.D.) of the peak area of the five marker constituents are 0.30, 2.50, 2.01, 0.28 and 2.46% for danshensu, protocatechuic acid, protocatechualdehyde, paeoniflorin and ferulic acid, respectively.

When the same sample solution was repeatedly injected for five times at an interval of 5 h, R.S.D. of the retention times were 1.07, 0.87, 1.07, 1.04 and 0.98% for danshensu, protocatechuic acid, protocatechualdehyde, paeoniflorin and ferulic acid, respectively. R.S.D. of peak areas were 0.57, 1.19, 2.04, 0.55 and 2.65% for danshensu, protocatechuic acid, protocatechualdehyde, paeoniflorin and ferulic acid, respectively. The results revealed good robustness of the proposed method.

3.5. Recovery test

Recovery experiments were carried out by spiking different amounts of authentic standards to Le–Mai granule with known contents of marker constituents. Then the samples were treated according to the sample preparation procedure. The results are shown in Table 3. It can be seen that the proposed method has an adequate degree of accuracy for the

Table 3
Recovery of each marker constituent in Le–Mai granule

Compound	Added (μg/g)	Found (μg/g)	Recovery (%)	R.S.D. (%) ($n = 3$)
Danshensu	770	756	98.2	0.29
	1540	1552	100.8	0.29
Protocatechuic acid	11.5	11.3	98.3	3.93
	34.5	34.6	100.3	2.03
Protocatechualdehyde	50.0	50.7	101.4	1.29
	100.0	100.1	100.1	0.94
Paeoniflorin	2100	2153	102.5	1.87
	3250	3183	97.9	2.64
Ferulic acid	37.5	37.1	98.9	0.94
	100.0	96.2	96.2	1.24

Table 4
Contents of marker constituents in three lots of Le–Mai granule

Compound	Content (μg/g) (mean ± S.D.)		
	0109 ($n = 3$)	0206 ($n = 3$)	030111 ($n = 5$)
Danshensu	1394 ± 21.08	1342.67 ± 38.79	1422.4 ± 8.17
Protocatechuic acid	13.67 ± 1.10	18.57 ± 0.40	23.99 ± 0.30
Protocatechualdehyde	107.67 ± 1.53	83.70 ± 0.80	102.62 ± 2.08
Paeoniflorin	6326.67 ± 64.66	6283.67 ± 18.04	6564.25 ± 36.36
Ferulic acid	67.95 ± 0.49	85.03 ± 0.93	87.18 ± 2.42

determination of the five marker constituents in the samples.

3.6. Sample analysis

The newly established method has been applied to the determination of the five marker constituents in three lots of Le–Mai granule. The contents ($n = 3$) of the five marker constituents in Le–Mai granules are shown in Table 4. For the three lots of Le–Mai granule, the measured values were quite different from each other. This might be due to the inconsistencies of the sources of crude herbs, the fluctuation of the processes of extraction, concentration, drying and granulation. Furthermore, the temperature and humidity of storage can also influence the contents of marker constituents in Le–Mai granule.

4. Conclusions

A multi-component HPLC method was developed for the simultaneous determination of five marker constituents in traditional Chinese medicinal preparation Le–Mai granule. The established method was precise and accurate and the sample preparation procedure was relatively simple. It should be a good application in the quality control of the manufacturing process of Le–Mai granule in the future.

Acknowledgements

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