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METHANDROSTENOLONE METABOLISM IN HUMANS: POTENTIAL PROBLEMS ASSOCIATED WITH ISOLATION AND IDENTIFICATION OF METABOLITES

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Summary—Methandrostenolone dose (amount and duration) and methods of isolation from urine can influence the identification and quantitation of methandrostenolone metabolites. Long-term use of methandrostenolone at high dosages led to the appearance of unmetabolized drug in the urine and contributed to the identification of a previously unreported metabolite, 3β,6§,17β-trihydroxy-17α-methyl-5§-1-androstene. Exposure of methandrostenolone *in vitro* to acid conditions induced a retropinacol rearrangement in the D-ring of the methandrostenolone molecule, causing the formation of 18-nor-17,17-dimethyl-1,4,13(14)-androstatrien-3-one in large amounts. The same acidic conditions led to the addition of a hydroxyl at the 6 position of the B-ring of either the retropinacol rearrangement products or native methandrostenolone resulting in the formation of 6β-hydroxy-18-nor-17,17-dimethyl-1,4,13(14)-androstatrien-3-one,6α-hydroxy-18-nor-17,17-dimethyl-1,4,13(14)-androstatrien, 6β-17α-methyl-1,4-androstadien-3-one and 6α,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one. Hydroxylation of native methandrostenolone at the 6 position also occurs endogenously. However, no evidence of an endogenous retropinacol rearrangement was found. Silylating agents alone can induce the formation of small amounts of 6β,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one. Discrepancies between previously published reports on methandrostenolone metabolism in man are discussed and compared with an animal model.

INTRODUCTION

Anabolic steroids, in spite of the concern that they may cause adverse effects in man, have apparently been gaining in popularity in those groups where size (football, bodybuilders) or strength (powerlifters) are important considerations [1]. In the course of our earlier studies on the physiological effects of anabolic agents, we investigated the metabolism of several anabolic steroids in bodybuilders who were using large doses (>25 mg/day) for long periods of time (>3 weeks) [2, 3]. In the Colorado region, Dianabol (methandrostenolone; 17α-methyl-17β-hydroxy-1,4-androstadiene-3-one) is one of the more popular agents. Initial reports [4, 5] had suggested that we should find six metabolites of methandrostenolone. In our studies, we found two of the previously reported major metabolites and found one previously unreported minor metabolite [2]. In an attempt to resolve the differences between these two reports we extended our investigations concerning the metabolism of methandrostenolone. We now report additional metabolites of methandrostenolone which are not in complete agreement with the above mention studies [4, 5] nor with a recent report by Masse *et al.* [6]. The cause of some of these apparent metabolic differences may be, in part, a result of the way the drugs were administered or may be artifacts generated by the isolation of the steroids. The isolation procedures, in particular, may play a major role in affecting the anabolic steroid metabolites detected. For example, the extraction procedures used by Durbeck and Buker [5] exposed urinary steroids to relatively harsh acid and basic conditions. Our experiments indicate that acid conditions alone can induce structural rearrangements in methandrostenolone, which is in agreement with previous reports concerning other 17-methylated anabolic steroids [7, 8]. As many anabolic agents are extensively metabolized, the identification of the true metabolites and physiological factors affecting their metabolism is central to any program trying to either identify the unauthorized use of these agents in athletes or for the study of drug elimination. Therefore it is an important concern that the effects of the administration regimen of methandrostenolone and the analytical procedures be well characterized.

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EXPERIMENTAL

Urine samples (24 h collection) from body builders who were using at least 25 mg/day of methandrostenolone, for longer than three weeks, were stored at −20°C. All glassware used throughout the analytical procedure was acid washed and silanized.

Extraction and derivatization of urine samples was conducted as previously reported [9]. Briefly, a urine sample was allowed to completely thaw and aliquots of 1–5 ml removed. 1 μg of 5β-dihydro-epitestosterone (Steraloids) diluted in ethanol was added to each millilitre of sample urine as an internal standard for quantitation. The aliquots were then passed through a C_{18} Sep-Pak (Waters Assoc., Milford, Mass) and the steroid-rich fraction eluted with methanol [10]. Solvent was removed under a stream of nitrogen. To hydrolyze the steroid conjugates, the dried methanolic extracts were dissolved in 2 ml of 0.1 M acetate buffer, pH 4.6, followed by 100 μl of Helix pomatia digestive juice (Calbiochem-Behring, La Jolla, Calif.). The mixture was incubated for 14 h at 55°C. Neutral steroids were extracted successively with 4 ml methylene chloride and 4 ml ethyl acetate. The combined extracts were washed with 1 ml of saturated solution of sodium bicarbonate followed by 2 × 2 ml of distilled water and residual solvent removed *in vacuo* at 60°C. Methyloxime derivatives were prepared by the method of Thenot and Horning [11] as follows: 100 μl of 2% methoxamine HCl in pyridine were added to the dried extract and allowed to derivatize at 60°C overnight and the pyridine was removed under nitrogen. Trimethylsilyl ethers were prepared by reacting the extract with 100 μl Tri-Sil-TBT reagent [composed of *N*-(trimethylsilyl)-imidazole:(TMSI)-*N*,*O*-bis(trimethylsilyl) acetamide: (BSA)-trimethylchlorosilane (TMCS) (3:3:2, v/v)] (Pierce Chemical Co., Rockford, Ill.) for 2 h at 100°C. Excess reagent was removed under a stream of nitrogen. The derivitized extract equivalent to 5 ml of urine was diluted to 250 μl with the chromatography solvent (cyclohexane:pryridine: hexamethyl-disilazane; 98:1:1) and 1 μl injected.

To investigate the effect of acid treatment on methandrostenolone structure, a tablet of methandrostenolone (5 mg; Dianabol, Ciba, Summit, N.J.) was dissolved in distilled water and passed through a C_{18} Sep-Pak cartridge. The steroid was eluted with methanol, evaporated to dryness under nitrogen, reconstituted with ethyl acetate and washed with distilled water to remove the food coloring in the tablet. The purified steroid sample was then split into four equal size aliquots and placed into glass tubes and the ethyl acetate evaporated. One sample was derivatized with methyloxime and Tri-Sil-TBT as above. The second sample was silylated only with Tri-Sil-TBT. 1 M HCl (2 ml) was added to the third tube and refluxed for 3 h at 60°C. The acidified sample was extracted from the HCl with methylene chloride and ethyl acetate, then derivatized with Tri-Sil-TBT. The fourth steroid aliquot received acetate buffer, pH 4.6 and the Helix pomatia enzyme preparation and in all subsequent steps treated exactly as the urine samples detailed above.

Gas chromatography

The analysis of urinary steroid extracts was performed on a Hewlett-Packard 3710A gas chromatograph fitted with a modified Van den Berg falling needle injector [12]. This was coupled to a 30 m × 0.25 mm, film thickness 0.25 μm, DB1 fused silica open tubular column (J & W Scientific, Cordova, Calif.). The carrier gas was helium, velocity 20 cm/s and the make-up gas nitrogen with a flow-rate of 20 ml/min. Hydrogen and air flow-rates were 30 ml/min and 300 ml/min, respectively. The injector and flame ionization detector temperatures were 300°C.

GC analysis of urinary steroid extracts was performed with an initial column temperature of 200°C for 4 min, then increased to a final temperature of 300°C at a rate of 4°C/min. The recorder chart speed was 1.25 cm/min.

Gas chromatography–mass spectrometry

Analyses were performed on both a Varian 3400 (split/splitless injector) and a Pye Unicam 204 gas chromatograph fitted with a modified Van den Berg injector. The GC column and carrier gas conditions were the same as reported above (gas chromatograph). Make-up gas was not required since the column was directly inserted into the ion source. The injection block and transfer line temperatures were 300°C.

The gas chromatograph was interfaced to either a Finnigan Ion Trap or to a Vacuum Generator (VG) MM-16 low resolution magnetic sector instrument. The VG multiplier was 1.75 kV, with a gain of 2×10^{-6}. The electron energy was 70 eV, source temperature 200°C and an accelerating voltage of 4 kV. The scan rate was 1 s per decade. Results were output to a VG 2050 data system.

GC–MS analysis of urinary steroid extracts was performed using the same temperature conditions as stated above.

Patients

After obtaining informed consent and after an initial interview where patients were advised of potential dangers accompanying use of anabolic agents, we obtained a patient history. This includes the length of time that they had used anabolic agents, the drugs used, the doses and the dose schedule. For the results reported in this work, we only accepted patients that were taking a single agent during the study period (e.g. no "stacking" of multiple steroids).

RESULTS

A typical chromatogram of total urinary steroids following long-term use of methandrostenolone is

shown in Fig. 1A. Major endogeneous urinary steroids are identified by name. Peaks representing methandrostenolone metabolites are numbered. The ion fragmentogram of ion m/z 143 which is characteristic of cleavage of 17-methyl-17-*O*-TMS from the D-ring of anabolic steroids is also shown in Fig. 1B. The major ions of the methandrostenolone metabolites identified and their structures are shown in Table 1. The mass spectra of several of these metabolites are different from previous reports [6] due to the methoxime derivative used in the present study. The full mass spectra of a previously unreported methandrostenolone metabolite, 3β,6§,17β-trihydroxy-17α-methyl-5§-1-androstene (peak number 5 in Fig. 1) is shown in Fig. 2. Ion m/z 143 was a prominent ion in the mass spectrum of every methandrostenolone metabolite identified. Examination of the total ion chromatogram (TIC) did

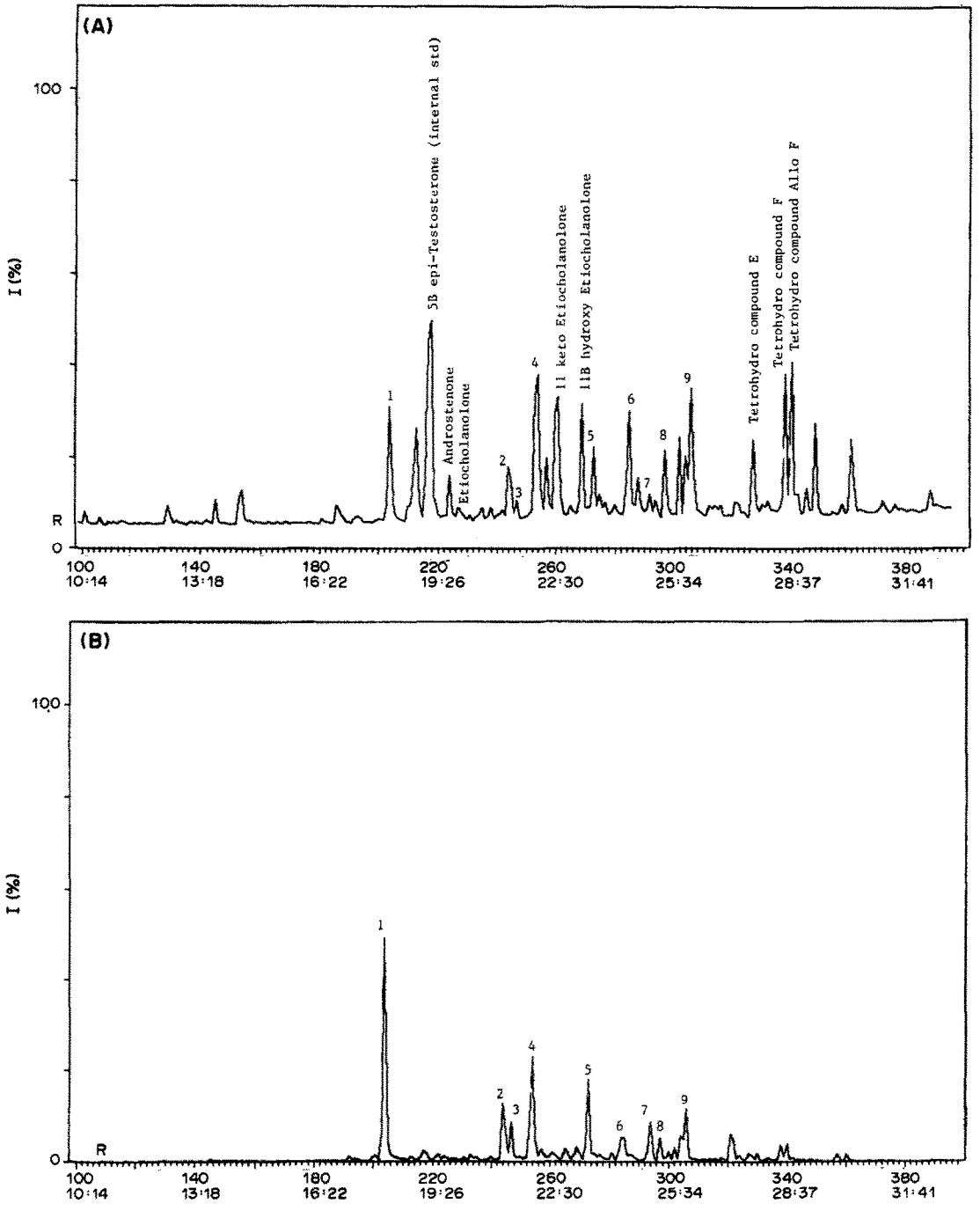
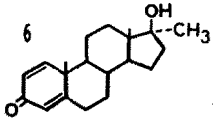
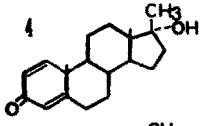
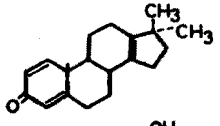
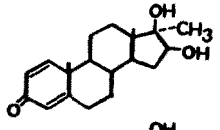
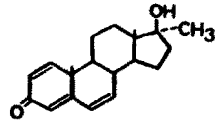
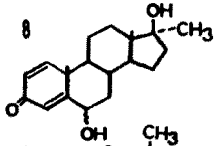
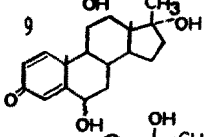
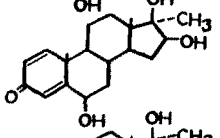
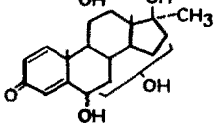
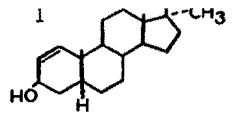
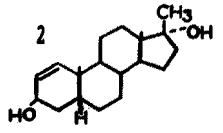
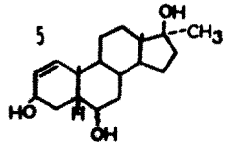
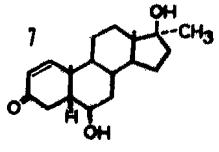
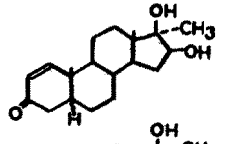
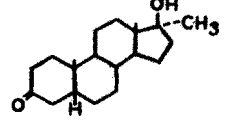
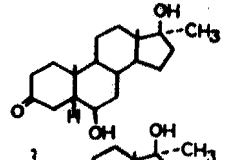
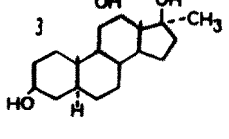


Fig. 1. Total ion chromatogram (A) of MOX/TMS derivatized urinary steroids from an individual using methandrostenolone. Endogenous urinary steroids are identified by name above each peak. Methandrostenolone metabolites are identified by numbers above each peak in the TIC and in the m/z 143 ion fragmentogram (B). The name, structure and major ions of each metabolite are listed in Table 1.

Table 1. Methandrostenolone metabolites identified in urine samples by various investigators

Steroid structure	Name	Major ions	Identification			
			Harission (1989)	Masse (1980)	Durbeck (1980)	Templeton (1984)
6 	17β-hydroxy-17α-methyl androsta-1,4-dien-3-one (methandrostenolone)	*143, 280, 311, 370, 386, 401 (M+)	Yes 3/4‡	No	No	No
4 	17α-hydroxy-17β-methyl-androsta-1,4-dien-3-one (epi-methandrostenolone)	*143, 280, 311, 370, 386, 401 (M+)	Yes 3/4‡	No	Yes	Yes
	18-nor-17,17 dimethyl-1,4,13(14)androstatrien 3-one	**91, 147, 122, 161, 267, 282 (M+)	No	Yes	Yes	No
	16β,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one	†265, 283, 298, 316 (M+)	No	No	No	Yes
	17α-methyl-17β-hydroxy-1,4,6-androstatrien-3-one	**143, 159, 211, 265, 280, 370 (M+)	No	No	Yes	No
8 	6§,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one	*143, 278, 368, 399, 458, 489 (M+)	Yes 3/4‡	Yes	Yes	Yes
9 	6§,17α-dihydroxy-17β-methyl-1,4-androstadien-3-one	*143, 278, 368, 399, 458, 489 (M+)	Yes 3/4‡	Yes	Yes	No
	6§,16β,17β-trihydroxy-17α-methyl-1,4-androstadien-3-one	**143, 209, 218, 231, 281, 548 (M+)	No	Yes	No	Yes
	Hydroxylated analogue of 6β-hydroxy methandrostenolone	**143, 209, 218, 231, 281, 548 (M+)	No	Yes	No	No

1 	5β,17β-dihydroxy-17α-methyl-5§-1-androstene	*143, 216, 268, 311, 358, 448 (M+)	Yes 4/4‡	Yes	No	No
2 	3β,17α-dihydroxy-17β-methyl-5§-1-androstene	*143, 216, 268, 311, 358, 448 (M+)	Yes 4/4‡	Yes	No	No
5 	3β,6§,17β-trihydroxy-17α-methyl-5§-1-androstene	*143, 197, 266, 356, 446, 536 (M+)	Yes 2/4‡	No	No	No
7 	6§,17β-dihydroxy,17α-methyl-5§-1-androsten-3-one	*143, 311, 361, 401, 460, 491 (M+)	Yes 2/4‡	Yes	No	No
	16β,17β-dihydroxy-17α-methyl-5§-androst-1-en-3-one	†265, 283, 298, 316 (M+)	No	No	No	Yes
	17β-hydroxy-17α-methyl-5β-androstan-3-one	**376 (M+)	No	Yes	No	No
	6§,17β-dihydroxy-17α-methyl-5§-androstan-3-one	**143, 464 (M+)	No	Yes	No	No
3 	3β,17β-dihydroxy-17α-methyl-5α-androstane	*143, 255, 345, 360, 435, 450 (M+)	Yes 3/4‡	Yes	No	Yes

The § symbol indicates that the orientation of the structure (α or β) is not known. The number left of the steroid structures designates the peak number from the chromatogram in Fig. 1 of the present study.
*Methoxime/TMS derivative.
**TMS derivative.
†Not derivatized; urine samples from rabbits administered methandrostenolone.
‡Urine samples from different individuals containing the metabolite/total number of samples.

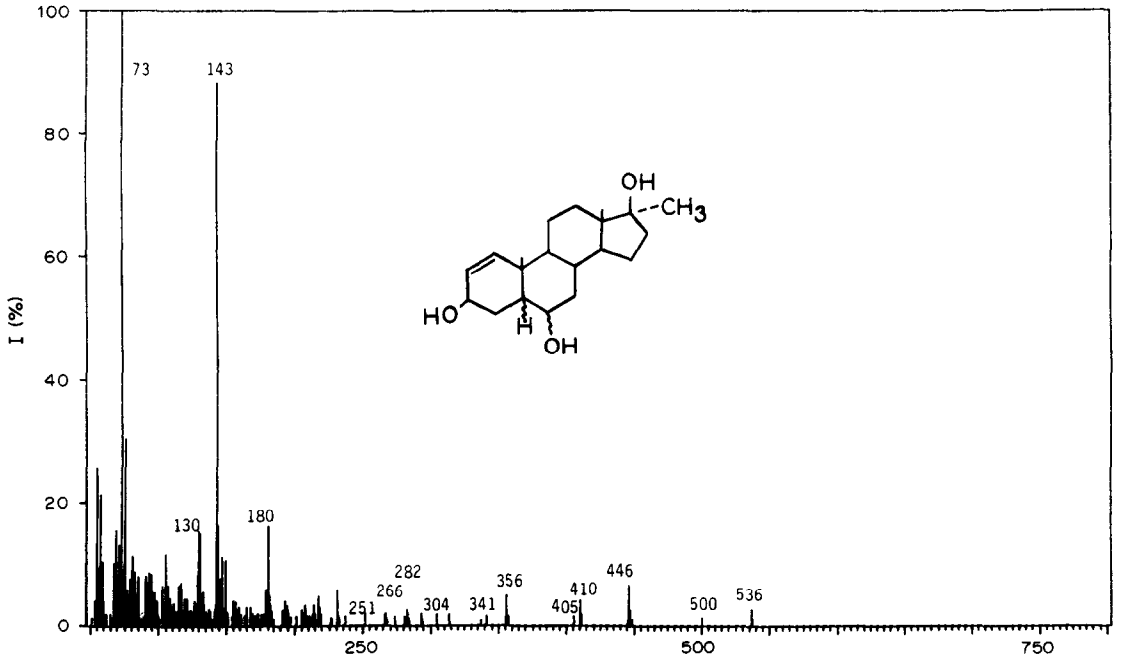


Fig. 2. Mass spectra of methandrostenolone metabolite 3β,6§,17β-trihydroxy-17α-methyl-5§-1-androstene (peak 5 in Fig. 1). The mass spectrum shown is of the tri-TMS derivative with the molecular weight 536.

not reveal additional mass spectra (with m/z 143) which could be interpreted as methandrostenolone metabolites.

Treatment with 1 M HCl prior to derivatization caused the formation of several structral changes, most notably a large amount (46% of total steroid added) of 18-nor-17,17-dimethyl-1,4,13(14)-androstatrien-3-one (Fig. 3C). There was no evidence of these compounds in the native steroid isolated from the tablet (Fig. 3A), the Tri-Sil-TBT derivatizing reagent alone induced the conversion of 2% of the methandrostenolone to 6§,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one and its 17β-methyl epimer (Fig. 3B). Converting the steroid keto functions to methoximes prior to silylation did not limit the formation of 6-hydroxy methandrostenolone by Tri-Sil-TBT. Likewise, the hydrolysis procedures used for urinary steroids did not further alter the structure of purified methandrostenolone.

DISCUSSION

The proper identification of metabolites from any drug is an important step in our understanding of the biochemical pathways used by the organism to modify and dispose of the drug and is a necessary step for developing monitoring procedures to follow pharmacokinetics or use. In the case of anabolic steroids, a large fraction of these compounds may undergo many biotransformation steps before being excreted into the urine. The metabolites measured for any given compound can vary depending on the dose and on the activation of certain enzymatic pathways (i.e. *P*-450 etc.).

Major studies which have attempted to elucidate the metabolites of methandrostenolone biotransformations, including the present study, are not in agreement [5, 6]. Table 1 summarizes the results from those studies which have investigated the metabolism.

In our studies on methandrostenolone, we found that the epimerization of the C-17 position was an important and major transformation. This finding is in agreement with two earlier studies [5, 13] and the reaction presumably is catalyzed by the same enzyme that rapidly converts other 17β-hydroxy steroids to their 17-keto analogues (i.e. testosterone→androstenedione). Masse *et al.* [6], discuss identifying epimethandrostenolone, but do not indicate its presence in the chromatogram and structures accompanying their paper. In contrast to previous studies in humans [5, 6] unmetabolized, native methandrostenolone was detected in the urine of our subjects. The duration and dosage of methandrostenolone used by the volunteers in this study may have provided more substrate than the body is capable of metabolizing, resulting in spillage of excess methandrostenolone into the urine. In a study of rabbits given multiple administrations of oral methandrostenolone, Templeton *et al.* [13], also found unmetabolized methandrostenolone excreted in the urine.

Several major metabolites indentified in Fig. 1, including 6§,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one (peaks 8 and 9), 3β,17β-dihydroxy-17α-methyl-5§-1-androsten (peaks 1 and 2), 6§,17β-dihydroxy-17α-methyl-5§-1-androsten-3-one (peak 7) and 3β,17β-dihydroxy-17α-methyl-5α-androstane

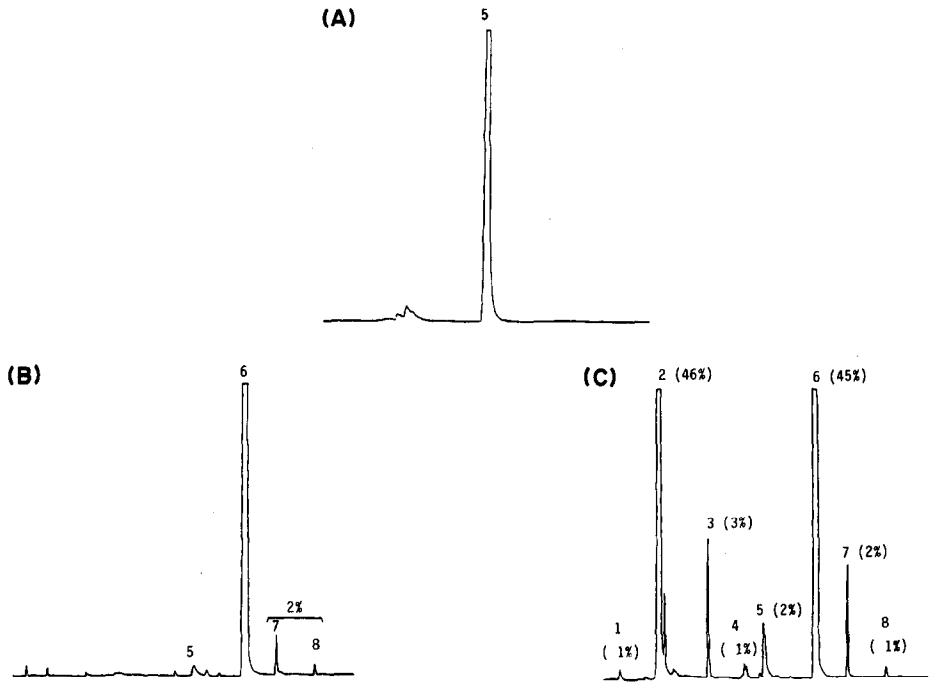


Fig. 3. Chromatograms of underivatized methandrostenolone (A), methandrostenolone derivatized with Tri-Sil-TBT (B), and acid-treated, Tri-Sil-TBT derivatized methandrostenolone (C). Major peaks are at saturation in order to get smaller peaks on scale. The number in brackets to the right of each peak (chromatogram C) is the percentage of the total drug added. Individual compounds and major ions are: (1) dehydrated methandrostenolone,91,121,133,171,267,282(M+); (2)18-nor-17,17-dimethyl-1,4,13(14)-androstatrien-3-one,91,147,122,161,267,282 (M+); (3) 6β-hydroxy-18-nor-17,17-dimethyl-1,4,13(14)-androstatrien-3-one,91,209,265,280,355,370 (M+); (4) 6α-hydroxy-18-nor-17,17-dimethyl-1,4,13(14)-androstatrien,91,209,265,280,355,370 (M+); (5) underivatized methandrostenolone,122,161,242,267, 282,300 (M+); (6) TMS derivatized methandrostenolone, 143,161,267,282,357,372 (M+); (7) 6β,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one, 143,281,355,370,445,460 (M+); (8) 6α,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one,143,281,355,370,445,460 (M+).

(peak 3) have been identified by both Masse *et al.* [6] and ourselves. These compounds all have A- or B-ring modifications of the steroid structure that represent known biotransformations in biological systems (i.e. change in saturation of the A-ring; hydroxylation at the 6 position).

On a qualitative basis the results from our laboratory and those from the study by Masse *et al.* [6] are somewhat similar. However on a *quantitative* basis they are very different. For example the largest metabolite peaks in this study (Fig. 1), 3β,17β-dihydroxy-17α-methyl-5§-1-androsten (peak 1) and epimethandrostenolone (peak 4) are not the major metabolites found by Masse *et al.* [6] who identified 17α-methyl-5β-androstan-3α,17β-diol (peak 3 in this study) and 6§,17β-dihydroxy-17α-methyl-1,4-androstadien-3-one (peak 8 in this study) as being quantitatively larger. These differences may be the result of the dose and duration of methandrostenolone use in each study or they could be caused by natural biochemical variations in human metabolism. Identification of these metabolites, which were not found by Durbeck and Buker [5], may be a consequence of the different extraction procedures used by these more recent reports or may be a function of the advances in GC–MS technology achieved in the past decade.

Our identification of a previously unknown methandrostenolone metabolite (3β,6§,17β-trihydroxy-17α-methyl-5§-1-androstene; see Fig. 2) in substantial quantity suggests the activation of enzyme system(s) that may be dependent on dose or duration of methandrostenolone use. Similarities in isolation, derivatization and GC–MS techniques between this research and Masse *et al.* [6] provide circumstantial evidence that the methandrostenolone treatment regimen prior to urine collection may have been the primary factor influencing the formation of this new metabolite rather than analytical differences.

The identification of the 18-nor-17,17-dimethyl-1,4-13(14)-androstantrien-3-one metabolite in other studies [5, 6] needs to be viewed cautiously. Previous reports by Segaloff and Gabbard [7] and Smith and Steele [8] have suggested that exposing 17β-hydroxy, 17α-methyl steroids in acidic, basic or dehydrating conditions will initiate a retropinacol rearrangement which results in the formation of a 13-en, 17,17-dimethyl compound. Our experiments with 1 M HCl acid provides specific confirmation that the retropinacol reaction occurs with methandrostenolone. 18-nor-17,17-dimethyl-1,4,13(14)-androstatrien-3-one is the major product of 1 M HCl-induced transformation of methandrostenolone (Fig. 3C). High acidity

appears to be effective in altering the A-B-rings of the steroid nucleus as well: epimeric pairs of 6-hydroxy methandrostenolone were formed upon treatment of the drug with HCl. This is presumably via a 1,4 Michael addition to the activated, conjugated ketone. Furthermore, the retropinacol rearrangement compound can also undergo the same 1,4 type addition and this leads to additional 6-hydroxy derivatives.

The acid conditions necessary to catalyze the retropinacol rearrangement could potentially dehydrate urinary 6-hydroxy-methandrostenolone and extend the original conjugation of the pi unsaturation in the A-ring, resulting in the formation of 17α-methyl-17β-hydroxy-1,4,6-triene compounds. This compound was found by Durbeck and Buker [5] but not by either ourselves or Masse *et al.* [6] where much milder conditions were used.

The lack of agreement between the various studies investigating methandrostenolone metabolism may be attributed to a number of factors including amount of anabolic steroid administered, duration of anabolic steroid use, route of administration (oral vs parenteral) and method for isolation and derivatization of the steroid metabolites. For example, in the present study 24 h urine samples were obtained from four individuals using $\geqslant$25 mg/day of methandrostenolone for greater than 3 weeks. In contrast, the investigations by Durbeck and Buker[5] and Masse *et al.*[6] used single doses of 10 and 50 mg methandrostenolone, respectively, with urine sample collection at 7 and 18 h, respectively, following administration. Moreover, there would appear to be considerable individual variation in how the drug is metabolized, as several of the metabolites identified were not found in every individual in the present study (see Table 1).

Even though the isolation and derivatization procedures used in the present study are relatively mild and are commonly used for steroid analysis, many commercially available derivatizing agents are acidic (pH $\leqslant$ 4) and could potentially effect steroid rearrangement. As we have shown in the present study, Tri-Sil-TBT alone is capable of inducing structural rearrangements in the methandrostenolone molecule. Therefore the identification of 6-hydroxy-methandrostenolone as a product of human steroid metabolism needs to be reexamined. While we have not attempted to identify all possible influences, it is not unreasonable to assume that varying combinations of time, type and amount of silylating agents would influence the magnitude to which 6-hydroxy-methandrostenolone is formed from methandrostenolone.

The lack of agreement between the various studies, expecially between the more recent reports, is of concern, as consistent identification of anabolic steroid metabolites is an important adjunct to any drug testing program. Failure to identify the conditions of drug administration or sample preparation that affect metabolite formation and detection may result in both false negatives and false positives. Thus, it would appear that further studies are needed to clearly define the conditions of methandrostenolone use and sample preparation which affect the kind and amount of metabolite excreted.

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