(26) Pimental, G. C., and McClellan, A. L., "The Hydrogen Bond," W. H. Freeman and Co., San Francisco, Calif., 1960.
(27) Sutor, D. J., *J. Chem. Soc.*, **1963**, 1105.
(28) Krimm, S., *Science* **158**, 530(1967).
(29) Shefter, E., *J. Pharm. Sci.*, **57**, 350(1968).
(30) Johnson, C. K., *Ortep, A Fortran Thermal—Ellipsoid Plot Program for Crystal Structure Illustrations*, U. S. Atomic Energy Commission Report ORNL-3794, 1965.

Keyphrases

Complexes—structural studies
Caffeine-5-chlorosalicylic acid complex
Crystal structure
Molecular structure

Comparative Study of a New Fluorometric Assay Mestranol Ether in Tablets with GLC and Colorimetric Assays

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Mestranol (17α-ethinylestradiol-3-methyl ether) has been found to react with methanol–sulfuric acid mixture of a critical composition to give a stable fluorophor of high intensity. This fluorescence permits the analysis of mestranol in the submicrogram range. The use of varying proportions of methanol and sulfuric acid enables the analyst to measure the fluorescence at several different wavelengths. The analyst can choose the reagent which gives him the selectivity necessary for his analytical problems. Colorimetric, GLC, and TLC methods for the determination of mestranol are also discussed and compared with the fluorometric method. The described method is satisfactory for both control and stability measurements of mestranol.

THE ANALYTICAL DETERMINATION of the estrogenic component of oral contraceptive tablets is of the utmost interest to the pharmaceutical industry. Control of manufacturing processes, quality control of the finished product, and stability measurements of the active ingredients require accurate and sensitive methods of analysis. The amounts of estrogen formulated into these tablets require analytical methods which are highly sensitive and selective in the microgram range. Other applications, such as the analysis of biological fluids, may require sensitivity in the submicrogram range.

Mestranol and ethinyl estradiol have been determined by colorimetric measurements for many years. Ganshirt and Polderman (1) developed a chromophor with mestranol using an aqueous sulfuric acid reagent. Shroff and Huettemann (2) used a phenol–sulfuric acid reagent to measure mestranol. The colorimetric determination of ethinyl estradiol by Tsilifonis and Chafetz (3) made use of a methanol–sulfuric acid reagent, which has been used in the determination of mestranol in this laboratory for several years.

The determination of mestranol by UV was described by Bastow (4). Shroff and Grodsky compared UV and GLC procedures (5), and an assay which was a combination of UV and TLC was compared to a GLC procedure by Schulz (6). France and Knox (7) assayed ethinyl estradiol by GLC without derivative formation, and Umbreit and Wisniewski (8) reported that mestranol could be assayed by GLC without thermal degradation.

Mestranol was determined fluorometrically by Hüttenrauch and Keiner (9) with antimony trichloride in glacial acetic acid. Bates and Cohen (10) heated steroids with aqueous sulfuric acid to develop fluorescence. Phosphoric acid was used by Boscott (11) to develop specific colors and fluorescence with several steroids. Comer *et al.* (12) and Cali and Khoury (13) have reported automated methods for mestranol.

The fluorometric procedure described by the authors is quite simple. The reagent develops a fluorophor at room temperature, which is highly intense and stable for at least 30 min. This

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paper will compare the newly developed fluorometric procedure with the authors' versions of the colorimetric and GLC assays. The ability of these methods to measure stability will also be discussed. TLC has been used to detect degradation products as well as to demonstrate and confirm the quantitative results of the other procedures, and a brief discussion is included. Because of the simplicity and sensitivity of this method, it will find application in biological analysis, trace analysis, and contamination control.

EXPERIMENTAL

General Procedure for Tablet Extraction

The tablets used for assay evaluation in this paper were labeled to contain 80 mcg. mestranol per tablet. The bulk of the tablet excipient material is sodium bicarbonate.

One tablet was weighed accurately and placed in a 50-ml. volumetric flask. Three drops of a 20% aqueous solution of sodium hydroxide and 2 ml. of water were added to the flask. The tablet was then dissolved by placing the flask in an ultrasonic cleaner for about 5 min. Approximately 25 ml. of chloroform was added to the flask, which was then shaken for 30 min. on a wrist-action shaker. Sufficient chloroform was added to raise all of the aqueous layer into the neck of the flask. The aqueous layer was aspirated and discarded, and the chloroform was transferred to a 100-ml. volumetric flask through anhydrous sodium sulfate. The content of the flask was diluted to volume with chloroform. This is the sample solution containing approximately 0.8 mcg. mestranol per ml. which is used in each of the described assays.

Fluorescence Assay Procedure

Apparatus—An Aminco-Bowman spectrophotofluorometer equipped with a xenon lamp, a IP21 photomultiplier tube, and silica cells was used to check all activation and emission wavelengths. A Turner model 110 filter fluorometer equipped with a G.E. F4T4 lamp and matched borosilicate cells was employed for all quantitative fluorometric measurements. Kodak Wratten filter 47B which has peak transmission at 430 mμ was used as the primary filter, and Corning filter 3385 which cuts off below 480 mμ as the secondary filter.

Reagent—To develop fluorescence, a 50% v/v mixture of absolute methanol and concentrated sulfuric acid (50% reagent) was used. To prevent heat build-up, the 50% reagent was prepared in an ice bath by adding sulfuric acid dropwise to a chilled flask of methanol.

Tablet Assay Procedure—Two milliliters of the sample solution containing about 1.6 mcg. of mestranol was pipeted into a test tube (18 × 120 mm.) and carefully evaporated to dryness using air and mild heat. The sample was redissolved with 0.1 ml. of methanol and then 5 ml. of the 50% reagent was added to the test tube. After shaking to mix thoroughly, the tube was allowed to stand at room temperature for 1 hr. to obtain maximum fluorescence development. The solution was then transferred to a Turner cell and the fluorescence measured against a reagent blank. To obtain on-scale readings with solutions in the concentration range of 0.1–0.4 mcg. per ml., a 1× aperture setting was used and a 10% transmittance density filter was combined with the secondary filter.

Colorimetric Assay Procedure

Apparatus—A Beckman model DU spectrophotometer with 1-cm. cells was used.

Reagent—The reagent used to develop the chromophor was 30% v/v mixture of methanol in concentrated sulfuric acid (30% reagent). The reagent was prepared in the same manner as the 50% reagent.

Tablet Assay Procedure—A 20-ml. aliquot of the sample solution, corresponding to 16 mcg. of mestranol, was pipeted into a 25-ml. volumetric flask. This solution was evaporated to dryness under a current of air and with gentle warming. Four drops of methanol, from a 1-ml. pipet, was added to redissolve the residue, and then 10 ml. of 30% reagent was pipeted into the flask. The contents were mixed well and the absorbance was read after 10 min. at 545 mμ against a reagent blank. A mestranol standard of about the same concentration was treated in the same way.

Gas-Liquid Chromatographic Procedure

Apparatus—F and M model 402 equipped with dual hydrogen flame detectors was used.

Column—A U-shaped glass column, 61 cm. (2 ft.) × 4 mm. i.d. was used. The column was cleaned with chromic acid cleaning solution and silanized before packing.

Liquid Phase—A silicone gum rubber, W-98 (F and M Scientific Corp., cat. No. LP-166).

Inert Support—Diatoport S, 80/100 mesh (a diatomaceous earth specially treated and silanized by F and M Scientific Corp.). The glass wool used to plug the ends of the column was also silanized.

Preparation of Column Packing—A 1% solution of W-98 in toluene was prepared by dissolving 3.0 Gm. of W-98 in 300 ml. of toluene with constant stirring on a steam bath. Fifty grams of 80/100 mesh Diatoport S was added to the W-98 solution in a liter filter flask. Vacuum was applied to degas the solution and it was allowed to stand for 5 min. The support was then separated from the solution by vacuum filtration. The support was spread and air dried for 24 hr. To remove the last traces of toluene, the support was heated at 100° for 1–2 hr.

Column Conditioning—The column was "no-flow" conditioned for 1 hr. at 325°. After cooling to room temperature, the carrier gas flow was started and the column was conditioned at 275° for 24 hr.

Instrumental Parameters—The instrument was operated isothermally at a column temperature of 205°. The injection port temperature was set at 230° and the detector temperature at 260°. Helium was used as carrier gas with the flow rate at 60–70 ml./min. Oxygen was used for operating the hydrogen flame detector. The range setting was at 10, and the attenuation at 16×.

Preparation of Mestranol Standard Solution—An accurately weighed and diluted solution of pure mestranol in chloroform containing 10 mcg./ml. was used in most cases.

Internal Standard—Cholestane (5α-cholestane

TLC homogeneous, Mann Research Laboratories, Inc.) was selected as the internal standard because of the stability, solubility, and retention time relative to mestranol. A cholestane solution at a concentration of 25 mcg./ml. of chloroform was used.

Preparation of Standard Curve—Internal standard solution in the amount of 2.0 ml., corresponding to 50 mcg. of cholestane, was pipeted into each of five 100-ml. beakers. Then 1.0, 2.0, 3.0, 4.0, and 5.0-ml. aliquots of 10 mcg./ml. of mestranol standard solution were pipeted into each beaker, respectively. Each solution was mixed and evaporated to dryness under a stream of air and mild temperature. The residue was redissolved with 1.0 ml. of chloroform, and approximately 5 µl. of the solution was injected into the gas chromatograph. The peak heights of mestranol and cholestane were measured to the nearest 0.5 mm. from the top of the peak to the corrected base line. The standard ratio of the mestranol peak height to the cholestane peak height was calculated.

Tablet Assay Procedure—Two milliliters of the 25 mcg./ml. cholestane solution was pipeted into a 100-ml. beaker. Then 50 ml. of the sample solution was pipeted into the same beaker. This mixture was then carefully evaporated to dryness under a stream of air and with gentle warming. One milliliter of chloroform was added to the dried residue to redissolve the sample, and 5 µl. of this solution was injected into the gas chromatograph. The sample ratio was calculated in the same way that the standard ratios were calculated and was used to determine the tablet strength.

Thin-Layer Chromatography

Up to 100 mcg. of material was applied to a 250-µ Silica Gel F_{254} plate supplied by Brinkmann Instruments Inc. The plate was developed in a saturated chamber containing chloroform–ethanol, 29:1. The solvent front was allowed to travel 15 cm. To visualize the spots the plate was sprayed with 30% reagent, heated at 110° for 10 min., and then viewed under long-wavelength UV light.

RESULTS AND DISCUSSION

Reagent Composition and Fluorescence Formation—Table I summarizes the characteristics of fluorescence formed by reacting mestranol with reagents of varying methanol–sulfuric acid concentration. It was found that two and possibly three distinct fluorophors are formed by mestranol. Using other reagent proportions than those described here, no other fluorophor was found. When the fluorophor formed with 40% reagent was examined, the spectra indicated that a mixture of the fluorophors of 30 and 50% reagents had been formed. The fluorophor of 60% reagent was the same as that of 50% reagent, but was less intense.

Table I—Color and Fluorescence Characteristics of Mestranol

	Color	Fluorescence, mµ Max. Activ.	Max. Emiss.
Sulfuric acid, concentrated	Orange-red	525	555
30% Reagent	Pink	545	565
50% Reagent	None	468	498

The activation and emission curves of these fluorophors were plotted using an Aminco-Bowman spectrophotofluorometer. In order to compare the intensity of fluorophors, instrument settings, and sample concentrations were the same for all of the spectra (0.2 mcg./ml.). The spectra of mestranol in concentrated sulfuric acid, in 30 and 50% reagents are shown in Fig. 1. These spectra are not energy corrected. The formation of two different and highly intense fluorophors, may allow the analyst to selectively eliminate fluorescence interferences due to other steroids by making a choice of either 30 or 50% reagent. Work is in progress on application of these reagents to the analysis of other steroids.

Detection of Mestranol by Fluorescence—To determine the lowest detection limits of mestranol by this procedure, aliquots of a 0.01 mcg./ml. solution of mestranol were evaporated and treated with 50% reagent. With the aperture set at 10 and no density filter, a solution of 0.001 mcg. (1 nanogram) mestranol per ml. of 50% reagent gave a reading of 22 galvanometer units. The linearity of the measurements at different concentrations in the nanogram range is shown in Fig. 2. Since the tablet assay method described here is in the microgram range, the instrument sensitivity had to be decreased. This was done by using an aperture of 1× and a 10% transmittance density filter in conjunction with the secondary filter. It was found to be more convenient to do this, than to make additional sample dilutions. The linearity of the measurements up to 2 mcg. is shown in Fig. 2. To improve

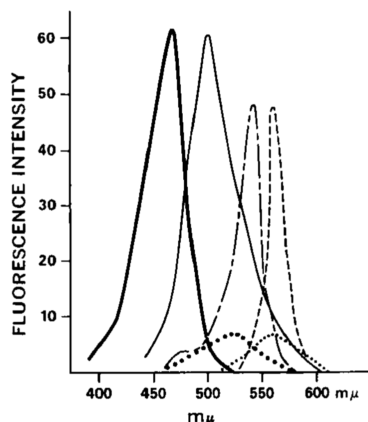

Fig. 1—Comparative activation and emission spectra of mestranol. Key: ——, activation in 50% reagent; ——, emission in 50% reagent; —·—, activation in 30% reagent; ---, emission in 30% reagent; •••, activation in sulfuric acid; · · ·, emission in sulfuric acid

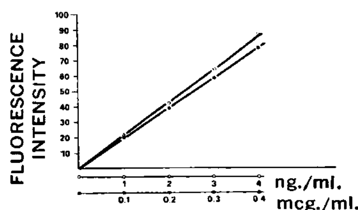

Fig. 2—Linearity of measurements of mestranol by fluorescence.

the reproducibility of the assay it was necessary to dissolve the residue of mestranol in a small volume (0.1 ml.) of methanol before adding the 50% reagent. Insufficient heat was evolved to require cooling, and the reagent properties were not adversely affected by additional methanol. The calculation of precision on a series of five standards assayed by this technique gave a relative standard deviation of ±0.63%.

Fluorescence Development—The reaction of the 50% reagent and mestranol to form the fluorophor does not go to completion at once. At room temperature, the intensity of the fluorescence increased until it reached maximum intensity about 50 min. after addition of the reagent to the sample. The maximum fluorescence was maintained from 50 to 90 min., when it began to decrease slowly as seen in Fig. 3. Based on these findings, it was decided to read all samples about 1 hr. after the addition of the reagent.

Colorimetric Determination—Colorimetric analysis with the 30% reagent has been the standard method in these laboratories for several years. It has been used along with various extraction techniques for both control and stability assays. The assay requires a sample concentration of about 2.0 mcg./ml. of 30% reagent. The calculation of precision on a series of five standards assayed by this technique gave a relative standard deviation of ±0.30%.

Gas Chromatographic Determination—Gas chromatography is a more specific technique for measuring mestranol, because it is a separation technique as well as a quantitation technique. Using the conditions described here, mestranol was chromatographed with no evidence of decomposition on the column. This was done without resorting to derivative formation, which would complicate the procedure. No problems were experienced due to interference from tablet excipients. They were apparently eliminated in the extraction procedure or during the chromatography. A typical chromatogram of mestranol and cholestane is shown in Fig. 4. Since only 0.25 mcg. of mestranol was injected on column in this procedure, the sensitivity of detection should make the procedure applicable in cases where much less sample is available to the analyst. An aliquot of the sample solution containing 40–50 mcg. of mestranol was usually taken for the assay. The procedure proved to be linear in response from 10 to 50 mcg. The precision of measurement by GLC was determined by calculating the standard ratios of 10 sample injections from the same solution, and gave a relative standard deviation of ±0.63%.

Measurement of Stability—In order to evaluate the application of these methods for stability measurement, tablets were artificially degraded with heat and humidity. Several tablets were placed in an area of high humidity at a temperature of about 65° for 14 days (Group I tablets). Another group of tablets was placed in an oven at 105° for 3 days (Group II tablets). These tablets were then

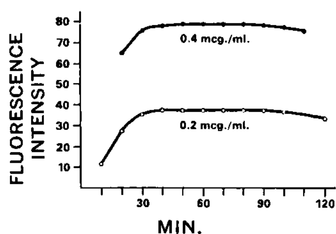

Fig. 3—Development and stability of fluorophor.

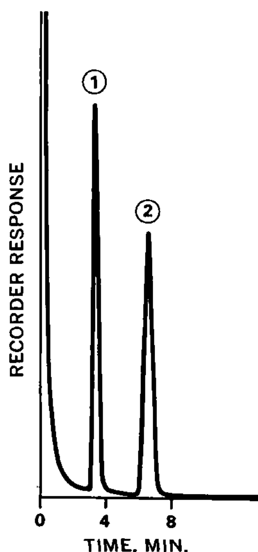

Fig. 4—Chromatogram of mestranol–cholestane mixture. Key: 1, mestranol; 2, cholestane.

TABLE II—COMPARISON OF METHODS FOR STABILITY MEASUREMENTS

	mcg. per Tablet		
	Color	Fluorescence	GLC
Tablets before aging	84.7	85.9	85.6
Group I	47.8	48.4	46.7
Group II	9.0	4.5	3.3

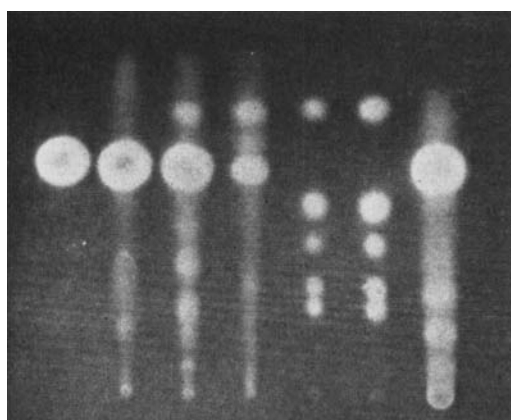

Fig. 5—TLC of mestranol and related steroids. Key: lane 1, (left to right) mestranol standard; lane 2, extract of fresh mestranol tablet; lane 3, extract of Group I tablets; lane 4, extract of Group II tablets; lane 5, mixture of steroids containing estrone methyl ether (highest R_f), estradiol methyl ether, estrone, ethinyl estradiol, and estradiol and appear in that order —1 mcg./steroid; lane 6, mixture of steroids—2 mcg./steroid; lane 7, methanol solution of mestranol exposed to Hanovia irradiation.

TABLE III—STATISTICAL COMPARISON OF METHODS[a]

Analyst	Color	Fluorescence	GLC	Analyst Means
A	706.5	716.5	714.1	712.4
B	690.7	701.7	706.3	699.6
C	699.8	695.6	—	697.7
				Grand mean
Method means	699.1	704.6	710.1	704.0
Estimates of Variation				
Variance[b] s_x^2	45.09	66.78	61.55	
Standard deviation s_x	6.72	8.17	7.85	
Rel. standard deviation c_x	0.96%	1.16%	1.10%	

[a] Results in mcg./Gm. [b] Individual tablets "within" analysts.

assayed by the three procedures described here and the results were compared as shown in Table II. Group I tablets retained only about 55% of their original strength, while in Group II tablets only trace amounts of mestranol remain. These observations were confirmed by the use of TLC.

By the GLC technique all known breakdown products of mestranol are separated from the parent compound. Because of this specificity the GLC assay is a positive measure of stability. Since the fluorometric and colorimetric results correlate very closely to the GLC results, all three methods are considered to be satisfactory stability assays.

Thin-Layer Chromatography—This technique was used as a qualitative assay to confirm the results by the other methods. The tablets were extracted in the same way as for the GLC measurements, then the desired portion of the tablet extract was applied to the plate. As is shown in Fig. 5, degradation of mestranol is observed in the tablets of Groups I and II. The figure also shows the result of exposing a methanol solution of mestranol raw material to irradiation by UV lamp. By this artificial means of accelerating decomposition, it was possible to form at least five decomposition products of mestranol, none of which corresponded to the related steroids chromatographed in lanes 5 and 6. However, the extract from the heated tablets, as seen in lane 4, contains components identifiable by this procedure as estrone methyl ether and 17α-ethinylestradiol. These results are not in agreement with the conclusions of Shroff and Grodsky (5) who state that "mestranol does not undergo any decomposition when stored under various conditions."

Comparison of Assay Methods—In order to evaluate the tablet assay procedures described in this paper, a study was run to compare the three different methods of measurement. The assays were performed by three different analysts. All samples were taken from a single lot of tablets. Each analyst extracted six single-tablet samples and then used aliquots of the extract for the different measurement procedures. The results were reported in mcg. of mestranol per Gm. to e'iminate the effect of tablet weight differences. The results are summarized in Table III. The means of six determinations by each analyst are shown.

An analysis of variance suggested that the variation of individual determinations within each of the three methods appeared to be consistent and that no significant difference among assay methods was shown. The grand mean of the experiment (n = 48) was not significantly different from the theory of 705.3 mcg. mestranol per Gm.

Assay of Ethinyl Estradiol—Ethinyl estradiol is the other estrogen besides mestranol which is widely used in oral contraceptive tablets. All three of the procedures applied to mestranol in this paper are applicable to the assay of ethinyl estradiol. The fluorescence spectrum of ethinyl estradiol is almost identical with that of mestranol in 50% reagent. Ethinyl estradiol forms a pink color with 30% reagent that has an absorbance maximum at 538 mμ instead of the 545 mμ of mestranol. It may also be assayed by GLC with the same operating parameters described for mestranol. It can be chromatographed without derivative formation and is eluted approximately 30 sec. after mestranol under the stated instrumental conditions.

REFERENCES

(1) Ganshirt, H. G., and Polderman, J., *J. Chromatog.*, **16**, 510(1964).
(2) Shroff, A. P., and Huettemann, R. E., *J. Pharm. Sci.*, **56**, 654(1967).
(3) Tsilifonis, D. C., and Chafetz, L., *ibid.*, **56**, 625(1967).
(4) Bastow, R. A., *J. Pharm. Pharmacol.*, **19**, 41(1967).
(5) Shroff, A. P., and Grodsky, J., *J. Pharm. Sci.*, **56**, 460 (1967).
(6) Schulz, E. P., *ibid.*, **54**, 144(1965).
(7) France, J. T., and Knox, B. S., *J. Gas Chromatog.*, **4**, 173(1966).
(8) Umbreit, A. R., and Wisniewski, J. V., *Facts and Methods (F & M Scientific Corp.)*, **5**, 9(1964).
(9) Hüttenrauch, R., and Keiner, I., *Pharmazie*, **20**, 242 (1965).
(10) Bates, R. W., and Cohen, H., *Endocrinology*, **47**, 166 (1950).
(11) Boscott, R., *Nature*, **162**, 577(1948).
(12) Comer, J. P., Hartsaw, P. E., and Stevenson, C. E., *J. Pharm. Sci.*, **57**, 147(1968).
(13) Cali, L. J., and Khoury, A. J., "Automation in Analytical Chemistry, Technicon Symposium 1966," vol. I, 1966, p. 196.

Keyphrases

Mestranol tablets—analysis
Fluorometry—analysis
Sulfuric acid-methanol—reagent
GLC, colorimetry, fluorometry—comparison
TLC—analysis confirmation
Degradation, mestranol—detected