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Rapid, Sensitive Colorimetric Method for Determination of Ethinyl Estradiol

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Abstract □ A colorimetric procedure, based on the formation of an azo dye by condensation of diazotized 5-chloro-2,4-dinitroaniline with ethinyl estradiol, was developed. An alkaline solution of ethinyl estradiol is reacted with the reagent, and the resulting color is measured at 450 nm. Absorbance *versus* concentration is linear up to 10 μg/ml; the lower limit of detection is 1 μg/ml under the conditions studied. Replicate analysis showed good agreement, and an average recovery of 99.6 ± 0.3% was obtained for analyses of synthetic mixtures. Vitamins and minerals likely to be present along with ethinyl estradiol in certain geriatric formulations, as well as ordinary tablet excipients and coating materials, do not interfere with the precision of the method or development of the color. The method is applicable to progestin–estrogen preparations. Assay results on various single-component as well as contraceptive commercial samples are reported.

Keyphrases □ Ethinyl estradiol in progestin–estrogen and vitamin/mineral geriatric preparations—colorimetric analysis □ Progestin–estrogen contraceptive tablets—colorimetric analysis of ethinyl estradiol □ Geriatric formulations containing vitamins, minerals, and estrogens—colorimetric analysis of ethinyl estradiol □ Colorimetry—analysis, ethinyl estradiol in progestin–estrogen and vitamin/mineral geriatric preparations

Several methods for the analysis of ethinyl estradiol are available in the literature, including UV (1), GLC after suitable derivatization (2, 3), and colorimetric methods (4–8). The USP XVIII colorimetric procedure (9), which is a modification of the Kober reaction (4), suffers from several disadvantages exhibited by the rather limited solubility of ethinyl estradiol in isooctane. These disadvantages might be circumvented by using an isooctane–chloroform mixture instead as modified in the first supplement to USP XVIII. The Kober reaction, in spite of many modifications (10–12), is time consuming, and the color development is critically dependent upon reagent composition, reaction time, and temperature. Interference by other nonphenolic steroids also can occur (13).

The suitability of diazotized 4-amino-6-chloro-*m*-benzenedisulfonamide for the determination of a number of estrogens has been investigated (14). The method, although suitable for routine analysis, has rather low sensitivity. The lower limit of detection is 0.05 mg of estrogen/ml of sample solution. Commercially available dosage forms, especially geriatric formulations, are usually of very low dosage (0.008–0.05 mg/tablet or capsule). Consequently, the sensitivity of this procedure is such that single-tablet analysis of most commercial dosage forms is not feasible.

In spite of its high sensitivity, the Liebermann–Burchard reaction-based fluorometric method of James (15), with 0.5 μg/ml as a lower limit for detection, is not applicable to progestin–estrogen preparations. Progestational steroids produce a color in the Liebermann–Burchard reaction that quenches the fluorescence of ethinyl estradiol.

It has been observed in these laboratories that diazotized 5-chloro-2,4-dinitroaniline couples with phenolic compounds to form products with extremely stable colors; this finding offers the basis for a rapid and sensitive method for the determination of pharmaceuticals containing a phenolic hydroxy group (16). The suitability of this reagent for the determination of ethinyl estradiol was investigated, and a rapid and sensitive colorimetric method for the determination of ethinyl estradiol was developed. This method eliminates the disadvantages of the Urbanyi and James methods, as well as those of the Kober reaction.

EXPERIMENTAL[1]

5-Chloro-2,4-dinitroaniline—This compound was prepared by a reported procedure (17). Several crystallizations from ethanol yielded an analytical sample, mp 174°.

Reagents and Solutions—The following were used: 5-chloro-2,4-dinitroaniline (0.2% ethanolic solution), sodium nitrite (3% aqueous solution), 1 *N* hydrochloric acid, 2 *N* sodium acetate, 0.1 and 1 *N* sodium hydroxide, and absolute ethanol.

[1] A Hungarian-made Spektromom 203 spectrophotometer was used.

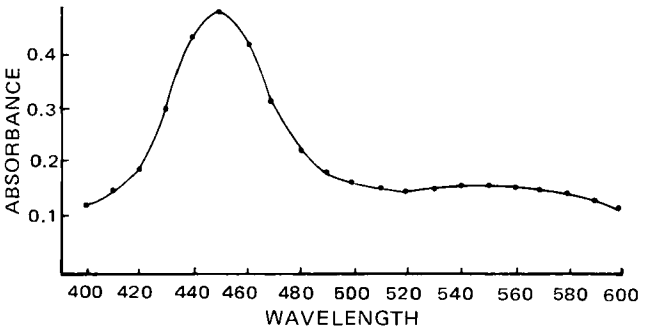

Figure 1—*Absorption spectrum of ethinyl estradiol with diazotized 5-chloro-2,4-dinitroaniline.*

Standard Ethinyl Estradiol Solution—Weigh accurately about 20 mg of ethinyl estradiol USP into a 10-ml volumetric flask and dissolve in ethanol. Dilute to volume with ethanol and then dilute 1 ml of this solution to 10 ml with 0.1 *N* sodium hydroxide. Pipet appropriate volumes so as to give solutions containing 1–10 μg/ml after color development.

Preparation of Assay Solutions—Extract an accurately weighed sample of the powdered synthetic mixture or tablets equivalent to about 0.5 mg of ethinyl estradiol with three 5-ml portions of ethanol. Centrifuge each portion, filter the ethanol extracts into a small beaker, and evaporate the ethanol to dryness. Transfer the contents of the beaker quantitatively with 0.1 *N* sodium hydroxide to a 10-ml volumetric flask and dilute to volume with additional 0.1 *N* sodium hydroxide. This solution is the assay solution.

For Contraceptive Tablets—Prepare as directed for the assay solutions. Transfer the contents of the beaker quantitatively with 0.1 *N* sodium hydroxide to a 10-ml volumetric flask, shake vigorously, transfer to a centrifuge tube, and centrifuge for 10 min. The clear solution is the assay solution for contraceptive tablets.

For Geriatric Formulations Containing Ethinyl Estradiol—Extract an accurately weighed sample of the powdered synthetic mixture equivalent to about 0.5 mg of ethinyl estradiol three times each with 5 ml of alcohol-free chloroform (18), centrifuge for 10 min, collect the extracts in a small beaker, and evaporate to dryness. Transfer the contents of the beaker quantitatively with 0.1 *N* sodium hydroxide to a 10-ml volumetric flask, and dilute to volume with additional 0.1 *N* sodium hydroxide. This solution is the assay solution.

Color Development—Into separate 10-ml volumetric flasks, pipet successively 1.0 ml each of 5-chloro-2,4-dinitroaniline, sodium nitrite, and hydrochloric acid solutions and mix well. Allow to stand for 2 min and add 1 ml of ethanol and 1 ml of standard solution, 1 ml of the assay solution, or 1 ml of 0.1 *N* sodium hydroxide solution to separate flasks. Add 2.0 ml of sodium acetate solution to each flask, and allow to stand for 20 min. Dilute each flask to volume with 1 *N* sodium hydroxide. Allow to stand for 30 min and determine the absorbance of the standard and sample solution at 450 nm in 1-cm cells against the blank using a suitable spectrophotometer.

RESULTS AND DISCUSSION

Initial investigations using lower concentrations of sodium nitrite solution resulted in inconsistent and nonreproducible spectral intensities for standard solutions. Accordingly, a study of the effect of sodium nitrite concentration on diazotization of 5-chloro-2,4-dinitroaniline and subsequent coupling with ethinyl estradiol was carried out using 1, 2, 3, 4, and 5% sodium nitrite solution. Use of the 3% sodium nitrite solution gave the highest color intensity. Destruction of the excess nitrous acid was not necessary since no interference in the subsequent color development and measurement was observed.

The normality of the sodium hydroxide used affected both the development of the color and the reproducibility of the method. Higher concentration of alkali resulted in lower absorption intensities. The most intense and reproducible absorption was obtained when ethinyl estradiol was dissolved in 0.1 *N* sodium hydroxide, coupled, and diluted to volume with 1 *N* sodium hydroxide.

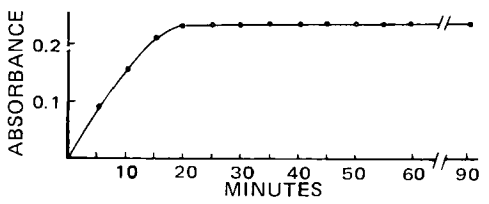

Figure 2—*Effect of coupling time on the absorbance of the final solution.*

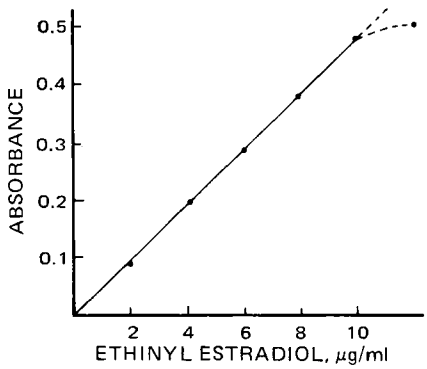

Figure 3—*Standard calibration curve for ethinyl estradiol with diazotized 5-chloro-2,4-dinitroaniline.*

The addition of 1 ml of ethanol to the diazotized solution of 5-chloro-2,4-dinitroaniline was necessary to avoid the turbidity observed in the absence of ethanol. The alcohol probably helps keep the coupling product in solution.

The absorption spectrum of the alkaline solution of the coupled product, resulting from the reaction of ethinyl estradiol with diazotized 5-chloro-2,4-dinitroaniline, is shown in Fig. 1 (absorption maximum at 450 nm). The molar absorptivity as calculated from the curve is 11.284×10^3.

The effect of coupling time under these conditions, after the addition of the alkali, on the absorbance of the final solution at 450 nm is shown in Fig. 2. Color intensity increased during the first 20 min and remained constant for at least 90 min.

The relationship between absorbance at 450 nm and concentration was linear up to 10 μg of ethinyl estradiol/ml of sample solution (Fig. 3).

The specificity of the method for ethinyl estradiol in the presence of several frequently encountered excipients is shown in Table I. A placebo was prepared containing some common tablet excipients (*e.g.*, lactose and starch); several different weights of the excipients, each approximating the sample weight of commercial tablets, were spiked with a known amount of ethinyl estradiol and the assay was performed. The average recovery was 100 ± 0.20%.

Multiple analyses were performed on different commercial samples of varying potencies. Agreement between replicate analyses was good, and all samples assayed 99.6% or better (Table II).

The method presented here is applicable to progestin–estrogen preparations. Synthetic mixtures, in which the ratio of the progestin norethindrone acetate to ethinyl estradiol was that of the average found in commercial formulations, as well as commercial coated contraceptive tablets containing this progestin along with ethinyl estradiol were satisfactorily analyzed by this method. Percentage recoveries were 99.86 ± 0.06%.

Other phenols will, of course, interfere in the determination of ethinyl estradiol by this method, as they would in the Kober reaction and the Urbanyi method. Although such interferences would not normally be expected, interference due to pyridoxine hydrochloride, which possesses a similar phenolic hydroxy group, was encountered. This problem was circumvented by extraction of ethinyl estradiol with alcohol-free chloroform in which pyridoxine hydrochloride is almost insoluble (19). Ascorbic acid does interfere with the development of the color and this interference is removed by the same method. Accordingly synthetic mixtures containing the minerals and vitamins shown in Table I, which are likely to be

Table I—Determination of Ethinyl Estradiol in the Presence of Common Excipients, Minerals, Vitamins, and Norethindrone Acetate[a]

	Ethinyl Estradiol		Ethinyl Estradiol
Lactose		Thiamine hydrochloride	
Added, mg/g	2.00	Added, μg/5 mg	10.0
Found, mg/g	2.00	Found, μg/5 mg	10.0
Recovery, %	100 ± 0.1	Recovery, %	100 ± 0.2
Starch		Niacinamide	
Added, mg/g	2.00	Added, μg/5 mg	10.0
Found, mg/g	2.00	Found, μg/5 mg	9.98
Recovery, %	100 ± 0.2	Recovery, %	99.8 ± 0.7
Norethindrone acetate		Pyridoxine hydrochloride	
Added, mg/50 mg	2.00	Added, μg/1.0 mg	10.0
Found, mg/50 mg	1.99	Found, μg/1.0 mg	9.98
Recovery, %	99.5 ± 0.3	Recovery, %	99.8 ± 0.4
Ascorbic acid		Cobalt sulfate	
Added, μg/50 mg	10.0	Added, μg/120 μg	10.0
Found, μg/50 mg	9.98	Found, μg/120 μg	10.05
Recovery, %	99.8 ± 0.4	Recovery, %	100.5 ± 0.2
Copper sulfate		Manganese sulfate	
Added, μg/1.26 mg	10.0	Added, μg/1.55 mg	10.0
Found, μg/1.26 mg	10.1	Found, μg/1.55 mg	9.98
Recovery, %	101 ± 0.2	Recovery, %	99.8 ± 0.6
Ferrous sulfate		Magnesium sulfate	
Added, μg/20 mg	10.0	Added, μg/2 mg	10.0
Found, μg/20 mg	9.98	Found, μg/2 mg	10.05
Recovery, %	99.8 ± 0.3	Recovery, %	100.5 ± 0.2
Zinc sulfate			
Added, μg/1 mg	10.0		
Found, μg/1 mg	10.0		
Recovery, %	100 ± 0.6		

[a] Values are averages of three determinations.

Table II—Determination of Ethinyl Estradiol in Single-Component and Contraceptive Tablets

Sample Type	Ethinyl Estradiol Declared, mg	Amount Found, mg/Tablet[a]	Recovery, %
Single component	0.050	0.0496	99.6 ± 0.4
Contraceptive	0.050	0.0499	99.96 ± 0.06

[a] Average of four determinations.

present along with ethinyl estradiol in certain geriatric formulations (20), were satisfactorily analyzed by this method. The amounts chosen were the averages found in commercial preparations (20).

The sensitivity of the method is such that single-tablet analysis, even at the lowest dosage, is feasible; this sensitivity is an improvement over the diazotized 4-amino-6-chloro-*m*-benzenedisulfonamide method (14). Another advantage is the applicability of the method to progestin–estrogen combinations, which is an improvement over the fluorometric method (15).

These improvements make this method more suitable for the determination of ethinyl estradiol and related estrogens in biological fluids, as well as for the determination of other compounds of pharmaceutical interest containing a hydroxy group (to be discussed in a future report).

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