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Computer-assisted optimization of the gas chromatographic separation of equine estrogens

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ABSTRACT

The pharmaceutically important conjugated estrogens of the type excreted by pregnant mares were baseline-resolved by gas chromatography (GC) on a SE-30 fused-silica open tubular column after acid hydrolysis and conversion to their *tert.*-butyldimethylsilyl derivatives. The temperature-programmed conditions were optimized with the aid of DryLab GC software with excellent agreement between the predicted and experimental results. The composition of conjugated estrogens in Premarin tablets is described as an application of the method.

INTRODUCTION

The estrogens are steroidal hormones responsible for the development and maintenance of the female sexual organs. Synthetic estrogens, or more commonly conjugated estrogens derived from pregnant mares' urine, are used in estrogen replacement therapy to treat symptoms of menopause, in postmenopausal women to help prevent bone loss due to osteoporosis, for some treatments of the advanced stages of breast and prostate cancer, and to control uterine bleeding due to a hormonal imbalance [1,2].

Pharmaceutical estrogenic preparations consist mainly of conjugated and esterified estrogens of the type excreted by pregnant mares. As defined in the US Pharmacopeia conjugated estrogens tablets contain not less than 73% and not more than 95% of the labeled amount of conjugated estrogens as the total of sodium estrone sulfate and sodium equilin sulfate [3]. The ratio

of sodium equilin sulfate to sodium estrone sulfate in the tablets is not less than 0.35 and not more than 0.65. As well as the sulfate conjugates of estrone and equilin, various, although lesser, amounts of the conjugates of equilenin, 17 α -estradiol and 17 α -dihydroequilin are usually present, together with minor concentrations of 17 β -estradiol, 17 β -dihydroequilin, 17 α - and 17 β -dihydroequilenin and estriol. Average concentrations of individual estrogens from natural sources vary widely owing to individual biological variations between animals and significant changes in composition associated with the timing of the uterus cycle. In February 1990, the United States Food and Drug Administration proposed withdrawing marketing applications for generic conjugated estrogen products because of compositional differences from registered drugs which might affect their safety and effectiveness [1]. For these reasons an effective and economic analytical method is required to monitor the composition of estrogenic products used for pharmaceutical applications.

The naturally occurring estrogens likely to be

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present in pharmaceutical products all possess an estrane skeleton with aromatization of the A ring and a phenolic hydroxy group at C-3 (Fig. 1). Individual estrogens differ primarily by the position and/or number of double bonds in the B ring, by the presence of a ketone or hydroxyl group at the C-17 position, by the absolute conformation of the hydroxyl group at C-17, and, in the case of estriol, by the presence of an α -hydroxyl group at the C-16 position.

Existing methods of analysis of estrogens in pharmaceutical products include colorimetric, fluorescence, and infrared techniques; these can be criticized for their lack of specificity [3,4]. Chromatographic methods have emerged as the preferred choice when identification and quantitation is important. Thin-layer chromatographic methods are the most economical for routine screening procedures [5,6] while column liquid chromatographic methods have emerged as the

preferred technique for the separation of the intact conjugates [7,8]. The baseline resolution of the free estrogens by column liquid chromatography requires critical separation conditions or chemical manipulation of the sample which limits its routine use [9–12]. Zweig *et al.* [13] and Pillai and McErlane [14] obtained partial separation of the equine estrogens as their trimethylsilyl derivatives on glass open tubular columns coated with cyanosilicone stationary phases. A complete separation of all ten equine estrogens as their oxime-trimethylsilyl derivatives [13] and trimethylsilyl derivatives [15] was obtained on open tubular columns coated with cyanosilicone and dimethylsilicone stationary phases, respectively. Robinson *et al.* [16] reported the determination of plasma levels of estrone and equilin by open tubular column gas chromatography (GC) and negative-ion chemical ionization mass spectrometry of their folphemesyl derivatives in volunteers who had taken conjugated estrogens tablets. In this report we propose an optimized separation of the equine estrogens by temperature-programmed open tubular column GC employing a computer simulation package, DryLab GC, to assist in establishing the optimum separation conditions.

The DryLab GC software assumes that band migration in temperature-programmed GC can be approximated as the sum of a series of (small) isothermal steps, each successive step being carried out at a slightly higher temperature [17–19]. The resulting integral equation, which has no explicit solution, is solved approximately by use of the so-called linear-elution-strength approximation. Band widths are calculated in a similar fashion assuming that the column dead time and plate count are temperature-independent. In spite of what might seem rather crude approximations good agreement between predicted and observed separations have been found [20–22]. It is not our intention to describe the theoretical basis of the model here or operation of the PC-based software, for that information the original references should be consulted [17–19]. In practice DryLab GC requires as input the retention times from two experimental linear temperature-programmed separations performed over the

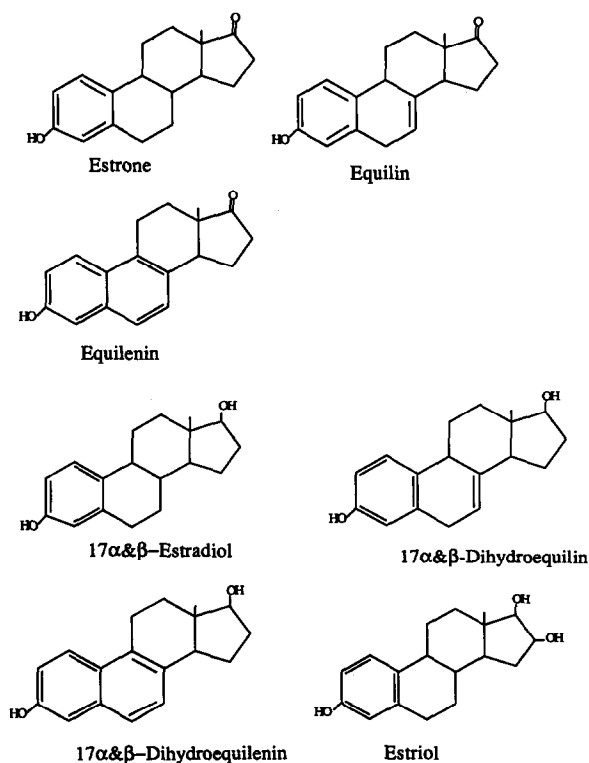


Fig. 1. Structures of the principal estrogens excreted in pregnant mares' urine.

same temperature range with two different program rates. (Ideally the program rates should differ by at least a factor of 3.) Computer simulations can then be employed to find either the optimum isothermal temperature for the separation or to optimize the initial and final temperatures, program rate, and shape of a multi-ramp program for the separation. Since computer simulations can be performed in a small fraction of the time required for typical experimental separations it enables optimum separation conditions to be identified much more rapidly than experimental trial-and-error procedures that are normally used. This presupposes that the results predicted by computer simulation are sufficiently reliable to qualitatively or quantitatively predict the results obtained by experiment. The second purpose of this report was to evaluate the reliability of computer simulation techniques in aiding the optimization of the separation of equine estrogens by temperature-programmed GC.

EXPERIMENTAL

Estrone, equilin, equilenin, 17α -estradiol, 17β -estradiol, and estriol were obtained from Sigma (St. Louis, MO, USA). 17α -Dihydroequilin, 17β -dihydroequilin, 17α -dihydroequilenin, and 17β -dihydroequilenin were a gift from Wyeth-Ayerst Research (Princeton, NJ, USA). Premarin tablets, containing 2.5 mg of conjugated estrogens per tablet, were obtained from a local pharmacy. *tert*-Butyldimethylchlorosilane was obtained from Hüls America (Piscataway, NJ, USA) and methoxylamine hydrochloride and bis(trimethylsilyl)acetamide from Aldrich (Milwaukee, WI, USA). All solvents were Omnisolv grade from EM Science (Gibbstown, NJ, USA); other chemicals were obtained from a variety of sources in the highest purity available.

Sample preparation

The sugar coating layer of the conjugated estrogen tablets was carefully removed with a moist paper towel down to the shellac layer and the tablets dried on filter papers [3]. Twenty tablets were weighed and finely powered in a mortar and

pestle to pass a 60-mesh sieve. An amount of powder containing 25 mg of conjugated estrogens was mixed with 20 ml of methanol, sonicated, centrifuged, and the methanol layer was collected.

To a round-bottom flask fitted with a reflux condenser were added the methanol extract, deionized and distilled water (20 ml), concentrated hydrochloric acid (4 ml), and a few boiling chips. The mixture was then heated under reflux for 15 min and allowed to cool to room temperature.

After acid hydrolysis the free estrogens were extracted with chloroform (2×10 ml and 1×5 ml) and the chloroform extracts combined, washed with water (5 ml), and passed through a short column of anhydrous sodium sulfate. The chloroform was removed on a rotary evaporator and the residue dissolved in acetonitrile and made up to 25 ml. The standard stock solution was divided into 5-ml vials and stored in a refrigerator at 4°C until used.

Preparation of derivatives (refs. 23–25)

Trimethylsilyl derivatives. An amount of stock solution equivalent to 0.5 mg of conjugated estrogen extract or estrogen standards was added to a 1.0-ml Reacti-Vial (Pierce, Rockford, IL, USA) and evaporated to a residue with a stream of nitrogen. To the residue were added 500 μ l of bis(trimethylsilyl)acetamide and the mixture was heated in a block heater at 70°C for 3 h.

Trimethylsilyl-methoxime derivatives. An amount of stock solution equivalent to 1.0 mg of conjugated estrogen extract or estrogen standards was added to a 5.0-ml Reacti-Vial and evaporated to a residue with a stream of nitrogen. To the residue were added 2.0 ml of anhydrous pyridine containing 5.0 mg of methoxylamine hydrochloride. The solution was allowed to stand overnight, evaporated to dryness under nitrogen, and the residue partitioned between ethyl acetate (2.0 ml) and 10% (w/v) aqueous sodium chloride containing 5% (v/v) hydrochloric acid (2.0 ml). The ethyl acetate layer was washed with 10% (w/v) sodium chloride solution containing 5% (w/v) sodium bicarbonate. The ethyl acetate

layer was dried over molecular sieves and evaporated to dryness under nitrogen. The residue was dissolved in acetonitrile (2.0 ml) for GC. Trimethylsilyl derivatives were prepared by dissolving the residue from the methoxime reaction in 1.0 ml of bis(trimethylsilyl)acetamide and heating the solution at 70°C for 3 h.

tert.-Butyldimethylsilyl derivatives. An amount of stock solution equivalent to 2.0 mg of conjugated estrogen extract or estrogen standards was added to a 5.0-ml Reacti-Vial and evaporated to a residue with a stream of nitrogen. To the residue were added 2.0 ml of dimethylformamide containing 2.5 mg of imidazole and 3.5 mg of *tert.*-butyldimethylsilyl chloride and the mixture was heated in a block heater at 60°C for 1 h. The solution was cooled to room temperature, diluted with an equal volume of methyl *tert.*-butyl ether and washed with water (2 × 1.0 ml). The ether layer was passed through a short column containing anhydrous sodium sulfate and evaporated to a residue with a stream of nitrogen. The residue was dissolved in acetonitrile (1.0 ml) for GC.

Equipment

Separations were performed on a Siemens Si-Chromat-2 gas chromatograph from ES Industries (Berlin, NJ, USA) fitted with a split injector and flame ionization detector. The column was a 15 m × 0.25 mm I.D. fused-silica open tubular column coated with a 0.25- μ m film of SE-30 from Alltech (Deerfield, IL, USA). The carrier gas was hydrogen at a flow-rate of 1.1 ml/min. The injector and detector temperatures were maintained at 300°C.

Computer simulations of temperature-programmed separations were performed on an Epson Apex 200 computer using the DryLab GC software from LC Resources (Lafayette, CA, USA).

RESULTS AND DISCUSSION

Pharmaceutical estrogenic preparations consist mainly of conjugated estrogens of the type excreted by pregnant mares. Before analysis it is

necessary to hydrolyse the conjugates which are otherwise too involatile or unstable for analysis by GC. The free estrogens are poorly resolved on the SE-30 column. After optimization of the separation conditions the ketone-containing estrogens (estrone, equilin and equilenin) are incompletely resolved and the 17 α - and 17 β -hydroxyl isomers of dihydroequilin, dihydroequilenin and estradiol co-elute. Derivatization techniques can be used to enhance the separation between the various estrogens through chemical reactions at the ketone and hydroxyl groups.

Derivatization of the phenolic group at C-3 and the hydroxyl groups at C-17 (also C-16 in the case of estriol) of the estrogens by bis(trimethylsilyl)acetamide improves the resolution and peak shapes for all estrogens (Fig. 2). Baseline resolution is obtained for all compounds except for estrone and equilin, which are nearly resolved to baseline. The separation of estrone and equilin, the major components in estrogenic preparations, is inadequate for reliable quantitation at the high column loadings that exist when it is desired to simultaneously determine the other estrogens present as minor components. The individual estrogens are also bunched into a narrow region of the chromatogram increasing the probability of interferences from other substances that may be found in estrogenic preparations. Anticipated losses in column performance as the column deteriorates over time with use would quickly render the separation of certain isomers inadequate, and the separation, therefore, lacks the ruggedness required for routine applications.

Since the temperature program was optimized for the separation of the trimethylsilyl derivatives improvements in resolution can be obtained only by optimizing stationary phase selectivity (choose another column type), by increasing the column length (but this will result in a significant increase in the separation time), or by selecting a different derivative with more favorable separation characteristics in the chromatographic system used to separate the trimethylsilyl derivatives. We chose the last alternative as the more flexible and simpler choice.

Methoxime derivatives were formed in the

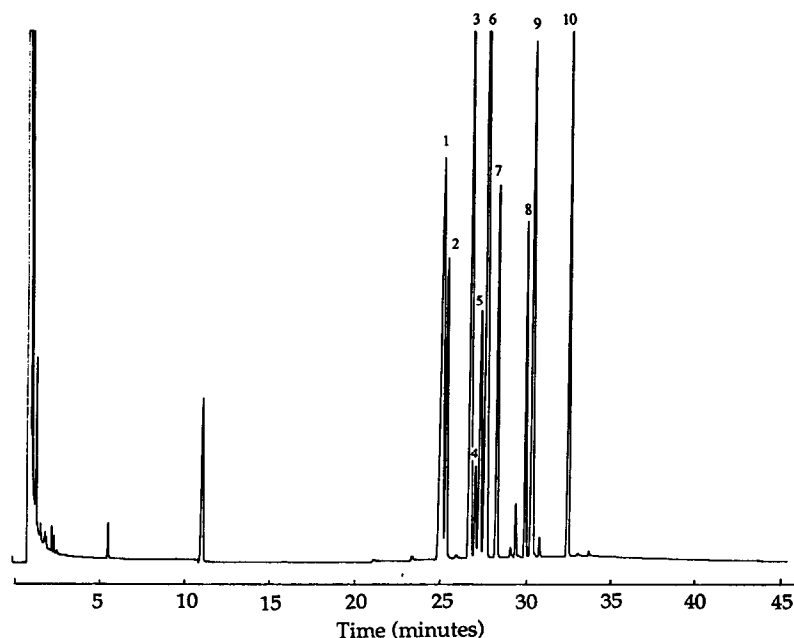


Fig. 2. Separation of the trimethylsilyl derivatives of a mixture of standard estrogens (4–10 ng of each standard on column). Peaks: 1 = estrone; 2 = equilin; 3 = equilenin; 4 = 17α -estradiol; 5 = 17α -dihydroequilin; 6 = 17β -estradiol; 7 = 17β -dihydroequilin; 8 = 17α -dihydroequilenin; 9 = 17β -dihydroequilenin; 10 = estriol. Temperature program: 200°C for 18 min then programmed at $5^{\circ}\text{C}/\text{min}$.

hope of improving the separation between estrone and equilin and equilenin and 17α -estradiol. The methoxime derivatives increase the retention of estrone and equilin but do not significantly improve the resolution of these estrogens. A double derivatization to form methoxime-trimethylsilyl derivatives is required if the other estrogens in conjugated estrogenic preparations are to be separated. Compared to the separation of estrogens in Fig. 2, under optimized separation conditions the methoxime-trimethylsilyl derivatives of estrone and equilin are moved closer in retention time to the other estrogens without being better resolved from each other, and the derivatives of equilenin and 17α -dihydroequilenin are no longer baseline-resolved. Formation of methoxime derivatives does not improve the separation of the ketone-containing estrogens on the SE-30 column used in these studies.

The formation of the trimethylsilyl derivatives was helpful in differentiating between the hydroxyl isomers at the C-17 position of the estrogen skeleton. Formation of the bulkier *tert*-bu-

tyldimethylsilyl derivatives further improved the resolution of the various C-17 hydroxyl isomers without deteriorating the selectivity for the separation of estrone and equilin (Fig. 3). All ten estrogens are baseline-resolved with the resolution between estrone and equilin depending on the sample loading. The resolution of estrone and equilin is sufficient for reliable quantitation in the simultaneous determination of the ten principal estrogen components found in pharmaceutical conjugated estrogens products.

The separation of the *tert*-butyldimethylsilyl derivatives of the estrogens in a hydrolysed extract from Premarin tablets is shown in Fig. 4. Adequate separation of the individual estrogens from each other and minor unidentified sample components is obtained except for 17β -estradiol, which is only partly resolved from an unknown sample component, and therefore is quantified with less certainty than the other identified estrogens. Since 17β -estradiol is itself a minor component in Premarin tablets this is not a serious limitation of the method for the assay of tablet formulations.

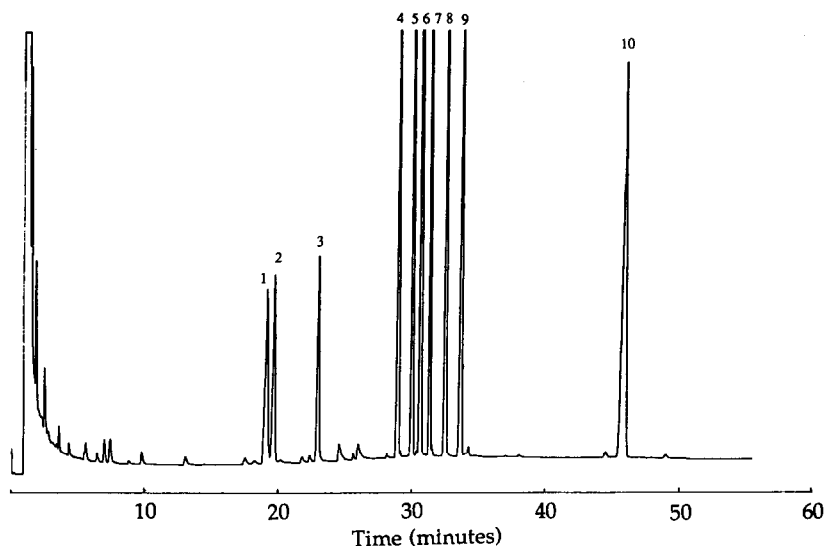


Fig. 3. Separation of the *tert.*-butyldimethylsilyl derivatives of a mixture of standard estrogens (4–10 ng of each standard on column). Peaks: 1 = estrone; 2 = equilin; 3 = equilenin; 4 = 17α -estradiol; 5 = 17α -dihydroequilin; 6 = 17β -estradiol; 7 = 17β -dihydroequilin; 8 = 17α -dihydroequilenin; 9 = 17β -dihydroequilenin; 10 = estriol. Temperature program: 235°C for 22 min then programmed at $5^{\circ}\text{C}/\text{min}$ to 290°C .

The amounts of the ten estrogens identified in Premarin tablets are summarized in Table I. The major components are estrone and equilin, which account for 89.1% of the total weight of the ten estrogens. Of the C-17 hydroxyl isomers, the estrogens with a 17α -hydroxyl group predominate,

but only account for 5.4% of the total weight of the identified estrogens. The estrogens exist primarily in the conjugated form, as demonstrated by the difference in the amount of estrogens found before and after acid hydrolysis (see Table I). The amount of estrogens as the total of sodi-

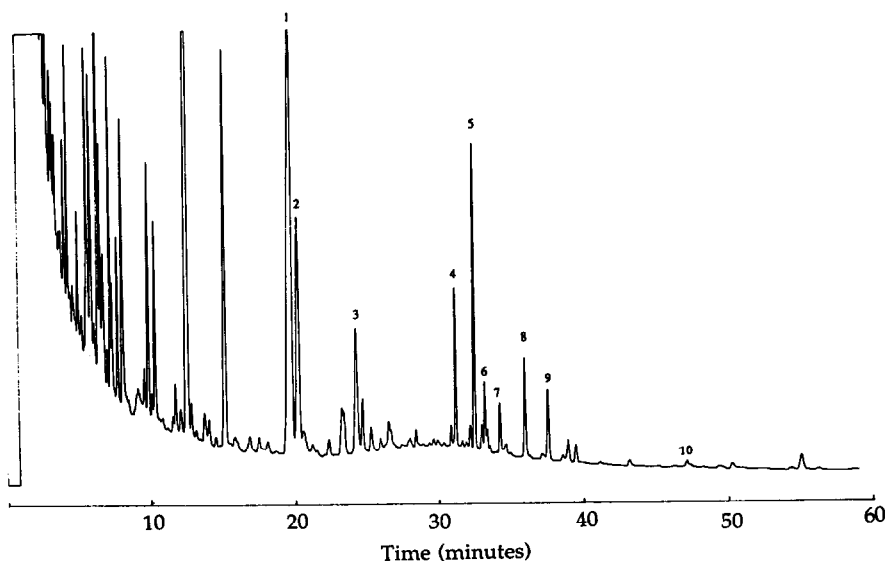


Fig. 4. Separation of *tert.*-butyldimethylsilyl derivatives of a hydrolysed extract from Premarin tablets. The identification of estrogens is the same as in Fig. 3. Temperature program: 235°C for 19 min then programmed at $5^{\circ}\text{C}/\text{min}$ to 290°C .

TABLE I
COMPOSITION OF PREMARIN TABLETS CONTAINING
2.5 mg OF CONJUGATED ESTROGENS

Estrogenic substance	Amount present after hydrolysis (mg)	Relative percentage
Estrone	1.043	65.6
Equilin	0.374	23.5
Equilenin	0.062	3.9
17 α -Estradiol	0.063	4.0
17 α -Dihydroequilin	0.018	1.1
17 α -Dihydroequilenin	0.004	0.3
17 β -Estradiol	0.013	0.8
17 β -Dihydroequilin	0.002	0.1
17 β -Dihydroequilenin	0.001	<0.1
Estriol	0.0007	<0.1

um estrone sulfate and sodium equilin sulfate, 89.7%, and the ratio of sodium equilin sulfate to sodium estrone sulfate, 0.36, conform to the specifications stated in the US Pharmacopeia for conjugated estrogens tablets [1].

Computer simulations using the DryLab GC software were used to aid the optimization of the temperature program conditions reported in these studies. The required input data are the retention times for the identified peaks obtained in two temperature-programmed separations with significantly different program rates, peak areas, asymmetry factors and the column plate count to simulate the appearance of the experimental chromatogram under different sets of experimen-

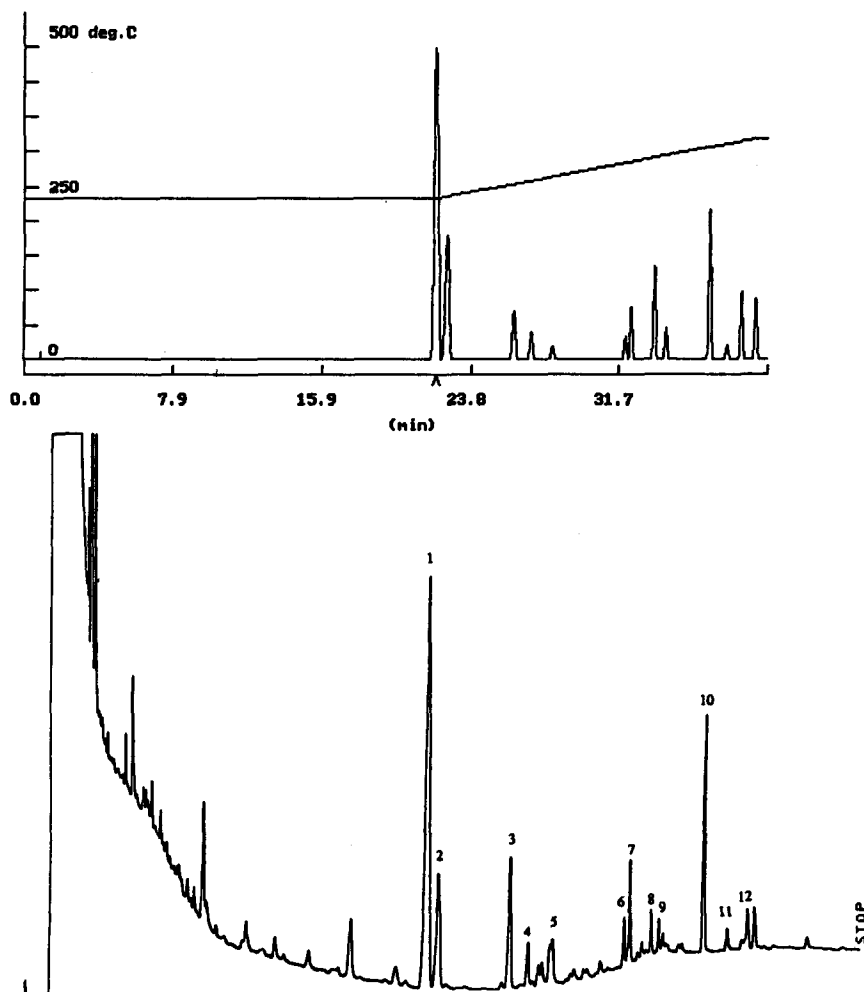


Fig. 5. Comparison of a computer-simulated chromatogram (top) with the equivalent experimental results (bottom) for a separation of the *tert.*-butyldimethylsilyl derivatives of a hydrolysed extract from Premarin tablets. The input data for the simulation is given in Table II.

TABLE II

COMPARISON OF THE PREDICTED AND EXPERIMENTAL RETENTION TIMES FOR THE SEPARATION OF *tert*-BUTYLDIMETHYLSILYL DERIVATIVES OF ESTROGENS IN PREMARIN TABLETS

Initial program runs used to generate the data for computer simulations: (1) 225°C, 0 min, to 280°C at 1°C/min; (2) 225°C, 0 min, to 280°C at 3°C/min. Program: 235°C for 22 min then 5°C/min to 320°C.

Substance	Retention time (min)	
	Predicted	Experimental
Estrone	21.9	21.5
Equilin	22.5	22.3
Equilenin	26.1	26.1
Unknown 1	27.0	27.0
Unknown 2	28.2	28.4
17 α -Estradiol	32.1	32.2
17 α -Dihydroequilin	32.4	32.4
17 β -Estradiol	33.6	33.6
17 β -Dihydroequilin	34.2	34.2
17 α -Dihydroequilenin	36.6	36.5
Unknown 3	37.5	37.5
17 β -Dihydroequilenin	38.3	38.4
Unknown 4	39.0	39.4

tal conditions. In all cases for the derivatized estrogens it was observed that the separation of estrone and equilin to baseline was the critical step which could best be achieved by an initial isothermal period at the start of the program. Conversely, estriol was always well separated from the other estrogens and mainly influenced the separation time. There was an optimum program rate that maximized the band spacing for the other estrogens with a C-17 hydroxyl group. We used the DryLab GC software to identify the optimum multi-ramp program for the separations as well as trial and error procedures to test whether a superior separation could be obtained away from those conditions predicted by computer simulation. The latter proved not to be the case, and given the speed with which simulations can be performed compared to actual experimental runs, computer simulations are a considerable help in optimizing temperature-programmed separations by GC.

An example of a computer simulation of a separation of estrogens (as *tert*-butyldimethylsilyl derivatives) and comparison to an experimental run where the two initial temperature programs used as input are substantially different to the conditions established as optimum is shown in Fig. 5. The predicted and experimental retention times are summarized in Table II. These results are fairly typical of the large number of simulations performed and compared to experimental results. Gross differences between predicted and experimental results were rarely found, and in those cases it was for minor components in the Premarin extract and most likely arose from incorrect peak assignments in the two initial temperature-programmed separations used as input for the computer simulations. Agreement for the separation of standards was always satisfactory.

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