for this work, one can reasonably expect that other drugs in the same or different vehicles could be similarly studied in various animal species in order to discover fundamental factors affecting drug absorption rate from the s.c. site.

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Technical Articles

Automatic Determination of Ethinyl Estradiol in Pharmaceutical Preparations

By A. J. KHOURY* and L. J. CALI†

An automated method for determining ethinyl estradiol or ethinyl estradiol methyl ether is described. The method is based on the fluorescence exhibited by ethinyl estradiol when it is treated with 90 per cent sulfuric acid. The nonvarying time, temperature, and volume characteristics of an automatic analyzer (AutoAnalyzer) system have been used to study a number of variables which might affect the data. Specificity of the fluorescence method with respect to degradation products, interference from other estrogens and progestational agents, as well as repeatability and sensitivity of the method are discussed. Also presented are data on trade packages of a number of antifertility formulations. Samples can be analyzed using the automated method at a rate of 20 samples per hour as opposed to one or two samples per hour by manual techniques.

SINCE THE USE of progesterone and ethisterone (17α-ethinyltestosterone), was first described by Inhoffen and Hohlweg (1) a number of antifertility products have become available. All of these products contain ethinyl estradiol (EE) or its methyl ether (EEME) in combination with a progestational steroid hormone. EE is usually formulated into tablets at concentrations as low as 0.05% of the total tablet weight along with another steroid whose concentration is 50 to 500 times greater than that of EE. The analysis of EE in this environment has presented a difficult analytical problem. The U.S.P. method of analysis (2) is very time consuming, while the colorimetric method described by Urbanyi and Rehm (3) does not distinguish between EE and other estrogens, and also is not applicable to the methyl ether of EE. Paper chromatographic

Received January 20, 1967, from Wyeth Laboratories, Philadelphia, PA 19101
Accepted for publication May 22, 1967.
Presented to the Drug Standards, Analysis and Control Section, A.PH.A. Academy of Pharmaceutical Sciences, Las Vegas meeting, April 1967.
The authors gratefully acknowledge the technical assistance of Dr. Jay G. Rutgers during preparation of this paper.
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methods (4) have been applied with some success but are limited due to the lack of accuracy and precision generally afforded by such procedures. Spectrofluorometric methods (5) while generally yielding specific data at low levels of concentration, can yield inaccurate data due to fluorescence quenching. Methods based on the Kober reaction (6) are inapplicable unless a separation technique is first applied. Talmage (7) described a gas chromatographic method for EE, but this procedure does not lend itself to analysis of large numbers of samples. A method which has not been reported previously but which is applicable to the analysis of EE in tablets of low dosage has been developed. The method is based on the chemically induced fluorescence which is manifested by EE in 90% H_2SO_4. The specificity afforded by this reaction makes it possible to directly apply this method to the analysis of EE in oral contraceptive preparations or in preparations containing similar estrogenic substances. Although the procedure may be carried out by conventional techniques, application of the method to the automatic analyzer[1] provides a method of analysis which can be used to obtain unit-to-unit variation of formulations containing as little as 5 mcg. of EE.

EXPERIMENTAL

Materials—Automatic analyzer equipment was used for the study. (*a*) Solid-prep sampler; volume set at 75 ml. and programmed at 20 samples per hour. (*b*) Proportioning pump. (*c*) Continuous filter; speed 3. (*d*) Fluorometer; primary filter (513 mμ) is a combination of Corning No. 1–60 (nearest lamp) and Wratten No. 58. Secondary filter (545 mμ) is a Wratten No. 23A. Sensitivity settings; activation source 30X; slit-3. (*e*) Linear recorder. (*f*) Acid flex tubing; conditioned by allowing the system to flush for 1 hr. Assemble the apparatus as shown in the flow diagram of Fig. 1.

Reagents and Solutions—Ninety per cent sulfuric acid in water (v/v); denatured alcohol (ethyl–methyl, 10:1) with water 40%.

Standard Ethinyl Estradiol Solutions—Prepare solutions of ethinyl estradiol in the denatured alcohol to contain 35, 40, 45, 50, and 55 mcg. of EE per ml. Place 1 ml. of the standards in the cups of the Solid-prep unit and obtain a standard curve.

Preparation of Samples—Place a weighed, finely powdered sample containing the equivalent of about 50 mcg. of EE in each sample cup. Obtain the reading from the recorder and calculate the number of micrograms of EE per weight of sample. For study of unit-to-unit variation, place 1 tablet in each of the sample cups.

RESULTS AND DISCUSSION

Linearity—An actual working curve which was obtained by analyzing standards of known concentration may be found in Fig. 2. A linear relationship exists for fluorescence and EE concentration in the range which was studied.

[1] AutoAnalyzer. Technicon Corp., Ardsley, N. Y.

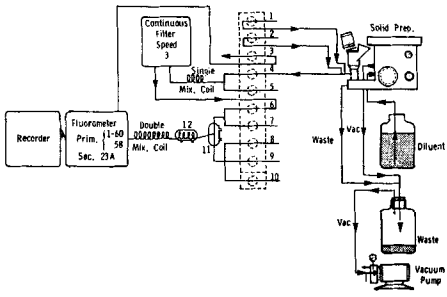

Fig. 1—Flow diagram for EE or EEME. Key: 1, 0.8 ml./min. air; 2, 4.06 ml./min. SDA No. 30; 3, 2.76 ml./min. debubbler; 4, 2.76 ml./min. sample; 5, 2.03 ml./min. SDA No. 30; 6, 2.76 ml./min.; 7, 2.0 ml./min. air; 8, 9, and 10, 2.76 ml./min. 90% H_2SO_4; 11, A-7 water-cooled TEE; 12, water-cooled mixing coil. All tubing is Acidflex.

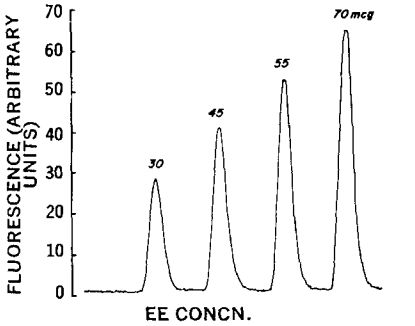

Fig. 2—Actual working curve for EE standards.

Precision—Repeatability of the procedure was demonstrated by assaying equal aliquots of an EE standard solution. These data, which may be found in Table I, indicate an average repeatability standard deviation of 1.3%

Table I—Repeatability: Twenty Individual Assays on Equal Aliquots from an EE Standard Solution[a]

Recovery, %	
1, 100.1	*11*, 102.1
2, 96.9	*12*, 104.0
3, 101.0	*13*, 100.7
4, 100.7	*14*, 101.0
5, 100.4	*15*, 98.9
6, 99.6	*16*, 100.6
7, 99.6	*17*, 99.4
8, 100.1	*18*, 100.0
9, 100.0	*19*, 101.0
10, 101.0	*20*, 100.0

[a] 1.0 mcg./ml., 95% alcohol.

Sensitivity—The automated method which has been developed was done so with the application of the method to individual EE dose variation in mind. The flow diagram of Fig. 1 is therefore applicable

to the analysis of samples which contain 25–100 mcg. of EE. The final concentration of the EE in the flow diagram indicated is about 0.06 mcg./ml. However, the practical limit of detectability has been shown to be 0.004 mcg./ml. of EE.[2]

Specificity—It has been known for some time that phenolsteroids (estrogens) when treated with sulfuric acid give rise to derivatives having a characteristic fluorescence. This effect was first observed in 1929 by Weiland, Straub, and Dorfmuller (8). The absorption and/or excitation spectra of these estrogen–H_2SO_4 chromogens exhibit maxima with broad peaks ranging from 410–450 mμ. The fluorescence characteristics of the EE–H_2SO_4 chromogen are vastly different from those of the other estrogens. EE has its activation maximum displaced at least 60 mμ toward the long wave region of the spectrum (λ_A = 513 mμ), and its fluorescence maximum at 545 mμ is displaced about 45 mμ. Figure 3 indicates the difference between

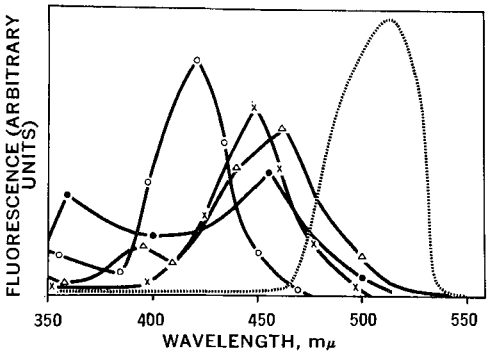

Fig. 3—Activation spectra of estrogen-sulfuric acid chromogens. Key: O, β-estradiol; ●, equilin; △ equilenin; X, estrone; ...., ethinyl estradiol.

the activation spectrum of the EE–H_2SO_4 chromogen and the activation spectra of other typical estrogen–H_2SO_4 chromogens.[3] This is quite significant if one considers that the only structural difference between EE and a typical estrogen, *e.g.*, β-estradiol, is the ethinyl moiety in position 17. When synthetic samples of EE plus a tenfold excess of equilin, equilenin, estrone, and β-estradiol were analyzed, less than 3% interference was contributed by these estrogenic substances.

The specificity of the reaction with respect to ethinyl estradiol degradation products was demonstrated by thin-layer chromatography.

Thin-layer plates were prepared from Silica Gel G HR after Stahl. The solvent system consisted of 1:1 hexane–ethylacetate. Two spray reagents, phosphomolybdic acid (9) and 90% sulfuric acid, were used.

Samples of thermally and photochemically degraded raw material exhibit a number of spots when phosphomolybdic acid detection is employed. This is illustrated in the chromatoplate on the left side of Fig. 4. Phosphomolybdic acid is a nonspecific reagent and is used only to indicate the absence or presence of components other than intact EE. When 90% H_2SO_4 was used for visualization of the same degraded raw material, one fluorescent component which corresponds in R_f to pure EE was detected. This is illustrated in the chromatoplate on the right side of Fig. 4. This indicates that the

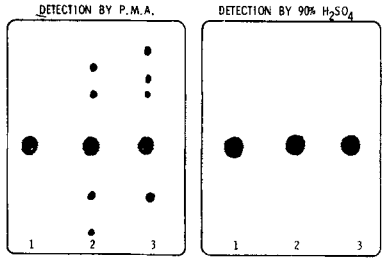

Fig. 4—Thin-layer chromatoplates of EE raw material. Key: 1, 20 mcg. of EE raw material; 2, 20 mcg. of the same raw material after exposure to direct U.V. light for 1 month; 3, 20 mcg. of the same raw material after storage at 100° for 1 month.

specificity of the sulfuric acid reagent eliminates the need for lengthy separation techniques. Further proof of H_2SO_4 specificity with respect to raw material EE decomposition products was obtained by quantitatively evaluating the degraded raw material by the automatic analyzer method and also by native fluorescence and the U.S.P. colorimetric methods. The data which are contained in Table II indicate that comparable results are obtained by all three assay procedures.

TABLE II—QUANTITATIVE ANALYSIS OF ETHINYL ESTRADIOL RAW MATERIAL BY THREE ASSAY PROCEDURES

Sample No.	Storage Condition	Native Fluorescence, % of Theory	Modified Kober, % of Theory	Automatic Analyzer, % of Theory
1	1 mo. 75° C.	67.6	69.0	69.8
2	1 wk. direct U.V. light exposure	94.3	94.6	93.9
3	1 mo. direct U.V. light exposure	88.8	89.1	88.9

Compressed tablets containing only EE (50 mcg./tablet) were subjected to accelerated conditions of heat and light. When these tablets were evaluated by the automated procedure and by the same two referee methods which were used previously, the results in Table III were obtained. These data indicate that the automatic analyzer method gives results which agree with native fluorescence and the colorimetric procedure.

The progestational type steroid hormone which is commonly found in oral contraceptive products

[2] Established on an Aminco-Bowman spectrophotofluorometer.

[3] These spectra are uncorrected and were obtained with an Aminco-Bowman spectrophotofluorometer.

Table III—Comparison of Data Obtained on EE Stability Samples by Three Assay Procedures

Sample No.	Storage Condition	% of Claim: Native Fluorescence	Colorimetry	Automatic Analyzer
1	3 mo., D.S.	60.0	65.3	66.0
2	1 mo., D.S.	73.0	76.0	76.0
3	1 mo., 75°C.	89.9	90.0	90.5
4	1 mo., 75°C.	83.1	79.8	81.0
5	12 mo., R.T.	97.1	96.9	99.0
6	12 mo., R.T.	93.1	97.2	97.0

along with EE is generally of the Δ^4-3-keto structural variety. The concentration of this steroid is such that it may usually be determined by direct ultraviolet spectrophotometric methods, with no interference from EE. Usually the Δ^4-3-ketosteroid will be 50–500 times more concentrated than EE in an individual dosage unit. In spite of this large difference in concentration, significant interference was not encountered when a number of antifertility products were examined by the automated procedure. Table IV indicates a tabulation of data which were obtained when a number of commercial products were assayed by the automated procedure. These four products contained 3 different progestational steroids ranging in concentration from 2 to 10 mg. per tablet.

Table VI—Comparison of Results by Colorimetric, Native Fluorescence, and Sulfuric Acid Fluorescence of EE-Δ^4-3-Ketosteroid Formulation

Aged Sample No.	Ethinyl Estradiol, mcg.: Natural Fluorescence Before Extn.	Natural Fluorescence After Extn.	Colorimetric	Sulfuric Acid Fluorescence
1	36.2	46.7	46.8	47.2
2	33.0	44.8	45.0	44.7

In order to study Δ^4-3-ketosteroid interference with the method, EE was separated from the progestational steroid by solvent extraction, and then assayed by the automated method. The data in Table V show that results with and without extraction are equivalent, indicating little or no interference from the progestational steroid.

Two trade materials were not amenable to the automated sulfuric acid fluorescence assay. One contains a Δ^5-3-ketosteroid which interferes with the sulfuric acid fluorescence causing high results. Another product contains a material in the formulation which quenches the fluorescence and causes low results. In the latter case, a chloroform extraction removed the active ingredients away from the interfering substances. The chloroform was removed by evaporation and the residue was analyzed by the method described earlier. This separation technique was not successful for the Δ^5-3-ketosteroid containing products.

Table IV—Determination of Ethinyl Estradiol in Currently Manufactured Contraceptives

% of Claim by Company No. 1[a]		2[a]		3		4	
100.0	103.0	99.0	101.0	104.0	106.1	97.0	102.0
104.0	104.0	93.9	100.0	100.5	100.5	97.0	101.2
99.0	105.0	99.0	91.7	101.0	94.5	98.0	100.0
102.0	105.0	94.6	98.0	96.5	101.0	102.0	88.6
99.0	102.0	96.5	100.0	98.0	100.5	101.0	104.0
104.0	99.0	105.0	94.5	100.0	96.0	112.0	97.8
105.0	98.0	97.0	97.0	100.0	103.4	101.0	99.8
102.0	103.5	99.2	97.0	96.5	99.1	99.2	100.6
103.0	103.0	95.8	93.5	95.0	95.9	97.8	99.8
99.0	104.0	99.0	97.0	99.5	96.7	98.2	99.2
Av. value (ϕ) = 102.2%		ϕ = 97.4%		ϕ = 99.3%		ϕ = 99.8%	
S. D. (σ) = 2.1%		σ = 3.1%		σ = 3.6%		σ = 4.2%	

[a] Ethinyl estradiol 3-methyl ether.

Table V—Comparison of Results with and without Extraction on Samples Containing 10 mg. of a Δ^4-3-Ketosteroid

No Extraction, mcg. EE	Extraction, mcg. EE
42.5	41.0
40.5	46.0
40.5	37.1
39.0	39.4
38.5	41.0
42.5	41.5
40.0	42.0
40.5	45.0
46.5	42.5

This sulfuric acid fluorescence method was used successfully in controlling developmental samples of a new Wyeth (10) antifertility product. This product contains 0.5 mg. of a new progestational steroid,[4] in combination with 50 mcg. of ethinyl estradiol. Since this quantity of progestational steroid is much lower than that found in other commercial products (2–25 mg.), attempts were made to analyze these products for EE by the native fluorescence method mentioned earlier. This provided a striking example of the problems that can occur when assaying by the native fluorescence procedure.

[4] WY-3707, 13-β-ethyl-17α-ethinyl-17-hydroxygon-4en-3-one.

Two aged samples of the combination tablets were assayed by the native fluorescence method, the colorimetric method, and the sulfuric acid fluorescence method. The data in Table VI show that the results by the native fluorescence method are much lower indicating that there may be a quenching effect operative. When the ethinyl estradiol was extracted from these sample mixtures and then assayed by the native fluorescence method, the results agreed with those obtained by the other two methods. This quenching effect was not noted with tablets containing only ethinyl estradiol.

SUMMARY

Application of the automatic analyzer to the analysis of ethinyl estradiol has been accomplished. The nonvarying volume, time, and temperature characteristics of this system have enabled the development of a repeatable assay procedure even for very low concentrations of EE. The procedure has been shown to be specific for intact EE in the presence of thermal and photochemical decomposition products. The procedure was also demonstrated to be specific for EE in combination with large amounts of the usual progestational steroid hormones and in the presence of large amounts of other similar estrogenic substances. A number of trade packages of antifertility products were successfully analyzed by the automated method. Twenty samples per hour can be handled easily as opposed to one to two samples per hour by conventional methods. This laboratory has found the automated method very convenient for determining unit-to-unit variation in formulations.

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New System of Disk Electrophoresis Using Large Acrylamide Gels

By ROBERT C. PETERSON

The disk electrophoretic method has been modified to use gels of 22-mm. diameter. A gel tube base was designed to produce a gel with a convex base, and a special buffer cell was provided. With the future goal of preparative electrophoresis, a quenched fluorescence technique was developed which locates the major protein zones without dyeing. A transverse destainer was designed for rapid removal of unbound dye from stained protein patterns.

THE THEORY, methods, and applications of gel electrophoresis have been well reviewed (1, 2). It has been our purpose to develop a simple, convenient method of columnar electrophoresis which could be adapted to preparative electrophoresis. Preliminary work has indicated that this approach is very promising.

The method of Ornstein (3) and Davis (4) has been modified to give gels 22 mm. in diameter by about 11.4 cm. in length.

METHODS AND MATERIALS

The pilot compound for this work was N.F. trypsin crystallized reference standard, lot 6040, 3226 N.F. units/mg. (5). The pH 5 buffer, pH 4.3 gel, and solutions for a gel concentration of 15% have been described.[1] Acrylamide, *N,N'*-methylenebisacrylamide, and β-alanine were of good purity.[2] *N,N,N',N'*-Tetramethylethylenediamine was practical grade, redistilled.[3] The ammonium persulfate and riboflavin were reagent grade.[3] The acetic acid was reagent grade.[4]

The gel tube was placed in the new base (see Fig. 1), and 40 ml. of lower gel ingredients[1] were introduced with the usual water layering. Teflon was selected for the base material in order to give less wear and a minimum of contamination. The 12.7-cm. long glass tube fits snugly within the O-ring and on the shelf in plug C.

When plug C is threaded into block B, the concavity in plug C forms the gel base for glass tube A. This rounded gel base (convex on the gel) is neces-

Received March 27, 1967, from the Analytical Research Department, Eli Lilly and Co., Indianapolis, IN 46206
Accepted for publication July 21, 1967.
The author expresses his gratitude for guidance to Dr. Harry Rose and Mr. Steven Mundy of these laboratories for competent technical assistance.

[1] Canal Industrial Corp., Bethesda, Md.
[2] Eastman Organic Chemicals, white label.
[3] Matheson Coleman and Bell.
[4] Baker and Adamson.