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Chiral resolution of enantiomeric steroids by high-performance liquid chromatography on amylose tris(3,5-dimethylphenylcarbamate) under reversed-phase conditions

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Abstract

Amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak AD) was used under reversed-phase conditions for the chiral separation of 26 steroids. It was found that the chiral separation behavior of Chiralpak AD under reversed-phase conditions differs clearly from those under normal-phase conditions thereby extending the range of application of this chiral stationary phase. The substitution of methanol for acetonitrile in the aqueous eluent did not grossly change the chiral separation behavior. The enantioselectivity under reversed-phase conditions is strongly dependent on the water concentration in the eluent up to 10%. The influence of the temperature on the chiral separation is discussed from a practical point of view. A HPLC method for the quantification of the unnatural enantiomer of estradiol in drug substances applying Chiralpak AD under reversed-phase conditions was developed and successfully validated.

Keywords: Enantiomer separation; Amylose tris(3,5-dimethylphenylcarbamate) stationary phases; Chiral stationary phases, LC; Steroids; Estradiol

1. Introduction

Steroid hormones are an important class of pharmaceuticals which are applied for oral contraception and hormone replacement therapy. Since steroids possess a number of stereogenetic centers they are optically active. Because enantiomers can exhibit different pharmacological activities the registration of chiral drugs requires information about their enantiomeric purity. This in turn 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 (CSPs) has gained increasing importance to accomplish this task [1]. Few chromatographic separations of enantiomeric steroids have been reported in the literature. Ladanyi et al. [2] separated 8-azagonane-12-one derivatives by use of 1-(*S*)-10-camphorsulphonic acid as chiral mobile phase modifier. The majority of the published papers reported on the separation of the enantiomers of norgestrel by use of cyclodextrins either as mobile phase modifier or bound to a silica support as chiral stationary phase [3–11]. In a previous study we

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separated the enantiomers of 11 different steroids on permethylated cyclodextrins and on Chiralpak AD [12]. During our further work we concentrated on the Chiralpak AD CSP due to its ability to separate a broad range of compounds.

Chiralpak AD columns consist of amylose tris(3,5-dimethylphenyl carbamate) coated on silica gel. 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 structure of higher order of the derivatized amylose, a left-handed helix with a chiral groove along the main chain [14].

The supplier of the Chiralpak AD column (Daicel) recommends its use with eluents comprising mixtures of an alkane and an alcohol. Dingenen showed that this column tolerates prolonged use under reversed-phase (RP) conditions with eluents comprising acetonitrile and water [15]. He compared the chiral separation of the enantiomers of metomidate and etomidate under normal-phase (NP) and reversed-phase (RP) conditions. Although enantioselectivities were not calculated, the chromatograms indicate different chiral separation behavior in both chromatographic modes. McCarthy separated the four stereoisomers of nadolol under normal- and reversed-phase conditions and reported the same elution order in both systems [16]. Inoue et al. presented a ''new'' Chiralpak AD-column which could be used under RP conditions in 1996 [17], but there are no significant differences in the design to the conventional columns. Shibata et al. established reversed-phase conditions for a related material [cellulose tris(3,5-dimethylphenyl carbamate), ''Chiralcel OD''] [18]. They investigated the influence of the type and concentration of mobile phase additives (for example salts, organic modifier).

In our previous study we compared the enantioselectivities on Chiralpak AD under normal- and reversed-phase conditions [12]. In this work we extended the range of investigated steroids by 15 compounds. We studied the influence of temperature and eluent composition under reversed-phase conditions on the chiral resolution. In order to verify the aptitude of reversed-phase chromatography on Chiralpak AD we validated a high-performance liquid chromatography (HPLC) method for the determination of the unnatural enantiomer of estradiol (*ent*-estradiol) in drug substances.

2. Experimental

2.1. Instrumentation

HPLC was carried out on Shimadzu LC-8A and LC-10A instruments. The Chiralpak AD columns (250×4.6 mm, 10 μm) were obtained from Baker.

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 under NP conditions [19]) was obtained from Fluka. The column dead time under RP conditions was determined by injecting water.

Steroids were prepared in the laboratory, except for compounds **6** (norgestrel, U.S.P.C., Rockville, USA) 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., Ref. [20]). Their purity was checked by RP chromatography, thin-layer chromatography (TLC), 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-nuclear magnetic resonance (NMR).

The steroids used for chiral separations were racemates, except for **7, 8, 19, 20** and **21**, 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 for the chiral

Table 1
Chromatographic conditions on Chiralpak AD

	NP	RP (acetonitrile)	RP (methanol)
Eluent	*n*-Hexane–2-propanol	Acetonitrile–water	Methanol–water
Flow	1 ml/min	1.4 ml/min	1 ml/min
Temperature	35°C	35°C	35°C
Detection	UV at λ_{max}	UV at λ_{max}	UV at λ_{max}
Sample solvent	*n*-Hexane–2-propanol 50:50 (v/v)	Acetonitrile	Methanol
Injection	4–13 μg in 10 μl	5–16 μg in 10 μl	5 μg in 10 μl

separations are listed 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, the 1:1 ratio of the peak areas was checked, which did not change upon changing the detection wavelength [21].

To switch from NP to RP conditions the new Chiralpak AD columns were first flushed with 2-propanol as intermediate solvent and then with acetonitrile–water. The columns were left in the RP mode and never switched back to NP conditions. There are contradictory opinions about the question whether these columns can be switched between the chromatographic modes multiple times [15,22]. We did not investigate into this question but instead used two separate columns for NP and RP chromatography.

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 seven 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 involves the entire molecule instead of single chiral centers.

3.1. Comparison of enantioselectivities in normal-phase mode vs. reversed-phase mode on Chiralpak AD

Table 2 summarizes the chromatographic results of the separations of compounds **1** to **26** on the Chiralpak AD under NP and RP conditions. The results of the chiral separations of compounds **1** to **11** with *n*-hexane–2-propanol and acetonitrile–water were taken from our previous work [12] and added here for the sake of comparison. The corresponding results of compounds **12** to **26** confirm the observations from that work, i.e., sharp differences in enantioselectivity between both modes. RP chromatography enables the chiral separation of compounds which cannot be sufficiently resolved under NP conditions (compounds **15**, **20**-**24**). On the other hand compounds **13**, **16** and **17** can only be resolved under NP conditions. To a certain degree both chromatographic modes complement each other. The use of Chiralpak AD in the RP mode therefore extends the range of application of this CSP.

3.2. Influence of the mobile phase composition on enantioselectivity under RP conditions on Chiralpak AD

The chromatographic results of the chiral separation with methanol–water are listed in Table 2.

Fig. 2 compares graphically the enantioselectivities achieved with acetonitrile–water and methanol–water. The overall separation characteristics are similar. That is compounds which can be separated with high enantioselectivity in acetonitrile–water in general show the same behavior with methanol–water (e.g., **3**, **11**, **20**). The same conclusion applies to the compounds showing low enantioselectivity. The elution orders in both mobile phases could be compared for 15 compounds (Table 2). The elution order did not change upon substituting methanol for acetonitrile, except in two instances (**5** and **12**). This again underlines the similar separation characteristics in both mobile phases. Since acetonitrile and methanol possess different hydrogen bonding capabilities but on the other hand yield similar chiral separations

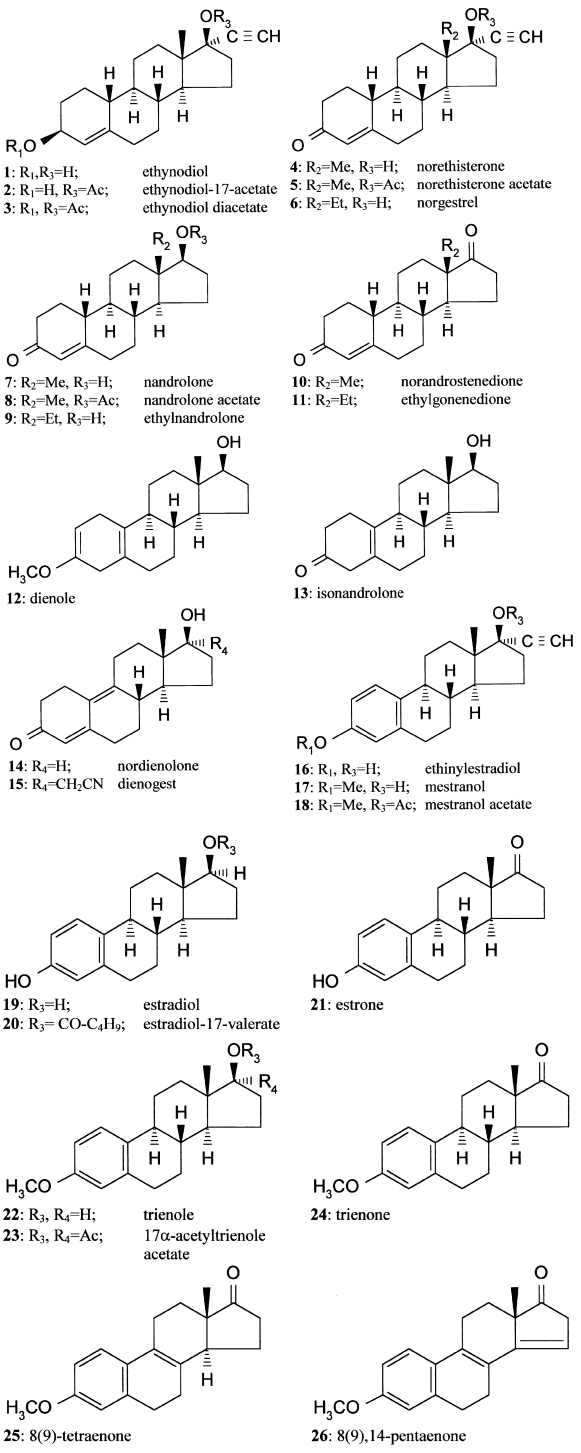

Fig. 1. Structures and trivial names of the investigated steroids.

it can be inferred that hydrogen bonding does not play a major role in the enantioselective retention on Chiralpak AD under RP conditions.

There are differences in enantioselectivity between acetonitrile–water and methanol–water for some compounds (e.g., **1**, **5**, **6**, **8**, **23**). A common structural feature which leads to these differences could not be found. In practice it is worthwhile to test the substitution of methanol for acetonitrile since this sometimes enables the chiral separation. Compare for example the resolutions of nandrolone acetate (**8**) in both eluents (Table 2).

In order to learn more about the behavior of Chiralpak AD columns under RP conditions we investigated into the influence of the water contents in the mobile phase on the chiral separation. Fig. 3 shows the retention factors and enantioselectivity for the chiral separation of *rac*-ethylgonenedione (**11**) as a function of water content in the acetonitrile–water eluent. In the range from 0 to 1% the retention factor of the last eluted enantiomer (k_2') drops slightly. The retention factor of the first eluted enantiomer (k_1') decreases distinctly with increasing water concentration from 0 to 10%. This behavior is unusual for RP chromatography. Above 10% water the expected retention behavior is observed for both enantiomers. Enantioselectivity increases sharply with increasing water concentration and reaches a plateau above 10%. A strong increase in enantioselectivity was also observed for *rac*-ethynodiol diacetate (**3**) up to 5% water. Beyound that concentration the measurement of enantioselectivity was thwarted by the extreme retention of the last eluted enantiomer.

The strong influence of water on enantioselectivity in the range from 0 to 10% could be caused by an alteration of the structures of higher order of the amylose tris(3,5-dimethylphenylcarbamate).

3.3. Influence of temperature on the chiral separation

The relationship between enantioselectivity and temperature is described by the following equation [23]:

$$\ln \alpha = \frac{\Delta\Delta H}{-RT} + \frac{\Delta\Delta S}{R} \quad (1)$$

In the temperature range normally accessible in

Table 2
Chromatographic results of the chiral separations on Chiralpak AD

Compound No.	NP						RP (acetonitrile)						RP (methanol)					
	2-PrOH (%)	k_1'[a]	k_2'[b]	α[c]	R_s[d]	First eluted[e]	CH_3CN (%)	k_1'	k_2'	α	R_s	First eluted	MeOH (%)	k_1'	k_2'	α	R_s	First eluted
1	10	4.21	5.92	1.41	5.4	N	95	0.60	1.20	2.00	4.1	*ent*	90	1.30	1.30	1.00		
2	10	2.46	3.17	1.29	3.7	[f]	95	0.68	1.17	1.71	3.0	[f]	90	0.91	1.39	1.53	2.3	
3	10	0.61	0.71	1.17	0.9	*ent*	95	0.22	6.54	30.0	8.9	N	95	1.04	5.05	4.85	7.5	N
4	10	3.31	4.48	1.35	4.6	N	95	0.67	0.67	1.00			85	1.75	1.75	1.00		
5	10	2.16	2.16	1.00			95	0.58	0.99	1.72	2.4	N	85	3.87	4.08	1.06	<0.8	*ent*
6	10	2.93	3.16	1.08	1.1	*ent*	95	0.49	0.59	1.22	1.0	*ent*	85	2.20	2.20	1.00		
7	10	3.40	4.79	1.41	4.1	N	95	1.13	2.96	2.63	6.5	N	95	0.68	0.98	1.45	2.9	N
8	10	1.58	1.91	1.21	2.2	N	95	3.48	3.48	1.00			95	3.36	4.09	1.21	2.3	N
9	10	3.38	4.43	1.31	4.3	N	95	1.21	4.35	3.59	10.4	N	95	0.70	1.37	1.95	5.4	N
10	10	2.88	4.82	1.68	8.1	N	95	1.50	3.03	2.02	5.0	N	95	1.77	2.89	1.63	5.8	N
11	10	2.32	2.32	1.00			95	1.70	4.84	2.85	7.3	N	95	1.69	6.61	3.92	14.1	N
12	3	1.85	1.98	1.07	<0.8	N	95	1.21	1.43	1.18	0.9	N	90	2.15	2.45	1.14	1.4	*ent*
13	10	2.12	2.51	1.18	2.2	*ent*	95	1.46	1.46	1.00			90	1.18	1.23	1.04	<0.8	
14	10	3.56	5.05	1.42	5.4	N	95	0.69	1.69	2.46	6.6	N	95	0.42	0.58	1.40	2.2	N
15	10	1.12	1.12	1.0			40[g]	2.80	3.75	1.34	3.4	N	[f]					
16	10	5.95	7.15	1.20	3.0	N	40[h]	3.79	3.79	1.00			85	0.78	0.78	1.00		
17	3	3.23	3.57	1.10	1.7	N	50	4.09	4.29	1.05	<0.8	*ent*	85	2.55	2.55	1.00		
18	3	1.24	1.45	1.17	2.0	N	95	0.19	0.32	1.63	1.2	N	95	0.93	1.06	1.14	1.0	N
19	10	4.06	4.47	1.10	1.5	N	95	0.26	0.33	1.30	<0.8	*ent*	95	0.21	0.34	1.61	1.9	*ent*
20	3	6.86	6.86	1.00			95	0.59	1.54	2.60	5.5	*ent*	95	1.37	5.61	4.11	9.7	*ent*
21	3	12.05	12.05	1.00			95	0.22	0.51	2.30	2.7	*ent*	95	0.54	0.94	1.74	4.1	*ent*
22	3	2.80	2.80	1.00			95	0.48	0.68	1.40	1.4	*ent*	95	0.64	1.05	1.63	4.2	*ent*
23	3	1.72	1.82	1.06	<0.8	*ent*	95	0.46	0.61	1.34	1.2	*ent*	95	1.86	1.86	1.00		
24	3	1.44	1.55	1.07	1.0	*ent*	95	0.43	0.90	2.08	3.8	*ent*	95	1.48	2.45	1.65	5.9	*ent*
25	3	1.62	1.62	1.00			70[h]	1.70	1.81	1.07	<0.8	N	95	1.41	1.55	1.10	1.1	N
26	3	1.29	1.29	1.00			70[h]	1.79	1.79	1.00			85	3.39	3.58	1.06	<0.8	N

[a] k' of first eluted enantiomer.
[b] k' of last eluted enantiomer.
[c] Selectivity.
[d] Resolution: $R_s = 1.18\ (t_2 - t_1)/(w_1 + w_2)$; t: retention time, w: peakwidth at half-height.
[e] N: Enantiomer with natural absolute configuration; *ent*: enantiomer with unnatural absolute configuration [25].
[f] Not measured.
[g] 0.6 ml/min.
[h] 1.0 ml/min.

HPLC (below the isoelution temperature) the chiral separation is enthalpically controlled, i.e., lowering the temperature leads to higher enantioselectivity. Fig. 4 shows this for the RP separation of *rac*-norethisterone (**4**). The chromatogram at 35°C shows only one peak. After a decrease in temperature down to 15°C resolution has increased somewhat and the peak due to the other enantiomer becomes distinguishable. However, resolution is still not sufficient, since the gain in enantioselectivity is in this case partly compensated for by the lower separation efficiency, caused by the reduced rate of mass transfer at the lower temperature. We conclude that a decrease in temperature has only a limited benefit for chiral resolution on Chiralpak AD under RP conditions.

On the contrary, our experience with RP chromatography on Chiralpak AD tells us that it is often beneficial for a separation with already sufficient enantioselectivity to use higher temperatures. Fig. 5 illustrates this for the separation of norgestrel (**6**). The increase in temperature from 20°C to 40°C leads to a decrease in enantioselectivity from 1.5 to 1.3. However, the loss in enantioselectivity is compen-

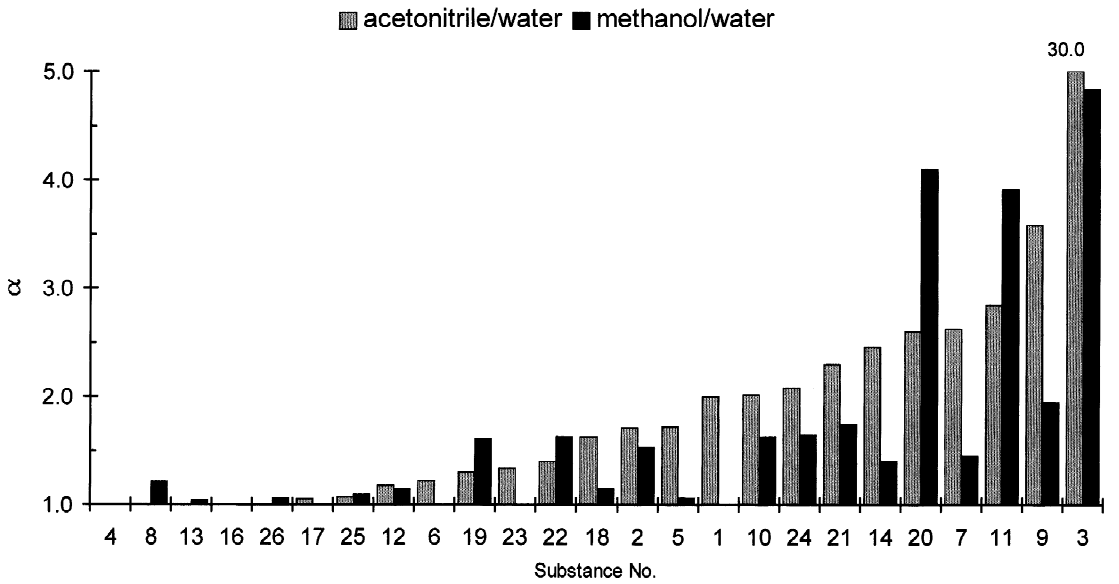

Fig. 2. Comparison of enantioselectivities on Chiralpak AD under RP conditions between methanol–water and acetonitrile–water.

sated for by the gain in chromatographic efficiency. The net result is a somewhat higher resolution. One advantage of the higher temperature for this separation is a shorter analysis time. Furthermore the higher chromatographic efficiency leads to higher peaks and thus increases sensitivity.

3.4. Development and validation of a HPLC method for the determination of ent-estradiol in drug substances

In order to verify the aptitude of Chiralpak AD for RP chromatography we developed a HPLC method for the determination of *ent*-estradiol (**19**) in batches

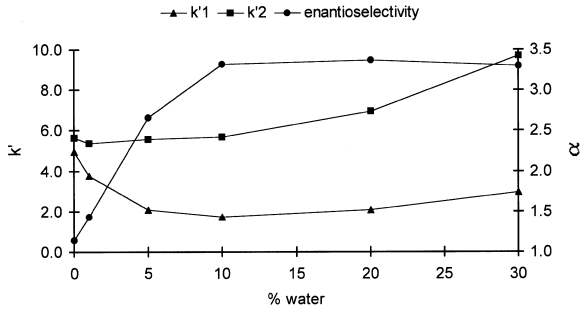

Fig. 3. Retention factors of the enantiomers of ethylgonenedione (**11**) and enantioselectivity as function of water content in the acetonitrile–water eluent.

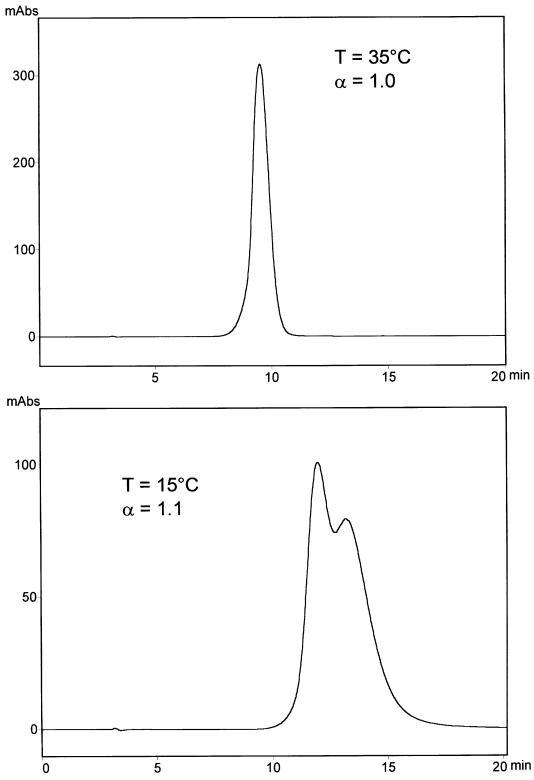

Fig. 4. Chromatograms of *rac*-norethisterone (**4**) on Chiralpak AD at different temperatures. Eluent: acetonitrile–water (60:40), flow: 1 ml/min, detection wavelength: 240 nm.

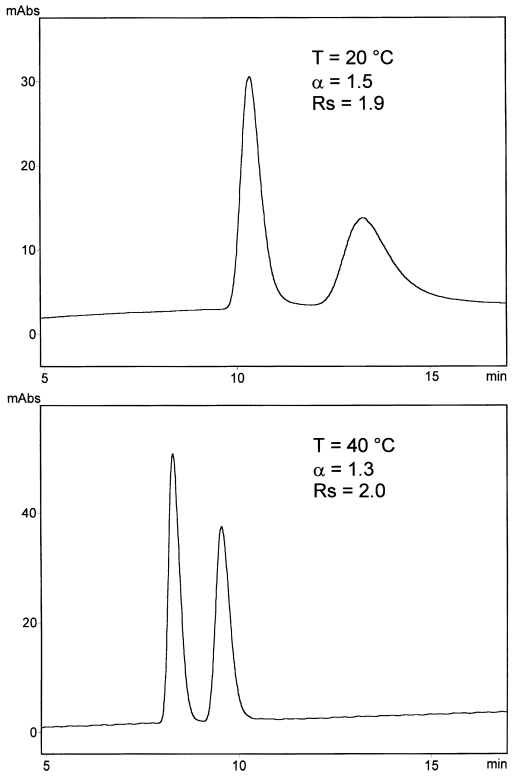

Fig. 5. Chiral separation of norgestrel (**6**) on Chiralpak AD at different temperatures. Eluent: acetonitrile–water (70:30), flow: 0.8 ml/min, detection wavelength: 240 nm.

of estradiol drug substances. The following conditions were chosen: eluent: acetonitrile–water (50:50), flow: 1 ml/min, $T = 40$°C, detection wavelength = 278 nm, sample solvent: acetonitrile, injection: 5 μg in 10 μl.

The temperature was chosen as high as possible for reasons explained in the previous section. The flow-rate represents a compromise between analysis time and the column pressure limit (50 bar).

The method was validated according to the guidelines of the ICH [24]. Specificity, linearity, precision, accuracy, detection and quantitation limit were evaluated.

The method yields a resolution between the estradiol enantiomers of 2.5. The resolution between *ent*-estradiol and an earlier eluted impurity is 1.8. The method is therefore specific.

A linear regression was calculated for eight different concentration levels in the range from 0.05% to 4.8%. From the results it can be concluded that the relationship between the concentration of *ent*-estradiol and the area of the corresponding peak is linear (slope = 19034, intercept = −58, $r^2 = 0.99995$).

Table 3
Comparison of spiked and found amount of *ent*-estradiol and recoveries

Spiked amount of *ent*-estradiol (%)	*ent*-Estradiol found (%)	Recovery (%)
0.099	0.100	100.7
0.198	0.203	102.4
0.494	0.495	100.2
1.005	1.012	100.7
1.990	1.991	100.1
4.830	4.850	100.4
	Mean	100.8

In order to determine the accuracy estradiol was spiked with different amounts of *ent*-estradiol. The concentration of *ent*-estradiol was determined by the HPLC method and the found value compared to the added concentration. The results are summarized in Table 3. From the recoveries of approximately 100% we conclude that the method is sufficiently accurate.

Precision was determined by repeatedly (7×) injecting a sample spiked with 0.20% *ent*-estradiol. A relative standard deviation of 2.6% was calculated for the found concentrations.

Quantitation and detection limits were determined from the signal-to-noise ratio (S/N). Fig. 6 shows the chromatogram of an estradiol sample spiked with 0.05% *ent*-estradiol, yielding a S/N of about 10. This

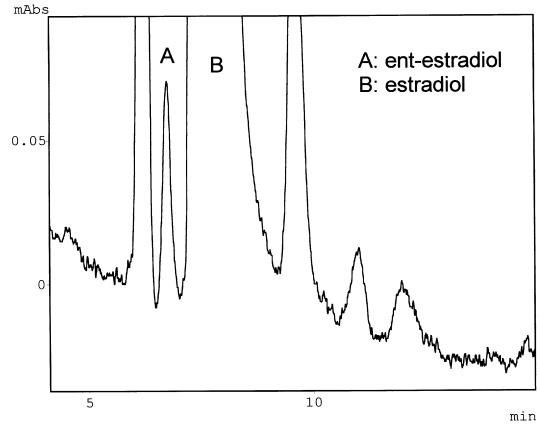

Fig. 6. Chromatogram of a batch of estradiol drug substance spiked with 0.05% *ent*-estradiol (chromatographic conditions: see Section 3.4).

concentration level represents therefore the quantitation limit. The detection limit was found to be at 0.02% *ent*-estradiol, yielding S/N ca. 3.

From the results of the validation we conclude that the HPLC method is suitable for the determination of *ent*-estradiol in batches of drug substances in the range from 0.05% to 4.8%. It is therefore possible to apply Chiralpak AD columns routinely under reversed-phase conditions for the quantification of enantiomers in drug substances.

4. Summary

It is possible to use Chiralpak AD columns with aqueous eluents. The use under RP conditions enhances the applicability of this CSP since it enables the chiral separation of many compounds, which cannot be resolved under NP conditions. It also simplifies its possible use for biological samples since a transfer from an aqueous to a non-aqueous sample solvent is no longer necessary.

The substitution of methanol for acetonitrile in the aqueous eluent did not dramatically change the general separation characteristics. This points to a minor role of hydrogen bonding as retention mechanism on Chiralpak AD under RP conditions. In particular cases the use of methanol–water instead of acetonitrile–water can improve the chiral separation.

For a particular racemate enantioselectivity increased with increasing concentration of water in the eluent up to 10%. An eluent consisting of acetonitrile–water (90:10) is therefore deemed a suitable starting point for the development of a chiral RP-HPLC method for new compounds.

The expected dependence of enantioselectivity from temperature for an enthalpically controlled chiral separation was observed, i.e., increasing enantioselectivity with decreasing temperature. However, RP separations on Chiralpak AD should be run at the highest possible temperature in order to gain efficiency, if the loss of enantioselectivity can be tolerated.

A RP-HPLC method for the quantification of *ent*-estradiol (**19**) in batches of estradiol drug substances was developed and successfully validated thus demonstrating the aptitude of Chiralpak AD columns for RP chromatography.

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