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Journal of Chromatography A, 668 (1994) 413–417

JOURNAL OF CHROMATOGRAPHY A

Short Communication

Effect of temperature on separation of norgestrel enantiomers by high-performance liquid chromatography

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Abstract

The influence of mobile phase composition, concentration of β-cyclodextrin and temperature on the high-performance liquid chromatographic separation of norgestrel was studied. In studies of the effect of temperature on the enantioselectivity of (±)-norgestrel, acetonitrile–water (25:75, v/v) modified by the addition of β-cyclodextrin (14 m*M*) was applied as the mobile phase. Enantiomers were detected using UV detection at 240 nm. The capacity factors were measured over a wide range of column temperatures from −5 to 70°C.

1. Introduction

Plots of the logarithms of capacity factors against the reciprocal of absolute temperature are usually linear and are known as Van 't Hoff plots [1–3]. Nevertheless, any reversible process that alters the enthalpy or entropy of adsorption in principle gives rise to non-linear Van 't Hoff plots. Among others, changes in conformation and changes in the extent to which the mobile phase interacts with either the analyte or the stationary phase are examples of such reversible behaviour [4,5]. Moreover, the presence of multiple types of retention mechanisms or multiple types of binding sites also leads to non-linearity of the Van 't Hoff plots. Particularly in chiral recognition, multiple types of retention and the importance of conformation can be expected, and therefore the effect of temperature on retention might be very complex.

Cyclodextrins (CDs) are toroidal-shaped cyclic oligomers of α-1,4-D-glucopyranose units and they are well known as chiral selectors [6,7]. The name "cyclodextrins" includes a large group of α-, β- and γ-CDs together with numerous derivatives, but β-cyclodextrin (β-CD) is the most often used cyclodextrin. In HPLC cyclodextrins have been used both chemically bonded to a stationary phase and added to the mobile phase [8,9].

Despite the number of papers dealing with various applications of CDs in chromatography, including chiral separations, the knowledge of the stereoselectivity and structural relationships between CDs and guest molecules is poor. Many factors seems to be responsible for the separation including the type of CD used [10], its concentration in the mobile phase [10,11], the type of mobile phase [10–12] and the temperature of the separation process [10]. In this paper we report the influence of mobile phase composition, concentration of β-CD and temperature on the separation of norgestrel enantiomers, which were chosen as a model compounds. Norgestrel [(±)-13β-ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one] is an interesting ster-

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0021-9673/94/$07.00
SSDI 0021-9673(93)E0039-W

oidal compound with progestogenic activity which is commonly used as a contraceptive agent. Enantiomers of this compound have different intrinsic pharmacological activity [13].

2. Experimental

2.1. Reagents

Norgestrel racemic mixture (Prempak) was supplied by Ayerst Laboratories (Andover, UK) and D-(−)norgestrel (Levonorgestrel) by Sigma (St. Louis, MO, USA).

Acetonitrile (Merck, Darmstadt, Germany) was of HPLC grade. Water was purified by double distillation. Mobile phases were filtered through a 1.5-μm membrane prior to use.

β-Cyclodextrin was supplied by Chinoin (Budapest, Hungary) and was purified by recrystallization from boiling water.

2.2. Chromatography

The liquid chromatograph, consisting of an analytical solvent pump, UV–Vis spectrophotometer and linear recorder, was a product of Knauer. A Rheodyne Model 7125 injection valve was used for sample introduction. Two types of column were used, Knauer ODS-1 (120 × 4.6 mm I.D.) and Supelco ODS (150 × 4.6 mm I.D.). The capacity factors were not influenced by the type of column used.

The flow-rate was varied, depending on the column and cyclodextrin concentration used, from 1 to 3 ml/min and the dead retention time from 0.64 to 0.22 min. The sample size was 20 μl and the concentration of the solutes was 10 μg/ml.

The column temperature was controlled by immersing the column in a stirred constant-temperature bath containing ethanol–water (30:70, w/w) used as a heat-exchange medium. The bath was connected to a thermostat adjustable from −5 to 70°C. Temperature was controlled with an accuracy of ±0.5°C. Additionally, the bottle with mobile phase was thermostated 1 h before the experiment in order to obtain proper temperature equilibrium.

Acetonitrile–water of various compositions was used as the mobile phase.

The void volume was determined by injecting sodium nitrate solution. The UV detector was operated at 240 nm. The capacity factors were calculated in the usual manner and are based on the average of at least five independent determinations of each solute.

3. Results and discussion

A plot of the capacity factors *versus* acetonitrile–water composition for norgesterel racemic mixture is shown in Fig. 1. The mobile phase was used without a chiral selector. As one normally expects, increasing the organic modifier concentration in the mobile phase hastens the elution of the analyte. However, owing to the low solubility of β-CD in mobile phases with a high concentration of organic component, a composition of acetonitrile–water (25:75, v/v) was chosen for further investigations.

The influence of the β-CD concentration on the capacity factors is exemplified in Fig. 2 by

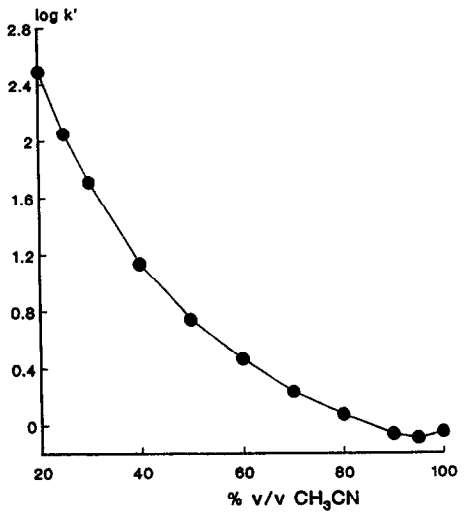

Fig. 1. Plot of log k' *vs.* mobile phase composition for D-(±)-norgestrel racemic mixture. The mobile phase (acetonitrile–water) was used without a chiral selector. Column, Knauer ODS-1 (120 × 4.6 mm I.D.) at 40°C.

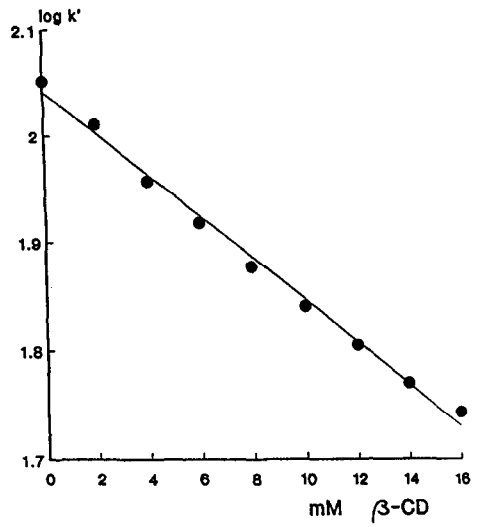

Fig. 2. Plot of log k' *vs.* concentration of β-CD with acetonitrile–water (25:75, v/v) as the mobile phase for D-(±)-norgestrel racemic mixture. Column, Knauer ODS-1 (120 × 4.6 mm I.D.) at 40°C.

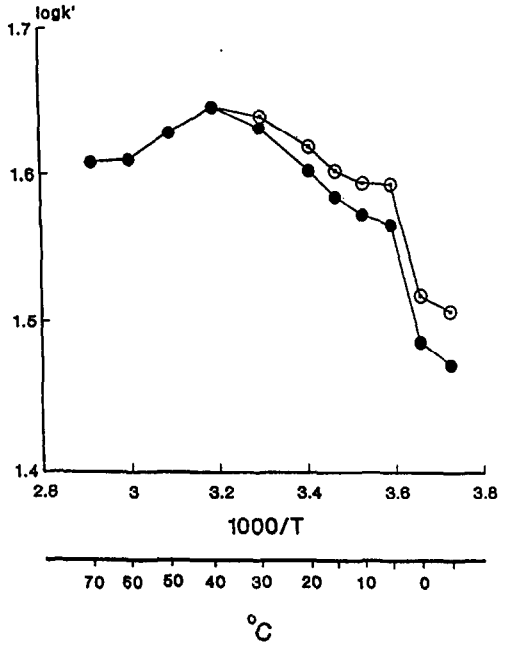

Fig. 3. Plots of log k' *vs.* $1000/T$ for the enantiomers of norgestrel. Column, Supelco ODS (150 × 4.6 mm I.D.). Mobile phase, acetonitrile–water (25:75, v/v) modified with the addition of 14 m*M* β-cyclodextrin. ○ = D-(−)- and ● = D-(+)-norgestrel.

the behaviour of norgestrel racemic mixture. The measurements were performed at 40°C (313 K) and no chiral resolution was observed over the whole range of β-CD concentrations. It is often observed that an increase in the concentration of β-CD leads to a concomitant decrease in the capacity factor [10–12,14] and, as can be see in Fig. 2, for norgestrel racemic mixture this is also this case. The observed effect suggests that the adsorption of guest–CD complexes on the reversed stationary phase is very close to zero for both enantiomers of norgestrel and that the predominating mechanism for retention is the formation of guest–CD complexes in the mobile phase. The plot is linear over the range of β-CD concentrations investigated. The shortest retention was observed using a 16 m*M* concentration of β-CD. However, owing to the extreme solubility conditions (the solubility of β-CD in water at 25°C is 16.3 m*M*) at such a high concentration, a mobile phase with the addition of 14 m*M* β-CD was chosen for further studies of the temperature effect.

Plots of the logarithms of the capacity factors against the reciprocal of absolute temperature are shown in Fig. 3. A linear Van 't Hoff behaviour is observed in the range between 70 and 40°C. In this region stereoselectivity is not observed. The temperature at which the deviation from linear Van 't Hoff behaviour, together with chiral separation, begins is 40°C. When the temperature decreases down to the sub-ambient region the retention also decreases. The best chiral separation was achieved in the range from −5 to 0°C. Chromatograms obtained at 40, 20 and 0°C are shown in Fig. 4. Although, chiral recognition is maintained at 20°C, baseline separation is observed at 0°C.

The effect of temperature on enantioselectivity in HPLC systems with mobile phases modified with the addition of CDs has not been described previously, with the exception of recent work by Seidel *et al.* [15]. They studied effect of temperature, in a narrow range from 65 to 35°C, on the separation of the mycotoxins ochratoxin A and zearalenone. Both compounds are not enantiomers, but the mobile phase used was modified by the addition of β-CD. The result obtained was a

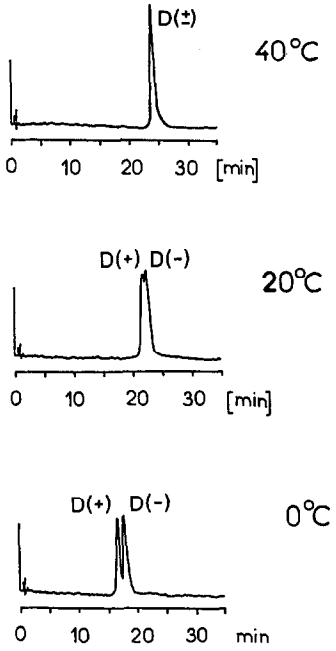

Fig. 4. Separation of D-(±)-norgestrel enantiomers at 40, 20 and 0°C. Other chromatographic conditions as in Fig. 3.

typical Van 't Hoff plot which corresponds to the first part of the plot presented in Fig. 3 from 70 to 40°C. On the other hand, we recently observed that for 1,8-dimethylnaphthalene the retention decreases when the temperature decreases [10], whereas other dimethylnaphthalenes behave typically, *i.e.*, the retention increases when the temperature decreases. These studies were performed in the temperature range 25–70°C. The unusual behaviour of 1,8-dimethylnaphthalene can be explained by considering that an increase in temperature always decreases CD complexation. Moreover, the increase in complexation is followed by a decrease in capacity factors. The second phenomenon can be easily explained by considering the better solubility of guest–CD complexes in the mobile phase. In contrast, the degree of complexation for other dimethylnaphthalenes is much lower and therefore the increase in complexation when the temperature decreases does not influence the k' *vs.* $1/T$ plot in the temperature range studied.

Considering the experimental results described

Table 1
Separation factors (α) and resolutions (R_s) for norgestrel enantiomers at various temperatures

Column temperature (°C)	α	R_s
30	1.02	0.08
20	1.04	0.15
15	1.04	0.23
10	1.05	0.30
5	1.06	0.44
0	1.08	0.60

in both previous papers [10,15], the following suggestions on the separation mechanism of norgestrel enantiomers on a molecular level can be given. In the temperature range 70–40°C the degree of complexation with β-CD for both enantiomers is very low and therefore the typical Van 't Hoff plot in Fig. 3 is observed. The phenomenon that retention decreases with increase in temperature has been observed many times in both HPLC and GC. On a molecular level it can be easily explained by the faster migration of the solute molecules through the chromatographic column and their lower affinity to the stationary phase. With norgestrel enantiomers in this temperature range, the complexation with β-CD is not enantioselective. Below 40°C the inclusion mechanism starts to be important. As evidence, deviation from the Van 't Hoff plot is observed in Fig. 3 and also the norgestrel enantiomers start to differ in their affinity to the β-CD molecule, as exemplified in Fig. 4. Table 1 lists the separation factors α [$\alpha = k'_{(-)}/k'_{(+)}$] and resolutions defined as $R_s = [t_{R(-)} - t_{R(+)}]/2[\sigma_{(-)} + \sigma_{(+)}]$. On a molecular level, the enantioselectivity in this temperature range, can probably be interpreted as being due to slower rotation of the guest and host molecules and hence a steric fit is possible.

The results presented here also suggest that the predominant mechanism for retention is the formation of guest–CD complexes in the mobile phase.

4. Acknowledgement

This work was supported in part by grant 202429101 from the State Committee for Scientific Research.

5. References

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