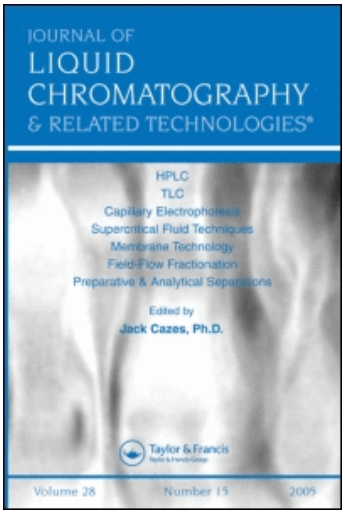


Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Utility of Cyclodextrin in Mobile Phase for High-Performance Liquid Chromatographic Separation of Bufadienolides

Kazutake Shimada[a]; Youko Kurata[a]; Tomoyuki Oe[a]
[a] Faculty of Pharmaceutical Sciences Kanazawa University, Kanazawa, Japan

To cite this Article Shimada, Kazutake , Kurata, Youko and Oe, Tomoyuki(1990) 'Utility of Cyclodextrin in Mobile Phase for High-Performance Liquid Chromatographic Separation of Bufadienolides', Journal of Liquid Chromatography & Related Technologies, 13: 3, 493 — 504
To link to this Article: DOI: 10.1080/01483919008051801
URL: http://dx.doi.org/10.1080/01483919008051801

JOURNAL OF LIQUID CHROMATOGRAPHY, 13(3), 493-504 (1990)

UTILITY OF CYCLODEXTRIN IN MOBILE PHASE FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF BUFADIENOLIDES

KAZUTAKE SHIMADA*, YOUKO KURATA, AND TOMOYUKI OE

Faculty of Pharmaceutical Sciences
Kanazawa University
13-1 Takara-machi
Kanazawa 920, Japan

ABSTRACT

The chromatographic behavior of bufadienolides including conjugates was examined by the addition of γ-cyclodextrin to the mobile phase in reversed-phase high-performance liquid chromatography. The capacity factor of sulfate or glucuronide was decreased sharply by the additive. The separation of bufadienolides, unseparable under the usual method, was much improved by the addition of γ-cyclodextrin.

INTRODUCTION

In previous papers we reported the use of cyclodextrin (CD) in the mobile phase which is of great advantage in the separation of isomeric steroids (estrogens [1, 2], bile acids [3]) and their fluorescence detection in reversed-phase high-performance liquid chromatography (HPLC).

Cardiac steroids obtained from natural sources were divided into two categories, cardenolide and bufadienolide having a five-

or six-membered lactone ring at the 17 β-position, respectively. We examined the retention behavior of cardenolides in the inclusion chromatography using CD as an additive in the mobile phase and clarified that the steroid A/B ring junction is the most important factor in the choice of the optimum CD to be added [4, 5]. We also suggested that the obtained information on cardenolides may be applicable to the separation of bufadienolides [5], the assumption was verified by the determination of in vitro metabolites of bufalin (**1**), one of the representative bufadienolides [6]. As a continuation of these works, the present paper clarifies the retention behavior of bufadienolides in the inclusion chromatography and the advantage of this chromatography to the separation of these compounds.

MATERIALS AND METHODS

Materials

CDs were kindly supplied by Nihon Shokuhin Kako Co. (Tokyo, Japan). Bufadienolides were isolated from the natural source [7]. 1-Anthroyl cyanide was purchased from Wako Pure Chem. Ind., Ltd. (Osaka, Japan). Solvents were purified by distillation prior to use.

Apparatus

HPLC was carried out on a JASCO TRI ROTAR chromatograph equipped with a JASCO UVIDEC-100-II ultraviolet detector (UV; 300 nm)(Japan Spectroscopic Co., Ltd., Tokyo) or Hitachi F-1000 fluorescence detector (FL; λex 370 nm, λem 470 nm)(Hitachi Ltd., Tokyo) at a flow rate of 1 ml/min. A Develosil ODS-5 column (5 μm; 15 cm x 0.4 cm i.d.) (Nomura Chemical Co., Seto, Japan) was

used at ambient temperature unless otherwise stated. The pH of the mobile phase was adjusted with H_3PO_4. The dead volume was determined by the use of $NaNO_3$ (UV) or MeOH (FL).

Derivatization Methods

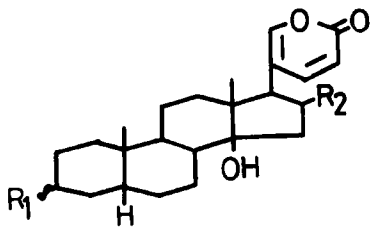
The derivatization of 3-epibufalin (**2**) with 1-anthroyl cyanide was done according to the procedure described by Goto et al. [8].

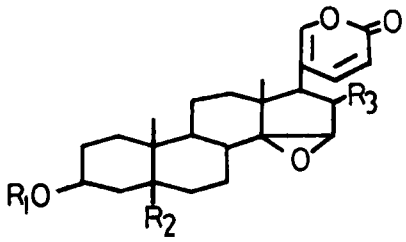
RESULTS AND DISCUSSION

Separation of Bufogenins

The chromatographic attempts to separate bufogenins obtained from toad venom have been reported in several papers, but the results of which are not necessarily satisfactory [9]. In previous paper we reported the HPLC separation of bufogenins on reversed phase column (μBondapak C_{18}) [10]. The separation of three pairs of bufogenins (resibufogenin (**9**), cinobufagin (**11**); marinobufagin (**14**), cinobufotalin (**16**); bufotalin (**8**), deacetylcinobufagin (**13**))(Figure 1) was unsatisfactory in that experiment using acetonitrile as an organic modifier. In this paper the inclusion chromatography using the mobile phase fortified with CD was used for the separation of these compounds. It has previously been disclosed that γ-CD was the most effective modifier for the retention of cardiac steroids having an A/B cis ring junction [5]. These data prompted us to use γ-CD in this chromatography.

The effects of γ-CD on the capacity factor (k') and resolution (Rs) of bufogenins are illustrated in Figure 2. Rapid elution and good separation of these compounds were obtained with





1. R_1=⁻OH, R_2=H
2. R_1=⁻H, R_2=H
3. R_1=⁻H, R_2=H
4. R_1=⁻OCO$(CH_2)_n$COArg·OH, R_2=H (**n=2–6**)
5. R_1=⁻OCO$(CH_2)_n$COOH, R_2=H (**n=2,6**)
6. R_1=⁻OSO$_3$H, R_2=H
7. R_1=⁻O-glucuronyl, R_2=H
8. R_1=⁻OH, R_2=OAc
9. R_1=R_2=R_3=H
10. R_1=CO$(CH_2)_2$COOH, R_2=R_3=H
11. R_1=R_2=H, R_3=OAc
12. R_1=CO$(CH_2)_2$COOH, R_2=H, R_3=OAc
13. R_1=R_2=H, R_3=OH
14. R_1=H, R_2=OH, R_3=H
15. R_1=CO$(CH_2)_6$COArg·OH, R_2=OH, R_3=H
16. R_1=H, R_2=OH, R_3=OAc
17. R_1=CO$(CH_2)_6$COArg·OH, R_2=OH, R_3=OAc

FIGURE 1. Structures of bufadienolides.

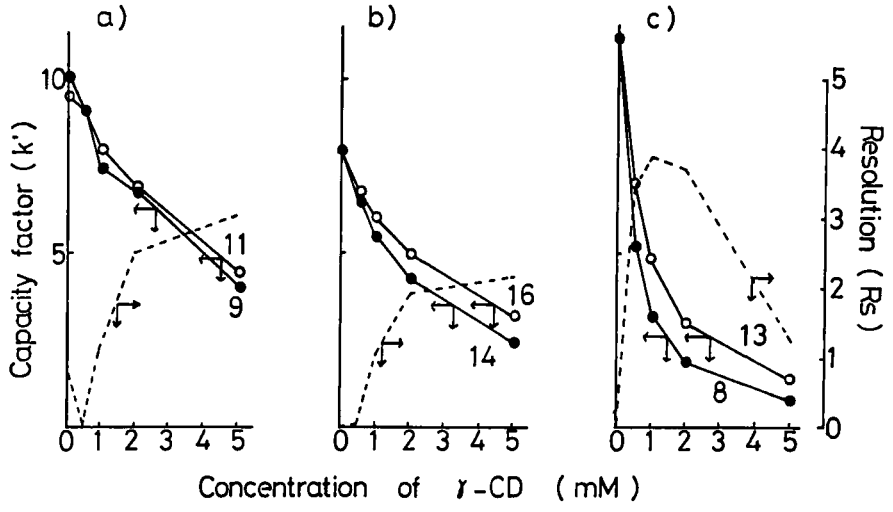


FIGURE 2. Effect of γ-CD on the retention and separation of bufogenins.
Conditions: mobile phase, CH_3CN-H_2O (a, 2:3; b, 1:2; c, 2:5) containing γ-CD as indicated; detection, UV.
The numbers referred to the structures as shown in Figure 1.

increasing concentration of γ-CD. One of the typical chromatograms is shown in Figure 3b, in which complete separation of **14** and **16** was observed by the addition of γ-CD in the mobile phase.

Effect of CD on the Retention of Bufadienolides Differing in Conjugation

Bufadienolides were obtained from toad venom as a bufogenin and conjugated one. The latter contained bufotoxin (bufogenin 3-suberoylarginine ester (**4**, **n=6**)), its homologs (**4**, **n=2-5**), bufogenin 3-fatty acid esters (**5**) and 3-sulfates (**6**)[7]. It is of interest to examine the effect of conjugation on the retention of these compounds in the inclusion chromatography. On this point of

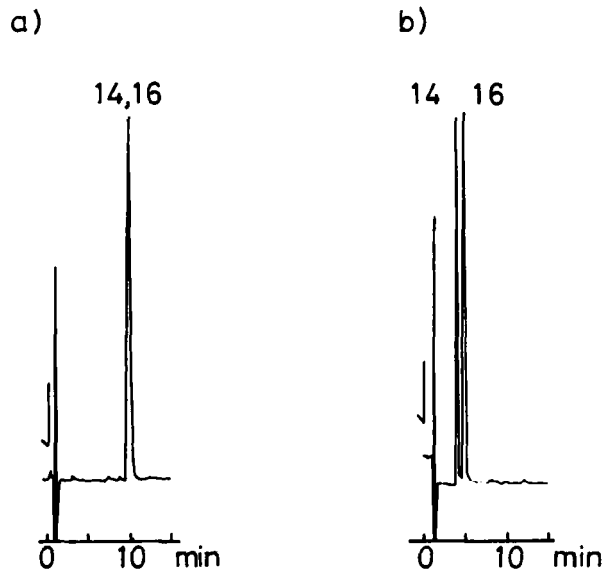


FIGURE 3. Separation of marinobufagin (**14**) and cinobufotalin (**16**). Conditions: mobile phase, a) CH_3CN-H_2O (1:2) b) CH_3CN-H_2O (1:2) containing γ-CD (5 mM).

view, the effects of γ-CD contents in the mobile phase on the k' values of various conjugated bufadienolides were investigated. All the k' values of the compounds examined obtained without CD were kept at greater than 9.1 by changing the concentration of the organic modifier, acetonitrile. Since the organic modifier competes with the solute for the hydrophobic CD cavity, a change in the proportion of organic modifier may influence the solute interaction with the CD. However, a change in the proportion was unavoidable to get the appropriate k' value for detection and characterization of the effect of CD [5].

Comparing the relative capacity factors (Rk') of bufalitoxin (**4**, **n=6**) with those of its homologs (**4**, **n=2-5**), the values were

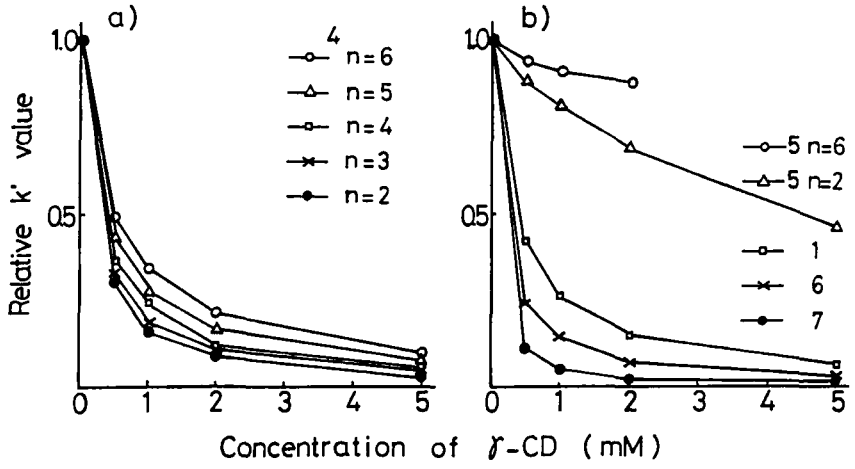


FIGURE 4. Effect of γ-CD on the retention of bufalin conjugates. a) Bufalitoxin and its homologs (**4**) b) other bufalin conjugates.
Conditions: mobile phase, CH_3CN-0.5% NaOAc(pH 4.0), ratio (**4 n=6**, 1:2; **4 n=5**, 4:9; **4 n=4**, 2:5; **4 n=3**, 10:27; **4 n=2**, 5:14; **5 n=6**, 2:1; **5 n=2**, 1:1; **1**, 1:2; **6**, 3:10; **7**, 5:21); detection, UV. The to (min) and k' values obtained without γ-CD, taken as 1.0 for the calculation of the Rk' value, are as follows (**4 n=6**, 1.2, 10.2; **4 n=5**, 1.2, 12.1; **4 n=4**, 1.2, 12.0; **4 n=3**, 1.2, 10.6; **4 n=2**, 1.3, 9.6; **5 n=6**, 1.1, 9.1; **5 n=2**, 1.1, 10.5; **1**, 1.1, 11.8; **6**, 1.3, 11.4; **7**, 1.3, 9.7).
The numbers referred to the structures as shown in Figure 1.
The data of **5 n=6** at the more than 3 mM of additive has not been obtained because of the solubility.

more influenced by the homologs having the shorter methylene units (Figure 4a). Among the examined other bufalin conjugates, Rk' value of glucuronide (**7**) was most influenced by the addition of CD, followed by that of sulfate (**6**)(Figure 4b). It is likely that the hydrophilic group on the conjugated moiety will be important factor for the formation of the inclusion complex from the solute and CD. The significant interaction, e.g., hydrogen bonding may occur between solutes bearing OH groups and the hydroxyl groups of CD [11].

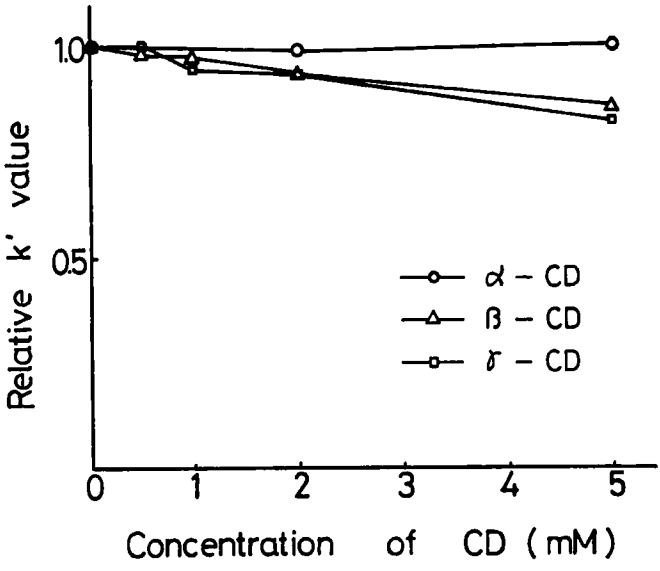


FIGURE 5. Effect of CD on the retention of 3-epi-(1-anthroyl) bufalin (3).
Conditions: column, YMC-GEL CN; mobile phase, CH_3CN-H_2O (10:11) containing CD as indicated; detection, FL; to 1.5 min; The k' value (9.4) obtained without CD was taken as 1.0 for the calculation of the Rk' values.

In previous paper we reported the chromatographic behavior of cardenolides labeled with 1-anthroyl cyanide was not so influenced by the addition of methylated-CD to the mobile phase. But the mobile phase containing more than 75% of organic modifier together with YMC-GEL C_8 column was used at that experiment [5]. High percentage of organic modifier may interfere the solute interaction with CD. In order to clarify this ambiguity, that of labeled 3-epibufalin (3) was examined by using YMC-GEL CN (5 μm;15 cm x 0.4 cm i.d.)(Yamamura Chem. Lab. Co., Ltd., Kyoto, Japan) and acetonitrile-water (10:11) containing α-, β- or γ-CD as a column and mobile phase, respectively (Figure 5). In spite

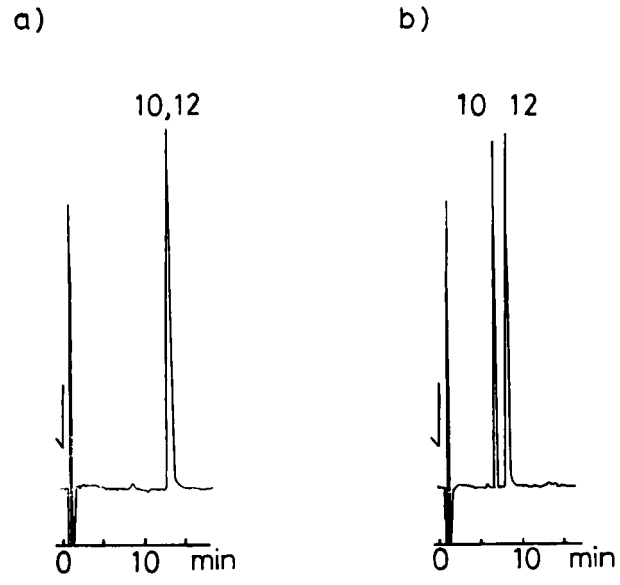


FIGURE 6. HPLC chromatograms of resibufogenin- and cinobufagin-3-succinates (**10, 12**).
Conditions; mobile phase, a) CH_3CN-0.5% NaOAc (pH 4.0)(3:4) b) CH_3CN-0.5% NaOAc (pH 4.0)(3:4) containing Y-CD(5 mM); detection, UV.

of more than 50% of water in the mobile phase, Rk' values were not so influenced by these CDs. The data suggested that bulky anthracene residue may interfere with the formation of the inclusion complex.

Separation of Conjugated Bufogenins

In the previous paper we reported the separation of bufogenin conjugates by the conventional HPLC but the results were not necessarily satisfactory [12]. The application of the inclusion chromatography to the separation of these compounds gave satisfactory results. The couple of examples were shown in

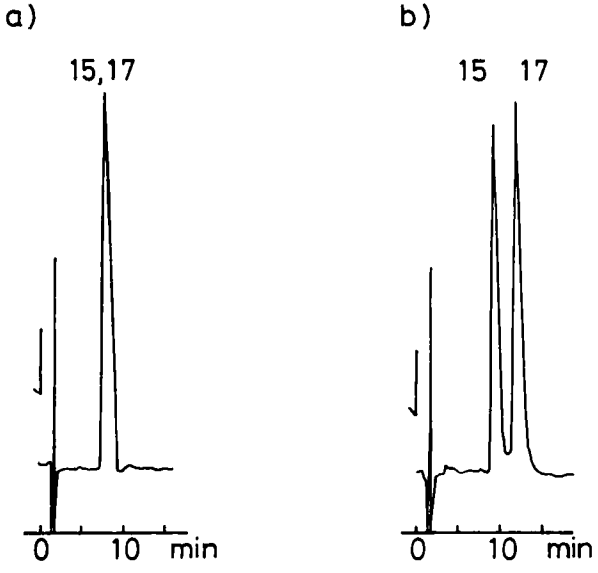


FIGURE 7. HPLC chromatograms of marinobufotoxin (**15**) and cinobufotalitoxin (**17**).
Conditions; mobile phase, a) CH_3CN-0.5% NaOAc (pH 3.0)(1:2) b) CH_3CN-0.5% NaOAc (pH 3.0)(2:5) containing γ-CD (3.85 mM); detection, UV.

Figures 6b and 7b, in which the complete separation has been observed on resibufogenin- and cinobufagin-3-succinates (**10**, **12**), marinobufotoxin (**15**) and cinobufotalitoxin (**17**), respectively. The separation of other pair of bufotoxin homologs (e. g., resibufogenin- and cinobufagin-3-succinylarginine esters) was also much improved by the additive.

In conclusion the use of γ-CD in the mobile phase affords an advantage to the separation of bufadienolides in the reversed-phase HPLC. Extraction of the eluate from HPLC with organic solvent (e.g., chloroform) followed by washing with water recovered the bufadienolide without CD. The method is applicable

not only to the determination but also the isolation of these compounds from the natural source.

Further applications of this chromatography to other compounds are being conducted in these laboratories, and the details will be reported elsewhere.

ACKNOWLEDGEMENTS

We thank Dr. Y. Kamano (Arizona State Univ.) for providing cinobufotalin. This work was supported by grants from the Ministry of Education, Science and Culture, Japan, Project Research Found from Graduate School of Natural Science and Technology, Kanazawa University and Takeda Research Foundation, which are gratefully acknowledged.

REFERENCES

1) Shimada, K., Masue, T., Toyoda, K., Takani, M. and Nambara, T., The utility of cyclodextrin in mobile phase for high-performance liquid chromatographic separation of isomeric estrogens, J. Liquid Chromatogr., 11, 1475 (1988).

2) Shimada, K., Masue, T. and Chiba, H., Application of inclusion chromatography to the determination of in vitro metabolites of estriol, J. Chromatogr. Sci., 27, 557 (1989).

3) Shimada, K., Komine, Y. and Oe, T., Chromatographic behavior of bile acids using cyclodextrin in mobile phase of high performance liquid chromatography, J. Liquid Chromatogr., 12, 491 (1989).

4) Shimada, K., Oe, T., Kanno, C. and Nambara, T., Utility of cyclodextrin in mobile phase for high performance liquid chromatographic separation of cardenolides, Anal. Sci., 4, 377 (1988).

5) Shimada, K., Oe, T., Hirose, Y. and Komine, Y., Retention behaviour of cardiac steroids using cyclodextrin in the mobile phase in high-performance liquid chromatography, J. Chromatogr., 478, 339 (1989).

6) Shimada, K., Hirose, Y. and Oe, T., Application of inclusion chromatography to the determination of in vitro metabolites of bufalin, J. Liquid Chromatogr., in press.

7) Shimada, K., Fujii, Y., Yamashita, E., Niizaki, Y., Sato, Y and Nambara, T., Studies on cardiotonic steroids from the skin of Japanese toad, Chem. Pharm. Bull., 25, 714 (1977).

8) Goto, J., Goto, N., Shamsa, F., Saito, M., Komatsu, S., Suzaki, K. and Nambara, T., New sensitive derivatization of hydroxysteroids for high-performance liquid chromatography with fluorescence detection, Anal. Chim. Acta, 147, 397 (1983).

9) Inoue, M., Kamano, Y., Pettit, G.R. and Smith, C.R., Separation of cinobufotalin by preparative liquid chromatography, J. Liquid Chromatogr., 10, 2453 (1987) and references cited therein.

10) Shimada, K., Hasegawa, M., Hasebe, K., Fujii, Y. and Nambara, T., Separation and determination of cardiac steroids by high-pressure liquid chromatography, Chem. Pharm. Bull., 24, 2995 (1976).

11) Armstrong, D.W. and DeMond, W., Cyclodextrin bonded phases for the liquid chromatographic separation of optical, geometrical and structural isomers, J. Chromatogr. Sci., 22, 411 (1984).

12) Shimada, K., Hasegawa, M., Hasebe, K., Fujii, Y. and Nambara, T., Separation of cardiotonic steroid conjugates by high-performance liquid chromatography, J. Chromatogr., 124, 79 (1976).