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Journal of Pharmaceutical and Biomedical Analysis 46 (2008) 442–448
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Simultaneous determination and pharmacokinetics of five bufadienolides in rat plasma after oral administration of Chansu extract by SPE-HPLC method

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Received 29 June 2007; received in revised form 1 November 2007; accepted 1 November 2007

Abstract

A sensitive and rapid solid-phase extraction-high performance liquid chromatography (SPE-HPLC) method has been developed for the determination of five bufadienolides, arenobufagin, teliocinobufagin, cinobufotalin, cinobufagin and resibufogenin in rat plasma and applied to a pharmacokinetic study in rats after oral administration of Chansu extract (*Venenum Bufonis*). Plasma samples were pretreated with solid-phase extraction using Extract-Clean™ cartridges, and the extracts were analyzed by a reversed-phase C_{18} column on a HPLC system with photodiode array detection (DAD). The calibration curves were linear over the range of 0.10–1.66 μg/ml for arenobufagin, 0.03–1.20 μg/ml for telocinobufagin, 0.01–0.62 μg/ml for cinobufotalin, 0.03–0.70 μg/ml for cinobufagin and 0.02–2.57 μg/ml for resibufogenin, respectively. The limit of quantification was 1.1 ng/ml for arenobufagin, 0.3 ng/ml for telocinobufagin, 9.7 ng/ml for cinobufotalin, 8.8 ng/ml for cinobufagin, 7.7 ng/ml for resibufogenin, respectively. The established method could be easily applied to the determination and pharmacokinetic studies of five bufadienolides in rat plasma after oral administration of Chansu extract.
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Keywords: *Venenum Bufonis*; Bufadienolides; SPE-HPLC; Pharmacokinetics; Rat plasma

1. Introduction

Chansu (*Venenum Bufonis*), is the dried white venom prepared from the skin secretions of toad (*Bufo bufo gargarizans* Cantor) [1,2]. It is an important traditional Chinese medicine used in China and other Asian countries for centuries to treat a number of diseases, such as sore throat, swells, pains, heart failure, skin problems, cancers, etc. [3]. Arenobufagin (Arg), telocinobufagin (Tel), cinobufotalin (Ctl), cinobufagin (Cbg) and resibufogenin (Reg) are the major active bufadienolides isolated from Chansu. Since the first bufadienolide was isolated from squill, more than 250 natural products of this structure type have been found [4,5]. They have been reported to show significant activities in inducing the apoptosis against endometriosis and bladder carcinoma [6,7], inhibiting the proliferation of prostate cancer cell lines [8], the colchicine-resistant primary liver carcinoma cell line PLC/PRF/5 [9] and exhibiting antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* [10]. Meanwhile, as cardioactive steroids, bufadienolides are also observed to possess the adverse effects such as contribution to cardiomyopathy [11], impairment of renal tubular sodium–calcium exchange efficiency [12], and inhibition of testosterone secretion in male rat [13], etc. Therefore, the detection and quantification of bufadienolides concentrations in plasma are very important for drug monitoring on preclinical studies and further investigations.

There are several articles published on the quantification of bufadienolides in Chansu crude drug, Chinese patent medicine containing Chansu and human liver tissues [14–16]. We have carried out comprehensive biotransformation studies on the Chansu bufadienolides and a series of transformed

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doi:10.1016/j.jpba.2007.11.001

products were obtained [17–28]. However, pharmacokinetic studies of bufadienolides are scarcely reported. PK study of only cinobufagin, one of these bufadienolides, has been done [29,30].

The present study reports, for the first time, the development and validation of a sensitive and rapid solid-phase extraction-high performance liquid chromatographic (SPE-HPLC) method for the simultaneous determination of five bufadienolides in rat plasma and its pharmacokinetics after oral administration of Chansu extract.

2. Experimental

2.1. Chemicals and reagents

Crude Chansu was purchased from Kang-Tai Traditional Chinese Medicine Trade Company, Qingdao, Shandong province.

Arg, Tel, Ctl, Cbg and Reg (Fig. 1) were isolated from Chansu in our laboratory. The internal standard (I.S.), 3-epi-16α-hydroxyl resibufogenin (Rph) (Fig. 1), was prepared by microbial transformation from *M. polymorphosporus* [27]. Their structures were identified on the basis of NMR and MS spectral analysis. Their purities were >99.5% as determined by HPLC.

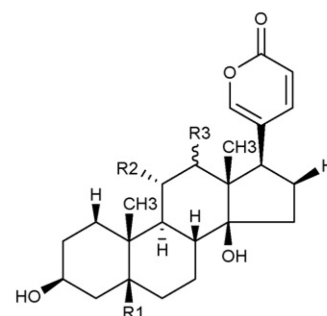
HPLC grade acetonitrile (Burdick & Jackson, MI, USA), acetic acid (Tedia, OH, USA) and the deionized water, prepared from Millipore water purification system was used for all analysis. The methanol for sample preparation was of HPLC grade, purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Chromatography

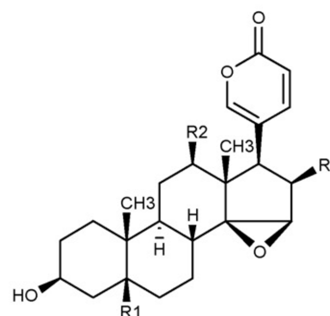
The analyses were performed on an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) with diode-array detector, using Extend-C_{18} column (5 μm, 4.6 mm × 250 mm, Agilent, Waldbronn, Germany). The mobile phase consisted of acetonitrile–water containing 0.3% (v/v) acetic acid. A gradient program was used as follows: a linear gradient of acetonitrile–water (28:72, v/v) for the first 13 min, then hold at acetonitrile–water (53:47, v/v) for 17 min. The mobile phase flow rate was 0.7 ml/min, and column temperature was set at 30 °C. The DAD detector recorded UV spectra in the range from 190 to 400 nm, and HPLC chromatogram was obtained at 296 nm.

2.3. Standard solution

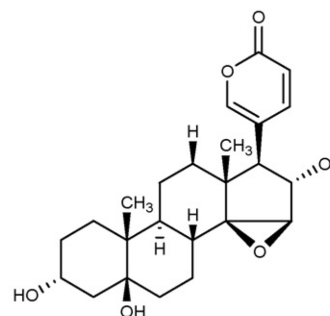
Stock solutions of Arg, Tel, Ctl, Cbg and Reg and internal standard (Rph) were prepared separately at concentrations of 1040, 1200, 970, 1100, 960 and 244 μg/ml, respectively in water–acetonitrile (50:50, v/v). All stock solutions were further diluted to give a series of concentrations for plasma calibration standards. The stock solution of internal standard (Rph) was also diluted to give a final concentration of 6.1 μg/ml with water–acetonitrile (50:50, v/v).



Arenobufagin R1=H, R2=OH, R3=O
Telocinobufagin R1=OH, R2=R3=H



Cinobufotalin R1=OH, R2=H, R3=OAc
Cinobufagin R1=R2=H, R3=OAc
Resibufogenin R1=R2=R3=H



3-epi-16α-hydroxyl resibufogenin

Fig. 1. Structures of six bufadienolides.

2.4. Sample preparation

Extract-Clean™ cartridges (Alltech Associates Inc.) were applied for pre-concentration and extraction of bufadienolides from rat plasma. Firstly, the conditioning was done by flushing the cartridge with methanol followed by deionized water. A mixture of 500 μl plasma samples containing 0.244 μg/ml I.S. (Rph) was transferred into a SPE column cartridge. After washing the cartridge with methanol–water (20:80, v/v), the compounds were eluted with 5 ml of methanol–water (60:40, v/v). The eluate was evaporated to dryness at 40 °C under vacuum and then dis-

solved in 150 μl mobile phase. Twenty microliters of the sample was injected into HPLC system for analysis.

2.5. Preparation of calibration standards and quality control samples

Plasma calibration standards were prepared by adding 20 μl of the appropriate standard solution to 500 μl drug-free rat plasma in a tube containing 0.122 μg I.S. The concentrations of five bufadienolides covering the range of 0.10–1.66 μg/ml for Arg, 0.03–1.20 μg/ml for Tel, 0.01–0.62 μg/ml for Ctl, 0.03–0.70 μg/ml for Cbg, 0.02–2.57 μg/ml for Reg and 0.24 μg/ml for Rph (I.S.), respectively. Quality control (QC) samples were prepared from blank plasma at high, medium and low concentrations for the five bufadienolides. All plasma samples were processed according to the procedure described above.

2.6. Assay validation

QC samples were used to evaluate the intra-day and inter-day precision and accuracy. The intra-day variance was determined by assaying the six replicates on the same day while inter-day variance was assayed on four consecutive days. Accuracy was determined by comparing the calculated mean concentrations to nominal concentrations. Precision was calculated as the relative standard deviation of measurements. The quality control samples were prepared on the day of analysis in the same way as calibration standards. During each analytical run, QC samples were included and processed as the calibration and unknown samples. The recoveries in rat plasma were determined by comparing the resulting peak area ratios with those obtained by direct injection of solutions at the same three concentrations as QC samples.

2.7. Preparation of Chansu extract

Ten grams of Chansu powder was extracted twice with methanol (1:20, w/v) in an ultrasonic water bath at room temperature for 1 h and then the extract was filtered. The filtered extract was then concentrated and lyophilized to yield a dry powder, which was stored at −20 °C before use.

To calculate the administered dose of five bufadienolides, their contents in Chansu extract were quantitatively determined. The lyophilized extract of Chansu was dissolved in water–acetonitrile (50:50, v/v) and diluted to the concentration of 2.17 mg/ml. Then 20 μl of this solution was injected into HPLC system for analysis. The contents of five bufadienolides (Arg, Tel, Ctl, Cbg and Reg) in the lyophilized extract of Chansu were determined to be 4.94, 1.85, 3.68, 7.47 and 5.98%, respectively, from the peak area ratios using an equation for linear regression obtained from the calibration curve.

2.8. Plasma collection

Male Sprague-Dawley rats (200–220 g) were obtained from the SLAC Laboratory Animal Co. LTD. of Shanghai Institutes for Biological Sciences (CAS, Shanghai). They were kept in environmentally controlled breeding room for 4 days until the time of the experiment. They were fed with standard laboratory food and water and fasted overnight before the administration.

Chansu extract was orally administered to the rats at a dose of 120 mg/kg. Venous blood samples (500 μl) were collected at 5, 15, 30, 45, 60, 120, 180, 300, 480 and 720 min after oral administration. All blood samples were immediately centrifuged and the separated plasma was stored at −80 °C until analysis.

2.9. Stability of bufadienolides in rat plasma

The stability of bufadienolides in rat plasma was tested on five freeze–thaw cycles with QC samples at three concentration levels. These samples were stored frozen at −20 °C and analyzed on days 0, 1, 3, 5 and 7 to give the evaluations.

2.10. Application to pharmacokinetic studies

Pharmacokinetic analysis of five bufadienolides was carried out according to a standard non-compartmental method using the WinNonlin (Pharsight Co., CA, Version 3.0).

3. Results and discussion

3.1. Method development

In the present study, comparative studies of three different methods of plasma preparations were carried out to achieve a maximum recovery and a best resolution of each analyte. A preliminary study for recoveries of analytes was performed by comparing protein precipitation by acetonitrile and liquid–liquid extraction method. The utilization of acetonitrile for protein precipitation gave no chromatographic peak information in rat plasma, while liquid–liquid extraction resulted in symmetrical peaks of all detectable compounds, including five analytes. Unfortunately, the blank plasma after liquid–liquid extraction gives rise to interfering substance within the analysis. Therefore, SPE method, using Extract-Clean™ cartridges (Alltech Associates Inc.), was developed before HPLC analysis. Compared with the liquid–liquid extraction, SPE method could not only clean up the interfering substances in samples, but also gave better recoveries of all five analytes. HPLC condition was developed to give the best peak resolution of each analyte.

3.2. Selection of internal standard

3-Epi-16α-hydroxyl resibufogenin (Rph) was selected as the I.S. due to the similarity of its structure to that of the analytes and its excellent chromatographic properties. Cinobufotalin when O-deacetylated and cinobufagin and resibufogenin when hydroxylated at R1 and/or R3 might be converted theoretically to the I.S., but no such results have been found when analyzing the plasma before adding I.S., i.e. no interfering substance exists at the retention time of I.S. Although I.S. was prepared by microbial transformation from *M. polymorphosporus* [27], due

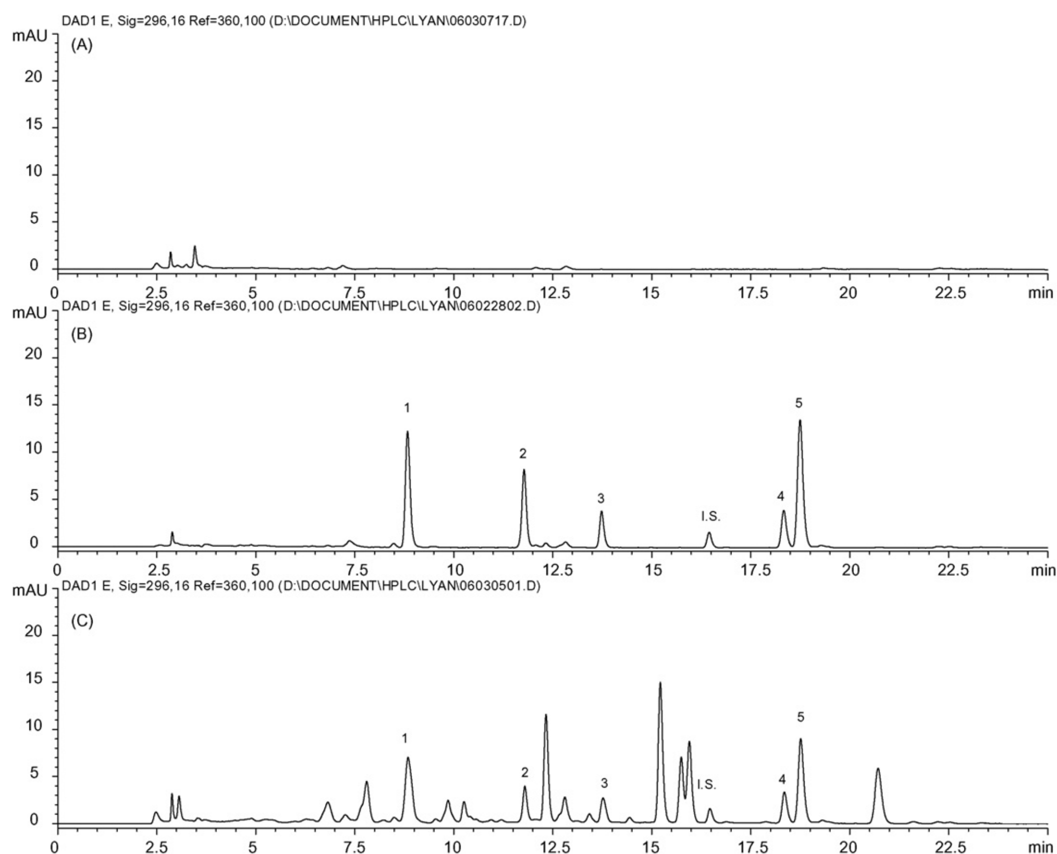


Fig. 2. Chromatograms of rat plasma samples: (A) blank plasma; (B) plasma spiked with arenobufagin (1), telocinobufagin (2), cinobufotalin (3), cinobufagin (4), resibufogenin (5) and their internal standards (I.S.); (C) plasma sample at 15 min after oral administration of Chansu extract.

Table 1
Intra-day and inter-day accuracy and precision of bufadienolides in rat plasma

Spiked concentration (μg/ml)	Intra-day ($n = 6$)			Inter-day ($n = 4$)		
	Measured concentration (μg/ml, mean ± S.D.)	Accuracy (%)	R.S.D. (%)	Measured concentration (μg/ml, mean ± S.D.)	Accuracy (%)	R.S.D. (%)
Arg						
1.040	1.118 ± 0.053	107.5	4.76	1.121 ± 0.048	107.8	4.31
0.520	0.443 ± 0.002	85.2	0.50	0.442 ± 0.003	85.0	0.61
0.166	0.153 ± 0.001	92.0	0.80	0.151 ± 0.001	90.8	0.61
Tel						
0.270	0.261 ± 0.016	96.8	6.01	0.265 ± 0.017	98.1	6.31
0.150	0.124 ± 0.001	82.8	0.95	0.132 ± 0.006	87.8	4.60
0.030	0.028 ± 0.003	93.7	11.59	0.032 ± 0.001	106.5	4.58
Ctl						
0.388	0.351 ± 0.009	90.4	2.52	0.347 ± 0.007	89.3	1.98
0.155	0.132 ± 0.001	85.3	1.03	0.132 ± 0.001	85.4	1.27
0.039	0.033 ± 0.001	85.0	4.40	0.033 ± 0.001	85.9	2.08
Cbg						
0.352	0.269 ± 0.028	76.6	10.50	0.262 ± 0.020	74.3	7.70
0.264	0.243 ± 0.002	92.1	0.97	0.241 ± 0.002	91.6	0.97
0.070	0.064 ± 0.001	90.8	1.68	0.062 ± 0.002	88.5	3.51
Reg						
1.200	1.041 ± 0.078	86.7	7.51	1.023 ± 0.052	85.2	5.07
0.600	0.600 ± 0.003	100.0	0.49	0.599 ± 0.002	99.9	0.41
0.096	0.105 ± 0.001	108.9	1.15	0.103 ± 0.001	107.1	0.73

Table 2
Recovery of bufadienolides from rat plasma ($n = 3$)

Compound	Spiked concentration (μg/ml)	Measured concentration (μg/ml, mean ± S.D.)	Recovery (%)	R.S.D. (%)
Arg	1.040	1.094 ± 0.054	105.2	4.92
	0.520	0.445 ± 0.002	85.6	0.36
	0.166	0.151 ± 0.001	90.6	0.88
Tel	0.270	0.254 ± 0.014	94.2	5.71
	0.150	0.136 ± 0.001	90.7	0.77
	0.030	0.031 ± 0.0003	103.3	0.90
Ctl	0.388	0.352 ± 0.010	90.8	2.88
	0.155	0.133 ± 0.0005	86.0	0.39
	0.039	0.034 ± 0.001	87.0	4.07
Cbg	0.352	0.274 ± 0.030	77.8	11.00
	0.264	0.242 ± 0.002	91.8	0.96
	0.070	0.060 ± 0.001	85.5	2.94
Reg	1.200	1.055 ± 0.080	88.0	7.61
	0.600	0.602 ± 0.003	100.3	0.45
	0.096	0.102 ± 0.001	106.1	0.95

to the difference in metabolic pathways between microorganism and mammal 3-epi-16α-hydroxyl resibufogenin is qualified to be used as the I.S.

3.3. Selectivity and chromatography

Typical chromatography of blank plasma, plasma spiked with five bufadienolides and I.S. and plasma obtained 15 min after oral administration of Chansu extract are shown in Fig. 2. There were no coeluting peaks in the vicinity of the five bufadienolides and I.S. peaks in the chromatogram of blank plasma. The retention times of Arg, Tel, Ctl, Cbg and Reg and I.S. (Rph) were 8.8, 11.8, 13.7, 18.4, 18.8 and 16.5 min, respectively. The analytes were well separated from co-extracted materials under the described chromatographic conditions.

3.4. Calibration curve

Evaluation of the assay was performed with a six-point calibration curve over the concentration range of 0.10–1.66 μg/ml for Arg, 0.03–1.20 μg/ml for Tel, 0.01–0.62 μg/ml for Ctl, 0.03–0.70 μg/ml for Cbg, 0.02–2.57 μg/ml for Reg with $r^2 > 0.99$. The limit of quantification (LOQ) was defined as the lowest drug concentration which can be determined with an intra-day relative standard deviation (R.S.D.) of 20%. The LOQ were found to be 1.1 ng/ml for Arg, 0.3 ng/ml for Tel, 9.7 ng/ml for Ctl, 8.8 ng/ml for Cbg and 7.7 ng/ml for Reg in rat plasma, respectively. The limit of detection (LOD) considering a signal-to-noise ratio 3:1 was estimated to be 0.8 ng/ml for Arg, 0.2 ng/ml for Tel, 6.5 ng/ml for Ctl, 7.3 ng/ml for Cbg and 6.4 ng/ml for Reg, respectively in rat plasma.

3.5. Precision and accuracy

The precision and accuracy of the assay were evaluated with quality control (QC) samples at low, medium and high concentrations. Results are summarized in Table 1. The intra- and inter-day precision calculated as the relative standard deviation (R.S.D.) were <12%. The accuracies of five bufadienolides were within the range of 74.3–108.9%.

3.6. Recovery

The extraction recovery from spiked plasma was calculated by comparing peak areas with QC samples ($n = 3$) at three different concentration levels. The corresponding values are shown in Table 2. All recoveries were within the range of

Table 3
Stability of bufadienolides in rat plasma ($n = 5$)

Spiked concentration (μg/ml)	Measured concentration (μg/ml, mean ± S.D.)	Variation coefficient (%)
Arg		
1.040	1.113 ± 0.044	4.00
0.520	0.450 ± 0.013	2.84
0.166	0.153 ± 0.002	1.54
Tel		
0.270	0.261 ± 0.015	5.69
0.150	0.134 ± 0.005	3.92
0.030	0.031 ± 0.003	8.90
Ctl		
0.388	0.349 ± 0.009	2.67
0.155	0.133 ± 0.001	0.72
0.039	0.033 ± 0.003	9.07
Cbg		
0.352	0.267 ± 0.025	9.31
0.264	0.241 ± 0.005	2.06
0.070	0.062 ± 0.002	4.06
Reg		
1.200	1.037 ± 0.069	6.69
0.600	0.598 ± 0.010	1.75
0.096	0.103 ± 0.003	3.21

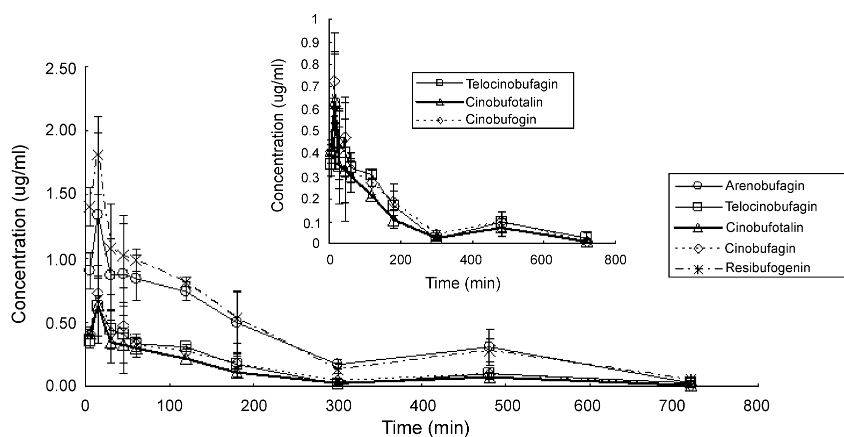


Fig. 3. Mean plasma concentration–time plots of Arg, Tel, Ctl, Cbg and Reg after a single oral dose of Chansu to rats. Each point represents mean ± S.D. ($n = 6$).

Table 4
Pharmacokinetic parameters of five bufadienolides after oral administration of Chansu extract in rat, each value represents the mean ± S.D. (n=6)

Parameter	Arg	Tel	Ctl	Cbg	Reg
C_{max} (μg/ml)	1.57 ± 0.59	0.69 ± 0.26	0.68 ± 0.33	0.77 ± 0.12	1.81 ± 0.34
T_{max} (min)	28 ± 21	35 ± 18	17 ± 15	20 ± 12	15 ± 0
$T_{1/2}$ (min)	153 ± 21	135 ± 43	119 ± 26	138 ± 30	154 ± 13
CL/F (L/(min kg))	0.025 ± 0.006	0.027 ± 0.009	0.066 ± 0.020	0.093 ± 0.017	0.028 ± 0.004
Vz/F (L/kg)	5.408 ± 1.240	4.745 ± 0.517	10.829 ± 1.898	18.045 ± 2.908	6.240 ± 0.836
AUC_{0-t} (min μg/ml)	242.620 ± 46.975	84.053 ± 18.236	68.834 ± 18.042	92.160 ± 13.767	266.846 ± 36.653
$AUC_{0-\infty}$ (min μg/ml)	249.837 ± 47.529	89.963 ± 22.856	71.920 ± 20.147	98.688 ± 17.700	278.138 ± 38.026
MRTINF (min)	229.323 ± 28.261	212.660 ± 77.965	196.826 ± 51.246	227.863 ± 66.785	227.537 ± 19.480

77.8–106.1%. The extraction recovery of Rph (internal standard) was 91.6%.

3.7. Stability

The results of stability of bufadienolides in rat plasma after five freeze–thaw cycles are shown in Table 3. The coefficient of variation was 0.72–9.31%. These results indicated that bufadienolides were stable for at least 7 days in rat plasma stored at −20 °C.

3.8. Application to pharmacokinetic study

After oral administration of Chansu extract (120 mg/kg) to rats, plasma concentrations of five bufadienolides were simultaneously determined by the described HPLC method. Pharmacokinetics was estimated by using the non-compartmental method. The mean plasma concentration–time profiles of Arg, Tel, Ctl, Cbg and Reg are shown in Fig. 3, and the pharmacokinetic parameters are shown in Table 4. All five bufadienolides were below the limit of detection after 12 h of administration of Chansu extract. They were rapidly absorbed and the rat plasma concentrations for Arg, Tel, Ctl, Cbg and Reg peaked at 28 ± 21, 35 ± 18, 17 ± 15, 20 ± 12 and 15 ± 0 min, respectively after oral dosing of 120 mg/kg Chansu extract. The mean maximum concentrations (C_{max}) for Arg, Tel, Ctl, Cbg and Reg in rat plasma were 1.57, 0.69, 0.68, 0.77 and 1.81 μg/ml, respectively. Half-life ($T_{1/2}$) values for Arg, Tel, Ctl, Cbg and Reg were 153, 135, 119, 138 and 154 min, respectively.

4. Conclusion

The analytical method described above is a sensitive and rapid solid-phase extraction-high performance liquid chromatography assay for the simultaneous quantification of five bufadienolides in rat plasma after oral administration of Chansu extract. Recovery, precision, sensitivity and linearity were satisfactory in the range studied. All five bufadienolides were assayed under the same chromatographic conditions. The method was validated to meet the requirements of the pharmacokinetic investigation of five analytes in rat plasma after oral administration of Chansu extract.

Acknowledgements

This work was supported by the National Supporting Program for TCM from Ministry of Science and Technology of China and Shanghai Commission of Science and Technology (2006BAI08B03-03).

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