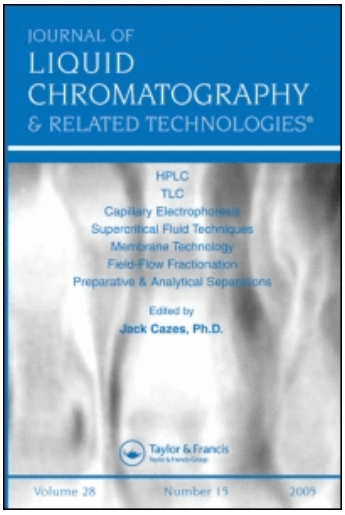

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SEPARATION OF TOAD POISON BUFADIENOLIDES BY HYDROPHOBIC GEL

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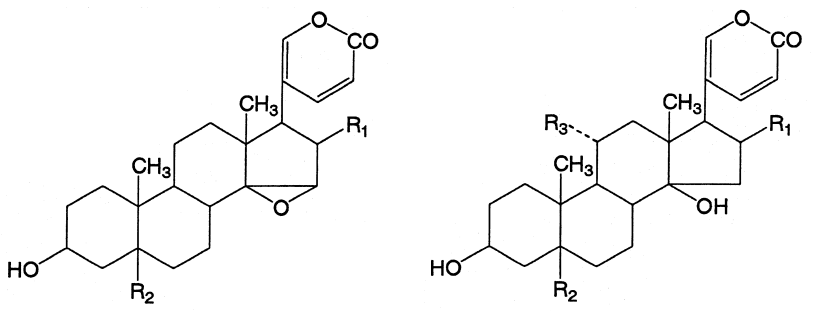
ABSTRACT

We have examined the column chromatography using hydrophobic gel carriers, Sephadex LH-20 and HP-Cellulofine, for separation of ten toad poison bufadienolides: resibufogenin, cinobufagin, bufalin, bufotalin, cinobufotalin, telocinobufagin, desacetyl-cinobufagin, desacetyl-bufotalin, desacetyl-cinobufotalin, and gamabufotalin (Figure 1). As a result, the use of developing solvents such as n-hexane/CH_2Cl_2/MeOH(4:5:1), n-hexane/$CHCl_3$/MeOH(4:5:1), and n-hexane/toluene/ MeOH (3:2:1) provided the effective separation of bufadienolides. For separation, we have established the flow speed of developing solvents and the relationship of separation with the diameter and length of column, in addition to the comparison of separation by Sephadex LH-20 and HP-Cellulofine and the relationship with silica gel thin-layer chromatography. Also, we have tested the separability of hydrophobic gel by repeated use and the recovery of bufadienolides on hydrophobic gel column by repeated use.



resibufogenin(R) R_1=R_2=H
cinobufagin(C) R_1=$OCOCH_3$, R_2=H
cinobufotalin(Cbt) R_1=$OCOCH_3$, R_2=OH
desacetyl-cinobufagin(DC) R_1=OH, R_2=H
desacetyl-cinobufotalin(DCbt) R_1=R_2=OH

bufalin(B) R_1=R_2=R_3=H
bufotalin(Bt) R_1=$OCOCH_3$, R_2=R_3=H
telocinobufagin(T) R_1=R_3=H, R_2=OH
desacetyl-bufotalin(DBt) R_1=OH, R_2=R_3=H
gamabufotalin(G) R_1=R_2=H, R_3=OH

Figure 1. Structure of bufadienolides.

INTRODUCTION

The toad poison bufadienolides[2] have a novel steroidal A/B *cis* and C/D *cis* structure with an α-pyrone ring at C17-position and exhibit a range of biological activities, such as cardiotonic, blood pressure stimulating, respiratory, antiviral, and antineoplastic activities. To obtain bufadienolides, separation and purification from natural sources such as the skin secretions of toads or the Chinese drug Ch'an Su were necessary. The Ch'an Su, which is called "Senso" in Japanese, is a product of the skin secretions of local toads such as *Bufo bufo gargarizans* Cantor or *Bufo melanostrictus* Schneider, and is the best source of bufadienolides.

Until now, for analytical separation of bufadienolides, gas liquid chromatography,[3] thin-layer chromatography,[4] and high performance liquid chromatography[5] were reported. Recently, we have reported the separation of bufadienolides by displacement thin-layer chromatography.[6] Also, for separation and purification of the complex mixture or large quantity of bufadienolides, the alumina or silica gel column chromatography by a wet or dry method is still ordinarily used.

Here, we report about the systematic separation of ten bufadienolides - resibufogenin(R), cinobufagin(C), bufalin(B), bufotalin(Bt), cinobufotalin(Cbt), telocinobufagin(T), desacetyl-cinobufagin(DC), desacetyl-bufotalin(DBt), desacetyl-cinobufotalin(DCbt), and gamabufotalin(G) (Figure 1) - which are the major components of Ch'an Su, using hydrophobic gel Sephadex LH-20 and HP-Cellulofine as carriers, in addition to the establishment of some necessary basic conditions.[1]

EXPERIMENTAL

Chemicals and Reagents

The solvents and the reagents were purchased from commercial sources of analytical grade. Sephadex LH-20 and HP-Cellulofine were purchased from Pharmacia Biotec and Seikagaku Kogyo Co. Ltd (Japan), respectively. Ch'an Su was obtained in the Hong Kong folk-medicinal market and extracted with CH_2Cl_2. The extract was concentrated *in vacuo*, and the residue was used. As the authentic samples, the ten bufadienolides - resibufogenin(R), cinobufagin(C), bufalin(B), bufotalin(Bt), cinobufotalin(Cbt), telocino-bufagin(T), desacetyl-cinobufagin(DC), desacetyl-bufotalin(DBt), desacetyl-cinobufotalin(DCbt) and gamabufotalin(G) - which were isolated in our laboratory, were utilized.

Thin-layer chromatography was conducted on precoated silica gel GF254 plate (UNIPLATE), which was purchased from ANALTEC. INC. Spots were obtained by ultraviolet light and heating of the TLC plate after a spray of 5% H_2SO_4-EtOH solution.

Chromatographic Procedure

As a typical technique, for swelling, Sephadex LH-20 was left to stand for 3 hr at room temperature in a developing solvent. The gel was poured carefully on the column (ϕ 19 mm wide and 500 mm length) without the intermixing of air. At the top of the setting column, the sample solution in 1-2 mL of the developing solvent was placed and eluted, carefully. In the case that the sample is insoluble in the developing solvent, the solvant mixture which was adjusted to the solubility of sample and changed the ratio of amount of developing solvent could be used.

The flow speed was set to about 2.0 mL/hr. The eluate was collected with each 150 drops (approximately 1.8 mL) by the use of a fraction collector (ADVANTEC, SF-2120, SUPER FRACTION COLLECTOR). For the dropping, a Teflon tube (width of hole: 1 mm) was used. For each chromatography, the first 15 mL of developing solvent was collected before the use of the fraction collector. Each eluate was determined by comparison with the authentic sample on TLC.

For establishment of the flow speed, the same two columns described above were set with n-hexane/CH_2Cl_2/MeOH (4:5:1). The flow speed was set up to 2.0 mL/hr (2-3 drops/min) and 40 mL/hr (50-60 drops/min), respectively. As a sample, CH_2Cl_2 extract of Ch'an Su was used.

For establishment of the diameter and length of column, the three columns- (1) ϕ19 mm X 500mm as standard column, (2) ϕ9 mm X 850 mm as narrow column and (3) ϕ30 mm X 195mm as wide column with n-hexane/ CH_2Cl_2/MeOH (4:5:1) - were set up by the same procedure as described above. 500 mg, 180 mg and 500 mg of CH_2Cl_2 extract of Ch'an Su were used as the samples of columns (1), (2), and (3), respectively.

Selection of Developing Solvents and Relationship with Thin-Layer Chromatographic Separation

For selection of developing solvents, the 500 mg of CH_2Cl_2 extract of Ch'an Su was used as the sample. Columns were prepared as described in the Chromatographic Procedure section. As the developing solvents, the following ten solvents were tested.

(A) n-hexane/CH_2Cl_2/MeOH (4:5:1)
(B) n-heptane/CH_2Cl_2/MeOH (4:5:1)
(C) cyclohexane/CH_2Cl_2/MeOH (4:5:1)
(D) n-hexane/$CHCl_3$/MeOH (4:5:1)
(E) n-hexane/CCl_4/MeOH (3:2:1)
(F) n-hexane/EtOAc/MeOH (4:5:1)
(G) n-hexane/toluene/MeOH (3:2:1)
(H) n-hexane/CH_2Cl_2/EtOH (4:5:1)
(I) n-hexane/CH_2Cl_2/2-propanol (4:5:1)
(J) n-hexane/CH_2Cl_2/acetone (3:3:4)

Eluates with solvents B, C, D, F, G, and H were collected with each 200 drops (approximately 2.4 mL). For solvents E, I, and J, every 400 drops (4.8mL) was used. These solvents were selected from TLC experiments with bufadienolides.

TLC was carried out with developing solvents, A-J. The ratio of solvents, E and G, was changed from 3:2:1 to 4:5:1 to enable separation on TLC plates. Two new solvents are marked as E' and G' respectively.

Examination of Separability of Hydrophobic Gel and Recovery Test of Compounds

The mixture of cinobufagin (20mg) and bufalin (20mg) was eluted with n-hexane/CH_2Cl_2/MeOH (4:5:1) by the same method as described in the Chromatographic Procedure section. Yields of the eluted samples were measured exactly. Then, a mixture of them was again eluted on the same column as the first one. By repeating this procedure three times, the separability of hydrophobic gel and also the recovery of compounds were examined.

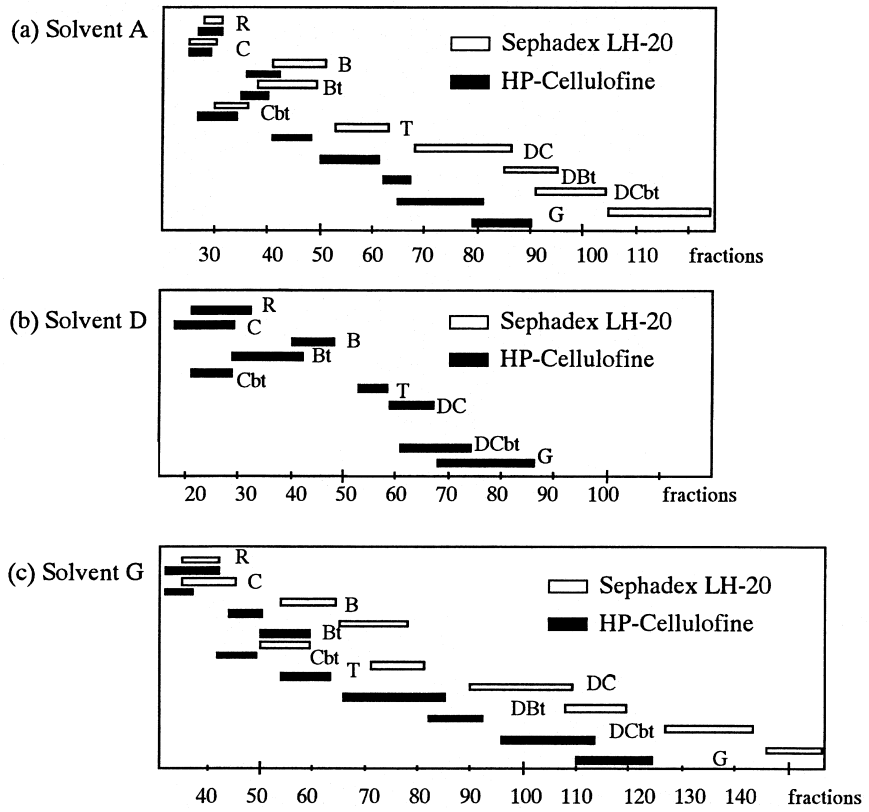


Figure 2. Best separation of bufadienolides by hydrophobic gel. Developing solvent: (a) n-hexane/CH_2Cl_2/MeOH 4:5:1 (Solvent A), (b) n-hexane/$CHCl_3$/MeOH 4:5:1 (Solvent D), (c) n-hexane/toluene/MeOH 3:2:1 (Solvent G). Carriers: Sephadex LH-20 or HP-Cellulofine. Compounds: R, C, B, Bt, Cbt, T, DC, DBt, DCbt and G were shown in Experimental. Column size was ϕ19 mm wide and 500 mm length. Sample: CH_2Cl_2 extract of Ch'an Su, 500 mg. 1 fraction: (a) 150 drops (approximately 1.8 mL), (b) and (c) 200 drops (approximately 2.4 mL).

Comparison of Separation by Sephadex LH-20 and HP-Cellulofine

For the comparison of separation by Sephadex LH-20 and by HP-Cellulofine, developing solvents A, D, and G were examined. 500 mg of CH_2Cl_2 extract of Ch'an Su was used as the sample. The preparation of columns was described in the Chromatographic Procedure section. In the chromatography with solvent D and G, each fraction was collected with 200 drops (approximately 2.4 mL).

Table 1

The Eluted Fraction Number of Bufadienolides by Hydrophobic Gel Chromatography

	Solvents									
Compounds	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
R	27-31	23-28	18-24	21-32	37-46	16-26	35-42	21-25	16-20	24-34
C	25-30	21-28	17-23	18-29	38-47	15-18	35-45	21-25	14-17	21-31
B	41-51	29-41	24-32	40-48	49-58	20-42	54-64	33-40	24-32	35-49
Bt	38-49	29-39	23-29	29-42	57-67	28-42	65-78	28-35	22-27	35-43
Cbt	30-36	24-28	18-23	21-28	51-58	16-30	50-59	22-27	16-22	26-36
T	53-63	41-47	28-35	53-58	59-69	42-58	81-91	42-49	36-43	45-55
DC	68-86	48-57	33-43	59-67	73-86	57-82	90-109	53-74	47-57	55-71
DBt	85-95	56-63	43-45	---	86-96	80-88	108-119	74-83	56-63	70-84
DCbt	91-104	61-77	45-53	61-75	99-104	91-111	127-143	82-111	70-90	85-96
G	105-124	74-91	52-61	68-86	113-134	91-116	146-156	101-121	77-100	82-96
Drops/1fr.	150	200	200	200	400	200	200	200	400	400
Carrier	S	H	H	H	H	S	S	H	H	S

Carrier: S-Sephadex LH-20, H-HP-Cellulofine. Compounds: R, C, B, Bt, Cbt, T, DC, DBt, DCbt and G are same as in Figure 2. Solvents: A-J are shown in the Experimental section.

RESULTS AND DISCUSSION

Best Separation of Bufadienolides by Hydrophobic Gel

In developing solvents A- J, the use of solvent A provided the best separation on the whole, as shown in Figure 2(a). In this case, the eluted order of the bufadienolides was as follows: cinobufagin ≥ resibufogenin ≥ cinobufotalin > bufotalin ≥ bufalin > telocinobufagin > desacetyl-cinobufagin > desacetyl-bufotalin ≥ desacetyl-cinobufotalin > gamabufotalin. Similar good separations were obtained by the use of solvents D and G, as shown in Figure 2(b) and (c), respectively. Also, results of separation by other solvents (B, C, E, F, and H-J) are indicated in Table 1, with the number of fractions.

Although separation of resibufogenin and cinobufagin was not completed in all cases, cinobufagin was eluted slightly earlier than resibufogenin, except in the case of solvent E. However, by repeated separation with solvent A, it is found that both compounds could be isolated. Separation of bufalin and bufotalin with solvent A was not completed perfectly, while the use of solvents D, E, and G provided the perfect separation of these compounds. In addition, separation with solvent H was good, though the perfect separation was not obtained. In the case of solvents E and G, bufalin was earlier to elute than bufotalin, but the use of solvents D and H caused reverse elution.

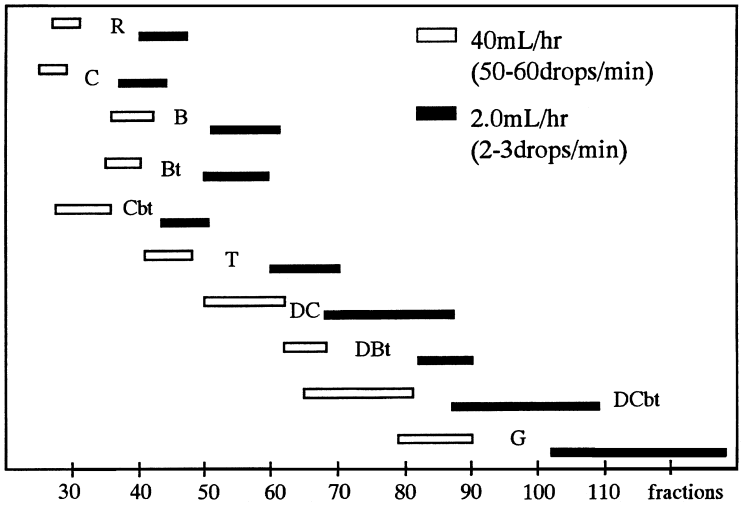


Figure 3. The effect of flow speed for separation. Flow speed: 40 mL/hr (50-60 drops/min) or 2.0 mL/hr (2-3 drops/min). Developing solvent: n-hexane/CH_2Cl_2/MeOH 4:5:1 (Solvent A). Carriers: HP-Cellulofine. Compounds, column size and sample were same as Figure 2. 1 fraction: 150 drops (approximately 1.8 mL).

Therefore, in the cases of solvents E and G, bufalin overlapped with cinobufotalin (Figure 2c). On the other hand, separations with solvents D and H were excellent with eluted order cinobufotalin > bufotalin > bufalin (Figure 2b). In the ten bufadienolides, separation of polar compounds - telocinobufagin, desacetyl-cinobufagin, desacetyl-bufotalin, desacetyl-cinobufotalin and gamabufotalin - was good. Interestingly, gamabufotalin was earlier to elute than desacetyl-cinobufotalin by use of solvent J.

Relationship Between Elution Speed and Separation

Figure 3 showed the results of separation by low speed (2.0mL/hr, 2-3drops/min) and high speed (40mL/hr, 50-60drops/min) respectively. Although both separations were good, the separation by low speed seemed to be better than that by high speed. However, in the low speed separation, it was observed that the band spread of compounds and the resulting elution took more time.

Relationship Between The Diameter and Length of Column and Separation

Figure 4 showed the result of the effect of diameter and length of column for separation. Separation on the wide column was the worst in the three kinds

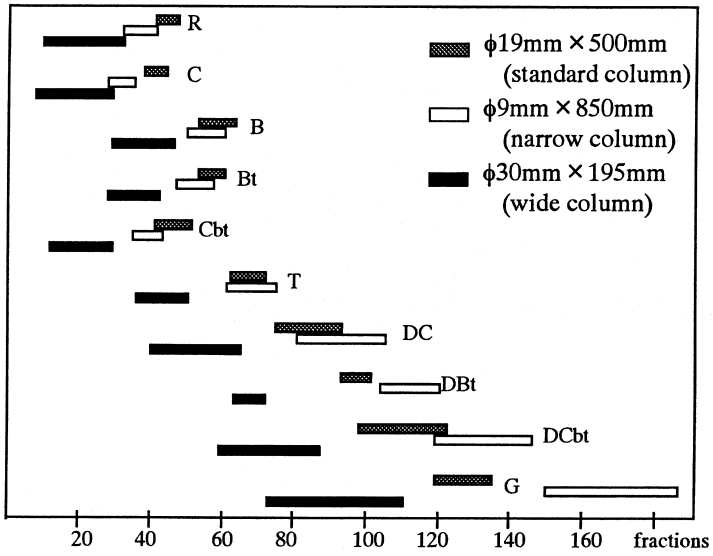


Figure 4. The effect of diameter and length of column on separation. Column size: ϕ 19 mm wide and 500 mm length (standard column), ϕ 9 mm wide and 850 mm length (narrow column), ϕ 30 mm wide and 195 mm length (wide column). Developing solvent: n-hexane/CH_2Cl_2/MeOH 4:5:1 (Solvent A). Carrier: HP-Cellulofine. Compounds were same as Figure 2. Sample: CH_2Cl_2 extract of Ch'an Su, 500 mg for standard and wide columns, 180 mg for narrow column. 1 fraction: 100 drops (approximately 1.2 mL).

of columns. By narrowing the column, separation was improved. However, the narrow column gave the spread of band and elution. The result was similar to that in the Best Separation of Bufadienolides section above. This systematic result also seemed to be related to the characteristics of hydrophobic gel.

Relationship Between Thin-Layer Chromatography and Hydrophobic Gel Chromatography

Table 2 showed the Rf values of ten bufadienolides on TLC with the same solvents as elution by hydrophobic gel. The elution order of bufadienolides by hydrophobic gel was found to be similar to the developing order on TLC. The result means that the eluting solvents of hydrophobic gel could be chosen by the examination of TLC. The elution of cinobufagin was earlier than that of resibufogenin on hydrophobic gel column with solvent A, whereas the Rf value of resibufogenin was higher than that of cinobufagin on TLC with same solvent A (see Figure 2, 3, and 4 for hydrophobic gel separation).

Table 2

Rf Values of Bufadienolides on TLC Plates

Compounds	Solvents A	B	D	E'	F	G'	H	I	J
R	0.59	0.56	0.60	0.13	0.62	0.14	0.65	0.62	0.67
C	0.57	0.55	0.57	0.12	0.63	0.14	0.63	0.61	0.64
B	0.48	0.46	0.49	0.07	0.06	0.09	0.53	0.51	0.60
Bt	0.44	0.42	0.45	0.05	0.51	0.08	0.49	0.45	0.53
Cbt	0.44	0.44	0.47	0.07	0.52	0.10	0.52	0.49	0.52
T	0.37	0.33	0.37	0.04	0.41	0.06	0.33	0.24	0.33
DC	0.36	0.33	0.36	0.04	0.35	0.06	0.34	0.23	0.35
DBt	0.35	0.31	0.35	0.04	0.37	0.04	0.34	0.24	0.32
DCbt	0.27	0.25	0.29	0.02	0.24	0.04	0.19	0.11	0.19
G	0.25	0.23	0.26	0.02	0.36	0.03	0.23	0.13	0.29

Compounds: R, C, B, Cbt, T, DC, DBt, DCbt, and G are the same as Figure 2. Solvents A-J are shown in the Experimental section.

On the other hand, the separation of bufalin and bufotalin was excellent on TLC plates in all cases, but not on hydrophobic gel (see Figure 2a). Also, the Rf values of cinobufotalin and bufotalin were close on TLC, whereas both compounds were separated excellently on hydrophobic gel in the all cases of developing solvents. Separation of the polar compounds - telocinobufagin, desacetyl-cinobufagin, desacetyl-bufotalin, desacetyl-cinobufotalin, gamabufotalin - on hydrophobic gel column was very effective, while the Rf values of polar compounds on TLC were close to each other.

Comparison of Separation by Sephadex LH-20 and HP-Cellulofine

The results of separation by Sephadex LH-20 and HP-Cellulofine under the same chromatographic condition were shown in Figures 2a and 2c, respectively. In both cases of chromatography, the difference between separation by Sephadex LH-20 and by HP-Cellulofine was not observed. However, in the case of separation by HP-Cellulofine, the compounds were eluted in the short band and the elution was completed in a short time.

Separability of Hydrophobic Gel and Recovery of Compounds on Hydrophobic Gel

Figure 5 showed the test results for the separability of Sephadex LH-20 as hydrophobic gel in the separations of cinobufagin and bufalin, respectively, by a three-time repetition. Separations of both compounds indicated a tendency to

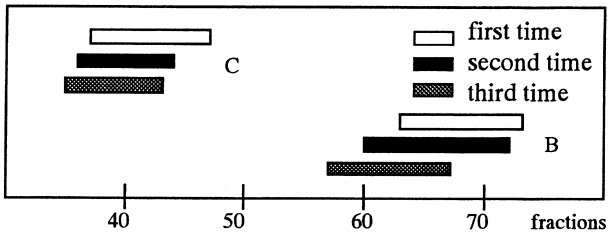


Figure 5. Separation of cinobufagin and bufalin with three time repetition. Developing solvent: n-hexane/CH_2Cl_2/MeOH 4:5:1 (Solvent A). Compounds : C and B were cinobufagin and bufalin, respectively. Column size and 1 fraction were same as Figure 3. Sample: mixture of cinobufagin (20 m) and bufalin (20 mg).

Table 3

Recovery Test of Cinobufagin and Bufalin on Hydrophobic Gel

Compounds	First	Second	Third
Cinobufagin	100.0%	99.7%	97.8%
Bufalin	96.5%	94.0%	88.0%

reduce from repeating. This means that hydrophobic gel could be used repeated three times. Recovery of cinobufagin and bufalin by three times repeated separation on the same hydrophobic gel, indicated in Best Separation of Butadienolides by Hydrophobic Gel section above, was tested. Yields (%) of both compounds are shown in Table 3. Although cinobufagin was stable on the gel, bufalin was unstable and yields of that was reduced. Therefore, for separation of unstable compounds, the careful use of hydrophobic gel was necessary.

CONCLUSION

The investigation shows that the use of hydrophobic gel, Sephadex LH-20, and HP-Cellulofine, is very useful for separation of the toad poison bufadienolides. It is effective as compared with the use of silica gel, because by hydrophobic gel chromatography, a constant separation is obtained in a short time and quantities of eluting solvent are small. The use of solvent A, n-hexane/CH_2Cl_2/MeOH (4:5:1), as developing solvent was effective for the separation of bufadienolides.

The eluting order of bufadienolides with this solvent is as follows: cinobufagin ≥ resibufogenin ≥ cinobufotalin > bufotalin ≥ bufalin > telocinobufagin > desacetyl-cinobufagin > desacetyl-bufotalin ≥ desacetyl-cinobufotalin > gamabufotalin. Also, for separation, the use of a combination of solvent A with other developing solvents is suggested, in addition to the repeated use of the same solvent. Especially, for the separation of bufalin and bufotalin, the use of solvent G, n-hexane/toluene/MeOH (3:2:1), was effective. In the comparison of separation by Sephadex LH-20 and by HP-Cellulofine, although both hydrophobic gels provided similar elution of bufadienolides on the whole, separation by HP-Cellulofine tended to elute with short bands for each compound than that of Sephadex LH-20. Finally, we expect that these results are useful for a comprehensive treatment of Ch'an Su, and also for the separation of other bufadienolides and related compounds such as cardenolides.

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