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Short communication

Determination and pharmacokinetics of DT-13 in rat plasma by LC–MS

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ABSTRACT

A sensitive and specific LC–MS assay for DT-13 in rat plasma was developed. DT-13 is an active steroidal saponin present in Liriopes Radix and is developed as an anti-tumor drug candidate. The samples were extracted by acetonitrile-mediated plasma protein precipitation. The chromatographic separation was carried out using a Ultimate C_{18} column (250 mm × 4.6 mm, i.d., 5 μm) with a mobile phase composed of acetonitrile: 5 mmol/L aqueous ammonium acetate (60:40, v:v). The method was validated and the specificity, linearity (r^2 = 0.9980 within 10–1000 ng/mL), lower limit of quantitation (LLOQ, 10 ng/mL), precision (intra- and inter-day <12.3%), accuracy (93.4–106.3%), recovery (91.0 ± 4.7%) and stability were determined. The method was applied to the pharmacokinetic study of DT-13 in rat plasma after intravenous and intragastric administration. The results showed DT-13 underwent a prolonged absorption and slow elimination with a low oral bioavailability (5.51%) in rats.

1. Introduction

Liriopes Radix, an important traditional Chinese medicine (TCM), is the dried tuber of *Liriope muscari* (Decne.) Baily. It has been applied as a yin-nourishing remedy and used to treat cough and cardiovascular disease for a long time [1]. A large group of steroidal saponins were found in *L. muscari* and other plants of *Liriope* Lour., which possess a broad range of interesting bioactivity [2]. The saponin monomer 13, namely 25(R, S) ruscogenin 1-O-[β-D-glucopyranosyl (1 → 2)][β-D-xylopyranosyl (1 → 3)]-β-D-fucopyranosy (DT-13, Fig. 1), is the major saponin monomer isolated from *L. muscari* [3]. Pharmacological studies have revealed that DT-13 exerts wide variety of activities such as cardioprotective, immunopotentiating, anti-inflammatory, and anti-tumor effects [4–10].

With the in-depth study of its anti-tumor activity, experiments displayed that DT-13 could inhibit growth, migration and adhesion of human breast cancer cell, as well as the lung metastases of B16 melanoma [11]. Some evidences suggested that DT-13 inhibited cancer cells metastasis via regulation of tissue factor, which was different from other anti-metastatic drugs [12,13]. Currently, there is no effective therapy for most cancer in the advanced stages after metastasis, thus the potent effect of DT-13 against tumor metastases with a very low toxicity drew much interest of researchers [14], which could be considered using in the conventional chemotherapy to provide a palliative cure. For the development of DT-13 as a new drug, information about its pharmacokinetics is required. However, there are still no reports on the sensitive assay and pharmacokinetic study of DT-13 so far. In this paper, a LC–MS method was developed for the quantitative determination of DT-13 in rat plasma, and the characterization of pharmacokinetics of DT-13 in rat was investigated to achieve this aim.

2. Experimental

2.1. Chemicals and animals

DT-13 (purity > 98%) was separated from Liriopes Radix by the authors, and the structure was validated by comparing the chemical and spectroscopic (UV, NMR and MS) data with those reported in literatures [3]. Ophiopogonin D (purity > 98%) used as internal standard (IS) was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China).

HPLC grade acetonitrile was from Tedia (Fairfield, OH, USA) and water was purified by a Milli-Q academic water purification system (Milford, MA, USA). All the other reagents were of analytical grade.

Male pathogen-free SD rats (220–250 g) were provided by the Experimental Animal Center of China Pharmaceutical University (Nanjing, China). Animal studies were carried out in accordance with the Guidelines for Animal Experimentation of China Pharmaceutical University (Nanjing, China) and the procedure was approved by the Animal Ethics Committee of this institute.

2.2. LC–MS instruments and conditions

The HPLC was performed on an Agilent 1200 system (Palo Alto, CA, USA). Separations were achieved by an Ultimate C_{18} column

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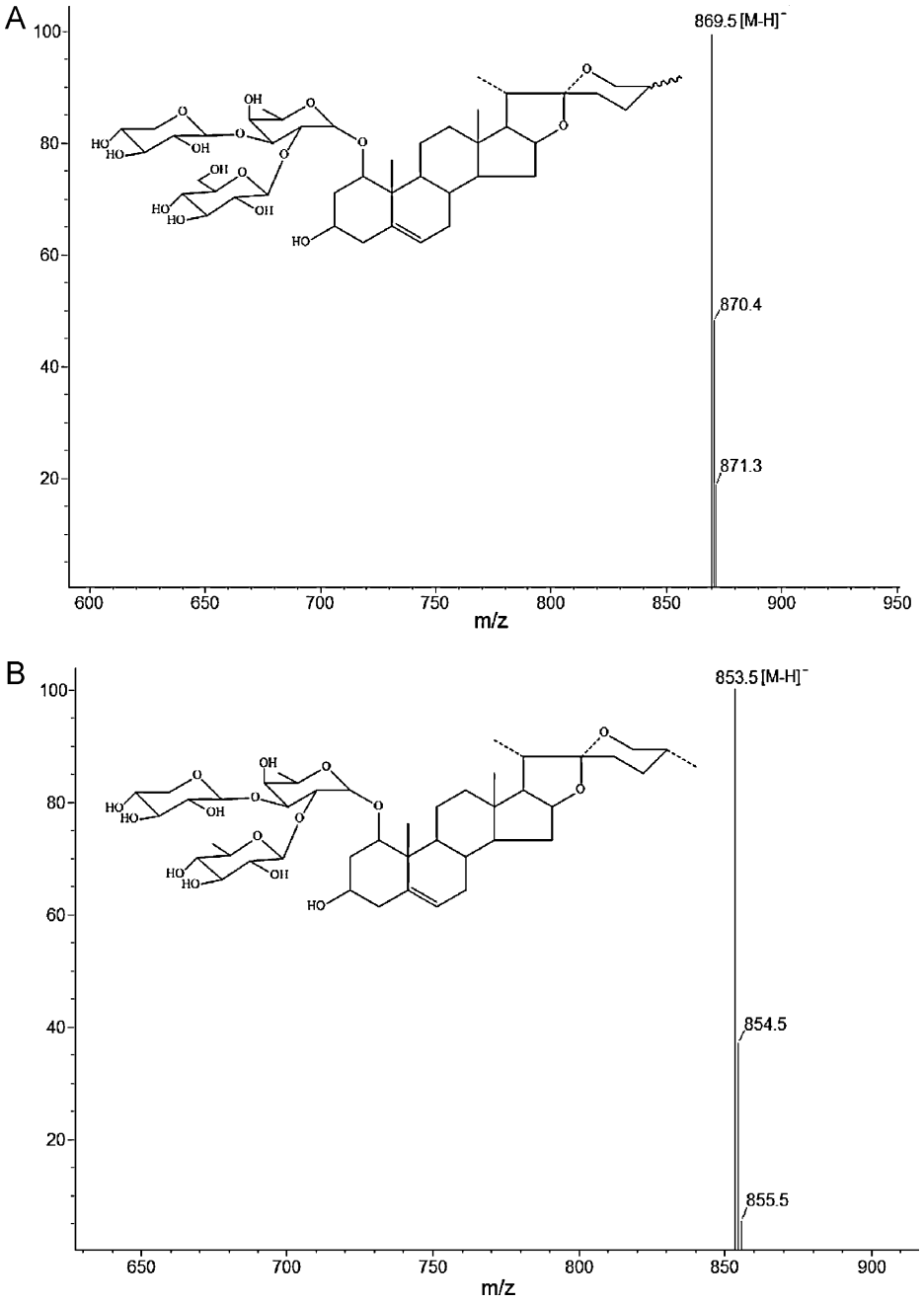

Fig. 1. Negative ion electrospray mass scan spectra of DT-13 (A, $C_{44}H_{70}O_{17}$, MW = 870) and ophiopogonin D (B, $C_{44}H_{70}O_{16}$, MW = 854).

(250 mm × 4.6 mm, i.d., 5 μm) (Welch Materials Inc., USA) at room temperature. The mobile phase consisted of acetonitrile–5 mmol/L aqueous ammonium acetate (60:40, v:v) with a flow rate of 1.0 mL/min in a run time of 10 min. The injection volume was 20 μL. The HPLC system was coupled in line to an Agilent 6110 Quadrupole LC/MS, which was operated by an ESI source in the negative mode. Drying gas temperature was set at 350 °C with a flow rate of 12.0 L/min and nebulising pressure was of 35 psi. Capillary voltage was at 3000 V and fragmentor voltage was set at 90 V. The assay was carried out by selected ion monitoring for DT-13 $[M-H]^-$ at *m/z* 869.50 and ophiopogonin D $[M-H]^-$ at *m/z* 853.50.

2.3. Sample preparation

A 100 μL aliquot of plasma and 10 μL of IS (10 μg/mL) were vortex-mixed for 30 s followed by protein precipitation with 0.4 mL of acetonitrile. The mixture was vortex-mixed for 1 min, and then the tubes were centrifuged for 5 min at 2000 × *g*. Finally, the supernatant was transferred to a clean glass tube and dried with nitrogen at 37 °C. The residue was re-dissolved in 100 μL of methanol, and 20 μL was injected into the LC–MS system for analysis after centrifugation for 10 min at 9000 × *g*.

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

Stock solution of DT-13 was prepared in methanol at concentration of 1000 μg/mL. Working solutions of analyte between 0.10 and 10 μg/mL were prepared by diluting the stock solution with methanol. A 10 μg/mL working solution of IS was similarly prepared in methanol. All solutions were stored at 4 °C and brought to room temperature before use.

Calibration samples, which covered the concentration range at 10, 20, 50, 100, 200, 500 and 1000 ng/mL, were prepared by freshly spiking working solutions into blank plasma. QC samples were prepared at 20, 100, and 500 ng/mL, and then treated as indicated below.

2.5. Method validation

The specificity of the method was assessed by preparing and analyzing six different batches of drug-free rat plasma. Each blank sample was tested for endogenous interference using the proposed extraction procedure and LC–MS conditions. The chromatogram of a blank plasma sample was compared with those obtained with a solution at the concentration of lower limit of quantification (LLOQ). The signal intensity at this concentration was at least 10 times higher response than that of blank plasma samples.

The calibration curves for DT-13 in plasma were generated by plotting the peak area ratio (y) of DT-13 to IS versus nominal concentrations (x) of DT-13 in plasma with $1/x^2$ weighted regression. Evaluation of the assay was performed with a seven-point calibration plot in the concentration range 10–1000 ng/mL.

The QC samples (20, 100 and 500 ng/mL) were analyzed to validate the accuracy and precision. Six replicates were analyzed in each of three analytical runs. 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.

Recoveries of DT-13 and IS were determined by comparing peak areas of the analyte and IS in extracted QC samples with those in post-extracted blank samples spiked with the corresponding concentrations of the two compounds. The extraction recovery and matrix effect at three QC concentrations were assayed in sets of six replicates. Extraction recovery was calculated by comparing the peak area of analytes added to plasma from untreated plasma and then extracted, with analytes added into preextracted plasma. The matrix effect was evaluated by comparing the peak area of analytes added into preextracted plasma from untreated rats, with analytes dissolved in matrix component-free reconstitution solvent.

The stability of DT-13 in rat plasma was evaluated by analyzing replicates ($n = 3$) of QC samples at three levels, which were exposed to different conditions of time and temperature. The short-term stability was determined after the exposure of the spiked samples at 25 °C for 12 h, and the ready-to-inject samples in the autosampler rack (15 °C) for 12 h. The long-term stability was assessed after storage of the samples at −20 °C for 30 days. The freeze/thaw stability was evaluated after three complete freeze/thaw cycles (−20 to 25 °C) on consecutive days. Concentrations following storage were compared to freshly prepared samples of the same concentrations.

2.6. Pharmacokinetic study

The method was applied for the pharmacokinetic studies of DT-13 by two different administrations: intravenous (1.0 mg/kg) and intragastric (25, 50 and 100 mg/kg) administration. Before drug administration, rats were fasted overnight and allowed free access to water. The suspension for oral administration was prepared by dispersing DT-13 in 0.5% sodium carboxymethyl cellulose (CMC-Na) solution. The solution for tail intravenous administration was obtained by dissolving DT-13 in isotonic saline with 5% HP-β-CD. Venous blood samples (0.3 mL) were withdrawn to heparinized tubes at 0.033, 0.083, 0.167, 0.5, 1, 1.5, 2, 3, 4, 6 and 8 h after intravenous administration and at 1, 2, 4, 6, 8, 10, 12, 18, 24, 36 and 48 h after intragastric administration, then immediately centrifuged at 2000 × g for 5 min. The plasma samples were stored at −20 °C.

3. Results and discussion

3.1. LC–MS optimization

In order to develop a sensitive and specific LC–MS method for quantification of DT-13 in rat plasma, some potentially factors were investigated and optimized. Both positive and negative ionization modes were tested for the analysis, the negative ionization mode was found to be more appropriate for the detection. The mass spectra of DT-13 and IS are shown in Fig. 1. Both the analyte and IS generated deprotonated molecular ions $[M-H]^-$ in the full scan spectra after direct injection, with $m/z = 869.50$ and 853.50, respectively.

During the optimization of chromatographic conditions, acetonitrile was found superior to methanol as the mobile phase in order to decrease column pressure and lower background noise. Furthermore, modifiers such as formic acid, acetic acid, and ammonium acetate alone or in combination in different concentrations were compared. The responds of analytes were greatly improved by adding ammonium acetate in mobile phase and the best peak shape and ionization were achieved using 5 mM ammonium acetate in water phase.

3.2. Method validation

3.2.1. Specificity, sensitivity and linearity

Typical chromatograms of blank plasma, blank plasma spiked with DT-13 and IS, and the rat plasma samples were presented in Fig. 2. There were no significant endogenous interferences observed at the retention time of the analyte (6.5 min) and IS (8.2 min).

The linear regression of the peak area ratios against concentration was fitted over the concentration range 10–1000 ng/mL in rat plasma. Calibration curves for the plasma assay were prepared by plotting the peak-area ratios (y) of drug to internal standard against the drug concentration (x). For DT-13 in rat plasma, a regression equation of $y = 0.002x - 0.036$ and correlation coefficient (r^2) of 0.9980 were obtained. The LLOQ of DT-13 was 10 ng/mL.

3.2.2. Precision and accuracy

The intra- and inter-day precision and accuracy results are shown in Table 1. The intra- and inter-day precision values (R.S.D.%) were both less than 12.3%, while the assay accuracies ranged from 93.4% to 106.3%. The method was proved to be highly accurate and precise.

3.2.3. Recovery and matrix effect

The extraction recoveries of DT-13 were 91.4 ± 7.3% for 20.0 ng/mL, 90.7 ± 4.9% for 100.0 ng/mL, 90.8 ± 3.5% for 500.0 ng/mL, and 84.8 ± 3.2% for the IS. The mean recovery for DT-13 was 91.0 ± 4.7%. The average matrix effect values were 89.7 ± 6.7%, 95.5 ± 7.2%, and 92.3 ± 5.0% for DT-13 at the three QC concentration levels and 91.6 ± 5.8% for the IS. These results indicated that no endogenous substances significantly influenced the ionization of DT-13 and IS.

3.2.4. Stability

A serial of stability experiments were performed and the results are summarized in Table 2. The analyte in rat plasma samples was stable for 12 h at ambient temperature, after three freeze–thaw cycles, and at −20 °C for 1 month. Treated samples were found to be stable at 15 °C in the autosampler for at least 12 h.

3.3. Pharmacokinetic application

The presented method was successfully applied to determine DT-13 in the plasma of six rats following the administration. All

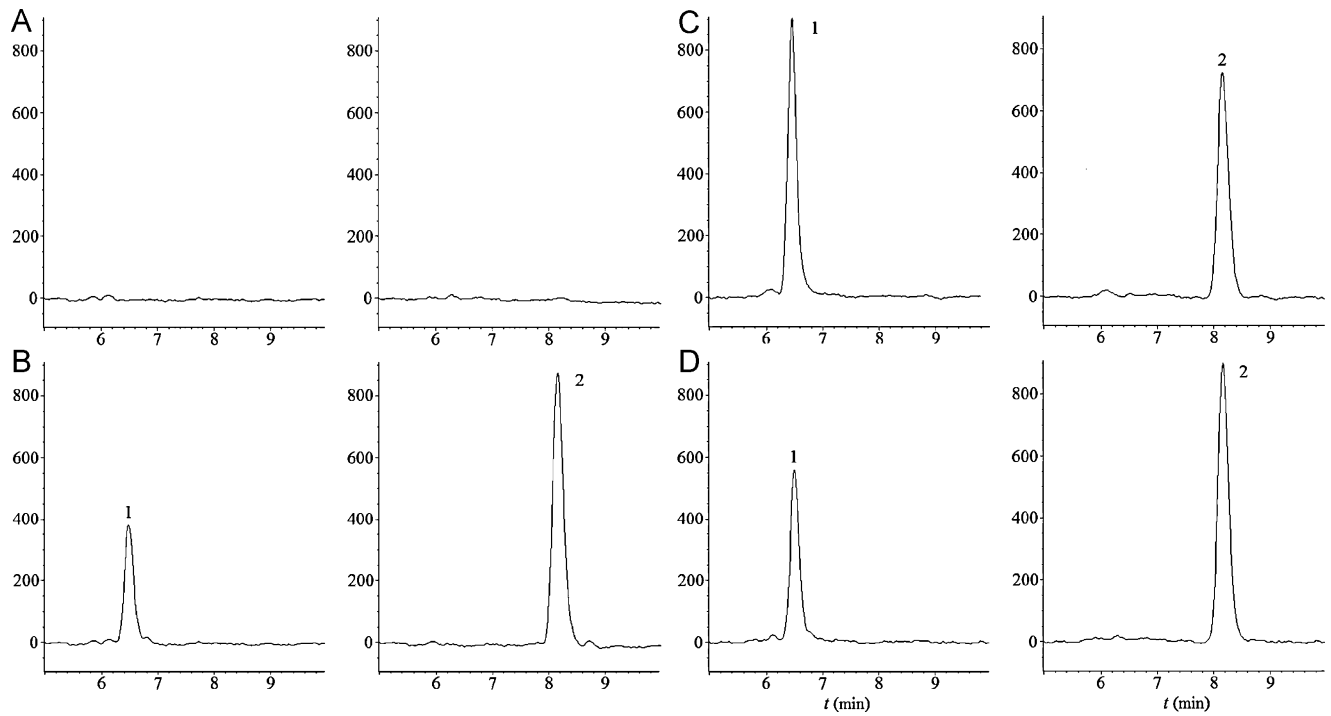

Fig. 2. SIM chromatograms of blank rat plasma (A); blank rat plasma spiked with DT-13 (1) and IS (2) (B); a rat plasma sample (0.5 h, i.v.) (C); a rat plasma sample (12 h, i.g.) (D).

Table 1
Precision and accuracy for the determination of DT-13 in rat plasma ($n = 6$).

QC sample	Intra-day			Inter-day		
	Measured (ng/mL)	Precision (%)	Accuary (%)	Measured (ng/mL)	Precision (%)	Accuary (%)
20	20.1 ± 2.0	9.7	100.6	18.7 ± 2.3	12.3	93.4
100	98.8 ± 4.4	4.5	98.8	96.3 ± 5.1	5.3	96.3
500	531.7 ± 19.5	3.7	106.3	493.3 ± 34.3	7.0	98.7

Table 2
Stability tests of DT-13 in rat plasma ($n = 3$).

Storage conditions	Concentration (ng/mL)		R.S.D. (%)	RE (%)
	Spiked	Measured		
12 h at room temperature	20	19.04	12.9	−5.0
	100	95.09	5.4	−4.9
	500	484.9	6.7	−3.2
1 month at −20 °C	20	22.06	9.5	9.3
	100	105.38	2.1	5.1
	500	486.67	1.6	−2.7
Three freeze/thaw cycles	20	21.99	4.5	9.1
	100	96.29	2.8	−3.9
	500	509.18	4.4	1.8
Autosampler (15 °C for 12 h)	20	19.80	13.4	−2.1
	100	105.45	4.3	5.2
	500	475.56	4.0	−5.1

Table 3
The main pharmacokinetic parameters of DT-13 after intravenous and intragastric administration to rats ($n = 6$).

Parameters	i.v. (mg/kg)		i.g. (mg/kg)	
	1	25	50	100
C_{max} (ng/mL)	684.52 ± 218.81	108.97 ± 36.25	193.61 ± 43.79	417.93 ± 101.79
T_{max} (h)	0.142 ± 0.183	7 ± 2.757	6.33 ± 4.46	7 ± 3.521
$t_{1/2}$ (h)	2.83 ± 1.93	18.92 ± 4.64	17.36 ± 6.96	16.06 ± 4.09
$AUC_{(0-t)}$ (ng h/mL)	1231.71 ± 503.34	1549.82 ± 400.50	3295.49 ± 607.43	7011.22 ± 1554.82
$AUC_{(0-\infty)}$ (ng h/mL)	1395.06 ± 646.30	1882.19 ± 472.35	3817.58 ± 690.52	7901.52 ± 1560.28
$MRT_{(0-\infty)}$ (h)	3.20 ± 1.78	27.13 ± 4.91	24.65 ± 5.24	22.46 ± 4.86
F (%)	–	5.40 ± 1.35	5.47 ± 0.99	5.66 ± 1.11

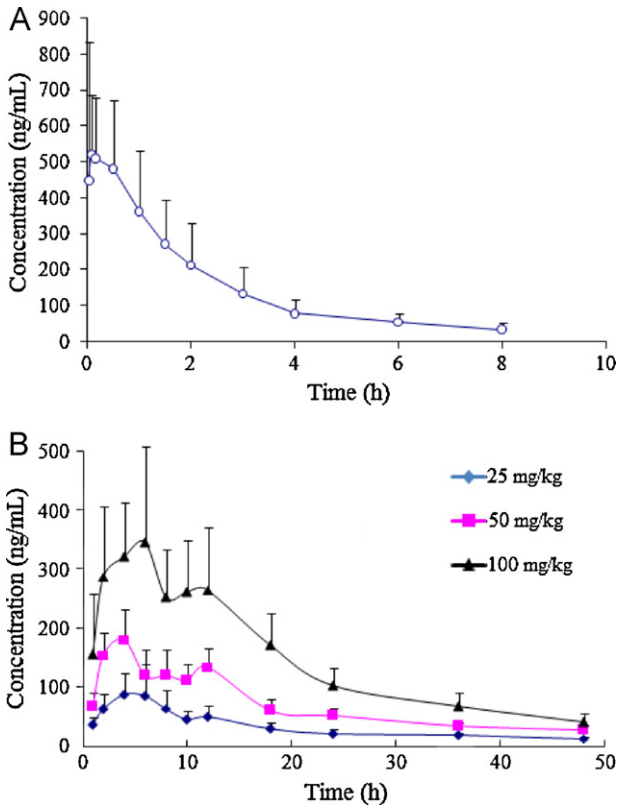

Fig. 3. Mean plasma concentration–time profile of DT-13 in rats (mean ± SD, $n = 6$). (A) After an intravenous administration dose of DT-13 (1 mg/kg) and (B) after an intragastric administration dose of DT-13 (25, 50 and 100 mg/kg).

the data was calculated by DAS 2.0 statistical software (Pharmacology Institute of China). The concentration versus time profile is shown in Fig. 3. The major pharmacokinetic parameters of DT-13 (Table 3) were calculated using non-compartmental analysis.

After an intravenous dose, the T_{max} and $t_{1/2}$ of DT-13 was about 0.14 and 2.83 h, respectively, which is much longer than that of some analogs [15]. Following oral administration, plasma levels of DT-13 reached maximum at about 7, 6.33 and 7 h with half-life of 18.92, 17.36 and 16.06 h for the dose of 25, 50 and 100 mg/kg, respectively. These results indicated that the drug was absorbed and eliminated slowly in rats. After oral dosing there is an excellent linear relationship between *AUC* and dose and also between C_{max} and dose, with correlation coefficients (r^2) of 0.999 and 0.995, respectively, indicating a dose proportionality of DT-13 after oral administration. It is obvious that the drug plasma level was low, although a high oral dose was given. By comparing the $AUC_{(0-\infty)}$ after oral and intravenous administration, the average oral bioavailability of DT-13 was estimated to be about 5.51%, however, contrasting with the lack of oral absorption for steroidal saponins it showed a relatively high value [16].

4. Conclusion

A simple and sensitive LC–MS method was developed and validated for the determination of DT-13 in rat plasma, this method has been successfully applied to pharmacokinetic study of DT-13 following intravenous and oral administration to rats. This method and pharmacokinetic study may be helpful in preclinical studies to establish appropriate dose and frequency for DT-13.

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