GCMTI RD-1:2024

Determination of Ginsenoside Re in Proprietary Chinese Medicines containing Psoraleae and Ginseng by High Performance Liquid Chromatograph-Diode Array Detector (HPLC-DAD)

GCMTI Method Publications

Determination of Ginsenoside Re in Proprietary Chinese Medicines containing Psoraleae and Ginseng by High Performance Liquid Chromatograph-Diode Array Detector (HPLC-DAD)[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 ginsenoside Re in pCm containing psoraleae and ginseng by high performance liquid chromatograph-diode array detector (HPLC-DAD).

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. n-Butanol.

2.4. Milli-Q water.

2.5. Ammonium hydroxide, 28% (w/v).

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

2.7. Extraction solvent

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

[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. Reagents for sample clean-up (n-butanol extraction)

2.8.1. Mix thoroughly 500 mL of n-butanol (Clause 2.3) with 500 mL of water (Clause 2.4) and allow the mixture to stand for layer separation.

2.8.1.1. Upper layer: n-butanol (*saturated with water*)

2.8.1.2. Lower layer: water (*saturated with n-butanol*)

2.8.2. 40% (v/v) Ammonia solution

Mix 200 mL of ammonium hydroxide (Clause 2.5) with 300 mL of water (Clause 2.4). Add 500 mL of n-butanol (*saturated with water*) (Clause 2.8.1.1) to the solution and mix thoroughly. Allow the mixture to stand for layer separation. The lower aqueous layer is then ready to use.

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

2.9.1. 10% (v/v) Methanol

Use 10 mL of methanol (Clause 2.2) and make up to 100 mL with water.

2.9.2. 30% (v/v) Methanol

Use 30 mL of methanol (Clause 2.2) and make up to 100 mL with water.

2.9.3. 20% (v/v) Acetonitrile

Use 20 mL of acetonitrile (Clause 2.1) and make up to 100 mL with water.

2.10. Preparation of standard solutions

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

Weigh accurately about 10 mg of ginsenoside Re (Clause 2.6) into a 10-mL volumetric flask, dissolve and make up to the mark with methanol (Clause 2.2).

2.10.2. Intermediate standard solution (ca. 100 µg/mL)

Transfer 1 mL of stock standard solution into a 10-mL volumetric flask and make up to the mark with extraction solvent (Clause 2.7).

2.10.3. Calibration standard solutions, CS1 – CS5

A series of calibration standard solutions are prepared by transferring an appropriate amount of intermediate standard solution into 10-mL volumetric flasks and make up to the mark with extraction solvent (Clause 2.7). Suggested volumes of standard solution used for the preparation are listed in the table below.

Calibration standard	Volume of intermediate standard solution (mL)	Final Volume (mL)	Conc. of Ginsenoside Re (µg/mL)
CS1	1.00	10	10
CS2	2.00	10	20
CS3	3.00	10	30
CS4	4.00	10	40
CS5	5.00	10	50

2.10.4. 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 ginsenoside Re into a 10-mL volumetric flask, dissolve and make up to the mark with methanol (Clause 2.2).

2.10.5. Intermediate ICV standard solution (ca. 100 µg/mL)

Transfer accurately 1 mL of stock ICV standard solution into a 10-mL volumetric flask and make up to the mark with extraction solvent (Clause 2.7).

2.10.6. ICV working standard solution (ca. 30 µg/mL)

Transfer 3 mL of intermediate ICV standard solution into a 10-mL volumetric flask and make up to the graduation mark with extraction solvent (Clause 2.7).

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

Refer to stock standard solution (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.

3.4. Volumetric pipettes, 1-mL.

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

3.6. Round-bottom flasks, 50-mL.

3.7. Flat-bottom flasks, 100-mL.

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

3.9. Centrifuge tubes, 15-mL and 50-mL.

3.10. Vortex mixer.

3.11. Ultrasonic bath.

3.12. Rotary evaporator

3.13. PTFE membrane filters, 0.45 μm.

3.14. LC glass vials.

3.15. SPE column: Polymeric reversed phase, 33μm, 6-mL SPE column, containing 100 mg sorbent, Phenomenex Strata-X or equivalent.

3.16. LC column: Inertsil NH_2 5 μm, 4.6 mm × 250 mm, GL Sciences or equivalent.

3.17. High performance liquid chromatograph-diode array detector (HPLC-DAD) 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 0.5 g of 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 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 100-mL flat-bottom flask.

4.1.6. Repeat Clauses 4.1.3 to 4.1.5 twice with 5 mL of extraction solvent (Clause 2.7). Collect all supernatants in the same flat-bottom flask.

4.2. Sample clean-up

4.2.1. n-Butanol extraction

4.2.1.1. Evaporate the collected supernatants (Clause 4.1.6) using rotary evaporation at 50°C to near dryness. Reconstitute the residue with 1 mL of extraction solvent (Clause 2.7).

4.2.1.2. Transfer the sample solution (Clause 4.2.1.1) to a 15-mL centrifuge tube. Rinse the flask with 5 mL of n-butanol (*saturated with water*) (Clause 2.8.1.1) and transfer the rinsing solution to the same 15-mL centrifuge tube. Add 5 mL of water (*saturated with n-butanol*) (Clause 2.8.1.2) to the 15-mL centrifuge tube.

4.2.1.3. Vortex the mixture for 40 seconds. Centrifuge the mixture at 4000 rpm for 2 minutes for layer separation. Carefully collect the upper organic layer (n-butanol extract) in a 50-mL centrifuge tube.
Remark:
Re-centrifuge with longer duration or higher revolution speed if the upper layer remains turbid after centrifugation.

4.2.1.4. Repeat Clause 4.2.1.3 twice with 5 mL of n-butanol (*saturated with water*) (Clause 2.8.1.1). Collect the upper organic layers (n-butanol extract) (*ca*. 15 mL in total) in the same 50-mL centrifuge tube.

4.2.1.5. Add 5 mL of 40% (v/v) ammonia solution (Clause 2.8.2) to the n-butanol extract (Clause 4.2.1.4). Vortex the mixture for 40 seconds and centrifuge the mixture at 4000 rpm for 2 minutes for layer separation. Discard the lower aqueous layer.
Remark:
Re-centrifuge with longer duration or higher revolution speed if the upper layer remains turbid after centrifugation.

4.2.1.6. Repeat Clause 4.2.1.5. Carefully transfer the n-butanol extract to a 100-mL flat-bottom flask and rinse the centrifuge tube with 5 mL of extraction solution (Clause 2.7) and transfer the rinsing solution to the same flat-bottom flask.

4.2.1.7. Evaporate the solvent using rotary evaporation at 55°C to near dryness.
Remark:
A small portion (ca. 1-2 mL) of extraction solution (Clause 2.7) could be added to the flask to facilitate the preconcentration process if complete

evaporation of n-butanol cannot be achieved.

4.2.2. Solid phase extraction (SPE) clean-up

4.2.2.1. Condition the SPE column (Clause 3.15) with 5 mL of methanol (Clause 2.2) followed by 5 mL of water (Clause 2.4).

4.2.2.2. Reconstitute the residue (Clause 4.2.1.7) with 1 mL of extraction solvent (Clause 2.7) and dilute the solution with 9 mL of water (Clause 2.4).

4.2.2.3. Load the diluted sample solution (Clause 4.2.2.2) onto the SPE column.

4.2.2.4. Rinse the flask with 5 mL of 10% (v/v) methanol (Clause 2.9.1) and load the rinsing solution onto the SPE column.

4.2.2.5. Wash the SPE column with 10 mL of 30% (v/v) methanol (Clause 2.9.2).

4.2.2.6. Elute the analytes with 10 mL of 20% (v/v) acetonitrile (Clause 2.9.3) and collect the eluent in a 50-mL round-bottom flask.

4.2.2.7. Evaporate the eluent using rotary evaporation at 50°C to near dryness. Reconstitute the residue to 1 mL with extraction solvent (Clause 2.7) using volumetric pipette (Clause 3.4).

4.2.3. Filter the sample solution with 0.45 μm PTFE membrane filter into a LC glass vial. The solution is ready for HPLC-DAD analysis.
Remark:
Dilute the sample solution with extraction solvent (Clause 2.7) if the concentration of analyte(s) is not within the calibration range.

4.3. HPLC-DAD analysis

4.3.1. Operate the HPLC-DAD 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 HPLC-DAD conditions:

HPLC system	:	Waters Alliance e2695 HPLC system or equivalent performance
Column	:	GL Sciences Inertsil NH_2, 5 μm, 4.6 mm × 250 mm or equivalent

Column temperature	:	35 ºC		
Flow rate	:	1 mL/min		
Injection volume	:	10 µL		
Mobile phase	:	A: Water		
		B: Acetonitrile		
Gradient	:	Time (min)	A%	B%
		0.0	5	95
		5.0	5	95
		15.0	12	88
		25.0	13	87
		65.0	13	87
		65.5	25	75
		70.0	25	75
		71.0	5	95
		80.0	5	95
DAD wavelength	:	203 nm		

4.3.3. Calibrate the HPLC-DAD system using at least 5 calibration standards (Clause 2.8.3).

4.3.4. Perform HPLC-DAD 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

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.

5.2. 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:

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

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

6. Reference

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

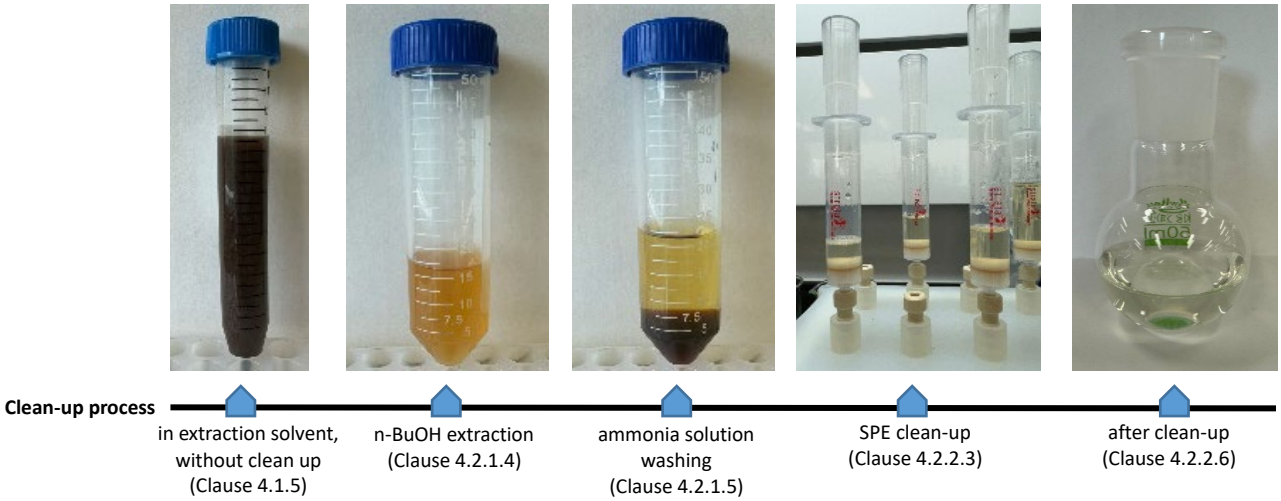
6.2. "Quantifying Uncertainty in Analytical Measurement", Eurachem/ CITAC Guide CG4, 3rd Edition, 2012.

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.

Annex A
(informative)

Further information on the clean-up process

A1. The following diagram illustrates the sample solutions obtained at different stages during the clean-up process.



A2. The stacked chromatogram of sample solutions obtained at different stages during the clean-up process.