Determination of Diosmetin-7-*o*- β -d-Glucoside in Rat Plasma by UPLC–MS/MS

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In this study, we used UPLC–MS/MS to determine diosmetin-7-*o*- β -d-glucoside in rat plasma and investigated its pharmacokinetics in rats. Six rats were given diosmetin-7-*o*- β -d-glucoside (5 mg/kg) by intravenous (i.v.) administration. The blood (150 μ L) was withdrawn from the caudal vein after administration. Diazepam was used as an internal standard (IS), and a one-step acetonitrile precipitation method was used to process the plasma samples. Chromatographic separation was achieved using a UPLC BEH C18 column using a mobile phase of acetonitrile–0.1% formic acid with gradient elution. Electrospray ionization (ESI) tandem mass spectrometry in multiple reaction monitoring (MRM) mode with positive ionization was applied, 463.1 $\rightarrow$ 301.0 for diosmetin-7-*o*- β -d-glucoside, m/z 285.1 $\rightarrow$ 193.0 for diazepam (IS). Intra-day and inter-day precision of diosmetin-7-*o*- β -d-glucoside in rat plasma were less than 14%. The method was successfully applied in the pharmacokinetics of diosmetin-7-*o*- β -d-glucoside in rats after intravenous administration. The $t_{1/2}$ of diosmetin-7-*o*- β -d-glucoside is 1.4 ± 0.4 h, which indicates the quick elimination.

Keywords: diosmetin-7-*o*- β -d-glucoside, pharmacokinetics, rat, UPLC–MS/MS

Introduction

The diosmetin-7-*o*- β -d-glucoside is a new compound obtained by dehydrogenating hesperidin to produce diosmin and then removing the rhamnose from the molecule by diosmin [1, 2]. Diosmin is a flavonoid derivative that improves microcirculation [3], regulates lymphatic function, reduces capillary permeability, improves elasticity and fragility, and inhibits edema formation [4–7]. It is a commonly used drug for the treatment of acne and chronic venous insufficiency. In addition, diosmin has a similar effect on vitamin P, which can reduce vascular fragility and reduce abnormally high permeability, so it is also used for the prevention and treatment of hypertension and adjuvant treatment of arteriosclerosis [8–10]. Because diosmin is not easily soluble in water and common organic solvents, its bioavailability is low. After the structure was transformed into diosmetin-7-*o*- β -d-glucoside, it was superior to diosmin in terms of solubility, absorption, distribution, and metabolism, and the bioavailability was greatly improved.

Ultra-high performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) combined technology combines the high resolution of liquid chromatography with the high sensitivity and high selectivity of mass spectrometry [11–20]. It has been widely used in medicine in recent years, including drugs and its metabolite analysis, chemical analysis of natural products, and the need for complex sample processing, and played a significant role in pharmacokinetic studies [21–23].

It was reported that the metabolites of diosmin and diosmetin were identified using an ultra-high-performance liquid chromatography coupled with linear ion trap-orbitrap multi-stage mass spectrometry (UHPLC–LTQ-orbitrap MSⁿ) strategy coupled with multiple metabolite templates, extracted ion chromatograms (EICs), and diagnostic product ions (DPIs) [24]. As a result, 46 diosmetin metabolites and 64 diosmin metabolites were respectively identified in rat biological samples. Diosmin and diosmetin-7-*o*- β -d-glucoside identified in urine and feces, as well as their subsequent metabolites accounted for a substantial part of all the diosmin metabolic products. However, to the best of our knowledge, the pharmacokinetics of diosmetin-7-*o*- β -d-glucoside had not been reported. In this study, we presented an UPLC–MS/MS method for the determination of diosmetin-7-*o*- β -d-glucoside in rat plasma with the lower limit of quantification (LLOQ) of 1 ng/mL and investigated its pharmacokinetics in rats, using the simple and fast one-step acetonitrile precipitation method to process the plasma samples, and only needed 50 μ L rat plasma.

Materials and Methods

Chemical and Animals. Diosmetin-7-*o*- β -d-glucoside (purity >98%, Figure 1) was purchased from Chengdu Mansite Bio-Technology Co., Ltd. (Chengdu, China). HPLC-grade methanol and formic acid were purchased from Merck (Darmstadt, Germany). Ultrapure water was prepared by a Millipore Milli-Q water system (Bedford, MA, USA). Sprague Dawley (SD) rats (male, body weight 200–220 g) were obtained from Animal Experimental Center of Wenzhou Medical University. Blank rat plasma was prepared for 6 lots of healthy SD rats.

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(A)

(B)

Figure 1. Chemical structure of diosmetin-7-*o*- β -d-glucoside (a) and the IS (B)

(A)

(B)

Figure 2. Mass spectrum of diosmetin-7-*o*- β -d-glucoside (a) and the IS (B)

Figure 3. UPLC–MS/MS spectra of diosmetin-7-*o*- β -d-glucoside and the IS in rat plasma: (A) blank plasma, (B) blank plasma spiked with diosmetin-7-*o*- β -d-glucoside (LLOQ) and the IS, and (C) rat plasma after administration of diosmetin-7-*o*- β -d-glucoside

Table 1. Accuracy, precision, matrix effect and recovery of diosmetin-7-*o*- β -d-glucoside in rat plasma ($n = 6$)

Concentration (ng/mL)	Accuracy (%)		Precision (%RSD)		Matrix effect (%)	Recovery (%)
	Intra- day	Inter- day	Intra- day	Inter- day		
1	97.0	101.9	13.8	13.1	101.1	89.8
50	100.2	94.3	4.8	8.6	100.1	90.0
900	98.2	99.0	8.8	3.5	104.8	90.9
1800	97.6	101.6	4.4	3.8	102.2	86.8

Instrument and Condition. A ACQUITY H-Class UPLC and a XEVO TQS-micro triple quadrupole mass spectrometer (Waters Corp, Milford, MA, USA) were used in this study. UPLC BEH C18 (2.1 mm $\times$ 50 mm, 1.7 μ m) was used at 40 °C. The mobile phase was composed of acetonitrile and 0.1% formic acid in gradient elution, and the flow rate was set at 0.6 mL/min. The gradient elution was as follows: 0–0.2 min, acetonitrile 10%, 0.2–1.0 min, linear acetonitrile from 10% to 90%; 1.0–2.0 min, acetonitrile 90%; 2.0–2.1 min, linear acetonitrile from 90% to 10%; 2.1–3.0 min, acetonitrile 10%.

Nitrogen was used as desolvation gas (800 L/h) and nebulizing gas. The capillary voltage was set to be 2.2 kV, the ion source temperature was 150 °C, and the desolvation temperature was 400 °C. Electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode with positive ionization was used: 463.1 $\rightarrow$ 301.0 for diosmetin-7-*o*- β -d-glucoside, m/z 285.1 $\rightarrow$ 193.0 for diazepam (IS) (Figure 2).

Sample Preparation. In a 1.5-mL centrifuge tube, 50 μ L plasma was added, further diluted with 200 μ L acetonitrile (containing IS 50 ng/mL), mixed by a vortexer for 1.0 min, and centrifuged at 13,000 rpm for 10 min at 4 °C. The supernate (100 μ L) was transferred into a liner pipe in vial, and the injection volume was set at 1 μ L.

Method Validation. The verification method was established in accordance with the US Food and Drug Administration (FDA) bioanalytical method validation guidelines. Validation projects included selectivity, matrix effects, linearity, precision, accuracy, recovery, and stability, six replicates at LLOQ, and 3 different concentrations (QCs).

Pharmacokinetics. Six rats were given diosmetin-7-*o*- β -d-glucoside (5 mg/kg) by intravenous (i.v.) administration. The

blood (100 μ L) was withdrawn from the caudal vein at 5 and 15 min and 1, 2, 4, 6, 8, and 12 h after administration and collected into 1.5-mL centrifuge tubes containing heparin. Plasma (50 μ L) was collected after centrifuging at 3000 rpm for 10 min and stored at -20 °C. Pharmacokinetic parameters were all analyzed using DAS 2.0 software (China Pharmaceutical University).

Results and Discussion

Method Optimization. The choice of positive and negative electrodes for ESI is often evaluated in the methodology [25, 26]. Diosmetin-7-*o*- β -d-glucoside was more suitable for ESI positive electrode detection. We optimized the ionization conditions of diosmetin-7-*o*- β -d-glucoside and the IS, and the highest abundance of the apigenin fragment was m/z 301.0, and the highest abundance of the IS fragment ion was m/z 193.0.

The removal of protein and interference out of the plasma was a key point of LC–MS/MS analysis [27, 28]. In this paper, acetonitrile protein precipitation method, methanol protein precipitation method, and ethyl acetate liquid–liquid extraction methods were investigated. Extract recoveries for acetonitrile protein precipitation, methanol protein precipitation, and ethyl acetate liquid–liquid were 90.1%, 80.4%, and 72.3%, respectively. The acetonitrile protein precipitation method was chosen as a pretreatment method for the samples because of its best extract efficiency.

Liquid chromatography conditions separate diosmetin-7-*o*- β -d-glucoside and IS as much as possible from the endogenous interfering substances [29–32]. In the investigation of chromatographic conditions, the mobile phase systems such as methanol–water, acetonitrile–water, methanol–0.1% formic acid, and acetonitrile–0.1% formic acid were investigated. The acetonitrile–0.1% formic acid resulted in the best chromatographic peak and suitable retention time.

Method Validation. The selectivity of the method was accessed by blank plasma, blank plasma, spiked with diosmetin-7-*o*- β -d-glucoside, and a rat plasma sample. Figure 3 illustrates the UPLC–MS/MS chromatograms of blank plasma spiked with diosmetin-7-*o*- β -d-glucoside. There were no obvious impurities and endogenous substances that had intervened in the detection of diosmetin-7-*o*- β -d-

Figure 4. Plasma concentration-time curve of diosmetin-7-*o*- β -d-glucoside after intravenous (5 mg/kg) administration

Table 2. Main pharmacokinetic parameters of diosmetin-7-*o*- β -d-glucoside in rats

Parameters	Unit	i.v. 5 mg/kg
AUC _(0-t)	Ng/mL*h	7412.9 $\pm$ 2498.1
AUC _(0-∞)	Ng/mL*h	7423.8 $\pm$ 2484.0
MRT _(0-t)	h	0.9 $\pm$ 0.3
MRT _(0-∞)	h	1.0 $\pm$ 0.3
<i>t</i> _{1/2z}	h	1.4 $\pm$ 0.4
V _z	L/kg	2.1 $\pm$ 2.8
CL _z	L/h/kg	2.8 $\pm$ 4.5
C _{max}	Ng/mL	0.7 $\pm$ 0.2

glucoside, suggesting the selectivity of the method was acceptable.

The concentration of diosmetin-7-*o*- β -d-glucoside standard curve in rat plasma was within the range of 1–2000 ng/mL. The LLOQ of diosmetin-7-*o*- β -d-glucoside in rat plasma was 1 ng/mL. The standard curve equation of diosmetin-7-*o*- β -d-glucoside in the rat plasma was $Y = 0.0029C + 0.0021$, $r = 0.9981$, where Y represents the the peak area ratio of diosmetin-7-*o*- β -d-glucoside to the IS, and C represents the concentration of diosmetin-7-*o*- β -d-glucoside in rat plasma.

The intra-day and inter-day precisions of diosmetin-7-*o*- β -d-glucoside in rat plasma were less than 14%. The accuracy range was between 94.3% and 101.9%, and the matrix effect was between 100.1% and 104.8%, the recovery was higher than 86.8%, Table 1. These data met the pharmacokinetic study requirements of diosmetin-7-*o*- β -d-glucoside.

For the rat plasma at room temperature for 2 h, at -20°C for 30 days, and under the freeze–thaw stability test, the variation of diosmetin-7-*o*- β -d-glucoside was within $\pm 14\%$, and the RSD was less than 12%, which indicated that the stability of diosmetin-7-*o*- β -d-glucoside was acceptable.

It was found that there are slight changes in various system parameters, such as the organic component of the mobile phase ($\pm 5\%$), the mobile phase flow rate (± 0.1 mL/min), and the column oven temperature ($\pm 2^{\circ}\text{C}$) [33]. In all the deliberately varied chromatographic conditions, the selectivity as well as the performance of the method was unchanged, which proves the method was robust.

Pharmacokinetics. Figure 4 displays the plasma concentration–time curves of diosmetin-7-*o*- β -d-glucoside. The main pharmacokinetic parameters analyzed by the non-compartment model were listed in Table 2. The $t_{1/2}$ of diosmetin-7-*o*- β -d-glucoside is 1.4 ± 0.4 h, which indicates quick elimination. The pharmacokinetic profile of diosmetin-7-*o*- β -d-glucoside in rats was characterized for the first time in this study. The UPLC–MS/MS method in this study was validated for the determination of diosmetin-7-*o*- β -d-glucoside in rat plasma with a total run time of 3 min.

Conclusion

In this study, we developed a fast UPLC–MS/MS method with good selectivity for diosmetin-7-*o*- β -d-glucoside in rat plasma. The linear range was 1–2000 ng/mL, and 50 μL plasma was used and processed by the acetonitrile precipitation method. This method was applied in pharmacokinetics of diosmetin-7-*o*- β -d-glucoside in rats.

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