Modified Procedure for Assay of Equine Conjugated Estrogens in Tablets and Powdered Concentrates

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The procedure developed by Carol *et al.* for the analysis of equine conjugated estrogenic preparations has been substantially modified at two major points: (*a*) the preparation of the extract of total estrogens and (*b*) the technique of color development. The net result of these changes along with other minor changes is a considerable saving of time and labor required to perform the analysis.

THE CHEMICAL assay of the complex mixture of estrogenic substances derived from the urine of gravid mares has been studied at length in our laboratory and elsewhere. In 1961 Carol *et al.* (1) proposed a colorimetric method for the determination of estrone, equilin, and total estrogens in commercial dosage forms of these substances. The routine requirement of 2 days to complete this assay was not considered excessive in view of the complexity of these preparations. After further work in this laboratory, several steps in this procedure were modified, resulting in a definite saving of time and labor.

DISCUSSION

The first of the two major changes is the technique employed to convert the dicyclohexylamine acetate complex of the conjugates to the free phenols. This conversion is accomplished at room temperature by allowing the eluate from the sample column to pass directly into a second column containing an intimate mixture of siliceous earth and concentrated hydrochloric acid. The assay solution obtained from this eluate is of sufficient purity not to require the alkali extractions and associated manipulations of the procedure by Carol *et al.* The subsequent isolation of the ketosteroid fraction from this solution remains essentially unchanged.

The second major change is the method adopted for color development. This modification allows the test aliquots to be evaporated as a part of the heating period, thus eliminating the use of glass-stoppered tubes and the need to evaporate and vacuum-dry the residues prior to color development as is required in the procedure by Carol *et al.* The technique for the determination of total estrogens and estrone uses a two-stage color development procedure which is based on the bicolor character of the Kober reaction (2). The formation of the initial yellow color is greatly accelerated for the common steroid estrogens by increasing the concentration of sulfuric acid in the N.F. XI Iron-Kober reagent. Subsequent dilution of this yellow phase with 33% sulfuric acid and a brief reheating produces the red color. Our experience with these modifications has shown that stable, reproducible, and reliable colors are obtained with relative ease and rapidity.

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With the exception of equilin, the responses of the individual estrogens relative to estrone when the open tube two-stage technique is used are lower by a small percentage when compared to the responses obtained in the stoppered tube one-stage heating technique. The two-stage technique employed for the determination of total estrogens reduces the response of equilin from 50 to 22% relative to estrone. Therefore, a correction of 78% rather than 50% of the equilin content must be added to the calculation of total estrogens.

Estrone produces a stable red color in the two-stage procedure in a total heating period of 25 min. However, in its determination in the ketosteroid fraction, equilin and equilenin interfere, as in the procedure by Carol *et al.* Similarly, by increasing the total heating period to 65 min. the effect of equilenin can be reduced drastically without causing much change in the estrone color. The effect of equilenin is then ignored, and a suitable correction is made for the effect of equilin.

(Since the publication of the procedure by Carol *et al.*, some manufacturers have incorporated excipients into their tablet formulations which cause significant losses of the conjugates in the chloroform prewash of the sample column used in the method. These losses are avoided when water-saturated ether is used for the prewashing agent.)

Assay

Reagents.—Iron-Kober reagent N.F. XI, p. 129; modified Iron-Kober reagent (mix thoroughly 5 vol. of Iron-Kober reagent with 3 vol. of concentrated sulfuric acid); Girard reagent T U.S.P. XVI, p. 1061; dicyclohexylamine acetate [see Carol *et al.* (1)].

Standard Estrone.—Accurately weigh 4.5–5.0 mg. of U.S.P. estrone reference standard and dissolve in benzene[1] with warming, if necessary. Cool, transfer quantitatively to a 100-ml. volumetric flask, and dilute to volume with benzene.

Standard Equilin.—Accurately weigh 1.5–2.0 mg. of purified equilin, dissolve in benzene (warming if necessary), cool, transfer quantitatively to a 100-ml. volumetric flask, and dilute to volume with benzene.

[1] On several occasions, spectra were obtained which required positive base line corrections. It was observed that this occurred when samples of benzene had been used that subsequently were found to produce a pronounced yellow color in concentrated sulfuric acid placed in contact with it at room temperature for 30 min. The extent of contamination was found to be variable within given lots from three commercial sources, including the spectral grades. Except in extreme cases, refluxing with concentrated sulfuric acid for 1 hr. and subsequent distillation is sufficient to render a contaminated lot usable. No difficulty should be experienced if the same sample of benzene showing only slight contamination is used throughout the procedure.

Procedure

Extraction of Total Estrogens.—Using a glass mortar and pestle, grind together until uniform 2 Gm. of siliceous earth and a counted number of tablets equivalent to 6–7 mg. of sodium estrone sulfate. Add 6 Gm. of siliceous earth and mix until uniform. For powdered concentrates or freshly ground tablet composites, thoroughly mix the equivalent of 6–7 mg. of sodium estrone sulfate with 8 Gm. of siliceous earth in a 150-ml. beaker. Add 4 ml. of water and mix until uniform. Transfer quantitatively in two portions, with the aid of a small amount of dry siliceous earth, to a 22 × 150-mm. glass chromatographic column containing a plug of fine glass wool. Tamp each portion moderately and complete the transfer with a small pledget of glass wool. Prewash the column with 125 ml. of water-saturated ether and discard the eluate. (The second column is conveniently prepared during the prewashing of the sample column.) Thoroughly mix 20 Gm. of siliceous earth and 12 ml. of concentrated hydrochloric acid with a glass rod. Transfer this mixture in several portions, tamping each moderately, to a 22 × 300-mm. glass chromatographic column containing a plug of fine glass wool. Hold the top layer in place with glass wool. Dilute 85 ml. of chloroform to 250 ml. with ether. Dissolve 250 mg. of dicyclohexylamine acetate in 200 ml. of this solvent. When the prewash of the sample column is complete, add 30–40 ml. of the chloroform–ether mixture to the hydrochloric acid column and quickly connect the sample column to the top of it with a suitable rubber stopper.[2]

Using a separator as a reservoir, elute the columns with the 200 ml. of the dicyclohexylamine acetate solution. Collect the eluate in a 300-ml. separator after discarding the first 10 ml. of eluate.[3] To avoid channeling, a layer of liquid should be maintained above the surface to the hydrochloric acid column until the elution of the sample column is complete. Loosen, but do not remove, the sample column from the lower column when the last of the eluant passes into it, and allow the columns to drain completely. Wash the eluate with two 25-ml. portions of water and combine the washes in a separator. Continue washing the eluate with 25-ml. portions of 2% sodium carbonate solution until the washings are alkaline. Combine these washes with the water washes. Render the combined washes alkaline with sodium carbonate, if necessary, and back-wash with 20 ml. of benzene. Discard the aqueous phase and wash the benzene with 10 ml. of water. Discard the water and add the benzene to the eluate in the first separator. Pass this solution through 15–20 Gm. of anhydrous sodium sulfate into a 300-ml. conical flask. Carefully evaporate the solution to dryness with gentle warming under a current of air. Dissolve the residue in about 40 ml. of benzene with warming. Cool and quantitatively transfer this solution to a 100-ml. volumetric flask and dilute to volume with benzene. (This is solution *A*, total estrogens.)

Isolation of Ketosteroids.—Evaporate a 50.0-ml. aliquot of solution *A* to about 5 ml. and quantitatively transfer it to a 25-ml. conical flask with a minimum volume of chloroform. Carefully evaporate to dryness with gentle heating and a current of air so that all of the residue is deposited on the bottom of the flask. [Reserve the remainder of solution *A* for the determination of total estrogens. This solution is also used for the paper chromatographic test. See Carol *et al.* (1).] Add 100 mg. of Girard reagent T and 0.5 ml. of glacial acetic acid to the residue, cover the flask with a small watch glass, and heat on a steam bath for 5 min. with occasional swirling to insure complete conversion. Cool the solution and quantitatively transfer with 50 ml. of cold water to a 125-ml. separator which contains 10 ml. of 5% sodium acetate solution. Wash the solution immediately with three 10-ml. portions of chloroform and combine the washings in a second separator. Back-wash the chloroform with 5 ml. of water, discard the chloroform, and add the aqueous phase to the first separator. Add 7 ml. of 33% sulfuric acid solution, mix gently, and immediately draw off and discard any chloroform that may separate. Allow the solution to stand for 30 min.; then extract it by shaking vigorously with 20 ml. of benzene for 1 min. Pass the aqueous phase into a second separator containing 15 ml. of benzene and extract similarly. Discard the aqueous phase. Wash the benzene extracts serially with 10 ml. of 2% sodium carbonate solution. Discard the wash and transfer the contents of the second separator to the first, completing the transfer by flushing with about 20 ml. of water. Shake the separator and discard the water. Wash the solution with an additional 20 ml. of water and discard the water. Pass the benzene solution through 4–6 Gm. of anhydrous sodium sulfate into a 50-ml. volumetric flask. Rinse the separator with several small portions of benzene, passing each through the sodium sulfate. Dilute to volume with benzene. (This is solution *B*, ketosteroid fraction.)

Color Development.—*General Comments.*—In order to obtain completely developed colors yielding reproducible and precise absorbances in the determinations below, the following general requirements for the heating sequences must be met in each. (*a*) The heating bath must be vigorously boiling; (*b*) the tubes should be immersed in the heating bath to a depth not less than two-thirds of their length to insure complete expulsion of the solvent; (*c*) the holder for the tubes must not allow any condensate to enter the tubes (a disk of sufficient diameter to cover completely the opening of the bath and drilled to support the tubes by their lips is adequate; unused holes should be plugged); and (*d*) the holder should be fitted with a sturdy handle to facilitate the inserting, swirling, and removing of all the tubes in the baths at the same instant.

Determination of Total Estrogens as Sodium Estrone Sulfate.—Deliver duplicate 1.0-ml. aliquots of solution *A* and *Standard Estrone* directly to the bottom of 18 × 150-mm. test tubes. Prepare a blank, using 1.0 ml. of benzene. Add 1.0 ml. of modified Iron-Kober reagent[4] to each tube. Heat the tubes in the boiling water bath for exactly 20 min. After the first 5 min. (all of the benzene should have been expelled by this time), collectively swirl the tubes to insure complete mixing. Cool the

[2] The rubber stopper portion of a Walter-type Gooch crucible holder is suitable.

[3] This forerun will generally be yellow due to the iron present in the siliceous earth; it is usually removed in this volume of solvent.

[4] The Iron-Kober reagent is conveniently dispensed from a large-bore graduated pipet.

TABLE I.— COMPARISON OF ANALYSIS OF EQUINE CONJUGATED ESTROGENS IN TABLETS BY TWO METHODS

Total Estrogens Declared, mg./Tablet	Sodium Estrone Sulfate, mg./Tablet		Sodium Equilin Sulfate, mg./Tablet		Total Estrogens, mg./Tablet	
	Modified	Carol	Modified	Carol	Modified	Carol
0.3	0.211	0.198	0.084	0.081	0.314	0.321
0.625	0.525	0.503	0.043	0.046	0.573	0.568
0.625	0.666	0.650	0.058	0.058	0.726	0.744
1.25	0.693	0.680	0.570	0.579	1.54	1.47
1.25	0.771	0.772	0.322	0.325	1.30	1.25

tubes in an ice-water bath and add 10.0 ml. of 33% sulfuric acid solution to each tube. Remove each tube from the bath and thoroughly mix the contents with a stirring rod (or, more conveniently, with a Vortex mixer) and return it to the ice bath. Reheat the tubes for exactly 5 min. in the boiling water bath and cool in the ice-water bath to room temperature. Record the absorbance spectra of the sample and standard solutions relative to the blank between 370 and 700 mμ. Calculate the total estrogens in the sample by:

$$\frac{A_1 C_1}{S_1 W} \times 138 + 0.78\,(NQS)$$

in which

A_1 = base line corrected absorbance of sample solution at 520 mμ
C_1 = concentration of estrone standard (mg./ml.)
S_1 = base line corrected absorbance of standard
W = number of tablets taken or weight of sample (Gm.)
NQS = sodium equilin sulfate content as determined

Determination of Equilin as Sodium Equilin Sulfate.—Deliver duplicate 1.0-ml. aliquots of solution *B* and of *Equilin Standard* directly to the bottom of 18 × 150-mm. test tubes, add exactly 0.6 ml. of alcohol to each, and mix thoroughly. (If the sample has a low equilin content, concentrate a suitable aliquot, not to exceed 20 mcg. total, in the test tube to no more than 0.1 ml. and add 0.6 ml. of alcohol. Rotate the tube to dissolve any ketosteroids adhering to the walls of the tube, then add 1.0 ml. of benzene, and mix thoroughly.) Prepare a blank, using 1.0 ml. of benzene and 0.6 ml. of alcohol. Add 1.0 ml. of Iron-Kober reagent slowly down the wall of each tube so that the reagent stratifies on the bottom of the tube with a minimum of mixing. (*Critical!*) Carefully place the tubes in the holder and heat in the boiling water bath for 29 min. Collectively swirl the tubes briefly but thoroughly (*critical!*) after the first 5 min. of heating. Cool the tubes in cold water to room temperature, dilute each with 3.0 ml. of 33% sulfuric acid solution, and mix well. Record the absorbance spectrum of each solution relative to the blank from 370 to 700 mμ. (The sample will show maxima at about 620 mμ and at about 520 mμ; a standard equilin solution that exhibits any other maxima or a pronounced shoulder between 600 and 450 mμ is incompletely developed.)

Calculate the sodium equilin sulfate (NQS) content of the sample by:

$$\frac{A_2 C_2}{S_2 W V} \times 138$$

in which

A_2 = absorbance of sample solution at the maximum at about 620 mμ
C_2 = concentration of equilin standard (mg./ml.)
S_2 = absorbance of standard equilin solution
V = volume of sample aliquot

Determination of Estrone as Sodium Estrone Sulfate.—Deliver duplicate 1.0-ml. aliquots of solution *B*, and of *Estrone Standard* and *Equilin Standard* directly to the bottom of 18 × 150-mm. test tubes. Prepare a blank, using 1.0 ml. of benzene. Add 1.0 ml. of modified Iron-Kober reagent to each tube, place in the holder, and heat for exactly 60 min. in the boiling water bath. Proceed as directed under the *Determination of Total Estrogens*, beginning, "After the first 5 min...." Record the absorbance spectra of the sample and standards relative to the blank from 400 to 600 mμ. Calculate the sodium estrone sulfate content of the sample by the formula:

$$\frac{A_3 - \left(\frac{S_3 A_2}{S_2}\right)}{S_4 W} \times 138\ C_1$$

in which

A_3 = absorbance of sample solution at 520 mμ
S_3 = absorbance of equilin standard at 520 mμ
S_4 = absorbance of estrone standard at 520 mμ

RESULTS

A comparison of analyses for sodium estrone sulfate, sodium equilin sulfate, and total estrogens as sodium estrone sulfate in several commercial tablet preparations by this method and by the procedure of Carol *et al.* is presented in Table I.

REFERENCES

(1) Carol, J., *et al.*, *J. Pharm. Sci.*, **50**, 550(1961).
(2) Haenni, E. O., *J. Am. Pharm. Assoc., Sci. Ed.*, **39**, 544(1950).