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Study of the determination and pharmacokinetics of bufadienolides in dog's plasma after administration of Liu-Shen-Wan by high performance liquid chromatography time-of-flight mass spectrometry

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Abstract

A sensitive and reliable high performance liquid chromatography-tandem time-of-flight mass spectrometry method (HPLC/TOF MS) has been developed to determine three active bufadienolides from Liu-Shen-Wan (LSW) in dog's plasma. Enhanced selectivity and sensitivity in comparison with traditional HPLC/DAD method could be obtained through this method. Bufodienolides could be well separated and distinguished from its nominally isobaric endogenous components by HPLC/TOF MS, with the linear calibration range covering from 0.5 ng/mL to 100 ng/mL and Limit of Detection (LOD) being about 0.15 ng/mL. This method was also proved to be quite stable, with the intra-day precision and the inter-day precision results being lower than 6.39% and 7.44%, respectively. Meanwhile HPLC/TOF MS was successfully used in the pharmacokinetic study of LSW. For resibufogenin, the major pharmacokinetic parameters AUC_{0-t}, C_{max} and $t_{1/2\alpha}$ were 160.72 ± 21.97 ng/mL min, 2.35 ± 0.71 ng/mL and 20.74 ± 5.89 min, respectively, and for bufalin the corresponding parameters were 55.55 ± 7.55 ng/mL min, 0.91 ± 0.15 ng/mL and 25.45 ± 13.28 min, respectively.

Keywords: Liu-Shen-Wan; Pharmacokinetics; Bufadienolides; HPLC/TOF MS

1. Introduction

Liu-Shen-Wan (LSW), a well-known heatclearing and detoxicating formula, is mainly composed of animal and mineral derived medicines including Musk (*Moschus berezovskii* Flerov), bezoar (*Bos taurus domesticus* Gmelin), toad venom (*Bufo bufo gargarizans* Cantor), borneol (*Dryobalanops aromatica* Gaertner. f.), pearl (*Pteria martensii* Dunker) and realgar (*Realgar*). This ancient prescription was confirmed to be effective in treating infectious inflammatory diseases such as diphtheria, scarlet fever, acute tonsillitis, purulent parotitis, encephalitis B, viral pneumonia and throat pain for more than 200 years [1–3].

Bufadienolides (Fig. 1) are the major effective constituents in Toad venom [4,5]. Bufadienolide is a type of steroid with a characteristic α-pyrone ring at C-17, and show significant cardiotonic, blood-pressure-stimulating, anesthetic, and antitumor activities [6,7].

The detection and accurate measurement of bufadienolides in body fluids are in especially urgent need for therapeutic drug monitoring of LSW. Recent literature revealed that several methods have been used to determine bufadienolides in toad venom [8–10], in traditional Chinese medicines formula [11–13] and in human liver [14]. In all of these reports, reversed-phase HPLC/UV was used as the quantitative determination technique. However, HPLC/UV is not sufficiently sensitive to determine bufadienolides in biological matrix for pharmacokinetics study. Recently, high performance liquid chromatography coupled with atmospheric pressure chemical ionization mass spectrometry tandem mass spectrometry technique was applied to the study of Chansu (toad venom), but it was just used as a identification method [9,10].

To our knowledge, no method has been reported for the simultaneous quantitative determination of these active constituents by using HPLC/MS for pharmacokinetic investigation. Being a useful tool for analysing compounds in complex matrices,

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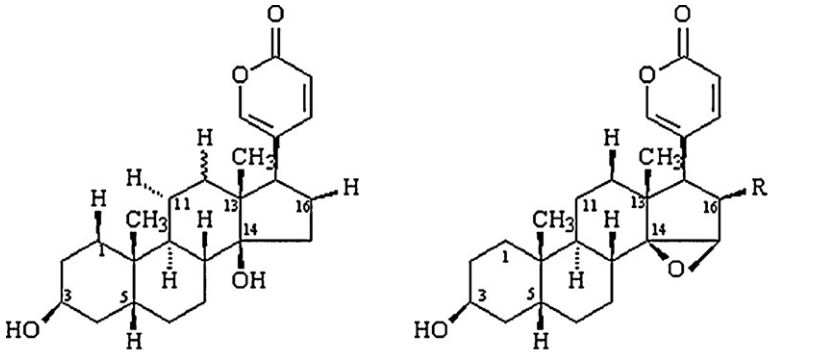

Fig. 1. Structures of three bufadienolides.

high performance liquid chromatography coupling with time-of-flight mass spectrometry (HPLC/TOF MS) has advantages over HPLC/UV, for it has high resoluteion and narrow extracted-ion windows (±0.02 *m/z*), which can then lead to reduced background, lower detection limit, and higher confidence. Further more, TOF MS has highly linear, wide dynamic range, and lock-mass correction. By extracting Nominal Mass Chromatograms from the complex biological matrix, bufodienolides could be detected and distinguished from the nominally isobaric endogenous components. All these advantages make it suitable for quantitative analysis of dog plasma after administration of LSW.

In this paper, a HPLC/TOF MS method in combination with solid phase extraction (SPE), for simultaneous quantitative determination of the three major bufadienolides in dog's plasma after administration of LSW is described.

2. Experimental

2.1. Chemicals and reagents

LSW (3.125 mg/pill, Lot No. 20050110) was supplied by Shanghai Lei Yun Shang Pharmaceutical Company (Shanghai, China). Reference standard Bufalin and resibufogenin were purchased from DELTA Information Centre For Natural Organic Compounds (Xi-an, China, 99% purity), and cinobufagin was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Lot.803-9202 Beijing, China, 99% purity). 5α-dihydrotestosterone, which was used as the internal standard (I.S.), were obtained from Sigma Co. (St. Louis, MO 63178 USA, 98% purity). Solid-phase extraction columns (Oasis® HLB, 1 cc/30 mg 30 μm) were obtained from Waters (Milford, MA, USA). Acetonitrile and methanol were of HPLC grade and purchased from Merck (Darmstadt, Germany) and formic acid of HPLC grade was obtained from Fluka (Buchs, Switzerland). Ultra-pure water was prepared from a Milli-Q system (Millipore, Milford, MA). All other reagents and solvents were of analytical grade and obtained from Beijing Chemical Company (Beijing, China).

2.2. HPLC condition

HPLC/DAD analysis was carried out on an Agilent 1100 series HPLC system (Agilent Series 1100, Palo Alto, CA, USA) comprising a binary pump, thermostated column compartment, a 1200 series injector with temperature controller, and a diode array detector. Separation was performed using a reversed-phase column (250 mm × 4.6 mm i.d., 5.0 μm, Alltima C18) with an Alltech RP18guard column (3.9 mm × 20 mm) at 30 °C. For qualitative investigations a binary gradient mobile phase was used, comprising 0.2% formic acid (solvent A) and acetonitrile (solvent B). Initially the proportions were 70% A and 30% B and then altered in a linear gradient to 56% A and 44% B over the period of 0–8 min at flow rate of 0.9 mL/min, followed by a linear gradient to 30% A and 70% B at the same flow rate in the subsequent 17 min. This gradient elution allowed the efficient separation both of the analytes and interfering components present in the plasma. After the 25 min analysis process, the column was washed with 95% acetonitrile for 5 min and then equilibrated with starting mobile phase (70% A and 30% B) for 10 min prior to the next run. One-third of the eluent was introduced into the TOF-MS system with a split valve. An injection volume of 20 μL was used throughout, and the injector was maintained at 4 °C during the analysis.

2.3. TOF-MS conditions

The HPLC system was coupled to an Agilent 1100 HPLC/MSD TOF (Agilent Corp, Waldbronn, Germany) equipped with an electrospray interface. The electrospray source includes dual nebulizers, one nebulizer for the HPLC eluent and the other for the internal reference solution. The reference nebulizer, along with the HPLC/MSD TOF's automated celebrant delivery system (CDS), provides continuous introduction of reference mass standards into the ion source for automated mass calibration. Accurate mass measurements were obtained with this CDS and thus enhanced accuracy was achieved.

The HPLC conditions for the HPLC/TOF-MS analysis were the same as those used in the HPLC analysis. TOF-MS analysis was performed in positive (ESI+) ion mode under the following operation parameters: capillary voltage 4.0 kV; nitrogen drying gas 9 L/min; nebulizer 40 psi; gas temp. 350 °C; fragmentor voltage 215 V (ESI+); skimmer voltage 60 V; octopole dc1 30 V (ESI+); octopole RF 250 V. Reference masses: 149.0233 and 922.0098 *m/z*. Data files were acquired in continuum (profile) mode, and spectras were stored from *m/z* 50–1000.

Analyst QS software (Applied Biosystems, Framingham, MA) was used to process the accurate mass data. Exact masses corresponding to particular elemental compositions were also calculated by the formula calculator in this software. Daily instrument tuning was carried out using the tuning solution (G1969-85000, Agilent Corp, USA) to ensure no more than 2 ppm mass error prior to run samples.

Quantitative and qualitative procedures: Nominal mass chromatograms (NMC, ±0.25 Da) were extracted from total-ion chromatograms (TIC). The concentrations of bufadienolides in plasma samples were then calculated from the equation of standard curve.

When accurate mass measurements were used to distinguish bufadienolides from the endogenetic components with similar *m/z* value, the NMC of the analyte was obtained firstly, then by scanning the top of the chromatographic peak an extracted accurate mass chromatogram (AMC) was obtained. AMC corresponding to particular elemental compositions was calculated by the formula calculator. Formula calculator factors were set as follows: C = 50, H = 50, O = 10; electronic state even, number of changes +1 and tolerance 3 ppm.

2.4. Standard solutions and calibration standard

Reference standard resibufogenin, bufalin, and cinobufagin were accurately weighed and dissolved in methanol to make stock solutions of 100 μg/mL, respectively. In a clean, dry 10 ml volumetric flask, internal standard 5α-dihydrotestosterone 10 mg was accurately weighed and dissolved in methanol to make a stock solution. This solution was further diluted with methanol to prepare internal standard working solution containing 120 ng/mL 5α-dihydrotestosterone. All the working solutions were stored at 4 °C.

A series of plasma samples were spiked with bufadienolides at concentrations ranging from 0.5 to 100 ng/mL. Standard calibration plots for resibufogenin, bufalin, cinobufagin were constructed by plotting analyte/I.S. peak-area ratios against nominal concentrations of these bufadienolides.

2.5. Drug administration and sample collection

The pharmacokinetic study was based on a single-dose design. In the morning after an overnight fast (10 h), six dogs (weighing from 10 to 11 kg, three male and three female) were given a single dose of LSW (48.6 mg/kg) by intragastric gavage, respectively. No food was given within 2 h after oral administration. Blood samples (approx. 2 mL) were collected from the foreleg vein at 0 min, 5 min, 10 min, 20 min, 30 min, 40 min, 50 min, 1 h, 2 h, 3 h, 4 h and 6 h after dose administration. Plasma was separated by centrifugation at 3000 × *g* for 10 min.

2.6. Sample preparation

The plasmas (800 μL) were prepared immediately after collection. Internal standard working solution (40 μL, 300 ng/mL) was added to 0.8 mL dog's plasma. Methanol (3 mL) was added and the mixture was vortex-mixed for 2 min followed by centrifugation at 3000 × *g* for 10 min. The supernatant was transferred to a polypropylene tube. 3 mL methanol was then added to the precipitate, then the mixture was vortex-mixed and centrifuged as described above. The supernatants were pooled and dried under a nitrogen current at 40 °C. The residue was dissolved in 100 μL methanol and diluted with 900 μL ultra-pure water. The mixture was vortex-mixed for 2 min and centrifuged at 3000 × *g* for 10 min. The supernatant was applied to Oasis® HLB cartridge, which had been conditioned with 1 mL methanol followed by 1 mL ultra-pure water. After washing the cartridge with 1 mL 50% methanol and 1 mL 2% ammonia in 50% methanol successively, the bufadienolides were eluted using 1 mL 1% acetic acid in methanol solution. The eluate was dried at 40 °C under a stream of nitrogen. The residue was dissolved in 100 μL mobile phase (water with 0.2% formic acid:acetonitrile 2:3), and stored in polypropylene tubes at 4 °C until the analysis. The samples were centrifuged at 5000 × *g* for 10 min before injection.

3. Results and discussion

3.1. Identification and specification

Representative NMC for blank plasma are shown in Fig. 2, in which panel-a (*m/z* 385.24), panel-b (*m/z* 387.25) and panel-c (*m/z* 443.24) represent NMC of endogenous substances in plasma, and panel-e (*m/z* 291.25) shows NMC for IS spiked. It was necessary to examine the endogenous components which fit bufadienolides nominal mass measurement. All these endogenous components were scanned to give AMC, and examined by the formula calculator as described above, through which the endogenous components were further distinguished from bufadienolides.

Fig. 3 shows representative NMC for plasma sample spiked with bufadienolides, and representative dosed plasma sample is shown in Fig. 4. The retention time for resibufogenin (*m/z*: 385.24 Da), bufalin (*m/z*: 387.25 Da) and cinobufagin (*m/z*: 443.24 Da) were 20.7 min, 17.2 min and 19.6 min, respectively.

3.2. Linearity

The plasma calibration curve was constructed from eight calibrators (0.5–100 ng/mL). The linear equations were $Y = 0.0954X + 0.4579$, $Y = 0.0767X + 0.2157$, and $Y = 0.1666X + 1.0937$ for resibufogenin, bufalin, and cinobufagin, respectively. The correlation coefficients (r) were 0.9983, 0.9987, and 0.9988, respectively.

3.3. Sensitivity

Limit of Detection (LOD) using HPLC/TOF-MS were 0.10 ng/mL, 0.15 ng/mL, 0.10 ng/mL (S/N = 3) for resibufogenin, bufalin, and cinobufagin, respectively, and the corresponding low Limit of Quantification were 0.30 ng/mL, 0.45 ng/mL, and 0.30 ng/mL (S/N = 10), respectively.

The UV LOD (296 nm, maximum absorption wavelength) for bufadienolides were about 10 ng/mL. Most of the concentrations

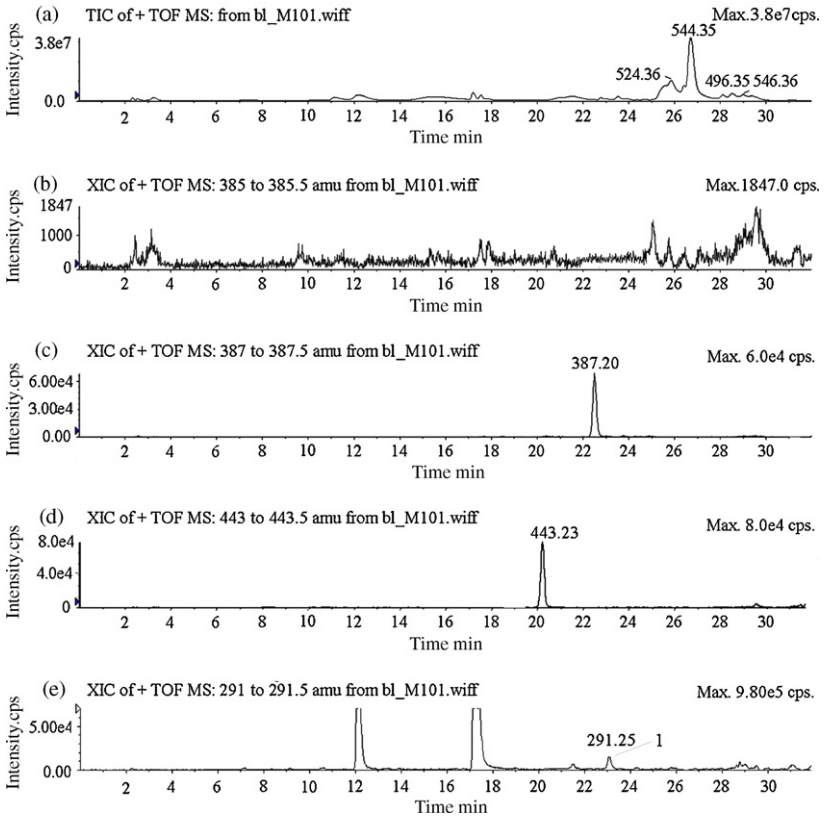

Fig. 2. The positive total ion chromatography (TIC) (a) and nominal mass chromatograms (b–e) of blank plasma at 385 Da, 387 Da, 443 Da and 291 Da, respectively. Peak 1 is IS. spiked.

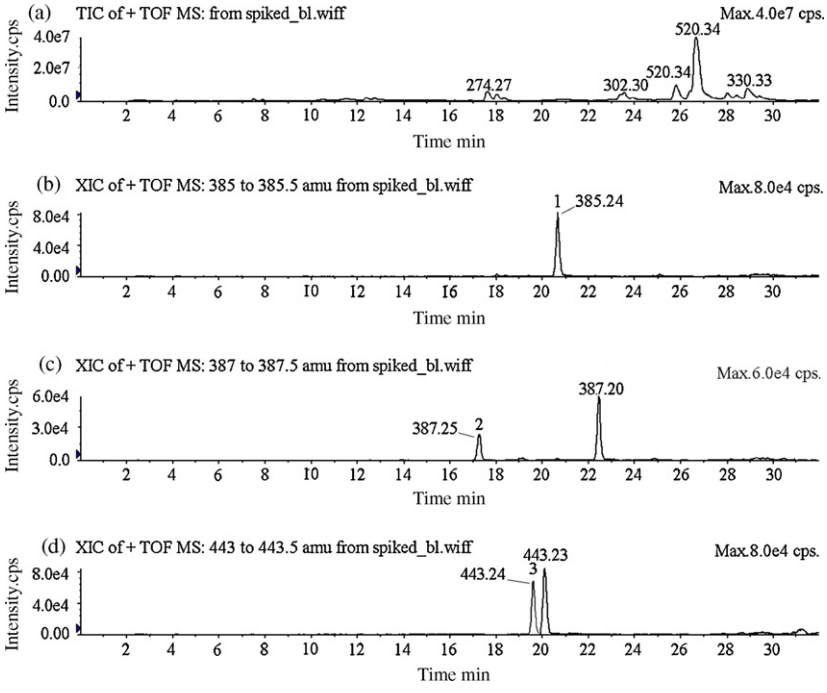

Fig. 3. The positive total ion chromatography (TIC) (a) and nominal mass chromatograms (b–d) of plasma sample spiked with bufadienolides. Peak 1 is resibufogenin (*m/z*: 385 Da), peak 2 is bufalin (*m/z*: 387 Da) and peak 3 is cinobufagin (*m/z*: 443 Da).

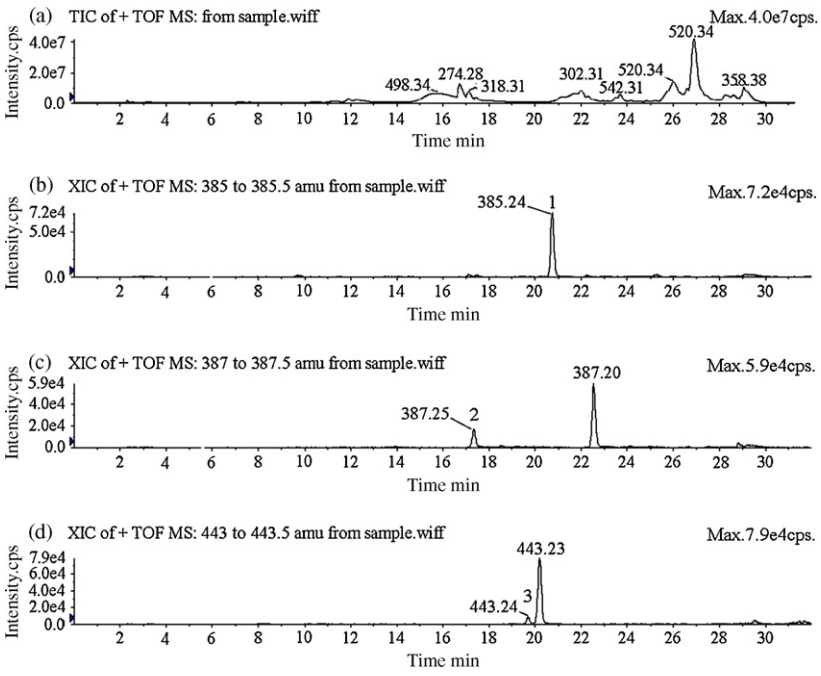

Fig. 4. The positive total ion chromatography (TIC) (a) and nominal mass chromatograms (b–d) of representative dosed plasma. Peak 1 is resibufogenin (*m/z*: 385 Da), peak 2 is bufalin (*m/z*: 387 Da) and peak 3 is cinobufagin (*m/z*: 443 Da).

of bufadienolides in dog's plasmas were lower than 10 ng/mL, so DAD detector was employed as a complementary method to verify retention times and resolution for bufadienolides from the HPLC column.

3.4. Precision and accuracy

The precision and accuracy of the method were evaluated using spiked plasmas at concentrations of 1.0, 15, and 90 ng/mL, respectively, and the spiked plasmas were prepared as sample preparation section. For measurement of intra-day precision and accuracy, six replicate samples at each concentration were assayed on the same day. Inter-day assay precision and accuracy were determined by analyzing the samples at each concentration on three successive days. During the period of time for precision and accuracy study, the HPLC–MS system ran continuously for 3 days. As listed in Table 1, the intra-day precision RSD was lower than 6.39%, with the accuracy ranging from 88.17

Table 1
Intra-day and inter-day precision and accuracy of bufadienolides spiked in dog's plasma

Determined concentration (ng/mL)	Bufadienolides	Detected concentration (ng/mL)	Precision RSD (%)	Accuracy (%)
Intra-day precision and accuracy ($n = 6$ replicate samples)				
1.0	Resibufogenin	0.98 ± 0.05	5.16	97.77
	Bufalin	1.00 ± 0.06	6.39	100.4
	Cinobufagin	0.99 ± 0.05	4.95	99.25
15	Resibufogenin	14.73 ± 0.84	5.71	98.22
	Bufalin	13.22 ± 0.57	4.30	88.17
	Cinobufagin	14.09 ± 0.70	4.98	93.90
90	Resibufogenin	90.54 ± 3.21	3.55	100.6
	Bufalin	89.36 ± 3.76	4.21	99.29
	Cinobufagin	84.37 ± 4.02	4.76	93.74
Inter-day precision and accuracy ($n = 3$ days of replicate samples)				
1.0	Resibufogenin	0.86 ± 0.05	6.20	85.80
	Bufalin	0.93 ± 0.06	6.63	93.08
	Cinobufagin	0.99 ± 0.06	6.34	98.69
15	Resibufogenin	13.86 ± 0.84	6.07	92.39
	Bufalin	14.07 ± 0.67	4.77	93.81
	Cinobufagin	14.26 ± 1.06	7.44	95.05
90	Resibufogenin	86.69 ± 2.73	3.15	96.32
	Bufalin	86.22 ± 2.88	3.34	95.80
	Cinobufagin	86.81 ± 3.75	4.32	96.45

Table 2
The recovery of determination results ($n = 3$)

Bufadienolides	Concentration added (ng/mL)	Mean concentration detected(ng/mL)	Mean recovery (%)	RSD (%)
Resibufogenin	1	0.87	86.97	6.81
Bufalin		0.84	84.02	3.29
Cinobufagin		0.94	93.55	5.47
Resibufogenin	15	13.24	88.25	4.32
Bufalin		12.82	85.46	3.98
Cinobufagin		12.79	85.28	4.31
Resibufogenin	90	80.60	89.55	3.76
Bufalin		79.31	88.12	4.45
Cinobufagin		79.06	87.84	6.08

to 100.6%, and the inter-day precision was less than 7.44%, with the accuracy ranging from 85.80 to 98.69%. These results indicate that the method is reliable, reproducible and accurate.

3.5. Recovery (extraction efficacy)

Spiked plasma samples were prepared in triplicate at concentrations of 1.0, 15, and 90 ng/mL, and assayed as described above. Recovery (extraction efficacy) was calculated by comparing the peak area obtained from the extracted sample with that from unextracted standard solution containing the same concentration of bufadienolides. For plasma concentrations of 1.0, 15 and 90 ng/mL the mean recovery of bufadienolides was more than 84.02%, 85.28, and 88.12% ($n = 3$), respectively (as seen in Table 2).

3.6. Stability of analytes

The stability of bufadienolides plasma sample was assessed by spiking blank dog's plasma with bufadienolides standard solution at three different concentrations (1.0, 15 and 90 ng/mL). The plasma samples were extracted by following the sample-preparation procedure, and analyzed as described above. The bufadienolides were found to be stable over at least 24 h, during that period of time the concentrations of the three bufadienolides ($n = 3$) were found to be within the range 97.34–99.75% of their respective initial concentrations.

3.7. Pharmacokinetic study

This HPLC/TOF MS method yielded satisfactory results for the determination of bufadienolides in dog's plasma samples, and could be successfully used to the pharmacokinetic study of bufadienolides after oral administration of LSW. The mean plasma concentration–time profiles for bufadienolides are shown in Fig. 5. Pharmacokinetic parameters were estimated by DAS software package (version 1.0, TongJi Medical University) and the results are given in Table 3.

Pharmacokinetic parameters obtained here for bufadienolides are accorded with previously published data. As early as in 1904, Kravkov [15] observed a deceleration of the heart rate and an enhancement of cardiac contractions in dogs injected with the toad venom, by which the author speculated that the toad venom may have a "digitalis-like" effect. However, a prominent cardiologist Chen [16], when study on the cardiotonic activity of the toad venom, failed to prove its practical application, as its effect on the heart was brief, though pronounced. This report was consistent with our result that $t_{1/2\alpha}$ and $t_{1/2\beta}$ of bufadienolides were 10–40 min, and the C_{max} appeared at around 30 min in the pharmacokinetic curves. The pharmacokinetic parameters investigated also suggested that bufadienolides can be used as marker compounds to characterize some profiles of this TCM formula.

Unlike single-entity drugs whose absorption, distribution, metabolism and excretion can be tracked by using chromatographic/spectrometric methods, a viable and robust technology to measure global compounds from LSW in complex matrices

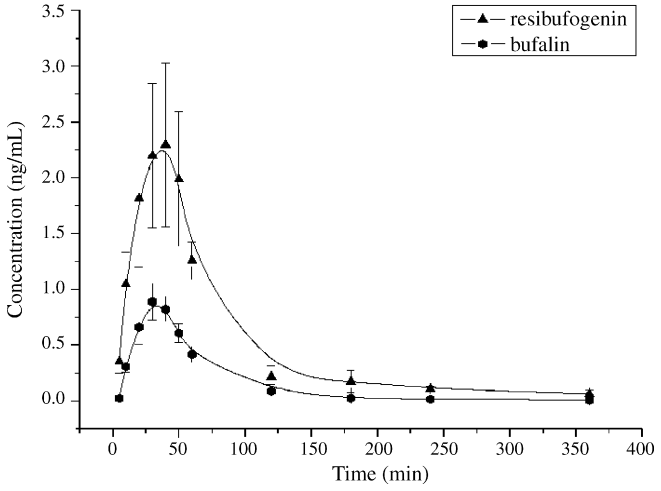

Fig. 5. Plasma concentration–time profiles for resibufogenin and bufalin in dogs after oral administration of Liu-Shen-Wan (48.6 mg/kg).

Table 3
Pharmacokinetic data (mean ± SD, $n = 6$) for resibufogenin, bufalin in dogs after oral administration of LSW (48.6 mg/kg)

Property	Resibufogenin	Bufalin
AUC_{0-t} (ng/mL min)	160.72 ± 21.97	55.55 ± 7.55
$AUC_{0-\infty}$ (ng/mL min)	163.33 ± 21.98	55.72 ± 7.47
C_{max} (ng/mL)	2.35 ± 0.71	0.91 ± 0.15
t_{max} (min)	38.33 ± 4.08	33.33 ± 5.16
$t_{1/2\alpha}$ (min)	20.74 ± 5.89	25.45 ± 13.28
$t_{1/2\beta}$ (min)	35.30 ± 12.65	32.11 ± 11.99

is not yet available. This HPLC/TOF MS method is an appropriate technological platform enabling the acquisition of the preliminary pharmacokinetic/dynamic (PK/PD) data of bufadienolides in plasma. And the further study about the PK-PD will be reported in a separate report.

Although cinobufagin (*m*/*z*: 443.24 Da), which was identified by standard solution, was observed in dog's plasma, its concentration in plasma was too low to be sufficiently determinated for pharmacokinetic study, since it was metabolized in plasma in less than 30 min.

4. Conclusion

A novel method has been established for quantitative determination of bufadienolides in dog's plasma after oral administration of LSW. By using the HPLC/TOF MS method, the LOD for bufadienolides were about 0.15 ng/mL and Limit of Quantification were about 0.45 ng/mL. The validated analytical method has been demonstrated to be suitable in pharmacokinetic study of dogs after administration of LSW.

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