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Determination of protodioscin in rat plasma by liquid chromatography–tandem mass spectrometry

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Abstract

Protodioscin (3-O-[α-L-rhamnopyranosyl-(1 → 2)-{α-L-rhamnopyranosyl-(1 → 4)}-β-D-glucopyranosyl]-26-O-[β-D-glucopyranosyl]-(25 R)-furost-5-ene-3 β,26-diol) is a naturally occurring saponin present in many oriental vegetables and traditional medicinal plants, which has been associated with potent bioactivity. However, there is no specific and sensitive assay for quantitative determination of protodioscin in biological samples. We have established a rapid, sensitive and selective LC-ESI-MS/MS method to measure protodioscin in rat plasma and investigated the pharmacokinetics of protodioscin after intravenous administrations. Plasma samples were prepared after plasma protein precipitation, and a aliquot of the supernatant was injected directly onto an analytical column with a mobile phase consisted of acetonitrile–water–formic acid (80:20:0.1, v/v/v). Analytes were detected with a LC-ESI-MS/MS system in positive selected multiple reaction-monitoring mode. The lower limit of quantification (LLOQ) was 20.0 ng/mL and a linear range of 20–125,000 ng/mL. The intra- and inter-day relative standard deviation (R.S.D.) across three validation runs over the entire concentration range was <8.0%. Accuracy determined at three concentrations (50, 5000 and 50,000 ng/mL for protodioscin) ranged from 0.2 to 1.8% as terms of relative error (R.E.). Each plasma sample was chromatographed within 3.5 min. This LC-ESI-MS/MS method allows accurate, high-throughput analysis of protodioscin in small amounts of plasma.

Keywords: Liquid chromatography; Mass spectrometry; Protodioscin; Pharmacokinetics

1. Introduction

Furostanol glycosides constitute a large group of steroidal saponins. They exist extensively in nature and have a broad range of interesting bioactivity. Protodioscin (3-O-[α-L-rhamnopyranosyl-(1 → 2)-{α-L-rhamnopyranosyl-(1 → 4)}-β-D-glucopyranosyl]-26-O-[β-D-glucopyranosyl]-(25 R)-furost-5-ene-3β, 26-diol)represents a typical example of a furostanol saponin that has been isolated from a number of oriental vegetables and medicinal plants.

Protodioscin was cytotoxic against most cell lines from leukemia and solid tumor in the NCI's human cancer panel [1]. Protodioscin displayed strong growth inhibitory effect against HL-60 cells [2]. The experiments were done by Gauthaman et al., Adaikan et al., Gauthaman et al., Arsyad [3–6], respectively. In China, the saponin mixture extracted from the roots of *Dioscorea Nipponica* Makino, in which protodioscin is a major component, is commercially available as an formulation (Chuan - Shan - Long Injection®).

To our knowledge, no analytical method was reported for the determination of protodioscin in biological samples. Analytical methods for the determination of protodioscin included a colorimetric method [8], high performance liquid chromatography and evaporative light scattering detection (HPLC–ELSD) [9], precolumn derivatization HPLC [10] and liquid chromatography–mass spectrometry method [11]. However, these methods are less sensitive, selective or involve complicated procedure.

In the present study, we have developed and validated a high-performance liquid chromatography–tandem mass spectrometric (LC-ESI-MS/MS) method for the determination of protodioscin in rat plasma samples. The method was applied

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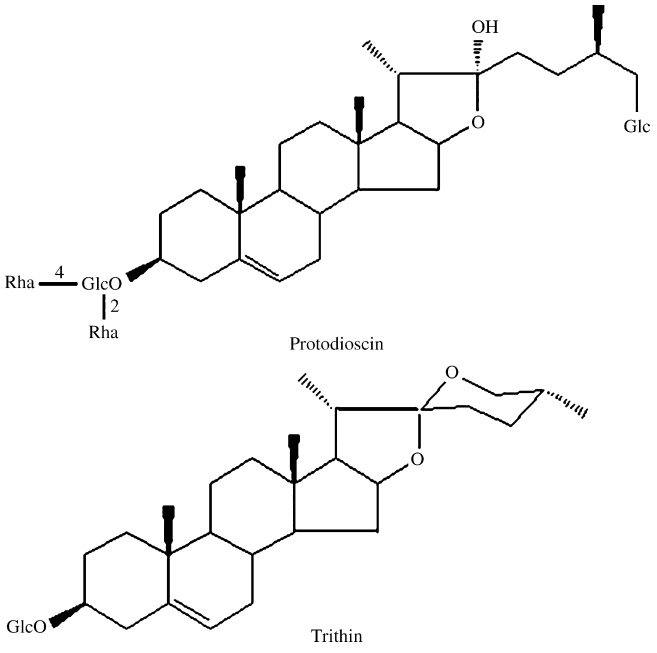

Fig. 1. Structures of protodioscin and trillin (IS) "Rha" means Rhamnose. "Glc" means Glucose. "4" means the linkage of Rha I at the 4-position (C-4) of Glc. "2" means the linkage of Rha II at the 4-position (C-4) of Glc.

to determine the pharmacokinetics of protodioscin after intravenous administration in rats.

2. Experimental

2.1. Materials and animals

Protodioscin (Fig. 1; 99.5% purity) was isolated and purified from the roots of *Dioscorea Nipponica Makino,* and its structure was confirmed by UV, IR, MS and NMR spectroscopy [7]. The internal standard (IS) trillin (Fig. 1; 98.8% purity) was supplied by Liaoning Bio-medical Technology Co. Ltd. (Dalian, China). Acetonitrile and methanol (HPLC grade) were purchased from Fisher Chemicals (Fair Lawn, NJ, USA), and water was doubly distilled in the laboratory. All other chemicals were purchased from commercial sources and used as received. Sprague-Dawley rats (male, mean body weight 220 ± 10 g) were purchased from the Animal Center of Guangdong Province (Guangzhou, China). Animals were allowed to acclimatize with access to food and water ad libitum for a 48 h period before use and were group-housed under a 12 h light/dark cycle in an environmentally controlled animal facility. All procedures were carried out under strict compliance with the national guide for the care and use of laboratory animals under a protocol approved by General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China.

Intravenous (I.V.) doses were given by tail vein injection. The formulation for I.V. administration (0.5, 1.0 and 3.0 mg/mL) was prepared by dissolving protodioscin in injection water. A dose of 1 mg/kg body weight is a suitable therapeutical dose for rat, which is converted from Chuan - Shan - Long Injection® [12].

2.2. Instrumentation

A Micromass Quattro (Micromass, Beverly, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface, a waters 2695 pump solvent system and an autoinjector was used for the LC-ESI-MS/MS analysis. The data were processed using MassLynx version 4.0 (Micromass) software.

2.3. Chromatographic and mass spectrometric conditions

Chromatographic separation was achieved on a Carbosorb ODS-3 column (50 mm × 2.0 mm i.d., 5 μm; Varian, USA) and a SecurityGuard C_{18} guard column (4 mm × 2.0 mm i.d., Phenomenex, Torrance, CA, USA) using a mobile phase of acetonitrile–water–formic acid (80:20:0.1, v/v/v). The liquid flow rate was set at 0.2 mL/min. The column temperature was maintained at 30 °C. The HPLC system was connected to the mass spectrometer via an ESI interface operating in the positive ion detection mode. The mass spectrometer source was maintained at a temperature of 105 °C and the desolvation temperature at 300 °C with drying gas (nitrogen) at a flow rate of 300 L/h and cone gas at a flow rate of 40 L/h. The sample cone energy was set at 35 and 20 V for protodioscin and trillin (IS), respectively, with RF lens at 0.5 V (RF Lens voltage describes focuses ions toward the center of the quadrupole).The mass spectrometer was programmed to monitor the transitions (selected reaction monitoring, SRM) *m/z* 1032.6 → *m/z* 869.7 (for protodioscin) and *m/z* 577.1 → *m/z* 253.1 (for IS), with collision induced fragmentation (collision gas argon at 2.6×10^{-3} mbar). The dwell time was 0.1 s, and the collision energy was set at 15 and 10 V for protodioscin and the IS, respectively. The electron multiplier setting was 650 V. The autoinjector temperature was maintained at 15 °C.

2.4. Calibration standards and quality controls

Standard stock solutions of protodioscin and IS were prepared at 400 μg/mL in methanol. The solution of protodioscin was then serially diluted with the mobile phase to obtain the desired concentrations. All stock solutions and working solutions were kept refrigerated (4 °C). The IS working solution (5 μg/mL) was also prepared by diluting the 400 μg/mL stock solution of IS with the mobile phase.

Calibration curves were prepared by spiking 20 μL of the appropriate standard solution to 40 μL of blank rat plasma. The obtained plasma concentrations were 20, 50, 100, 500, 1000, 10,000, 20,000, 50,000 and 12,5000 ng/mL. The quality control samples (QCs) were prepared using a different stock solution of protodioscin to obtain the plasma concentrations of 50, 5000 and 50,000 ng/mL, representing low, medium and high concentration levels, respectively. These QCs were stored in glass tubes at −20 °C until analysis. The spiked plasma samples (standards and quality controls) were extracted and assayed on each analytical batch along with the unknown samples.

2.5. Sample preparation

Frozen plasma samples were thawed to room temperature prior to extraction. Protodioscin was determined after plasma protein precipitation, which was performed by adding 100 μL of acetonitrile to 40 μL of plasma following the addition of 20 μL of trillin and 20 μL of methanol (20 μL of the appropriate standard solution for calibration and QC samples). Subsequently, the mixture was vortex-mixed for 60 s, followed by centrifugation for 10 min at 4000 × *g*. A 20 μL aliquot of the supernatant was injected onto the LC–MS–MS system.

2.6. Method validation

To evaluate linearity, plasma calibration curves were prepared and assayed in duplicate on three separate days. The calibration curves were fitted by least-square regression using $1/x^2$ as weighting factor of the peak area ratio of protodioscin to IS versus protodioscin plasma concentrations.

Accuracy and precision were assessed by determining the concentration of protodioscin in QC samples using six replicate preparations of plasma samples at three concentration levels on three separate days, using three separate sources of rat plasma. Precision was calculated as the relative standard deviation (R.S.D.) within a single run and between different assays, and the accuracy was calculated as the percentage of deviation between nominal and measured concentrations.

The lower limit of quantification of protodioscin was experimentally defined as the lowest concentration in plasma samples at which the within- and between-run precision were <20% and the accuracy varied between 80 and 120%.

It is very important to investigate the matrix effects in order to develop a reliable and reproducible LC–MS–MS method. The matrix effect was evaluated by comparing the absolute peak areas of the post-spiked standards with those of the neat standards prepared in mobile phase. Recovery was determined by comparing the absolute peak areas of the pre-spiked standards with those of the post-spiked standards. The pre-spiked standards were prepared by the procedure described in Section 2.5. The post-spiked samples were the drug-free control plasma prepared by the same procedure and then spiked with working standard stock solutions. The concentrations of 50, 5000 and 50,000 ng/mL for protodioscin and 5 μg/mL concentrations for I.S. were prepared for matrix effect and recovery assessment.

The stability of protodioscin in the plasma was assessed by analyzing triplicate QC samples at 50, 5000 and 50,000 ng/mL stored for 24 h at ambient temperatures and also following three cycles of freezing at −20 °C and thawing. Concentrations following storage were compared to freshly prepared samples of the same concentrations. The stability of protodioscin after protein precipitation was investigated following storage at ambient temperature for 8 h.

2.7. Pharmacokinetics study

Three groups of rats ($n = 6$) were given an I.V. dose of protodioscin (0.5, 1.0 and 3.0 mg/mL) by tail vein injection. Blood samples (150 μL) were collected into heparinized tubes by puncture of the retro-orbital sinus under ether anesthesia at 0 (predose), 2, 10, 30, 60, 90, 120, 180, 240,360 and 480 min after administration. Plasma was obtained by centrifugation at 4000 × *g* for 10 min and samples were stored at −20 °C until analysis.

Protodioscin plasma-concentration data for individual rats were analyzed by noncompartmental analysis using the TopFit software package (Gustav Fisher, Stuttgart, Germany, Version 2.0).

3. Results and discussion

3.1. Sample preparation

In the early methods at development stage, we investigated several liquid–liquid extraction (LLE) methods by using *n*-hexane–dichloromethane–isopropanol (20:10:1, v/v/v) and ethyl acetate as the extraction solvents. It was found that the recoveries of both the analyte and the internal standard were <20% when using any of the pretreatment conditions described above, maybe for the polarity reason. The polar and water-soluble character of protodioscin makes it difficult to extract it from plasma by technique such as conventional liquid–liquid extraction. Comparatively, protein precipitation (PPT) is simple and easy to operate. Finally protein precipitation method was chosen. Adequate sensitivity could be achieved by injecting the supernatant directly onto the LC-ESI-MS/MS system for analysis.

3.2. LC-ESI-MS/MS selection

Although both ESI and APCI sources produced significant signal for quantification, ESI was chosen because the sensitivity and linearity for the analyte were better than that of the APCI. By positive ESI mode, the analyte formed predominately protonated molecules $[M-18+H]^+$ in Q1 full scan mass spectra (suggesting a loss of H_2O). The major product ion, *m/z* 869.6 $[M\text{-}18\text{-}C_6H_{12}O_6+H]^+$ (suggesting a loss of glucose), was mon-

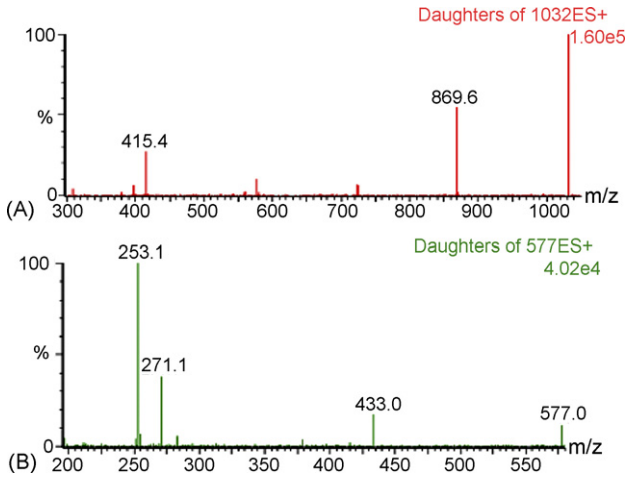

Fig. 2. Product ion mass spectra of (A) protodioscin and (B) trillin.

itored in the SRM successive analysis. For trillin, the precursor ion *m/z* 577.1 $[M+H]^+$ was paired with the product ion *m/z* 253.1 in the SRM analysis. Fig. 2 displays the product ion spectra of $[M\text{-}18+H]^+$ of protodioscin (A) and $[M+H]^+$ of trillin (B).

To achieve symmetrical peak shapes and short chromatographic cycle times, the mobile phase consisting of acetonitrile and aqueous formic acid was used with the Carbosorb ODS-3 material column.

3.3. Internal standard selection

Although a stable isotope-labeled internal standard is preferred for LC-ESI/MS/MS quantitative assays,this is not available for protodioscin. In our case, the analogs of protodioscin, e.g., trillin, Ginsenoside Rh2 and sarsaparilloside could be utilized as internal standard, as they all showed the similar behavior as the analyte in the entire sample preparation, chromatographic elution and mass spectrometric detection. But Ginsenoside Rh2 was not chosen due to its instability. As a result, trillin was chosen as the internal standard in this experiment.

3.4. Method validation

3.4.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank rat plasma with the corresponding spiked plasma. Fig. 3 shows the typical chromatograms of a blank plasma sample, blank plasma sample spiked with protodioscin (50 ng/mL) and IS, and a plasma sample from a rat 3 h after intravenous administration. No significant interference from endogenous substances with analyte or trillin was detected.

3.4.2. Linearity and lower limit of quantification

The linear regression of the peak area ratios versus concentrations was fitted over the concentration range of 20–125,000 ng/mL in rat plasma. A typical equation of the calibration curve was as follows: $y = 1.02x - 129.28$ ($r^2 = 0.993$),

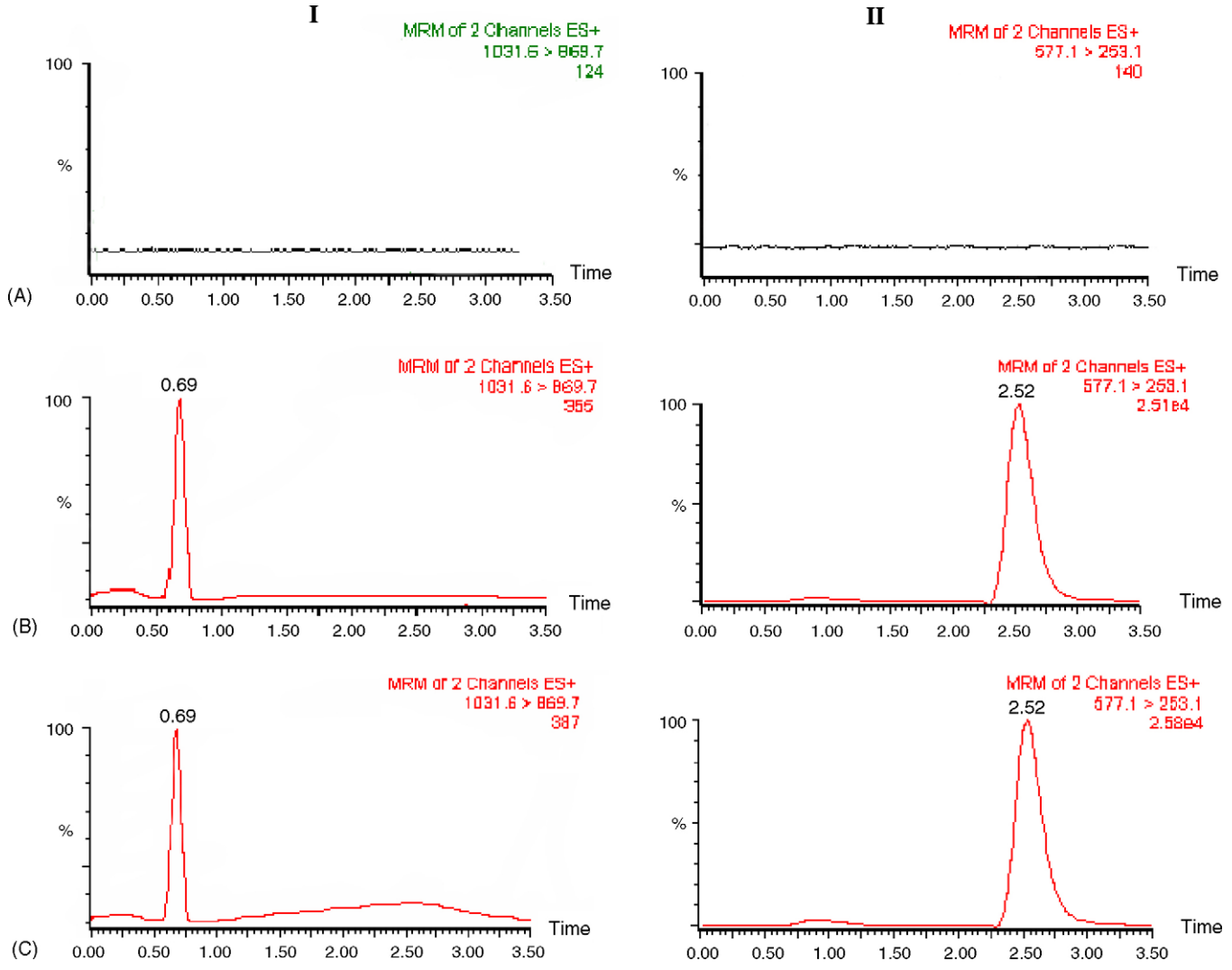

Fig. 3. Representative SRM Chromatograms: (A) Blank rat plasma sample; (B) blank plasma sample spiked with protodioscin (I, 50 ng/mL) and internal standard (II, 5000 ng/mL); (C) plasma sample (I, 51 ng/mL and II, 5000 ng/mL) from a rat 480 min after intravenous administration with 1 mg/kg of protodioscin.

Table 1
Precision and accuracy for the analysis of protodioscin in rat plasma ($n = 3$ day, 6 replicates per day)

Added C (ng/mL)	Found C (ng/mL)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Accuracy (%)
50.0	50.9	6.7	4.7	1.8
5000.0	506.8	6.5	4.6	0.2
50000.0	50212.4	7.8	6.8	0.4

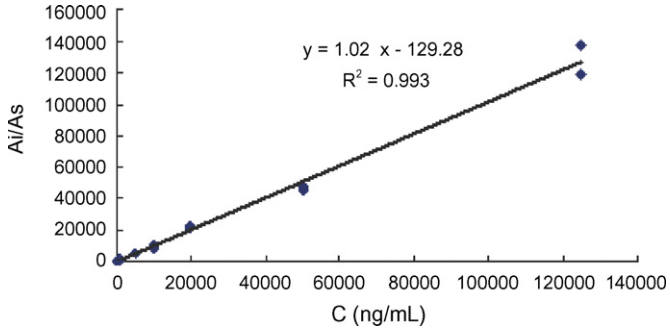

Fig. 4. Calibration curve of protodioscin in rat plasma.

where y is the peak area ratio of analyte to IS and x is the plasma concentration of protodioscin. Calibration curve of protodioscin is shown in Fig. 4.

The lower limit of quantification was 20 ng/mL for determination of protodioscin in plasma. At the LLOQ level, the within- and between-day precision were 6.5 and 8.3%, respectively and the accuracy was 98.3%. Under the present LLOQ, the protodioscin concentration could be determined in plasma samples up to 480 min after a single intravenous dose of 1.0 mg/kg protodioscin, which is sensitive enough to investigate the pharmacokinetic behavior of the drug.

3.4.3. Precision and accuracy

Table 1 summarizes the within- and between-day precision and accuracy for protodioscin evaluated by assaying the QC samples. In this assay, the intra- and inter-day precision ranged from 6.5 to7.8% and from 4.7 to 6.8% for each QC level, respectively. The accuracy was within ± 1.8%.

3.4.4. Recovery and matrix effect

The recovery of protodioscin, determined at three concentrations (50, 5000, 50,000 ng/mL), were 85.4 ± 5.4%, 83.9 ± 6.6% and 81.1 ± 5.8% ($n = 6$), respectively. The recovery of trillin was investigated as 83.1 ± 4.7% ($n = 6$). Less than 10% matrix suppression was observed. The matrix effect should not have a significant impact on assay performance.

3.4.5. Stability

No significant changes in the protodioscin concentrations were measured after three freeze–thaw cycles; the corresponding relative errors from the same three concentrations were 3.2, −4.1, and −2.8%, and the storage was for 24 h at ambient temperature (RE < 3.7%). The analyte was stable in the supernatant after protein precipitation at ambient temperature for at least 8 h (RE < −8.7%).

3.4.6. Application in pharmacokinetics study

The presented method was successfully applied to quantify protodioscin in the plasma of six rats for 480 min following three intravenous doses. A proportional increase in the area under the plasma concentration–time curve ($AUC_{0\text{-}t}$) with increasing protodioscin dose was observed in rats. A linear relationship was observed as follows: $y = 1.388 \times 10^3 x + 28.81$, $r = 0.997$, where

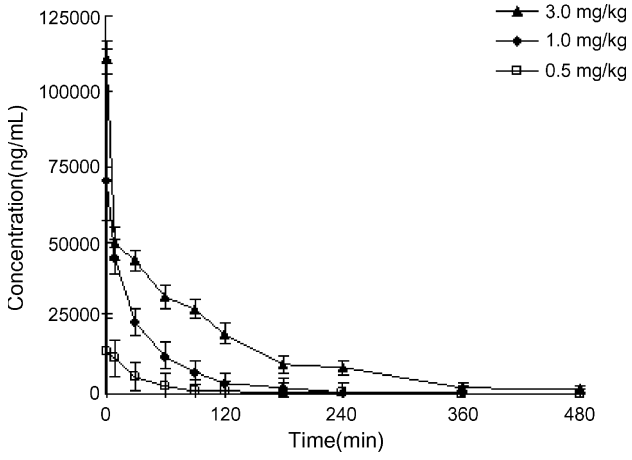

Fig. 5. Mean plasma concentration–time curve of protodioscin in rats after intravenous administrations ($n = 6$ for 0.5, 1.0 and 3.0 mg/kg, $\bar{x}$ ± S.D.).

Table 2
Pharmacokinetic parameter of protodioscin in rats after intravenous administrations)

Parameter	Dose (mg/kg)		
	0.5	1	3
$t_{1/2}$ (min)	78 ± 12	58 ± 11	27 ± 19
k_e (1/min)	0.0089 ± 0.0056	0.012 ± 0.042	0.029 ± 0.009
C_{max} (μg/mL)	70 ± 9	116 ± 15	378 ± 10
V_{ss} (mL/kg)	71.6 ± 13	49 ± 21	23.5 ± 9
CL (mL/min/kg)	0.637 ± 0.19	0.598 ± 0.05	0.681 ± 0.06
$AUC_{0\text{-}t}$ (μg min/mL)	732 ± 82	1406 ± 107	4196 ± 245
$AUC_{0\text{-}inf}$ (μg min/mL)	785 ± 94	1673 ± 195	4406 ± 209

y is the area under the plasma concentration–time curve and x is the intravenous dose. The concentration versus time profile is shown in Fig. 5. Pharmacokinetic parameters are shown in Table 2.

4. Conclusion

A rapid and precise LC-ESI-MS/MS method was developed and validated for the determination of protodioscin in rat plasma, and applied to a pharmacokinetics study of protodioscin in rats after intravenous administrations. The method is sensitive and specific, which further enhances its utility as an analytical method for use in pharmacokinetic studies of protodioscin. Moreover, given the relatively small sample requirement (40 μL) and sensitivity of this method (S/N ≥ 11.3 at 20 ng/mL), it may also be useful for in vitro studies of protodioscin (e.g., tissue culture studies) where low concentrations or small sample volumes may be expected. In addition, by using a narrow-bore column instead of 4.6 i.d. column with lower flow rate, this assay reduces solvent costs and minimizes environmental impact of the toxic solvent. Finally, we have successfully applied this LC-ESI-MS/MS method by measuring protodioscin in rat plasma from a pharmacokinetic study after intravenous administrations.

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