Chem. Pharm. Bull. 24(12)2995—3000(1976)

UDC 547.92.02.05 : 543.544.061

Separation and Determination of Cardiac Steroids by High-Pressure Liquid Chromatography[1]

Kazutake Shimada, Masatoshi Hasegawa, Kumiko Hasebe, Youichi Fujii,[2] and Toshio Nambara[2a]

Pharmaceutical Institute, Tohoku University[2]

(Received March 26, 1976)

A method for the simultaneous analysis of cardiac steroids by high-pressure liquid chromatography has been developed. Reversed phase chromatography on a μ-Bondapak C_{18} column was particularly effective for the separation of sixteen bufadienolides and nine cardenolides. Determination of the principal bufogenins in the fresh venom of the Japanese toad was carried out by the internal standard method. The relationship between chemical structures and retention values is also discussed.

Numerous papers dealing with the separation, characterization and determination of cardiac steroids have already been reported.[3-6] These methods, however, are not necessarily satisfactory with respect to sensitivity, rapidity and simplicity. In recent years considerable attention has been directed toward the application of high-pressure liquid chromatography (HPLC) to the separation and purification of physiologically active natural products.[7] In these laboratories the isolation and characterization of cardiac steroids from the Japanese toad has been carried out.[8] For the comparative biochemical studies a method for the simultaneous analysis of cardiac steroids in the toad venom has become an essential prerequisite. The present paper describes a new procedure for the separation of cardiac steroids without derivatization by HPLC and its application for the analysis of the venom secreted from the Japanese toad.

An initial study was focused on the chromatographic separation of the common cardiac steroids under the various conditions. The retention times relative to bufalin or digitoxigenin used as a reference were determined with sixteen bufadienolides and nine cardenolides. It is evident from the data listed in Table I and II that reversed phase chromatography on a μ-Bondapak C_{18} column using methanol/water and tetrahydrofuran/water as a mobile phase gave a complete separation of all the steroids. In particular, two pairs of epimeric 3-hydroxylic and 14,15-epoxy compounds were distinctly resolved, when chromatographed on a μ-Porasil or Corasil I column with hexane/tetrahydrofuran.

1) Part CXV of "Studies on Steroids" by T. Nambara; Part CXIV: K. Shimada, M. Hasegawa, K. Hasebe, Y. Fujii, and T. Nambara, *J. Chromatog.*, **124**, 79 (1976).
2) Location: *Aobayama, Sendai*; *a*) To whom any inquiries should be addressed.
3) E. Iseli, M. Kotake, Ek. Weiss, and T. Reichstein, *Helv. Chim. Acta*, **48**, 1093 (1965).
4) M. Komatsu, Y. Kamano, and M. Suzuki, *Bunseki Kagaku*, **14**, 1049 (1965); M. Komatsu and T. Okano, *ibid.*, **15**, 1115 (1966).
5) K. Sakurai, E. Yoshii, H. Hashimoto, and K. Kubo, *Chem. Pharm. Bull.* (Tokyo), **16**, 1140 (1968).
6) C. Züllich, K.H. Damm, and B.P. Lisboa, *J. Chromatog.*, **115**, 117 (1975).
7) J.J. Kirkland, "Modern Practice of Liquid Chromatography," Wiley-Interscience, John Wiley & Sons, Inc., New York, 1970, p. 386.
8) K. Shimada, Y. Fujii, E. Mitsuishi, and T. Nambara, *Tetrahedron Letters*, **1974**, 467; *idem*, *Chem. Ind.* (London), **1974**, 342; K. Shimada, Y. Fujii, and T. Nambara, *ibid.*, **1974**, 963; *idem*, *Tetrahedron Letters*, **1974**, 2767; K. Shimada, Y. Fujii, Y. Niizaki, and T. Nambara, *ibid.*, **1975**, 653; Y. Fujii, K. Shimada, Y. Niizaki, and T. Nambara, *ibid.*, **1975**, 3017; Y. Fujii, K. Shimada, and T. Nambara, *Chem. Ind.* (London), **1976**, 614 (1976); K. Shimada, Y. Sato, Y. Fujii, and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **24**, 1118 (1976).

Table I. Relative Retention Times of Bufadienolides

Compound	μ-Bondapak C_{18}			μ-Porasil	Corasil I	
	MeOH/H_2O (2:1) 1 ml/min	CH_3CN/H_2O (1:1) 1 ml/min	THF/H_2O (2:3) 1 ml/min	hexane/THF (1:1) 1.5 ml/min	hexane/THF (3:1) 1.5 ml/min	$CHCl_3$/AcOEt/cyclohexane (1:1:1) 1.5 ml/min
Gamabufotalin	0.46	0.46	0.41	1.79	3.44	4.55
Hellebrigenin	0.54	0.50	0.44	2.10	4.48	6.45
Arenobufagin	0.55	0.48	0.54	1.36	1.96	2.41
Desacetylcinobufotalin	0.58	0.51	0.41	4.87	17.44	13.95
Desacetylbufotalin	0.62	0.63	0.49	1.79	4.07	4.34
Resibufagin	0.66	0.82	0.59	1.68	2.22	1.55
Bufotalin	0.71	0.69	0.71	1.19	1.48	1.77
Telocinobufagin	0.72	0.68	0.61	1.78	2.82	4.02
Cinobufotalin	0.78	0.82	0.71	1.46	1.82	1.77
Marinobufagin	0.78	0.82	0.66	1.50	1.67	1.73
Desacetylcinobufagin	0.82	0.68	0.55	2.90	8.19	5.32
Cinobufagin	1.20	1.33	1.15	1.19	1.37	0.91
Resibufogenin	1.20	1.45	1.08	1.09	1.00	1.02
Bufalone	0.92	0.83	1.00	0.87	0.78	0.82
3-Epibufalin	0.99	0.95	0.92	1.18	1.33	1.32
Bufalin	1.00 (8.2 min)	1.00 (9.4 min)	1.00 (14.0 min)	1.00 (3.3 min)	1.00 (2.7 min)	1.00 (2.2 min)

Table II. Relative Retention Times of Cardenolides

Compound	μ-Bondapak C_{18}			μ-Porasil	Corasil I	
	MeOH/H_2O (2:1) 1 ml/min	CH_3CN/H_2O (2:3) 1 ml/min	THF/H_2O (1:2) 1.5 ml/min	hexane/THF (1:1) 1.5 ml/min	hexane/THF (3:1) 1.5 ml/min	$CHCl_3$/AcOEt/cyclohexane (1:1:1) 1.5 ml/min
Sarmentogenin	0.50	0.33	0.37	1.96	4.13	3.45
Digoxigenin	0.54	0.36	0.38	1.92	4.00	3.45
Gitoxigenin	0.71	0.53	0.57	1.91	4.03	2.88
14α,15α-Epoxy-"β"-anhydrodigitoxigenin	1.02	1.36	1.08	0.90	0.73	0.80
14β,15β-Epoxy-"β"-anhydrodigitoxigenin	1.15	1.52	1.15	1.13	1.22	0.80
"β"-Anhydrodigitoxigenin	2.31	3.81	2.76	0.79	0.53	0.60
Digitoxigenone	0.96	1.28	0.94	0.85	0.83	0.80
3-Epidigitoxigenin	1.04	1.01	1.00	1.27	1.73	1.40
Digitoxigenin	1.00 (6.9 min)	1.00 (12.6 min)	1.00 (11.1 min)	1.00 (3.9 min)	1.00 (3.0 min)	1.00 (2.0 min)

Examinations were then made on the retention value contributions for the representative functional groups. The retention times relative to each reference were estimated under two different conditions and were expressed in a logarithm (see Table III). There can be seen a close similarity in decrement or increment due to a functional group between bufadienolides and cardenolides. The constancy of functional group value implies that an additive logarithmic relationship for the relative retention time may be valid, when the absence of intramolecular interaction between functional groups is presupposed. The retention value contributions for the functional groups on the reversed phase column may be in the following sequential order: 11α-OH<12β-OH<16β-OH<5β-OH=16β-OAc; 14α,15α-epoxy<14β,15β-epoxy.

Effects of the unsaturated lactone ring at C-17 on the retention value were also determined with bufadienolides and cardenolides employing the μ-Bondapak C_{18} and μ-Porasil columns.

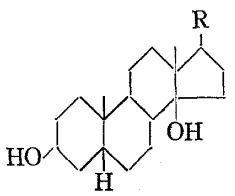
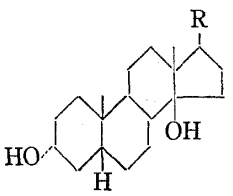
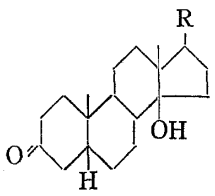
As can be seen in Table IV the retention time ratios of bufadienolides to cardenolides determined under the definite conditions are nearly constant irrespective of the remaining structures. It is to be noted that the α-pyrone ring contributes toward the retention value to greater extent than the butenolide in reversed phase chromatography with methanol/water as a mobile phase, while this elution order is turned in normal phase chromatography with hexane/tetrahydrofuran.

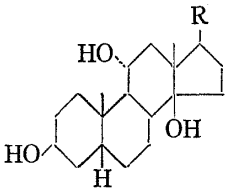
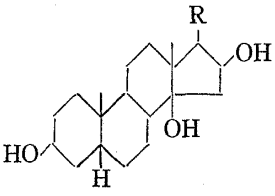
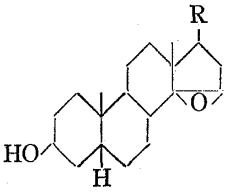
TABLE III. Retention Time Contributions for Functional Groups[a]

Functional group	Bufadienolide		Cardenolide	
	1	2	1	2[b]
5β-OH	−0.14	0.40		
11α-OH	−0.34	0.42	−0.30	0.50
12β-OH			−0.27	0.44
16β-OH	−0.21	0.47	−0.15	0.47
16β-OAc	−0.14	0.13		
14α,15α-Epoxy			0.01	0.03
14β,15β-Epoxy			0.04	0.11

a) Figures represent the logarithm of retention time relative to an internal standard (IS). Bufalin and digitoxigenin were employed as IS for bufadienolides and cardenolides, respectively.
b) Conditions used were as follows: 1) μ-Bondapak C_{18} column, methanol/water (2: 1), 1 ml/min; 2) μ-Porasil column, hexane/tetrahydrofuran (3: 2), 2 ml/min.

TABLE IV. Retention Time Ratios of Bufadienolides to Cardenolides

Condition[a]			
1	1.20	1.13	1.15
2	0.95	0.77	0.90

Condition[a]			
1	1.12	1.09	1.23
2	0.83	0.88	0.79

a) Conditions used were the same as given in Table III.

On the basis of these data the separation and determination of cardiac steroids in the fresh venom secreted from the parotid glands of the Japanese toad was then undertaken. The steroidal constituents were separated by thin-layer chromatography (TLC) into three fractions (zone 1, 2 and 3), which in turn were submitted to HPLC on a μ-Bondapak C_{18} column, respectively. As illustrated in Fig. 1, 2 and 3 thirteen kinds of bufogenins were unequivocally characterized. Cinobufotalin and marinobufagin showed similar chromatographic behaviors, but they could be differentiated with ease when tetrahydrofuran/water was employed as a solvent system.

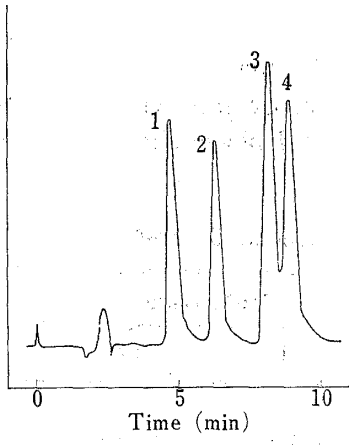


Fig. 1. Chromatogram of Bufogenins in the Venom of Japanese Toad (Zone 1)

1: compound S (IS), 2: bufalin, 3: cinobufagin, 4: resibufogenin
conditions: μ-Bondapak C_{18} column; mobile phase acetonitrile/water (1:1), 1.5 ml/min; detection 280 nm

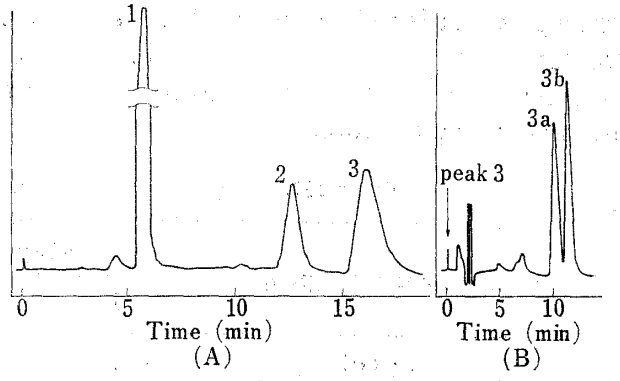


Fig. 2. Chromatogram of Bufogenins in the Venom of Japanese Toad (Zone 2)

1: arenobufagin, 2: bufotalin, 3a: marinobufagin, 3b: cinobufotalin
conditions: μ-Bondapak C_{18} column; mobile phase A) methanol/water (5:4), B) tetrahydrofuran/water (1:2), 1.5 ml/min; detection 280 nm

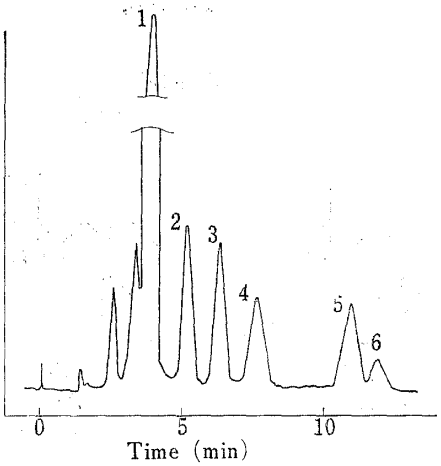


Fig. 3. Chromatogram of Bufogenins in the Venom of Japanese Toad (Zone 3)

1: gamabufotalin, 2: hellebrigenin, 3: desacetylcinobufotalin, 4: desacetylbufotalin, 5: telocinobufagin, 6: desacetylcinobufagin
conditions: μ-Bondapak C_{18} column; mobile phase methanol/water (5:4), 1.5 ml/min; detection 280 nm

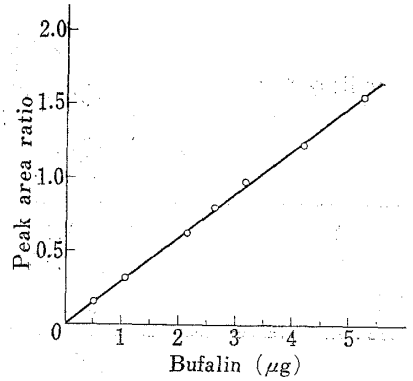


Fig 4. Calibration Curve for Bufalin

The present method was then applied to the determination of bufalin, one of the principal bufogenins in the toad venom. The calibration curve was constructed by plotting the ratio of the peak area of bufalin to that of compound S (17α,21-dihydroxypregn-4-ene-3,20-dione), an internal standard, against the weight of bufalin; good linearity was observed in the range 0.5—5 μg of bufalin (see Fig. 4). Quantitation of bufalin by the internal standard method was performed with the venom sample obtained from the Japanese toad in the manner as previously established. As shown in Table V the amount of bufalin was determined to be 3.47 μg per milligram of the dry venom with an excellent reproducibility. A known amount of bufalin added to the toad venom was satisfactorily recovered as collected in Table VI. The weight ratios of other principal bufogenins in the toad venom, arenobufagin, gama-

Table V. Reproducibility of the Present Method for Determination of Bufalin in the Toad Venom[a]

Found (μg)						Mean±S.D. (μg)
3.53	3.55	3.53	3.36	3.46	3.36	3.47±0.087

a) Figures represent the amount of bufalin per milligram of a dry venom sample.

Table VI. Recovery Test for Bufalin added to the Toad Venom

In toad venom[a] (μg)	Added (μg)	Found (μg)				Recovery rate Mean±S.D. (%)
123	52	175	189	171	186	
123	105	252	239	239	—	104±4.5

a) Figures represent the amount of bufalin determined with 35.6 mg of a dry venom sample.

bufotalin, cinobufagin and resibufogenin, to bufalin were estimated to be 6.27, 2.81, 2.00 and 1.42, respectively. The results on the determination of cardiac steroids were confirmed by the use of three different mobile phases, methanol/water, acetonitrile/water and tetrahydrofuran/water. It is noteworthy that arenobufagin was found most abundant among bufogenins in the venom of the Japanese toad.

The present procedure appears to be of greater advantage than the hitherto known methods, since a variety of cardiac steroids can be separated, characterized and determined simultaneously under the mild conditions without any derivatization. It is hoped that the proposed method may also serve for the analysis of cardiac steroids in the Chinese toad venom preparation, *Ch'an Su*.

Experimental

Instruments——The apparatus used was a Waters Model ALC/GPC 202 R401 high-pressure liquid chromatograph (Waters Associates Inc., Milford) equipped with an ultraviolet detector monitoring absorbance at 254 nm for cardenolides and 280 nm for bufadienolides. The samples were injected with a Model U6K sample loop injector (Waters Associates Inc.) with an effective volume of 2 ml. The μ-Porasil (1′×1/4″ i.d.), μ-Bondapak C_{18} (1′×1/4″ i.d.) and Corasil I (2′×1/8″ i.d.) columns (Waters Associates Inc.) were used under ambient conditions.

Samples and Reagents——"β"-Anhydrodigitoxigenin, 14α,15α-epoxy-"β"-anhydrodigitoxigenin and 14β,15β-epoxy-"β"-anhydrodigitoxigenin were prepared by the method of Hofer, *et al.*[9] Digitoxigenone and 3-epidigitoxigenin were also synthesized by the method of Yamada.[10] Other steroids were separated from the skin of Japanese toad or kindly donated by Drs. Y. Kamano, M. Okada, and H. Ishii. Compound S used as an internal standard was recrystallized repeatedly from MeOH/ether. All the reagents employed were of analytical grade. Solvents were purified by distillation prior to use.

Preparation of Toad Venom Sample——The fresh venom was collected from the parotid glands of ten Japanese toads (*Bufo vulgaris formosus* Boulenger) by squeezing with a pincette, immediately dissolved in $CHCl_3$/MeOH (1: 1) (100 ml) and divided into ten portions. To each portion containing 40.8 mg of the toad venom in dry weight was added compound S (505.6 μg) in MeOH (1 ml) and concentrated *in vacuo*. The residue obtained was submitted to preparative TLC using benzene/AcOEt (1: 1) as a developing solvent. The adsorbent corresponding to *Rf* 0.6 (zone 1), *Rf* 0.4 (zone 2) and *Rf* 0.2 (zone 3) was eluted with AcOEt/$CHCl_3$ (1: 1) and the eluate was then evaporated *in vacuo*, respectively. The residue was redissolved in MeOH (1 ml), a 20 μl aliquot of which was injected with a microsyringe into the chromatograph equipped with the μ-Bondapak C_{18} column.

Determination of Bufalin and Other Bufogenins in Toad Venom——Bufalin, cinobufagin, resibufogenin (zone 1), arenobufagin (zone 2) and gamabufotalin (zone 3) were determined by the internal standard method.

9) P. Hofer, H. Linde, and K. Meyer, *Helv. Chim. Acta*, **15**, 1041 (1962).
10) A. Yamada, *Chem. Pharm. Bull.* (Tokyo), **8**, 18 (1960).

The calibration curve was constructed by plotting the ratio of the peak area of each bufogenin to that of compound S against the weight of each sample, whereby satisfactory linearity was observed, respectively.

Recovery Test for Bufalin——To a dry toad venom sample (35.6 mg) were added compound S (526.5 μg) in MeOH (1 ml) and bufalin (52 μg, 105 μg) in MeOH (4 ml) and then evaporated *in vacuo*. The residue obtained was submitted to preparative TLC, followed by HPLC in the manner as described above.

Acknowledgement The authors express their sincere thanks to Dr. Y. Kamano, Taisho Pharmaceutical Co., Ltd., Dr. M. Okada, Tokyo Biochemical Research Institute, and Dr. H. Ishii, Shionogi & Co., Ltd., for generous gifts of precious samples. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture which is gratefully acknowledged.