Quantitative Determination of Ethinyl Estradiol by Gas Chromatography, and a Comparison of Gas Chromatographic and U.S.P. Procedures

By O. D. BOUGHTON, RHYS BRYANT, W. J. LUDWIG, and D. L. TIMMA

Ethinyl estradiol was extracted from other tablet or granulation excipients by a modified U.S.P. procedure. It was then quantitatively determined by gas chromatography of its trimethylsilyl ether. Estrone was used as an internal standard. A comparison of this gas chromatographic method with the U.S.P. method indicated that, in general, the precision of the U.S.P. method was superior to that of the gas chromatographic method.

THE ACCURATE analytical determination of estrogenic and progestational substances is of interest to all pharmaceutical manufacturers, especially those concerned with the now well-established oral contraceptives. Analysis of the progestational agent is usually much easier than analysis of the estrogen, because of the necessity for a larger amount of the former in tablets.

The authors' main interest has been in the analysis of the estrogen ethinyl estradiol. The accepted standard method for assay of this material has been that of the U.S.P. XVII (1). This method is time-consuming and is nonspecific in that it may be used for any estrogen characterized by a phenolic steroidal A ring (2).

Subsequently, alternative methods using gas chromatography have been applied to the problem. Schulz (3) reported the estimation of ethinyl estradiol-3-methyl ether in the presence of other steroids by gas chromatography. More recently, Talmage, Penner, and Geller (4) reported a gas chromatographic technique for determination of ethinyl estradiol in sesame oil solutions and solid dosage forms. The ethinyl estradiol appears to have been uncontaminated with other steroids and was estimated as its acetate.

A quantitative gas chromatographic method for determination of ethinyl estradiol in which the active ingredient is ethinyl estradiol alone, or ethinyl estradiol plus 17β-hydroxy-6α-methyl-17-(1-propynyl)-androst-4-en-3-one (dimethisterone) has been developed.

A comparison of the gas chromatographic method *versus* the U.S.P. method to gain insight into the precision of the two methods has also been made. (No comparison of the two methods has been made until now, although Talmage, Penner, and Geller stated (4), with no supporting data, that the U.S.P. method was no more accurate than $\pm 10\%$.)

The method of Talmage *et al.* depends on initial preparation of the 3-acetate of ethinyl estradiol, and subsequent gas chromatography of this compound. In attempting to apply this method to the problems reported here, two distinct disadvantages in the method were discovered. Preparation of the acetate involves the use of acetic anhydride which must subsequently be removed by evaporation at an elevated temperature. Even under the conditions stated in the original paper (evaporation under nitrogen on the steam bath) (4), this step is time consuming and introduces a possible source of error. Second, the authors wished to analyze for at least 50% less ethinyl estradiol than Talmage, Penner, and Geller had analyzed, and for our purposes the acetate-derivative did not give sufficient response on the chromatograph.

In this method the ethinyl estradiol, after extraction, is converted to its trimethylsilyl ether and chromatographed. Advantages of the use of the trimethylsilyl ether are that it is readily prepared, quantitatively, at room temperature, and is sufficiently volatile to give an excellent chromatographic response. (Quantities of ethinyl estradiol as low as 1 mcg. after trimethylsilylation are readily detectable.) Details of the procedure are presented under *Experimental.*

The comparison of the gas chromatographic and U.S.P. methods of analysis took the form of two experiments. In experiment *A*, the authors took a small amount of granulation and further homogenized it using a mortar and pestle. It was then assayed 10 times by both U.S.P. (1) and gas chromatographic (GLC) methods. In experiment *B* the authors allowed conditions to approach those usually found in analysis of a production batch, when the possibility of a certain amount of inhomogeneity cannot be ruled out. Both tablets and granulations were

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analyzed 10 times by each method. The results of these experiments were then analyzed statistically in order to obtain the comparisons required.

GAS CHROMATOGRAPHIC METHOD

Experimental

Apparatus.—Gas chromatograph, F and M model 609, with flame ionization detector. Aerograph model 471 digital integrator.

Column.—Stainless steel column (6 ft. × 1/4 in.) packed with 3.8% SE-30 on Anakrom ABS, 60/70 mesh. The liquid phase was deposited by the filtration technique (5) from a 2% solution of SE-30 in methylene chloride. The column was "no flow" preconditioned (no carrier gas flowing) for 0.5 hr. at 325°. The carrier gas used was nitrogen.

Chromatography Conditions.—Column temperature, 260°; injection port temperature, 285°; detector temperature, 265°; hydrogen flow, 7; air flow, 7; nitrogen flow, 9 (these flows may vary according to the instrument); attenuation, 800×.

Standard Ethinyl Estradiol Solution.—*Solution A*.—A 100.0-mg. quantity of reference standard ethinyl estradiol U.S.P. was dissolved in 100 ml. absolute ethanol.

Solution B.—A 10.0-ml. quantity of solution *A* was diluted to 100 ml. with absolute ethanol. (The ethanolic solutions are stable and require no special precautions.)

Solution C.—A 5.0-ml. quantity of solution *B* was diluted to 100 ml. with chloroform. Twenty milliliters of standard solution *C* contained 0.1 mg. of ethinyl estradiol. Solution *C* was prepared fresh daily.

Internal Standard (Estrone) Solution.—*Solution A*.—A 150.0-mg. quantity of estrone U.S.P. was dissolved in 100 ml. absolute ethanol.

Solution B.—A 5.0-ml. quantity of solution *A* was diluted to 100 ml. with absolute ethanol. (The ethanol solutions are stable and require no special precautions.)

Solution C.—A 10.0-ml. quantity of solution *B* was diluted to 100 ml. with chloroform. Ten milliliters of solution *C* contained 0.075 mg. estrone. Solution *C* was prepared fresh daily.

Etherification Reagents.—In a screw-cap vial was placed a mixture[1] of anhydrous pyridine[2] (4.5 ml.), hexamethyldisilazane[3] (1.5 ml.), and anhydrous trimethylchlorosilane[3] (0.5 ml.). No purification of reagents was necessary. The tube was capped tightly and the contents mixed thoroughly. It is essential to exclude atmospheric or other moisture, which destroys the reagent. The etherification reagent was prepared fresh daily.

Extraction Procedure.—An amount of tablet or granulation equivalent to 0.1 mg. of ethinyl estradiol was placed in a 125-ml. separator containing sulfuric acid (30 ml., 1 *N*), and the suspension extracted with chloroform (3 × 20 ml.). After filtration through a small quantity of cotton, the combined chloroform solution was evaporated to low bulk (about 5 ml.). Petroleum ether (25 ml., b.p. 30–60°) was added to the hot solution. It was cooled to room temperature and transferred to a 125-ml. separator with the aid of several small portions of petroleum ether. The solution was extracted with 10% aqueous sodium hydroxide (3 × 10 ml.). The extract was acidified with dilute sulfuric acid (6 ml., 1:1 v/v) and cooled. The acid solution was extracted with chloroform (2 × 20 ml.), which was passed through a column (10 × 1.2 cm.) of anhydrous sodium sulfate. The column was rinsed with chloroform (1 × 10 ml.). All column eluates were combined, 10.0 ml. of internal standard solution (estrone) was added, and the solution was evaporated to dryness. Chloroform (1–2 ml.) was added to the hot residue. The resulting solution, after cooling, was transferred to a 1-dr. screw-cap vial and evaporated to dryness under a gentle air stream. It was finally dried at 80° for 5 min. *in vacuo*.

Etherification Step.—To the cooled ethinyl estradiol extract in the vial was added 10 drops (0.1 ml.) of etherification reagent. The vial was tightly stoppered and the contents thoroughly mixed. After 30–60 min. excess reagent was evaporated with a gentle air stream (about 15 min.). (The reaction time was varied from 15 min. to 60 min. with no effect. The reaction is obviously complete in less than 15 min. A lapse-time of 30–60 min. was chosen arbitrarily to allow simultaneous assay of a number of samples.) Immediately prior to chromatography, chloroform (10 drops) was added and the resulting solution mixed thoroughly. This solution (5–6 μl.) was injected into the chromatograph. Duplicate injections of each sample are to be preferred.

Standard.—A standard was carried through the procedure with each set of samples. Twenty milliliters of standard ethinyl estradiol solution was shaken with dilute sulfuric acid (30 ml., 1 *N*). The chloroform solution was drained through cotton, and the sulfuric acid extracted with chloroform (2 × 20 ml.). The combined chloroform solution was then treated as for the samples.

Calculation.—The peaks due to estrone and ethinyl estradiol have retention times of approximately 4.5 min. and 7 min., respectively. The ratio (R) of the peak areas is given by:

$$R = \frac{\text{area of ethinyl estradiol peak}}{\text{area of estrone peak}}$$

For tablets containing 0.1 mg. ethinyl estradiol:

$$\text{mg. of ethinyl estradiol} = \frac{R \text{ for sample}}{R \text{ for standard}} \times 0.100$$

For granulations containing 0.04% ethinyl estradiol:

$$\%\ \text{ethinyl estradiol} = \frac{R \text{ for sample}}{R \text{ for standard}} \times 0.0400$$

RESULTS

Reproducibility of the Etherification Step.—Four aliquots of a standard solution of ethinyl estradiol, after addition of estrone, were etherified and each aliquot chromatographed in duplicate.

[1] This mixture is available commercially from Applied Science Laboratories, Inc.

[2] Analytical reagent grade pyridine supplied by Mallinckrodt was used. It was dried with molecular sieve, type 4A.

[3] Hexamethyldisilazane and trimethylchlorosilane were obtained from Peninsular Chemresearch, Inc.

TABLE I.—ETHERIFICATION STEP

	R	Av. R
Std. 1	1.755	1.740
Std. 1	1.725	
Std. 2	1.695	1.720
Std. 2	1.745	
Std. 3	1.655	1.635
Std. 3	1.615	
Std. 4	1.650	1.685
Std. 4	1.720	

TABLE II.—STANDARD THROUGH EXTRACTION PROCEDURE[a]

	R	Av. R	% Recovery
Sample 1	1.710	1.685	99.4
Sample 1	1.660		
Sample 2	1.690	1.710	101
Sample 2	1.730		
Sample 3	1.720	1.670	98.5
Sample 3	1.620		
Sample 4	1.695	1.730	102
Sample 4	1.765		
Sample 5	1.670	1.680	99.1
Sample 5	1.690		

Av. recovery = 100%, $\sigma = \pm 1.5$

[a] All samples were aliquots of the same standard ethinyl estradiol solution.

Table I incorporates the results. The average R value was 1.695, $\sigma = \pm 0.014$.

Reproducibility of the Extraction.—This was checked (*a*) by taking a known quantity of ethinyl estradiol through the extraction procedure; (*b*) by adding a known quantity of ethinyl estradiol to the tablet placebo (containing all excipients including dimethisterone, but without the ethinyl estradiol) and taking this through the procedure; and (*c*) by taking the placebo through the procedure, and then adding a known quantity of ethinyl estradiol. The samples were then etherified and chromatographed in the normal manner. The results were compared with the average R value (1.695), obtained by chromatography of the standards which had not been taken through the method, to give the percentage recovery. (See Tables II–IV.)

Reproducibility of the Method.—Table V summarizes the results obtained when 4 separate aliquots of the same granulation (containing only ethinyl estradiol as active ingredient) were taken completely through the method. The average percentage of ethinyl estradiol was 0.0404, $\sigma = \pm 0.0025$.

Table VI gives similar results for 5 aliquots of another granulation, which contained both ethinyl estradiol and dimethisterone. In this case, the percentage of ethinyl estradiol was found to be 0.0412, $\sigma = \pm 0.0018$.

General Applicability.— Some results of the applicability of the method to tablets and granulations are included in Table VII (EE = formulations containing ethinyl estradiol; MEE = formulations containing both dimethisterone and ethinyl estradiol).

COMPARISON OF THE METHODS

Experiment *A*

Samples.—Two granulations were used, one containing ethinyl estradiol as sole active ingredient (EE granulation); the other containing both dimethisterone and ethinyl estradiol (DMEE granulation).

Preparation of Samples.—A small sample (11 Gm.) of the EE granulation was finely ground in a mortar. (The sample size was restricted in order to ensure as high a degree of homogeneity as

TABLE III.—STANDARD PLUS PLACEBO THROUGH EXTRACTION PROCEDURE[a]

	R	Av. R	% Recovery
Sample 1	1.785	1.765	104
Sample 1	1.745		
Sample 2	1.680	1.710	101
Sample 2	1.740		
Sample 3	1.900	1.890	112
Sample 3	1.880		
Sample 4	1.705	1.715	101
Sample 4	1.720		
Sample 5	1.680	1.675	98.7
Sample 5	1.670		

Av. recovery = 103%, $\sigma = \pm 5.2$

[a] All samples were aliquots of the same standard solution.

TABLE IV.—PLACEBO THROUGH PROCEDURE, THEN ADDITION OF STANDARD[a]

	R	Av. R	% Recovery
Sample 1	1.735	1.695	100
Sample 1	1.655		
Sample 2	1.830	1.850	109
Sample 2	1.870		

Av. recovery = 104%

[a] Samples were aliquots of the same standard solution.

TABLE V.—METHOD APPLIED TO ALIQUOTS OF ONE GRANULATION[a]

	Theory	% Ethinyl Estradiol
Sample 1	0.040%	0.0377
Sample 2	0.040	0.0410
Sample 3	0.040	0.0390
Sample 4	0.040	0.0436

Av. % ethinyl estradiol = 0.0404, $\sigma = \pm 0.0025$

[a] Containing only ethinyl estradiol as active ingredient.

TABLE VI.—METHOD APPLIED TO ALIQUOTS OF ONE GRANULATION[a]

	Theory	% Ethinyl Estradiol
Sample 1	0.040%	0.0396
Sample 2	0.040	0.0406
Sample 3	0.040	0.0414
Sample 4	0.040	0.0443
Sample 5	0.040	0.0404

Av. % ethinyl estradiol = 0.0412, $\sigma = \pm 0.0018$

[a] Containing both ethinyl estradiol and dimethisterone.

TABLE VII.—RESULTS

	Theory	Found
EE tablet 1	0.100 mg.	0.093 mg.
EE tablet 2	0.100 mg.	0.091 mg.
EE tablet 3	0.100 mg.	0.094 mg.
EE tablet 4	0.100 mg.	0.108 mg.
DMEE tablet 1	0.100 mg.	0.102 mg.
DMEE tablet 2	0.100 mg.	0.106 mg.
DMEE tablet 3	0.100 mg.	0.094 mg.
DMEE tablet 4	0.100 mg.	0.097 mg.
DMEE tablet 5	0.100 mg.	0.098 mg.
DMEE tablet 6	0.100 mg.	0.088 mg.
DMEE tablet 7	0.100 mg.	0.100 mg.
DMEE granulation 1	0.040%	0.0399%
DMEE granulation 2	0.040%	0.0417%
DMEE granulation 3	0.040%	0.0398%
DMEE granulation 4	0.040%	0.0403%

TABLE VIII.—PERCENTAGE ETHINYL ESTRADIOL IN GRANULATIONS

EE Granulation		DMEE Granulation	
U.S.P. Method	GLC Method	U.S.P. Method	GLC Method
0.0383%	0.0431%	0.0404%	0.0382%
0.0369	0.0410	0.0408	0.0371
0.0408	0.0351	0.0414	0.0327
0.0422	0.0386	0.0445	0.0382
0.0449	0.0366	0.0400	0.0399
0.0417	0.0351	0.0382	0.0387
0.0425	0.0346	0.0383	0.0336
0.0376	0.0431	0.0387	0.0387
0.0377	0.0406	0.0413	0.0391
0.0397	0.0406	0.0458	0.0377

possible.) All 10 assays by the U.S.P. method and all 10 assays by the gas chromatographic method were done on this 11-Gm. sample.

An 11-Gm. sample of DMEE granulation was treated similarly, and all 20 assays performed on it.

Method of Assay.—*a.*—The U.S.P. method was carried out by a single operator experienced with the method using 0.500 Gm. of sample for each assay. Not more than four assays (two EE and two DMEE) were undertaken on the same day.

b.—The gas chromatographic method was performed by a single operator experienced with the method using 0.250 Gm. of sample for each assay. Again, not more than four assays were done on a single day.

Results.—The results obtained in experiment *A* are shown in Table VIII. (Theoretically, granulations should contain 0.040% ethinyl estradiol.)

Statistical Analysis.—The results of the statistical calculations are included in Table IX.

Experiment *B*

Samples.—Four samples were used: 1, EE granulation; 2, DMEE granulation; 3, EE tablets; and 4, DMEE tablets.

Preparation of Samples.—*a.*—From the batch of EE tablets, 200 tablets were retained as the stock of tablets. From this stock each day, 20 tablets were taken and pulverized in a mortar; 0.500 Gm. of this sample was used for the U.S.P. method, and 0.250 Gm. of the sample was used for the gas chromatographic method. A separate lot of 20 tablets was used each day, and 0.500 and 0.250-Gm. samples removed from it.

TABLE IX.—STATISTICAL COMPARISON OF THE TWO METHODS

	EE Granulation		DMEE Granulation	
	U.S.P. Method	GLC Method	U.S.P. Method	GLC Method
Av. value, %	0.04023	0.03884	0.04094	0.03738
Variance (σ^2)	6.86×10^{-6}	10.89×10^{-6}	6.35×10^{-6}	5.57×10^{-6}
S.D. (σ)	±0.0026	±0.0033	±0.0025	±0.0024
S.D. as %	±6.5	±8.5	±6.1	±6.4

TABLE X.—COMPARISON OF THE U.S.P. AND GAS CHROMATOGRAPHIC METHODS

	EE Granulation		DMEE Granulation		EE Tablet		DMEE Tablet	
Day	U.S.P. Method	GLC Method	U.S.P. Method	GLC Method	U.S.P. Method	GLC Method	U.S.P. Method	GLC Method
1	0.0359	0.0431	0.0409	0.0405	0.108	0.108	0.106	0.104
2	0.0390	0.0342	0.0390	0.0438	0.108	0.111	0.106	0.111
3	0.0353	0.0352	0.0450	0.0374	0.099	0.096	0.101	0.100
4	0.0392	0.0358	0.0412	0.0401	0.100	0.103	0.105	0.107
5	0.0366	0.0341	0.0362	0.0418	0.100	0.090	0.102	0.104
6	0.0386	0.0388	0.0404	0.0411	0.108	0.105	0.119	0.110
7	0.0405	0.0348	0.0394	0.0492	0.104	0.110	0.105	0.112
8	0.0391	0.0371	0.0410	0.0575	0.098	0.116	0.100	0.131
9	0.0398	0.0380	0.0410	0.0397	0.109	0.102	0.098	0.107
10	0.0382	0.0412	0.0388	0.0450	0.100	0.105	0.100	0.106

TABLE XI.—STATISTICAL COMPARISON OF THE METHODS FOR GRANULATIONS

	EE Granulation		DMEE Granulation	
	U.S.P. Method	GLC Method	U.S.P. Method	GLC Method
Av. value, %	0.03822	0.03723	0.04029	0.04361
Variance (σ^2)	2.97×10^{-6}	9.37×10^{-6}	5.09×10^{-6}	34.6×10^{-6}
S.D. (σ)	±0.0017	±0.0030	±0.0023	±0.0059
S.D. as %	±4.5%	±8.1%	±5.7%	±13.5%

Table XII.—Statistical Comparison of the Methods for Tablets

	EE Granulation		DMEE Granulation	
	U.S.P. Method	GLC Method	U.S.P. Method	GLC Method
Av. value, mg.	0.1034	0.1046	0.1042	0.1092
Variance (σ^2)	19.8×10^{-6}	56.4×10^{-6}	35.1×10^{-6}	71.8×10^{-6}
S.D. (σ)	±0.0045	±0.0075	±0.0059	±0.0085
S.D. as %	±4.4%	±7.2%	±5.7%	±7.8%

A stock of DMEE tablets was treated in exactly the same way.

b. Granulations.—From the batch of EE granulation, 160 Gm. was removed. It was passed through a sample splitter to give 2 × 80-Gm. portions. Each 80-Gm. portion was passed separately through the sample splitter to yield 4 × 40-Gm. portions. Each 40-Gm. portion was similarly split, and so on, with each subsequent 20-Gm. and 10-Gm. portion until 32 × 5-Gm. portions were obtained. Of these 32 × 5-Gm. portions, 10 × 5 Gm. were used for the current experiment.

Each day one of the 5-Gm. portions was taken and again pulverized using a mortar and pestle; 0.500 Gm. was taken for the U.S.P. assay, and 0.250 Gm. was taken for the gas chromatographic assay.

The DMEE granulation was treated in the same way.

Method of Assay.—The U.S.P. method was performed by a single operator experienced with the method. Each day for a total of 10 days, one each of the EE tablet, DMEE tablet, EE granulation, and DMEE granulation was assayed.

The gas chromatographic method was performed by a single experienced operator. Each day, for a total of 10 days, one each of the EE and DMEE tablets, and EE and DMEE granulations was assayed.

Results.—The results obtained in experiment *B* are summarized in Table X. Granulations are quoted as percentages; tablets as mg./tablet. (Theoretical content of granulations is 0.040%; and of tablets 0.100 mg.)

Statistical Analysis.—The average values obtained and the variance (σ^2) and standard deviations (σ) of the methods are reported in Tables XI (for granulations) and XII (for tablets).

DISCUSSION

The Gas Chromatographic Method.—Dimethisterone had a retention time of 12 min. and in no way interfered with the other steroid peaks. It was therefore unnecessary to extract the ethinyl estradiol completely from dimethisterone, as is required by the U.S.P. XVII procedure.

The use of a short column of sodium sulfate helped to remove any trace of acid which might be occluded in the chloroform and subsequently interfere with the etherification step.

Comparison of the Methods.—In all cases, the standard deviation (as per cent) is lower for the U.S.P. method than for the gas chromatographic method. While it is also obvious that the average value obtained differs between the methods, statistically (using the *t* test) only in the case of the DMEE granulation (experiment *A*) is this significant. Also, only in the case of the DMEE granulation (experiment *B*), and possibly the EE granulation (experiment *B*), can any significance be attached to the difference in variances (*F* test).

SUMMARY

The Gas Chromatographic Method.—Ethinyl estradiol was extracted into chloroform from an acid suspension and separated from dimethisterone by alkaline extraction. With estrone as an internal standard, the ethinyl estradiol was converted to its trimethylsilyl ether and this was chromatographed on a SE-30 column.

Evaluation of the results contained in Table VII shows that, based on an expected value of 0.0400% ethinyl estradiol, the average value found was 0.039%, σ = ±0.002. Based on an expected value of 0.100 mg./250 mg. tablet, the average found value was 0.098 mg., σ = ±0.002.

The Comparison of Methods.—Under the conditions of our comparisons, the U.S.P. method in all cases showed a standard deviation lower than that of the gas chromatographic method. The over-all average standard deviation for the U.S.P. method was ±5.5% and for the GLC method ±8.6%. Although the U.S.P. method would appear to give a better precision, in most cases the difference in variances is not statistically significant.

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