Determination of Paeoniflorin, *Z*-Ligustilide, Ferulic Acid and/or Rehmannioside D 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 28 formulations, which contain Radix Paeoniae Alba (白芍), Radix Angelicae Sinensis (當歸) and/or Radix Rehmanniae (地黃). The main cores of these Chinese herbal medicines (Chm) are the principal or assistant drug, or as a major component. The formulations for each main core are listed in the tables below.

1.3. For Radix Paeoniae Alba (白芍) as the main core, 12 formulations have been validated in the method. The analysed chemical marker is paeoniflorin (Pae).

Item	Formulation	Ancient Chinese Medicine Bibliography
1.	真武湯	傷寒論
2.	麻子仁丸	傷寒論
3.	固經丸	丹溪心法
4.	桂枝加龍骨牡蠣湯	金匱要略
5.	痛瀉藥方	丹溪心法
6.	獨活寄生湯	備急千金要方
7.	十全大補湯	太平惠民和劑局方
8.	荊芥連翹湯	沈氏尊生方
9.	人參養榮湯	三因極一病證方論
10.	當歸芍藥散	金匱要略
11.	黃芪建中湯	金匱要略
12.	聖愈湯	醫宗金鑒

1.4. For Radix Angelicae Sinensis (當歸) as the main core, 10 formulations have been validated in the method. The analysed chemical markers are *Z*-ligustilide (Li) and ferulic acid (FA).

Item	Formulation	Ancient Chinese Medicine Bibliography
1.	聖愈湯	醫宗金鑒
2.	當歸四逆湯	傷寒論
3.	濟川煎	景嶽全書
4.	補陽還五湯	醫林改錯
5.	蠲痹湯	濟生方
6.	當歸芍藥散	金匱要略
7.	調經丸	證治準繩
8.	消風散	外科正宗
9.	溫經湯	金匱要略
10.	人參養榮湯	三因極一病證方論

1.5. For Radix Rehmanniae (地黃) as the main core, 12 formulations have been validated in the method. The analysed chemical marker is rehmannioside D (Reh D).

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

1.6. This method has been validated for qualitative and/or quantitative determination of four analytes, *viz.* Pae, Li, FA and Reh D in compound Chinese medicine granules using LC-MS/MS. Hydrocortisone is used as an internal standard (I.S.) in the analysis. The validated working ranges for each analyte are tabulated as below.

Analyte	**Concentration Ranges**	
	μg/L in Solution	**μg/g in Solid**
Pae	20–500	100–2500
Li	0.2–5	1–25
FA	2–50	50–1250
Reh D	2–50	50–1250

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. Acetonitrile (ACN), LC-MS grade.

2.4. 50 % MeOH Solution

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

2.5. 5 % ACN Solution

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

2.6. Formic acid, LC-MS grade.

2.7. 0.1 % Formic Acid Solution

Mix ~1 mL of formic acid and ~999 mL of water in a 1-L measuring cylinder.

2.8. Hydrocortisone, CAS. No.: 50-23-7.

2.9. Pae, CAS. No.: 23180-57-6.

2.10. Li, CAS. No.: 81944-09-4.

2.11. FA, CAS. No.: 537-98-4.

2.12. Reh D, CAS. No.: 81720-08-3.

2.13. I.S. Solutions

2.13.1. Stock I.S. Solution (~2000 µg/mL)

Weigh accurately ~20 mg of hydrocortisone into a 10-mL volumetric flask. Dissolve and make up to the graduation mark with methanol.

2.13.2. Intermediate I.S. Solution 1 (IS1, ~10 µg/mL)

Pipette 0.05 mL of stock I.S. solution into a 10-mL volumetric flask. Make up to the graduation mark with ACN.

2.13.3. Intermediate I.S. Solution 2 (IS2, ~100 µg/L)

Pipette 0.1 mL of **IS1** into a 10-mL volumetric flask. Make up to the graduation mark with 5 % ACN solution.

2.13.4. Intermediate I.S. Solution 3 (IS3, ~5 µg/L)

Pipette 0.025 mL of **IS1** into a 50-mL volumetric flask. Make up to the graduation mark with 5 % ACN solution.

2.14. Standard Solutions

2.14.1. Individual Stock Standard Solutions

Weigh accurately the suggested amount of reference materials in 10-mL volumetric flasks respectively. Dissolve and make up to the graduation mark with MeOH. The suggested concentrations of individual stock standard solutions are listed below.

Individual Stock Standard Solution	Weight of Reference Material (mg)	Final Vol. (mL)	Conc. (µg/mL)
Pae	~50	10	~5000
Li	~10	10	~1000
FA	~20	10	~2000
Reh D	~20	10	~2000

2.14.2. Mixed Intermediate Standard Solution 1 (INT1)

Pipette appropriate volume from the individual stock standard solutions from Clause 2.14.1 into a 10-mL volumetric flask. Make up to the graduation mark with ACN solution. The suggested concentration of the INT1 is listed below.

INT1	Vol. of the Stock Standard Solution (mL)	Final Vol. (mL)	Conc. (μg/mL)
Pae	2.0	10	~1000
Li	0.1		~10
FA	0.5		~100
Reh D	0.5		~100

2.14.3. Mixed Intermediate Standard Solution 2 (INT2)

Pipette 0.1 mL from INT1 into a 10-mL volumetric flask. Make up to the graduation mark with 5 % ACN solution. The suggested concentration of the INT2 is listed below.

INT2	Vol. of the INT1 Solution (mL)	Final Vol. (mL)	Conc. (μg/L)
Pae	0.1	10	~10000
Li			~100
FA			~1000
Reh D			~1000

2.15. Calibration Standard Solutions

Freshly prepare at least six calibration standard solutions from INT2 and/or CS6. Make up with 5 % 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 INT2 (mL)	Final Conc. (μg/L)				Vol. of IS2 (mL)	Final Conc. (μg/L)	Final Vol. (mL)
		Pae	Li	FA	Reh D		I.S.	
CS3	0.1	100	1	10	10	0.5	5	10
CS4	0.2	200	2	20	20	0.5	5	10
CS5	0.3	300	3	30	30	0.5	5	10
CS6	0.5	500	5	50	50	0.5	5	10

Calibration Standard Solution	Vol. of CS6 (mL)	Final Conc. (μg/L)				Vol. of IS3 (mL)	Final Conc. (μg/L)	Final Vol. (mL)
		Pae	Li	FA	Reh D		I.S.	
CS1	0.4	20	0.2	2	2	9.6	5	10
CS2	1.0	50	0.5	5	5	9.0	5	10

Remark: *Other concentrations may be used as appropriate. Other sequences of dilution may be employed to achieve the required concentrations. The concentration of I.S. in the calibration standard solutions shall be prepared at ~5 μg/L. Record the details in the data sheets.*

2.16. Initial Calibration Verification (ICV) Standard Solutions

2.16.1. Stock ICV Standard Solutions

Prepare stock ICV standard solutions according to Clause 2.14.1 using an independent source of reference materials (another brand or another batch of standard with same brand as appropriate).

2.16.2. Mixed Intermediate ICV Standard Solution I (ICV I)

Prepare ICV I using stock ICV standard solutions according to the suggested volumes in Clause 2.14.2. Make up to the graduation mark with ACN solution.

2.16.3. Mixed Intermediate ICV Standard Solution II (ICV II)

Prepare ICV II using ICV I according to Clause 2.14.3. Make up to the graduation mark with 5 % ACN solution.

2.16.4. ICV Working Standard Solution

Prepare the ICV working standard solution according to the CS3 or CS4 in Clause 2.15 using ICV II instead of INT2. Make up to the graduation mark with 5 % ACN solution.

2.17. Calibration Check Standard

Calibration check standard shall be the CS3 or CS4 of the calibration standard solutions.

2.18. Method Blank

The method blank shall be carried out through the complete sample preparation procedures as per Clauses 4.1 to 4.2 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-, 25- and 50-mL.

3.3. Measuring cylinder, 1-L.

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

3.5. Autopipettes, 100-, 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. PTFE membrane filters, 0.20-µm or equivalent.

3.11. LC glass vials.

3.12. UPLC column, Acquity HSS T3 Column (2.1 × 150 mm, 1.8 µm) or equivalent.

3.13. LC-MS/MS, Dionex Ultimate 3000 UPLC with Triple Quad 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. Filter the sample solution with 0.20-µm PTFE filter disc.

4.1.9. Dilute the sample solution 100 times by pipetting 0.01 mL of the sample solution as per Clause 4.1.8 and 0.05 mL of IS2 (~100 µg/L of I.S.) into a LC glass vial. Make up to 1.0 mL with 5 % ACN solution. The sample solution is ready for the analysis of Pae and Li.

4.1.10. Further dilute the sample solution 5 times by pipetting 0.2 mL of sample solution as per Clause 4.1.9 and 0.8 mL of IS3 (~5 µg/L of I.S.) into a LC glass vial. The sample solution is ready for the analysis of FA and Reh D.

Remark: *Further dilute the sample solution with IS3 (~5 µg/L of I.S.) if the concentration of analyte(s) is not within the calibration range. The concentration of I.S. in the sample solution shall be maintained at ~5 µg/L.*

4.2. Analysis of LC-MS/MS

4.2.1. 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.

4.2.2. Suggested LC conditions:

LC System	**:**	Dionex UltiMate 3000 HPLC System or equivalent performance
Column	**:**	Acquity HSS T3 Column (2.1 × 150 mm, 1.8 µm) or equivalent
Mobile Phase A	**:**	0.1 % Formic Acid Solution
Mobile Phase B	**:**	ACN
Elution Program	**:**	Time (min) / A (%) / B (%) 0.0 / 99 / 1 1.0 / 99 / 1 2.0 / 95 / 5 3.0 / 95 / 5 8.0 / 90 / 10 9.0 / 80 / 20 17.0 / 80 / 20 19.0 / 35 / 65 25.0 / 35 / 65 27.0 / 5 / 95 32.0 / 5 / 95 33.0 / 99 / 1 40.0 / 99 / 1
Flow Rate	**:**	0.2 mL/min
Injection Volume	**:**	10 µL

Reported Retention Time	**:**	~7.5 min for Reh D; ~14.0 min for Pae; ~17.5 min for FA; ~25.9 min for Li; and ~21.0 min for I.S.
Column Temperature	**:**	30 ºC

4.2.3. Suggested MS/MS conditions:

MS/MS System	**:**	Triple Quad 6500+ System or equivalent performance
Ionisation Mode	**:**	Electrospray ionisation
Polarity	**:**	Positive and Negative
Ionspray Voltage	**:**	4500 and –4500
Source Temperature	**:**	500 ºC
Ion Source Gas 1 (GS1)	**:**	50
Ion Source Gas 2 (GS2)	**:**	50
Curtain Gas (CUR)	**:**	30
Collision Gas (CAD)	**:**	9
Scan Type	**:**	MRM

4.2.4. Suggested MRM acquisition parameters:

Analyte	**MRM Transition**	**Polarity**	**Dwell Time (msec)**	**DP**	**EP**	**CE**	**CXP**
Pae	479.1→120.8*	–ve	50	–35	–10	–32	–10
	479.1→77.0^		50	–35	–10	–70	–4
Li	191.0→173.0*	+ve	50	+100	+10	+22	+22
	191.0→145.0^		50	+100	+10	+23	+18
FA	193.0→133.9*	–ve	50	–30	–10	–24	–15
	193.0→177.9^		50	–30	–10	–18	–15
Reh D	685.2→263.1*	–ve	50	–130	–10	–28	–15
	685.2→179.0^		50	–130	–10	–34	–11
I.S.	363.0→120.9*	+ve	50	+70	+10	+30	+18
	361.0→331.3*	–ve	50	–90	–10	–17	–24

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 relative retention time of the detected peak (RRT_{sample}) with that of the average relative retention time (RRT) from calibration standards. The RRT_{sample} shall not differ from that of the average RRT from calibration standards by more than 2.5 % 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. For each analyte, establish a calibration curve by plotting the relative area ratio (Area of Analyte/Area of I.S.) against the concentration of 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. (2025). *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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