Selective Colorimetric Determination of Microgram Amounts of Quinestrol and Ethinyl Estradiol in Dosage Forms

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Ethinyl estradiol and its 3-cyclopentyl ether, quinestrol, react with a methanol–sulfuric acid reagent of critical composition to form a stable color with $\lambda_{max.}$ 540 mμ and ε 36,000. The chromogenic reaction appears to be specific for these estrogens and the related steroid, mestranol. Separations required utilize only simple solvent extraction, and the use of the nonpolar solvent, petroleum ether, affords additional selectivity in the assay of dosage forms. Application of the method for the assay of quinestrol and ethinyl estradiol in representative dosage forms is described. The assay has sensitivity, precision, and accuracy suitable for its use in content uniformity testing of dosage forms declaring 10 mcg. or more without resort to micro techniques.

A NEED FOR a facile and accurate means for assaying microgram amounts of quinestrol in the numerous formulations produced in the course of process evaluation, stability testing, and clinical studies led to the examination of procedures for isolating and quantitating the estrogen from individual dosage units. Work on the assay of quinestrol, the 3-cyclopentyl ether of ethinyl estradiol, led to a superior assay for ethinyl estradiol itself. The procedure appears equally applicable to assay of mestranol, the 3-methyl ether of ethinyl estradiol.

The Iron-Kober assay for ethinyl estradiol used in U.S.P. XVII (1) is equally applicable to quinestrol, but the procedure requires meticulous technique for satisfactory results, and it is insufficiently rapid and precise for the analysis of single tablets. The azo dye method for phenolic estrogens (2) is a general chromogenic reaction for phenols, inapplicable to quinestrol determination and lacking in sensitivity adequate for process control studies. Gas-liquid chromatographic assays for ethinyl estradiol and mestranol (3–7) provide both sensitivity and specificity, but the procedures suffer from excipient interference, and the precision reported (7) was unsatisfactory for our purposes. Thin-layer chromatography (3, 8) and paper chromatography (9) were less suitable from the standpoints of time, convenience, and accuracy than the colorimetric method reported here.

Treatment of many estrogens with concentrated sulfuric acid results in a red color with green fluorescence (10), a procedure used in U.S.P. as an identification reaction for ethinyl estradiol. Reaction of ethinyl estradiol or quinestrol with the ethanol–sulfuric acid reagent described by Heusghem and Jehotte (11) affords an intense red color with green fluorescence, but the color fades rapidly with detriment to the precision and accuracy of the method. Study of this reaction led to the development of a methanol–sulfuric acid chromogenic reagent of definite and critical proportions which provided a stable and intense color suitable for measurement of microgram amounts of the estrogens.[1] The reaction was found surprisingly specific for ethinyl estradiol and its 3-ethers among a number of representative estrogens, although noninterfering chromophores were produced with three other estrogenic steroids.

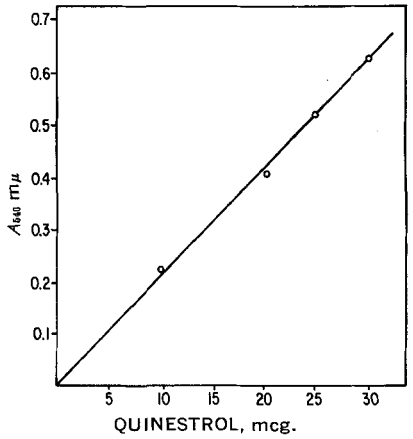

Fig. 1—Beer's law plot for the quinestrol chromophore.

EXPERIMENTAL

Equipment and Supplies—Absolute methanol and sulfuric acid were analytical reagent grade. The petroleum ether should give no color with concentrated sulfuric acid (Mallinckrodt's Nanograde was

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[1] Subsequent to presentation of this manuscript we learned that a similar method has been used for mestranol at Eli Lilly and Co. (12).

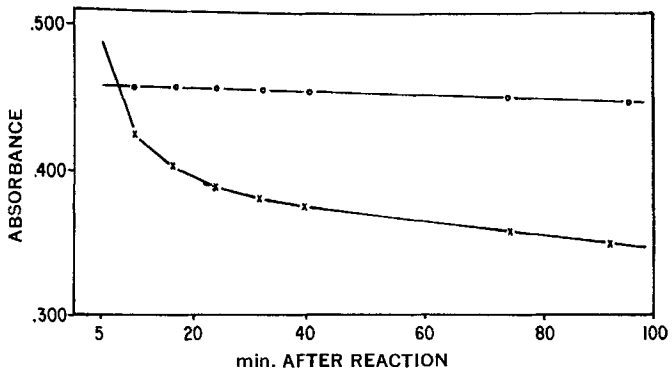


Fig. 2—Stability of color produced in the Heusghem and Jehotte and in this procedure for 20 mcg. of ethinyl estradiol. Key: ×, Heusghem and Jehotte; O, this procedure.

used). The separators used were fitted with Teflon stopcocks. A Beckman DU spectrophotometer and a Beckman DK-2 recording spectrophotometer were employed with 1-cm. silica cells for absorbance measurements.

The steroids used were official grades or comparable purity. These included reference standard quinestrol, ethinyl estradiol U.S.P., mestranol (Syntex), estrone N.F., estrone 3-cyclopentyl ether (Vismara Terapeutici, Casatenovo, Como, Italy), and β-estradiol N.F. α-Estradiol, equilin, 17-α-dihydroequilin, and equilinin were obtained from Ayerst Laboratories.

Chromogenic Reagent—Cautiously add concentrated sulfuric acid to 30.0 ml. of chilled absolute methanol in a 100-ml. volumetric flask. Adjust to room temperature and take the solution to the mark with acid. The reagent appears stable for at least 7 days.

Standard Preparation—Dissolve about 50 mg. of quinestrol or ethinyl estradiol, accurately weighed, in methanol and dilute to 100.0 ml. Further dilute 5.0 ml. to 50.0 ml. with absolute methanol to obtain a solution containing about 25 mcg. in 0.50 ml.

Assay of Tablets and Capsules—Determine the average unit weight of not less than 10 units for composite sample assay, and reduce the sample to a fine powder in a glass mortar. Transfer a weighed sample equivalent to about 25 mcg. of quinestrol or ethinyl estradiol to a 125-ml. separator containing 10 ml. of water, add 4–5 drops of concentrated hydrochloric acid, and extract the mixture with two 75-ml. vol. of petroleum ether, shaking vigorously for 2 min. during each extraction. Combine the solvent extracts in a second separator, and wash with one 10-ml. vol. of 1% sodium carbonate to remove colorants, if present. Discard the aqueous phase, and quantitatively transfer the combined extracts to a third, *scrupulously dry* separator through a cotton pledget filter with the aid of an additional 10 ml. of petroleum ether. Add exactly 0.5 ml. of anhydrous methanol, then add exactly 5.0 ml. of chromogenic reagent. Shake the mixture vigorously for 4 min., and allow the phases to separate. Discard the first few drops of the colored lower phase through the bore of the stopcock, and deliver about 4.5 ml. of the lower phase into a dry test tube. Withdraw exactly 4.0 ml. of the solution by pipet into a dry glass-stoppered centrifuge tube, add exactly 0.3 ml. of anhydrous methanol, mix well, and clarify if necessary by centrifugation. Determine the absorbance of the solution at the maximum, about 540 mμ, designating the absorbance A_u.

Concomitantly develop a color with the standard preparation, using 0.5 ml. of the standard preparation and 150 ml. of petroleum ether in a separator and following the procedure described above, beginning with "... add exactly 5.0 ml. of chromogenic reagent." Designating the concentration of the standard preparation in mcg./0.50 ml. as C and the absorbance of the color produced from it as A_s:

$$\text{estrogen, mcg./unit} = A_u/A_s \times C \times \text{mg. av. unit wt./mg. sample wt.}$$

Individual Unit-Dose Assay—Use the procedure described above with an individual intact tablet or the contents of one capsule as sample in place of the composite, adjusting the volumes of reagents appropriately for dosage forms containing more than 25 mcg. of estrogen.

Assay of Ethinyl Estradiol in a Complex Dosage Form—The example chosen was a tablet[2] declaring 5 mcg. of ethinyl estradiol, 2.5 mg. of methyltestosterone, and 16 mg. of thyroglobulin. Transfer an accurately weighed amount of pulverized tablet composite equivalent to about 15 mcg. of ethinyl estradiol to a 250-ml. separator containing 10 ml. of water, add 1-ml. of concentrated hydrochloric acid, and mix thoroughly. Extract the mixture with two 100-ml. vol. of petroleum ether, shaking about 2 min. during each extraction. Combine the extracts in a second 250-ml. separator, and wash the extract with three 5-ml. vol. of water, discarding the washings. Extract the organic phase with two 10-ml. vol. of 10% sodium hydroxide, shaking for 5 min. each time. Transfer the aqueous phases through a small cotton pledget into a third separator containing 7 ml. of concentrated hydrochloric acid. Discard the organic phase, then continue as described above for the general procedure, starting with "... extract with two 75-ml. vol. of petroleum ether. ..."

RESULTS AND DISCUSSION

Validity of Beer's Law at 540 mμ—Concentration was found to be proportional to absorbance for the chromophore produced by reaction of quinestrol or ethinyl estradiol with the chromogenic reagent. A typical Beer's law plot is shown in Fig. 1. It may be noted that assay of individual 10-mcg. tablets affords

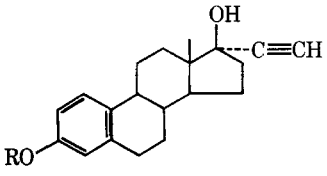
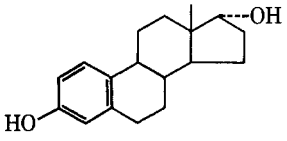
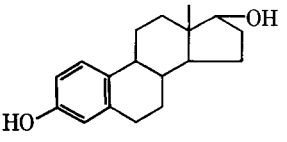
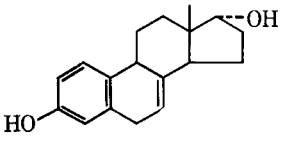
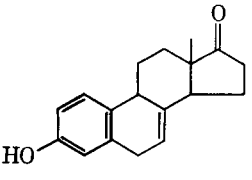
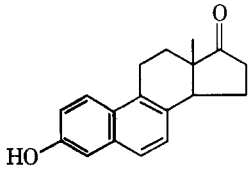
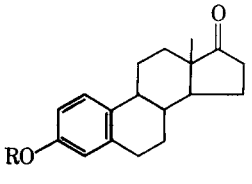
[2] Marketed as Plestran Tablets by Warner-Chilcott Laboratories.

absorbance values at the low end of the optimum range for spectrophotometric accuracy. While the use of micro cells would have afforded better absorbance values by permitting smaller volumes of chromogenic reagent, this expedient was not resorted to in this study.

Color Stability—The stability of the color produced in the Heusghem and Jehotte procedure (11) and the color produced in the procedure reported here are compared in Fig. 2. The initial rapid fading of the color in the published procedure led to erratic results in routine assays. The difference in the methods was more marked with assay samples than with standards, for the petroleum ether solvent affords more selectivity than the more polar and hygroscopic diethyl ether used in the published method. Moreover, the new procedure obviates a solvent evaporation step.

Selectivity of the Color Reaction—The remarkable selectivity of the chromogenic reaction is illustrated in Table I. The absorptivity values reported are approximations obtained by assuming no volume changes on mixing reagents. Spectra of the ethinyl estradiol chromophore and the yellow chromophore from 17-α-dihydroequilin are shown in Fig. 3. The reaction is more selective than reactions using similar

TABLE I—VISIBLE SPECTRAL DATA FOR THE METHANOL–SULFURIC ACID CHROMOPHORE OF VARIOUS ESTROGEN STEROIDS

	Estrogen	λ max., mμ	a	ε
	Ethinyl estradiol (R = H)	540	110.2	36000
	Quinestrol (R = cyclopentyl)	540	92.8	36000
	Mestranol (R = methyl)	540	110	34000
	α-Estradiol	452	91.4	24000
	β-Estradiol	Nil	...	...
	17-α-Dihydroequilin	452	155	42000
	Equilin	452	67.2	18000
	Equilinin	Nil	...	...
	Estrone (R = H)	Nil	...	...
	Estrone 3-cyclopentyl ether (R = cyclopentyl)	Nil	...	...

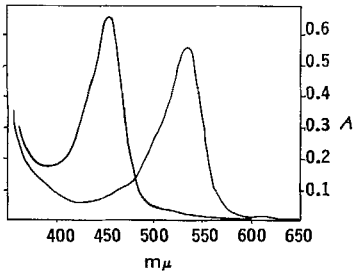


Fig. 3—Visible spectra of the chromophore from 25 mcg. of ethinyl estradiol (peak at right) and 17-α-dihydroequilin.

reagents, such as the Kober reaction to which all estrogens respond (13) and the Wintersteiner-Pfiffner green fluorescence in sulfuric acid (14). The lack of color formation in the reaction with estrone or its 3-ether suggests that the chromogenic reaction involves the groups at the 17-position of ethinyl estradiol and its ethers, but it is more difficult to adduce a structural feature common to the three other estrogens which results in the yellow chromophore. Elucidation of the apparent selectivity of the reactions of estrogens with methanol–sulfuric acid awaits characterization of the chromophores.

A possible, although not observed, degradation route for quinestrol in dosage forms is ether hydrolysis to form ethinyl estradiol. The latter might also be a by-product of quinestrol or mestranol synthesis. Extraction from alkaline solution, where any ethinyl estradiol would be retained as phenolate ion, would provide a selective assay for quinestrol or mestranol. As noted above, use of a nonpolar solvent in the isolation procedure confers a high degree of selectivity. No interference was encountered from red colorants used in tablet formulations, and spectrophotometric scans of the chromophore indicated absence of interferences in every formulation assayed.

Effect of Water on the Chromogenic Reaction—The necessity for employing dry reagents and glassware in the reaction was tested by adding varying amounts of water in the reaction of 20 mcg. of ethinyl estradiol with the chromogenic reagent. The effect is evident in Table II.

Recovery of Quinestrol Added to Placebo Tablets—Essentially quantitative recovery of added quinestrol was obtained. This was demonstrated by experiments using 20.0 mcg. of quinestrol and 150 mg. of excipients. These data are presented in Table III.

Table II—Effect of Added Water on the Color Obtained with 20 mcg. Ethinyl Estradiol

Water Added, ml.	0	0.05	0.10	0.15	0.20
A_{540} mμ	0.455	0.430	0.410	0.390	0.335

Table IV—Sextuplicate Assays of Commercial Ethinyl Estradiol Tablets

Brand	Declared, mcg.	Found, mcg.	±S.D.	Relative S.D., %
A	10.0	10.5	0.12	1.2
B	20.0	19.8	0.39	2.0

Table V—Assay of Individual Quinestrol Tablets

	Declared, mcg./Tablet	
	10.0	25.0
	Found, mcg./Tablet	
1	10.0	24.2
2	9.3	24.2
3	10.3	24.8
4	9.5	23.2
5	9.5	25.2
6	11.0	23.2
7	9.8	26.0
8	9.5	24.2
9	10.5	23.4
10	9.3	24.8
	Av., mcg./Tablet	
	9.9	24.3
	Relative S. D., %	
	5.8	3.4

Assay of Quinestrol in Tablets—Seventeen trials on composites of tablets declaring 25 mcg. gave a mean of 24.4 mcg. (97.6%) with a relative standard deviation of 0.5 mcg. (2%). Fifteen trials on tablets declaring 10 mcg. provided a mean value of 9.6 mcg. Here the relative standard deviation from the mean was 0.28 mcg. or 2.9%.

Assay of Ethinyl Estradiol Tablets—Composite samples of two commercially available tablet formulations of ethinyl estradiol marketed by different reputable domestic manufacturers were assayed by the proposed procedure in sextuplicate. Table IV shows the results obtained. The precision compares quite favorably with that obtained in other procedures. Boughton *et al.* (7) reported over-all standard deviations of ±5.5% by the U.S.P. method and ±8.6% for their gas chromatographic procedure in assays of ethinyl estradiol tablets declaring 100 mcg. Heusser (8) reported a ±5% precision for assays of 20 mcg. tablets using colorimetry following a thin-layer chromatographic separation of the drug.

Content Uniformity Assays—Since process control studies require the assay of individual dosage units to insure uniform distribution of microgram amounts of drugs in mg. amounts of excipient matrix, the proposed procedure was applied to quinestrol tablets. The results of individual tablet assay on two typical quinestrol formulations are presented in Table V. Since the precision obtained in recovery

Table III—Recovery of 20.0 mcg. of Added Quinestrol

Trial	1	2	3	4	5	6	7
Found, mcg.	19.5	19.8	20.2	20.2	20.0	19.3	20.0

Av. recovery ± S.D. = 19.9 ± 0.34 mcg. or 99.5 ± 1.7%

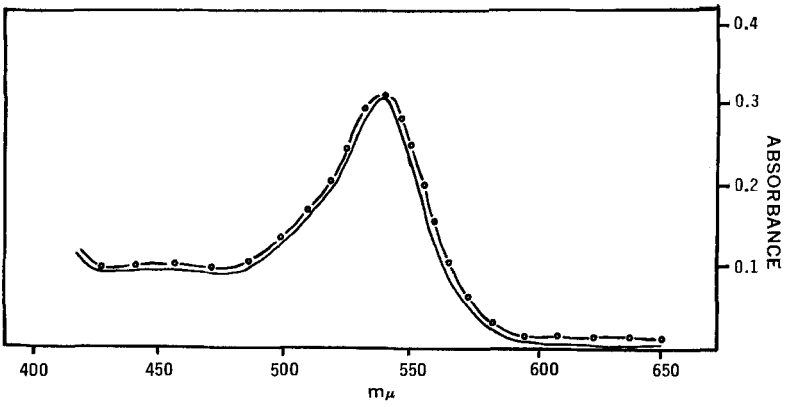


Fig. 4—Recovery of 15 mcg. of ethinyl estradiol from the tablets. (See Footnote 2). *Key: —, ethinyl estradiol standard; -·-·-·-·-, sample.*

experiments with quinestrol was 1.7%, the assay appears quite suitable for use in process control studies.

Ethinyl Estradiol Assay in the Estrogen–Androgen–Thyroglobulin Tablets—Recovery experiments for 10.0 mcg. of ethinyl estradiol added to the equivalent of two placebo tablets yielded values of 10.1 and 9.9 mcg.; for 15.0 mcg. added to the equivalent of three placebo tablets, a value of 15.6 mcg. was obtained. Assay of a production lot of tablets provided a value of 5.03 mcg./tablet or 102% of the declared amount. The absorption spectra of the sample and standard shown in Fig. 4 indicate the procedure provides clean separation from interferences.

SUMMARY AND CONCLUSION

Ethinyl estradiol, its 3-cyclopentyl ether, quinestrol, and its 3-methyl ether, mestranol, react with a methanol–sulfuric acid reagent of critical composition to form a stable chromophore with $\lambda_{max.}$ 540 mμ and ϵ 3.6 X 10^4. The chromogenic reaction appears to be specific for these estrogens among a number of others tested. A noninterfering yellow color with $\lambda_{max.}$ 452 mμ and varying absorptivities is produced by the reagent with α-estradiol, equilin, and 17-α-dihydroequilin, but no color is obtained with β-estradiol, estrone, estrone 3-cyclopentyl ether, and equilinin.

Examples are given of the use of the reaction for colorimetric assay of microgram amounts of quinestrol and ethinyl estradiol in dosage forms. The sensitivity, precision, accuracy, and manipulative ease of the method compare favorably with the U.S.P. colorimetric assay for ethinyl estradiol and the various chromatographic assays for it and mestranol in the literature. Solvent extraction with petroleum ether is used for isolation of the estrogens from dosage forms; this procedure itself affords considerable selectivity, since most excipients and many co-formulated drugs are excluded. An example of the selectivity of the procedure is presented in the assay of mcg. amounts of ethinyl estradiol in the presence of mg. amounts of methyltestosterone and thyroglobulin in a commercial dosage form.

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