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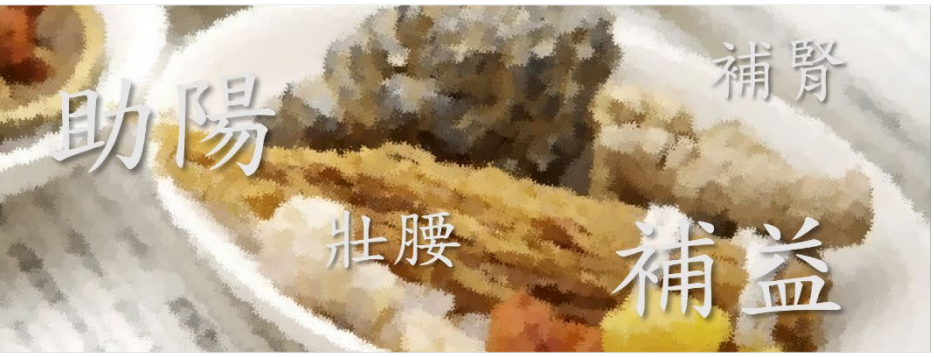
**Determination of Ginsenosides (Re, Rg1, Rf & Rb1)
in Proprietary Chinese Medicines containing Psoraleae and Ginseng
by Liquid Chromatograph-Tandem Mass Spectrometer
(LC-MS/MS)**

GCMTI Method Publications



Determination of Ginsenosides (Re, Rg1, Rf & Rb1) in Proprietary Chinese Medicines containing Psoraleae and Ginseng 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. Proprietary Chinese medicines (pCm) containing psoraleae (補骨脂) and ginseng (人參) for nourishing and Yang invigorating are commonly found in Hong Kong. Nevertheless, analysis of the chemical markers of psoraleae and ginseng is a great challenge since it is susceptible to interference from matrix and other chemical components.

1.2. This method describes the procedures for qualitative and quantitative determination of ginsenosides (Re, Rg1, Rf & Rb1) in pCm containing psoraleae and ginseng 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. Acetonitrile, LC-MS grade.

2.2. Methanol, LC-MS grade.

2.3. Milli-Q water.

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

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

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

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

[1] This method intends to provide a reliable analytical method that can be used as a 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 testing their pCm products when adopting this method.

2.8. Extraction solvent

Methanol : water (7:3 v/v).

2.9. Reagents for sample clean-up (Solid Phase Extraction (SPE) clean-up)

2.9.1. 95% (v/v) Acetonitrile

Use 95 mL of acetonitrile (Clause 2.1) and make up to 100 mL with water.

2.9.2. 70% (v/v) Acetonitrile

Use 70 mL of acetonitrile (Clause 2.1) and make up to 100 mL with water.

2.10. Preparation of standard solutions

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

Weigh accurately about 10 mg of ginsenosides (Clause 2.4 - 2.7) into separate 10-mL volumetric flasks, dissolve and make up to the mark with methanol (Clause 2.2), respectively.

2.10.2. Mixed intermediate standard solution I (ca. 100 µg/mL)

Transfer 1 mL of each individual stock standard solutions into a 10-mL volumetric flask and make up to the mark with methanol (Clause 2.2).

2.10.3. Mixed intermediate standard solution II (ca. 10 µg/mL, freshly prepared)

Transfer 1 mL of mixed intermediate standard solution I into a 10-mL volumetric flask and make up to the mark with methanol (Clause 2.2).

2.10.4. Mixed intermediate standard solution III (ca. 100 ng/mL, freshly prepared)

Transfer 0.1 mL of mixed intermediate standard solution II into a 10-mL volumetric flask and make up to the mark with extraction solvent (Clause 2.8).

2.10.5. Calibration standard solutions, CS1 – CS5

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

Calibration standard	Volume of mixed intermediate standard solution III (mL)	Final Volume (mL)	Conc. of Ginsenosides Re/Rb1/Rg1/Rf (ng/mL)
CS1	0.20	10	2
CS2	0.50	10	5
CS3	1.00	10	10
CS4	1.50	10	15
CS5	2.00	10	20
CS6	2.50	10	25

Remark:
The calibration curves were established with the ranges as suggested below:

Analyte	Calibration standards used	Concentration range (ng/mL)
Ginsenoside Re	CS1 – CS6	2 - 25
Ginsenoside Rb1	CS2 – CS6	5 - 25
Ginsenoside Rf	CS1 – CS6	2 - 25
Ginsenoside Rg1	CS1 – CS6	2 - 25

2.10.6. Individual stock initial calibration verification (ICV) standard solutions (ca. 1000 μg/mL)

Prepare individual stock ICV standard solutions, from source different from that of the calibration standard. Weigh accurately about 10 mg of ginsenosides (Clause 2.4 - 2.7) into separate 10-mL volumetric flasks, dissolve and make up to the mark with methanol (Clause 2.2), respectively.

2.10.7. Mixed intermediate ICV standard solution I (ca. 100 μg/mL)

Transfer 1 mL of each individual stock ICV standard solutions into a 10-mL volumetric flask and make up to the mark with methanol (Clause 2.2).

2.10.8. Mixed intermediate ICV standard solution II (ca. 10 μg/mL, freshly prepared)

Transfer 1 mL of mixed intermediate ICV standard solution I into a 10-mL volumetric flask and make up to the mark with methanol (Clause 2.2).

2.10.9. Mixed intermediate ICV standard solution III (ca. 100 ng/mL, freshly prepared)

Transfer 0.1 mL of mixed intermediate ICV standard solution II into a 10-mL volumetric flask and make up to the mark with extraction solvent (Clause 2.8).

2.10.10. ICV working standard solution (ca. 15 ng/mL, freshly prepared)

Transfer 1.5 mL of mixed intermediate ICV standard solution III into a 10-mL volumetric flask and make up to the mark with extraction solvent (Clause 2.8).

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

Refer to individual stock standard solutions (Clause 2.10.1).

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 water and then with acetone twice.

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 glass vials.

3.11. SPE column: Aminopropyl (NH2) Cartridge, 55-105µm, 6-mL SPE column, containing 500 mg sorbent, Waters or equivalent.

3.12. LC column: InertSustain C18, 5 µm, 2.1 mm × 250 mm, GL Sciences or equivalent.

3.13. Liquid chromatograph-tandem mass spectrometer (LC-MS/MS) system.

4. Procedures

4.1. Sample extraction

4.1.1. Grind and homogenise solid samples using grinder or blender.

4.1.2. Weigh accurately about 0.25 g of 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 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 of extraction solvent (Clause 2.8). Collect all supernatant in the same 25-mL volumetric flask and make up to the mark with extraction solvent (Clause2.8).

4.2. Solid phase extraction (SPE) clean-up

4.2.1. Condition the SPE column (Clause 3.11) with 5 mL of acetonitrile (Clause 2.1) followed by 5 mL of 95% (v/v) acetonitrile (Clause 2.9.1).

4.2.2. Dilute 0.5 mL of sample solution (Clause 4.1.6) with 9.5 mL of acetonitrile (Clause 2.1).

4.2.3. Load the diluted sample solution (Clause 4.2.2) onto the SPE column.

4.2.4. Rinse the flask with 5 mL of 95% (v/v) acetonitrile (Clause 2.9.1) and load the rinsing solution onto the SPE column for washing.

4.2.5. Elute the analytes with 8 mL of 70% (v/v) acetonitrile (Clause 2.9.2) and collect the eluate in a 10-mL volumetric flask. Make up to the graduation mark with 70% (v/v) acetonitrile (Clause 2.9.2).

4.2.6. Dilute the sample solution (Clause 4.2.5) by 2.5-fold with extraction solvent (Clause 2.8) (overall 50-fold dilution from sample solution (Clause 4.1.6)).

4.2.7. Filter the diluted sample solution with 0.2 μm PTFE membrane filter into a LC glass vial. The solution is ready for LC-MS/MS analysis.
Remark:
Dilute the sample solution with extraction solvent (Clause 2.8) if the concentration of analyte(s) is not within the calibration range.

4.3. LC-MS/MS analysis

4.3.1. 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.

4.3.2. Suggested LC conditions:

LC system	:	Thermo Scientific UltiMate 3000 UHPLC or equivalent performance		
Column	:	GL Sciences InertSustain C18, 5 μm, 2.1 mm × 250 mm or equivalent		
Column temperature	:	40 ºC		
Flow rate	:	0.3 mL/min		
Injection volume	:	5 μL		
Mobile phase	:	A: Water B: Methanol		
Gradient	:	Time (min)	A%	B%
		0.0	60	40
		2.0	60	40
		16.0	30	70
		23.0	30	70
		23.1	5	95
		25.0	5	95
		25.1	60	40
		28.0	60	40

4.3.3. Suggested MS/MS conditions:

MS/MS system	:	SCIEX 6500+ system
Ionization mode	:	Electrospray ionization (ESI)
Polarity	:	Negative mode
Ionspray voltage	:	-4500V
Source temperature	:	350ºC
Ion source gas 1 (GS1)	:	40
Ion source gas 2 (GS2)	:	40
Curtain gas (CUR)	:	25
Collision gas (CAD)	:	Medium
Scan type	:	MRM

4.3.4. Suggested MRM acquisition parameters:

Analyte	MRM transition	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.*

4.3.5. Calibrate the LC-MS/MS system using at least 5 calibration standards (Clause 2.10.5).

4.3.6. 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 requirement

5.1.1. Identify the target analyte in the sample by comparison of the retention time of the detected peak (RT_{sample}) with that of the average retention time (RT) of the calibration standards. The RT_{sample} shall not differ from that of the average RT of calibration standards by more than 5% for positive identification.

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 the calibration standards in linear calibration mode.

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

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

where C = Conc. of analyte obtained from calibration curve (in ng/mL)
V = Final volume (mL)
D = Dilution factor
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.