Gas Chromatographic Assay of 17α-Ethynylestradiol-3-methylether in Oral Progestational Agents—Comparison with Thin-Layer Chromatographic Assay

By E. P. SCHULZ

A gas chromatographic method, using 1 per cent QF-1 as a liquid phase, has been developed which permits the rapid quantitation of 17α-ethynylestradiol-3-methylether in the presence of 17α-ethynyl-19-nortestosterone or 6-chloro-6-dehydro-17α-acetoxy-progesterone. The results obtained are compared to those achieved by an equally reliable but slower thin-layer chromatographic method. Using the two described methods, assay values are also presented for the determination of the above estrogen in pharmaceutical dosage forms containing oral progestational agents.

THE NEED for a rapid accurate method to control the quantity of 17α-ethynylestradiol-3-methylether (EEME), formulated as an active component with oral progestational agents, has led to the development of a gas chromatographic assay based on prior elimination of progestational agents using cyclohexane as a selective solvent for the estrogen. Methods currently employed in the analysis of estrogens are generally based on ultraviolet absorption (1) or on colorimetric methods such as the 2,6-dibromoquinone chlorimide (2) or Kober (3) reactions. Although these methods are sufficiently sensitive, each lacks the specificity required for a rapid analytical method. Both the ultraviolet and the colorimetric methods are dependent on the phenolic ring A, a pronounced common structural feature of estrogens. Specificity can, of course, be imparted to the above methods through prior isolation of the estrogen by column partition or paper chromatography. However, because of the large number of manipulations, low results may be obtained using partition methods. The use of paper chromatography, as experienced in these laboratories to isolate the above estrogen from progestational agents, has generally resulted in low and irreproducible assay values.

Modification of a recently published thin-layer chromatographic (4) method for steroids does offer the desired specificity that the above methods lack. However, the gas chromatographic method described in this paper, besides being as specific and accurate as the presented thin-layer modification, is less time consuming. Analysis of EEME can be performed in 1.5 hours by the gas chromatographic method, while the described thin-layer method requires 3–4 hours. Moreover, the thin-layer method is a more tedious technique, requiring the application of 10 separate 50-μl. portions of extract to a small area of the thin layer.

REAGENTS AND APPARATUS

Instrument.—A Barber-Colman model 10 gas chromatograph, equipped with a beta-ray ionization detector and Ra^{226} source, was utilized.

Column.—A U-shaped glass column, 1.8 M. × 5 mm. i.d., packed with the coated support, was employed.

Liquid Phase.—A fluorosilicone liquid (Dow Corning Corp., No. FS-1265) designated as QF-1 was used.

Inert Support.—Untreated siliconized diatomaceous earths (120–130 mesh Anakrom-ABS or 80–100 mesh Diatoport-S) were suitable as support materials after 1% coating with QF-1 from acetone solution. Acid-base washed gas chrom P, commercially supplied, was unsuitable for this type of analysis. Treatment of acid-base washed gas chrom P in the following manner rendered it equivalent to the siliconized supports.

Gas chrom P (100–140 mesh acid-base washed material commercially supplied) was overlaid with concentrated HCl and allowed to stand in a closed system for a 24-hour period. The dried support, after washing with water and acetone, was shaken for 2 hours with 5.6% KOH in methanol. The support, after washing with methanol, was shaken with chloroform and the fine particles decanted off. The support was then covered with 1% QF-1 using acetone as the solvent for the application of the liquid phase.

EXPERIMENTAL

Column Conditioning.—The packed column was conditioned by passing argon carrier gas for about 24 hours at a rate of 20–25 ml. per minute at a column temperature of 230°.

Instrumental Parameters.—In the development of the analytical method a column temperature of 221°, a cell temperature of 240°, and a flash heater temperature of 260° were used. Using a conventional bubble-counter, the flow rate was maintained at a rate of 80 ml. per minute. A detector voltage of 750 and sensitivity range of 30 were used. Argon carrier gas was used exclusively.

EEME Reference Standard.—Throughout this work a reference standard of EEME was used which met the following standards: (*a*) the E 1%, 1 cm. value at 279 mμ was 67.4 in cyclohexane; (*b*) using 90:10 benzene–ethyl ether as the developer solvent, a thin-layer chromatogram—silica gel G impregnated (4) with a phosphor to facilitate detection of spots—showed only one spot (R_f 0.32) after 10 cm.

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development. Using a Black Light Cabinet with a 254 mμ excitation source, the EEME was clearly visible as a single blue fluorescent spot.

Sample Preparation.—The oral progestational agents used in this investigation were 17α-ethynyl-19-nortestosterone (ENT) (5) and 6-chloro-6-dehydro-17α-acetoxy-progesterone (CDAP) (6). About 2000 mg. of either above steroid, containing 0.1 to 0.4% EEME, was weighed to the nearest 0.1 mg. and transferred to a 50-ml. reflux flask containing 20.0 ml. of cyclohexane. The contents were then refluxed for 0.5 hour. After cooling to room temperature, the extract was filtered through Whatman No. 42 paper. Clear cyclohexane extract (5.0 ml.) was evaporated to dryness in a 50-ml. glass-stoppered flask. Just prior to analysis by gas chromatography, 5.0 ml. of acetone was added to the flask. The tightly stoppered contents were then shaken to insure complete solution of the residue.

Gas Chromatographic Analysis of EEME in ENT Samples.—Using a clean 10-μl. syringe, 8.0 μl. of reference standard EEME in acetone (10.0 mg. per 100 ml.) was injected. This was repeated with two additional 8.0-μl. injections of standard, with a waiting period for each EEME peak to emerge before making the subsequent injection. Before each sampling, the syringe was rinsed thoroughly with acetone, vacuum dried, and examined before each injection to insure the absence of air bubbles.

Three separate 8.0-μl. injections of the sample extract from the cyclohexane residue were treated as above, with a waiting period for both the EEME and ENT to emerge before proceeding with the second and third injections.

$$\% \text{ EEME} = \frac{A_x}{A_s} \cdot \frac{B}{G}$$

where A_x is the average area of three sample injections; A_s is the average area of three EEME standard injections; B is the microgram weight of EEME standard (about 200 mcg.) per 2.0 ml. of acetone; and G is the milligram weight of ENT extracted with 20.0 ml. of cyclohexane.

Gas Chromatographic Analysis of EEME in CDAP Samples.—The procedure was exactly as described under *Gas Chromatographic Analysis of EEME in ENT Samples*. However, after the three injections of EEME standard, the analyst need only await the emergence of the EEME peak (from the CDAP extract) before proceeding with the two remaining injections of sample extract, since the retention time of CDAP is 1 hour compared to 6 minutes for ENT. The calculation presented in the previous paragraph may be used, where G becomes the milligram weight of CDAP extracted with 20.0 ml. of cyclohexane.

Gas Chromatographic Analysis of EEME Tableted with ENT or CDAP.—A number of thoroughly ground tablets equivalent to 1000 mcg. of EEME were refluxed for 0.5 hour with 50.0 ml. of cyclohexane. The cooled extract was filtered through Whatman No. 42 paper, and 10.0 ml. of filtrate was evaporated to dryness as above. The residue, dissolved in 2.0 ml. of acetone, was analyzed by gas chromatography as described under the two preceding sections.

$$\text{mcg. EEME/tablet} = \frac{A_x}{A_s} \cdot \frac{5B}{N}$$

where A_x, A_s, and B remain as defined above; and N is the number of ground tablets extracted with 50.0 ml. of cyclohexane.

Determination of Peak Areas.—Peak areas were measured using a base-line drawn tangent to both EEME minima. The areas were merely calculated as the product of peak-height times width at half peak-height. The band widths were accurately measured using a Bausch & Lomb No. 81-34-97-20 spectrum measuring magnifier.

Thin-Layer Chromatographic Assay of EEME in Formulated and Nonformulated Samples.—Cyclohexane extracts of EEME, containing about 500 mcg. of EEME, were evaporated to dryness in a 50-ml. glass-stoppered flask. Exactly 1.0 ml. of methanol was added to the cooled flask, and the stoppered contents were thoroughly swirled to effect solution of the residue. Using a 50-μl. syringe, 10 separate 50-μl. portions of the methanol solution were applied to a silica gel G thin layer (5 × 20 cm.) impregnated with a phosphor according to the procedure described in *Reference 4*. After drying the applied spot, the plate was developed for a distance of 10 cm. using 90:10 benzene–ethyl ether as the developing solvent. The chromatographed spots were detected as blue fluorescent areas using a 254 mμ excitation source. A square area encompassing the EEME spot (R_f 0.32) was scraped with a razor blade and transferred to a 25-ml. glass-stoppered flask containing 5.0 ml. of spectrograde methanol. After 5 minutes of magnetic stirring, the extract was filtered through a fine porosity sintered-glass funnel, collecting at least 3.5–4 ml. of methanol in a test tube inserted in the vacuum flask.

The absorbance, E_x, of the above solution (1 cm. path) was determined at 278 mμ, using as a blank a portion of the chromatoplate treated as above. The eluted blank was cut out adjacent to the EEME spot and approximated the EEME spot both in area and R_f value. The absorbance, E_s, of 250 mcg. of EEME reference standard treated as above was determined at 278 mμ. Exactly 100 μl. of EEME standard in methanol (25.0 mg. per 10.0 ml.) was applied. The EEME content of nonformulated samples was determined using the following relationship

$$\% \text{ EEME} = \frac{E_x}{E_s} \cdot \frac{1000}{VW}$$

where E_x and E_s are defined above; W is the milligram weight of sample extracted with 20.0 ml. of cyclohexane (see *Sample Preparation*); and V is the volume of cyclohexane (milliliters) evaporated to dryness and containing 500 mcg. of EEME.

The EEME content of formulated samples was calculated using

$$\text{mcg. EEME/tablet} = \frac{E_x}{E_s} \cdot \frac{25000}{VN}$$

where E_s and E_x remain as defined above; N is the number of ground tablets extracted with 50.0 ml. of cyclohexane as under *Gas Chromatographic Analysis of EEME Tableted with ENT or CDAP;* and V is the volume of cyclohexane (milliliters) evaporated to dryness and containing 500 mcg. of EEME.

RESULTS AND DISCUSSION

Gas Chromatography.—Efforts to use 1% QF-1 on untreated gas chrom P (acid-base washed as com-

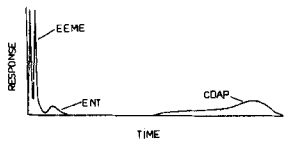

Fig. 1.—Gas chromatograms of acetone injections of residues from cyclohexane extracts of EEME from EEME–ENT and EEME–CDAP samples.

mercially supplied) were met with about a 70% loss in sensitivity compared to the response obtained with 1% QF-1 on treated gas chrom P, Diatoport-S, or Anakrom-ABS. The use of SE-30 instead of QF-1 on treated gas chrom P led to inadequate separation of the EEME peak from the solvent peak. Attempts to resolve this difficulty by varying the column temperature and flow rate failed to effect the desired separation of solvent and EEME peaks.

Initial attempts to determine EEME by direct injection of acetone, chloroform, or methanol extracts of ENT were fruitless because of the effects of large amounts of ENT extracted along with the EEME. Reproducible quantitation of EEME could not be attained presumably because of detector or column overloading effects. In the course of this investigation it was noted that cyclohexane would dissolve EEME, selectively, while only trace amounts of either ENT or CDAP—quantities insufficient to interfere in quantitative measurements—were soluble in cyclohexane. The relative retention time of ENT:EEME shown in Fig. 1 is 4:1, while the relative retention time for CDAP:EEME is about 40:1. Because of the asymmetry of the CDAP peak, the latter is presented as an approximation only.

Attempts to inject cyclohexane extracts of EEME directly were abandoned in favor of acetone injections of the residues from cyclohexane extracts because considerable variation of the cyclohexane peak width was noted on repeated injections. In some instances, the peak width was sufficiently wide to contribute to the EEME peak areas. Acetone injections of the residues from the cyclohexane extracts, however, only exhibited narrow acetone peaks which did not interfere in EEME area measurement.

Since the ENT and CDAP peaks appear at distances greater than 4 sigma units from the EEME peak, the contribution of either ENT or CDAP to the EEME peak area (7) will be less than 0.04%. A sigma unit is defined (7) as the peak width of ENT or CDAP at 0.882 peak height. To confirm fully that no decomposition of EEME occurred during vapor phase analysis, the EEME was collected after gas chromatography and rechromatographed on silica gel G thin layers. As a developer solvent, 90:10 benzene–ethyl ether was used for a distance of 10 cm. That no significant decomposition of EEME occurred during gas chromatography might be inferred from the following observations.

(*a*) The two expected decomposition products of EEME—estrone (R_f 0.16) and its 3-methylether (R_f 0.46)—were not detectable on thin-layer chromatograms of the residue collected after gas chromatography, using a solvent system (90:10 benzene–ether) capable of separating all three estrogens. (*b*) Only one estrogenic spot (R_f 0.32) was detected on the thin layer.

Reproducibility of Gas Chromatographic Areas.—Injections in the range of 0.08 mcg. EEME deviated about 10% from a mean value, while the mean average deviation in the range 0.4 to 4.2 mcg. of EEME was plus or minus 2%. To determine the reproducibility of repeated injections using the same syringe and the same EEME solution, 10 injections indicated that the injection error is plus or minus 3.4%.

Interferences.—Of the excipients used to formulate oral progestational tablets (lactose, cornstarch, polyvinylpyrrolidone, magnesium stearate, and stearic acid), only stearic acid seriously interfered with the measurement of peak areas. The stearic acid, because of its preponderance, completely masked the presence of EEME. This problem was, however, partially resolved by converting the stearic acid to cyclohexane insoluble sodium stearate. The filtered cyclohexane extract (40 ml.) containing the EEME and stearic acid was shaken with 2.0 ml. of 1 *N* sodium hydroxide, and the refiltered cyclohexane phase was treated as described in the procedure for the analysis of tablets. Despite this treatment, assay results were invariably 10 to 15% above the expected values. For formulations containing polyvinylpyrrolidone, it was necessary to stir the cooled extract (after refluxing with cyclohexane) for 15 minutes with 4.0 ml. of distilled water to effect quantitative extraction of the EEME. Without the latter step, recoveries were in the order of 70 to 80% EEME. The addition of water serves to dissolve the cyclohexane insoluble polymer and to release EEME physically incorporated within the polymer. The separated cyclohexane phase was filtered and treated as described under *Gas Chromatographic Analysis of EEME Tableted with ENT or CDAP*.

Recovery Data.—The results for the analyses of tableted EEME are presented in Table I. Two determinations of EEME/ENT tablets resulted in recovery values of 95.8 and 97.1% EEME by gas chromatography, while analysis of the same sample by thin-layer chromatography resulted in a 98.5% recovery. Two determinations of EEME/CDAP

Table I.—Analysis of Tableted EEME:Progestational Combinations

Formulation	mcg. EEME Found/Tablet: Gas Chromatography	Thin-Layer Chromatography
Tablets containing 10 mg. ENT and 60 mcg. EEME	57.4 and 58.3	59.1
Tablets containing 2 mg. CDAP and 80 mcg. EEME	77.1 and 79.4	76.4

Table II.—Analysis of Unformulated EEME:Progestational Combinations

Synthetic Mixture, %	Gas Chromatography	Thin-Layer Chromatography
EEME in ENT, 0.40	0.413, 0.394, 0.386	0.391
EEME in ENT, 0.10	0.0971, 0.0944, 0.0986	0.104
EEME in CDAP, 0.10	0.0995, 0.0960, 0.0967	0.103

tablets resulted in recovery values of 96.4 and 98.2% EEME by gas chromatography, while analysis by thin-layer chromatography led to a 95.5% recovery value. The above tablets were prepared by adding levels of EEME (from a spectrophotometrically standardized stock solution of EEME in methanol) to an appropriate amount of an excipient mixture containing either CDAP or ENT. After drying, the mixture was tableted and assayed. The excipients used were cornstarch, lactose, and magnesium stearate.

The results of analyses of synthetic mixtures of ENT plus EEME or CDAP plus EEME are shown in Table II, an indication that cyclohexane extracts EEME quantitatively in the presence of either CDAP or ENT.

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4,4′-Dibromodiphenyldisulfimide as a Reagent for the Identification of Organic Bases I. Preparation of the Reagent and Derivatives of Some Antihistamines

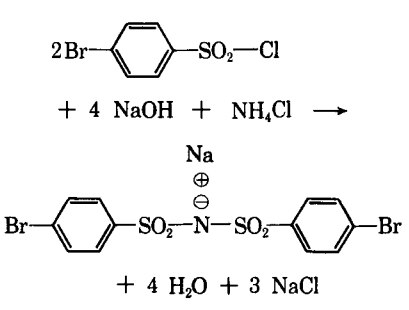
By EDWARD A. JULIAN* and ELMER M. PLEIN

A procedure for the synthesis of a new reagent, 4,4′-dibromodiphenyldisulfimide, which reacts with all classes of amines and produces crystalline derivatives suitable for the determination of various physical properties of value in analytical work, is reported. Melting points for the 4,4′-dibromodiphenyldisulfimide derivatives of 21 antihistamines are also presented.

DISULFIMIDE compounds ($R\text{-}SO_2\text{-}NH\text{-}SO_2\text{-}R$) have been suggested as reagents for the preparation of derivatives of some medicinal amines (1, 2). It is the purpose of this paper to report the synthesis of a new disulfimide reagent, 4,4′-dibromodiphenyldisulfimide, which reacts with primary, secondary, and tertiary amines and to report on the use of this reagent in the preparation of some medicinal amine derivatives of antihistamine drugs.

The synthesis of disulfimide compounds and their use in preparing derivatives were reported as early as 1854 (3). Most of the disulfimides synthesized (4–6) have been aromatics with halide substitutions in positions 3 and 4. Procedures reported in the literature for the preparation of disulfimide compounds (7, 8) were not applicable for the preparation of 4,4′-dibromodiphenyldisulfimide, so a new method was devised.

Two moles of *p*-bromobenzenesulfonyl chloride was reacted with ammonium chloride and sodium hydroxide to give sodium-4,4′-dibromodiphenyldisulfimide.



The sodium salt of the reagent can be treated with acid for conversion to the acid imide.

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