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

Ke Li[a], Yingwu Wang[b], Jingkai Gu[b], Xiaoyan Chen[a], Dafang Zhong[a,b,*]

[a] *Laboratory of Drug Metabolism and Pharmacokinetics, Shenyang Pharmaceutical University, Wenhua Road 103, Shenyang 110016, PR China*
[b] *Research Center for Drug Metabolism, Jilin University, Changchun, PR China*

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Abstract

A sensitive and specific high-performance liquid chromatography–tandem mass spectrometric (LC–MS/MS) assay for dioscin in rat plasma was developed. Ginsenoside Rh2 was employed as an internal standard. Dioscin is a naturally occurring saponin present in many traditional Chinese medicinal plants. Dioscin was determined after the acetonitrile-mediated plasma protein precipitation. The mobile phase consisted of acetonitrile:10 mmol/l aqueous ammonium acetate (95:5, v:v), which was pumped at 0.8 ml/min. The analytical column (100 mm × 4.6 mm i.d.) was packed with Hypersil ODS material (5 μm). The standard curve was linear from 1 to 100 ng/ml. The assay was specific, accurate (percentage deviations from nominal concentrations were <10%), precise and reproducible (within- and between-day coefficients of variation <10%). Dioscin in rat plasma was stable over three freeze–thaw cycles and at ambient temperatures for 24 h. The utility of the assay was demonstrated by determining dioscin plasma concentrations in five rats for 120 h following a single oral gavage dose of 90 mg/kg.

Keyword: Dioscin

1. Introduction

Dioscin, diosgenyl 2,4-di-*O*-a-L-rhamnopyranosyl-*p*-D-glucopyranoside (Fig. 1), is one of the most common saponins occurring in plants and has been isolated from a number of oriental vegetables and traditionally medicinal plants. The bioactivities of dioscin, such as antitumor [1–7], antifungal [8,9], have been reported.

It was proved that HL-60 cells underwent differentiation and apoptosis after dioscin treatment [2]. Further studies indicated that dioscin was also a potent apoptosis inducer in Hela cells [4], suggesting that dioscin could have anticancer activity. In China, the saponin mixture extracted from the roots of *Dioscorea Nipponica Makino*, in which dioscin is a major component, is commercially available as an oral formulation (Shu-Yu-Zao-Gan Tablet®). However, the pharmacokinetics of dioscin is absent. In this paper, a specific LC–MS/MS assay was developed, and the pharmacokmetic behavior of dioscin in rats was characterized after a single oral gavage dose.

2. Materials and methods

2.1. Materials

Dioscin (99.4% pure) was obtained from Hengrui Pharmaceutical Co. Ltd. (Jiangsu, China). Ginsenoside Rh2 (98.8% pure) was supplied by the College of Chemistry, Jilin University (Jilin, China). Acetonitrile and methanol (HPLC grade) were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). Water was doubly distilled in the laboratory. All other chemicals were purchased from commercial sources and used as received.

* Corresponding author. Tel.: +86 24 2390 2539; fax: +86 24 2390 2539.
E-mail address: zhongdf@china.com (D. Zhong).

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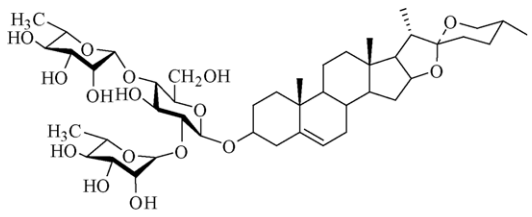

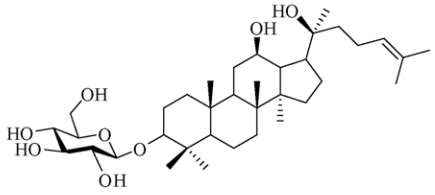

Fig. 1. Structures of dioscin and ginsenoside Rh2 (internal standard).

2.2. Chromatography and mass spectrometry

The high-performance liquid chromatography was performed on an Agilent 1100 system (Palo Alto, CA, USA) equipped with a G1313A autosampler, a vacuum degasser unit, and a G1312A binary pump. The mobile phase consisted of acetonitrile–10 mmol/1 aqueous ammonium acetate (95:5, v:v), without the adjustment of pH. This was delivered at a flow rate of 0.8 ml/min. The injection volume was 20 μL. The analytical column used was packed with Hypersil ODS material (100 mm × 4.6 mm i.d., 5 μm) from Agilent. The chromatography was performed at ambient temperatures. The HPLC system was coupled in line to an API 4000 mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) via a TurboIonSpray ionization interface. Following optimization of the setting, the instrument was operated in the negative mode with an ion spray voltage of −4.5 kV, curtain gas pressure of 10 psi, nebulizer gas pressure of 30 psi, heater gas pressure of 15 psi, and the source temperature was set at 500 °C. The curtain, nebulizer, heater and collision gases were all nitrogen. The fragmentation transitions for the multiple reaction monitoring (MRM) were *m*/*z* 867.5→721.5 amu for dioscin, and *m*/*z* 621.5→161.5 amu for Rh2. Data were collected and analyzed by the Analyst 1.3 Data Acquisition and Processing software (Applied Biosystems/MDS Sciex).

2.3. Preparation of stock and working solutions

The stock solution of dioscin (1 mg/ml) was prepared in methanol and serially diluted to produce a 2 μg/ml stock solution, which was diluted to give working solutions of 2, 4, 8, 20, 40, 80, 160, and 200 ng/ml in methanol. Aqueous (approximately 10% methanol) solutions of 4, 20 and 160 ng/ml were used in stability testing by diluting the working solutions of 40, 200 ng/ml and 2 μg/ml with doubly distilled water, respectively. An 800 ng/ml solution of the internal standard, ginsenoside Rh2, was similarly prepared in methanol. All stock solutions and working solutions were stored at 2–8 °C.

2.4. Preparation of calibration standards and quality control (QC) samples

Calibration standards and QC samples of dioscin were prepared by spiking 20 μl of the working solutions and 20 μl of Rh2 to 40 μl of drug-free rat plasma. Calibration standards were prepared at concentrations of 1, 2, 4, 10, 20, 40, 80, and 100 ng/ml of dioscin in plasma, while QC samples were prepared at 2, 10, and 80 ng/ml, and then treated as indicated below.

2.5. Sample treatment

Dioscin 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 Rh2 and 20 μl of methanol. Subsequently, the mixture was vortex-mixed for 30 s, followed by centrifugation for 10 min at 4000 × *g*. The supernatant was transferred to a clean glass tube for analysis.

2.6. Method validation

Standard curves ranging from 1 to 100 ng/ml of dioscin were run on three separate days. The integrated ion chromatogram peak areas of dioscin and Rh2 were used to construct a standard curve from the peak area ratio versus nominal dioscin concentration using linear regression analysis with $1/x^2$ weighting. Six replicates of QC samples at 2, 10, and 80 ng/ml of dioscin were included in each run to determine the within- and between-run precision of the assay. The accuracy was determined as a percent difference between the mean detected concentrations and the nominal concentrations. The relative standard deviation (R.S.D.) was used to report the precision. The lower limit of quantitation (LLQ) was assessed by analyzing 18 plasma samples spiked with 1 ng/ml of dioscin in three runs, the lowest concentration on the standard curve.

2.7. Stability

The stability of dioscin in the plasma was assessed by analyzing triplicate QC samples at 2, 10, and 80 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 dioscin after protein precipitation was investigated for 8 h at ambient temperatures.

2.8. Application of the method

Wistar rats (250 ± 20 g), used in the pharmacokinetics study of dioscin, were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). Rats were administered 90 mg/kg dioscin by oral gavage (10 ml/kg) after an overnight fasting period. The drug was formulated by diluting a dimethyl sulfoxide solution of dioscin (1:19, v:v) with bean oil:Tween 80:water (7:11:1, v:v). Blood samples (150 μl) were collected into heparinized tubes from each rat by the puncture of the retro-orbital sinus. This was performed at 0 (predose), 1, 2, 4, 8, 12, 24, 36, 48, 60, 72, 96 and 120 h after administration. Blood was immediately processed for plasma by centrifugation at 3000 × *g* for 10 min. Plasma samples were frozen and maintained at −20 °C until analysis.

3. Results and discussion

3.1. LC–MS/MS optimization

Acquisitions of mass spectrometry data were made in negative full scan mode on dioscin standard in continuous infusion. The deprotonated molecule was *m/z* 867.5 $[M-H]^-$. The major product ion, *m/z* 721.5 $[M-H–C_6H_{10}O_4]^-$ (suggesting a loss of rhamnose), was monitored in the MRM successive analysis. The product ion mass spectrum of the $[M-H]^-$ precursor ion is shown in Fig. 2A. Acquisitions in positive mode were also tried, but over 20-fold lower MS signal of $[M+H]^+$ ion was observed than the response of $[M-H]^-$ ion. Fig. 2B shows the MS/MS spectrum of the I.S., ginsenoside Rh2, in continuous infusion. The precursor ion *m/z* 621.5 $[M-H]^-$ was poised with the product ion *m/z* 161.5 $[M-H–aglycone]^-$ in the MRM analysis.

During the optimization of chromatographic conditions, dioscin and I.S. were extensively retained on the columns. To achieve symmetrical peak shapes and short chromatographic cycle times, the mobile phase consisting of acetonitrile and aqueous ammonium acetate was used with the Hypersil ODS column.

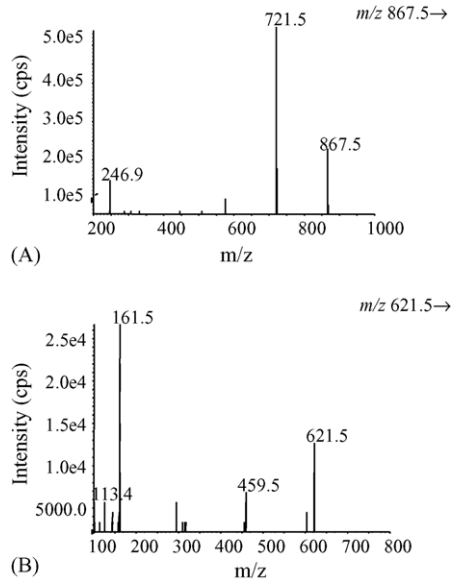

Fig. 2. Product ion mass spectra of (A) dioscin and (B) ginsenoside Rh2 with each $[M-H]^-$ ion.

3.2. Assay specificity

The LC–MS/MS method has high specificity because only ions derived from the analytes of interest are monitored. MRM chromatograms of dioscin and Rh2 for a drug-free plasma sample, a plasma sample spiked with 1 ng/ml of dioscin (the LLQ) and 400 ng/ml of Rh2 and a plasma sample (2.1 ng/ml) 96 h after oral gavage administration of 90 mg/kg of dioscin spiked with 400 ng/ml of Rh2 are shown in Fig. 3. No significant peaks interfering with dioscin or the internal standard were observed in the drug-free rat plasma.

Matrix effects from the endogenous substances provide another possible source of problems regarding assay specificity. The ion suppression effect was evaluated by comparing the peak areas of dioscin (1 ng/ml) and Rh2 (400 ng/ml) in six QC samples with those of the standard solutions, which were prepared in the same way as QC samples except water

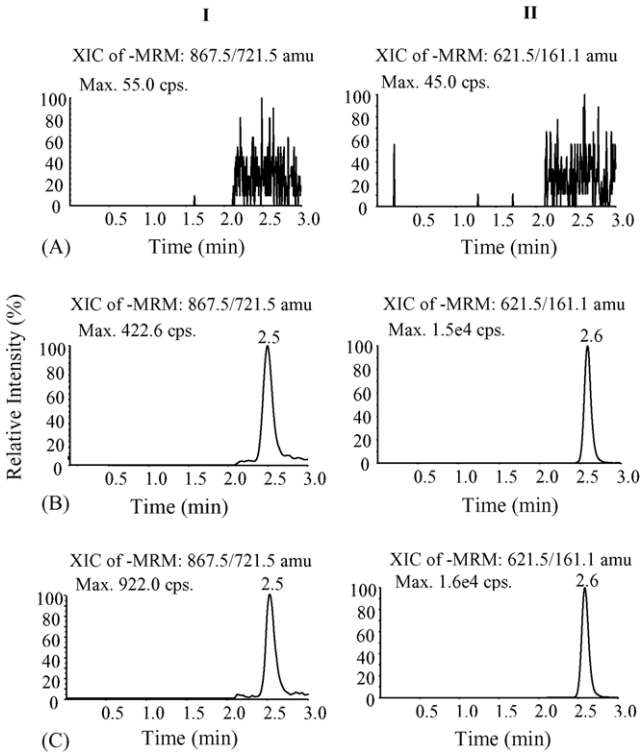

Fig. 3. MRM chromatograms (I: *m/z* 867.5–721.5 amu; II: *m/z* 621.5–161.1 amu) for (A) drug-free plasma, (B) plasma spiked with 1 ng/ml of dioscin (LLQ) and 400 ng/ml Rh2 and (C) a plasma sample (2.1 ng/ml) 96 h after the oral gavage administration to a rat with 90 mg/kg of dioscin.

Table 1
Precision and accuracy results for dioscin in rat plasma (3 days, six replicates per day)

Nominal plasma concentration (ng/ml)	Mean measured concentration (ng/ml)	Accuracy (%)	Within-run precision (%)	Between-run precision (%)
1.00	0.97	−3.4	3.7	6.3
2.00	2.06	3.0	5.2	2.7
10.0	10.2	2.3	2.5	4.9
80.0	77.5	−3.1	1.6	7.1

Table 2
Stability data for dioscin

Stability test	Initial concentration (ng/ml)	Recovery (%)	R.S.D. (%)
Stability after three freeze–thaw cycles	1.9	99.0	3.6
	9.7	103.5	2.4
	80.2	102.6	6.5
Stability in rat plasma at ambient temperature for 24 h	1.9	102.3	1.7
	9.7	96.0	3.2
	80.2	104.3	2.6
Stability after protein precipitation at ambient temperature for 8 h	1.9	101.4	4.5
	9.7	96.5	1.4
	80.2	102.1	7.3

substituted for drug-free plasma. For dioscin, the mean peak area from the six samples had relative error of −5.6% compared with that from standard solutions. For Rh2, the relative error was 4.8%. It was indicated that no endogenous substances significantly influenced the ionization of dioscin and Rh2.

3.3. Validation

The correlation coefficients for the standard curves ranged from 0.9962 to 0.9990. The mean (S.D.) slope and intercept for the regression lines of best fit were 0.0175 (0.0016) ml/ng and 0.0149 (0.0055), respectively ($n = 3$). The validation concentration range was from 1 to 100 ng/ml, using 40 μl of plasma. The assay performance for the determination of dioscin is shown in Table 1. The accuracies for all tested concentrations were within 10% of nominal and both the within- and between-run precisions were acceptable [10].

3.4. Stability

A number of stability experiments were performed and the results are summarized in Table 2. No significant changes in the dioscin concentrations were measured after three freeze–thaw cycles and storage for 24 h at ambient temperature. The analyte was stable in the supernatant after protein precipitation in the autosampler at ambient temperature for at least 8 h.

3.5. Application of the method

The presented method was successfully applied to quantify dioscin in the plasma of five rats for 120 h following a single 90 mg/kg oral gavage dose. The concentration versus time profile is shown in Fig. 4. The mean maximum plasma concentration (S.D.) of dioscin was 18.6 (3.8) ng/ml occurring at 12 h post dose. The mean apparent plasma half-life (S.D.) was 25.2 (2.6) h. The mean (S.D.) area under the plasma concentration versus time curve was 962.9 (53.6) ng h/ml.

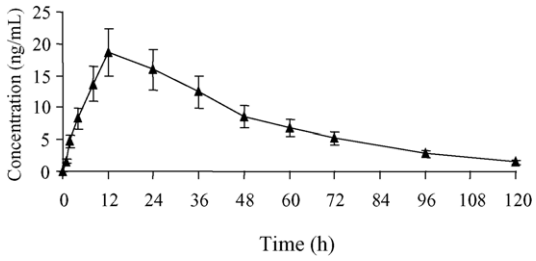

Fig. 4. The mean plasma concentration-time profile of dioscin after an oral dose of 90 mg/kg to rats ($n = 5$).

It appeared that the drug plasma level was very low, although a high oral dose was given. This method was somewhat modified and applied in the study following an intravenous administration. The preliminary results revealed an extremely low oral bioavailability (0.2%). Generally, bioavailability of drugs following oral administration is determined by several factors such as solubility, GI stability, intestinal permeability and first-pass extraction in the gut and/or by the liver. At present, dioscin is under investigation to clarify its absorption metabolism in our laboratory. Analytical methods, including this assay described here, will be applied in these studies.

4. Conclusion

The LC–MS/MS assay described here allowed quantitation of dioscin in the range of 1–100 ng/ml and was shown to be applicable to the study of the pharmacokinetics of dioscin following a single 90 mg/kg oral gavage dose to rats. The as-

say is sensitive, specific, precise, and accurate according to commonly accepted criteria.

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