Determination of Seven Compounds in Tang Maikang Granule by High-Performance Liquid Chromatography

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Abstract

An accurate and sensitive high-performance liquid chromatography method is developed and applied to the determination of seven compounds in a kind of traditional Chinese medicinal preparation of Tang Maikang Granule. The method is performed on Hypersil C_{18} column (250- × 4.6-mm i.d., 5 µm), and different mobile phases and detectors are selected according to the various compounds. For astragaloside IV, an evaporative light scattering detector (ELSD) is used with a gradient of methanol–water at an eluent gas rate of 2.0 mL/min, under a drift tube temperature of 80°C. Formononetin and calycosin are also eluted by a gradient of methanol–water, but a photodiode array (PDA) detector is used at a wavelength of 254 nm for formononetin and calycosin. A PDA detector at a wavelength of 230 nm is used for paeoniflorin, with methanol–water (30:70, v/v) as the mobile phase. For danshensu and protocate chualdehyde, an eluent of methanol–0.5% acetic acid (12:88, v/v) is used, with PDA detection at 280 nm. For berberine, methanol and water containing 0.1% sodium dodecanesulphonate (SDS) and 0.1% phosphorous acid (70:30, v/v) is employed as the mobile phase, also using a PDA detector, but the detection wavelength is 265 nm. The intra- and interrun precision (relative standard deviation) of this method is less than 5% for seven analytes.

Introduction

Traditional Chinese medicine as a kind of natural drug played an important role in the prevention and treatment of diseases. Up until now, traditional Chinese medicine has been made into a concentrated preparation, which is more efficient, easier, and more convenient to the patient in China, but the quality of the concentrated preparation produced by different pharmaceutical factories (even by the same factory) varies with crude herbs, who the cultivar was, where it was planted, when it was harvested, and so on. That is to say, the quality control of the concentrated preparation of traditional Chinese medicine is a very urgent problem to be solved.

Generally, the quality control of traditional Chinese drugs has involved determining only one or two compounds, which are not often their own special compound and could also be contained in other crude herbs (e.g., ursolic acid is present in both fructus corni and fructus crataegi). Thus, more compounds in traditional Chinese drugs should be determined at the same time to improve quality control (1).

Tang Maikang Granule is used as a remedy for diabetes II and its complication (2). Its formulation consists of the following crude herbs: radix astragali, radix paeoniae rubra, radix salviae miltiorrhizae, coptis root, radix achyranthis bidentatae, radix ophiapogonis, rhizome polygonati, and radix rehmanniae. The quality control of Tang Maikang Granule has mainly used thin-layer chromatography to determine astragaloside IV, paeoniflorin, protocate chualdehyde, berberine, oleanolic acid, etc. (2). Up to the present, a high-performance liquid chromatography (HPLC) method has not been applied to quantitate those compounds. A simpler, more sensitive, and accurate quality control technique for Tang Maikang Granule needs to be established.

In this paper, a multirun HPLC procedure to determine the compounds of astragaloside IV, formononetin, calycosin, paeoniflorin, danshensu, protocate chualdehyde, and berberine in Tang Maikang Granule is developed. The method establishes a more sensitive and accurate method to perform quality control for Tang Maikang Granule.

Experimental

Reagents and chemicals

Astragaloside IV, paeoniflorin, danshensu, protocate chualdehyde, and berberine hydrochloride were obtained from the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). Formononetin (99.83%) and calycosin (99.37%) were obtained from Shanghai University of Traditional Chinese Medicine (Shanghai, China). The structures of the compounds are shown in Figure 1. Tang Maikang Granule was produced by Joint-wit Pharmaceutical in the Chinese Academy of Traditional Chinese Medicine (Lot No.: 030315 and 030610) (Chendu, China). The water used was double-distilled. Methanol was HPLC grade (Yuwang, China). Other reagents were analytical grade (Beijing, China).

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Apparatus and chromatographic conditions

The HPLC system consisted of a M32 ADD-ON single system (Waters, Milford, MA), 2996 photodiode array (PDA) detector, evaporative light scattering detector (ELSD) (Alltech, Deerfield, IL), and Waters 717 autosampler equipped with a 10 µL injection loop (Waters). A Hypersil ODS-1 column (250- × 4.6-mm i.d., 5 µm) (Dalian Elite Analytical Instruments Co. Ltd., Dalian, China) and Kromasil ODS guard column (10- × 4.6-mm i.d., 5 µm) (Zirchrom Science Instrument Co. Ltd., Tianjing, China) were used.

Astragaloside IV was eluted with a mobile phase consisting of methanol and water by a methanol gradient from 70% to 100% for 20 min, using ELSD at eluent gas rate of 2.0 mL/min under a drift tube temperature of 80°C. The column temperature was 25°C.

Formononetin and calycosin were detected by a PDA detector at 254 nm. The mobile phase was water and methanol with a water step gradient from 40% to 50% for 10 min, then from 50% to 60% for 20 min, and finally maintained under 60% water concentration for another 20 min at a flow rate of 1.0 mL/min. The column temperature was kept at 25°C.

Paeoniflorin was analyzed with a constant methanol–water (30:70, v/v) eluent, and the detection wavelength was 230 nm, other conditions were the same as used in the determination of formononetin and calycosin.

For danshensu and protocate chualdehyde, an eluent of methanol–0.5% acetic acid (12:88, v/v) was used with PDA detection at 280 nm. The flow rate was 1.0 mL/min, and the column temperature was 35°C.

For berberine, methanol and water containing 0.1% sodium dodecanesulphonate (SDS) and 0.1% phosphorous acid (70:30, v/v) were employed using PDA detection at 265 nm. The flow rate was 1.0 mL/min, and the column temperature was 25°C.

Standard stock solution and calibration curve preparation

Astragaloside IV, formononetin, calycosin, and paeoniflorin were prepared by dissolving in methanol, reaching a final concentration of 40.3, 94.0, 93.0, and 92.5 µg/mL, respectively. Danshensu (96.4 µg/mL) and protocate chualdehyde (312.0 µg/mL) were obtained by dissolving in 60% methanol, and berberine hydrochloride (1125.0 mg/mL) was obtained by dissolving in 30% methanol.

For working solution preparation, astragaloside IV was diluted to a series of concentration from 4.0 to 28.2 µg/mL; formononetin and calycosin ranged from 0.5 to 2.8 µg/mL, and paeoniflorin varied from 18.5 to 92.5 µg/mL. The danshensu dilution concentration was between 1.9 and 9.2 µg/mL, and protocate chualdehyde was in the range of 15.6 to 249.6 µg/mL, and berberine hydrochloride was 9.0 µg/mL ~ 54.0 µg/mL. The calibration curve for each analyte was built by injecting the working solution, respectively.

Sample preparation

A sample (5.0000 g) of Tang Maikang Granule was ground into powder, then 40 mL of methanol was added. The sample was extracted three times by an ultrasonic device for 40 min (Tianjing Hengao Instrument Co., Tianjing, China). After filtration, the resultant was evaporated to dryness, and the dried residues were redissolved in 30 mL of water and extracted four times by adding 40, 30, 20, and 20 mL of *n*-butanol, respectively. The organic layers were mixed together and evaporated to dryness again; the dried residues were filtrated after dissolving in 10 mL methanol.

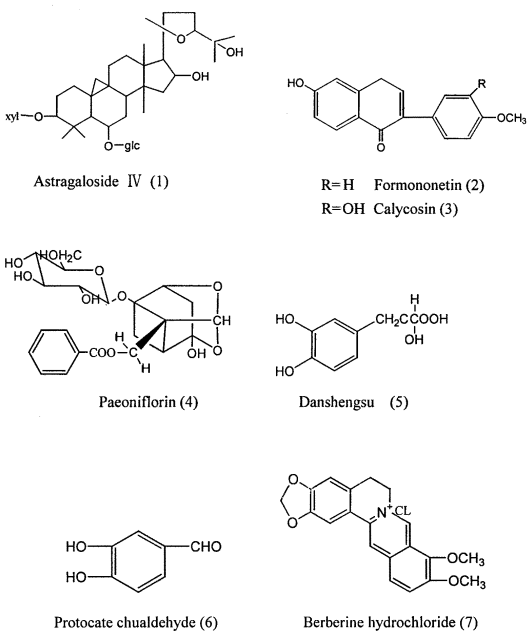

Figure 1. Structures of the seven components.

Table I. Linearity for Seven Components

Component	Equation of the calibrations curve	Range studies (µg /mL)	Correlation coefficient
Astragaloside IV	$A = 1.28 \times 10^4 C - 4.26 \times 10^4$	4.0~28.2	0.9994
Formononetin	$A = 2.30 \times 10^5 C - 1.86 \times 10^5$	0.5~2.8	0.9995
Calycosin	$A = 2.42 \times 10^5 C - 1.93 \times 10^5$	0.5~2.8	0.9998
Paeoniflorin	$A = 1.39 \times 10^5 C - 1.43 \times 10^5$	18.5~92.5	0.9995
Danshengsu	$A = 8.20 \times 10^4 C - 7.78 \times 10^4$	1.9~9.2	0.9999
Protocate chualdehyde	$A = 1.37 \times 10^5 C - 1.44 \times 10^5$	15.6~249.6	0.9999
Berberine hydrochloride	$A = 1.78 \times 10^5 C - 2.04 \times 10^5$	9.0~54.0	0.9999

Table II. Content of Seven Components in Tang Maikang Granules (*n* = 6)

Component	Batch: 030315	Batch: 030610
Astragaloside IV (mg/g)	0.044 ± 0.005	0.042 ± 0.001
Formononetin (µg/g)	1.896 ± 0.003	1.977 ± 0.006
Calycosin (µg/g)	0.962 ± 0.003	0.990 ± 0.006
Paeoniflorin (mg/g)	0.465 ± 0.008	0.456 ± 0.001
Danshengsu (mg/g)	4.110 ± 0.036	3.912 ± 0.034
Protocate chualdehyde (mg/g)	0.090 ± 0.001	0.088 ± 0.002
Berberine (mg/g)	0.088 ± 0.001	0.090 ± 0.001

The resulting sample solution was stored at 4°C for the determination of astragaloside IV, formononetin, calycosin, and paeoniflorin.

Samples (15.0000 g) of Tang Maikang Granule were also powdered in order to analyze danshensu and protocate chualdehyde, followed by ultrasonic extraction three times by adding 60 mL of 80%, 60%, or 50% methanol for 40 min each time. The resulting solution was evaporated to dryness after all extraction solution was filtrated. The dried residues were filtrated after dissolving in 10 mL of 80%, 60%, or 50% methanol; the resulting sample solution was stored at 4°C.

For the analyte berberine, another 15.0000 gram of Tang Maikang Granule was weighed and ground, then treated by ultrasonic wave using 60 mL of 20%, 30%, 50%, 60%, or 80% methanol as solvent for 40 min, separately. The procedure of filtrating the extraction elution and evaporating to dryness was described previously. Finally, the residues were redissolved in 10 mL of 20%, 30%, 50%, 60%, or 80% methanol. The prepared sample was stored under 4°C after filtrating the resulting solution.

Precision, repetition, recovery test, and limit of detection

The intraday precision of the method was studied by determining the highest and lowest concentration of all seven compounds, and the inter-day precision was studied each day by quantitating the concentration of the seven samples for five days.

The repetition test was performed by injection the four samples three times, respectively. The recovery test was carried out by adding various known amounts of standard solution into each sample three times. The limit of detection (LOD) was defined as the concentration of standard of each compound that produce analytical signals equal to three times the noise signals.

Results and Discussion

Sample extraction

The Tang Maikang Granule samples for analyzing danshensu and protocate chualdehyde were prepared with 80%, 60%, or 50% methanol as solvent. The resulting solutions were analyzed by HPLC. The results of danshensu were 3.550, 3.912, and 3.500 mg/g, respectively, and the results for protocate chualdehyde were 0.084, 0.088, and 0.078 mg/g, respectively. The results suggest that the desired compound content was at the highest level in the prepared solution when using 60% methanol as solvent. Therefore, 60% of methanol was selected to prepare the sample solution.

For berberine, 20%, 30%, 50%, 60%, or 80% methanol was used as the solvent, and the results were 0.076, 0.089, 0.043, 0.039, and 0.038 mg/g. It was obvious to select 30% methanol as the solvent.

Chromatographic conditions

Among the seven compounds, only astragaloside IV had UV absorption on the end of the UV spectrum at 200 nm. Thus, it could not be determined by PDA detection, and ELSD was chosen as the detector to analyze astragaloside IV.

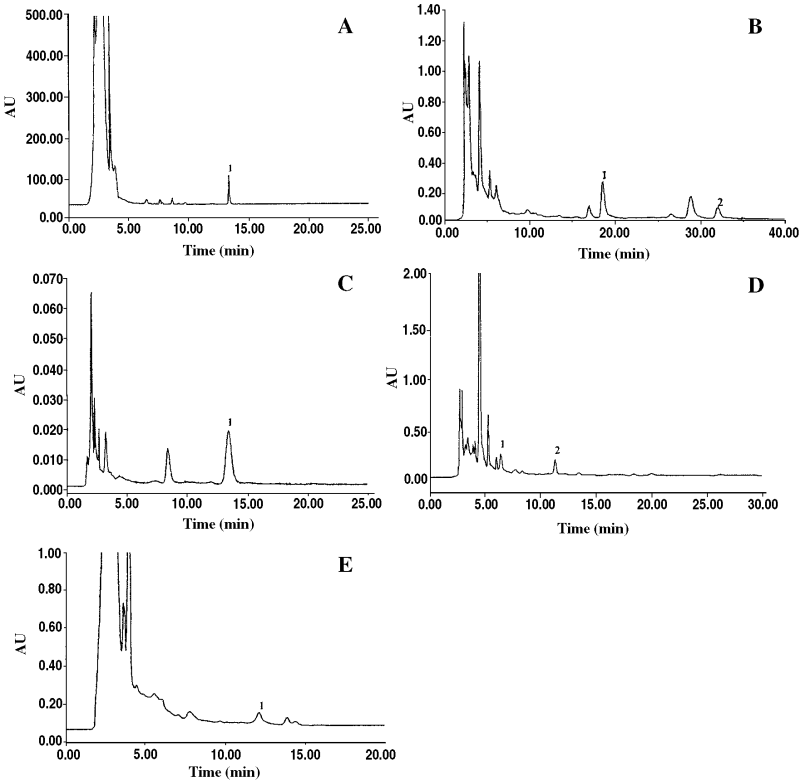

Figure 2. Chromatograms of determination of seven compounds in sample (peaks identified with standard): astragaloside IV (A); formononetin and calycosin (B) (peak 1: formononetin and peak 2: calycosin); paeoniflorin (C); danshengsu and protocate chualdehyde (D) (peak 1: danshengsu and peak 2: protocate chualdehyde); and berberine (E).

Table III. Precision, Repetition Values, and Detection of Limits for Seven Components

Component	Precision (standard) Intraday (n = 5) RSD%	Precision (standard) Interday (5 days) RSD%	Repetition (sample) (n = 4) RSD%	LOD (ng/µL)	Quantity of detection (ng)
Astragaloside IV	1.81	3.36	1.19	1.34	4.03
Formononetin	0.49	0.83	0.29	3.23	18.80
Calycosin	0.12	0.44	1.44	3.11	18.60
Paeoniflorin	0.83	1.13	1.33	2.26	7.40
Danshengsu	0.67	0.75	0.97	85.89	173.52
Protocate chualdehyde	0.19	0.41	0.42	4.06	3.12
Berberine hydrochloride	0.67	1.49	0.26	11.60	9.00

In addition, the concentration of paeoniflorin was almost 100 times as much as the concentration of formononetin and calycosin in sample, so those three compounds could not be measured simultaneously. After analyzing formononetin and calycosin, the sample for measuring was diluted by 25 times to quantitate the content of paeoniflorin.

Furthermore, berberine was alkaloid, and the peak would tail with the eluent just after injection. The problem was easily solved by adding 0.1% SDS into the mobile phase.

Calibration and linearity

The calibration curves were obtained by plotting the peak area against the concentration of corresponding standards. Equations for a linear least square regression fit of each analyte of interest along with the concentration range and correlation coefficients are provided in Table I. Table II summarizes theresults for assays on two different batches of Tang Maikang Granule. Chromatograms of sample are shown in Figure 2.

Five negative control samples were prepared without using radix astragali, radix paeonie rubra, radix salviae miltiorrhizae, and coptis root according to the formulation of Tang Maikang, then treated as Tang Maikang Granule sample. The resultant solution was determined by the HPLC method described in this paper. No peaks were found at the retention time of all standard compounds. Those results indicated that other ingredients in Tang Maikang Granule did not disturb the determination of the seven compounds. Therefore, the operating conditions were fit for quantitating the seven desired compounds.

Precision, repetition, recovery test, and limits of detection

The results of the precision and repetition test indicated that the method is reliable, with relative standard deviation percentage being less than 2, except the interday result of astragaloside IV, which was 3.36 (Table III).

The average recovery of three analyses of all seven compounds ranged from 95.70% to 104.61% (Table IV), and these can be considered to be satisfactory results. Table III also showed the LOD of the seven compounds.

Table IV. Recovery Test for Seven Components (*n* = 3)

Component	Found	Added	Observed	Recovery (%)	Average recovery (%)	RSD (%)
Astragaloside IV (mg/g)	0.042	0.040	0.085	105.56	104.18	1.53
	0.042	0.081	0.125	102.76		
	0.042	0.122	0.170	104.91		
Formononetin (μg/g)	1.977	2.350	4.462	105.72	104.61	1.76
	1.977	4.700	6.795	102.49		
	1.977	1.410	9.425	105.63		
Calycosin (μg/g)	0.990	2.325	3.370	102.33	99.05	2.39
	0.990	4.650	5.489	96.85		
	0.990	6.975	7.823	97.96		
Paeoniflorin (mg/g)	0.456	0.185	0.743	97.97	99.98	2.40
	0.456	0.370	0.836	102.64		
	0.456	0.555	1.007	99.32		
Danshengsu (mg/g)	3.912	0.868	4.745	96.02	95.70	0.55
	3.912	1.736	5.572	95.99		
	3.912	3.472	7.214	95.10		
Protocate chualdehyde (mg/g)	0.0880	0.0157	0.0846	97.80	98.42	0.72
	0.0880	0.0312	0.125	99.38		
	0.0880	0.0624	0.170	98.09		
Berberine hydrochloride (mg/g)	0.990	2.325	3.370	102.33	99.05	2.39
	0.990	4.650	5.489	96.85		
	0.990	6.975	7.823	97.96		

Conclusion

The concentration of astragaloside IV, formononetin, calycosin, paeoniflorin, danshensu, protocate chualdehyde, and berberine in Tang Maikang Granule can be determined by HPLC. This paper demonstrates that the method for determining the seven compounds of interest was simple, accurate, sensitive, rapid, and that this method can be applied for routine quality control of Tang Maikang Granule.

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