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Simultaneous determination of cytotoxic bufadienolides in the Chinese medicine ChanSu by high-performance liquid chromatography coupled with photodiode array and mass spectrometry detections

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Abstract

ChanSu (toad venom) is a traditional Chinese medicine for the treatment of serious liver and gastric cancers. The major cytotoxic compounds in ChanSu are bufadienolides. In this paper, a strategy combining qualitative LC/MS analysis and quantitative HPLC determination of major bufadienolides was used for global quality control of ChanSu crude drug. Majority of the bufadienolides in methanol extract of ChanSu were unambiguously characterized by high-performance liquid chromatography coupled with atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS/MS), and by comparing with pure compounds. In addition, eight major bufadienolides were simultaneously determined in one single HPLC run within 30 min with photodiode array detection (DAD). All compounds showed good linearity in a wide concentration range, and their limits of detection (LOD) were around 1 ng. Thus, >95% of the bufadienolides in ChanSu could be characterized, and >90% of them were quantitated. The established method is rapid, simple and sensitive, and could be used for the routine analysis of ChanSu crude drug and its preparations. This research sets a good example for the comprehensive quality control of traditional medicine.

Keywords: Bufadienolide; ChanSu; Chinese medicine; Cytotoxicity; HPLC/DAD/APCI-MS; Quality control

1. Introduction

Traditional medicines, which are prepared from plants, animals or minerals, have been used in countries such as China and India for thousands of years [1]. However, they still cannot be accepted by most advanced countries as therapeutic agents, although a big proportion of today's new drugs come directly or indirectly from traditional medicines [2,3]. A major reason is that the chemical components of traditional medicines could not be explicitly elucidated since they contain a complex mixture of compounds. Moreover, the contents of these compounds may vary significantly depending on plant species, geographical sources, harvesting, processing and storage [4]. All these factors make it fairly difficult for traditional medicines to fulfill the requirements for a modern drug: safety, efficacy and stability. Therefore, desirable qualitative and quantitative analyses, or quality control, seem to be the first step for the modernization and globalization of traditional medicines [5].

Currently, typical quality control of a traditional medicine involves qualitative and quantitative analyses of one or few of the major compounds, while the less abundant or minor compounds, which are commercially unavailable, were usually neglected [6]. However, one essential difference of traditional medicines from chemical drugs is that their therapeutic effects are due to the joint contribution of multi-components, not only the major ones [7]. Therefore, a more comprehensive and global strategy, which could cover most of the chemical constituents, is valuable for the quality control of traditional medicine.

High-performance liquid chromatography (HPLC) is currently the most frequently used separation technique, and is capable of resolving complex chemical mixtures including the crude extract of traditional medicines. Mass spectrometry is a rapid and sensitive technique for structural elucidation, and

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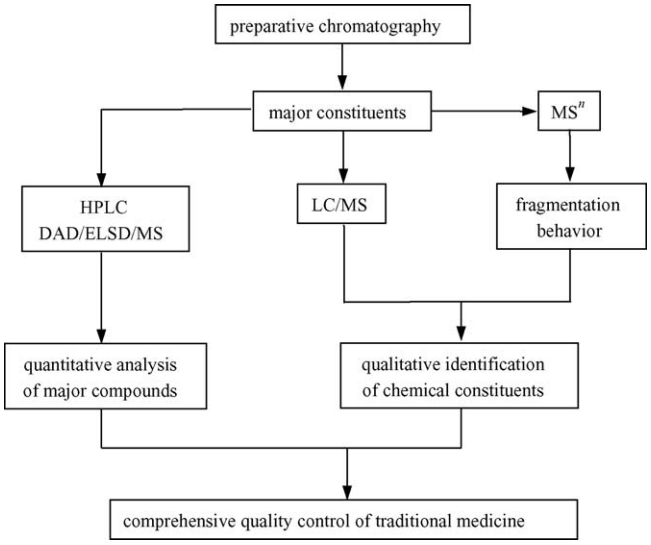

Fig. 1. Our strategy combining HPLC and LC/MS for the comprehensive quality control of traditional medicine.

its connection to HPLC could facilitate high-throughput and informative screening of chemical constituents in traditional medicines, especially those compounds present at trace levels and are difficult to obtain by conventional phytochemical means [8]. In our laboratory, we are trying to combine the qualitative capability of LC/MS and quantitative power of HPLC for the quality control of traditional medicine. Our research strategy is depicted in Fig. 1. First, pure compounds are isolated and subjected to tandem mass spectrometry (MS^n) analysis, which could lead to their fragmentation rules. Then, the crude drug extract is analyzed by on-line LC/MS. The structures of known and unknown compounds could be elucidated based on their MS information. On the other hand, the major constituents could be quantitatively determined by HPLC coupled with DAD, ELSD (evaporative light scattering detection) or MS detectors, depending on the characteristics of the analytes. In this way, the combination of qualitative and quantitative analyses could allow a global quality control of traditional medicine.

ChanSu (toad venom) is a traditional Chinese medicine used to treat heart failure and pains. It is prepared from the skin secretions of giant toads, including *Bufo bufo gargarizans* Cantor and *B. melanostictus* Schneider. Recently, it has been found that ChanSu also possesses potent antitumor activities, and the major active constituents are bufadienolides, a class of C-24 steroids with a characteristic α-pyrone ring at C-17 [9,10]. The major bufadienolides from ChanSu include bufalin, cinobufagin, and resibufogenin, each showing significant cytotoxic activities against human liver and gastric cancer cells, with IC_{50} values of 10^{-2} to 10^{-3} μmol/l [11,12].

The currently available quality control procedure of ChanSu only involves TLC qualitative analysis and HPLC quantitative analysis of bufalin, cinobufagin and resibufogenin [13,14]. However, a lot more bufadienolides were isolated from the crude drug in our recent chemical investigation, and their contents have never been described in detail before. In this paper, we report the isolation, structural elucidation, and cytotoxic evaluation of 12 bufadienolides from ChanSu. All the major bufadienolides were characterized in the methanol extract by LC/MS, and eight of them were quantitated simultaneously by HPLC/UV in a single gradient run within 30 min. This is the first example for the comprehensive quality control of ChanSu, and could be applied to routine analysis of this drug.

2. Experimental

2.1. Chemicals and reagents

The ChanSu crude drug for bufadienolides isolation was purchased from Anguo, Hebei, and authenticated by the author. It appears as dark brown cake (8 cm in diameter, 1 cm thick).

Silica gel (200–300 mesh) for column chromatography was purchased from Qingdao Marine Chemical Corporation (Qingdao, China). HPLC grade acetonitrile (Fisher, Fair Lawn, NJ) and ultra-pure water were used for HPLC analysis. All other chemical solvents were of analytical grade or higher from Beijing Chemical Corporation (Beijing, China).

2.2. Isolation and purification of bufadienolides

An amount of 50 g of ChanSu was dissolved in 2 l of water and extracted with an equal volume of ethyl acetate twice. The organic extracts were combined and concentrated in a rotary evaporator at 40 °C to give a brown solid (12.4 g). It was subjected to silica gel column chromatography and eluted with petroleum ether–acetone (4:1, 2:1, 1:1, v/v). The obtained fractions were further purified by preparative liquid chromatography. A SpectraSERIES HPLC apparatus (Thermo Quest) was used. Samples were separated on a YMC ODS-A column (5 μm, Ø 10 mm × 250 mm) and eluted with methanol–water (45:55, 55:45, 58:42, v/v). The flow rate was 2.0 ml/min, and the detection wavelength was 296 nm.

2.3. HPLC conditions

The analyses were performed on an Agilent 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, a diode-array detector, an autosampler, and a column compartment. The samples were separated on a Zorbax Eclipse XDB-C_{18} column (5 μm, Ø 4.6 mm × 250 mm, Agilent). The mobile phase consisted of acetonitrile (MeCN) and water containing 0.3% (v/v) acetic acid. A gradient program was used as follows: a linear gradient from 28% (v/v) to 54% MeCN in the first 15 min, then isocratic at 54% for additional 15 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 nm to 400 nm, and the HPLC chromatogram was monitored at 296 nm.

The above HPLC conditions were used for all LC/MS and HPLC analyses. The data were processed with a Finnigan Xcalibur 1.3 Software.

2.4. LC/MS analysis

A Finnigan LCQ Advantage ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) was connected to the Agilent 1100 HPLC instrument via an APCI interface. The parameters were the same as we had described in previous report [15].

For sample preparation, 0.1 g of ChanSu drug powder (100 mesh) was extracted with 20 ml of methanol in an ultrasonic water bath at room temperature for 30 min. The solution was then diluted to 25 ml with methanol and filtered through a 0.22-μm micropore membrane (Jinteng Corp., Tianjin, China) before use. A volume of 10 μl was injected for analysis.

2.5. HPLC/UV quantitation

2.5.1. Stock solution

An amount of 24.8 mg of cinobufagin, 11.6 mg of resibufogenin, 10.7 mg of bufalin, 9.4 mg of arenobufagin, 8.0 mg of bufotalin, 4.6 mg of telocinobufagin, 9.8 mg of cinobufotalin, and 5.1 mg of gamabufotalin were accurately weighed, mixed and dissolved in 25 ml of acetonitrile to make a stock solution.

2.5.2. Working solutions

A volume of 5 ml, 4 ml, 3 ml, 2 ml, 1 ml, 0.5 ml, 250 μl, and 50 μl of the stock solution was transferred to a 5-ml volumetric flask, respectively, and diluted to volume with acetonitrile to give eight serial concentrations.

2.5.3. Calibration curve

A volume of 10 μl of each of the eight working solutions was injected into the HPLC instrument for analysis. The peak areas of each standard compound monitored at 296 nm were plotted against their individual concentrations to work out the calibration curves.

2.5.4. Method validation

Precision of the method was evaluated by analyzing the same sample for five consecutive times. Relative standard deviation (RSD) of peak areas for each of the eight bufadienolides was calculated, respectively.

The same sample was put at room temperature, and analyzed at 0 h, 12 h, 24 h, 36 h, and 48 h, respectively, to test stability of the bufadienolides.

For recovery test, 50 mg of ChanSu powder was extracted with 20 ml of methanol in an ultrasonic water bath at room temperature for 30 min. The solution was spiked with 2 ml of stock solution, and then diluted to 25 ml with methanol. A portion of 10 μl was injected for analysis. Recovery rate of each bufadienolide was calculated according to the following equation: (detected amount − original amount)/added amount × 100%.

2.5.5. Sample test

The drug sample solutions were prepared in the same way as described above for LC/MS analysis, and 10 μl was injected. All analyses were monitored at 296 nm.

3. Results and discussion

3.1. Isolation and structural elucidation of bufadienolides from ChanSu crude drug

A total of 12 pure compounds were isolated from ChanSu by preparative chromatography, and their structures were identified by nuclear magnetic resonance (NMR) and mass spectrometry (MS): resibufogenin (90.8 mg), cinobufagin (164.2 mg), bufalin (73.5 mg), arenobufagin (12.6 mg), bufotalin (21.3 mg), telocinobufagin (15.8 mg), ψ-bufarenogin (7.2 mg), bufotalinin (2.9 mg), 3-*oxo*-cinobufotalin (2.6 mg), 19-*oxo*-cinobufagin (3.4 mg), cinobufotalin (24.8 mg), and gamabufotalin (38.8 mg). Their chemical structures are given in Fig. 2. The NMR data of bufotalin, ψ-bufarenogin, bufotalinin, 19-*oxo*-cinobufagin and 3-*oxo*-cinobufotalin were fully assigned for the first time on the basis of 2D NMR spectra (Tables 1 and 2). Purities of the compounds were determined to be >99% by HPLC/UV analysis.

Bufotalin: ^{1}H NMR(DMSO-d_6, 500 MHz): 8.16 (1H, d, J = 10.0 Hz, H-22), 7.50 (1H, s, H-21), 6.18 (1H, d, J = 10.0Hz, H-23), 5.37 (1H, t, J = 9.0 Hz, H-16), 4.36 (1H, s, 14-OH), 4.17 (1H, d, J = 3.0 Hz, 3-OH), 3.88 (1H, *br*s, H-3), 2.86 (1H, d, J = 9.0 Hz, H-17), 2.60 (1H, dd, J = 15.0 Hz, 9.0 Hz, H-15), 1.77 (3H, s, -OAc), 0.84 (3H, s, 19-CH_3), 0.64 (3H, s, 18-CH_3).

19-*oxo*-Cinobufagin: ^{1}H NMR(DMSO-d_6, 500 MHz): 9.50 (1H, s, 19-CHO), 7.85 (1H, d, J = 10.0 Hz, H-22), 7.46 (1H, s, H-21), 6.23 (1H, d, J = 10.0 Hz, H-23), 5.45 (1H, d, J = 9.5Hz, H-16), 4.42 (1H, d, J = 2.5 Hz, 3-OH), 3.91 (1H, *br*s, H-3), 3.75 (1H, s, H-15), 2.86 (1H, d, J = 9.5 Hz, H-17), 1.81 (3H, s, -OAc), 0.75 (3H, s, 18-CH_3).

Bufotalinin: ^{1}H NMR(DMSO-d_6, 500 MHz): 10.00 (1H, s, 19-CHO), 7.74 (1H, dd, J = 10.0Hz, 2.5 Hz, H-22), 7.52 (1H, d, J = 2.5 Hz, H-21), 6.26 (1H, d, J = 10.0 Hz, H-23), 5.36 (1H, d, J = 3.0 Hz, 3-OH), 5.19 (1H, s, 5-OH), 4.02 (1H, *br*s, H-3), 3.62 (1H, s, H-15), 0.64 (3H, s, 18-CH_3).

3.2. Cytotoxic activities of bufadienolides

The *in vitro* cytotoxic activities of bufadienolides against three human cancer cell lines were determined by the MTT method [16,17]. As shown in Table 3, all the tested compounds exhibited significant inhibitory effects. Besides the well-known cinobufagin, bufalin and resibufogenin, several other bufadienolides including arenobufagin, bufotalin, telocinobufagin and gamabufotalin were also obtained in good yields and even showed more potent cytotoxicities. However, these compounds were seldom taken into account in the quality control of ChanSu in previous reports. Even in the Chinese Pharmacopoeia, only cinobufagin and resibufogenin were determined as the marker compounds [18]. Given the yields and cytotoxicities of these bufadienolides, it is obviously unreasonable that their presence in ChanSu were neglected. To establish a new method that reflects quality and stability of ChanSu in a more comprehensive way is thus greatly necessitated.

Table 1
^{13}C NMR spectral data of bufadienolides (125 MHz, DMSO-d_6)

C	Arenobufagin	Bufotalin	Telocinobufagin	ψ-Bufarenogin	Bufotalinin	3-*oxo*-cinobufotalin	19-*oxo*-cinobufagin	Cinobufotalin	Gamabufotalin
1	31.6t	29.5t	24.9t	29.5t	16.9t	30.3t	20.0t	24.8t	31.9t
2	28.3t	27.5t	27.3t	27.9t	26.2t	36.8t	25.8t	27.2t	28.5t
3	64.6d	64.6d	66.5d	64.3d	65.5d	210.9s	63.5d	66.5d	64.8d
4	33.5t	33.0t	36.6t	33.6t	37.3t	48.9t	31.8t	36.5t	33.7t
5	37.4d	35.6d	73.5s	36.0d	74.6s	76.3s	28.4d	73.4s	37.3d
6	26.5t	26.4t	34.9t	26.8t	35.2t	34.1t	27.0t	33.8t	26.9t
7	21.3t	20.7t	23.3t	20.7t	22.7t	22.6t	19.9t	22.2t	21.2t
8	38.9d	41.3d	40.2d	39.2d	33.2d	31.8d	32.9d	31.7d	41.0d
9	39.4d	34.5d	38.1d	45.3d	41.2d	40.9d	36.5d	41.1d	40.3d
10	36.7s	34.8s	40.3s	37.0s	54.5s	40.0s	50.6s	40.4s	36.2s
11	73.3d	20.7t	21.5t	213.9s	21.7t	21.2t	20.6t	21.0t	66.9d
12	213.4s	39.9t	40.0t	82.0d	38.0t	38.8t	38.7t	38.8t	50.3t
13	62.1s	48.9s	47.9s	53.9s	44.3s	44.5s	44.5s	44.4s	48.3s
14	84.1s	82.5s	83.4s	82.1s	73.7s	71.9s	71.6s	72.0s	82.8s
15	31.8t	39.1t	31.9t	33.3t	59.3d	59.4d	59.4d	59.4d	32.1t
16	27.6t	73.7d	28.4t	27.2t	31.4t	74.4d	74.4d	74.4d	28.2t
17	40.0d	55.9d	49.9d	45.0d	45.8d	48.8d	48.9d	48.8d	49.8d
18	17.3q	16.6q	16.8q	18.2q	16.3q	17.0q	16.7q	16.6q	17.7q
19	23.4q	23.7q	16.6q	24.1q	208.9d	16.9q	207.1d	16.8q	23.9q
20	120.8s	117.2s	122.7s	119.7s	121.8s	116.0s	116.0s	116.0s	122.3s
21	150.2d	151.4d	149.2d	150.8d	150.6d	152.2d	152.2d	152.2d	149.3d
22	147.1d	150.1d	147.3d	148.1d	147.3d	148.4d	148.5d	148.4d	147.2d
23	114.6d	111.6d	114.2d	113.2d	114.1d	112.9d	112.9d	112.8d	114.1d
24	161.1s	161.1s	161.3s	161.2s	161.0s	160.8s	160.8s	160.8s	161.3s
$O\underline{C}OCH_3$		169.4s				169.4s	169.3s	169.3s	
$OCO\underline{C}H_3$		20.6q				20.2q	20.2q	20.2q	

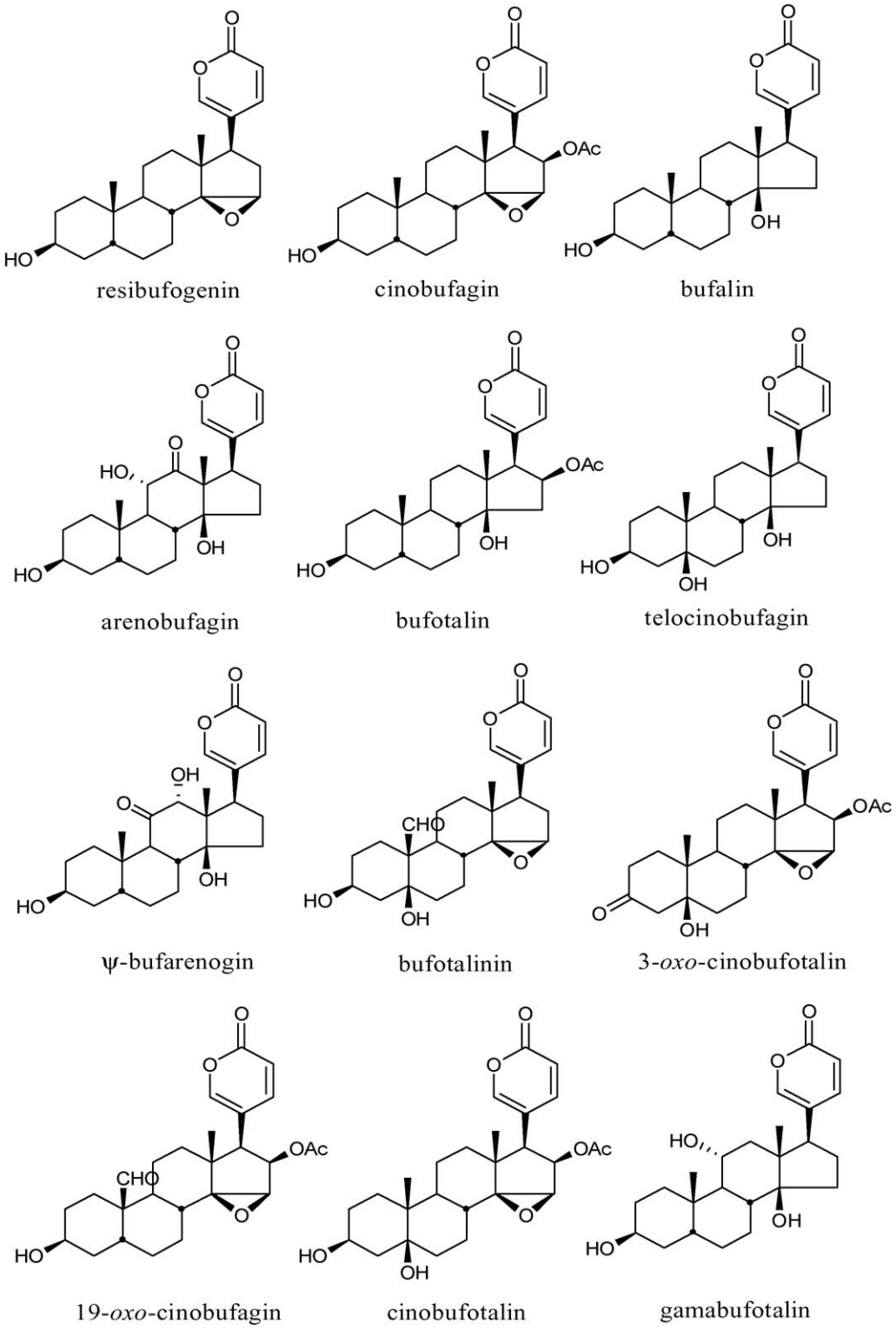

Fig. 2. Chemical structures of bufadienolides isolated from ChanSu.

3.3. Qualitative identification of bufadienolides in ChanSu crude drug by LC/MS

In our previous report, we have investigated the APCI-MS fragmentation behaviors of bufadienolides, and identified 35 compounds from the methanol extract of ChanSu, including major components and those only present at trace levels [15]. However, characterization of main compounds closely related with therapeutic effects is good enough for drug quality control. Using the same HPLC/DAD/APCI-MS/MS procedure, all the significant peaks (relative peak area >1%) corresponding to bufadienolides in the HPLC/UV chromatogram

Table 2
^{1}H NMR spectral data of bufadienolides (500 MHz, DMSO-d_6)

H	Arenobufagin	ψ-Bufarenogin	Gamabufotalin	Cinobufotalin
1	2.27*br*d (11.0)	1.62	2.33	1.81
	1.34	1.44	1.32	1.65
2	1.58	1.73	1.72	2.34
	1.26	1.40	1.28	1.92
3	3.84*br*s	3.90*br*s	3.88*br*s	
4	1.72	1.74	1.80	3.02d (15.0)
	1.15	1.24	1.18	1.84
5	1.72	1.70	1.60	
6	1.80	1.82	1.74	1.58
	1.14	1.20	1.14	1.20
7	1.80	1.80	1.78	1.48
	1.20	1.16	1.16	0.96
8	1.97	2.24*br*t (12.0)	1.62	2.05
9	1.68	2.42d (12.0)	1.48	2.00
11	4.25dd (11.0, 5.0)		3.50*br*s	1.52
				1.28
12		3.93d (3.5)	1.54	1.70
			1.49	1.55
15	1.64	1.58	2.00	3.78s
	1.21	1.52	1.60	
16	1.85	1.82	2.06	5.46d (9.0)
	1.58	1.52	1.62	
17	3.96t (8.5)	2.35	2.48	2.88d (9.5)
18	0.78 (3H, s)	0.80 (3H, s)	0.61 (3H, s)	0.72 (3H, s)
19	1.06 (3H, s)	0.94 (3H, s)	0.96 (3H, s)	0.88 (3H, s)
21	7.57d (2.0)	7.52d (2.0)	7.54d (2.0)	7.47d (1.5)
22	7.80dd (10.0, 2.0)	7.64dd (10.0, 2.0)	7.89dd (10.0, 2.0)	7.80dd (10.0, 1.5)
23	6.32d (10.0)	6.22d (10.0)	6.28d (10.0)	6.24d (10.0)
3-OH	4.13d (3.0)	4.23d (3.0)	4.09d (2.0)	
5-OH				4.56s
11-OH	4.47d (5.0)		4.01d (6.0)	
12-OH		5.11*br*s		
14-OH	4.95s	4.42s	4.11s	
$OCOCH_3$				1.80 (3H, s)

Note: Those peaks whose multiplicity was not designated are multiplets (m).

Table 3
Cytotoxic activities of bufadienolides against human cancer cells ($n = 3$)

Compound	IC_{50} (μmol/l)		
	BGC-823[a]	Bel-7402[b]	HeLa[c]
Resibufogenin	1.1×10^{-1}	1.3×10^{-1}	1.0×10^{-2}
Cinobufagin	6.5×10^{-2}	5.4×10^{-2}	7.4×10^{-2}
Bufalin	4.5×10^{-2}	7.0×10^{-3}	2.8×10^{-2}
Arenobufagin	3.6×10^{-3}	1.9×10^{-3}	1.6×10^{-3}
Bufotalin	1.8×10^{-2}	2.6×10^{-2}	2.0×10^{-3}
Telocinobufagin	2.7×10^{-2}	3.7×10^{-2}	1.9×10^{-3}
ψ-Bufarenogin	2.7	1.5	5.5×10^{-2}
Bufotalinin	7.1×10^{-2}	6.9×10^{-2}	3.2×10^{-2}
Cinobufotalin	4.7	>100	1.8
Gamabufotalin	1.0×10^{-2}	5.3×10^{-3}	1.5×10^{-3}

[a] Human gastric cancer cells.
[b] Human hepatoma cells.
[c] Human cervical carcinoma cells.

of methanol extract of ChanSu were unambiguously characterized based on their UV spectra, full scan mass spectra and MS/MS spectra, and by comparing with pure compounds (Fig. 3). The identification of gamabufotalin is given as an example in Fig. 4. Its UV spectrum gave an absorption maximum at 296 nm, resulting from the α-pyrone ring. The MS and MS/MS spectra were consistent with our previously reported data.

Fortunately, all the major bufadienolides in ChanSu were isolated from the crude drug and served as pure standards for qualitative identification, except desacetylcinobufagin which was prepared by enzymatic modification [19]. It is noteworthy that 3-*oxo*-cinobufotalin could not be detected in ChanSu extract even in selected-ion-monitoring (SIM) mode. Considering its fairly low yield, this compound could be an artifact arising from dehydrogenation of cinobufotalin in the purification process.

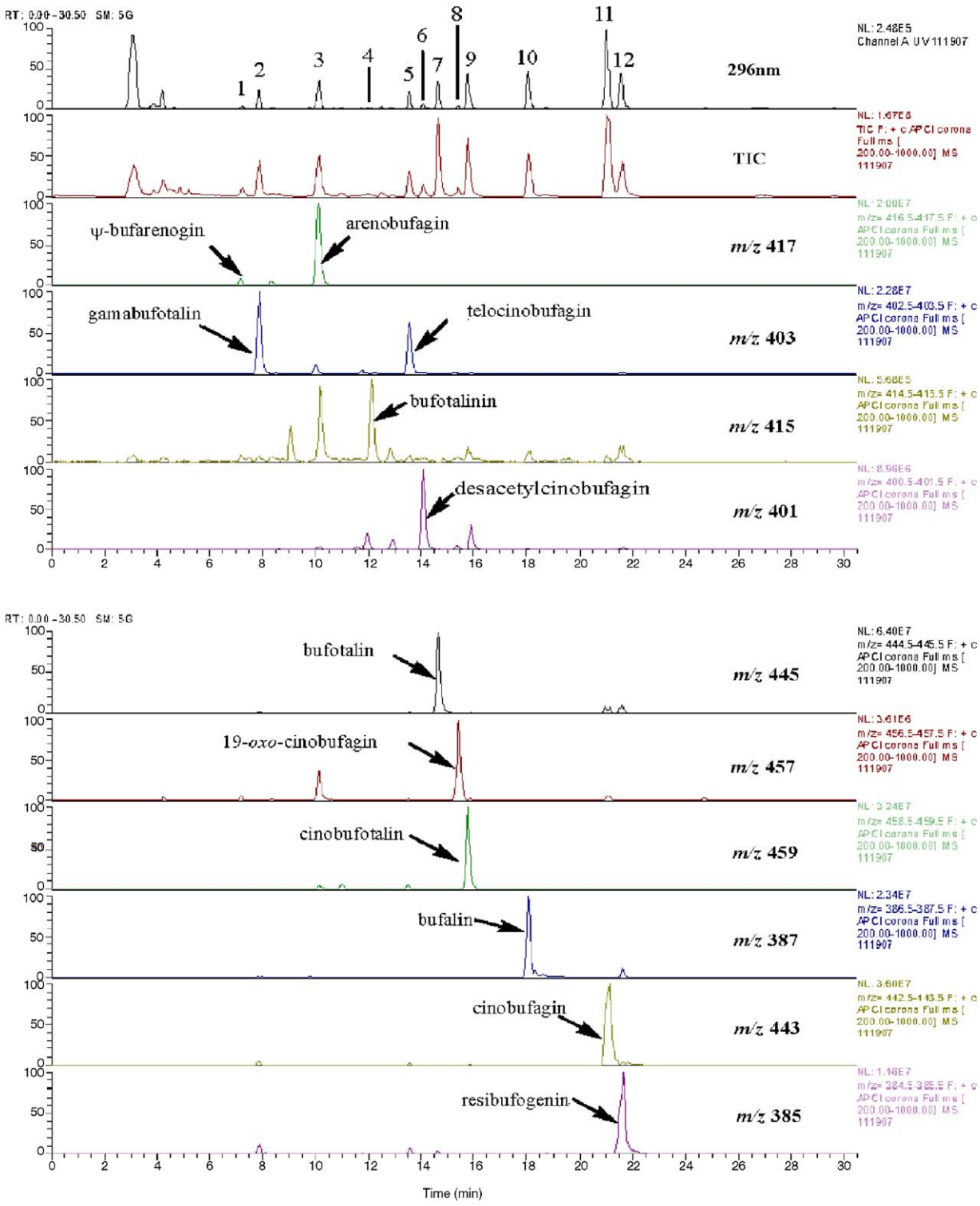

Fig. 3. Identification of bufadienolides in ChanSu by LC/MS analysis. From top to bottom, the chromatograms represent HPLC/UV profile monitored at 296 nm, LC/MS total ion current (TIC), and extracted ion chromatograms for different $[M+H]^+$ ions. For peak identification: 1, ψ-bufarenogin; 2, gamabufotalin; 3, arenobufagin; 4, bufotalinin; 5, telocinobufagin; 6, desacetylcinobufagin; 7, bufotalin; 8, 19-*oxo*-cinobufagin; 9, cinobufotalin; 10, bufalin; 11, cinobufagin; 12, resibufogenin.

Thus, all the significant peaks in the HPLC/UV profiles of ChanSu were unambiguously characterized, and they constitute >95% of total bufadienolides in the crude drug extract, preliminarily calculated on the basis of peak area.

3.4. Quantitative analysis of major bufadienolides in ChanSu crude drug by HPLC/UV

There have been a number of papers dealing with the quantitative analysis of bufadienolides in ChanSu and its preparations

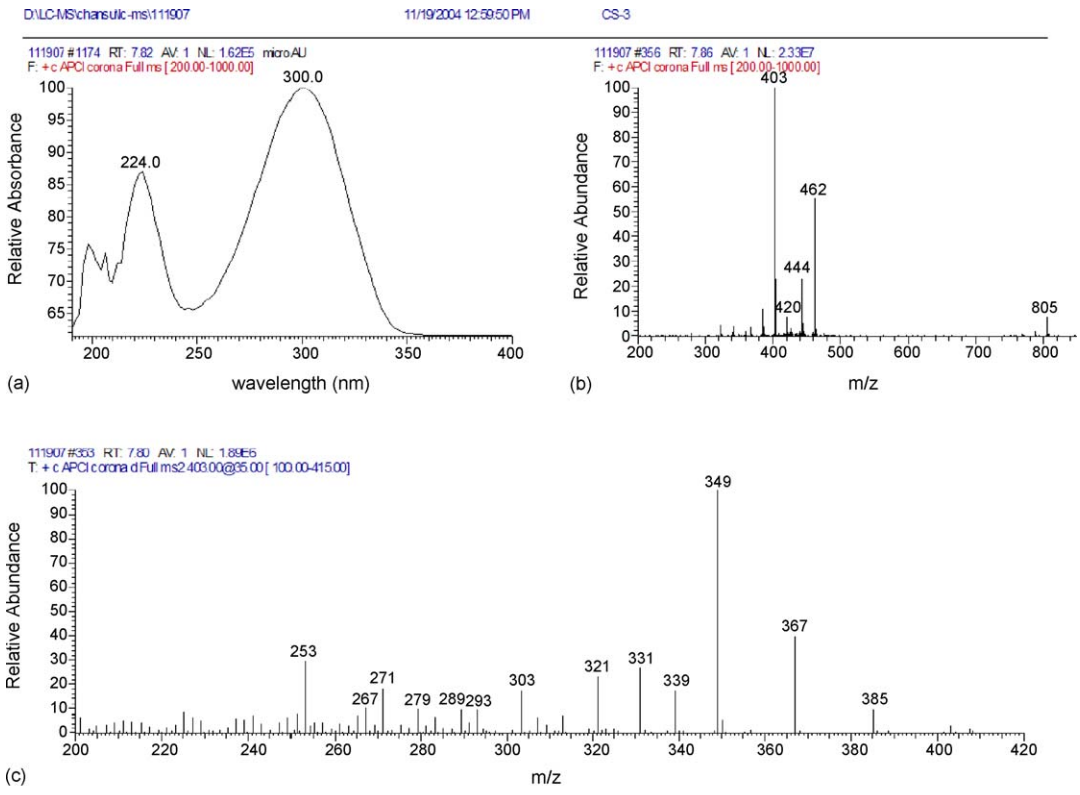

Fig. 4. Identification of gamabufotalin by HPLC/DAD/APCI-MS/MS: (a) online UV spectrum; (b) full scan mass spectrum, showing molecular adduct ions including $[M+H]^+ = m/z$ 403; $[M+18]^+ = m/z$ 420; $[M+42]^+ = m/z$ 444; $[M+60]^+ = m/z$ 462; and $[2M+H]^+ = m/z$ 805; (c) MS/MS spectrum for the $[M+H]^+$ ion at m/z 403.

by HPLC/UV. However, they only focused on cinobufagin, bufalin, and resibufogenin, which are commercially available, while the other bufadienolides were seldom involved [13,14]. In the present investigation, eight major bufadienolides were determined simultaneously in one single run within 30 min (Fig. 5). The optimization of HPLC conditions has been discussed in our previous report [15]. All the determined compounds could be completely separated from neighboring peaks except arenob-

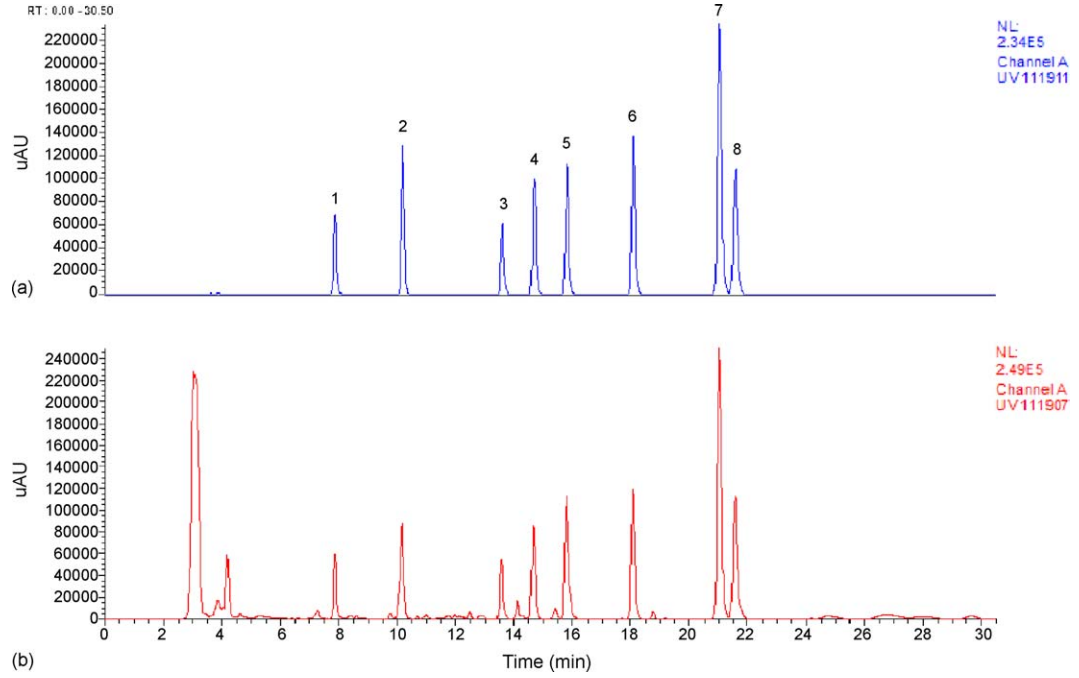

Fig. 5. HPLC/UV quantitation of major bufadienolides in ChanSu crude drug: (a) chromatogram of eight bufadienolide standards; (b) chromatogram of ChanSu methanol extract: 1, gamabufotalin; 2, arenobufagin; 3, telocinobufagin; 4, bufotalin; 5, cinobufotalin; 6, bufalin; 7, cinobufagin; 8, resibufogenin.

Table 4
Regression equations of bufadienolides for HPLC/UV quantitation ($n = 8$)

Retention time (min)	Compounds	$y = ax + b$		r^2	Concentration range (μg/ml)	LOD (ng)	LOQ (ng)
		Slope (a)	Intercept (b)				
7.89	Gamabufotalin	11583	−4761	0.9995	2–204	1.02	4.08
10.12	Arenobufagin	11437	12269	0.9999	4–376	1.75	3.76
13.59	Telocinobufagin	11149	5018	0.9999	2–184	0.92	3.68
14.62	Bufotalin	10703	9862	0.9999	3–320	1.60	3.84
15.71	Cinobufotalin	10078	11554	0.9999	4–392	0.78	3.92
18.07	Bufalin	11946	17753	0.9999	4–428	1.28	4.28
21.00	Cinobufagin	10330	52926	0.9997	1–992	1.19	4.96
21.45	Resibufogenin	11202	15256	0.9999	5–464	0.93	4.64

Note: In the regression equation $y=ax+b$, x refers to the concentration of pure bufadienolides (μg/ml); y the peak area; r, the correlation coefficient; LOD, limit of detection; LOQ, limit of quantitation.

ufagin, which had been found to be co-eluted with two minor bufadienolides by LC/MS [15]. Still, the peak at 10.12 min was calculated as pure arenobufagin because it constituted >80% of the peak area and it was difficult to be separated from the two interfering compounds. Purity of the other bufadienolides was confirmed by their on-line full scan MS spectra (Fig. 3).

Regression equations of the eight standards are given in Table 4. They all showed good linearity in broad concentration ranges. The limit of detection (LOD) was obtained as the amount to give a signal-to-noise ratio (S/N) of 3, and limit of quantitation (LOQ) to give S/N of 10.

Precision of the method was measured by analyzing the same sample five times (sample no. 3, as described below). The relative standard deviation (RSD) of peak area of each bufadienolide was <1.0%. The recovery test was carried out by adding a known amount of pure compounds to the sample, and the calculated recovery rates for all bufadienolides were in the range of 96.9–101.8% (Table 5).

To test stability of the analytes, the same sample was analyzed at an interval of 12 h for up to 48 h. Peak area RSD for all of the eight monitored bufadienolides were <3.0%, suggesting it is safe to analyze the sample within 2 days. However, the peak area of desacetylcinobufagin increased by around 10% after 30 days of storage at room temperature, and the content of cinobufagin decreased accordingly. This result suggested the gradual deacetylation of cinobufagin in normal conditions.

Five samples of ChanSu crude drug, including a toad skin sample were analyzed. Samples 1–4 were purchased in September to October of 2004 from drug stores in China, and sample 5 was obtained from China Institute for Control of Pharmaceutical and Biological Products (CICPBP) as a reference material. Samples 1, 2, 3 and 5 appeared as hard brown cake. Sample 4 was prepared from toad skin and presented as dark thin sheets. Voucher specimens were deposited in the authors' laboratory. Peak areas at 296 nm of the eight bufadienolides were recorded, and their contents in the crude drug were calculated according to the regression equations. The results are shown in Table 6.

The four ChanSu samples showed very similar HPLC profiles, except that their bufadienolide contents varied greatly. The total contents of samples 1, 3 and 5 were 13–18% (w/w), complying with the standards specified by Chinese Pharmacopoeia that contents of cinobufagin and resibufogenin should be >6% [18]. The bufadienolide contents of sample 2, however, were significantly lower. We consider that adulterant materials were artificially added to this sample during its processing, which is a serious factor that affects quality of TCM crude drugs in the Chinese herb market [4]. In the toad skin sample (no. 4), none of the eight bufadienolides was detected. Thus, toad skin should not be used as a source of ChanSu despite its long history of medicinal use.

Table 5
Recovery test for the determination of bufadienolides in ChanSu crude drug ($n = 3$)

Compounds	Detected (μg)	Original (μg)	Calculated (μg)	Added (μg)	Recovery (%)
Gamabufotalin	0.3381	0.1744	0.1637	0.1632	100.3
Arenobufagin	0.6347	0.3315	0.3032	0.3008	100.8
Telocinobufagin	0.3163	0.1717	0.1446	0.1472	98.2
Bufotalin	0.5538	0.2926	0.2612	0.2560	102.0
Cinobufotalin	0.7938	0.4895	0.3043	0.3136	97.0
Bufalin	0.7331	0.3907	0.3424	0.3424	99.9
Cinobufagin	1.9082	1.1200	0.7882	0.7936	99.3
Resibufogenin	0.8970	0.5276	0.3694	0.3712	99.5

Note: calculated amount = detected amount − original amount; recovery = calculated amount/added amount × 100%. The original amount was calculated according to the calibration.

Table 6
Contents of eight major bufadienolides in ChanSu drug samples (mg/g)

Compounds	Sample 1	Sample 2	Sample 3	Sample 4 (toad skin)	Sample 5
	Source				
	Beijing	Beijing	Zhejiang	Zhejiang	CICPBP
Gamabufotalin	9.13	0.82	8.73	0	6.57
Arenobufagin	15.32	0.64	16.60	0	16.09
Telocinobufagin	6.86	0.43	8.60	0	4.31
Bufotalin	14.03	1.01	14.66	0	7.54
Cinobufotalin	21.89	1.50	24.52	0	15.37
Bufalin	18.19	1.82	19.57	0	13.07
Cinobufagin	55.17	5.01	56.11	0	36.84
Resibufogenin	27.38	3.20	26.43	0	32.48
Total	168.02	14.45	175.25	0	132.30

4. Conclusions

A new strategy for global quality control of traditional medicine was proposed in this paper, and it was applied to the analysis of ChanSu, an antitumor Chinese medicine. In one single HPLC/DAD/APCI-MS/MS run within 30 min, >95% of the cytotoxic bufadienolides in methanol extract of ChanSu were qualitatively characterized by LC/MS, and >90% of them were quantitatively determined by HPLC/UV. The procedure is simple, rapid and accurate, and could be used for the routine analysis of ChanSu and its preparations.

Quality control has been a bottleneck issue for the modernization and globalization of traditional medicine. Our success in ChanSu analysis with this new strategy suggested its feasibility for the quality control of traditional medicine. Following this procedure, majority of the bioactive constituents of a crude drug could be clearly elucidated on both qualitative and quantitative basis, which is crucially important for stability and safety of a traditional medicine. HPLC-fingerprinting is a widely accepted technique for the analysis of traditional medicine [8,20]. However, only when its major peaks were identified could a fingerprint be valuable. Obviously, this kind of "informative" fingerprint could be closely correlated with pharmacological activities of the drug, and facilitate the discovery of essential components that take the therapeutic effects. Therefore, the LC/MS method established in this study could also be used for the fingerprinting analysis of ChanSu crude drug.

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