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Simultaneous determination of three sesquiterpene lactones from *Herba Inula* extract in rat plasma by LC/MS/MS and its application to pharmacokinetic study

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ABSTRACT

A rapid and sensitive liquid chromatography–tandem mass spectrometry method has been developed and validated for the determination of 1-acetoxy-6α-hydroxyeriolanolide, 1β-hydroxyalantolactone and ivangustin from *Herba Inula* extract in rat plasma. Plasma samples were pretreated by protein precipitation with methanol. Chromatographic separation was accomplished on a TOSOH TSKgel ODS column with mobile phase consisting of methanol and 0.3% formic acid (80:20, v/v). The detection was carried out by multiple-reaction monitoring mode under positive electrospray ionization. The quantification was performed using the transitions of *m/z* 309.1/185.0 for 1-acetoxy-6α-hydroxyeriolanolide, *m/z* 249.0/231.1 for 1β-hydroxyalantolactone and ivangustin and *m/z* 285.0/193.0 for diazepam, respectively. Calibration curves were linear over the concentration range of 4–800 ng/mL for 1-acetoxy-6α-hydroxyeriolanolide, 8–500 ng/mL for 1β-hydroxyalantolactone and ivangustin. The limit of detection (LOD) was 1 ng/mL for 1-acetoxy-6α-hydroxyeriolanolide, 1.6 ng/mL for 1β-hydroxyalantolactone and ivangustin (S/N = 3). The intra-day and inter-day precisions (RSD%) for the three compounds were less than 7.8% and 8.6%, and the accuracy (RE%) ranged from −4.6 to 6.8%. The method was successfully applied to pharmacokinetic studies of the three sesquiterpene lactones after oral administration of 300 mg/kg *Herba Inula* extract to rats, the $t_{1/2}$ of 1-acetoxy-6α-hydroxyeriolanolide, 1β-hydroxyalantolactone and ivangustin was 9.65 ± 1.43, 14.88 ± 0.82 and 13.93 ± 2.74 (h). The $AUC_{(0-t)}$ of 1-acetoxy-6α-hydroxyeriolanolide, 1β-hydroxyalantolactone and ivangustin was 1102.46 ± 247.04, 808.92 ± 117.53 and 990.35 ± 275.49 (ng h/mL), respectively.

1. Introduction

Traditional Chinese medicine (TCM) has a long history of therapeutic practice in China. Nowadays, with the improving methods and advanced technology, more attention has been paid to the clinical use of TCM. *Herba Inulae* (Jinfeicao), a Chinese folk medicine, is derived from the aerial parts of *Inula japonica* Thunb belonging to the family of Asteraceae, and has been listed in the Pharmacopeia of the People's Republic of China [1]. It has been used for the treatment of dispersing phlegm, relieving coughing, treating bronchitis, trachitis and parotitis [1,2]. The main bioactive components of *Herba Inulae* are sesquiterpene lactones, and 37 of which have been isolated and identified by now [3–5].Pharmacological studies have shown that 1-acetoxy-6α-hydroxyeriolanolide, 1β-hydroxyalantolactone and ivangustin (Fig. 1), which are abundant in *Herba Inula*, have diverse biological activities, such as antifungal, antibacterial, anti-inflammatory, antidiabetic, as well as insecticidal effects [6–12]. In addition, the compound 1β-hydroxyalantolactone has been patently for the treatment of rheumatoid arthritis [13].

To our knowledge, few reports described a method for the determination of the three compounds in biological samples, and there was no publication on the pharmacokinetic study of the three sesquiterpene lactones in animals. Three papers reported the assay of three compounds' analogs, atractylenolide I, atractylenolide II and atractylenolide III in vivo [14–16], which have similar effects [17–19] of the three compounds we have analyzed in this paper. Furthermore, there's one paper reporting the determination of costunolide and dehydrocostuslactone [20], the well know sesquiterpene lactones, in rat plasma recently. Since sesquiterpene lactones have various biological activities and more new compounds [5,11] will be isolated, there's a need to learn more metabolic behaviors of them for the further study. As the

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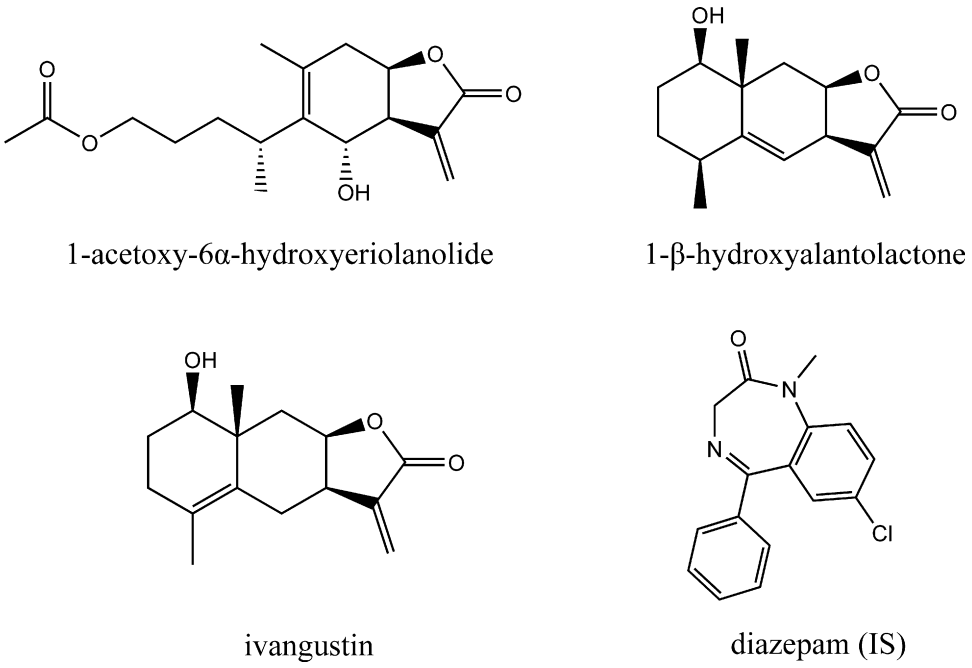

Fig. 1. Chemical structures of 1-acetoxy-6α-hydroxyeriolanolide, 1β-hydroxyalantolactone, ivangustin and diazepam (IS).

three sesquiterpene lactones have poor ultraviolet absorptions, high-performance liquid method with ultraviolet detection (HPLC-UV) is not sensitive enough for the pharmacokinetic study of these three compounds. Therefore, the aim of this study was to develop and validate a rapid, simple, and sensitive LC/MS/MS method for the determination of the three analytes in rat plasma for the first time. The present method is simple, sensitive and reproducible, and thus could be successfully applied to pharmacokinetics study after oral administration of *Herba Inulae* extract.

2. Experimental and methods

2.1. Materials

Reference standards of 1-acetoxy-6α-hydroxyeriolanolide, 1β-hydroxyalantolactone, ivangustin with purity more than 98% were isolated and purified in our laboratory and confirmed by comparing the IR, ^{1}H and ^{13}C nuclear magnetic resonance data with reported Refs. [21,22]. Diazepam (internal standard, purity > 98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Chemical Co. Ltd. (Beijing, China). MS-grade methanol was purchased from Merck (Merck Company, Germany), ultra pure water was generated from the Milli-Q system (Millipore Corp., USA). HPLC-grade formic acid was purchased from the Tedia Company Inc. (Tedia, Fairfield, OH, USA). All the other reagents were of analytical grade.

2.2. LC–MS/MS conditions

The LC–MS/MS system consisted of an Agilent 1200 mod. liquid chromatography system (Agilent, MA, USA) equipped with a binary solvent delivery system, an autosampler, a column compartment and a 6410 triple quadrupole mass spectrometer with electrospray ionization (ESI) source. Data were analyzed by MassHunter software (Agilent Corporation, MA, USA).

The chromatographic separation was achieved on an TOSOH TSKgel ODS column (15 cm × 4.6 mm, 3 μm) from TOSOH (TOSOH Corporation, Japan) at 30 °C. A binary mobile phase system consisted of (A) methanol and (B) 0.3% formic acid in water (80:20, v/v) at a flow rate of 0.6 mL/min.

All analytes, including the IS, were monitored under positive ionization conditions and quantified by MRM mode with transitions of protonated precursor ion at m/z 309.1 → 185.0 for 1-acetoxy-6α-hydroxyeriolanolide, at m/z 249.0 → 231.1 for both 1β-hydroxyalantolactone and ivangustin, and at m/z 285.0 → 193.0 for IS, respectively (Table 1). High purity nitrogen was employed as both the nebulizing and drying gas. Other parameters of the mass spectrometer were set to obtain highest intensity of protonated molecules of the three analytes as follows: drying gas flow 10.0 L/min; drying gas temperature, 350 °C; nebulizer pressure, 50 psi; capillary voltage, 4000 V.

2.3. Preparation of Herba Inulae extract

The powdered *Herba Inulae* (300 g) was extracted with 3 L ethanol by infiltration. The filtrate was concentrated under reduced pressure to 300 mL, then the residual liquid was extracted with ethyl acetate (900 mL). After that, the extract was evaporated to dryness using rotary evaporator, yielding 2.5 g *Herba Inulae* extract.

2.4. Preparation of standards, internal standard and quality control (QC) samples

Stock solutions at 1.0 mg/mL for each compound were separately prepared by dissolving the accurately weighed reference compound in methanol and stored at 4 °C. A series of working solutions of these analytes were freshly prepared by diluting standard solutions with methanol at appropriate concentrations. The IS was prepared in methanol at a concentration of 100 μg/mL and was further diluted to 100 ng/mL as a working solution.

The calibration standard and QC samples were prepared by spiking 100 μL working standard solutions into 100 μL blank rat plasma. The ranges of concentrations for working standards were 4–800 ng/mL for 1-acetoxy-6α-hydroxyeriolanolide, 8–500 ng/mL for 1β-hydroxyalantolactone and ivangustin. Solutions of 10, 100 and 600 ng/mL for 1-acetoxy-6α-hydroxyeriolanolide, 16, 80 and 400 ng/mL for 1β-hydroxyalantolactone and ivangustin were

Table 1
ESI-MS/MS parameters on the parent and daughter ions (m/z) and collision energy of the three analytes and IS.

Analyte	m/z	Product ion	Fragmentor energy (V)	Collision energy (eV)
1-Acetoxy-6α-hydroxyeriolanolide	309.1 → 185.0	$[M+H-H_2O-C_2H_4O_2-CO-H_2O]^+$	80	5
1β-Hydroxyalantolactone	249.0 → 231.1	$[M+H-H_2O]^+$	80	2
Ivangustin	249.0 → 231.1	$[M+H-H_2O]^+$	80	2
Diazepam (IS)	285.0 → 193.0	$[M+H-C_6H_5-CH_3]^+$	150	35

prepared as QC samples. The calibration standard and QC samples were stored at −20 °C until analysis.

2.5. Sample preparation

To a 100 μL aliquot of rat plasma, 50 μL internal standard solution (100 ng/mL) and 250 μL methanol were added. After vortex-mixing for 1 min and centrifugation (14,000 × g) for 10 min, the supernatant was separated out and evaporated to dryness under vacuum at 45 °C. Then the residue was reconstituted in 100 μL mobile phase, vortex-mixed briefly, and finally 15 μL of the sample solution was injected for LC/MS/MS analysis.

2.6. Method validation

The method validation process was carried out following the Food and Drug Administration (FDA) guidelines [23].

2.6.1. Selectivity

To investigate potential interferences of endogenous compounds co-eluting with analyte and IS, blank rat plasma from six different sources were tested. Chromatographic peaks of analytes and IS were identified based on their retention times and MRM responses.

2.6.2. Matrix effect and recovery

The matrix effect was measured by comparing the peak responses of samples spiked post-extraction with those of standard solutions evaporated and reconstituted in mobile phase. The matrix effect of the three analytes were determined at three QC levels in six replicates, respectively, whereas the matrix effect of IS was determined at a single concentration.

The extraction efficiency of the three compounds was determined by analyzing six replicates of plasma samples at three QC concentration levels. The extraction recovery was calculated by comparing the peak areas of plasma extracts spiked with analytes before extraction with those of the post-extraction spiked samples.

2.6.3. Calibration curves and LLOQ (lower limit of quantification)

Calibration curves were constructed by plotting peak-area ratios of each analyte to IS versus plasma concentrations using a $1/X^2$ weighted linear least-squares regression model in duplicate on three consecutive days. LLOQ is defined as the lowest concentration point of the calibration curve at which an acceptable accuracy within ±20% and precision below 20% can be obtained. Analyte response at the level of LLOQ should be at least five times the blank plasma.

2.6.4. Precision and accuracy

Precision was expressed as the relative standard deviation (R.S.D.) and accuracy was calculated as the relative error (R.E.). Three levels of QC samples in six replicates were analyzed during the same day using the same calibration curve to determine the intra-day precision. Three batches of QC samples were analyzed on three consecutive days to evaluate the inter-day precision and accuracy. The intra-day and inter-day precisions were required to be less than 15%, and the accuracy to be within ±15%.

2.6.5. Stability

Stability of the analytes was investigated at three QC levels. The short-term stability of untreated QC samples stored for 6 h at room temperature was determined. The long-term stability was assessed after the untreated QC samples had been stored at −20 °C for two weeks. The freeze–thaw cycles stability was determined after three freeze–thaw cycles (−20 °C to room temperature as one cycle). The post-preparative stability was determined by reanalyzing QC samples after keeping them under the conditions of 4 °C for 12 h. The analyte was considered stable in the biological matrix when 85–115% of the initial concentrations were found.

2.7. Applications in pharmacokinetics studies

The experimental protocol was approved by the Animal Ethics Committee of the Second Military Medical University. Six male Wistar rats (200–220 g) were kept in air-conditioned animal quarters at 22 ± 2 °C and a relative humidity of 50 ± 10%, with free access to food and water until 12 h prior to the experiment. *Herba Inulae* extract was dissolved with 10% ethanol yielding a concentration of 300 mg/mL. The rats were orally given *Herba Inulae* extract 300 mg/kg body weight equaling to a dose of 1-acetoxy-6α-hydroxyeriolanolide 120 mg/kg body weight, 1β-hydroxyalantolactone 30 mg/kg body weight, and ivangustin 38 mg/kg body weight. Blood samples of 200 μL were collected in heparin containing tubes from epicanthic veins of rats by capillary tube predose (0 h) and at 0.083, 0.167, 0.333, 0.5, 1, 1.5, 2, 3, 4, 6, 9, 12, 24 h post dose, and centrifuged at 14,000 × g for10 min at 4 °C. The plasma were collected and frozen at −20 °C until analysis.

Plasma concentration–time data were analyzed by the non-compartmental method to obtain pharmacokinetic parameters. Maximum plasma drug concentration (C_{max}), and the time to reach maximum plasma drug concentration (T_{max}) were taken directly from the observed data. The elimination half-life ($t_{1/2}$) was calculated as $0.693/k_e$, where k_e, the elimination rate constant, was calculated from the slope of the logarithm of the plasma concentration versus time for the final four measurable points. The area under concentration–time curve (AUC_{0-24}) was calculated by the trapezoidal rule. The $AUC_{0-\infty}$ was calculated by AUC_{0-24} plus $AUC_{24-\infty}$ ($AUC_{24-\infty}$ = concentration of the last experimental point/k_e). The values were calculated by Microsoft Excel (Microsoft, Seattle, WA, USA).

3. Results and discussion

3.1. Chromatographic conditions

1β-Hydroxyalantolactone and ivangustin are isomers, so they have the same parent ion. In our research, they almost have the same daughter ions in the mass spectrometry. However, among these daughter ions, those that can obtain higher responses in MS are the same to both 1β-hydroxyalantolactone and ivangustin. Therefore, chromatographic separation has become critical to determine the isomers. Different chromatographic conditions

Table 2
Main pharmacokinetic parameters of 1-acetoxy-6α-hydroxyeriolanolide, 1β-hydroxyalantolactone and ivangustin after oral administration of 300 mg/kg *Herba Inula* extract ($n = 6$, mean ± S.D).

Parameter	1-Acetoxy-6α-hydroxyeriolanolide	1β-Hydroxyalantolactone	Ivangustin
t_{max1} (h)	0.53 ± 0.24	0.19 ± 0.07	0.19 ± 0.07
C_{max1} (ng/mL)	609.03 ± 165.43	236.74 ± 43.02	278.88 ± 28.49
t_{max2} (h)	6.33 ± 0.82	7.00 ± 1.55	6.00 ± 0.00
C_{max2} (ng/mL)	117.02 ± 61.36	71.93 ± 6.73	102.17 ± 35.89
$t_{1/2}$ (h)	9.65 ± 1.43	14.88 ± 0.82	13.93 ± 2.74
$AUC_{(0-24)}$(ng h/mL)	1102.46 ± 247.04	808.92 ± 117.53	990.35 ± 275.69
$AUC_{(0-24)}$/administered dose (ng h/mL mg)	45.94 ± 10.29	134.82 ± 19.59	130.31 ± 36.28
$AUC_{(0-\infty)}$ (ng h/mL)	1166.36 ± 238.96	1011.50 ± 119.51	1186.80 ± 296.72

were investigated in order to get appropriate retention time, satisfactory ionization, better resolution and sensitivity. The addition of formic acid was to enhance the sensitivity and to get better peak shape. The optimized chromatographic conditions provided a short runtime, with no significant endogenous interference, and could be used to separate the two isomers effectively.

3.2. Mass spectrometric conditions

To optimize the responses of the three compounds and IS, the positive ionization was used for good sensitivity in our research. In the full scan mass spectra, the protonated molecular ions $[M+H]^+$ of the isomers, 1β-hydroxyalantolactone and ivangustin (m/z 249.0), were stable. However, under the product ion scan mode, the most intensive ion was $[M+H\text{-}H_2O]^+$ (m/z 231.1) for both of them, and there's no relative more intensive ion to either of them.

3.3. Sample preparation

Different extraction methods were attempted during our experiment. Liquid liquid extraction, LLE was initially developed with ethyl acetate, however, the matrix effect of 1-acetoxy-6α-hydroxyeriolanolide was found to be above 150%. Protein precipitation of the three analytes and IS from plasma samples was then explored. Different organic precipitation solvents were also evaluated. Although the analytes and IS could be extracted with methanol and acetonitrile, the results showed that methanol produced higher recoveries as precipitation agent than acetonitrile. Moreover, protein precipitation method was much simpler, less time-consuming than LLE, therefore could be utilized in this study.

3.4. Method validation

3.4.1. Selectivity

The typical chromatograms of a blank sample, a plasma sample spiked with the three analytes at LLOQ and IS, and a plasma sample from a rat 1 h after oral administration of *Herba Inulae* extract are shown in Fig. 2. Three channels were used for recording, channel 1 for 1-acetoxy-6α-hydroxyeriolanolide with a retention time of 3.8 min, channel 2 for 1β-hydroxyalantolactone and ivangustin with retention time of 4.1 and 4.5 min and channel 3 for IS with a retention time of 5.8 min. As shown in Fig. 2, no interfering peaks from endogenous substances were observed at the times of the analyte and IS.

3.4.2. Matrix effect and recovery

In our study, the estimation of the matrix effect was conducted following the procedures described above. The ratios (peak areas) were between 110.6% and 104.9% for 1-acetoxy-6α-hydroxyeriolanolide, between 102.9% and 104.7% for 1β-hydroxyalantolactone and between 100.2% and 102.2% for ivangustin at three QC levels, respectively. The matrix effect for IS was 96.1%. These results indicated that no apparent interference was found under the present chromatographic, mass spectrometric and extraction conditions.

The recovery was in the range of 90.0% and 97.8% for 1-acetoxy-6α-hydroxyeriolanolide, in the range of 79.2% and 80.6% for 1β-hydroxyalantolactone, and in the range of 80.4% and 83.2% for ivangustin at three QC levels, respectively. The recovery of IS was 85.3 ± 2.2%.

3.4.3. Calibration curves and LLOQ

The calibration curves were ranged from 4 to 800 ng/mL for 1-acetoxy-6α-hydroxyeriolanolide, from 8 to 500 ng/mL for 1β-hydroxyalantolactone and ivangustin. The correlation coefficients (r^2) were 0.9995, 0.9983 and 0.9985 for 1-acetoxy-6α-hydroxyeriolanolide, 1β-hydroxyalantolactone and ivangustin, respectively, and the calibration curves were $y = 0.0098x + 0.0432$, $y = 0.0036x + 0.0144$ and $y = 0.0025x + 0.0095$, where y is the peak area ratio of the component to the IS, and x (ng/mL) is the plasma concentration of the three compounds.

3.4.4. Precision and accuracy

In this assay, the intra- and inter-day precisions were less than 7.8% and 8.6%, and the accuracy was within ±6.8% for the three analytes. All were within the acceptable range, indicating that the current method was highly accurate and precise, and reproducible.

3.4.5. Stability

The three compounds were found to be stable in the plasma samples through three freeze–thaw cycles, and after being stored at −20 °C for two weeks or being stored at room temperature at 6 h. On the other hand, no significant degradation was observed when processed samples were kept at 4 °C for 12 h.

3.5. Pharmacokinetic study

The mean plasma concentration–time profile is presented in Fig. 3. It showed that the concentrations of the analytes in rat plasma were quantifiable at least 24 h after oral administration and all of the three analytes displayed double peaks. This phenomenon was probably due to enterohepatic circulation and re-absorption. The main parameters were reported for the first time in Table 2. The two isomers had similar pharmacokinetic behaviors, which were different from the other analyte, 1-acetoxy-6α-hydroxyeriolanolide. All the C_{max} of the three were within 35 min, which are similar to those of atractylenolide I, II and III reported previously [13–15], demonstrating they were absorbed quickly in rat plasma. Moreover, the two isomers were absorbed more quickly than the other compound, which may be due to their higher lipophilicity. In addition, the normalized $AUC_{(0-24)}$ seems to indicate that the two isomers are better absorbed than 1-acetoxy-6α-hydroxyeriolanolide. On the contrary, the $t_{1/2}$ of 1-acetoxy-6α-hydroxyeriolanolide was shorter than that of the two isomers, indicating that 1-acetoxy-6α-hydroxyeriolanolide was easier to be eliminated. The successful application of LC/MS/MS

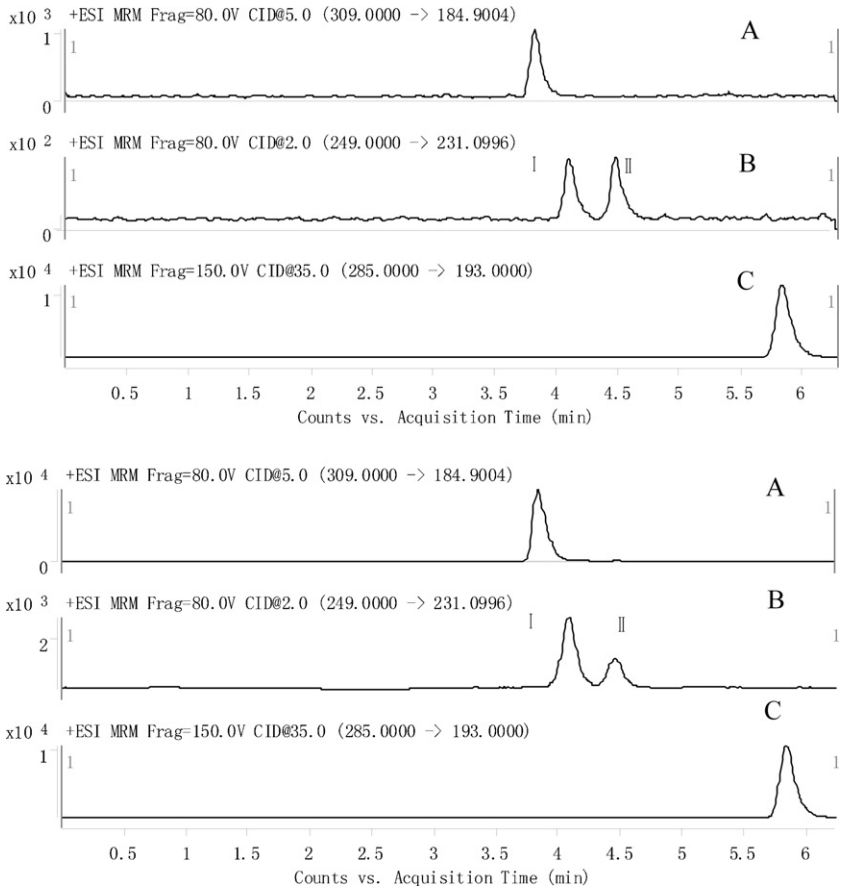

Fig. 2. Representative MRM chromatograms of 1-acetoxy-6α-hydroxyeriolanolide (A), 1β-hydroxyalantolactone (I) and ivangustin (II) (B), diazepam (C, IS) in rat plasma sample. Above: plasma sample of LLOQ, (A) 4 ng/mL, (B) 8 ng/mL for both compounds, (C) 100 ng/mL. Below: 1 h after oral administration of *Herba Inulae* 300 mg/kg.

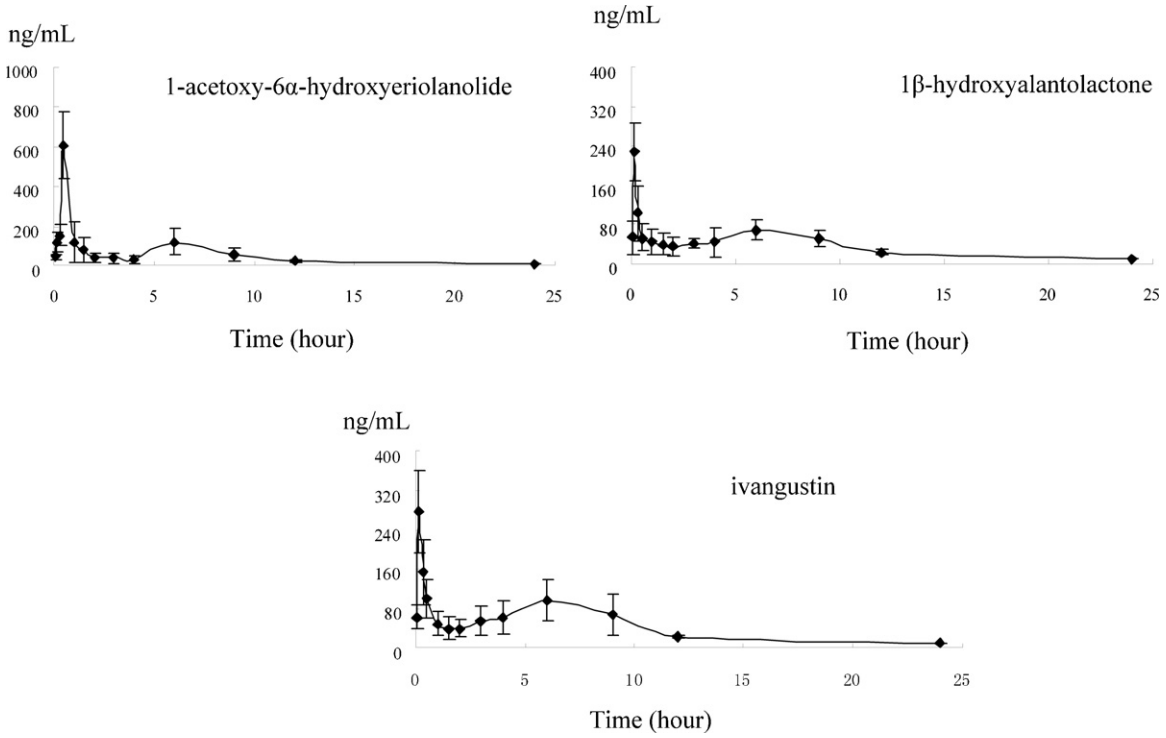

Fig. 3. Mean (±S.D., $n = 6$) plasma concentrations of 1-acetoxy-6α-hydroxyeriolanolide, 1β-hydroxyalantolactone, and ivangustin as a function of time following oral administrations (dose at 300 mg/kg) of *Herba Inulae* extract to rats.

method to pharmacokinetic study indicated that the established bioanalytical method was suitable for pharmacokinetic study.

4. Conclusion

A rapid and sensitive LC/MS/MS method has been developed, for the first time, for the simultaneous determination of 1-acetoxy-6α-hydroxyeriolanolide, 1β-hydroxyalantolactone and ivangustin in rat plasma after oral administration of 300 mg/kg *Herba Inulae* extract. The method had excellent selectivity, linearity, accuracy and precision, and the pharmacokinetic study revealed that the three compounds had similar pharmacokinetic behaviors. Most importantly, it will provide an applicable reference for determination of other sesquiterpenes of TCM in plasma.

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