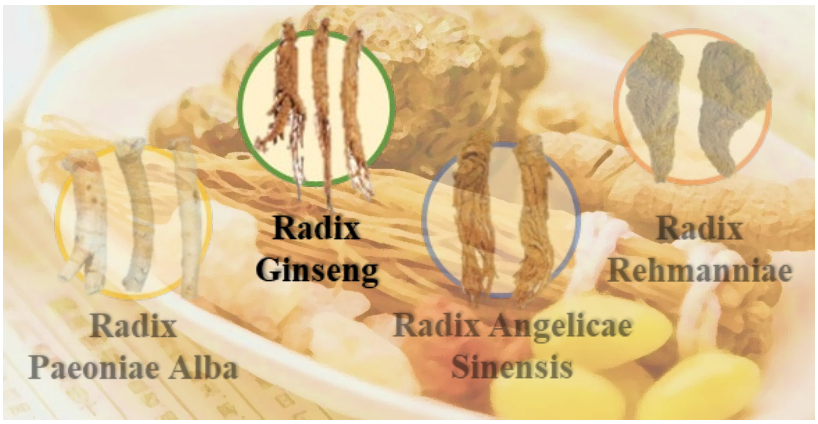
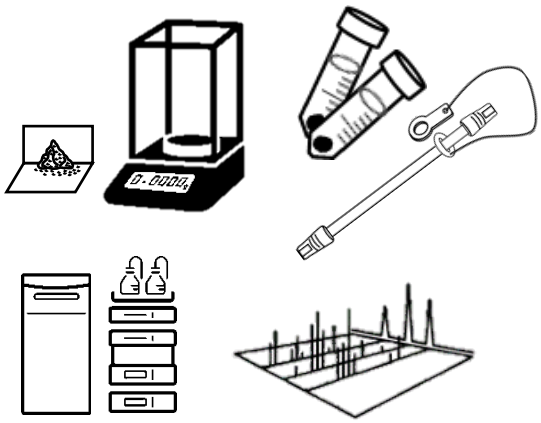
GCMTI RD-2: 2025

Determination of Ginsenosides in Compound Chinese Medicine Granules by LC-MS/MS

GCMTI Method





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Determination of Ginsenosides in Compound Chinese Medicine Granules by LC-MS/MS[1]

Safety precautions: *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 vapor.*

1. Introduction

1.1. Compound Chinese medicine granules are one of the proprietary Chinese medicines (pCm) sold in a variety of formulations in Hong Kong. Most of them are formulated in accordance with ancient Chinese medicine bibliographies. In general, they are produced by pulverising, decorating, filtering and concentrating a series of Chinese herbal medicines into a blend of water-soluble powders.

1.2. This method utilising the technique of liquid chromatograph (LC) coupled with a tandem mass spectrometer (MS/MS) is developed to analyse 10 formulations with Radix Ginseng (人參) as the principal or assistant drug, or as a major component. The 10 formulations are listed below.

Item	Formulation	Ancient Chinese Medicine Bibliography
1.	聖愈湯	醫宗金鑒
2.	舉元煎	景嶽全書
3.	理中湯	傷寒論
4.	人參養榮湯	三因極一病證方論
5.	大建中湯	金匱要略
6.	六君子湯	醫學正傳
7.	附子理中湯	三因極一病證方論
8.	人參敗毒散	太平惠民和劑局方
9.	麥門冬湯	金匱要略
10.	溫經湯	金匱要略

1.3. This method has been validated for qualitative and/or quantitative determination of four analytes, *viz.* ginsenoside Re (Re), ginsenoside Rg1 (Rg1), ginsenoside Rf (Rf) and ginsenoside Rb1 (Rb1) in compound Chinese medicine granules using LC-MS/MS. The validated working range is 1–50 μg/L in solution, which corresponds to 50–2500 μg/g in the original sample.

2. Reagents

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

2.1. Water, Milli Q.

2.2. Methanol (MeOH), LC-MS grade.

2.3. Ethanol (EtOH), absolute grade.

2.4. Acetonitrile (ACN), LC-MS grade.

2.5. 50 % MeOH Solution

Mix ~500 mL of MeOH and ~500 mL of water in a 1-L measuring cylinder.

2.6. 95 % ACN Solution

Mix ~950 mL of ACN and ~50 mL of water in a 1-L measuring cylinder.

2.7. 70 % ACN Solution

Mix ~700 mL of ACN and ~300 mL of water in a 1-L measuring cylinder.

2.8. Re, CAS. No.: 52286-59-6.

2.9. Rg1, CAS. No.: 22427-39-0.

2.10. Rf, CAS. No.: 52286-58-5.

2.11. Rb1, CAS. No.: 41753-43-9.

2.12. Standard Solutions

2.12.1. Stock Standard Solutions (~2000 µg/mL)

Weigh accurately ~20 mg of Re, Rg1, Rf and Rb1 into separate 10-mL volumetric flasks. Dissolve and make up to the graduation mark with MeOH.

2.12.2. Mixed Intermediate Standard Solution 1 (INT1, ~100 µg/mL)

Pipette 0.5 mL of the stock standard solutions into a 10-mL volumetric flask. Make up to the graduation mark with MeOH.

2.12.3. Mixed Intermediate Standard Solution 2 (INT2, ~1 µg/mL)

Pipette 0.1 mL of INT1 into a 10-mL volumetric flask. Make up to the graduation mark with MeOH.

2.12.4. Mixed Intermediate Standard Solution 3 (INT3, ~100 µg/L)

Pipette 2.5 mL of INT2 into a 25-mL volumetric flask. Make up to the graduation mark with 50 % MeOH solution.

2.13. Calibration Standard Solutions

Freshly prepare at least six calibration standard solutions from INT3. Make up with 50 % MeOH solution in 10-mL volumetric flasks. Suggested concentrations of calibration standard solutions used in the analysis are listed below.

Calibration Standard Solution	**Vol. of INT3 (mL)**	**Final Vol. (mL)**	**Final Conc. of Re, Rg1, Rf and Rb1 (µg/L)**
CS1	0.1	10.0	1
CS2	0.5	10.0	5
CS3	1.0	10.0	10
CS4	2.0	10.0	20
CS5	3.0	10.0	30
CS6	5.0	10.0	50

Remark: *Other concentrations may be used as appropriate. Other sequences of dilution may be employed to achieve the required concentrations. Record the details in the data sheets.*

2.14. Initial Calibration Verification (ICV) Standard Solutions

2.14.1. Stock ICV Standard Solutions (~2000 µg/mL)

Prepare stock ICV standard solutions according to Clause 2.12.1 using an independent source of reference materials (another brand or another batch of standard with same brand as appropriate).

2.14.2. Mixed Intermediate ICV Standard Solution I (ICV I, ~100 µg/mL)

Pipette 0.5 mL of the stock ICV standard solutions into a 10-mL volumetric flask. Make up to the graduation mark with MeOH.

2.14.3. Mixed Intermediate ICV Standard Solution II (ICV II, ~1 µg/mL)

Pipette 0.1 mL of ICV I into a 10-mL volumetric flask. Make up to the graduation mark with MeOH.

2.14.4. Mixed Intermediate ICV Standard Solution III (ICV III, ~100 µg/L)

Pipette 1.0 mL of ICV II into a 10-mL volumetric flask. Make up to the graduation mark with 50 % MeOH solution.

2.14.5. ICV Working Standard Solution (~10 or 20 µg/L)

Pipette 1.0 or 2.0 mL of ICV III into a 10-mL volumetric flask. Make up to the graduation mark with 50 % MeOH solution.

2.15. Calibration Check Standard

Calibration check standard shall be the CS3 or CS4 of the calibration standard solutions, *viz.* ~10 or 20 µg/L.

2.16. Method Blank

The method blank shall be carried out through the complete sample preparation procedures as per Clauses 4.1.3 to 4.3 and shall contain the same amount of reagents as the sample solutions.

3. Apparatus

Note: *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. Volumetric flasks, 10- and 25-mL.

3.3. Measuring cylinder, 1-L.

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

3.5. Autopipettes, 300-, 1000-µL and 10-mL.

3.6. Centrifuge tubes, 15-mL with polypropylene screw cap.

3.7. Ultrasonic bath.

3.8. Vortex mixer.

3.9. High speed centrifuge with rotation speed settable at least 4000 rpm.

3.10. Freezer, capable of operating at ~–20 °C.

3.11. Glass test tubes.

3.12. Centrifugal evaporator or equivalent performance.

3.13. Aminopropyl (NH_2) SPE cartridges, 55- to 105-µm, 6-mL SPE column, containing 500 mg sorbent or equivalent.

3.14. PTFE membrane filters, 0.20-µm or equivalent.

3.15. LC glass vials.

3.16. UPLC column, InertSustain C_{18} Column (2.1 × 250 mm, 5 µm) or equivalent.

3.17. LC-MS/MS, Dionex Ultimate 3000 UPLC with QTRAP 6500+ System or System with equivalent performance.

4. Procedures

4.1. Sample Preparation

4.1.1. Grind and homogenise solid samples using grinder or blender before analysis, if necessary.

4.1.2. Accurately weigh ~0.5 g of the sample into a 15-mL screw cap centrifuge tube.

4.1.3. Add 10 mL of 50 % MeOH solution into the centrifuge tube. Vortex the sample mixture in the centrifuge tube for ~1 min.

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

4.1.5. Centrifuge the sample mixture at ~4000 rpm for ~10 min to settle the residue. Transfer the supernatant solution to a 25-mL volumetric flask.

4.1.6. Repeat Clauses 4.1.3 to 4.1.5 twice with 4 mL of 50 % MeOH solution.

4.1.7. Collect all supernatant in the same 25-mL volumetric flask and make up to the mark with 50 % MeOH solution.

4.1.8. Transfer 2 mL of the sample solution to a new 15-mL centrifuge tube.

4.1.9. Pipette 6 mL of EtOH into the centrifuge tube and vortex for ~30 s.

4.1.10. Store the sample solution (Clause 4.1.9) in the freezer at ~–20 °C for ~10 min.

4.1.11. Centrifuge the sample solution at ~4000 rpm for ~10 min to settle the dispersing solids or top-floating layer.

4.1.12. Pipette 4 mL of supernatant to a glass test tube.

4.1.13. Evaporate the supernatant to dryness at ~80 °C by centrifugal evaporator, and then reconstitute with 1 mL of 50 % MeOH solution.

4.1.14. Pipette 0.5 mL of EtOH and then 8.5 mL of ACN into the glass test tube.

4.1.15. Vortex the sample solution for ~30 s.

4.2. Sample Cleanup

4.2.1. Condition a NH_2 SPE cartridge with ~5 mL of ACN. Equilibrate with 5 mL of **95 % ACN solution**. The eluant is discarded.

4.2.2. Totally transfer the sample solution as per Clause 4.1.15 to the NH_2 SPE cartridge. The eluant is discarded.

4.2.3. Rinse the glass test tube with 5 mL of **95 % ACN solution** and then totally transfer to the NH_2 SPE cartridge. The eluant is discarded.

4.2.4. Further add 5 mL of **70 % ACN solution** to the NH_2 SPE cartridge and collect the eluant in a new glass test tube.

4.2.5. Evaporate the eluant to dryness at ~80 °C by centrifugal evaporator, and then reconstitute with 1 mL of 50 % MeOH solution.

4.2.6. Filter the sample solution with 0.20-μm PTFE filter disc and collect the filtrate into a LC glass vial.

4.2.7. Further dilute the sample solution 1000 times with 50 % MeOH solution.

Remark: *Further dilute the sample solution with 50 % MeOH solution if the concentration of analyte(s) is not within the calibration range.*

4.3. Analysis of LC-MS/MS

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

4.3.2. Suggested LC conditions:

LC System	**:**	Dionex UltiMate 3000 HPLC System or equivalent performance
Column	**:**	InertSustain C_{18} Column (2.1 × 250 mm, 5 µm) or equivalent
Mobile Phase A	**:**	Water
Mobile Phase B	**:**	MeOH
Elution Program	**:**	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
Flow Rate	**:**	0.3 mL/min
Injection Volume	**:**	5 µL
Reported Retention Time	**:**	~12.5 min for Re; ~12.8 min for Rg1; ~17.3 min for Rf; and ~20.6 min for Rb1
Column Temperature	**:**	40 °C

4.3.3. Suggested MS/MS conditions:

MS/MS System	**:**	QTRAP 6500+ System or equivalent performance
Ionisation Mode	**:**	Electrospray ionisation
Polarity	**:**	Negative
Ionspray Voltage	**:**	–4500
Source Temperature	**:**	350 °C
Ion Source Gas 1 (GS1)	**:**	40
Ion Source Gas 2 (GS2)	**:**	40
Curtain Gas (CUR)	**:**	30
Collision Gas (CAD)	**:**	Medium
Scan Type	**:**	MRM

4.3.4. Suggested MRM acquisition parameters:

Analyte	MRM Transition	Dwell Time (msec)	DP	EP	CE	CXP
Re	945.6→637.4*	100	–240	–10	–54	–39
	945.6→475.4^	100	–240	–10	–70	–25
Rg1	799.6→637.4*	100	–205	–10	–34	–27
	799.6→475.4^	100	–205	–10	–50	–27
Rf	799.6→475.4*	100	–225	–10	–54	–29
	799.6→637.4^	100	–225	–10	–44	–39
Rb1	1107.6→945.5*	100	–255	–10	–60	–55
	1107.6→783.5^	100	–255	–10	–66	–45

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

5. Calculation/Result Interpretation

5.1. Identification Requirement

5.1.1. Identify the analyte(s) in the sample by comparison of the retention time of the detected peak (RT_{sample}) with that of the average retention time (RT) from calibration standards. The RT_{sample} shall not differ from that of the average RRT from 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 curves by plotting the peak area against the concentration for each analyte in linear calibration mode. Obtain the slope, y-intercept and the coefficients of determination (r^2) from the calibration curve.

5.3. Calculate the concentration of analyte in sample (μg/g) as follows:

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

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

6. Reference

6.1. Chinese Pharmacopoeia Commission. (2020). *Pharmacopoeia of the People's Republic of China.* China Medical Science Press.

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

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