Determination of Micro Amounts of Estrogens in Anabolic Vitamin Tablets by Quantitative Paper Chromatography

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A quantitative paper chromatographic method has been developed which is used routinely as a stability assay for estrogenic hormones in anabolic vitamin tablets. The method involves (*a*) extraction of the estrogenic hormone into ether from an alcohol-water suspension of the ground tablets, (*b*) clarification of the extract by chromatography on an alumina column, (*c*) paper chromatographic separation of the estrogenic hormone present in the column eluate from interfering degradation products, (*d*) location of the estrogenic hormone on the chromatogram by the guide strip technique employing a chromogenic agent, (*e*) elution of the estrogenic hormone from the chromatogram, and (*f*) quantitative spectrophotofluorometric analysis of the eluate. Recoveries in excess of 95 per cent are obtained by chromatographing simultaneously replicates of standard and sample solutions on the same chromatogram. A detailed account of the procedure is presented using the assay of ethinyl estradiol in concentrations of 0.0014 per cent in anabolic vitamin tablets as an example.

ANABOLIC VITAMIN tablets are oral preparations containing small amounts of anabolic steroid hormones combined with vitamins and minerals.

While the androgen–estrogen ratio in such tablets is proportioned to induce the desired anabolic action, the physiological potency of estrogens necessitates that they be present in micro amounts.

A quantitative paper chromatographic–spectrophotofluorometric method for the analysis of estrogens in castor oil and tablet formulations (1) serves as the basis for the quantitation of estrogens in anabolic vitamin tablets. However, the multiplicity of components present in such preparations requires extensive extraction and clarification prior to paper chromatographic quantitation.

The estrogen is extracted into ether from an alcohol-water suspension of the ground tablets, and is then adsorbed on an alumina column for separation from some interfering constituents. After elution from the column, the solvent is evaporated, and the estrogen residue is dissolved in a small volume of 95% ethanol. Aliquots of this extract are subjected to paper chromatography, followed by quantitation of the estrogen by spectrophotofluorometry.

The method is described in detail using, as an example, the determination of ethinyl estradiol in concentrations of 0.0014% in anabolic vitamin tablets[1] (Table I).

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[1] Oral preparation providing therapeutic amounts of ethinyl estradiol and methyltestosterone plus protective amounts of vitamins and added minerals. Marketed as Dumogran by E. R. Squibb & Sons, New Brunswick, N. J.

EXPERIMENTAL

Reagents and Equipment.—Analytical grade ether, chloroform (reagent grade), aqueous acetone solution (add 1% water to reagent grade acetone), 95% ethanol (redistilled for paper chromatography), toluene (reagent grade), and propylene glycol (reagent grade) were used. Aluminum oxide (Woelm, neutral activity, grade 1 for chromatography, Alupharm Chemicals, New Orleans, La.) was also employed. (Keep tightly stoppered and store in a desiccator to prevent moisture pickup.) Sea sand (Merck reagent No. 7478) was used. (Store in a dessicator.)

Chromatographic columns—the column body is approximately 9.5 mm. O.D. and 150 mm. long equipped with a capillary tip (approximately 6 mm. O.D. by 1 to 2 mm. I.D. and 40 mm. long) at one end and a solvent reservoir (approximately 38 mm. O.D. × 100 mm. long) at the other end. An electric mortar

TABLE I.—COMPOSITION OF ANABOLIC VITAMIN TABLET

	mg.
Anabolic Hormones	
Methyltestosterone	4.0
Ethinyl estradiol	0.008
Vitamins	
A	2,500 U.S.P. units
D	250 U.S.P. units
B_1	2.5
B_2	1.5
B_6	1.0
B_{12} as cobalamin concentrate	0.001
Folic acid	0.1
Niacinamide	10.0
Calcium pantothenate	2.5
C	37.5
E	2.5 Int. units
Minerals	
Iron	5.0
Iodine	0.05
Copper	0.5
Manganese	0.5
Magnesium	3.0
Zinc	0.5

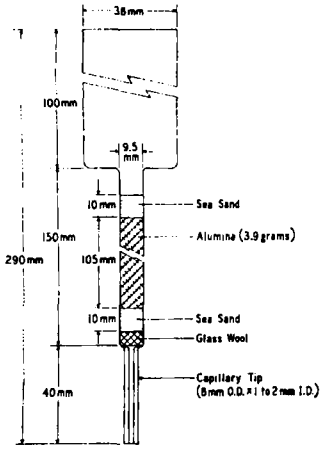

Fig. 1.—Column used in the purification of ethinyl estradiol.

grinder (similar to model MG-2, Torsion Balance Co., Clifton, N. J.), and glass syringes, 50 ml. (B & D Luer Lok 50 YL) with attachable 14 gauge, 5 in. long cannulae (B & D No. 1250 NR, Becton, Dickinson and Co., Rutherford, N. J.) were employed. A manometer bulb and release valve (No. 5000 BV, Becton, Dickinson and Co., Rutherford, N. J.) attached *via* rubber tubing to glass tubing in a single hole No. 7 rubber stopper was provided. The paper chromatographic equipment and reagents were previously described (1), except the filter paper was ethanol washed four times.

Preparation of Column.—Fill the column with the chloroform so that it reaches up into the reservoir about 5 cm., and seal the outlet with the finger tip. Tamp a small plug of glass wool into the bottom of the column. Add approximately 2 cm. of sea sand on top of the plug. Tap in some alumina, remove the finger seal, and stir the column contents as the alumina descends to insure the release of trapped air bubbles. Maintain the chloroform level at 5 cm. into the reservoir.

Perform the same operation with succeeding portions of alumina until an alumina column of about 10.5 cm. (3.9 Gm. of alumina) is obtained. Top off the column with 1 cm. of sea sand, and allow the chloroform to drain to about 2 cm. above the top of the column. Pressure is applied at the top of the column with the rubber stopper and manometer bulb to accelerate drainage and remove air bubbles.

Do not allow the column to run dry. Figure 1 shows a typical column.

Preparation of Standard.—The reference standard solution should contain 0.2 mg. of ethinyl estradiol per milliliter of solution. In a volumetric flask, the appropriate amount of the standard is dissolved in the smallest volume of *N,N*-dimethylformamide. Then methyl isobutyl ketone is added to the mark.

Preparation of Sample.—Grind 120 tablets in the mortar grinder for 5 to 10 minutes until a fine powder is obtained. An amount of powder which contains, based on theory, 400 mcg. of ethinyl estradiol is weighed and transferred to a 250-ml. tall form glass-stoppered bottle (2 in. diameter). Add, with swirling, 14 ml. of 95% ethanol followed by 40 ml. of water. Stopper and shake. Add 100 ml. of ether, stopper, invert twice, remove stopper to release pressure, then restopper, and shake vigorously for 15 to 20 seconds. Place bottle in centrifuge cup, and spin for 10 minutes at 2000 r.p.m.

Transfer the ether layer with a syringe and cannula to a 250-ml. separator containing 20 ml. of 0.1 *N* HCl. Extract the suspended tablet powder further with 2 × 50 ml. of ether, and transfer the ether layers (after centrifugation) to the separator.

Stopper the separator, and shake once. With the separator inverted, release ether pressure by opening the stopcock. Close the stopcock, and shake vigorously 10 to 15 times. Discard the lower aqueous layer. Wash the pooled ether extract with another 20 ml. of 0.1 *N* HCl, then with 1 × 5 ml. of distilled water.

Transfer the washed ether extract to a 500-ml. conical flask. Evaporate the ether at 60° under a stream of nitrogen. Redissolve the residue in 10 ml. of anhydrous ether, and take to dryness as before. Repeat the ether addition and evaporation a second time. Toward the end of the evaporation, insure a completely anhydrous and solvent-free residue by using a vigorous stream of nitrogen and rotating the flask to spread the oil layer. Remove the source of heat, and flush the flask with nitrogen for 5 minutes.

Column Chromatography.—Dissolve the oily residue obtained in the sample preparation in 10 ml. of chloroform, and transfer to the prepared alumina column. Allow this first solution to drain to within 2 cm. of the top of the column. Rinse the flask with two successive 10-ml. portions of chloroform, and transfer to the column, allowing each wash to descend to within 2 cm. of the top of the column. The manometer bulb and rubber stopper may be used to maintain a flow rate of 1–2 drops per second.

Elute the ethinyl estradiol from the column with 75 ml. of acetone containing 1% added water, and collect the eluate in a 200-ml. volumetric flask. Evaporate the acetone eluate to dryness at 60° with a current of nitrogen. Rinse down the sides of the flask with 5 ml. of acetone, and take down to dryness in the same manner. Allow the flask to cool under nitrogen. Add 4 ml. of 95% ethanol. Shake well to insure complete solution of the ethinyl estradiol. This solution is then assayed by paper chromatography.

Paper Chromatography.—Two chromatograms are run per sample. The slotted filter paper described previously (2) contains six 0.75-in. strips which are spotted as follows:

Origin: *1* *2* *3* *4* *5* *6*

1, Sample = 0.2 ml. *4*, Sample = 0.2 ml.
2, Standard = 0.1 ml. *5*, Sample = 0.2 ml.
3, Standard = 0.1 ml. *6*, Paper blank

Two-tenths-milliliter capacity blow-out pipets, graduated in 0.01 ml., are used to apply the standards and the sample to the paper. The spotting technique has been described in detail previously (2).

The spotted strips are impregnated with propylene glycol by dipping them into a 25% solution of propylene glycol in chloroform. The origin end of the paper is dipped first just up to the origin, and blotted. The remainder of the strip is then passed through the dipping solution with a rapid pass over the origin.

Following the dipping step, the strips are hung in a hood until the odor of chloroform can be detected no longer, then they are placed in the chromatography chamber, the bottom of which is covered with toluene. The tab ends of the strip are placed into the troughs containing the developing solvent—toluene saturated with propylene glycol.

TABLE II.—DATA FOR ETHINYL ESTRADIOL ASSAY[a]

Chromatogram	Flask No.	Cell No.	Spot, ml.	Photometer Readings: Uncorrected	Corrected for Blank	Av. St. = 21.0 mcg. Sa. = 0.2 ml.	Concn., mg./Tablet[b]
	1	1	St. = 0.10	69	63		
						63.5	
	2	2	St. = 0.10	70	64		0.0078
1	3	3	Sa. = 0.20	64	58		
						58.5	
	4	4	Sa. = 0.20	65	59		
	5	5	Paper blank	6	...		
	6	1	St. = 0.10	68	62		
						62.5	
	7	2	St. = 0.10	69	63		0.0078
2	8	3	Sa. = 0.20	64	58		
						58	
	9	4	Sa. = 0.20	64	58		
	10	5	Paper blank	6	...		

[a] Project: 22-654. Sample: anabolic vitamin tablets. Control: ZC73831. Assay: ethinyl estradiol. Sample weight: 27890 mg./4 ml. Av. tablet weight = 559 mg. Weight standard: 21.0 mg./100 ml. Filter paper: ethanol-washed finger strips. Impregnated with propylene glycol (25% in $CHCl_3$). Developing solvent: toluene saturated with propylene glycol. Developing time: 20 hours. Drying conditions: 20 minutes at 90° C. Eluting solution: 10 ml. redistilled 95% EtOH. Eluting time: 30 minutes. Spectrophotofluorometer specifications: instrument, Aminco-Bowman (catalog No. 4-8100); cells silica, 10 mm.; tube, 1P28; slit, No. 3; activate, 280 mμ; fluorescence, 310 mμ. [b] Av. 0.0078 mg./tablet.

After a 20-hour descending solvent development, the strips are removed from the chamber and dried in a mechanical convection oven for 20 minutes at 90°.

The positions of the ethinyl estradiol spots on the chromatograms are located by employing the guide strip technique. The strip corresponding to the *1* spot on each chromatogram is cut out, dipped into a solution consisting of 1 part of Folin-Ciocalteu phenol reagent and 4 parts of water (3), then placed into a chamber containing concentrated ammonium hydroxide for 5 minutes. Ethinyl estradiol appears on the chromatogram as a blue zone against a light gray background.

The color-developed guide strip is air-dried, then realigned with the untreated portion of the chromatogram. The portions of the ethinyl estradiol zones are marked off with a solder pencil, cut out and folded, and placed in 50-ml. conical flasks. A paper blank, equal in area to the standard and sample segments, is included for each chromatogram. Ten milliliters of redistilled 95% ethanol are added to each flask and the ethinyl estradiol eluted off the filter paper segment by shaking on a reciprocating shaker for 30 minutes (2).

The amount of ethinyl estradiol present in the eluates is determined by spectrophotofluorometry using an Aminco-Bowman spectrophotofluorometer (catalog No. 4-8100) as previously described (1).

Calculations.—The photometer readings are recorded on a data sheet having all information necessary to calculate the concentration of ethinyl estradiol in the sample. Table II gives the data obtained in a typical analysis.

For each of the two chromatograms, readings are obtained for the standard and sample at one level in duplicate. The readings of the two standards for each chromatogram are averaged as are the two sample readings.

The concentration of ethinyl estradiol per tablet is calculated from

$$\text{mg. Ethinyl Estradiol per Tablet} = \frac{A \times B \times C \times D}{E \times F \times G}$$

where A = average photometer reading of 10-ml. eluate of 0.2-ml. chromatographed sample extract, B = weight, in milligrams, of chromatographed 0.1-ml. standard, C = average weight, in milligrams, of one tablet, D = volume, in milliliters, of final sample extract, E = average photometer reading of 10-ml. eluate of 0.1-ml. chromatographed standard, F = weight, in milligrams, of sample, and G = volume, in milliliters, of chromatographed sample extract.

The final concentration of ethinyl estradiol per tablet is obtained by averaging the values obtained for each of the two chromatograms.

Sample Calculation.—Table II, chromatogram No. 1:

$$\text{mg. Ethinyl Estradiol per Tablet} = \frac{58.5 \times 0.021 \times 559 \times 4}{63.5 \times 27890 \times 0.2} = 0.0078$$

RESULTS AND DISCUSSION

Recoveries of 95% or better must be obtained if any analytical method is to serve as an effective stability assay. The assay procedure described here meets this requirement.

This was demonstrated by subjecting four known aliquots of ethinyl estradiol to the complete analytical procedure. The average of the four individual assays gave a figure deviating by 5% from the theoretical concentration of 103 mcg./ml. (Table III).

TABLE III.—ACCURACY AND PRECISION OF METHOD[a]

Aliquot No.	Chromatogram, mcg./ml. 1	2	Av., mcg./ml.	Deviation from Theory,[b] %
1	98.4	96.2	97.3	−5.5
2	96.3	99.5	97.9	−5.0
3	98.2	96.8	97.5	−5.4
4	96.7	100.3	98.5	−4.4
Av.			97.8	−5.0
S.D.			0.44	
Coefficient of variation, %			0.45	

[a] Known concentrations of ethinyl estradiol subjected to the complete analytical procedure. [b] Theoretical value = 103 mcg./ml.

The analysis of ethinyl estradiol in tablets gave even better results, as shown in the data presented in Table IV.

Recovery experiments where known amounts of ethinyl estradiol were added to the ground tablet powder confirm that the assay yields results of 95% or better (Table V).

The water content of the acetone used to elute the ethinyl estradiol from the column is critical. The data presented in Table VI indicate that a water content of 1 to 2% is necessary to achieve maximum recovery of the ethinyl estradiol from the column.

It is advisable not to perform the column chromatographic step in a very humid atmosphere. At high humidity, moisture pickup by the chloroform and alumina deactivates the alumina, so that some of the ethinyl estradiol is eluted with the chloroform used as a purification solvent.

To determine the effect of moisture on the recovery of ethinyl estradiol, a 400-mcg. quantity was subjected to the procedure using alumina to which 6% water had been added. Only 84% of the ethinyl estradiol was recovered in the acetone eluate (Table VII), while the remainder was found in the chloroform wash.

Glassware used in the chromatographic purification should be dried carefully before use. The chloroform used to prepare the alumina column and to transfer the partially clarified sample to the column should be free of moisture (less than 0.02% water). In a second experiment, the chloroform used in these steps was saturated with water. The subsequent elution of ethinyl estradiol off the column yielded only 92% of the total introduced onto the column (Table VII).

The 0.1 *N* HCl removes a considerable amount of yellow pigment from the ether extracts. The chromatographic purification separates the fat-soluble vitamins, methyltestosterone, and some yellow pigment from the estrogen. Several attempts to perform the paper chromatographic isolation without prior column chromatographic purification resulted in elongated smears unsuitable for stability analysis.

Attempts to perform a batching assay on the column chromatographic acetone eluate by colorimetry in ethanol–concentrated sulfuric acid (1:1) and by fluorimetry for the estrogen *per se* or by the Ittrich modification of the Kober reaction (4) gave erroneous results. Only the combination of column chromatography purification and paper chromatography quantitation gave a satisfactory assay.

TABLE IV.—ANALYSES OF ETHINYL ESTRADIOL IN ANABOLIC VITAMIN TABLETS

Sample No.	Found, mg./Tablet	Deviation from Theory,[a] %
1	0.0081	+1.2
2	0.0082	+2.5
3	0.0079	−1.2
4	0.0078	−2.5
5	0.0078	−2.5
6	0.0076	−5.0
7	0.0077	−3.8

[a] Theoretical concentration: 0.008 mg. per tablet.

TABLE V.—RECOVERY OF ETHINYL ESTRADIOL ADDED TO ANABOLIC VITAMIN TABLETS

Sample No.	Added, mcg./Tablet	Found, mcg./Tablet	Net Recovery, mcg./Tablet	Recovery, %
1	0	8.2	—	—
1	7.9	15.8	7.6	96.2
2	0	7.9	—	—
2	8.3	15.7	7.8	94.0
3	0	7.4	—	—
3	7.9	14.8	7.4	93.8
4	0	7.6	—	—
4	7.9	15.1	7.5	95.0
5	0	7.7	—	—
5	7.9	15.9	8.2	103.8

TABLE VI.—RECOVERY OF ETHINYL ESTRADIOL FOLLOWING CHROMATOGRAPHY ON ALUMINA AND THE EFFECT OF WATER CONTENT OF ACETONE USED AS ELUTING SOLUTION

Water Added to Acetone,[a] %	Recovery of Ethinyl Estradiol, %
0	67
0.25	90
0.5	92
1.0	98
2.0	98
4.0	93
6.0	89

[a] Acetone (Merck No. 7106) contained 0.28% water by gas chromatographic analysis.

TABLE VII.—RECOVERY OF ETHINYL ESTRADIOL FOLLOWING CHROMATOGRAPHY ON ALUMINA AND EFFECT OF WATER CONTENT OF ALUMINA

Water Content, %	Recovery of Ethinyl Estradiol, %
6	84
Zero, followed by wash with water-saturated chloroform	92

SUMMARY

1. A method combining quantitative paper chromatography and spectrophotofluorometry has been developed for the assay of micro amounts of estrogens in anabolic vitamin tablets.

2. The procedure is used routinely to measure the stability of ethinyl estradiol at a concentration of 8 mcg. per tablet or 0.0014%.

3. Prior to quantitative paper chromatography, the estrogen is extracted into ether from an alcohol–water suspension of the ground tablets, then adsorbed on an alumina column to separate it from some interfering constituents.

4. The estrogen is eluted off the column using acetone to which 1% water has been added. The concentration of water in the eluting solution is a critical factor in recovering the estrogen off the column.

5. Accuracies in excess of 95% are obtained by chromatographing simultaneously replicates of standard and sample solutions on the same chromatogram.

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