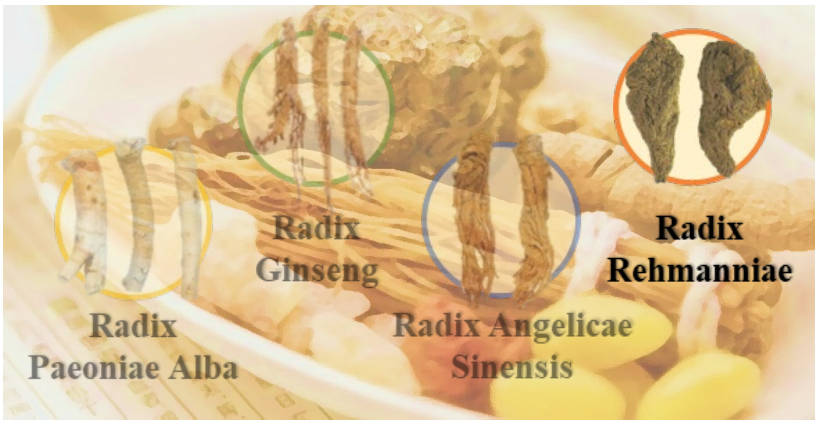
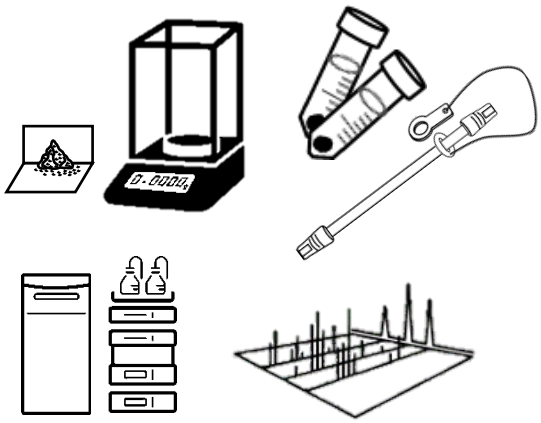
GCMTI RD-3: 2025

Determination of Rehmannioside D in Compound Chinese Medicine Granules by HPLC/UPLC-DAD

GCMTI Method





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Determination of Rehmannioside D in Compound Chinese Medicine Granules by HPLC/UPLC-DAD[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, composing two techniques *viz.* high-performance liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC) coupled with a diode array detector (DAD), is developed to analyse 11 formulations with Radix Rehmanniae (地黃) as the principal or assistant drug, or as a major component. The 11 formulations and the specific technique of HPLC-DAD or UPLC-DAD utilised are listed below.

Item	Formulation	Ancient Chinese Medicine Bibliography	Technique
1.	聖愈湯	醫宗金鑒	HPLC-DAD
2.	養陰清肺湯	重樓玉鑰	HPLC-DAD
3.	清胃散	脾胃論	HPLC-DAD
4.	小薊飲子	濟生方	HPLC-DAD
5.	當歸六黃湯	蘭室秘藏	HPLC-DAD
6.	左歸丸	景嶽全書	UPLC-DAD
7.	八味地黃丸	博青主女科·產後編	UPLC-DAD
8.	玉泉散	血證論卷八	HPLC-DAD
9.	大補陰丸	丹溪心法	HPLC-DAD
10.	芎歸膠艾湯	金匱要略	HPLC-DAD
11.	消風散	外科正宗	HPLC-DAD

1.3. This method has been validated for qualitative and/or quantitative determination of rehmannioside D in the 11 formulations using HPLC-DAD or UPLC-DAD. The validated working range for HPLC-DAD (Curve 1) is 2–80 μg/mL in solution, which corresponds to 50–2000 μg/g in the original sample. The validated working range for UPLC-DAD (Curve 2) is 2–50 μg/mL in solution, which corresponds to 50–1250 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. Acetonitrile (ACN), LC-MS grade.

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

2.4. Ethanol (EtOH), Absolute grade.

2.5. Chloroform, AR grade.

2.6. Butan-1-ol, AR grade.

2.7. 1 % ACN Solution

Mix ~10 mL of ACN and ~990 mL of water in a 1-L measuring cylinder.

2.8. 50 % MeOH Solution

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

2.9. Mixture of Chloroform and Butan-1-ol (4:1, v/v)

Mix ~80 mL of chloroform and ~20 mL of butan-1-ol in a 100-mL measuring cylinder.

2.10. Rehmannioside D, CAS. No.: 81720-08-3.

2.11. Standard Solutions

2.11.1. Stock Standard Solution (STO, ~5000 µg/mL)

Weigh accurately ~50 mg of rehmannioside D into a 10-mL volumetric flask. Dissolve and make up to the graduation mark with MeOH.

2.11.2. Intermediate Standard Solution (INT, ~100 µg/mL)

Pipette 0.5 mL of the stock standard solution into a 25-mL volumetric flask. Make up to the graduation mark with 50 % MeOH solution.

2.12. Calibration Standard Solutions for Curve 1

Freshly prepare at least six calibration standard solutions from INT or STO. Make up with 1 % ACN solution in 10-mL volumetric flasks. Suggested

concentrations of calibration standard solutions used in the analysis are listed below.

Calibration Standard Solution	Vol. of INT (mL)	Vol. of STO (mL)	Final Vol. (mL)	Final Conc. (μg/mL)
CS1	0.2	---	10.0	2
CS2	0.5	---	10.0	5
CS3	1.0	---	10.0	10
CS4	2.0	---	10.0	20
CS5	5.0	---	10.0	50
CS6	---	0.16	10.0	80

Note: *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.*

2.13. Calibration Standard Solutions for Curve 2

Freshly prepare at least six calibration standard solutions from INT. Make up with 1 % ACN solution in 10-mL volumetric flasks. Suggested concentrations of calibration standard solutions used in the analysis are listed below.

Calibration Standard Solution	Vol. of INT (mL)	Final Vol. (mL)	Final Conc. (μg/mL)
CS1	0.2	10.0	2
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 Solution (~5000 μg/mL)

Prepare a stock ICV standard solution according to Clause 2.11.1 using an independent source of reference materials (another brand or another batch of standard with same brand as appropriate).

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

Pipette 0.2 mL of the stock ICV standard solution into a 10-mL volumetric flask. Make up to the graduation mark with 50 % MeOH solution.

2.14.3. ICV Working Standard Solution (~10 or 20 µg/mL)

Pipette 1.0 or 2.0 mL of ICV I into a 10-mL volumetric flask. Make up to the graduation mark with 1 % ACN 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/mL.

2.16. Method Blank

The method blank shall be carried out through the complete sample preparation procedures as per Clauses 4.1.3 to 4.4 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. Disposable glass droppers.

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

3.12. Round bottom flasks, 50-mL.

3.13. Rotary evaporator or equivalent performance.

3.14. PTFE membrane filters, 0.20-µm, 13-mm or equivalent.

3.15. LC glass vials.

3.16. HPLC column, Zorbax Eclipse XDB-C_{18} Column (4.6 × 250 mm, 5 µm) or equivalent.

3.17. UPLC column, Acquity UPLC BEH C_{18} Column (2.1 × 150 mm, 1.7 µm) or equivalent.

3.18. HPLC-DAD, Dionex UltiMate 3000 HPLC System or equivalent performance.

3.19. UPLC-DAD, Acquity H-Class UPLC System or 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.

4.2. Liquid-liquid Extraction

4.2.1. Pipette 2.0 mL of the sample solution (Clause 4.1.7) into a 15-mL screw cap centrifuge tube.

4.2.2. Add ~5 mL of chloroform and butan-1-ol (4:1, v/v) mixture.

4.2.3. Vortex the sample solution in the centrifuge tube for ~1 min.

4.2.4. Centrifuge the sample solution at ~4000 rpm for ~10 min to settle the two layers.

4.2.5. Totally transfer the upper layer with a disposable glass dropper into a new 15-mL screw cap centrifuge tube.

4.3. EtOH Precipitation

4.3.1. Pipette 6 mL of EtOH to the sample solution in the 15-mL screw cap centrifuge tube.

4.3.2. Store the sample solution in the freezer at ~–20 °C overnight.

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

4.3.4. Totally transfer the supernatant to a 50-mL round bottom flask.

4.3.5. Evaporate the supernatant to dryness at ~45 °C by rotary evaporator, and then re-dissolve with 1 mL of 1 % ACN solution.

4.3.6. Filter the sample solution with 0.2-μm with 13-mm PTFE filter disc into a LC glass vial.

Remark: *Further dilute the sample solution with 1 % ACN solution if the concentration of analyte is not within the calibration range.*

4.4. Analysis of HPLC/UPLC-DAD

4.4.1. Operate the HPLC-DAD or UPLC-DAD 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.

4.4.2. Suggested HPLC-DAD conditions:

HPLC System	**:**	Dionex UltiMate 3000 HPLC System or equivalent performance
Column	**:**	Zorbax Eclipse XDB-C_{18} Column (4.6 × 250 mm, 5 µm) or equivalent
Mobile Phase A	**:**	Water
Mobile Phase B	**:**	ACN
Elution Program	**:**	Time (min) / A (%) / B (%) 0.0 / 99 / 1 3.0 / 99 / 1 15.0 / 97 / 3 30.0 / 95 / 5 40.0 / 95 / 5 42.0 / 90 / 10 45.0 / 50 / 50 48.0 / 50 / 50 49.0 / 5 / 95 55.0 / 5 / 95 56.0 / 70 / 30 58.0 / 70 / 30 59.0 / 99 / 1 70.0 / 99 / 1
Flow Rate	**:**	0.5 mL/min
Injection Volume	**:**	10 µL
Reported Retention Time	**:**	~36.4 min for rehmannioside D
Column Temperature	**:**	25 °C
DAD wavelength	**:**	203 nm

4.4.3. Suggested UPLC-DAD conditions:

UPLC System	**:**	Acquity H-Class UPLC System or equivalent performance
Column	**:**	Acquity UPLC BEH C_{18} Column (2.1 × 150 mm, 1.7 µm) or equivalent
Mobile Phase A	**:**	Water
Mobile Phase B	**:**	ACN
Elution Program	**:**	Time (min) / A (%) / B (%) 0.0 / 99 / 1 3.0 / 99 / 1 20.0 / 97 / 3 40.0 / 95 / 5 45.0 / 95 / 5 46.0 / 90 / 10 47.0 / 50 / 50 48.0 / 50 / 50 49.0 / 5 / 95 55.0 / 5 / 95 56.0 / 70 / 30 58.0 / 70 / 30 59.0 / 99 / 1 70.0 / 99 / 1
Flow Rate	**:**	0.2 mL/min
Injection Volume	**:**	10 µL
Reported Retention Time	**:**	~30.0 min for rehmannioside D
Column Temperature	**:**	25 °C
DAD wavelength	**:**	203 nm

5. Calculation/Result Interpretation

5.1. Identification Requirement

Identify the analyte 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 RT from 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 linear calibration mode. Obtain the slope, y-intercept and the coefficient of determination (r^2) from the calibration curve.

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

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

where C = Concentration of analyte obtained from calibration curve (μg/mL);
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.

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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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