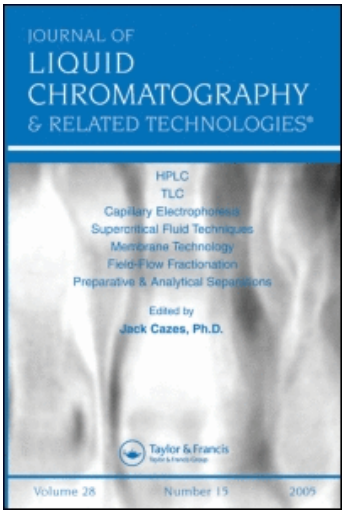

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Simultaneous Determinations of Cinobufagin and Its Metabolites by Reverse-Phase High Performance Liquid Chromatography in Rat Serum and Urine

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SIMULTANEOUS DETERMINATIONS OF CINOBUFAGIN AND ITS METABOLITES BY REVERSE-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN RAT SERUM AND URINE

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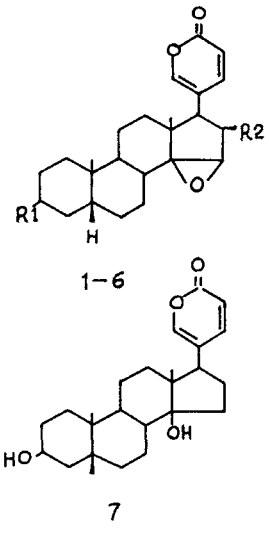
ABSTRACT

A high-performance liquid chromatography was developed for simultaneous determinations of Cinobufagin (CB) and its metabolites deacetylcinobufagin, 3-epideacetylcinobufagin, 3-ketodeacetylcinobufagin, 3-epicinobufagin and 3-ketodeacetylcinobufagin in serum and urine of rat. The biological samples were extracted with diethylether-ethylacetate (4:1v/v) in the presence of bufalin as the internal standard. Recoveries for CB and all other compounds were in the range of 80.8%-100.2%. Excellent resolution was obtained by reversed-phase chromatography on a 4.6mmI.D.x150 mm ODS column using acetonitrile:water (50:50 v/v) as the mobile phase at a flow-rate of 0.7 ml/min with UV detector at 300 nm. Standard curve data revealed linearity over a range of 10-500 ng per ml serum and urine. The detection limits of CB and its metabolites were less than 10 ng/ml. Coefficients of variation for the analysis were less than 10. CB and its some metabolites were observed in

serum and urine of rats treated with CB at an intravenous dose of 0.5mg/kg. Simplicity, accuracy and reproduction of the method were found to be acceptable. This method could be used for studying pharmacokinetics and metabolic pathway of CB.

INTRODUCTION

Chan'Su in China has been using for centuries as cardiotonic, local anesthetic and treatment of ecphyma. It has been found that the effective components of this chinese medicine are bufadienolides (1-3). Cinobufagin (CB) is a prominent component of bufadienolides. Its pharmacological activities, such as cardiotropic effect (4-6), respiratory-stimulating action (7), blood pressure-increasing effect (8) and anaesthetic action (9) have been reported to date. However, only a few studies concerning metabolic fate and pharmacokinetics of CB have been carried out. Toma et al found the two CB metabolites, deacetylcinobufagin (M1) and 3-epideacetylcinobufagin (M2) in serum of rats (10). At present, it is not clear whether CB is converted to other metabolites in addition to M1 and M2. One of the reasons for the lack of information concerning the metabolism of CB could be due, if not all, to a lack of available and sensitive method to analyze CB and its metabolites in biological materials. In this respect, Aoki et al reported the highly sensitive enzyme immunoassay for CB (11), however, this method is not



No	Compound	R1	R2
1	CB	βOH	$OCOCH_3$
2	M1	βOH	OH
3	M2	αOH	OH
4	M3	=O	OH
5	M4	αOH	$OCOCH_3$
6	M5	=O	$OCOCH_3$
7	Bufalin		

FIGURE 1. Chemical structure of CB and its metabolites.

suitable for simultaneous determinations of CB and its metabolites in biological samples.

This paper therefore describes a simple one-step solvent extraction procedure, selectivity, sensitivity and accuracy for the simultaneous determinations of CB and its metabolites including 3-ketodeacetylcinobufagin (M3) and 3-epicinobufagin (M4) and 3-ketocinobufagin (M5) as possible metabolites in biological materials. The chemical structures of CB and its metabolites examined in this study are shown in Fig.1.

MATERIALS AND METHODS

Chemicals

CB and bufalin were isolated from chinese toad venom preparation Chan'Su (Shibata pharmaceutical Co., Ltd., Tokyo,Japan) by column chromatography according to the method of Komatsu and Okano (12). M1, M2 and M3 were synthesied by the method of Pettit and Kamano (13). Except M1 and M3, all compounds were crystallized to constant melting points which agreed to the reported values. M1 and M3 were identified by H^1-NMR, and each gave a single peak by HPLC. Methanol and acetonitrile (HPLC grade), diethylether and ethylacetate (analytical grade) were obtained from Wako pure chemical industries Ltd., Osaka, Japan.

Apparatus and HPLC conditions

The chromatographic system consisted of a model CCPM pump, a model 8000 variable wavelength UV detector (TOSOH Co.,Ltd.,Tokyo, Japan) and a model 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A). The stainless steel chromatographic column (150 mm x 4.6 mm I.D.) was packed with 5 um octadecyl-bonded silica (TSK gel ODS 80Tm, TOSOH Co.,Ltd.,Japan). A model Sic 7000A integrator recorder (System instruments Co.,Tokyo, Japan) was used to determine the peak-area ratio for all quantitative measurements. The mobile

phase consisted of acetonitrile:water (50:50) with a flow rate of 0.7 ml/min. The analytical wavelength was set at 300 nm. All chromatographic experiments were carried out at room temperature.

Standard solution and calibration curve

22.1 mg of CB was accurately weighted, quantitatively transfered to a 10 ml volumetric flask, and dissolved in methanol. The stock solutions for M1, M2, M3 M4, M5 and bufalin were made by dissolving 10.0 mg of the compounds in 10.0 ml methanol and were stored at 0-4°C. The working standards was prepared from the stock solutions by making serial dilutions to the concentration required for the standard curve.

Calibration curves for CB and its metabolites were constructed by transferring appropriate aliquots of the above stock solutions to blank serum and urine to concentrations of 2, 10, 25, 50, 100, and 500 ng/ml serum or urine, respectively. These standard solutions were then treated in the same manner as described below. The internal standard (IS) used for the experiments with serum and urine studies was 25 ng/ml. The standard curves for CB and its metabolites were made by plotting peak-area ratios against the amount of standards added.

Extraction procedure

In 0.5 ml of serum or urine, 0.025ml (0.01 mg/ml) of bufalin solution was added as IS. The serum or urine was extracted with 5.0 ml of diethylether-ethylacetate (4:1 v/v) by mechanically shaking for 15 min, and then centrifuged at 3000 rpm for 10 min. The upper organic layer (4.0 ml) was evaporated to dryness under a gentle stream of nitrogen at room temperature.

The extraction efficiency was determined in spiked samples of serum and urine containing IS. The peak-area ratios of CB, M1, M2, M3, M4, M5 and IS before and after extraction were compared. The instrument and HPLC conditions were identical to those described above.

in vivo metabolism of CB

Seven-week old male Wistar rats (Nippon Seibutu Zairyo Co.,Tokyo, Japan) were acclimatized to the animal house at room temperature ($22\pm2^{o}C$) under a 12 hr light/dark cycle for at least 1 week before use. The serum and urine were collected from rats at appropriate times after an intravenous (tail vein) injection of CB (0.5 mg/kg). The collected urine was then acidified to PH 4-5 by the addition of hydrogen chloride.

RESULTS and DISCUSSION

Selectivity

Fig.2 shows typical chromatogram of a standard spiked with 0.4 ug/ml of CB, M1, M2, M3, M4, M5 and 0.25 ug/ml

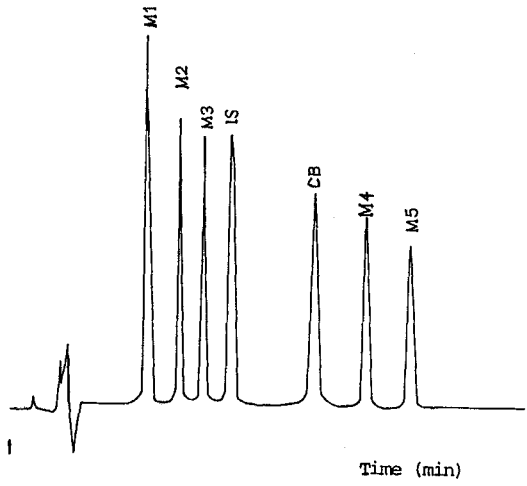


FIGURE 2. Chromatogram of standard solution.

of bufalin as the internal standard. Their retention times were 5.29, 6.57, 7.64, 12.17, 14.89, 17.16 and 8.69 min, respectively.

Fig.3 depicts representative chromatograms of the extracts from drug-free serum (A), serum spiked with 0.4 ug/ml of CB, M1, M2, M3, M4, M5 and IS (B), and rat serum sample taken 10 min after i.v. administration of CB at a dose of 0.5 mg/kg (C). As can be seen in Fig.2, CB and its metabolites were well resolved with resolution factor of 4.5 between M1 and M2, 2.7 between M2 and M3, 2.6 between M3 and IS, 7.3 between IS and CB, 5.2 between CB and M4, 5.0 between M4 and M5,

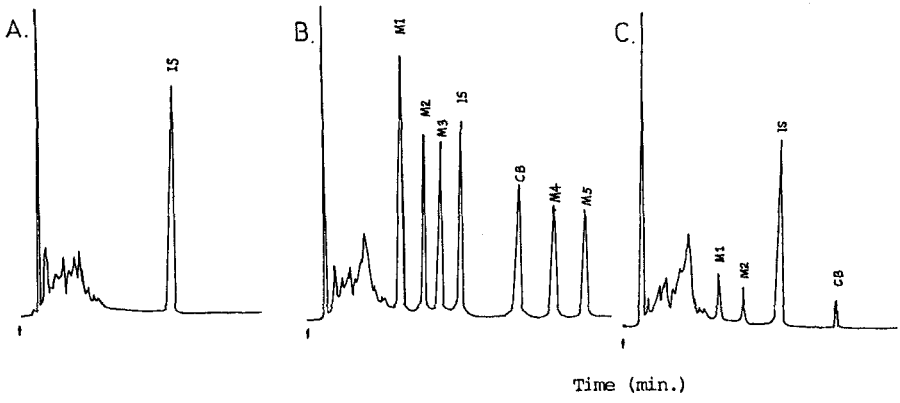


FIGURE 3. Chromatogram of the extract of drug-free serum of rat (A); serum spiked with 0.4 ug/ml M1, M2, M3, IS, CB, M4 and M5 (B); rat serum sample taken 10 min after i.v administration of CB (0.5mg/kg) (C).

respectively. The base line was stable and free from interference.

Fig.4 shows the typical chromatogram of the extract of drug-free urine (A), urine standard spiked with 0.4 ug/ml of CB, M1, M2, M3, M4, M5, 0.25 ug/ml of IS, 0.3 ug/ml of M4, 0.3 ug/ml of M5 (B), and rat urine sample taken 2 hr after i.v. administration of CB at a dose of 0.5 mg/kg (C). Blank urine sample showed no extranous peaks corresponding to the CB and its metabolites.

All of these results clearly indicate that the present HPLC method is acceptable for simultaneous

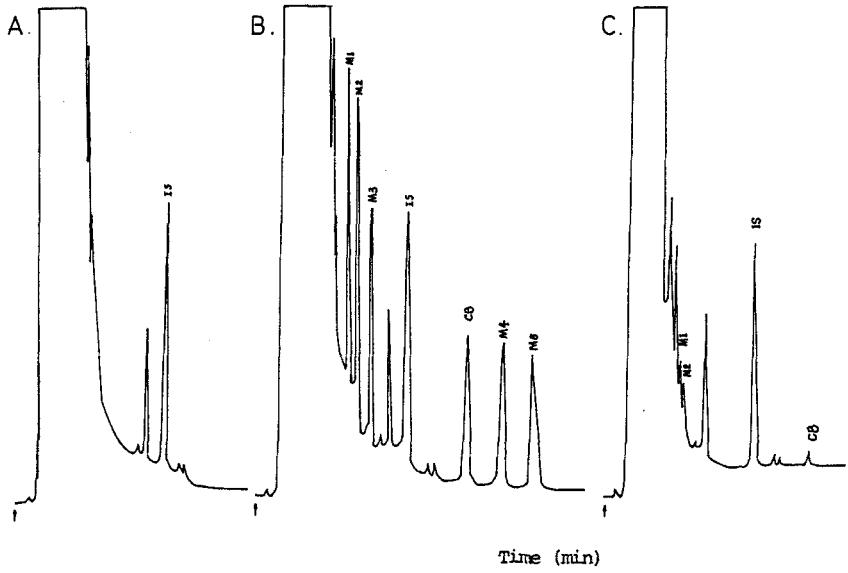


FIGURE 4. Chromatogram of the extract of drug-free urine of rat (A); urine standard spiked with M1, M2, M3, IS, CB, M4, M5 (B); and rat urine taken 2 hr after i.v administration of CB (0.5 mg/kg) (C).

determinnation of CB and its metabolites in biological samples.

Extraction efficiency and accuracy

The recovery of each compound was determined by comparing the peak area obtained by direct injection of standard solution to those obtained after extraction from serum and urine.

The recoveries for CB, its metabolites and internal standard after extraction from the serum and urine were

TABLE 1. Recovery of CB and Its Metabolites from Serum and Urine

Compound	Added (ng/ml)	Recovered (ng/ml)	Recovery (%)	CV (%)
From Serum	(n=10)			
M1	50.0	48.7	97.4	5.50
	250.0	240.6	96.2	5.99
M2	50.0	48.3	96.6	5.25
	250.0	250.2	100.1	7.50
M3	50.0	48.3	96.6	6.12
	250.0	247.8	99.1	1.25
CB	50.0	50.1	100.2	6.76
	250.0	250.4	100.2	2.30
	500.0	495.3	99.1	1.16
M4	50.0	47.5	95.0	9.76
	250.0	240.4	96.2	4.58
M5	50.0	42.2	84.4	8.21
	100.0	88.0	88.0	6.90
IS	25.0	23.5	95.8	5.76
	250.0	250.4	100.2	6.90
From Urine	(n=8)			
M1	25.0	25.7	102.8	7.18
	240.0	216.0	90.0	5.18
M2	25.0	23.1	92.4	5.70
	240.0	226.5	94.4	7.33
M3	25.0	21.6	86.4	8.03
	240.0	226.5	94.4	7.33
CB	25.0	22.6	90.4	4.48
	240.0	228.2	95.1	6.00
M4	25.0	23.3	93.2	4.50
	240.0	220.9	92.0	4.93
M5	25.0	20.2	80.8	8.51
	240.0	211.2	88.0	8.40
IS	25.0	23.3	93.2	3.99

greater than 84.4% and 80.8%, respectively, and were independent on the drug concentrations (Table 1).

By investigating other extraction conditions (data not shown), itwas found that CB and its metabolites were efficiently extracted with diethylether-ethylacetate (4:1 v/v) without adjustment of serum pH.

TABLE 2. Accuracy of Assays for CB and Its Metabolites in Serum and Urine

Compound	Within-day (n=10) Mean	SD ng/ml	CV%	Day to day (n=8) Mean	SD ng/ml	CV%
Serum						
M1	94.4	4.08	4.32	92.5	5.43	5.87
M2	90.5	6.71	7.41	88.6	6.69	7.55
M3	93.4	5.66	6.06	86.4	5.83	6.75
CB	91.6	4.62	5.04	93.2	4.49	4.82
M4	89.8	5.25	5.84	93.5	5.26	5.62
M5	86.5	3.04	3.51	84.5	5.83	6.90
Urine						
M1	44.4	2.75	6.19	44.6	3.03	6.80
M2	44.5	3.24	7.13	44.9	3.52	7.83
M3	34.2	1.94	5.68	37.3	2.49	6.68
CB	48.0	1.52	3.17	48.6	1.74	3.58
M4	50.0	2.26	4.51	50.1	2.48	4.95
M5	37.1	1.56	4.21	38.7	2.63	6.80

Satisfactory recoveries of CB and its metabolites added to urine were obtained when pH of urine sample was adjusted to 5 or more lower.

Table 2 shows the within-day and day to day variations in the dermination of CB and its metabolites by the present method. The coefficients of variation were less than 7.41% and 7.55% in serum, and 7.13% and 7.83% in urine, respectively, over the entire concentration ranges measured of each compound.

Detection limit

To evalute the detection limits the integrator was set at Attenuation 1. The highest snsitivity and the

TABLE3.The Regression Equations of Calibration Curves for CB and Its Metabolites in Serum and Urine

Compounds	Equation	r(n=8)
Serum Extracts		
M1	Y=0.0328(M1)+0.0253	0.9998
M2	Y=0.0277(M2)+0.0154	0.9999
M3	Y=0.0283(M3)+0.0115	0.9998
CB	Y=0.0332(CB)+0.0585	0.9996
M4	Y=0.0227(M4)+0.0443	0.9997
M5	Y=0.0158(M5)+0.0878	0.9988
Urine Extracts		
M1	Y=0.0350(M1)+0.0037	0.9998
M2	Y=0.0275(M2)+0.0086	0.9998
M3	Y=0.0265(M3)+0.0188	0.9997
CB	Y=0.0371(CB)+0.0446	0.9998
M4	Y=0.0254(M4)+0.0057	0.9991
M5	Y=0.0202(M5)+0.0098	0.9996

Internal standard : 25 ng/ml bufalin. r= Correlation coefficient. Y=Peak-area ratio versus internal standard. Independent variable in each equation is in parenthesis and in ng of CB and its metabolites per 1 ml of serum or urine. Numerical constants are the slope and intercept, respectively, for each compound.

noise for baseline was measured. A properly diluted standard solution was injected into the chromatograph and the peak area of CB and its metabolites were measured. The detection limits of CB, M1, M2, M3, M4 and M5 in serum were 2 ng/ml, 2 ng/ml, 2 ng/ml, 3 ng/ml and 3 ng/ml, respectively, based on signal-to-noise radio of 3. The detection limits of CB, M1, M2, M3, M4 and M5 in urine were 7 ng/ml, 5 ng/ml, 5 ng/ml, 2 ng/ml, 3 ng/ml and 3 ng/ml, respectively.

Linearity and sensitivity

Calibration curves of each compound were linear in the concentration ranges of 2.5-500 ng/ml serum and 5-500 ng/ml urine, respectively, with correlation coefficients of a greater than 0.9991 (Table 3).

Application of the method

As shown in Fig.3C and Fig.4C, CB, M1 and M2 were detected in serum and in urine after i.v. administration of CB at a dose of 0.5 mg/ kg. Semilogarithmic plots of the serum levels of CB, M1 and M2 versus time after i.v. administration of CB to rats are shown in Fig.5. CB was quickly metabolized after the injection with maximum concentration of 109.3 ng/ml at five minute. The metabolic rate of M1 and M2 showed biphasic pattern, one being accumulation phase and other an elimination phase. The maximum concentrations of M1 and M2 measured were 151 ng/ml at 10 min and 129 ng/ml at 20 min, respectively. After 1 hr, the levels of CB and M1 became too low to detect (< 3 ng/ml), while the concentration of M2 was 11.1 ng/ml at 2 hr. The serum levels of CB, M1 and M2 were not detected at 24 hr after injection of CB.

As shown in Fig.3 c, the urine levels of CB, M1 and M2 were very low. The concentrations of CB, M1 and M2 were 25 ng/ml, 30 ng/ml and 20 ng/ml, respectively, at

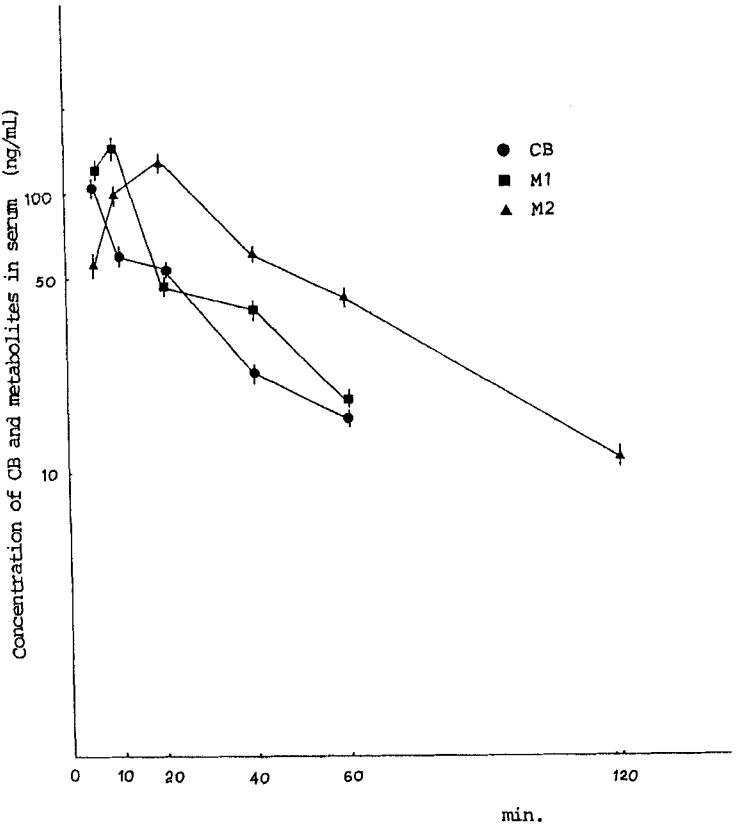


FIGURE 5. Semilogarithmic plots of serum level of CB and metabolites versus time in rat after i.v administration of CB (0.5 mg/kg). The data are expressed as the means offour-five animals per plot ± S.E.M (shown as bars).

2 hr after the injection. Such low excretion of CB and its metabolites in urine suggest that those compounds could be further metabolized to other polar metabolites. Possibly, reduction of double-bound at 17 unsaturated lactone of CB and its metabolites could be

occured, because the determinations of these compounds in serum and urine were carried out by monitoring 17-unsaturated lactone ring moiety at 300 nm.

With respect to metabolism of digoxigenin, Ganlt et al have shown that the drug is mainly converted to polar metabolites through 3-ketodigitoxigenin and subsequently to 3-epidigitoxigenin in human (14), however, we could not detect 3-keto metabolites of CB in serum and urine of the rat. Therefore, further detailed studies on metabolism of CB will be required. In conclusion, the present HPLC method would be useful to determine and analyze metabolic pathway and pharmacokinetics of the drug.

REFERENCES

1. Fieser, L.F., Fieser, M., : Steroides, Reinhold. Publ. Corp., New York, N.Y., 1959, p.787-797.

2. Komatsu, M., : Chan'Su, Sougou Rinshou, 6, 1063, 1967.

3. Tamm, Ch., :Proceedings of the First International Pharmacological Meeting vol.3, ed. by Wilbrandt, W., Lindgren, P., Pergamm Press, London 1963, p.11-26.

4. Chen, K. K., Anderson, R. C., and Henderson, F. G., : Comparision of Cardiac action of Bufalin, Cinobufolin, and Telocinobufagin with Cinobufagin, Proc. Soc. Exp. Biol.Med., 76, 372, 1951.

5. Okada, M., and Suga, T., : Pharmacology of the Princioles Isolated from Senso (Chan'Su), the dried venom of the Chinese Toad, Asian Medical Journal, 5, 353, 1962.

6. Morishitd, S., Saito, T., Mishima, Y., Mizutani, A., Hirai, Y.and Kawakami, M., : Pharmacological Studies of Cinobufagin, in Comparison with Digitoxin, Nippon Yakurigaku Zasshi, 86, 269, 1985.

7. Chen, K. K. and Kovarikova, A., : Pharcology and TOxicology of Toad venom, J. Pharmaceu. sci., 56, 1535, 1967.

8. Yoshida, S., Kamano,Y. and Sakai, T., : Studies of the surface anesthetic activity of bufadiennolides isolated from Ch'an Su, Chem. Pharmaceu. Bull., 24, 1714, 1976.

9. Suga, T., : Respiration and Blood Pressure Action of Bufogenin and Other bufadiennolides. Ochanomizu Igaku zasshi, 7, 2653, 1959.

10. Tooma,S., Morishita,S., Kuronuma,K., MIshima,Y., Hiraz, Y., and Kawakami, M., : Metabolism and Pharmacokinetics of Cinobufagin, Xenobiotica, 17, 1195, 1987.

11. Aoki, K., Aoki, M., Hirai, Y. and Kuroiwa, Y., : Enzyme Immunoassay for Cinobufagin, Chem. Pharm. Bull. 34, 1184, 1986.

12. Komatsu, M. and Okano, T., : Separation of Bufosteroids by Eluting Dry Column Chromatography on Silica Gel, Yakugaku zasshi 87, 712, 1967.

13. Pettit, G. and Kamano, Y., : Bufadienolides. 21. Synthesis of Cinobufagin from Bufatalin, J. Org. Chem., 37, 4040, 1972.

14. Ganlt, H., Kalra, J., Longerich, L., Dawe, M., : Digoxigenin Biotransformation, Clin. Pharmacol. Ther., 31, 695, 1982.