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Note

Temperature programming in high-performance liquid chromatography

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Recently there has been renewed interest in temperature as one of the operating parameters in high-performance liquid chromatography (HPLC). Several reports indicate that resolution may be improved by increasing the column temperature, but we found the opposite, particularly with steroids. We have previously reported that resolution improves on cooling the column[1]. The separation of compounds which are difficult to separate at ambient temperature can be improved using either aqueous or non-aqueous mobile phases[2–4].

Temperature programming in HPLC has been largely ignored and there are few reports in the literature[5]. This paper describes the increased resolution in the separation of steroids as a result of temperature programming from ambient to subambient temperatures using both aqueous and non-aqueous mobile phases. The results indicate that an investigation of the successive lowering of the column temperature together with manipulation of the mobile phase composition may increase the ability to separate compounds difficult to resolve.

EXPERIMENTAL

All chemicals were of analytical-reagent grade and all solvents were EM Omnisolve products (EMScience, Cherry Hill, NJ, U.S.A.). Solutions of estrone (E_1), 17β-estradiol (E_2), cortisone (E), cortisol (F) and desoxycorticosterone (D) (Sigma) were prepared in methanol and kept at 4°C.

A modular liquid chromatograph equipped with an LDC pump, a Kratos Spectroflow 773 variable-wavelength detector and a Rheodyne loop injector was used. A Whatman Partisil 10 ODS-3 reversed-phase C_{18} (25 cm × 4.6 mm I.D.) column with methanol–water (65:35, v/v) as the mobile phase was used at a flow-rate of 1.5 ml/min. Acetonitrile–methanol (65:35, v/v) was used as the non-aqueous mobile phase; all conditions were the same as for the aqueous solvent system. All steroids were injected after stabilization of the column temperature. The column temperature was decreased stepwise in each experiment and samples were injected at the selected temperature after stabilization. The temperature range was ambient to −15°C for the aqueous mobile phase and ambient to −50°C for the non-aqueous mobile phase. Liquid nitrogen bled into a glass jacket was used to cool the insulated chamber in

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which the column was suspended. The absorption was recorded at 245 nm for corticoids and 280 nm for estrogens.

For temperature gradients mixtures of model steroids were injected at various stages during cooling the column from ambient to subambient temperatures The column was cooled at the rate of 5°C min^{-1} for E_1–E_2 and 3.5°C min^{-1} of E–F pairs, together with desoxycorticosterone (D). Another experiment followed the same protocol except for elevation of the column temperature, which was increased from −50°C to ambient at the rate of 1.0°C min^{-1}.

Graphs were plotted of retention time (t_R) and resolution (R_s) against temperature. The rate of decrease or increase of column temperature was calculated by using the arithmetic mean value:

$$\text{Rate of temperature decrease/or increase} = \frac{\text{column temperature (initial} - \text{final)}}{\text{retention time}}$$
$$= (T_i - T_f)/t_R$$
$$= \Delta T/t_R$$

RESULTS AND DISCUSSION

The effect of column temperature on resolution is shown in Figs. 1 and 2. It can be seen that the resolution is dependent on temperature, in addition to other HPLC parameters. The straight-lines in Figs. 1 and 2 indicate that the pairs estrone–17β-estradiol (E_1–E_2) and cortisone–cortisol (E–F) were not resolved at ambient temperature (25°C) with either the aqueous or non-aqueous mobile phase. The resolution gradually improved as the column temperature decreased and was maximal at −15°C with the aqueous mobile phase. The column back-pressure was between 1400 and 1500 p.s.i. With the non-aqueous mobile phase, initially E and F appeared as a single peak and D, which was used as an internal standard, was isolated at ambient temperature. Similar and successive improvements in resolution were observed as the temperature decreased from ambient to −50°C, at which the column back-pressure did not exceed practical limits.

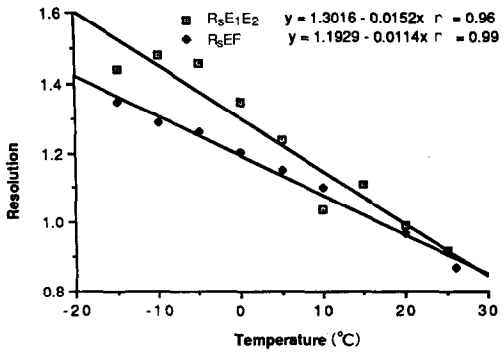

Fig. 1. Resolution–temperature curves for the aqueous mobile phase [methanol–water (65:35,v/v)]. Temperature range = ambient to −20°C. Column = Whatman Partisil 10 ODS-3 C_{18} (25 cm × 4.6 mm I.D.). R_s = Resolution. E_1 = estrone; E_2 = 17β-estradiol; E = cortisone; F = cortisol.

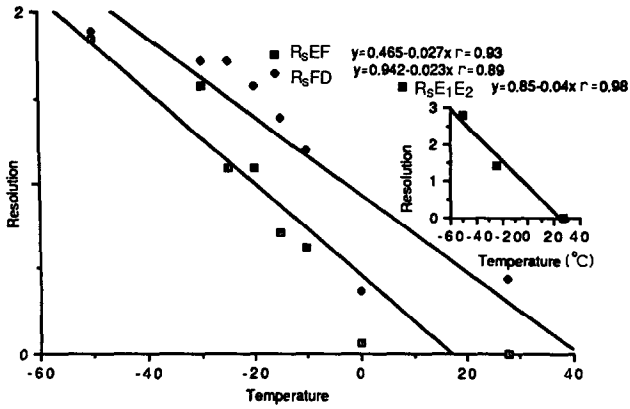

Fig. 2. Resolution–temperature curves for the non-aqueous mobile phase [acetonitrile–methanol (65:35, v/v)]. Temperature range = ambient to −50°C. D = Desoxycorticosterone. Other parameters as in Fig. 1.

With the aqueous mobile phase t_R increases were E_1 34.8, E_2 43.5, E 26, F 34.9 and D 82.9% when the column was cooled from ambient to −15°C, and with the non-aqueous mobile phase 25.28, 39.7, 15.4, 21.3 and 38.3%, respectively, from ambient to −50°C. The increase in t_R was due to an increase in the mobile phase viscosity[6,7].

The temperature gradients shown in Figs. 3 and 4 are plots of R_s and t_R against temperature from ambient to subambient to ambient. These curves indicate that the improvement in R_s increases with decrease in temperature, which is inversely proportional to the column temperature, which is the reverse of the situation in gas chromatography (GC). The situation in GC is different in that there is interaction

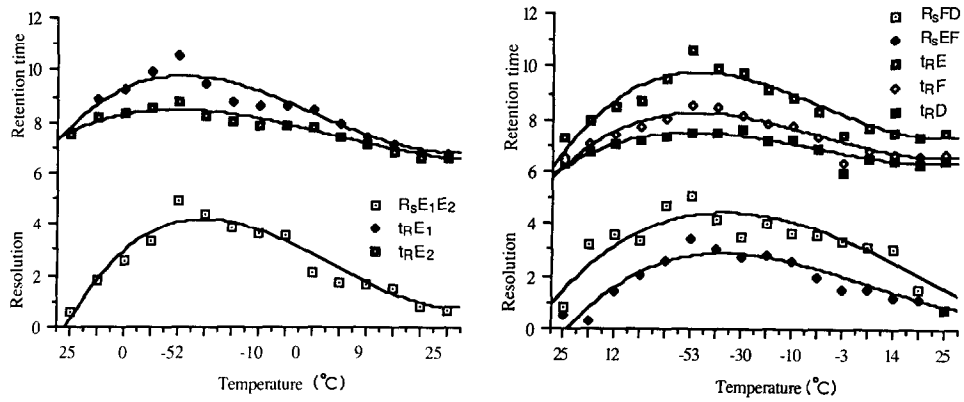

Fig. 3. Resolution, retention and temperature programming for estrogens. Temperature range = 25 to −52 to 25°C; t_R = retention = time difference between injection and appearance of specific peak during development. $R_sE_1E_2$ = resolution of E_1 and E_2. Other parameters as in Figs. 1 and 2.

Fig. 4. Resolution, retention and temperature programming for corticoids. Temperature range = 25 to −52 to 25°C. R_sEF = resolution of E and F; R_sED = resolution of E and D. Other parameters as in Fig. 2.

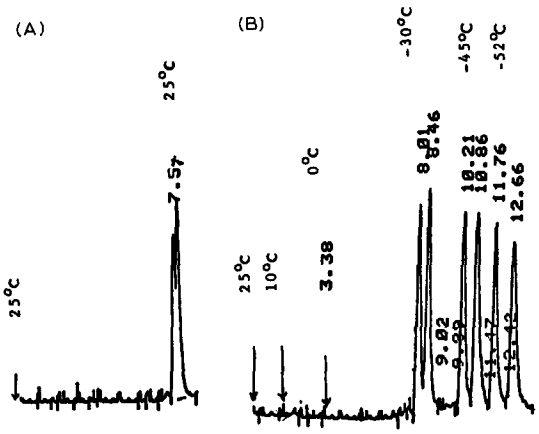

Fig. 5. Temperature programming; (A) sample injected at 25°C and chromatogram developed at 25°C; (B) steroid pair sample E_1–E_2 injected at 25, 10 and 0°C. Peaks were seen at −30, −45 and −52°C. See Fig. 3. See Fig. 1 for key to designations.

between gas and liquid phases and both the solute and mobile phase are in gas form, but in HPLC the interaction is between liquid and liquid phases.

These graphs of retention and resolution *versus* temperature confirmed that the lower the temperature the better is R_s, which reached a maximum at −15 and −50°C with the aqueous and non-aqueous mobile phase, respectively. An interesting feature was that at both ends (ambient temperature) R_s was minimal (no separation achieved).

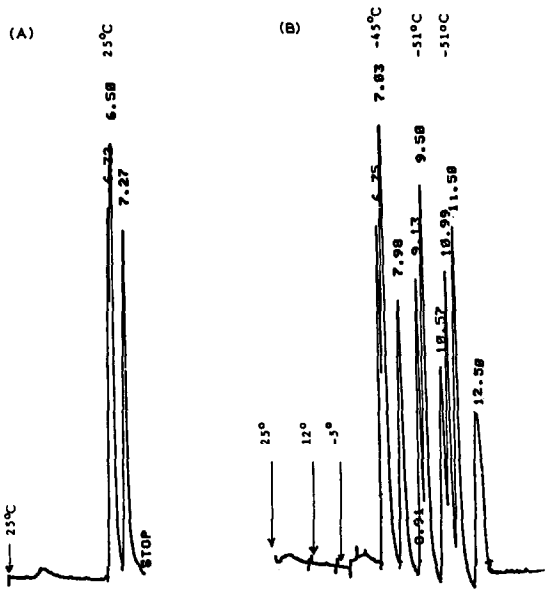

Fig. 6. Temperature programming: (A) sample injected at 25°C and chromatogram developed at 25°C; (B) steroid pair sample E–F and D injected at 25, 12 and −5°C. Peaks were seen at −45 and −51°C. See Fig. 4. See Fig. 1 for key to designations. D = Deoxycorticosterone.

The chromatograms shown in Figs. 5 and 6 summarize the effect of the temperature gradient on R_s. The sample mixture was injected in Fig. 5B at 25, 10 and 0°C. Resolved pairs of E_1–E_2 peaks were seen at −30, −45 and −52°C with t_R = 8.10, 8.46; 10.21, 10.86; and 11.76, 12.66 min. R_s was significantly improved compared with the chromatogram in Fig. 5A for ambient temperature. Similar results were obtained for the corticoid E–F–D mixture. Fig. 6A shows E and F as a shoulder at ambient temperature, but when the column temperature was programmed all components were resolved very clearly at −52°C with t_R = 10.86, 11.76 and 12.66 min, as shown in Fig. 6B.

It was observed that with increase in column temperature the resolution started to decrease and at ambient temperature the compounds were not resolved.

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