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Resolution of enantiomeric steroids by high-performance liquid chromatography on chiral stationary phases

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Abstract

The chiral separation of 11 steroids was investigated on amylose tris(3,5-dimethylphenyl carbamate) (Chiralpak AD). This chiral stationary phase was subjected to both normal-phase and reversed-phase conditions. Enantioselectivities were higher in the reversed-phase mode for the majority of steroids. The influence of structural modifications of the steroid molecule on the chiral separation was investigated. Acetylation of hydroxyl groups decreased enantioselectivity in the normal-phase mode. However, some acetates exhibited higher enantioselectivities in the reversed-phase mode. The type of alkyl substitution had opposite effects under normal-phase and reversed-phase conditions. The reversed-phase eluent probably changes the chiral recognition characteristics of amylose tris(3,5-dimethylphenyl carbamate). All 11 steroids were also chromatographed on permethylated β- and γ-cyclodextrin columns. Enantioselectivities were lower compared to the amylose column, with the β-cyclodextrin being superior to the γ-cyclodextrin.

1. Introduction

The increasing awareness of the fact that enantiomers of a substance can exhibit different pharmacological activities has led to research efforts with the goal to be able to detect and quantify small amounts of the "wrong" enantiomer in pharmaceuticals.

Liquid chromatography utilizing chiral stationary phases (CSP) has gained increasing importance to accomplish this task [1].

Few separations of enantiomeric steroids have been reported in the literature. Ladanyi et al. separated 8-azagonane-12-one derivatives by use of 1(S)-10-camphorsulphonic acid as chiral mobile phase modifier [2]. The majority of the published papers reported on the separation of racemic-norgestrel by use of cyclodextrins either as mobile phase modifier or bound to a silica support as CSP [3–11].

Due to first promising results we investigated the chiral separation of 11 steroids on amylose tris(3,5-dimethylphenyl carbamate) ("Chiralpak AD") [12]. Adsorption on this material is believed to occur by interaction of the polar groups of the solute with the carbamate groups of the CSP via hydrogen bonding or dipole–dipole interaction [13]. Chiral recognition is probably brought about by the higher order structure of the derivatized amylose, a left-handed helix with a chiral groove along the main chain [12].

For the following compound classes the influence of the solute structure on the observed enantio-

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selectivity on amylose tris(3,5-dimethylphenyl carbamate) was studied: amino acid derivatives [14], cannabinoids [15,16], β-amino alcohols [17]. In our study we tried to elucidate which structural elements on the steroid backbone affect the chiral separation on amylose tris(3,5-dimethylphenyl carbamate).

According to the supplier of the "Chiralpak AD" column (Daicel), it is to be used with eluents comprising mixtures of n-hexane and 2-propanol or ethanol. But in practice it has been shown that this column tolerates prolonged use under reversed-phase conditions with eluents comprising acetonitrile and water [18]. Ishikawa and Shibata established reversed-phase conditions for a related material [cellulose tris(3,5-dimethylphenyl carbamate), "Chiralcel OD"] [19].

In this work, we systematically compared the enantioselectivities of this material in the normal-phase and reversed-phase modes.

For comparison, the chiral separation of all 11 steroids was also investigated on permethylated β- and γ-cyclodextrin (PM-β-CD, PM-γ-CD).

2. Experimental

2.1. Instrumentation

High-performance liquid chromatography (HPLC) was carried out on a Shimadzu LC-8A instrument. The chiral columns used were: Chiralpak AD (250× 4.6 mm, 10 μm) from Baker, Nucleodex β- and γ-PM (200×4.0 mm, 5 μm) from Macherey–Nagel.

2.2. Materials

HPLC grade solvents were obtained from Merck (Darmstadt, Germany). Water was purified by use of an Elgastat water purification system. 1,3,5-Tri-tert.-butylbenzene (for column dead time measurement on the Chiralpak AD [20]) was obtained from Fluka.

Steroids were prepared in house, except for compounds 6 (rac-norgestrel, U.S.P.C., Rockville, USA), 11 (rac-ethylgonenedione, S&D Chemicals) and the natural enantiomer of 1 (ethynodiol, Steraloids, Wilton, NH, USA).

The laboratory-made compounds used in this study are mainly the result of efforts in the field of total synthesis of steroids (e.g. [21]). Their purity was checked by reversed-phase chromatography, thin-layer chromatography, melting point and measurement of optical rotation. The optical rotation of the racemates was zero, for the enantiomers the values agreed with the literature.

The structures of the laboratory-made compounds were confirmed by ^{13}C and ^{1}H NMR. All steroids used for chiral separations were racemates, except for 7 and 8, where mixtures of the enantiomers were injected. For all compounds the elution orders were determined by injecting the pure enantiomers, except for ethynodiol-17-acetate, where only the racemate was available.

2.3. Chromatographic conditions

The chromatographic conditions are summarized in Table 1.

All separations were repeated at least twice in order to ensure reproducibility. All separations were monitored at two different wavelengths. To verify that indeed enantiomers were separated it was checked if the ratio of the peak areas of 1:1 did not change by changing the detection wavelength [23].

3. Results and discussion

Fig. 1 shows the structures of the investigated steroids. It should be kept in mind that steroids possess a multitude of chiral centers (for example, compound 1 has 7 chiral centers). Therefore chirality should be treated as a feature of the molecule as a whole. As a consequence, chiral recognition at the used CSPs probably includes the entire molecule instead of single chiral centers.

3.1. Separations on the cyclodextrin phases

Table 2 summarizes the chromatographic results of the separations on the CD columns. Fig. 2 shows the chromatograms of compound 9 (ethylnandrolone) as a typical example.

On comparison, it turns out that for 8 out of 11 steroids the selectivity on the PM-β-cyclodextrin is greater than or at least comparable to, the selectivity on the PM-γ-cyclodextrin column. This is in contrast

Table 1
Chromatographic conditions

Parameter	PM-CD columns	Chiralpak AD	
		Normal-phase mode	Reversed-phase mode
Eluent	acetonitrile–water (30:70, v/v)	n-hexane–2-propanol (90:10, v/v)	acetonitrile–water (95:5, v/v)
Flow (ml/min)	1	1	1.4
Temp. (°C)	35	35	35
Detecton	UV at 240 and 215 nm	UV at 240 and 215 nm	UV at 240 and 215 nm
Sample solvent	acetonitrile	n-hexane–2-propanol (50:5, v/v)	acetonitrile
t_0 (min)	1.6 (est. [22])	3.2	2.5
Injection	3–9 μg in 10 μl	4–13 μg in 10 μl	5–16 μg in 10 μl

to the predominant opinion in the literature which claims γ-cyclodextrins to be more suitable for the chiral separation of steroids because of their greater cavity size [3,8]. Among the 3 steroids which perform better on the PM-γ-cyclodextrin column is

norgestrel [6], which was the typical test compound in former studies.

On inspection of the elution orders, it is striking that on the PM-γ-cyclodextrin column the enantiomers with the unnatural absolute configuration are
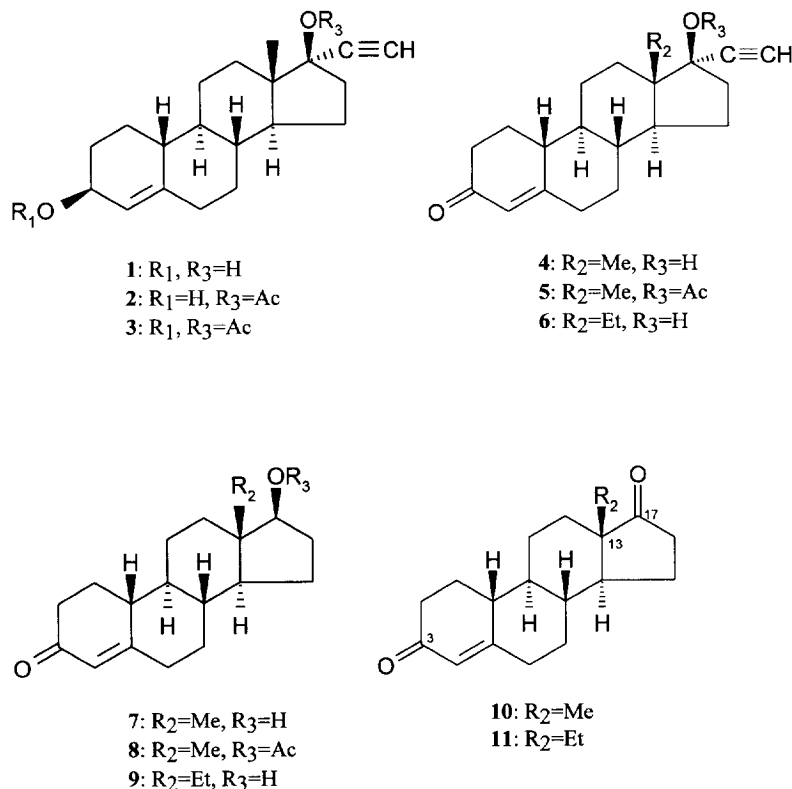

1: R_1, R_3=H
2: R_1=H, R_3=Ac
3: R_1, R_3=Ac

4: R_2=Me, R_3=H
5: R_2=Me, R_3=Ac
6: R_2=Et, R_3=H

7: R_2=Me, R_3=H
8: R_2=Me, R_3=Ac
9: R_2=Et, R_3=H

10: R_2=Me
11: R_2=Et

Fig. 1. Structures of the investigated steroids.

Table 2
Chromatographic results of the separations on the cyclodextrin phases

Compound	Trivial name	β-PM-cyclodextrin					γ-PM-cyclodextrin				
		k'_1 [a]	k'_2 [b]	α [c]	R_s [d]	first eluted [e]	k'_1	k'_2	α	R_s	first eluted
1	Ethynodiol	6.97	7.77	1.11	1.3	N	4.20	4.22	1.01	<0.8	ent
2	Ethynodiol-17-acetate	13.56	13.56	1.00	–		7.72	8.28	1.07	<0.8	[g]
3	Ethynodiol diacetate	8.81	10.56	1.20 [f]	2.2	ent	21.50	23.75	1.10	1.0	ent
4	Norethisterone	3.63	3.94	1.09	1.1	ent	3.00	3.31	1.10	1.4	ent
5	Norethisterone acetate	6.31	8.13	1.29	3.6	ent	5.63	6.44	1.14	2.4	ent
6	Norgestrel	4.63	5.00	1.08	1.1	N	4.06	4.56	1.12	1.6	ent
7	Nandrolone	3.75	4.00	1.07	0.9	ent	2.19	2.38	1.09	0.8	ent
8	Nandrolone acetate	12.09	12.09	1.00	–		4.66	5.12	1.10	1.0	ent
9	Ethylnandrolone	3.99	4.64	1.16	1.5	N	3.03	3.31	1.09	<0.8	ent
10	Norandrostenedione	2.69	3.00	1.12	1.4	ent	1.81	1.94	1.07	<0.8	ent
11	Ethylgonenedione	2.94	3.19	1.09	1.0	N	2.50	2.63	1.05	<0.8	ent

[a] k' of first eluted enantiomer.
[b] k' of last eluted enantiomer.
[c] Selectivity.
[d] Resolution: $R_s = 1.19\ (t_2 - t_1)/(w_1 + w_2)$; t, retention time; w, peakwidth at half-height.
[e] N, enantiomer with natural absolute configuration; ent, enantiomer with unnatural absolute configuration [26].
[f] 40% Acetonitrile.
[g] Not measured.

eluted first. This is an indication that the test compounds are all separated by the same mechanism on this column, maybe by an inclusion-type mechanism.

No clear pattern is observed on the PM-β-cyclodextrin column.

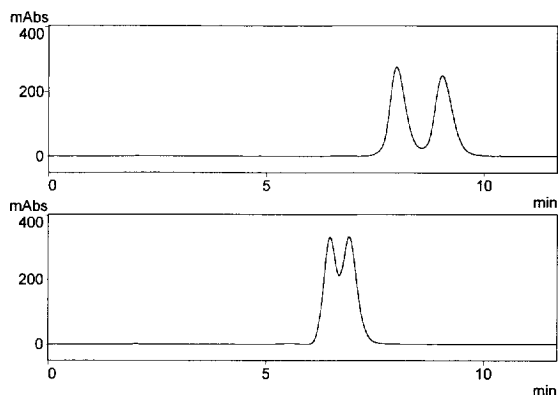Baseline resolution could be achieved with only 4

Fig. 2. Chromatograms of ethylnandrolone (9) on PM-β-cyclodextrin (above) and PM-γ-cyclodextrin column (below). Eluent: acetonitrile–water (30:70, v/v). Flow: 1 ml/min. $T = 35°C$. Detection wavelength: 240 nm.

compounds. However, after careful optimization of the chromatographic conditions for each single compound this situation would probably improve somewhat.

3.2. Separations on Chiralpak AD

Table 3 summarizes the chromatographic results of the separations on the Chiralpak AD. The two test eluent compositions were chosen based upon previous experience. Compounds 5, 11, 4 and 8 could not be resolved by use of these eluents. It was tried to achieve separation by weaker eluents (i.e. lower 2-propanol content for normal-phase separation and lower acetonitrile content for reversed-phase separation). Only after these attempts failed were the compounds declared non-separable in the two test eluents.

3.3. Comparison of enantioselectivities in normal-phase mode vs. reversed-phase mode

Upon inspection of the results in Table 3 it can be seen that for a majority of 9 out of 11 compounds the

Table 3
Chromatographic results of the separations on Chiralpak AD

Compound	Trivial name	β-PM-cyclodextrin					γ-PM-cyclodextrin				
		k'_1 [a]	k'_2 [b]	α [c]	R_s [d]	first eluted [e]	k'_1	k'_2	α	R_s	first eluted
1	Ethynodiol	4.21	5.92	1.41	5.4	N	0.60	1.20	2.00	4.1	ent
2	Ethynodiol-17-acetate	2.46	3.17	1.29	3.7	[g]	0.68	1.17	1.71	3.0	[g]
3	Ethynodiol diacetate	0.61	0.71	1.17	0.9	ent	0.22	6.54	30.0	8.9	N
4	Norethisterone	3.31	4.48	1.35	4.6	N	0.67	0.67	1.00	–	
5	Norethisterone acetate	2.16	2.16	1.00	–		0.58	0.99	1.72	2.4	N
6	Norgestrel	2.93	3.16	1.08	1.1	ent	0.49	0.59	1.22	1.0	ent
7	Nandrolone	3.40	4.79	1.41	4.1	N	1.13	2.96	2.63	6.5	N
8	Nandrolone acetate	1.58	1.91	1.21	2.2	N	3.48	3.48	1.00	–	
9	Ethylnandrolone	3.38	4.43	1.31	4.3	N	1.21	4.35	3.59	10.4	N
10	Norandrostenedione	2.88	4.82	1.68	8.1	N	1.50	3.03	2.02	5.0	N
11	Ethylgonenedione	2.32	2.32	1.00	–		1.70	4.84	2.85	7.3	N

[a] k' of first eluted enantiomer.
[b] k' of last eluted enantiomer.
[c] Selectivity.
[d] Resolution: $R_s = 1.19 (t_2 - t_1)/(w_1 + w_2)$; t, retention time; w, peakwidth at half-height.
[e] N, enantiomer with natural absolute configuration; ent, enantiomer with unnatural absolute configuration [26].
[f] 40% Acetronitrile.
[g] Not measured.

enantioselectivity is higher in the reversed-phase than in the normal-phase mode.

Separation in the reversed-phase mode was not possible for compounds 4 and 8. But in those two cases the normal-phase mode worked.

In the normal-phase mode, compounds 5 and 11 could not be resolved. However, the reversed-phase mode provided resolution.

Summing up, it may be stated that facile baseline resolution for all steroids was achieved by first trying a Chiralpak AD column in the reversed-phase mode and resolving the remaining compounds on a Chiralpak AD in the normal-phase mode.

Fig. 3 shows the chromatograms of compound 4 in the normal-phase and reversed-phase modes as an example. An extreme difference between the chromatographic modes is observed with compound 3. From the gross differences in the separation characteristics, it can be concluded that the aqueous eluent changes the nature of the CSP. Chiralpak AD columns under normal-phase and reversed-phase conditions must therefore be regarded as different CSPs.

In six cases, the elution order of the enantiomers could be measured in both chromatographic modes. In two of the six known cases, a reversal of elution order occurred by changing the mode.

3.4. Influence of structural changes on enantioselectivity

Levin et al. recently studied the role of free hydroxyl groups in the chiral recognition of cannabinoids on amylose tris(3,5-dimethylphenyl carba-
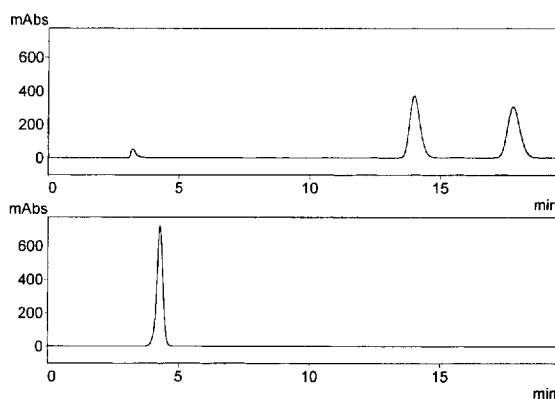

Fig. 3. Chromatograms of rac-norethisterone (4) on Chiralpak AD. Top: normal-phase conditions; eluent, n-hexane–2-propanol (90:10, v/v); flow, 1 ml/min; $T = 35°C$; detection wavelength, 240 nm. Bottom: reversed-phase conditions; eluent, acetonitrile–water (95:5, v/v); flow, 1.4 ml/min; $T = 35°C$; detection wavelength, 240 nm.

mate) in the normal-phase mode [16]. They found a drastic decrease in enantioselectivity on acetylation of the free hydroxyl groups. We observed a similar trend under normal-phase conditions for compounds 1, 2, 3 (ethynodiol+acetates), 4, 5 (norethisterone+acetate) and 7, 8 (nandrolone+acetate).

The separations of the above mentioned compounds in the reversed-phase mode do not follow such a simple pattern. Acetylation of ethynodiol (1) at position C_{17} yielding compound 2 leads to a decrease in enantioselectivity (2.00→1.71). But further acetylation at position C_3 (yielding compound 3) causes a drastic increase in enantioselectivity to $\alpha=30$. Acetylation of norethisterone (4) at position C_{17} to compound 5 enables enantioseparation. Nandrolone (7), on the other hand, shows the reverse trend, with the acetylation at position C_{17} (compound 8) leading to loss of separation. These data show that under reversed-phase conditions, hydrogen bonding via free hydroxyl groups of the solute is not as important for chiral recognition as under normal-phase conditions. Acetylation of hydroxyl groups can even improve enantioselectivity under reversed-phase conditions.

The type of alkyl substitution at C_{13} also affects enantioseparation. The enantioselectivities of 13-methyl steroids together with their corresponding ethyl homologues were extracted from Table 3 and summarized in Table 4 for more convenient comparison. Under normal-phase conditions, a methyl

homologue exhibits greater enantioselectivity compared to the corresponding ethyl homologue. However, under reversed-phase conditions the opposite behavior is observed.

These observations suggest that the steric fit of the solute into chiral cavities plays a role in chiral recognition [24]. The used acetonitrile–water eluent probably changes the nature of these cavities leading to the observed differences between the normal-phase and reversed-phase systems.

3.5. Column stability under reversed-phase conditions

The Chiralpak AD column for reversed-phase chromatography has been used extensively in our laboratory with acetonitrile–water eluents for more than two years. During this time only minor performance deterioration of the column was observed.

Fig. 4 shows as an example the chromatograms of *rac*-ethylgonenedione (11). The first chromatogram (top) was measured before and the second chromatogram (bottom) after 11 months of routine use. We conclude that Chiralpak AD columns should be stable for at least 1 year under reversed-phase conditions.

To switch from normal-phase to reversed-phase conditions, the new Chiralpak AD columns were first flushed with 2-propanol as intermediate solvent and then with acetonitrile–water. The columns were left

Table 4
Influence of alkyl substitution at C_{13} on enantioseparation of steroids on Chiralpak AD under normal-phase and reversed-phase conditions

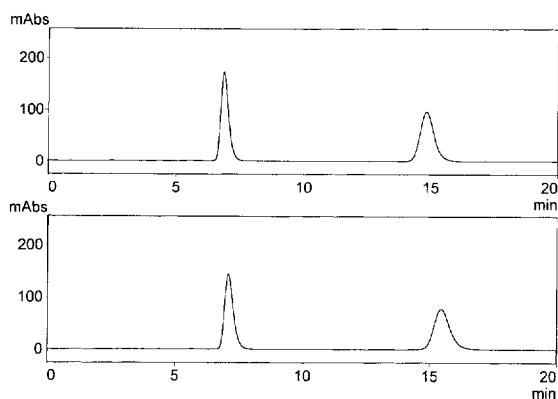|	α under normal-phase conditions	α under reversed-phase conditions
R_2=Me: Norethisterone (4)	1.4	1.0
		↓
	↑	
R_2=Et: Norgestrel (6)	1.1	1.2
R_2=Me: Nandrolone (7)	1.4	2.6
		↓
	↑	
R_2=Et: Ethylnandrolone (9)	1.3	3.6
R_2=Me: Norandrostenedione (10)	1.7	2.0
		↓
	↑	
R_2=Et: Ethylgonenedione (11)	1.0	2.8

Fig. 4. Chromatograms of *rac*-ethylgonenedione (11) on Chiralpak AD under reversed-phase conditions: eluent, acetonitrile–water (95:5, v/v); flow, 1.4 ml/min; $T=35°C$; detection wavelength, 240 nm. Bottom: after 11 months of routine use.

in the reversed-phase mode and never switched back to normal-phase conditions. There are contradictory opinions about the question as to whether these columns can be switched between the chromatographic modes multiple times [18,25]. We did not investigate this question but instead used two separate columns for normal-phase and reversed-phase chromatography.

4. Summary

For the majority of the studied steroids the permethylated β-cyclodextrin column gave higher enantioselectivities than the permethylated γ-cyclodextrin column.

Clearly superior to the cyclodextrin columns was the amylose tris(3,5-dimethylphenyl carbamate) column in the reversed-phase mode which provided baseline resolution for the majority of the investigated compounds. The remaining compounds could be resolved on amylose tris(3,5-dimethylphenyl carbamate) in the normal-phase mode.

The use of Chiralpak AD columns under reversed-phase conditions is a useful complement to their use under normal-phase conditions. It enables the separation of enantiomeric steroids otherwise difficult to resolve and yields the highest enantioselectivities for the majority of the investigated compounds compared to the other used CSPs.

In contrast to the normal-phase mode, hydrogen bonding of solute hydroxyl groups to the carbamate moieties does not play the major role under reversed-phase conditions.

Small changes in the structure of steroids can have an immense influence on enantioselectivity on Chiralpak AD. The steric fit of the steroid molecule into chiral cavities seems to affect chiral recognition in both chromatographic modes leading to different enantioselectivities towards methyl and ethyl homologues.

Further investigations are under way.

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