GCMTI RD-5:2022

Determination of Ginsenosides (Re, Rg1, Rf and Rb1) in Baifeng Wan by Liquid Chromatograph-Tandem Mass Spectrometer (LC-MS/MS)

GCMTI method publications

Determination of Ginsenosides (Re, Rg1, Rf and Rb1) in Baifeng Wan by Liquid Chromatograph-Tandem Mass Spectrometer (LC-MS/MS)[1]

Safety Precaution: *This procedure involves carcinogenic chemicals, corrosive chemicals and flammable solvents. Apply precautions when handling such chemicals, for example: use eye and hand protection and where necessary carry out the work in a fume cupboard to avoid inhalation of vapour.*

1. Introduction

1.1. Baifeng Wan is a prevalent proprietary Chinese medicine (pCm) in China and Hong Kong. It is commonly used for treating various diseases caused by blood deficiency or gynaecological disorders. Ancient Chinese medicines bibliography as well as Chinese Pharmacopeia (CP) have documented the major ingredients for the prescriptions. Nevertheless, in Hong Kong market there are numerous modified formulations of Baifeng Wan products with varying compositions. Among others, Chinese herbal medicines such as Ginseng Radix Et Rhizoma (人參), Angelicae Sinensis Radix (當歸), Chuanxiong Rhizoma (川芎), Cyperi Rhizoma (香附), Paeoniae Radix Alba (白芍), Rehmanniae Radix (地黃), Astragali Radix (黃芪), Salviae Miltiorrhizae Radix Et Rhizoma (丹參) and Glycyrrhizae Radix Et Rhizoma (甘草) are commonly found in different brands of Baifeng Wan products. The corresponding chemical markers are as follows:

Chinese Herbal Medicines	Common Chemical Markers
Ginseng Radix Et Rhizoma (人參)	Ginsenosides
Angelicae Sinensis Radix (當歸)	Z-ligustilide
Chuanxiong Rhizoma (川芎)	Z-ligustilide
Cyperi Rhizoma (香附)	α-cyperone
Paeoniae Radix Alba (白芍)	Paeoniflorin
Rehmanniae Radix (地黃)	Rehmannioside
Astragali Radix (黃芪)	Astragaloside IV
Salviae Miltiorrhizae Radix Et Rhizoma (丹參)	Tanshinone and salvianolic acid B
Glycyrrhizae Radix Et Rhizoma (甘草)	Liquiritin

[1] This method is intended to provide a reliable analytical method that can be used as quality control method for determining the targeted chemical marker(s) in the corresponding pCm product(s). It is the user's responsibility to assess the suitability of application to their pCm products when adopting this method.

1.2. This method specifies the procedures for qualitative and/or quantitative determination of ginsenosides (Re, Rg1, Rf and Rb1) in Baifeng Wan sample by liquid chromatograph-tandem mass spectrometer (LC-MS/MS).

2. Reagents

Note: All reagents used should be of analytical reagent grade or equivalent unless otherwise specified.

2.1. Methanol, LC-MS grade.

2.2. Acetonitrile, LC-MS grade.

2.3. Milli-Q water.

2.4. Ginsenoside Re, CAS. No.: 52286-59-6.

2.5. Ginsenoside Rg1, CAS. No.: 22427-39-0.

2.6. Ginsenoside Rf, CAS. No.: 2005-06-24.

2.7. Ginsenoside Rb1, CAS. No.: 41753-43-9.

2.8. Extraction solvent

Methanol : water (1:1 v/v).

2.9. Preparation of standard solutions

2.9.1. Individual stock standard solutions (ca. 1000 μg/mL)

Weigh accurately about 10 mg of ginsenosides (Re, Rg1, Rf and Rb1) into separate 10-mL volumetric flasks, dissolve and make up to the graduated mark with acetonitrile : water (1:1 v/v), respectively.

2.9.2. Mixed intermediate standard solution I (ca. 10 μg/mL)

Prepare the mixed intermediate standard solution I by transferring 0.1 mL of each individual stock standard solution into a 10-mL volumetric flask and make up to the graduated mark with extraction solvent (Clause 2.8.).

2.9.3. Mixed intermediate standard solution II (ca. 200 ng/mL)

Prepare the mixed intermediate standard solution II by transferring 0.2 mL of mixed intermediate standard solution I into a 10-mL volumetric flask and make up to the graduated mark with extraction solvent (Clause

2.8.).

2.9.4. Calibration standard solutions, CS1 – CS6

A series of calibration standard solutions are prepared by transferring an appropriate amount of mixed intermediate standard solution II into 10-mL volumetric flasks and make up with extraction solvent (Clause 2.8.). Suggested volumes of standard solution used for the preparation are listed in the table below.

Remark~~s~~: the calibration curve(s) shall include at least 5 calibration standards. The user may use calibration standards with appropriate concentrations to accommodate the working range of the analysis.

Calibration standards	**Volume of mixed intermediate standard solution II (mL)**	**Final volume (mL)**	**Conc. of ginsenosides (Re, Rg1, Rf and Rb1) (ng/mL)**
CS1	0.25	10	5
CS2	0.50	10	10
CS3	1.00	10	20
CS4	1.50	10	30
CS5	2.00	10	40
CS6	2.50	10	50

2.9.5. Individual stock ICV standard solutions (ca. 1000 μg/mL)

Prepare individual stock ICV standard solutions, from sources different from that of the calibration standard. Weigh accurately about 10 mg of ginsenosides (Re, Rg1, Rf and Rb1) into separate 10-mL volumetric flasks, dissolve and make up to the graduated mark with acetonitrile : water (1:1 v/v), respectively.

2.9.6. Mixed intermediate ICV standard solution I (ca. 10 μg/mL)

Prepare the mixed intermediate ICV standard solution I by transferring accurately 0.1 mL of each individual stock ICV standard solution into a 10-mL volumetric flask and make up to the graduated mark with extraction solvent (Clause 2.8.).

2.9.7. Mixed intermediate ICV standard solution II (ca. 200 ng/mL)

Prepare the mixed intermediate ICV standard solution II by transferring accurately 0.2 mL of mixed intermediate ICV standard solution I into a 10-mL volumetric flask and make up to the graduated mark with extraction solvent (Clause 2.8.).

2.9.8. ICV working standard solution (ca. 30 ng/mL)

Prepare the ICV working standard solution by transferring 0.15 mL of mixed intermediate ICV standard solution II into a 1-mL volumetric flask and make up to the graduated mark with extraction solvent (Clause 2.8.).

2.9.9. Spike standard solutions (ca. 1000 µg/mL)

Refer to individual stock standard solutions (Clause 2.9.1.).

3. Apparatus

All glassware shall be rinsed with acetone and washed with detergent solution as soon as practicable after use. After detergent washing, glassware shall be rinsed immediately, firstly with acetone and then with water. The water rinse shall be followed by another two more rinses with acetone, respectively.

3.1. Grinder or blender.

3.2. Analytical balance, capable of weighing to 0.01 mg.

3.3. Volumetric flasks, 1-mL, 10-mL and 25-mL.

3.4. Auto pipettes, 100-µL, 300-µL and 1000-µL.

3.5. Centrifuge with rotation speed of at least 4000 rpm.

3.6. Centrifuge tubes, 15-mL.

3.7. Vortex mixer.

3.8. Ultrasonic bath.

3.9. PTFE membrane filters, 0.2 µm.

3.10. LC glass vials.

3.11. LC column: Acquity UPLC® BEH, C18 1.7 µm, 2.1 mm × 100 mm, Waters or equivalent.

3.12. Liquid Chromatograph-Tandem Mass Spectrometer (LC-MS/MS) system.

4. Procedures

4.1. Sample preparation

4.1.1. Grind and homogenize solid samples using grinder or blender before analysis.

4.1.2. Weigh accurately about 0.25 g of Baifeng Wan sample into a 15-mL centrifuge tube.

4.1.3. Add 10 mL of extraction solvent (Clause 2.8.) into the centrifuge tube. Vortex the sample mixture in the centrifuge tube for 1 minute.

4.1.4. Sonicate the sample mixture in an ultrasonic bath for 20 minutes at room temperature.

4.1.5. Centrifuge the sample solution at 4000 rpm for 10 minutes. Carefully transfer the supernatant solution to a 25-mL volumetric flask.

4.1.6. Repeat clauses 4.1.3. to 4.1.5. twice with 5mL extraction solvent (Clause 2.8). Collect all supernatant in the same 25-mL volumetric flask and make up to mark with extraction solvent (Clause 2.8.). Dilute the sample solution by 20-fold with extraction solvent (Clause 2.8.).

4.1.7. Filter the diluted sample solution with 0.2 µm PTFE filter disc into a LC glass vial. The solution is ready for LC-MS/MS analysis.

Remarks: Further dilute the sample solution with extraction solvent (Clause 2.~~5~~8.) if the concentration of analyte(s) is not within the calibration range.

4.2. LC-MS/MS analysis

4.2.1. Operate the LC-MS/MS system in accordance with the instrument manual. Carry out analysis with the conditions as suggested below. It may be necessary to modify the operation conditions for optimal signal output. Record the actual experimental conditions in the worksheet.

4.2.2. Suggested LC conditions:

LC system	:	Thermo Scientific UltiMate 3000 UHPLC or equivalent performance
Column	:	Acquity UPLC® BEH, C18 1.7 µm, 2.1 mm × 100 mm, Waters or equivalent
Column temperature	:	40 ºC

Flow rate	:	0.3 mL/min		
Injection volume	:	5 µL		
Mobile phase	:	A: Water		
		B: Acetonitrile		
Gradient	:	Time (min)	A%	B%
		0.0	90	10
		6.0	90	10
		13.0	40	60
		13.5	5	95
		15.5	5	95
		16.0	90	10
		20.0	90	10

4.2.3. Suggested MS/MS conditions:

MS/MS system	:	AB SCIEX 6500+ system
Ionization mode	:	Electrospray ionization (ESI); Negative mode
Ionspray voltage	:	-4500V
Source temperature	:	350 ºC
Ion source gas 1 (GS1)	:	40
Ion source gas 2 (GS2)	:	40
Curtain gas (CUR)	:	20
Collision gas (CAD)	:	Medium
Scan Type	:	MRM

4.2.4. Suggested MRM acquisition conditions for the analysis of ginsenosides (Re, Rg1, Rf and Rb1):

Analytes	**MRM transitions**	**Dwell time msec**	**DP**	**EP**	**CE**	**CXP**
Ginsenoside Re	945.6 → 637.4*	50	-240	-10	-54	-39
	945.6 → 475.4^	50	-240	-10	-70	-25
Ginsenoside Rg1	799.6 → 637.4*	50	-205	-10	-34	-27
	799.6 → 475.4^	50	-205	-10	-50	-27
Ginsenoside Rf	799.6 → 475.4*	50	-225	-10	-54	-29
	799.6 → 637.4^	50	-225	-10	-44	-39
Ginsenoside Rb1	1107.6 → 945.5*	50	-255	-10	-60	-55
	1107.6 → 783.5^	50	-255	-10	-66	-45

*Remark: The quantification MRMs and the qualification MRMs are marked with * and ^ respectively.*

4.2.5. Calibrate the LC-MS/MS system using at least 5 calibration standards (Clause 2.9.4.).

4.2.6. Perform LC-MS/MS analysis for method blank(s), sample(s), sample duplicate(s), spike sample(s) and relevant check standard solution(s)

according to the quality control plan as established in the laboratory.

5. Calculation / result interpretation

5.1. Identification requirements:

5.1.1. For LC-MS/MS analysis, identify the target analyte(s) in the sample by comparison of the retention time(s) of the detected peak(s) (RT_{sample}) with that of the average retention time(s) (RT) of the calibration standards. The RT_{sample} shall not differ from that of the average RT of calibration standards by more than 5%.

5.1.2. The relative abundance of MRMs shall meet the tolerance for positive identification of the analyte(s) (with reference to that of the average relative abundance of the calibration standard):

Relative intensity to the base peak	% Allowable deviation
>50%	±20%
>20% to 50%	±25%
>10% to 20%	±30%
≤10%	±50%

5.2. Establish the calibration curve by plotting the peak area against the concentration of analyte in linear calibration mode.

5.3. Calculate the concentration of analyte in the sample, in μg/g, using the following equation:

$$\text{Concentration of analyte } (\mu g/g) = \frac{C \times V \times D}{1000 \times W}$$

where C = Conc. of analyte obtained from calibration curve (in ng/mL);
V = Final volume (mL);
D = Dilution factor; and
W = Sample weight (g).

5.4. If matrix effect is suspected when significant bias is detected in spike recovery, it may be minimized by (1) further dilution of the sample solution or (2) quantification using standard addition approach.

6. Reference

6.1. Chinese Pharmacopoeia Commission. Pharmacopoeia of the People's Republic of China Volume 1, 2020 ed. China Medical Science Press.

6.2. “Quantifying Uncertainty in Analytical Measurement”, Eurachem / CITAC Guide CG4, 3rd Edition, 2012.

6.3. V. J. Barwick and S. L. R. Ellision, “VAM Project 3.2.1 Development and Harmonisation of Measurement Uncertainty Principles Part (d): Protocol for Uncertainty Evaluation from Validation data”, LGC/VAM/1998/088 Version 5.1, January 2000.