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Note

Assay for conjugated estrogens in tablets using fused-silica capillary gas chromatography

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Pharmaceutical products containing conjugated estrogens, especially tablets, have been in use since 1942 for the treatment of estrogen deficiencies in post-menopausal women. The official compendial tests for the analysis of these products include a colorimetric assay for strength along with estimates of estrone and equilin content[1]. In addition, there is a gas chromatographic (GC) test that separates ten of the major estrogen components and provides a qualitative "identity" test. This GC procedure has also been applied to the quantitative analysis of conjugated estrogens in dosage forms[2]. However, it has not been adopted as an official compendial assay since collaborative studies have shown that the column is difficult to reproducibly prepare and a packing cannot be purchased which provides the necessary resolution of the ten estrogens. Also, the method involves retention times of up to one hour for a complete run.

In order to overcome these deficiencies in the packed column GC method and the shortcomings of the USP colorimetric methods, the use of glass capillary GC has been explored. Two recent papers have dealt with the resolution of estrogens using capillary column GC. Zweig *et al.*[3] used this procedure for separating the trimethylsilyl derivatives of the estrogens in pregnant mares' urine. However, the separations described did not resolve β-estradiol from α-dihydroequilin and did not show improved separation of the other estrogens as compared to the packed column procedure. Pillai and McErlane's approach[4] involves formation of a dual derivative (oxime–trimethylsilyl). Excellent separation of the estrogens is achieved. However, the reaction conditions used are quite strong, and the formation of *syn* and *anti* isomers due to the methoxime derivatives is a potential problem.

With the advent of fused-silica capillary column technology and the increased efficiency associated with this type of column, an investigation was undertaken to resolve the estrogen mixture using this recent development. The results are reported in this paper and the following advantages are noted: (1) separation of all ten estrogens, including β-estradiol from α-dihydroequilin; (2) formation of a single trimethylsilyl derivative at room temperature; (3) no need for temperature programming; (4) 28-min run time; (5) commercially available column; (6) acceptable accuracy and precision data on tablet samples; (7) column adaptability and ruggedness.

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EXPERIMENTAL

Apparatus

The instrument used was a Packard Model 421 gas chromatograph with a factory-equipped inlet splitter and flame-ionization detector assembly for capillary column use (Packard, Downers Grove, IL, U.S.A.). The column was a 10 m × 0.24 mm I.D.) fused-silica wall-coated open tubular column coated with SP-1000. It was a commercial column purchased from Quadrex (New Haven, CT, U.S.A.). Column temperature was 225°C. Inlet and detector temperatures were 280°C. Carrier gas was helium with a column pressure of 17 p.s.i. resulting in a flow-rate of about 1 ml/min. The splitting ratio was about 20 to 1. The detector purge rate was 50 ml helium/min and the injection size was 0.2 μl with an electrometer setting of $1 \cdot 10^{-10}$ A f.s. A Hewlett-Packard 3354B lab automation system was used for the data collection.

Method

The same sample and standard preparations are used as the previously published packed-column method[2] except that 1 ml of a 100 μg/ml solution of 1-methylestrone (Steraloids, Wilton, NH, U.S.A.) in methanol is substituted for testerosterone as the internal standard. This, in addition to the GC conditions, are the only changes which have been made.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of a standard mixture of the trimethylsilyl derivatives of the ten estrogens found in a typical Premarin® tablet (Ayerst Labs., New York, NY, U.S.A.) using the fused-silica column. Fig. 2 shows an actual tablet analysis (1.25-mg tablet). Compared to Fig. 3, which is the same tablet preparation using the DEGS packed column procedure, two distinct advantages are evident: (1) the capillary column decreases assay time from 69 to 28 min; (2) resolution is enhanced using the capillary column, especially in the diol region and estrone/equilin region of the chromatogram. These advantages are important when large numbers of samples are routinely assayed. Sample throughput is increased and quantitation is made easier due to the improved peak separation.

The efficiency of the SP-1000 column has been very acceptable considering the type of compounds involved. Estrogens are notoriously adsorptive in nature and extremely inert columns and connections are required to minimize tailing and band broadening. Using equilin as a reference peak, these columns have consistently yielded about 25,000 theoretical plates or about 2500 plates per meter. Greater efficiencies could be obtained by injecting smaller amounts of the estrogens since the amounts used in the method now are on the verge of overloading the column. However, smaller amounts mean higher sensitivities and the baseline at the solvent peak would be affected and make quantitation more difficult in the diol region of the chromatogram.

In addition, the columns have been found to be very stable, despite the fact that analyses are performed at the theoretical temperature limit of the liquid phase (225°C). It is very important that precautions are taken to exclude oxygen from the carrier gas during use and during storage. Carrier gas lines equipped with oxygen

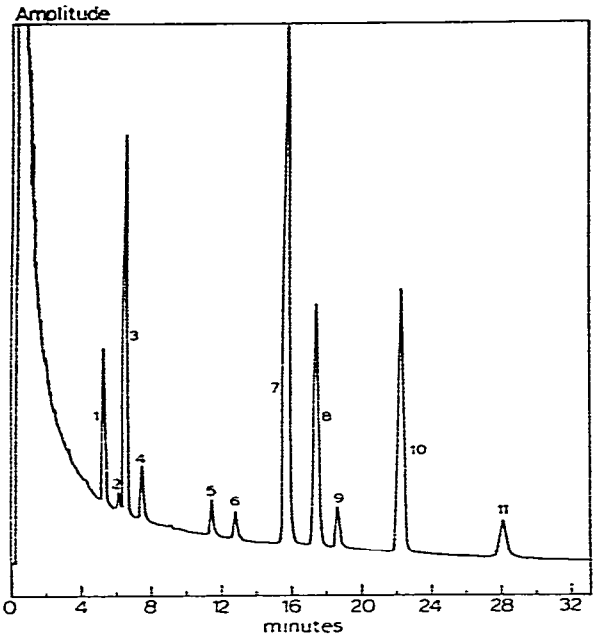

Fig. 1. Chromatogram of the ten estrogen standard mixture on the SP-1000 fused-silica column. 1 = α-Estradiol, 2 = β-estradiol, 3 = α-dihydroequilin, 4 = β-dihydroequilin, 5 = α-dihydroequilenin, 6 = β-dihydroequilenin, 7 = estrone, 8 = equilin, 9 = $\Delta^{8,9}$-dehydroestrone, 10 = 1-methylestrone, 11 = equilenin. (All estrogens present as the trimethylsilyl ethers).

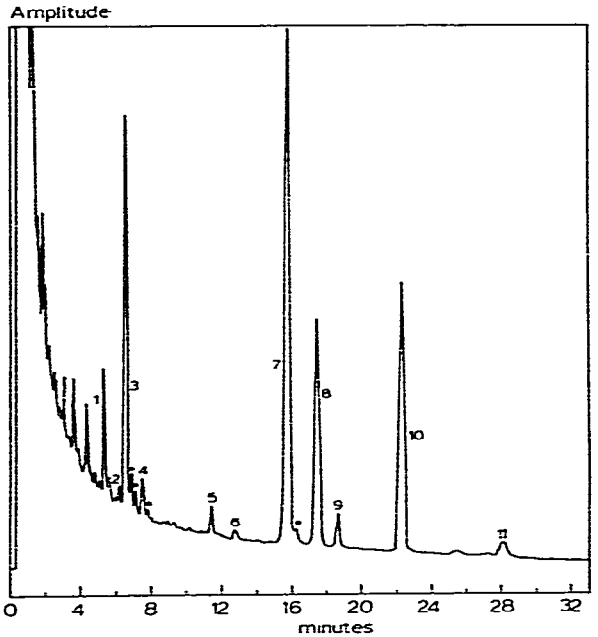

Fig. 2. Chromatogram of a typical Premarin tablet extract on the SP-1000 fused-silica column.

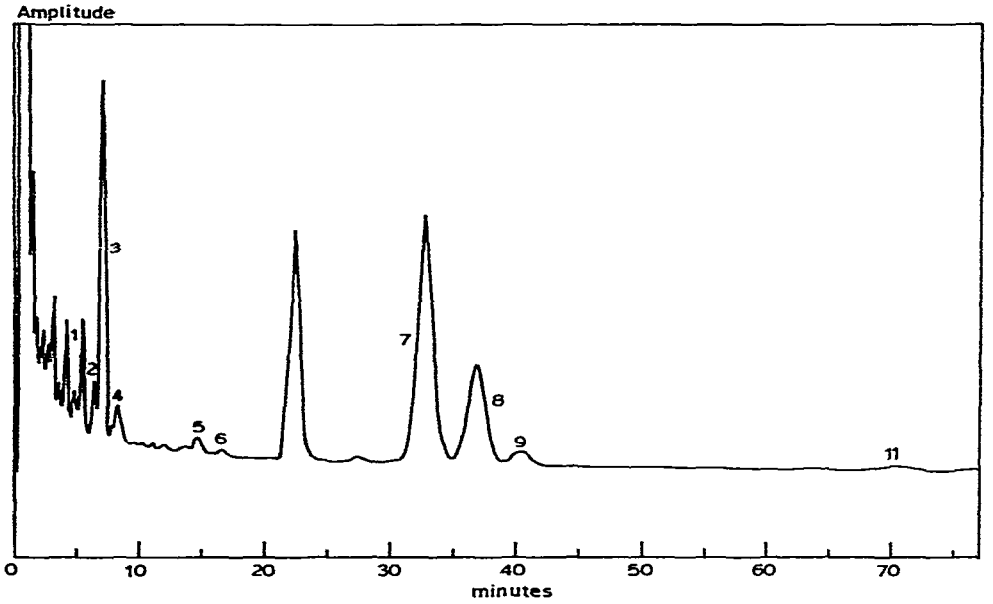

Fig. 3. Chromatogram of a typical Premarin tablet extract using the DEGS packed-column procedure. Column temperature = 200°C.

scrubbers are a necessity. During periods of storage outside the instrument, columns should be connected to an inert gas flow.

The ruggedness of these columns is also a feature which contributes to the efficiency of the system. Due to a fused-silica column's high resistance to breakage and its narrow O.D., the end of the column can actually be placed inside the flame tip, thus minimizing any dead volume in the detector connections. Also, the same column can be switched to different gas chromatographs without any modifications since the fused-silica material can be curved to almost any shape without breakage.

Precision experiments were performed to document the method. A batch of conjugated estrogen tablets* (1.25 mg) was analyzed five times on each of two days. Table I shows the results obtained from these experiments. The interday and intraday variation is considered very acceptable. The major estrogens such as estrone, equilin and α-dihydroequilin show very good coefficients of variation. As the amounts decrease for the other estrogens, the variation increases, which is to be expected. These compounds are present at only 8 to 40 μg per tablet.

Table II shows the comparison of the capillary column results with results obtained on the same tablet pool using the packed-column procedure. Overall, the results agree quite well. Some results, however, tend to be slightly lower by the capillary assay and can be explained by the increased resolution. α-Dihydroequilin, β-dihydroequilin and estrone have been separated from nearby components (indicated by asterisks). These peaks are included in the packed-column assay since they are either masked by the main component or are present as unresolved shoulders on the parent peaks. The retention times of these peaks do not correspond to a large number

* Premarin tablets. Ayerst Labs.

TABLE I

PRECISION OF ASSAY OF THE CAPILLARY COLUMN METHOD

Five tablet pools assayed on each of 2 days. C.V. = Coefficient of variations.

*Component**	*Day 1*		*Day 2*		*Overall*
	μg/tablet (average of 5 runs)	*C.V. (%)*	*μg/tablet (average of 5 runs)*	*C.V. (%)*	*C.V. (%)*
α-Estradiol	54	1.7	52	2.1	2.5
β-Estradiol	8.0	7.0	7.6	12.1	9.4
α-Dihydroequilin	195	1.6	191	1.7	1.9
β-Dihydroequilin	21	6.2	18	11.5	12.4
α-Dihydroequilenin	22	6.9	25	3.6	6.9
β-Dihydroequilenin	10	8.4	8.0	3.7	14.9
Estrone	710	1.5	688	1.1	2.1
Equilin	330	1.0	319	1.8	2.3
$\Delta^{8,9}$-Dehydroestrone	56	5.7	54	9.2	9.9
Equilenin	40	7.3	37	6.2	7.2

* Estrogens present as the sodium sulfate salts.

of typical estrogenic steroids for which standards are available[5]. The other component, for which some variation exists, is equilenin. This can be attributed to the nature of the peak itself. The packed column produces a broad flat peak which is extremely difficult to integrate accurately whereas the capillary column produces a sharp, easily quantitated peak.

The capillary method has revealed a peak pattern which might explain some of the small unknown peaks. α-Estradiol, α-dihydroequilin and β-dihydroequilin are all followed by a smaller peak. This repeating dual peak pattern is suggestive of the

TABLE II

COMPARISON PACKED-COLUMN AND CAPILLARY COLUMN ASSAY RESULTS ON A TABLET SAMPLE

*Component**	*μg/tablet*	
	Capillary column	*Packed column*
α-Estradiol	53	53
β-Estradiol	7.7	Not quantitated
α-Dihydroequilin	193	198
β-Dihydroequilin	19	32
α-Dihydroequilenin	24	19
β-Dihydroequilenin	9.0	8.0
Estrone	699	720
Equilin	324	330
$\Delta^{8,9}$-Dehydroestrone	55	51
Equilenin	39	31

* Estrogens present as the sodium sulfate salts.

presence of double bond isomers or possible different *cis–trans* ring junctions within the molecule. A number of isomers of α-dihydroequilin[6] and equilin[7] have already been identified. $\Delta^{8,9}$-Dehydroestrone, an isomer of equilin[8], and present in these tablets, is an example of this pattern. There is also a peak resolved from estrone. It is probably not an isomer of estrone and amounts to only about 1% of the total estrogenic content of the tablets.

The accuracy of the method was determined by spiking a placebo tablet pool with a known amount of sodium estrone sulfate. The equivalent of one tablet was spiked with 1 ml of an 837.3 μg per ml solution of sodium estrone sulfate in water and then carried through the procedure and compared to a known estrone standard workup. Duplicate workups yielded results of 831.1 and 829.0 μg sodium estrone sulfate for an average of 99.2% recovery.

CONCLUSION

A fused-silica capillary column GC method has been developed for the determination of conjugated estrogens in tablets. It offers several advantages as compared to the packed-column method, the primary ones being speed of analysis and increased resolution. The method, atlhough applied in this report only to tablets, should also be easily adapted to the assay of other pharmaceutical formulations such as injectables, creams and raw materials.

REFERENCES

1 *The United States Pharmacopeia*, Mack Publishing Co., Easton, PA, 20th rev. ed., 1980.
2 R. N. Johnson, R. P. Masserano, R. Haring, B. T. Kho and G. Schilling, *J. Pharm., Sci.*, 64 (1975) 1007.
3 J. S. Zweig, R. Roman, W. B. Hagerman and W. J. A. VandenHeuvel, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 3 (1980) 169.
4 G. K. Pillai and K. M. McErlane, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1981) 70.
5 R. N. Johnson, R. P. Masserano, B. T. Kho and W. P. Adams, *J. Pharm. Sci.*, 67 (1978) 1218.
6 T. Luukkainen and H. Adlercreutz, *Biochim. Biophys. Acta*, 107 (1965) 579.
7 W. Brown, J. W. Findlay and A. B. Turner, *Chem. Commun.*, (1968) 10.
8 R. Ramon, C. H. Yates, J. F. Millar and W. J. A. VandenHeuvel, *Can. J. Pharm. Sci.*, 10 (1975) 8.