Analytical Methods

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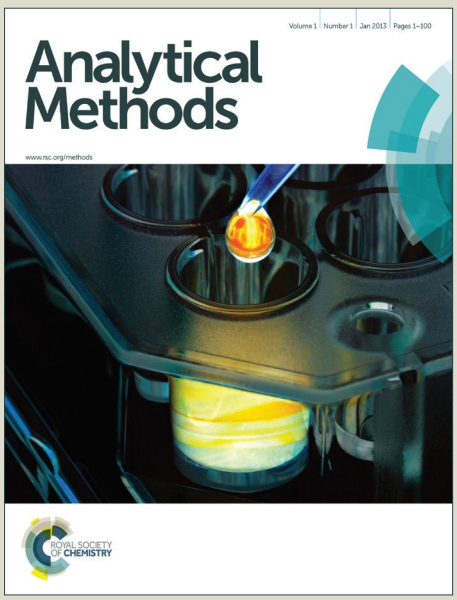

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Metabolic profiles of 11,13α-dihydroixerin Z in rats using High Performance Liquid Chromatography–LTQ–Orbitrap mass spectrometry

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A highly sensitive and specific HPLC-ESI-LTQ-Orbitrap combined with multiple mass defect filters (MMDF) method was used to profile and identify the metabolites of 11,13α-dihydroixerin Z (DIZ) in rats. Plasma was collected after intravenously administered of DIZ to rats (50 mg/kg). Based on the accurate mass measurements, the retention time and mass fragmentation patterns, in total, 40 metabolites were tentatively identified and characterized. The distribution of its metabolites in rats was reported for the first time. Hydroxylation, hydrolysis, methylation, cysteine conjugation, glutathione (GSH) conjugation, sulfate conjugation, N-acetylcysteine conjugation, and glucuronidation were found to comprise the major metabolic reaction of DIZ in rat. These results are very helpful for better comprehension of the metabolism and also can give strong indications on the effective forms of DIZ in *vivo*.

1. Introduction

11,13α-dihydroixerin Z, a sesquiterpene lactone, is a main active principal of *Ixeris sonchifolia* Hance (Bunge), [1] which are widely used as folk medicine in China for its remarkable medical effects, such as dissipating blood stasis and invigorating the circulation of blood.[2-5]

The Kudiezi Injection extracted and puried from the whole herb of *I. sonchifolia* has been approved by State Food and Drug Administration of China to treat cardiovascular diseases.[6-11] DIZ is one of the major sesquiterpene lactone compounds in Kudiezi Injection.[12]

In recent decades, DIZ has been reported to have various activities for instance,

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anti-inflammatory, anti-microbial, and anti-tumor activities, [13,14] although the detailed mechanisms of these actions are not clear. Therefore metabolic profiles of DIZ in *vivo* after intravenously administered are very important for exploring its pharmacological mechanism and truly effective forms.

Developing metabolic profiles for the trace amounts of metabolites in biological samples is a complication risk for the interferences from the matrix or the background. Recently, the technique of high-performance liquid chromatography coupled with mass spectrometry (HPLC/MS) has been extensively applied to analyze and identify metabolites in biological matrices due to its high efficiency, sensitivity, and selectivity.[15-18] Particularly, HPLC-ESI-LTQ-Orbitrap is suitable to achieve the profile of known and unknown metabolites as it possesses high sensitivity and high resolution. Moreover, for the identification of trace metabolites, a great amount of off–line LC–MS data mining methods have been successfully developed. For instance, a lot of data processing technologies have been deeply applied to the identification of complicated compounds or metabolites. Isotope pattern filtering (IPF), extracted ion chromatogram (EIC), product ion filtering (PIF), neutral loss filtering (NLF), multiple mass defect filters (MMDF) and mass defect filter (MDF) are included.[19-21]

Accordingly, in this work, a highly sensitive and specific HPLC-ESI-LTQ-Orbitrap combined with MMDF method was established to profile and identifies the metabolites in rats following intravenously administered of DIZ. To our best knowledge, it is the first time to report the metabolism of DIZ in rats.

2. Experimental

2.1. Chemicals and reagents

Ixerin Z (IZ) and 11,13α-dihydroixerin Z (DIZ) (Fig.1) were isolated from the water extracted of *Ixeris sonchifolia* Hance in our laboratory. Solid-phase extraction columns (3cc/60 mg, 30 μm) were obtained from Waters. Ultra-pure water was freshly prepared using a Milli-Q water purification system (Millipore, Billerica, MA, USA). HPLC-grade acetonitrile was supplied by Fisher Scientific Co. (NJ, USA). All other chemicals and regents were of analytical grade available and commercially available.

2.2. Animals and drug administration

Six male Sprague–Dawley rats, weighing 200–250 g, were obtained from Beijing Weitong Lihua Experimental Animals Company (Beijing, China) and housed with free access to food and water in a week for acclimatization. Before the experiment, all rats were fasted for 12 h and fed with water. DIZ (10 mg/mL) dissolved in deionized water was intravenously administered to each rat at a dose of 50 mg/kg body weight. 0.5 mL blood samples were collected from the retro-orbital venous plexus at 0, 0.5, 1, 2, 4h post-dose. The samples were immediately centrifuged at 3000 g for 15 min to obtain the plasma. After collecting blood samples at 4 h from the rats, the tissues, including heart, liver, spleen, lung, kidney, were rapidly dissected out and flushed with cold normal saline. All samples and tissues were stored at –20 °C until other pretreatment and analysis. The animal protocols were approved by the institutional Animal Care and Use Committee at Beijing University of Chinese Medicine (kj-dw-18-20140505-01).

2.3. Sample preparations

The plasma sample was pretreated by a solid-phase extraction (SPE) method. An SPE cartridge was pretreated with 4 mL of methanol and 4 mL of water, successively. A 1 mL sample of plasma was loaded on Oasis HLB solid phase extraction C18 column, and then was washed with 4 mL of water and 4 mL of methanol, successively. The methanol eluate was collected and evaporated to dry under N_2 at room temperature.

Each tissue was homogenized in normal saline in the ratio of 1:5 g/mL. The obtained tissue homogenate was centrifuged at 10,000 rpm for 10 min. The supernatant was separated out and evaporated to dry under N^2 at room temperature.

The residue of each biological sample was re-dissolved in 100 μL of acetonitrile–water (15/85, *v/v*), and vortexed at 3000 rpm for 3 min. The solution was then centrifuged at 14000 g at 4 ℃ for 15 min, and 10μL supernatant was injected into the HPLC system for analysis.

2.4. Instrumentation and conditions

All LC/MS analyses were performed on a Finnigan LTQ/Orbitrap (Thermo Electron, Bremen, Germany) coupled to an ESI source (Thermo Electron, Bremen, Germany). Metabolites were separated on a SB-C18 column (5 μm, 250 ×4.6 mm, Agilent Technologies, Germany) at room temperature and a flow rate of 1 mL/min. The mobile phase consisted of water (A) and acetonitrile (B) using a gradient elution of 15% to 19% B at 0 min to 10 min, 19 % to 21 % B at 10 min to 35

min, 21% to 80% B at 35 min to 40 min, 80% to 80% B at 40 min to 45 min, 80% to 15% B at 45 min to 46 min, and 15% B for equilibration of the column at 46 min to 55 min.

The optimized operating parameters in the negative ion mode were listed as follows: capillary voltage of 35 V, electrospray voltage of 3.0 kV, capillary temperature of 350℃, sheath gas flow rate of 30 (arbitrary units), auxiliary gas flow rate of 10 (arbitrary units), and tube lens of 110 V. High-resolution MS analysis was operated with a mass range of *m/z* 100–1200 at a resolving power of 30,000. Data-dependent MS^n scanning can be triggered by the fragmentation spectra of the target ions. Helium served as the collision gas. Collision-induced dissociation (CID) was performed with an isolation width of 2 Da, an activation q of 0.25 and activation time of 30 ms. The collision energy was set to 35%.

2.5. Data processing

Thermo Xcaliber 2.1 workstation (Thermo Fisher Scientific) was used for the data acquiring and processing. For computer-based MMDF approach, representative structure with predicted mass defect window were set as filtering template for homologous compounds screening. In order to obtain as many fragment ions as possible, the peaks detected with intensity over 10 000 were selected for identifications.

3. Results and discussion

3.1. Mass fragmentation behaviour of DIZ

For a better understanding of the MS^n fragmentation behaviors of the metabolites, the MS^n fragmentation pattern of the parent compound was examined for the first time by HPLC–LTQ–Orbitrap mass spectrometry. In negative ion mode, DIZ formed a deprotonated molecule $[M-H]^-$ at *m/z* 423.1666 (1.67 ppm, $C_{21}H_{27}O_9$). Fragmentation of this precursor ion provided a characteristic fragment ion at *m/z* 261.1133 (1.14 ppm, $C_{15}H_{17}O_4$) by the cleavage of glucosyl moiety ($C_6H_{10}O_5$) at the C-3 position. The fragment ion at *m/z* 217.1234 (1.06 ppm, $C_{14}H_{17}O_2$), *m/z* 187.0767 (1.32 ppm, $C_{12}H_{11}O_2$), *m/z* 199.1130 (1.24 ppm, $C_{14}H_{15}O$) and 379.1763(1.17 ppm, $C_{20}H_{27}O_7$) were yielded by the loss of $C_6H_{10}O_5 + CO_2$, $C_6H_{10}O_5 + 2CH_3+CO_2$, $C_6H_{10}O_5 + H_2O+ CO_2$, and CO_2 from the ion at the parent ion, which were useful information in metabolite identification. Besides, the fragments at *m/z* 217.1234 and *m/z* 187.0767 can be formed by loss of CO_2 and loss of $2CH_3$

from the ion at *m/z* 261.1132 in the MS^3 spectra. The proposed fragmentation pattern of DIZ was illustrated in Fig. 1.

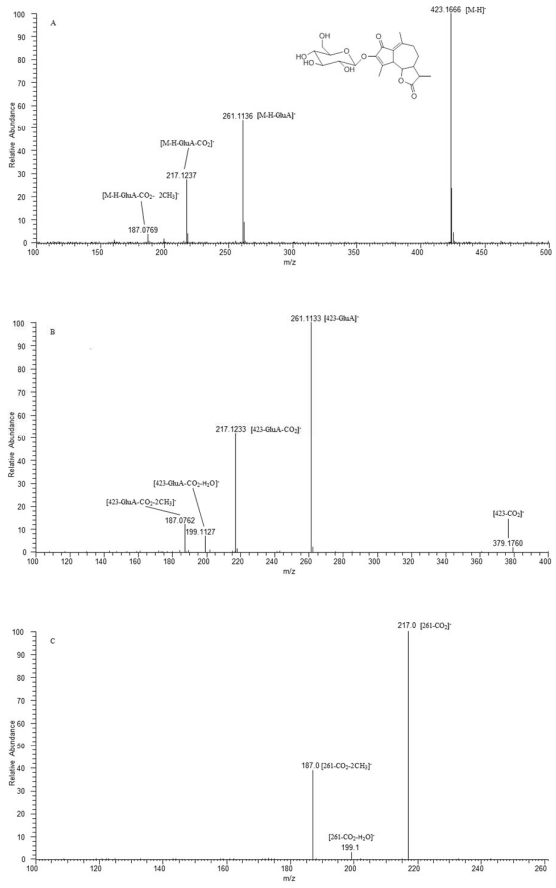


Fig.1. ESI-MS^n spectra of DIZ: (A) MS spectrum; (B) MS^2 spectrum (precursor-ion was m/z 423); (C) MS^3 spectrum (precursor-ion was m/z 261)

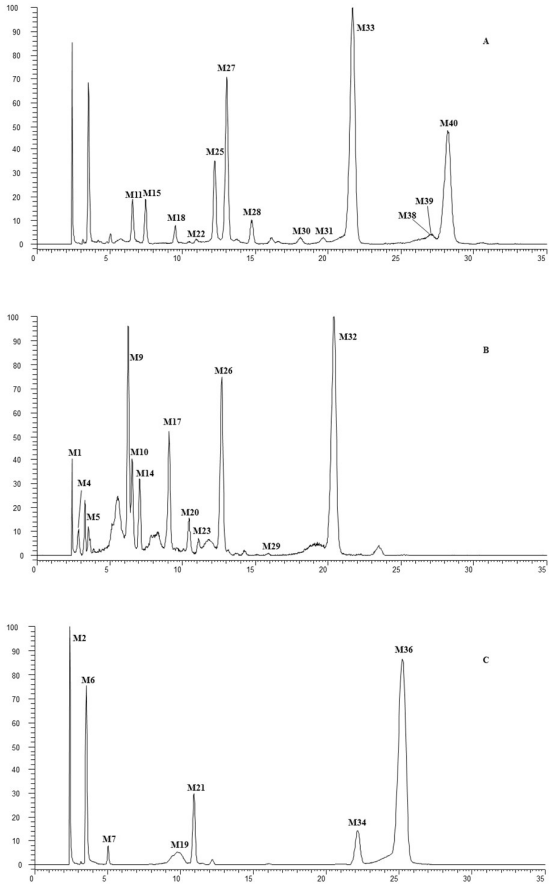
3.2. Analytical methods

The Combination of MMDF and high–resolution EIC (HREIC) methods were implemented for metabolite detection including low level of common and uncommon metabolites. The high–resolution EIC process is extensively adopted to detect common metabolites with predictive molecular weights, whereas the MMDF is highly effective in the detection of uncommon metabolites. For MMDF method, the first and important step was setting the MMDF templates, which are usually drug filter, substructure filer and conjugate filter. The second step was to confirm the mass range and mass defect range according to the templates mentioned above. Each MDF window was frequently set to ±50 mDa around the mass defects and a mass range of ±50 Da around the filter template masses. Finally, the parent drug filter template was based on the location

of DIZ ($C_{21}H_{27}O_9$) with a mass defect range from 115.0 to 215.0 mDa and mass range from 413 to 513 Da. Four types of conjugate filters, including GSH conjugation, cysteine conjugation, N-acetylcysteine conjugation, and sulfate conjugation were also established herein, including DIZ + GSH conjugation with a mass defect range from 200 to 300 mDa and mass range from 678 to 778 Da, DIZ + cysteine with a mass defect range from 133.2 to 233.2 mDa and mass range from 494 to 594 Da, DIZ + N-acetylcysteine with a mass defect range from 129.0 to 229.0 mDa and mass range from 534 to 634 Da, and DIZ + sulfate with a mass defect range from 71.1 to 171.1 mDa and mass range from 453 to 553 Da.

3.3. Detection and structural elucidation of metabolites

After comparison the HXEIC and MMDF of drug samples with corresponding black samples, a total of 40 metabolites as well as the parent drug was detected and identified. The HXEIC of drug samples are shown in Fig 2. The chromatographic and mass spectrometric data of the parent drug and its metabolites are shown in Table 1.



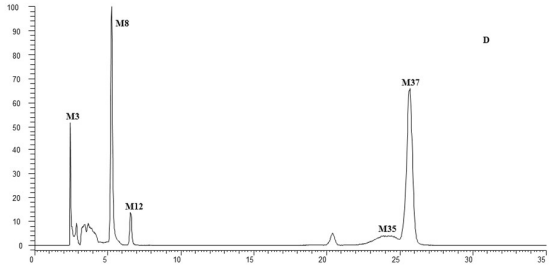


Fig.2. High resolution extracted ion chromatograms for the multiple metabolites in 10 ppm: (A) *m/z* 421.1493, 422.1268, 424.1424, 439.1599, 441.1755; (B) *m/z* 453.1391, 455.1548, 469.1704, 503.1218; (C) *m/z* 423.1650, 542.1681, 584.1796; (D) *m/z* 437.1442, 728.2331

Table 1. The characteristic fragment ions of DIZ metabolites in rats by HPLC-LTQ-Orbitrap MS.

Peak	t_R	Theoretical Mass (*m/z*)	Experimental Mass (*m/z*)	Error (ppm)	Formula $[M-H]^-$	MS/MS fragment	Identification/Reactions
M1	2.4	503.1218	503.1241	4.6	$C_{21}H_{27}O_{12}S$	MS^2[503]: 259.0974 (100), 215. 1076 (36) MS^3[259]: 215 (100)	Sulfate conjugation
M2	2.4	542.1681	542.1716	4.7	$C_{24}H_{32}O_{11}NS$	MS^2[542]: 421.1507 (100), 377.1607 (55), 380.1168(18), 4 55.1380 (18) MS^3 [421]: 259 (100), 377 (71), 215 (68), 257 (58)	Cysteine conjugation
M3	2.4	728.2331	728.2361	4.1	$C_{31}H_{42}O_{15}N_3S$	MS^2 [728]: 306.0764 (100)	GSH conjugation
M4	2.9	455.1548	455.1570	4.9	$C_{21}H_{27}O_{11}$	MS^2 [455]:193.0353(100) MS^3 [193]:131 (100), 175 (44)	Hydrolysis, glucuronidation hydrogenation and hydroxylation
M5	3.5	455.1548	455.1568	4.4	$C_{21}H_{27}O_{11}$	MS^2[455]:193.0352 (100) MS^3 [293]: 219 (100), 231 (20)	Hydrolysis, glucuronidation hydrogenation and hydroxylation
M6	3.5	542.1681	542.1716	4.7	$C_{24}H_{32}O_{11}NS$	MS^2 [542]: 421.1507 (100), 377.1606 (53), 380.1169 (18),455.1381 (17) MS^3 [421]: 259 (100), 215 (66), 377 (63), 257 (60)	Cysteine conjugation
M7	5.1	542.1681	542.1714	4.3	$C_{24}H_{32}O_{11}NS$	MS^2 [542]: 421.1505 (100) MS^3 [421]: 259 (100), 241 (67), 215 (66), 197 (40)	Cysteine conjugation
M8	5.3	728.2331	728.2354	3.1	$C_{31}H_{42}O_{15}N_3S$	MS^2 [728]: 306.0747 (100)	GSH conjugation
M9	6.3	453.1391	453.1413	4.8	$C_{21}H_{25}O_{11}$	MS^2 [453]: 193.0356 (100), 435.1299 (26), 391.1400 (14) MS^3 [193]: 131 (100), 113 (45), 175 (40)	Hydrolysis, glucuronidation and hydroxylation
M10	6.5	455.1548	455.1570	4.9	$C_{21}H_{27}O_{11}$	MS^2 [455]: 293.1036 (100), 249.1137 (62), 231.1027 (62),	Dihydroxylation

M11	6.6	439.1599	439.1619	4.6	$C_{21}H_{27}O_{10}$	411.1642 (54), MS^3 [293]: 219 (100), 231 (20) MS^2 [439]: 215.1071 (100), 277.1082 (35)	Hydroxylation
M12	6.6	728.2331	728.2366	4.8	$C_{31}H_{42}O_{15}N_3S$	MS^2 [728]: 306.0747 (100)	GSH conjugation
M13	6.7	441.1755	441.1776	4.7	$C_{21}H_{29}O_{10}$	MS^2 [441]:423.1659(100), 279.1235(25), MS^3 [423]:187 (100), 217 (39), 261 (24), 199 (17)	Hydrogenation and hydroxylation
M14	7.1	453.1391	453.1411	4.3	$C_{21}H_{25}O_{11}$	MS^2 [453]: 193.0352 (100) MS^3 [193]: 131 (100), 157 (67), 175 (52)	Hydrolysis, glucuronidation and hydroxylation
M15	7.5	439.1599	439.1617	4.2	$C_{21}H_{27}O_{10}$	MS^2 [439]: 277.1082 (100), 215.1076 (31), 233.1082 (15)	Hydroxylation
M16	7.9	441.1755	441.1773	4.0	$C_{21}H_{29}O_{10}$	MS^2 [441]: 279.1235 (100), 235.1324 (24) MS^3 [279]: 261 (100), 234 (52)	Hydrogenation and hydroxylation
M17	9.1	453.1391	453.1414	5.0	$C_{21}H_{25}O_{11}$	MS^2 [453]: 193.0354 (100) MS^3 [193]: 175 (100), 131 (66), 113 (55)	Hydrolysis, glucuronidation and hydroxylation
M18	9.5	439.1599	439.1619	4.6	$C_{21}H_{27}O_{10}$	MS^2 [439]: 277.1084 (100), 215.1076 (51) MS^3 [277]: 203 (100), 233 (33), 215 (29)	Hydroxylation
M19	9.8	584.1796	584.1815	3.2	$C_{26}H_{34}O_{12}NS$	MS^2 [584]: 455.1383 (100), 421.1508 (70) MS^3 [455]: 377 (100)	N-acetylcysteine conjugation
M20	10.5	455.1548	455.1567	4.2	$C_{21}H_{27}O_{11}$	MS^2 [455]: 293.1036 (100), 249.1130 (70), 231.1024 (68) MS^3 [293]: 219 (100), 231 (20)	Dihydroxylation
M21	10.9	584.1796	584.1825	4.9	$C_{26}H_{34}O_{12}NS$	MS^2 [584]: 455.1378 (100), 421.1510 (12)	N-acetylcysteine conjugation
M22	11.1	439.1599	439.1619	4.6	$C_{21}H_{27}O_{10}$	MS^2 [439]: 215.1077 (100), 277.1082 (67), 421.1508 (53) MS^3 [215]: 200 (100), 213 (41)	Hydroxylation

M23	11.1	455.1548	455.1569	4.6	$C_{21}H_{27}O_{11}$	MS^2 [455]: 293.1038 (100), 219.0606 (29) MS^3 [293]: 219 (100), 231 (13)	Dihydroxylation
M24	11.5	441.1755	441.1777	4.9	$C_{21}H_{29}O_{10}$	MS^2[441]:279.1235 (100), 235. 1341 (22) MS^3[279]: 261 (100), 217 (19)	Hydrogenation and hydroxylation
M25	12.2	439.1599	439.1617	4.2	$C_{21}H_{27}O_{10}$	MS^2 [439]: 421.1507 (100), 259.0974 (48), 277.1080 (34) MS^3 [421]: 259 (100), 215 (39)	Hydroxylation
M26	12.7	453.1391	453.1411	4.3	$C_{21}H_{25}O_{11}$	MS^2 [453]: 259.0973 (100), 193.0354 (58), 277.1082 (25), 435.1298 (21) MS^3 [259]: 215 (100)	Hydrolysis, glucuronidation and hydroxylation
M27	13.0	439.1599	439.1617	4.2	$C_{21}H_{27}O_{10}$	MS^2 [439]: 277.1082 (100), 215.1076 (31), 233.1082 (15) MS^3 [277]: 205 (100), 215(87), 233 (87)	Hydroxylation
M28	14.7	439.1599	439.1617	4.2	$C_{21}H_{27}O_{10}$	MS^2 [439]: 263.1287 (100), 359.0194 (8) MS^3 [263]: 219 (100)	Hydrolysis, glucuronidation and hydrogenation
M29	15.9	469.1704	469.1721	3.5	$C_{22}H_{29}O_{11}$	MS^2 [469]: 275.0923 (100), 231.1028 (55), 193.0352 (43) MS^3 [275]: 231 (100)	Hydrolysis, glucuronidation, hydrogenation, hydroxylation and methylation
M30	18.1	439.1599	439.1617	4.2	$C_{21}H_{27}O_{10}$	MS^2 [439]: 263.1290 (100)	Hydrolysis, glucuronidation and hydrogenation
M31	19.7	421.1493	421.1508	3.5	$C_{21}H_{25}O_9$	MS^2 [421]: 259.0980 (100), 215.1077 (41) MS^3 [259]:215 (100), 187 (12)	Dehydrogenation
M32	20.4	469.1704	469.1721	3.5	$C_{22}H_{29}O_{11}$	MS^2 [469]: 437.1450 (100),193.0352 (28) MS^3 [437]:193 (100)	Hydrolysis, glucuronidation, hydrogenation, hydroxylation and methylation
M33	21.7	421.1493	421.1509	3.8	$C_{21}H_{25}O_9$	MS^2 [421]: 259.0973	Dehydrogenation

						(100), 215.1075 (57), 241.0867 (42)	
M34	22.2	423.1650	423.1665	3.6	$C_{21}H_{27}O_9$	MS^3 [259]: 215 (100) MS^2 [423]: 261.1129 (100), 217.1233 (18) MS^3 [261]:217 (100), 187 (33)	Parent Drug isomer
M35	24.4	437.1442	437.1463	4.8	$C_{21}H_{25}O_{10}$	MS^2 [437]: 193.0354 (100) MS^3 [193]:131 (100),113 (43) ,89 (37)	Hydrolysis and glucuronidation
M36	25.2	423.1650	423.1667	4.1	$C_{21}H_{27}O_9$	MS^2 [423]: 261.1132 (100), 217.1234 (57), 187.0766 (14) MS^3 [261]:217 (100), 187(37)	Parent Drug
M37	25.7	437.1442	437.1464	5.0	$C_{21}H_{25}O_{10}$	MS^2 [437]: 193.0355 (100) MS^3 [193]: 131 (100) ,113 (48),175 (42)	Hydrolysis and glucuronidation
M38	27.1	422.1268	422.1289	5.0	$C_{20}H_{24}O_7NS$	MS^2 [422]: 259.0961 (100),162.0233 (67), 215.1070 (59)	Dehydrogenation, hydrolysis and N-acetylcysteine conjugation
M39	27.1	424.1424	424.1446	5.1	$C_{20}H_{26}O_7NS$	MS^2 [424]: 162.0228 (100) MS^3 [162]: 84 (100)	Hydrolysis and N-acetylcysteine conjugation
M40	28.2	422.1268	422.1290	5.2	$C_{20}H_{24}O_7NS$	MS^2 [422]: 162.0233 (100) MS^3 [162]: 84 (100), 120(8)	Dehydrogenation, hydrolysis and N-acetylcysteine conjugation

3.3.1. Metabolites M31, M33, M34 and M36

Metabolites M33 and M36 was identified by comparing the retention time, accurate MS and MS^n spectra with the authentic reference IZ and DIZ, respectively. Metabolites M31 and M34 showed the same deprotonated molecule ion with M33 and M36, suggesting that M31 and M34 were isomers of M33 and M36, respectively.

3.3.2. Metabolite M1, M3, M8, M12, M19, M38, M39 and M40

The metabolite M1 showed an HPLC profile with a retention time at 2.4 min and an ESI-MS spectrum which gave a deprotonated molecular ion at *m/z* 503.1241(4.6 ppm, $C_{21}H_{27}O_{12}S$), 80 Da more than that of DIZ, suggesting that M1 was DIZ sulfate. Metabolites M3, M8 and M12 showed the same $[M-H]^-$ ion at m/z 728.24 ($C_{31}H_{42}O_{15}N_3S$) and then yielded $[GSH-H]^-$ at *m/z* 306.0764 (3.2 ppm, $C_{10}H_{16}N_3O_6S$) , 306.0747 (-2.4 ppm, $C_{10}H_{16}N_3O_6S$), and 306.0747 (-2.4 ppm, $C_{10}H_{16}N_3O_6S$), which suggested that they were glutathione addition of DIZ.

M19 and M21 eluted at 9.8 and at 10.9 min possessed the same $[M-H]^-$ ($C_{26}H_{34}O_{12}NS$) at m/z 584.18, 161 Da more than that of parent drug, suggesting that they were acetylcysteine conjugate, and the fragment ion at *m/z* 421 show the nuclear parent were the Ixerin Z isomers. Therefore the metabolites M9 and M21 were identified as DIZ acetylcysteine isomers.

Metabolites M38 and M40 eluted at the retention time of 27.1 and 28.2 min showed the deprotonated molecule at *m/z* 422.1289 (5.0 ppm, $C_{20}H_{24}O_7NS$) and *m/z* 422.1290 (5.2 ppm, $C_{20}H_{24}O_7NS$) in the full-scan spectra, which was 162 Da less than metabolites M9 and M21, suggesting the nucleus structuce was 3-hydroxydehydroleucodin, which was formed by loss a glucose of DIZ. The ions at *m/z* 162.0233 (8.4 ppm, $C_5H_8O_3NS$) was observed in the MS^2 spectra, suggesting the presence of one acetylcysteine. Therefore the metabolites M38 and M40 were identified as 11,13α-dihydrodehydroleucodin acetylcysteine isomers.

Metabolites M39 eluted at 27.1 min with the deprotonated molecule ion at *m/z* 424.1446 (5.1ppm, $C_{20}H_{26}O_7NS$), 2 Da more than metabolites M38 and M40. The ions at *m/z* 162.0228 (5.3 ppm, $C_5H_8O_3NS$) was observed in the MS^2 spectra, suggesting the presence of one acetylcysteine. Therefore the metabolite M39 was identified as hydrogenation of 11,13α-dihydrodehydroleucodin acetylcysteine.

3.3.3. Metabolites M9, M14, M17 and M26

Metabolites M9, M14, M17 and M26 were eluted at 6.3, 7.1, 9.1 and 12.7 min, respectively. All of them showed a deprotonated molecule ion at *m/z* 453.14 ($C_{21}H_{25}O_{11}$), 30 Da more than that of DIZ. The fragment ion at *m/z* 193.0356 (6.8 ppm, $C_6H_9O_7$), *m/z* 193.0354 (5.8 ppm, $C_6H_9O_7$) in the MS^2 spectra of M9, M17 respectively, show the existence of glucuronide in. Therefore, M9 and M17 were tentatively identified as glucuronide of hydroxylation 11,13α-dihydrodehydroleucodin isomers. The fragment ion at *m/z* 277.1081 was observed by loss 176.0330(8.3 ppm, $C_6H_9O_7$) Da than the precursor ion at *m/z* 453.1411 in M14 MS^2 spectra, suggesting the presence of glucuronide. Therefore, M14 was tentatively identified as glucuronide of hydroxylation 11,13α-dihydrodehydroleucodin isomers. In metabolite M26 MS^2 spectra, fragment ions at m/z 291.0872 was showed by loss 162.0519 (-2.3ppm, $C_6H_{10}O_5$) Da than the precursor ion at *m/z* 453.1411, suggesting the presence of glucose. Therefore, M26 was tentatively identified as hydroxylation and methoxylation 11,13α-dihydrodehydroleucodin isomers

3.3.4. Metabolites M11, M15, M18, M22, M25, M27, M28and M30

Metabolites M11, M15, M18, M22, M25, M27 , M28and M30 were eluted at 6.6, 7.5, 9.5, 11.1, 12.2, 13.0, 14.7, and 18.1min with the quasi-molecular ions of *m/z* 439.1619 (4.6ppm, $C_{21}H_{27}O_{10}$), *m/z* 439.1617 (4.2ppm, $C_{21}H_{27}O_{10}$), *m/z* 439.1619 (4.6ppm, $C_{21}H_{27}O_{10}$), *m/z* 439.1619 (4.6ppm, $C_{21}H_{27}O_{10}$), *m/z* 439.1617 (4.2ppm, $C_{21}H_{27}O_{10}$), *m/z* 439.1617 (4.2ppm, $C_{21}H_{27}O_{10}$), *m/z* 439.1617 (4.2ppm, $C_{21}H_{27}O_{10}$), and *m/z* 439.1617 (4.2ppm, $C_{21}H_{27}O_{10}$), 16 Da more than that of DIZ.

The fragment ion at *m/z* 277.1082, *m/z* 277.1082, *m/z* 277.1082, *m/z* 277.1082, *m/z* 277.1080, *m/z* 277.1082, *m/z* 277.1080, and *m/z* 277.1082 was observed by loss 162.0537(8.8 ppm, $C_6H_{10}O_5$), 162.0537(8.8 ppm, $C_6H_{10}O_5$), 162.0537(8.8 ppm, $C_6H_{10}O_5$), 162.0537(8.8 ppm, $C_6H_{10}O_5$), 162.0535(7.6 ppm, $C_6H_{10}O_5$), 162.0537(8.8 ppm, $C_6H_{10}O_5$), 162.0535(7.7 ppm, $C_6H_{10}O_5$), and 162.0537(8.8 ppm, $C_6H_{10}O_5$)Da than the precursor ion at *m/z* 439 in M11, M15, M18, M22, M25, M27, M28 and M30 MS^2 spectra, suggesting the presence of glucose. Therefore, they were tentatively identified as hydroxylation DIZ.

3.3.5. Metabolites M2, M6 and M7

Metabolites M2, M6 and M7 showed the same deprotonated molecular ion $[M–H]^-$ at *m/z* 542.17($C_{24}H_{32}O_{11}NS$), 119 Da more than that of DIZ. This characteristic ion at *m/z* 421 was

observed by loss 121 Da than the precursor ion at *m/z* 542 in their MS^2 spectra, suggesting the presence of one cysteine residue in. Therefore, M2, M6 and M7 were tentatively identified as cysteine of DIZ.

3.3.6. Metabolites M4, M5, M10, M20, and M23

Metabolites M4, M5, M10, M20, and M23 were eluted at 2.9, 3.5, 6.5, 10.5, and 11.1min. All of them showed a deprotonated molecule ion at *m/z* 455.15 ($C_{21}H_{27}O_{11}$), 32 Da more than that of DIZ. The fragment ion at *m/z* 293.1037, *m/z* 293.1044, *m/z* 293.1036, *m/z* 293.1036, and *m/z* 293.1038 was observed by loss 162.0533(6.3 ppm, $C_6H_{10}O_5$), 162.0524 (0.8 ppm, $C_6H_{10}O_5$), 162.0534(6.9 ppm, $C_6H_{10}O_5$), 162.0534(6.9 ppm, $C_6H_{10}O_5$), and 162.0532(5.7 ppm, $C_6H_{10}O_5$) Da than the precursor ion at *m/z* 439 in M4, M5, M10, M20, and M23 MS^2 spectra, suggesting the presence of glucose. Therefore, they were tentatively identified as dihydroxylation DIZ.

3.3.7. Metabolites M35 and M37

Metabolites M35 and M37, eluted at 24.4 and 25.7 min possessed a deprotonated molecular ion at *m/z* 437.1463 (4.8 ppm, $C_{21}H_{25}O_{10}$) and *m/z* 437.1464(5.0 ppm, $C_{21}H_{25}O_{10}$), 14 Da more than that of DIZ. The fragment ion at *m/z* 193.0354 (5.8 ppm, $C_6H_9O_7$), *m/z* 193.0355 (6.3 ppm, $C_6H_9O_7$) in the MS^2 spectra of M35, M37 respectively, show the existence of glucuronide in. Therefore, they were tentatively identified as glucuronide of 11,13α-dihydrodehydroleucodin.

3.3.8. Metabolites M13, M16 and M24

Metabolites M13, M16 and M24 were eluted at 6.7, 7.9, and 11.5 min, with the deprotonated molecule ion of *m/z* 441.17 ($C_{21}H_{29}O_{10}$). The fragment ion at *m/z* 279.1235 (2.9 ppm, $C_{15}H_{19}O_5$) in the MS^2 spectra of M33-M34, respectively, which were 162 Da ($C_6H_{10}O_5$) less than that of metabolites M38-M41 showed the present of glucosyl group. Therefore, we deduced the change of H_2O was happened at aglycone of DIZ. Finally, they were tentatively identified as hydroxylation and hydrogenation, DIZ isomers.

3.4. Distribution of the metabolites of DIZ in rats

In the present work, the distribution of 40 metabolites in rat tissues was studied for the first time. The results showed that 10 metabolites were observed in liver, including M5, M10, M14, M17, M30, M35, M36-M38, and M40. In the kidney, 9 metabolites (M5, M11, M14, M15, M17, M25, M26, M28, and M37) were found. In the spleen, 6 metabolites (M5, M10, M14, M17, M36,

and M38) were detected. In the Heart, 4 metabolites (M14, M17, M36, and M38) were found, and 3 metabolites (M14, M36, and M38) were found in the lung of rats. None were found in the brain of rats.

In addition, M14, M17, M36 and M38 were distributed more widely than the other metabolites. M14, M36, and M38 existed in the heart, liver, spleen, lung, and kidney; M17 was detected in the heart, liver, spleen, and kidney. Therefore, these four metabolites might play an important role in exerting pharmacological effects of DIZ in *vivo*, and their pharmacological actions deserve further investigation.

4. Conclusion

In this reports, the metabolites of DIZ in rats was fully investigated by HPLC-ESI-LTQ-Orbitrap combined with MDF technique for the first time. A total of 40 metabolites as well as parent drug were profiled and identified, after using two MMDF filter templates. The metabolic reactions of DIZ in rats were Hydroxylation, hydrolysis, methylation, cysteine conjugation, glutathione (GSH) conjugation, sulfate conjugation, N-acetylcysteine conjugation, and glucuronidation.

In addition, the distribution of 40 metabolites of DIZ in rats was reported for the first time, and the result showed that the liver was the major tissue for the distribution of these metabolites, followed by the kidney.

In conclusion, this work thoroughly profiled the metabolites of DIZ in rats and revealed the distribution of 40 metabolites, which is very helpful to understand the in *vivo* metabolic fate, effective forms and pharmacological and toxic actions of DIZ.

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