Selective Colorimetric Determination of Ethynodiol Diacetate in Oral Estrogen-Progestin Combination

RODOLFO PASINI and GILBERTO GAVAZZI

Abstract □ A colorimetric method has been developed for the rapid characterization and precise determination of ethynodiol diacetate in an estrogen-progestin combination using antimony trichloride as reagent. The results obtained demonstrate the specificity of this reaction for ethynodiol diacetate compared with other substances with progestinic action often used in these combinations. Furthermore, the results prove the reliability and practicality of this reaction as well as the complete lack of interferences from the estrogen fraction present in these formulations.

Keyphrases □ Estrogen-progestin combination—analysis □ Ethynodiol diacetate—determination □ Antimony trichloride—color reagent □ Colorimetric analysis—spectrophotometer

Many analytical techniques such as gas chromatography (1–5), thin-layer chromatography (TLC) (1–6), UV spectrophotometry (4–6), fluorometry (5, 7, 8), and colorimetry (5, 8–10) have been used for the determination of the estrogen component (ethinylestradiol or mestranol) present in the estrogen-progestin tablets and the procedures are abundantly described in literature.

On the other hand, there are few reports regarding the determination of the progestin component perhaps because of the ever-increasing numbers of hormones with a progestative function used in oral formulations. For some of these, chlormadinone acetate (6–11), lynestrenol (6), norethynodrel (6, 12, 13), norethisterone (6), allylestrenol (14), and megestrol acetate (6), the analytical problem has been solved utilizing particular colorimetric reactions or, even better, UV absorption.

Determination of ethynodiol diacetate by means of UV readings after extraction from the tablets is not possible because the low maximum (203–205 mμ) of the substance makes it almost impossible to eliminate the interference produced by the extraction products. Keay (6) suggested for the determination of ethynodiol diacetate, a method of separation by TLC with quantitative determination of the steroid in reference to a series of standards. A saturated solution of antimony trichloride in chloroform was used as the developer and the color intensity of the spots was read with a densitometer.

This work served as the basis for the present study in which the developer for the TLC was used as the reagent for the identification and quantitative colorimetric determination.

EXPERIMENTAL

Apparatus—Spectrophotometer with 1-cm. cells (Zeiss model PMQ 11).

Reagents—*Chloroform*—Ethanol-free, analytical reagent grade, stored in a cool, dark place over anhydrous sodium sulfate. The ethanol, usually present as stabilizer, must be eliminated by refluxing the chloroform over sodium for about 4 hr. Then the chloroform should be distilled through a Vigreu column of 80–90 cm. to eliminate the initial fractions.

Acetic Anhydride—Analytical reagent grade.

Reagent—Antimony trichloride solution (25%), in dry ethanol-free chloroform containing 1% acetic anhydride. The standardization of the reagent preparation method is extremely important because remarkable differences in results have been observed when the reagent is prepared in different ways.

Twenty-five grams of antimony trichloride, perfectly dry, is placed in a 100-ml. glass-stoppered volumetric flask, containing 60–70 ml. of ethanol-free chloroform. Shake continuously until the antimony trichloride is dissolved, and make to volume. During the solubilization process, warming must be avoided. The solution obtained should be filtered rapidly and 1 ml. of acetic anhydride added. The reagent must be kept in a dark cool place and stored on anhydrous sodium sulfate.

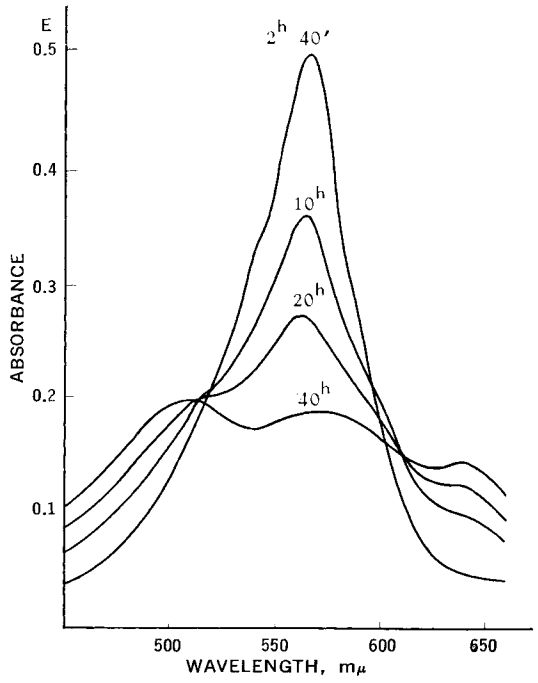

Figure 1—*Change with time of the visible absorption spectrum of the reaction.*

Reference Standards—*Identity Test*—Twenty milligrams of reference standard ethynodiol diacetate was dissolved in dry chloroform containing 1% acetic anhydride and diluted to 50 ml. to obtain a solution containing about 200 mcg./ml. Solutions of chlormadinone acetate, norethynodrel, norethisterone, lynestrenol, ethisterone, medroxyprogesterone acetate, ethinylestradiol, and mestranol were prepared in the same way. The purity of the substances was determined by TLC.

Assay—Twenty-five milligrams of ethynodiol diacetate, accurately weighed, was dissolved in dry chloroform containing 1% acetic anhydride, and diluted to 100 ml. Further dilutions of 3, 4, and 5 ml. to 50 ml. were made to obtain three solutions containing exactly 15, 20, and 25 mcg./ml.

Identity Test—The identification of ethynodiol diacetate contained in tablets was made as follows: the equivalent of four ground tablets, corresponding to about 4 mg. of ethynodiol diacetate was placed into a 50-ml. glass-stoppered conical flask, 20 ml. of dry chloroform containing 1% acetic anhydride was added, and the mixture shaken for about 30 min. on a mechanical shaker, then rapidly filtered. Two milliliters of this solution, corresponding to about 400 mcg. of ethynodiol diacetate, was pipeted into a 10-ml. glass-stoppered test tube, and 3 ml. of reagent was added. An intense violet color, with a max. at 565 mμ was produced after a few minutes.

Assay Procedure for Formulated Ethynodiol Diacetate—Tablets containing 1 mg. of ethynodiol diacetate and 100 mcg. of mestranol were assayed by the following procedure.

The average unit weight of no less than 20 units for composite sample assay was determined and the sample was reduced to a fine powder in a glass mortar. A quantity, exactly weighed, of the powder, equivalent to about two ground tablets was transferred in a 100-ml. glass-stoppered volumetric flask. A 60–70-ml. quantity of dry chloroform containing 1% acetic anhydride was added and the mixture was shaken for about 30 min. on a mechanical shaker. A quantity of solvent required for dilution to exactly 100 ml. was added and the solution containing theoretically 20 mcg./ml. of ethynodiol diacetate, was filtered rapidly. A 2-ml. portion of the solution, corresponding to about 40 mcg. of ethynodiol diacetate was pipeted into a 10-ml. glass-stoppered test tube; a standard curve was prepared measuring 2 ml. of the standard solutions corresponding to 30, 40, and 50 mcg. of steroid into three 10-ml. glass-stoppered test tubes. After the addition of 3 ml. of reagent to each test tube, they were stoppered, shaken for a short time, and kept in a dark place at 25 ± 0.5°. Color development was allowed for 2 hr. 40 min., and the contents of each stoppered test tube were transferred to 1-cm. glass cells. Absorbance values at 565 mμ were measured immediately using, as a reference blank, dry chloroform containing 1% acetic anhydride. The quantity of ethynodiol diacetate in the reaction was determined by reading the concentration from a Beer-Lambert plot. The percentage of ethynodiol diacetate contained in the tablets was calculated from this quantity.

RESULTS AND DISCUSSION

The concentration optimum of the reagent was determined using concentrations of 15, 20, 25, and 30% of antimony trichloride. The best results were obtained with the reagent at 25%. The exact concentration of the reagent is very important because any variations may lead to considerable changes in the color intensity.

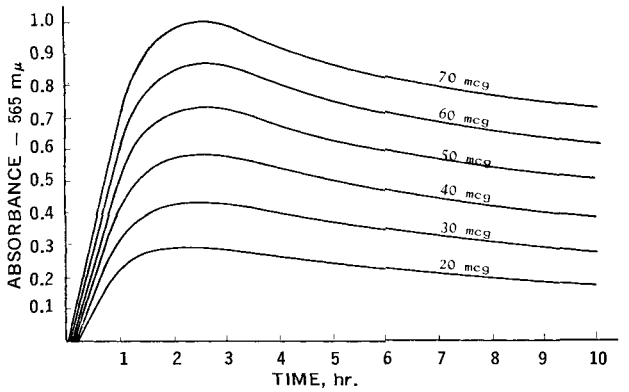

Figure 2—*Plot of absorbance* versus *time for specific concentrations of ethynodiol diacetate.*

Therefore, the concentration may be checked by titration of the chlorides adding 1 ml. of reagent to about 100 ml. of 10% tartaric acid solution and titrating with a silver nitrate solution. The concentration optimum of the solution is between 24.8 and 25.2%.

The reaction time has been determined following the color development. The reaction reaches a peak after 2 hr. 20 min. and remains stable for several minutes. After 3 hr., the beginning of the degradation is observed and this degradation tends to modify the visible absorption spectrum until, after 10 hr., an inflection appears at a wavelength of 520 mμ.

After 20 hr. from the beginning of the reaction, there is a levelling of the visible absorption spectrum, while after 40 hr. the absorbance value of $\lambda_{max.}$ at 510 mμ is higher than the absorbance value of $\lambda_{max.}$ at 565 mμ and the visible absorption spectrum shows a $\lambda_{min.}$ at wavelength 540 mμ (Fig. 1).

The interference of light in the development of the reaction has been shown. Contrasting extinction values have been observed in solutions exposed to light, while such anomalies have not occurred in solutions stored in dark places. Finally, the independence of the concentration from the reaction time has been studied. It has been found that the reaction time is similar at the different concentrations; this means that the maximum color intensity is reached at all concentrations after 2 hr. 40 min. (see Fig. 2).

Specificity—The specificity of the reaction for ethynodiol diacetate has been studied in comparison with other progestin and estrogen molecules generally used in estrogen-progestin combinations. This research has shown the specificity of the ethynodiol diacetate reaction in comparison with the different substances tested. After 2 hr. 40 min. of reaction, the extinction values of the solutions have been determined at their $\lambda_{max.}$ and at 565 mμ. In this manner, it has been possible to determine the ratio between the reaction intensity of ethynodiol diacetate and the one of other substances. The results of this research are shown in Table I and Fig. 3.

Repeatability—Six experiments have been performed at the same time on four different concentrations using the same standard solution and dry reagent and working in a dry place at 25 ± 0.5°. The average deviation of the extinction values of ethynodiol diacetate ranged from ±0.50%. This limit can be lowered working with larger volumes; that is, using 10 ml. of solution and 15 ml. of re-

Table I—Colorimetric Reaction with Time of Some Steroids Usually Present in Oral Estrogen-Progestin Combination

Substance	Initial Color	After 30 min.	After 1 hr.	After 120 min.	After 180 min.	Max.[a]
Ethynodiol diacetate	Red-violet	Violet (strong)	Violet (strong)	Violet (strong)	Violet (strong)	565[b]
Chlormadinone acetate	Colorless	Colorless	Colorless	Colorless	Colorless	No[c]
Norethynodrel	Slightly opal	Slightly opal	Slightly opal	Slightly opal	Slightly opal	No
Lynestrenol	Colorless	Colorless	Pale pink	Pale pink	Pink	530
Ethisterone	Colorless	Colorless	Colorless	Colorless	Colorless	No
Medroxyprogesterone ac.	Colorless	Colorless	Colorless	Colorless	Colorless	No
Norethisterone	Colorless	Colorless	Colorless	Colorless	Colorless	No
Ethinylestradiol	Pale green	Colorless	Colorless	Colorless	Pale violet	565
Mestranol	Colorless	Colorless	Colorless	Colorless	Pale blue	595

[a] Blank = chloroform + 1% acetic anhydride. [b] Spectrophotometric determination of ethynodiol diacetate solution was possible after dilution 1:50 with chloroform. [c] No = colorless solution.

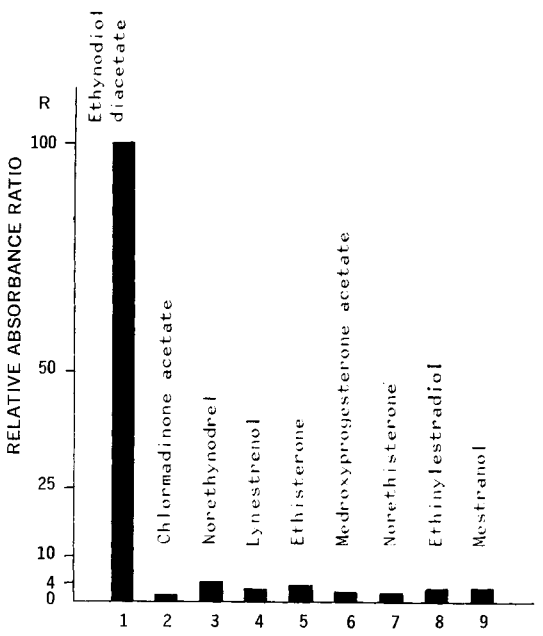

Figure 3—*Colorimetric reaction of some steroids usually present in oral estrogen-progestin combination. Ratio absorbance value at* λ = *565 mμ.*

agent instead of 2 ml. of solution and 3 ml. of reagent. The results are shown in Table II.

Interferences—It was found that none of the excipients (cornstarch, lactose, Mg stearate, talc, *etc.*), interfered in the determination. It has been stated that the moisture sometimes present in the solutions or in the glassware interferes with the development of the reaction. Therefore the solution must be kept on dry sodium sulfate and the glassware dried before use. The preparation must be made quickly and during the reaction development the solution must be kept in a dry place and in glass-stoppered test tubes.

Linearity—The linearity of the reaction has been investigated in a concentration range between 5 and 90 mcg. of ethynodiol diacetate at a volume of 5 ml. or between 25 and 450 mcg. at a volume of 25 ml. The developed color gives absorbance at 565 mμ which is linear with concentration in a range of 5–60 mcg. (or 25–300 mcg. for tests at the volume of 25 ml.), but with higher concentrations the relationship is not linear.

Recovery of Ethynodiol Diacetate Added to Placebo Tablets—Essential quantitative recovery of added ethynodiol diacetate was obtained and has been demonstrated by experiments using 0.5, 1, 2, and 5 mg. of ethynodiol diacetate and 100 mg. of excipients. These data are reported in Table III.

Assay of Ethynodiol Diacetate Tablets—The analytical method described here has been used for the analysis of ethynodiol diacetate

Table II—Reproducibility of Absorbance Values of Replicate Ethynodiol Diacetate Determinations

Ethynodiol Diacetate Standard (mcg. in Reaction)[a]			
Sample 1[b] (20 mcg.)	Sample 2[b] (30 mcg.)	Sample 3[b] (40 mcg.)	Sample 4[b] (50 mcg.)
0.283	0.426	0.576	0.718
0.285	0.424	0.575	0.721
0.283	0.428	0.571	0.720
0.282	0.426	0.571	0.720
0.282	0.427	0.574	0.723
0.285	0.428	0.573	0.719
$\bar{X}$ = 0.283	0.427	0.573	0.720
SD = ±0.0014	±0.0015	±0.0021	±0.0017
RSD = ±0.50%	±0.35%	±0.36%	±0.24%

[a] Determinations at the volume of 5 ml. [b] All samples were aliquots of the same standard ethynodiol diacetate solution.

Table III—Recovery of Ethynodiol Diacetate from Various Quantities of Ethynodiol Diacetate Standard Added to Placebo Tablets[a]

Ethynodiol Diacetate, mg. Added	Found	Recovery, %
0.5	0.5065	101.3
0.5	0.4970	99.4
0.5	0.5015	100.3
1	1.033	103.3
1	1.000	100.0
1	1.016	101.6
2	2.032	101.6
2	1.994	99.7
2	2.000	100.0
5	5.065	101.3
5	4.985	99.7
5	5.065	101.3
$\bar{X}$ = 100.8%		
RSD = ±1.1%		

[a] Average value of duplicate assay.

contained in two commercial preparations. The assay has been performed on eight samples and the results obtained, which have been reported in Table IV, show the precision of the analytical technique. The relative standard deviation remains within the limit of ±1.03 and 1.74%.

Table IV—Assay of Ethynodiol Diacetate Tablets

Declared (1000-mcg./Tablet)	
Brand A, % Found	Brand B, % Found
99.8	100.8
100.8	98.1
99.8	101.0
101.8	98.3
99.2	98.2
102.0	102.0
99.7	98.1
100.0	101.6
$\bar{X}$ = 100.4%	99.76%
SD = ±1.034	±1.735
RSD = ±1.03%	±1.74%

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