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RESEARCH ARTICLE

Identification of the Metabolites of Scutebarbatine A in Rat Plasma, Bile, Urine, and feces by Using Ultra-high-performance Liquid Chromatography Coupled with Q Exactive Hybrid Quadrupole-orbitrap High-resolution Mass Spectrometry

Lei Zhao[1], Hongjin Wang[2], Nannan Yuan[2], Guochun Yang[3], Jinwei Gao[4,*] and Lixin Sun[2,*]

[1]*School of Traditional Chinese Pharmacy, Shenyang Pharmaceutical University, Shenyang, 110016, China;* [2]*School of Pharmacy, Shenyang Pharmaceutical University, Shenyang, 110016, China;* [3]*School of Pharmacy, Jiangsu Vocational College of Medicine, Jiangsu, 224005, China;* [4]*School of Pharmacy, Shenyang Medical College, Shenyang, 110034, China*

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Abstract: ***Background:*** Scutebarbatine A is a new neo-Clerodane Diterpenoid Alkaloids from Scutellaria barbata, which has many pharmacological effects, such as anti-tumor, antibacterial, and anti-inflammatory. However, there are no studies on the metabolism of Scutebarbatine A.

Objective: The objective of this study is to explore the metabolism of Scutebarbatine A in the body, the bile, plasma, urine, and feces samples of rats after administration were investigated.

Methods: The biological samples were investigated using ultra-high-performance liquid chromatography coupled with Q Exactive hybrid quadrupole-orbitrap high-resolution mass spectrometry (UPLC-Q-Orbitrap-HRMS).

Results: A total of 20 metabolites were identified: 16 phase I metabolites and 4 phase II metabolites. The main metabolic pathways were hydrolysis, oxidation, hydrogenation, dehydration, and combination with sulfate.

Conclusion: This study further elucidates the metabolism of Scutebarbatine A in rats and provides a reference for the study of its pharmacodynamic material basis and pharmacological mechanism.

Keywords: Scutebarbatine A, metabolite identification, metabolic pathway, UPLC-Q- Orbitrap-HRMS, plasma, bile, urine, feces.

1. INTRODUCTION

Scutellaria barbata is the dry whole plant of Scutellaria barbata D.Don, a plant of the scutellaria barbata D.Don in the labiatae. It has the effects of antipyretic and detoxification, removing blood stasis and stopping bleeding, diuresis, and swelling, and is now widely used in the treatment of various malignant tumors as a recognized anti-cancer Chinese medicine [1]. At present, various chemical components such as flavonoids, diterpenoids, polysaccharides, and volatile oils have been isolated from Scutellaria barbata. They have antitumor, antiviral, antibacterial, antioxidant, immune regulation, and other pharmacological effects. Among them, diterpenoids and their lactones are one of its main active ingredients. Many studies have shown that it has cytotoxic effects on different types of cancer cells and shows good anti-inflammatory and antiviral activities [2]. Diterpenoids can be divided into crosane-type diterpenoids and diterpene alkaloids, both of which have anti-tumor activity, such as Scutellaria barbata Linquan alkaloid D, barbatellarine E, Scutebarbatine A, *et al.* [3, 4]. Among them, Scutebarbatine A is a new cropane-type diterpenoid extracted from Scutellaria barbata, and a variety of pharmacological activities have been proven. At present, the research based on the pharmacological activity of Scutellaria barbata and its mechanism of action were mostly aimed at a certain type of ingredients, and there are few studies on a single ingredient. Therefore, the study of the single ingredient of Scutebarbatine A is of certain significance.

Nowadays, tumor treatment has become a knotty problem, and the development and application of anti-tumor drugs are very important. Scutebarbatine A has anti-tumor, anti-inflammatory [5], antioxidant, and other pharmacological activities. Studies have shown that Scutebarbatine A can effectively treat hepatic carcinoma, lung cancer and other oncological diseases by inhibiting cell proliferation and growth [6, 7], regulating immune function [8], stimulating apoptosis [9], and inhibiting protein expression [10]. In addition, Scutebarbatine A can also achieve anti-inflammatory effects by effectively inhibiting the production of NO and has a significant ability to protect cells from H_2O_2 [11-13]. At present, most of the researches on Scutebarbatine A focus on the study of pharmacological effects and its mechanism, and there is no research on its metabolism in the body. Research on drug metabolites in the body is not only an essential element of drug metabolism research, but it is also a necessary source of novel drug development.When modifying the structure of a drug, it is often essential to master the laws of drug metabolism, such as drug metabolism sites, metabolic enzyme types, metabolic forms and pathways, metabolites, *etc.* [14, 15]. Drug metabolism studies can provide references or a basis for structural modification, which has important practical significance in discovering novel drugs with curative effects and lower side effects. Therefore, this study aimed to provide some ideas for the development of related new drugs in the future through the study of the metabolism of Scutebarbatine A *in vivo*.

In this study, UHPLC-Q-Exactive technology was used to quickly and sensitively analyze chitin A metabolites. A total of 21 compounds were identified, including 1 prototype and 20 metabolites. The metabolic pathways were oxidation, reduction, dehydration, hydrolysis, sulfation, and their complex reactions. This result

*Address correspondence to these authors at the School of Pharmacy, Shenyang Pharmaceutical University, Shenyang, 110016, China;
E-mail: sunlixin67@yahoo.com
School of Pharmacy, Shenyang Medical College, Shenyang, 110034, China;
E-mail: gaojinwei0903@163.com

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can provide important information for the metabolism of Scutebarbatine A in the body and provide a reference for follow-up research.

2. MATERIAL AND METHODS

2.1. Chemicals and Reagents

Scutebarbatine A (purity > 99 %) was extracted by the laboratory; HPLC grade acetonitrile, methanol, and formic acid were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA); purified water was purchased from Hangzhou Wahaha Group Co. Ltd. (Hangzhou, Zhejiang Province, China).

2.2. Instrument and Analytical Conditions

2.2.1. UPLC–MS/MS Instrumentation

Vanquish Flex Ultra-Performance Liquid Chromatography (Thermo Fisher Scientific, MA, USA) was used for the liquid system, and ACQUITY UPLC® BEH C_{18} column (2.1 × 100 mm, 1.7 μm) was used as the chromatographic column. Mass spectrometry was performed on a UPLC-Q-Exactive Orbitrap MS (Thermo Fisher Scientific, MA, USA) with a heated electrospray ion source.

2.2.2. Chromatographic Conditions

Chromatographic separations were carried out on an ACQUITY UPLC® BEH C_{18} column (2.1 × 100 mm, 1.7 μm) at 30 °C. The mobile phase was 0.1 % formic acid water (A) – acetonitrile (B). The flow rate was set at 0.2 mL min^{-1}. Injection volume: 5 μL. The gradient procedure was as follows: 0 - 6 min, 10 % - 30 % B; 6 - 14 min, 30 % - 55 % B; 14 - 17 min, 55 % - 85 % B; 17 - 20 min, 85 % - 95 % B; 20 - 20.1 min, 95 % - 10 % B; 20.1 - 23.1 min, 10 % B.

2.2.3. MS Conditions

Mass spectrometry conditions were as follows: ion source: electrospray ionization source; ionic mode: positive ion detection mode; sheath flow rate: 30 arb; auxiliary flow rate: 10 arb; spray voltage: 3.8 kV; capillary temperature: 320 °C; auxiliary gas temperature: 310 °C. The data acquisition mode was Full MS – $ddMS^2$ (Top N, N=10), the mass scanning range of the first mass spectrum was *m/z* 100.0-1500; the resolution was 70000; the mass scanning range of the second mass spectrum was *m/z* 100.0-1500.0; the resolution was 17500; collisional gas: high purity nitrogen; collisional energy: 20, 30, 40 eV.

2.2.4. Animals and Drug Administration

Twelve male SPF SD (Sprague Dawley) rats ((bodyweight 200 g-240 g, Animal production certificate No. SCXK (Liao) 2020-00011; Animal ethics No. SYXK (Liao) 2018-0009) purchased from Liaoning Changsheng Biotechnology Co. Ltd. were raised by the Animal Experiment Center of Shenyang Pharmaceutical University. The feeding conditions were as follows: temperature 22 ± 2 °C, humidity 50 %-60 %, light duration 12 h, except for the 12 h fasting the night before the experiment, free drinking, and eating during the rest of the time.

Twelve healthy male rats were randomly divided into 4 groups (n = 3), which were numbered K, Y1, Y2, and Y3. Group K was used to collect corresponding blank biological samples; groups Y1 and Y2 were used to collect biological samples of plasma and bile after administration, and group Y3 was used to collect urine and feces biological samples after administration. Before the experiment, all animals were bred adaptively in the SPF animal room for 7 days, fasted for 12 h before administration, and free drinking water during the whole process. All experiments were carried out in accordance with the animal experiment guidelines of Shenyang Pharmaceutical University, and the protocol was approved by the animal ethics committee.

Scutebarbatine A solution was prepared with 0.5 % sodium carboxymethyl cellulose. The administration group was given intragastric administration of 40 mg/kg; the blank group was given intragastric administration of 0.5 % sodium carboxymethyl cellulose solution at the same dosage.

2.3. Samples Collection

2.3.1. Plasma Sample

Blood was taken from the rat orbital venous plexus at 0.5, 1.5, 3, 6, 9, 12, 24, and 48 h after administration, and placed in a centrifuge tube treated with sodium heparin, and then the supernatant was centrifuged for 10 min at 4000 rpm and stored in a sample tube at -20 °C.

2.3.2. Urine and Feces Samples

The 3 rats in the Y3 group were placed in metabolic cages, and urine and feces samples were collected at 0-2, 2-4, 4-6, 6-12, 12-24, and 24-48 h after administration of feces sample. The collected feces samples were placed to air dry naturally at room temperature. All samples were stored at -20 °C for testing.

2.3.3. Bile Samples

The rats after administration were anesthetized with pentobarbital, and then bile samples were collected for 0-2, 2-4, 4-6, 6-12, 12-24, and 24-48 h through bile cannulation. The samples were sealed and stored at -20 °C.

2.4. Sample Pretreatment

2.4.1. Plasma Sample Pretreatment

200 μL of plasma samples at each time point were mixed, and precipitated the protein with 3 times methanol. The mixed sample was centrifuged at 4 °C (12 000 r/min) for 10 min, and the supernatant was taken and dried with nitrogen at room temperature. The residue was reconstituted with 200 μL 60 % methanol, centrifuged at 4 °C (12000 r/min) for 10 min, and the supernatant was taken for analysis.

2.4.2. Urine and Bile Samples Pretreatment

The urine and bile samples were taken according to the volume ratio of the samples collected in each time period; a total of 1 mL and 3 mL of methanol was added for extraction. The samples were centrifuged for 10 min at 4 °C and 12 000 rpm. The supernatant was taken and dried under nitrogen at room temperature. The residue was reconstituted with 200 μL of 60 % methanol and centrifuged at 12 000 rpm at 4 °C for 10 min. The supernatant and samples were taken for analysis.

2.4.3. Feces Samples Pretreatment

The feces samples were dried and ground at room temperature and mixed at different time points according to the mass ratio. The mixed sample of 1.0 g was extracted with 5 mL 60 % methanol for 30 min. The supernatant was centrifuged at 4 °C (12 000 r/min) for 10 min and then dried by nitrogen gas at room temperature. The residue was reconstituted with 200 μL 60 % methanol, centrifuged at 4 °C (12000 r/min) for 10min, and the supernatant was taken for analysis.

3. RESULT AND DISCUSSION

3.1. Fragmentation of Scutebarbatine A

There are always many similarities between the parent compound and its metabolites in structure and fragmentation mode. Therefore, understanding the fragmentation pathway and characteristic fragments of the parent compound is of great significance to the identification of metabolites.

The molecular formula of M0 was $C_{32}H_{35}N_2O_7$. In the positive ion mode, the quasi-molecular ion $[M+H]^+$ was *m/z* 559.2399. In the MS^2 spectrum, it can be found that *m/z* 559.2399 continuously lost two molecules of $C_6H_5NO_2$ after a collision to produce fragment ions of *m/z* 436.2095, 313.1798, and 124.0392. The MS^2 spectrum and its fragmentation pathway are shown in Fig. (**1**).

3.2. Identification of Metabolites

First, possible metabolites based on the structure of the parent compound were speculated. Secondly, TraceFinder 4.1 General

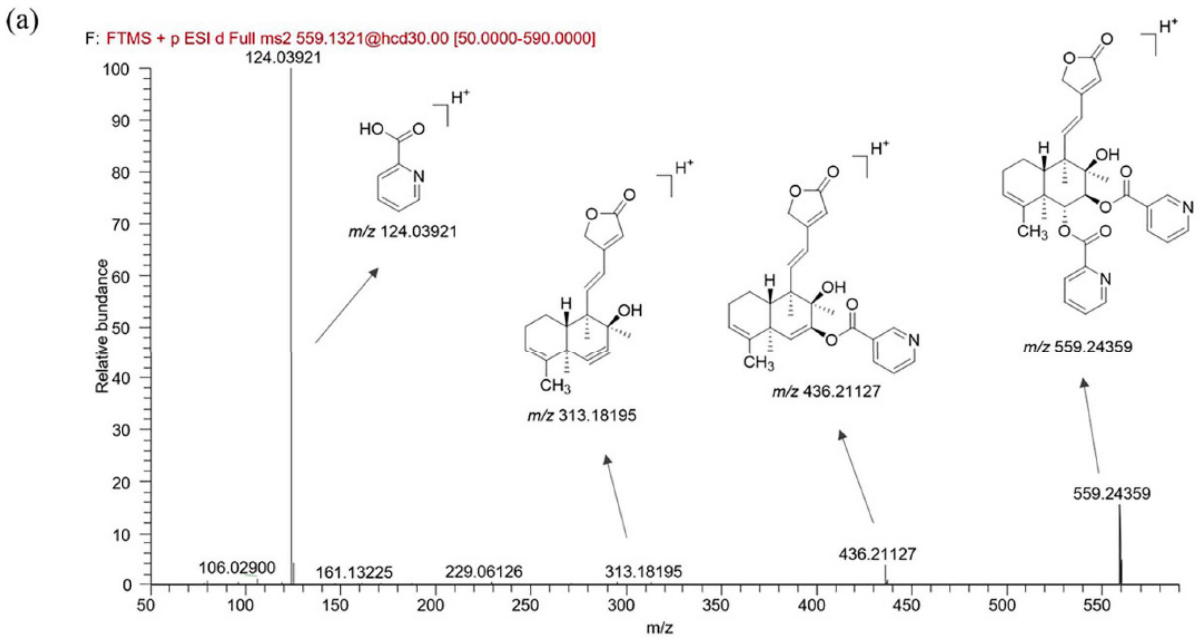

Fig. (1a). MS^2 spectrum of scutebarbatine A in positive ion mode.

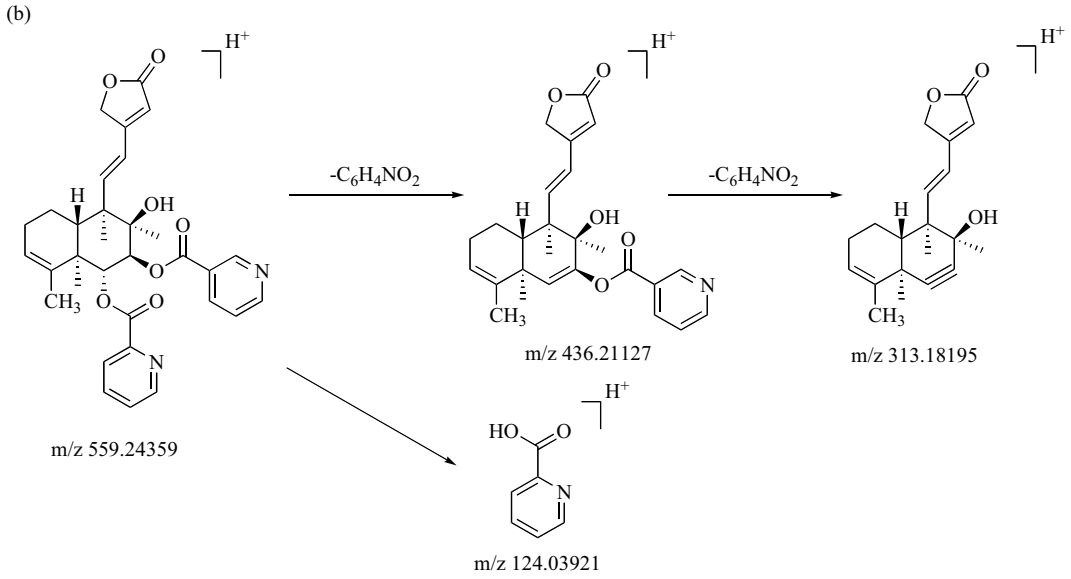

Fig. (1b). The fragmentation pathway of Scutebarbatine A in positive ion mode.

Quan data processing software was used to obtain the total ion current chromatogram of the drug administration sample after subtracting the background and extracting the mass spectrum information of the metabolites from it. Combining the structural characteristics, characteristic fragments, and lysis rules of the parent compound, the inferred metabolites were further verified and identified. The total ion chromatogram subtracted background of metabolites of Scutebarbatine A in rats is shown in Fig. (**2**).

3.2.1. Phase I Metabolites

M1(t_R = 9.84 min) produced the ion peak $[M+H]^+$ at *m/z* 575.2342 ($C_{32}H_{34}N_2O_8$), which was 16 Da more than M0. It was preliminarily inferred that it might be the oxidation product of methyl on M0 or nitrogen on the pyridine ring. Since fragment ions of *m/z* 140.0334 were not detected in the MS^2 spectrum, it was speculated that the reaction was methyl oxidation. The fragment ion *m/z* 452.2049 produced after M1 lost one molecule of $C_6H_5NO_2$ was 16 Da more than the fragment ion *m/z* 436.2095 produced under the same conditions of M0, which further verifies the above inference. Fragment ions *m/z* 557.2249 and 434.1949 were formed by the loss of one molecule of H_2O from *m/z* 575.2342 and 452.2049, respectively. Finally, it was concluded that M1 was the product of the oxidation of the methyl group on the M0 core to alcohol.

The retention time of metabolite M2 was 14.73 min, and the exact mass was 575.2362, which was 16 Da more than M0. It was speculated that it was a metabolite of M0 with one oxygen added, and the molecular formula was $C_{32}H_{34}N_2O_8$. The specific reaction may be methyl oxidation or N oxidation on the pyridine ring, and there were fragment ions with *m/z* 140.0334, so it was speculated that M2 was the nitrogen oxidation product on the M0 pyridine ring. The fragment ions *m/z* 452.2053, 436.2099, and 124.0387 could further verify this speculation.

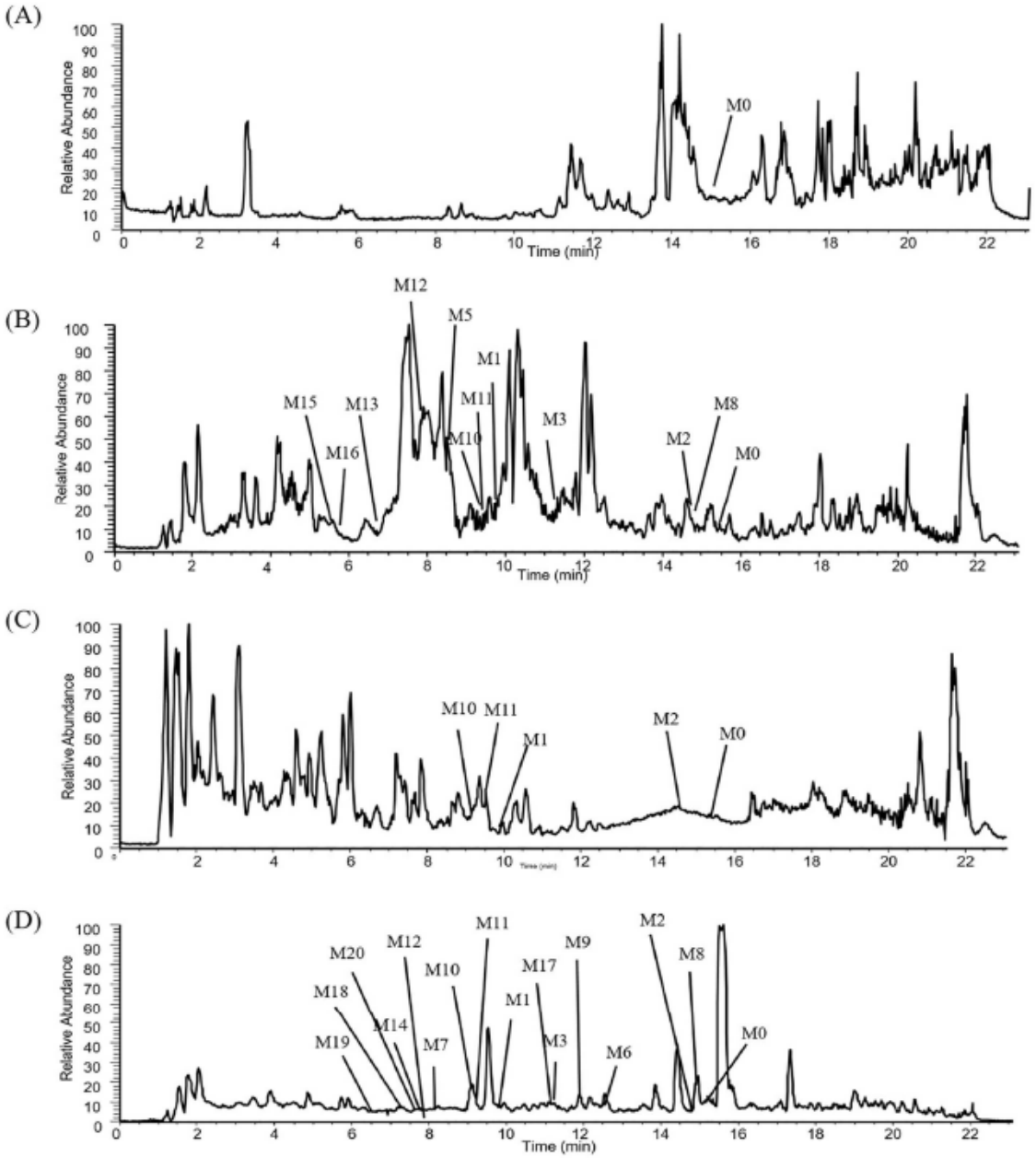

Fig. (2). The total ion chromatogram subtracted backgroung of metabolites of scutebarbatine A in rat (**A**): plasma (**B**): bile (**C**): urine (**D**): feces.

M3 (t_R = 11.15 min) exhibited a $[M+H]^+$ ion at *m/z* 573.2189 ($C_{32}H_{32}N_2O_8$), which was 14 Da more than M0 and 2 Da less than M1 and M2. According to its structural characteristics, no fragment ion of *m/z* 140.03 was detected, it is speculated that M3 may be the product of oxidation of the methyl group of M0 to aldehyde or the product of dehydrogenation of M1. Its fragment *m/z* 450.1905 was 14 Da more than the fragment ion *m/z* 436.2095 in M0 and 2 Da less than the fragment ion *m/z* 452.2049 in M1, which further validates the above speculation.

M4 (t_R = 9.86 min) exhibited a $[M+H]^+$ ion at *m/z* 589.2153, which was 16 Da more than M3. It was speculated that the aldehyde in M3 continues to be oxidized to acid or nitrogen oxidation occurs on the pyridine ring. There was no fragment ion of *m/z* 140.03, so it was speculated that M4 was the metabolite of the aldehyde group in M3 that continues to be oxidized to the carboxyl group.

The retention time of M5 was 8.04 min, and the $[M+H]^+$ was *m/z* 589.2154, which was 16 Da more than M3 like M4. It may be an oxidation reaction of M3, and *m/z* 140.0334 was detected in the secondary fragments. Therefore, it was speculated that M5 was a metabolite of oxidation of the nitrogen of the pyridine ring on the basis of M3.

M6 (t_R = 12.45 min) produced the ion peak $[M+H]^+$ at *m/z* 577.2509, which was 2 Da more than M1 and M2. As there were no *m/z* 140.03 fragments, it was speculated that M6 was the olefin hydrogenation reduction product of M1. The fragment ions *m/z* 454.2179 and 313.1783 were both 2 Da larger than M1 in mass, further confirming that M6 was the olefin hydrogenation reduction product of M1.

The elution time of M7 was 8.13 min, $[M+H]^+$ was *m/z* 595.2611, which was 36 Da more than M0. The fragment ions were *m/z* 436.2093, 313.1781, and 124.0387, which were consistent with the characteristic fragment ions of M0. No fragment ion of *m/z* 140.03 was detected, so it was considered a reaction product on the mother nucleus. It was speculated that M7 might be the product of

the conjugated diene on the nucleus after the oxidation of the alicyclic internal olefin or the product of the continuous reduction of the alicyclic internal olefin after the oxidation of the chain olefin.

The retention time of M8 was 14.88 min, $[M+H]^+$ was *m/z* 557.2243, which was 18 Da less than M1. It was consistent with the fragment ion *m/z* 557.2249 produced by dehydration of M1 and M2. Fragment ion *m/z* 140.03 was not detected. So it was speculated that it was a dehydration product of M1. The remaining fragment ions *m/z* 434.1938 and 124.0387 were consistent with the fragment ions in M1, further verifying the above speculation results. Finally, it was speculated that M8 was the dehydrated product of M1.

M9 (t_R = 11.87 min) produced the $[M+H]^+$ ion at *m/z* 579.2661, which was 2 Da more than M6. The fragment ions *m/z* 456.2364 and 438.2258 were both 2 Da more than M6. It was speculated that M9 was the hydrogenation reduction product of M6.

M10 (t_R = 9.31 min) and M11 (t_R = 9.49 min) produced the same theoretical ion peak $[M+H]^+$ at *m/z* 591,2297. Their molecular mass was 2 Da more than M4 and M5. Compared with M10 and M11, the fragment ion of *m/z* 140.03 was detected in M11 but not in M10. So it was inferred that M10 was the hydrogenation product of M4. Finally, it was speculated that M10 and M11 were hydrogenated products of M4 and M5, respectively.

The theoretical precursor ion peaks $[M+H]^+$ of M12 and M13 both were *m/z* 607.2247, which was 16 Da more than M10 and M11, and the retention times were 7.96 and 6.81 min, respectively. In their MS^2 spectrum, there were fragment ions with *m/z* 140.03 in the secondary fragments of M13 but not in M12. Therefore, it was speculated that M12 and M13 were the oxidation products of M10 and M11, respectively. In addition, the fragment ions *m/z* 589.2128, 484.1919, and 466.1846 of M12 and the fragment ions *m/z* 468.2026 and 452.2044 of M13 could further confirm the above inference.

The elution time of M14 ($C_{32}H_{36}N_2O_9$) was 7.82 min, the precursor ion peak $[M+H]^+$ was *m/z* 593.2431. The secondary fragment *m/z* 575.2348 was consistent with M1 and M2. It was inferred that M14 was a metabolite of M1 or M2. M14 was 18 Da more than M1 and M2, and there was *m/z* 140.0334 in the secondary fragments, which proved that nitrogen oxidation reaction occurred on the pyridine ring. It was speculated that it might be an additional H_2O molecule on the basis of M2. The secondary fragment ions *m/z* 470.2207, 140.0334, and 124.0387 could verify this speculation. Finally, it was speculated that M14 might be the reaction product of the addition of water molecule on olefin of M2 or the olefin hydrogenation reaction after the oxidation of methyl to alcohol.

M15 (t_R = 5.67 min) produced the $[M+H]^+$ ion at *m/z* 623.2197, which was 16 Da more than M12 and M13. Because there was *m/z* 140.0334 in the secondary fragments, it indicated that the reaction might continue to be nitrogen oxidation on the basis of M12. The fragment ions *m/z* 484.1946, 466.1802, and 124.0387 were consistent with M12, which could verify the above speculation.

M16 (t_R = 5.72 min) produced the $[M+H]^+$ ion at *m/z* 625.2351, which was 2 Da more than M15. The fragment ions *m/z* 607.2259, 502.2046, 486.2108, and 468.1964 were all 2 Da more than the fragment ions obtained by the same fragmentation method of M15. The fragment ions *m/z* 140.0334 and *m/z* 124.0387 were consistent with M15. Thus, it was finally speculated that M16 was the hydrogenation product of M15.

3.2.2. Phase II Metabolites

M17 (t_R = 11.02 min) exhibited a $[M+H]^+$ ion at *m/z* 641.2135, which was 82 Da more than M0. Fragment ions with *m/z* 559.2399 (M0) in the MS^2 spectrum were present. It was speculated that M17 might be the product of M0 hydrogenation reduction that continues to occur after the sulfation reaction. The fragment ions *m/z* 436.2093, 313.1793, and 124.0389 were consistent with the fragment ions of M0, which could further verify the above speculation.

M18 exhibited a $[M+H]^+$ ion at *m/z* 655.1896, which was 80 Da more than M1 or M2. Because the fragment ion *m/z* 140.03 was not detected in the secondary fragments, it was speculated that M18 might be the product of the sulfated reaction of M1. The fragment ion *m/z* 575.2404, 434.1954, and 124.0392 were consistent with the M1, so it was inferred that M18 was the sulfated product of M1.

M19 was 2 Da more than M18, the excimer ion peak $[M+H]^+$ was *m/z* 657.2098, and the retention time was 6.69 min. It was speculated that M19 was the hydrogenation reduction product of M18. The secondary fragments *m/z* 575.2377, 434.1957, and 124.0392 were consistent with M18, verifying the above speculations.

The retention time of M20 was 7.76 min, and the precursor ion peak $[M+H]^+$ was *m/z* 673.1999, which was 82 Da more than M10 and M11. Fragment ions of *m/z* 140.03 were not found in M20, so it was speculated that M20 might be sulfated on M10 and continue to be hydrogenated and reduced. The secondary fragment ions *m/z* 591.2305 of M20 were consistent with the precursor ions of M10. The secondary fragment ions *m/z* 575.2359, 468.1966, and 452.2045 were 2 Da more than the fragment ions produced by the same fragmentation pathway of M10. The above results were consistent with the inferred results. Therefore, it was inferred that M20 was the product of continuous hydrogenation reduction after sulfuric acid esterification of M10.

3.2.3. Metabolic Pathways of Scutebarbatine A

In this paper, the metabolites and metabolic pathways of Scutebarbatine A in rat plasma, bile, urine, and feces were identified. A total of 20 metabolites and 1 prototype were identified. In the plasma, only the prototype compound M0 was identified; in the bile, in addition to the prototype compound M0, 12 metabolites (M1~M5, M8, M10~M13, M15, M16) were also identified, all of which were phase I metabolite; in the urine, besides the prototype, 4 phase I metabolites (M1, M2, M10, M11) were also identified; In feces, 16 metabolites were identified in addition to M0, including 12 phase I metabolites (M1~M4, M6~M12, M14) and 4 phase II metabolites (M17~M20). In the entire metabolic process, the phase I metabolic pathway mainly involves the oxidation of hydrocarbon groups, the oxidation of olefins, N-oxidation, the reduction of olefins, and the hydrolysis of ester bonds, while the phase II metabolic pathway only involves the reaction with sulfuric acid. The specific metabolic pathway was shown in Fig. (**3**); mass spectrometry data for each metabolite are shown in Table **1**.

4. DISCUSSION

In this experiment, we adopted UHPLC-Q-Exactive technology with higher resolution, better quality stability, higher sensitivity, and faster analysis speed for complex matrices. After consulting the literature, it was found that there was no related literature on the pharmacodynamics and toxicology of Scutebarbatine A; therefore,the maximum tolerated dose of Scutebarbatine A is unknown. The dosage (about 4.0 mg/kg) of the pharmacokinetic experiment conducted by the research group was referred, and finally, the dosage was determined to be 40 mg/kg. The prototype of Scutebarbatine A and 20 metabolites were detected and identified, including 12 in bile, 4 in urine, and 16 in feces, while only the prototype was detected in plasma. By comparing the peak area of each compound, it was found that M12, M10, and a small amount of M0 were mainly detected in bile; M10 and part of M0 were mainly detected in urine; M7, M12, and a large amount of M0 were mainly detected in feces. It was guessed that the dose given might be too high. The results showed that Scutebarbatine A could be directly absorbed as a prototype in rats, and the main excretion pathway was feces.

Table 1. Mass data of Scutebarbatine A (M0) and its metabolites in rat.

No.	Description	t_R/min	Molecular Formula	Calculated m/z	Detecte m/z	Error ppm	MS/MS	Source			
								Plasma	Bile	Urine	Feces
M0	M0	15.34	$C_{32}H_{35}N_2O_7$	559.23996	559.23999	0.1	436.20959,313.17981, 124.03921	+	+	+	+
M1	Oxidation	9.84	$C_{32}H_{34}N_2O_8$	575.23487	575.23425	-1.1	557.2491,452.20493,434.19498,311.16229,124.03879		+	+	+
M2	Oxidation	14.73	$C_{32}H_{34}N_2O_8$	575.23487	575.23621	2.3	557.22644,452.20532,436,20993, 313.17926,140.03342,124.03875		+	+	+
M3	Oxidation	11.15	$C_{32}H_{32}N_2O_8$	573.21922	573.21893	-0.5	450.19052,327.16019,124.03928		+		+
M4	Oxidation	9.86	$C_{32}H_{32}N_2O_9$	589.21414	589.21535	2.1	571.32031,466.18298,450.19080, 124.03878		+		
M5	Oxidation	8.04	$C_{32}H_{32}N_2O_9$	589.21414	589.21545	2.2	466.18405,450.18903,140.03342, 124.03875		+		
M6	Oxidation+ reduction	12.45	$C_{32}H_{36}N_2O_8$	577.25052	577.25092	0.7	559.24078,454.21790,436.21063, 313.17831,124.03874				+
M7	Oxidation+reduction	8.13	$C_{32}H_{42}N_2O_9$	595.26109	595.26117	0.1	577.25220,472.22971,454.21954, 331.18802,313.17819,124.03875				+
M8	Dehydration	14.88	$C_{32}H_{36}N_2O_7$	557.22431	557.22437	0.1	434.19388,311.16479,124.03874		+		+
M9	Oxidation+reduction	11.87	$C_{32}H_{38}N_2O_8$	579.26617	579.26617	0	561.25647,456.23642,438.22589, 333.20493,315.19440,124.03883				+
M10	Oxidation+reduction	9.31	$C_{32}H_{34}N_2O_9$	591.22979	591.22986	0.1	573.21375,466.23193,450.18954, 124.03881		+	+	+
M11	Oxidation+reduction	9.49	$C_{32}H_{34}N_2O_9$	591.22979	591.22900	-1.3	573.22119,468.19827,452.20428, 140.03346,124.03880		+	+	+
M12	Oxidation+reduction	7.96	$C_{32}H_{34}N_2O_{10}$	607.2247	607.22406	-1.1	589.21283,484.19196,466.18466, 124.03876		+		+
M13	Oxidation+reduction	6.81	$C_{32}H_{34}N_2O_{10}$	607.2247	607.22571	1.7	589.21735,484.19504,468.20267, 140.03348,124.03381		+		
M14	Oxidation+hydrolysis/Oxidation+ reduction	7.82	$C_{32}H_{36}N_2O_9$	593.24554	593.24315	-4.0	575.23486,557.22626,470.22073, 452.20764,434.19446,311.16330, 140.03340,124.03876				+
M15	Oxidation+reduction	5.67	$C_{32}H_{34}N_2O_{11}$	623.21961	623.21973	0.2	605.21021,500.19016,484.19467, 466.18027,140.03343,124.03876		+		
M16	Oxidation+reduction	5.72	$C_{32}H_{36}N_2O_{11}$	625.23526	625.23517	-0.1	607.22595,502.20468,486.21082, 468.19641,140.03340,124.03875		+		
M17	Sulfation+reduction	11.02	$C_{32}H_{36}N_2O_{10}S$	641.21242	641.2135	1.7	559.24091,436.20932,124.03897				+
M18	Oxida-tion+Sulfation	7.31	$C_{32}H_{34}N_2O_{11}S$	655.19169	655.18962	-3.2	575.24042,557.22827,434.19543,124.03928				+
M19	Oxida-tion+Sulfation+eduction	6.69	$C_{32}H_{36}N_2O_{11}S$	657.20734	657.20984	3.8	639.29480,575.23779,534.17914,452.20517,124.03927				+
M20	Oxida-tion+reduction+Sulfation	7.76	$C_{32}H_{36}N_2O_{12}S$	673.20225	673.19995	-3.4	591.23056,575.23590,468.19669,434.19305,124.03878				+

The symbol "+" means this metabolite can be detected in the biological sample.

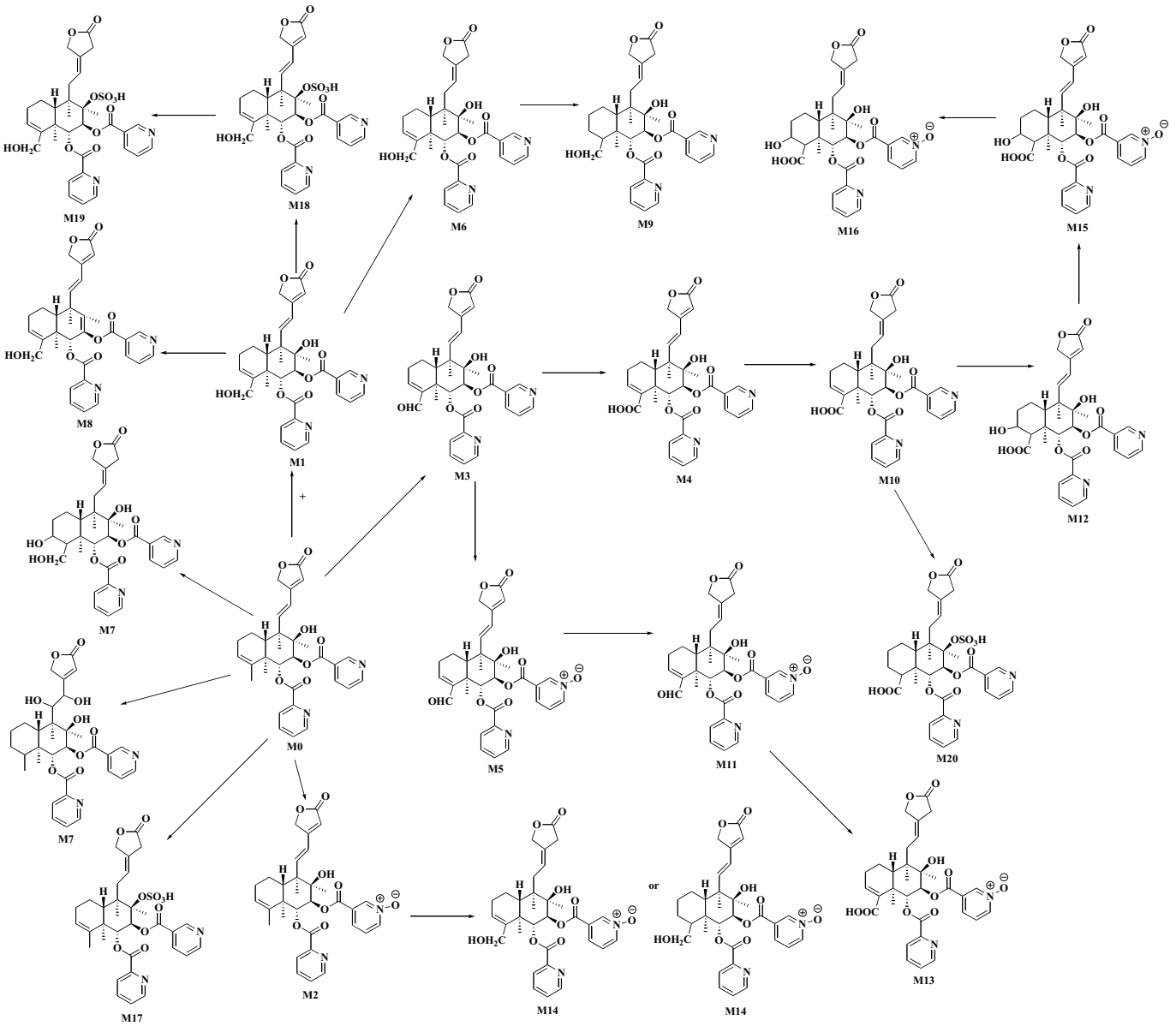

Fig. (3). Proposed metabolic pathways of Scutebarbatine A in rat.

The analysis method and sample processing method were optimized: (1) Analysis method optimization: According to the structure of the compound, the positive ion mode was first considered for detection. At the same time, plasma samples were also tested in negative ion mode. It was found that the response was low in the negative ion mode and the fragment information was very little, so the sample was finally tested in the positive ion mode; the follow-up will focus on its research in negative ion mode. During the optimization process of the spray voltage, it can be seen that as the spray voltage value increases, the response also increases. After reaching the optimal value, the continuation to increase was not required.Therefore, the minimum voltage value of 3.8 kV after the maximum and stable response was selected. The collision energy was adjusted from 20, 40, and 60 eV to 20, 30, and 40 eV according to the response value of the fragment ions. The response of fragment ions increased from 1.55×10^6 to 1.97×10^7. (2) Sample processing method optimization: Because the response was low during point-by-point detection, in order to improve the response, samples collected at different time points were mixed and tested. The influence of different ratios of reconstituted solvents on the identification results was investigated, and it was found that 60 % methanol-water solution could extract and identify more metabolites. Finally, it was determined that the reconstituted solvent was 60 % methanol-water solution.

CONCLUSION

In this paper, UHPLC-Q-Orbitrap-HRMS was used to analyze and identify the metabolites of Scutebarbatine A in rats. By analyzing the precise molecular masses of these metabolites, mass spectrometry fragmentation rules, and characteristic fragment ions, one prototype and twenty metabolites were screened and identified from rat plasma, bile, urine, and feces. This is the first time that the metabolites of Scutebarbatine A in rats have been identified, and twenty of them were all new compounds.The metabolic pathways included phase I and phase II metabolism, which included methyl oxidation, olefin oxidation, N-oxidation, olefin reduction, ester bond hydrolysis, sulfation, and their complex reactions. It provided important information for further study on the metabolites and mechanism of Scutebarbatine A.

LIST OF ABBREVIATION

HRMS = High-Resolution Mass Spectrometry

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The animal study was approved by the Institutional Animal Ethics Committee and performed according to the Regulations of Experimental Animal Administration issued by the State Commission of Science Technology of the People's Republic of China, (SYXK (Liao) 2018-0009).

HUMAN AND ANIMAL RIGHTS

All animal research procedures were followed in accordance with the standards set forth in the eighth edition of Guide for the Care and Use of Laboratory Animals (published by the National Academy of Sciences, The National Academies Press, Washington, D.C.).

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analysed during this study are included in this published article.

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CONFLICTS OF INTEREST

The authors have declared no conflict of interest, financial or otherwise.

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