J. Pharm. Pharmacol. 1992, 44: 1023–1026
Received October 8, 1991

Simultaneous Determination of Bufadienolides in the Traditional Chinese Medicine Preparation, Liu-Shen-Wan, by Liquid Chromatography*

Z. HONG‡, K. CHAN†‡§ AND H. W. YEUNG‡¶

‡*The Chinese Medicinal Materials Research Centre, and Departments of* §*Pharmacology, and* ¶*Biochemistry, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong*

Abstract—The bufadienolide compounds (bufalin, cinobufagin and resibufogenin), major constituents of Chansu in Liu-Shen-Wan (LSW), were determined by reverse phase high performance liquid chromotography. The procedure involves a preliminary extraction of the bufadienolides from LSW with chloroform using ultrasonication and subsequent evaporation to dryness of the chloroform extract. The residue of the chloroform extract was dissolved in methanol and separated on a Merck LiChrosorb RP-18 column. Methanol : water (74 : 26) was used as mobile phase. The compounds were satisfactorily separated with good chromatographic peaks. Good coefficients of correlation ($r > 0.999$) were obtained from the calibration of peak areas with concentrations for the 3 bufadienolides. Results of analysis showed that there were differences between the contents of bufadienolides in 11 LSW samples of different origin available to the public in Hong Kong where at present there is no legal control over the sale of traditional Chinese medicines. The variability of quantities of bufadienolides in Chansu may be a hazard to the public.

The bufadienolide compounds, bufalin, cinobufagin and resibufogenin, are the major steroidal compounds isolated from Chansu (*Venenum bufonis*), the medicinal toad venom, which is the dried white secretion of the auricular glands and the skin glands of *Bufo melanostictus* Schneider or *Bufo bufo gargarizans* Gantor as shown in Fig. 1. Chansu is included as

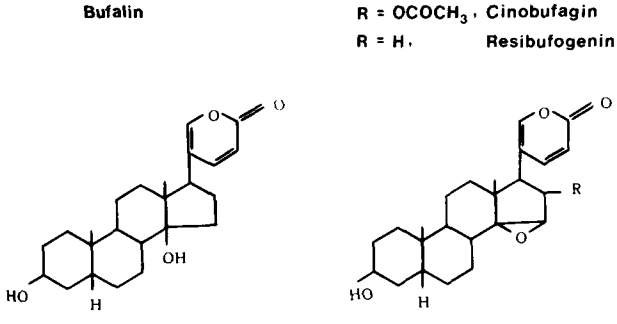

FIG. 1. Structures of bufadienolides in Chansu.

a major component in Liu-Shen-Wan (LSW), a proprietary traditional Chinese medicine, which is translated as "pill of six ingredients with magical effects" and used for treatment of tonsillitis, sore throat, furuncle and telephium. The other five ingredients are pearl, musk, realgar, borneol and cow-bezoar (*Calculas bovis*). LSW is also claimed to possess local anaesthetic and antibiotic activities. At high doses it is extremely toxic resulting in acute poisoning with cardiac arrhythmia, breathlessness, convulsion, coma and other severe side-effects. Even so, it is claimed that this traditional medicine is safe to take. The proprietary product is freely available to the public. In fact, the supply of most traditional Chinese medicines to the public in Hong Kong is not controlled by the Pharmacy Ordinance if these products contain only substances from herbal sources. The quality and safety of these products is not officially controlled in Hong Kong. We now report the analysis of the bufadienolides in some of these products using reversed phase high performance liquid chromatography (RP-HPLC).

* A part of the results was presented at the British Pharmaceutical Conference held in Merseyside, September 1991.

† Present address and correspondence: K. Chan, Liverpool School of Pharmacy, Byrom Street, Liverpool L3 3AF, UK.

Materials and Methods

Apparatus

The liquid chromatography system consisted of a Waters 510 pump linked to a U6k injector and a Waters 484 tunable absorbance detector. Chromatograms were recorded on a Waters 746 data module (Waters Associates, Milford, MA, USA). Analyses were performed on a reverse-phase C18 column (Hibar, LiChrosorb, RP-18, 250 × 4·0 mm i.d., 5 μm, Merck) linked to a C18 pre-column (RP-18, 40 × 4·0 mm i.d., 10 μm, Merck). The operational conditions for the HPLC system were: mobile phase, methanol–water (74:26 v/v) which was degassed in an ultrasonicator for 30 min and was run at a flow rate of 0·7 mL min^{-1} at ambient temperature (20 ± 2°C). The eluates were detected at 300 nm and the sensitivity scale was set at 0·0·01 aufs.

Other apparatus included 15 mL glass centrifuge tubes with well fitting screw caps, 5 mL glass centrifuge tubes and 10 mL volumetric flasks. All glassware was cleaned and silanized according to a procedure previously described, to avoid drug loss (Chan & Dehghan 1978).

Materials

The following materials were used: Analar grade organic solvents, chloroform and methanol (Merck, Darmstadt, Germany), water, double-distilled in a glass apparatus; bufalin and cinobufagin, purchased from Sigma Chemical Co. (St Louis, MO, USA); resibufogenin purchased from Beijing, China; other LSW products were purchased from local traditional Chinese medicine shops in Hong Kong and China.

Table 1. Correlation between mean peak-area (±s.d.) and concentration of bufalin, cinobufagin and resibufogenin.

Bufalin (n = 3)		Cinobufagin (n = 3)		Resibufogenin (n = 3)	
Weight (ng)	Peak area	Weight (ng)	Peak area	Weight (ng)	Peak area
7·7	17·7 ± 3·2	8·5	26·6 ± 3·0	9·1	24·3 ± 0·6
38·7	76·8 ± 1·2	42·7	81·9 ± 5·1	45·4	92·7 ± 3·3
77·3	150·5 ± 5·8	85·3	158·3 ± 3·9	90·7	184·0 ± 3·8
116·0	221·0 ± 6·4	128·0	232·4 ± 10·0	136·1	270·8 ± 9·3
154·9	297·4 ± 2·7	170·6	307·0 ± 3·6	181·4	362·5 ± 3·2
193·3	375·2 ± 5·1	213·3	393·8 ± 4·6	226·8	448·1 ± 9·1
*Batch standard at 116·0 (n = 8) Calibration graph Y = 1·901X + 1·653 r = 0·9995	221·0 ± 5·7	*Batch standard at 128.0 (n = 8) Calibration graph Y = 1·816X + 2·238 r = 0·9996	232·4 ± 7·7	*Batch standard at 136·1 (n = 8) Calibration graph Y = 1968X + 3·671 r = 0·9991	270·0 ± 8·3

* Batch standard = a measure of between-day precision.

Preparation of reagents, standards and quantitation

Standard methanolic solutions of bufalin, cinobufagin and resibufogenin were prepared at 0·58, 0·64 and 0·68 mg mL^{-1}, respectively, and stored at 0°C before analysis. Standards calculated as ng of drug per 20 μL in methanol were made and diluted using methanol to cover the calibration range 0–200 ng for each of the bufadienolides. Analysis and measurement at each concentration point of the calibration line was performed three times. Calibration graphs were constructed by plotting the peak-areas corresponding to the three compounds against the known concentrations. Correlation between peak-areas and concentrations were calculated and correlation coefficients were obtained. Quantitation of unknown samples was achieved by the respective peak-area to obtain the concentration from the calibration graph.

Recovery study

The recovery of bufadienolide using the general procedure was assessed by adding the standards to samples of powdered LSW in a centrifuge tube. For comparison, the same weight of LSW was prepared in another centrifuge tube. These samples were extracted and assayed as described under the general procedure. The concentrations were obtained by the peak-areas from the calibration graph and average recovery was calculated by difference between the samples with and without added standard.

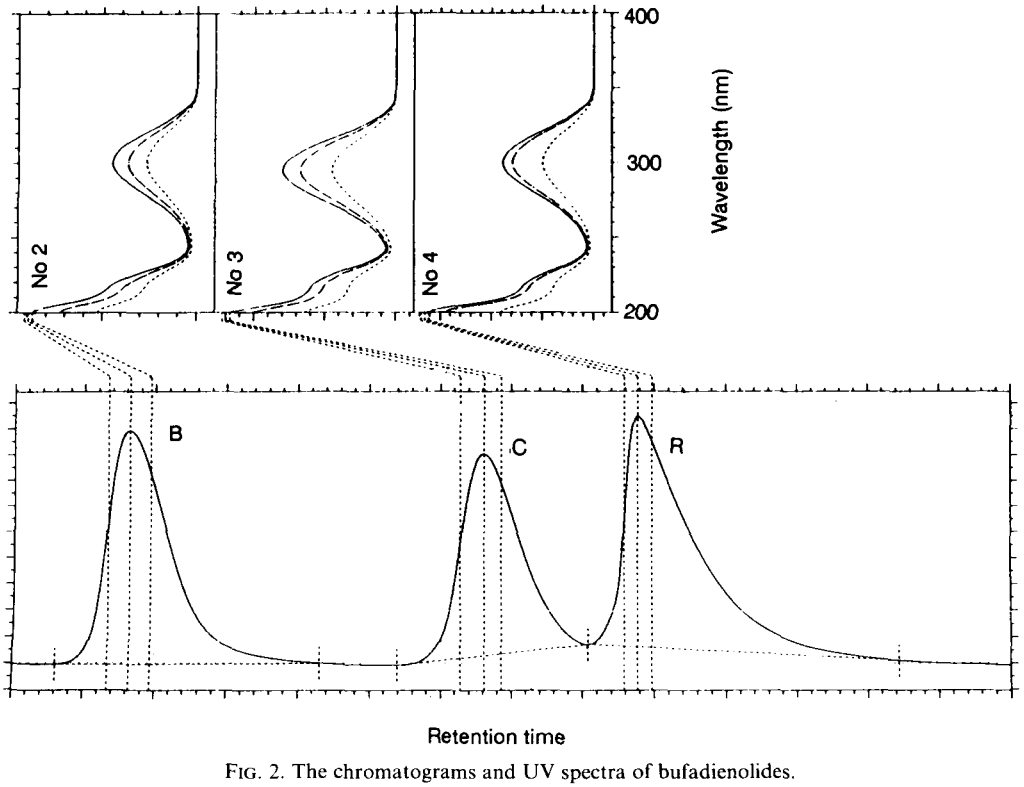

FIG. 2. The chromatograms and UV spectra of bufadienolides.

General procedure

In a 15 mL glass centrifuge tube, an accurately weighed (100 mg) sample of pulverized LSW was extracted twice with 2 mL of chloroform by shaking the tube vertically using a vortex mixer for 30 s and vibration in an ultrasonicator for 30 min. The contents of the tube were centrifuged for 15 min at 2500 *g*. The chloroform extract was transferred into a 10 mL volumetric flask. The residue was washed twice with 2 mL of chloroform using a vortex mixer for 30 s. After centrifugation for 10 min at 2500 *g*, the chloroform extracts were combined into the same volumetric flask, and diluted to volume with chloroform. A 0·1 mL sample of chloroform extract was placed into a 5 mL glass centrifuge tube and evaporated to dryness in a water bath at 40°C under a gentle stream of nitrogen. The residue of the chloroform extract was redissolved in 1 mL of methanol and centrifuged for 15 min at 3000 *g*. Finally a 20 μL sample of the methanolic solution was injected into the HPLC system for analysis.

Table 2. Percentage of bufalin, cinobufagin and resibufogenin in eleven Liu-Shen-Wan proprietary products.

Liu-Shen Wan samples*	Content (%) (n = 3)		
	Bufalin	Cinobufagin	Resibufogenin
Chansu†	2·844	6·264	6·122
Hong Kong 1	0·303	0·827	0·451
Hong Kong 2	0·362	1·003	0·546
Hong Kong 3	0·634	1·582	0·679
Shanghai	0·228	0·545	2·384
Guangzhou	0·251	0·455	0·581
Wuhan	0·163	0·319	0·213
Suzhou	0·397	0·851	0·921
Sichuan	0·264	0·401	0·812
Chengdu	0·206	0·295	0·604
Chuongqing	0·266	0·434	0·696
Hangzhou	0·376	0·404	1·312

* Samples were manufactured from various factories in Hong Kong and China. † Chansu is the medicinal toad venom (*Venenum bufonis*).

Results and Discussion

Abel & Macht (1912) first isolated two crystalline substances, bufagin and adrenaline, from the skin secretion of a tropical toad *Bufoaqua*. Later, Chen & Jensen (1929) isolated from Chansu (a commercial preparation of toad venom), adrenaline, cholesterol and two more digitalis-like products, cinobufagin and cinobufotoxin.

Several methods of analysis of bufadienolides have been reported in Chinese or Japanese (Kuchi 1977; Nei et al 1980; Zhang et al 1984). We have modified some of these assays for the simultaneous determination of bufalin, cinobufagin and resibufogenin.

The UV spectra of the bufadienolides

For optimal UV detection the wavelength was set at 300 nm which is the maximum absorbance wavelength for the three compounds as shown in Fig. 2. These UV spectra were obtained using the Waters 994 programmable photodiode array detector. At this wavelength, the maximum sensitivity and selectivity for detection was obtained for the bufadienolides while other substances did not interfere with the chromatograms.

Performance of the HPLC system

Fig. 3 shows chromatograms of the bufadienolides in standard solutions and extracts from Chansu and the 11 different LSW products. The analytical peaks were satisfactorily separated with good peak shapes. The respective retention times of bufalin, cinobufagin and resibufogenin were 9·5, 11·2 and 13·0 min. The overall analysis time was 16 min.

Calibration and recovery

The calibration graph relating peak area and concentration

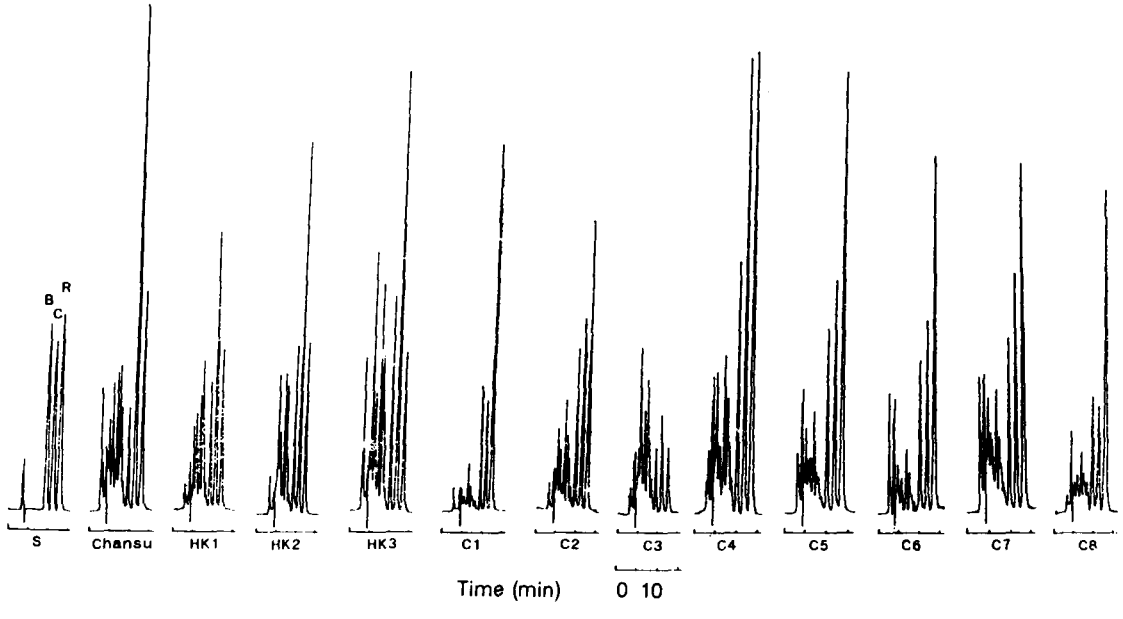

FIG. 3. HPLC of extracts of LSW samples. B, bufalin; C, cinobufagin; R, resibufogenin.

of each of the bufadienolides was linear over the calibration range (Table 1). The correlation coefficients were satisfactory. The interbatch variation was 2·6, 3·3 and 3·1% at 221·0, 232·4 and 270·8 ng mL^{-1} of bufalin, cinobufagin and resibufogenin, respectively (Table 1).

The average recovery of bufalin, cinobufagin and resibufogenin from the preparations after chloroform extraction was 102·4% (CV = 3·6%; n = 3), 101·6% (CV = 2·8%; n = 3) and 103·5% (CV = 4·4%; n = 3), respectively.

Application

In Hong Kong, China and some south-east Asian countries, LSW has been accepted by the public and traditional herbalists as a useful and safe remedy for treatment of minor ailments including inflammation, bacterial infection, pain and influenza. The dose recommended usually ranges from 8 to 10 pills once or twice per day. Most of these products do not include an expiry date. Chansu is the most toxic of the 6 ingredients included in LSW; realgar is also toxic. In a recent review of the literature reporting toxicity of LSW from 1964 to 1989, Yuen & Tan (1990) observed that of 27 cases, 11 were overdosed (including 4 deaths), 11 cases were related to allergic reactions (including two of anaphylactic shock), 3 contact dermatitis (after topical application), one suffered from difficulty in swallowing and one complained of dizziness, shortage of breath and violent vomiting with severe stomach cramp. Some of these symptoms are related to the cardiotoxicity of Chansu. The HPLC procedure was used to determine the concentrations of bufadienolides in LSW. Results suggest a wide variation in the amounts of the three compounds in the 11 different LSW samples (Table 2). The first 3 samples were obtained from Hong Kong manufacturers while the rest were purchased from China. Such variation may be due to the source of the toad venom used, the amount included in the formulation and variation in the manufacturing process. Thus, we believe the amount of toxic substances in LSW products should be quality controlled. It is recommended that the public should be aware of potential hazard of non-legal medicines and that legislation in controlling this type of medicine is required.

The present HPLC method has several advantages. The extraction procedure is simple to operate. The analytical peaks are well resolved, and good coefficients of correlation between peak areas and concentrations were obtained without the use of an internal marker. Satisfactory average recoveries of the bufadienolides from various products were achieved. The HPLC analysis is sensitive and the assay only used a small amount of LSW sample. This method can be used to quality control Chansu in LSW or other traditional Chinese medicines containing these toxins. Development of other analytical techniques is required for the quantitation of the other five ingredients. Such assays are needed for setting up guidelines for quality control of these types of herbal medicines.

Acknowledgement

We are grateful to Mr N.S. Lee who allowed one of us (Z. Hong who is a visiting scientist from West China University of Medical Sciences, China) to spend some time in the Hong Kong Government Laboratories for familiarization with the analytical work.

References

Abel, J. J., Macht, D. D. (1912) Two crystalline pharmacological agents obtained from the tropical toad Bufoaqua. J. Pharmacol. Exp. Ther. 3: 319–377

Chan, K., Dehghan, A. (1978) The isolation and determination of neostigmine, pyridostigmine and their metabolites in human biological fluids. J. Pharmacol. Methods 1: 311–320

Chen, K. K., Jensen, H. (1929) Crystalline principles from Chan Su, the dried venom of the Chinese toad. Proc. Soc. Exp. Biol. Med. 26: 278–280

Kuchi, K. (1977) Studies on the quality estimation of drugs of animal origins 1. The determination of bufadienolides in Chansu (Senso) and its preparation. Yakuzaku Zasshi 97: 274 (in Japanese)

Nei, R.-H., Zhang, Q.-M., Qian, J. (1980) Separation and determination of bufadienolides compounds in Chansu. Chinese Traditional Pat. Med. 5: 41–44 (in Chinese)

Yuen, W.-N., Tan, D.-J. (1990) Undesirable reactions caused by Liu Shen Wan. Ibid. 12: 24–25 (in Chinese)

Zhang, Y.-Z., Yang, F., Wang, Z.-L. (1984) TLC densitometric determination of steroidal contents in Chinese medicines containing Chansu. Chinese J. Pharmac. Anal. 4: 321–323 (in Chinese)