SUPPLEMENTARY MATERIAL

Multi-components determination by single reference standard and HPLC fingerprint analysis for *Lamiophlomis rotata* Pill

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A validated HPLC method was developed to evaluate the quality of *Lamiophlomis rotata* Pill combined the multi-components analysis by single reference standard with HPLC fingerprint analysis. Five bioactive components (shanzhiside methyl ester, loganin, 8-*O*-acetylshanzhiside methyl ester, forsythoside B and luteolin-7-*O*- β -D-glucopyranoside) were selected as makers to control the quality of *Lamiophlomis rotata* Pill. The results revealed that the chromatographic fingerprint method coupled with multi-components analysis provides an effective and feasible way to determine the components in *Lamiophlomis rotata* Pill.

Keywords *Lamiophlomis rotata* Pill, multi-components determination, fingerprint.

1. Experimental

1.1. Materials, chemicals and reagents

Shanzhiside methyl ester, loganin, 8-*O*-acetylshanzhiside methyl ester, forsythoside B were purchased from the National Institutes for Food and Drug Control (Beijing, China), and luteolin-7-*O*- β -D-glucopyranoside were obtained from Chengdu Pufeide Biological Technology Co., Ltd. (Chengdu, China). The purity of five compounds, assessed by HPLC, was all over 98%. *Lamiophlomis rotata* Pill was collected from different pharmacy stores.

Methanol and acetonitrile of HPLC grade were provided by Concord Technology Co., Ltd. (Tianjin, China) and acetic acid was supplied by Kermel Chemical Reagent Co., Ltd. (Tianjin, China). Distilled water for the HPLC mobile phase was purified by redistillation, all reagents and samples were passed through a 0.45 μ m membrane filter before use.

1.2. Apparatus and chromatographic conditions

The chromatographic analysis was carried out on two liquid chromatography systems: SHIMADZU HPLC system (SHIMADZU Corporation, Japan) consisting of two SPD-10AT plus pumps, a UV-Vis detector, an AT-330 Column heater and a Rheodyne 7725i injector with a 20 μL sample loop; JASCO HPLC system (JASCO Corporation, Japan) consisting of two JASCO-PU-2080 plus pumps, a UV-Vis detector, an AT-330 Column heater and a Rheodyne 7725i injector with a 20 μL sample loop. Separation of the **makers** was achieved on three columns: Inertsil ODS-3 column (250 mm $\times$ 4.6 mm i.d., 5 μm); Agela C₁₈ (250 mm $\times$ 4.6 mm i.d., 5 μm); Shodex C₁₈ (250 mm $\times$ 4.6 mm i.d., 5 μm).

The mobile phase consisted of A (acetonitrile) and B (0.5% acetic acid aqueous solution). The partitioned gradient profile was: 0-16 min, 11% A; 16-21 min, 11-15% A; 21-31 min, 15-18% A; 31-45 min, 18-22% A. Finally, returning to the starting conditions (11% A) for 10 min to allow equilibration of the column to the initial conditions. The mobile phase degassed before being used in the system. The column temperature was maintained at 30°C. The injection volumes of sample and reference standard solution were 20 μL and the HPLC chromatograms were monitored by UV detection at 254 nm, the effluent flow rate was set at 0.8 mL $\cdot\text{min}^{-1}$.

1.3. Preparation of solutions

Standard solutions

Stock solution of shanzhiside methyl ester, loganin, 8-*O*-acetylshanzhiside methyl ester, forsythoside B and luteolin-7-*O*- β -D-glucopyranoside was prepared by dissolving accurately weighed standards with 70% methanol to obtain the concentrations of 61.2 $\mu\text{g}\cdot\text{mL}^{-1}$, 36.4 $\mu\text{g}\cdot\text{mL}^{-1}$, 78.5 $\mu\text{g}\cdot\text{mL}^{-1}$, 47.6 $\mu\text{g}\cdot\text{mL}^{-1}$, 114.5 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively. The mixed solution was stored in a 25 mL flask at 4°C.

Sample solutions

An amount of 250 mg powdered drug precisely which was obtained by pulverizing 20

pills in a 100 mL glass-stoppered conical flask, 50 mL 70% methanol was added, then the total weight was measured. Additional 70% methanol was added to compensate for any loss after sonicating for 30 min. The extracts were filtered with 0.45 µm membrane filter prior to injection.

1.4. Data analysis of SSDMC method

The content of internal reference standard 8-*O*-acetylshanzhiside methyl ester was calculated through the ESM method and the contents of other components were obtained by conversion factors. The conversion factors (F_x) based on the linearity data can be calculated as follow:

$$F_x = \frac{A_s / C_s}{A_{sx} / C_{sx}} \quad (1)$$

Where A_s and C_s were the peak area and the concentration of internal reference standard 8-*O*-acetylshanzhiside methyl ester; A_{sx} and C_{sx} were the peak area and the concentration of other analytes.

The relative retention time of the analyte (RRT_x) was calculated as the ratio of retention time of the analyte (T_x) and 8-*O*-acetylshanzhiside methyl ester (T_s):

$$RRT_x = \frac{T_x}{T_s} \quad (2)$$

1.5. The systematically quantified fingerprint method

The software Digitized Evaluation System for Super-Information Characteristics of TCM Chromatographic Fingerprints 4.0 (Software certificate No. 0407573, China) based on SQFM was applied to assess the quality of herbal preparations. And the values of the macro qualitative similarity (Sm), the macro quantitative similarity (Pm) and the variation coefficient (α), which determined by SQFM, were used together for evaluation.

The values of Sm defined as eqn (3) was used to describe both the number and distribution of chemical components between the sample and reference fingerprints. The values of α described in eqn (4) was able to control the range limits of the

fingerprint peak leveling differences between the sample fingerprint (SFP) and reference fingerprint (RFP). P_m was defined as the ratio of the apparent weight of RFP and the weight of the sample, which was corrected for the weight of samples based on eqn (5), where x_i and y_i are the peak areas of the component peaks in the sample and reference fingerprints, respectively.

$$S_m = \frac{1}{2}(S_F + S_{F'}) = \frac{1}{2} \left(\frac{\sum_{i=1}^n x_i y_i}{\sqrt{\sum_{i=1}^n x_i^2} \sqrt{\sum_{i=1}^n y_i^2}} + \frac{\sum_{i=1}^n \frac{x_i}{y_i}}{\sqrt{n \sum_{i=1}^n \left(\frac{x_i}{y_i}\right)^2}} \right) \quad (3)$$

$$\alpha = \left| 1 - \frac{P}{C} \right| \quad (4)$$

$$P_m = \frac{1}{2}(C + P) = \frac{1}{2} \left(\frac{\sum_{i=1}^n x_i y_i}{\sum_{i=1}^n y_i^2} + \frac{\sum_{i=1}^n x_i}{\sum_{i=1}^n y_i} S_F \right) \times 100\% \quad (5)$$

1.6. Method validate

Calibration curves, LOD and LOQ

The mixed standard solution was diluted into five appropriate concentrations for the construction of calibration curves. Then calibration curves were drawn and the regression equations were calculated via partial least squares. The results showed that each analyte had good linear relationship over the concentration range. The LOD and LOQ for each analyte were determined by diluting stock solutions to a series of appropriate concentrations giving equivalent signal-to-noise ratios of 3 and 10, respectively (Table S1).

< Table S1 >

Precision, repeatability and accuracy

Intra-day and inter-day precision was analyzed by replicating the injection of the

standard solution on 1 day (n=6) and on three consecutive days. The results showed the intra-day RSD values of the five components were less than 2.3%, and the calculated mean inter-day RSD values were less than 2.9%. Repeatability was tested by injecting six independently prepared samples. RSD values of component contents for shanzhiside methyl ester, loganin, 8-*O*-acetylshanzhiside methyl ester, forsythoside B and luteolin-7-*O*- β -D-glucopyranoside in samples were 1.8%, 2.3%, 1.9%, 2.8% and 1.5%, respectively. For the accuracy, which was carried out to investigate the percentage of recovery by spiking known amounts of the mixed reference standard solutions to samples, was tested by six independently prepared samples of the same concentration. It was found that the recovery was between 98.5-101.2% with the RSDs less than 3.1%.

Stability

The stability study was determined with the same sample solution at room temperature for 0, 4, 8, 12, 24, 36, 48h. The values of RSD were less than 3.2%, which demonstrated that the sample solution was stable within 48h.

Calculation of conversion factor and relative retention time

The values of F_x and RRT_x calculated by formula (1) and formula (2) of five marker substances were shown in Table S1 with 8-*O*-acetylshanzhiside methyl ester as internal reference standard. The values of conversion factor for shanzhiside methyl ester, loganin, forsythoside B and luteolin-7-*O*- β -D-glucopyranoside were 1.108, 1.034, 1.791 and 0.452. The values of relative retention time were 0.381, 0.734, 1.145 and 1.332, respectively. It was proved that the F_x and RRT_x of each analyte were stable at different concentrations.

Robustness

As the most important role in SSDMC method, the stability of the conversion factor should be tested strictly. Thus, several parameters including environmental parameters and operational parameters were investigated with five concentration

standard solutions of linearity.

Environmental parameters were investigated on two instruments (SHIMADZU and JASCO) with the same parameters (Inertsil ODS-3 (250 mm × 4.6 mm i.d., 5μm) column with the column temperature of 30°C and 0.5% acetic acid.), three columns (Inertsil ODS-3 (250 mm × 4.6 mm i.d., 5μm), Agela C18 (250 mm × 4.6 mm i.d., 5μm) and Shodex C18 (250 mm × 4.6 mm i.d., 5μm)) with the same parameters (the column temperature of 30°C and 0.5% acetic acid on SHIMADZU).

The operational parameters were investigated at three column temperatures (25, 30 and 35°C) and three different concentrations (0.2, 0.4 and 0.5%) of acetic acid in mobile phase with the same environmental parameters (Inertsil ODS-3 (250 mm × 4.6 mm i.d., 5μm) column and SHIMADZU HPLC system).

According to the data displayed in Table S2, for the value of RRT_x , different operational parameters (S3, S4) varied in a narrow range with the maximum SD of 0.009. However, different environment parameters (S1, S2), especially different columns, were fluctuated with the maximum SD of 0.038. It was speculated that diverse packing would affect retention behavior of different substances. For the value of F_x , the maximum SD was 0.029, which indicated good ruggedness both under environmental parameters and operational parameters. In addition, we also investigated the flow rate (0.8 and 1.0 mL·min⁻¹), which was sensitive to the resolution of five makers, should be strictly followed in the experiment.

< Table S2 >

1.7. Comparison of the external standard method and SSDMC

The ESM and established SSDMC method were applied to simultaneously determine five active substances in eleven batches of *Lamiophlomis rotata* Pill samples. For the SSDMC method, the internal reference standard 8-*O*-acetylshanzhiside methyl ester was analyzed by the ESM method. The contents of shanzhiside methyl ester, loganin, forsythoside B and luteolin7-*O*-β-D- glucopyranoside were calculated according to their conversion factors. The contents of five compounds calculated by two methods were summarized in Table S3. The figures of the sample and mixed references are

showed in Fig.S1.

< Table S3 >

< Fig S1 >

1.8. HPLC fingerprint and similarity analysis

Eleven batches of *Lamiophlomis rotata* Pill samples were used to developed HPLC fingerprint and analysis as shown in Fig.S2. And the results carried out by the SQFM method were displayed in Table S4. According to the data, Sm of all eleven samples were more than 0.9 and α were less than 0.2, which indicated that the whole sample has high similarity with the reference fingerprint. However, Pm of sample S1-S5 and S7 were less than 75%, indicating an unacceptable result of these samples. The result was also consistent with the contents of five components. Therefore, it can be indicated that the SQFM could be applied as an auxiliary method for the quality control of *Lamiophlomis rotata* Pill.

< Fig.S2 >

< Table S4 >

Figure captions

Fig.S1 Mixed reference standards (A); sample of *Lamiophlomis rotata* Pill (B); shanzhiside methyl ester (1), loganin (2), 8-*O*-acetylshanzhiside methyl ester (3), forsythoside B (4) and luteolin-7-*O*- β -D-glucopyranoside (5).

Fig.S2 HPLC fingerprints of 11 batches of *Lamiophlomis rotata* Pill

Fig S1

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Fig S2

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Table S1Regression equation, correlation, linearity range, LOD, LOQ, F_x and RRT_x (n = 3).

Analyte	Regression equation	R^2	Range ($\mu\text{g}\cdot\text{mL}^{-1}$)	LOD ($\mu\text{g}\cdot\text{mL}^{-1}$)	LOQ ($\mu\text{g}\cdot\text{mL}^{-1}$)	F_x	RRT_x
Shanzhiside methyl ester	$y=9165x - 3436$	0.9997	6.115-61.15	0.20	0.60	1.108 ± 0.020	0.381 ± 0.001
Loganin	$y=9448x - 2541$	0.9994	3.644-36.44	0.40	1.00	1.034 ± 0.029	0.734 ± 0.001
8- <i>O</i> -acetylshanzhiside methyl ester	$y=9635x - 4324$	0.9998	7.854-78.54	0.78	1.56	1.000 ± 0.000	1.000 ± 0.000
Forsythoside B	$y=6521x - 2092$	0.9996	4.763-47.63	0.80	2.40	1.791 ± 0.033	1.145 ± 0.001
Luteolin-7- <i>O</i> - β -D- glucopyranoside	$y = 20873x - 18728$	0.9992	11.45-114.5	0.62	1.86	0.452 ± 0.016	1.332 ± 0.003

Table S2

Fx and RRT_x in robustness test for SSDMC method. $(\bar{x} \pm \bar{s})$

Analyte	Fx				RRT_x			
	S_1^a	S_2^b	S_3^c	S_4^d	S_1^a	S_2^b	S_3^c	S_4^d
Shanzhiside methyl ester	1.104±0.021	1.119±0.013	1.107±0.011	1.096±0.015	0.376±0.013	0.386±0.008	0.379±0.003	0.370±0.005
Loganin	1.040±0.027	1.028±0.031	1.036±0.013	0.055±0.029	0.727±0.005	0.724±0.004	0.725±0.002	0.728±0.003
8- <i>O</i> -acetylshanzhi side methyl ester	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000
Forsythoside B	1.759±0.022	1.758±0.025	1.810±0.013	1.813±0.026	1.150±0.003	1.306±0.038	1.147±0.004	1.149±0.006
Luteolin-7- <i>O</i> - β -D-glucopyranoside	0.481±0.026	0.450±0.018	0.502±0.024	0.458±0.029	1.352±0.019	1.306±0.038	1.338±0.009	1.332±0.007

^a S_1 was performed on two instruments (SHIMADZU and JASCO), Inertsil column with the column temperature of 30°C and 0.5% acetic acid.

^b S_2 was performed on three columns (Inertsil, Agela and Shodex), the column temperature of 30°C and 0.5% acetic acid on SHIMADZU.

^c S_3 was performed on Inertsil column with the three column temperatures (25, 30, 35°C), 0.5% acetic acid on SHIMADZU.

^d S_4 was performed on Inertsil column with the three column temperatures of 30°C, three concentrations of acetic acid (0.2, 0.4 and 0.5%) on SHIMADZU.

Table S3

Contents of five components in eleven *Lamiophlomis rotata* Pill samples.

	Lot number	ESM ^a content (mg•g ⁻¹)					SSDMC ^b content (mg•g ⁻¹)				
		SM ^c	L ^d	ASM ^e	FB ^f	LGR ^g	SM ^c	L ^d	ASM ^e	FB ^f	LGR ^g
S1	1120611	3.41	2.34	4.51	2.76	5.18	3.35	2.40	4.51	2.70	5.31
S2	1130116	3.13	1.38	3.90	3.63	3.76	3.10	1.41	3.90	3.57	3.86
S3	1130306	2.02	1.03	3.63	3.49	3.58	1.97	1.05	3.63	3.47	3.62
S4	1131205	3.19	1.81	3.49	3.18	4.51	3.13	1.86	3.49	3.09	4.63
S5	1140205	2.73	1.20	3.53	3.28	3.35	2.67	1.23	3.53	3.19	3.44
S6	20141213-1	4.55	2.67	5.26	4.44	5.67	4.46	2.73	5.26	4.35	5.81
S7	20140111-1	3.14	1.71	3.41	3.08	4.45	3.06	1.75	3.41	2.99	4.56
S8	20140111-2	4.91	2.66	5.89	3.54	7.87	4.79	2.72	5.89	3.48	8.06
S9	20140111-3	4.90	2.77	5.89	3.70	7.82	4.81	2.84	5.89	3.64	8.01
S10	20141213-2	4.94	2.71	5.95	3.43	8.15	4.85	2.79	5.95	3.37	8.33
S11	20141213-3	5.05	3.44	6.10	3.80	6.14	4.92	3.54	6.10	3.71	6.30
	Average	3.82	2.16	4.69	3.48	5.50	3.74	2.21	4.69	3.41	5.63

^aESM . external standard method.

^bSSDMC . single standard to determine multi-components method.

^cSM. Shanzhiside methyl ester ^dL. Loganin ^eASM. 8-*O*-acetylshanzhiside methyl ester ^fFB. Forsythoside B ^gLGR.Luteolin-7-*O*-β-D-glucopyranoside.

Table S4

The evaluation results of 11 batches of *Lamiophlomis rotata* Pill.

Para.	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	RFP
Sm	0.956	0.971	0.971	0.981	0.976	0.991	0.986	0.998	0.998	0.996	0.992	1
Pm%	70.1	64.1	31.2	68.2	61.5	95.5	67.7	114.7	115.4	115.4	103.3	100
α	0.001	0.091	0.097	0.022	0.105	0.055	0.026	0.022	0.012	0.042	0.021	0
Grade	5	6	8	6	6	2	6	3	3	3	1	1