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Simultaneous determination of five main active bufadienolides of Chan Su in rat plasma by liquid chromatography tandem mass spectrometry

Wen Xu [a], Heng Luo [b], Yaping Zhang [b], Lei Shan [a], Haiyun Li [a], Ming Yang [b], Runhui Liu [a], Weidong Zhang [a,c,*]

[a] *Department of Natural Medicinal Chemistry, School of Pharmacy, Second Military Medical University, No. 325 Guohe Rd., Shanghai 200433, PR China*
[b] *Key Laboratory of Modern Preparation of TCM, Ministry of Education, Jiangxi University of Traditional Chinese Medicine, Nanchang 330004, PR China*
[c] *School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200030, PR China*

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Abstract

To study the pharmacokinetics of Chan Su, a sensitive and selective method was developed and validated for the determination of five main bufadienolides (cinobufagin, resibufogenin, bufalin, bufotalin and arenobufagin) in rat plasma. The analytes were extracted by liquid–liquid extraction with ethyl acetate after internal standard (IS, caudatin) spiked. The separation was performed by a ZORBAX SB-C_{18} column (3.5 μm, 2.1 mm × 100 mm) and a C_{18} guard column (5 μm, 4.0 mm × 2.0 mm) with an isocratic mobile phase consisted of acetonitrile–water–formic acid (50:50:0.05, v/v/v) at a flow rate of 0.3 mL/min. The Agilent G6410A triple quadrupole LC/MS system was operated under the multiple reaction monitoring mode (MRM) using the electrospray ionization technique in positive mode. The nominal retention times for cinobufagin, resibufogenin, bufalin, bufotalin, arenobufagin and caudatin were 3.07, 3.55, 2.30, 1.62, 1.22 and 3.43 min, respectively. All analytes showed good linearity in a wide concentration range ($r > 0.995$) and their lower limits of quantification (LLOQ) were all 1.0 ng/mL. The method was linear for all analytes with correlation coefficients >0.995 for all analytes. The average extract recoveries of the five analytes from rat plasma were all over 85%, the precisions and accuracies determined were all within 15%. This method has been successfully applied to pharmacokinetic study of Chan Su in rats following oral administration.

Keywords: Liquid chromatography tandem mass spectrometry; Chan Su; Bufadienolides; Rat plasma

1. Introduction

Chan Su is the dried venom of toad (*Bufo bufo gargarizans* Cantor) and a widely used traditional Chinese medicine as a cardiotonic and local anesthetic agent in China and some other Asian countries. Bufadienolide type cardiotonic steroids are the major active components and marker compound of the drug [1], while the toxic effect of the drug may also be due to this kind of components [2,3]. To study the pharmacology activity, quality control and toxicity of Chan Su, we extracted more than 10 bufadienolides from this drug and our MTT assay results (unpublished data) showed that 5 main bufadienolides (cinobufagin, resibufogenin, bufalin, bufotalin and arenobufagin, Fig. 1) had more obvious anticancer activity than water-soluble partition of Chan Su which has already been prepared into injection for the clinical treatment of several kind of cancer [4]. Because of the dose-dependent and time-dependent anticancer activity of the five bufadienolides, it is necessary to study the pharmacokinetics of them. So a sensitive and accurate analytical method is required. Furthermore, it is also necessary to develop a convenient, accuracy and sensitive method for the clinical monitor of the drug due to its high toxicity.

For the pharmacokinetic study and clinical monitoring of Chan Su, several methods had already been developed. An LC-UV method for the determination of bufalin with a LLOQ of 0.1 μg/mL using 1 mL plasma [5] and an LC-UV method for the

* Corresponding author at: Department of Natural Medicinal Chemistry, School of Pharmacy, Second Military Medical University, No. 325 Guohe Rd., Shanghai 200433, China. Tel.: +86 21 25070386; fax: +86 21 25070386.
E-mail address: wdzhangy@hotmail.com (W. Zhang).

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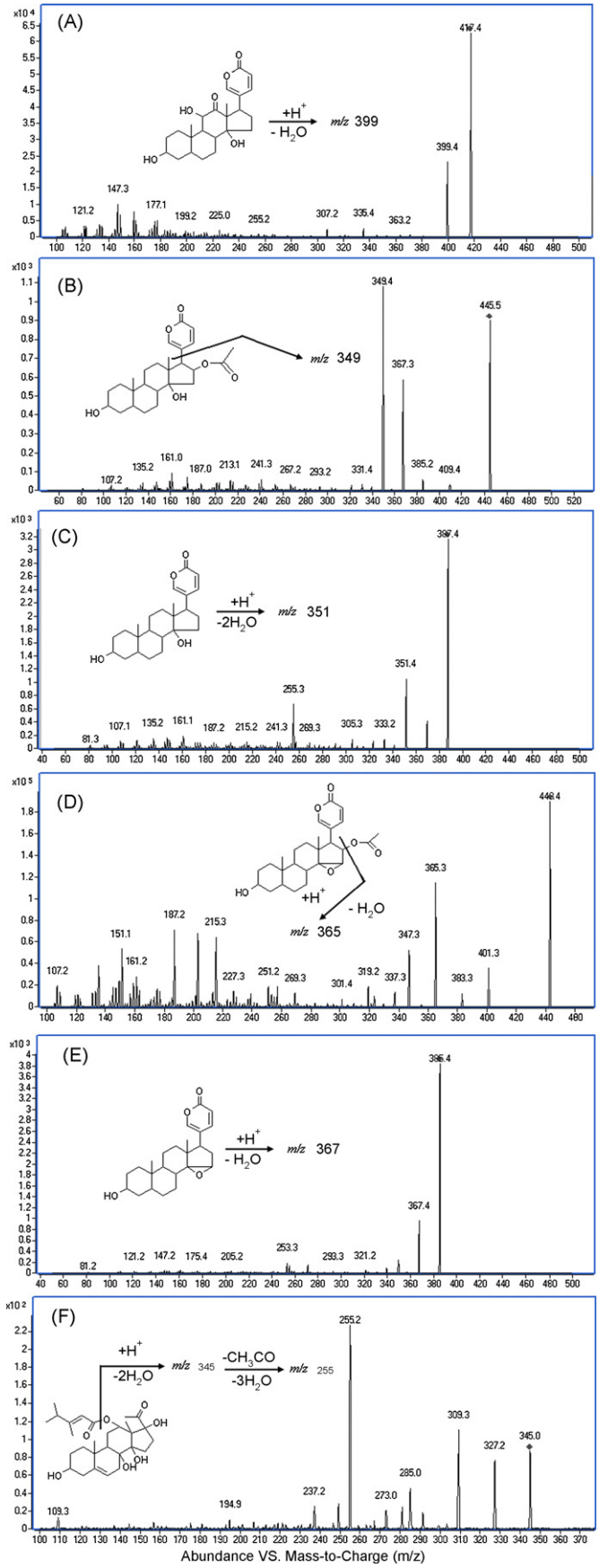

Fig. 1. Chemical structures and full scan product ion of precursor ions of arenobufagin (A), bufatalin (B), bufalin (C), cinobufagin (D), resibufogenin (E), and caudatin (F).

simultaneous determination of cinobafagin and resibufogenin in rat plasma using 0.2 mL plasma with LLOQs of 6.8/7.7 ng/mL [6] had been reported. However, these LC-UV methods were not sensitive enough for the determination of these bufadienolides in biological sample after they were orally administrated, furthermore, too much blood loss will interfere the in vivo metabolism and pharmacokinetics of the drug. More sensitive fluoroimmunoassay method and enzyme immunoassay methods had been developed for the determination of bufalin, cinobufagin, and resibufogenin separately [7–9]. But lack of commercial assay kit limits the usage of those methods. Recently, an LC/TOF MS method was developed for the determination of cinobufagin, resibufogenin and bufalin in dog plasma [10]. The reported method was sensitive enough for pharmacokinetic study and clinical drug monitoring, but the running time was about 30 min which was too long when a large amount of samples needed to be assayed. Moreover, the costliness of the instrument and strict requirement for sample preparation limit the wildly usage of the method. Furthermore, the other two main and active components of Chan Su, bufotalin and arenobufagin, had not been assayed in biological samples by published methods. And up to now, no LC–MS/MS method had been reported for the quantitative determination of components of Chan Su in biological samples. So a high selective LC–MS/MS method for the determination of bufadienolides in biological sample had been developed by us and described in this paper. This method is sensitive, simple and five main active bufadienolides of Chan Su can be rapidly assayed simultaneously. The reported method is proposed for the pharmacokinetic study and clinical monitoring of Chan Su as well as its complex dosage form. And this method had been successfully applied for the determination of plasma drug concentrations after Chan Su was intragastric administrated to rats.

2. Experimental

2.1. Chemicals and reagents

Cinobufagin, resibufogenin, bufalin, bufotalin and arenobufagin were extracted from Chan Su and refined in our laboratory (>99% purity, identified by NMR and MS). Caudatin was extracted from *Cynanchum bungei* Decne and refined in our laboratory (>99% purity, dentified by NMR and MS, IS). Chan Su was purchased from Nanjing Medicine Nantong Kambridge Co. Ltd. (Nantong, China, with contents of 4.21, 2.63, 1.40, 1.15 and 0.48% (w/w) of cinobufagin, resibufogenin, bufalin, bufotalin and arenobufagin, detected by LC-UV). HPLC-grade ethyl acetate was purchased from Tianjin Kermel Chemical Reagents Development Centre (Tianjin, China). HPLC-grade acetonitrile was purchased from Merck Company (Darmstadt, Germany). HPLC-grade formic acid was purchased from Tedia Company Inc. (Fairfield, USA). All other reagents were of analytical grade.

2.2. Instrumentation

An Agilent 6410A triple quadrupole LC–MS system (Agilent Corporation, MA, USA) equipped with G1311A quaternary pump, G1322A vacuum degasser, G1329A autosampler and G1316A therm. Column compartments was used for all analyses. The system was controlled by MassHunter software (Agilent Corporation, MA, USA).

2.3. Chromatographic conditions

The separation was performed by a ZORBAX SB-C_{18} column (3.5 μm, 2.1 mm × 100 mm, Agilent Corporation, MA, USA) and a C_{18} guard column (5 μm, 4.0 mm × 2.0 mm, Phenomenex, CA, USA) with an isocratic mobile phase consisted of acetonitrile–water–formic acid (50:50:0.05, v/v/v) at a flow rate of 0.3 mL/min. The column temperature was maintained at 35 °C and the injection volume was 10 μL.

2.4. Mass spectrometric condition

Ionization was achieved using electrospray in the positive mode with the spray voltage set at 4000 V. Nitrogen was used as nebulizer gas and nebulizer pressure was set at 40 psi with a source temperature of 105 °C. Desolvation gas (nitrogen) was heated to 350 °C and delivered at a flow rate of 10 L/min. For collision-induced dissociation (CID), high purity nitrogen was used as collision gas at a pressure of about 0.1 MPa. Quantitation was performed using multiple reaction monitoring (MRM) mode, Table 1 shows the optimized MRM parameters for detected drugs and IS. The peak widths of precursor and product ions were maintained at 0.7 amu at half-height in the MRM mode.

2.5. Preparation of stock and working solutions

The standard stock solutions of cinobufagin, resibufogenin, bufalin, bufotalin, arenobufagin and caudatin were prepared in methanol to final concentrations of 100 μg/mL for each analyte. The stock solution of each analyte was further diluted with water to achieve standard working solutions at concentrations of 5.00, 25.0, 50.0, 250.0, 500.0, 2500 and 5000 ng/mL. The working solution is the mixture of all the five analytes. Internal standard working solution (1500 ng/mL) was prepared by diluting the stock solution of caudatin with water. All the working solutions were kept at 4 °C.

2.6. Preparation of standard and quality control (QC) samples

Calibration curves were prepared by spiking 100 μL of blank plasma each with 20 μL of one of the above mentioned working solutions to produce the calibration point equivalent to 1.00, 5.00, 10.0, 50.0, 100.0, 500.0 and 1000 ng/mL of each analyte.

The QC samples were prepared using a separately weighted stock solution of each analyte to obtain the plasma concentrations of 5.00, 50.0 and 500.0 ng/mL, representing low, medium and high concentration of QC samples, respectively. The spiked plasma samples (standard and QC samples) were pretreated and detected on each analytical batch along with the unknown samples.

2.7. Sample preparation

To a 100 μL aliquot of plasma sample, 20 μL of water and 20 μL of the IS working solution were added. Samples were then vortex-mixed for 30 s and extracted with 3.5 mL ethyl acetate by vortex-mixing for 1.0 min. After centrifugation at 2000 × *g* for 10 min, the upper organic layer was transferred to another tube and evaporated to dryness at 45 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL mobile phase followed by vortex-mixing for 1.0 min and centrifugation at 2000 × *g* for 10 min. Then, a 10 μL aliquot of supernatant was injected onto the LC–MS/MS system.

2.8. Assay validation

Comparison of the chromatograms of the blank and the spiked rat plasma was used to assay the selectivity of the method. In order to develop a reliable and reproducible method, the matrix effect was also investigated. The matrix effect was evaluated by the following experiment. Triplicates of QC samples at three levels of all the five analytes and IS were added into 0.1 mL mixed blank rat plasma and water separately, and then the spiked samples were pretreated with exactly the same procedure as described in Section 2.7.

Calibration curves ranging from 1.00 to 1000 ng/mL of each analytes were run on 3 separate days. Calibration curves were constructed from the peak-area ratios of each analyte to IS versus plasma concentrations using a $1/x^2$ weighted linear least-squares regression model.

Six replicates of QC samples at three levels of each analyte were included in each run to determine the intra-day and inter-

Table 1
Optimized multiple reaction monitoring (MRM) parameters for arenobufagin, bufotalin, bufalin, cinobufagin, resibufogenin and caudatin

	Precursor ion	Fragmentor energy (V)	Collision energy (eV)	Product ion
Arenobufagin	417	220	25	399
Bufotalin	445	160	18	349
Bufalin	387	190	20	351
Cinobufagin	443	140	18	365
Resibufogenin	385	140	12	367
Caudatin	345	140	12	255

day precision of the assay. The accuracy was determined as the percentage difference between the mean detected concentrations and the nominal concentrations. The lower limit of quantification (LLOQ) is defined as the lowest concentration of standard that can be measured with an acceptable accuracy and precision (≤20% for both parameters).

The extraction recoveries of the five analytes at three QC levels were determined by comparing peak areas obtained from plasma samples with those found by direct injection of a standard solution of the same concentration.

The stability of five analytes in plasma was assessed by analyzing triplicate QC samples stored for 6 h at ambient temperatures, three cycles of freezing at −20 °C and thawing and stored for 1 month at −20 °C, respectively. The stability of five analytes as well as IS in reconstituted extract at room temperature for 24 h was also assessed. Concentrations following storage were compared with freshly prepared samples of the same concentrations.

2.9. Application of the analytical method

Male Wistar rats, weighing approximately 230–250 g, were provided by Shanghai SLAC Lab Animal Co., Ltd. (Shanghai, China). The animal experimentation was approved by the Second Military Medical University Animal Ethics Committee (Shanghai, China). Six rats received an intragastric administration of 100 mg/kg Chan Su (5 mL/kg). The drug was suspended in 0.5% CMC-Na (w/v). Blood samples (250 μl) were collected in heparinized tubes from each rat at 0, 0.17, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0 and 9.0 h after administration. Blood samples were immediately centrifuged and then the plasma samples were stored at −20 °C until analysis. Plasma collected from six vehicle-administrated rats served as blank.

All pharmacokinetic parameters were determined by non-compartmental analysis. The peak plasma level (C_{max}) and the time to reach the peak plasma concentration (t_{max}) were obtained directly from the concentration-time data. The elimination rate constant (K_e) was calculated from the slope of the logarithm of the plasma concentration versus time using the final four points. The apparent elimination half-life ($t_{1/2}$) was calculated as $0.693/K_e$. The area under the plasma concentration–time curve (AUC) and the area under the first moment curve (AUMC) were calculated by the trapezoidal rule. The mean residence times (MRT) were calculated by dividing AUMC by AUC. The values were calculated by Microsoft Excel (Microsoft, Seattle, Washington, USA) and each value is expressed as mean ± S.D.

3. Results and discussion

3.1. LC–MS/MS optimization

Cinobufagin, resibufogenin, bufalin, bufotalin, arenobufagin and caudatin (IS) were at first characterized by MS^2 scan and MS–MS product ions to ascertain their precursor ions and to select product ions for use in MRM mode, respectively. To get the richest relative abundance of precursor ions and product ions, the parameters for fragmentor energies and collision energies were optimized, and the MRM transition were chosen to be *m/z* 443 → 365 for cinobufagin, *m/z* 385 → 367 for resibufogenin, *m/z* 387 → 351 for bufalin, *m/z* 445 → 349 for bufotalin, *m/z* 417 → 399 for arenobufagin and *m/z* 345 → 255 for caudatin. Fig. 1 shows the spectra of full scan product ion of precursor ions of the five analytes and IS.

Formic acid was added to the mobile phase for suppressing the production of $[M + Na]^+$ ions and enhancing the abundance of $[M + H]^+$ ions. After optimization, a proper concentration of formic acid was chosen. Further addition of formic acid would do little help for the production of precursor ions and would do more harm to the column.

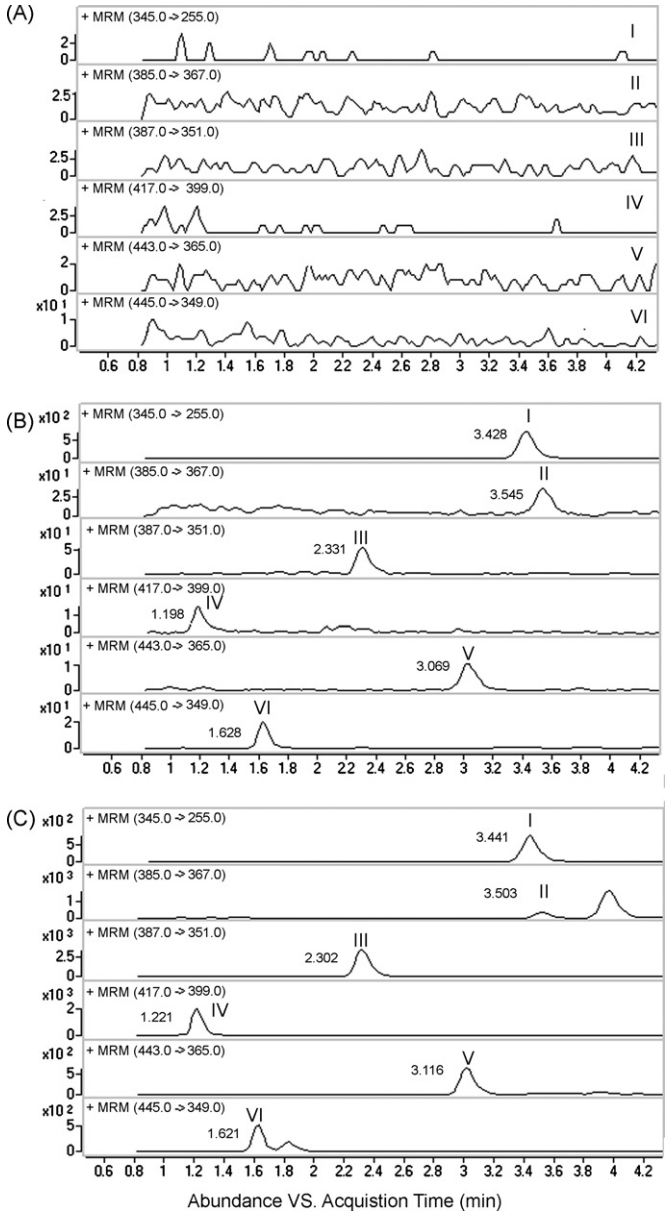

Fig. 2. Representative MRM chromatograms of resibufogenin(II), bufalin(III), arenobufagin(IV), cinobufagin(V), bufotalin(VI) and caudatin(I, IS) in rat plasma. (A) A blank plasma sample, (B) a blank plasma sample spiked with resibufogenin, bufalin, arenobufagin, cinobufagin, bufotalin at the lower limit of quantification and IS, and (C) plasma sample from a rat 0.25 h after intragastric administration of Chan-Su total extract at a dose of 100 mg/kg.

Table 2
Precision and accuracy from QC samples of rat plasma extracts ($n = 3$ days and six replicates per day)

Analyte	Added C (ng/mL)	Found C (ng/mL)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Relative error (%)
Arenobufagin	5.00	5.21	10.6	9.5	4.2
	50.0	51.7	4.4	8.3	3.4
	500.0	489.5	5.1	4.7	−2.1
Bufotalin	5.00	4.79	8.1	10.1	−4.2
	50.0	49.3	5.4	4.8	−1.4
	500.0	502.8	1.6	5.7	0.6
Bufalin	5.00	5.17	12.4	14.7	3.4
	50.0	48.6	8.1	10.4	−2.8
	500.0	519.5	3.4	6.4	3.9
Cinobufagin	5.00	5.26	8.1	10.8	5.2
	50.0	47.9	5.4	6.7	−4.2
	500.0	512.8	3.7	2.7	2.6
Resibufogenin	5.00	5.19	11.8	13.7	3.8
	50.0	48.5	6.1	10.6	−3.0
	500.0	510.6	4.6	5.7	2.1

3.2. Selection of internal standard

An internal standard should be used when performing MS quantitation. An appropriate internal standard will control for extraction, HPLC injection and ionization variability. In this method caudatin was chosen as internal standard for its similar chemical structure, extraction recovery, ionization response in ESI mass spectrometry and chromatographic retention time with those of bufadienolides. It was also stable during the period of pretreatment and assaying of the plasma samples. Moreover, caudatin is not a component of Chan Su and Chan Su is seldom co-administrated with herbal drugs containing caudatin, so this method does not tend to be interfered when the method is adapted for wider application.

3.3. Method validation

3.3.1. Assay selectivity and matrix effect

LC–MS/MS method has high selectivity because only selected ions produced from selected precursor ions are monitored. Comparison of the chromatograms of the blank and the spiked rat plasma (see Fig. 2) indicated no significant interference at the retention times of the analytes and the IS.

The results of matrix effect experiments showed that there was no significant difference between the peak areas of samples prepared from rat plasma and from water which indicated that no co-eluting unseen compounds significantly influenced the ionization of analytes and IS.

3.3.2. Linearity of calibration curves and lower limit of quantification

The calibration curve for spiked rat plasma containing each analyte was linear over the range 1.00–1000 ng/mL with a correlation coefficient (r) > 0.995. Typical equations for the calibration curves were as follows:

Arenobufagin	$Y = 1.05 \times 10^{-2}X + 2.17 \times 10^{-3}$	$r = 0.9986$
Bufotalin	$Y = 3.29 \times 10^{-2}X + 3.76 \times 10^{-3}$	$r = 0.9995$
Bufalin	$Y = 1.51 \times 10^{-2}X + 1.20 \times 10^{-3}$	$r = 0.9982$
Cinobufagin	$Y = 1.43 \times 10^{-2}X + 3.71 \times 10^{-3}$	$r = 0.9976$
Resibufogenin	$Y = 1.62 \times 10^{-2}X + 1.73 \times 10^{-3}$	$r = 0.9968$

The lower limit of quantification for determination of each analyte in plasma, defined as the lowest concentration analyzed with accuracy within ±20% and a precision ≤20%, were all 1.00 ng/mL. These limits are sufficient for the pharmacokinetic studies of the five analytes following an intragastric administration of Chan Su to rats.

3.3.3. Assay precision and accuracy

Intra- and inter-day precision was assessed from the results of QCs by using a one-way analysis of variance (ANOVA). The mean values and R.S.D. for QC samples at three concentration levels were calculated over three validation runs. Six replicates of each QC level were determined in each run. The accuracy of the method was determined by calculating the percentage deviation observed in the analysis of QCs and expressed as the relative error (R.E.). Table 2 summarizes the intra- and inter-day precision and accuracy for the five analytes from the QC samples. All intra- and inter-day precision and accuracy were acceptable.

Table 3
Extraction recovery of the liquid–liquid extraction method ($n = 6$) for arenobufagin, bufotalin, bufalin, cinobufagin and resibufogenin

	Extraction recovery ± S.D. (%)		
	5.00	50.0	500.0
Arenobufagin	85.3 ± 9.5	88.6 ± 7.8	88.5 ± 5.8
Bufotalin	93.5 ± 8.1	91.8 ± 5.3	93.2 ± 3.4
Bufalin	98.1 ± 7.5	97.6 ± 4.9	98.3 ± 2.6
Cinobufagin	94.8 ± 6.4	95.5 ± 4.5	95.2 ± 3.7
Resibufogenin	91.7 ± 6.6	91.0 ± 5.6	92.9 ± 3.5

Table 4
Pharmacokinetic parameters of arenobufagin, bufotalin, bufalin, cinobufagin and resibufogenin after intragastric administration of Chan Su extract to rats at a dose of 100 mg kg^{-1} $n = 6$

Parameter	Values				
	Arenobufagin	Bufotalin	Bufalin	Cinobufagin	Resibufogenin
T_{max} (h)	0.46 ± 0.19	0.42 ± 0.20	0.28 ± 0.11	0.33 ± 0.13	0.33 ± 0.13
C_{max} (ng/mL)	314.6 ± 69.2	24.9 ± 16.2	635.6 ± 151.3	41.0 ± 20.6	73.5 ± 19.4
K_e (h^{-1})	0.40 ± 0.05	0.19 ± 0.05	0.27 ± 0.01	0.20 ± 0.04	0.31 ± 0.03
$t_{1/2}$ (h)	1.75 ± 0.21	3.85 ± 1.10	2.58 ± 0.11	3.54 ± 0.67	2.26 ± 0.25
$AUC_{0\rightarrow t}$ (ng h/mL)	597.0 ± 55.5	47.9 ± 17.7	1596.0 ± 634.7	66.3 ± 7.3	106.5 ± 28.2
$AUC_{0\rightarrow \infty}$ (ng h/mL)	612.5 ± 59.0	57.2 ± 16.9	1746.6 ± 727.2	78.2 ± 9.3	113.3 ± 30.9
$AUMC_{0\rightarrow t}$ (ng h^2/mL)	1438.5 ± 211.2	274.9 ± 71.4	6155.5 ± 3313.7	361.0 ± 76.8	339.3 ± 131.9
MRT (h)	2.34 ± 0.17	4.99 ± 1.42	3.41 ± 0.52	4.59 ± 0.60	2.94 ± 0.52

3.3.4. Extraction recovery

The extraction recoveries of the analytes under the liquid–liquid extraction conditions were summarized in Table 3. The recovery of the internal standard was 90.7 ± 2.6% in rat plasma ($n = 6$).

The extraction recoveries were high and stable for all analytes and IS. But when small amount of organic solvent was added into the unextracted sample, such as methanol and acetonitrile, the extraction recoveries for all analytes would decline. Especially for bufotalin and arenobufagin, their extraction recoveries were around 30%. To guarantee the high and stable extraction recovery, all analytes and IS working solutions were diluted by water instead of pure organic solvent or mobile phase. Our stability test result showed that all the analytes and IS solutions remained stable during the period of experiment.

3.3.5. Analyte stability

The stability of cinobufagin, resibufogenin, bufalin, bufotalin and arenobufagin in rat plasma and mobile phase were investigated. The analyte was found to be stable in rat plasma stored for 1 month at −20 °C and in reconstituted mobile phase at room temperature for 24 h (<5% reduction). After storage at 1–4 °C for 2 month, no obvious reduction was found in the stock and working solutions. The analyte was found to be stable after three freeze-thaw cycles with a reduction of less than 15%. The analytes were also shown to be stable in rat plasma at room temperature for at least 6 h with a reduction of less than 15%.

3.4. Application of the analytical method to pharmacokinetic studies

The LC–MS/MS method developed was used to investigate the pharmacokinetics of cinobufagin, resibufogenin, bufalin, bufotalin and arenobufagin after an intragastric administration of 100 mg/kg Chan Su suspension to six rats. Fig. 3 shows the mean plasma concentration–time curves of the five analytes after the intragastric administration. The main pharmacokinetics parameters were calculated and summarized in Table 4.

Among the five main active bufadienolides of Chan Su, the AUC values for bufalin and arenobufagin were high, while those for the mark compounds of Chan Su, cinobufagin and resibufogenin, as well as for bufotalin were low. Bufalin has attracted interests of many researchers for its toxicity and obvious pharmacological activities. But up to know, little paper has been reported about the pharmacological activity and toxicity of arenobufagin. Our unpublished MTT assay data showed that arenobufagin has obvious anticancer activity, add to the high plasma concentration of this drug in rats' plasma, this drug needs to be further studied about its toxicity and pharmacological activity.

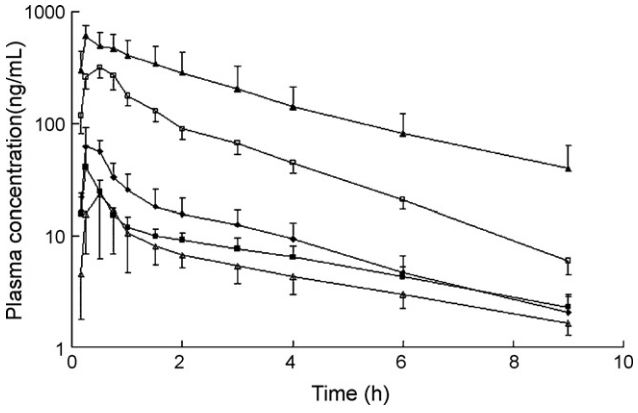

Fig. 3. Plasma concentration–time curves of arenobufagin (□), bufotalin (△), bufalin (▲), cinobufagin (■), and resibufogenin (♦) after intragastric administration of Chan Su extract to rats at a dose of 100 mg/kg. Each value is expressed as mean ± S.D. $n = 6$.

4. Conclusion

An LC–MS/MS method was developed for the simultaneous determination of cinobufagin, resibufogenin, bufalin, bufotalin and arenobufagin in rat plasma. This method was sensitive, rapid, with high accuracy and met all requirements in bioanalytical method. The analytical method has been successfully applied to assay plasma concentration of five bufadienolides in rat plasma. Since all the five assayed bufadienolides are all toxic agents, and when they are taken overdose, severe adverse effect might happen, it is necessary to monitor them when high dose of Chan Su is taken. The described method can be adapted for clinical drug monitoring.

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