

SIMPLE SEPARATION OF THE BUFADIENOLIDE DERIVATIVES AND ISOMERS BY THE HYDROPHOBIC GEL SEPHADEX LH-20

Ayano Yamashita, Toshihiko Nogawa, and Yoshiaki Kamano*

Faculty of Science, Kanagawa University, 2946 Tsuchiya, Hiratsuka,
Kanagawa 259-1293, Japan

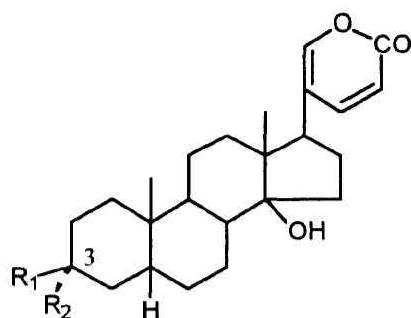
ABSTRACT

We have examined the column chromatography using hydrophobic gel, Sephadex LH-20, for separation of the derivatives and isomers of the bufadienolide ; bufalin, cinobufagin, and resibufogenin. The use of *n*-hexane/CH₂Cl₂/MeOH (4:5:1) and *n*-hexane/EtOAc/MeOH (4:5:1) as developing solvents provides the effective separation of the bufadienolide derivatives such as 3-ester, 3-oxo, Δ^4 , and $\Delta^{1,4}$ derivatives and isomers at 14-position. By this study with previous separation for natural bufadienolides, the use of hydrophobic gel was found to be useful in the point of less-energy and economic aspect, besides the good separation.

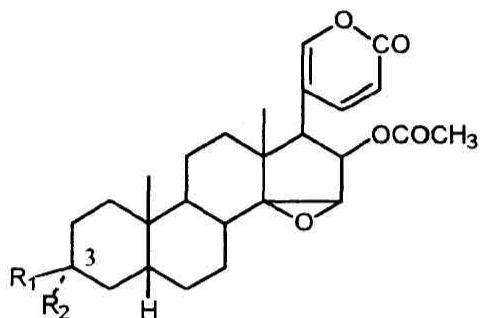
1. INTRODUCTION

The toad poison bufadienolides¹ have a novel steroidal A/B *cis* and C/D *cis* structure with an α -pyrone ring at C-17 position and exhibits a range of biological activities such as cardiotoxic, blood pressure stimulating, respiration, antiviral and antineoplastic activities. Also, we have reported that bufadienolide derivatives and isomers have cytotoxic activity as well².

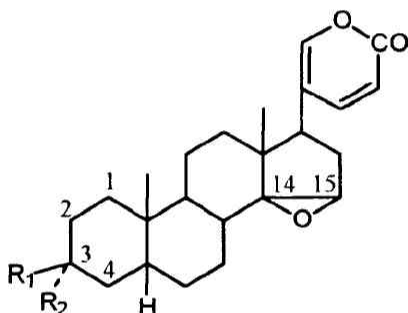
For analytical separation of bufadienolides, gas liquid chromatography³, thin-layer chromatography⁴ and high performance liquid chromatography⁵ were reported. Recently, we have reported the separation of bufadienolides by displacement thin-layer chromatography⁶. Furthermore, for separation and purification of bufadienolides, we have reported the chromatography by the hydrophobic gel, Sephadex LH-20 and HP-Cellulofine⁷. By this experiment, we found the use of hydrophobic gel is useful for separation of bufadienolides and is economic in the aspect of less-energy. Then, we applied this gel chromatography to separation of bufadienolide derivatives such as 3-ester, 3-oxo, Δ^4 and $\Delta^{1,4}$ derivatives and isomers at 14-position of bufadienolides. As a result, this method provided excellent separation for bufadienolide derivatives and isomers.



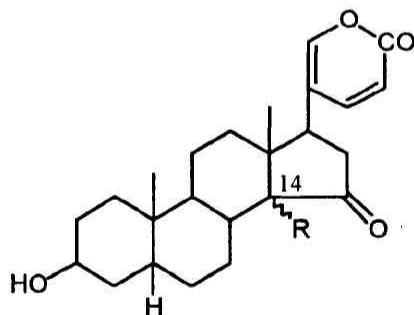
bufalin (**B**) : $R_1=OH$, $R_2=H$
 3-acetyl-bufalin (**AB**) : $R_1=OCOCH_3$, $R_2=H$
 3-oxo-bufalin (**OB**) : $R_1+R_2=O$
 bufalin-3-suberate (**BSub**) : $R_1=OCO(CH_2)_6COOH$, $R_2=H$



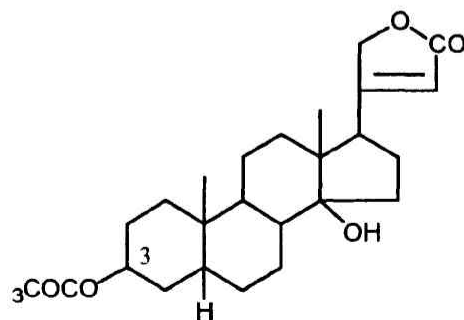
cinobufagin (**C**) : $R_1=OH$, $R_2=H$
 3-acetyl-cinobufagin (**AC**) : $R_1=OCOCH_3$, $R_2=H$
 3-oxo-cinobufagin (**OC**) : $R_1+R_2=O$
 cinobufagin-3-succinate (**CSuc**) : $R_1=OCO(CH_2)_2COOH$, $R_2=H$
 cinobufagin-3-suberate (**CSub**) : $R_1=OCO(CH_2)_6COOH$, $R_2=H$



resibufogenin (**R**) : $R_1=OH$, $R_2=H$
 14 α ,15 α -epoxy-resibufogenin (**α ER**) : $R_1=OH$, $R_2=H$, 14 α ,15 α -epoxy
 3-acetyl-resibufogenin (**AR**) : $R_1=OCOCH_3$, $R_2=H$
 3-oxo-resibufogenin (**OR**) : $R_1+R_2=O$
 Δ^4 -3-oxo-resibufogenin (**Δ^4 OR**) : $R_1+R_2=O$, $\Delta^{4(5)}$
 $\Delta^{1,4}$ -3-oxo-resibufogenin (**$\Delta^{1,4}$ OR**) : $R_1+R_2=O$, $\Delta^{1(2),4(5)}$
 resibufogenin-3-suberate (**RSub**) : $R_1=OCO(CH_2)_6COOH$, $R_2=H$



14 α -artebufogenin (**14 α AG**) : $R=\alpha-H$
 14 β -artebufogenin (**14 β AG**) : $R=\beta-H$



3-acetyl-digitoxigenin (**AD**)

Figure 1. Structures of Bufadienolide Derivatives and Isomers.

2. EXPERIMENTAL

2.1 Chemicals and Reagents

The solvents and the reagents were purchased from commercial sources of analytical grade. Sephadex LH-20 was purchased from Pharmacia.

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 TLC plate after a spray of 5% H_2SO_4 -EtOH solution.

2.2 Materials

The natural products, bufalin (**B**), cinobufagin (**C**), and resibufogenin (**R**) were isolated from *Ch'an Su* (Figure 1). All derivatives and isomers : 3-acetyl-bufalin (**AB**), 3-oxo-bufalin (**OB**), bufalin-3-suberate (**BSub**), 3-acetyl-cinobufagin (**AC**), 3-oxo-cinobufagin (**OC**), cinobufagin-3-succinate (**CSuc**), cinobufagin-3-suberate (**CSub**), 14 α ,15 α -epoxy-resibufogenin (α **ER**), 3-acetyl-resibufogenin (**AR**), 3-oxo-resibufogenin (**OR**), Δ^1 -3-oxo-resibufogenin (Δ^1 **OR**), $\Delta^{1,4}$ -3-oxo-resibufogenin ($\Delta^{1,4}$ **OR**), resibufogenin-3-suberate (**RSub**), 14 α -artebufogenin (**14 α AG**), 14 β -artebufogenin (**14 β AG**), and 3-acetyl-digitoxigenin (**AD**) (Figure 1) have used the stored sample, which were already synthesized from corresponding natural products in our laboratory².

2.3 Chromatographic Procedure

As a typical technique, Sephadex LH-20 was left to stand for about 3hrs at room temperature in developing solvent for swelling. The gel was poured carefully on the column without the intermixing of air. At the top of the setting column, the sample solution in 1-2 mL of developing solvent was placed and eluted carefully. In the case the sample is insoluble in developing solvent, the solvent mixture which was adjusted to the solubility of sample, and changed ratio of amount of developing solvent could be used. The flow speed was set to about 2.0mL/hr. The eluate was collected with suitable number of drops by use of a fraction collector (ADVANTEC, SF-2120, SUPER FRACTION COLLECTOR). For the dropping, a Teflon tube (diameter of hole : 1mm) was used. As a developing solvent, MeOH 100%, *n*-hexane/ CH_2Cl_2 /MeOH (4:5:1) (Solvent A), or *n*-hexane/EtOAc/MeOH (4:5:1) (Solvent B) was used.

2.4 Separation of Bufadienolide Derivatives and Isomers

For separation, the derivatives and isomers divided in the following nine groups and examined.

- I. Bufalin derivatives : **B**, **AB**, and **OB**
- II. Cinobufagin derivatives : **C**, **AC**, and **OC**
- III. Resibufogenin derivatives : **R**, **AR**, and **OR**
- IV. Resibufogenin derivatives introduced some double bonds on A ring : **R**, **OR**, Δ^1 **OR**, and $\Delta^{1,4}$ **OR**
- V. Resibufogenin and Its 14 α ,15 α -isomer : **R** and α **ER**
- VI. 14 α -artebufogenin and Its 14 β -isomer : **14 α AG** and **14 β AG**

VII. Bufadienolide and Cardenolide : **AB** and **AD**

VIII. Cinobufagin and Its dicarboxylic acid monoesters : **C**, **CSuc**, and **CSub**

IX. Bufalin-, Cinobufagin-, and Resibufogenin-3-suberate : **BSub**, **CSub**, and **RSub**

In Table 1, the separation conditions such as sample weight, used solvent, column size, and collected drop numbers/fraction were summarized. In the groups, Group I was examined using two kinds of MeOH (100%) and Solvent A, respectively. Also, separation of Group V was examined by the use of two kinds of Solvents, A and B.

Table 1 Separation Conditions of Bufadienolides

| | compounds ¹⁾ | sample weight | solvent ²⁾ | column size ³⁾ | drops/fr. |
|-------------------|---|---------------|-----------------------|---------------------------|-----------|
| Group I | B, AB, OB | 10mg each | Me and A | (a) | 100 |
| Group II | C, AC, OC | 5.0mg each | A | (b) | 100 |
| Group III | R, AR, OR | 10mg each | A | (a) | 100 |
| Group IV | R, OR, Δ^4 OR, $\Delta^{1,4}$ OR | 10mg each | A | (c) | 100 |
| Group V | R, α ER | 10mg each | A and B | (d) | 200 |
| Group VI | 14 α AG, 14 β AG | 2.5mg each | A | (d) | 50 |
| Group VII | AB, AD | 10mg each | A | (a) | 100 |
| Group VIII | C, CSuc, Csub | 10mg each | A | (a) | 100 |
| Group IX | Bsub, Csub, Rsub | 3.0mg each | B | (e) | 50 |

1) compounds were refer to Figure 1.

2) Me is MeOH 100%, A and B were Solvent A : *n*-hexane/CH₂Cl₂/MeOH (4:5:1), and Solvent B : *n*-hexane/EtOAc/MeOH (4:5:1), respectively.

3) (a), (b), (c), (d), and (e) were ϕ 23mmX480mm, ϕ 12mmX530mm, ϕ 20mmX600mm, ϕ 23mmX450mm, ϕ 12mmX260mm, respectively.

3. RESULTS AND DISCUSSION

3.1 Separation of Bufadienolide Derivatives

Results of Groups, I, II, III, and IV are indicated in Table 2, with the number of fractions. For separation of bufalin derivatives (Group I), the use of Solvent A constructed with *n*-hexane/CH₂Cl₂/MeOH (4:5:1) as developing solvent provided a perfect separation, although the elution with MeOH did not give a good separation. Similar good separations of Groups, II, III and IV, were obtained by the use of Solvent A, respectively. In separation of Group IV, the elution of resibufogenin (**R**) was last. Interestingly, with the increase of double bond, late elution was observed.

**Table 2 Eluted Fraction Number of Bufadienolide Derivatives
by Hydrophobic Gel Chromatography**

| Group I | | | Group II | | Group III | | Group IV | |
|---------|------------------------|------------------------|----------|------------------------|-----------|------------------------|---------------------|------------------------|
| compd. | fraction ¹⁾ | fraction ²⁾ | compd. | fraction ²⁾ | compd. | fraction ²⁾ | compd. | fraction ²⁾ |
| AB | 14-24 | 55-71 | AC | 15-19 | AR | 49-57 | OR | 57-67 |
| OB | 18-27 | 74-84 | OC | 17-21 | OR | 60-75 | Δ ⁴ OR | 65-75 |
| B | 17-27 | 139-155 | C | 22-29 | R | 93-108 | Δ ^{1,4} OR | 77-87 |
| | | | | | | | R | 103-115 |

compounds were refer to Figure 1, 1) eluted with MeOH 100%, 2) eluted with Solvent A

3.2 Separation of Bufadienolide Isomers

Chromatograms of resibufogenin (**R**) and 14α, 15α-epoxy-resibufogenin (α**ER**) with Solvent A constructed with *n*-hexane/CH₂Cl₂/MeOH (4:5:1) and Solvent B constructed with *n*-hexane/EtOAc/MeOH (4:5:1) on SiO₂ TLC plates is shown in Figure 2. In the case of Solvent A, these compounds were completely overlapped. On the other hand, in the use of Solvent B, 14α, 15α-epoxy-resibufogenin (α**ER**) was developed slightly higher than resibufogenin (**R**) with Δ*R_f* = 0.014. From these results, it is expected that separation of these compounds is difficult by traditional SiO₂ column chromatography. But, the use of hydrophobic gel, Sephadex LH-20 provided perfect separation with Solvent B as shown in Figure 3a. In the case of Solvent A resibufogenin (**R**) was eluted slightly earlier than 14α, 15α-epoxy-resibufogenin (α**ER**), however separation was failed. Eluted order on hydrophobic gel chromatography with Solvent B was resibufogenin (**R**) > 14α, 15α-epoxy-resibufogenin (α**ER**), this order was reverse for developing order on SiO₂ plate.

In separation of 14α-artebufogenin (14α**AG**) and 14β-artebufogenin (14β**AG**), 14β-artebufogenin (14β**AG**) was eluted earlier than an isomer (14α**AG**) and the result was good (Figure 3b).

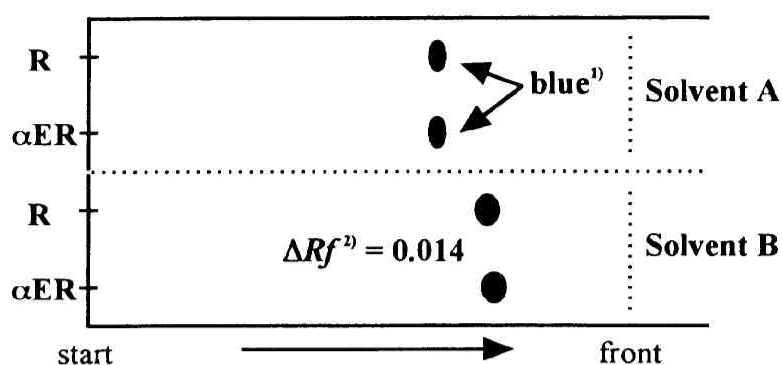


Figure 2. Thin-layer chromatograms of resibufogenin (**R**) and 14 α ,15 α -epoxy-resibufogenin (α **ER**) on SiO₂ TLC plate using Solvent A constructed *n*-hexane/CH₂Cl₂/MeOH (4:5:1) and Solvent B constructed *n*-hexane/EtOAc/MeOH (4:5:1) as mobile phase. 1) Detected colors after spraying 5% H₂SO₄-EtOH solution and heating. 2) ΔR_f is difference between R_f value of 14 α ,15 α -epoxy-resibufogenin and that of resibufogenin.

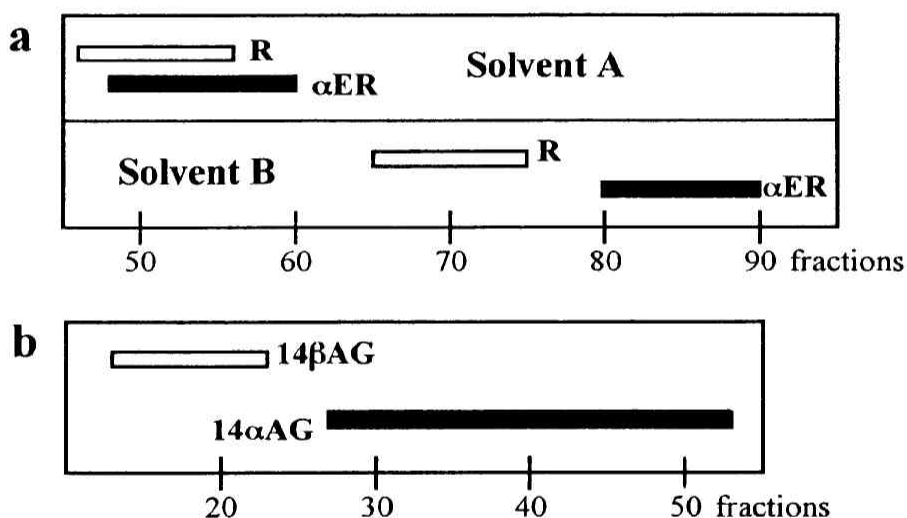


Figure 3. Separation of bufadienolide isomers. **a** shows separations of resibufogenin (**R**) and 14 α ,15 α -epoxy-resibufogenin (α **ER**) using Solvent A and Solvent B as developing solvent (Group V). **b** shows separation of 14 α -artebufogenin (14 α **AG**) and 14 β -artebufogenin (14 β **AG**) (Group VI). Chromatographic conditions refer to Table 1.

3.3 Separation of Group VII

In separation of bufadienolide and cardenolide, acetyl-bufalin (**AB**) was eluted earlier than acetyl-digitoxigenin (**AD**) and the separation was good, as shown in Figure 4. This result shows that hydrophobic gel chromatography applies to separation of cardenolides as well as that of bufadienolides.

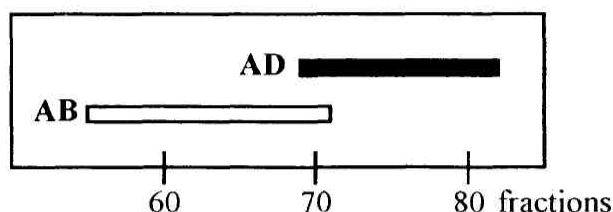


Figure 4. Separation of bufadienolide and cardenolide. Separation of acetyl-bufalin (**AB**) and acetyl-digitoxigenin (**AD**) is shown. Chromatographic conditions refer to Table 1.

3.4 Separations of Group VIII and Group IX

In separation of cinobufagin and its dicarboxylic acid monoesters, the use of Solvent A provided a good separation eluting with the following order : cinobufagin-3-suberate > cinobufagin > cinobufagin-3-succinate, as shown in Figure 5.

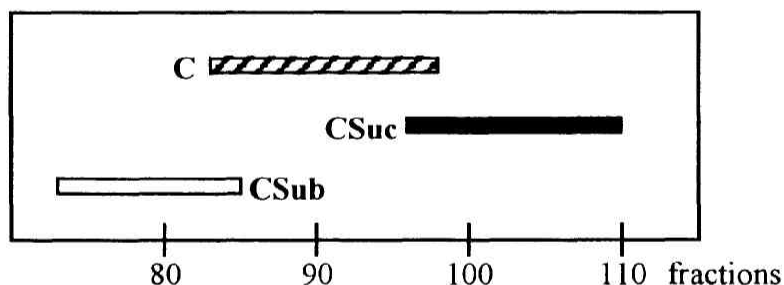


Figure 5. Separation of cinobufagin, cinobufagin-3-succinate, and cinobufagin-3-suberate (**C**, **CSuc**, and **CSub**, respectively). Chromatographic conditions refer to Table 1.

Chromatograms of bufalin-, cinobufagin-, and resibufogenin-3-suberates (**BSub**, **CSub**, and **RSub**) on SiO_2 TLC plate with Solvent B were illustrated in Figure 6a. Separation of these compounds by SiO_2 column chromatography using this solvent might be impossible, because on SiO_2 TLC plate, tailing of sample spot from starting point was observed for each compounds, and R_f values were close. But eluate by hydrophobic gel, Sephadex LH-20 column chromatography using same solvent (Solvent B) provided excellent separation as shown in Figure 6b.

These bufadienolide dicarboxylic acid monoester derivatives may be difficult to separate on traditional SiO_2 column chromatography, because of absorption to silica gel and tailing of eluted samples. On the other hand, in the hydrophobic gel chromatography, these compounds were eluted in a short time, and good separation was obtained.

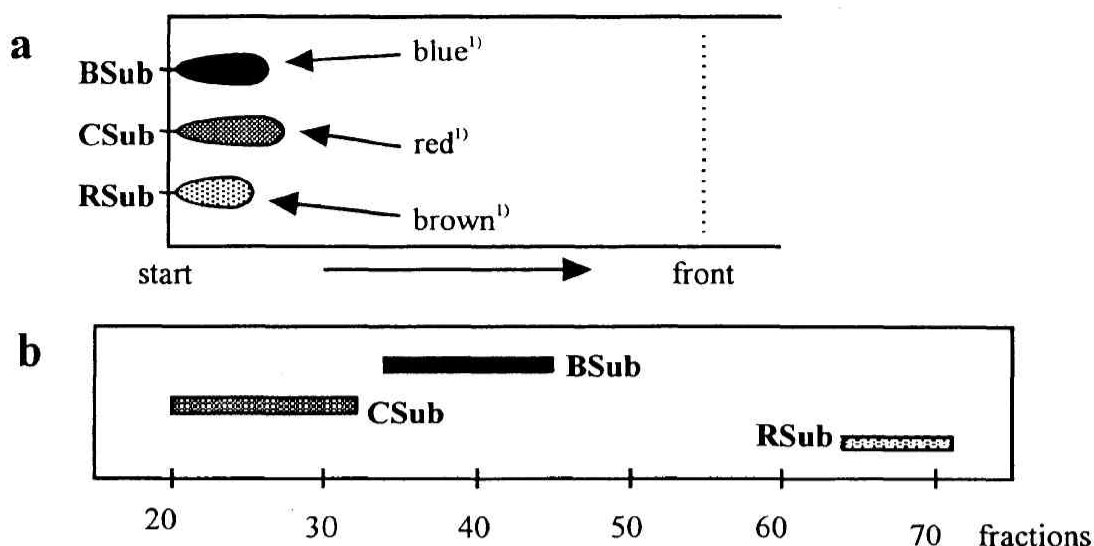


Figure 6. Separation of bufalin-, cinobufagin-, resibufogenin-3-suberates (**BSub**, **CSub**, and **RSub**, respectively). **a** shows chromatograms of **BSub**, **CSub**, and **RSub** on SiO_2 plates using Solvent B as mobile phase. 1) Detected colors after spraying 5% H_2SO_4 -EtOH solution and heating. **b** shows separation of **BSub**, **CSub**, and **RSub** on hydrophobic gel chromatography. Chromatographic conditions refer to Table 1.

3.5 Relationship between Elution and Structure

Relationships between elution and structure by hydrophobic gel chromatography are summarized in a-e in below.

- a. $3\text{-OAc} > 3\text{-C=O} > 3\text{-OH}$
- b. $3\text{-C=O} > \Delta^4 3\text{-C=O} > \Delta^{1,4} 3\text{-C=O} > 3\text{-OH}$
- c. $14\beta, 14\beta\text{-epoxy} > 14\alpha, 15\alpha\text{-epoxy}$
- d. $14\beta\text{-H} > 14\alpha\text{-H}$
- e. bufadienolide > cardenolide

From the results of c and d, 14β -isomers (C/D *cis*) were eluted earlier its α -isomer (C/D *trans*). Also, the elution of cardenolide was later than that of bufadienolide (e). These results were similar to those obtained previously with natural products⁷.

4. CONCLUSION

The investigation shows that the chromatography on hydrophobic gel, Sephadex LH-20 using *n*-hexane/ CH_2Cl_2 /MeOH (4:5:1) or *n*-hexane/EtOAc/MeOH (4:5:1) as solvents is very useful for separations of bufadienolide derivatives and isomers. Thus, the use of Sephadex LH-20 is a convenient method for separation in the point of elution time, and also, is an economic way in the aspect of that the gel is able to use repeatedly. The resulting use of hydrophobic gel was found to be an economic method in the aspect of less-energy. We expect that proposed method may serve for the separation of other bufadienolides and related compounds such as cardenolides.

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