detecting reagents. Degradation was perceived by the gradual appearance of a yellow color at the point of sample application.

In conjunction with the R_f values, further verification of the identity of the anticoagulants considered in this study was ascertained by spraying the chromatograms with either I or II. The first reagent gave characteristic colors with each of the coumarins, whereas the latter only reacted with ethyl biscoumacetate (pink color) and warfarin (pink-violet color). In addition, certain spots were easily identified by their characteristic appearance under 365- and 254-nm. UV light after development with Solvent 2, 4, 7, or 12 (Table II and Fig. 1). As little as 1 mcg. of sample could be detected with iodine vapors, I, or II. The detection limit for phenprocoumon was about 2–3 mcg. III was the least sensitive of all the reagents tested.

The excellent one-dimensional chromatographic separation obtained with the majority of solvents obviates the use of the two-dimensional technique for the resolution of a mixture of coumarin anticoagulants. In general, separation was better than has been reported in other laboratories and the time of development with the solvents employed was much shorter.

This work may be useful as an adjunct tool for the rapid identification and confirmation of coumarin anticoagulants in experimental pharmacology, biochemistry, and quality control as well as in forensic and clinical medicine.

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Fluorometric Determination of Ethinyl Estradiol in Tablets

THERON JAMES

Abstract □ A fluorometric procedure, based on the Liebermann–Burchard reaction, was developed for assaying ethinyl estradiol in tablets. A chloroform extract of ethinyl estradiol is reacted with acetic anhydride and sulfuric acid, and the resulting fluorophor is measured at 400 nm. while exciting at 324 nm. Fluorescence *versus* concentration is linear up to 10 mcg./ml.; the lower limit of detection is 0.5 mcg./ml. under the conditions studied. Replicate analyses showed good agreement, and an average recovery of 100.0 ± 0.94% was obtained for 10 analyses of a synthetic mixture. Assay results on eight different commercial samples (0.01–0.50 mg./tablet) are reported.

Keyphrases □ Ethinyl estradiol tablets—fluorometric analysis, Liebermann–Burchard reaction □ Spectrophotofluorometry—analysis, ethinyl estradiol tablets, Liebermann–Burchard reaction

Commercially available tablets of ethinyl estradiol are usually of very low dosages (0.01–0.05 mg./tablet). Consequently, quantitative analysis of these products has been difficult. The USP (1) colorimetric procedure, a modification of the Kober reaction, has several disadvantages:

1. Ethinyl estradiol is only slightly soluble in isooctane, so it may not be completely extracted in the USP procedure. Indeed, the first supplement to the USP XVIII, official in November 1971, modified the original isooctane extraction by using an isooctane–chloroform mixture instead.

2. The method involves numerous transfers and extractions, making it both time consuming and cumbersome.

3. There are several very critical variables in the color formation (moisture, purity of reagents, preparation of the sulfuric acid–methanol reagent, *etc.*).

Other colorimetric procedures (2–6), which are also variations of the Kober reaction, suffer similar handicaps.

UV (7) and GLC (8, 9) methods for determining ethinyl estradiol also have been reported. These methods require extensive cleanup procedures. Furthermore, the GLC methods usually involve derivatization.

The sensitivity of fluorescence prompts an investigation of its application to the analysis of ethinyl estradiol. Fluorescence methods reported to date (10–14), however, are again extensions of the Kober reaction. Little work has been done on the natural fluorescence of ethinyl estradiol or other forms of induced fluorescence.

Albers and Lowry (15), using a modification of the Liebermann–Burchard reaction, developed a fluorometric procedure for cholesterol. They also examined other steroids under similar conditions. Several steroids exhibited fluorescence, but they differed from cholesterol and from each other both in intensity and spectral characteristics.

Since estrone was among those steroids quoted, this approach was investigated for its applicability to the analysis of ethinyl estradiol. In the proposed method, a portion of the powdered tablet is incorporated into an acid–base column. After a preliminary wash, ethinyl estradiol is eluted with chloroform and the residue from this chloroform extract is subjected to the Liebermann–Burchard reaction. The resulting fluorophor is linear up to 100 mcg. ethinyl estradiol in the final solution.

EXPERIMENTAL

Apparatus—A recording spectrophotofluorometer[1] with 1-cm. cells was used, with the following instrument parameters: xenon lamp; meter multiplier, 0.3; sensitivity, 20–29; 1P21 photomultiplier; slit arrangement No. 4; excitation wavelength, about 324 nm.; and emission wavelength, about 400 nm.

Reagents—Redistilled heptane, spectrograde chloroform[2] (alcohol free), reagent grade concentrated sulfuric acid (96%), and acid-washed diatomaceous earth[3] were used. A chloroform–acetic anhydride reagent (5:1) was prepared by mixing 5 parts chloroform with 1 part fresh or recently opened acetic anhydride.

Standard Preparation—Prepare a solution containing about 10 mcg. of ethinyl estradiol USP, accurately weighed, per milliliter in chloroform. Pipet a 4.0-ml. aliquot into a 125-ml. conical flask and evaporate to dryness. Proceed as in *Procedure*, beginning with: "Pipet 10.0 ml. of chloroform–acetic anhydride reagent into the flask. . . ."

Procedure—Place a pledget of glass wool in the base of a chromatographic column. To 3 g. of diatomaceous earth in a beaker, add 2 ml. of 10% sodium hydroxide, mix until fluffy, and pack moderately in the column.

Grind at least 20 tablets to pass a 60-mesh sieve. Accurately weigh a portion of powder equivalent to about 40 mcg. of ethinyl estradiol, and transfer to a 100-ml. beaker. Add 2–3 ml. of 2 *N* HCl, swirl to effect solution, and place in an ultrasonic bath for about 15 min. Finally, add 3 g. of diatomaceous earth, mix until fluffy, and add to the chromatographic column, packing moderately. Place a pledget of glass wool above the column packing and wash the column with 100 ml. of *n*-heptane. Discard the heptane wash.

Change the receiver to a 125-ml. conical flask, and elute the ethinyl estradiol with 100 ml. of water-washed chloroform. Evaporate the chloroform eluate to dryness and cool. Pipet 10.0 ml. of chloroform–acetic anhydride reagent into the flask, swirl to mix, and let stand 15 min. Add exactly 0.5 ml. concentrated sulfuric acid, stopper, mix, and let stand 2 hr. Read within 30 min. after 2-hr. development time.

Adjust the spectrophotofluorometer to about 70% fluorescence intensity at 400 nm. with the standard solution. Scan the sample and standard solutions from 350 to 550 nm., reading the maximum at about 400 nm. Use 10 ml. of chloroform–acetic anhydride and 0.5 ml. sulfuric acid as a blank.

RESULTS AND DISCUSSION

Initial investigations, using freshly prepared reagents, resulted in inconsistent and nonreproducible spectral intensities for standard solutions. The chloroform used in these early experiments contained 1% ethanol, which apparently caused the erratic results; alcohol-free chloroform produced a more stable and reproducible fluorophor. Spectrograde chloroform or chloroform that has been passed through silica gel[4] is suitable for the analysis. In addition, it was discovered, fortuitously, that allowing the chloroform–acetic anhydride reagent to stand at least 20 hr. resulted in stable, reproducible spectra. If the chloroform contains ethanol, the ethanol will react with acetic anhydride on prolonged standing, thereby effectively eliminating it as an interference in the final solution.

Fluorescence intensity reaches a maximum in about 2 hr. and is fairly stable for 30 min. Fluorescence is proportional to concentration from 0.5 to 10 mcg./ml. (Fig. 1), and ordinary tablet excipients do not interfere. As suggested by Albers and Lowry (15), however, there does appear to be some photodecomposition on prolonged irradiation.

The column cleanup procedure eliminates several sources of errors or interferences:

1. The strong acid neutralizes basic tablet excipients and acidifies the sample mixture, thereby permitting better extraction of ethinyl estradiol.
2. The sodium hydroxide layer serves as a trap to prevent loss of ethinyl estradiol in the heptane wash.

Table I—Recovery of Ethinyl Estradiol from Various Weights of a Placebo

Placebo Weights, mg.	Ethinyl Estradiol Added, mcg.	Recovery, %
50	46	98.4, 99.6
100	46	99.9, 99.6
300	46	99.7, 99.6
500	46	101.1, 101.0
800	46	101.6, 100.0
		Average = 100.0
		SD = 0.94%

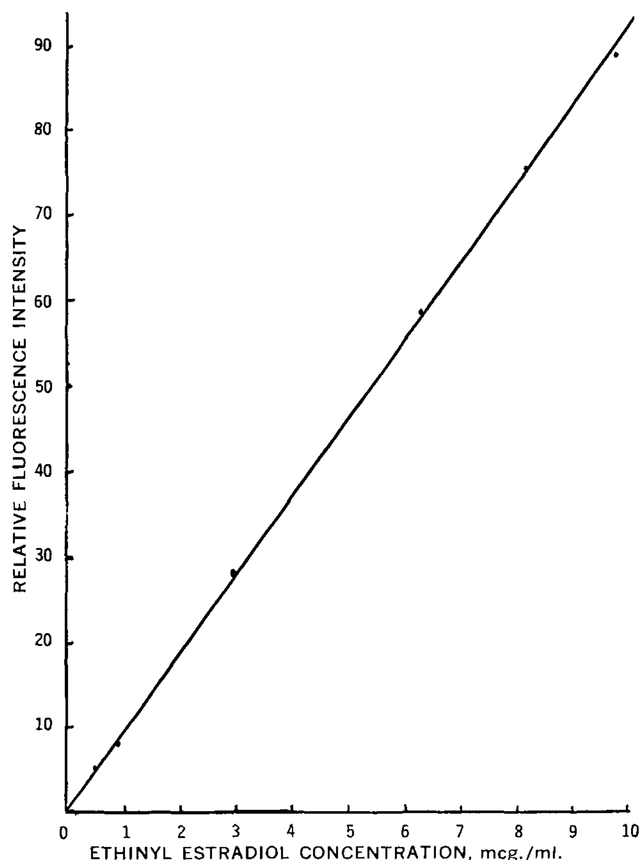

Figure 1—*Standard curve for the fluorometric determination of ethinyl estradiol by the Liebermann–Burchard reaction.*

[1] Aminco-Bowman spectrophotofluorometer, American Instrument Co., Inc., Silver Spring, Md.
[2] Spectroquality chloroform, Matheson, Coleman & Bell, Los Angeles, Calif.
[3] Celite 545, Johns-Manville Corp., New York, N. Y.
[4] Silica Gel Indicating (6–16 mesh), E. H. Sargent & Co.

Table II—Assay of Commercial Ethinyl Estradiol Tablets

Sample	Manufacturer	—Ethinyl Estradiol, mg./Tablet— Declared	Found
1	A	0.01	0.009, 0.009
2	B	0.02	0.019, 0.018 0.019
3	C	0.02	0.020, 0.020 0.019, 0.020
4	D	0.02	0.018, 0.018
5	A	0.05	0.045, 0.043
6	D	0.05	0.053, 0.055
7	E	0.1	0.099, 0.099 0.100, 0.098
8	D	0.5	0.495, 0.503

3. The heptane wash removes some organic tablet excipients and coating material.
4. The acid-base column retains water-soluble dyes and coloring agents.

A placebo was prepared to contain some common tablet excipients (*e.g.*, lactose, starch, and magnesium stearate). Several different weights of this mixture, each approximating sample weights of commercial tablets, were spiked with a known amount of ethinyl estradiol and the assay was performed. The results are shown in Table I. The average recovery was 100 ± 0.94%.

Multiple analyses were performed on eight different commercial samples of varying potencies. These samples represent five different manufacturers, and three of the samples were coated tablets. Agreement between replicate analyses was good, and all samples assayed 90% or better (Table II).

The Liebermann-Burchard reaction is a general reaction for steroids and sterols. However, each differs in fluorescence characteristics, sensitivity, and time of reaction. Consequently, the proposed method does offer a degree of specificity. Additionally, the sensitivity is such that single-tablet analysis, even at the lowest dosage form, is quite feasible.

The method presented here is not applicable to progestin-estrogen preparations. Because they are present in much higher concentrations than ethinyl estradiol, progestational steroids produce a color in the Liebermann-Burchard reaction that quenches the fluorescence of ethinyl estradiol. An adequate separation of the two components would resolve this problem.

Preliminary investigations indicate that an additional cleanup procedure appears to give the desired separation. This cleanup consists of diluting the concentrated chloroform extract with isooctane, extracting ethinyl estradiol with sodium hydroxide, and reextracting ethinyl estradiol into chloroform. Optimum parameters and exact details have not been established, but work is continuing along these lines.

SUMMARY

A fast, facile, fluorometric method for determining ethinyl estradiol in tablets is described. The procedure, based on the Liebermann-Burchard reaction, is sensitive enough for the lowest dosage forms available and offers a certain degree of specificity. It is applicable to a variety of commercially available, single-component preparations, and ordinary tablet excipients and coating materials do not interfere.

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