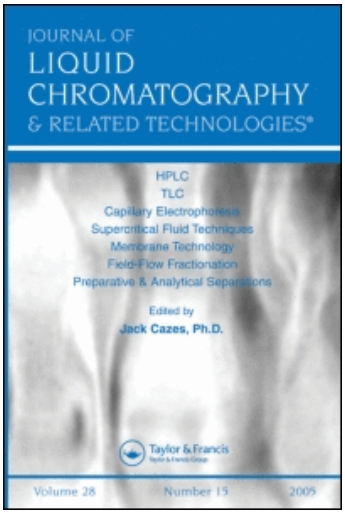


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# HPLC of Steroids in Non-aqueous Mobile Phase at Subambient Temperature

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# HPLC OF STEROIDS IN NON-AQUEOUS MOBILE PHASE AT SUBAMBIENT TEMPERATURE

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## ABSTRACT

Increased resolution of steroid mixtures was found in high performance liquid chromatography of steroids at subambient temperatures. With aqueous mobile phase using a reversed phase column it was not possible to decrease temperatures below -10°C due to increased viscosity. This report describes further increase in resolution at -50°C using a non-aqueous mobile phase for the separation of steroids. Retention times were shorter several fold while resolution was improved.

## INTRODUCTION

The effect of cooling on resolution in liquid chromatography has been reported from this laboratory (1). Other reports indicate improved resolution of some compounds at subambient temperatures (2-4). Mazzo et al (5) and Beyer et al (6) separated enantiomers at lower temperatures in HPLC, either cooling

the mobile phase or reducing the column temperature. Bishop et al (7) resolved racemic drugs at subambient temperature.

The present report is a continuation of our previously reported work showing increased separation of steroids cortisone, cortisol, desoxycorticosterone and estrone and estradiol-17β pairs at subambient temperatures with methanol - water (65 : 35 v/v) as mobile phase. A comparative study has been done with a non-aqueous mobile phase in which column temperature down to -50°C was used. It was not possible with the aqueous phase to use this temperature due to increase of column back pressure and increase of viscosity. In the theory of chromatography, constant temperature is always assumed, because the diffusion coefficients, viscosities and capacity ratios are a function of temperature (8). Snyder (9) has summarized the changes in retention as a result of elevation of temperture.



## MATERIALS AND METHODS

All chemicals were reagent grade. All chromatographic solvents including water were HPLC grade. Stock solutions of estrone (Ayerst McKenna and Harrison Ltd.), estradiol-17β (Sigma Chemical, Co), cortisone, cortisol, and desoxycorticosterone (Mann Research Laboratories, NY, NY) were dissolved in methanol and kept at 4°C.

A modular liquid chromatograph equipped with a LDC pump, a KRATOS spectroflow 773 variable wavelength detector, and a Rheodyne loop injector was used. Absorption was recorded at 245 nm and 280 nm with a mobile phase flow of 1.5 ml/min with a reversed phase HPLC (Whatman Partisil 10,ODS-3-(4.6 nm x 25 cm) column. All steroids were injected after stabilization of column temperatures. Liquid nitrogen bleeding into a glass jacket was used for cooling in an insulated chamber in which the column was suspended. Column

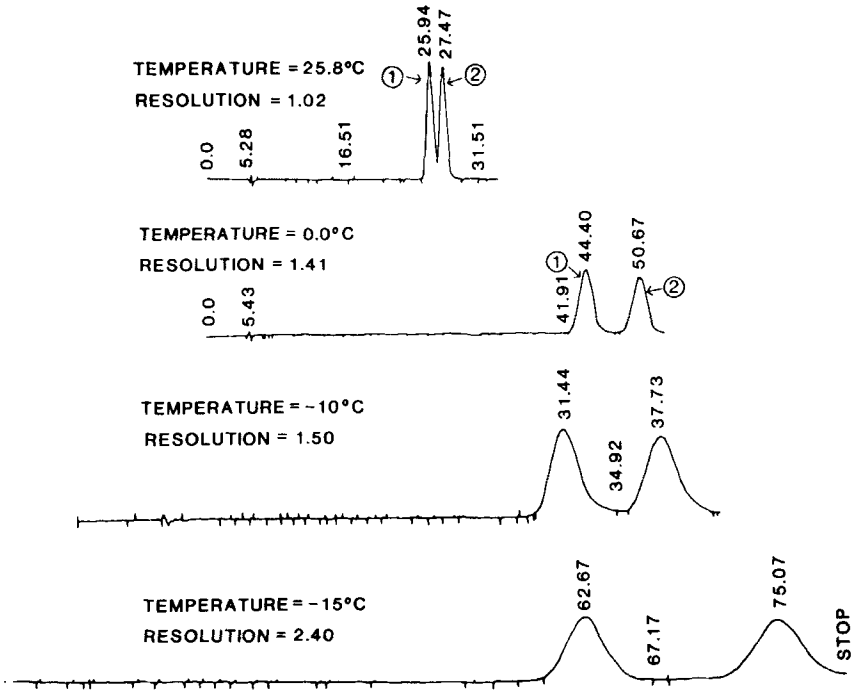


Figure 1. Separation of Estrogens at Subambient Temperatures. Mobile Phase: Methanol-Water 65:35 v/v.



temperature was ± 0.5°C from ambient to subambient temperature as assessed by a VWR digital thermo-couple. All conditions of the mobile phases and pressure were identical for flow rate (1.5 ml/min) and various temperatures for the methanol-water (65:35 v/v) and acetonitrile-methanol (65:35 v/v) systems.

## RESULTS AND DISCUSSION

The data for resolution obtained on decreasing the temperature from ambient (25°C) to -15°C for aqueous and non-aqueous phases are shown in Table 1. Table 1 indicates that both pairs were poorly resolved at ambient

Table 1

EFFECT OF SUBAMBIENT TEMPERATURES ON RESOLUTION OF STEROIDS

Mobile Phase Flow. 1.5 mL/min.

Detection. 245nm and 280 nm.

| Temperature °C | Resolution $R_sEF$ | Resolution $R_sE_1E_2$ |
|---|---|---|
| | Mobile Phase: Methanol : Water (65 : 35 v/v | |
| 25 | 1.30 | 0.95 |
| 10 | 1.56 | 1.21 |
| 0 | 1.70 | 1.41 |
| -10 | 2.14 | 1.50 |
| -15 | - | 2.40 |
| | Mobile Phase: Acetonitrile : Methanol (65 : 35 v/v) | |
| 25 & 10 | -* | -* |
| 0 | 0.64 | 0.72 |
| -10 | 0.74 | 0.91 |
| -20 | 1.02 | 1.11 |
| -30 | 1.20 | 1.90 |
| -40 | 1.32 | 2.31 |
| -50 | 1.65 | 2.80 |

* No separation.

E = Cortisone, F = Cortisol, and D = Desoxycorticosterone.

$E_1$ = Estrone and $E_2$ = Estradiol - 17β

Column: Whatman $C_{18}$ Partisil 10 ODS-3 (4.6 mm x 25 cm)

$R_s$ = Resolution.

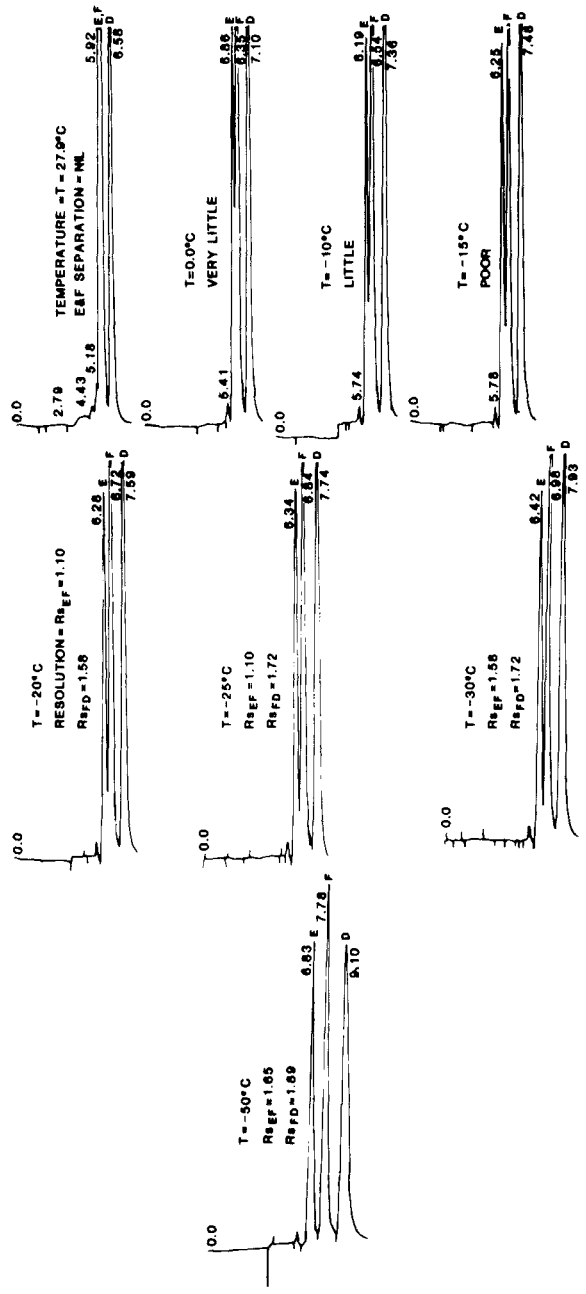


Figure 2. Separation of Corticoids at Subambient Temperatures. Mobile Phase: Acetonitrile-Methanol 65:35 v/v.

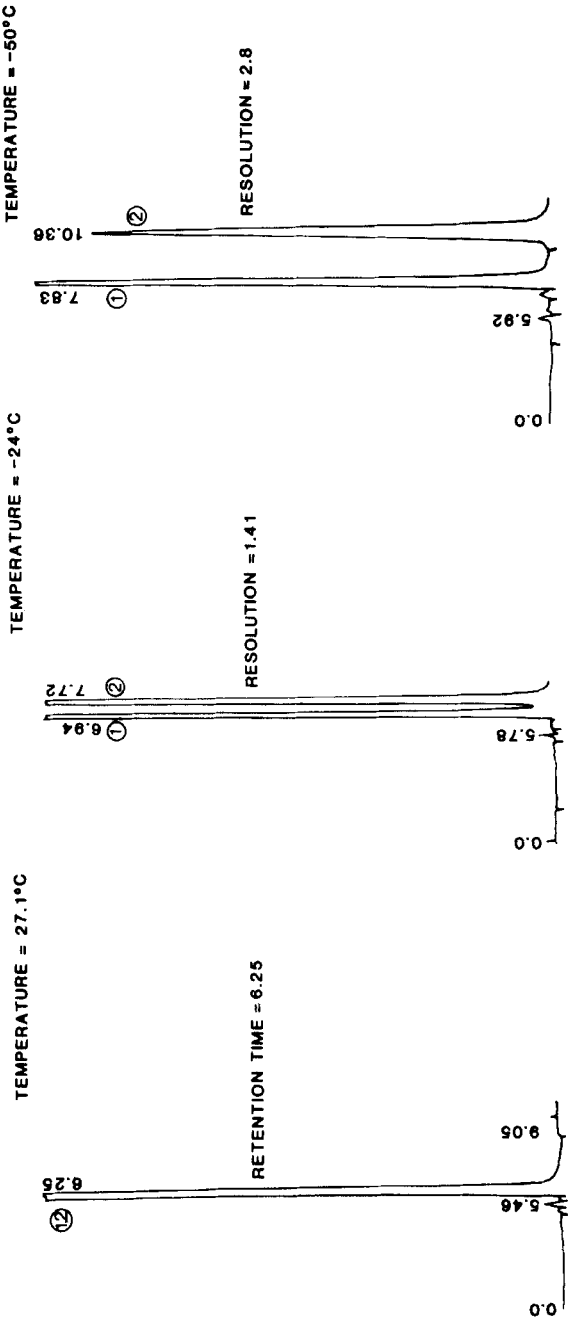


Figure 3. Separation of Estrogens at Subambient Temperatures. Mobile Phase: Acetonitrile-Methanol 65:35 v/v.

temperature with either mobile phase. Decreases of temperature with aqueous phases increased the resolution more than two fold at -15°C but further cooling caused increase of column back pressure presumably due to increase of viscosity. At subambient temperatures retention time ($t_R$) increased in all cases as shown in Figures 1-3. The resolution ($R_S$) at -10°C for cortisone, cortisol $R_S$EF was 2.14 and at -15°C for estrone, estradiol-17β, $R_sE_1E_2$ was 2.40 with $t_R$D 46.5 and $t_RE_2$ 75.07 minutes respectively.

With the non-aqueous mobile phase no separation was observed from 25°C to 10°C; E,R, and $E_1$, $E_2$ pairs showed single peak. At 0°C the beginning of a separation in both the pairs was noticed and they were completely resolved at -50°C. The resolution shown in Figures 2 and 3 indicate $R_s$EF 1.65, $R_sE_1E_2$ 2.80. The $t_R$D 9.10 and $t_RE_2$ 10.36 respectively which were several times less than with the aqueous mobile phase at -15°C.

With decrease in temperature resolution increased with both mobile phases. Values are summarized in Table 1 and the separation trend is shown in the chromatograms. The improvement of resolution is in contrast to reports that increase of column temperature improves the efficiency (9). Jinno and Hirata (2) report that a decrease in temperature improves selectivity while decreasing efficiency. Beyer et al (6) reported that resolution was reduced to zero with the increase of temperature and it was unchanged between 10°C to 65°C. These findings indicate that resolution can be enhanced by optimizing mobile phase-temperature interrelationships in HPLC. However, as Katz et al (10) point out for more accurate measurements and control of column temperature the use of a microbore column may be desirable.



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