Determination of 16 Functional Components in Orchid Medicinal Materials Traditional Chinese Medicine by UPLC-MSMS

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Abstract: A method for the determination of 16 functional components in orchid medicinal materials traditional Chinese medicine by UPLC-MSMS was established. Samples were extracted with 50% ethanol, isolated by ACQUITY UPLC®HSS T3 chromatographic column (1.8μm, 2.1×100mm), making for 5mmol/L ammonium acetate containing 0.1 % (v /v) formic acid water and acetonitrile solution as mobile phase, and detected by UPLC-MSMS after gradient separation. The results showed that the linear correlation coefficients of 16 components were all greater than 0.990, the relative standard deviation of the method precision is less than 10%, the detection limit of the method is less than 1.2 mg/kg, and the limit of quantification is less than 4 mg/kg. The recovery rate of the method is in the range of 79.90%-103.80% when the added scalar is 0.001mg/kg-10mg/kg. Thus, this method is accurate and reliable, and can quickly and accurately determine the contents of 16 functional components in orchid medicinal materials Chinese herbal medicines simultaneously.

1. Introduction

Orchid medicinal materials, recorded in the 2020 edition of the "Pharmacopoeia of the People's Republic of China",[1] include Pseudobulb of Appendiculate Cremastra, Gastrodia elata, Dendrobium, Bletilla Striata, Illicium difengpi, Dendrobium officinale, Rhizome of Conic Gymnadenia.

Pseudobulb of Appendiculate Cremastra[2] is the dried Pseudobulb of Cremastra Appendiculata, Pleione Bulbocodioides or Pleione Yunnanensis. It has the effect of clearing away heat and detoxifying, reducing swelling and dissipating knots. It is used in carbuncle swollen boils poison, scrofula phlegm nucleus, snake and insect bites and Zhengjia ruffian mass.

Gastrodia Elata[3] is the dried tuber of the Orchid Gastrodia Elata. Modern pharmacological studies have shown that Gastrodia elata has sedative and hypnotic, anti-convulsant, anti-anxiety, neuroprotective, analgesic, enhanced blood circulatory system function, enhanced immunity, anti-amnesia and anti-aging effects.

Dendrobium[4] is the fresh or dried stems of the orchid plants Dendrobium Nobile, Dendrobium Chrysotoxum or Dendrobium Fimbriatum, and the fresh or dry stems of similar species of the same genus. It has the functions of nourishing stomach and promoting body fluid, nourishing yin and clearing away heat.

Bletilla Striata[5] is the dry tuber of orchid medicinal materials. It mainly has pharmacological effects such as hemostasis, antibacterial, anti-tumor, anti-inflammatory and analgesic, promoting wound healing and inhibiting melanin production.

Illicium Difengpi[6] is an octagonal plant of the magnolia family, a dried bark of the magnoliaceae plant, and the bark of the ground maple bark is a traditional Chinese medicine for dispelling wind and dehumidification, which is mainly used for the treatment of rheumatic joint pain, lumbar muscle strain and other symptoms.

Dendrobium Officinale[7] is an orchid herb, also known as Tiepilan, Chlorophytum and Lishucao. It has the effects of promoting body fluid and nourishing the stomach, nourishing yin and clearing heat, moistening the lungs and benefiting the kidneys, improving eyesight and strengthening the waist.

Rhizome of Conic Gymnadenia[8] is the tuber of orchid orchid ginseng, which has the functions of relieving cough and relieving asthma, invigorating kidney and spleen, regulating qi and blood, and relieving pain.

The above orchid medicinal materials contain gastrodin, adenosine, uridine, adenine, uracil, p-

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hydroxybenzyl alcohol, p-hydroxybenzoic acid, militarine and other active ingredients. The efficacy of traditional Chinese medicine is mainly due to the synergistic effect of multiple components. In order to more comprehensively evaluate the orchid medicinal materials and their active ingredients and quality in vivo, the simultaneous determination method of various medicinal components in the orchid medicinal materials was studied.

The existing technologies mainly focus on high performance liquid chromatography and high performance liquid chromatography-mass spectrometry [9] [10] [11]. Among them, high performance liquid chromatography is more commonly used, but due to the limitation of its detector resolution, some functional components may not be separated from other impurities, resulting in inaccurate quantification. Recently, there have been some reports on the functional components of orchid medicinal materials by high performance liquid chromatography-mass spectrometry, but these methods are all research methods for detecting one or a few components of a certain medicinal material in orchid medicinal materials. There is no method in the existing data that specifically uses liquid chromatography tandem triple quadrupole mass spectrometry to simultaneously determine a variety of medicinal components in orchid medicinal materials. Therefore, the method utilizes liquid chromatography tandem triple quadrupole mass spectrometry to analyze various functional components in orchid medicinal materials, and can more comprehensively evaluate orchid medicinal materials and active components as well as their quality in vivo.

2. Materials and Methods

2.1 Reagents and Consumables

Chromatographically pure methanol (CNW), chromatographically pure acetonitrile (CNW), mass spectrometry-pure formic acid (fisher 50mL), mass spectrometry-pure ammonium acetate (fisher 50g), water was self-made first-grade water (Milibo), and the standards were purchased from Alta Technology Co., Ltd.

2.2 Equipment

AB 4500 ultra-high performance liquid chromatography-mass spectrometry/mass spectrometer (SCIEX, USA), ultrasonic cleaner (Tianjin Aotesiens Instrument Co., Ltd.), ME204E electronic analytical balance (Mettler, Switzerland), 80 mesh sieve (No. 5 sieve).

2.3 Experiment Method

2.3.1 Reagent preparation

50% ethanol solution: accurately pipette 50 mL of absolute ethanol, and dilute to 100 mL with water.

2.3.2 Preparation of standard solutions

(1) Preparation of standard stock solutions of 16 functional ingredients: Accurately weigh an appropriate amount of 16 functional ingredient standard substances, dissolve and dilute with methanol water (1:1 volume ratio) to a concentration of 4 μg/mL of p-hydroxybenzaldehyde; p-hydroxybenzyl alcohol, The concentration of guanosine is 400 μg/mL; the concentration of gastrodin, adenosine, cytidine, uridine, guanine, cytosine, dactylorhin A, and Militarine is 2 μg/mL; the concentration of Parishin A, Parishin B, Parishin C, Parishin E is 200 μg /mL; stock solution with a thymine concentration of 80 μg/mL.

(2) Preparation of standard working solution of 16 functional components: draw standard stock solution respectively, dilute with 50% ethanol solution to hydroxybenzaldehyde concentration of 1ng/mL, 2ng/mL, 4ng/mL, 10ng/mL, 20ng/mL, 40ng/mL mL; p-hydroxybenzyl alcohol, guanosine concentration of 100ng/mL, 200ng/mL, 400ng/mL, 1000ng/mL, 2000ng/mL, 4000ng/mL; gastrodin, adenosine, cytidine, uridine, guanine , Cytosine, dactylorhin A, Militarine at concentrations of 0.5 ng/mL, 1ng/mL, 2 ng/mL, 5ng/mL, 10 ng/mL, 20ng/mL; Parishin A, Parishin B, Parishin C, Parishin E at concentrations of 50ng/mL, 100ng/mL, 200ng/mL, 500ng/mL, 1000ng/mL, 2000ng/mL;

thymidine concentrations are 40ng/mL, 80ng/mL, 200ng/mL, 400ng/mL, 800ng/mL.

2.3.3 Sample preparation

Weigh 0.5g (accurate to 0.0001g) of the sample that has passed through an 80-mesh sieve into a 50mL centrifuge tube, add 20mL of 50% methanol aqueous solution, shake on a vortex mixer for 5 minutes, extract by ultrasonic for 50 minutes, cool to room temperature, 8000 r/min Centrifuge for 5 min, transfer the supernatant to a 50 mL volumetric flask, repeat the extraction of the residue with 20 mL of 50% methanol aqueous solution, combine the two extracts, make up to the mark with 50% methanol aqueous solution, and take the supernatant over 0.22 μm for testing. If it exceeds the linear range, it should be diluted with 50% methanol aqueous solution to be tested.

2.3.4 Liquid chromatography-tandem mass spectrometry conditions

(1) Liquid chromatography conditions

ACQUITY UPLC®HSS T3 column (1.8μm, 2.1×100mm); injection volume 5 μL; flow rate: 0.4mL/min; column temperature 40℃; gradient elution: eluent A is 5mmol/L ammonium acetate containing 0.1 % (v/v) formic acid water, eluent B contains acetonitrile, elution procedure: 0~1.50min, A: 75%~75%; 1.50~3.00min, A: 75%~20%; 3~5min , A: 20%~20%; 5.01~7.00min A: 75%~75%.

(2) MS conditions

Air curtain gas flow rate: 30 L/min; atomizing gas flow rate (GS1): 50 L/min; auxiliary heating gas flow rate (GS2): 50 L/min; collision gas (CAD): medium intensity (medium); auxiliary heating gas Temperature: 500 ℃; Spray voltage: 5000 V (ESI+)/-4500 V (ESI-); Scanning mode: Multiple reaction monitoring mode.

3. Results and Analysis

3.1 Optimization of mass spectrometry parameters

Table 1: Mass spectrometry detection parameters of target compounds

Number	Project	Parent Ion (M/Z)	Daughter Ion (M/Z)	Declustering Voltage / V	Injection Voltage /V	Collision Voltage / V	Injection Voltage /V
1	P-Hydroxybenzaldehyde	120.8	91.8*	-65	-10	-32	-13
		120.8	93	-65	-10	-26	-13
2	P-Hydroxybenzyl Alcohol	142.1	107*	60	10	11	13
		142.1	124	60	10	7	13
3	Gastrodin	304.2	107*	35	10	15	13
		304.2	180.2	35	10	11	13
4	Adenosine	268	136*	50	10	22	13
		268	119	50	10	60	13
5	Cytidine	244.2	112.2*	35	10	14	13
		244.2	244.2	35	10	6	13
6	Uridine	245.2	113*	35	10	13	13
		245.2	245.2	35	10	3	13
7	Guanine	152.1	135*	80	10	35	13
		152.1	110	80	10	36	13
8	Guanosine	284.1	152*	110	10	15	13
		284.1	284.1	110	10	3	13
9	Cytosine	112.1	112.1*	60	10	9	13
		112.1	95.1	60	10	25	13
10	Thymine	127.1	110*	50	10	20	13
		127.1	84	50	10	21	13
11	Parisonside A	995.2	727*	-150	-10	-36	-13
		995.2	459	-150	-10	-52	-13
12	Parisonside B	727.1	423.1*	-100	-10	-35	-13
		727.1	441	-100	-10	-29	-13
13	Parisonside C	727.1	423.1*	-100	-10	-35	-13
		727.1	441	-100	-10	-29	-13
14	Parisonside E	459.2	111*	-80	-10	-25	-13
		459.2	173	-80	-10	-21	-13
15	Militarine	771.3	457.1*	-60	-10	-29	-13
		725.3	457.1	-110	-10	-24	-13
16	Dactylorhin A	887.3	619.2*	-80	-10	-30	-13
		887.3	439.2	-80	-10	-45	-13

Note: * is quantitative ion

The 16 functional components were divided into standard solutions with a concentration of 200μg/L, and the full-scan detection of mass spectrometry was carried out by continuous injection of

needle pump flow injection, and the first-level mass spectrum of the tested compounds was obtained as well as the corresponding parent ions of each compound were found. Then, a secondary mass spectrometry scan was performed to obtain the corresponding product ions. Two pairs of characteristic ions were found for each compound, and the collision energy and cluster removal voltage were optimized respectively. Finally, the best spectral parameters of 16 efficacy components including p-hydroxybenzaldehyde, p-hydroxybenzyl alcohol, gastrodin, adenosine, cytidine, uridine, guanine, guanosine, cytosine, thymine, parisonin A, parisonin B, parisonin C, Parisonin E, Dactylorhin A and Militarine were obtained.

P-hydroxybenzaldehyde and Dactylorhin A have good response in anion mode, so anion [M-H]- is used as the parent ion of this compound. Militarine has a daughter ion with good response under [M-H]- and [M+HCOOH]-. Therefore, the ion pairs in [M-H]- mode are used as quantitative ions. Adenosine, cytidine, uridine, guanosine, cytosine, thymine, adenine and guanine have a good response under [M+H]+ positive ion mode. Therefore, positive ion [M+H]+ was used as the parent ion of the compound. P-hydroxybenzyl alcohol and gastrodin have good response in [M+NH4]+ positive ion mode, so positive ion [M+NH4]+ is used as the parent ion of these compounds. Mr Leeson glycosides from gastrodia elata and citric acid formed by the junction of ester glucoside keys, Barry's glycosides from 3 A molecular on the combination of gastrodine glycosides and lemon, glycosides B, Barry Barry sen sen glycosides C by 2 molecules on the combination of gastrodine glycosides and lemon, Barry's glycosides from 1 E molecules on the combination of gastrodine glycosides and lemon, under the mode of negative ions [M - H] - all have high response, Therefore, negative ion [M-H]- is used as the parent ion of these compounds. Ms has the function of positive and negative ion switching monitoring, which can be carried out simultaneously in the same needle sample operation. The ms detection parameters of target compounds are shown in Table 1.

3.2 Mobile phase and column optimization

In order to obtain good peak form and ionization efficiency, the experiment compares 0.1% (V/V) formic acid water-methanol, 0.1% (V/V) formic acid water-acetonitrile, 5mmol/L ammonium acetate containing 0.1% (V/V) formic acid water-methanol and 5mmol/L ammonium acetate containing 0.1% (V/V) formic acid water-acetonitrile. And the results showed that: as the mobile phase, 5mmol/L ammonium acetate containing 0.1% (V/V) formic acid water-acetonitrile showed a good response to most of the functional components. After comprehensive consideration, 5mmol/L ammonium acetate containing 0.1% (V/V) formic acid water-acetonitrile was selected as the mobile phase. In comparison with the peaks and separations of BEH C18 column, HSS T3 column and Hilic column, the two isomers of parisin B and parisin C could not be separated well by C18 and Hilic column under 5mmol/L ammonium acetate containing 0.1% (v/v) formic acid water-acetonitrile mobile phase. The use of T3 column can well separate the two, and the chromatogram of parisin B and parisin C was shown in Figure 1. Under optimized chromatographic and mass spectrometry conditions, the total ion flow chromatogram of the 16 functional components was shown in Figure 2.

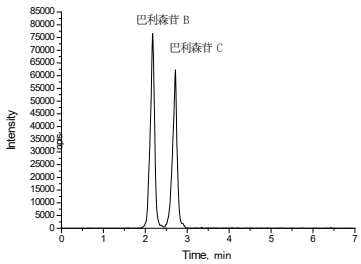

Figure 1: Chromatograms of parisonside B and parisonside C.

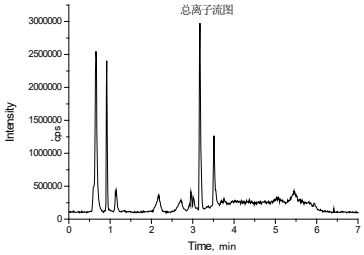

Figure 2: Total ion current map of 16 functional components.

3.3 Optimization of pretreatment conditions

3.3.1 Selection of extraction solvent and optimization of conditions

In the Chinese pharmacopoeia (part I) of the 2020 edition, dilute ethanol solution (49.5% ~ 50.5%

ethanol aqueous solution by volume ratio) and 0.05% formic acid methanol solution are extracted by hot reflux, and the extracted solution is concentrated to nearly dry. Then the residue is dissolved to prepare the test solution. The operation is relatively complicated, but the extraction rates of water-soluble nucleosides and bases such as cytosine were lower under these conditions. There are also ultrasonic extraction using dilute ethanol solution. In this study, the extraction effects of ultrasonic extraction of 20% methanol, 50% methanol, 80% methanol, 100% methanol, 20% ethanol, 50% ethanol, 80% ethanol, 100% ethanol were compared. The sample of Gastrodia elata was weighed through the fifth sieve and 0.5g sample (accurate to 0.0001g) was put into a 50mL centrifuge tube and 20 mL extraction solution was added. The vortex mixer was oscillated for 3 min and ultrasonically extracted for 1h. The results showed that the extraction efficiency of most of the active ingredients was best when 50% ethanol solution was used as extraction solvent. Therefore, 50% ethanol was used as the extraction solvent of 16 effective ingredients.

3.3.2 Optimization of ultrasonic extraction time

In order to select the optimal extraction time and efficiency, the extraction efficiency of ultrasonic extraction time of 10min, 20min, 30min, 40min, 50min, 60min, 70min and 80min was compared at (200 W, 40 kHz). The results showed that when the ultrasonic extraction time was lower than 30min, The extraction efficiency of 14 active ingredients increased slowly, and the content of most active ingredients reached the maximum at 50min, 60min and 70min. Therefore, 50min was selected as the extraction time considering the efficiency.

3.4 Limits of detection and limits of quantitation

The concentration at SNR 3 was taken as the detection limit, and the concentration at SNR 10 as the quantification limit. The results are shown in Table 2.

Table 2: Limits of Detection and Quantitation

Number	Project	Concentration (Ng/Ml)	Signal to noise ratio	The detection limit(mg/kg)	The quantitation limit(mg/kg)
1	Cytosine	0.1	11.6	≤0.006	≤0.02
2	Gastrodin	0.5	150	≤0.006	≤0.02
3	Adenosine	0.5	26.2	≤0.006	≤0.02
4	Cytidine	0.5	16.8	≤0.006	≤0.02
5	Uridine	0.5	10.6	≤0.006	≤0.02
6	Guanine	0.5	17.0	≤0.006	≤0.02
7	P-Hydroxybenzaldehyde	1	13.8	≤0.012	≤0.04
8	Thymine	20	15.8	≤0.24	≤0.8
9	Parishin A	50	32.5	≤0.6	≤2
10	Parishin B	50	17.5	≤0.6	≤2
11	Parishin C	50	12.9	≤0.6	≤2
12	Parishin E	50	17	≤0.6	≤2
13	P-Hydroxybenzyl Alcohol	100	10.9	≤1.2	≤4
14	Guanosine	100	12.7	≤1.2	≤4
15	Militarine	0.5	11.8	≤0.006	≤0.02
16	Dactylorhin A	0.5	15.9	≤0.006	≤0.02

As can be seen from the table, the detection limits of the 16 functional components are between 0.006mg/kg-1.2mg/kg, the quantification limits are between 0.02mg/kg-4mg/kg, and the detection limits and quantification limits are both low.

3.5 Method accuracy and precision

The added recovery and precision of dried gastrodia elata were studied. According to the method of sample preparation, an appropriate amount of reference substance was added, and 6 times of parallel determination was carried out according to the optimized pre-treatment detection method,

and the recovery rate was calculated. When the cytosine level was 0.02mg/g, the average recovery was 91.99% and RSD was 4.70%. The average recovery was 93.79% with RSD of 3.43% when gastrodin was 4mg/g. The average recovery was 86.73% and RSD was 5.41% when adenosine level was 0.1mg/g. The average recovery was 92.45% with RSD of 6.45% when the level of cytidine was 0.01mg/g. When uridine was 0.025mg/g, the average recovery was 95.89% and RSD was 4.96%. When guanine was 0.001mg/g, the average recovery was 87.89% and RSD was 7.63%. When p-hydroxybenzaldehyde dosage was 0.15mg/g, the average recovery was 93.30% and RSD was 6.21%. When the thymine dosage was 0.03mg/g, the average recovery was 92.16% and RSD was 3.69%. When the dosage of Parishin A was 10mg/g, the average recovery was 99.18% and RSD was 2.33%. When the dosage of Parishin B was 10mg/g, the average recovery was 88.07% with RSD of 4.86%. When the dosage of Parishin C was 4mg/g, the average recovery was 93.40% and RSD was 5.18%. When the dosage of Parishin E was 10mg/g, the average recovery was 93.88% and RSD was 7.05%. When the dosage of p-hydroxybenzyl alcohol was 1.5mg/g, the average recovery was 89.50% and RSD was 3.89%. When the dosage of guanosine was 0.1mg/g, the average recovery was 86.77% and RSD was 4.12%. When the Militarine dosage was 0.0002mg/g, the average recovery was 96.30% and RSD was 2.31%. When the dosage of Dactylorhin A was 0.0002mg/g, the average recovery was 91.00% and RSD was 2.58%.

The above results showed that the samples had good parallelism, RSD was less than or equal to RSD≤10%, and the recoveries were 79.90%-103.80%, which met the verification requirements of relevant methods.

3.6 Actual sample determination

A batch of Gastrodia Elata, Rhizome of Conic Gymnadenia and Dendrobium officinale were immediately purchased in the market, and the detection work was carried out according to this method. The detection results of 16 functional components were shown in Table 3 (unit mg/g):

Table 3: Actual sample measurement data (n=3)

Sample	Gastrodia Elata	Rhizome of Conic Gymnadenia	Dendrobium officinale
Cytosine	0.0191	0.00117	0.0138
Gastrodin	3.55	0.741	-
Adenosine	0.0423	0.18	0.39
Cytidine	0.00812	0.0036	0.0589
Uridine	0.0189	0.0607	0.0582
Guanine	0.00237	0.00119	0.006
P-Hydroxybenzaldehyde	0.184	0.0567	0.00695
Thymine	0.0115	0.0109	0.023
Parishin A	9.12	-	-
Parishin B	6.07	-	-
Parishin C	0.977	-	-
Parishin E	5.032	-	-
P-Hydroxybenzyl alcohol	2.27	0.444	-
Guanosine	0.0511	0.243	0.729
Militarine	-	2.19	-
Dactylorhin A	-	1.79	-

Note: "-" means less than the detection limit.

4. Conclusion

Through the optimization of the extraction method of orchid medicinal materials samples and solvent standard, the experiments use liquid chromatography series triple quadrupole mass

spectrometer detection, retention and ion fragment abundance ratio time qualitative and quantitative ion peak area external standard method to simultaneously determine 16 components of orchid medicinal materials. After optimization, the recoveries of 16 components in orchid medicinal materials reaches 79.90%-103.80%, the RSDS of precision and stability within 24 h were ≤10%, the linear range is between 0.04mg/g and 160mg/g, and the limits of detection and quantification could meet the minimum requirements of relevant standards.

The method has good isolation and specificity, low detection limit and quantitation limit, good linear range, good repeatability, accuracy and reliability, which can be popularized and applied to the detection of functional components in orchid medicinal materials.

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