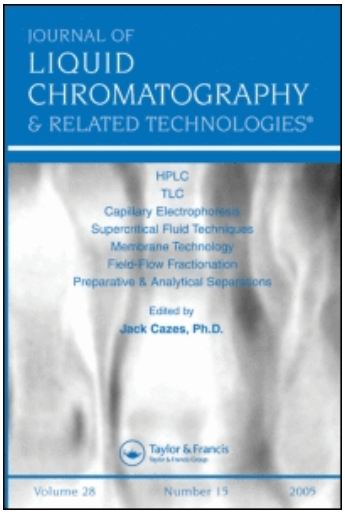

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STUDIES ON NEUROSTEROIDS. II. RETENTION BEHAVIOR OF DERIVATIZED 20-OXOSTEROIDS AND THEIR SULFATES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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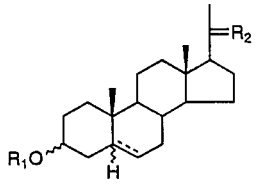
ABSTRACT

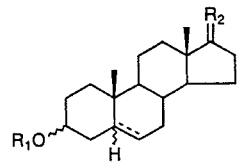
The retention behavior of five 20-oxosteroids (pregnenolone, 3 β - hydroxy- 5 α -pregnan-20-one, 3 β -hydroxy- 5 β - pregnan-20-one, 3 α -hydroxy- 5 α -pregnan-20-one, and 3 α - hydroxy-5 β -pregnan-20-one) and their sulfates derivatized with 4-(*N,N*-dimethylaminosulfonyl)- 7-hydrazino-2,1,3-benzoxadiazole were examined using reversed-phase high-performance liquid chromatography with fluorescence detection. Inclusion chromatography using cyclodextrin as a mobile phase additive was also used for this purpose and found effective in separating the isomeric derivatized 20-oxosteroids and their sulfates.

INTRODUCTION

In the previous paper in this series, we have clarified the retention behavior of five 17-oxosteroids derivatized with a fluorogenic reagent, 5-dimethylamino-1-naphthalenesulfonic hydrazide ($DNSNHNH_2$) or 4- (*N, N* - dimethylaminosulfonyl) - 7 - hydrazino- 2, 1, 3 - benzoxadiazole (DBDH) using reversed-phase HPLC and found that inclusion chromatography using cyclodextrin (CD) as a mobile phase additive was effective for the separation of these derivatives [1]. These data are helpful in establishing the method of determination of 17-oxosteroids (kinds of neurosteroids found in the mammalian brain) using HPLC with fluorescence detection as described in the previous paper [1]. The derivatization rate of 17-oxosteroids with DBDH was higher than that with $DNSNHNH_2$ (data not shown), and several 20-oxosteroids and sulfates of 17- and 20-oxosteroids were also found in the mammalian brain [2]. These data prompted us to clarify the retention behavior of 20-oxosteroids and their sulfates derivatized with DBDH.

In this paper, the retention behavior of five 20-oxosteroids [pregnenolone (I a), 3 β -hydroxy-5 α -pregnan-20-one (II a), 3 α - hydroxy-5 α -pregnan-20-one (III a), 3 α - hydroxy-5 β - pregnan-20-one (IV a), and 3 β -hydroxy-5 β -pregnan-20-one (V a)] derivatized with DBDH is examined using reversed-phase HPLC with fluorescence detection. Those of 20- and 17-oxosteroid sulfates (I - X b) derivatized with DBDH are also examined (Fig. 1). Inclusion chromatography using cyclodextrin (CD) as a mobile phase additive is also used for this purpose.





I : 3β-OR$_1$, Δ^5

II : 3β-OR$_1$, 5α-H

III : 3α-OR$_1$, 5α-H

IV : 3α-OR$_1$, 5β-H

V : 3β-OR$_1$, 5β-H

VI : 3β-OR$_1$, Δ^5

VII : 3β-OR$_1$, 5α-H

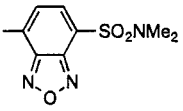
VIII : 3α-OR$_1$, 5α-H

IX : 3α-OR$_1$, 5β-H

X : 3β-OR$_1$, 5β-H

a : R$_1$=H, R$_2$=O

b : R$_1$=SO$_3$H, R$_2$=O

c : R$_1$=H, R$_2$=NNH- 

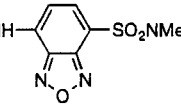
d : R$_1$=SO$_3$H, R$_2$=NNH- 

Figure 1. Structures of examined oxosteroids and their derivatives

MATERIALS AND METHODS

Materials

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). 20-Oxo- (I-Va) and 17-oxo-steroids (VI-Xa) were kindly donated by Teikoku Hormone Mfg. (Tokyo). The sulfates (I-Xb) of the above oxosteroids were synthesized in our

laboratory in the usual way using a chlorosulfonic acid/pyridine complex. DBDH was purchased from Tokyo Kasei Kogyo (Tokyo).

Derivatization Procedure

The derivatization of the oxosteroids (I - X a) and their sulfates (I - X b) with DBDH has been done using previously described procedures [1,3] to give the corresponding derivatives (I - X c,d).

Apparatus

HPLC was carried out using a JASCO TRI ROTAR chromatograph equipped with an Hitachi F-1000 fluorescence (FL: λ ex. 450 nm, λ em. 550 nm)(Hitachi, Tokyo) detector. A TSKgel ODS 80 Tm (5 μ m) 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 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 DBD-20-oxosteroids

The separation of five DBD-20-oxosteroids (I-Vc) *via* reversed-phase HPLC using MeOH or MeCN as an organic modifier was examined, but satisfactory 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 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 Fig. 3. The former host compound was more effective than the

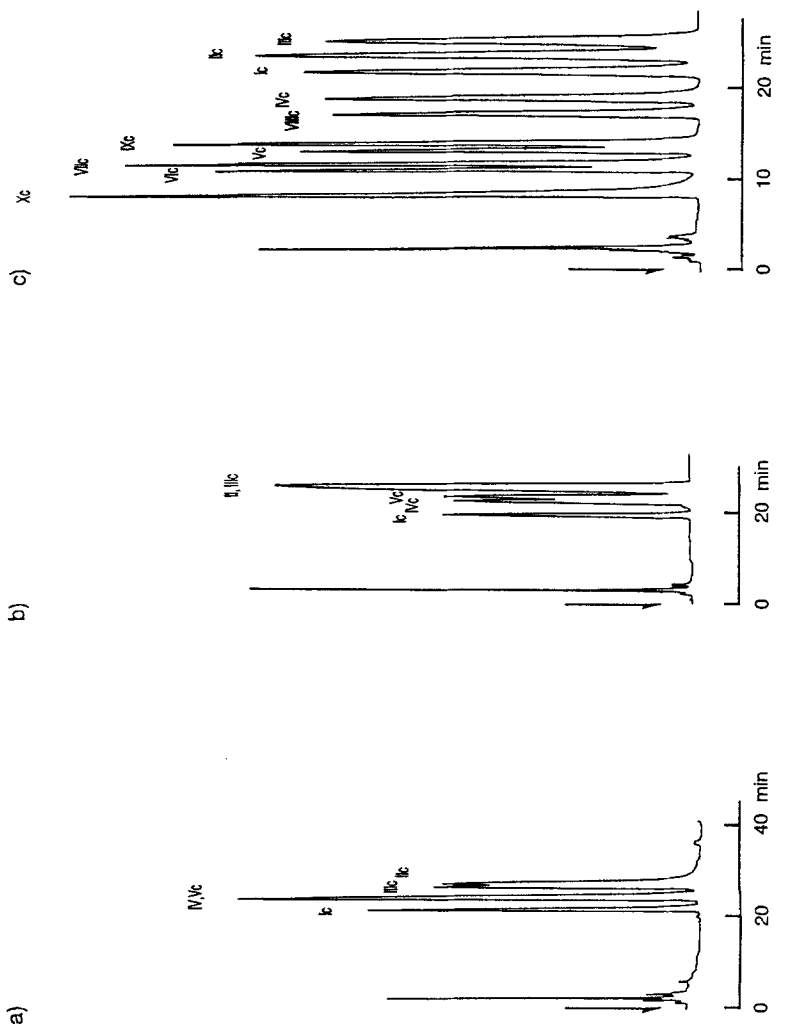


Figure 2. Separation of DBD-20-oxosteroids
Conditions: mobile phase, a) MeOH-H_2O (7:2)
b) MeCN-H_2O (2:1) c) MeOH-H_2O (3:1) containing γ-CD (4mM).

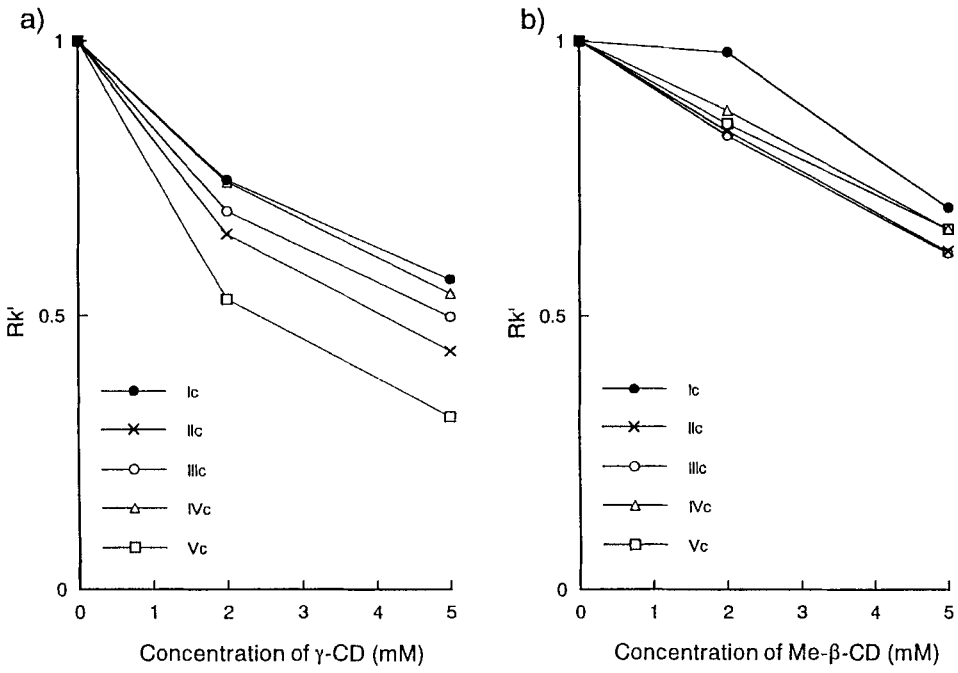


Figure 3. Effect of CD on the retention of DBD-20-oxosteroids
Conditions: mobile phase, MeOH-H_2O (a; 7:2 b; 4:1) containing CD as indicated. The k' values obtained without CD, taken as 1.0 for the calculation of Rk' value, are as follows: a) Ⅰc 12.8 Ⅱc 14.1 Ⅲc 15.9 Ⅳc 15.3 Ⅴc 13.5; b) Ⅰc 8.8 Ⅱc 9.6 Ⅲc 11.1 Ⅳc 10.8 Ⅴc 9.5. t_0 1.7 min.

latter, and the Rk' of Vc (3 β, 5 β -isomer) was decreased more by the addition of γ - CD in the mobile phase (Fig. 3a). These data are compatible with the previously obtained data on cardiac steroids [4] and derivatized 17-oxosteroids [1], that is, γ - CD is remarkably more effective than the other CD in decreasing the k' values of compounds having an A/B cis ring junction and a 3 β -hydroxy group. Satisfactory separation of DBD-20-oxosteroids together with DBD-17-oxosteroids was done by the addition of γ -CD in the

mobile phase using MeOH as an organic modifier, and the chromatogram is shown in Fig. 2c.

Retention Behavior of DBD-20-oxosteroid Sulfates

The use of MeOH or MeCN as an organic modifier together with 0.25% KH_2PO_4 (pH 3.0) as an ion suppressor each gave a single symmetrical peak but did not give satisfactory separation of the five DBD-20-oxosteroid sulfates as shown in Fig. 4a, b. The effect of γ - and Me-β-CD on the Rk' of these derivatives is shown in Table 1. The latter host compound was more effective than the former, and the Rk' of IVd (3α, 5β-isomer) was decreased more by the addition of this host compound in the mobile phase. The same phenomena were also observed when MeOH was used as an organic modifier (data not shown). These data are incompatible with those obtained with unconjugated derivatized 17- or 20-oxo-steroids as shown above. The complete separation of five derivatives was then obtained as shown in the chromatogram (Fig. 4c). All of the above data prompted us to examine the retention behavior of DBD-17-oxosteroid sulfates (Table 2). Me-β-CD was more effective than γ - CD, and the Rk' of IXd (3α, 5β - isomer) was decreased more by the addition of Me-β-CD in the mobile phase. These phenonena are compatible with those obtained with DBD-20-oxosteroid sulfates. Satisfactory separation is shown in Fig. 5.

Conclusions

In order to establish the determination method for neurosteroids, the chromatographic behavior of derivatized 20-oxosteroids and their sulfates has been examined with reversed-phase HPLC

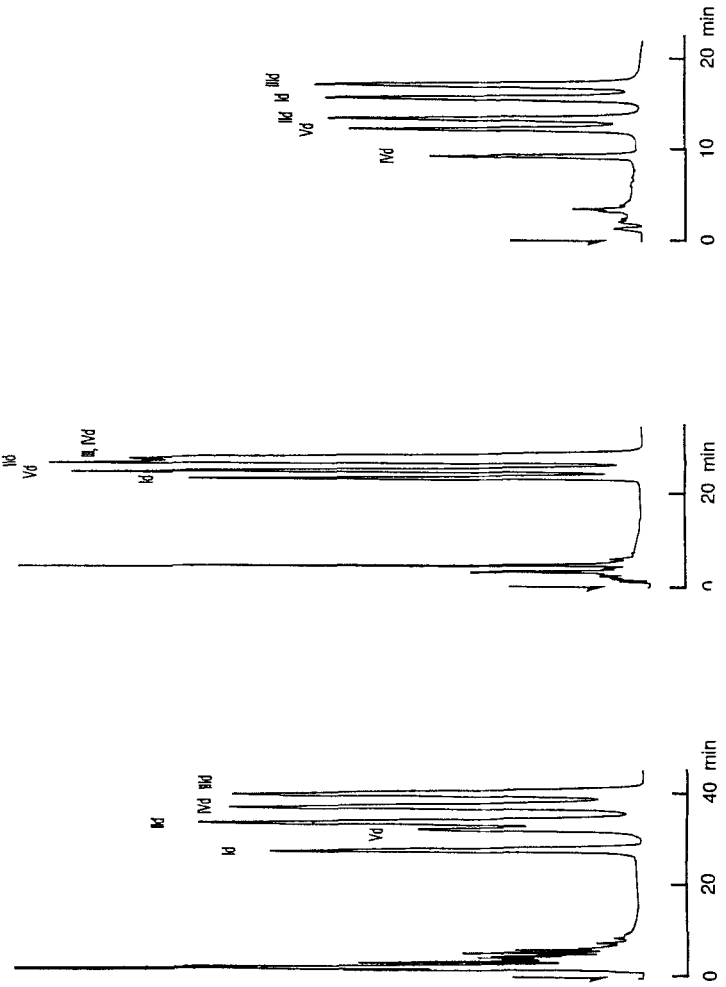


Figure 4. Separation of DBD-20-oxosteroid sulfates
Conditions: mobile phase, a) MeOH-0.25% KH_2PO_4 (pH 3.0) (5:2)
b) MeCN-0.25% KH_2PO_4 (pH 3.0) (9:10) c)MeCN-0.25% KH_2PO_4
(pH 3.0) (9:10) containing Me-β-CD (2 mM).

Table 1. Effect of CD on the Rk' value of DBD-20-oxosteroid sulfates

		Rk' [a]			
		γ - CD [b]		Me-β-CD	
	k' [c]	2 mM	4 mM	2 mM	5 mM
Id	10.2	0.89	0.79	0.71	0.47
IId	11.9	0.80	0.66	0.52	0.29
IIId	12.2	0.84	0.72	0.66	0.41
IVd	12.2	0.62	0.41	0.34	0.17
Vd	11.1	0.63	0.45	0.51	0.28

Conditions: mobile phase, MeCN-0.25% KH_2PO_4 (pH 3.0) (1:1) 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 1.4 min.

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

		Rk' [a]			
		γ - CD [b]		Me-β-CD	
	k' [c]	2 mM	3 mM	2 mM	5 mM
VId	10.8	0.94	0.81	0.73	0.48
VIId	12.5	0.86	0.70	0.52	0.31
VIIId	15.9	0.92	0.76	0.66	0.42
IXd	16.9	0.71	0.53	0.39	0.21
Xd	12.9	0.69	0.52	0.55	0.32

Conditions: mobile phase, MeCN-0.25% KH_2PO_4 (pH 3.0) (4: 5) 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.

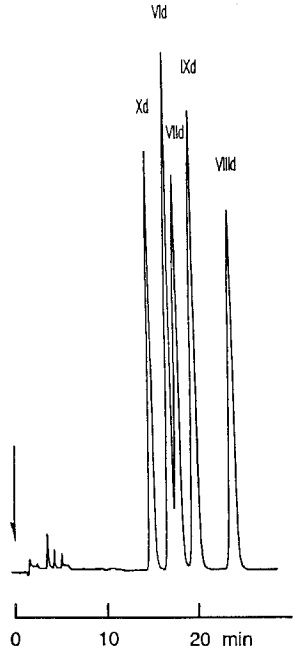


Figure 5. Separation of DBD-17-oxosteroid sulfates
Conditions: mobile phase, MeCN-0.25% KH_2PO_4 (pH 3.0) (4:5) containing γ -CD (2 mM).

including inclusion chromatography. The addition of a host compound in the mobile phase was effective in separating these isomers, and these derivatized oxosteroids or their sulfates were satisfactorily separated by this method. Among the host compounds used, γ - CD and Me- β -CD are more effective than the other CD in decreasing the k' value of the derivatized oxosteroids and their sulfates, respectively. Among these compounds, oxosteroids having the 3 β -, 5 β - configuration and the sulfates having the 3 α -, 5 β - configuration are most affected in their retention behavior by the addition of γ - CD and Me- β -CD, respectively. These data

indicate that the functional group at the 3 position may play an important role in the inclusion phenomenon. The development of the determination of neurosteroids in the mammalian brain is now under investigation in our laboratory, and the details will be reported in the near future.

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