Simultaneous Determination of Seven Components in Qibaomeiran Pill by HPLC–DAD

Chunying Wang, Qiao Wang, Zhifang Yuan, Wei Yang, Fengli Xie, and **Lantong Zhang***
Department of Pharmaceutical Analysis, School of Pharmacy, Hebei Medical University, Shijiazhuang, 050017, P. R. China

Abstract

A high-performance liquid chromatography coupled with photo diode array detection method is developed for simultaneous determination of seven active components in Qibaomeiran pill, including rutin, 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glycoside, ferulic acid, psoralen, isopsoralen, emodin, and physcion. The analysis is performed on a C_{18} column using a mobile phase composed of A (0.1% acetic acid) and B (acetonitrile) with linear gradient elution. Four wavelengths at 245, 290, 320, and 350 nm were chosen as the monitoring wavelength to determine seven active components, respectively. All the compounds show good linearity (r > 0.999). The developed method is fully validated in respect to precision, repeatability, and accuracy. The proposed method is successfully applied to quantify the seven active components in different Qibaomeiran pill samples. The results indicate that the developed assay could be considered as a suitable quality control method for the Qibaomeiran pill.

Introduction

The Qibaomeiran pill, a well-known composite formula of traditional Chinese medicine (TCM) consists of seven herbs: Radix Polygoni Multiflori, Fructus Psoraleae, Radix Angelicae Sinensis, *Semen Cuscutae*, Poria, *Fructus Lycii*, and Radix Achyranthis Bidentatae. The combination of these medicinal materials have several beneficial effects including deficiency of blood marked by dizziness, blurred vision, premature grey hair, and lassitude of the loins and knees through synergistic interaction. In the clinical practice of traditional Chinese medicine, the pill has been used to treat dementia praesenilis (1–4), infertility in men (5–6), alopecia (7), aplastic anemia (8), osteoporosis (9), and dermatosis (10).

From the published literature, several components, such as rutin (RT) (11–13) from *Semen Cuscutae*, 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glycoside (TSG) (14–17) from Radix Polygoni Multiflori, ferulic acid (FA) (18–22) from Radix Angelicae Sinensis, psoralen (PR) (23) from Fructus Psoraleae, isopsoralen (IPR) from Fructus Psoraleae, emodin (ED) (24–26) from Radix Polygoni Multiflori, and physcion (PS) (27) from Radix Polygoni Multiflori, have been reported to be biological active components contributing to the therapeutic effects of the Qibaomeiran pill (see Figure 1 for their chemical structures). Moreover, these components are derived from different medical material and are often considered as their marker compounds. Therefore, it is significant to determine the seven components to ensure the quality of the Qibaomeiran pill.

Several methods such as high-performance liquid chromatography (HPLC)(28–29), thin layer chromatography (30–31), nonaqueous capillary electrophoresis (32), fluorescence spectrometry (33), and capillary zone electrophoresis (34) have been developed for the determination of these constituents in herbs and Chinese medicinal preparations, and some methods have been reported for quantification of ED (35), TSG (36–37), PR, and IPR (38) in the Qibaomeiran pill. However, most of them quantified just one or two components and could not control the quality of the Qibaomeiran pill overall. The aim of this work was to develop a new reverse-phase high-performance liquid chro-

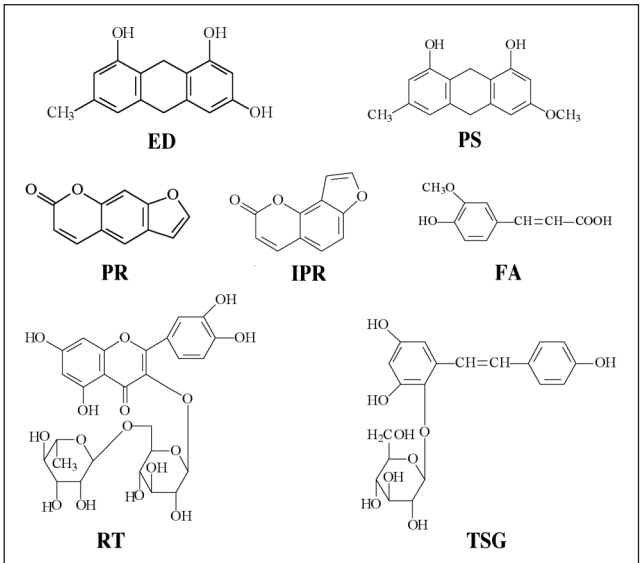

Figure 1. Chemical structures of seven bioactive compounds determined.

*Author to whom correspondence should be addressed: email zhanglantong@263.net

matography (RP-HPLC) method for simultaneous determination of seven biological active components with diode array detection (DAD). The developed assay could be considered as a suitable quality control method for the Qibaomeiran pill.

Experimental

Materials and reagents

The standards of RT, TSG, FA, PR, IPR, ED, and PS were purchased from National Institute for the Control of Pharmaceutical and Biological Products in Beijing, China. HPLC-grade acetonitrile was purchased from Sigma (St. Louis, MO). Ultrapure water was prepared from Milli-Q purification system (Millipore Corp., Molsheim, France). Other reagents were of analytical grade.

Nine Qibaomeiran pill samples were collected from three Chinese medicine manufacturers, manufactures A–C, whose actual names had been removed in order to preserve confidentiality. Sample 1–3, 4–6, and 7–9 were produced from manufactures A–C, respectively.

Apparatus

The HPLC analysis was performed on a Waters 600 liquid chromatograph, equipped with a Nitrogen fumes degasser, a photo DAD (Waters 2996), a quaternary pump, a column compartment, and a manual injection system with a 20 µL loop, connected to Waters Empower software.

Chromatographic conditions

A Dikma Diamonsil C_{18} column (250 × 4.6 mm i.d., 5 µm) was used. The mobile phase was composed of 0.1% (v/v) acetic acid solution (A) and acetonitrile (B). The applied elution conditions were: 0–5 min, isocratic 25% (v/v) B; 5–7 min, linear gradient 25–65% (v/v) B; 7–15 min, isocratic 65% (v/v) B; 15–20 min, linear gradient 65–100% (v/v) B; 20–30 min, isocratic 100% (v/v) B. Then, the column was reconditioned with 25% (v/v) B isocratic for 10 min. The flow rate was 1.0 mL/min and the column compartment was kept at the temperature of 30ºC. Four detection wavelengths of 245, 290, 320, and 350 nm were chosen simultaneously to record chromatograms.

Sample preparation

Qibaomeiran pill samples were first ground into fine powder. Fine powder (2.5 g, 60 mesh) was accurately weighed and was transferred into a round bottom flask, mixed with 10 mL of dehydrated ethanol, and the total weight was accurately weighted. The flask was then connected to the refluxing apparatus. The extraction was performed with a water bath for 2 h. After that, it was maintained at room temperature for 30 min. Dehydrated ethanol was added into the extracted solution to replenish the decreased weight. After centrifugation at 4000 × g for 10 min, the supernatant was passed through a 0.45-µm membrane filter prior to injection into the HPLC system.

Calibration curves

A stock solution containing all seven standards was prepared by dissolving accurately weighted portions of the standards in methanol to a final concentration of 1073 µg/mL for RT, 2250 µg/mL for TSG, 2625 µg/mL for FA, 567 µg/mL for PR, 685 µg/mL for IPR, 232 µg/mL for ED and 88 µg/mL for PS, respectively, and was stored at 4ºC. Working solutions of the analytes were prepared by diluting the mixture stock solution with methanol. The solutions were brought to room temperature and filtered through a 0.45 µm membrane before HPLC analysis. Each calibration curve consisted of six different concentrations and was performed in triplicate. All calibration curves were constructed from peak areas of the standards versus their concentrations. The amounts of these seven components in the Qibaomeiran pill could be quantified according to the regression equations.

Limits of detection and quantification

Stock solution containing seven standards compounds was diluted to a series of appropriate concentrations with methanol, and an aliquot of the diluted solutions was injected into HPLC

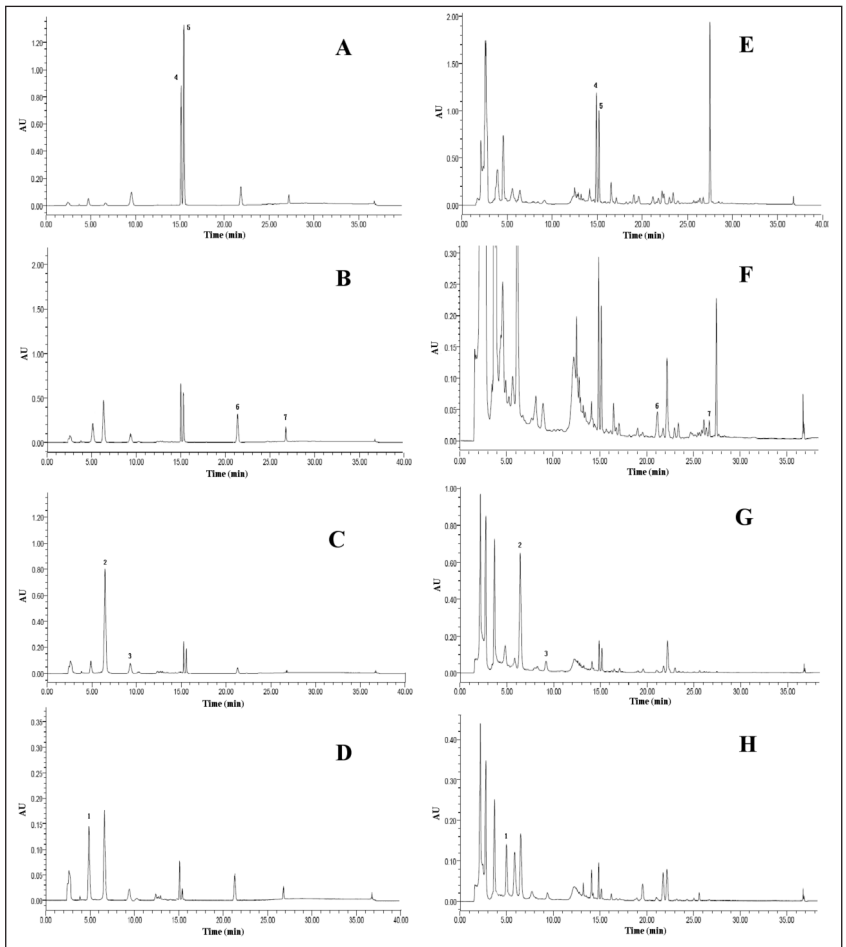

Figure 2. HPLC–DAD chromatograms of: standard mixture (245 nm) (A); standard mixture (290 nm) (B); standard mixture (320 nm) (C); standard mixture (350 nm) (D); Qibaomeiran pill (245 nm) (E); Qibaomeiran pill (290 nm) (F); Qibaomeiran pill (320 nm) (G); Qibaomeiran pill (350 nm) (G).

for analysis. The limits of detection (LOD) and quantification (LOQ) for each analyte were calculated with corresponding standard solution on the basis of a signal-to-noise ratio (S/N) of 3 and 10, respectively.

Precision, accuracy, and stability

The intra- and inter-day variations were measured to determine the precision of the developed method. The intra-day variability was examined on six different extracted solutions prepared according to the method mentioned previously from the same sample within one day, and the inter-day reproducibility was determined for three consecutive days.

To further evaluate the accuracy of the method, a recovery test was carried out by spiking 10 mL of dehydrated ethanol solution, having a known amount of investigated compounds, into an accurately weighed portion of fine powder (1.25 g, 60 mesh) sample in which the contents of seven analytes have been calculated according to the peaks corresponding to their standards. Then, the resultant samples were extracted and analyzed using the method described earlier. The average recoveries were estimated by the formula: recovery (%) = (amount found – original amount)/spiked amount × 100%, and relative standard deviation (%) = (SD/mean) × 100%.

Stability study was performed with the same sample solution after storage at room temperature for 2, 4, 8, 12, and 24 h. Variations were expressed by RSD.

Results and Discussion

Chromatographic conditions optimization

The effect of mobile phase composition was examined. It was found that when methanol was used, RP and IRP could hardly be resolved from each other, and EP was overlapped with some other unknown component. However, when methanol was replaced by acetonitrile, the situation was greatly improved and satisfactory resolution was obtained. The addition of acid in the mobile phase was found to be useful for the improvement of peak shape, but the type and concentration of acids seem to have no serious effect on the separation.

Maximally efficient detection can be obtained by selecting the wavelength where the component has the maximum absorption. These seven analytes belong to five different structural types, namely coumarins (RP and IRP), anthraquinone (EP and PS), organic acids (FA), flavone glycoside (RT), and stilbenes (TSG), which have rather different UV absorption properties. In this study, detection wavelengths of 245 for RP and IRP, 290 for EP and PS, 320 for FA and TSG, and 350 nm for RT were chosen to record chromatograms. Representative chromatograms at 245, 290, 320, and 350 nm for the seven standard analytes and the sample are shown in Figure 2.

Figure 2A–2D displays that the seven standard analytes were well separated, and the resolution between any two compounds was greater than 1.5. Other compounds in the sample did not interfere with the analysis of the seven analytes, as shown in Figure 2E–2H. The chromatographic peaks were identified by comparing their retention time and the spectrograms of DAD (Figure 3) with those of each reference compound. In addition, spiking samples with the reference compounds showed no additional peaks, which further confirmed the identities of the peaks of analytes.

Extraction method

In order to obtain satisfactory extraction efficiency, extraction method, extraction solvent, and extraction time were investigated. The results suggested that refluxing was better than ultrasonic extraction; so refluxing was used in further experiments.

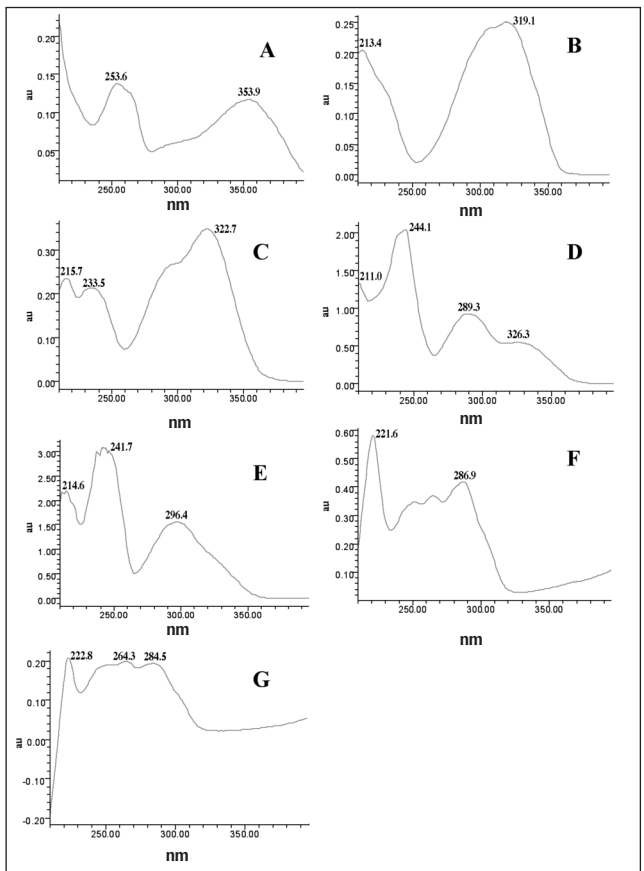

Figure 3. DAD spectrograms of: rutin (4.76 min) (A); 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glycoside (6.67 min) (B); ferulic acid (9.55 min) (C); psoralen (15.13 min) (D); isopsoralen (15.44 min) (E); emodin (21.84 min) (F); physcion (27.18 min) (G).

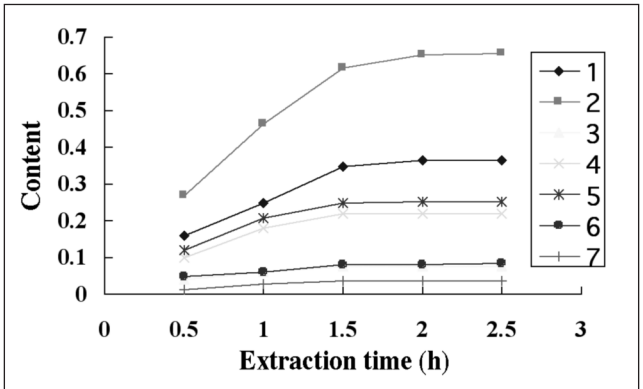

Figure 4. Extraction efficiency of different extraction time: rutin, 1; 2,3,5,4′-tetrahydroxy-stilbene-2-O-β-D-glycoside, 2; ferulic acid, 3; psoralen, 4; isopsoralen, 5; emodin, 6; physcion, 7.

Fifty percent methanol, 70% methanol, methanol, and dehydrated ethanol were performed as extraction solvents to analyze the effect of the solvent on extraction efficiency. The results showed that methanol and dehydrated ethanol had the same extraction efficiency. For safety precautions, dehydrated ethanol was the most suitable extraction solvent. Then 2.5 g samples were extracted with 10 mL dehydrated ethanol by refluxing for 0.5, 1.0, 1.5, 2.0, and 2.5 h, respectively, to determine optimal extraction time. As shown in Figure 4, the maker compounds were almost completely extracted within 2.0 h. Hence, 2.0 h was chosen as optimal extraction time.

Method validation

Under current chromatographic conditions, all calibration curves showed good linear regression ($r > 0.999$). The results are given in Table I. The LOD ranged from 0.17 to 2.10 ng for the seven compounds, and the LOQs were less than 4.68 ng, indicating that this method is sensitive for the quantitative evaluation of the Qibaomeiran pill. Validation studies of the method proved that this assay had good reproducibility, and the intra-day and inter-day variations were less than 3.0% for all analytes. This indicated that the newly developed analytical method was accurate with the recovery in the range of

Table I. Regression Data, LODs, and LOQs for the Components Determined

Peak No.*	Components	Monitoring wavelength (nm)	Regression equation†	Correlation factors	Linear range (mg/mL)	LOD (ng)	LOQ (ng)
1	RT	340	$y = 1.3362 \times 10^7x + 2.9532 \times 10^5$	0.9999	0.0215–0.536	1.39	3.68
2	TSG	320	$y = 4.555 \times 10^7x + 3.953 \times 10^5$	0.9994	0.0450–1.125	0.64	2.10
3	FA	320	$y = 5.737 \times 10^7x + 2.730 \times 10^5$	0.9992	0.0052–0.131	0.82	2.05
4	PR	245	$y = 5.215 \times 10^7x - 2.625 \times 10^3$	0.9997	0.0113–0.283	0.17	0.57
5	IPR	245	$y = 3.085 \times 10^7x + 1.70 \times 10^4$	0.9991	0.0137–0.342	0.46	1.06
6	ED	290	$y = 5.948 \times 10^7x + 8.234 \times 10^3$	0.9993	0.0046–0.116	0.88	2.93
7	PS	290	$y = 4.774 \times 10^7x + 5.100 \times 10^4$	0.9999	0.0018–0.044	2.10	4.68

* Peak numbers are from Figure 2.
† y is the peak area, and x is the concentration.

Table II. Precision and Accuracy Date of the Validation Tests for the Proposed HPLC–DAD Method

		Precision (n = 5)		Accuracy* (n = 5)				
Peak no.	Compounds	Intra-day RSD (%)	Inter-day RSD (%)	Original (mg) (mean ± SD)	Spiked (mg)	Found (mg) (mean ± SD)	Recovery*	RSD (%)
1	RT	0.79	1.56	0.455 ± 0.002	0.429	0.893 ± 0.008	102.1 ± 1.73	1.70
2	TSG	0.85	0.87	0.816 ± 0.002	0.900	1.729 ± 0.011	101.4 ± 1.03	1.01
3	FA	2.09	2.46	0.096 ± 0.002	0.079	0.176 ± 0.001	100.4 ± 1.20	1.19
4	PR	1.59	1.15	0.276 ± 0.002	0.227	0.505 ± 0.004	100.9 ± 1.59	1.57
5	IPR	1.71	2.68	0.313 ± 0.002	0.343	0.657 ± 0.004	100.0 ± 1.34	1.34
6	ED	1.78	1.82	0.102 ± 0.002	0.093	0.196 ± 0.001	100.2 ± 0.74	0.74
7	PS	1.09	2.79	0.044 ± 0.002	0.035	0.079 ± 0.001	99.99 ± 1.66	1.66

* Accuracy (%) = (found–original) / spiked amount × 100%

Table III. Quantitative Analytical Results of Various Qibaomeiran Pill Samples

	A				B				C				
Compounds	1	2	3	RSD (%)	4	5	6	RSD (%)	7	8	9	RSD (%)	RSD (%)
RT (mg/g)	0.3635	0.3590	0.3495	2.01	0.1362	0.1465	0.1387	3.93	0.2325	0.2401	0.2368	1.71	38.50
TSG (mg/g)	0.6521	0.6627	0.6474	1.22	0.6212	0.6065	0.6132	1.21	0.7134	0.7421	0.7365	2.11	7.85
FA (mg/g)	0.0769	0.0761	0.0745	1.71	0.1165	0.1143	0.1204	2.75	0.0652	0.0613	0.0626	3.24	28.75
PR (mg/g)	0.2203	0.2184	0.2192	0.54	0.1577	0.1623	0.1648	2.33	0.1958	0.1924	0.2012	2.39	13.16
IPR (mg/g)	0.2503	0.2496	0.2495	0.23	0.1896	0.1954	0.1843	3.02	0.2351	0.2238	0.2342	2.85	12.05
ED (mg/g)	0.0819	0.0826	0.0795	2.08	0.0686	0.0698	0.0642	4.48	0.1143	0.1192	0.1235	3.96	26.03
PS (mg/g)	0.0353	0.0354	0.0350	0.67	0.0231	0.0221	0.0214	3.94	0.0515	0.0467	0.0486	5.01	32.85

99.99–102.1%, with RSDs less than 2.0%. Table II shows the results of the tests of precision and accuracy. The analytes in the sample solution were stable within 24 h, with a relative standard deviation of 1.57–3.28%.

Analysis of samples

The developed HPLC–DAD analytical method was subsequently applied to simultaneously determine the seven bioactive compounds in nine Qibaomeiran pill samples, and the calculated contents of analytes were shown in Table III. The results indicate that the concentrations of each component in the samples from the same manufacturer have much lower RSDs, but there is notable difference among the manufacturers. There are some factors, such as preparation procedures and the quality of herbal materials, that lead to the variance of these components.

Conclusion

The proposed method makes it possible to simultaneously determine multiple components in one run with acceptable levels of linearity, precision, stability, and accuracy. The method has been applied successfully to simultaneously quantify seven bioactive components in the Qibaomeiran pill samples. The results demonstrate that the proposed method could be readily utilized as quality control for the Qibaomeiran pill.

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