Metabolism of Saikosaponin a in Rats: Diverse Oxidations on the Aglycone Moiety in Liver and Intestine in Addition to Hydrolysis of Glycosidic Bonds

Guoqiang Liu, Yuan Tian, Geng Li, Lei Xu, Rui Song, and Zunjian Zhang

Key Laboratory of Drug Quality Control and Pharmacovigilance (Gu.L., Y.T., Ge.L., L.X., R.S., Z.Z.) and State Key Laboratory of Natural Medicines (Z.Z.), China Pharmaceutical University, Nanjing, China

ABSTRACT

The main objective of the present study was to completely characterize the metabolites of the triterpenoid saikosaponin a (SSa) in rats. To this aim, we compared the metabolites in plasma, bile, urine, and feces samples following oral and i.v. routes of administration using liquid chromatography–diode array detector coupled with hybrid ion trap–time-of-flight mass spectrometry. As a result, besides 2 known metabolites, prosaikogenin f and saikogenin f, 15 new metabolites were detected in all. It was found that SSa is metabolized mainly in phase I manner, i.e., hydration and mono- and diene. Further, these saikosaponins are hydrolyzed into their isomeric derivatives with the allyl oxide linkage in the 13,28-position of aglycone forming into a heteroannular or homoannular diene. Further, these saikosaponins are hydrolyzed into their corresponding prosaikogenins and saikogenins by the hydrolyzing activities of intestinal bacteria, such as Eubacterium sp. A-44 (Kid et al., 1997), which were also essential for the appearance of prosaikogenin a and saikogenin a in rat plasma after oral administration of saikosaponin b1 (Kid et al., 1998). Although the total recovery of excreted saikosaponin a (SSa), prosaikogenin f (PSGf), and saikogenin f (SGf) in the feces of conventional rats was >50% of the starting dose in a period over 0 to 24 hours after the oral administration of SSa (Shimizu et al., 1985a), the disposition of <50% remained unexplained. The whole metabolic profile of SSa cannot be reflected by only deglycosylation metabolites absorbance from the intestine when taken orally or the toxicity induced by marked hemolysis when administered by injection, especially i.v. (Segal et al., 1978). Better understanding of the efficacy and safety of saikosaponins requires the elucidation of their biologic fates in the body.

Although the pharmacokinetics of saikosaponin a, b1, b2, c, and d in rats after i.v. or oral administration has been reported (Shimizu et al., 1984; Fujiwara and Oghara, 1986; Kid et al., 1998; Tang et al., 2007; Xu et al., 2012), there was limited knowledge about the in vivo metabolism of these compounds. Study of the metabolism of saikosaponins has mainly been focused on the artificial isomers in gastric conditions and deglycosylation metabolites by intestinal bacteria (Shimizu et al., 1985a). Japanese researchers reported that saikosaponins are unstable in the gastric juice and prone to convert into their isomeric derivatives with the allyl oxide linkage in the 13,28 position of aglycone breaking into a heteroannular or homoannular diene. Further, these saikosaponins are hydrolyzed into their corresponding prosaikogenins and saikogenins by the hydrolyzing activities of intestinal bacteria, such as Eubacterium sp. A-44 (Kid et al., 1997), which were also essential for the appearance of prosaikogenin a and saikogenin a in rat plasma after oral administration of saikosaponin b1 (Kid et al., 1998). Although the total recovery of excreted saikosaponin a (SSa), prosaikogenin f (PSGf), and saikogenin f (SGf) in the feces of conventional rats was >50% of the starting dose in a period over 0 to 24 hours after the oral administration of SSa (Shimizu et al., 1985a), the disposition of <50% remained unexplained. The whole metabolic profile of SSa cannot be reflected by only deglycosylation metabolites.
such as PSGf and SGf, and so can that of the other saikosaponins. Thus we chose SSa as a representative of the saikosaponins to clarify its metabolic fate in rats using the liquid chromatography–diode array detector coupled with hybrid ion trap–time-of-flight mass spectrometry (LC-DAD-IT-TOF-MS) technique based on the work of pioneering researchers (Shimizu et al., 1984, 1985a; Fujiwara and Ogihara, 1986; Kida et al., 1997, 1998).

Materials and Methods

Chemicals and Reagents

SSa reference standard (purity, >98%) was purchased from the National Institutes for Food and Drug Control (Beijing, China). Analytical-grade ethyl carbamate was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). High-performance LC-grade acetonitrile and methanol were purchased from Merck KGaA (Darmstadt, Germany). Analytical-grade Tween 80 was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Analytical-grade ethyl acetate was purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Twice-distilled water prepared in-laboratory was used throughout the study.

Dosing Procedure

Male Sprague-Dawley rats weighing 220 ± 20 g were purchased from the Qinglongshan Experimental Animal Center (Nanjing, China). The animals were housed under standard conditions with food and water provided ad libitum. Animal studies were conducted under the approval of the Animal Ethics Committee of the China Pharmaceutical University.

After acclimating in stainless steel rat metabolism cages individually for 1 week, the animals were fasted for 12 hours with free access to water before the experiment. SSa dissolved in 5% Tween 80 was administrated either i.v. via the tail vein at a dose of 15 mg/kg or through intragastric gavage at a dose of 50 mg/kg. The vehicle with equal volumes was administered by either i.v. injection or intragastric gavage to the remaining half of the animals as the control.

Sample Collection

**Plasma Collection.** Twelve rats were divided into four groups with three animals per group. Blood samples (about 0.5 ml) were collected in 1.5 ml sodium heparinized tubes 0.083, 0.167, 0.333, 0.5, 1, 1.5, 3, 4.5, 6, 7.5, 9, 10.5, and 12 hours after i.v. or oral administration of the SSa or vehicle. Plasma samples of each animal were separated by centrifugation at 3000g for 10 minutes at 4°C in an Eppendorf centrifuge 5430 R (Eppendorf, Hamburg, Germany) and pooled over 0.083 to 12 hours.

**Bile Collection.** Twelve rats divided into four groups with three animals per group were implanted with a PE-10 cannula (Smith Medical, Ashford, UK) into the bile duct under anesthesia by ethyl carbamate. Bile samples of each animal were collected on ice and pooled over 0 to 24 hours after i.v. or oral administration of the SSa or vehicle.

**Urine and Feces Collection.** The urine and feces were collected separately on ice over 24 hours from another 12 rats after i.v. or oral administration of the SSa or vehicle. All of the collected plasma, bile, urine, and feces samples were stored at −20°C before analysis.

Sample Preparation

Plasma (2 ml) was loaded on the C18 cartridge (250 mg), washed with 5 ml water, and eluted with 3 ml acetonitrile. After the evaporation of the combined acetonitrile under a stream of nitrogen gas in water bath at 35°C, the residue was dissolved in 500 µl acetonitrile–water solution (50:50 [v/v]) for LC-DAD-IT-TOF-MS analysis. The bile and urine samples were treated by the same procedure as the plasma samples.

The feces specimens (0.5 g) were homogenized in 5 ml ice-cold acetonitrile–water solution [50:50 (v/v)] by a homogenizer (Shanghai Jinda Biochemical Equipment Co. Ltd., Shanghai, China). The homogenate was centrifuged at 2130g for 10 minutes to separate the supernatant, which was extracted with three times its volume of ethyl acetate. After centrifugation at 2130g for 10 minutes, the ethyl acetate layer was separated and vaporized under a stream of nitrogen gas in water bath at 35°C. The residue was dissolved in 500 µl acetonitrile–water solution (50:50 [v/v]) for LC-DAD-IT-TOF-MS analysis.

LC-DAD-IT-TOF-MS Analysis

LC experiments were conducted on a Shimadzu (Kyoto, Japan) high-performance LC system consisting of an LC-30AD binary pump, a DGU-20As degasser, a SIL-30AC autosampler, and a CTO-20AC column oven. Chromatographic separation was achieved on a Shimadzu VP-ODS column (150 mm × 2.0 mm, 2.2 µm) (Shimadzu) at 40°C. The mobile phase (delivered at 0.4 ml/min) comprised solvent A, acetonitrile–methanol solution (80:20 [v/v]), and solvent B, 0.06% HCOOH in water. A gradient elution was performed: 20–40% A for 0–20 minutes, 40–50% A for 20–60 minutes, 95% A for 60–70 minutes, and 20% A for 70–90 minutes. The UV absorption from 190 to 400 nm was recorded by an SPD-M20A diode array detector (Shimadzu) to determine whether the allyl oxide linkage in the 13,28 position of the aglycone moiety was broken into a heteroannular or homoannular diene (Kubota et al., 1966; Kubota and Hinoh, 1968).

MS analyses were conducted on a Shimadzu IT-TOF-MS (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization source. MS1 analysis was carried out in positive and negative mode simultaneously, followed by MS2 analyses in positive mode and MS3 analyses in negative mode, respectively. Some important equipment parameters were as follows: electrospray voltage, +4.5 kV for positive mode and −3.5 kV for negative mode; nebulizer gas (N2) flow, 1.5 l/min; curved desolvation line temperature, 200°C; heat block temperature, 200°C; drying gas (N2) pressure, 100 kPa; detector voltage, 1.7 kV; ion accumulated time, 50 milliseconds; positive scan range [mass-to-charge ratio (m/z)], 350–1350 for MS1 and 100–900 for MS2; negative scan range (m/z), 350–1350 for MS3, 300–1000 for MS2, and 200–800 for MS3; collision energy, 30% both for positive and negative MS. After the data acquisition, MetID solution 1.2 (Shimadzu) was used to screen metabolite candidates. SSa, PSGf, and SGf were chosen as three templates in the mass defect filter method (Zhu et al., 2006; Zhang et al., 2009) with a mass defect window of ±0.050 Da.

Results

Fragment Pattern of SSa

At first, both the positive and negative MS fragment patterns of SSa were analyzed. Besides the typical adduct ion [M+K]⁺ at m/z 819.4267 (C₁₂H₆O₆K⁺), [M+Na]⁺ at m/z 803.4572 (C₁₂H₆O₆Na⁺), we observed a large amount of fragment ions such as [M-H₂O+H]⁺ at m/z 763.4635 (C₁₂H₆O₅), [M-2H₂O+H]⁺ at m/z 745.4511 (C₁₂H₆O₄), [M-H₂O-glc+H]⁺ (glc, glucose) at m/z 601.4083 (C₁₅H₂₀O₆), [M-2H₂O-glc+H]⁺ at m/z 583.3982 (C₁₅H₂₉O₈), [M-H₂O-glc-fuc+H]⁺ (fuc, fucose) at m/z 455.3534 (C₁₅H₂₇O₈), and [M-2H₂O-glc-fuc+H]⁺ at m/z 437.3402 (C₁₅H₂₅O₇, 100% relative signal intensity) in the positive full-scan mass spectrum. Further, it was confirmed that 455.3534 comes from 763.4635 rather than 803.4572 or 819.4267 after comparing the MS2 spectra of the precursor ions at m/z 763.4635, 803.4572, and 819.4267. The MS³ spectrum obtained from the precursor ion at m/z 455.3458 showed 437.3398 (C₁₅H₂₇O₈, 455-H₂O), 419.3295 (C₁₅H₂₅O₇, 455-2H₂O), 401.3224 (C₁₅H₂₃, 455-3H₂O), 425.3399 (C₁₅H₂₄O₇, 455-CH₂O), McLaugherty rearrangement, 407.3296 (C₁₅H₂₄O₇, 455-CH₂O-H₂O), and 389.3199 (C₁₅H₂₂, 455-CH₂O-2H₂O) in the high-m/z range. More importantly, in the low-m/z range appeared the characteristic fragment ions at m/z 223.1687 (C₁₀H₁₄O₂), named L1, ring C cleavage, 205.1584 (C₁₀H₁₃O₂, L1-H₂O), and 187.1484 (C₁₀H₁₂O₂, L1-2H₂O, 100% relative signal intensity) corresponding to the AB ring (ABCD or ABCDE were labelled on the structure of ion at m/z 763 in Fig. 2) part; and those coming from the CDE ring part such as 299.2361 (C₁₁H₁₅O₃, named R1, ring B cleavage); 285.2213 (C₁₀H₁₄O₂, named R2, ring B cleavage), 267.2123 (C₁₀H₁₃, R2-H₂O), 283.2049 (C₁₀H₁₂O₂, named R3, ring B cleavage), 265.1946 (C₁₀H₁₂, R3-H₂O), 271.2073 (C₁₀H₁₁O₂, named R4, ring B cleavage), 253.1961 (C₉H₁₃, R4-H₂O), 241.1945 (C₉H₁₂, R₄-CH₂O), 245.1915 (C₁₅H₂₄O₇, R₄-CH₂O, retro-Diels-Alder reaction),
227.1687 (C17H23, R4-C2H2-H2O); 269.1914 (C16H22O, named R5), 251.1805 (C19H23, R5-H2O), 239.1811 (C18H23, R5-CH2O); 233.1897 (C16H22O, named R6), 215.1789 (C16H23, R6-H2O); and 231.1755 (C16H23O, named R7, ring C cleavage), 213.1648 (C16H23, R7-H2O), 201.1634 (C16H23, R7-CH2O), 205.1584 (C16H23O, R7-C2H5, retro-Diels-Alder reaction, coincided with L1-H2O), 187.1484 (C16H30, R7-C2H2-H2O, coincided with L1-H2O) (see Fig. 1). The mass fragmentation pathway is proposed in Fig. 2.

In the negative full-scan mass spectrum, two major ions, the \([\text{M}+\text{HCOO}]^-\) at m/z 825.4654 (C43H69O15, 100% relative signal intensity) and the \([\text{M}-\text{H}]^-\) at m/z 779.4593 (C42H67O13), were observed. The anionic-adduct molecular ion transitioned to the predominant \([\text{M}-\text{H}]^-\) at m/z 779.4566 (C42H67O13) in the MS2 spectrum, which in turn gave the basic \([\text{M}-\text{glc-H}]^-\) at m/z 617.4067 (C36H57O8) in the MS3 spectrum. In the MS4 experiment, isolation and fragmentation of 617.4067 resulted in the \([\text{M}-\text{glc-fuc-H}]^-\) at m/z 471.3464 (C30H47O4, 100% relative signal intensity), the \([\text{M}-\text{glc-fuc-CH}_3\text{OH-H}]^-\) at m/z 439.3207 (C29H43O3), and the one involving the ring-cross cleavage of fucose at m/z 541.3513 (C33H49O6). This fragmentation was in agreement with the previous findings (Huang et al., 2008).

The positive and negative MSn analysis was applied to identify the structures of the aglycone part and glycosyl group, respectively, in this experiment.

Fig. 1. The positive MSn spectra of SSa. (A) Full-scan mass spectrum; (B) MS2 of the precursor ion at m/z 763.4635; (C) MS3 of the precursor ion at m/z 455.3534.
Screening of Metabolites

With the aid of the MetID solution 1.2 software, the metabolites were screened by comparing the full-scan mass spectra of the plasma, bile, urine, and feces of rats after administration of SSa with that of the control samples of rats after administration of vehicle. A total of 17 metabolites were detected besides the parent drug. The extracted ion chromatograms of the typical biosamples are shown in Fig. 3. Information on the parent compound and metabolites is summarized in Tables 1 and 2.

Structure Elucidation of Metabolites

**M0 (Parent Drug).** M0 in samples was unambiguously identified by comparing the retention time and MS and UV data with the standard reference.

**M13 (Hydration).** Based on the [M+Na]⁺ at m/z 821.4632 (C₄₂H₇₀NaO₁₄) in the positive full-scan mass spectrum and the [M-H]⁻ at m/z 797.4706 (C₄₂H₆₉O₁₄) and the [M+HCOO]⁻ at m/z 843.4772 (C₄₃H₇₁O₁₆, 100% relative signal intensity) in the negative full-scan mass spectrum, the molecular formula (MF) of M13 was
determined as C_{42}H_{70}O_{14}, with one H_{2}O more than M0. The MS^{2} spectrum of deprotonated molecular ion showed the predominant [M-glc-H]^{-} at m/z 635.4135 (C_{36}H_{59}O_{9}), which subsequently provided the major fragment ions including the [M-glc-fuc-H_{2}O-H]^{-} at m/z 471.3479 (C_{30}H_{47}O_{4}, 100% relative signal intensity), the [M-glc-fuc-H_{2}O-CH_{3}OH-H]^{-} at m/z 439.3214 (C_{29}H_{43}O_{3}), and the one involving ring-cross cleavage of the fucose moiety at m/z 559.3655 (C_{33}H_{51}O_{7}) in the MS^{3} spectrum. This fragment pattern was identical to that of hydroxysaikosaponin a (HOSSa) as reported previously (Huang et al., 2008). Further, we validated this inference by comparison of its retention time with that of HOSSa derived from SSa in aqueous condition. Thus M13 was identified as HOSSa. The conversion of SSa
to HOSSa, which can occur to a limited extent in aqueous solvent, may accelerate in vivo.

**M14 and M15 (Monoxydation).** From the [M±Na]+ at m/z 819.4527 (C42H68NaO13) in the positive full-scan mass spectrum and the [M-H]− at m/z 795.4561 (C42H68O13) and the [M+HCOO]− at m/z 841.4623 (C42H68O16, 100% relative signal intensity) in the negative full-scan mass spectrum, their MF was determined as C42H68O13 with one oxygen more than M0. Their predominant fragment ion [M-glc-fuc-CH3OH-H]− at m/z 663.4124 (C42H68NaO13) in the positive full-scan mass spectrum and the [M-H]− at m/z 633.4018 (C42H68NaO13) and the [M+HCOO]− at m/z 679.4031 (C37H57O11, 100% relative signal intensity) in the negative full-scan mass spectrum. The MS/MS spectrum of the precursor ion at m/z 633.4018 gave the major fragment ions including the [M-fuc-H]− at m/z 487.3447 (C30H47O5, 100% relative signal intensity) and the [M-fuc-CH3OH-H]− at m/z 453.3378 (C30H45O3) in the positive full-scan mass spectrum, the MF of M3 and M4 was supposed as C30H46O4, with one oxygen more than M0. Their predominant fragment ion [M-glc-H]− at m/z 633.3976 (C30H68O14) from the precursor ion at m/z 795.4561 in the MS2 spectrum transitioned to the major fragment ion [M-glucosidic bond]− at m/z 487.3436 (C30H68NaO14, 100% relative signal intensity) in the MS3 spectrum. In addition, the [M-glc-fuc-CH3OH-H]− at m/z 453.3172 (C30H68O15, 100% relative signal intensity) in the MS4 spectrum. The MS/MS spectrum of the precursor ion at m/z 453.3172 in the MS 2 spectrum. Their fragment ions at m/z 221.1550 (C14H21O2, named L2) and 203.1428 (C14H19O, L2-H2O, 100% relative signal intensity) were 2 Da less than ion L1 and L1-H2O, respectively. These results indicated that the MS 5 spectrum of the precursor ion at m/z 453.3172 to that of the parent compound suggested that M2 has the same aglycone as the parent compound, which was also confirmed by the UV end absorption. So M2 was identified as SSGf as reported previously (Shimizu et al., 1985b).

**M2 (Hydrolysis of β-Fucosidic Bond).** The MF of M2 was determined as C30H68O14 based on the [M+H]++ at m/z 455.3527 (C30H68NaO14) in the positive full-scan mass spectrum and the [M+HCOO]− at m/z 517.3513 (C31H59O7) in the negative full-scan mass spectrum. The similar positive MS2 spectrum of the precursor ion at m/z 517.3513 to that of the parent compound showed the linkage of a dezoxyschisose to the aglycone. These spectral data were in agreement with those of SSSg as reported in the literature (Huang et al., 2008). M1 was identified as PSGf.

**M16 and M17 (Monoxydation and Hydrolysis of β-Glucosidic Bond).** The MF of M16 and M17 was determined as C30H68O14, with one oxygen more than M1, according to the [M±Na]+ at m/z 657.3997 (C30H68NaO14) in the positive full-scan mass spectrum and the [M-H]− at m/z 633.4018 (C30H68O13) and the [M+HCOO]− at m/z 679.4031 (C37H57O11, 100% relative signal intensity) in the negative full-scan mass spectrum. The MS/MS spectrum of the precursor ion at m/z 633.4018 gave the major fragment ions including the [M-fuc-H]− at m/z 487.3447 (C30H47O5, 100% relative signal intensity) and the [M-fuc-CH3OH-H]− at m/z 453.3378 (C30H45O3), while the one involving ring-cross cleavage of the fucose moiety at m/z 557.3489 (C33H60O7) appeared in the MS2 spectrum of M16 rather than M17. This fragmentation was very similar to that of M14 and M15. We supposed that M16 and M17 derived from M14 and M15, respectively, through hydrolyzing the terminal glucose moiety according to their MS2 spectra and retention time.
TABLE 2
LC-DAD-IT-TOF-MS analysis of SSa and its observed metabolites in rat biosamples

<table>
<thead>
<tr>
<th>No.</th>
<th>T_R</th>
<th>UV</th>
<th>Positive MS ²</th>
<th>Negative MS ³</th>
<th>Positive MS ³</th>
<th>Negative MS ³</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13</td>
<td>22.9</td>
<td>190</td>
<td>821.4632</td>
<td>797.4706, 843.4772²</td>
<td>[797.4706] → [635.4135] → 559.3655, 471.3479³, 439.3214</td>
<td>HOSS³</td>
<td></td>
</tr>
<tr>
<td>M14</td>
<td>15.2</td>
<td>190</td>
<td>819.4527</td>
<td>795.4561, 841.4623³</td>
<td>[795.4561] → [633.3976] → 557.3457, 487.3436³, 455.3172</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M17</td>
<td>24.5</td>
<td>190</td>
<td>657.3968</td>
<td>633.4018, 679.4031, 633.4029, 679.4047³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M12</td>
<td>30.1</td>
<td>190</td>
<td>483.3123³, 501.3207</td>
<td>517.3184³</td>
<td>[501.3207] → 483.3087³</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HOSS, hydroxysaikosaponin; LC-DAD-IT-TOF-MS, liquid chromatography–diode array detector coupled with hybrid ion trap–time-of-flight mass spectrometry; PSGF, prosaikogenin f; SSa, saikosaponin a.  
² Of 100% relative signal intensity.  
³ With standard.  
⁰ With data in reference.
185.1335 (C_{14}H_{17}, L_{3}-2H_{2}O) with 2 Da less than L_{1}-2H_{2}O indicated that the dehydrogenation of M4 could only have occurred on ring A with two hydroxyl groups remaining. Most likely, M4 should be Δ^1-SGf. Some key MS fragmentation of M3 and M4 is proposed in Fig. 4, A and B, respectively.

**M5 and M6 (Hydroxylation and Hydrolysis of β-Fucosidic Bond).** The MF of M5 and M6 was determined as C_{30}H_{48}O_{5}, with one oxygen more than M2 from the [M-H-2H_{2}O+H]^{+} at m/z 471.3457 (C_{30}H_{47}O_{4}) and the [M-2H_{2}O+H]^{+} at m/z 453.3369 (C_{30}H_{45}O_{3}, 100% relative signal intensity) in the positive full-scan mass spectrum and the [M+HCOO]^{-} at m/z 533.3499 (C_{31}H_{49}O_{7}) in the negative full-scan mass spectrum. Their AB ring part structure didn’t change according to the fragment ions at m/z 223.1697 (C_{14}H_{23}O_{5}, L_{1}), 205.1601 (C_{13}H_{21}O, L_{1}-H_{2}O), and 187.1489 (C_{12}H_{19}, L_{1}-2H_{2}O, 100% relative signal intensity) in the MS^{2} spectrum of the precursor ion at m/z 471.3457 of M5 and 453.3369 of M6. The fragment ions of

![Proposed fragmentation pathways of part of metabolites.](image-url)
M5 at m/z 249.1861 (C_{16}H_{25}O_{2}, named R8), 231.1739 (C_{16}H_{23}O, R8-H_{2}O), and 213.1651 (C_{16}H_{21}, R8-2H_{2}O) resulting from the precursor ion [M-H_{2}O+H]^+ at m/z 471.3457 were in agreement with R6+O, R6+O-H_{2}O, and R6+O-2H_{2}O, respectively. We supposed that hydroxylation occurred on the CDE ring part structure of M5 and the introduced hydroxyl function may locate at C21, C22, or C27. The key MS fragmentation of M5 is illustrated in Fig. 4C. For M6, the fragment ions at m/z 229.1584 (C_{16}H_{23}, named R9) and 211.1477 (C_{16}H_{19}, R9-H_{2}O) obtained from the precursor ion [M-2H_{2}O+H]^+ at m/z 453.3369 were consistent with R7+O-H_{2}O and R7+O-2H_{2}O, respectively, indicating that hydroxylation took place on the CDE ring part structure of M6 and the added hydroxyl group may be at C19, C21, C22, C27, C29, or C30. The key MS fragmentation of M6 is shown in Fig. 4D.

M7(Carboxylation and Hydrolysis of β-Fucosidic Bond). Based on the [M-H_{2}O+H]^+ at m/z 485.3250 (C_{30}H_{45}O_{5}) and the [M-2H_{2}O+H]^+ at m/z 467.3153 (C_{30}H_{43}O_{4}, 100% relative signal intensity) in the positive full-scan mass spectrum and the [M-H]^2 at m/z 501.3234 (C_{30}H_{45}O_{6}) in the negative full-scan mass spectrum, the MF of M7 was determined as C_{30}H_{46}O_{6}, with two oxygens more and two hydrogens fewer than M2. In the MS^2 spectrum of the precursor ion at m/z 485.3250, the fragment ions at m/z 223.1692 (C_{14}H_{23}O_{2}, L1), 205.1594 (C_{14}H_{21}O_{2}, L1-H_{2}O), and 187.1482 (C_{13}H_{19}O_{2}, L1-2H_{2}O, 100% relative signal intensity) showed the intact AB ring part structure. Moreover, the fragment ions at m/z 263.1567 (C_{14}H_{23}O_{2}, named R10, in line with R6+2O-2H), 245.1433 (C_{14}H_{21}O_{2}, R10-H_{2}O), and 199.1510 (C_{14}H_{19}O_{2}, R10-H_{2}O-HCOOH) showed one carboxyl function and two hydroxyl groups on the CDE ring part structure and eliminated the possibility of carboxylation at C29 or C30. The end absorption in UV spectrum ruled out the possibility of carboxylation at C28, which otherwise needed the 13,28-oxide bridge to break into a heteroannular or homoannular diene. Taken together, M7 was tentatively characterized as SGf-27-oic acid, and its key MS fragmentation is presented in Fig. 4E.

M8 and M10 (Hydroxylation, Carboxylation, and Hydrolysis of β-Fucosidic Bond). As an isomer of M7, the MF of M8 was also determined as C_{30}H_{46}O_{6}, with two oxygens more and two hydrogens fewer than M2, based on the positive and negative full-scan mass spectra. The CDE ring part structure of this metabolite remained unchanged according to its fragment ions at m/z 233.1907 (C_{16}H_{23}O_{2}, R6) and 215.1805 (C_{16}H_{21}O_{2}, R6-H_{2}O) from the precursor ion at m/z 485.3273 in the MS^2 spectrum. Additionally, the fragment ions at m/z 253.1451 (C_{14}H_{21}O_{4}, named L4, consistent with L1+2O-2H), 235.1340 (C_{14}H_{19}O_{3}, L4-H_{2}O), 217.1224 (C_{14}H_{17}O_{2}, L4-2H_{2}O), and 171.1169 (C_{13}H_{15}, L4-2H_{2}O-HCOOH, 100% relative signal intensity) suggested that carboxylation happened on the AB ring part.

Fig. 5. The major metabolic pathway of SSa in rats.
structure. Further, the carboxyl group should not locate at C25 or C26 because of the fragment ions at m/z 233.1907 (C16H25O, R6) and 215.1805 (C16H23, R6-H2O) responsible for the CDE ring part structure. We supposed that carboxylation took place at 23-CH2OH, accompanied by hydroxylation at C1, C2, or C24. The key MS fragmentation of M8 is illustrated in Fig. 4F.

The MF of M10 was determined as C30H46O7, with three oxygens more and two hydrogens fewer than M2 according to the [M-H2O+H]+ at m/z 501.3199 (C30H45O6, 100% relative signal intensity) and the [M-2H2O+H]+ at m/z 483.3980 (C30H43O5) in the positive full-scan mass spectrum and the [M-H]+ at m/z 517.3171 (C30H45O7) in the negative full-scan mass spectrum. In the MS2 spectrum of the precursor ion at m/z 501.3199, the major fragment ions at m/z 253.1447 (C14H21O4, L4), 235.1345 (C14H19O3, L4-H2O), 217.1235 (C14H17O2, L4-2H2O), and 171.1167 (C13H15, L4-2H2O-HCOOH, 100% relative signal intensity) suggested that M10 has the same AB ring part structure as M8, while the fragment ions at m/z 249.1584 (C16H25O2, R8), 231.1713 (C16H23O, R8-H2O), and 213.1560 (C16H21, R8-2H2O) indicated that it has the same CDE ring part structure as M5.

The accurate mass data with error (ppm) for fragment ions of all metabolites are listed in Supplemental Table 1.

Discussion

The main objective of the present study was to completely characterize the metabolites of the triterpenoid SSa in rats. To this aim, we compared the metabolites in plasma, bile, urine, and feces samples following oral and i.v. routes of administration. Our results showed that SSa is metabolized diversely. The metabolic profile is proposed in Fig. 5. The observed routes of SSa metabolism are some types of oxidation such as dehydrogenation, hydroxylation, carboxylation, and combinations of these steps on the aglycone moiety, in addition to the previously reported hydrolysis of glycosidic bonds (Shimizu et al., 1985a; Kida et al., 1998).

The detected metabolites in biosamples differ via different routes of administration, as summarized in Table 1. So a relative comprehensive information of in vivo experience of SSa was deduced from the complementary results after different routes of administration, as shown in Fig. 6. After intravenous dosing, SSa was hydrolyzed into M14 and M15 in the liver, as indicated by their detection in the bile. Interestingly, the β-glucosidic bond of SSa, M14, and M15 was hydrolyzed to produce M1, M16, and M17, respectively, as detected in the bile, suggesting that the hydrolyzing activity for the β-glucosidic bond exists not only in the intestinal bacteria but also in the liver. This inference is supported by other reports that various soluble and membrane-bound β-glucosidase with broad specificity catalyzing such reactions had been detected in most mammalian tissues (Robinson, 1956; Abrahams and Robinson, 1969; Glew et al., 1976; Daniels et al., 1981). SSa and M13–M17 excreted through the bile duct were metabolized further in the intestine, according to their disappearance in the feces. In the intestine, SSa was metabolized through hydrolysis mediated by the intestinal flora following oxidation catalyzed by the metabolizing enzymes in the intestinal mucosal cells (Kaminsky and Zhang, 2003; Zhang et al., 2007). Both the renal and biliary routes were observed for the excretion of SSa and its metabolites. In the case of oral administration, M1–M4 and M7–M12 produced in the intestine were partly absorbed into the systemic circulation, as indicated by their excretion in the urine. This may be due to their improved permeability through the intestinal membrane after hydrolysis of glycosidic bonds because the permeability decreased in the order of saikogenin a, prosaikogenin a, and saikosaponin b1, as reported previously (Kida et al., 1998). Additionally, our results showed that no saikosaponin b1 or g, prosaikogenin a or h, or saikogenin a or h was detected in all the biosamples after oral administration of SSa, suggesting the negligible effect of gastric juice on the allyl oxide linkage in the 13,28 position, which was consistent with the findings of Shimizu et al. (1985a).

Fig. 6. The proposed disposition of SSa in rats after oral and i.v. administration.
Furthermore, various bioactivities of saikosaponins might be linked closely to their interaction with biomembranes of cells which appeared to intimately parallel their degree of hemolytic activity (Ahn and Sok, 2006). Previous structure-activity relationship research showed that for these activities the ether linkage between C13 and C28, the 23-CH2OH, the configuration of the hydroxyl group at C16, and the proper polar balance between the sugar moiety (polar position) and the aglycone moiety (nonpolar position) is important (Abe et al., 1980; Yokoyama et al., 1981; Nose et al., 1989a,b; Kumazawa et al., 1990; Ahn et al., 1998; T sai et al., 2002). In this experiment, the ether linkage between C13 and C28 of all identified metabolites except M13 didn’t cleave according to their UV spectra. The configuration of the hydroxyl group at C16 of SSSa was β type and so should be that of its metabolites. The polar balance of metabolites had been broken after the hydrolysis of glycosidic bonds but was compensated to some degree by the addition of hydroxyl and carboxyl groups on the aglycone moiety. It is believed that the addition of functional groups such as –OH, –COOH, –SH, –O-, or –NH2 by the phase I enzymes can dramatically alter the biologic properties of the drug (Brunton et al., 2008). As to saikosaponins, the –OH was proven important for their interaction with the cell surface based on the fact that whereas the transformation of the 23-methyl group (saikosapoin e) to CH2OH (SSSa) resulted in twofold enhancement in gastric juice and intestinal flora. Shimizu K, Amagaya S, and Ogihara Y (1984) Quantitative analysis of the metabolites of saikosaponins. Planta Med 60:139–143.

184 Liu et al.

References


Kim BM and Hong SH (2011) Sequential caspase-2 and caspase-8 activation is essential for saikosaponin a-induced apoptosis of human colon carcinoma cell line. Apoptosis 16:184–197.


Address correspondence to: Dr. Zunjian Zhang, Key Laboratory of Drug Quality Control and Pharmacovigilance, China Pharmaceutical University, 24 Tongjiexiang, Nanjing 210009, China. E-mail: zunjianzhangcpu@hotmail.com