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Determination of Salvianolic Acid B in Baifeng Wan by Liquid Chromatograph-Tandem Mass Spectrometer (LC-MS/MS)

GCMTI method publications

Determination of Salvianolic Acid B 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 salvianolic acid B 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. Formic acid, LC-MS grade.

2.5. Salvianolic acid B, CAS. No.: 121521-90-2.

2.6. 0.1% Formic acid solution

Use 1 mL of formic acid and make up to the 1 L with water (Clause 2.3).

2.7. Extraction solvent

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

2.8. Dilution solvent

Acetonitrile : water (2:8 v/v)

2.9. Preparation of standard solutions

2.9.1. Stock standard solution (ca. 1000 μg/mL)

Weigh accurately about 10 mg of salvianolic acid B into a 10-mL volumetric flask, dissolve and make up to the graduated mark with methanol.

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

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

2.9.3. Intermediate standard solution II (ca. 1000 ng/mL) (Freshly prepared)

Prepare intermediate standard solution II by transferring 1 mL of

intermediate standard solution I into a 10-mL volumetric flask and make up to the graduated mark with dilution solvent (Clause 2.8.).

2.9.4. Calibration standard solutions, CS1 – CS5 (Freshly prepared)

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

Calibration standard	**Volume of intermediate standard solution II (mL)**	**Final volume (mL)**	**Conc. of salvianolic acid B (ng/mL)**
CS1	0.25	10	25
CS2	0.50	10	50
CS3	1.00	10	100
CS4	1.50	10	150
CS5	2.00	10	200

Remark: It is suggested to recalibrate the instrument with freshly prepared calibration standard solutions at least once every 12 hours.

2.9.5. Stock initial calibration verification (ICV) standard solutions (ca. 1000 μg/mL)

Prepare stock ICV standard solution, from source different from that of the calibration standard. Weigh accurately about 10 mg of salvianolic acid B into a 10-mL volumetric flask, dissolve and make up to the graduated mark with methanol.

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

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

2.9.7. Intermediate ICV standard solution II (ca. 1000 ng/mL) (Freshly prepared)

Prepare intermediate ICV standard solution II by transferring accurately 1 mL of intermediate ICV standard solution I into a 10-mL volumetric flask and make up to the graduated mark with dilution solvent (Clause 2.8.).

2.9.8. ICV working standard solution (ca. 100 ng/mL) (Freshly prepared)

Prepare ICV working standard solution by transferring 1 mL of intermediate ICV standard solution II into a 10-mL volumetric flask and make up to the graduated mark with dilution solvent (Clause 2.8.).

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

Refer to stock standard solution (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, 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 polypropylene (PP) 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 2.7.) 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 5 mL extraction solvent (Clause 2.7.). Collect all supernatant in the same 25-mL volumetric flask and make up to mark with extraction solvent (Clause 2.7.). Dilute the sample solution by 20-fold with dilution solvent (Clause 2.8.).

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

Remarks:
Analyze the sample solutions within 12 hours after preparation. Otherwise, keep the sample solutions at 4°C or below immediately after preparation.

Further dilute the sample solution with dilution solvent (Clause 2.8.) 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 or equivalent
Column temperature	:	20 ºC
Flow rate	:	0.25 mL/min
Injection volume	:	5 μL
Mobile phase	:	A: 0.1% Formic acid buffer (Clause 2.6.) B: Acetonitrile

Gradient :	Time (min)	A%	B%
	0.0	80	20
	2.0	80	20
	3.0	50	50
	5.0	50	50
	5.5	5	95
	7.5	5	95
	8.0	80	20
	12.0	80	20

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	:	400 ºC
Ion source gas 1 (GS1)	:	30
Ion source gas 2 (GS2)	:	30
Curtain gas (CUR)	:	20
Collision gas (CAD)	:	9
Scan Type	:	MRM

4.2.4. Suggested MRM acquisition conditions:

Analyte	MRM transition	Dwell time msec	DP	EP	CE	CXP
Salvianolic acid B	717.1 → 518.8*	50	-35	-10	-28	-29
	717.1 → 320.8^	50	-35	-10	-48	-47

*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 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 (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.