

Determination of Isoetosendanin in Rat Plasma by Liquid Chromatography–Tandem Mass Spectrometry: Application to Pharmacokinetics Study

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A rapid, sensitive and accurate liquid chromatography–tandem mass spectrometry (LC–MS–MS) method was developed and validated for the quantification of isotoosendanin, an important bioactive component isolated from *Meliae cortex*. A Capcell PAK C18 column (100 × 4.6 mm) was used for the chromatographic elution using methanol–10 mM ammonium acetate–formic acid (80:20:0.1, v/v/v) as mobile phase at the flow rate of 0.6 mL/min. MS–MS analysis was performed on a triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization source in positive ion mode. Extraction of isotoosendanin and genistein (internal standard, IS) from rat plasma was determined by precipitating protein treatment. Quantification was performed by MS in the multiple reaction monitoring mode with positive ionization at m/z 557 → 437 for the analyte and m/z 271 → 215 for IS, respectively. Linear isotoosendanin calibration curves were obtained between 2.0–2,000 ng/mL with a correlation coefficient greater than 0.99. Acceptable precision and accuracy were acquired for concentrations over the standard curve range. Satisfactory results were achieved for sensitivity, specificity, recovery, freeze/thaw and stability. This analytical method was successfully applied to determine the pharmacokinetic parameters of isotoosendanin after an oral administration of 200 mg/kg to rats.

Introduction

Meliae cortex, locally known as “Ku-lian-pi,” is a traditional Chinese medicine (TCM) recorded in the Chinese Pharmacopoeia (1), which has been widely used in China for the treatment of ascariasis, oxyuriasis and abdominal pain (2). Phytochemical investigation in our laboratory and others has shown that limonoid-type compounds were the principle and characteristic constituents of *Meliae cortex* (3, 4), and the bioactivity studies of *Meliae cortex* have primarily focused on limonoids and their effects on pesticidal, antibacterial, anti-inflammatory and cytotoxic activities (5, 6). Research from our laboratory has demonstrated that isotoosendanin showed significant cytotoxicity against three types of human cancer cells (BEL7402 cell, $IC_{50} = 8.57 \mu\text{g/mL}$; H460 cell, $IC_{50} = 4.10 \mu\text{g/mL}$; SGC-7901 cell, $IC_{50} = 9.25 \mu\text{g/mL}$; unpublished data). Additionally, the significant anti-inflammatory effects of isotoosendanin have been reported (7). More recently, our laboratory reported the pharmacokinetic properties in Sprague-Dawley (SD) rats of methyl kulonate, which was a prominently active limonoid isolated from *Meliae cortex* (8). In our previous research, methyl kulonate, as one of the active components of *Meliae cortex*, was used to evaluate its pharmacokinetic characterization following oral administration of methyl kulonate to male SD rats. However, isotoosendanin (Figure 1), the isomer of toosendanin, has not been determined by any

chromatographic method in biological samples, while toosendanin has been measured by liquid chromatography–flame ionization detection (LC–FD) from the extract of fruit of *Meliazazedarach* (9).

In the current study, an analytical method employing liquid chromatography–tandem mass spectrometry (LC–MS–MS) was validated and developed to determine the plasma concentration following an oral dose of 200 mg/kg isotoosendanin to rats. This is the first chromatography article for isotoosendanin and the first pharmacokinetics study for isotoosendanin.

Experimental

Chemicals and reagents

Standard isotoosendanin was isolated from *Meliae cortex* and characterized by spectroscopic methods [proton nuclear magnetic resonance ($^1\text{H-NMR}$), carbon-13 NMR ($^{13}\text{C-NMR}$) and mass spectrometry (MS)], and further measured for its purity by the high-performance liquid chromatography (HPLC) system in our laboratory. HPLC-grade methanol and formic acid were obtained from Sigma-Aldrich (St. Louis, MO). Ammonium acetate was purchased from Tedia (Fairfield, OH). Ultrapure water was prepared using a Milli-Q system (Millipore, Bedford, MA). Other reagents were of analytical-reagent grade.

HPLC operating conditions

Purity detection of standard isotoosendanin was performed on a Capcell MG C18 column (50 × 4.6 mm; Shiseido, Tokyo, Japan) with a UV detector at 230 nm. The mobile phase was 0.1% formic acid in methanol (A) and 0.1% formic acid in water (B) at a constant flow rate of 0.6 mL/min. The solvent gradient was linearly increased from 20% A to 100% A within 8 min. The column temperature was kept at 30°C.

Chromatographic separation for the quantification of isotoosendanin in rat plasma samples was performed with a Capcell PAK C18 column (100 × 4.6 mm; Shiseido) with a mass spectrometric detector. The mobile phase contained methanol–10 mM ammonium acetate–formic acid (80:20:0.1, v/v/v). The flow rate gradient was 0.6 mL/min and injection volume was 20 μL . The column temperature was 30°C and the total running time was no more than 4.0 min.

Mass spectrometry operating conditions

The MS full scan for purity check of standard isotoosendanin was performed on an Agilent 1100 HPLC system coupled with a 6310 MSD Trap XCT Ultra (Agilent Technologies, Waldbronn, Germany). The MS was equipped with an electrospray ionization (ESI) source in positive ion mode. The instrument

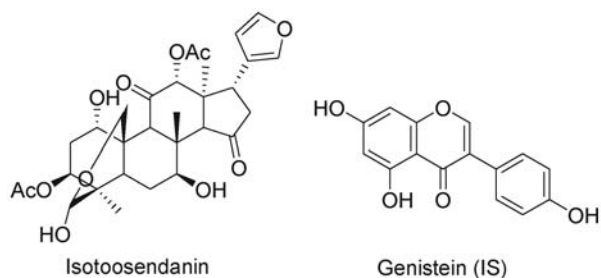


Figure 1. The chemical structures of isotoosendanin and genistein (IS).

parameters were as follows: dry gas temperature, 350°C; dry gas, 10 L/min; nebulizer pressure, 30 psi; spray capillary voltage, 3,500 V. Data acquired were controlled by Agilent Chemstation Rev. A.09.03 software.

The LC–MS system for quantification was an Agilent 6410 Triple Quad equipped with an Agilent 1200 binary pump and Agilent 6410B mass selective detector with an atmospheric pressure chemical ionization (APCI) source (Agilent Technologies, Santa Clara, CA). The positive ion detection mode was chosen. Data analysis was performed using Agilent Mass Hunter B 01.03 software. The mass detector was used in multiple reaction monitoring (MRM) mode for the highest selectivity and sensitivity, and also for the lowest detection limits. Nitrogen gas was used for drying and collision. The MS detector parameters were set at the following values: gas temperature, 350°C; gas flow, 5 L/min; vaporizer, 350°C; nebulizer pressure, 20 psi; capillary voltage, 4,500 V; capillary current, 4 μ A. Fragmentor energies (FE) of Q1 were 135 V for isotoosendanin and 155 V for the internal standard (IS), and optimized collision energies (CE) were 15 V for isotoosendanin and 25 V for IS, respectively. Two transitions were selected for quantifications of isotoosendanin (m/z 557 \rightarrow 437) and IS (m/z 271 \rightarrow 215), respectively with each 400 ms scan time.

Preparation of stock solution, calibration standards and quality control samples

Two primary stock solutions of isotoosendanin (1.0 mg/mL) were prepared in methanol from separate weightings for preparation of calibration standard and quality control (QC) samples. The IS stock solution of 1.0 mg/mL was also prepared in methanol. One stock solution of isotoosendanin was successively diluted with methanol–water (50:50, v/v) to obtain working solutions to prepare calibration curves for isotoosendanin at 2.00, 5.00, 20.0, 100, 200, 500, 1,000 and 2,000 ng/mL, respectively. Another set of working stock solution of isotoosendanin was diluted from the other primary stock in methanol–water (50:50, v/v) for preparation of QC solutions at concentrations of 5.00, 100 and 1,800 ng/mL. The IS working solution (2,000 ng/mL) was prepared by diluting 1.0 mg/mL of genistein with methanol–water (50:50, v/v). All working solutions were kept in refrigeration at 4°C.

Calibration curves were prepared by mixing 50 μ L of the working solutions into 50 μ L of blank plasma; the resultant plasma concentrations were from 2.00 to 2,000 ng/mL.

Plasma sample preparation

Simple protein precipitation treatment was used for processing of calibration standards, QC samples and test samples. In brief,

a 50- μ L aliquot of plasma samples was added to 50 μ L of methanol and 50 μ L of IS working solution (2,000 ng/mL) in 1.5-mL Eppendorf tubes. The mixture was vortexed for 30 s and centrifuged at 11,000 rpm for 5 min, and a 20- μ L aliquot of the supernatant was injected directly into the LC–MS–MS system.

Assay validation

To evaluate linearity, calibration curves with eight levels ranging from 2.00 to 2,000 ng/mL of isotoosendanin were prepared and analyzed in duplicate on three consecutive days. The curves were fitted by a weighted ($1/x^2$) least squares linear regression method through the determination of the peak-area ratios of the analyte to IS.

QC samples at three different concentration levels (5.00, 100 and 1,800 ng/mL) were analyzed to calculate the accuracy and precision. These samples were prepared in six replicates on three consecutive days. The accuracy was expressed by relative error (RE) and the precision by relative standard deviation (RSD). According to the values by using one-way analysis of variance (ANOVA), the accuracy and precision were required to be within $\pm 15\%$.

The lower limit of quantification (LLOQ), which was measured by analyzing samples prepared in six replicates in three different batches, was less than or equal to 20%.

Extraction recoveries of isotoosendanin at three QC levels ($n = 6$) were determined by comparing peak-area ratios of the analyte to IS in samples that were spiked with the analyte before extraction with samples to which the analyte was added post-extraction. The recovery of IS was determined in a similar way, using the medium concentration of QC samples as a reference.

Matrix effects were investigated by comparing the peak areas of isotoosendanin neat solution with those of post-preparation spiked blank plasma at two low and high concentration levels (5.00 and 1,800 ng/mL), respectively. The inter-subject variability of matrix effect at each concentration level should be less than 15% (10).

The stability of isotoosendanin in rat plasma was assessed by analyzing replicates ($n = 3$) of plasma samples at the concentrations of 5.00 and 1,800 ng/mL. The samples were exposed to different conditions, such as after the exposure of the spiked samples to room temperature for 2 h by exposure of the ready-to-inject samples (after protein precipitation) to the autosampler rack at room temperature for 24 h, after storage of the standard spiked rat samples at -20°C for nine days and after three complete freeze/thaw cycles (-20 to 25°C) on consecutive days. The analyte was considered to be stable in plasma when 85–115% of the initial concentrations were measured.

Pharmacokinetics study

Six male SD rats (220 ± 20 g) were purchased from Shandong University of Traditional Chinese Medicine (Jinan, China). Animals were housed in well-ventilated cages and kept at 20–25°C on a natural 12 h light–dark cycle. All animal experiments were approved by the Institutional Ethics Committee. Aqueous suspension of isotoosendanin was prepared in 0.5% carboxymethyl cellulose sodium (CMC–Na) and the concentration of

the oral formulation was 20 mg/mL. After an oral dose of 200 mg/kg to rats, blood samples were collected pre-dose and at 0.083, 0.167, 0.5, 1, 2, 3, 5, 8, 12 and 24 h post-dose in heparinized plastic tubes via the orbital vein. Plasma was harvested by centrifuging the blood at 3,000 rpm for 5 min and was stored frozen at -20°C until LC-MS-MS analysis. Animals were allowed to feed 2 h post-dose of isotoosendanin.

Oral pharmacokinetics parameters for isotoosendanin in rats were calculated by non-compartmental analysis of data using DAS 2.0 software (Drug and Statistics, Mathematical Pharmacology Professional Committee of China, Shanghai, China),

Results and Discussion

Purity and NMR spectral data of isotoosendanin

Isotoosendanin was obtained as a white powder from the MeOH extract of *Meliae cortex*, and its purity was determined at 99.3% by HPLC at 230 nm (Figure 2). The $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectral data were consistent with the previous report (7).

Method development

In developing the method, suitable precursor ions and product ions of the analytes and IS were selected for MRM analysis through a syringe pump infusion. At the first step, an LC-ESI-MS interface was used for detection and MS response was observed to be not stable for isotoosendanin. In the present study, an APCI source in positive mode was used, which showed a strong and stable signal. Standard solutions (2,000 ng/mL) of the analyte and IS were separately injected into the MS to obtain their precursor ions and to further optimize mass parameters such as FE and CE. The precursor ion $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$ at m/z 557 of isotoosendanin and protonated molecule $[\text{M} + \text{H}]^+$ at m/z 271 of IS were exhibited higher abundance in the MS full scan mode. Under the product ion scan mode, the product ion spectra of the precursor ions displayed fragment ions at m/z 437 for isotoosendanin and at m/z 215 for IS (Figure 3).

Chromatographic conditions were optimized to obtain maximum peak response and short run time without interfering with the MS response of the analyte in the APCI source. A

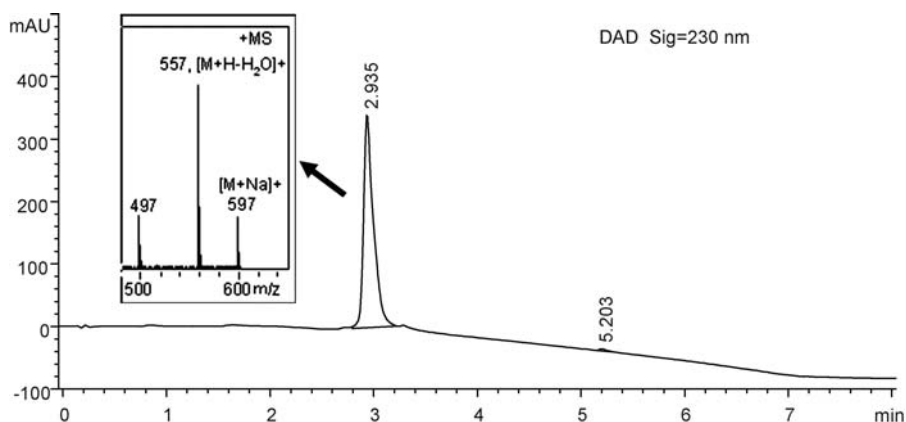


Figure 2. Ultraviolet spectra (230 nm) of the isotoosendanin standard isolated from *Meliae cortex* and the full scan MS spectra of isotoosendanin at 2.935 min.

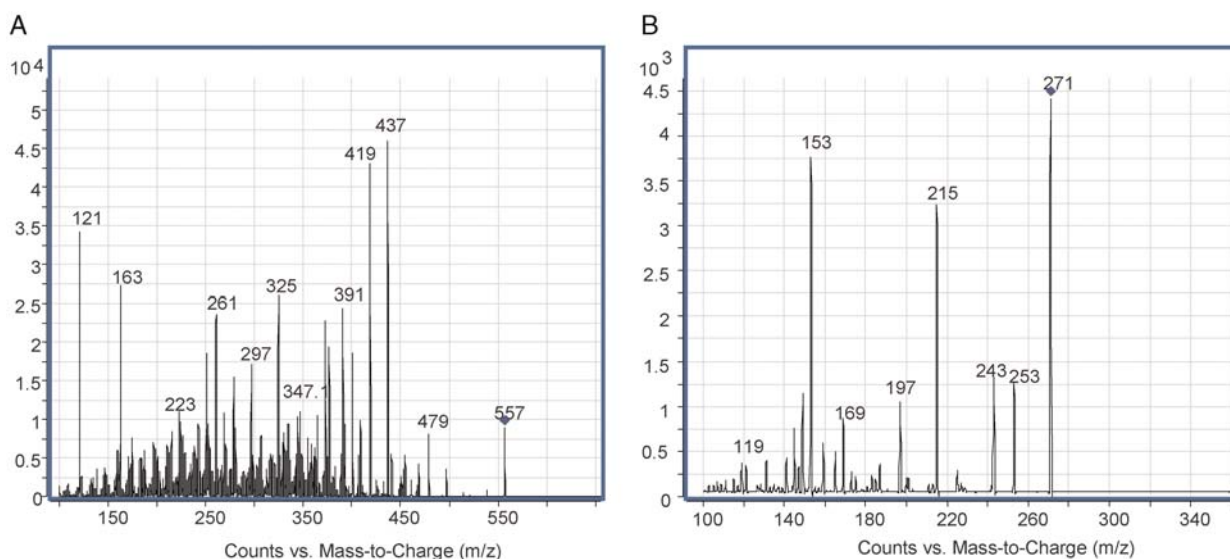


Figure 3. MS-MS spectra of: $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$ at m/z 557 of isotoosendanin (A); $[\text{M} + \text{H}]^+$ at m/z 271 of genistein (B).

perfect peak shape and suitable retention time were observed when employing a mobile phase consisting of methanol–10 mM ammonium acetate–formic acid (80:20:0.1, v/v/v).

Method validation

Selectivity and matrix effect

Figure 4 shows the typical chromatographic peaks of blank plasma, blank plasma spiked with isotoosendanin and IS, and rat plasma at 2.0 h postdose. No interfering endogenous substances were observed at the respective retention times of the analyte and IS.

The matrix effects for isotoosendanin at concentrations of 5.00 and 1,800 ng/mL were measured to be 88.0 ± 6.5 and $89.8 \pm 11.0\%$ ($n = 6$), respectively (Table I). The matrix effects for IS (2,000 ng/mL in rat plasma) were $71.8 \pm 2.8\%$ ($n = 6$). Although ion suppression was observed for IS from rat plasma, the isotoosendanin concentration was accurately determined without influence.

Calibration curve and sensitivity

Linear responses were obtained for isotoosendanin ranging from 2.00 to 2,000 ng/mL. Typical equations for the calibration curves were: $y = 8.923 \times 10^{-5}x + 1.037 \times 10^{-4}$, $r^2 = 0.9960$ for isotoosendanin, where y is the peak area ratio of isotoosendanin to the IS, and x (ng/mL) is the plasma concentration of isotoosendanin. The LLOQ used in the construction of the calibration curve was set at 2.00 ng/mL with RE and RSD not exceeding 20%.

Precision and accuracy

Accuracy and precision of the assay are summarized in Table II. Intra-day and inter-day precisions for all three tested concentrations (low, medium and high) were no more than 13.2%, and the accuracy of the method ranged from -2.0% to 1.9%. These results indicated that the accuracy and precision of the assay were within acceptable range.

Recovery

In the present study, the extraction recoveries for isotoosendanin at 5.00, 100 and 1,800 ng/mL were $105.6 \pm 8.6\%$, $117.4 \pm 10.0\%$ and $93.4 \pm 5.7\%$ ($n = 6$), respectively (Table I). The extraction recovery of the IS was $119.8 \pm 0.7\%$ ($n = 6$). These data indicated that the recoveries for the analyte and IS were high (partly exceeding 100%), which was acceptable.

Stability

The data of stability experiments indicated that isotoosendanin was stable under storage for 2 h at ambient temperature, after three freeze-thaw cycles and under storage for nine days at -20°C . Isotoosendanin was also stable after protein precipitation at ambient temperature for 24 h (Table III).

Pharmacokinetics study

The validated method was successfully applied to the determination of isotoosendanin in rat plasma after a single oral dose of 200 mg/kg of isotoosendanin. The mean plasma concentration versus time profile of isotoosendanin is shown in Figure 5. The observed maximum plasma concentration (C_{\max}) was 156.0 ± 40.1 ng/mL at 1.83 ± 0.4 h post-dose. The area under

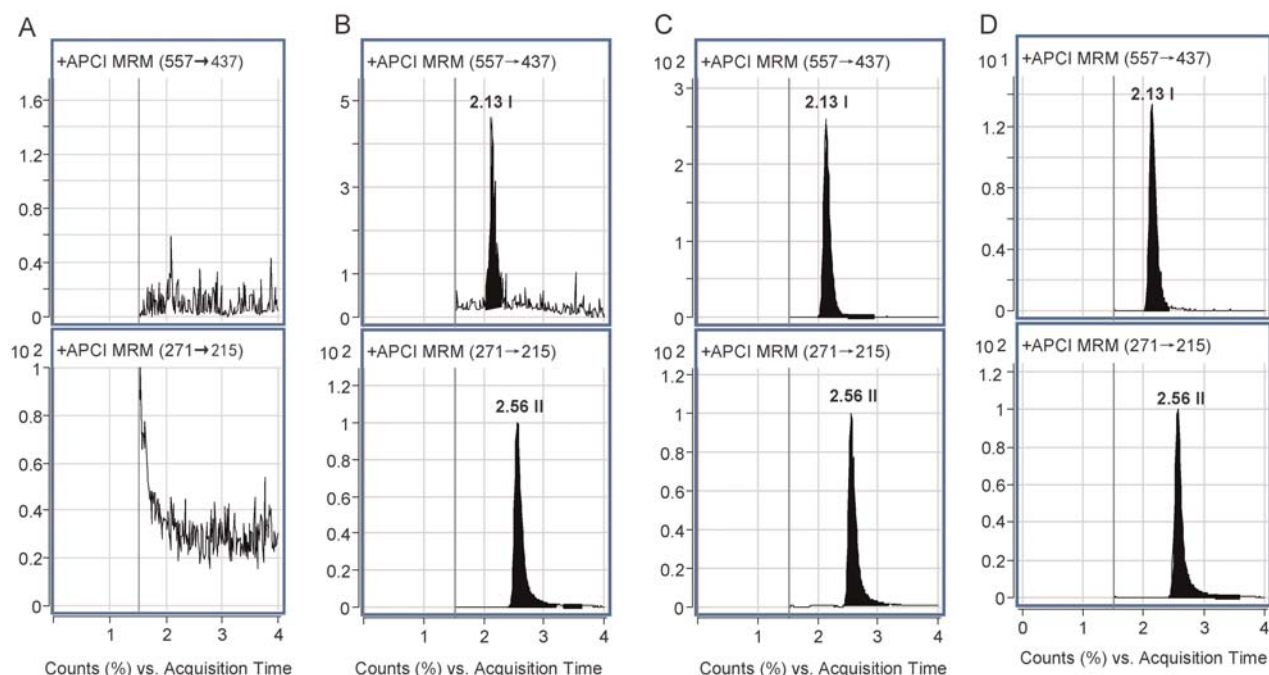


Figure 4. Typical MRM chromatograms of isotoosendanin and IS in rat plasma: blank plasma (A); plasma spiked with isotoosendanin at 2.00 ng/mL and IS at 2,000 ng/mL (B); plasma spiked with isotoosendanin at 2,000 ng/mL and IS at 2,000 ng/mL (C); plasma at 2.0 h after an oral dose of 200 mg/kg isotoosendanin (D). Peaks I and II refer to isotoosendanin and IS, respectively.

Table I
Recovery and Matrix Effects of Isozoosendanin and Genistein (IS) in Rat Plasma*

| Analyst | Nominal concentration (ng/mL) | Mean extraction recovery (n = 6) | Mean matrix effect (n = 6) |
|----------------|-------------------------------|----------------------------------|----------------------------|
| Isozoosendanin | 5.00 | 105.6 ± 8.6% | 88.0 ± 6.5% |
| | 100 | 117.4 ± 10.0% | — |
| | 1,800 | 93.4 ± 5.7% | 89.8 ± 11.0% |
| Genistein | 2,000 | 119.8 ± 0.7% | 71.8 ± 2.8% |

*Note: Data are mean values ± SD (n = 6).

Table II
Precision and Accuracy Data for Isozoosendanin in Rat Plasma*

| Concentration (ng/mL) | | Precision (RSD%) | | Accuracy (RE%) |
|-----------------------|-------------|------------------|-----------|----------------|
| Added | Found | Intra-day | Inter-day | |
| 5.00 | 5.09 ± 0.4 | 7.1 | 13.2 | 1.9 |
| 100 | 99.4 ± 7.9 | 8.1 | 7.2 | -0.6 |
| 1,800 | 1,764 ± 120 | 5.3 | 8.8 | -2.0 |

*Note: Data are expressed in pre-study validation, three days, six replicates per day.

Table III
Stability of Isozoosendanin Exposed to Various Storage Conditions*

| Concentration (ng/mL) | Ambient, 2 h | - 20°C, 9 days | Three freeze/thaw | Autosampler ambient, 24 h |
|-----------------------|--------------|----------------|-------------------|---------------------------|
| 5.00 | 4.99 ± 0.5 | 5.00 ± 0.5 | 5.01 ± 0.4 | 4.68 ± 0.6 |
| 1,800 | 1,767 ± 94.9 | 1,716 ± 179.4 | 1,706 ± 165.7 | 1,661 ± 11.0 |

*Note: Data are mean values ± SD (n = 3).

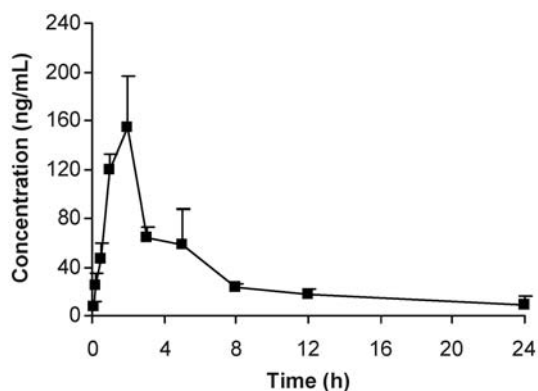


Figure 5. Mean plasma concentrations of isozoosendanin after a single oral dose of 200 mg/kg to rats. Each point represents mean ± SD (n = 6).

the mean plasma concentration versus time curve from 0 to 24 h (AUC_{0–24h}) was 788.2 ± 71.5 ng h/mL (Table IV).

Conclusions

The quantification of isozoosendanin in rat plasma samples was successfully achieved using LC-MS-MS. The method gave satisfactory validation characteristics with respect to specificity,

Table IV
Primary Pharmacokinetic Parameters of Isozoosendanin After Oral Administration of 200 mg/kg to Rats*

| Parameters | Mean ± SD |
|----------------------------------|--------------|
| T _{max} (h) | 1.83 ± 0.4 |
| C _{max} (ng/mL) | 156.0 ± 40.1 |
| AUC _[0–24h] (ng h/mL) | 788.2 ± 71.5 |
| AUC _{[0–∞)} (ng h/mL) | 822.9 ± 80.7 |
| t _{1/2} (h) | 5.4 ± 1.5 |
| MRT _[0–24h] (h) | 6.5 ± 1.3 |
| MRT _{[0–∞)} (h) | 7.5 ± 2.2 |

*Note: Data are mean values ± SD (n = 6).

sensitivity, precision, accuracy, matrix effects and recoveries. Therefore, we conclude that the assay was suitable for a pre-clinical pharmacokinetic study of isozoosendanin after a single oral administration of 200 mg/kg of isozoosendanin to rats.

Acknowledgments

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