Validation of Methanol-Sulfuric Acid Colorimetry as a Stability Assay Method for Quinestrol by Comparison with a Gas Chromatographic Procedure

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Excellent correlation was obtained between assay values by the methanol-sulfuric acid colorimetric method and a highly selective gas-chromatographic procedure, the specificity of which was demonstrated by separations on two different columns. Quinestrol, ethinyl estradiol 3-cyclopentyl ether, was assayed by both methods after intentional degradation by heating long periods in hydrogen peroxide, hydrochloric acid, or sodium hydroxide solutions, and the identifiable products were measured quantitatively by gas chromatography. The colorimetric method was found to be a valid stability assay for quinestrol.

COLORIMETRIC DETERMINATION of quinestrol, ethinyl estradiol 3-cyclopentyl ether, provides a facile and accurate means for measuring microgram amounts of the estrogen in dosage forms. Since colorimetry eliminates the need for specialized apparatus and technique and provides a considerable advantage in speed, it is preferred to the use of chromatographic techniques for routine and, especially, for occasional assays. The purpose of the study reported here was to determine the validity of the colorimetric assay used in these laboratories for determination of quinestrol in the presence of its degradation products.

The pink color formed by reaction of ethinyl estradiol or its 3-ethers, quinestrol and mestranol, with a methanol–sulfuric acid reagent was shown to be a highly selective means for determining these estrogenic steroids by Tsilifonis and Chafetz (1); several closely related estrogens give either no color or noninterfering yellow colors under the prescribed conditions. Khoury and Cali (2, 3) recently described a fluorometric assay for ethinyl estradiol and mestranol which makes use of 90% sulfuric acid as the fluorogenic reagent. The reaction product with either reagent appears to be identical. Excitation and fluorescence spectra obtained with methanol–sulfuric acid reagent and ethinyl estradiol and its ethers are identical with those reported by use of 90% sulfuric acid, and the methanol–sulfuric acid fluorophore has been used to measure nanogram amounts of quinestrol contamination in other drug dosage forms here.

Khoury and Cali (2, 3) inferred that sulfuric acid-induced fluorescence is specific for undegraded ethinyl estradiol from their finding that only the undegraded steroid gave a fluorescent spot on a thin-layer chromatogram of thermally or photochemically degraded material. This report describes validation of the previously reported colorimetric assay (1) as a stability method for quinestrol dosage forms. A linear correlation is demonstrated between assay results obtained by the colorimetric method and a specific gas chromatography procedure on quinestrol samples partly degraded by heating with acid, alkali, or hydrogen peroxide. A colorimetric procedure which obviates interference from ethinyl estradiol, the gas chromatographic method, and observations on the stability of quinestrol is presented here.

EXPERIMENTAL

Equipment and Supplies—Absolute methanol, Phillips "pure" grade isooctane, and common laboratory reagents were used. Ethinyl estradiol USP, estrone 3-cyclopentyl ether (Warner-Vister Institute), estrone NF, and quinestrol reference standard were used in this study. The separators used in the colorimetric assay were fitted with Teflon stopcocks. Absorbance measurements were made in 1-cm. silica cells in a Beckman DU instrument and a Cary model 14 recording spectrophotometer. The gas chromatographic instrument and operating parameters are described below.

The *chromogenic reagent* was made by cautiously

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adding concentrated sulfuric acid to 30.0 ml. of chilled methanol in a 100-ml. volumetric flask, in small increments and with frequent agitation so that the mixture was made to the mark at room temperature. Too rapid addition of acid to alcohol results in a yellow reagent.

Degradation with Peroxide—Mixtures of 2.0 ml. of 1 mg./ml. methanolic quinestrol were heated at reflux in 250-ml. flasks with 50 ml. of methanol and 50 ml. of water containing 0.5, 1.0, 1.5, or 2.0 ml. of 30% hydrogen peroxide solution for about 22 hr. in one set of experiments and about 40 hr. for another. The mixtures were evaporated to dryness under reduced pressure in a rotary-film apparatus at about 40°. The residue was taken up in 15 ml. of chloroform and washed three times with 5-ml. volumes of water to remove residual peroxide. The washed chloroform was evaporated to dryness, and the residue was dissolved in 3.0 ml. of chloroform. The samples were apportioned for colorimetric and gas chromatographic assay.

Acid and Base Degradation—Mixtures of 2.0 ml. of 1 mg./ml. quinestrol in methanol were heated at reflux in 250-ml. flasks with 50 ml. of methanol and 50 ml. of 0.1 *N*, 1.0 *N*, 3.0 *N*, or 6 *N* hydrochloric acid or with 0.1 *N* or 1 *N* sodium hydroxide for periods of about 20 hr. The solutions were neutralized and evaporated to dryness at about 40°. The residues were extracted with 15 ml. of chloroform, the extracts were evaporated to dryness, and the residues were taken up in 3 ml. of chloroform. Portions of each sample were taken as assay samples in each method.

Colorimetric Method—Prepare the *standard preparation* by the following procedure: accurately weigh about 50 mg. of reference standard quinestrol, dissolve it in methanol, and dilute the solution to 100 ml. Further dilute 2.0 to 50 ml. with methanol to obtain a 20 mcg./ml. solution.

Dilute 1.0 ml. of the chloroform sample obtained by partial degradation to 25 ml. with methanol. Transfer 1 ml. containing the equivalent of about 20 mcg. of quinestrol to a 125-ml. separator. Add 10 ml. of water and 5 drops of concentrated hydrochloric acid. Extract the mixture with about 75 ml. of isooctane, shaking for 2 min. Allow the phases to separate, and discard the lower layer. Quantitatively transfer the isooctane to a second, *scrupulously dry* separator through a solvent-wetted cotton pledget filter, using about 10 ml. of fresh isooctane to effect the transfer. Equilibrate the isooctane solution with exactly 5.0 ml. of *chromogenic reagent* by shaking 2 min. Discard 2–3 drops of the pink lower phase through the stopcock bore, and collect about 4.5 ml. of the pink solution into a 15-ml. glass-stoppered centrifuge tube. Withdraw by pipet exactly 4.0 ml. and add exactly 0.4 ml. of absolute methanol. Mix thoroughly, and centrifuge the contents to dispel air bubbles. Determine the absorbance of the solution, A_u, *versus* a solvent blank at the maximum, about 540 mμ.

Concomitantly develop a color with 1.0 ml. of the *standard preparation*, beginning with, "Add 10 ml. of water and 5 drops of concentrated hydrochloric acid." Designate the concentration of quinestrol in the standard in mcg./ml. as C and the absorbance obtained as A_s.

$$\text{quinestrol, mcg./sample} = A_u/A_s \times C \times \text{dilution factors}$$

Gas Chromatography—Place a volume of the sample used for the colorimetric assay equivalent to 10–100 mcg. of quinestrol in a 7-ml. glass vial. Add 1.0 ml. of an 80 mcg./ml. chloroform solution of *n*-dotriacontane,[1] the *internal standard*. Evaporate the solvent on the steam bath with the aid of a gentle stream of nitrogen. Take up the cooled residue with 0.2 ml. of chloroform. Mix thoroughly and inject 5 μl. on the column of the gas chromatograph. The following conditions were used:

1. A 1.2-m. 6-mm. (4 ft. 1/4 in. o.d.) glass U-tube column packed with 4% OV-17[1] on 80/100-mesh Gas Chrom Q[1] was used in an F & M Scientific model 402 instrument, which provides on-column injection and dual columns and detectors. The temperature was maintained isothermally at 253° for the column and 263° for the flame-ionization-detector chamber. Helium carrier gas flow rate was maintained at 120 ml./min. and hydrogen gas flow to the detector was set at 60 ml./min.

2. A second system was used as a check on separations, where all of the operating parameters were identical with the first, but the column used was a 1.2-m. 6-mm. o.d. glass U-tube packed with 3% SE-30 on 80/100 mesh Diatoport S.[2]

The response factor for quinestrol was determined by injecting 10-, 50-, and 100-mcg. amounts of quinestrol with the internal standard. Where the response of quinestrol is R_Q, the internal standard response R_I, the weight of internal standard W_I, and the weight of quinestrol standard W_Q:

$$\text{Factor } (F) = R_Q/R_I \times W_I/W_Q$$

The amount of quinestrol in the sample is calculated:

$$\text{mcg. quinestrol} = R_Q/R_I \times W_I/F$$

Response factors were determined in a similar manner for ethinyl estradiol, estrone, and estrone-3-cyclopentyl ether.

RESULTS AND DISCUSSION

Specificity of the Gas Chromatographic Assay—Quantitative results were obtained using the OV-17 column described in system 1 above, however, the separations were checked on the alternate column given in system 2. Correspondence of sample peak retention times to those of the standard substances in two systems provides a high degree of specificity of identification. The retention times obtained for the standard substances on the two columns are given in Table I. The only quinestrol degradation products found in significant amounts were ethinyl estradiol and, in one case, estrone-3-cyclopentyl ether (*v.i.*). Quantitative measurements were made by the peak height method.

Comparison of Methods—Both methods provided recoveries of 98%. Relative standard deviations were 1.7% for the colorimetric assay and 1.6% for the gas chromatographic procedure. As noted earlier (1), the colorimetric method does not distinguish between quinestrol and ethinyl estradiol, and it has been used in these laboratories with a thin-layer limit test for ethinyl estradiol. (A procedure for selective colorimetric assay of quinestrol in the presence of ethinyl estradiol is described

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[2] F & M Scientific, Division of Hewlett-Packard, Avondale, Pa.

below.) Ethinyl estradiol was found in significant amounts, however, only in the experiments in which quinestrol was heated in at least 0.5 *M* hydrochloric acid for 20 hr. Figure 1 shows the correlation obtained by plotting the quinestrol assay value by the colorimetric method against the total of quinestrol and ethinyl estradiol (as quinestrol) determined by the gas chromatographic procedure. The close correspondence obtained between assay values on the same samples establishes the empirical colorimetric assay as a valid stability-indicating method for quinestrol, if provision is made to measure ethinyl estradiol by an independent method or eliminate it in the isolation scheme.

Degradation with Peroxide—Results obtained by both assay methods on the peroxide-degraded samples are presented in Table II. The pot temperature of the 50% methanol solutions was about 77°. Poor correlation was obtained between the amount of peroxide added and the percent of quinestrol which remained. This may reflect differences in handling the work-up after the reflux period, for residual peroxide was present in every case, and it would be concentrated during the evaporation steps. Failure to wash out peroxide residues led to low assays by the gas chromatographic procedure, indicating further reaction on the hot chromatographic column. The experimental design does not permit any estimates of the rate of quinestrol degradation by hydrogen peroxide, nor was this within the scope of the present study. Summation of the measurable peaks does not provide material balance; unidentified peaks were noted at retention times of 3.4 and 31.4 min. and on the reverse slope of the quinestrol peak (about 28 min.) using the OV-17 column.

Degradation with Acid—Heating quinestrol at reflux with 0.05 *M* hydrochloric acid in 50% methanol led to no detectable degradation. The amount of ethinyl estradiol formed by hydrolysis of the ether function increased with acid concentration as shown in Table III. The pot temperature was again about 77°. Unidentified peaks were noted in chromatograms run on the OV-17 column with the two higher concentrations of acid. A peak with a retention time of 34.6 min. was observed after heating with 1.5 *M* acid. Additional peaks were observed after heating with 3 *M* hydrochloric acid at 5.8, 14.6, 16.6, 41.8, and 50.4 min.

Table I—Retention Times of Standard Steroids on Two Columns

Compound	Retention Time, min. Column I	Retention Time, min. Column II
Estrone	8.0	3.2
Ethinyl estradiol	9.4	4.0
n-Dotriacontane	11.4	18.0
Estrone-3-cyclopentyl ether	22.4	9.0
Quinestrol	27.4	11.6

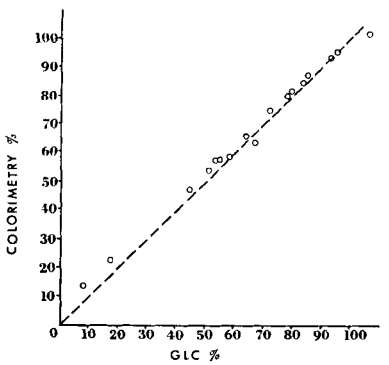

Fig. 1—Correlation of colorimetric and gas chromatographic assay values on degraded quinestrol samples. See text. Dashed line represents perfect correlation.

Degradation in Alkali—Heating quinestrol in 0.05 *M* alkali gave values of 95% quinestrol by the colorimetric method and 95.2% by gas chromatography. In this concentration of base, less than 1% of quinestrol was found converted to estrone-3-cyclopentyl ether, but unidentified peaks were noted with retention times of 1.6, 2.2, 3.2, and 5.2 min. In 0.5 *M* methanolic sodium hydroxide, 58% intact quinestrol was found by the colorimetric method and 54.7% by gas chromatography. Estrone-3-cyclopentyl ether was found in 6.5% concentration (expressed as quinestrol), and less than 1% estrone was seen. The unidentified peaks noted were the same as in the lower base concentration experiment. Talmage *et al.* (4) and Boughton and his co-workers (5) used estrone as the internal standard for gas chromatographic determination of ethinyl estradiol. It would appear that this is a poor choice, for the estrone is both a synthesis precursor and a possible degradation product. Langecker (6) has demonstrated the presence of estrone and acetylene after treating ethinyl estradiol with 1 *N* sodium hydroxide for 30 min.

Selective Colorimetric Assay for Quinestrol—The previously reported procedure (1) has been modified to provide fewer manipulations and to eliminate interference from ethinyl estradiol. The following procedure is recommended.

Transfer a weighed sample equivalent to about 20 mcg. of quinestrol to a 125-ml. separator containing 10 ml. of water and 1 ml. of methanol. Add 4–5 drops of concentrated hydrochloric acid, and extract the mixture with about 75 ml. of isooctane, shaking for 2 min. Allow the layers to separate, and discard the lower phase. Wash the isooctane layer with 5 ml. of 1 *N* sodium hydroxide, shaking the mixture about 1 min. Discard the alkali layer, and transfer the isooctane phase quantitatively to a second, *scrupulously dry* 125-ml. separator through a solvent-wetted cotton pledget filter, using about 10 ml. of fresh isooctane to effect the transfer. Continue the procedure as described above under *Colorimetric Method*, beginning with, "Equilibrate the isooctane solution with exactly 5.0 ml. of *chromogenic reagent*. . . ."

Use of 1 *N* sodium hydroxide to remove any ethinyl estradiol present was checked by adding 20- and 30-mcg. amounts of ethinyl estradiol to 20-mcg. amounts of quinestrol and going through the procedure described. There was no difference in absorbance values for quinestrol to which an equal or 1.5-fold excess of ethinyl estradiol was added and samples without added ethinyl estradiol. Recoveries of quinestrol added to placebo tablets were identical whether the alkali wash was used or water was employed.

SUMMARY AND CONCLUSIONS

Assay of quinestrol solutions partly degraded by heating with hydrogen peroxide, hydrochloric acid,

TABLE II—RESULTS OF PEROXIDE DEGRADATION OF QUINESTROL

30% Peroxide, ml.	Hr.	Percent Quinestrol Remaining				
		Colorimetric	GLC	EE[a]	ES[b]	EsCPE[c]
0.5	22	87.4	84.0	trace	trace	—
1.0	22	85.1	83.6	trace	trace	—
2.0	22	81.4	78.6	trace	trace	—
1.5	22	80.2	77.9	trace	trace	—
0.5	22	75.0	71.3	—	trace	—
1.0	22	65.4	63.9	—	<1%	—
1.5	22	58.6	58.4	—	<1%	—
1.0	40	58.0	53.0	trace	trace	<1%
2.0	40	54.0	51.3	trace	trace	trace
2.0	22	46.7	44.0	trace	<1%	—
0.5	40	21.9	18.2	trace	<1%	<1%
1.5	40	12.9	8.0	trace	trace	trace

[a] Ethinyl estradiol. [b] Estrone. [c] Estrone-3-cyclopentyl ether.

TABLE III—DEGRADATION OF QUINESTROL IN HYDROCHLORIC ACID

HCl	Percent Intact Quinestrol					
	Colorimetric	GLC	EE[a]	Q + EE[b]	ES[c]	EsCPE[d]
0.5 *M*	101	106	1.8	108	—	—
1.5 *M*	93	82.7	9.8	92.5	trace	1%
3 *M*	64	36.2	30.9	67.1	trace	1%

[a] Ethinyl estradiol calculated as quinestrol. [b] Total of quinestrol and ethinyl estradiol calculated as quinestrol. [c] Estrone. [d] Estrone-3-cyclopentyl ether.

or sodium hydroxide by a colorimetric method (1) and by a highly selective gas chromatographic method shows excellent correlation in values. The colorimetric method has thus been established as a valid stability-indicating assay for quinestrol, providing that ethinyl estradiol is measured by an independent limit test or eliminated in the isolation scheme. Ethinyl estradiol was found in significant amount only in those samples degraded by heating in 0.5 *M* or stronger hydrochloric acid. The specificity of the gas chromatographic procedure used was established by obtaining separations on two different columns. Both provided excellent separations of quinestrol from ethinyl estradiol, estrone, estrone-3-cyclopentyl ether, and unidentified peaks noted in several of the chromatograms.

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Keyphrases

Quinestrol—analysis
Stability assay—quinestrol dosage forms Colorimetric analysis
Methanol–sulfuric acid—color reagent
GLC—analysis