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Determination of helicidum and its metabolites in dog plasma by LC/UV/MS/MS and its application to pharmacokinetic studies

Qingfei Liu *, Xiangdong Liu, Guoan Luo, Weiwei Tian, Yiming Wang

Institute of Material Medica, Tsinghua University, Beijing 100084, China

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Abstract

A simple, rapid and reliable method was developed for the identification and quantification of helicidum and its metabolites in beagle dog plasma by liquid chromatography/ultra-violet/electrospray ionization-ion trap mass spectrometry (LC/UV/ESI-ITMS). Two metabolites were identified by MS: formylphenyl-*O*-β-D-pyranosyl alloside (I) and hydroxylmethylphenyl-*O*-β-D-pyranosyl alloside (II). UV was used for concentration determination with the wavelength of 270 nm. Liquid–liquid extraction was used and the extraction recovery exceeded 90%. Kromacil C_{18} column (5 μm, 4.6 mm i.d. × 250 mm) was used as the analytical column. Linear detection responses were obtained for helicidum concentration ranging from 1.76×10^{-4} to 70.4×10^{-4} μmol/mL (0.050–2.00 μg/mL). The precision and accuracy data, based on intra- and inter-day variations over 3 days, were less than 5%. The limit of determination and quantitation (LOD, LOQ) for helicidum was 0.010 and 0.030 μg/mL, respectively. Pharmacokinetic data of helicidum and the two metabolites were obtained with this method after administration of intravenous injection and a single oral dose of tablets to six beagle dogs, respectively.
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Keywords: Helicidum; Metabolite; HPLC/UV/ESI-ITMS; Dog plasma; Pharmacokinetics

1. Introduction

Helicidum, formaldehydephenlyl-*O*-β-D-pyranosyl alloside, is the main composition from fruits of *Helicid hilagirica Beed*. The chemical structure of helicidum is shown in Fig. 1A. In china, it is often used in clinic to treat with neurasthenic syndromes, vascular headache, and trigeminalache with high efficiency and low side effect and toxicity [1–5]. Up to now, there was no method reported to determine helicidum and its metabolites in plasma after intravenous or oral administration. Chen et al. [6,7] reported the method of ultra-violet (UV) spectrophotometric analysis to determine the content and release of helicidum from helicidum tablets and hydrophile matrix tablets, respectively. However, the method was not sensitive enough for detecting helicidum and its metabolites in the plasma of dogs after administration of helicidum. Therefore, we developed a simple, sensitive high performance liquid chromatography/ultra-violet/electrospray ionization-ion trap mass spectrometry (HPLC/UV/ESI-ITMS) method for the simultaneous determination of helicidum and its metabolites in beagle dog plasma following intravenous administration of helicidum injection and oral administration of helicidum tablets at a single dose for the first time, respectively. MS was used for identification of helicidum and its metabolites and UV for concentration determination.

2. Experimental

2.1. Chemicals and reagents

Helicidum (>99% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Helicidum tablets (labeled as 25 mg/tablet, real concentration of 25.02 mg/tablet determined by HPLC/UV) were purchased from Yunnan Yuxi Pharmaceutical Co. Ltd. (Yunnan, China). Helicidum injection was prepared in sterilized saline solution (2.50 mg/mL). Acetonitrile was of HPLC grade (Fisher, USA). Distilled, deionized water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA). All other chemicals were of analytical reagent grade.

* Corresponding author. Tel.: +86 10 62794103; fax: +86 10 62772263.
E-mail address: liuqf@mail.tsinghua.edu.cn (Q. Liu).

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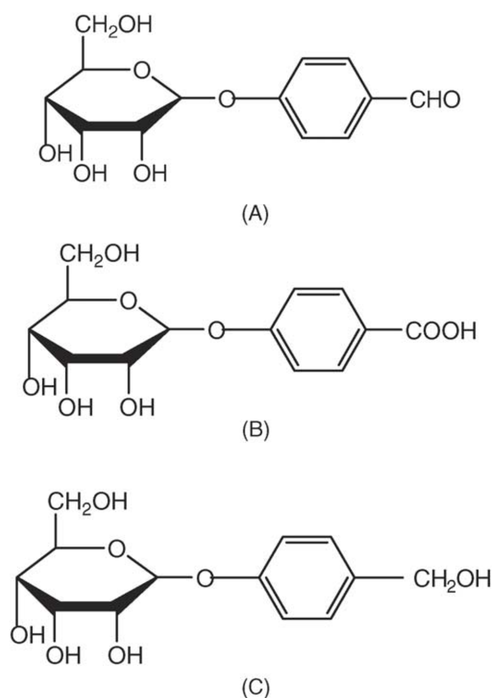

Fig. 1. Chemical structures of helicidum (A) and its metabolites (B and C). (A) Helicidum (formaldehydephenyl-*O*-β-D-pyranosyl alloside); (B) formylphenyl-*O*-β-D-pyranosyl alloside; (C) hydroxylmethylphenyl-*O*-β-D-pyranosyl alloside.

2.2. Equipment and chromatographic conditions

All analytical procedures were performed on an Agillent 1100 LC–MS system (Agillet Co., USA), with UV detector and ESI-ITMS detector. Separation was carried out by a Kromacil C_{18} column (5 μm, 4.6 mm × 250 mm, Dikma Co., Sweden) maintained at 30 °C. The mobile phase was acetonitrile–ice acetic acid–water (5:1:94, v/v/v) at the flow rate of 1.0 mL/min. Wavelength of determination was 270 nm. The injection volume was 20 μL.

The following optimized MS conditions were selected: Nebulizer gas (high-purity nitrogen gas, 99% purity, Beijing Gas Co. Ltd., China.) was 25.0 psi. Dry gas flow was 8.0 L/min and dry temperature was 350 °C. The voltage on the ESI interface was maintained at −3.5 kV in the negative ion mode. In the full scan mode, the mass spectrometer was operated over a range of *m/z* 50–500 and the target mass was 300 *m/z*. Compound stability was 100% and trap drive level was 100%. A divert valve was used to prevent MS contamination when running LC–MS. The selective ion monitoring (SIM) was used.

2.3. Animals and blood sample preparation

Six beagle dogs (three males, three females), weighing 13.8 ± 0.4 kg (mean ± S.D.), were purchased from Beijing Mars Biotechnology Co. Ltd. The animals were housed and cared for under a constant temperature at (22 ± 1 °C), humidity at (50 ± 10%). Diet was prohibited for 12 h before the experiment while water was taken freely. They were intravenously injected helicidum injection by the dose of 1 mg helicidum per kg body weight. Blood samples (about 1 mL) were collected in 1.5 mL heparinized polythene tubes at 0, 5, 15, 30, 45, 60, 90, 120, 180, 240, 360, and 540 min after administration.

After 1 month, each animal was retreated by oral administration of helicidum tablets with six tablets containing 150 mg helicidum. Blood samples (about 1 mL) were collected in 1.5 mL heparinized polythene tubes at 0, 5, 15, 30, 45, 60, 90, 120, 180, 240, 360, and 540 min after administration.

Each collected blood sample was immediately centrifuged at 4500 rpm for 10 min at 4 °C (TGL 16C, Medical centrifuge Co., Beijing, China). The resulting plasma (200 μL) and 100 μL of water–ice acetic acid (94:1) was added to a 5 mL polythene centrifuge tube. They were vortex-mixed for 1 min and then mixed with 1.5 mL methanol using vortex for 2 min. The tube was centrifuged at 4500 rpm for 10 min. The upper layer was transferred to another tube. The extraction from plasma was repeated twice. The upper layers were combined and evaporated to dryness at 35 °C under a gentle stream of nitrogen. The residue was reconstituted in 0.4 mL mobile phase, and centrifuged at 4500 rpm at 4 °C for 15 min. The supernatant (20 μL) was injected into the HPLC/UV/MS system for analysis. The same sample handling process was used for recovery and precision determinations in plasma.

2.4. Identification of helicidum and its metabolites by MS

Blood samples obtained after administration of helicidum preparations were detected by MS. According to the mass spectrometry data, helicidum and its metabolites were identified.

2.5. Standard solution, calibration curve, and quality control samples

The standard stock solution of 40 μg/mL of helicidum was prepared in methanol. A series of standard working solutions with concentrations in the range of 0.10–4.00 μg/mL for helicidum were obtained by further dilution of the standard stock solution with methanol. All solutions were stored at 4 °C. A series of 0.2 mL standard working solutions were evaporated to dryness at 35 °C under a gentle stream of nitrogen. The residues were reconstituted in 0.2 mL of blank plasma to prepare the calibration standards containing 2.00, 1.50, 1.00, 0.500, 0.200, 0.100, and 0.050 μg/mL for helicidum by the same method of blood sample preparation.

Quality control (QC) samples were prepared in the same way as calibration standards with blank plasma, and concentrations were 0.05, 0.20, and 1.00 μg/mL. The samples were stored at −80 °C until analysis.

2.6. Validation of the method

2.6.1. Specificity

Specificity is a measurement of the degree of interference in the analysis of complex sample mixtures. Chromatograms of blank blood sample, drug-contained blood samples collected at 60 min after intravenous and oral administration, respectively, were compared. The assay was considered adequately specific

when no endogenous plasma components eluted at the same retention times of the analytes of interest.

2.6.2. Linearity

Linearity of calibration was tested by extraction and assayed ($n = 3$). Calibration curve in the concentration range of 1.76×10^{-4} to 70.4×10^{-4} μmol/mL (0.050–2.00 μg/mL) for helicidum was determined by plotting the peak area versus helicidum concentration in beagle dog plasma.

2.6.3. Recovery

To determine the recovery of helicidum by the liquid–liquid extraction method, plasma samples were spiked with helicidum concentrations of 0.05, 0.20, and 1.00 μg/mL. The resulting peak area was compared with that of the standards prepared in mobile phase to provide the recovery.

2.6.4. Precision and accuracy

Intra-day accuracy and precision (each $n = 3$) were tested by analysis of the QC samples at different times during the same day. Inter-day accuracy and precision were determined by repeated analysis of the same samples over three consecutive days. The concentration of each sample was determined using calibration standards prepared on the same day. Accuracy of the method was determined by relative error (RE), which was calculated by the equation [8]:

$$\mathrm{RE} = \frac{C_1 - C_2}{C_2} \times 100\%,$$

where C_1 was mean of determined concentration, C_2 was actual concentration. Precision was determined by relative standard deviation (%R.S.D.).

2.6.5. LOD and LOQ

The limit of detection (LOD) was determined at the lowest concentration to be detected taking into consideration a 1:3 baseline noise-calibration point ratio. Signals 10 times higher than the peak noise height were regarded as the limit of quantification (LOQ). It was repeated five times for confirmation.

2.6.6. Stability

The stability of helicidum for 4 and 8 h at room temperature in plasma was evaluated, respectively, by repeated analysis ($n = 3$) of the QC samples. The amount of helicidum in the plasma samples was determined using a newly prepared calibration curve.

2.7. Assay application

The present method was used to determine concentration–time profiles of helicidum and its metabolites in beagle dog plasma after intravenous administration of helicidum injection and oral administration of helicidum tablets, respectively. The concentration of helicidum was determined by using the equation of linear regression obtained from the calibration curve. The concentration of the metabolites of helicidum was determined by the method of semi-quantification with the equation of linear regression for helicidum.

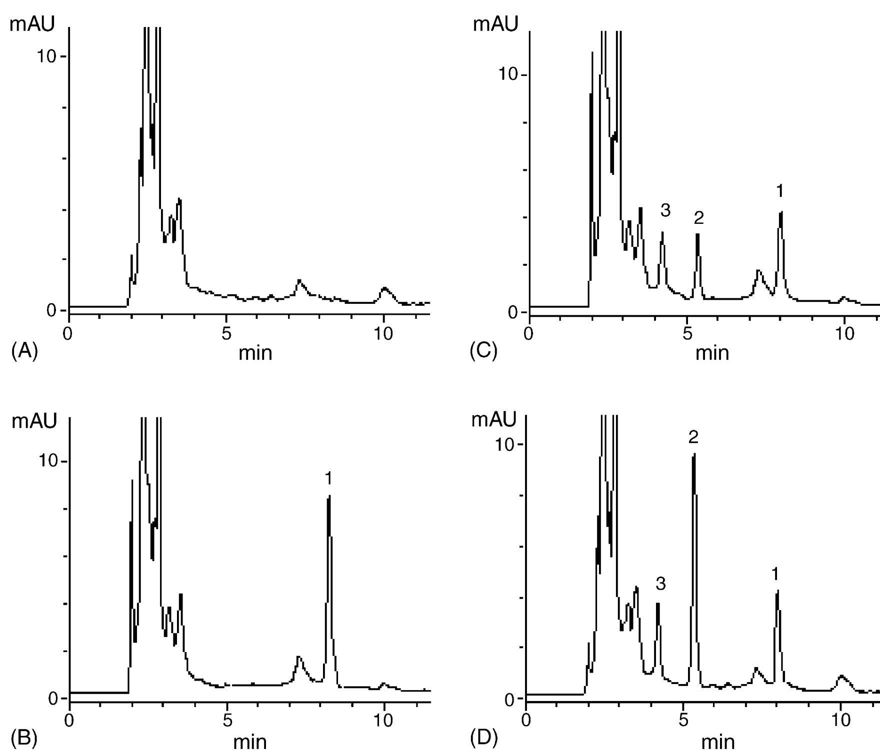

Fig. 2. Chromatograms of helicidum and its metabolites in dog plasma: (A) blank dog plasma; (B) blank plasma spiked with helicidum; (C) a dog plasma sample at 1 h after intravenous administration; (D) a dog plasma sample at 1 h after oral administration (1, helicidum; 2, formylphenyl-*O*-β-D-pyranosyl alloside; 3, hydroxylmethylphenyl-*O*-β-D-pyranosyl alloside).

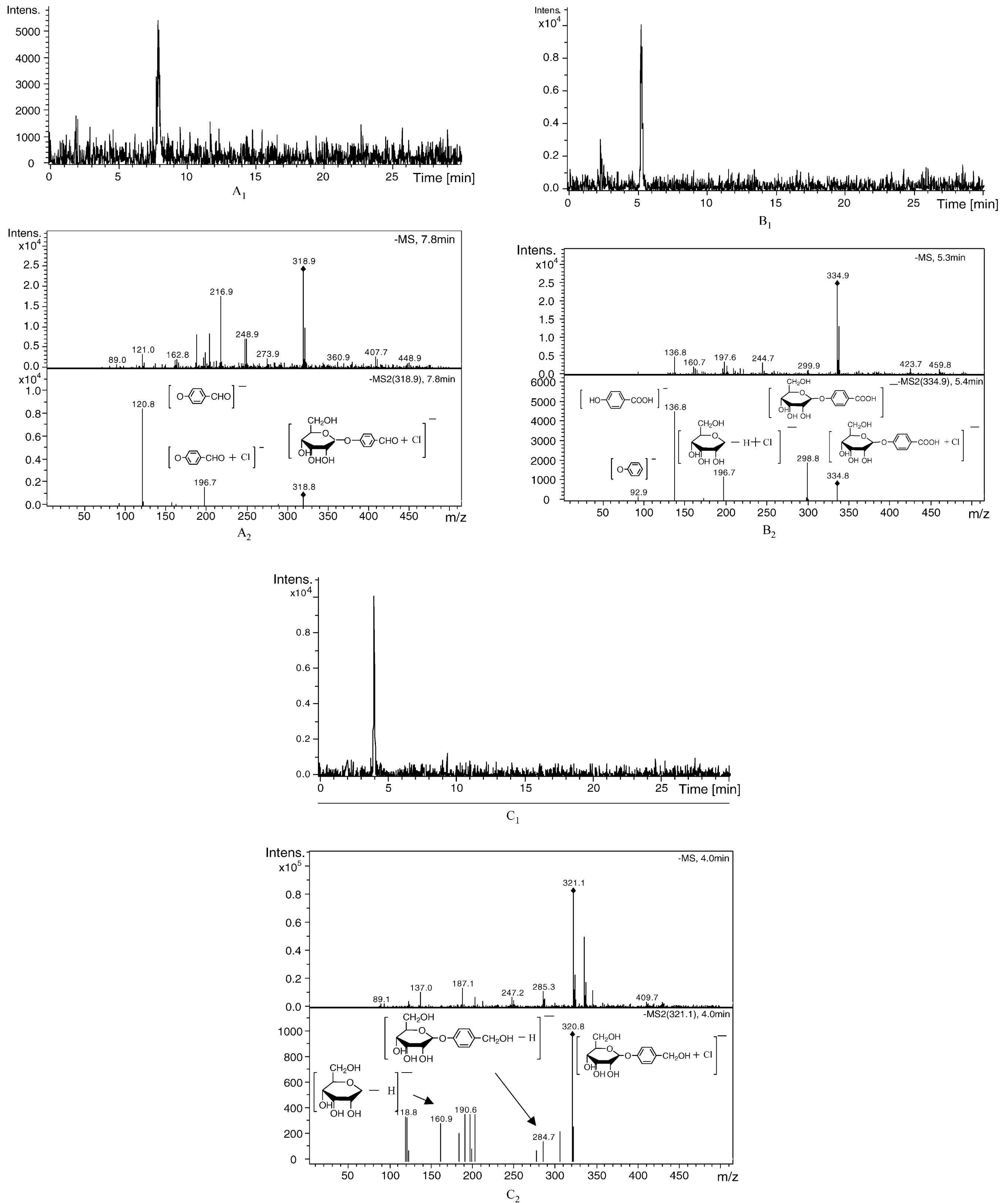

Fig. 3. EIC and MS of helicidum and its metabolites in dog plasma: (A_1) EIC of helicidum; (A_2) MS of helicidum; (B_1) EIC of metabolite B; (B_2) MS of metabolite B; (C_1) EIC of metabolite C; (C_2) MS of metabolite C.

3p97 pharmacokinetic software (provided by Chinese Pharmacology Association) was used to describe the compartment-model of the plasma concentration–time curve by compartmental analysis and calculate main pharmacokinetic parameters. The area under the concentration–time curve ($AUC_{0–t}$) was calculated by the trapezoidal method. Pharmacokinetic parameters were derived from the means of the individual results.

3. Results and discussion

3.1. Chromatography and mass spectrum

The liquid chromatograms and the mass spectra of helicidum and its metabolites were showed in Figs. 2A–D and 3A–C, respectively. The analytes formed predominantly negative ionized molecules $[M–H]^-$ in full scan spectra, and negative ionized molecules $[M–H + Cl]^-$ were detected at *m/z* 319 for $[284-H + Cl]^-$, *m/z* 321 for $[286-H + Cl]^-$, and *m/z* 335 for $[300-H + Cl]^-$. From the fragments of MS2, it could be identified that the molecule with *m/z* 319 was helicidum conjugated with Cl, *m/z* 321 was mylphenyl-*O*-β-D-pyranosyl alloside conjugated with Cl, and *m/z* 335 was hydroxylmethylphenyl-*O*-β-D-pyranosyl alloside conjugated with Cl. Hence, it could be identified that peak 1 in chromatograms of Fig. 2 was helicidum, peak 2 was formylphenyl-*O*-β-D-pyranosyl alloside, oxidized metabolite of helicidum, and peak 3 was hydroxylmethylphenyl-*O*-β-D-pyranosyl alloside, reduced metabolite of helicidum. The chemical structures of the two metabolites were shown in Fig. 1B and C, respectively. As shown in Fig. 2C, the retention times of peaks 1, 2, and 3 were approximately 7.8, 5.3, and 4.0 min, respectively. The assay was considered adequately specific for no endogenous plasma components eluted at the same retention times of the analytes of interest.

3.2. Calibration curve

The calibration curve of helicidum was constructed in the range 1.76×10^{-4} to 70.4×10^{-4} μmol/mL (0.050–2.00 μg/mL). The regression equation of the curve was calculated as follows: $y = 25979.8x - 191.0$ (correlation coefficient $r = 0.9999$), where x was concentration of helicidum (μmol/mL) and y was peak area of helicidum. It showed good liner relationships between the peak areas and the concentrations.

3.3. LOD, LOQ, and recovery

Under the condition, the LOD and LOQ of helicidum were 0.010 and 0.030 μg/mL, respectively. For the different samples with concentration of 0.050, 0.200, and 1.00 μg/mL, recoveries of helicidum were 90.6 ± 3.86, 90.7 ± 2.84, and $89.3 \pm 1.98\%$, respectively ($n = 3$). The mean recovery was $90.19 \pm 0.80\%$, more than 90%, showing that the liquid–liquid extraction method established could extract the most of helicidum from plasma.

Table 1
Intra-day and inter-day assay variations of helicidum in beagle dog plasma ($n = 3$)

	Spiked concentration (μg/mL)	Measured concentration (μg/mL)	RE (%)	R.S.D. (%)
Intra-day	0.050	0.051 ± 0.002	2.00	3.92
	0.200	0.198 ± 0.008	−1.00	4.04
	1.00	1.01 ± 0.03	0.70	3.28
Inter-day	0.050	0.048 ± 0.002	−4.00	4.17
	0.200	0.203 ± 0.009	1.50	4.43
	1.00	0.987 ± 0.036	−1.30	3.65

3.4. Accuracy and reproducibility

Analytical accuracy and precision data were shown in Table 1. The intra-day and inter-day %R.S.D. data of helicidum assays at low to high concentrations were less than 5%. Clearly, there was no significant difference between the intra- and inter-day precision, at least over the limited 3-day period investigated here. Assay accuracy, assessed by RE, ranged from −4.00 to 2.00%. For each concentration of helicidum, RE of intra-day was lower than that of inter-day. For either intra-day or inter-day, accuracy increased with decreased RE when concentration varied from low concentration to high.

3.5. Stability

Stability was expressed as a percentage of nominal concentration. The result was shown in Table 2. The mean recoveries from the nominal concentration were 96.00–97.60% at 4 h and 92.00–93.50% at 8 h. Helicidum was stable for at least 4 h at room temperature in plasma samples. The reason for the loss of helicidum was that there was a group of –CHO in the chemical structure of helicidum which was easily turned into –COOH or $–CH_2OH$ at room temperature. So the samples should be determined within 4 h or stored under −80 °C.

3.6. Application

With the similar chemical structures compared with helicidum, the UV absorption of the two metabolites B and C was similar to that of helicidum. So the contents of the two metabolites in plasma were determined using the regression equation of helicidum. The plasma concentration–time profiles of helicidum

Table 2
Stability of helicidum in beagle dog plasma ($n = 3$)

Spiked concentration (μg/mL)	Time (h)	Measured concentration (μg/mL)	Accuracy (%)
0.050	4	0.048 ± 0.002	96.00 ± 4.00
	8	0.046 ± 0.003	92.00 ± 6.00
0.200	4	0.195 ± 0.006	97.50 ± 3.00
	8	0.187 ± 0.009	93.50 ± 4.50
1.00	4	0.976 ± 0.027	97.60 ± 2.70
	8	0.927 ± 0.034	92.70 ± 3.40

Table 3
Pharmacokinetic parameters of helicidum and its metabolites after single intravenous/oral dose of helicidum to beagle dogs ($n = 6$)

Parameters	Intravenous injection			Oral tablets		
	Helicidum	Metabolite B	Metabolite C	Helicidum	Metabolite B	Metabolite C
T_{max} (min)	5	30	30	60	60	90
C_{max} (mg/L)	2.30 ± 0.71	0.71 ± 0.20	0.47 ± 0.15	0.78 ± 0.23	2.07 ± 0.64	0.40 ± 0.11
$t_{1/2}$ (min)	4.34 ± 0.39	78.29 ± 12.64	119.57 ± 14.08	60.85 ± 21.17	33.69 ± 7.85	108.94 ± 20.67
AUC_{0-t} (mg/L min)	258 ± 70	50 ± 14	57 ± 14	114 ± 29	164 ± 31	60 ± 17
MRT_{0-t} (min)	143 ± 49	55 ± 15	132 ± 23	129 ± 30	85 ± 15	153 ± 36

and its metabolites were shown in Fig. 4A and B. Pharmacokinetic parameters were shown in Table 3.

After intravenous administration of helicidum injection, the two metabolites B and C could be detected at 5 and 15 min, respectively, indicating that metabolism of helicidum rapidly occurred.

After oral administration of helicidum tablets, the time to reach the maximum plasma concentrations (T_{max}) of helicidum was 60 min and C_{max} was 0.78 ± 0.23 mg/L. As for the two metabolites B and C, T_{max} were 60 and 90 min, and C_{max}, calculated as helicidum, were 2.07 ± 0.64 and 0.40 ± 0.11 mg/L, respectively. Fig. 4B showed that the metabolite B was produced and eliminated rapidly after oral administration.

For intravenous injection, ratio of AUC_{0-t} of helicidum and its metabolites B and C was 1:0.19:0.22; however, for oral tablets, ratio was 1:1.44:0.53, showing that the metabolite B was the main product after oral administration.

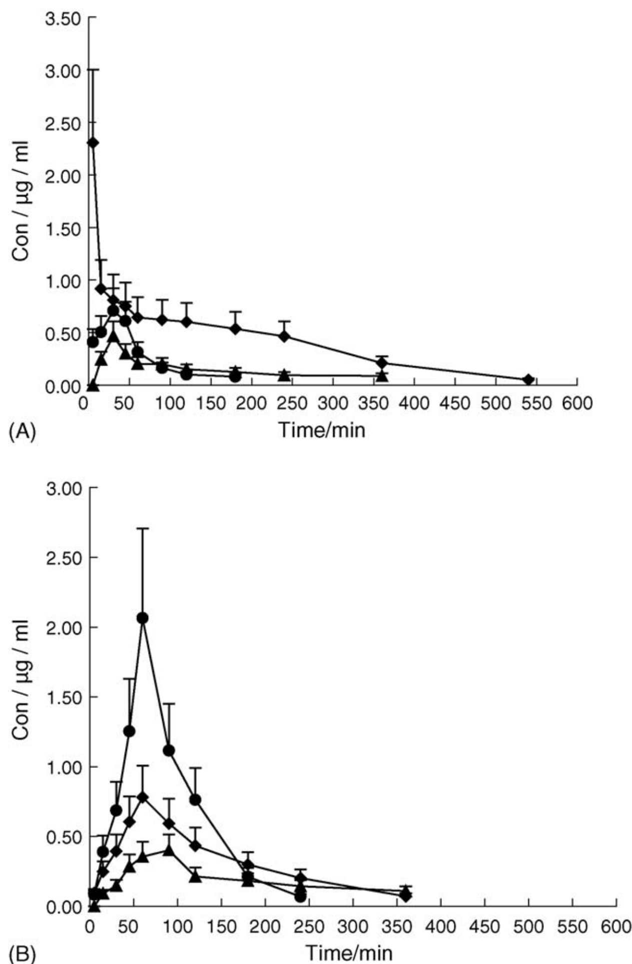

Fig. 4. Plasma concentration–time profiles of helicidum and its metabolites following intravenous helicidum injection (A) and oral helicidum tablets (B) ((♦) helicidum, (▲) formylphenyl-*O*-β-D-pyranosyl alloside, (●) hydroxylmethylphenyl-*O*-β-D-pyranosyl alloside).

4. Conclusion

A method was developed for the identification and quantification of helicidum and its metabolites in dog plasma by HPLC/UV/MS. MS was used for identification of helicidum and its metabolites and UV for concentration determination. The method was rapid, for identification and quantification could be done at the same time. Liquid–liquid extraction was used and the extraction recovery exceeded 90%. The assay had a good sensitivity and repeatability to fit the need of determination. The selectivity of this method was shown to be excellent, with no interference from other analytes. The results obtained using this method showed that the intra-day precision and inter-day precision for the analytes were less than 5% R.S.D. Furthermore, the HPLC column used for this work remained stable throughout our study.

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