Metabolites identification of ganoderic acid D by ultraperformance liquid chromatography/quadrupole time-of-flight mass spectrometry

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Running Title: Metabolites Identification of Ganoderic Acid D

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Number of text pages: 26
Number of tables: 1
Number of figures: 5
Number of references: 18
Number of words in the abstract: 192
Number of words in the introduction: 426
Number of words in the discussion: 1583

Abbreviations:
GD, ganoderic acid D; UPLC/Q-TOF, ultraperformance liquid chromatography/quadrupole time-of-flight; MS, mass spectrometry; MDF, mass defect filter.
Abstract

Ganoderic acid D (GD) is the major active triterpenoid in *Ganoderma lucidum*, a daily-used medicinal fungus. However, the metabolic fate of GD remains unknown. In order to know whether GD is extensively metabolized, we first investigated the metabolism of GD in vitro and in vivo. The metabolic profiles of the bile samples obtained from rats in vivo were almost the same as those obtained in vitro. Using ultraperformance liquid chromatography/quadrupole time-of-flight mass spectrometry, totally 25 metabolites were identified from the bile sample. Few metabolites were found in the urine samples. These results indicated that the biliary rather than renal clearance was the major route of excretion. The major metabolites were identified by comparing with the standard reference compounds. Metabolites at low concentrations were identified by interpreting the mass spectra. Both phase I and phase II metabolites were observed. The metabolic transformation included reduction, monohydroxylation, dihydroxylation, trihydroxylation, oxidation, desaturation, sulfation and glucuronidation. The main metabolic soft spots in the chemical structure of GD were 3-carbonyl group, angular methyl groups, 7-hydroxy group and 26-carboxylic acid moiety. Over all, this study gives us an insight into the metabolism of GD, an active oxygenated tetracyclic triterpenoid.
Introduction

Ganoderic acid D (GD), a highly oxygenated tetra-cyclic triterpenoid, is the main active component of *Ganoderma lucidum* which has been widely used as supplementary dietary or prescribed in clinic in eastern countries to cure many kinds of diseases (Shiao, 2003; Paterson, 2006; Zhou et al., 2006; Boh et al., 2007; Olaku and White, 2011). Previous studies from our group revealed that 48 hours of GD treatment inhibited the proliferation of HeLa human cervical carcinoma cells with an IC50 value of 17.3±0.3μM. Flow cytometric analysis and DNA fragmentation analysis indicated that GD induced G2/M cell cycle arrest and apoptosis. The cytotoxic effect of GD was associated with regulated expression of 21 proteins (Yue et al., 2008).

Currently, the metabolic fate of GD still remains unknown. In order to investigate the metabolism of GD and take an insight into the metabolic pathways of the natural oxygenated tetra-cyclic triterpenoids (Guo et al., 2012), as one part of our research, we performed in vivo and in vitro metabolite identification study for GD in rat using the ultraperformance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF MS) coupled with a metabolic identification specific software provided from the instrument vendor. This UPLC/Q-TOF MS coupled with chemical intelligent software workflow has proven to be a fast and effective approach for metabolic identification for xenobiotics in recent years (Bateman et al., 2007;...
In the metabolism studies, it is very important to identify and characterize the chemical structure of metabolites (Leveson et al., 2005; Holčapek et al., 2008). In present study, the main metabolites were easily identified by comparing fragmentation ions obtained from MS and chromatographic behaviors of the authentic standards. However, the assignment of the minor unknown metabolites was a challenge due to the complication of the mass fragmentation patterns in the tetracyclic skeleton of GD. Fortunately, we had systematically studied the fragmentation pathways of oxygenated tetracyclic triterpenoids (Cheng et al., 2011) and found out that the mass cleavages of the ring skeleton were very characteristic, and related to the chemical structure (Figure 1). These fragmentation patterns can be used as a critical clue during the study of GD metabolism. In the end, 25 metabolites were identified from rat bile, and the metabolic pathways of GD were proposed. To the best of our knowledge, this is the first report ever on the full metabolite profiles of GD, a highly oxygenated tetracyclic triterpenoid. In addition, this study also exhibits the effectiveness of high resolution mass spectrometry in metabolic studies of the complicated drug, especially the natural product related drugs.

**Materials and Methods**

**Chemicals and Reagents**

Ganoderic acid D and the reference compounds (ganoderic acid B and ganoderic
acid E) were isolated from the fruiting bodies of *G. lucidum*. Their chemical structures were unambiguously identified by the comparison of their NMR and MS spectra data with those reported in the literature (Kubota et al., 1982; Kikuchi et al., 1985; Kohda et al., 1985; Cheng et al., 2010). Their purities were over 98% determined by HPLC/UV analysis. Acetonitrile, methanol and formic acid were of HPLC grade (Burdick & Jackson, Honeywell International Inc., USA). Ultra-pure water was prepared using a Milli-Q water purification system (Millipore, MA, USA). Pooled rat liver microsomes were purchased from In Vitro Technologies (Maryland, USA). 1,1,1-tris(hydroxymethyl)aminomethane (50-100 mM) and the NADPH-regenerating system were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were analytical reagent grade (Sinopharm Chemical Reagent Co. Ltd., China).

**Animals**

Male Sprague-Dawley rats (180-220g) were obtained from the Laboratory Animal Center of Shanghai Institute of Materia Medica. They were kept in an environmentally controlled animal room (20±2°C, 60±5% humidity; 12-h dark/light cycle) for 7 days before starting the experiments. Rats were fed standard laboratory food with water ad libitum and fasted overnight before the test. The animal facilities and protocols were approved by Animal Care and Use Committee of Shanghai Institute of Materia Medica.

**Drug administration and sample preparation**
Pure GD (14 mg) was dissolved in 1 ml acetone. Stearic acid (28 mg) and soybean lecithin (54 mg) were dissolved in 3 ml CH$_2$Cl$_2$. The two solutions were mixed and evaporated at 40˚C to dryness in vacuum. The residue was dissolved in 1% tween-80 (6 ml), and sonicated for 2 hours. The homogeneous solution was given to rats orally at 10 mg/kg.

Under light anesthesia, bile fistulas in 3 rats were cannulated with polyethylene-5 tubing for bile collection. The bile was collected for 12 h after dosing. The rats were allowed to recover from anesthesia before the administration. Blank bile was collected prior to dosing. HCl solution (2%) was added to the bile samples (300 μl) until pH=2. The solution was extracted twice, each time with 600 μl ethyl acetate. The combined extracted solution was blown to dryness with nitrogen stream. The residue was dissolved with 200 μl methanol and filtered through a 0.22 μm microporous membrane prior to injection.

Rats were held in the metabolism cages, and urine samples (including the blank) were collected for 24 h. The pH of the urine (15 ml) was adjusted to 2 with HCl solution (2%) before it was extracted with ethyl acetate (30 ml×2). The combined extracted solution was blown to dryness with nitrogen stream. The residue was dissolved with 200 μl methanol and filtered through a 0.22 μm microporous membrane prior to injection.

Blood was collected from the angular vein at 0.25, 0.5, 1, 2 h. Sample from each time point was first centrifuged at 4000 rpm for 10 min, then 400 μl of the plasma was taken for further sample preparation. Each of the plasma sample was treated with 200
μl of 2% HCl and 1.2 ml acetonitrile to adjust the pH and to precipitate the protein. After centrifuging at 4000 rpm for 5 min, the supernatant was blown to dryness with nitrogen stream. The residue was dissolved with 200 μl methanol and filtered through a 0.22 μm microporous membrane prior to injection.

**In vitro metabolism studies**

The rat liver microsomes were carefully thawed on ice before the experiment. GD (100 μM) was mixed with the microsomes (the concentrations of microsomal protein were 1.0 mg/ml) in 100 mM 1,1,1-tris(hydroxymethyl)aminomethane buffer (pH 7.4). The total incubation volume was 1 ml. After 3 min of preincubation at 37 °C, the incubation reaction were initiated by the addition of NADPH (3.0 mM) and UDPGA (3.0 mM). After a 1 h incubation, the reactions were terminated with 1 ml ice-cold acetonitrile. The mixture was centrifuged at 12000 rpm for 15 min, 10 μl of the supernatant was directly injected for UPLC/Q-TOF MS experiments. Control samples containing no NADPH or substrates were prepared. Each of the incubations was performed in duplicate.

**LC-MS methodology**

Chromatographic separation was achieved using an Acquity UPLC system (Waters, Milford, MA) on an Acquity UPLC HSS T3 column (1.8 μm, 2.1×100 mm; Waters). The mobile phase was a mixture of 0.05% formic acid in water (A) and acetonitrile (B). The gradient elution started from 98% A to 40% A linear in 16 minutes, then
reach 0% A linear at 17 minute, equilibrated at 98% A for 3 minute. The column temperature was set at 45 °C, and the flow rate was 0.6 ml/min. The mass spectrometry detection was conducted using a Waters Xevo G2 Q-TOF high resolution mass spectrometer operated in negative ion electrospray mode. Nitrogen and argon were used as desolvation gas and collision gas, respectively. Data acquisition was from 50-1200 Da with a source temperature set at 120 °C, a desolvation temperature set at 550 °C, and cone voltage set at 16 V. A centroid data collection mode was used. Lock spray standard was 400 ng/ml leucine enkephalin (m/z 554.2615 in ESI-) solution infused at 5 μl/min. A MS^E data acquisition was used. The collision energy was 2 V for the low collision energy scan, and 30-70 V for the high collision energy scan. The mass spectrometer and UPLC system were controlled by MassLynx 4.1 software (Waters, Milford, MA). Data processing was by MetaboLynx XS 2.0 (Waters, Milford, MA). As a result of the MS^E data acquisition, for each sample, both precursor ion and fragment ion information were obtained from a single LC injection. During data processing, MetaboLynx XS would first apply exact mass defect filter (MDF), and then compare all peaks (still remaining after applying MDF) from dose sample with control sample. The delta mass employed for the mass defect filter is 50 mDa. Potential metabolites were reported from the MetaboLynx browser, which could be studied further for structural elucidation by simply review the fragment ions obtained from the same injection.

Results
Characterization of GD and the metabolites in rat bile

Thirty-two potential metabolites were listed from the MetaboLynx Browser after the UPLC/Q-TOF MS<sup>5</sup> data acquisition and the data processing by MetaboLynx XS 2.0 (Supplemental Figure 1). As a result, the general metabolic patterns were tentatively assigned (Supplemental Figure 2). After further review of the fragment ion information, a total of 25 metabolites of GD were identified from rat bile sample (shown in Figure 2). The structures of metabolites were confirmed by the comparison of chromatographic retention times and mass spectra with available reference standards or by study of the characterized mass spectral fragmentation patterns. Table 1 listed the detailed information of these metabolites. Proposed structures of the metabolites were shown in Figure 3 and Figure 4.

Parent compound M0. The retention time of the parent compound GD was 11.3 min. The high collision energy mass spectrum of GD was shown in Figure 1. In negative ion mode, a series of important fragment ions were obtained. The proposed fragmentation pathways were depicted in Figure 1. After the prominent loss of H<sub>2</sub>O and CO<sub>2</sub>, the mass fragmentation occurred on the ring skeleton. The ion of m/z at 301.180 (C<sub>19</sub>H<sub>25</sub>O<sub>3</sub>, calculated m/z 301.180) was formed by the cleavage of the ring D. This ion further lost a molecule of H<sub>2</sub>O to generate the ion at m/z 283.169 (C<sub>19</sub>H<sub>23</sub>O<sub>3</sub>, calculated m/z 283.169). The ion of m/z at 451.283 (C<sub>29</sub>H<sub>39</sub>O<sub>4</sub>, calculated m/z 451.285) went through a ring C cleavage to form the ion at m/z 247.133 (C<sub>15</sub>H<sub>19</sub>O<sub>3</sub>, calculated m/z 247.133), and further cleaved at ring A to generate the ion...
of m/z at 149.060 (C₉H₉O₂⁻, calculated m/z 149.060).

Metabolite M1. Metabolite M1 (retention time tᵣ = 9.9 min) was the most abundant metabolite detected in vivo. It showed a [M-H]⁻ ion at m/z 515.302 (Table 1), 2 Da heavier than the parent compound. The fragment ions of m/z at 303.197 (C₁₉H₂₇O₃⁻, calculated m/z 303.196) and 249.149 (C₁₅H₂₁O₃⁻, calculated m/z 249.149) generated from ring D cleavage and ring C cleavage respectively, were 2 Da heavier than the parent compound; while 149.060 resulted from ring C and ring A cleavages remained the same. These results indicated that the modification occurred on the 3-carbonyl group of ring A. Therefore M1 was identified as 3,7-dihydroxy-11,15,23-trioxolanost-8-en-26-oic acid. This conclusion was confirmed by comparing the retention time and high collision energy mass spectrum with those of the authentic standard (ganoderic acid B). M1 and M0 could also be detected from the bile sample at 252 nm using a LC-DAD instrument.

Metabolite M2. Metabolite M2 (retention time tᵣ = 11.5 min) exhibited a [M-H]⁻ ion at m/z 511.270, 2 Da lighter than the parent compound. The fragment ion at m/z 299.166 (C₁₉H₂₃O₃⁻, calculated m/z 299.165) generated by the cleavage of the ring D and the loss of methyl radicals from m/z 449.269 (C₂₉H₃₇O₄⁻, calculated m/z 449.269) to 434.246 (C₂₈H₃₄O₄⁻, calculated m/z 434.246) or 419.222 (C₂₇H₃₁O₄⁻, calculated m/z 419.222), indicated the presence of the 7,11-dioxo skeleton (Cheng et al., 2011). Therefore, M2 was identified as 3,7,11,15,23-pentaoxolanost-8-en-26-oic acid. The structure of M2 was confirmed by comparing with the authentic standard (ganoderic acid E).
Metabolites M9 and M10. Both metabolites M9 and M10 (retention time $t_R = 8.9$ min and 8.7 min) exhibited a [M-H]$^-$ ion at $m/z$ 529.281, 16 Da heavier than the parent compound. The high collision energy mass spectra of M9 and M10 were the same, suggested that they were a pair of isomers. As shown in Figure 5, except the ions formed by the loss of H$_2$O, CO$_2$ or HCHO, the key fragment ions of $m/z$ at 287.166 (C$_{18}$H$_{23}$O$_3^-$, calculated $m/z$ 287.165) and 233.118 (C$_{14}$H$_{17}$O$_3^-$, calculated $m/z$ 233.118) were generated from the ring D cleavage and ring C cleavage, respectively, followed by the loss of a molecule of HCHO. The key ion of $m/z$ at 149.062 was generated from ring C and ring A cleavages. These data indicated that the hydroxylation occurred on angular methyl group of ring A (position 28 or 29). Therefore, M9 and M10 were tentatively characterized as 7,28(or 29)-dihydroxy-3,11,15,23-tetraoxolanost-8-en-26-oic acid.

Metabolite M23. Metabolite M23 (retention time $t_R = 8.5$ min) had a [M-H]$^-$ ion at $m/z$ 595.258. The ions of $m/z$ at 285.185 (C$_{19}$H$_{25}$O$_2^-$, calculated $m/z$ 285.186) and 231.140 (C$_{15}$H$_{19}$O$_2^-$, calculated $m/z$ 231.139) were formed from the ring D cleavage and ring C cleavage, respectively, followed by the loss of a molecule of H$_2$SO$_4$. The ion of HSO$_4^-$ ($m/z$ 96.960) was also observed. In the chemical structure of GD derivatives, the 7-hydroxy-15-oxo groups are the crucial features for the cleavage of ring C (Cheng et al., 2011). The existence of the ring C cleavage suggested that the 7-OH remained intact, and the sulfation occurred on 3-OH. A reduction reaction first occurred on 3-carbonyl group, then the 3-hydroxy group was conjugated with sulfuric acid. Therefore, M23 was tentatively identified as 7-hydroxy-11,15,23-trioxo-3-
sulfooxylanost-8-en-26-oic acid.

The detailed interpretation of all other metabolites can be found in the supplemental data.

Characterization of the metabolites in rat plasma and urine

In the UPLC/Q-TOF MS chromatograms of the plasma and urine samples, the main metabolite M1 (ganoderic acid B) and the parent drug (ganoderic acid D) could be detected in the plasma (Supplemental Figure 3 and Supplemental Figure 4). However, no metabolite or the parent compound could be detected in the urine sample (Supplemental Figure 5 and Supplemental Figure 6).

Characterization of the metabolites of GD in vitro in rat liver microsomes

The metabolites detected in the rat liver microsomes incubations were similar to those found in the bile samples. The metabolite M1 formed by the reduction was the most abundant metabolite in vitro. The monohydroxylated metabolites in the incubations included M4, M5, M8, M9, and M10. The dihydroxylated metabolites detected in vitro included M15 and M20. The metabolite of M13 formed by the hydroxylation and reduction was observed. The phase II metabolites of M24 and M25 formed by the glucuronidation reaction were also identified in the in vitro samples.

Discussion
Metabolism of GD in rat

In the present study, we have first investigated the metabolites of GD from rat bile, plasma, and urine. By using the UPLC/Q-TOF MS scan coupled with the MetaboLynx XS screening, 25 metabolites were tentatively identified from the bile, and one metabolite from the plasma. There was no metabolite or parent drug found in the urine. These results indicated that the hepatocyte metabolism was the major route of clearance for this compound. This result was in agreement with the theory that the drugs and metabolites whose molecular weight ranged from 500-600 Da preferentially excreted into the bile (the molecular weight of GD was 514 Da) (Ghibellini et al., 2006). Because the volume of the blood sample was low, only ganoderic acid B and the parent compound (GD) were found in the plasma sample.

The metabolic profiles of the in vivo samples were in agreement with those of the in vitro samples. All phase I and phase II metabolites found in vitro were also observed from the bile samples. Because the analyte concentrations of the bile samples were higher, more low-level metabolites, such as monohydroxylated metabolite (M6), dihydroxylated metabolite (M17) and trihydroxylated metabolite (M21), were found from the in vivo samples. Both the in vivo and in vitro experiments indicated that liver played an important role in the clearance of GD in rat.

Proposed metabolic pathways of GD were depicted in Figure 4. Both phase I and phase II metabolites were observed. The formation of the phase I metabolites in rats involved reduction (M1), hydroxylation (M4-M10, M15-M22), desaturation (M3), and oxidation (M2). Among phase I metabolites, the product of reduction (ganoderic
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acid B) was the most abundant metabolite. This compound was a natural compound separated from *G. lucidum*. The hydroxylation products were also extensively observed. This type of reaction involved the addition of one, two, or even three hydroxyl groups to the parent drug. In some metabolites, two metabolic transformations were simultaneously occurred. For example, metabolite M11 was formed by the desaturation reaction followed by a hydroxylation reaction. The phase II biotransformation observed included sulfation and glucuronidation. The phase II metabolite M23 was formed by a reduction reaction followed by a sulfation reaction. M24 and M25 were formed by the conjugation between the parent compound and glucuronic acid. The glucuronidation metabolites of metabolite M1 (ganoderic acid B) were also detected by the low collision energy scans and predicted by the MetaboLynx software (Supplemental Figure 2), however, the signals of the corresponding high collision energy mass spectra were too weak to give a reasonable chemical structure.

The chemical structure of GD is comprised of a highly oxygenated tetra-cyclic ring skeleton and an acidic side chain (Figure 1). The 3-carbonyl group is the most vulnerable metabolic soft spot. It can be quickly transformed to hydroxyl group by reduction reaction. However, this type of reaction wasn’t observed on other carbonyl group in the chemical structure of GD. The angular methyl groups (on position 19, 28, 29, 30) easily suffer from the hydroxylation reactions. Interestingly, the hydroxylation reaction hasn’t been observed to occur on the the C-H bond of the ring (except C-2). For example, the 12-OH derivatives of GD are extensively found from the extraction
of *G. lucidum* (Cheng et al., 2011), however, this type of compounds haven’t been detected in the bio-samples of GD. This phenomenon may be caused by the spatial structure of the GD. The angular methyl groups are on the outside of the chemical structure, which are easily exposed to the large size enzyme that mediates the hydroxylation reactions. Due to the high steric hindrance, the C-H bonds on the ring cannot be associated with the active site of the enzyme and do not suffer from the hydroxylation reaction. The 7-hydroxyl group can be oxidized to carbonyl group or conjugated to the glucuronic acid. The C16-C17 can go through desaturation reaction to form the α,β-unsaturated carbonyl group. The 26-carboxylic acid group can form O-glucuronide. The α or β position of the carboxylic acid group can also undergo hydroxylation reactions. In short, the main metabolic soft spots of the chemical structure of GD are the 3-carbonyl group, angular methyl groups, 7-hydroxyl group and 26-carboxylic acid moiety.

**Interpretation of the mass spectra and the importance of the high resolution mass spectrometry**

The characterization of the metabolites of GD was a challenge due to the complication of the fragment ions generated by the cleavages of the ring skeletons. Previously, we had systematically studied the fragmentation pathways of oxygenated tetracyclic triterpenoids. We found that the fragmentation of the triterpenoid acids were rather characteristic in negative ion mode. After the prominent loss of H₂O and CO₂, cleavages took place on the A, B, C and D rings. The cleavage site was highly
dependent on the positions of the carbonyl groups and hydroxyl groups in the
tetracyclic skeleton (Cheng et al., 2011). The GD is a 7-hydroxy-11,15-dioxo
derivative. The main fragmentation pathways of this type of compounds are the
cleavages of ring C and ring D. Different substitutions on the chemical structure will
result in different \( m/z \) of the fragment ions. These decomposition features are
extremely useful in distinguishing the isomers generated by the metabolic
transformation.

The fragment ions generated by the Q-TOF mass spectrometry are abundant. The
spectra generated can be divided into two areas as depicted in Figure 5. The \( m/z \) of the
first area ranges from 50-350. The fragment ions in this area are very characteristic
and generated by the ring cleavages. Therefore, they are used to determine the
chemical structure of the ring skeleton. The other area with \( m/z \) ranged from 350-550
is used as important data to determine the side chain of the chemical structure.

The high resolution mass spectrometry is very important in the metabolic studies.
The Q-TOF instrument offers high sensitivity in the full scan MS and MS\(^E\) modes of
operation. More importantly, it can measure the exact mass of the precursor ion and
fragment ions, and provides elemental composition of the corresponding ions. There
are opinions that the low resolution mass spectrometry instruments, such as ion-trap,
triple-quadrupole, and quadrupole-ion trap, are enough for the identification of the
metabolites. However, the correct interpretation of the low resolution mass spectra
becomes increasingly difficult when we confront with complicated chemical structure,
especially the natural drugs.
The present study exhibits the importance of the high resolution mass spectrometry and proves where its value exists. For example, the $\Delta m$ of the loss of two methyl radicals and a molecule of HCHO are both 30 Da. In the low resolution mass spectrometry, we cannot distinguish the two species from one another. In high resolution mass spectrometry, the exact mass of two methyl radicals is 30.047, while the exact mass of HCHO is 30.011. The two species can be easily distinguished. In Figure 5, the $\Delta m$ between the two ions of $m/z$ at 511.271 and 481.260 is 30.011. This data suggests that a molecule of HCHO lost from the fragment ion of $m/z$ at 511.271, and indicates the existence of a hydroxymethyl group connected to a quaternary carbon. Without the information the high resolution mass spectrometry provided, we might make a mistake, and consider it ($\Delta m=30$) to be the loss of two methyl radicals and lead to the false identification of a 7,11-dioxo derivative.

When performing metabolites identification for biological samples, the most difficult challenge is the interferences of the endogenous compounds from the biological matrix. This is where the exact mass capability from the time of flight mass spectrometer plays a crucial role. During data processing, MetaboLynx XS will apply a mass defect filter to the raw data set so that only the ingredients with mass defects that fits into the parameter requirement will be included for further data mining, all others will be excluded. The mass defect parameters are set based on the parent drug mass defect plus all mass detects from the fragments that potentially could become the sub-parent form metabolites. Supplemental Figure 1 shows the effects of the exact mass defect filter. Many irrelevant peaks are excluded.
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The software will further process the MDF filtered raw data files to compare all peaks from dose sample with control sample. Potential metabolites are displayed in MetaboLynx browser (Supplemental Figure 2), and can be further studied for the structural elucidation. Most of the metabolites in the list can be characterized, only the structures of several minor metabolites can’t be assigned due to the weak signal of the fragment ions.

Conclusion

The present study reports for the first time on the comprehensive metabolic profile of GD in vivo and in vitro. The results reveal that the hepatocyte metabolism is the major route of clearance for GD. By using UPLC/Q-TOF MS/MetaboLynx XS workflow, a total of 25 metabolites were identified from the rat bile samples. GD could undergo extensive phase I and II metabolism in rat before excreted into bile. The phase I metabolism included reduction, hydroxylation, oxidation, and desaturation. The reduction product (ganoderic acid B), which is also a naturally occurring compound, was the main metabolite. The hydroxylation metabolites, including monohydroxylation products, dihydroxylation products, and trihydroxylation products, were extensively found. The phase II metabolism included sulfation and glucuronide conjugation. The main metabolic soft spots of the chemical structure of GD were 3-carbonyl group, angular methyl groups, 7-hydroxyl group and 26-carboxylic acid moiety. This study has increased our knowledge of the metabolic
pathways of an oxygenated tetra-cyclic lanostane triterpenoid. These results lay a solid foundation in the further metabolic and clinical studies of ganoderic acids.
Authorship Contributions

Participated in research design: Guo, Cheng, and Yang.

Conducted experiments: Cheng, Yang, Pang, Yu, and Tao.

Contributed new reagents or analytical tools: Cheng, Yang, Yu, and Millar.

Performed data analysis: Cheng and Yang.

Wrote or contributed to the writing of the manuscript: Cheng, Yang, Guan, and Guo.


Guo X, Han J, Ye M, Ma X, Shen X, Xue B, Che Q (2012) Identification of major compounds in rat bile after oral administration of total triterpenoids of *Ganoderma lucidum* by high-performance liquid chromatography with


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*Communications in Mass Spectrometry* **25**: 3103-3113.


Footnotes

This work was financially supported by the National Natural Science Foundation of China [Grant 30701077]; the National Science & Technology Major Project “Key New Drug Creation and Manufacturing Program”, China [Grants 2009ZX09308-005, 2009ZX09311-001, 2009ZX09502-020, 2009ZX09304-002]; and the Major Projects of Knowledge Innovation Program of the Chinese Academy of Sciences [Grant KSCX2-YW-R-166].

C.C. and M.Y. contributed equally to this work.
Legends for figures

**Figure 1.** High collision energy mass spectrum of ganoderic acid D (obtained on Q-TOF mass spectrometry) and the proposed fragmentation pathways of the ganoderic acid D.

**Figure 2.** Total ion chromatogram of the bile sample collected after oral administration of ganoderic acid D at 10 mg/kg (A); Extracted ion chromatogram of the glucuronide conjugates of ganoderic acid D in rat bile sample (B); Extracted ion chromatogram of the dihydroxylation metabolites of ganoderic acid D in rat bile sample (C); Extracted ion chromatogram of the monohydroxylation metabolites of ganoderic acid D in rat bile sample (D).

**Figure 3.** Chemical structures of metabolites M4-M10 and M12-M22.

**Figure 4.** Proposed metabolic pathways of ganoderic acid D in rats.

**Figure 5.** Mass spectrum of metabolite M9 obtained by using product ion scan on Q-TOF mass spectrometry; proposed fragmentation patterns were signed.
Table 1. Characterization of in vivo metabolites of ganoderic acid D in rat bile by UPLC/Q-TOF

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<th>Metabolite</th>
<th>Description</th>
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<th>Calculated mass</th>
<th>Fragment ions</th>
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<td>529.280</td>
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<td>529.280</td>
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<td>monohydroxylation</td>
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<td>529.281</td>
<td>529.280</td>
<td>149.096, 113.058</td>
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<td>M11</td>
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<td>527.265</td>
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<td>531.296</td>
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<td>439.247, 317.181, 263.130, 149.067, 87.008</td>
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<td>545.275</td>
<td>483.273, 453.259, 409.201, 303.159, 273.149, 233.114, 149.063</td>
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<td>561.270</td>
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<td>trihydroxylation</td>
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<td>561.270</td>
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<td>595.258</td>
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<td>9.5</td>
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<td>689.317</td>
<td>495.276, 451.287, 301.181, 247.133, 193.036, 175.025, 113.024, 85.029</td>
</tr>
</tbody>
</table>
Figure 2

(A)

(B)

(C)

(D)
Figure 3

M4 and M5
M6
M7
M8
M9 and M10
M11
M12
M13 and M14
M15
M16
M17
M18
M19
M20
M21
M22
Figure 5

Bile P.O. 321 (529)

- Key area to determine the ring skeleton
- Ring C cleavage - HCHO
- Ring D cleavage - HCHO
- Ring C and ring A cleavage
- 149.061
- 233.117
- 287.164
- 299.163
- 300.171
- Ring D cleavage
- 437.268
- 467.282
- Ring D cleavage - H₂O
- 481.258
- 511.270
- -H₅O
- Ring A cleavage
- 512.272
- Ring D cleavage
- 513.276

M9