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Determination of Astragaloside IV in Baifeng Wan by Liquid Chromatograph-Tandem Mass Spectrometer (LC-MS/MS)

GCMTI method publications

Determination of Astragaloside IV 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 astragaloside IV 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. Ammonia solution, 28%.

2.5. Formic acid, LC-MS grade.

2.6. Ammonium formate.

2.7. Astragaloside IV, CAS. No.: 84687-43-4.

2.8. Ammonium formate solution, 5M

Dissolve 63.1 g of ammonium formate in 200 mL of water (Clause 2.3.).

2.9. Ammonium formate buffer solution, 10mM with 0.1% formic acid

Mix 2 mL of 5M ammonium formate solution (Clause 2.8.) with 1 mL of formic acid (Clause 2.5.) and make up to 1 L with water (Clause 2.3.).

2.10. Dilution solution

Methanol : water (8:2 v/v).

2.11. Extraction solution

Prepare extraction solution by mixing 40 mL ammonia solution (Clause 2.4.) with 960 mL dilution solution (Clause 2.10.).

2.12. Preparation of standard solutions

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

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

2.12.2. 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 solution (Clause 2.10.).

2.12.3. Intermediate standard solution II (ca. 200 ng/mL)

Prepare intermediate standard solution II by transferring 0.2 mL of intermediate standard solution I into a 10-mL volumetric flask and make up to the graduated mark with dilution solution (Clause 2.10.).

2.12.4. Calibration standard solutions, CS1 – CS5

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 solution (Clause 2.10.). 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 astragaloside IV (ng/mL)**
CS1	0.1	10	2
CS2	0.2	10	4
CS3	0.4	10	8
CS4	0.8	10	16
CS5	1.0	10	20

2.12.5. Stock initial calibration verification (ICV) standard solution (ca. 1000 μg/mL)

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

2.12.6. 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 solution (Clause 2.10.).

2.12.7. Intermediate ICV standard solution II (ca. 200 ng/mL)

Prepare intermediate ICV standard solution II by transferring accurately 0.2 mL of intermediate ICV standard solution I into a 10-mL volumetric flask and make up to the graduated mark with dilution solution (Clause 2.10.).

2.12.8. ICV working standard solution (ca. 10 ng/mL)

Prepare ICV working standard solution by transferring 0.5 mL of intermediate ICV standard solution II into a 10-mL volumetric flask and make up to the graduated mark with dilution solution (Clause 2.10.).

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

Refer to stock standard solution (Clause 2.12.1.).

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.

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

3.5. Flat-bottom flasks, 50-mL.

3.6. Glass stoppers.

3.7. Glass pipette, 25-mL.

3.8. Heating mantle.

3.9. Reflux condenser.

3.10. Water chiller, capable to maintain a flow of water below 15 ºC.

3.11. PTFE membrane filters, 0.2 μm.

3.12. LC glass vials.

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

3.14. 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 50-mL flat-bottom flask.

4.1.3. Transfer accurately 25 mL of extraction solution (Clause 2.11.) with glass pipette and add a stir-bar to the flask. Stopper the flask to avoid solvent loss. Measure and record the weight of the whole set-up.

4.1.4. Connect the condenser to the water chiller and initiate the water flow. Maintain the water temperature below 15 ºC.

4.1.5. Connect the flask to the condenser and extract the sample mixture by reflux for 60 ± 5 min.

4.1.6. After cooling to ambient temperature, disassemble the flask from condenser and stopper the flask.

4.1.7. Restore the weight of the whole set-up (Clause 4.1.6.) to the original value (as measured in Clause 4.1.3.), to the nearest 0.01 g, by adding the extraction solution (Clause 2.11.).

4.1.8. Filter the sample solution with 0.2 μm PTFE membrane filter and dilute the sample solution by 20-fold using dilution solution (Clause 2.10.).

4.1.9. The solution is ready for LC-MS/MS analysis.

Remark: Further dilute the sample solution with dilution solvent (Clause 2.10.) 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	:	30 ºC
Flow rate	:	0.25 mL/min
Injection volume	:	10 µL
Mobile phase	:	A: NH_4HCO_2 buffer (Clause 2.9.) B: Acetonitrile

Gradient :

Time (min)	A%	B%
0.0	80	20
2.0	80	20
17.0	50	50
18.0	5	95
19.0	5	95
19.5	80	20
21.0	80	20

4.2.3. Suggested MS/MS conditions:

MS/MS system	:	AB SCIEX 6500+ system
Ionization mode	:	Electrospray ionization (ESI); Positive mode
Ionspray voltage	:	5500V
Source temperature	:	300 ºC
Ion source gas 1 (GS1)	:	30
Ion source gas 2 (GS2)	:	30
Curtain gas (CUR)	:	20
Collision gas (CAD)	:	Medium
Scan Type	:	MRM

4.2.4. Suggested MRM acquisition conditions:

Analyte	MRM transition	Dwell time msec	DP	EP	CE	CXP
Astragaloside IV	785.4 → 143.1*	100	41	10	17	18
	785.4 → 455.3^	100	41	10	19	18

*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 2.12.4.).

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.