Anal.—Calcd. for $C_{16}H_{28}ClNOS$: C, 60.57; H, 8.80; N, 4.39. Found: C, 60.46; H, 8.72; N, 4.10. U.V. max. 233 mμ (5110); 238 mμ shed (5900); 245 mμ shed (3020).

3 - Dimethylamino - 2 - methyl - 1 - (3 - thienyl)-1-benzyl-1-propanol Hydrochloride.—Benzylmagnesium bromide was prepared from 3.4 Gm. (0.02 mole) of benzyl bromide, 730 mg. (0.03 mole) of magnesium turnings, and anhydrous ether. The Grignard solution was stirred at room temperature during the dropwise addit on of 2.9 Gm. (0.015 mole) of 3-(3-dimethylamino-2-methylpropanoyl) thiophene in 25 ml. of dry ether. The reaction mixture was stirred and refluxed for 1 hour; it was then decom posed by the addition of 7 ml. of water. The ether layer was separated, the reaction mixture was extracted with two 25-ml. portions of ether, and the ether layers were combined and dried over Drierite. The hydrochloride was prepared as indicated above. After recrystallization from an ethanol-ether mixture, a yield of 3.8 Gm. (78%) of a compound which melted at 207° was obtained.

Anal.—Calcd. for $C_{17}H_{24}ClNOS$: C, 62.46; H, 7.40; N, 4.29. Found: C, 62.12; H, 7.19; N, 4.38.

3 - Dimethylamino - 1 - propyl - 1 - (3 - thienyl)-2-methyl-1-propanol Methiodide.—Three hundred and ninety-four milligrams (0.002 mole) of 3-(3-dimethylamino-2-methylpropanoyl) thiophene was treated with an equal molar quantity of propylmagnesium bromide in 15 ml. of dry ether; this mixture was refluxed for 2 hours and then decomposed with the addition of water. The ether .ayer was separated and extracted with two 10-ml. portions of ether. The ether layers were combined, dried over Drierite, and then evaporated to dryness. The residue was dissolved in 10 ml. of acetone. The quaternary salt of the free base was formed by the addition of 1 ml. of methyl iodide; 5 ml. of ether was added to precipitate the salt. A yield of 5.2 Gm. (68%) of the methiodide, m.p. 120–127°, was thus obtained.

Anal.—Calcd. for $C_{14}H_{26}INOS$: C, 43.87; H, 6.84. Found: C, 43.38; H, 6.56.

Type III Compound

4 - Dimethylamino - 1 - phenyl - 2 - (3 - thienyl)-3-methyl-2-propanoyloxybutane Hydrochloride.—To 20 ml. of dry dioxane, 46 mg. (0.002 mole) of sodium metal was added, and the mixture was stirred and heated at reflux temperature until sodium sand resulted. Five hundred and eighty milligrams (0.002 mole) of 3-dimethylamino-2-methyl-1-(3-thienyl)-1-benzyl-1-propanol was added slowly and refluxed for 6 hours. The solution was cooled, and 186 mg. (0.0022 mole) of propionyl chloride was added. After refluxing for 6 hours, the mixture was cooled, water was added, and the mixture was made basic to litmus with sodium carbonate. The solution was extracted three times with 20-ml. portions of ether; the combined extracts were dried over anhydrous potassium carbonate, and the compound was converted in the usual manner to the hydrochloride. A yield of 297 mg. (39.4%) of a compound, m.p. 217°, was thus obtained.

Anal.—Calcd. for $C_{20}H_{28}ClNO_2S$: C, 62.89; H, 7.39; N, 3.67. Found: C, 62.61; H, 7.33; N, 3.73.

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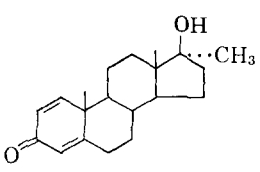
Quantitative Fluorometric Determination of Methandrostenolone

By F. TISHLER, P. B. SHETH, M. B. GIAIMO, and W. J. MADER

A fluorometric procedure is described for the determination of methandrostenolone (17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one). The method is based on the flluorogen formed when the steroid is heated with hydrochloric acid at 100°. In order to determine the selectivity of this reaction, a number of related steroids has been studied.

METHANDROSTENOLONE[1] is a new tissue-building compound prepared in these laboratories. Its structural formula is illustrated:



Received March 22, 1962, from the Research Department, Ciba Pharmaceutical Co., Summit, N. J.
Accepted for publication April 11, 1962.
[1] Ciba's trade name for methandrostenolone is Dianabol.

The ultraviolet spectrum of methandrostenolone in methanol appears in Fig. 1 and can be used for the quantitative determination of the compound; however, ultraviolet analysis in methanol cannot be employed when methyltestosterone is present as an impurity since each steroid exhibits a maximum around 241 mμ.

Fluorometry offers a means to the qualitative and quantitative determination of microgram amounts of steroids. Sulfuric acid has been employed by many workers (1–4) to form the fluorogen used for the determination of various steroids. Recently Touchstone and Murawec (5) found that certain steroids exhibited a greater fluorescent intensity when heated with a 2*N* methanolic potassium hydroxide solution prior to being dissolved in sulfuric acid than when dissolved in sulfuric acid alone. The use of hydrochloric acid to induce fluorescence in steroids has also been reported (6–8).

It has been observed in these laboratories that when methandrostenolone is heated at 100° with hydrochloric acid a fluorogen is formed, which is the basis of the method of analysis to be described. Under the same conditions, methyltestosterone exhibits no fluorescence, thereby offering a means of determining methandrostenolone in which methyltestosterone might be present as an impurity. Optimum conditions for the hydrochloric acid-induced fluorescence have been determined and a number of related steroids have been studied in order to determine the selectivity of this reaction.

EXPERIMENTAL

Apparatus.—All measurements are made on an Aminco-Bowman spectrophotofluorometer with a quartz cell having a 1-cm. light path using a $^1/_{16}$-in. defining slit (band pass = 6 mμ) and an RCA 1-P-21 photomultiplier. The excitation and fluorescent spectra are obtained on an Electro model 101 XY recorder. The instrument is calibrated against a quinine solution by the method of Sprince and Rowley (9). Absorbance measurements are made with a Cary recording spectrophotometer.

Reagents.—All steroids used in the study are of reference standard quality, either assayed by phase solubility (10) or purchased from the U.S.P. as reference standards. All other reagents are reagent grade. Standard methandrostenolone solutions are prepared containing 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0 mcg./ml. in methanol.

Procedure.—Weigh a counted number of not less than 20 tablets and reduce them to a fine powder. Transfer an accurately weighed portion of the powder, equivalent to 10 mg. of methandrostenolone, to a 100-ml. volumetric flask. Add 75 ml. of methanol, shake for 10 minutes, dilute to volume with methanol, and mix well. Centrifuge a portion of the mixture. Transfer a 5.0-ml. aliquot sample of the clear solution to a 100-ml. volumetric flask, dilute to volume with methanol, and mix well. (Concentration = 5.0 mcg./ml.)

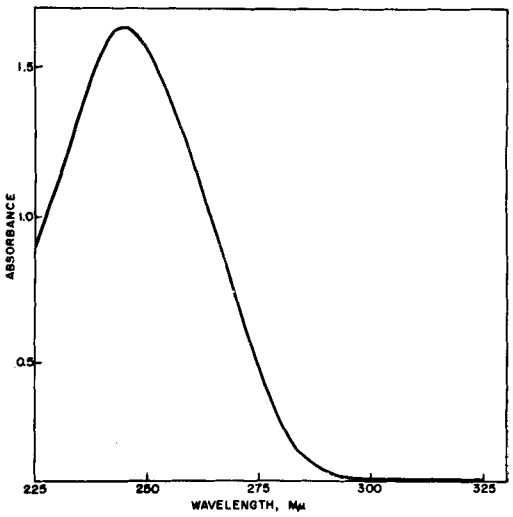


Fig. 1.—Ultraviolet absorption spectrum of methandrostenolone in methanol, 0.01 mg./ml.

Pipet 2.0 ml. of the sample preparation, 2.0 ml. of a standard solution (5.0 mcg./ml.), and 2.0 ml. of methanol into separate 25-ml. volumetric flasks. Add 5 ml. of concentrated hydrochloric acid and heat in an oven at 100°. After 20 minutes, wash down the sides of the volumetric flasks with 2 ml. of methanol and continue heating for an additional 20 minutes. Cool the flasks to room temperature, dilute to volume with methanol, and mix well. Read the fluorescent intensity of the blank, sample, and standard solutions at an excitation of 280 mμ and a fluorescence of 345 mμ after setting the photometer sensitivity to full and the meter multiplier to 0.03. Calculate the amount of methandrostenolone in each tablet by the following formula

$$\frac{A_o - A_b}{A_s - A_b} \times 10 \times \frac{Wt}{Ws} =$$

mg. of methandrostenolone/tablet

where Ao = fluorescent intensity of the sample, As = fluorescent intensity of the standard, Ab = fluorescent intensity of the blank, Wt = average weight per tablet in Gm., and Ws = weight of sample in Gm.

DISCUSSION

When exhibited, excitation and fluorescent maxima were obtained for all steroids used in this study. The excitation and fluorescent spectra for methandrostenolone appear in Fig. 2. In order to determine the optimum conditions for the formation of the fluorogen, the fluorescence of the final solution as a function of time and milliliters of hydrochloric acid added was investigated. From Figs. 3 and 4 it can be seen that at 100°, a constant maximum fluorescence is obtained after 20 minutes using 4 ml. of hydrochloric acid. To be sure of maximum fluorescent intensity, 5 ml. of acid was used in the procedure. It was found that, due to evaporation of the solvent over the required heating period, reproducible results were not always obtained be-

cause of unreacted methandrostenolone on the sides of the flask. For this reason, a second heating period was employed after washing the flasks with 2 ml. of methanol. A plot of fluorescent intensity *vs.* concentration was found to be linear up to 30 mcg./25 ml. of final solution (Fig. 5).

Temperatures below 100° were unsatisfactory, and at room temperature the fluorogen was not formed. Dilution with water or dilute hydrochloric acid rather than methanol decreased the fluorescent intensity in all cases.

As a test of the applicability of the fluorometric procedure to pharmaceutical preparations, several

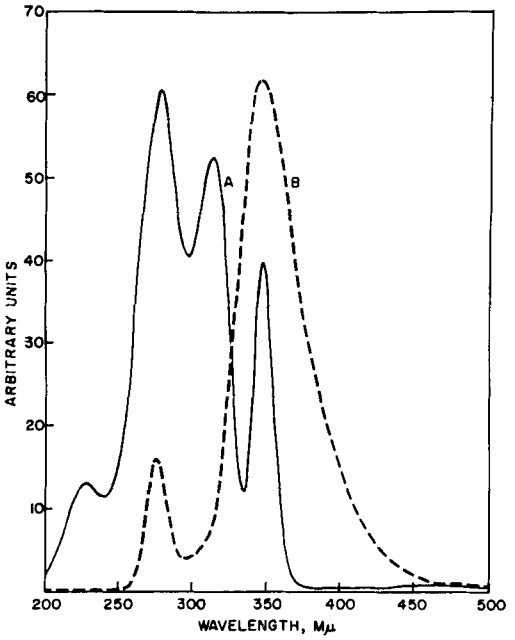


Fig. 2.—Excitation and fluorescent spectra of methandrostenolone. *A*, Excitation scan, fluorescence held at 345 mμ; *B*, fluorescent scan, excitation held at 280 mμ.

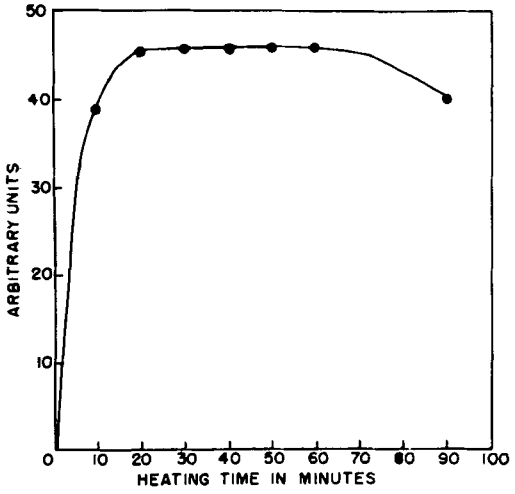


Fig. 3.—Plot of fluorescences of final solution against heating time of reaction.

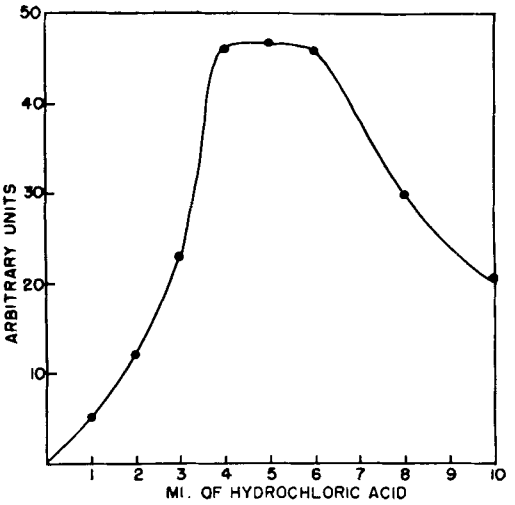


Fig. 4.—Plot of fluorescences of final solution against milliliters of concentrated hydrochloric acid.

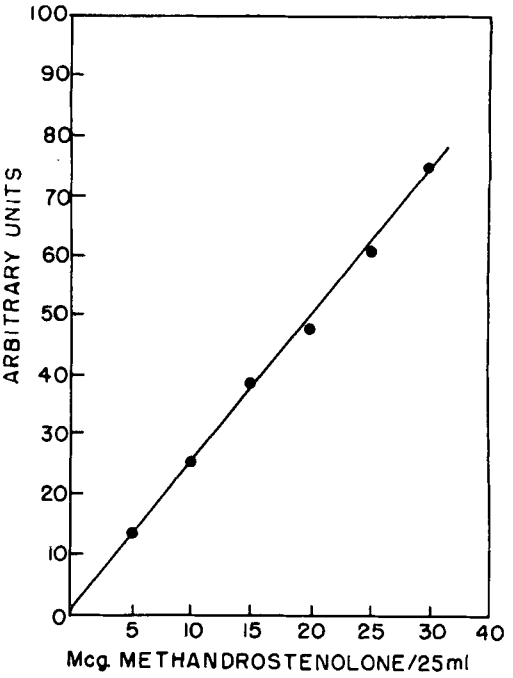


Fig. 5.—Plot of fluorescences of final solution against concentration of methandrostenolone (0.1 meter multiplier).

lots of tablets containing methandrostenolone were analyzed by both the proposed method and by ultraviolet analysis. The results appear in Table I. The results on several commercial lots, performed in triplicate, showed a precision of ±2%.

Common tablet excipients did not interfere with the fluorescent technique. In order to determine whether methyltestosterone interfered with the fluorometric procedure, several samples of methandrostenolone containing up to 10% of methyltestosterone were assayed for methandrostenolone. Methyltestosterone itself showed no fluorescence

TABLE I.—COMPARISON OF TABLETS BY FLUORESCENT AND ULTRAVIOLET PROCEDURES

Sample	Description and Declaration, mg.	Methandrostenolone Found, mg. Fluorescent	Ultraviolet
1	2.5	2.47	2.49
2	2.5	2.48	2.50
3	5.0	4.94	5.02
4	1.0 with 9α-fluoro-16α-methylprednisolone[b]	1.05	1.04[a]

[a] A preliminary extraction and separation is necessary before U.V. analysis can be employed. [b] Ciba's trade name for 9α-fluoro-16α-methylprednisolone is Gammacorten.

with hydrochloric acid but when present in concentrations greater than 8%, complete development of the fluorogen was inhibited.

A number of related steroids was studied in order to determine the selectivity of the reaction and the effect of various groups on the molecule (see Table II). Of the various steroids studied, only methandrostenolone, ethinyl estradiol, 17α-methylestradiol, and 17α-ethyl-17β-hydroxyandrosta-1,4-dien-3-one showed fluorescence under the conditions employed. The excitation spectrum which is the same for ethinyl estradiol and 17α-methylestradiol appears in Fig. 6. From Figs. 2 and 6, it can be seen that methandrostenolone can be readily distinguished from ethinyl estradiol and 17α-methylestradiol as the latter compounds show no maximum at 310 mμ; however, methandrostenolone cannot be distinguiushed from 17α-ethyl-17β-hydroxyandrosta-1,4-dien-3-one as both excitation and fluorescent spectra are very similar.

It is, therefore, indicated that hydrochloric acid-induced fluorescence is applicable only to methandrostenolone and certain structurally related steroids. The reaction appears to be selective for $\Delta^{1,4}$-dien-3-one or $\Delta^{1,3,5,(10)}$-trien-3-ol steroids which have both a 17β-hydroxy and 17α-alkyl or alkyne substitution. Further studies are being carried out to determine the chemistry of the reaction of hydrochloric acid-induced fluorescence and to determine the effect of various other substitutions at the 17α-, 17β-, and 3-positions.

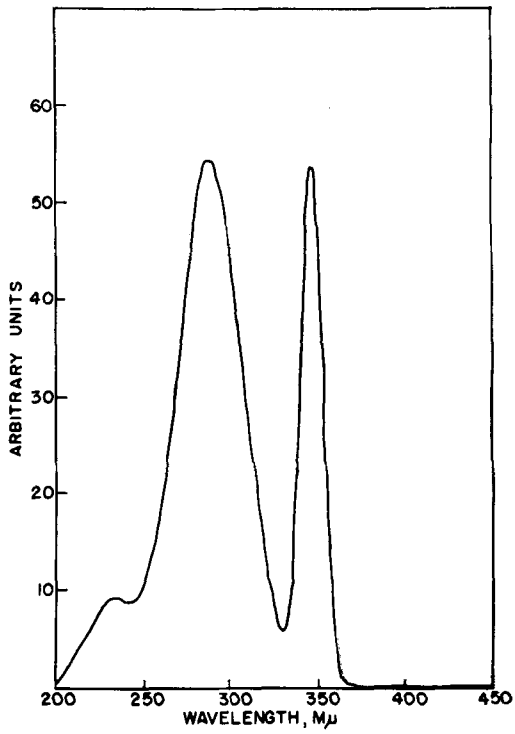


Fig. 6.—Excitation spectrum for both ethinyl estradiol and 17α-methylestradiol, fluorescence held at 345 mμ.

SUMMARY

1. A method is described for the quantitative fluorometric determination of methandrostenolone.

2. A number of related steroids is studied to determine the selectivity of hydrochloric acid-induced fluorescence

TABLE II.—FLUORESCENCE OF 17α-METHYL-17β-HYDROXYANDROSTA-1,4-DIEN-3-ONE AND SOME RELATED COMPOUNDS

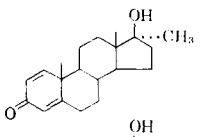
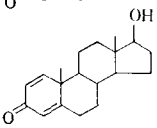
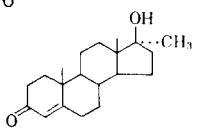
Compound	Structure	Relative Fluorescent Intensity
17α-Methyl-17β-hydroxyandrosta-1,4-dien-3-one		100
17β-Hydroxyandrosta-1,4-dien-3-one		Nil
Methyltestosterone		Nil

TABLE II (*Continued*)

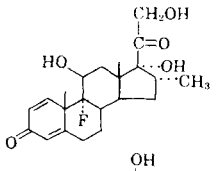
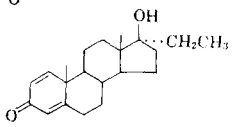
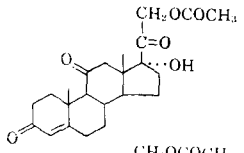
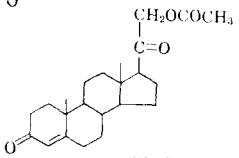
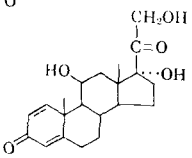
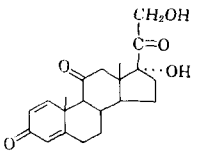
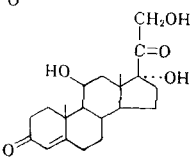
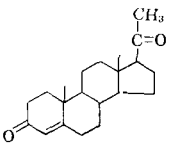
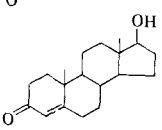
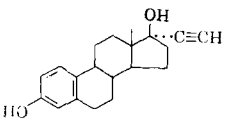
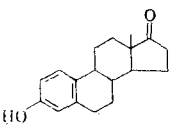
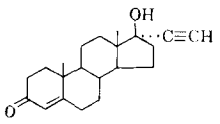
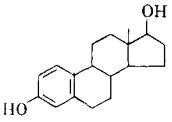
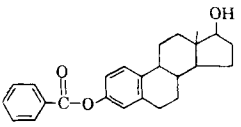
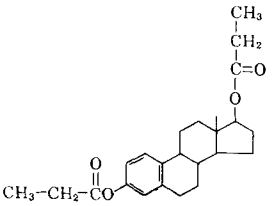
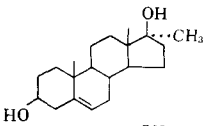
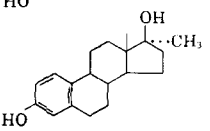
Compound	Structure	Relative Fluorescent Intensity
9α-Fluoro-16α-methylprednisolone		Nil
17α-Ethyl-17β-hydroxyandrosta-1,4-dien-3-one		80
Cortisone acetate		Nil
Desoxycorticosterone acetate		Nil
Prednisolone		Nil
Prednisone		Nil
Hydrocortisone		Nil
Progesterone		Nil
Testosterone		Nil
Ethinyl estradiol[a]		48
Estrone		Nil

TABLE II (*Continued*)

Compound	Structure	Relative Fluorescent Intensity
Ethisterone		Nil
β-Estradiol		4
Estradiol benzoate		Nil
Estradiol dipropionate		Nil
17α-Methyl-5-androstene-3β,17β-diol		11
17α-Methylestradiol[a]		81

[a] A maximum fluorescent reading is obtained at an excitation of 290 mμ and a fluorescence of 345 mμ.

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