Determination of Dienestrol in Pharmaceuticals

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A polarographic method and an ultraviolet irradiation method for the determination of dienestrol in pharmaceuticals are presented. Both methods are capable of producing results of a higher degree of accuracy and precision than those obtainable by the U.S.P. XVI method.

A VARIETY OF methods have been proposed for the determination of the synthetic estrogens including nitration, diazotization, reduction, and the formation of addition compounds (1–9). Wessely and Wratil (10) and Goulden and Warren (11) determined *cis*- and *trans*-stilbene polarographically; however, α,β-dimethylstilbene, diethylstilbestrol, and dienestrol were not reduced polarographically. Gry (12) polarographically determined dienestrol and diethylstilbestrol in addition to some steroidal estrogens by means of their nitroso derivatives according to the methods reported by Baggesgaard-Rasmussen (13–17). Gottlieb (18) presented basic data on the nitrosophenol reaction for several synthetic estrogens and subsequently developed a colorimetric procedure for the determination of diethylstilbestrol in pharmaceuticals (19). Goodyear *et al.* (20) introduced an ultraviolet irradiation technique for the determination of diethylstilbestrol in tablets which was later modified by Banes (21), who observed that dienestrol also produced a yellow color upon ultraviolet irradiation.

The U.S.P. XVI procedure for dienestrol in tablets utilizes the Folin-Denis reagent in a colorimetric procedure. This reaction is not particularly specific because difficulties are encountered in the presence of reducing substances. This investigation was undertaken in an effort to develop a method of greater specificity than that based on the Folin-Denis color reaction.

EXPERIMENTAL

Polarographic Assay

Apparatus

Sargent model XXI recording polarograph: both the large (20-ml. sample volume) and small (3-ml. sample volume) H-type cells were used. The saturated calomel electrode (SCE) is separated from the sample compartment by an agar plug and fritted glass disk. The electrode capillary delivered 1.136 mg. of mercury per second at a column height of 45 cm. with a drop time of 4.20 sec.

Assay

Buffer Solution.—Dissolve 1.5 Gm. of potassium hydroxide in 10 ml. of water contained in a 100-ml. volumetric flask, add 80 ml. of glacial acetic acid, dilute to volume, and mix.

Electrolyte Solution.—Transfer 5.4 Gm. of ammonium chloride and 45 ml. of stronger ammonia water to a 100-ml. volumetric flask, dilute to volume, and mix.

Standard Preparation.—Dissolve a suitable quantity, accurately weighed, of N.F. dienestrol reference standard[1] in methanol; dilute quantitatively and stepwise with methanol to obtain a solution of known concentration containing about 200 mcg./ml. Pipet 5 ml. of this solution into a glass-stoppered conical flask and evaporate to dryness on a steam bath with the aid of a current of air. Proceed as directed under *Assay Preparation*, beginning with "Dissolve the residue in 5.0 ml. of buffer solution. . . ."

Assay Preparation.—Pack a pledget of fine glass wool in the base of a chromatograpnic tube, 25 × 300 mm., with the aid of a tamping rod. To 2 Gm. of acid-washed chromatographic siliceous earth[2] in a 150-ml. beaker, add 1 ml. of approximately 1 *N* tribasic potassium phosphate and mix until uniform. Transfer the mixture to the column and tamp to compress.

Weigh and finely powder not less than 20 dienestrol tablets. Weigh accurately a portion of the powder equivalent to about 1 mg. of dienestrol and transfer it to a 150-ml. beaker. Add 6 Gm. of acid-washed chromatographic siliceous earth and mix thoroughly. Add 4 ml. of approximately 1 *N* tribasic potassium phosphate and mix until the mixture is fluffy. Transfer it quantitatively to the column in two portions, tamping each portion to compress the material to a uniform mass. Place a pledget of glass wool on the top of the column. Wash the column with 100 ml. of iso-octane and discard the washings. Elute the column with 150 ml. of water-washed chloroform, collecting the eluate in a 250-ml. glass-stoppered conical flask. Evaporate the solution to dryness on a steam bath with the aid of a current of air.

Dissolve the residue in 5.0 ml. of *Buffer Solution*, stopper the flask, and shake vigorously. Pipet 4.0 ml. of this solution into a 25-ml. volumetric flask, add 3 drops of sulfuric acid and 5 drops of a freshly prepared saturated solution of sodium nitrite, and mix. Allow the reaction to proceed for 30 min. Immerse the flask in an ice bath and add about 20

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[1] Reference is made to N.F. reference standard material since dienestrol will be official in N.F. XII.
[2] Acid-washed Celite, Johns-Manville Corp.

ml. of *Electrolyte Solution*. After about 1 min., remove the flask, allow the solution to come to room temperature, and dilute to volume with *Electrolyte Solution*.

Procedure.—Transfer a portion of the *Assay Preparation* to a polarographic cell immersed in a water bath regulated at 24.5° to 25.5° and deaerate by bubbling purified nitrogen through the solution for 10 min. Insert the dropping mercury electrode of a suitable polarograph and record the polarogram from −0.2 to −1.25 v., using a saturated calomel electrode as the reference electrode. Determine the height of the diffusion current at −0.95 v. Calculate the quantity, in milligrams, of $C_{18}H_{18}O_2$ in the portion of the tablets taken by the formula $0.0313\ C[(id)_u/(id)_s]$, in which C is the concentration, in micrograms per milliliter, of N.F. dienestrol reference standard in the *Standard Preparation*, $(id)_u$ is the observed diffusion current value of the *Assay Preparation*, and $(id)_s$ is that determined similarly on the *Standard Preparation*.

Ultraviolet Irradiation Assay

Apparatus

A Beckman DK-2 or other recording spectrophotometer, operative between 600 and 370 mμ, was used. The irradiation apparatus was constructed from a vertically mounted tubular (about 18 in. long) 15-w. germicidal ultraviolet lamp (General Electric) passing through the center of a fixed rigid circular disk. Holes of sufficient diameter to support the tubes were drilled in the disk at regular intervals on a 6-cm. radius. The position of the disk relative to the ends of the lamp was such that the assay solutions to be irradiated would encircle the lamp near its center. The exposed portion of the lamp above the disk was simply enclosed in a suitable opaque covering; below the disk, the irradiation chamber was formed by fitting an ordinary desk blotter lined with aluminum foil (dull side facing the lamp) around the circumference of the disk. The disk may be constructed with holes drilled on a series of radii; the disk used in this laboratory has holes also on radii of 10 and 13 cm.

Assay

Acetate Buffer.—Transfer 133 Gm. of anhydrous sodium acetate and 20 ml. of glacial acetic acid to a 1000-ml. volumetric flask, dilute to volume, and mix.

Methanolic Buffer Solution.—Transfer 25 ml. of *Acetate Buffer* to a 100-ml. volumetric flask and dilute to volume with methanol.

Standard Preparation.—Dissolve in methanol a suitable quantity of N.F. dienestrol reference standard, accurately weighed, and dilute quantitatively and stepwise to obtain a solution of known concentration containing about 40 mcg./ml. Transfer to a suitable vessel 10.0 ml. of this solution and add 10.0 ml. of *Methanolic Buffer Solution*.

Assay Preparation.—(Proceed as directed under *Assay Preparation* in the proposed polarographic assay for dienestrol tablets through the evaporation of the eluate to dryness.) Add 25.0 ml. of methanol to the residue and let stand for 15 min. with frequent swirling. Add 25.0 ml. of *Methanolic Buffer Solution* and mix.

Standardization of Irradiation Procedure.—(*Caution:* Protect the eyes from direct rays of ultraviolet light in the following procedure.) Use Vycor 7900 tubes, 18 × 150 mm. or equivalent, the transmittance characteristics of which have been tested as follows.

Transfer a convenient volume of the *Standard Preparation* to each of the tubes, stopper each, place them 6.0 cm. from a 15-w. germicidal lamp, and irradiate the solutions transversely for about 30 min. Record the spectrum of the yellow solutions in 1-cm. cells between 600 and 370 mμ and measure the absorbance of the maximum at about 410 mμ, using water as the blank. Again irradiate the solutions for 5-min. intervals. Note the total time required for which the change in absorbance between successive intervals does not exceed 5%. Select those tubes which show substantially identical maximum absorbances (about 0.550) after irradiating for this total period of time.

Procedure.—Transfer convenient volumes of the *Standard Preparation* and of the *Assay Preparation* to separate tubes selected as described under *Standardization of Irradiation Procedure*. Irradiate the solutions for the optimum time interval established. Record the spectrum of the yellow solutions between 600 and 370 mμ, using water as the blank. Correct the absorbance of the maximum at about 410 mμ for background absorbance by linearly extrapolating that portion of the spectrum between 450 and 550 mμ just beyond the maximum and subtract the absorbance at the point on this line directly under the maximum from the total absorbance at about 410 mμ. Calculate the quantity, in milligrams, of $C_{18}H_{18}O_2$ in the portion of the tablets taken by the formula $0.05\ C\ (A_u/A_s)$, in which C is the exact concentration, in micrograms per milliliter, of N.F. dienestrol reference standard in the *Standard Preparation*, A_u is the baseline absorbance of the irradiated *Assay Preparation*, and A_s is the baseline absorbance of the irradiated *Standard Preparation*.

RESULTS AND DISCUSSION

The U.S.P. XVI assay procedure for dienestrol tablets does not provide for the separation of the estrogen from interfering substances prior to quantitation. The presence of lactose in a formulation will yield high assay values, indicated by the results in Table I. By modification of the U.S.P. XVI procedure to remove lactose, more accurate results are obtained. The procedure was modified by diluting an aliquot of the U.S.P. assay preparation with water and extracting with ether, filtering the ether extracts, and evaporating to dryness. The residue then was dissolved and made to volume with 50% ethanol and determined as directed in the U.S.P. XVI assay.

Polarographic Method

The formation of stable nitroso compounds has been used by a number of investigators, in addition

TABLE I.—ASSAY OF 0.1-mg. TABLETS

U.S.P. XVI, % of Declared	Modified U.S.P., % of Declared
141	100.6[a]
145	101.2[b]

[a] Aliquot of U.S.P. XVI assay preparation diluted with water and extracted with ether. [b] Soxhlet extraction of sample with ether.

TABLE II.—COMPARISION OF RESULTS BY PROPOSED METHODS AND MODIFIED U.S.P. METHOD

	Polarography, % of Declared	Irradiation, % of Declared	Modified U.S.P., % of Declared
Simulated tablet mixture			
0.1 mg./tablet	99.5	100.3	
	99.4	100.3	
0.5 mg./tablet	99.9	100.2	
	99.5	100.8	
Commercial tablets			
0.1 mg.	90.9	91.0	91.0[a]
	91.3	92.0	92.0
	91.5	90.3	94.0[b]
	91.6	90.0	89.0
			91.0[c]
			85.0
0.5 mg., *A*[d]	104.6	102.6	105.8[c]
	105.2	104.6	103.8
	105.0		
	104.8		
0.5 mg., *B*[d]	95.0	95.6	95.6[c]
	94.8	95.3	94.0
	94.8	95.2	

[a] Absolute alcohol extraction. [b] Acetone extraction. [c] Methylene chloride extraction (Soxhlet). [d] *A* and *B* signify different lots.

to those already cited, for the polarographic determination of pharmaceuticals (22–26). Using the method suggested by Gry (12) for the determination of dienestrol, reproducible results could not be obtained, primarily because of the distorted wave produced at the low concentration levels used. To use larger amounts, as suggested in the method, would not be practical because of the relatively low concentration of dienestrol in commercial tablet preparations. The excipient material present, especially stearates, must be removed; otherwise, they interfere with the polarographic analysis. Liquid-liquid extraction techniques, such as the modified U.S.P. XVI procedure, are unsatisfactory since they do not separate stearic acid from dienestrol.

The conversion of dienestrol to its nitroso derivative is completed in 30 min.; after dilution to volume with the recommended electrolyte solution, it is stable for several hours. The amount of ammonium hydroxide solution specified in the electrolyte solution is essential for stopping the reaction. In the polarographic method proposed, the diffusion current was found to be proportional to dienestrol concentration within the range of 8 mcg./ml. and 90 mcg./ml. As shown by the results in Table II, the experimental error by this method of analysis was found to be ±1%, with a precision of ±1%.

Irradiation Method

Uniform and reproducible irradiation conditions are essential to this method. The irradiation apparatus described above facilitates these requirements by (*a*) maintaining at all times a fixed distance between the tubes and lamp and (*b*) reducing the effect of possible variable light intensity along the axis of the lamp, particularly near its ends. Good results can be obtained from a simpler apparatus made from a test tube rack and a germicidal lamp fitted with a reflector, provided the above requirements are met carefully.

The yellow irradiation product of dienestrol forms considerably more slowly than that obtained from diethylstilbestrol under any conditions yet employed; however, the two substances appear to be quite similar. The infrared spectrum of this product from dienestrol indicates no phenolic hydroxyl, but shows an intense absorption at 6.02 μ and is similar to the spectrum reported by Banes (21) for the yellow product from diethylstilbestrol. Banes considered this product to be a tricyclic conjugated ketone of the phenanthrene series. A further similarity of these two substances is their reaction with $NaHSO_3$ and dilute HCl; the infrared spectra of the colorless products formed show the loss of the carbonyl absorption and the reappearance of hydroxyl absorption. The ultraviolet spectra are suggestive of substituted phenanthrene diols.

Thin-layer chromatography of irradiated dienestrol solutions indicates a mixture of substances, the complexity of which is greatly affected by the reaction medium. It is hoped that continued investigations will culminate in the isolation and identification of the substances as well as the yellow product and their relation to it, thus supporting the hypothesis that dienestrol is isomerized slowly to a stilbene, which then cyclizes to a phenanthrene derivative in the manner proposed by Hugelshofer *et al.* (27).

In the ultraviolet irradiation procedure proposed, the color produced is proportional to concentration in the range between 5 and 30 mcg./ml. As shown by the results in Table II, the experimental error by this method of analysis was also found to be ±1%, with a precision of ±1%.

SUMMARY

Commercial dienestrol tablets were analyzed by both an ultraviolet irradiation and a polarographic procedure and were compared to the U.S.P. XVI assay. More accurate results are obtained than with the U.S.P. assay. The proposed methods compare favorably with each other. Therefore, the choice of which procedure to employ is left to individual preference concerning technique.

The polarographic method depends upon the formation of a stable nitroso derivative of dienestrol and its subsequent reduction, while the ultraviolet irradiation procedure relies upon conversion of dienestrol to a yellow nonphenolic substance and its determination by spectrophotometry.

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Technical Articles

Stabilization of Fluoride Solutions in Glass Containers by Aluminum

By J. R. MELLBERG

The addition of sodium fluoride to liquid multivitamin preparations at acid pH in glass containers results in a reaction with glass. The addition of equimolar amounts of aluminum chloride and ethylenediaminetetraacetic acid to an ascorbic acid solution has shown no fluoride–glass reaction over a period of 5 months. Ascorbic acid stability remained good, and *in vivo* absorption of fluoride from a similarly stabilized multivitamin solution was approximately 80 per cent.

The addition of fluoride to liquid vitamin preparations has become widely accepted, especially for pediatric use. It has been shown, however, that when sodium fluoride is added to multivitamin solutions in glass containers at the most acceptable pH range of 2.5 to 4.5, a precipitate, which can be identified as sodium fluorosilicate (Na_2SiF_6) (1), occurs within 24 hr. Because of this, most vitamin–fluoride preparations are dispensed now in plastic bottles. Since glass has certain advantages, such as clarity and lack of oxygen transmission, which are superior to plastic, it was desired to find a way to stabilize fluoride in such containers. Of the usual inorganic elements known to complex fluoride (2), aluminum has the widest use in human medication and is known for its low toxicity. The addition of metals, like aluminum to vitamin solutions, however, is likely to increase the rate of decomposition of ascorbic acid. Therefore, an agent to complex aluminum was needed also. A combination of aluminum ion and ethylenediaminetetraacetic acid (EDTA) was found to provide both ascorbic acid and fluoride stability. Other fluoride or aluminum complexing agents having stability constants of the proper magnitude could be used also with the obvious limitations of low toxicity and solution compatibility.

The physiological availability of the aluminum complexed fluoride was determined *in vivo* by analysis of urinary fluoride after ingestion of known quantities of the vitamin–fluoride preparation.

Table I.—Effect of Various Concentrations of $AlCl_3$ and EDTA on Ascorbic Acid Stability in an Ascorbic Acid Preparation at pH 4.3

mmoles/L. F	mmoles/L. Al	mmoles/L. EDTA	Ascorbic Acid Concn., mg./ml. 4 Days	2 Wk.	1 Mo.	5 Mo.
Room Temperature						
43.9	0	0	50.2	48.6	49.5	43.3
43.9	0	97.8	51.8	50.3	48.1	43.3
43.9	43.9	43.9	52.4	48.6	47.2	43.7
43.9	43.9	0	51.4	49.1	47.2	40.6
43.9	97.8	0	46.2	44.8	43.7	36.6
43.9	195.6	0	38.3	32.9	32.3	28.3
43.9	195.6	195.6	51.0	49.1	48.5	44.2
43.9	195.6	97.8	45.8	41.4	40.1	36.2
50°C.						
43.9	0	0	47.1	39.7	32.7	8.4
43.9	0	97.8	48.4	41.9	33.2	8.9
43.9	43.9	43.9	...	40.7	31.4	9.3
43.9	43.9	0	44.0	35.0	22.7	4.9
43.9	97.8	0	40.9	29.9	15.7	2.8
43.9	195.6	0	33.4	25.2	12.6	4.0
43.9	195.6	195.6	...	42.3	31.4	9.7
43.9	195.6	97.8	...	33.3	20.9	4.4

EXPERIMENTAL

Determination of Ascorbic Acid and Fluoride Stability.—An ascorbic acid solution was prepared by dissolving 35 Gm. of ascorbic acid in 175 ml. of glycerol, 105 ml. of propylene glycol, 52.5 ml. of polysorbate 80,[1] and 200 ml. of distilled water

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[1] Marketed as Tween 80 by Atlas Chemical Industries, Wilmington, Del.