Preparative Isolation of Bufalin and Cinobufagin from Chinese Traditional Medicine ChanSu

Zhen Yang*, Huafei Luo, Hao Wang, and **Huimin Hou**
National Pharmaceutical Research Center, Shanghai Institute of Pharmaceutical Industry, Shanghai, P.R. China

Abstract

In this investigation, a method is developed for the isolation and purification of bufadienolides (resibufogenin, cinobufagin, and bufalin) from the Chinese traditional medicine ChanSu. The crude ChanSu extract is prepared with solvent refluxing under optimal conditions. A new chromatographic approach for separating bufadienolides utilizes silica gel column chromatography with isocratic elution using cyclohexane–acetone (5:1) as the mobile phase to separate resibufogenin, followed by reversed-phase C18 preparative HPLC column using isocratic elution of methanol–water (72:28) to separate bufalin and cinobufagin. Combining these two methods, bufalin and cinobufagin could be completely separated in high purity and recovery, and the amounts obtained were 1.9 and 3.1 g, respectively, from 500 g of ChanSu. These two compounds have been identified by mass spectrometry and 1H NMR, and their purities were quantitated by HPLC at 99% and 98%, respectively.

Introduction

ChanSu is a famous Chinese traditional medicine obtained from the dry, white secretion of the auricular and skin glands of Bufo bufo gargarizans (a toad), and is officially listed in the Chinese Pharmacopeia 2005 (16). ChanSu is also a major component of other Chinese medicines, including liu-shen-wan (a specific pill of six ingredients treating various diseases of the mouth cavity and throat), she-xiang-bao-xin-wan (curing coronary heart disease and angina), and niu-huang-xiao-yan-wan (eliminating inflammation and relieving pain) (1,2). Traditional uses of ChanSu include the stimulation of myocardial contraction, pain relief, and treatment of tonsillitis, sore throat, and palpitations (3). The cardiotonic effect of ChanSu is due to its major bufadienolides components, which are natural steroids with potent anti-tumor activities (4). It has been shown that bufadienolides exhibit significant inhibitory activities against human myeloid leukemia cells (K562, U937, ML1, and HL60), human hepatoma cells (SMMC7221), and prostate cancer cells (LNCaP, DU105, and PC3) (5–7). These activities are mediated by the induction of cell apoptosis and cell differentiation, and the regulations of a variety of genes and proteins involved in the process (8–10). Bufalin, cinobufagin, and resibufogenin are three major components of bufadienolides, and their contents in the crude drug could be as high as 5% of the dry weight (10–12). It is widely reported that bufalin blocks vasodilation and increases vasoconstriction, vascular resistance, and blood pressure via an inhibition of Na+/K+-ATPase. It has also been evaluated for interleukin-6 (IL-6) antagonistic activity due to growth-inhibitory activities on IL-6-dependent MH-60 cells (14,15). Due to structural similiarities, cinobufagin has almost the same pharmacological activity as bufalin, but is less potent; resibufogenin has the weakest pharmacological effect among the three components (13). The chemical structures of the three major compounds are shown in Figure 1.

Due to the definite pharmacological effect of pure compounds or a mixture of bufadienolides, there is a huge demand for bufalin and cinobufagin in their pure forms. There are detailed

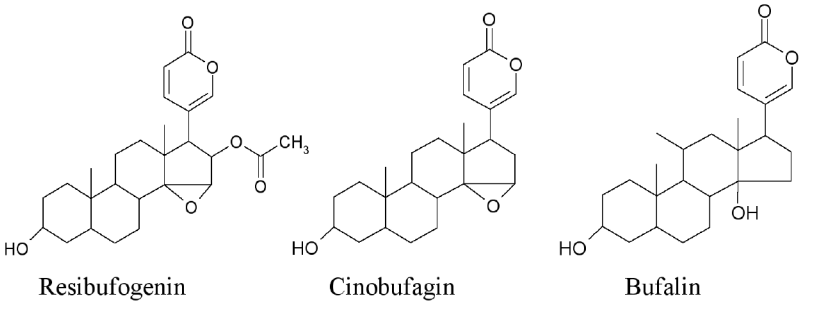

Figure 1. Chemical structures of the three major compounds of bufadienolides.

* Author to whom correspondence should be addressed: email zyang21@mail.uh.edu.

descriptions about separation conditions of ChanSu using silica gel column chromatography, but this process is tedious, time-consuming, and yields a lower recovery (16). After separation from silica gel column chromatography, a reasonably high purity fraction is achieved, but at the expense of the yield. Ye et al. (17) have analyzed dozens of different components in ChanSu by analytical-scale HPLC–MS, which does not provide sufficient amounts for further pharmaceutical development and pharmacology research.

This study is aimed at developing an efficient technique for the preparative isolation of bufadienolides with high purity and yield. Reversed-phase preparative HPLC is currently an alternative method for separation and purification of multiple compounds, especially during the investigation of herbal medicine. The entire procedure for isolating bufadienolides from ChanSu is represented in the flow diagram depicted in Figure 2. This improved method for the separation of bufadienolides not only provides a high purity and yield (as compared to repeated elution by silica gel column chromatography) but can also be extended to large-scale preparative separations.

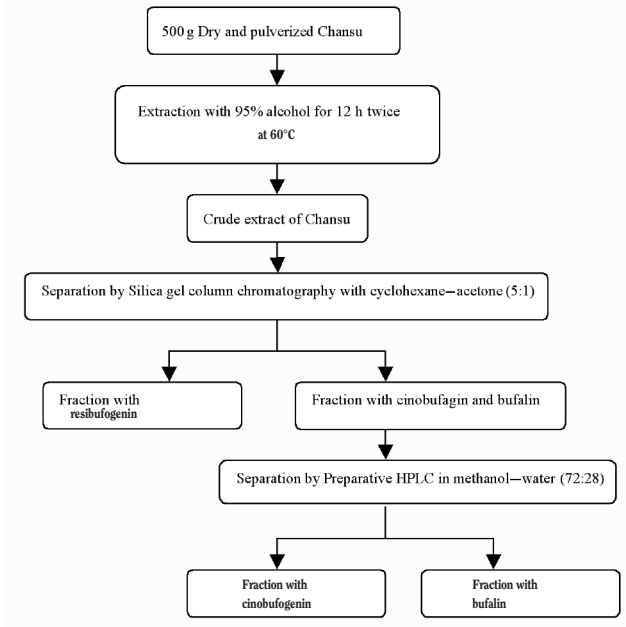

Figure 2. Flow diagram of the entire isolation procedure of bufadienolides from ChanSu.

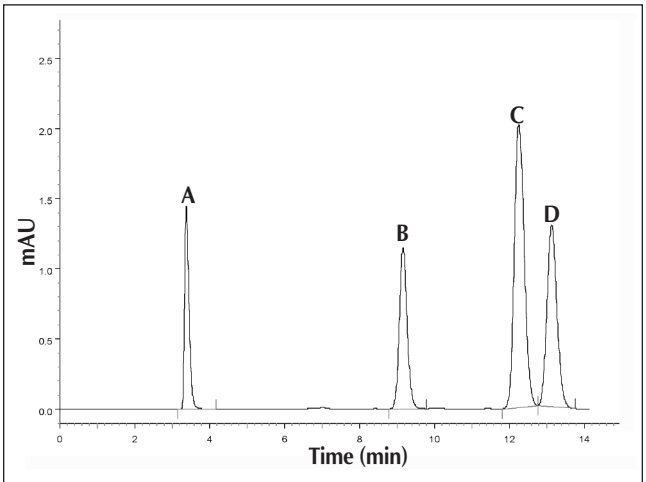

Figure 3. Analytical-scale HPLC chromatogram of the three major bufadienolides in fraction 3: acetone (A), bufalin (B), cinobufagin (C), resibufogenin (D).

Experiment

Instrumentation

Preparative HPLC was performed using a Shimadzu HPLC system (Kyoto, Japan), equipped with LC-8A pump, SCL-10Avp system controller, SPD-M10Avp UV–vis diode array detector (DAD), SIL-10ADvp degasser, SCL-10A series auto sampler, and fraction collector FRC-10A. The size of Preparative RP C18 column (Shimadzu) was 280 × 20 mm and the size of the Analytical RP C18 column (Diamonsil, Dikma Technologies, Beijing) was 250 × 4.6 mm. The Shimadzu ClassVp HPLC software was used for instrument control, data acquisition, and data analysis.

Materials and reagents

A custom-made glass column was 800 × 100-mm i.d. silica gel (1000 g, 200–300 mesh), analytical grade, was purchased from Qingdao Marine Chemical Factory (Qingdao, China). All organic solvents used for crude extraction and silica gel column chromatography were of analytical grade and were provided by Shanghai Chemical Reagents Co. (Shanghai, China). HPLC-grade methanol was purchased from Dima Technology Inc. (Richmond Hill, VA). Standard of bufalin was purchased from Sigma Chemical Co. (St. Louis, MO). Standards of cinobufagin and resibufogenin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The dry ChanSu was purchased from Shanghai Huayu Pharmaceutical Co. (Shanghai, China).

Preparation of crude extract

Prior to extraction, a batch of 500 g dry ChanSu was pulverized into powder by an electrical comminuter. The ChanSu powder and 1 L 95% ethanol were placed in a 2000-mL flask, and the solution was refluxed in water bath at 60°C for 12 h. The extraction process was repeated twice, then the extract was collected and evaporated to dryness in a rotary evaporator (60°C), to yield about 30 g of residue. The residue was stored in a dessicator for separation and purification studies, or until further use.

Separation by silica gel chromatography column

Approximately 30 g of crude extract was dissolved in 30 mL acetone and mixed with 10 g silica gel, which was then loaded on top of the silica gel column. The column was eluted with cyclohexane–acetone (5:1) at 3 mL/min and effluents were collected in 50 mL fractions. Each fraction was analyzed by analytical HPLC, and the elution order of the three major bufadienolides was $t_{resibufogenin} < t_{cinobufagin} < t_{bufalin}$. The HPLC chromatogram of the three components is shown in Figure 3.

Analytical-scale HPLC conditions

Chromatography was performed using a Shimadzu LC-10ADvp HPLC system with an analytical C18 Diamonsil column (250 × 4.6 mm i.d. × 5-µm particle size), and the mobile phase was isocratic, using acetonitrile–water (50:50) with a flow rate of 1 mL/min. The chromatography was performed at room temperature. Effluents were monitored on UV–vis DAD at a wavelength of 296 nm. The injection volume was 20 µL.

Separation by preparative HPLC

Chromatography was performed using a Shimadzu LC-10ADvp HPLC system equipped with a preparative C18 Shimadzu column (280 × 20 mm i.d. × 10 µm particle size). Injection volume was 2 mL, and the elution was isocratic, using methanol–water (72:28) with a flow rate of 5 mL/min and performed at room temperature for a total run time of 50 min. Effluents were monitored using a UV–vis DAD at 296 nm.

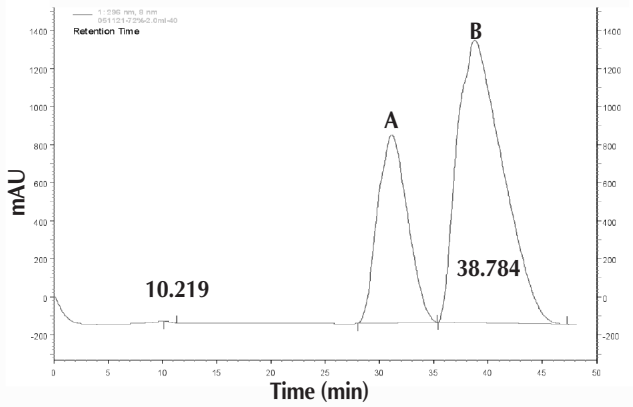

Figure 4. Preparative HPLC chromatogram of cinobufagin (A) and bufalin (B).

Processing procedures of effluent fractions

Through analytical HPLC of effluent fractions, resibufogenin was resolved from the other components. The solvents from these fractions were removed and the resibufogenin was dissolved in a small amount of acetone at 45°C, recrystallized by gradually cooling down to 4°C, and stored overnight. The overall weight of the resibufogenin was 2.5 g.

Cinobufagin and bufalin cannot be separated from each other in effluent fractions due to their similar chemical structures, which results in similar retention behavior in silica gel chromatography. After optimization of eluted solution, (decreasing the elution capability of solvent or utilizing gradient elution) there is little improvement on the poor resolution. Therefore, the effluent fractions containing these two components were collected. Finally, approximately 8 g residue was obtained after the solvent was evaporated in the rotary evaporator at 60°C. The residue was stored in a dessicator until further use. The 8 g sample containing cinobufagin and bufalin was dissolved in 160 mL methanol to form a concentration of 50 mg/mL solution for isolation by HPLC. The separation by preparative HPLC is shown in Figure 4.

The fractions of bufalin and cinobufagin were collected at 28–35 min and 36–45 min, respectively, and the mobile phase was removed by evaporating the solvent by rotary evaporation at 60°C. The residues were recrystallized in acetone and methanol, respectively, by gradually cooling to 4°C and were stored overnight. The yield was 3.1 g cinobufagin and 1.9 g bufalin.

Results and Discussion

The structural identification

The structural identification of cinobufagin and bufalin was carried out by UV, MS, 1H NMR spectra as follows:

Compound 1: needle crystal (MeOH); mp: 234–236°C; $UV_{max\ (MeOH)}$: 298 nm. ESI–MS: [M +H]+ (*m/z* 387), [M +H-18]+ (*m/z* 369), [M +H-36]+ (*m/z* 351), [M +H–36–96]+ (*m/z* 255), [M +H–36–28]+ (*m/z* 323), [M +H-54]+ (*m/z* 333), [M +H–54–28]+ (*m/z* 305). 1H NMR (500 MHz, D-acetone) δ: 0.75 (3H, s, 18-CH3), 0.94 (3H, s, 19-CH3), 2.55 (1H, t, 17-H), 3.12 (1H, s, 3-H), 3.32 (1H, s, 3-OH), 4.04(1H, s, 14-OH), 6.16 (1H, d, 23-H), 7.40 (1H, s, 21-H), 7.96 (1H, d, 22-H). The results were similar to those in reference (18,19). Therefore,

Table I. Comparisons of Different Extract Solvents and Extract Modes on Extract Recovery (%) (*n* = 3)

	Extract solvent			Extract mode	
	Chloroform	95% Alcohol	Acetic ether	Heat reflux	Cold soak
Bufalin	45.2 ± 4.6	78.5 ± 10.2	17.9 ± 3.4	78.5 ± 6.3	40.1 ± 4.2
Cinobufagin	41.3 ± 3.1	68.4 ± 7.3	15.5 ± 2.3	68.4 ± 4.5	36.7 ± 5.3

* Recovery (%) = the amount of compound in crude extract/the amount of compound in ChanSu.

Table II. Effects of Different Ratios of Elution System on Separation (*n* = 3)

Ratios of cyclohexane–acetone	Recovery of three components in different sections of elution fraction (%)					
	R	R + C	C	C + B	B	R + C + B
9:1	23.5 ± 3.4	–	8.8 ± 0.9	7.5 ± 1.1, 6.4 ± 1.4	11.9 ± 2.0	–
5:1	39.3 ± 5.6	10.3 ± 1.7, 9.7 ± 1.5	11.7 ± 2.3	38.7 ± 5.8, 35.6 ± 6.8	10.4 ± 1.1	–
3:1	11.2 ± 2.1	–	–	–	5.9 ± 1.3	41.6 ± 7.3, 58.6 ± 8.7, 43.3 ± 7.9

* B represents bufalin, C represents cinobufagin, and R represents resibufogenin. Recovery (%) = the amount of compound in collected fractions/the amount of compound in crude extract.

compound 1 corresponded to bufalin.

Compound 2: colorless crystal (acetone); mp: 213-215°C; $UV_{max\ (MeOH)}$: 295 nm. ESI–MS (*m/z*): [M+H]+ (*m/z* 443), [M +H-42]+ (*m/z* 401), [M+H-60]+ (*m/z* 383), [M+H–60–18]+ (*m/z* 365), [M+H–60–36]+ (*m/z* 347), [M+H–60–18–28]+ (*m/z* 337), [M+H–60–18–56]+ (*m/z* 319). 1H NMR (500 MHz, D-acetone) δ: 0.84 (3H, s, 18-CH3), 0.99 (3H, s, 18-CH3), 1.85 (3H, s, 17-OAC), 2.92 (1H, s, 17-H), 3.75 (1H, s, 15-H), 4.02 (1H, s, 3-OH), 5.52 (2H, d, 16-H), 6.12 (1H, d, 23-H), 7.32 (1H, s, 21-H), 7.93 (1H, d, 22-H). The results were similar to those in reference (18,19). Therefore, compound 2 corresponded to cinobufagin.

Optimization of extract conditions

Different kinds of solvents were used and different extract modes were employed for extraction. The results indicated that 95% alcohol and heat reflux were the best extraction conditions. The comparison of extraction recovery is given in Table I.

Optimization of silica gel column chromatography conditions

The extract sample of ChanSu was eluted by silica gel column with different ratios of cyclohexane–acetone at the flow rate of 3 mL/min (16). The elute was monitored by analytical HPLC. The recovery of the three components in elution fractions on separation with different ratios of cyclohexane–acetone is shown in Table II.

The elute capability of cyclohexane–acetone (9:1) was limited in that the three major components could not be completely eluted, and the separation recovery of the three major components was only 23.5 ± 3.4%, 8.8 ± 0.9%, and 11.9 ± 2.0%, respectively. When cyclohexane–acetone (5:1) was used as the elution solvent, five sections of effluent fractions were collected from the column. The third section was a mixture containing cinobufagin (38.7 ± 5.8%) and bufalin (35.6 ± 6.8%). Resibufogenin could be roughly separated using this elution system, but only 11.7% and 10.4% of cinobufagin and bufalin, respectively, could be completely isolated, due to their poor resolution in silica gel chromatography. The elution capability of cyclohexane–acetone (3:1) was so strong that a large amount of the three major components were mixed together in effluent fractions that did not achieve separation. The condition was not able to thoroughly isolate bufalin and cinobufagin in the silica gel chromatography column. In comparison, the use of cyclohexane–acetone (5:1) had achieved the largest amounts of crude separation mixtures including bufalin and cinobufagin. The crude separation mixtures containing bufalin and cinobufagin were left for separation by preparative HPLC.

Optimization of chromatography conditions for preparative HPLC separation

The optimization of chromatography conditions for preparative HPLC is very important because the isolation of several grams of high purity compounds in a laboratory needs dozens of hours, even several days, to complete. Therefore, productivity rate is a major element to be considered, along with selectivity, resolution, purity, and yields, when optimizing chromatography conditions. In order to shorten the cycle time and increase the productivity rate of separation while retaining high yield and purity, chromatography conditions were optimized in following aspects: mobile phase and injection volume.

Effect of different ratios of mobile phase on preparative separation

In order to optimize the separation of the two components, methanol–water (65:35), (70:30), (72:28), and (75:25), and four different ratios of mobile phases were tested for optimization. Methanol is typically used for preparative chromatography because it is usually cheaper than acetonitrile. Effects of different ratios of mobile phase on run-time and separation resolution are shown in Figure 5.

With the increase of methanol in the mobile phase, the run-time is reduced while the resolution is decreased. A 46 min isocratic run consisting of methanol–water 72:28 provided a resolution of 1.2.

Table III. Comparison of Separation Efficiency of Bufadienolides Between the Two Methods (*n* = 3)

	Purified by preparative HPLC		Purified by silica gel column chromatography	
	Bufalin	Cinobufagin	Bufalin	Cinobufagin
HPLC purity (%)	99.8 ± 3.2	99.2 ± 3.6	94.9 ± 4.3	93.6 ± 5.2
Absolute purity (%)	97.9 ± 4.5	97.1 ± 4.2	89.7 ± 8.4	88.5 ± 5.4
Recovery (%)*	32.2 ± 6.7	35.1 ± 7.8	10.4 ± 4.8	11.7 ± 6.2

* Recovery (%) = the amount of a component in purified product/the amount of a component in crude extract.

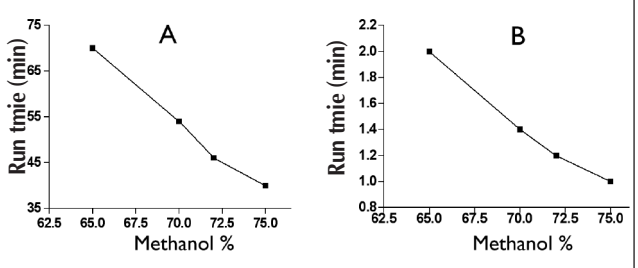

Figure 5. Effects of different ratios of mobile phase on run time (A) and resolution (B) of separation. Injection volume: 1.5 mL.

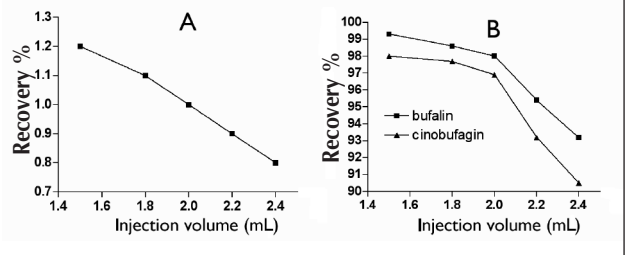

Figure 6. Effects of different injection volume on resolution (A) and recovery (B). Mobile phase is methanol–water (72:28) and the purity of two components which exhibit online are demanded >98%.

Effect of different injection volumes on preparative separation

After the chromatography column, mobile phase, rate, and other conditions were determined, the injection volume was the most important element which determined the resolution and recovery of separation. Several different injection volumes (1.5, 1.8, 2, 2.2, and 2.4 mL) were tested for optimization. Effects of different injection volumes on resolution and recovery are shown in Figure 6.

As the injection volume increased, the resolution decreased accordingly. When the injection volume > 2 mL, the two components could not be separated completely, which resulted in a lower recovery of the preparative separation (< 97%). The purity of bufalin is higher than that of cinobufagin because the previous fraction of bufalin can influence the later fraction of cinobufagin due to tailing phenomenon when the resolution is close to 1. In order to keep a high purity (> 97%) of two components, the latter part of fraction of bufalin and the front part of fraction of cinobufagin were not collected. Therefore, 2.0 mL was chosen as the final injection volume, which provided the best balance between a large injection volume and high separation recovery.

Comparison of separation efficiency of bufalin and cinobufagin between preparative HPLC and silica gel column chromatography

Preparative HPLC for the separation and purification of bufalin and cinobufagin has several advantages over traditional silica gel column chromatography. Firstly, the peak purity of these two components exhibited in preparative HPLC is as high as 0.99%, and the absolute purity is above 97%. In contrast, the purity of bufalin and cinobufagin obtained from the silica gel column is 89%. Table II lists the purity, recovery, and yield of bufalin and cinobufagin obtained by the two different separation techniques. This clearly indicates that the compound separated by preparative HPLC has higher purity and recovery than the same compound obtained only from silica gel column chromatography. Secondly, due to its short cycle time and high sample load, the separation efficiency and production rate of HPLC are much higher than in silica gel column chromatography, and it can be extended to large-scale preparative separation. Lastly, HPLC can exhibit the selectivity and resolution of fraction peak online, which made the optimization of preparative HPLC condition and monitoring effluent fractions very easy and efficient as a result.

Conclusion

Isolation and purification of a compound through preparative HPLC is not a novel method in herbal chemistry, but applications for preparative HPLC are limited. The methodology developed for isolation of bufalin and cinobufagin provided high recovery and purity compared to traditional silica gel column chromatography. It provides an alternative method for isolating bufalin and cinobufagin more efficiently and with high purity.

Reference

1. Z. Hong, K. Chan, and H.W. Yeung. Simultaneous determination of bufadienolides in the traditional Chinese medicine preparation, liu-shen-wan, by liquid chromatography. *J. Pharm. Pharmacol.* **44:** 1023–1026 (1992).
2. E. Akiko, R. Mun-Chual, K. Kanki, and H. Masahiko. Inhibitory effects of bufadienolides on interleukin-6 in MH-60 cells. *Notes. J. Nat. Prod.* **67:** 2070–2072 (2004).
3. K.K. Chen and A. Kovarikove. Pharmacology and toxicology of toad venom. *J. Pharm. Sci.* **67:** 376–380 (1967).
4. A.B. David, D. Pradip, W. Alice, and D. Amitava. Falsely elevated serum digitoxin concentrations due to cross-reactivity of water-extractable digitoxin-like immunoreactivity of Chinese medicine ChanSu: elimination of interference by use of a chemiluminescent assay. *Clin. Chim. Acta* **300:** 151–158 (2000).
5. Y. Masuda, N. Kawazoe, S. Nakajo, T. Yoshida, Y. Kuroiwa, and K. Nakaya. Bufalin induces apoptosis and influences the expression of apoptosis-related genes in human leukemia cells. *Leukemia Res.* **19:** 549–556 (1995).
6. S. Hashimoto, Y. Jing, N. Kawazoe, Y. Masuda, S. Nakajo, T. Yoshida, Y. Kuroiwa, and K. Nakaya. Bufalin reduces the level of topoisomerase II in human leukemia cells and affects the cytotoxicity of anticancer drugs. *Leukemia Res.* **21:** 875–883 (1997).
7. M.B. Pamnani, S. Chen, and H.J. Bryant. Effect of three sodium-potassium adenosine triphosphate inhibitors. *Hypertension* **18:** 316–324 (1991).
8. W.Y. Chan, T.B. Ng, and H.W. Yeung. Examination for toxicity of a Chinese drug, the total grandular secretion product ChanSu in pregnant mice and embryos. *Biol. Neonate.* **67:** 376–380 (1995).
9. L. Krenn and B. Kopp. Bufadienolides from animal and plant sources. *Phytochem.* **48:** 1–29 (1998).
10. P.S. Steyn and F.R. van Heerden. Bufadienolides of plant and animal origin. *Nat. Prod. Rep.* **15:** 397–413 (1998).
11. T. Nogawa, Y. Kamano, A. Yamashita, and G.R. Pettit. Isolation and structure of five new cancer cell growth inhibitory bufadienolides from the Chinese traditional drug ChanSu. *J. Nat. Prod.* **64:** 1148–1152 (2001).
12. J.Y. Yeh, W.J. Huang, S.F. Kan, and P.S. Wang. Effects of bufalin and cinobufagin on the proliferation of androgen dependent and independent prostate cancer cells. *Prostate* **54:** 112–124 (2003).
13. L.S. Zhang, K. Nakaya, T. Yoshida, and Y. Kuroiwa. Bufalin as a potent inducer of differentiation of human myeloid leukemia cells. Biochem. Biophys. *Res. Commun.* **178:** 686–693 (1991).
14. L.S. Zhang, K. Nakaya, T. Yoshida, and Y. Kuroiwa. Induction by bufalin of differentiation of human leukemia cells HL60, U937, and ML1 toward macrophage/monocyte-like cells and its potent synergistic effect on the differentiation of human leukemia cells in combination with other inducers. *Cancer Res.* **52:** 4634–4641 (1992).
15. C.H. Xiao. Chemistry of Chinese traditional medicine. 1st edition. Shanghai Science and Technical Publication, Shanghai 1998, pp. 523–526.
16. M. Ye and D.A. Guo. Analysis of bufadienolides in the Chinese drug ChanSu by high-performance liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry. Rapid Commun. *Mass Spectrom.* **19:** 1881–1892 (2005).
17. K. Yoshiaki, Y. Hiroshi, T. Yoshihiro, and K. Manki. The isolation and structure of new bufadienolides, 3-(hydrogensuberates) of resibufogeninin, cinobufagin and bufalin the structures of the so-called "bufotoxins". *Tetrahedron Lett.* **54:** 5673–5676 (1968).
18. Z. Wang, J. Wen, J. Zhang, M. Ye, and D. Guo. Simultaneous determination of four bufadienolides in human liver by high-performance liquid chromatography. *Biomed. Chromatogr.* **18:** 318–322 (2004).

Manuscript received October 10, 2006;
revision received February 12, 2007.