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

Kazutake SHIMADA*, Tomoyuki OE*, Chiyomi KANNO and Toshio NAMBARA****

**Faculty of Pharmaceutical Sciences, Kanazawa University, Takaramachi, Kanazawa 920*
***Pharmaceutical Institute, Tohoku University, Aobayama, Sendai 980*

Cyclodextrin was used as a component of mobile phase for the separation of cardenolides by reversed-phase high performance liquid chromatography. The addition of γ-cyclodextrin to the mobile phase improved the separation of isomeric cardenolides. The proposed method was applied to the isolation of cardiac glycosides having a doubly linked sugar from plant material and to the clarification of ring C ketol rearrangement products of cardiac glycosides.

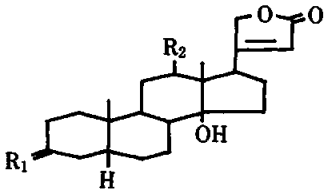
Keywords High performance liquid chromatography, reversed-phase column, cyclodextrin, cardenolide, cardiac glycoside, inclusion complex

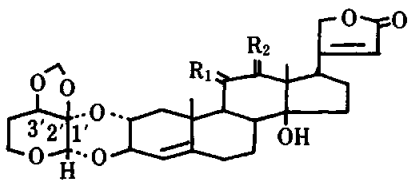
In the previous paper[1] we reported the use of cyclodextrin (CD) in the mobile phase, which is of great advantage to the separation of isomeric estrogens and their fluorescence detection in reversed-phase high performance liquid chromatography (HPLC). CD is readily available and exerts no influences on the reproducibility of retention time and column life.[1] These data prompted us to investigate further the separation of several cardenolides (Fig. 1) by HPLC on a reversed-phase column with the mobile phase containing CD. The present paper deals with the applications of this method to the isolation of cardiac glycosides from plant material and the clarification of ring C ketol rearrangement products of cardiac glycosides.

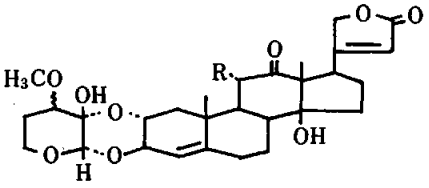
Experimental

Materials

CDs were kindly supplied by Nihon Shokuhin Kako







1 : R_1=<O-(digitoxose)$_3$, R_2=H
2 : R_1=<O-(digitoxose)$_3$, R_2=OH
3 : R_1=<OH, R_2=H
4 : R_1=<OH, R_2=OH
5 : R_1=O, R_2=OH
6 : R_1=<H, R_2=OH

7 : R_1=R_2=H_2
10 : R_1=<H, R_2=O
11 : R_1=O, R_2=<H
12 : R_1=R_2=O
13 : R_1=O, R_2=<OH
14 : R_1=O, R_2=H_2
15 : R_1=H_2, R_2=O

8 : R=OH, 3′β-OCH_3
9 : R=H, 3′α-OCH_3

Fig. 1 Structures of cardenolides.

Co., Ltd. (Tokyo). Cardiac glycosides having a doubly linked sugar were obtained from the natural source in these laboratories as reported previously.[2,3] Other cardenolides were prepared from digitoxin and digoxin (Nakarai Chemicals, Ltd., Kyoto) by known methods.[4] 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 (240 nm) (Japan Spectroscopic Co., Ltd., Tokyo) at a flow rate of 1 ml/min. A Develosil ODS-5 (5 μm) column (15 cm×0.4 cm i.d.) (Nomura Chemical Co., Seto) was used at ambient temperature. The void volume was determined by the use of sodium nitrate.[1]

Quantitative determination of ketol rearrangement products

A glycoside (3 mg) was dissolved in methanol–acetone (1:1, 1 ml), adsorbed on alumina (200 mg, Aluminum oxide 90, E. Merck, Darmstadt), allowed to stand at room temperature for 3 d and then eluted with methylene chloride–ethyl acetate (1:1). After evaporation of the solvent, the residue was subjected to HPLC to determine the amount ratios of the products. Each compound (500 μg) spiked to the blank was recovered at a rate of more than 89.3% (n=5, relative standard deviation ≤4.5%).

Results and Discussion

Effect of CDs in the mobile phase on the retention

The effects of α-, β- and γ-CD contents in the mobile phase on capacity factor (k') of digitoxin (**1**), digitoxigenin (**3**) and elaeodendroside D (**7**)[2] were investigated (Table 1). Among CDs examined, γ-CD was most effective for decreasing the k' values of the former two cardenolides. On the contrary, the k' values of **7** were more significantly depressed by the addition of β-CD. It has previously been disclosed that β-CD was the most effective modifier for the retention of estrogens.[1] Although no plausible explanation is at present available for these phenomena, it is likely that ring juncture, 17β-butenolide ring, or doubly linked sugar in **7** will be important features for the formation of the inclusion complex from the solute and CD. It is well known that β-CD is much less soluble in water than other CDs.[5] These findings led us to use γ-CD as the modifier for further study.

Separation of digoxigenin, 3-dehydrodigoxigenin and 3-epidigoxigenin

Digoxigenin (**4**), one of the major metabolites of digoxin (**2**), undergoes further biotransformation in living animals.[6] 3-Dehydrodigoxigenin (**5**) and 3-epidigoxigenin (**6**) are regarded as metabolic intermediates, but the details still remain unclear due to the lack of a reliable analytical method. The separation of these compounds by HPLC on a reversed-phase column was examined by the addition of γ-CD to the mobile phase using acetonitrile as the organic modifier. The relative k' values of the 3-hydroxyl compounds (**4**, **6**) were more remarkably influenced than the 3-dehydro compound (**5**) with increasing concentration of γ-CD in the mobile phase (Fig. 2). The complete separation (R_s≥2) was attained within 5 min as illustrated in Fig. 3. The proposed method may be applicable to the study on metabolic pathways of digoxin.

Table 1 Effect of CDs in the mobile phase on the retention of cardiac stereoids

CD/mM		k' Digitoxin (1)	Digitoxigenin (3)	Elaeodendroside D (7)
	0	7.3[a]	7.1[b]	9.2[c]
α-CD	1	8.0	7.5	9.2
	2	7.7	7.5	9.2
	5	8.2	7.4	9.0
β-CD	1	5.8	4.9	2.9
	2	5.2	4.0	1.8
	5	3.7	2.4	0.8
γ-CD	0.5	4.0	3.3	9.0
	1	3.1	2.0	8.1
	2	1.9	1.2	7.1
	5	0.8	0.6	5.4

Conditions; mobile phase, a) acetonitrile–H_2O (2:3, t_0 1.2 min), b) acetonitrile–H_2O (1:2, t_0 1.1 min), c) acetonitrile–H_2O (6:7, t_0 1.2 min). Each system contained CD as indicated.

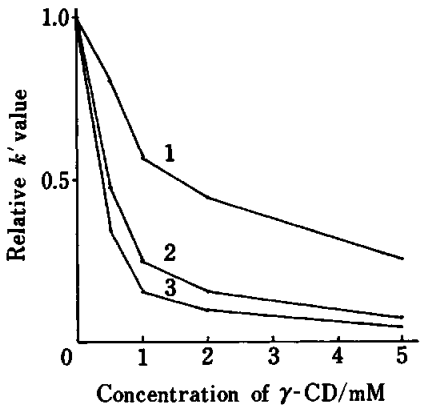


Fig. 2 Effect of γ-CD on the retention of digoxigenin, 3-dehydrodigoxigenin and 3-epidigoxigenin. 1: 3-dehydrodigoxigenin (**5**), 2: 3-epidigoxigenin (**6**), 3: digoxigenin (**4**). Conditions: mobile phase, acetonitrile–H_2O (2:9) containing γ-CD as indicated, t_0 1.1 min. Each k' value (1: 14.3, 2: 12.3, 3: 7.2) obtained without γ-CD was taken as 1.0.

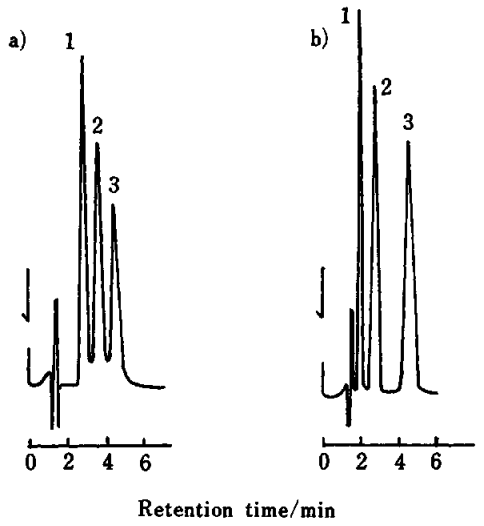


Fig. 3 Separation of digoxigenin, 3-dehydrodigoxigenin and 3-epidigoxigenin. 1: digoxigenin (**4**), 2: 3-epidigoxigenin (**6**), 3: 3-dehydrodigoxigenin (**5**). Conditions: mobile phase, a) acetonitrile–H_2O (1:3), b) acetonitrile–H_2O (1:3) containing 1.16 mM γ-CD.

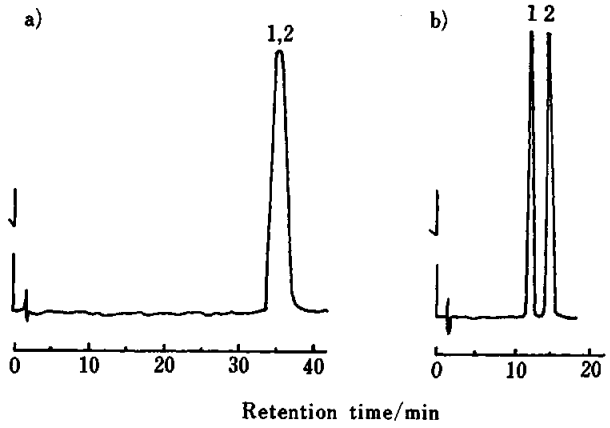


Fig. 4 Separation of elaeodendroside P and R. 1: elaeodendroside P (**8**), 2: elaeodendroside R (**9**). Conditions: mobile phase, a) acetonitrile–H_2O (2:7), b) acetonitrile–H_2O (2:7) containing 7.71 mM γ-CD.

Separation of elaeodendroside P and R

In the preceding papers, we reported the isolation and characterization of twelve cardiac glycosides having a doubly linked sugar named elaeodendroside A–L from seeds of *Elaeodendron glaucum* PERS.[2,3] Further studies on this plant material gave a mixture of new cardiac glycosides named elaeodendroside P (**8**) and R (**9**) having a doubly linked sugar. The separation of these two, however, could not be attained by conventional reversed-phase HPLC (Fig. 4a). The addition of γ-CD (7.71 mM) to the mobile phase provided the satisfactory separation of the two compounds (Fig. 4b). The cardiac glycosides were

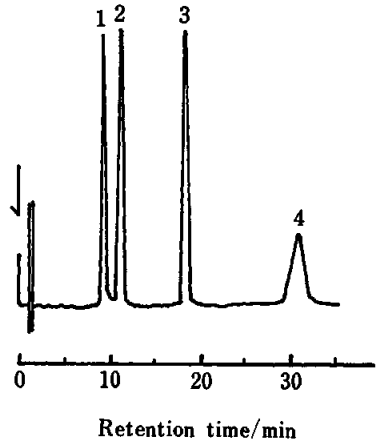


Fig. 5 Separation of elaeodendroside A, J, N and M. 1: elaeodendroside N (**13**), 2: elaeodendroside J (**11**), 3: elaeodendroside A (**10**), 4: elaeodendroside M (**12**). Conditions: mobile phase, acetonitrile–H_2O (2:5) containing 7.71 mM γ-CD.

recovered from the eluate by extraction with chloroform followed by washing with water. The presence of γ-CD in the mobile phase did not interfere with extraction of these compounds.[7]

Separation of cardiac glycosides having ring C substituent

It has been clarified that elaeodendroside A (**10**) underwent ketol rearrangement to yield elaeodendroside J (**11**) and elaeodendroside M (**12**) having a diosphenol structure.[2,8-10] On being treated with alumina or silica gel, **10** and **11** became interconvertible.[2,10] The stability of elaeodendroside N (**13**), recently isolated from the same plant material[7], has not yet been clarified. The separation of **11** and **13** could not be accomplished by the usual HPLC method. The addition of γ-CD (7.71 mM) to the mobile phase, however, gave satisfactory separation of compounds **10**–**13** (Fig. 5). After treatment of **10**, **11**, or **13** with alumina in methanol–acetone for 3 d[2], the reaction mixture was subjected to HPLC with mobile phase containing γ-CD. Treatment of **10** afforded **11**, **12** and unchanged starting material in the amount ratio of 3:1:7, while **11** gave **10**, **12** and unchanged **11** in the amount ratio of 2:1:18. On the contrary, the starting material was recovered almost quantitatively when **13** was treated in the manner described above. These results imply that **13** is a much more stable compound and undergoes no ketol rearrangement under this condition.

The separation of 11- and 12-oxo compounds (**14**, **15**)[2] was also attempted. Although their k' values were not remarkably affected by the addition of γ-CD (6.94 mM) to the mobile phase, these two were completely resolved (R_s 1.5), as shown in Fig. 6. In the previous paper we reported that Na^+ and K^+-ATPase inhibitory activities of cardiac glycosides are significantly affected by the presence of ring C

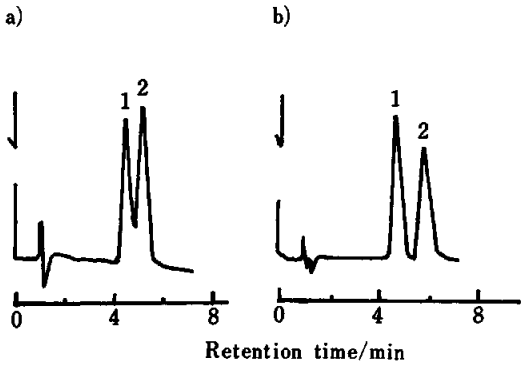


Fig. 6 Separation of cardiac glycosides having 11- or 12-oxo group. 1: compound **14**, 2: compound **15**. Conditions: mobile phase, a) acetonitrile–H_2O (3:4), b) acetonitrile-H_2O (3:4) containing 6.94 mM γ-CD.

substituent.[11] The biological activities of these newly isolated compounds seem to be of particular interest.

In conclusion the use of γ-CD in the mobile phase is of great advantage to the separation of isomeric cardenolides in reversed-phase HPLC. Further applications of this method to other steroids and related compounds are being conducted in these laboratories, and the details will be reported elsewhere.

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