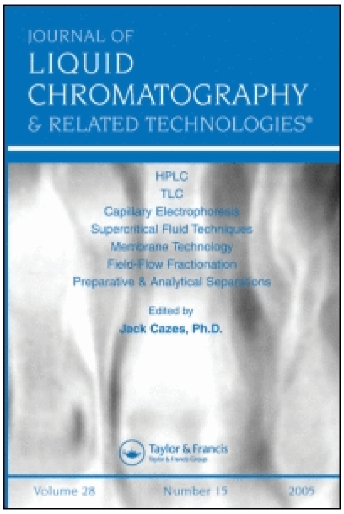

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STUDIES ON NEUROSTEROIDS. I. RETENTION BEHAVIOR OF DERIVATIZED 17-OXOSTEROIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The retention behavior of five 17-oxosteroids (dehydroepiandrosterone, epiandrosterone, androsterone, 5β-androsterone, and 5β-androstane-3β ol-17-one) derivatized with 5-dimethylamino-1-naphthalenesulfonic hydrazide, 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole or *p*-nitrophenylhydrazine are examined using reversed-phase high-performance liquid chromatography. Inclusion chromatography using cyclodextrin as a mobile phase additive is also used for this purpose and found effective in separating the isomeric derivatized 17-oxosteroids.

INTRODUCTION

Since the discovery of dehydroepiandrosterone (Ia) in rat brain, several 17- and 20-oxosteroids, called "neurosteroids", have been elucidated in mammalian brain [1]. In the last few years, major progress has been made towards the elucidation of the molecular mechanism of action of steroid hormones in the brain and anterior pituitary. The determination of neurosteroids (< 70 ng/g of brain) has been usually done by gas chromatography-mass spectrometry or radioimmunoassay, but these methods have some problems in their simplicity and versatility [2]. High-performance liquid chromatography (HPLC) is promising for the establishment as a convenient determination method, but highly sensitive derivatization is necessary because neurosteroids are not very responsive to the commonly used detectors. In order to establish a reliable determination method, fluorometric derivatization is a promising technique and is necessary to clarify the retention behavior of the derivatized neurosteroids and their isomers.

In this paper, the retention behavior of five 17-oxosteroids [Ia, epiandrosterone (IIa), androsterone (IIIa), 5β- androsterone (IVa), and 5β-androstane-3β-ol-17-one (Va)] derivatized with fluorogenic reagent, 5-dimethylamino-1-naphthalenesulfonic hydrazide ($DNSNHNH_2$) or 4- (*N, N*- dimethylaminosulfonyl) - 7 - hydrazino- 2, 1, 3 -benzoxadiazole (DBDH), is examined using reversed-phase HPLC (Fig. 1). Inclusion chromatography using cyclodextrin (CD) as a mobile phase additive is also used for this purpose [3], and the *p*-nitrophenylhydrazones (NP) of these steroids are used to clarify the inclusion behavior during this chromatography.

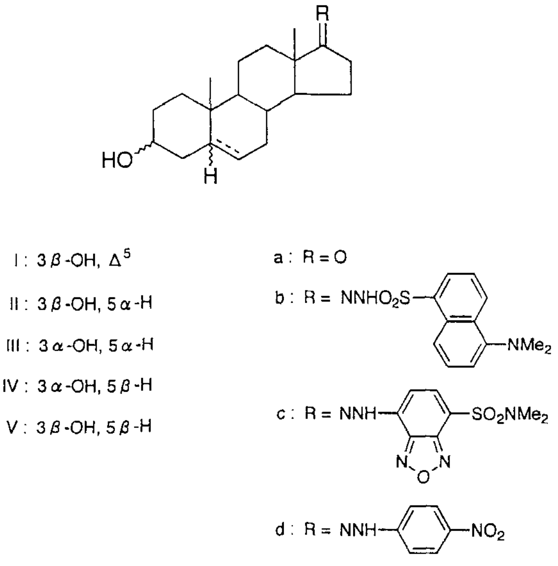

Figure 1. Structures of 17-oxosteroids and their derivatives
Ia: dehydroepiandrosterone IIa: epiandrosterone
IIIa: androsterone IVa: 5β-androsterone
Va: 5β-androstane-3β-ol-17-one

MATERIALS AND METHODS

Materials

α-, β-, and γ-CDs were kindly supplied by Nihon Shokuhin Kako (Tokyo, Japan). Heptakis - (2,6 - di - O - methyl)-β-CD (Me-β-CD; 10.5 methyl residues/mol) was prepared and donated by Kao (Tokyo). 17-Oxosteroids were kindly donated by Teikoku Hormone Mfg. (Tokyo). DNSNHNH$_2$, DBDH, and *p*-nitrophenylhydrazine (NPH) were purchased from Tokyo Kasei Kogyo (Tokyo).

Derivatization Procedure

The derivatization of the 17-oxosteroids with $DNSNHNH_2$ and DBDH has been done using previously described procedures [4,5] to give the corresponding hydrazones (DNS- and DBD-17-oxosteroids), respectively. The derivatization with NPH has been done in the usual way using HCl as the catalyst.

Apparatus

HPLC was carried out using a JASCO TRI ROTAR chromatograph equipped with a UVIDEC-100-II UV (JASCO, Tokyo) or Hitachi F-1000 fluorescence (FL)(Hitachi, Tokyo) detector. A TSKgel ODS 80 TM (5 μm) column (15 cm x 0.46 cm i.d.)(TOSOH, Tokyo) was used at ambient temperature at a flow rate of 1 ml/min, and the void volume was measured with $NaNO_3$ (UV 210 nm) or MeOH (λ ex 280 nm, λ em 320 nm). The pH of the mobile phase containing KH_2PO_4 was adjusted with H_3PO_4.

RESULTS AND DISCUSSION

Retention Behavior of DNS-17-oxosteroids

$DNSNHNH_2$ is widely used as the fluorometric derivatization reagent for carbonyl compounds and several methods for the determination of 17-oxosteroids in biological fluids using this reagent have been reported [4]. The separation of five DNS-17-oxosteroids (I-Vb) *via* reversed-phase HPLC using MeOH or MeCN as an organic modifier was examined. The use of the ion suppressor [0.25% KH_2PO_4 (pH 3.0)] was effective in giving a symmetrical and single peak, but the complete separation has not been done as shown in Fig. 2a, b. These data prompted us to try inclusion chromatography using CD as the mobile phase additive for

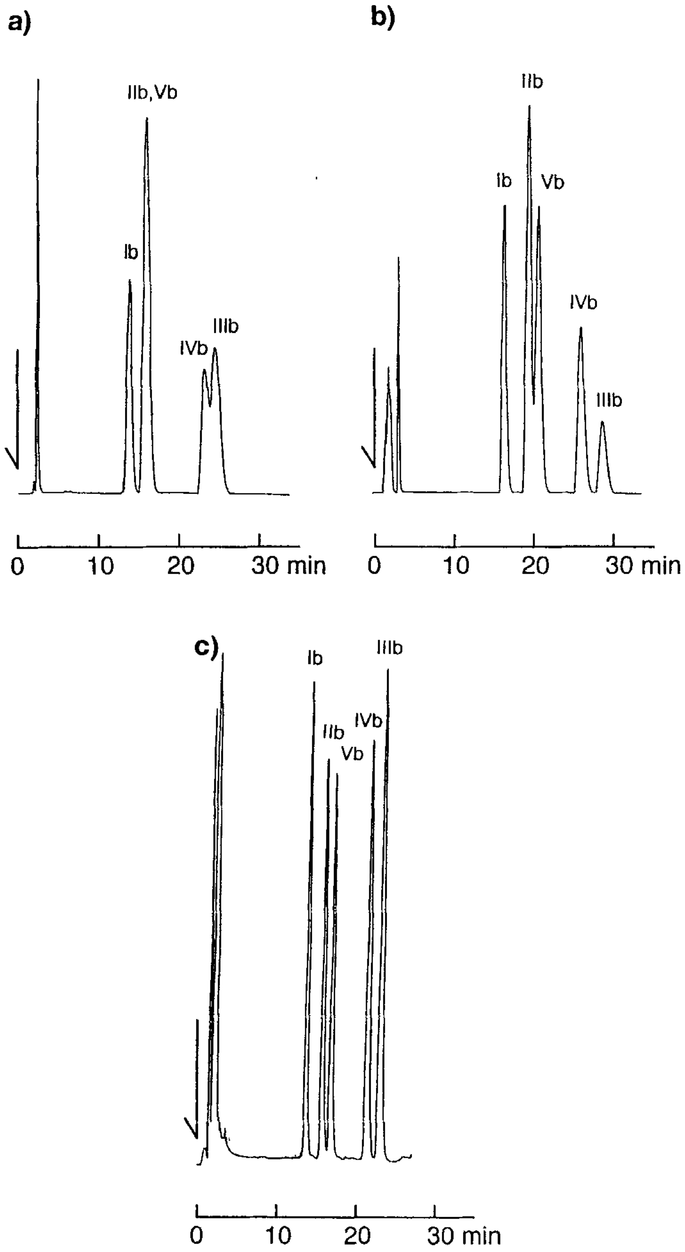

Figure 2. Separation of DNS-17-oxosteroids

Conditions: mobile phase, a) MeOH-0.25% KH_2PO_4 (pH 3.0)(3:1) b) MeCN-0.25% KH_2PO_4 (pH 3.0)(3:2) c) MeCN-0.25% KH_2PO_4 (pH 3.0)(3:2) containing Me-β-CD (2 mM); t_0, a) 1.8 min b) 1.5 min c) 1.6 min; detection, FL (λ ex 340 nm, λ em 525 nm).

Table 1. Effect of CD on the Rk' value of DNS-17-oxosteroids

		Rk' [a]			
		γ - CD		Me-β-CD	
	k' [b]	2 mM	5 mM	2 mM	5 mM
Ib	6.7	0.81	0.69	0.77	0.73
IIb	7.7	0.77	0.63	0.76	0.68
IIIb	12.8	0.77	0.66	0.76	0.71
IVb	12.1	0.75	0.63	0.78	0.75
Vb	8.0	0.63	0.44	0.78	0.75

Conditions: mobile phase, MeOH-0.25% KH_2PO_4 (pH 3.0) (3 : 1) containing CD as indicated. a) The k' value obtained without CD was taken as 1.0 . b) The k' value obtained without CD. t_0 1.8 min.

the separation of these derivatives. The effect of γ - and Me-β-CD on the relative capacity factor (Rk') of these derivatives is shown in Table 1. The former host compound is more effective than the latter one and Rk' of Vb (3β, 5β-isomer) was most decreased by the addition of γ - CD in the mobile phase. These data are compatible with the previously obtained data on cardiac steroids [6], that is, γ - CD is remarkably more effective than the other CDs in decreasing the k' values of compounds having an A/B cis ring junction and 3β-hydroxy group. The complete separation of the five derivatives was done by the addition of Me-β -CD and the chromatogram is shown in Fig. 2c.

Retention Behavior of DBD-17-oxosteroids

Recently, DBDH has been developed as a fluorogenic labeling reagent for aldehydes and ketones [5]. The resultant hydrazones were separated on a reversed-phase column and fluorometrically detected at sub-pmol levels. Furthermore, the hydrazones were more

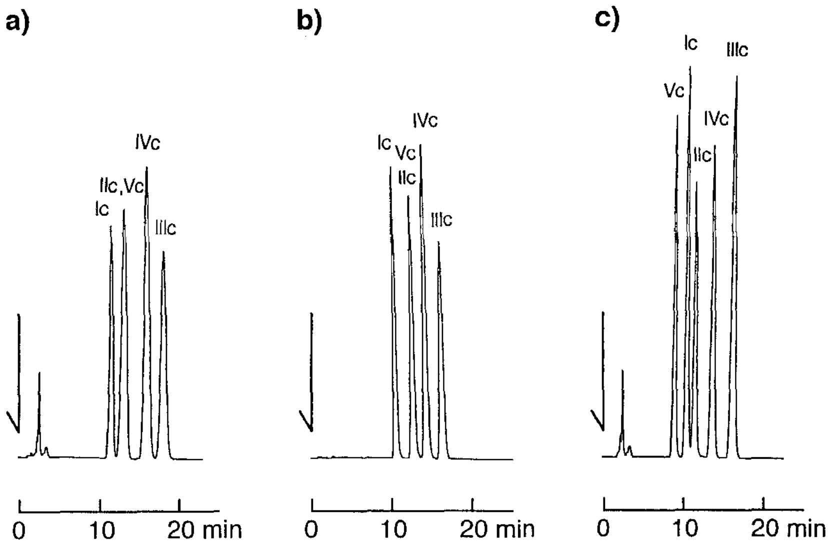

Figure 3. Separation of DBD-17-oxosteroids
Conditions: mobile phase, a) MeOH-H_2O (7:2)
b) MeCN-H_2O (2:1) c) MeOH-H_2O (7:2) containing γ -CD
(2 mM); t_0, a) 1.7 min b) 1.5 min c) 1.7 min; detection, FL
(λ ex 450 nm, λ em 550 nm).

sensitively detected by peroxyoxalate chemiluminescence than by fluorescence [5]. These data prompted us to use DBDH as a derivatization reagent for the 17-oxosteroids and the obtained hydrazones (I-Vc) were subjected to separation on reversed-phase HPLC. The use of MeOH or MeCN as an organic modifier each gave a symmetrical and single peak but did not give satisfactory separation of the five DBD-17-oxosteroids as shown in Fig. 3a, b. The addition of γ -CD in the mobile phase produced a sharp decrease in the Rk' of Vc, which is compatible with that of the DNS derivative (Table 2), and the complete separation of five derivatives was then obtained as shown in the chromatogram (Fig. 3c).

Table 2. Effect of CD on the Rk' value of DBD-17-oxosteroids

		Rk' [a]		
		γ - CD [b]	Me-β-CD	
	k' [c]	2 mM	2 mM	5 mM
Ic	4.0	0.82	0.82	0.71
IIc	4.7	0.78	0.80	0.67
IIIc	6.4	0.80	0.78	0.70
IVc	5.7	0.77	0.81	0.69
Vc	4.5	0.64	0.80	0.68

Conditions: mobile phase, MeOH-H_2O (4: 1) containing CD as indicated. a) The k ' value obtained without CD was taken as 1.0 . b) Due to its solubility, the experiment with 5 mM has not been done. c) The k ' value obtained without CD. t_0 1.7 min.

Retention behavior of NP-17-oxosteroids

The above described retention behavior of DNS- and DBD-17-oxosteroids in inclusion chromatography showed that γ - CD is more effective than Me-β -CD for decreasing the Rk ' values of these derivatives. In spite of the derivatization residue (DNS and DBD) at the 17-position, the Rk ' values of derivatized V having a 3β , 5β - configuration sharply decreased with the addition of γ -CD in the mobile phase. These data prompted us to examine the retention behavior of PN-17-oxosteroids (I-Vd) using inclusion chromatography. If the inclusion selectively occurs at the introduced fluorophore at the 17-position, the retention behavior of the derivatives having the NP residue, which is smaller than DNS or DBD residue, should be more affected by the addition of Me- β -CD having an inner diameter smaller than γ -CD. But γ -CD is more effective than Me-β -CD as in the case of the DNS- and DBD-derivatives (Table 3). The complete separation of these

Table 3. Effect of CD on the Rk' value of NP-17-oxosteroids

		Rk' [a]		
		γ - CD [b]	Me- β -CD	
	k' [c]	2 mM	2 mM	5 mM
Id	8.6	0.66	0.91	0.74
IId	10.1	0.61	0.87	0.67
IIId	12.7	0.65	0.98	0.76
IVd	11.0	0.65	0.99	0.74
Vd	9.9	0.52	0.86	0.65

Conditions: mobile phase, MeOH-H_2O (4: 1) containing CD as indicated. a) The k ' value obtained without CD was taken as 1.0 . b) Due to its solubility, the experiment with 5 mM has not been done. c) The k ' value obtained without CD. to 1.8 min.

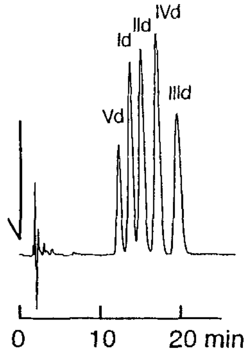

Figure 4. Separation of NP-17-oxosteroids

Conditions: mobile phase, MeOH-H_2O (4:1) containing γ -CD (2 mM); t_0 2.0 min; detection, UV (254 nm).

derivatives were also done by the addition of γ-CD in the mobile phase (Fig. 4).

Conclusions

In order to establish the determination method for neurosteroids, the chromatographic behavior of derivatized 17-oxosteroids has been examined with reversed-phase HPLC including inclusion chromatography. The addition of a host compound in the mobile phase is effective in separating these isomers, and five derivatized 17-oxosteroids were clearly separated by this method. In spite of the introduced derivatization residue (DNS, DBD, and NP) at the 17-position, γ-CD is more effective than Me-β-CD in decreasing the k' value of these compounds. The compound having the 3β-, 5β-configuration is most affected in its retention behavior by the addition of γ-CD. These data indicate that inclusion with a steroid moiety rather than the introduced derivatization residue may play an important role in this chromatography procedure.

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REFERENCES

1) Mathur, C., Prasad, V.V.K., Raju, V.S., Welch, M., and Lieberman, S., Steroids and their conjugates in the mammalian brain, *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 85 (1993).

2) Akwa, Y., Morfin, R.F., Robel, P., and Baulieu, E.-E., Neurosteroid metabolism. 7 α -Hydroxylation of dehydroepiandrosterone and pregnenolone by rat brain microsomes. *Biochem. J.*, **288**, 959 (1992).

3) Shimada, K., Mitamura, K., Ishitoya, S., and Hirakata, K., High performance liquid chromatographic separation of sensitive fluorescent derivatives of bile acids with cyclodextrin-containing mobile phase, *J. Liquid Chromatogr.*, **16**, 3965 (1993).

4) Kawasaki, T., Maeda, M., and Tsuji, A., Determination of 17-oxosteroids in serum and urine by fluorescence high-performance liquid chromatography using dansyl hydrazine as a pre-labeling reagent, *J. Chromatogr.*, **226**, 1 (1981).

5) Uzu, S., Imai, K., Nakashima, K., and Akiyama, S., Determination of medroxyprogesterone acetate in serum by HPLC with peroxyoxalate chemiluminescence detection using a fluorogenic reagent, 4-(*N,N*-dimethylaminosulphonyl)-7-hydrazino-2,1,3-benzoxadiazole, *J. Pharm. Biomed. Anal.*, **10**, 979 (1992).

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

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