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RETENTION BEHAVIOUR OF CARDIAC STEROIDS USING CYCLODEXTRIN IN THE MOBILE PHASE IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The retention behaviour of twenty cardiac steroids and four fluorescent derivatives was examined by the addition of cyclodextrin to the mobile phase in reversed-phase high-performance liquid chromatography. The addition of a suitable cyclodextrin improved the separation of isomeric cardiac steroids. The steroid A/B ring junction is the most important factor in the choice of the optimum cyclodextrin to be added; the C/D ring junction is less important. The hydroxyl group at the 3- or 12-position of the steroid enhanced the changes in retention times of these compounds. The effect of an unsaturated lactone ring at the 17β-position on the retention in the presence of cyclodextrin was also determined with cardenolide (five-membered ring) and bufadienolide (six-membered ring) but little difference was observed. Isomeric cardiac steroids, whose separation has not been done by the conventional method, were clearly separated by this method. The fluorescence intensity of 3-(1-anthroyl)-cardiac steroid was enhanced by the addition of cyclodextrin to the mobile phase.

INTRODUCTION

Cyclodextrins (CDs) are toroidal-shaped cyclic oligosaccharides consisting of α-1,4-linked D-glucopyranose units. They exhibit an highly stereoselective ability to form inclusion complexes with a variety of molecules and ions. Some attempts to utilize this phenomenon have been made in gas and liquid chromatography[1]. A CD-bonded column or CD-containing mobile phase in high-performance liquid chromatography (HPLC) is often preferable to conventional ones for the separation of optical, geometrical and structural isomers[2–8].

In previous papers we reported the use of CD in the mobile phase, which is of great advantage in the separation of isomeric steroids (oestrogens[9], bile acids[10]) and their fluorescence detection in reversed-phase HPLC. As a continuation of this work, the present paper deals with the retention behaviour of twenty cardiac steroids and four fluorescent derivatives using CD as a component of the mobile phase in HPLC[a]. The effect of CD on the fluorescence detector response has also been investigated.

[a] Part of this work has been published as a preliminary report[11].

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EXPERIMENTAL

Materials

CDs were kindly supplied by Nihon Shokuhin Kako (Tokyo, Japan). Heptakis(2,6-di-O-methyl-β-CD) (Me-β-CD) was prepared and donated by Kao (Tokyo, Japan). Octakis(2,6-di-O-methyl-γ-CD) (Me-γ-CD) was prepared by the method reported by Casu *et al.*[12]. Cardiac steroids were isolated from the natural source[13] or synthesized from digitoxin and digoxin (Nakarai Tesque, Kyoto, Japan) by known methods[14]. 1-Anthroyl cyanide was obtained from Wako (Tokyo, Japan). Solvents were purified by distillation prior to use.

Apparatus

HPLC was carried out on a Shimadzu LC-6A chromatograph equipped with a Shimadzu SPD-6AV ultraviolet (UV) detector (Shimadzu, Kyoto, Japan) or an Hitachi F-1000 fluorescence (FL) detector (Hitachi, Tokyo, Japan) at a flow-rate of 1 ml/min. A Develosil ODS-5 (5 μm) column (15 cm × 0.4 cm I.D.) (Nomura Chemical, Seto, Japan) was used at ambient temperature unless stated otherwise. The void volume was determined by the use of $NaNO_3$ (UV) or methanol (FL).

Derivatization

Cardiac steroids were derivatized with 1-anthroyl cyanide according to the procedure described by Goto *et al.*[15].

RESULTS AND DISCUSSION

Effect of CD on the retention of cardiac steroids differing in A/B ring junction and/or 3-substituent

In our preliminary work it was suggested that the retention behaviour of cardiac steroids in HPLC is influenced by differences in the steroid A/B ring junction and 3-substituent[11]. On the basis of these findings, the effects of the α-, β- and γ-CD contents in the mobile phase on the capacity factors, k', of compounds **1**–**10**, which differ in the A/B ring junction and/or 3-substituent, were investigated (Figs. 1 and 2). All the k' values of the compounds examined obtained without CD were kept at greater than 6.81 by changing the concentration of the organic modifier. 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.

Among the CDs examined, α-CD had little effect on the k' values of all the compounds examined. On the contrary, the k' values of these compounds decreased with increasing concentration of β- or γ-CD in the mobile phase. This phenomenon can be explained by the cavity size of the CD, that of α-CD being too small to include the cardiac steroid. Regarding the A/B ring junction, γ-CD was remarkably more effective than β-CD in decreasing the k' values of compounds having a A/B *cis* ring junction. A/B *trans* and 5-ene series slightly prefer β-CD to γ-CD, but the reverse effect has been observed with 4-ene series.

Irrespective of the A/B ring junction, the effect of the 3-substituent on the

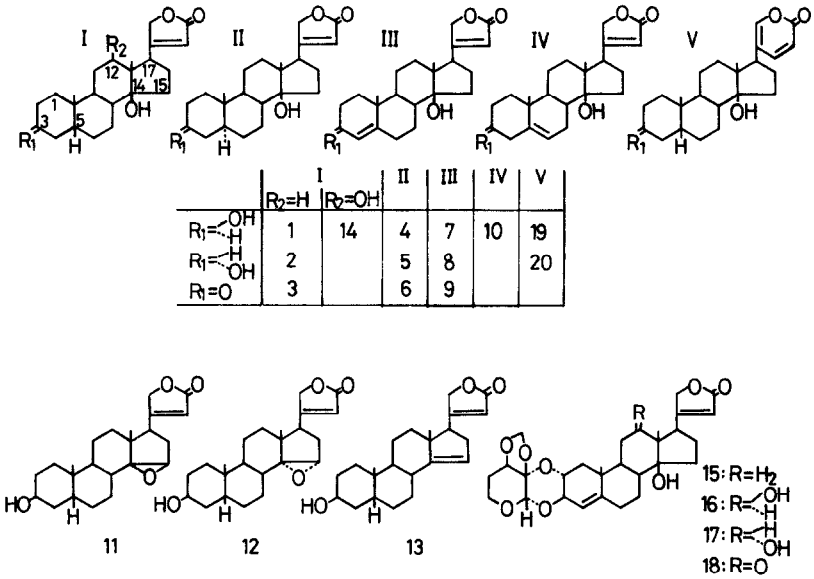

Fig. 1. Structures of cardiac steroids.

relative k' value decreased in the order of 3 β-OH, 3 α-OH and 3-oxo groups with increasing concentration of CDs. The relative k' values of the 3-hydroxyl compounds were influenced more markedly than those of the corresponding 3-oxo compounds in both the A/B *cis* and *trans* series, but only a slight difference was observed in the 4-ene series.

Separation of digitoxigenin (1) and uzarigenin (4)

The above data prompted us to separate digitoxigenin (**1**) and uzarigenin (**4**), whose separation has not been done by the conventional method (Fig. 3a). The effects of β- and γ-CD on the k' values and resolution, R_s, of **1** and **4** are illustrated in Fig. 4. Rapid elution and good separation of geometric isomers were obtained with increasing concentration of β-CD. The addition of 5 mM γ-CD gave rapid elution of the isomers, and although the R_s value decreased it was still 5. Conversely, a lower γ-CD concentration (2mM) affords the best resolution (R_s 9.0) with a corresponding increase in k'. The k' value of compound **1** or **4** was influenced more markedly than that of **4** or **1** with increasing concentration of γ- or β-CD, respectively. A complete separation was attained with shortening of retention time, as shown in Fig. 3b and c. A retention reversal is observed in these chromatograms, and would be helpful in identifying peaks in chromatograms of biological samples.

Effect of CD on the retention of cardiac steroid differing in C/D ring junction, 12- or 17-substituent

Cardiac steroids obtained from natural sources were divided into two categories, cardenolide and bufadienolide having a five- or six-membered lactone ring at the 17β-position, respectively. Many cardiac steroids differing in the C/D ring junction or 12-substituent were synthesized or isolated from natural sources to determine their

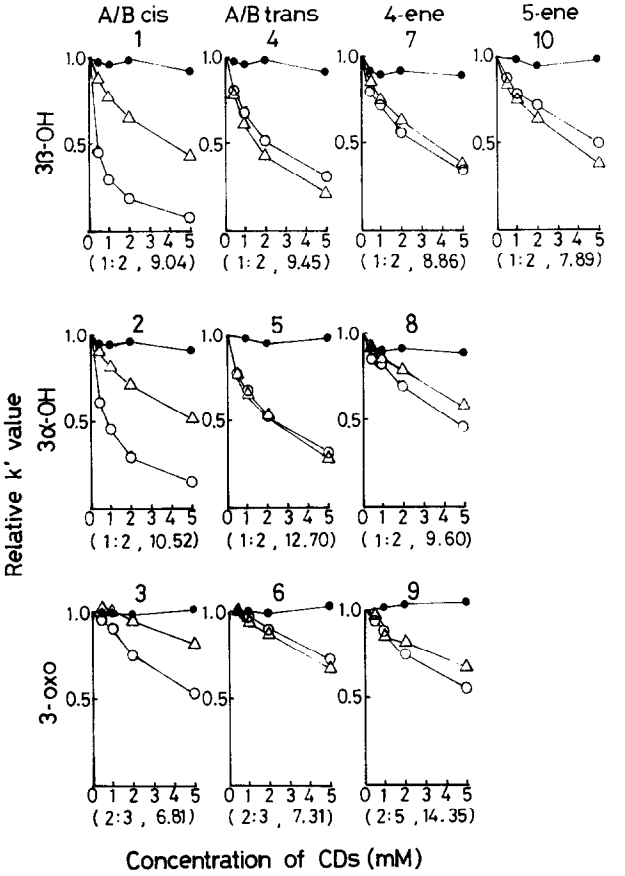

Fig. 2. Effect of CD on the retention of cardiac steroids differing in the A/B ring junction and/or 3-substituent; ●, α-CD; △, β-CD; ○, γ-CD. Conditions: mobile phase, acetonitrile–water containing CD as indicated; detection, UV 240 nm; $t_0 \doteq 1.10$ min. Compound numbers indicated at the top of each figure. The ratio of acetonitrile–water and the k' value obtained without CD, taken as 1.0 for the calculation of the relative k' value, are indicated in parentheses.

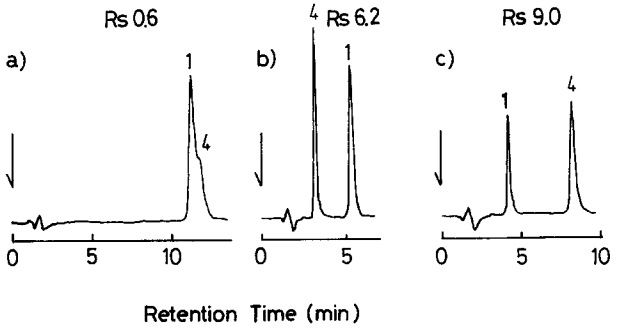

Fig. 3. Separation of digitoxigenin (**1**) and uzarigenin (**4**). Conditions: mobile phase, (a) acetonitrile–water (1:2), (b) and (c) containing β-CD (5.0 m*M*) and γ-CD (1.0 m*M*); detection, UV 240 nm.

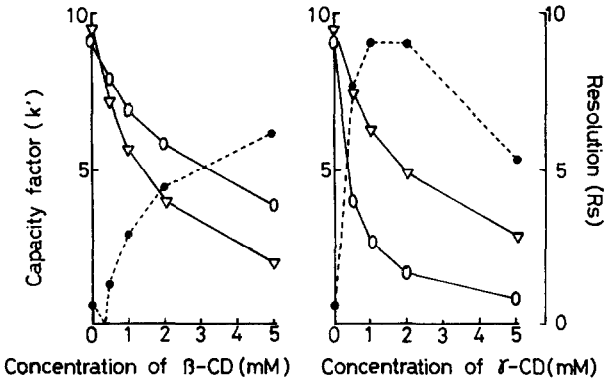

Fig. 4. Effect of CD on the retention and separation of digitoxigenin and uzarigenin; ○, digitoxigenin (**1**); ▽, uzarigenin (**4**); ●, resolution of **1** and **4**. Conditions; mobile phase, acetonitrile–water containing CD as indicated; detection, UV 240 nm.

biological activities. Structure–activity relationships of these compounds showed that these functions are the key structural features for the cardiotonic activity[13]. These data prompted us to examine the retention behaviour of cardiac steroids differing in the C/D ring junction, 12- or 17-substituent (Fig. 5). All the k' values of the compounds examined obtained without CD were kept at > 5.50 as described above. The relative k' values of digitoxigenin (**1**) and compounds **11**–**13** differing in the C/D ring junction were affected by the addition of γ-CD to the mobile phase, the contribution of the

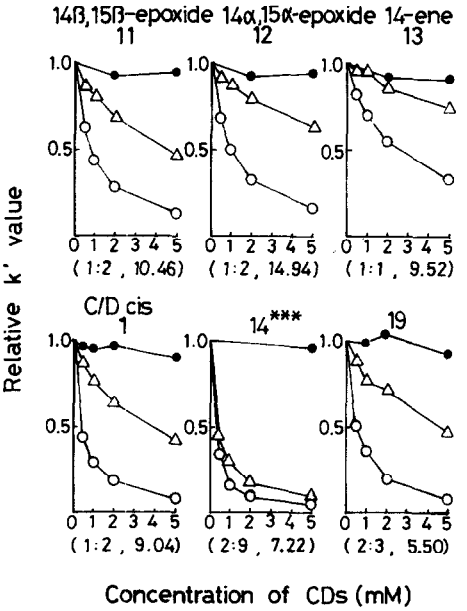

Fig. 5. Effect of CD on the retention of cardiac steroids differing in the C/D ring junction, 12- or 17-substituent: ●, α-CD; △, β-CD; ○, γ-CD. Conditions as in Fig. 2 except for compound **19** which was monitored at 300 nm. *** From ref. 11.

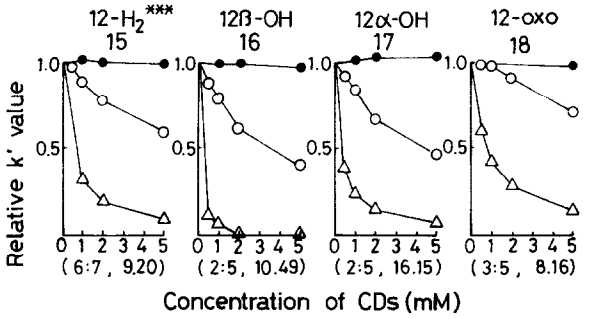

Fig. 6. Effect of CD on the retention of elaeodendroside derivatives differing in the 12-substituent; ●, α-CD; △, β-CD; ○, γ-CD. Conditions as in Fig. 2. *** From ref. 11.

structural feature to the decrease in k' value being in the order of C/D-*cis*> 14β,15β-epoxide> 14α,15α-epoxide> 14-ene. The A/B ring junction of all the compounds examined was *cis*, so γ-CD was a more effective modifier than β-CD for the retention as described above.

Next, the effect of the 12-substituent on the relative k' value was investigated with compound **1** and digoxigenin (**14**) having a 12β-hydroxyl group. The relative k' value of **14** was influenced more than that of **1** by the addition of γ-CD. Although the A/B ring junction of **14** was *cis*, β-CD was as effective as γ-CD in decreasing the k' value. Further investigation on the effect of the 12-substituent on the retention was made by using elaeodendroside derivatives, recently isolated from plant material in these laboratories (Fig. 6)[13]. The relative k' value of compounds having a 12β- or α-hydroxyl group (**16**,**17**) was influenced more than that of the unsubstituted

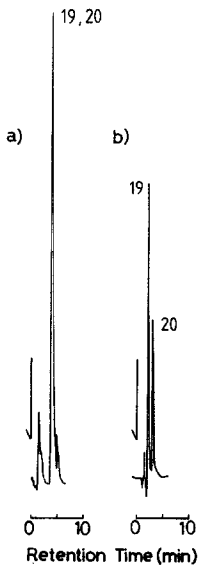

Fig. 7. Separation of bufalin (**19**) and 3-epibufalin (**20**). Conditions: mobile phase, (a) acetonitrile–water (1:1), (b) (5:8) containing 3.9 m*M* γ-CD; detection, UV 300 nm.

compound (**15**) by β- and γ-CDs. On the contrary, the relative k' value of 12-oxoelaeodendroside D (**18**) was affected less than that of **15** by the additives. It is likely that the hydroxyl group on the steroid moiety will be the important factor for the formation of the inclusion complex from the solute and CD. The significant interactions, *e.g.*, hydrogen bonding may occur between solutes bearing OH groups and the 2- and 3-hydroxyl groups of CD.

The effect of the unsaturated lactone ring at the 17β-position on the retention by each CD was also determined with compound **1** and bufalin (**19**). No remarkable difference in the effect on their relative k' values was observed as shown in Fig. 5. This suggested that the above information on cardenolides may be applicable to the separation of bufadienolides by HPLC using CD. The separation of compound **19** and 3-epibufalin (**20**), not achieved by the conventional method, was attempted on this assumption (Fig. 7a). γ-CD was used as an additive in the mobile phase, and gave a satisfactory separation of these compounds as shown in Fig. 7b.

Effect of CD on the retention and FL response of labelled cardenolides

The determination of the serum concentration of cardiac steroids is important in clinical chemistry[16]. Some derivatization methods have been developed for HPLC analysis, but the sensitivity is not so enough to monitor the concentration of this drug in serum. Recently Goto *et al.*[15] synthesized a new fluorescence derivatization agent, 1-anthroyl cyanide, and obtained satisfactory results in the determination of bile acids in biological fluids.

It has also been reported that CD serves to enhance and stabilize the fluorescence intensity of dansyl amino acids on a silica gel layer[17]. Fluorescence enhancement was also observed on inclusion of coumarin derivatives with β-CD[18]. Similar phenomena were observed for oestrogen and labelled bile acids as reported previously[9,10]. On the basis of these findings, the chromatographic behaviours of four cardenolides labelled with 1-anthroyl cyanide and their FL response were investigated (Fig. 8). Owing to the sparing solubility of CDs in the mobile phase containing more than 50% of organic modifier, Me-β- and Me-γ-CDs were used as a substitute for β- and γ-CDs, respectively. The effects of methylated CDs on the k' values of oestriol[9] and digitoxigenin (**1**) were not so different from those of CDs. The chromatographic behaviour is shown in Fig. 9. Only small changes in the retentions were observed with increasing concentration of Me-γ-CD. The addition of Me-β-CD gave some changes in the retention but much less than those of underivatized cardenolides. The bulky

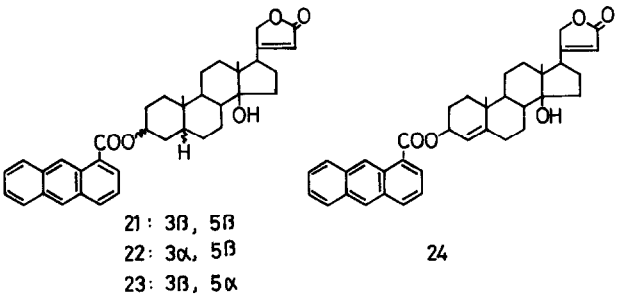

Fig. 8. Structures of 3-(1-anthroyl)cardenolides.

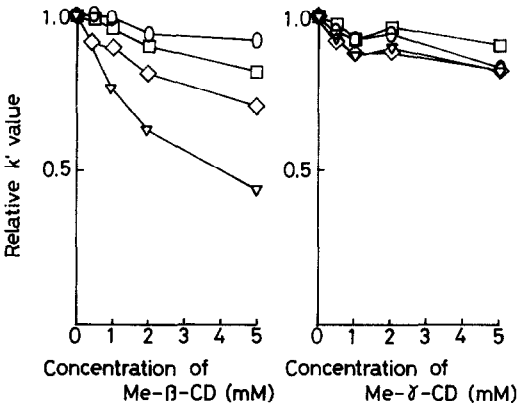

Fig. 9. Effect of CD on the retention of labelled cardenolides: ○, 3-(1-anthroyl)digitoxigenin (**21**); □, 3-epi-(1-anthroyl)digitoxigenin (**22**); ▽, 3-(1-anthroyl)uzarigenin (**23**); ◇, 3-(1-anthroyl)canarigenin (**24**). Conditions: column, YMC-GEL C_8 (5 μm; 15 cm × 0.4 cm I.D.) (Yamamura Chem. Lab., Kyoto, Japan); mobile phase, acetonitrile–water (3:1) containing CD as indicated; detection, FL excitation, 370 nm; emission, 470 nm); t_0 = 1.34 min. The k' value (**21**, 9.41; **22**, 9.61; **23**, 10.91; **24**, 9.45) obtained without CD was taken as 1.0 for the calculation of the relative k' values.

anthracene residue may interfere with the formation of the inclusion complex, but further studies are necessary to clarify this.

The fluorescence intensity of 3-(1-anthroyl)uzarigenin (**22**), whose k' value was most influenced by the additive, was enhanced approximately 1.15 times by the addition of 5 m*M* Me-β-CD to the mobile phase. This datum is compatible with our previous results[9,10].

CONCLUSION

The retention behaviour of twenty cardiac steroids and four fluorescence derivatives was demonstrated by HPLC using CD as the mobile phase additive. The present data show that: differences in the A/B ring junction are more effective than those in the C/D ring junction for changes in the k' values; hydroxyl group at the 3- and 12-positions enhance the decrease in k'. This information is useful for the separation of isomeric cardiac steroids whose separation has not been done by the conventional method. Also such a characteristic chromatographic behaviour reflecting chemical structure would be helpful in identifying the peaks in chromatograms of biological samples. The method is also of advantage in the detection of fluorescent derivatives. Further application of the present method to clarify the metabolic pathways of cardenolide and bufadienolide is being carried out in these laboratories and the details will be reported elsewhere.

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