Research Article

Metabolism Analysis of Alantolactone and Isoalantolactone in Rats by Oral Administration

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Alantolactone and isoalantolactone are the major active ingredients of *Inulae Radix*. Their metabolism *in vivo* and *in vitro* was investigated by UPLC-Q-TOF-MS for the first time. As a result, nine metabolites *in vivo* including cysteine conjugates, oxidates, dehydrogenates, and hydrates were detected in rat bile after oral administration. The metabolites produced *in vitro* by incubation with rat liver microsomes were found to be substantially identical to those detected *in vivo*. However, no metabolites were detected in the samples of plasma, feces, and urine or in the incubates of gastric juice, intestinal juice, and intestinal bacteria. These results reveal that the liver is the main metabolic organ for alantolactone and isoalantolactone, and the first pass effect of the liver appears to be the reason for the low oral bioavailability of the two lactones.

1. Introduction

Alantolactone and isoalantolactone, first isolated from *Inula helenium* [1], are the main active constituents in *Radix Inulae*, commonly used in a folk medicine called Tu-Mu-Xiang in China [2]. Current literature shows that alantolactone and isoalantolactone possess various antimicrobial [3], anti-inflammatory [4], and antitumor activities [5–10] both *in vivo* and *in vitro*. A recent study done by the team revealed that alantolactone and isoalantolactone could significantly relieve irritable bowel syndrome (IBS) by regulating neurotransmitters, gastrointestinal hormones, and intestine propulsive movement. This study suggested that alantolactone and isoalantolactone were promising candidates for IBS applications [11].

To further evaluate their potential use as drugs, some key pharmacokinetic parameters were investigated in the previous study. The results demonstrated that the absolute bioavailabilities of alantolactone and isoalantolactone were not high [12, 13], indicating that they may undergo metabolism *in vivo*. However, no metabolism studies on the two lactones have been reported. In the present study, their metabolic profiles *in vivo* in rats and *in vitro* were elucidated.

2. Experimental

2.1. Chemicals and Materials. Dried roots of *Inula helenium* L. were purchased from the herbal market in May 2015 in Sichuan Province, China, and identified by Professor Xiaobo Li. A specimen (SJTUIH 20150509) was deposited in the School of Pharmacy, Shanghai Jiao Tong University. HPLC-grade acetonitrile was purchased from Merck Company (Darmstadt, Germany). Petroleum ether (60–90°C), acetone, dichloromethane, alcohol, and silver nitrate were of analytical grade and purchased from China National Medicines Co. Ltd. (Shanghai, China). Ultra-pure water used in UPLC mobile phase was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Preparation of Alantolactone, Isoalantolactone, and Other Relevant Lactones. *Inula helenium* roots (2.1 kg) were extracted by refluxing with 9 L 95% ethanol twice (each 2 h). The extraction solution was completely evaporated under reduced pressure to afford a dark brown extractive (137 g). The extractive was suspended in 1500 mL water and extracted with dichloromethane (1000 mL × 3). Dichloromethane solution was evaporated under reduced pressure to leave

behind a brown residue (58 g). The residue was subjected to silica gel CC (90 cm $\times$ 8 cm) which was eluted with petroleum ether-acetone (100:0 to 95:5, v/v) to afford 34 fractions (Fr.1–Fr.34). Fr.6 (5.2 g) was further separated by silica gel (containing 10% silver nitrate) CC (70 cm $\times$ 5.5 cm) eluted with petroleum ether-acetone (98:2, v/v) to afford compounds **1** (266 mg) and **2** (183 mg). Fr.8 (847 mg) was further purified by silica gel CC (60 cm $\times$ 4.5 cm) eluted with petroleum ether-acetone (98:2 to 96:4, v/v) to afford compound **3** (18 mg). Fr.10 (562 mg) was further subjected to silica gel CC (60 cm $\times$ 4.5 cm) eluted with petroleum ether-acetone (97:3, v/v) to afford compound **4** (53 mg). Fr.13 (509 mg) was further purified by silica gel CC (50 cm $\times$ 4.5 cm) eluted with petroleum ether-acetone (95:5 to 92:8, v/v) to afford compounds **5** (22 mg) and **6** (13 mg). Fr.16 (419 mg) was further separated by silica gel (containing 10% silver nitrate) CC (50 cm $\times$ 4.5 cm) eluted with petroleum ether-acetone (85:15, v/v) to afford compounds **7** (11 mg) and **8** (5 mg).

2.3. Animal Experiment and Sample Preparation

2.3.1. Animal Experiment. Twelve male Sprague-Dawley rats (200 $\pm$ 20 g) were purchased from the Laboratory Animal Center of Fudan University (Shanghai, China). All experiments on rats were implemented in accordance with the guidelines set by Shanghai Jiao Tong University Animal Ethics Committee (Shanghai, China). The animals were randomly divided into alantolactone group, isosalantolactone group, and blank group (each with four rats), housed under controlled conditions (temperature: 20–25°C, humidity: 40–70%) with a natural light-dark cycle for 1 week before the experiment. All rats were fasted overnight with water allowed ad libitum, and then a single dose of alantolactone or isosalantolactone was given orally to rats at 90 mg/kg (blank group received oral administration of water). Plasma samples were collected from the orbital vein at 60 min after administration [13]. Urine and feces samples from the rats were collected during 24 h after administration. A cannular port system was surgically implanted on the rats, which allowed continuous collection of bile. Bile samples from rats were obtained during 12 h after dosing. The samples were stored at –20°C until further analysis.

2.3.2. Sample Preparation. Plasma sample (100 μ L) was added to 400 μ L acetonitrile in a 1.5 mL centrifuge tube. The tube was vortex-mixed for 1 min and centrifuged at 14,000 rpm for 10 min. The supernatant (2 μ L) was injected into the LC-MS analysis.

Feces sample was pulverized with a mortar and pestle. Five volumes of normal saline and acetonitrile (1:3, w/v) were added to 0.05 g pulverized feces and vortexed for 1 min. After centrifugation at 14,000 rpm for 10 min, 10 μ L supernatant was diluted with 1 mL acetonitrile. An aliquot of 2 μ L of the sample was injected into the LC-MS system for further analysis.

Urine sample (100 μ L) was added to 400 μ L acetonitrile in a 1.5 mL centrifuge tube. The tube was vortexed for 1 min. After centrifugation at 14,000 rpm for 10 min, the supernatant

was evaporated to dryness in a 40°C water bath by a nitrogen blowing instrument. The residue was reconstituted with 100 μ L mobile phase. All the dissolved samples were centrifuged at 14,000 rpm for another 5 min, and an aliquot of 2 μ L supernatant was injected into the LC-MS system for further analysis.

Bile sample (15 μ L) was added to 485 μ L acetonitrile in a 1.5 mL centrifuge tube, vortexed for 1 min, and centrifuged at 14,000 rpm for 10 min. An aliquot of 2 μ L supernatant was injected into the LC-MS system for further analysis.

2.4. Incubation In Vitro and Sample Preparation

2.4.1. Incubation with Artificial Gastric Juice and Intestinal Juice. Two milligrams each of alantolactone or isosalantolactone was added to 10 mL of artificial gastric juice (containing 10 mg/mL pepsin and 0.08 M HCl, pH 1.4) and artificial intestinal juice (containing 10 mg/mL trypsin and 0.05 M KH_2PO_4 , pH 6.8), respectively. The mixture was incubated at 37°C and one milliliter of incubated solution was transferred to a 10 mL volumetric centrifuge tube at 0, 1, 2, 4, and 6 h and adjusted to a pH of 7.0 with 0.1 M sodium hydroxide. A four-fold volume of methanol was added to the incubated solution immediately and mixed. The mixture was centrifuged at 4800 rpm for 20 min, and the supernatant was evaporated under reduced pressure (40°C) to produce a residue. The residue was dissolved in 1 mL of methanol, filtered through a 0.22 μ m membrane, and then analyzed by LC-MS.

2.4.2. Incubation with Rat Intestinal Bacteria. Fresh fecal samples from rats (2 g) were homogenized with 25 volumes of GAM broth. The sediments were removed by filtration through three pieces of gauze. The suspension was incubated at 37°C with 4 mg alantolactone or isosalantolactone in an anaerobic incubator in which the air had been replaced with a gas mixture (H_2 5%, CO_2 10%, and N_2 85%) for 48 h. The cultured mixture was taken out and extracted with water-saturated *n*-butanol at 0, 6, 12, 24, and 48 h, respectively. The extract was then evaporated and the residue dissolved in 0.5 mL methanol. The redissolved sample was analyzed with LC-MS.

2.4.3. Incubation with Rat Microsomes. Samples for incubation were prepared with 50 μ M alantolactone or isosalantolactone, 5 mM MgCl_2 , 1 mM NADPH, and 1 mg/mL microsomal protein in potassium phosphate buffer (0.1 M, pH 7.4). After preincubation at 37°C for 3 min, the incubations were initiated by the addition of NADPH and further incubated at 37°C for 60 min in a shaking water bath. The reactions were terminated with an equal volume of ice-methanol. The mixtures were then centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was analyzed for metabolite profiling by UPLC-MS.

2.5. UPLC-MS Conditions. Chromatographic separation was performed on an ACQUITY UPLC BEH C_{18} column (100 mm $\times$ 2.1 mm i.d., 1.7 μ m, Waters Corp., Milford, MA, USA) using an ACQUITY™ UPLC system equipped with a

FIGURE 1: Representative TIC chromatograms of bile collected during 24 h after oral administration of alantolactone and isovalantolactone. (1) Blank group; (2) alantolactone group; and (3) isovalantolactone group.

binary pump, a DAD detector, a vacuum degasser, and an autosampler (Waters Corp., Milford, MA, USA). The detection wavelength was set at 210 nm and the column oven was maintained at 40°C. The mobile phase contained acetonitrile (A) and 0.1% formic acid (B) solution, at a flow rate of 0.2 mL/min. For analysis of metabolites, the gradient system started with 5% A, programmed linearly to 80% A in 20 min, held for 2 min, followed by a decrease to 20% A in 1 min to reequilibrate the column. The samples were kept at 10°C in the autosampler.

Mass spectrometry was carried out using a Waters Synapt mass spectrometer (Waters Corp., Milford, MA, USA). Ionization was operated using an ESI source in the positive ion mode. The ionization source parameters were as follows: capillary voltage 1 kV, cone voltage 30 V, source temperature 110°C, desolvation temperature 350°C, desolvation gas flow 600 L/h, and cone gas flow 30 L/h. Nitrogen was used as the desolvation and cone gas, and argon was used as the collision gas. Data acquisition was performed in full-scan. All data acquired were processed using Masslynx v.4.1 software (Waters Corp., Milford, MA, USA).

3. Results and Discussion

3.1. Preparation of Alantolactone, Isoalantolactone, and Other Relevant Lactones. The repeated silica gel CC afforded alantolactone (1) and isovalantolactone (2). In addition, six relevant lactones 3,4-dehydroalantolactone (3), 5 α ,6 α -epoxyalantolactone (4), 6-hydroxyl alantolactone (5), 4-hydroxymethyl isovalantolactone (6), 11-carboxyl alantolactone (7), and 11-carboxyl isovalantolactone (8) were obtained. Their structures were confirmed by MS and NMR determination [14–19].

3.2. The Metabolites Analysis of Alantolactone and Isoalantolactone In Vivo. The metabolites in plasma, bile, feces, and urine of rats orally administrated with alantolactone and isovalantolactone were investigated by UPLC-Q-TOF-MS

analysis. Although no metabolites were detected in plasma, urine, and feces samples, 9 metabolites were found in bile samples (Figure 1 and Supplementary file available here). Their chemical structures were further deduced from their t_R values, exact masses, formulas, and characteristic fragmentation ions (Table 1).

Metabolites M1 and M6 both with a formula of $C_{15}H_{20}O_4$ were detected as protonated molecular ions $[M + H]^+$ at m/z 265 which were 32 Da higher than those of alantolactone and isovalantolactone, indicating that they could be peroxidation products of alantolactone and isovalantolactone. Product ions at m/z 247 were also observed, which suggested the successive loss of H_2O through the cleavage of the lactone ring. M1 and M6 were further confirmed to be 11-carboxyl alantolactone and 11-carboxyl isovalantolactone, by their identical retention times and the same molecular weights to compounds 7 and 8, respectively.

Metabolites M2 and M7 both with a formula of $C_{18}H_{27}NO_4S$ exhibited $[M + H]^+$ ions at m/z 354, which were 119 Da higher than those of alantolactone and isovalantolactone, indicating that M2 and M7 were their cysteine conjugates. The presence of a minor fragment ion at m/z 120 further indicated the existence of cysteine. Based on these results and the findings from the previous report [20], M2 and M7 were considered as the two-phase reaction metabolites of liver and speculated to be 13-cysteine alantolactone and 13-cysteine isovalantolactone, respectively.

Metabolite M3 with a retention time of 3.898 min and a formula of $C_{15}H_{20}O_3$ was observed as protonated molecular ion $[M + H]^+$ at m/z 249 which was 16 Da higher than the protonated ion of alantolactone, indicating that M3 was an epoxide of alantolactone. The MS spectrum of M3 also showed a product ion at m/z 231, which was generated from m/z 249 by loss of H_2O . M3 was further identified as 5 α ,6 α -epoxyalantolactone by its identical retention time and molecular weight to compound 4.

Metabolite M4 was observed as protonated molecular ion $[M + H]^+$ at m/z 231, which was 2 Da lower than the

TABLE 1: Parent compounds and metabolites detected in the bile of rats after oral administration of alantolactone and isoalantolactone.

Parent compound/metabolite	t_R (min)	Measured mass	Calculated mass	Formula	Formula change	Characteristic fragmentation ion	Identification
Alantolactone							
M1	6.216	232.1549	232.1463	$C_{15}H_{20}O_2$	/	233.1549 $[M + H]^+$, 215.1438 $[M + H - H_2O]^+$	/
	2.550	264.1457	264.1462	$C_{15}H_{20}O_4$	$[C_{15}H_{20}O_2] + O_2$	265.1457 $[M + H]^+$, 247.1350 $[M + H - H_2O]^+$, 229.1219 $[M + H - 2H_2O]^+$	Peroxidate
M2	3.296	353.1762	353.1661	$C_{18}H_{27}NO_4S$	$[C_{15}H_{20}O_2] + C_3H_7NO_2S$	354.1762 $[M + H]^+$	Cysteine conjugate
M3	3.898	249.1490	248.1412	$C_{15}H_{20}NO_3$	$[C_{15}H_{20}O_2] + O$	249.1490 $[M + H]^+$, 231.1390 $[M + H - H_2O]^+$	Epoxide
M4	5.247	230.1387	230.1307	$C_{15}H_{18}O_2$	$[C_{15}H_{20}O_2] - H_2$	231.1387 $[M + H]^+$, 203.1446 $[M + H - CO]^+$	Dehydrogenate
M5	5.724	250.2015	250.1569	$C_{15}H_{22}O_3$	$[C_{15}H_{20}O_2] + H_2O$	251.2015 $[M + H]^+$, 233.1988 $[M + H - H_2O]^+$, 215.1808 $[M + H - 2H_2O]^+$, 186.9544 $[M + H - 2H_2O - CO]^+$	Hydrate
Isoalantolactone							
M6	6.048	232.1558	232.1463	$C_{15}H_{20}O_2$	/	233.1558 $[M + H]^+$, 215.1441 $[M + H - H_2O]^+$, 265.1431 $[M + H]^+$, 247.1335 $[M + H - H_2O]^+$, 229.1234 $[M + H - 2H_2O]^+$	/
	2.314	264.1431	264.1462	$C_{15}H_{20}O_4$	$[C_{15}H_{20}O_2] + O_2$		Oxide
M7	3.209	353.1740	353.1661	$C_{18}H_{27}NO_4S$	$[C_{15}H_{20}O_2] + C_3H_7NO_2S$	354.1740 $[M + H]^+$	Cysteine conjugate
M8	4.612	230.1383	230.1307	$C_{15}H_{18}O_2$	$[C_{15}H_{20}O_2] - H_2$	231.1383 $[M + H]^+$, 186.9587 $[M + H - CO_2]^+$	Dehydrogenate
M9	5.725	250.2039	250.1569	$C_{15}H_{22}O_3$	$[C_{15}H_{20}O_2] + H_2O$	251.2039 $[M + H]^+$, 233.1923 $[M + H - H_2O]^+$, 215.1811 $[M + H - 2H_2O]^+$, 186.9593 $[M + H - 2H_2O - CO]^+$	Hydrate

FIGURE 2: Metabolism profiles of alantolactone and isoalantolactone incubated with rat liver microsomes. Data are presented as mean $\pm$ SD, $n = 3$. Metabolic ratio = $(P_0 - P_t)/P_0 \times 100\%$, P_0 : peak area of 0 min; P_t : peak area of t min.

FIGURE 3: The representative TIC chromatograms of alantolactone and isoalantolactone incubated with rat liver microsomes for 1 h. (1) Blank; (2) alantolactone; and (3) isoalantolactone; M1–M9, metabolites.

protonated ion of alantolactone, indicating that M4 was a dehydrogenate. The MS spectrum also showed a product ion at m/z 203, which was generated from m/z 231 by loss of CO. M4 was confirmed to be 3,4-dehydroalantolactone by the identical retention time and the same molecular weight to compound 3.

Metabolites M5 and M9 were detected as protonated molecular ions $[M + H]^+$ at m/z 251, which were 18 Da higher than the protonated ions of alantolactone and isoalantolactone, indicating that M5 and M9 could be their hydrates. MS spectra also showed fragment ions at m/z 233, corresponding to $[M + H - H_2O]^+$, suggesting the presence of a hydroxyl group. M5 and M9 were confirmed to be 6-hydroxyl alantolactone and 15-hydroxyl isoalantolactone by the identical retention times and molecular weights to compounds 5 and 6, respectively.

Metabolite M8 was observed at m/z 231 $[M + H]^+$ and also considered as a dehydrogenate. The MS spectrum showed a

product ion at m/z 203, which was generated from m/z 231 by loss of CO. Based on these results and the findings from our previous research [21], M8 was speculated to be 5, 6-dehydroisoalantolactone.

3.3. The Metabolic Organ of Alantolactone and Isoalantolactone. The results mentioned above suggested that liver was the main metabolic organ of alantolactone and isoalantolactone. To confirm this result, alantolactone and isoalantolactone each was incubated *in vitro* with gastric juice, intestinal juice, fecal bacteria, and liver microsomes. The lactones were found to be stable in gastric juice, intestinal juice, and fecal bacteria, respectively. However, the incubation of alantolactone and isoalantolactone with the liver microsomes from the experimental animal led to the rapid metabolism (Figure 2) and generated 9 metabolites identical to those found *in vivo* (Figure 3). This confirmed that the main

FIGURE 4: Possible metabolic pathways of alantolactone and isoalantolactone in rat.

metabolic organ for the two lactones was the liver. Possible metabolic pathways for the lactones are shown in Figure 4.

4. Conclusion

In the present study, metabolism of alantolactone and isoalantolactone *in vivo* (urine, feces, bile, and plasma) and *in vitro* (gastric juice, intestinal juice, fecal bacteria, and liver microsomes) were investigated. Nine metabolites were found in bile and liver microsomes, indicating that a first pass effect of the liver on the two lactones existed.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Supplementary Materials

Figure 1: HR ESI (+) MS spectrum of alantolactone detected in bile. Figure 2: HR ESI (+) MS spectrum of alantolactone metabolite M1 detected in bile. Figure 3: HR ESI (+) MS spectrum of alantolactone metabolite M2 detected in bile.

Figure 4: HR ESI (+) MS spectrum of alantolactone metabolite M3 detected in bile. Figure 5: HR ESI (+) MS spectrum of alantolactone metabolite M4 detected in bile. Figure 6: HR ESI (+) MS spectrum of alantolactone metabolite M5 detected in bile. Figure 7: HR ESI (+) MS spectrum of isoalantolactone detected in bile. Figure 8: HR ESI (+) MS spectrum of isoalantolactone metabolite M6 detected in bile. Figure 9: HR ESI (+) MS spectrum of isoalantolactone metabolite M7 detected in bile. Figure 10: HR ESI (+) MS spectrum of isoalantolactone metabolite M8 detected in bile. Figure 11: HR ESI (+) MS spectrum of isoalantolactone metabolite M9 detected in bile. (*Supplementary Materials*)

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