GCMTI RD-3:2021

Determination of Naringin and Liquiritin in Pei Pa Koa by High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) and High Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS)

GCMTI method publications

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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. Pei Pa Koa is a prevalent proprietary Chinese medicine in China and Hong Kong. It is used for the relief of sore throat, coughs, hoarseness and aphonia. The formulations and production procedures of Pei Pa Koa are varied with manufacturers, commonly it was made through a procedure by continuously decocting the Chinese herbal medicines including the fritillary bulb (Bulbus fritillariae cirrhosae, 川貝母), loquat leaf (Eriobotrya japonica, 枇杷葉), pomelo peel (Citrus maxima, 化橘紅), chinese bellflower root (Platycodon grandiflorum, 桔梗), bitter apricot kernel (Prunus armeniaca, 苦杏仁), licorice root (Glycyrrhiza uralensis, 甘草) and menthol (薄荷) , followed by addition of syrup and honey base in ethanol. The common chemical markers in these Chinese herbal medicines are as follows:

Name of Chinese Herbal Medicines	Name of Common Chemical markers
Bulbus fritillariae cirrhosae (川貝母)	Peimisine
Eriobotrya japonica (枇杷葉)	Oleanolic acid and ursolic acid
Citrus maxima (化橘紅)	Naringin
Platycodon grandiflorum (桔梗)	Platycodin D
Prunus armeniaca (苦杏仁)	Amygdalin
Glycyrrhiza uralensis (甘草)	Liquiritin and Glycyrrhizic acid
Menthol (薄荷)	Menthol

1.2. This method specifies the procedures for determination of naringin and liquiritin in Pei Pa Koa sample.

1.3. The chemical markers are qualitatively and/or quantitatively determined by high performance liquid chromatography-diode array detector (HPLC-DAD)

and high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS).

2. Reagents

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

2.1. HPLC-DAD standard solution preparation

2.1.1. Individual stock standard solution (~1000 μg/mL)

Prepare individual stock standard solutions by weighing accurately about 5 mg of naringin and 5 mg of liquiritin into two separate 5-mL volumetric flasks respectively, dissolve and make up to the graduated mark with methanol.

2.1.2. Mixed intermediate standard solution (~100 μg/mL)

Prepare the mixed intermediate standard solution by transferring 1 mL of each individual stock standard solution into a 10-mL volumetric flask and make up to the graduated mark with dilution solvent.

2.1.3. Calibration standards, CS1 – CS5

A series of calibration standard solutions of ~0.1, 0.5, 1.0, 2.5, 5.0 μg/mL are prepared by transferring an appropriate amount of mixed intermediate standard solution into 10-mL volumetric flasks and make up with dilution solvent. Suggested volumes of calibration standard solutions used for the preparation are listed below.

Calibration Standards	**Volume of mixed intermediate standard solution (mL)**	**Volume of CS5 (mL)**	**Final Volume (mL)**	**Conc. of naringin and liquiritin (μg/mL)**
CS1		0.2	10	0.1
CS2		1	10	0.5
CS3	0.1		10	1.0
CS4	0.25		10	2.5
CS5	0.5		10	5.0

2.1.4. Individual stock ICV solution (~1000 μg/mL)

Prepare the individual stock ICV solution by weighing accurately about 5 mg

of naringin and liquiritin into two separate 5-mL volumetric flasks respectively, dissolve and make up to the graduated mark with methanol.

2.1.5. Mixed intermediate ICV standard solution (~100 μg/mL)

Prepare the mixed intermediate ICV standard solution by transferring 1 mL of each individual stock ICV solution into a 10-mL volumetric flask and make up to the graduated mark with dilution solvent.

2.1.6. ICV working standard solution (~2.5 μg/mL)

Prepare the ICV working standard solution by transferring 0.25 mL of mixed intermediate ICV standard solution into a 10-mL volumetric flask and make up to the graduated mark with dilution solvent.

2.1.7. Mixed spike standard solution (~30 μg/mL for naringin; ~20 μg/mL for liquiritin)

Prepare the mixed spike standard solution by transferring 0.3 mL of naringin stock standard solution and 0.2 mL of liquiritin stock standard solution into a 10-mL volumetric flask and make up to the graduated mark with dilution solvent.

2.2. HPLC-MS/MS standard solution preparation

2.2.1. Individual stock standard solution (~1000 μg/mL)

Prepare individual stock standard solutions by weighing accurately about 5 mg of naringin and 5 mg of liquiritin into two separate 5-mL volumetric flasks respectively, dissolve and make up to the graduated mark with methanol.

2.2.2. Mixed intermediate standard solution I (~10 μg/mL)

Prepare the mixed intermediate standard solution I by transferring 0.1 mL of each individual stock standard solution into a 10-mL volumetric flask and make up to the graduated mark with dilution solvent.

2.2.3. Mixed intermediate standard solution II (~250 ng/mL)

Prepare the mixed intermediate standard solution II by transferring 0.25 mL of mixed intermediate standard solution I into a 10-mL volumetric flask and make up to the graduated mark with dilution solvent.

2.2.4. Calibration standards, CS1 – CS5

A series of calibration standard solutions of ~0.5, 1.0, 2.0, 5.0, 10 ng/mL are prepared by transferring an appropriate amount of mixed intermediate standard solution II into 10-mL volumetric flasks and make up with dilution

solvent. Suggested volumes of calibration standard solutions used for the preparation are listed below.

Calibration standards	Volume of mixed intermediate standard solution II (mL)	Volume of CS5 (mL)	Final Volume (mL)	Conc. of naringin and liquiritin (ng/mL)
CS1		0.5	10	0.5
CS2		1	10	1.0
CS3		2	10	2.0
CS4	0.2		10	5.0
CS5	0.4		10	10

2.2.5. Individual stock ICV solution (~1000 μg/mL)

Prepare the stock ICV solution by weighing accurately about 5 mg of naringin and liquiritin into two separate 5-mL volumetric flasks respectively, dissolve and make up to the graduated mark with methanol.

2.2.6. Mixed intermediate ICV standard solution I (~10 μg/mL)

Prepare the mixed intermediate ICV standard solution I by transferring 0.1 mL of each individual stock ICV solutions into a 10-mL volumetric flask and make up to the graduated mark with dilution solvent.

2.2.7. Mixed intermediate ICV standard solution II (~250 ng/mL)

Prepare the mixed intermediate ICV standard solution II by transferring 0.25 mL of mixed intermediate ICV standard solution I into a 10-mL volumetric flask and make up to the graduated mark with dilution solvent.

2.2.8. ICV working standard solution (~5 ng/mL)

Prepare the ICV working standard solution by transferring 0.2 mL of mixed intermediate ICV standard solution II into a 10-mL volumetric flask and make up to the graduated mark with dilution solvent.

2.2.9. Mixed spike standard solution (~30 μg/mL for naringin; ~20 μg/mL for liquiritin)

Prepare the mixed spike standard solution by transferring 0.3 mL of naringin stock standard solution and 0.2 mL of liquiritin stock standard solution into a 10-mL volumetric flask and make up to the graduated mark with dilution

solvent.

2.3. Methanol, LC-MS grade.

2.4. Acetonitrile, LC-MS grade.

2.5. Formic acid, analytical grade.

2.6. Milli-Q water.

2.7. Dilution solvent

Acetonitrile : 0.02% Formic acid (2:8).

3. Apparatus

All glassware shall be rinsed with acetone and washed with detergent solution as soon as practicable after use. After detergent whing, glassware shall be rinsed immediately, firstly with acetone and then with water. The water rinse shall be followed by another two more rinses with acetone, respectively.

3.1. Volumetric flasks, 5-mL and 10-mL.

3.2. Auto pipettes, 300-µL, 1000-µL, 5000-µL and 10000-µL.

3.3. Centrifuge tube, 15-mL.

3.4. Analytical balance, capable of weighing to 0.1 mg.

3.5. Ultrasonic bath.

3.6. PTFE membrane filters, 0.45 µm.

4. Procedures

4.1. HPLC-DAD analysis

4.1.1. Sample preparation

4.1.1.1. Weigh accurately about 0.2 g of Pei Pa Koa sample into a 15-mL

centrifuge tube.

4.1.1.2. Add 10 mL of dilution solvent into the centrifuge tube. The sample is mixed by vortexing the centrifuge tube for 1 minute.

4.1.1.3. The sample mixture is then sonicated in an ultrasonic bath for 10 minutes.

4.1.1.4. The supernatant solution is filtered by 0.45µm PTFE membrane filter.

4.1.1.5. Mix well prior to HPLC-DAD analysis.

4.1.2. HPLC-DAD analysis

4.1.2.1 Operate the HPLC-DAD system in accordance with the instrument manual. Analyse the samples with the following suggested conditions. It may be necessary to modify the operation conditions for optimum signal output.

4.1.2.2. Suggested HPLC conditions:

HPLC system	:	Waters Alliance e2695 HPLC system
Column	:	GL Science Intersil ODS-4, 4.6 × 250 mm, 5µm
Column temperature	:	25 ºC
Flow rate	:	1.0 mL/min
Injection volume	:	100 µL
Mobile phase	:	A: 0.02 % formic acid B: Acetonitrile

Gradient :

Time (min)	A %	B %	Flow (mL/min)
0.0	82	18	1.0
10.0	82	18	1.0
21.0	72	28	1.0

21.1	5	95	1.0
24.0	5	95	1.0
24.1	82	18	1.0
29.0	82	18	1.0

4.1.2.3. Suggested DAD conditions:

Detector wavelength : 278 nm

4.2. HPLC-MS/MS analysis

4.2.1. Sample preparation

4.2.1.1. Weigh accurately about 0.2 g of Pei Pa Koa sample into a 15-mL centrifuge tube.

4.2.1.2. Add 10 mL of dilution solvent into the centrifuge tube. The sample is mixed by vortexing the centrifuge tube for 1 minute.

4.2.1.3. The sample mixture is then sonicated in an ultrasonic bath for 10 minutes.

4.2.1.4. The supernatant solution is filtered by 0.45µm PTFE membrane filter and diluted with appropriate amount of dilution solvent prior to HPLC-MS/MS analysis.

4.2.1.5. The suggested dilution factor is 500-fold.

4.2.2. HPLC-MS/MS analysis

4.2.2.1 Operate the HPLC-MS/MS system in accordance with the instrument manual. Analyse the samples with the following suggested conditions. It may be necessary to modify the operation conditions for optimum signal output.

4.2.2.2. Suggested HPLC conditions:

HPLC system : Dionex UltiMate 3000 HPLC system

Column	:	GL Science Intersil ODS-4, 2.1 × 250 mm, 5µm
Column temperature	:	25 ºC
Flow rate	:	0.3 mL/min
Injection volume	:	5 µL
Mobile phase	:	A: 0.02 % formic acid B: Acetonitrile

Gradient :

Time (min)	A %	B %	Flow (mL/min)
0.0	80	20	0.3
4.0	80	20	0.3
9.0	70	30	0.3
14.0	50	50	0.3
16.0	5	95	0.3
18.0	5	95	0.3
18.1	80	20	0.3
22.0	80	20	0.3

4.2.3. Suggested MS/MS conditions:

MS/MS system	:	AB SCIEX 6500+ system
Ionization mode	:	Electrospray ionization (ESI) –ve mode
Ionspray voltage	:	-4500V
Source temperature	:	500 ºC
Ion source gas 1 (GS1)	:	60
Ion source gas 2 (GS2)	:	70
Curtain gas (CUR)	:	20
Collision gas (CAD)	:	9
Scan Type	:	MRM

4.2.4. Suggested MRM acquisition conditions for the analysis of naringin and liquiritin:

Analytes	**MRM transitions**		**Dwell time msec**	**DP**	**EP**	**CE**	**CXP**
Naringin	579.2 → 271.1	Quantifying MRM	30-300	-160	-10	-45	-15

	579.2 → 459.1	Qualifying MRM	30-300	-160	-10	-35	-15
Liquiritin	417.1 → 255.1	Quantifying MRM	30-300	-92	-10	-27	-15
	417.1 → 135.0	Qualifying MRM	30-300	-92	-10	-39	-15

5. Calculation / result interpretation

5.1. Identification requirements

5.1.1. For HPLC-DAD and HPLC-MS/MS analysis, targeted analyte in sample is identified by comparison of the retention time of the detected peak with that of the standard. The retention time (RT) of the analyte shall not differ from that of the standard by more than 5 %.

5.1.2. Calculate the relative abundance of ions (preferably including the quasimolecular ion) for

(i) at least two MRM ions for LC-MS/MS analyses; or

(ii) at least a pair of diagnostic ions for instrument capable of measuring accurate mass.

5.1.2. For HPLC-MS/MS, the relative abundance of MRM/diagnostic ions (qualifying MRM/quantifying MRM) shall meet the tolerance for the positive identification of the analyte (with reference to that of the standard solution or that of the average of the standard solutions):

Relative intensity to the base peak (%)	**% Allowable deviation**
> 50%	±20%
> 20 to 50%	±25%
> 10 to 20%	±30%
≤ 10%	±50%

5.2. Calibration curve

5.2.1. Plot the peak area against concentration (in μg/mL for HPLC-DAD;

ng/mL for HPLC-MS/MS) for each standard. Obtain the slope, y-intercept and the correlation coefficient (r) from the calibration curve.

5.2.2. Calculate the deviation of each calibration level using the following equation:

$$\text{Deviation of calibration level (\%)} = \frac{C - C_{theo}}{C_{theo}} \times 100\%$$

where C = Conc. of standard from the calibration curve (in μg/mL for HPLC-DAD; ng/mL for HPLC-MS/MS) and

C_{theo} = Theoretical conc. of the calibration standard (in μg/mL for HPLC-DAD; ng/mL for HPLC-MS/MS)

The value C shall be given by:

$$\text{C(ng or μg/mL)} = \frac{A - Y}{M}$$

where A = Peak area of standard;

Y = Y-intercept of the calibration curve; and

M = Slope of the calibration curve.

5.3. Calculate the content of analyte in the sample using the following equation:

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

where C = Conc. of analyte from calibration curve (in μg/mL for HPLC-DAD; ng/mL for HPLC-MS/MS);

V = Final volume (mL);

D = Dilution factor; and

W = Sample weight (g).

5.4. Calculate the average deviation from the mean (ADM) of the pair of duplicate samples using the following equation:

$$\text{ADM}\ (\%) = \frac{D_1 - (D_1 + D_2)/2}{(D_1 + D_2)/2} \times 100\%$$

where D1 = Value of sample 1 and
D2 = Value of sample 2.

6. Reference

6.1. Chinese Pharmacopoeia Commission. Pharmacopoeia of the People's Republic of China Volume 1, 2015 ed. China Medical Science Press.

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