Rapid, Sensitive Colorimetric Method for the Determination of Estrogens

By TIBOR URBANYI and C. R. REHM

A colorimetric method has been developed for the routine determination of estrogens in pharmaceutical preparations. The method is based on the formation of an azo dye from the condensation of diazotized 4-amino-6-chloro-*m*-benzene disulfonamide with estrogens which contain a phenolic hydroxyl group. The condensation product exhibits a red color with an absorption maximum at 500 mμ in alkaline solution. The most reproducible results are obtained when coupling is carried out at pH 5 buffered solution. The color formation obeys Beer's law over a range of 0.05–0.25 mg. of estrogens per ml. of sample solution. Optimum conditions for the color formation have been determined, and the application of this procedure to pharmaceutical products is given.

THE OFFICIAL U.S.P. procedure for the determination of ethinyl estradiol, estradiol benzoate, estradiol dipropionate, and estrone are based on the Kober reaction in which a violet color is developed on reaction of the estrogens in a sulfuric acid–phenol–iron reagent (1). The Kober reaction, in spite of many modifications over the years, still leaves much to be desired in an analytical procedure. The method is time-consuming, and the color development is critically dependent upon reagent composition, reaction time, and temperature. Furthermore, interference by other nonphenolic steroids can occur (2).

Colorimetric methods, based on the coupling of the phenolic hydroxyl group with diazotized amines, have been reported for a number of the estrogens. These include coupling with diazotized *p*-nitroaniline (3), with diazotized sulfanilic acid (4, 5) and its derivatives (6), and with tetrazotized dianisidine (7). These methods have been used primarily in the determination of estrogens in biological samples.

It has been observed in these laboratories that diazotized 4-amino-6-chloro-*m*-benzene disulfonamide couples with phenolic compounds to form products with extremely stable colors (8). The suitability of this reagent for the determination of a number of estrogens has been investigated. As a result of these studies, a rapid and sensitive colorimetric method for the determination of ethinyl estradiol, estradiol, estradiol esters, and estrone has been developed.

EXPERIMENTAL

A Beckman model DU spectrophotometer was used to determine the absorbance values reported. A Cary model 11 recording spectrophotometer was used to obtain the spectra presented.

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Determination of Ethinyl Estradiol and Other Phenolic Estrogens

Reagents and Solutions.—4-Amino-6-chloro-*m*-benzene disulfonamide, 0.2% methanolic solution; sodium nitrite, 1% aqueous solution; hydrochloric acid, 1 *N;* sulfuric acid, 18 *N;* sodium acetate, 2 *N* solution; sodium hydroxide, 0.1 and 1 *N* aqueous solutions; sodium hydroxide, 10% aqueous solution; sodium hydroxide, 10% in 80% methanol–water.

Standard Estrogen Solution.—Weigh accurately about 50 mg. of the estrogen into a 50-ml. volumetric flask and dissolve it in methanol. Make up to volume with methanol, then dilute 10.0 to 100 ml. with 0.1 *N* sodium hydroxide. Concentration: approximately 0.1 mg./ml.

Preparation of Assay Solutions.—*Solid Dosage Forms.*—Extract an accurately weighed sample of the powder or powdered tablets equivalent to about 5 mg. of the estrogen with three 10-ml. portions of methanol. Centrifuge each portion and filter the methanol extracts into a beaker and evaporate the methanol to dryness. Transfer the contents of the beaker quantitatively with 0.1 *N* sodium hydroxide to a 50-ml. volumetric flask and dilute to volume with additional 0.1 *N* sodium hydroxide. This is the assay solution

Oil Solutions.—Pipet 5.0 ml. of 10% sodium hydroxide solution in 80% methanol–water into a 40-ml. centrifuge tube. Accurately pipet a volume of the oily sample solution containing approximately 5 mg. of estradiol or estradiol esters and shake mechanically for a minimum of 15 min., add 20 ml. of petroleum ether and 5 ml. of 10% aqueous sodium hydroxide, shake for 2 min., and centrifuge. Remove the lower layer with a syringe equipped with a blunt, 14-gauge needle and filter the solution through a paper filter into a 50-ml. volumetric flask. Add 10 ml. of 10% sodium hydroxide to the centrifuge tube, shake 2 min., centrifuge, and filter the lower layer to the flask as before. Add sulfuric acid dropwise until the solution is approximately neutral (test with indicator paper, pH between 6 and 8). Dilute the solution to volume with 0.1 *N* sodium hydroxide. This is the assay solution.

Color Development.—Into separate 10-ml. volumetric flasks pipet successively 1.0 ml. each of 4-amino-6-chloro-*m*-benzene disulfonamide, sodium nitrite, and hydrochloric acid solutions, and mix well. Allow to stand 1–2 min. and add 2.0 ml. of the stand-

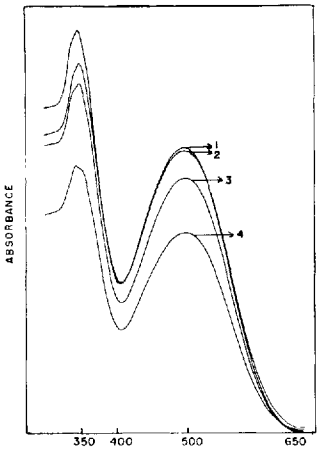

Fig. 1.—Absorption spectra of coupled products of: 1, estradiol; 2, estrone; 3, ethinyl estradiol; 4, estradiol dipropionate.

TABLE I.—MOLECULAR ABSORPTIVITIES

Compd.	E (500 mμ)
Estradiol	8.17×10^3
Ethinyl estradiol	7.93×10^3
Estradiol dipropionate	8.07×10^3
Estrone	8.04×10^3

TABLE II.—STABILITY OF COLOR OF COUPLING PRODUCT OF ESTRADIOL

Time, hr.	Absorbance at 500 mμ: Dark	Laboratory Lighting	Sunlight
0	.525	.525	.525
0.5	.525	.525	.525
1.0	.525	.525	.525
1.5	.525	.520	.525
2.0	.535	.535	.540
3.0	.530	.530	.540
4.0	.530	.530	.540

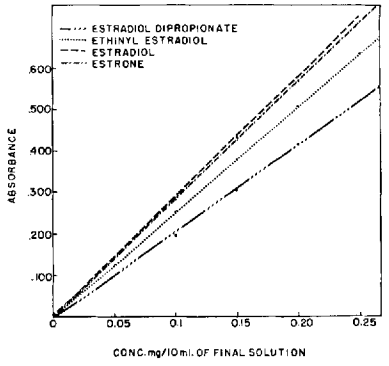

Fig. 4.—Plots of absorbance *vs.* concentration for diazo products of various estrogens.

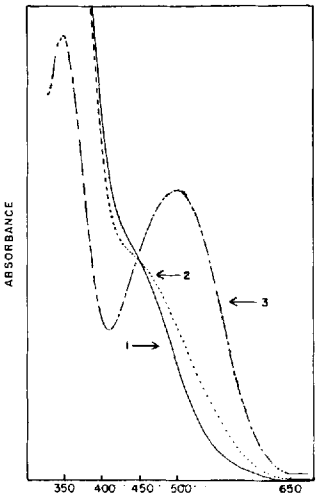

Fig. 2.—Effect of pH on the absorption spectrum of the coupled product of estradiol. Key: 1, pH 5.3; 2, pH 10.1; 3, pH 13.0.

ard solution, 2.0 ml. of the assay solution, and 2.0 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 exactly 6 min. Dilute each flask to volume with 1 *N* sodium hydroxide and determine the absorbance of the standard and sample solutions at 500 mμ in 1-cm. cells against the blank, using a suitable spectrophotometer.

DISCUSSION

The absorption spectra of alkaline solutions of the coupled products, resulting from the reaction of ethinyl estradiol, estradiol, estradiol dipropionate after saponification, and estrone with diazotized 4-amino-6-chloro-*m*-benzene disulfonamide are shown in Fig. 1. It is seen that similar spectra are obtained

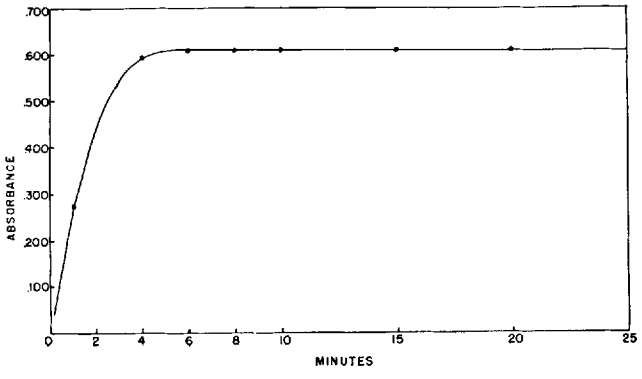

Fig. 3.—Absorbance of final solution *vs.* coupling reaction time for estradiol; coupling reaction carried out at pH 5.0.

TABLE III.—COLORIMETRIC DETERMINATION OF ESTROGENS IN THE PRESENCE OF COMMON EXCIPIENTS

Excipient	Estradiol	Ethinyl Estradiol	Estrone	Estradiol Dipropionate
Lactose				
Added, mg./Gm.	1.00	1.00	1.00	...
Found, mg./Gm.[a]	1.02	1.00	1.00	...
% Recovery	100	100	100	...
Starch				
Added, mg./Gm.	3.30	3.30	3.30	...
Found, mg./Gm.[a]	3.23	3.30	3.26	...
% Recovery	98	100	99	...
Sesame Oil				
Added, mg./Gm.	...	...	...	1.00
Found, mg./Gm.[a]	...	...	...	0.99
% Recovery	...	...	...	99

[a] Average of duplicate determinations.

for the four compounds, each exhibiting a broad absorption maximum at 500 mμ. Esters of estradiol exhibit spectra identical to that of estradiol when coupled with the reagent after saponification, as shown in Fig. 1. Saponification of mono- and diesters of estradiol proceeds rapidly in alkaline solution at room temperature and is complete in 10 min. or less in aqueous media which is 0.1 *M* or stronger in hydroxyl ion. The structure of the coupled products has not been determined; however, it is logical to assume that coupling occurs in one of the positions *ortho* to the phenolic hydroxyl group.

The molecular absorptivities of these four estrogens as calculated from the curves in Fig. 1 are listed in Table I.

It can be seen from these data that the molar absorptivities of the four compounds are nearly identical within experimental error.

The pH dependency of the absorption spectrum of the reaction product of estradiol with diazotized 4-amino-6-chloro-*m*-benzene disulfonamide is shown in Fig. 2.

It can be seen from these spectra that the red color of the coupled product is produced only in a highly alkaline medium (pH > 12). At pH 5 or below, the coupled product in solution exhibits a yellow color, the absorption spectrum exhibiting a shoulder at 450 mμ. At pH values between pH 5 and 12, varying shades of orange are produced. The absorption band at 500 mμ increases in intensity up to a pH of about 13; at pH values above this no further increase in intensity occurs. The spectra exhibit an isosbestic point at about 450 mμ as shown in Fig. 2. The absorption spectra of the coupled products of ethinyl estradiol and estrone with the diazotized disulfonamide exhibit similar pH dependencies.

The initial reaction between nitrous acid and 4-amino-6-chloro-*m*-benzene disulfonamide appears to be somewhat more complex than a simple diazotization reaction since it has been observed that approximately 2 moles of nitrous acid are consumed by the compound (8). Conditions for diazotization of the disulfonamide do not appear to be critical. Diazotization of the disulfonamide in 1 *N* hydrochloric acid is complete in 2 min. in the presence of excess nitrous acid. Destruction of the excess nitrous acid was not found to be necessary since no interference in the subsequent color development and measurement was observed. Coupling of the estrogens studied with diazotized 4-amino-6-chloro-*m*-benzene disulfonamide does not proceed readily in acid solution but was found to proceed rapidly when carried out at pH 5 or above. The most reproducible absorbance values were obtained when coupling was carried out at about pH 5. When coupling was carried out in alkaline solution, it was found that the resulting absorbance (color intensity) was critically dependent upon the rate of addition of alkali. This indicated a rapid destruction of the diazotized disulfonamide in highly alkaline solution. By coupling at pH 5, however, this problem is avoided and day to day variation in color intensity never exceeded 2%. This is consistent with the observation of Rehm and Smith, who found that coupling of the reagent with chromotropic acid under similar conditions of pH also gave highly consistent absorbance values (8). The effect of coupling time at pH 5 before the addition of alkali upon the absorbance of the final solution at 500 mμ is shown in Fig. 3. It is seen that color intensity increases during the first 6 min. and then remains constant over a period of at least 25 min. This indicates that the resulting diazo compound is quite stable at this pH.

The extreme stability of the red color as measured at 500 mμ is shown by the data in Table II. These data indicate no significant changes in absorbance of the final solution over a 4-hr. period in the dark and under normal laboratory illumination. An increase of about 3% in absorbance is noted after 2 hr. for solutions placed in direct sunlight.

The relationship between absorbance at 500 mμ and concentration was found to be quite linear for ethinyl estradiol, estradiol, estradiol dipropionate, and estrone as shown in Fig. 4.

The specificity of the method for these compounds in the presence of several frequently encountered excipients is demonstrated by the data shown in Table III. Recoveries of 98–100% were obtained for the various estrogens in the presence of lactose, starch, and sesame oil. Other phenols will, of course, interfere in the determination of these estrogens by this method as they would in the case of the Kober reaction; however, such interferences would not normally be expected.

The reproducibility, sensitivity, and rapidity of this procedure represent a considerable improvement over the presently used Kober reaction for the determination of these estrogens.

The application of this reaction to the determination of other compounds of pharmaceutical interest

containing phenolic hydroxyl groups such as pyridoxine will be presented in a future report.

SUMMARY

Diazotized 4-amino-6-chloro-*m*-benzene disulfonamide has been found to couple with various estrogens containing a phenolic hydroxyl group to yield a stable red color in alkaline solution.

This reaction is the basis of a rapid, sensitive, and reproducible method for the determination of estrogens such as estradiol, ethinyl estradiol, estrone, and estradiol dipropionate.

Commonly encountered excipients such as lactose, starch, and sesame oil were found not to interfere in the determination of these estrogens.

REFERENCES

(1) Kober, S., *Biochem. Z.*, **239**, 209(1931).
(2) Venning, E. H., Evelyn, K. A., Harkness, E. V., and Browne, J. S. L., *J. Biol. Chem.*, **120**, 225(1937).
(3) Schmulowitz, J. J., and Wylie, H. B., *J. Lab. Clin. Med.*, **21**, 210(1935).
(4) Talbot, N. B., Wolfe, J. K., MacLachlan, E. A., Karush, F., and Butler, A. M., *J. Biol. Chem.*, **134**, 319 (1940).
(5) Bender, A. E., and Wilson, A., *Biochem. J.*, **41**, 423 (1947).
(6) Mitchell, F. L., and Davies, R. E., *ibid.*, **56**, 690(1954).
(7) Lieberman, S., *J. Clin. Invest.*, **31**, 341(1952).
(8) Rehm, C. R., and Smith, J. B., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 386(1960).

Effects of Adsorbents on Drug Absorption II

Effect of an Antidiarrhea Mixture on Promazine Absorption

By DONALD L. SORBY and GRACE LIU

An antidiarrhea mixture containing attapulgite and citrus pectin was studied for its potential effect on the absorption of promazine from the human gastrointestinal tract. Test conditions were established so that results would have maximum applicability to the clinical use situation. Drug and adsorbent were not equilibrated prior to administration. Under these conditions, the antidiarrhea mixture decreased the rate and extent of absorption of the test drug. An *in vitro* adsorption study established that the antidiarrhea mixture had a strong affinity for the test drug. Results are in general agreement with the previous report in this series.

A PREVIOUS report (1) showed that activated charcoal and activated attapulgite both altered the absorption of promazine from the gastrointestinal tract. Prior to administration to human subjects, a 50-mg. quantity of promazine was equilibrated with the particular adsorbent material. The resulting test doses contained 38.5 mg. of the total 50-mg. quantity of promazine adsorbed to activated attapulgite or 24.7 mg. bound to activated charcoal. Under these conditions, activated attapulgite slowed the rate of absorption; however, it had no significant effect on the total availability of the drug. Activated charcoal, on the other hand, appeared not to release any of the adsorbed drug while within the gastrointestinal tract and only promazine, which was free in solution in the test dose at the time of administration, was absorbed. *In vitro* studies of desorption rates from adsorbates demonstrated that promazine release was rapid from activated attapulgite and very slow from activated charcoal.

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Several pharmaceutical products containing adsorbent materials are intended to control diarrhea by exerting their action within the gastrointestinal tract. In this respect they are thought to adsorb certain toxic amines produced by putrefaction or as by-products of bacterial metabolism, and thus prevent their undesirable actions on the human body (2). The question arises concerning whether the presence of such adsorbent materials within the gastrointestinal tract might also interfere with absorption of amine-type drugs, many of which are known to be strongly adsorbed *in vitro* by the materials included in antidiarrhea preparations (2–6).

The observed effects of adsorbents on drug absorption (1) are applicable only to the situation where drug and adsorbent are equilibrated before administration to test subjects. The question concerning whether the same effects will be obtained if drug and adsorbent do not meet until both are within the confines of the gastrointestinal tract was left unanswered.

The purpose of this research was to determine whether the presence of an adsorbent antidiarrhea mixture within the gastrointestinal tract would interfere with the absorption of an amine-type drug. Promazine hydrochloride (50