Metabolism of saikosaponin a in rats: Diverse oxidations on the aglycone moiety in liver and intestine besides hydrolysis of glycosidic bonds

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Abbreviations: SSa, saikosaponin a; PSGf, prosaikogenin f; SGf, saikogenin f; HOSSa, hydroxysaikosaponin a; LC, liquid chromatography; DAD, diode array detector; IT, ion trap; TOF, time of flight; MS, mass spectrometry; LC-DAD-IT-TOF-MS, liquid chromatography-diode array detector coupled with hybrid ion trap-time of flight-mass spectrometry; MD, mass defect; fuc, fucose; glc, glucose; MF, molecular formula; Mc, McLafferty rearrangement; RDA, retro-Diels-Alder reaction.
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 the plasma, bile, urine and feces samples following oral and intravenous routes of administration using liquid chromatography-diode array detector coupled with hybrid ion trap-time of flight-mass spectrometry (LC-DAD-IT-TOF-MS). As a result, besides two known metabolites, prosaikogenin f (PSGf) and saikogenin f (SGf), fifteen new metabolites were detected in all. It was found that SSa is metabolized mainly in phase I manner, i.e., hydration and mono-oxidation on the aglycone moiety and hydrolysis of β-glucosidic bond in the liver while sequential hydrolysis of β-glucosidic and β-fucosidic bonds followed by dehydrogenation, hydroxylation, carboxylation and combinations of these steps on the aglycone moiety in the intestinal tract. Both the renal and billiary routines were observed for the excretion of SSa and its metabolites. Further, a clear metabolic profile in rats was proposed in detail according to the results from the in vivo animal experiment after different routes of administration. Our results update the preclinical metabolism and disposition information of SSa, which is not only helpful in the future human metabolic study of this compound but also provides basic information for better understanding the efficacy and safety of prescriptions containing saikosaponins.
Introduction

Saikosaponins, the major bioactive ingredients of the genus Bupleurum with a long history of medicinal use, exert significant pharmacological activities such as antitumor targeting various cancer cells (Motoo and Sawabu, 1994; Ahn et al., 1998; Hsu et al., 2000; Tsai et al., 2002; Wu, 2003; Hsu et al., 2004; Kim and Hong, 2011), chemosensitization (Wang et al., 2010a), anti-inflammatory (Yokoyama et al., 1981; Nose et al., 1989a; Bermejo Benito et al., 1998), immunomodulatory (Kumazawa et al., 1989; Leung et al., 2005; Sun et al., 2009), hepatoprotective (Abe et al., 1980; Fan et al., 2007; Wu et al., 2008), antinephritic (Li et al., 2005; Hattori et al., 2008; Chen et al., 2008), antibacterial and antiviral (Kumazawa et al., 1990; Chiang et al., 2003; Cheng et al., 2006), estrogenic (Wang et al., 2010b), promoting amylase secretion in rat pancreatic acini (Yu et al., 2002). On the other hand, saikosaponins have also been reported to be responsible for toxicity induced by their hemolytic action (Abe et al., 1978a; Abe et al., 1978b; Nose et al., 1989b). Moreover, the poor selectivity resulted from the broad spectrum of pharmacological activity of saikosaponins put at risk the prescriptions containing them in the clinical applications, especially in combination with other drugs with the drug-drug interactions taken into consideration. In general, therapeutic application of saponins is very limited because of either the poor absorbance from the intestine when taken orally or the toxicity induced by marked hemolysis when administered by injection, especially intravenously (Segal et al., 1978). To better understand the efficacy and safety of saikosaponins needs their biological fates in body to be elucidated.

Although the pharmacokinetics of saikosaponin a, b1, b2, c, and d in rats after intravenous or oral administration has been reported (Shimizu et al., 1984; Fujiwara and Ogihara, 1986; Kida et al., 1998; Tang et al., 2007; Xu et al., 2012), there was limit knowledge about the in vivo metabolism of these compounds. Metabolism of saikosaponins has mainly been focused on the artificial isomers in gastric condition and desugar metabolites by intestinal bacteria.
Japanese researchers reported that saikosaponins are unstable in the gastric juice to convert their isomeric derives 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 (Kida et al., 1997) which were also essential for the appearance of prosaikogenin a and saikogenin a in the rat plasma after oral administration of saikosaponin b1 (Kida et al., 1998). Although the total recovery of excreted SSa, PSGf and SGf in the feces of conventional rats was more than 50% of the starting dose in a period over 0 to 24 h after the oral administration of SSa (Shimizu et al., 1985a), there remained less than 50% unexplained. It’s obvious that only PSGf and SGf detected in the plasma and feces were not enough to reflect the whole metabolic profile of SSa, and so were the other saikosaponins. Thus we chose SSa as a representative of the saikosaponins to clarify its metabolic fate in rats using LC-DAD-IT-TOF-MS technique based on the work of pioneer researchers (Shimizu et al., 1984; Shimizu et al., 1985a; Fujiwara and Ogihara, 1986; Kida et al., 1997; Kida et al., 1998).
Material 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). HPLC-grade acetonitrile and methanol were purchased from Merck (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 one week, the animals were fasted for 12 h with free access to water before experiment. SSa dissolved in 5% Tween 80 was administrated either through intravenous injection (iv) 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 administrated either by intravenous injection or intragastric gavage to the remaining half 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 h after intravenous or oral administration of the SSa or vehicle. Plasma samples of each animal were separated by centrifugation at 3000 × g for 10 min at 4 ºC in an Eppendorf centrifuge 5430 R (Eppendorf, Hamburg, Germany) and pooled over 0.083 to 12 h.
Bile collection. Twelve rats divided into four groups with three animals per group, were implanted with a PE-10 cannula into the bile duct under anesthesia by ethyl carbamate. Bile samples of each animal were collected on ice and pooled over 0 to 24h after intravenous or oral administration of the SSa or vehicle.

Urine and feces collection. The urine and feces were collected separately on ice over 0 to 24 h from another twelve rats after intravenous 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. 2 ml plasma 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 that of 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 2130 x g for 10 min to separate the supernatant which was extracted by three-time volume of ethyl acetate. After centrifugation at 2130 x g for 10 min, 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) HPLC 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 (150mmx2.0mm, 2.2µm) (Shimadzu, Kyoto, Japan) 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 min, 40%-50% A for 20-60 min, 95% A for 60-70 min and 20% A for 70-90 min. The UV absorption from 190 to 400nm was recorded by a SPD-M20A DAD (Shimadzu, Kyoto, Japan) to determine whether the allyl oxide linkage in the 13, 28-position of 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. MS\(^1\) analysis was carried out in positive and negative mode simultaneously followed by MS\(^n\) analyses in positive mode and MS\(^n\) 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 (N\(_2\)) flow, 1.5 l/min; CDL temperature, 200 °C; heat block temperature, 200 °C; drying gas (N\(_2\)) pressure, 100 kPa; detector voltage, 1.7 kV; ion accumulated time, 50 ms; positive scan range (m/z), 350–1350 for MS\(^1\) and 100–900 for MS\(^2\); negative scan range (m/z), 350–1350 for MS\(^1\), 300–1000 for MS\(^2\) and 200–800 for MS\(^3\); collision energy, 30% both for positive and negative MS\(^n\). After the data acquisition, MetID solution 1.2 was used to screen metabolite candidates. SSa, PSGf and SGf were chosen as three templates in the mass defect filter (MDF) method (Zhu et al., 2006; Zhang et al., 2009) with a mass defect window of ±0.050Da.
Fragment pattern of SSa. At first, both the positive and negative MS fragment patterns of SSa were analyzed.

Besides the typical adduct ion \([\text{M+K}]^+\) at m/z 819.4267 (C_{42}H_{68}KO_{13}) and \([\text{M+Na}]^+\) at m/z 803.4572 (C_{42}H_{68}NaO_{13}), we observed a large amount of fragment ions such as \([\text{M-H}_2\text{O}+\text{H}]^+\) at m/z 763.4635 (C_{42}H_{67}O_{12}), \([\text{M-2H}_2\text{O}]^+\) at m/z 745.4511 (C_{42}H_{66}O_{11}), \([\text{M-H}_2\text{O-glc}+\text{H}]^+\) at m/z 601.4083 (C_{36}H_{57}O_{7}), \([\text{M-2H}_2\text{O-glc}+\text{H}]^+\) at m/z 583.3982 (C_{36}H_{55}O_{6}), \([\text{M-H}_2\text{O-glc-fuc}+\text{H}]^+\) at m/z 455.3534 (C_{30}H_{47}O_{3}, 75) and \([\text{M-2H}_2\text{O-glc-fuc}+\text{H}]^+\) at m/z 437.3402 (C_{30}H_{45}O_{2}, 100% relative signal intensity) in the positive full scan mass spectrum. Further it’s 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 MS² spectrum obtained from the precursor ion at m/z 455.3458 showed 437.3398 (C_{30}H_{45}O_{2}, 455-H_2O), 419.3295 (C_{30}H_{43}O, 455-2H_2O), 401.3224 (C_{30}H_{41}, 455-3H_2O), 425.3399 (C_{29}H_{45}O_{2}, 455-CH_2O, Mc rearrangement), 407.3296 (C_{29}H_{43}O, 455-CH_2O-H_2O), 389.3199 (C_{29}H_{41}, 455-CH_2O-2H_2O) in the high m/z range. More importantly, in the low m/z range appeared the characteristic fragment ions at m/z 223.1687 (C_{14}H_{23}O_{2}, named as L1, ring C cleavage), 205.1584 (C_{14}H_{21}O, L1-H_2O) and 187.1484 (C_{14}H_{19}, L1-2H_2O, 100% relative signal intensity) corresponding to the AB ring part; and those coming from the CDE ring part such as 299.2361 (C_{17}H_{31}O, named as R1, ring B cleavage); 285.2213 (C_{19}H_{29}O, named as R2, ring B cleavage), 267.2123 (C_{19}H_{27}O, R2-H_2O); 283.2049 (C_{19}H_{25}O, named as R3, ring B cleavage), 265.1946 (C_{19}H_{23}O, R3-H_2O); 271.2073 (C_{19}H_{21}O, named as R4, ring B cleavage), 253.1961 (C_{19}H_{20}O, R4-H_2O), 241.1945 (C_{19}H_{20}O, R4-CH_2O), 245.1915 (C_{17}H_{23}O, R4-C_2H_2, RDA), 227.1687 (C_{17}H_{21}O, R4-C_2H_2-H_2O); 269.1914 (C_{19}H_{21}O, named as R5), 251.1805 (C_{19}H_{23}O, R5-H_2O), 239.1811 (C_{18}H_{25}O, R5-CH_2O); 233.1897 (C_{16}H_{23}O, named as R6), 215.1789 (C_{16}H_{23}O, R6-H_2O); and 231.1755 (C_{16}H_{23}O, named as R7, ring C cleavage), 213.1648 (C_{16}H_{21}O, R7-H_2O), 201.1634 (C_{16}H_{21}O, R7-CH_2O), 205.1584 (C_{16}H_{21}O, R7-C_2H_2, RDA, coincided with L1-H_2O), 187.1484 (C_{14}H_{19}, 100% relative signal intensity).
R7-C₃H₂-H₂O, coincided with L1-H₂O) (See Figure 1). The mass fragmentation pathway was proposed in Figure 2.

In the negative full scan mass spectrum, two major ions, the [M+HCOO]⁻ at m/z 825.4654 (C₄₃H₆₉O₁₅, 100% relative signal intensity) and the [M-H]⁻ at m/z 779.4593 (C₄₂H₆₇O₁₃) were observed. The anionic-adduct molecular ion transitioned to the predominant [M-H]⁻ at m/z 779.4566 (C₄₂H₆₇O₁₃) in the MS² spectrum which in turn gave the basic [M-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 [M-glc-fuc-H]⁻ at m/z 471.3464 (C₃₀H₄₇O₄, 100% relative signal intensity), the [M-glc-fuc-CH₃OH-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 indentify the structures of aglycone part and glycosyl group respectively in this experiment.

Screening of metabolites. With the aid of the MetID solution1.2 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 were shown in Figure 3. The information of the parent compound and metabolites were summarized in Table 1 and 2.

Structure elucidation of metabolites. M₀ (parent drug). M₀ in samples was unambiguously identified by comparing the retention time, 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 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 (mono-oxidation).** From the [M+Na]$^+$ at m/z 819.4527 (C$_{42}$H$_{68}$NaO$_{14}$) in the positive full scan mass spectrum and the [M-H]$^-$ at m/z 795.4561 (C$_{36}$H$_{67}$O$_{14}$) and the [M+HCOO]$^-$ at m/z 841.4623 (C$_{43}$H$_{69}$O$_{16}$, 100% relative signal intensity) in the negative full scan mass spectrum, their MF was determined as C$_{42}$H$_{68}$O$_{14}$ with one oxygen more than M0. Their predominant fragment ion [M-glc-H]$^-$ at m/z 633.3976 (C$_{36}$H$_{57}$O$_9$) from the precursor ion at m/z 795.4561 in the MS$^2$ spectrum transitioned to the major fragment ion [M-glc-fuc-H]$^-$ at m/z 487.3436 (C$_{30}$H$_{47}$O$_5$, 100% relative signal intensity) in the MS$^3$ spectrum. In addition the [M-glc-fuc-CH$_3$OH-H]$^-$ at m/z 455.3172 (C$_{29}$H$_{43}$O$_4$) and the one involving ring-cross cleavage of fucose moiety at m/z 557.3457 (C$_{33}$H$_{49}$O$_7$) were also observed in the MS$^3$ spectrum of M14 but not M15. These information indicated that both M14 and M15 were mono-oxidative metabolites of SSa and the adding oxygen should locate on the aglycone part. But we failed to assign the exact substitution.

**M1 (hydrolysis of $\beta$-glucosidic bond).** According to the [M-H$_2$O+H]$^+$ at m/z 601.4077 (C$_{36}$H$_{57}$O$_7$) in the positive full scan mass spectrum and the [M-H]$^-$ at m/z 617.4076 (C$_{36}$H$_{57}$O$_8$) and the [M+HCOO]$^-$ at m/z 663.4124
(C₃₇H₅₉O₁₀, 100% relative signal intensity) in the negative full scan mass spectrum, the MF of M1 was determined as C₃₆H₅₈O₈. The similar positive MS² spectrum of the precursor ion at m/z 455.3541 to that of the parent compound indicated that M1 has the same aglycone as SSa, which was confirmed by its UV end absorption. The same negative MS/MS spectra of the precursor ion at m/z 617.4076 as that of the parent compound showed the linkage of a deoxyhexose to the aglycone. These spectral data were in agreement with those of PSGf as reported in the literature (Huang et al., 2008). So M1 was identified as PSGf.

M16 and M17 (mono-oxidation and hydrolysis of β-glucosidic bond). The MF of M16 and M17 was determined as C₃₆H₅₈O₉ with one oxygen more than M1, according to the [M+Na]⁺ at m/z 657.3997 (C₃₆H₅₈NaO₉) in the positive full scan mass spectrum and the [M-H]⁻ at m/z 633.4018 (C₃₆H₅₇O₉) and the [M+HCOO]⁻ at m/z 679.4031 (C₃₇H₅₉O₁₁, 100% relative signal intensity) in the negative full scan mass spectrum. The MS/MS spectrum of 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 455.3178 (C₂₉H₄₃O₄) while the one involving ring-cross cleavage of fucose moiety at m/z 557.3489 (C₃₃H₄₉O₇) 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.

M2 (hydrolysis of β-fucosidic bond). The MF of M2 was determined as C₃₀H₄₈O₄ based on the [M-H₂O+H]⁺ at m/z 455.3527 (C₃₀H₄₇O₃) in the positive full scan mass spectrum and the [M+HCOO]⁻ at m/z 517.3513 (C₃₁H₄₉O₆) in the negative full scan mass spectrum. The similar positive MS² spectrum of the precursor ion at m/z 455.3527 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 SGf as reported previously (Shimizu et al., 1985b).
M3 and M4 (dehydrogenation and hydrolysis of β-fucosidic bond). According to the [M+Na]^+ at m/z 493.3279 (C_{30}H_{46}NaO_4) and the [M-H_2O+H]^+ at m/z 453.3378 (C_{30}H_{45}O_3) in the positive full scan mass spectrum, the MF of M3 and M4 was supposed as C_{30}H_{46}O_4 with two hydrogens less than M2. The CDE ring part structures of M3 and M4 remained intact according to the fragment ions at m/z 285.2207 (C_{30}H_{25}O, ion R2) and 267.2109 (C_{30}H_{27}, R2-H_2O) from the precursor ion at m/z 453.3378 in the MS^2 spectrum. Their fragment ions at m/z 221.1550 (C_{20}H_{29}O, named as L2) and 203.1428 (C_{14}H_{19}O, L2-H_2O, 100% relative signal intensity) were 2 Da less than ion L1 and L1-H_2O, respectively. These results indicated that the dehydrogenation both of M3 and M4 took place on the AB ring part structure. The fragment ion of M3 at m/z 175.1479 (C_{13}H_{19}, L2-H_2O-CO) resulting from neutral loss of CO from 203.1428 suggested that M3 has a ketone carbonyl group on the ring which should locate at C3. So M3 was inferred as 3-keto-SGf. As to M4, the fragment ion at m/z 185.1335 (C_{14}H_{17}, L3-2H_2O) with 2 Da less than L1-2H_2O indicated that the dehydrogenation of M4 could only occurred on ring A with two hydroxyl groups remained. Most likely, M4 should be Δ^1-SGf. Some key MS fragmentation of M3 and M4 were proposed in Figure 4A and 4B 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_2O+H]^+ at m/z 471.3457 (C_{30}H_{47}O_4) and the [M-2H_2O+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_{19}O_2, L1), 205.1601 (C_{14}H_{21}O, L1-H_2O) and 187.1489 (C_{14}H_{20}, L1-2H_2O, 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 M5 at m/z 249.1861 (C_{14}H_{23}O_2, named as R8), 231.1739 (C_{14}H_{21}O, R8-H_2O) and 213.1651 (C_{14}H_{19}O, R8-2H_2O) resulting from the precursor ion [M-H_2O+H]^+ at
m/z 471.3457 were in agreement to R6+O, R6+O-H2O and R6+O-2H2O respectively. We supposed that hydroxylation occurred on the CDE ring part structure of M5 and the introducing hydroxyl function may locate at C21, C22 or C27. The key MS fragmentation of M5 was illustrated in Figure 4C. For M6, the fragment ions at m/z 229.1584 (C16H21O, named as R9) and 211.1477 (C16H19, R9-H2O) obtained from the precursor ion [M-2H2O+H]+ at m/z 453.3369 were respectively consistent with R7+O-H2O and R7+O-2H2O, indicating that hydroxylation took place on the CDE ring part structure of M6 and the adding hydroxyl group may be at C19, C21, C22, C27, C29 or C30. The key MS fragmentation of M6 was shown in Figure 4D.

M7 (carboxylation and hydrolysis of β-fucosidic bond). Based on the [M-H2O+H]+ at m/z 485.3250 (C30H45O5) and the [M-2H2O+H]+ at m/z 467.3153 (C30H43O4, 100% relative signal intensity) in the positive full scan mass spectrum and the [M-H] at m/z 501.3234 (C30H45O6) in the negative full scan mass spectrum, the MF of M7 was determined as C30H46O6 with two oxygens more and two hydrogens less than M2. In the MS² spectrum of the precursor ion at m/z 485.3250, the fragment ions at m/z 223.1692 (C14H23O2, L1), 205.1594 (C14H21O, L1-H2O) and 187.1482 (C14H19, L1-2H2O, 100% relative signal intensity) showed the intact AB ring part structure. Moreover, the fragment ions at m/z 263.1567 (C16H23O3, named as R10, in line with R6+2O-2H), 245.1433 (C16H21O2, R10-H2O) and 199.1510 (C15H19, R10-H2O-HCOOH) showed one carboxyl function and two hydroxyl groups on the CDE ring part structure and expelled 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 was presented in Figure 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 C30H46O6 with two oxygens more and two hydrogens less 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_{25}O, R6) and 215.1805 (C_{16}H_{23}, R6-H_{2}O) from the precursor
ion at m/z 485.3273 in the MS² spectrum. Additionally, the fragment ions at m/z 253.1451 (C_{14}H_{21}O, named as L4,
consistent with L1+2O-2H), 235.1340 (C_{14}H_{19}O_{2}, 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
structure. Further, the carboxyl group should not locate at C25 or C26 because of the fragment ions at m/z
233.1907 (C_{16}H_{25}O, R6) and 215.1805 (C_{16}H_{23}, R6-H_{2}O) responsible for the CDE ring part structure. We supposed
that carboxylation took place at 23-CH_{2}OH accompanied by hydroxylation at C1, C2 or C24. The key MS
fragmentation of M8 was illustrated in Figure 4F.

The MF of M10 was determined as C_{30}H_{46}O_{7} with three oxygens more and two hydrogens less than M2
according to the [M-H_{2}O+H]^{+} at m/z 501.3199 (C_{30}H_{45}O_{6}) and the [M-2H_{2}O+H]^{+} at m/z 483.3980 (C_{30}H_{43}O_{5}, 100%
relative signal intensity) in the positive full scan mass spectrum and the [M-H]⁻ at m/z 517.3171 (C_{30}H_{45}O_{7}) 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 (C_{14}H_{21}O_{5}, L4), 235.1345 (C_{14}H_{19}O_{4}, L4-H_{2}O), 217.1235 (C_{14}H_{17}O_{2}, L4-2H_{2}O) and
171.1167 (C_{13}H_{15}, L4-2H_{2}O-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 (C_{16}H_{23}O_{3}, R8), 231.1713 (C_{16}H_{23}O_{4}, R8-H_{2}O) and
213.1560 (C_{16}H_{21}, R8-2H_{2}O) 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 were listed in the Supplemental
Table 1.
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 the plasma, bile, urine and feces samples following oral and intravenous routes of administration. Our results showed that SSa is metabolized diversely. The metabolic profile was proposed in Figure 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 besides 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 more comprehensive biological process of SSa in detail was deduced from the complementary results after different routes of administration as shown in Figure 6. After intravenous dosing SSa was oxidized 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 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-bounded β-glucosidase with broad specificity catalyzing such reactions had been detected in most mammalian tissues (Robinson et al., 1956; Abrahams and Robinson, 1969; Clew et al., 1976; Daniels et al., 1981). SSa, M13-M17 excreted through the bile duct have been metabolized further in the intestine according to their disappearance in the feces. In the intestine SSa was metabolized through the hydrolysis mediated by the intestinal flora following the oxidation catalyzed by the metabolizing enzyme in the intestinal mucosal cells (Kaminsky and Zhang 2003; Zhang et al., 2007). Both the renal and billiary routines 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 that 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, 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 (Shimizu et al., 1985a).

What’s more, 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). The previous structure–activity relationship researches showed that for these activities the ether linkage
between C13 and C28, the 23-CH₂OH, the configuration of the hydroxy group at C16 and the proper polar
balance between the sugar moiety (polar position) and the aglycone moiety (nonpolar position) are important (Abe
et al., 1980; Yokoyama et al., 1981; Nose et al., 1989a; Nose et al., 1989b; Kumazawa et al., 1990; Ahn et al., 1998;
Tsai 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 hydroxy group at C16 of SSa 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
group by the phase I enzymes can dramatically alter the biological properties of the drug (Brunton et al., 2008). As
to saikosaponins, the -OH was proven important for their interaction with cell surface based on the fact that while
the transformation of the 23-methyl group (saikosaponin e) to CH₂OH (SSa) resulted in 2-fold enhancement in the
anti-cell adhesive activity on the whole, the acetylation of this carbinol group to form 23-O-acetysaikosaponin-a
led to a substantial loss of action (Ahn et al., 1998). Thus, the introduction of the hydroxyl and carboxyl group on
the aglycone moiety may lead to the marked change of activities, which is of significance for the development of
saikosaponins in the pharmaceutical industry. In addition we also detected two dehydrogenating metabolites, M3
and M4, in both of feces and urine of rats after oral dosing of SSa. The new adding unsaturated groups on M3 and
M4 may make them function as electrophiles which can react with nucleophilic cellular macromolecules such as
DNA, RNA and protein. Further assessing for exposure and activities of these metabolite still need to be
conducted.

To summarize, the metabolic profile of SSa in rats has been characterized in detail by comparing the results
following different routes of administration using the LC-DAD-IT-TOF-MS. The observed routes of SSa
metabolism involve hydration and mono-oxidation on the aglycone moiety and hydrolysis of β-glucosidic bond in
the liver while some types of oxidation including dehydrogenation, hydroxylation and carboxylation on the
aglycone moiety following sequential hydrolysis both of β-glucosidic and β-fucosidic bonds in the intestinal tract.
SSa and its metabolites can be excreted through both the renal and biliary routines. These results may provide
valuable information for assessing the efficacy and safety of prescription containing saikosaponins in the clinical
applications. Moreover the discussion for oxidation on the aglycone moiety gives us some clues for structural
modification of these promising compounds.
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Authorship Contributions

Participated in research design: Liu, Tian and Zhang

Conducted experiments: Liu and Li

Performed data analysis: Liu, Xu and Song

Wrote or contributed to the writing of the manuscript: Liu, Tian and Zhang
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 lines. Apoptosis 16: 184-197.


DMD #48975


Footnotes

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zunjianzhangcpu@hotmail.com.
Legends of Figures

Figure 1. The positive MS² spectra of SSa. (A) Full scan mass spectra; (B) MS² of the precursor ion at m/z 763.4635; (C) MS² of the precursor ion at m/z 455.3534.

Figure 2. Proposed fragmentation pathway of SSa.

Figure 3. EICs of the typical biosamples of rats. (A) Control bile sample after intravenous administration; (B) Dosed bile sample after intravenous administration; (C) Control feces sample after oral administration; (D) Dosed feces sample after oral administration.

Figure 4. Proposed fragmentation pathways of part of metabolites. (A) M3; (B) M4; (C) M5; (D) M6; (E) M7; (F) M8.

Figure 5. The major metabolic pathway of SSa in rats.

Figure 6. The proposed disposition of SSa in rats after oral and intravenous administration.
Table 1. Metabolites of SSa detected by the MDF method with different templates.

<table>
<thead>
<tr>
<th>MDF Template</th>
<th>Metabolites</th>
<th>MF</th>
<th>Molecular Weight</th>
<th>ΔMD from Template</th>
<th>Metabolites appearance in biosamples</th>
<th>Intravenous</th>
<th>Oral</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Plasma</td>
<td>Bile</td>
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<td>- + - - -</td>
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<td>- - - + +</td>
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+, Detected; -, Undetected.
Table 2. LC-DAD-IT-TOF-MS analysis of SSa and its observed metabolites in rats biosamples.

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<th>No.</th>
<th>( T_R ) (min)</th>
<th>UV (nm)</th>
<th>Positive MS(^1)</th>
<th>Negative MS(^1)</th>
<th>Positive MS(^2)</th>
<th>Negative MS(^2)</th>
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<td>779.4593,</td>
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<td>797.4706</td>
<td>843.4772(^a)</td>
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<td>HOSSa(^c)</td>
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<td>795.4553</td>
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<tr>
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<td>517.3513&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[455.3527]→ 285.2210, 267.2118, 223.1703, 205.1597, 187.1497&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SGf&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
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<td>52.3</td>
<td>&lt; 190</td>
<td>435.3271&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>3-keto-SGf</td>
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<td>533.3499&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[471.3457]→ 249.1861, 231.1739, 223.1697, 213.1651, 205.1601, 187.1489&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td>453.3361&lt;sup&gt;a&lt;/sup&gt;, 471.3469</td>
<td>533.3467&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>&lt; 190</td>
<td>467.3153&lt;sup&gt;a&lt;/sup&gt;, 485.3250</td>
<td>501.3234&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[485.3250]→ 455.3161, 263.1656, 245.1533, 231.1374, 223.1692, 205.1594, 199.1493, 187.1482&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[501.3234]→ 453.2987&lt;sup&gt;a&lt;/sup&gt;, 437.3038</td>
<td>SGf-27-oic acid</td>
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<td>M8</td>
<td>42.2</td>
<td>&lt; 190</td>
<td>467.3167&lt;sup&gt;a&lt;/sup&gt;, 485.3273</td>
<td>501.3238&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[485.3273]→ 253.1451, 235.1340, 233.1907, 217.1224, 215.1805, 171.1169&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[501.3238]→ 453.2991&lt;sup&gt;a&lt;/sup&gt;, 437.3044</td>
<td>Carboxylation and hydroxylation of SGf</td>
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<td>M9</td>
<td>27.0</td>
<td>&lt; 190</td>
<td>467.3158&lt;sup&gt;a&lt;/sup&gt;, 485.3259</td>
<td>501.3217&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[485.3259]→ 467.3140&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[501.3217]→ 453.3016&lt;sup&gt;a&lt;/sup&gt;, 437.3069</td>
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<td>&lt; 190</td>
<td>483.3098&lt;sup&gt;a&lt;/sup&gt;, 501.3199</td>
<td>517.3171&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[501.3199]→ 253.1447, 249.1867, 235.1345, 231.1743, 217.1235, 213.1635, 171.1167&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[517.3171]→ 469.2965&lt;sup&gt;a&lt;/sup&gt;, 453.3023</td>
<td>Carboxylation and hydroxylation of SGf</td>
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<td>M11</td>
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<td>&lt; 190</td>
<td>483.3104&lt;sup&gt;a&lt;/sup&gt;, 501.3204</td>
<td>517.3152&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[501.3204]→ 483.3127&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[517.3152]→ 469.2932&lt;sup&gt;a&lt;/sup&gt;, 453.2989</td>
<td>Unidentified</td>
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<tr>
<td>M12</td>
<td>30.2</td>
<td>&lt; 190</td>
<td>483.3123&lt;sup&gt;a&lt;/sup&gt;, 501.3207</td>
<td>517.3184&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[501.3207]→ 483.3087&lt;sup&gt;a&lt;/sup&gt;</td>
<td>[517.3184]→ 469.2936&lt;sup&gt;a&lt;/sup&gt;, 453.2991</td>
<td>Unidentified</td>
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<sup>a</sup> Of 100% relative signal intensity; <sup>b</sup> With standard; <sup>c</sup> With data in reference.
Figure 2

This figure illustrates the molecular structures of various compounds, showing their fragmentation pathways and mass-to-charge ratios (m/z) at different stages. The compounds are denoted with A, B, C, D, E, etc., and the reactions include losses of water (\(-\text{H}_2\text{O}\)) and the addition of a deprotonated water molecule (\(-\text{H}_2\text{O}+\text{H}^+\)). The molecular formulas for each compound are indicated, with some labeled with R or L, and their corresponding m/z values are noted.
Figure 4

A. [(M3-H2O+H)+] at m/z 453

B. [(M4-H2O+H)+] at m/z 453

C. [(M5-H2O+H)+] at m/z 471

D. [(M6-H2O+H)+] at m/z 471

E. [(M7-H2O+H)+] at m/z 485

F. [(M8-H2O+H)+] at m/z 485
Figure 5
Figure 6

Liver

M13

Hydration

Mono-oxidation

M14 & M15

Bile Duct

M0 - M2, M5 - M11 in feces

Intestine

SSa

Hydrolysis

M1

Oxidation

M5 - M11

M1

Mono-oxidation

M16 & M17

Hydrolysis

SSa

Mono-oxidation

M14

Hydrolysis

M16

Bile Duct

M0 - M12 in feces

Liver

M1

Systemic Circulation

M0 & M1 in urine

Kidney

Systemic Circulation

M0 - M4, M7 - M11 in urine

Kidney

Intestine

SSa

Hydrolysis

M1

Oxidation

M5 - M11

i.g.

SSa

Mono-oxidation

M14

Hydrolysis

M16

Portal Vein

M3 - M12

SSa

Mono-oxidation

M14

Hydrolysis

M16