variety of complex samples and unusual techniques or critical control of the conditions are not necessary to obtain reliable and accurate results.

SUMMARY

A spectrophotometric method is described for the determination of the quaternary ammonium compound, poldine methylsulfate. The method is based on the ultraviolet absorbance of methanol solutions of the drug after separation of interfering substances by a reineckate derivative and regeneration of the conjugate base through ion-exchange chromatography. The method has been shown to be applicable to the determination of the compound in pharmaceutical preparations and in the presence of its hydrolytic degradation products. The stability of the compound in buffered aqueous systems under various conditions of pH and temperature was investigated utilizing the spectrophotometric procedure presented.

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Methandrostenolone

Mechanism of Hydrochloric Acid Induced Fluorescence

By F. TISHLER and S. M. BRODY

The main products formed when methandrostenolone (17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one) is heated with methanolic hydrochloric acid have been isolated by preparative thin-layer chromatography and identified by application of infrared, ultraviolet, and nuclear magnetic resonance spectra. A mechanism for the hydrochloric acid induced fluorescence is postulated.

IN A PREVIOUS PAPER by Tishler, *et al.* (1), a fluorometric procedure was described for the determination of methandrostenolone[1] (Compound I, Fig. 1) based on the fluorogen formed when the steroid was heated with a methanolic solution of hydrochloric acid at 100°. A number of related steroids were studied to determine the selectivity of the reaction. Under the conditions employed, the reaction appeared to be selective for $\Delta^{1,4}$-dien-3-one or $\Delta^{1,3,5(10)}$-trien-3-ol steroids which had both a 17β-hydroxy and a 17α-alkyl or alkyne substitution.

With the aid of preparative thin-layer chromatography it has been possible to isolate the main products formed during the reaction. Based upon the identification of these products, a mechanism for hydrochloric acid-induced fluorescence with methnadrostenolone and structurally related steroids is postulated.

DISCUSSION

A preparative thin-layer technique as described by Korzun, *et al.* (2), was used to isolate the desired compounds. Figure 2 shows a typical separation after the entire plate has been sprayed with a modified LeRosen reagent (3). A summary of the isolated fractions is given in Table I.

The total recovery was approximately 80%. Material was lost due to the development of a guide strip on the plates and due to strongly adsorbed material which was not eluted. The three major components, Compounds IV, V, and VIII (Fig. 1), were further purified by chromatographing them separately on a small alumina column. Figure 3 shows the purified steroids as chromaotgraphed on a standard thin-layer plate together with the reaction mixture.

It was evident from the ultraviolet spectrum (Fig. 4) of the reaction mixture that methandrostenolone had undergone a change in its structure. Since the reaction was selective for those steroids which had the structure previously described above, one could predict that the changes had occurred solely in ring A or D or in both rings.

Infrared and nuclear magnetic resonance (N.M.R.) data of Compounds IV, V, and VIII showed the loss of the hydroxyl group originally present in ring D of methandrostenolone. The N.M.R. spectra for the above compounds showed one or two bands between 55 and 61 cycles per second, a region characteristic of the $C_{17,17'}$ dimethyls (4). The integrated area of the bands indicated the presence of six hydrogens which is consistant for two methyl groups. It was apparent that the loss of the 17-hydroxyl group was followed by the migration of

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[1] Marketed as Dianabol by Ciba Pharmaceutical Co., Summit, N. J.

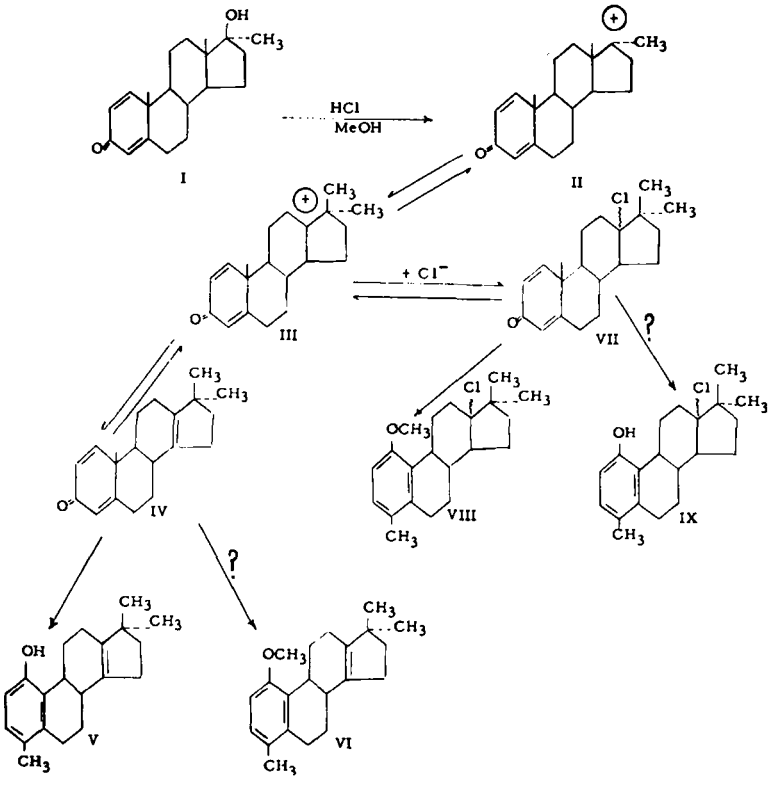
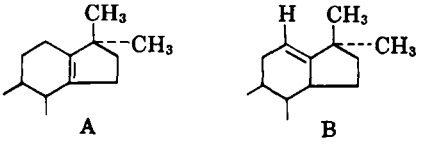


Fig. 1.—Postulated mechanism for hydrochloric acid-induced fluorescence of methandrostenolone.

the C_{18} methyl group from position 13 to 17 giving Species III (Fig. 3).

One pathway that Species III could follow is the loss of a proton at position 12 or 14 with the subsequent formation of a double bond either at position 13–14 (*A*) or 12–13 (*B*)



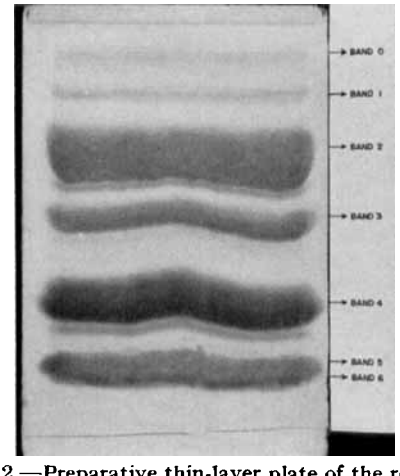


Fig. 2.—Preparative thin-layer plate of the reaction mixture after spraying with LeRosen reagent.

N. M. R. data for Compounds V and VIII showed that no olefinic hydrogens were present, while data for Compound IV showed no additional olefinic hydrogens were present besides those due to ring A which appear further down field in the spectrum, thus ruling out structure B (4, 5).

It was shown by spectral data that Compound IV was unchanged in ring A, *i.e.*, the $\Delta^{1,4}$-dien-3-one system was still present. In addition, the N.M.R. indicated that the C_{19} methyl group, which appears at about 75 cycles per second in structures of this type, was still at position 10 (5). From the above reasoning it can be inferred that Compound IV has the structure assigned in Fig. 1.

The data obtained for Compounds V and VIII

Table I.—Summary of Isolated Fractions

Band	Description	Wt., Gm.	R_f[b]	% Recovery
0	Origin	0.52	0.00	6.0
1	Mixture	0.24	0.00, 0.10	2.8
2	Compound IV	2.31	0.10	26.5
3	Unknown fraction	0.59	0.20	6.8
4	Compound V	1.97	0.35	22.6
5	Compound VIII	1.16	0.85	13.3
6	Unknown fraction	0.25[a]	0.90	2.9

[a] This weight includes unknown compound and Compound VIII. [b] These R_f values are those obtained from standard thin layer results.

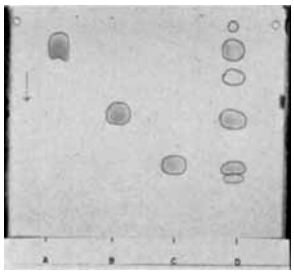


Fig. 3.—Standard thin-layer plate. Key: A, Compound IV; B, Compound V; C, Compound VIII; D, reaction mixture.

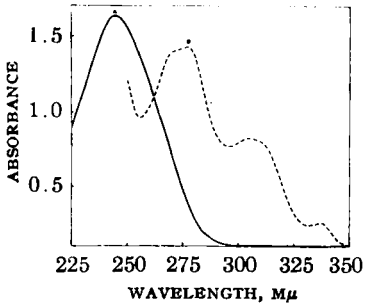


Fig. 4.—Ultraviolet absorption spectra of methandrostenolone *A*, 0.015 mg. per ml., and the reaction mixture *B*, 0.03 mg. per ml., in methanol.

indicated that there was no longer a ketone group, conjugated or unconjugated, present in the molecule. The N.M.R. spectra for these compounds in the aromatic region showed the splitting of the hydrogens to be 8.5 cycles per second which is indicative of ortho splitting (6). Since the N.M.R. indicated the loss of the 75 cycles per second band and I.R. and N.M.R. data indicated the presence of a hydroxyl group (phenolic) in Compound V, it was evident that Compound IV had undergone a dienone-phenol rearrangement (7–11) with the resulting structure assigned in Fig. 1.

Elemental analysis of Compound VIII showed that chlorine (Cl^-) had added to the steroid, thereby indicating that Species III could follow a second pathway as seen in Fig. 3. Although no chemical evdience is available to fix the location and configuration of the chlorine, a 13α-chloro is a most likely assignment (12). From I.R. and N.M.R. data it was shown that Compound VIII was a 1-methoxy-4-methyl steroid instead of a 1-hydroxy-4-methyl steroid. Elks and his co-workers (13) have reported the direct preparation of phenolic ethers by a dienone-phenol rearrangement in alcohol. This same mechanism can be applied to Compound VII with the subsequent formation of Compound VIII.

Further heating of Compounds IV, V, and VIII with methanoic hydrochloric acid showed by thin-layer chromatography that Compounds V and VIII were not changed, while Compound IV was converted, in large part, to Compounds V and VIII. Compounds V and VIII (10 mcg./25 ml. of methanol) possess native fluorescence at 325 mμ when activated at 280 mμ which is typical of estrogenic steroids; Compound IV exhibited no native fluorescence.

When Compounds V and VIII (10 mcg./25 ml.) were treated according to Tishler, *et al.* (1), for the fluorescent determination of methandrostenolone, the fluorescent readings at 345 mμ were increased 10–15-fold when the solution was activated at

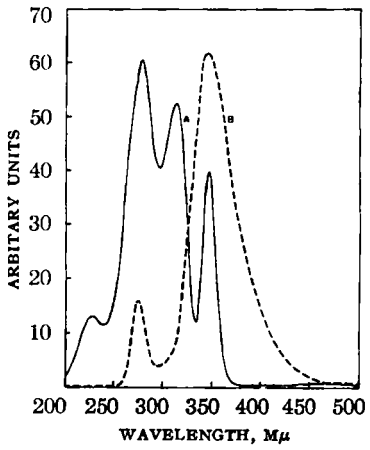
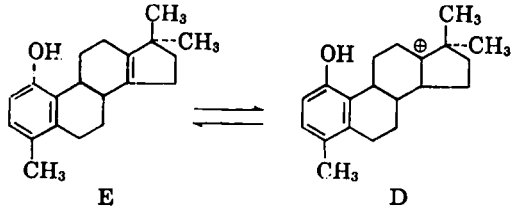
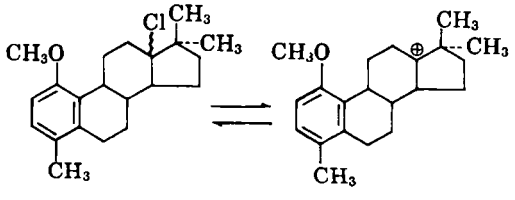


Fig. 5.—Excitation and fluorescent spectra of methandrostenolone, Compounds IV, V, and VIII Key: A, excitation scan—fluorescence held at 345 mμ; B, fluorescent scan—excitation held at 280 mμ.

280 mμ. Compound IV under the same conditions gave a fluorescent reading of the same increased intensity. The activation and fluorescent spectra which were obtained with Compounds IV, V, and VIII after heating with methanolic hydrochloric acid are the same as that obtained with methandrostenolone and appear in Fig. 5.

Based upon the above results, it is suggested that Species D and F are present in the methanolic hydrochloric acid solution after heating and are responsible for the fluorescent characteristic of methandrostenolone.





No explanation is available at present regarding why Compounds V and VIII rather than VI and IX are the predominant products formed. Further work is being carried out on structurally related steroids to learn more of hydrochloric acid-induced fluorescence.

EXPERIMENTAL

Apparatus

N.M.R. spectra were obtained in $CDCl_3$ on a Varian A-60 using tetramethylsilane as an internal reference standard. U.V. curves were made on a

Cary recording spectrophotometer, I.R. spectra were obtained with a Beckman I.R.-5. Excitation and fluorescent spectra were recorded on an Electro model 101 XY recorder in conjunction with an Aminco-Bowman spectrophotofluorometer.

Procedure

Methandrostenolone (1.0 Gm.) was dissolved in 15 ml. of methanol in a 50-ml. volumetric flask. Ten milliliters of concentrated hydrochloric acid was added and the mixture was placed in a 100° oven for 40 minutes. After cooling, the viscous oil (0.95 Gm.) formed during the reaction was separated from the remainder of the mixture by decantation.

Approximately 1.0 Gm. of the reacted material, dissolved in a minimum amount of chloroform-methanol (1:1), was applied with a pipet as a narrow band 8 in. long and at a point 1 in. from the end of the plate to eight glass plates (10 × 15 in.) coated to a thickness of 1 mm. with Silica Gel G (E. Merck, Darmstadt, Germany). The plates were placed at an angle in 12 × 12 × 24-in. rectangular chromatographic chambers and the solvent (benzene-chloroform [80:20]) was allowed to ascend to about 1 in. from the top of the plates. To obtain a satisfactory resolution of the mixture, the plates were removed, air dried, and then redeveloped once again by the above procedure. The two developments took from 6 to 8 hours. A guide strip 1 in. wide located along one edge of the plate was sprayed with a modified LeRosen reagent. The bands thus revealed were marked, scraped off the glass plates with a porcelain spatula, and eluted from the adsorbent with a 1:1 mixture of chloroform-methanol. Evaporation of the solvent afforded oils in all cases. The compounds were further purified by chromatographing them separately on a small alumina column.

Compound IV.—Isolation of this compound afforded a colorless oil which was resistant to crystallization; $\lambda_{max.}^{MeOH}$ 243 mμ, log ε 4.12; $\lambda_{max.}^{Nujol}$ 6.04 μ (ring ketone with αβ-α′β′ conjugation.

Anal.—Based on the 2,4-dinitrophenylhydrazone of Compound IV. Calcd. for $C_{26}H_{30}N_4O_4$: C, 67.53; H, 6.49; N, 12.12. Found: C, 67.49; H, 6.43; N, 11.88.

Compound V.—This material was recrystallized from aqueous methanol and yielded fine white needles; m.p. 106–108°; $\lambda_{max.}^{MeOH}$ 279 mμ, log ε 3.35; $\lambda_{max.}^{Nujol}$ 2.86 μ (⁻OH); 12.44 μ (*para*-type).

Anal.—Calcd. for $C_{20}H_{26}O$: C, 85.10; H, 9.22. Found: C, 84.96; H 9.22.

Compound VIII.—This material was recrystallized from methanol and yielded fine white needles; m.p. 53 to 54.5°; $\lambda_{max.}^{MeOH}$ 278 mμ, log ε 3.35 and $\lambda_{max.}^{MeOH}$ 284, log ε 3.32; $\lambda_{max.}^{Nujol}$ 8.00 and 9.56 μ (aromatic ether); 12.26 μ (*para*-type).

Anal.—Calcd. for $C_{21}H_{29}ClO$: C, 75.79; H, 8.72; Cl, 10.68. Found: C, 75.43; H, 8.77; Cl, 10.50.

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