# CAPILLARY GAS-CHROMATOGRAPHIC RETENTION BEHAVIOR AND PHYSICO-CHEMICAL PROPERTIES OF UNDERIVATIZED EQUINE ESTROGENS

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The capillary gas-chromatographic retention behavior of six underivatized equine estrogens (estrone, equilin, equilenin and their corresponding 17α-diols) on a mixed stationary phase (non-polar CP-Sil 5 CB and slightly polar CP-Sil 19 CB capillary columns coupled in series) was characterized by their Kovats retention indices and steroid numbers. In order to find whether a correlation exists between the chromatographic retention and some physico-chemical properties of the compounds, their octanol–water partition coefficients and dipole moments were measured. No straightforward correlation was observed between the physico-chemical properties and the retention behavior, which suggests that the GC separation of equine estrogens is governed by some specific electron donor–electron acceptor interactions of a chemical nature.

Estrone (*I*), equilin (*III*), equilenin (*V*), and their 17-α-diols: 17-α-estradiol (*II*), 17-α-dihydroequilin (*IV*) and 17-α-dihydroequilenin (*VI*) in the form of their sodium sulfate esters, are the main constituents of pharmaceuticals classified by USP XXII (ref.[1]) as "Conjugate Estrogens" or "Esterified Estrogens". The official USP XXII assay[1] as well as the GC methods developed for their separation and determination[2–6] involve enzymatic hydrolysis of the sodium esters and trimethylsilyl or methoxamine-trimethylsilyl or oxime-trimethylsilyl derivatization of the 3-phenolic forms of

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the compounds. Capillary GC separation and determination of underivatized equine estrogens on a mixed stationary phase has been studied recently[7].

For a quantitative structure–retention relationship (QSRR) study, the retention behavior of the compounds must be characterized by reliable retention parameters. The Kovats retention index (*I*) is the conventional retention parameter in gas chromatography. In the GC analysis of steroids, the steroid number (SN) has also proved to suit well.

The aim of this study was to find whether a correlation exists between the chromatographic retention and some physico-chemical properties of equine estrogens.

## EXPERIMENTAL

### Apparatus

The instrumentation used was as described in our previous paper[7]. The oven temperature mode was isothermal at 200, 205 and 210 °C or multi-ramp programmed at 170 °C–2 K/min–216 °C–0.1 K/min–217 °C–10 K/min–247 °C–5 K/min–255 °C.

### Chemicals and Samples

Stock solutions and standard mixtures containing equal amounts of the equine estrogens and an internal standard (mestranol, IS) were prepared as described previously[7].

A mixture of $C_{20}$ – $C_{24}$ n-alkanes (Laboratory of Synthetic Fuels, Prague Institute of Chemical Technology, Prague, The Czech Republic) and $C_{25}$ and $C_{28}$ n-alkanes (Eindhoven University of Technology, Eindhoven, The Netherlands) was used to provide reference values for determination of the Kovats indices.

5α-Androstane (5α-A) and 5α-pregnane (5α-P) were prepared by Huang–Minlou reduction of the corresponding 17-keto or 20-keto derivatives, respectively (Institute of Organic Chemistry and Biochemistry, Prague, The Czech Republic), whereas 5α-cholestane (5α-CH) was obtained from Sigma (St. Louis, U.S.A.). These steroidal hydrocarbons served as reference compounds for steroid number determination.

### Determination of Kovats Retention Indices

A standard mixture of the equine estrogens was chromatographed together with the n-alkane standards ($C_{20}$ – $C_{24}$, $C_{25}$ and $C_{28}$) at three different isothermal oven temperatures (200 °C, 205 °C and 210 °C). The Kovats retention indices were calculated on an Als-Pro Retention minicomputer (Analytical Laboratory Systems, Deidesheim, Germany).

### Determination of Steroid Numbers

A standard mixture of the equine estrogens was chromatographed together with the 5α-A, 5α-P, and 5α-CH standards at three different isothermal and one multi-ramp programmed oven temperatures. The steroid numbers were calculated by using both graphical and mathematical procedures.

Determination of Octanol–Water Partition Coefficients

One ml aliquots of stock solutions of *I*, *II*, *III*, *IV*, *V*, and *VI* were transferred separately in three test tubes, dried in a nitrogen stream, and dissolved in an octanol–water mixture. The total liquid volume was 6 ml and the octanol-to-water volume ratio was 1 : 5, 1 : 1, or 5 : 1. The samples were shaken vigorously for 10 min and centrifuged (20 min, 3 000/min). The organic and aqueous layers were transferred to separate test tubes, dried in a nitrogen stream and dissolved in 1 ml of a mestranol solution. Aliquots of 1 μl of the organic and aqueous extracts of each estrogen were GC analyzed in the multi-ramp temperature program mode.

Determination of Dipole Moments

The dipole moments (μ) of *I* and *II* were determined at 25 °C in a benzene solutions (five concentrations of each compound were prepared within the weight fraction range of 0.0002 to 0.0015) using the method of Halverstadt and Kumler[8]. The relative permittivities were measured on an instrument with direct frequency reading, designed by one of us (V. V.). The working frequency was 1.3 MHz. The densities were determined by using an Ostwald–Sprengler pycnometer. Molecular refractions were calculated based on published increments[9].

The dipole moments of all of the equine estrogens were calculated on a PC-AT 286 computer with a coprocessor by using the MM2 molecular mechanics program (version 1985) developed by Allinger[10,11].

## RESULTS AND DISCUSSION

### *Kovats Indices and Steroid Numbers*

Figure 1*a* shows a typical chromatogram of a standard mixture of equine estrogens chromatographed together with n-alkanes at a constant oven temperature of 205 °C. The calculated Kovats indices are given in Table I.

The chromatograms obtained at a constant oven temperature (205 °C) and in the multi-ramp programmed oven temperature mode are shown in Figs 1*b* and 1*c*, respectively.

W. J. A. Vanden Heuvel et al.[12] developed a graphical procedure for the determination of SN, whereas F. A. Vanden Heuvel et al.[13] suggested a mathematical equation. We calculated the SN values by means of the following equations:

$$\mathrm{SN}_i = 19 + 8\,\frac{\log r_{i,a}}{\log r_{c,a}} \qquad (1)$$

and

$$\mathrm{SN}_i = 21 + 6\,\frac{\log r_{i,p}}{\log r_{c,p}}\ , \qquad (2)$$

where $r_{x,y}$ ($r_{x,y} = t'_x/t'_y$) are the relative retention times of a steroid (subscript *i*) or 5α-CH (subscript *c*) to 5α-A (subscript *a*) or 5α-P (subscript *p*). The SN values

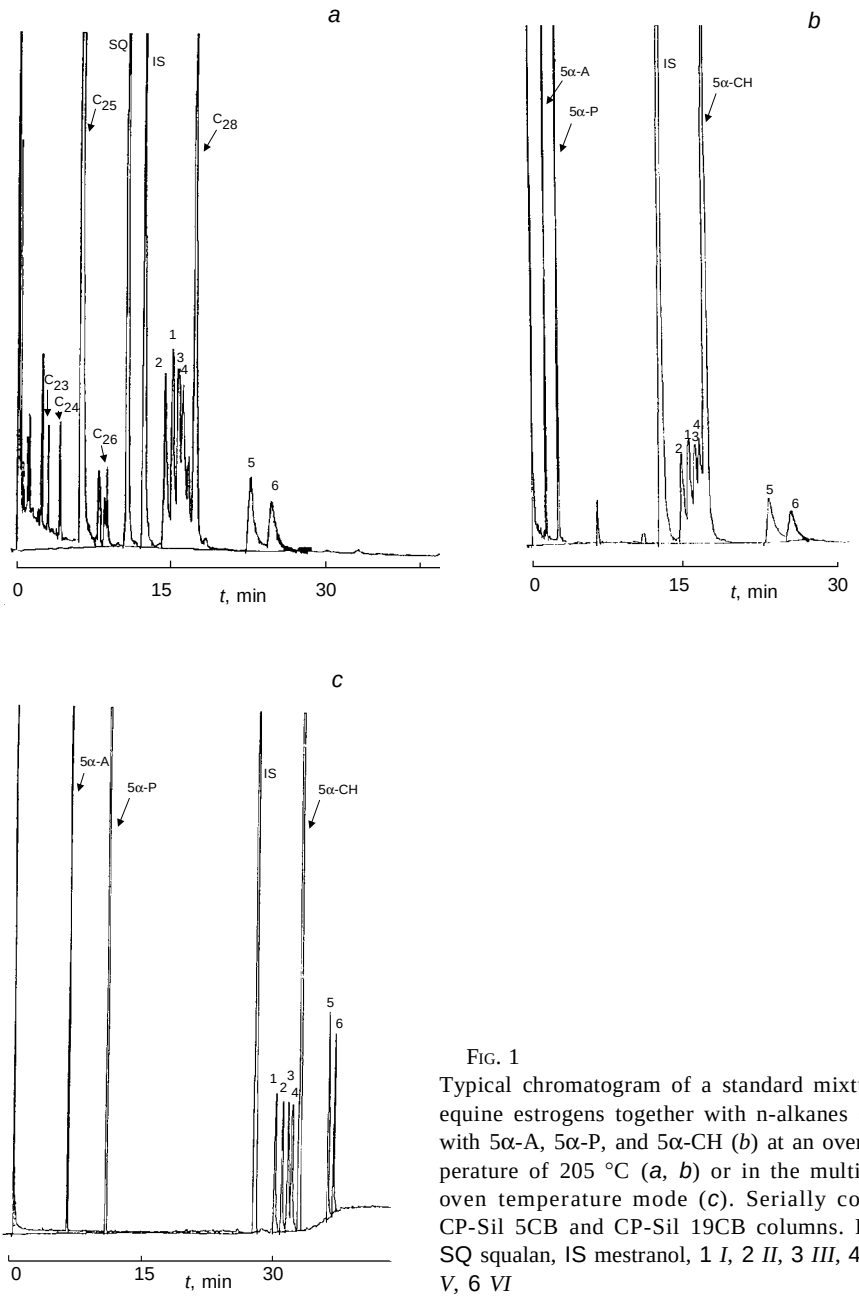


FIG. 1

Typical chromatogram of a standard mixture of equine estrogens together with n-alkanes (*a*) or with 5α-A, 5α-P, and 5α-CH (*b*) at an oven temperature of 205 °C (*a*, *b*) or in the multi-ramp oven temperature mode (*c*). Serially coupled CP-Sil 5CB and CP-Sil 19CB columns. Peaks: SQ squalan, IS mestranol, 1 *I*, 2 *II*, 3 *III*, 4 *IV*, 5 *V*, 6 *VI*

determined at 200, 205, and 210 °C and in the multi-ramp oven temperature program mode by using Eqs (*1*) and (*2*) are listed in Table II. Although the results by Vanden Heuvel et al.[14,15] suggest that the SN values are virtually temperature independent, a slight increase was observed with increasing oven temperature.

TABLE I
Kovats indices *I* of equine estrogens at three isothermal oven temperatures[a]

| Compound | *t*, °C | | |
|---|---|---|---|
| | 200 | 205 | 210 |
| *II* | 2 725.1 | 2 742.9 | 2 743.9 |
| *I* | 2 736.6 | 2 755.9 | 2 756.2 |
| *III* | 2 745.3 | 2 765.4 | 2 766.1 |
| *IV* | 2 751.6 | 2 772.7 | 2 772.5 |
| *V* | 2 837.2 | 2 863.3 | 2 862.6 |
| *VI* | 2 854.3 | 2 887.3 | 2 873.9 |

[a] Each value represents the mean of 3 measurements. The RSD values varied from 0.2 to 0.6%.

TABLE II
Steroid numbers of equine estrogens at different oven temperatures calculated by using Eqs (*1*) and (*2*)[a]

| Steroid | Equation (*1*) *t*, °C | | | | Equation (*2*) *t*, °C | | | |
|---|---|---|---|---|---|---|---|---|
| | 200 | 205 | 210 | multi-ramp | 200 | 205 | 210 | multi-ramp |
| *II* | 26.47 | 26.51 | 26.53 | 26.52 | 26.49 | 26.55 | 26.59 | 26.49 |
| *I* | 26.64 | 26.69 | 26.69 | 26.67 | 26.66 | 26.70 | 26.76 | 26.64 |
| *III* | 26.77 | 26.83 | 26.88 | 26.78 | 26.78 | 26.84 | 26.88 | 26.76 |
| *IV* | 26.87 | 26.93 | 27.00 | 26.88 | 26.88 | 26.94 | 27.00 | 26.84 |
| *V* | 28.10 | 28.14 | 28.20 | 27.47 | 28.05 | 28.09 | 28.15 | 27.50 |
| *VI* | 28.43 | 28.47 | 28.50 | 27.56 | 28.36 | 28.41 | 28.44 | 27.61 |

[a] Each value represents the mean of 3 measurements. The RSD values varied from 0.3 to 0.8%.

A linear correlation between the *I* and SN values was also observed for the equine estrogens, employing the data in Tables I and II. The linear regression parameters are given in Table III.

*Dipole Moments and Octanol–Water Partition Coefficients*

For investigating whether a correlation exists between the chromatographic data and the structures of the compounds studied, the polarity of their molecules was chosen as the physico-chemical property of interest and dipole moments were taken as the measured data. The dipole moments of all estrogens were calculated by using the MM2 molecular mechanics program (version 1985)[10,11]. The dipole moments of *I* and *II*, which constitute the representative ketone–diol pair, were also determined

TABLE III
Parameters of the linear relation $I = a + b(\mathrm{SN})$ of equine estrogens at different oven temperatures[a]

| *t*, °C | Equation (*1*) | | Equation (*2*) | |
|---|---|---|---|---|
| | *a* | *b* | *a* | *b* |
| 200 | 947.15 | 67.16 | 855.12 | 70.57 |
| 205 | 769.96 | 74.38 | 676.51 | 77.83 |
| 210 | 948.07 | 67.69 | 818.61 | 72.41 |

[a] Correlation coefficients varied from 0.9979 to 0.9999.

TABLE IV
Dipole moments (in $10^{30}$ C m) of equine estrogens calculated by molecular mechanics program and measured experimentally

| Compound | $\mu_{calc}$ | $\mu_{exp}$ |
|---|---|---|
| *I* | 7.77 | 10.07 |
| *II* | 5.34 | 6.84 |
| *III* | 9.01 | – |
| *IV* | 6.74 | – |
| *V* | 6.77 | – |
| *VI* | 4.67 | – |

experimentally. Since the structural data of the remaining four compounds are unavailable (see Cambridge Data Base), the X-ray data (atomic coordinates) of *I* (ref.[16]) and *II* (ref.[17]) were used as input data with modifications (for *III*, *IV*, *V*, and *VI*) or nonmodified (for *I* and *II*).

The calculated and observed dipole moments of the equine estrogens are given in Table IV. Significant differences in the dipole moments (i.e. polarities) are found between the hydroxy ketones and their corresponding diols, while the differences among hydroxy ketones or among diols are lower and can be accounted for by the different numbers of $C_{sp2}$–$C_{sp3}$ bonds in their molecules. The differences between the experimental and calculated dipole moments can be explained by unfitting parametrization of the MM2 program.

All of the compounds were distributed in the octanol phases, so that precise calculation of the octanol–water partition coefficients is impossible.

No straightforward correlation was found between the physico-chemical properties and the retention behavior of the equine estrogens, which suggests that some specific electron donor–electron acceptor interactions of a chemical nature play the most important role in the GC separation of the compounds under study.

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