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Study of a Set of Chromatographic Parameters for the Determination of Estrogens

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Optimum chromatographic parameters for the separation and detection of estrogen TMS derivatives are described.

Estrogens are best separated by using a 2 m glass column with 1 % SE-30 load; an 8′ iso temperature program, 230° C, 3° C/min, 296° C; 24 ml/min nitrogen flow; 33 ml/min hydrogen flow and 1.5 Kg/cm² air pressure.

Gas-liquid chromatograhy is one of the analytical techniques employed to determine the estrogens content of biological fluids for clinical purposes.

Methods have already been described to determine estrogens in urine from non pregnant (6) and pregnant women (1-3, 7, 8, 10).

In most of these methods, the technical procedures used for hydrolysis of the glucoronyl derivatives and subsequent extraction of the free estrogens with organic solvents, has been described in detail. Further, in some methods a purification is done before the sample is chromatographed. In contrast, very small attention has been devoted to the optimization of the chromatographic parameters that constitute the last step of the analytical procedure.

This paper describes a set of chromatographyc parameters that optimize the analysis of the estrogens, as trimethylsilyl derivatives, when using a column loaded with 1 % SE-30 as stationary phase.

Materials and Methods

Chemicals. All chemicals were of spectro grade and were purchased from commercial sources.

Gas chromatography. Gas-liquid chromatography was carried out with a Carlo Erba chromatograph equipped with a flame ionization detector. For silylation of pure steroids used as standards 50 µl of the silylating reagent; pyridine: bistrimethylsilylacetamide: trimethyl chlorosilane (6:10:1), were added to the dried residue and incubated at 60° C for 1 hour as described by CHAMBAZ and HORNING (4).

Two glass columns (2 m) with internal diameters of 45 mm were used to evaluate

a temperature programme according to DABRIO (5). Stationary phase was either 3 % OV-225 or 1 % SE-30, both on silanysed Gas Chrom P (mesh 80-100).

The nitrogen and hydrogen flows that produce the optimum response in the detector were calculated according to MC WILLIAM (9).

Results and Discussion

Evaluation of columns and temperature programmes. In order to find the optimum conditions in which the main estrogens present in urine samples could be separated, two different columns were injected with 3.6 μg of trimethylsilyl (TMS) derivatives of pure estrogens and steroids. Each derivative was run at four different temperatures, which were kept constant. The retention time (RT) for each one was then determined.

As it can be seen in figure 1, a linear relation between the log RT for each compound and temperature, was observed in both columns.

Moreover, with the data presented in figure 1, the optimum temperature and stationary phase to completely separate the estrogens was predicted.

The 1 % SE-30 stationary phase and a 8′ iso., 230° C, 3° C/min 296° C temperature programme, produced the best separations (fig. 2a).

However, if a shorter analysis is required, another temperature programme can be used (5′ iso., 250° C, 3° C/min 280° C) (fig. 2b). Although it permits an adequate separation of the estrogens, it is not as good as the first one.

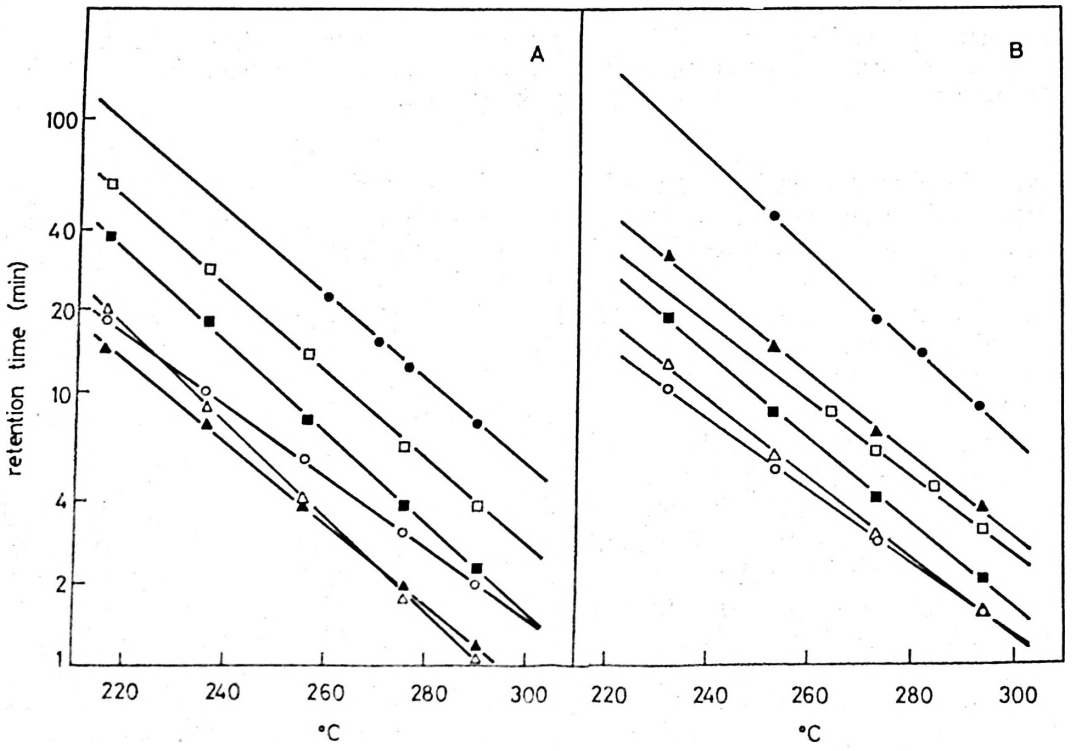

Fig. 1. *Retention time of estrogens at different temperatures.*
3.6 μg of TMS derivatives of pregnandiol ○; cholesterol n-butyrate ●; cholestanone □; estriol ■; estradiol △ or estrone ▲, were injected into columns loaded with A: 1 % SE-30, or B: 3 % OV-225, and the RT for each compound was measured at different temperatures.

An advantage of these temperature programmes, is that it allows the separation of pregnandiol from estrogens. Although using selective procedures for extracting estrogens from urine, pregnandiol is always present in sizeable amounts in urine extract (specially from urine extract of pregnant women).

Choice of nitrogen and hydrogen flow. Variable carrier gas and hydrogen flows can produce different responses in the detector as described by TRANCHANT (11). To find the optimum response, 3,6 μg of strone-TMS were injected at both 12 ml/min and 24 ml/min of nitrogen, and the hydrogen flow was changed. Air pressure was kept constant at 1.5 Kg/cm^2 in all cases. A nitrogen flow of 24 ml/min with a 33 ml/min hydrogen flow produced the maximum response in the detector (figure 3).

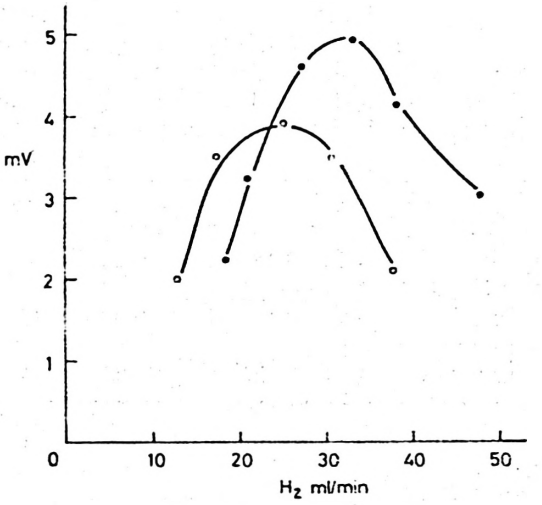

Fig. 3. *Detector response at variable hydrogen and nitrogen flow.*
3,6 μg of estrone-TMS was injected into a column loaded with 1 % SE-30 at 12 ml/min (○), and 24 ml/min (●) of nitrogen flow.

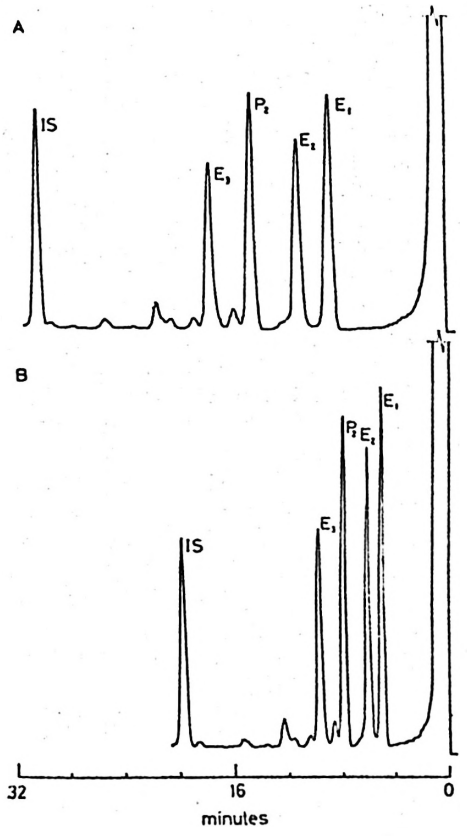

Fig. 2. *Gas chromatograms of a mixture of estrogens.*
4.8 μg of TMS derivatives of estrone, E_1; estradiol, E_2; pregnandiol, P; estradiol, E_3 and cholesterol n-butyrate, IS, on 1 % SE-30 ran at A: 8′ iso., 230° C, 3° C/min 296° C or B: 5′ iso., 250° C, 3° C/min 280° C.

Proportionally coefficient. As the resultant peak for different compounds can differ depending on the chemical nature of the compound (with equal quantities of each), it is desirable to correlate the peak obtained for a given amount of compound with that resulting from a known quantity of internal standard (IS) that will be kept constant within a set of experiments. An aliquot of 2.5 μl of mixture containing 2 μg/μl of estrone, estradiol, estriol and internal standard was chromatographed (table I). The proportionally coefficient for each estrogen is given by the ratio:

$$K = \frac{\text{estrogen area}}{\text{internal standard area}}$$

Table I. *Linearity and proportionally coefficients for estrogens.*
2.5 μl of a mixture containing 2 μg/μl of estrogens and internal standard as TMS derivatives were injected in a 1 % SE-30 column and ran at 8' iso., 230° C, 3° C/min 296° C with a nitrogen flow 24 ml/min and hydrogen flow 33 ml/min. The average of several determinations is given followed by the standard deviation. Number of determinations in each case is 5, except for K, that is 18.

	3,800 ng	1,600 ng	720 ng	290 ng	82 ng	r	k
Estrone	4.36±0.18	1.60±0.08	0.79±0.11	0.32±0.02	0.11±0.01	0.9982	1.24±0.07
Estradiol	4.33±0.07	1.79±0.12	0.45±0.17	0.35±0.008	0.11±0.01	0.9994	1.36±0.10
Estriol	3.48±0.12	1.56±0.02	0.86±0.08	0.34±0.008	0.09±0.01	0.9985	1.11±0.09
Cholesterol-n-butyrate	3.39±0.12	1.50±0.05	0.54±0.02	0.33±0.01	0.09±0.01	0.9986	1.00±0.00

r) Correlation coefficient; k) Proportionality coefficient as k = estrogen area/internal standard area.

Linearity. A linear relation between the injected quantity of a given estrogen and the peak area, corrected for K, obtained in a chromatogram was observed for values ranging from 82 ng to 3,800 ng (table I).

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Resumen

Se describen los parámetros cromatográficos óptimos que permiten la separación y detección de los derivados TMS de estrógenos.

Se evalúan dos columnas cromatográficas, OV-225, al 3 % y SE-30 al 1 % sobre Gas Chrom P (80-100 mallas), obteniéndose las mejores separaciones cuando se usa la fase estacionaria SE-30 1 % y un programa de temperatura de 8 minutos isoterma 230° C, incremento de 3° C/min hasta temperatura final de 296° C. La máxima respuesta del detector en estas condiciones se obtiene con un flujo de nitrógeno de 24 ml/min, flujo de hidrógeno de 33 ml/min y una presión de aire constante de 1,5 Kg/cm^2.

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