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Short Communication

Effect of derivatization of steroids on their retention behaviour in inclusion chromatography using cyclodextrin as a mobile phase additive

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ABSTRACT

The effect of derivatization of steroids on their retention behaviour in high-performance liquid chromatography using cyclodextrin as a mobile phase additive was examined by using as model compounds four 17-ketosteroids and twenty derivatives substituted at the 3- or 17-position with *p*-nitrobenzoyl chloride, *p*-nitrobenzoyl azide, 1-anthroyl azide, *p*-nitrophenylhydrazine and O-*p*-nitrobenzylhydroxylamine. The results suggested that separation reflecting the chemical structures of the steroid moieties is obtained by derivatizing a functional group which is located near the isomeric position, and derivatives having more hydrogen-bonding sites show greatly changed chromatographic behaviour.

INTRODUCTION

In recent years, considerable attention has been focused on inclusion chromatography using cyclodextrin (CD) as a mobile phase additive or stationary phase in high-performance liquid chromatography (HPLC) [1]. This type of chromatography is often preferable to conventional techniques for the separation of optical, geometric and structural isomers [2,3].

In previous papers we reported the use of CD as a mobile phase additive, which is of great advantage in the separation of isomeric steroids (oestrogens [4], bile acids [5], cardiac steroids [6,7]) in reversed-phase HPLC. The method was also applied to the separation of fluorescent derivatives. Bile acids derivatized with 1-anthroyl cyanide [8] or bromoacetylpyrene [9] showed a similar chromatographic behaviour to that of the underivatized compounds and satisfactory separation was also obtained by this mode of chromatography. However, the effect of the derivatization of steroids on their retention behaviour in this type of chromatography has not been examined fully.

In this work, the retention behaviour of four 17-ketosteroids [androsterone (**Ia**), epiandrosterone (**Ib**), etiocholanolone (**Ic**) and epietiocholanolone (**Id**)] and twenty

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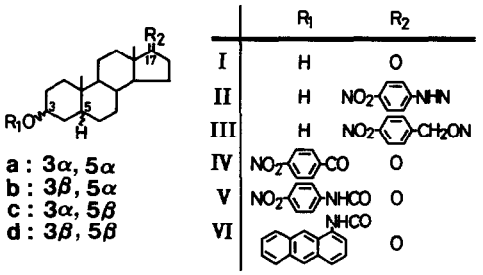

Fig. 1. Structures of 17-ketosteroids and their derivatives.

derivatives substituted at the 3- or 17-position (Fig. 1) were examined from the standpoints of structures of the steroids, derivatized position, derivatized form and mobile phase additive in order to obtain information on the appropriate methods for the derivatization of steroids in this type of chromatography.

EXPERIMENTAL

Materials

CDs and 17-ketosteroids were kindly supplied by Nihon Shokuhin Kako (Tokyo, Japan) and Teikoku Hormone (Tokyo, Japan), respectively. *p*-Nitrophenylhydrazine and O-*p*-nitrobenzylhydroxylamine · HCl were purchased from Nacalai Tesque (Kyoto, Japan) and *p*-nitrobenzoyl chloride from Tokyo Kasei Kogyo (Tokyo, Japan). *p*-Nitrobenzoyl azide was prepared from *p*-nitrobenzoyl chloride by a conventional method with sodium azide, and 1-anthroyl azide was synthesized according to the procedure described by Fujino *et al.* [10].

Apparatus

HPLC was carried out on a Shimadzu (Kyoto, Japan) LC-6A chromatograph equipped with a Shimadzu SPD-6AV ultraviolet (UV) detector at a flow-rate of 1.0 ml/min. A YMC-GEL CN (5 μm) column (15 cm × 0.4 cm I.D.) (YMC, Kyoto, Japan) was used at ambient temperature. The void volume was determined by the use of sodium nitrate. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained with a JEOL (Tokyo, Japan) JNM-FX 100S spectrometer at 100 MHz.

Derivatized 17-ketosteroids

The 17-ketosteroids 17-(*p*-nitrophenylhydrazone) (**II**), 17-(O-*p*-nitrobenzyloxime) (**III**), 3-(*p*-nitrobenzoate) (**IV**), 3-(N-*p*-nitrophenylcarbamate) (**V**) and 3-(N-1-anthrylcarbamate) (**VI**) were synthesized from the corresponding 17-ketosteroids (**I**) by using *p*-nitrophenyhydrazine, O-*p*-nitrobenzylhydroxylamine · HCl, *p*-nitrobenzoyl chloride, *p*-nitrobenzoyl azide and 1-anthroyl azide, respectively, by conventional methods. Although the *syn* and *anti* conformers were formed, the main product was used as the authentic sample in the case of hydrazone (**II**) and oxime (**III**) derivatives, which were obtained by purification by recrystallization and preparative thin-layer chromatography, respectively. All these structures were confirmed by the ^{1}H NMR spectra.

TABLE I

THE CAPACITY FACTORS OF 17-KETOSTEROIDS AND THEIR DERIVATIVES

The numbering corresponds to that in Fig. 1. Mobile phase, methanol–water: **I**, 2:3, t_0 0.82 min; **II–V**, 3:2, t_0 0.78 min; **VI**, 13:7; t_0 0.80 min. Detection, UV: **I**, 210 nm; **II–IV**, 254 nm; **V**, 280 nm; **VI**, 254 nm.

No.	a	b	c	d
I	14.1	11.7	12.5	11.2
II	15.5	14.7	14.6	13.5
III	9.6	8.9	8.6	9.3
IV	11.7	16.9	12.3	16.0
V	15.0	17.7	15.7	16.0
VI	6.7	12.6	10.3	10.1

RESULTS AND DISCUSSION

It is necessary to use a mobile phase containing water in inclusion chromatography using CD as a mobile phase additive in reversed-phase HPLC [1]. Steroids were eluted much earlier from a CN-coated than from an alkyl-coated column using the same solvent system in reversed-phase HPLC. It is possible for a CN-coated column to elute steroids with a mobile phase containing a large proportion of water [7]. According to these data, all the experiments were done with a YMC-GEL CN column.

The capacity factors (k') of all the compounds examined obtained without CD are listed in Table I, in which more than 35% of water was used in the mobile phase. The relative k' values obtained with the addition of 2 m*M* of β- or γ-CD are reported in Fig. 2. Although the data are not shown, α-CD had little effect on the k' values of any of the compounds examined as reported previously for other steroids [4–9]. On the contrary, the k' values of all these compounds decreased with increasing concentration of β- or γ-CD in the mobile phase. This effect can be explained by the cavity size of the CD, that of α-CD being too small to include these compounds.

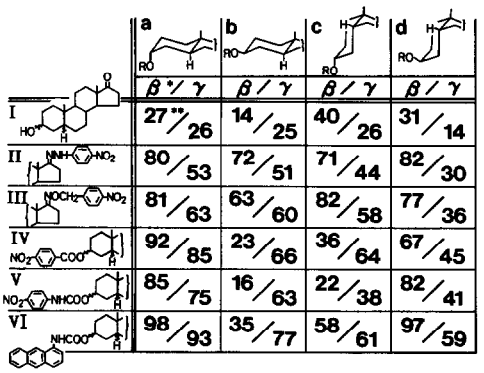

Fig. 2. Relative capacity factors of 17-ketosteroids and their derivatives. * Added CD (2 m*M*). ** Relative identification value (the identification value obtained without CD was taken as 100).

Effect of CD on the retention of 17-ketosteroids

Regarding the 5-position isomers, the k' value of the 5α-isomer (**Ia, b**) was influenced more than that of the 5β-isomer (**Ic, d**) with the addition of 2 m*M* β-CD. Although the relative k' values of **Ia** and **Ic** were the same (26), that (14) of the 5β-isomer (**Id**) was influenced more than that (25) of the 5α-isomer (**Ib**) with the addition of 2 m*M* γ-CD. The effect is more clearly observed with 3β-hydroxy compounds (**Ib, d**) than the corresponding 3α-isomers (**Ia, c**) (Fig. 2). These findings agree with our previous results obtained with cardiac steroids [6].

Effect of CD on the retention of derivatized 17-ketosteroids

The effect of derivatization at the 17-position on the retention behaviour was examined as above and similar results were observed with both hydrazone (**II**) and oxime (**III**) derivatives (Fig. 2), that is, the k' values of all these derivatives decreased more with the addition of γ-CD than β-CD. In 3β-hydroxy compounds, the k' value of the 5α-isomer (**IIb, IIIb**) was influenced more than that of the 5β-isomer (**IId, IIId**) with the addition of β-CD. In contrast, the relative k' value of the 5β-isomer (**IId, IIId**) became smaller than that of the 5α-isomer (**IIb, IIIb**) with the addition of γ-CD. This effect was also observed with 3α-hydroxy compounds (**IIa** *vs.* **IIc**; **IIIa** *vs.* **IIIc**), except for **IIa** (80) *vs.* **IIc** (71) with β-CD as mobile phase additive.

Next, the effect of derivatization at the 3-position was examined and the following results reflecting the chemical structures of steroid moiety were obtained (Fig. 2). The k' values of the 3α,5α-series (**IVa–VIa**) are least influenced in each instance moiety by either CD. In contrast, those of the 3α,5β-series (**IVc–VIc**) were substantially decreased with the addition of either CD. The k' value of the 5α- (**IVb–VIb**) or 5β-isomer (**IVd–VId**) of the 3β-series was decreased more than that of the 5β- or 5α-isomer with the addition of β- or γ-CD, respectively. The addition of β-CD decreased the k' values of compounds having a 3-equatorial substituent (**IVb,c–VIb,c**) more than those of the corresponding isomer (**IVa,d–VIa,d**). On the other hand, γ-CD decreased the k' values of derivatives having an A/B *cis* ring junction (**IVc,d–VIc,d**) more than those of the derivatives having an A/B *trans* ring junction (**IVa,b–VIa,b**).

These results indicate that β- and γ-CD distinguish substituents at the 3-position and the A/B ring junction, respectively. It may be difficult for the former host compound to include an axial substituent [3α,5α (a); 3β,5β (d) series]. Although a few exceptions have been observed, with respect to the structures of the derivatizing moiety, the k' values of the derivatives having more hydrogen bonding sites (**II, V**) are more influenced in most instances than the other derivatives (**III, IV**). Also, the k' values of the derivative having a larger substituent (**VI**) are smaller than those of the corresponding derivative having a smaller substituent (**V**).

CONCLUSIONS

The retention behaviour of four 17-ketosteroid isomers and twenty derivatives was demonstrated by HPLC using CD as a mobile phase additive. Compounds derivatized at the 3-position showed greater variations in chromatographic behaviour reflecting their configurational changes at the 3- and 5-positions than compounds derivatised at the 17-position. The results suggested that the separation reflecting the chemical structures of steroid moieties is obtained by derivatizing the functional

groups which are located near the isomeric position, and derivatives having more hydrogen-bonding sites show greatly changed chromatographic behaviour in this type of chromatography. These results should be helpful in choosing a derivatization method for steroids in this type of chromatography, and in identifying the peaks in chromatograms of biological samples.

Further investigations of the chromatographic behaviour of derivatized steroids in inclusion chromatography are being made in comparison with the conventional method [11].

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