THE IDENTIFICATION OF 17α-HYDROXY-17-METHYL-1,4-ANDROSTADIEN-3-ONE AS A METABOLITE OF THE ANABOLIC STEROID DRUG 17β-HYDROXY-17-METHYL-1,4-ANDROSTADIEN-3-ONE IN MAN

B.S. Macdonald, P.J. Sykes, P.M. Adhikary, and R.A. Harkness

Departments of Chemistry, Clinical Chemistry and Paediatric Biochemistry, University of Edinburgh, Scotland.

Received: August 3, 1971

ABSTRACT

The more polar of the two major urinary metabolites of methandrostenolone, 17β-hydroxy-17-methyl-1,4-androstadien-3-one, in man has already been identified as 6β-hydroxymethandrostenolone, 6β, 17β-dihydroxy-17-methyl-1,4-androstadien-3-one. The other metabolite has now been identified as the 17-epimer of methandrostenolone,17α-hydroxy-17-methyl-1,4-androstadien-3-one. The compound was isolated from the freely extractable neutral fraction of urine following the administration of 5 mg of the drug to normal men. The relevant chromatographic fractions from thin layer and gas liquid systems were identified by carbon skeleton chromatography. The 17-epimer has been synthesised, details of which are included, and the previously unidentified metabolite was found to be identical with the synthetic compound.

The characterisation of the 17-epimer defines a hitherto apparently unknown biochemical pathway.

INTRODUCTION

The metabolism of the anabolic steroid methandrostenolone (Dianabol) 17β-hydroxy-17-methyl-1,4-androstadien-3-one has been previously studied by Rongone and Segaloff (1). Extraction, after β-glucuronidase hydrolysis, of the urine from a woman with advanced adenocarcinoma of the lung following administration of 4 g of the drug afforded two metabolites which accounted for 7.25% of the dose. 80% of the extracted material was identified

as 6β-hydroxymethandrostenolone 6β, 17β-dihydroxy-17-methyl-1,4-androstadien-3-one. The structure of the second metabolite was not established but on the basis of the evidence available Rongone and Segaloff suggested that it was an isomer of methandrostenolone rather than an oxygenated product.

Adhikary and Harkness have studied the metabolism of small doses of methandrostenolone in normal man (2,3). They succeeded in recovering 5% of the dose as two major metabolites from the freely extractable neutral fraction of the urine. High temperature catalytic reduction with gas chromatography of the hydrocarbon products ('carbon skeleton chromatography' [4,5]) was used to locate the metabolites and to quantify them. The two compounds occurred in the ratio 2:1, the minor of the two being identified as 6β-hydroxymethandrostenolone (2). The 17-epimer of methandrostenolone 17α-hydroxy-17-methyl-1,4-androstadien-3-one has now been synthesised and has been shown to be identical with the major urinary metabolite (M) of methandrostenolone in man (6).

ISOLATION AND PURIFICATION

In each study, the normal therapeutic dose of one 5 mg tablet of methandrostenolone was administered to a male volunteer in good health, and the total urine output of the following 25 hr was collected. Extraction of the urine with chloroform gave the 'free fraction'. The extracts were chromatographed on silica gel G coated plates using chloroform-methanol 19:1 (v/v) as mobile phase. Chromatographic fractions were collected as eluates from 1 cm bands; these were halved, evaporated to dryness and one set of residues dissolved in 0.1 ml of ethanol. 10 μl of this solution from each fraction was used for high temperature catalytic reduction at 180-190°C using 1-3% w/w platinum catalyst. The reduction products were analysed by gas chromatography using SE-30 or NGA columns with a hydrocarbon standard derived from the drug by the same method. From these results, chromatographic fractions were selected which contained the same skeleton as the drug

(positive fractions). These fractions were then purified by further chromatography on thin layers of silica gel G and finally by preparative gas liquid chromatography. Additional confirmation of the carbon skeleton of the compound or compounds in these fractions was again obtained by gas chromatography after high temperature catalytic reduction.

The More polar of the two metabolites showed the same chromatographic behaviour as 6β-hydroxymethandrostenolone. The major metabolite behaved similarly to methandrostenolone on thin layer chromatography, but a slightly shorter retention time on gas chromatography (SE-30 or QF-1) indicated that the compound was not merely unchanged drug.

PREPARATION OF 17-EPIMETHANDROSTENOLONE

The preparation of 17α-hydroxy-17-methyl-4-androsten-3-one (17β-methylepitestosterone) has been described by Sondheimer et al. (7), using pregnenolone acetate as starting material. The synthesis of 17-epimethandrostenolone here described is essentially the same although different reagents were used and the starting material was 3β-hydroxy-5-androsten-17-one (dehydroisoandrosterone).

Experimental

17-Methylene-5-androsten-3β-ol (I).- Triphenylmethylphosphonium bromide (25.0 g) and potassium t-butoxide : t-butanol (1:1 complex, 13.04 g) were dissolved in dry dimethylsulphoxide (80 ml) under nitrogen with gentle warming. A solution of 3β-hydroxy-5-androsten-17-one (4.04 g) in dimethylsulphoxide (80 ml) was added. The reaction mixture was maintained at 80°C for 1hr, diluted with ice and 10% sodium chloride solution and extracted with ether. The ether layers were combined and washed with water, dried over anhydrous magnesium sulphate and evaporated to dryness in vacuo to give crude product (5.09 g) heavily contaminated with triphenylphosphine oxide. Recrystallisation from methanol yielded pure I, (3.17 g); m.p. 134-135° (lit. 133-134°C [7]): infra red (CS_2) 895, 1655 (17-methylene), 3450 cm^{-1}(3-OH); n.m.r. ($CDCl_3$) τ9.20 (C-18 methyl), 8.98 (C-19 methyl), 5.36 (J = 4 cps) (C-17 methylene), 4.64 (J = 6 cps) (6-H).

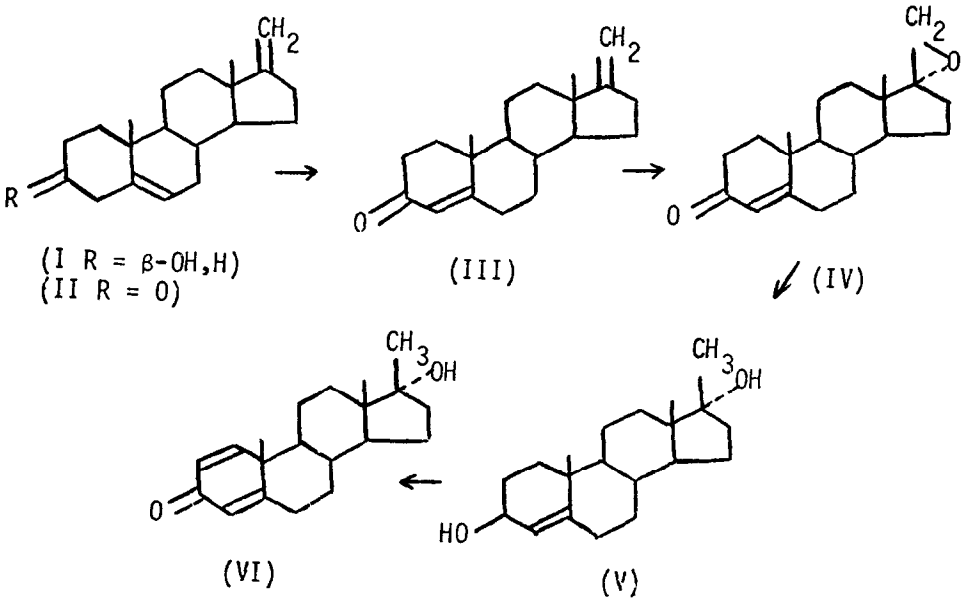

Figure 1

17-Methylene-5-androsten-3-one (II)- The alcohol (I, 3.07 g) was dissolved in ice-cold acetone (215 ml), maintained at 0°, and Jones Reagent (6.14 ml) was added with stirring. After 45 seconds the reaction was quenched with methanol (120 ml) water was added, the organic solvents were removed on a rotary evaporator and the steroid was extracted twice into ether. The combined ether extracts were washed with saturated sodium bicarbonate solution and then with water before being dried over anhydrous magnesium sulphate. Evaporation to dryness yielded a crude product (2.83 g) which after crystallisation from ethanol gave a mixture of II and III, (0.898 g); m.p. 109-117°C; infra red (CS_2) 895, 1655 (17-methylene), 1675 (carbonyl, III), 1715 cm^{-1} (carbonyl, II).

17-Methylene-4-androsten-3-one (III).- The mixture of enones (II and III, 0.85 g) from the previous stage was dissolved in ethanol (50 ml) and anhydrous oxalic acid (0.2 g) was added. The solution was held at 60°C for 30 mins, water was added and the

ethanol was removed. The steroid was extracted into ether, washed with saturated sodium bicarbonate solution, and water, and finally dried over anhydrous magnesium sulphate. Crystallisation from acetone afforded white needles of III, (0.76 g); m.p. 129-131° (lit. 129-131° [7]); infra red (CS_5) 895, 1655 (17-methylene), 1675 cm^{-1} (carbonyl); n.m.r. ($CDCl_3$) τ 9.16 (C-18 methyl), 8.80 (C-19 methyl), 5.36 (J = 4 cps) (C-17 methylene), 4.27 (J = 6 cps)-(4-H).

17,20-Oxido-21-nor-4-pregnen-3-one (IV) - The enone (III, 0.5 g) was dissolved in methylene chloride (20 ml) and m-chloroperoxybenzoic acid (0.35 g) was added. The reaction was allowed to proceed for 2 hrs at room temperature. Excess peracid was destroyed with 10% sodium sulphite solution until starch-iodide paper remained white when moistened with a drop of the reaction mixture. The solution was neutralised with saturated sodium bicarbonate solution, washed with water and dried over anhydrous magnesium sulphate. Evaporation to dryness and recrystallisation from acetone gave the oxide (0.45 g); m.p. 180-184° (lit. m.p. 179-182°C [7]); infra red (CS_2) 1675 cm^{-1} (carbonyl); n.m.r. ($CDCl_3$) τ 4.28 (4-H), 7.30 (epoxide), 8.82 (C-19 methyl) and 9.14 (C-18 methyl).

17-Methyl-4-androsten-3β,17α-diol (V) - The oxide (IV, 0.45 g) was dissolved in sodium-dried ether and was slowly added to lithium aluminium hydride (0.2 g) in ether (20 ml). The mixture was refluxed for 30 min and excess reagent was destroyed with water. The steroid was extracted into ether, washed with water and dried over anhydrous magnesium sulphate. Evaporation to dryness and recrystallisation from acetone gave the diol (0.36 g); m.p. 225-228°; infra red (CS_2) 3410 cm^{-1} (hydroxyl); n.m.r. ($CDCl_3$) τ 4.72 (4-H), 5.84 (3α-H), 8.82 (C-20 methyl), 8.94 (C-19 methyl), 9,31 (C-18 methyl); $[\alpha]_D$ + 8° (C = 0.18). Analysis by accurate mass measurement, calc. for $C_{20}H_{32}O_2$ 304.240217, found 304.239655.

17α-Hydroxy-17-methyl-1,4-androstadien-3-one (VI) - The diol (V, 0.35 g) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (0.57 g) in dry dioxan (20 ml) were refluxed for 6 hr. The solvent was then removed by evaporation, the residue was taken up in benzene and was filtered through a layer of alumina to remove insoluble quinol. The crude product was chromatographed on an alumina column (10 g. Spence type, activity II). Elution with benzene removed three compounds with no carbonyl function, but elution with benzene-chloroform (1:1) removed two polar compounds. Examination by n.m.r. and mass spectrometry identified the less polar compound as 17α-hydroxy-17-methyl-4-androsten-3-one (17β-methylepitestosterone) (0.04 g); m.p. 180-182° (lit. 182° [8]); infra red (CS_2), 1675 (carbonyl), 3410 cm^{-1} (hydroxyl); n.m.r. ($CDCl_3$) τ 4.28 (4-H), 8.81 (C-19 methyl, 8.81 (C-20 methyl), 9.28 (C-18 methyl) and the more polar compound as 17α-hydroxy-17-methyl-1,4-androstadien-3-one (17-epimethandrostenolone)(0.14 g); m.p. 221°; infra red (CS_2), 910, 1636 (alkene), 1665 (carbonyl), 3410 cm^{-1} (hydroxyl): n.m.r. ($CDCl_3$) 2.94 (J = 10 cps), (1-H), 3.73 (J = 2 cps)

and 3.83 (J = 2 cps), (2-H), 3.92 (J = 2 cps), (4-H), 8.77 (C-19 methyl), 8.82 (C-20 methyl), 9.26 (C-18 methyl); $[\alpha]_D$ + 3° (C = 0.50). Analysis by accurate mass measurement, calc. for $C_{20}H_{28}O_2$ 300.208919, found 300.210430.

Instruments used were: melting points (corrected) - Kofler block; infra red spectra - Unicam S.P. 200 spectrophotometer; n.m.r. spectra Varian HA 100; mass spectra - AEI, MS902; gas liquid chromatography - Perkin-Elmer model 801; optical rotations - measured at 20° in methanol using a Perkin Elmer model 141 automatic polarimeter.

IDENTIFICATION

The less polar metabolite, M, from several studies was pooled until 100 µg was available. However, even after preparative gas liquid chromatography it could not be obtained entirely pure. It therefore became necessary to investigate the efficiency of the various methods of detection available with regard to establishing conclusively the structure of M.

In view of the similarity between their structures, the spectral, chromatographic and other physical behaviour of methandrostenolone and its 17-epimer was examined. Pure methandrostenolone and 17-epimethandrostenolone prepared as previously described were used for this purpose.

The R_f values of the epimers on silica gel thin layer chromatography were identical in all the solvent systems investigated. Both compounds produced an orange-brown spot after development with 5% sulphuric acid in ethanol spray and heating and a blue spot with 8% w/v phosphomolybdic acid in methanol spray. The infra red spectra of the compounds in carbon disulphide solution were very similar except for slight differences in the fingerprint region, the adsorption maximum for the 1,4-dienone carbonyl being at 1665 $cm.^{-1}$

Rongone and Segaloff (1) quoted the melting point of their unidentified metabolite as 222-224°. The melting point of authentic 17-epimethandrostenolone is 221° and that of methandrostenolone is 163°C. Similar differences in the melting points of pairs of 17-epimers are shown by testosterone, m.p. 155° and 17-epitestosterone, m.p. 221° and by methyltestosterone m.p. 164° and 17β-methylepitestosterone, m.p. 182° (8).

The optical rotations of the following pairs of 17-epimeric compounds have been recorded:- methyltestosterone, +76° and 17β-methylepitestosterone, +67° (8); 17-methyl-4-androsten-3β,17β-diol, +14°, and 17-methyl-4-androsten-3β, 17α-diol, +8°; methandrostenolone, +12° and 17-epimethandrostenolone, +3°. The differences between the rotations of each pair of epimers are of the same sign and order of magnitude. Thus compounds V and VI and their respective epimers behave as

The analytical techniques which proved of greatest use in distinguishing the 17-epimers in this investigation were n.m.r., g.l.c. and mass spectrometry.

Nuclear Magnetic Resonance Spectroscopy

100 MHz spectra were run in deuteriochloroform solutions using a Varian HA 100 instrument. The spectra of the epimers are identical but for the position of the C-18 methyl resonance which is at 9.07 τ in the spectrum of methandrostenolone and at 9.26 τ in that of its 17-epimer. This difference in chemical shifts is due to the effect of the orientation of the 17-hydroxyl group on the 18-methyl group. Similar observations have been reported by Ananchenko *et al.* (10).

Gas Liquid Chromatography

G.l.c. was used initially to show that M was a metabolite of methandrostenolone rather than unchanged drug (3). However, this separation was difficult; in later experiments methandrostenolone and authentic 17-epimethandrostenolone were chromatographed on QF-1 and SE-30 columns, separately and as mixtures. Fractionally different retention times were obtained when the compounds were chromatographed separately, methandrostenolone having the longer retention time. Mixtures of equal amounts of the epimers gave fractional separation of the tops of the peaks, whereas mixtures of unequal amounts gave rise only to a single peak. No peak separation could be achieved using OV-1 columns. These steroids were, therefore, only separable on columns of very high resolving power, as in the initial experiments.

The trimethylsilyl ether (TMSE) derivatives of both epimers were prepared according to the method of Makita and Wells (11) and chromatographed on OV-1 columns at 235°C. The retention time of methandrostenolone TMSE derivative relative to methandrostenolone was 1.08 and of 17-epimethandrostenolone TMSE derivative relative to its parent compound was 0.78. The metabolite M was also converted to its TMSE derivative and was similarly chromatographed. The corresponding retention time ratio was 0.78, indicating M to be 17-epimethandrostenolone.

Mass Spectrometry

Mass spectrometry was carried out using an AEI MS 902 instrument operated at 70 e.V. Spectra of microgram samples of metabolite M were obtained by dissolving the entire sample in dry ether (10-20 µl). A syringe was used to transfer a portion (2-3 µl) of this solution to a quartz probe and the solvent was gently evaporated with a hair dryer. This process was repeated several times until sufficient material (ca. 10 µg) had been transferred to the probe, which was then inserted directly into the spectrometer. Spectra obtained in this way

showed that several compounds were generally present in the metabolite samples even after repeated preparative thin layer chromatography. It was established that the mass spectra of synthetic methandrostenolone and its 17-epimer were unaffected by gas chromatography of the compounds prior to spectrometry. Consequently, metabolite samples were purified by preparative gas chromatography, collected in glass tubes and transferred to the spectrometer probe as already described.

Prior to mass spectrometry of M is was necessary to compare the spectra of pure methandrostenolone Fig. 2(a) and authentic 17-epimethandrostenolone Fig. 2(c). The base peak of both compounds is m/e = 122 (12). The presence of this ion establishes the 1,4-dienone structure of the A-ring, since it must arise by B-ring fragmentation with the transfer of two protons to the charged species, Fig. 3 (13).

Under the conditions encountered within the mass spectrometer 17-epimethandrostenolone readily dehydrates to give a peak at m/e = 282 (M^+-H_2O) which is large compared with the molecular ion, M^+ at m/e = 300. It also gives a peak at m/e = 267 ($M^+-H_2O-CH_3$). Both of these peaks are present in the spectrum of methandrostenolone but are unimportant compared with M^+ and the ion at m/e = 242 which arises from fragmentation across the D-ring. It can be seen from these results that, although both epimers give rise to peaks at the same m/e values the intensities can be used to determine which epimer is present (6). Ananchenko et al. (10) obtained similar results in their study of the mass spectra of 17-epimeric oestranes and 19-nortestosterones and the corresponding D-homo-17a-epimeric compounds.

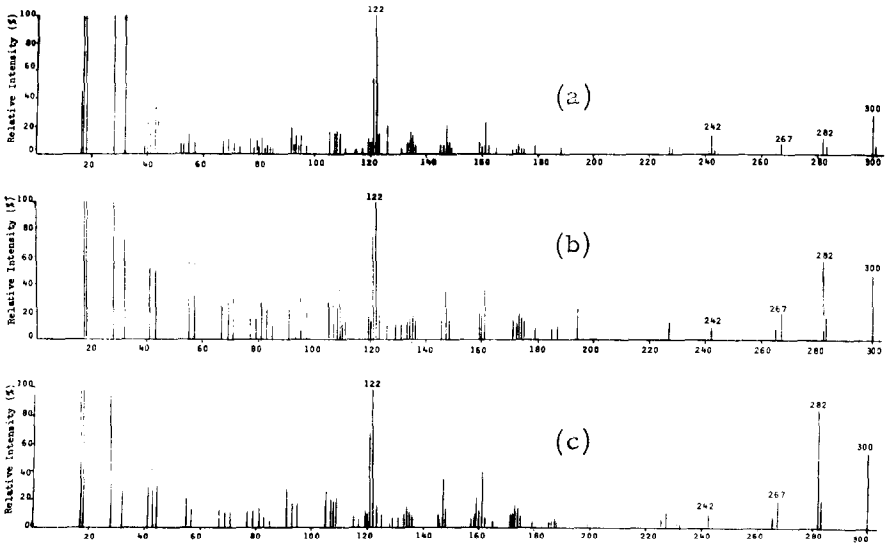

Figure 2

The mass spectrum of the metabolite M after purification by preparative gas chromatography is shown in Fig. 2(b). This spectrum adds further support to the earlier conclusions that M is not merely unchanged drug and verifies the assumption that it is 17-epimethandrostenolone.

For further identification of M the TMSE derivatives of the authentic epimers and M were subjected to mass spectrometry.

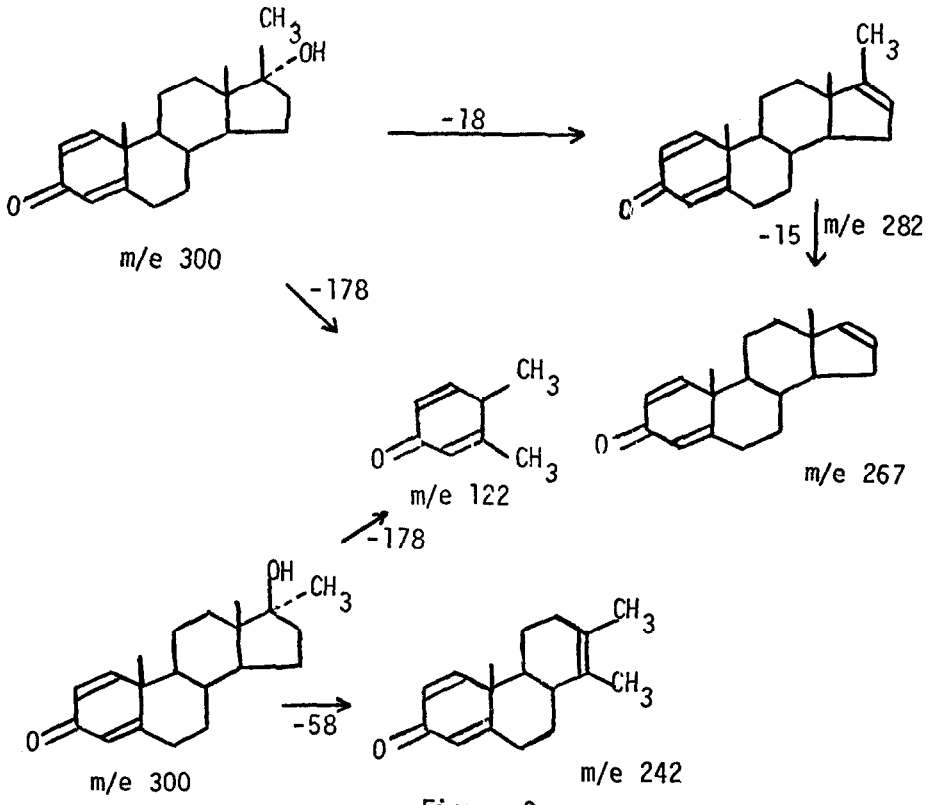

Figure 3

All three gave similar spectra with the molecular ion, M^+ at m/e = 372, indicating the successful formation of the derivative. The base peak is at m/e = 143, the origin of which is shown in fig. 4 (12). The only other ion of importance is that at m/e = 282.

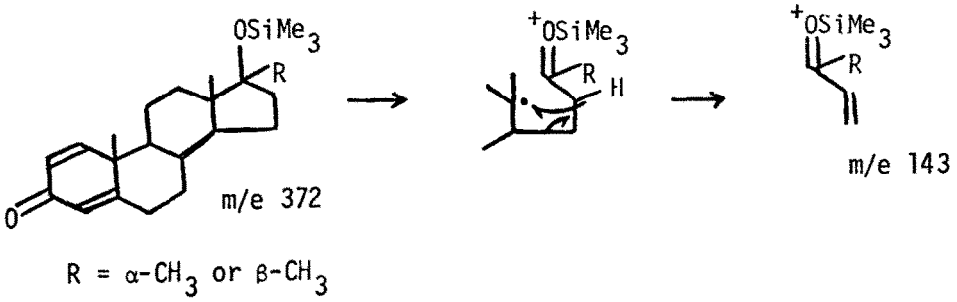

Figure 4

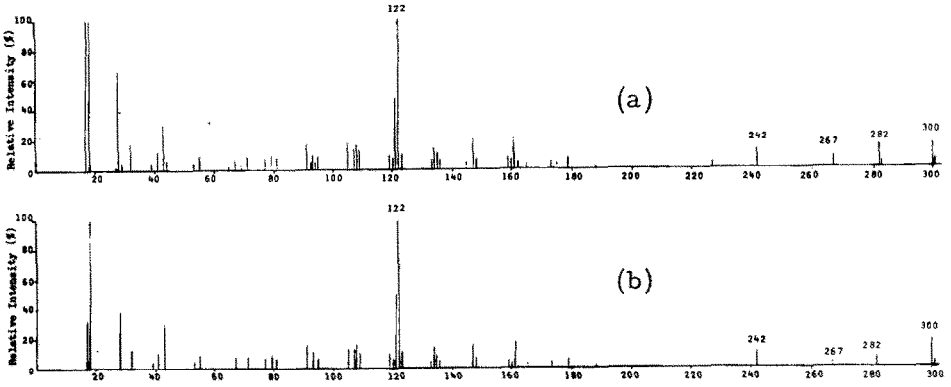

Figure 5

Examination of Commercial Methandrostenolone

Because less than 5% of administered methandrostenolone can be recovered as 17-epimethandrostenolone it seemed advisable to check that tablets of commercial methandrostenolone do not contain a small quantity of the epimer. Accordingly, ten 5 mg tablets of methandrostenolone (Dianabol, Ciba) were ground to a fine powder and extracted with benzene. The insoluble carrier material was removed by filtration and the solvent was removed on a rotary evaporator. Crystals, which had a melting point identical with that of pure methandrostenolone were obtained. Gas liquid chromatography of the TMSE derivative of the drug obtained from tablets showed no 17-epimer.

Fig.5(a) shows the mass spectrum of a synthetic mixture containing 95% pure methandrostenolone and 5% 17-epimethan-

drostenolone. The spectrum of methandrostenolone extracted from tablets is shown in fig.5(b). It is identical with the spectrum of pure methandrostenolone, fig.2(a), and differs from fig.5(a). No epimer was detectable after extracting methandrostenolone from water and subsequent thin layer chromatography during which the samples were exposed to light. The compound has already been shown to be unaltered after gas chromatography. Since the 17-epimer was not present in the starting material, nor was it produced by the isolation procedures, the 17-epimethandrostenolone isolated from urine after the administration of methandrostenolone must be of biochemical origin.

DISCUSSION

Proof that a major metabolite of methandrostenolone in normal man is 17-epimethandrostenolone raises the question as to how it arises. The epimerisation of secondary hydroxyl groups is well known chemically (14) whereas epimerisation of the tertiary 17-hydroxyl group is unknown (15). The epimerisation of secondary steroid alcohols has previously been reported in biochemical systems especially when there is an adjacent oxygen function which can assume an oxo-form (16) but such reactions generally constitute minor metabolic pathways. Biochemical epimerisation of tertiary alcohols, to our knowledge, has not been previously described.

The metabolism of methandrostenolone proceeds quite differently from that of 17α-methyltestosterone (17) where the 4(5)-double bond and the 3-oxo group are both reduced. In addition, there is no evidence of epimerisation at C-17. These findings suggest that the A-ring of methandrostenolone is more stable to the usual enzymatic conversions but may somehow facilitate the epimerisation at C-17. Similar facts

to these have also been observed by Schubert *et al.* (18) in their work on the metabolism of the 4-chloro analogue of methandrostenolone (Oral-Turinabol). These workers have isolated from the freely extractable fraction an unidentified metabolite present in relatively large amounts which may well be the 17-epimer of Oral-Turinabol.

The isolation of 17-epimethandrostenolone from the freely extractable fraction rather than the conjugated 'glucuronide' fraction of urine is unusual; little or in some cases no metabolites were obtained from urine after β-glucuronidase hydrolysis. In addition to the inhibition of glucuronyl transfer to the 17β-hydroxyl group by the 17α-methyl group the structure of the A-ring probably further reduces conjugation of the 17-hydroxyl group; this is consistent with findings of Hsia *et al.* (19) who found that 17β-hydroxy-17-methyl-1,4-androstadien-3-one was four times more effective than 17β-hydroxy-17-methyl-4-androsten-3-one in inhibiting conjugation of o-aminophenol *in vitro*.

The mechanism of formation of the 17-epimer is at present being studied.

ACKNOWLEDGEMENTS

We are grateful to Professor L.G. Whitby and Professor J.I.G. Cadogan for their encouragement, to Mr. A.M. Torrance and Mr. D. Thomas for technical assistance, to Dr. A. Segaloff for a supply of 6β-hydroxymethandrostenolone and to Ciba for the pure methandrostenolone used in this work.

REFERENCES

1. Rongone, E.L., and Segaloff, A., STEROIDS, 1, 179, (1963).

2. Adhikary, P.M., and Harkness, R.A., BIOCHEM.J., 112, 30P (1969).

3. Adhikary, P.M., Ph.D. Thesis, University of Edinburgh, 1969.

4. Adhikary, P.M., and Harkness, R.A., ANALYT.CHEM., 41, 470 (1969).

5. Adhikary, P.M., and Harkness, R.A., J.CHROMATOG., 42, 29 (1969).

6. Macdonald, B.S., Sykes, P.J., Adhikary, P.M., and Harkness, R.A., BIOCHEM.J., 122, 26P (1971).

7. Sondheimer, F., Mancera, O., Urquiza, M., Rosenkranz, G., J.AM.CHEM.SOC., 77, 4145 (1955).

8. Fieser, L.F., and Fieser, M., STEROIDS, Reinhold Publishing Corp., 1959, p. 519.

9. Klyne, W., DETERMINATION OF ORGANIC STRUCTURES BY PHYSICAL METHODS (Eds. Braude, E.A., Nachod, F.C.), 1955, p. 78.

10. Ananchenko, S.N., Leonov, V.N., Zaretskii, V.I., Wulfson, N.S., and Torgov, I.V., TETRAHEDRON, 20, 1279 (1964).

11. Makita, M., and Wells, W.W., ANALYT.BIOCHEM., 5, 523 (1963).

12. Brooks, C.J.W., Thawley, A.R., Rocher, P., Middleditch,B.S., Anthony, G.M., and Stillwell, Wanda G., ADVANCES IN CHROMATOGRAPHY, 1970, Editor A Zlatkis, Chromatography Symposium, University of Houston, Houston 1970, p. 262.

13. Peterson, L., ANALYT.CHEM., 34, 1781 (1962).

14. Chemistry of Carbon Compounds, IID, STEROIDS (Ed. Coffey), 1970, pages 39 and 48.

15. Chemistry of Carbon Compounds, IID, STEROIDS (Ed. Coffey), 1970, pages 271 to 276.

16. Raud, H.R. and Hobkirk, R., BIOCHEM.J., 103, 724, (1967).

17. Rongone, E.L., and Segaloff, A., J.BIOL.CHEM., 237, 1066, (1962).

18. Schubert, K., and Wehrberger, K., ENDOKRINOLOGIE, 55, 257, (1970).

19. Hsia, D.Y.Y., Riabov, S., and Dowden, R.M., ARCH.BIOCHEM. BIOPHYS., 103, 181 (1963).