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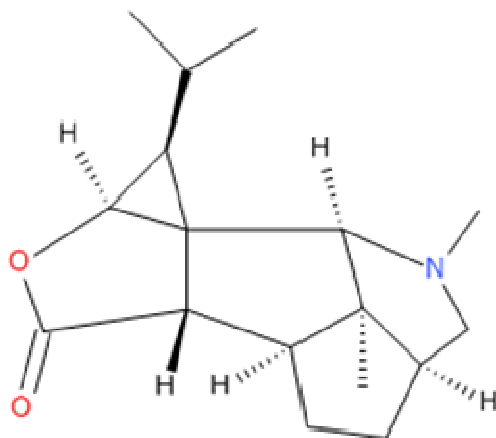
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A sensitive, simple and rapid UFLC-MS/MS method was firstly developed and validated for the determination of dendrobine in rat plasma.



Dendrobine

A simple, rapid and reliable UFLC-MS/MS method for the determination of dendrobine in rat plasma and its application to a pharmacokinetic study

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ABSTRACT : In this work, we established a simple, sensitive and rapid method for quantitative detection method for measurement of dendrobine levels in plasma using Ultra fast liquid chromatography with tandem mass spectrometry (UFLC–MS/MS). The plasma samples were performed by protein precipitation and then the supernatant was extracted by chloroform. An aliquot of extracted residues was injected into UFLC-MS/MS system with separation performed by an ACQUITY HSS T3 column. Acetonitrile and water containing 10 mM ammonium acetate was used as a mobile phase. The average extraction recoveries were greater than 80.5 % for dendrobine and 90.9 % for matrine (Internal standards, IS) from plasma. Good linearity ($R^2 > 0.992$) was observed in the range of 1–1000 ng/mL. For rat plasma, the overall accuracy of this method was 94.3–110.3 %. Intra-assay and inter-assay precision was less than 11.7 %. The lower limit of quantification and detection for dendrobine was 1.0 and 0.4 ng/mL, respectively. The practical utility of this new UFLC-MS/MS method was confirmed by pharmacokinetic studies followed by sublingual intravenous administration of three levels of dendrobine in rats.

Keywords: Dendrobine, UFLC–MS/MS, Pharmacokinetics

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1. Introduction

Dendrobine (Fig 1) was originally isolated from *Dendrobium nobile* and also can be found in *D. findlayanum*¹. The various prepared commodities made from *D. nobile* and *D. findlayanum* were popular in China and have a large market share in the health product field². They have received considerable attention in medical, health care and nutritional research^{3,4}. It is reported that they have various pharmacological effects, including antioxidation, antitumor activity, immunomodulation, neuroprotective activities and hepatoprotective activity⁵⁻⁹. Dendrobine was considered to be a main active alkaloid compound and used as a chemical marker for quality control and discrimination of *D. nobile* and *D. findlayanum*¹⁰⁻¹². The fact that few studies have investigated its pharmacokinetics has compromised the further utility of dendrobine.

HPLC and GC are reported to be a suitable instrument for the determination of dendrobine in herbs and prescriptions of traditional Chinese medicine (TCM). Yi *et al* developed a GC method for the determination of dendrobine and this method was suitable for analysis of dendrobine in *D. nobile* Linde¹². A HPLC method was also developed for the determination of dendrobine by Guo' group and Yi' group and was successfully applied to determine the content of dendrobine in different sources of *D. nobile* plants¹³. Li's group developed a UPLC method (LOD=2.44 µg/mL) to determine the content of dendrobine in different species of *Dendrobium*¹⁴. The quantitation method of dendrobine in rats was developed using HPLC-UV. Their results indicated that the linear range of dendrobine was 6.25-100.0 µg/mL¹⁵. Because the content of active compounds in TCM was relatively low in rat plasma, it

was necessary to develop a higher sensitive assay method. In our study we tried to use the HPLC–UV based method for the detection of dendrobine in plasma obtained from the rats. The HPLC–UV based method was not sensitive enough for the detection of relatively low levels of dendrobine. Therefore a sensitive and rapid method of
5 detection was required and developed using Ultra fast liquid chromatography with tandem mass spectrometry (UFLC–MS/MS) in order to obtain a higher sensitivity and shorter time. In this study, we describe a validated gradient UFLC-MS/MS method for the determination of dendrobine in rat plasma following administration of three different dosages and conducted a pharmacokinetic study of dendrobine in rats.

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2. Experimental

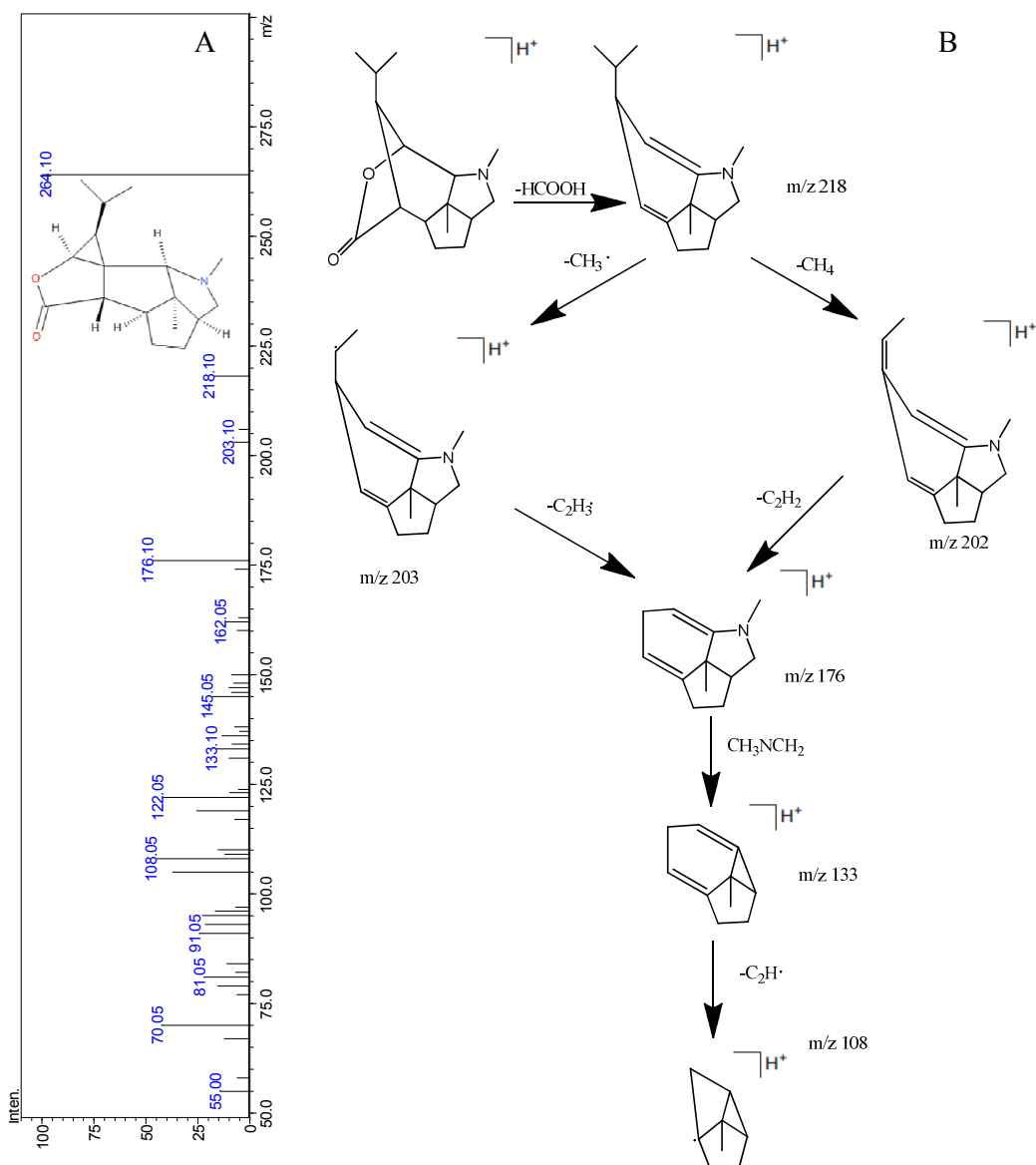
2.1 Chemicals and reagents

Dendrobine (Fig.1) and matrine (Internal Standard, IS) with 98.0% of purity were purchased from Chengdu MUST Bio-Technology Co. Ltd (Sichuan, China). HPLC
15 grade acetonitrile and methanol were purchased from Merck (Merck, Darmstadt, Germany). Ultra-pure water was prepared by Millipore Milli-Q system (Millipore, Bedford, MA, USA). Formic acid, chloroform, ammonium acetate and all of other reagents and solvents were of analytical grade.

2.2 Animals

20 Eighteen Sprague-Dawley male rats (220 ± 20 g) were purchased from the Experimental Animal Center of Wenzhou Medical College (Zhejiang, China). All animals were housed and cared under a constant temperature of 25 ± 1 °C and

humidity of 50 ± 10 %. The experimental rats were kept at a 12-12 hour light and dark interval. The ethical approval for the study was given by the Animal Care and Ethics Committee of Wenzhou Medical College.



5 Figure 1. The chemical structures and daughter scan ion spectra of dendrobine (A) and its proposed MS/MS main fragmentation pathway (B).

2.3 Chromatographic and mass spectrometric conditions

The chromatographic separation was carried out by a Shimadzu UFLC/MS-8030 plus

system. LC separation was performed with an ACQUITY HSS T3 (1.8 μm , 2.1 \times 150 mm) column with a guard column. The gradient mobile phases consisted of solvent A (10 mM ammonium acetate in water, pH=7.0) and solvent B (acetonitrile). The gradient elution was conducted with the following conditions: 40 % B (0-0.5 min), 40-90 % B (0.5-0.8 min), 90 % B (0.8-2.7 min), 90-40 % B (2.7-4.5 min), 40 % B (4.5-6.5 min). The flow rate was 0.3 mL/min and the injection volume was 5 μL . The column temperature was maintained at 40 $^{\circ}\text{C}$.

Mass spectrometric detection equipment was equipped with an electrospray ionization (ESI) source. The ESI source was set in positive ionization mode. The optimal MS parameters were as follows: capillary voltage, 3.5 kV; DL temperature, 250 $^{\circ}\text{C}$; heat block temperature, 400 $^{\circ}\text{C}$; nebulizing gas flow and drying gas flow were 3.0 L/min and 15.0 L/min, respectively. The optimized collision energy for dendrobine and matrine was 41 v and 40 v. Quantitation was performed using multiple reaction monitoring (MRM) of the transitions of m/z 264.4 \rightarrow m/z 122.1 for dendrobine and m/z 249.40 \rightarrow m/z 148.1 for IS, respectively.

2.4 Preparation of standard solution and quality control (QC) samples

Individual stock solutions of dendrobine (1.0 mg/mL) and IS (0.52 mg/mL) were prepared by dissolving the required amount of chemical reference substance in chloroform and methanol, respectively. The stock solution was diluted with methanol to obtain working standard solutions of desired concentrations. The IS solution was diluted to 5.2 $\mu\text{g/mL}$ with methanol. The plasma calibration standards were prepared

at concentrations of 1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL for dendrobine and 520 ng/mL for IS by adding appropriate amounts of working solutions to 100 μ L of blank rat plasma.

For the validation of this method, three levels of quality control (QC) samples at concentrations of low (2 ng/mL), medium (50.0 ng/mL) and high (400.0 ng/mL) for dendrobine together with 520 ng/mL for IS were prepared by spiking blank plasma samples. The spiked samples were then treated as described in the Section 2.5. All samples were stored at -20 $^{\circ}$ C until analysis.

10 2.5 Plasma sample preparation

A 100 μ L of plasma sample was transferred to a 2 mL tube, 10 μ L of IS solution (520 ng/mL) was added and mixed for 30 s, followed by an addition of 300 μ L acetonitrile. After vortex-mixing for 2 min, samples were centrifuged at 16627 g for 15 min to remove the protein. The supernatant (300 μ L) was separated and transferred to a 2 mL tube, followed with 600 μ L of chloroform. After vortex-mixing for 1 min, samples were again centrifuged at 16627 g for 10 min. The under layer (0.75 mL) was separated and liquid completely evaporated under nitrogen at 35 $^{\circ}$ C. The residue was reconstituted in 100 μ L of acetonitrile/water (40:60) and vortex-mixed for 2 min, followed by centrifugation at 16627 g for 15 min. A 5 μ L aliquot of the supernatant was injected into the UFLC-MS/MS system for analysis.

2.6 Method validation

The selectivity of the method was assessed by analyzing the blank plasma. The chromatograms of the blank plasma sample spiked with dendrobine, and a rat plasma sample after sublingual intravenous administration of dendrobine were compared in order to find potential interfering peaks within the range of the retention time of each sample. The linearity of dendrobine over the range from 1 to 1000 ng/mL was investigated with matrine as IS. The limit of detection (LOD) and lower limit of quantification (LLOQ) were defined as dendrobine responses which yielded a signal to noise ratio of greater than 3 and 10, respectively. The intra- and inter-day precisions and accuracy of the method were assessed by analyzing QC samples on the same day and on five consecutive days, respectively. The extraction recovery was calculated by comparing the analyte/internal standard peak area ratios obtained from extracted plasma samples with those originally dissolved with biological matrix extract. The matrix effect was evaluated by comparing the analyte/internal standard peak area ratios dissolved with blank matrix extract against those dissolved with menthol. QC samples at concentrations of 2.0, 50.0, 400.0 ng/mL were applied to investigate the stability of dendrobine in plasma under three storage conditions: (1) three freeze–thaw cycles of 24 h, (2) storage at -20 °C for 2 weeks, (3) keeping it in the autosampler (4 °C) for 24 h and (4) 12 h at room temperature.

20 **2.7 Pharmacokinetic study**

This validated method was applied to monitor the plasma concentrations of dendrobine in rats after sublingual intravenous administration of dendrobine at a dose

of 0.5, 2.0 and 5.0 mg/kg, respectively. The rats fasted for 12 h and had free access to water before dosing by sublingual intravenous administration. The blood samples (approximately 0.3 mL) were collected in heparinized polythene tubes from the rats' caudal veins at 0.083, 0.167, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10 and 12 h after dosing. After 5 centrifugation at 3000 rpm for 15 min, the plasma was separated and stored at -20°C until analysis. The pharmacokinetic parameters were analyzed by drug and statistics (DAS) software (version 2.1, Shanghai, China).

3. Results and Discussion

10 3.1 Method Development

Various combinations of acetonitrile, methanol, water, ammonium acetate and formic acid in water with variable volume content of each component were investigated and compared to identify the optimal mobile phase. Acetonitrile was chosen as the organic phase because it could provide lower column pressure compared to methanol. 15 Compared with 0.1 %, 0.2 % formic acid and 5 mM ammonium acetate, 10 mM ammonium acetate added into the water has a higher sensitivity and produce a sharper peak shape. Therefore 10 mM ammonium acetate in water was chosen as the aqueous phase. Gradient elution with the mobile phase consisted of acetonitrile and water containing 10 mM ammonium acetate provided better peak symmetry, proper 20 retention time, and avoided the matrix effects for the analyte and IS when compared with isocratic elution. A flow rate of 0.3 mL/min produced good peak shapes and permitted a run time of 6.5 min.

An efficient clean-up for bio-samples to remove protein and potential interferences prior to UFLC-MS analysis was an important step in these studies. Acetonitrile and chloroform were chosen as the protein precipitation and extract solvent, respectively, because they exhibited better acceptable recoveries. The assay were also performed on different types of columns, including BEH-C₁₈ (2.1 × 100 mm, 1.7 μm), BEH Shield RP18 (2.1 × 100 mm, 1.7 μm), shim-pack XR-ODS III column (2.0 × 75 mm, 1.6 μm) and ACQUITY HSS T3 (2.1 × 100 mm, 1.8 μm). The results indicated that HSS T3 column had more suitable retention time and exhibited a more symmetrical peak shape, and thus used for separation column in this assay.

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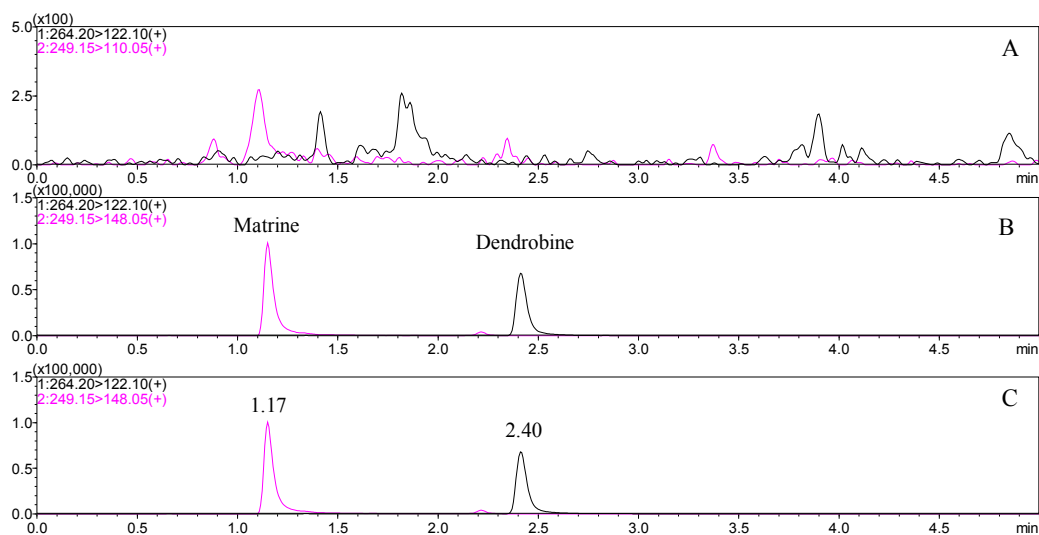


Figure 2. MS chromatograms of (A) blank rat plasma, (B) Blank rat plasma spiked with dendrobine (50 ng/mL) and IS (52 ng/mL) and (C) Rat plasma sample collected 30 min after i.v. injection of 0.5 mg/kg dendrobine.

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3.2 Selectivity, Recovery and Matrix Effect (ME)

Figure 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with dendrobine and IS, and a plasma sample. No interfering endogenous substances were observed at the retention times of the analyte and IS. Mean recoveries of dendrobine were greater than 80.5 % (Table 1). The recovery of the IS was better than 90.0 % ($n=6$). The ME for dendrobine in the plasma were measured over the range of 95.2 % to 105.2 % at each QC level ($n=6$) (Table 1). The ME for IS (520 ng/mL) was in the range of 93.2 % - 98.4 % ($n=6$). As a result, ME from the plasma was negligible in this method.

10 Table 1. Matrix effect and extraction recovery for the assay of dendrobine and IS in rat plasma ($n = 5$).

Norminal conc. (ng/mL)	Extraction recovery (% , mean \pm SD)	Matrix effect (% , mean \pm SD)
Dendrobine		
2	80.5 \pm 2.1	105.2 \pm 2.2
50	81.7 \pm 1.9	95.2 \pm 2.9
400	82.3 \pm 2.7	96.2 \pm 3.1
Matrine		
520	90.9 \pm 2.5	95.8 \pm 2.6

3.3 Calibration Curves and Sensitivity

15 The linear regressions of the peak area ratios versus concentrations were fitted over the concentration range of 1-1000 ng/mL for dendrobine by weighted ($1/x^2$) least squares linear regression. The typical regression equation of the calibration curve of dendrobine was $Y = 0.0202X + 0.0113$ ($R^2 = 0.992$), where y represents the ratios of

dendrobine peak area to that of IS and x represents the plasma concentration. For dendrobine, the present UFLC-MS/MS method gave an LLOQ of 1.0 ng/mL with an accuracy of 92.3 % and a precision of 14.8 % in terms of CV. The LOD, defined as a signal–noise ratio of >3 , was 0.4 ng/mL for dendrobine in the plasma.

5

3.4 Precision and Accuracy

The precision of the method was determined by calculating CV for QCs at three concentration levels over five validation days. Intra-day precision was less than 6.2 % and the inter-day precision was less than 11.7 % at each QC level. The accuracy of the method ranged from 94.3 % to 110.3 % at each QC level. Assay performance data is presented in Table 2. The above results demonstrated that the values were within the acceptable range and the method was accurate and precise for rat plasma samples.

Table 2. Intra- and inter-day accuracy and precision for the determination of dendrobine in rat plasma (n=5).

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	Nominal conc (ng/mL)	Calculated conc (ng/mL)	Accuracy (%)	Precision (% CV)
Intra-day	2	2.1 ± 0.1	104.0	5.9
	50	49.4 ± 3.1	98.7	6.2
	400	377.2 ± 17.6	94.3	4.7
Inter-day	2	2.4 ± 0.3	110.3	11.7
	50	52.7 ± 4.3	105.3	8.1
	400	412.4 ± 39.0	103.1	9.5

3.5 Stability

The stability of dendrobine during the sample storing and processing procedures was fully evaluated by analysis of QC samples. The results of stability tests indicated that

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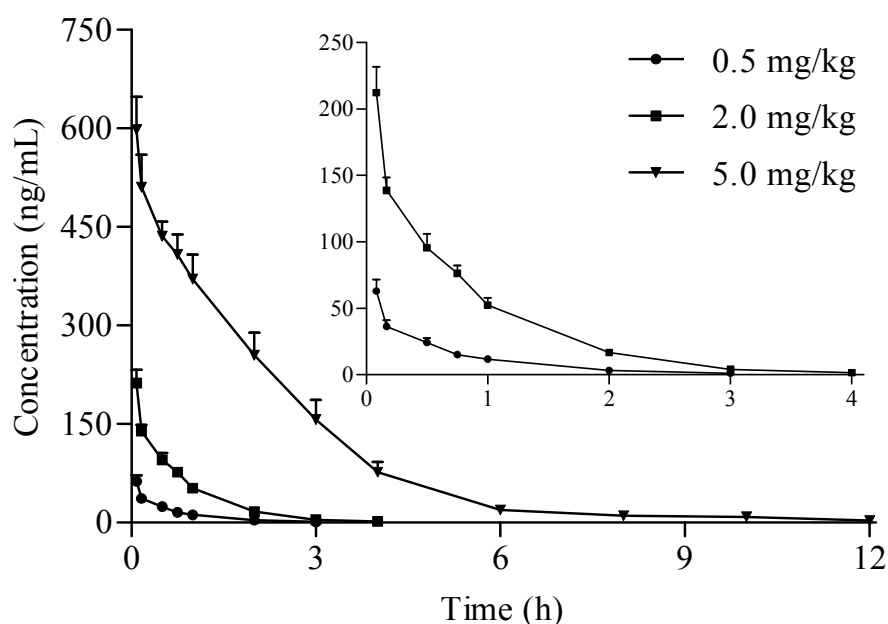
dendrobine was stable in plasma samples for 12 h at room temperature (RE: 0.4 % - 11.5 %, RSD < 3.4 %); three freeze–thaw cycles (RE: 0.3 % - 7.0 %, RSD < 0.9 %); keeping for 12 h in an autosampler (RE: –0.3 % - 7.8 %, RSD < 2.9 %) and long-term stability (RE: -5.4 % to 1.6 %, RSD < 13.1 %). This suggested the established method
5 was suitable for the pharmacokinetic study of dendrobine.

3.6 Pharmacokinetics

The sensitive and rapid UFLC-MS method was applied to the pharmacokinetic study in rats following intravenous administration of dendrobine after single-dose of 0.5, 2.0
10 and 5.0 mg/kg. Pharmacokinetic analyses and plasma concentration versus time data were analyzed by DAS software. Pharmacokinetic parameters were calculated by using the two-compartmental model and the results were summarized in Table 3. The mean plasma concentration–time curves after administration of dendrobine were shown in Fig. 3. As shown in Fig.3 and Table 3, the $t_{1/2}$ was 0.6 ± 0.2 and 0.5 ± 0.1 h
15 for administration doses of 0.5 and 2.0 mg/kg, revealing that the residence time of dendrobine *in vivo* was very short. However, due to the saturated metabolism, the $t_{1/2}$ was 1.7 ± 0.6 h for administrated doses of 5.0 mg/kg. It also showed that when using a higher dose of dendrobine, the time points of blood collection should be prolonged to more than 12 h in order to elucidate the profile of dendrobine in rats. When the dose
20 of dendrobine was increased from 0.5 to 2 mg and from 2 to 5 mg, the AUC(0–t) did not increase proportionately with the dose (Table. 3), which was indicative of the nonlinearity of the pharmacokinetics in the 5.0 mg/kg group. The possible cause of

the nonlinearity was considered to be associated with that the dendrobine was in a saturated metabolism or bound with plasma proteins.

The maximum plasma concentrations (C_{max}) area under the plasma concentration versus time curve from 0 h to the last measurable concentration ($AUC(0-t)$) and area under the plasma concentration versus time curve from 0 h to infinity ($AUC(0-\infty)$) were significantly different among three groups. This result indicates that absorption was increased with the administration of a large dose and C_{max} increased with the dose. The elimination half-time ($t_{1/2}$), total body clearance (CL_z), and mean residence time (MRT) did not differ significantly between the 0.5 and 2.0 mg/kg groups. The volume of distribution (V_{zF}) was not significantly different between all the groups. And the V_{zF} was about 5–25 L, indicating that the dendrobine mostly existed in the blood and extracellular fluid, lacking of specific tissue distribution.



15 Figure 3. Mean plasma concentration–time profiles for dendrobine after sublingual

intravenous administration of three different dosages.

Table 3. Pharmacokinetic parameters for dendrobine in rats following 0.5, 2.0 and 5.0 mg/kg administration of dendrobine (n = 6).

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Parameter	Unit	0.5 mg/kg	2.0 mg/kg	5.0 mg/kg
AUC(0-t)	ug/L*h	36.2 ± 10.7	154.3 ± 33.2	1359.3 ± 201.7*
AUC(0-∞)	ug/L*h	37.7 ± 11.2	155.4 ± 31.9	1365.9 ± 207.1*
MRT(0-t)	h	0.6 ± 0.1	0.7 ± 0.1	1.9 ± 0.5*
MRT(0-∞)	h	0.7 ± 0.1	0.8 ± 0.1	2.0 ± 0.5*
t _{1/2}	h	0.6 ± 0.2	0.5 ± 0.1	1.7 ± 0.6*
CL _Z	L/h/kg	14.2 ± 3.9	13.3 ± 2.4	3.7 ± 0.6*
V _Z	L/kg	13.3 ± 8.3	10.0 ± 2.2	9.1 ± 4.0
C _{max}	ug/L	62.9 ± 21.8	212.3 ± 48.0	637.0 ± 80.8*

* Compare with 0.5 and 2.0 mg group, $P < 0.05$.

4. Conclusion

A sensitive, rapid and specific UFLC-MS/MS method for the determination of dendrobine in rat plasma was developed and validated over the concentration range of 1-1000 ng/mL. This method is successfully used in the pharmacokinetic study of dendrobine in rat plasma. The pharmacokinetics of dendrobine in rats are fit to a two-compartmental model. The profile of concentration versus time was nonlinear and the pharmacokinetic parameters were analyzed. Although this being fundamental research, the results of this study provide valuable information for the development and clinical utility of dendrobine.

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