GLC Assay of Conjugated Estrogen Formulations

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Abstract □ A procedure is described for the enzyme hydrolysis and GLC assay of conjugated estrogens in commercial formulations. Resolution of up to nine of the components is achieved on a methyl phenyl cyanopropyl silicone-coated column using a dual-derivatization and dual-injection technique. Replicate results from analyses of a commercial tablet formulation yielded coefficients of variation between 1.0 and 17.2%, depending mainly on the quantity present; a coefficient of variation of 0.4% was obtained for the assay for total conjugated estrogens. Similar results were obtained with other commercial formulations.

Keyphrases □ Estrogens, conjugated—enzyme hydrolysis and GLC analysis, commercial formulations □ GLC—analysis, conjugated estrogens, commercial formulations □ Enzyme hydrolysis—for GLC analysis of conjugated estrogens, commercial formulations □ Hormones—conjugated estrogens, enzyme hydrolysis and GLC analysis, commercial formulations

According to the USP (1), conjugated estrogens contain "a mixture of the sodium salts of the sulfate esters of the estrogenic substances, principally estrone and equilin, that are of the type excreted by pregnant mares." The USP also requires that conjugated estrogens contain dihydroequilin-17α, dihydroequilin-17β, estradiol-17α, estradiol-17β, 8-dehydroestrone, and equilenin. In addition, other sulfate esters of dihydroequilenin-17α and dihydroequilenin-17β have been identified in the urine of pregnant mares (2).

The USP XIX assay for conjugated estrogens is a long and difficult procedure, in which only estrone (I) and equilin (II) are quantified after acid hydrolysis of the sulfates. Dihydroequilin-17α and 17β (III), estradiol-17α and 17β (IV), 8-dehydroestrone (V), and equilenin (VI) are separately identified only by GLC following enzyme hydrolysis. The assay for total conjugated estrogens is performed separately using a color-producing reagent.

Experience in these laboratories indicated that the acid hydrolysis of the sulfate may lead to decomposition of some estrogens, notably dihydroequilin.

Because of the complex nature of the product, many methods have been suggested to resolve the components including column (3–6), thin-layer (7, 8), paper (9–13), high-speed liquid (14), and gas–liquid (15–17) chromatographic techniques. Earlier studies (18) showed the feasibility of resolving the estrogen steroids by GLC using a dual-derivatization and dual-injection procedure on a methyl phenyl cyanopropyl silicone (OV-225) column.

In the present procedure, the sulfate esters of equine estrogens are hydrolyzed by enzyme incubation and subsequently quantified by GLC.

EXPERIMENTAL

Enzyme Hydrolysis—*Tablet Samples*—Twenty tablets were weighed and finely powdered, and an aliquot equivalent to 1.5 mg of conjugated estrogens was weighed directly into a 50-ml round-bottom, polytetrafluoroethylene[1]-lined, screw-capped centrifuge tube. Fifteen milliliters of acetate buffer (pH 5.2, 0.02 *M*) was added, and the tubes were shaken on a horizontal shaker for 30 min.

Injectable Samples —The contents of a multiple injection vial (25 mg) were transferred quantitatively to a 50-ml volumetric flask, using the acetate buffer, and brought to volume. A 3-ml aliquot (1.5 mg) of this solution was then placed in a 50-ml round-bottom, polytetrafluoroethylene-lined, screw-capped centrifuge tube containing 12 ml of acetate buffer.

Redistilled benzene (10 ml) was added to all tubes, and they were gently shaken on a horizontal shaker for 20 min. The tubes were centrifuged, and the top benzene layer was removed as completely as possible with a 10-ml pipet. An additional 10 ml of benzene was added, and the process was repeated. The tubes were then shaken to resuspend the solid material, and the contents were transferred to a 250-ml round-bottom flask; the tubes were rinsed with 2 × 2 ml of acetate buffer, which was added to the flask. By using a rotary evaporator and taking care to avoid frothing, the residual benzene was removed until its odor was no longer apparent. Traces of benzene remaining in the centrifuge tube were removed with a gentle stream of air.

The contents of the flask were then transferred back to the original centrifuge tube with 3 × 2 ml of acetate buffer, 7500 units of sulfatase enzyme was added, and the tubes were incubated for 30 min at 45° with occasional shaking. The tubes were then cooled to room temperature, 15 ml of pure redistilled chloroform was added, and the tubes were shaken for 30 min on a horizontal shaker.

After centrifuging, the chloroform layer was removed as completely as possible by driving the tip of a pipet down through the aqueous layer and gently blowing out any water that may have entered the pipet before drawing up the chloroform. The chloroform was transferred to a stoppered tube through a funnel containing a pledget of glass wool covered with a bed of anhydrous sodium sulfate. The free phenolic estrogens were stored in the refrigerator until required for derivatization. (Storage times did not exceed 2 days.)

Sample Preparation—Two 5-ml polytetrafluoroethylene-lined, screw-capped, conical vials[2] were used for each sample. One milliliter of an ethanolic solution of the internal standard, ethinyl estradiol[3] (VII), was added to one vial, and then 3 ml of the estrogen hydrolysate in chloroform was added to each vial. To the vial containing the estrogens and the internal standard, 0.1 ml of pyridine and 0.05 ml of a silylating reagent[4] were added; the sample was capped and heated for 10 min at 60°. To the other sample (no internal standard), 0.1 ml of a 2% solution of methoxamine in pyridine was added; this sample was heated at 60° for 3 hr. After this time, 0.05 ml of silylating reagent was added and the solution was further heated at 60° for 10 min.

At the completion of the silylation reaction, 2–4-μl aliquots of the samples were injected in the 3% OV-225 column.

Column Packing—Experience has shown that the method of column preparation is important in achieving good resolution. Therefore, the procedure is described in detail.

The coated support, 3% OV-225 on Chromosorb W (HP), 100–120 mesh[5], was prepared by uniformly depositing a chloroform solution (50 ml) of the stationary phase (0.75 g), using a Pasteur pipet, over the surface of a bed of the support phase (25 g) uniformly dispersed in a 13.97-cm (5.5-in.) petri dish. The vessel in which the stationary phase was prepared was rinsed with 2–3 ml of chloroform onto the support in the same manner.

The barely moist cake of coated support was then transferred to a fluidizer sitting on a hot plate. With a gentle stream of clean dry nitrogen flowing through the fluidizer, the temperature of the hot plate was raised until a thermometer inserted in the base of the fluidizer registered about 175°. Heating was continued for 0.5 hr or until the coated support tumbled freely and no odor of chloroform was detectable.

A 1.8-m × 0.63-cm (6-ft × 0.25-in.) o.d., glass, U-shaped column was filled with chromic acid and allowed to sit for 0.5 hr. The column was emptied, rinsed with water and methanol, and then dried by drawing air through it. It was then filled with 5% dimethylchlorosilane in dry toluene

[1] Teflon (du Pont).

[2] ReactiVials, Pierce Chemical Co., Rockford, Ill.
[3] USP reference standard.
[4] TRISIL TBT, Pierce Chemical Co., Rockford, Ill.
[5] Chromatographic Specialties, Brockville, Ontario, Canada.

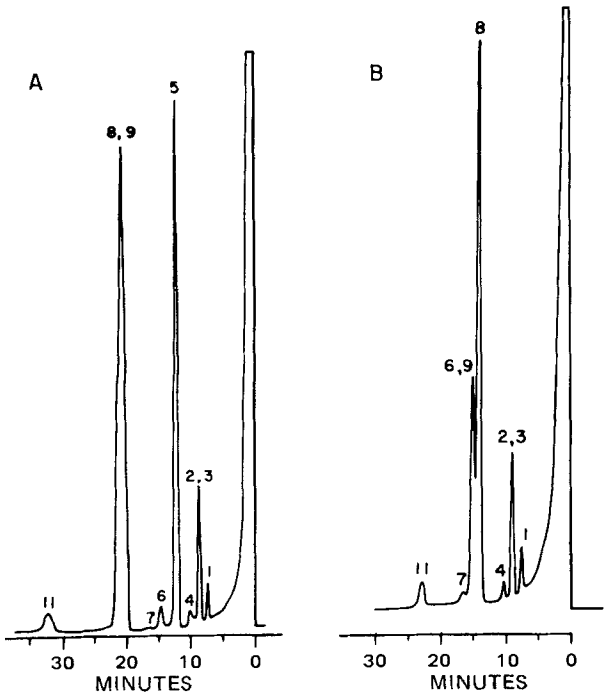


Figure 1—*Trimethylsilyl (A) and methoxamine–trimethylsilyl (B) derivatives of a synthetic composite of equine estrogens. Key: 1, estradiol-17α; 2, estradiol-17β; 3, dihydroequilin-17α; 4, dihydroequilin-17β; 5, ethinyl estradiol; 6, dihydroequilenin-17α; 7, dihydroequilenin-17β; 8, estrone; 9, equilin; and 11, equilenin.*

and allowed to stand for 1 hr. Then it was emptied, rinsed with methanol and acetone, and thoroughly dried as before.

The column was packed by applying vacuum on one end while adding about 15.2-cm (6-in.) sections of coated support to the other and gently vibrating each section.

The column was conditioned in the gas chromatograph oven at 175° with a nitrogen flow of 45 ml/min for 8 hr. The oven temperature was then raised to 235° at 3°/min and held for an additional 16 hr or until a stable baseline was achieved. An occasional injection of a mixture of *N,O*-bis-(trimethylsilyl)acetamide, trimethylsilyldiethylamine, and hexamethyldisilazane[6] shortened conditioning times.

RESULTS AND DISCUSSION

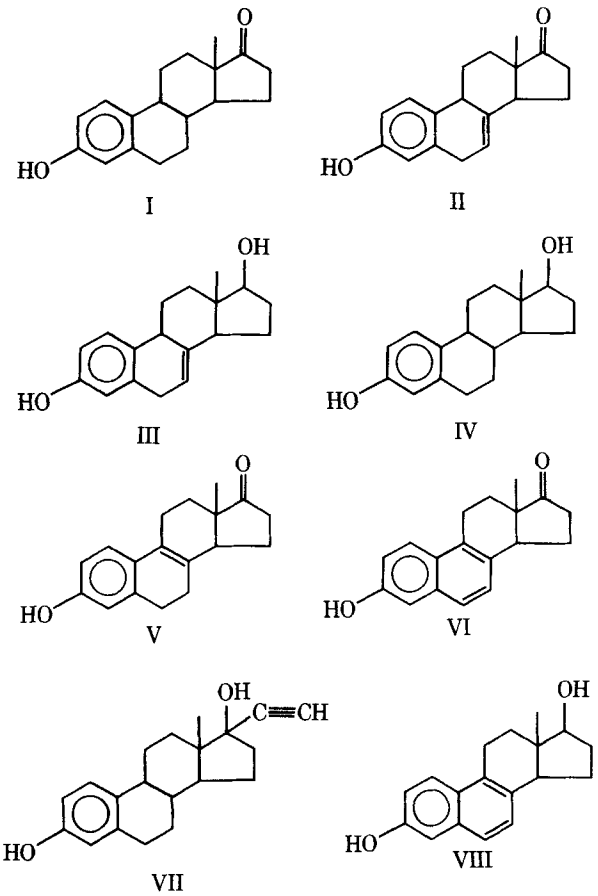
The resolution achieved using a 3% methyl phenyl cyanopropyl silicone (OV-225) column for the trimethylsilyl derivatives of a synthetic composite of sulfates of equine estrogens (I–VIII) is shown in Fig. 1A. In this chromatogram, baseline separation of all steroids is apparent except for the pairs estradiol-17β–dihydroequilin-17α and estrone–equilin within a total chromatographic analysis time of less than 55 min.

Separation of the two major steroids, estrone and equilin, was achieved by forming the methoxamine–trimethylsilyl derivatives of the keto steroids present in the mixture with the resulting resolution (R = 0.8) of the keto steroids as shown in Fig. 1B. The pair estradiol-17β–dihydroequilin-17α could not be resolved under any conditions employed. However, since the former is known to contribute little to the normal natural balance of the components of equine estrogens (2), little interference would be observed in the determination of dihydroequilin-17α. (The steroid 8-dehydroestrone was not included in the synthetic mixture due to a short supply.)

Quantitative Aspects—To utilize this resolution of estrogens as the basis of a quantitative method for the individual determination of most, if not all, of the components of conjugated estrogens, area response factors for the trimethylsilyl derivatives of the estrogens were determined in relation to an internal standard, ethinyl estradiol (Table I).

With the addition of a known amount of internal standard, the absolute amounts of each steroid in a mixture could then be determined from:

$$A = \frac{B \times C \times D}{E} \qquad \text{(Eq. 1)}$$



where A = weight of steroid, B = response factor, C = weight of internal standard, D = area of steroid, and E = area of internal standard.

The determination of the amounts of the equine estrogens observed in a sample from the foregoing calculation will yield a total for estrone and equilin. However, the proportions of these two steroids can be determined from the chromatogram of the methoxamine–trimethylsilyl derivatives (which does not contain any internal standard) by their relative peak heights. Peak height responses for estrone and equilin were identical. The peak observed for equilin as its methoxamine–trimethylsilyl derivative overlaps with that for the trimethylsilyl derivative of dihydroequilenin-17α (VIII). The contribution of the latter is most easily determined by the peak height ratio between the chromatogram of the trimethylsilyl derivatives and that for the methoxamine–trimethylsilyl derivatives by:

$$F = \frac{G \times H}{I} \qquad \text{(Eq. 2)}$$

where F = height of dihydroequilenin-17α in the methoxamine–trimethylsilyl chromatogram, G = height of dihydroequilenin-17α in the trimethylsilyl chromatogram, H = height of dihydroequilenin in the methoxamine–trimethylsilyl chromatogram, and I = height of dihydroequilin in the trimethylsilyl chromatogram.

Subtraction of the contribution of dihydroequilenin-17α from equilin

Table I—Response Factors for Equine Estrogens

Trimethylsilyl Steroid Ethers	Response[a]
Estradiol-17α	0.91
Estradiol-17β	1.04
Dihydroequilin-17α	1.04
Dihydroequilin-17β	1.03
Dihydroequilenin-17α	0.95
Dihydroequilenin-17β	1.04
Estrone	1.20
Equilin	1.20
8-Dehydroestrone	1.00
Equilenin	1.12

[a] Relative to ethinyl estradiol.

[6] Silyl-8, Pierce Chemical Co.

Table II—Assay of Synthetic Composite of Equine Estrogens

Steroid	Percent Added	Range Found, %	Average Found, %	CV, %
Estradiol-17α	1.9	1.7–1.8	1.8	3.2
Estradiol-17β–dihydro-equilin-17α	7.8	6.4–8.6	7.1	4.9
Dihydroequilin-17β	1.6	0.8–1.3	1.1	14.4
Dihydroequilenin-17α	2.0	2.2–2.4	2.2	6.8
Dihydroequilenin-17β	0.8	0.8–0.9	0.9	6.7
Estrone	58.4	59.1–59.5	59.3	0.3
Equilin	22.5	21.4–22.6	21.9	2.4
Equilenin	5.1	4.6–6.3	5.8	6.6
Assay[a]		(96.9–99.0)	(98.1)	0.9

[a] $n = 6$.

Table III—Assay of 2.5-mg Tablet

Steroid	Range, %	Average, %	CV, %
Estradiol-17α	4.5–4.8	4.7	3.1
Estradiol-17β–dihydro-equilin-17α	14.7–15.4	15.2	1.7
Dihydroequilin-17β	1.1–1.2	1.2	4.0
Dihydroequilenin-17α	1.9–2.3	2.0	5.8
Dihydroequilenin-17β	0.3–0.4	0.3	17.2
Estrone	50.7–52.0	51.4	1.0
Equilin	19.5–21.2	20.2	3.3
8-Dehydroestrone	1.6–1.9	1.8	5.2
Equilenin	2.8–3.7	3.4	9.6
Assay[a]	2.49–2.51 mg	2.5 mg	0.4

[a] $n = 6$.

yields the true height of the equilin peak. This value can then be used, along with the height of the peak observed for estrone, to determine the relative amounts of estrone and equilin from the total of these two estrogens obtained as their combined trimethylsilyl derivatives. The quantitation of the individual estrogens is then complete, and these values can simply be added to give the total assay amount of the free phenolic estrogens. The equivalent weight of the conjugates can then be calculated by:

$$\text{total assay} = \text{weight of free steroids} \times 1.38 \times \text{dilution factor} \quad \text{(Eq. 3)}$$

where 1.38 represents the weighted molecular weight ratio between free phenol and the sulfate ester.

To test the robustness of the 3% OV-225 column to varying conditions of the gas chromatograph, the column oven temperature was adjusted ±15° around the normal 225° used for the assay. The nitrogen flow was also adjusted ±15 ml/min around the 40 ml/min employed. None of these conditions, either singly or in combination, markedly affected the assay results of the individual steroids or the total assay. Under the extreme conditions of the column temperature and gas flow, the total chromatogram time for the trimethylsilyl derivatives varied between 20 and 45 min with no apparent lack of resolution.

To evaluate the variability of the GLC assay of a synthetic mixture of equine estrogens, a sample of the free phenolic steroids was prepared and then assayed according to the described method (Table II).

Enzyme Hydrolysis—Before the assay method could be applied to commercial conjugated estrogen formulations, the hydrolytic cleavage

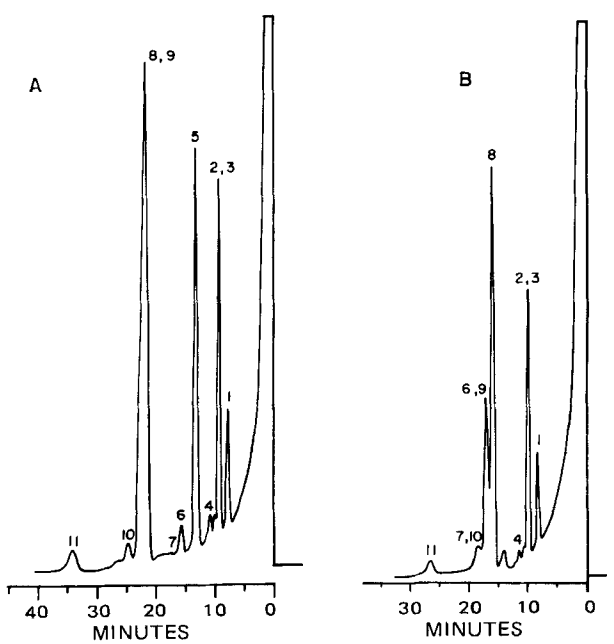


Figure 2—*Trimethylsilyl (A) and methoxamine–trimethylsilyl (B) derivatives of equine estrogens obtained from a 2.5-mg commercial tablet. Key: 1, estradiol-17α; 2, estradiol-17β; 3, dihydroequilin-17α; 4, dihydroequilin-17β; 5, ethinyl estradiol; 6, dihydroequilenin-17α; 7, dihydroequilenin-17β; 8, estrone; 9, equilin; 10, 8-dehydroestrone; and 11, equilenin.*

of the sodium sulfate esters of the phenolic steroids was investigated. The sulfatase enzyme derived from *Helix pomatia*[7] was subjected to varying time, temperature, buffer concentration, and pH conditions. Many methods (19, 20) showed the effectiveness of sulfatase enzyme in pH 5.2 acetate buffer at 37° toward the hydrolysis of estrone sulfate. However, the time interval quoted was frequently about 18 hr, and this time was deemed inappropriate for this study. Therefore, the variables of temperature (40, 45, 50, and 55°), pH (4.5, 5.2, 5.5, and 6.0), hydrolysis time (20, 30, and 40 min), and acetate buffer molarity (0.02, 0.1, and 0.2) were studied. The results indicated that quantitative recovery of the free steroid could be expected under all conditions and combinations of these conditions when potassium estrone sulfate was used as a substrate. As a laboratory convenience, commercial sulfate conjugates of equine estrogens were routinely hydrolyzed at 45° for 30 min in 0.02 *M* acetate buffer at pH 5.2.

When conjugated estrogen samples were initially hydrolyzed under these conditions and then extracted into chloroform (a single extraction was quantitative), the samples revealed, after evaporation and silylation, an extraneous peak in the chromatogram interfering with the less retentive diols. A benzene prewash of the formulation samples, after they had been extracted into the acetate buffer, removed the interference. This peak, appearing between the trimethylsilyl derivatives of estradiol-17α and dihydroequilin-17α, was purified by preparative GLC of the silylated prewash residue. Mass spectral analysis of the compound giving rise to the impurity showed an apparent molecular ion at *m/e* 396 with other prominent ions at *m/e* 381, 323, 277, 217, 216, 203, 189, 188, 187, and 159. Comparisons of the mass spectra with other similarly prepared estrogen samples failed to provide any similarities between the samples.

To ascertain that sufficient enzyme was being used to obtain complete hydrolysis of estrogen conjugates in formulations (phosphate inhibits the action of sulfatase enzyme and is used in tablet formulas), a series of tablet samples was prepared using 1000, 2000, 4000, 8000, and 10,000 units of sulfatase. In some cases, 1000 units of enzyme was sufficient to yield theoretical quantitative recovery of the estrogens, but other samples required at least 4000 units. Consequently, for new tablet formulations, each analysis was performed in triplicate using 1000, 5000, and 7500 units of enzyme to expose enzyme inhibition.

Formulation Quantitation—A commercial 2.5-mg tablet was assayed by obtaining the trimethylsilyl derivative of the steroid hydrolysate (Fig. 2A). The peak due to dihydroequilenin-17β is not noticeable in the chromatogram but was detected by the integrator. A peak following the estrone–equilin peak was identified as 8-dehydroestrone from chromatographic comparison with authentic material. The chromatogram of the methoxamine–trimethylsilyl derivative (Fig. 2B) displays the partial resolution of estrone and equilin, sufficient to allow their quantitation (Table III).

The chromatograms of the trimethylsilyl and methoxamine–trimethylsilyl derivatives of a 1.25-mg tablet obtained from the same manufacturer are shown in Figs. 3A and 3B, and the quantitative assay is given in Table IV. The similarity of relative estrogen amounts is quite obvious and would likely be related to the manufacturer's source of pregnant mare's urine. The label claim of 1.25 mg was exceeded in this instance by 16%, the USP maximum.

Similar results (Table V) were obtained for the analysis of an injectable preparation from the same manufacturer; again the similarity of the relative proportions of steroids points to a uniform source.

The method has been applied with equal facility to various tablet

[7] Type H-2, Sigma Chemical Co., St. Louis, Mo.

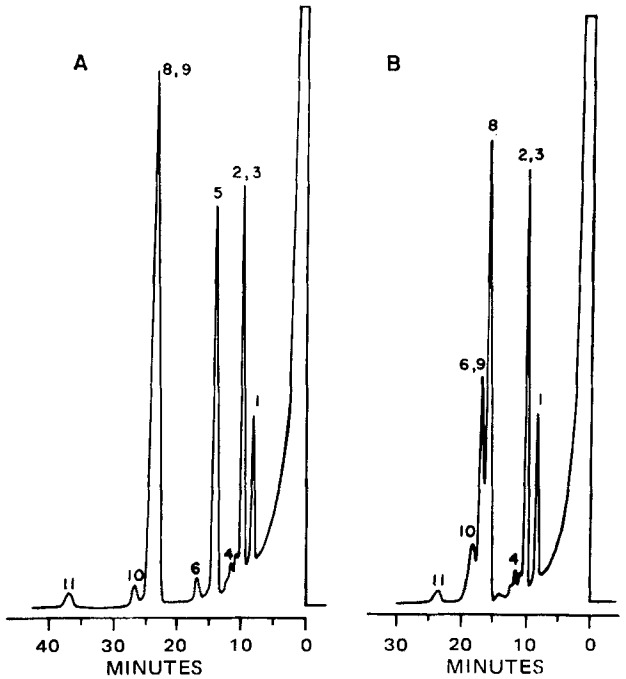


Figure 3—*Trimethylsilyl (A) and methoxamine–trimethylsilyl (B) derivatives of equine estrogens obtained from a 1.25-mg commercial tablet. Key: 1, estradiol-17α; 2, estradiol-17β; 3, dihydroequilin-17α; 4, dihydroequilin-17β; 5, ethinyl estradiol; 6, dihydroequilenin-17α; 8, estrone; 9, equilin; 10, 8-dehydroestrone; and 11, equilenin.*

formulations and has provided the basis of a GLC identification and quantitation of the main estrogenic components of conjugated estrogen preparations.

Comparison with Literature Methods—Other GLC methods for the quantitative analysis of esterified (16) and conjugated (17) estrogens have been reported. They are based on the resolution of the trimethylsilyl ether derivatives of the free equine estrogens on a diethylene glycol succinate-coated column. The steroids are resolved, except that the peak due to estradiol-17β appears as a shoulder on the dihydroequilin-17α peak. On an OV-225 column as used in the present study, the peak due to the minor constituent, estradiol-17β, is not resolved from dihydroequilin-17α.

The proposed OV-225 column is apparently indefinitely stable under conditions of normal usage. Diethylene glycol succinate columns, however, are limited to a useful lifetime to 2–3 weeks in equine estrogen analysis (2), probably due to column breakdown from oxidative processes and to bleed at the temperature of the analysis (21). In addition, there are reportedly batch-to-batch variations in the properties of diethylene glycol succinate (22), and these variations may affect the chromatographic behavior of the steroids.

The time and cost of the OV-225 and diethylene glycol succinate procedures are comparable. The time required for preparation of the additional samples for the OV-225 procedure is compensated for by the chromatographic time of 55 min as compared to 75 min for the diethylene glycol method. The peaks observed on the OV-225 column are sharp, whereas those seen on the diethylene glycol succinate are broadened due to the longer retention times; the peak due to equilenin, therefore, may be difficult to quantify. Calculations associated with the OV-225 method are marginally longer due to the double-injection technique, but this factor is not significant in practice[8].

Both the OV-225 and diethylene glycol succinate methods have advantages. For the analysis of a single sample, the preference may be for the diethylene glycol succinate method because of the slightly better resolution. For the long-term analysis of many samples, the stability and robustness of the OV-225 method appear to be highly advantageous.

Table IV—Assay of 1.25-mg Tablet

Steroid	Weight, mg	Percent
Estradiol-17α	0.08	5.5
Estradiol-17β–dihydro-equilin-17α	0.21	14.5
Dihydroequilin-17β	0.01	0.7
Dihydroequilenin-17α	0.03	1.7
Dihydroequilenin-17β	0.01	0.3
Estrone	0.74	50.6
Equilin	0.32	21.9
8-Dehydroestrone	0.03	2.0
Equilenin	0.04	2.7
Total	1.46 mg	(116% of label claim)

Table V—Assay of 25-mg Multiple Injection Vial

Steroid	Weight, mg	Percent
Estradiol-17α	0.81	3.0
Estradiol-17β–dihydro-equilin-17α	4.14	15.4
Dihydroequilin-17β	0.97	3.6
Dihydroequilenin-17α	0.36	1.3
Dihydroequilenin-17β	0.06	0.2
Estrone	12.66	47.2
Equilin	6.64	24.7
8-Dehydroestrone	0.56	2.1
Equilenin	0.68	2.5
Total	26.9 mg/vial	(107% of label claim)

REFERENCES

(1) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970.

(2) R. Roman, C. H. Yates, J. F. Millar, and W. J. A. VandenHeuvel, *Can. J. Pharm. Sci.*, **10**, 8 (1975).

(3) G. A. Grant and D. Beall, *Rec. Progr. Hormone Res.*, **5**, 307 (1950).

(4) K. Savard and B. Bierdules, *Endocrinology*, **68**, 411 (1961).

(5) B. R. Bhavani, R. V. Short, and S. Solomen, *ibid.*, **85**, 1172 (1969).

(6) G. J. Krol, R. P. Masserano, J. F. Carney, and B. T. Kho, *J. Pharm. Sci.*, **59**, 1483 (1970).

(7) L. E. Crocker and B. A. Lodge, *J. Chromatogr.*, **62**, 158 (1971).

(8) *Ibid.*, **67**, 419 (1972).

(9) J. E. Cox, *J. Chromatogr.*, **61**, 193 (1971).

(10) G. J. Krol, G. R. Bayden, R. H. Moady, J. C. Comeau, and B. T. Kho, *ibid.*, **61**, 187 (1971).

(11) E. Heltman, *Science*, **111**, 571 (1950).

(12) E. Heltman, *J. Am. Chem. Soc.*, **73**, 851 (1951).

(13) R. E. Oakey, *J. Chromatogr.*, **8**, 2 (1962).

(14) A. G Butterfield, B. A. Lodge, and N. J. Pound, *J. Chromatogr. Sci.*, **11**, 401 (1973).

(15) I. Schroeder, J. C. Medina-Scivedo, and G. Lopez-Sanches, *ibid.*, **10**, 183 (1972).

(16) R. Roman, C. H. Yates, J. F. Millar, and W. J. A. VandenHeuvel, *Can. J. Pharm. Sci.*, **10**, 12 (1975).

(17) R. Johnson, R. Masserano, R. Haring, B. Kho, and G. Schilling, *J. Pharm. Sci.*, **64**, 1007 (1975).

(18) K. M. McErlane, *J. Chromatogr. Sci.*, **12**, 97 (1975).

(19) M. F. Jayle, R. Scholler, P. Jarrige, and S. Metay, *Bull. Soc. Chim. Biol.*, **41**, 1593 (1959).

(20) J. Jarrige and M. F. Boyle, *ibid.*, **45**, 783 (1963).

(21) R. F. Kruppa, and R. S. Henly, Paper 144 presented at the Pittsburgh Conference, Cleveland, Ohio, Mar. 1974.

(22) J. R. Mann and S. T. Preston, *J. Chromatogr. Sci.*, **11**, 216 (1973).

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[8] A program for the Hewlett-Packard 9810 calculator is available on request.