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Identification of Ursolic acid in Pei Pa Koa by 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 the identification of the ursolic acid in Pei Pa Koa sample.

1.3. The chemical marker is qualitatively determined by 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. Individual stock standard solution (~1000 μg/mL)

Prepare individual stock standard solution by weighing accurately about 2 mg of ursolic acid into a 2-mL volumetric flask, dissolve and make up to the graduated mark with methanol.

2.2. Intermediate standard solution I (~10 μg/mL)

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

2.3. Intermediate standard solution II (~50 ng/mL)

Prepare the intermediate standard solution II by transferring 0.05 mL of intermediate standard solution I into a 10-mL volumetric flask and make up to the graduated mark with ethanol.

2.4. Working standard solutions (~1 ng/mL)

Prepare the working standard solutions of ~ 1 ng/mL by transferring 0.2 mL of intermediate standard solution II into a 10-mL volumetric flask and make up with dilution solvent.

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

Prepare the individual stock ICV solution by weighing accurately about 2 mg of ursolic acid into a 2-mL volumetric flask, dissolve and make up to the graduated mark with methanol.

2.6. Intermediate ICV standard solution I (~10 μg/mL)

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

2.7. Intermediate ICV standard solution II (~50 ng/mL)

Prepare the intermediate ICV standard solution II by transferring 0.05 mL of intermediate ICV standard solution I into a 10-mL volumetric flask and make up to the graduated mark with ethanol.

2.8. ICV working standard solution (~1 ng/mL)

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

2.9. Spike standard solution (~50 ng/mL)

Use the intermediate ICV standard solution II as a spike standard solution.

2.10. Detection limit (DL) calibration standard solution:

1 ng/mL or 10 ng/g

2.11. Methanol, LC-MS grade.

2.12. Ethanol, absolute ethanol.

2.13. Ammonium acetate, analytical grade.

2.14. Buffer solution, ammonium acetate (~10 mM)

Weigh accurately about 770.8 mg of ammonium acetate into a 1-L volumetric flask and make up to the graduated mark with Milli-Q water.

2.15. Milli-Q water.

2.16. Dilution solvent

Ethanol : buffer solution (1:1).

2.17. Extraction solvent

Ethanol : methanol (1:1)

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, 2-mL, 10-mL and 1000-mL.

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

3.3. Centrifuge tube, 15-mL.

3.4. Hot water bath, 60 °C.

3.5. Vortex mixer.

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

3.7. Ultrasonic bath.

3.8. PTFE membrane filters, 0.2 µm.

4. Procedures

4.1. Sample preparation

4.1.1. Weigh accurately about 1.0 g of Pei Pa Koa sample into a 15-mL centrifuge tube.

4.1.2. Add 1 mL of extraction solvent into the centrifuge tube. The sample is then placed into a hot water bath at 60 °C for 1 minute.

4.1.3. The sample is mixed by vortexing the centrifuge tube for 5 minutes.

4.1.4. Add 4 mL of ethanol into the centrifuge tube. The sample mixture is then sonicated in an ultrasonic bath for 10 minutes and store at freezer at -20 °C for 10 minutes.

4.1.5. The sample is centrifuged at 4000 rpm for 15 minutes. The supernatant solution is then filtered by 0.2 µm PTFE membrane filter.

4.1.6. Transfer 0.5 mL of sample solution and 0.5 mL of buffer solution into a 2-mL sample vial and mix well prior to HPLC-MS/MS

analysis. Make appropriate dilution if necessary.

4.2. Preparation of method blank

Prepare a method blank by carrying out the procedures as per clause 4.1 without sample.

4.3. Preparation of spike sample

4.3.1. Prepare a spiked sample by carrying out the procedures as per clause 4.1.1 to 4.1.3.

4.3.2. Add 0.2 mL of spike standard solution and 3.8 mL of ethanol into the centrifuge tube (or any amount of standard deemed appropriate).The sample mixture is then sonicated in an ultrasonic bath for 10 minutes and store at freezer at -20 °C for 10 minutes.

4.3.3. Follow the clause 4.1.5. to 4.1.6. to prepare the spike sample.

4.4. HPLC-MS/MS analysis

4.4.1 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.

4.4.2. Suggested HPLC conditions:

HPLC system	:	Dionex UltiMate 3000 HPLC system
Column	:	Waters Acquity UPLC BEH C_{18}, 2.1 × 100 mm, 1.7 µm
Guard column	:	Waters Acquity UPLC BEH C_{18}, 2.1 × 5 mm, 1.7 µm
Column temperature	:	25 ºC
Flow rate	:	0.4 mL/min
Injection volume	:	40 µL
Mobile phase	:	A: 10 mM ammonium acetate

B: Acetonitrile

Isocratic :

Time(min)	A %	B %	Flow
0.0	40	60	0.4
15.0	40	60	0.4

4.4.3. 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
CEM	:	2000

4.4.4. Suggested MRM acquisition conditions for the analysis of ursolic acid:

Analyte	**MRM transitions**		**Dwell time msec**	**DP**	**EP**	**CE**	**CXP**
Ursolic acid	455.4 → 407.4	Quantifying MRM	1000	-200	-10	-55	-10
	455.4 → 391.4	Qualifying MRM	1000	-200	-10	-59	-21

4.4.5. Repeat injection of detection limit (DL) calibration standard solution (~1 ng/mL) 3 times. Record retention time and signal response.

4.4.6. Calculate the average retention time (RT) and signal response for analyte using equation in clause 5.2.1.

4.4.7. Inject method blank, ICV working standard solution, sample

solution(s), duplicate sample and spike sample respectively.

4.4.8. Record the retention time of the detected peak(s) and the signal response.

4.4.9. Compare the signal response of the detected peak(s) with the average signal response of the analyte in the DL calibration standard solution.

5. Calculation / result interpretation

5.1. Identification requirements

5.1.1. The targeted analyte in sample is identified by comparison of the retention time (RT) of the detected peak with that of the standard. The retention time 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.

5.1.3. 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 Calculation of average RT and signal response

5.2.1 Calculate the average RT and signal response of base peak of the selected ions or ion pairs for analyte of the detection limit calibration standard solution by using the following equation:

$$\text{Average RT or signal response} = \frac{Inj_1 + Inj_2 + Inj_3}{3}$$

where Inj_1, Inj_2, Inj_3 = RT or signal response of base peak of the selected ions or ion pairs of 3 injections of detection limit calibration standard solution respectively.

5.2.2 Calculate the % RSD of RT and signal response by using the following equation:

$$\%\ \text{RSD} = \frac{s}{\bar{X}} \times 100\%$$

where s = Standard deviation of RT or signal response of 3 injections of detection limit calibration standard solution,

$\bar{X}$ = Average RT or signal response of 3 injections of detection limit calibration standard solution.

5.3. Result interpretation

5.3.1. If the signal response of the analyte found in the sample is equal to or greater than 70% of the average signal response of the analyte in the detection limit calibration standard solution, the analyte is considered to be "**POSITIVE**" in the sample.

6. Reference

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