URINARY METABOLITES OF ANABOLIC STEROIDS: IDENTIFICATION AND SYNTHESIS

BY
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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Doctor of Philosophy

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I dedicate this to

my wife Ping,

my little daughter Cathay,

and my parents.
ABSTRACT

The metabolism of several anabolic 17α-methyl steroids, namely formebolone, mestanolone, methandienone, methyltestosterone, oxandrolone, oxymetholone and stanozolol, were investigated. The identification of metabolites was based on GC/MS analysis of different derivatives, and chemical synthesis of corresponding reference steroids. Common metabolic reactions have been studied and structure-metabolism relationships were discussed. New biotransformation routes, such as oxidative pathways of 2-formyl and 2-hydroxymethylene steroids, have been unveiled. The mechanism of 17-epimerization of these 17β-hydroxy-17α-methyl steroids in vivo has also been clarified.

A simple and convenient method for the preparation of 17β-tertiary sulfate derivatives of these steroids has been developed. A stereoselective approach was used for the partial synthesis of reference steroids in order to confirm the stereochemical features of an acidic metabolite of oxymetholone.
RÉSUMÉ

Le métabolisme de plusieurs stéroïdes anabolisants 17α-méthyles, notamment la formébolone, la mestanolone, la méthandiènone, la méthyltestostérone, l’oxandrolone, l’oxymétholone et le stanozolol a été étudié chez l’homme. L’identification des métabolites urinaires a été réalisée à l’aide de la technique de chromatographie en phase gazeuse-spectrométrie de masse (CG-SM), par l’analyse de différents dérivés chimiques de ces derniers ainsi que par la synthèse partielle des stéroïdes de référence correspondants. Les voies métaboliques ont été étudiées et certaines relations entre la structure des stéroïdes et leurs voies métaboliques sont discutées. De nouvelles routes de biotransformation telles les voies d’oxydation des stéroïdes portant un groupe 2-formyl et 2-hydroxyméthylène ont été découvertes. Le mécanisme de la réaction d’épimérisation in vivo des stéroïdes 17β-hydroxy-17α-méthyles a aussi été étudiée.

Une méthode de synthèse simple et efficace des dérivés 17β-sulfate de ces stéroïdes a été développée. Une approche stéréoselective fut utilisée pour la synthèse partielle de stéroïdes de référence afin de confirmer la stéréochimie de certains substituants d’un des métabolites acides de l’oxymétholone.
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GLOSSARY OF ABBREVIATIONS

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<tr>
<td>br</td>
<td>broad</td>
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<tr>
<td>BSA</td>
<td>(N,O)-bis-(trimethylsilyl)-acetamide</td>
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<td>CI</td>
<td>chemical ionization</td>
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<tr>
<td>mCPBA</td>
<td>3-chloroperbenzoic acid</td>
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<tr>
<td>c.v.</td>
<td>coefficient of variation</td>
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<td>d</td>
<td>doublet</td>
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<td>d-d</td>
<td>doublet of doublets</td>
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<tr>
<td>DAC</td>
<td>diethylaluminum cyanide</td>
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<tr>
<td>DHEA</td>
<td>dehydroepiandrosterone (3β-hydroxy-androst-5-en-17-one)</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
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<tr>
<td>EI</td>
<td>electron-impact ionization</td>
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<td>FAB</td>
<td>fast atom bombardment</td>
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<tr>
<td>GC</td>
<td>gas chromatography</td>
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<td>GC/MS</td>
<td>gas chromatography/mass spectrometry</td>
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<td>h</td>
<td>hours</td>
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<td>HP</td>
<td>Hewlett-Packard</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>HRGC</td>
<td>high resolution gas chromatography</td>
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<td>HRMS</td>
<td>high resolution mass spectrometry</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>i.d.</td>
<td>inner diameter</td>
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<td>IOC</td>
<td>the International Olympic Committee</td>
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<td>IR</td>
<td>infra red</td>
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<td>ISTD</td>
<td>internal standard</td>
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<td>LDA</td>
<td>lithium disopropylamide</td>
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<td>LRMS</td>
<td>low resolution mass spectrometry</td>
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<td>m</td>
<td>multiplet</td>
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<td>M⁺⁺</td>
<td>molecular ion</td>
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<td>min</td>
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<tr>
<td>MMC</td>
<td>magnesium methyl carbonate</td>
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<td>MNNG</td>
<td>1-methyl-3-nitro-1-nitrosoguanidine</td>
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<tr>
<td>MP</td>
<td>melting point</td>
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<tr>
<td>ms</td>
<td>millisecond</td>
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<td>MSD</td>
<td>mass selective detector</td>
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<tr>
<td>MSTFA</td>
<td>N-methyl-N-(trimethylsilyl)-trifluoroacetamide</td>
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<tr>
<td>17α-MT</td>
<td>17α-methyltestosterone (methyltestosterone)</td>
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<tr>
<td>17α-MTSPS</td>
<td>17α-methyltestosterone sulfate pyridinium salt</td>
</tr>
<tr>
<td>M.U.</td>
<td>methylene unit</td>
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<tr>
<td>19-NA</td>
<td>19-norandrosterone</td>
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<td>19-NE</td>
<td>19-noretiocholanolone</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<td>19-NT</td>
<td>19-nortestosterone</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>RT</td>
<td>retention time</td>
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<td>RRT</td>
<td>relative retention time</td>
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<td>s</td>
<td>singlet</td>
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<td>s.d</td>
<td>standard deviation</td>
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<td>SIM</td>
<td>selective ion monitoring</td>
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<td>t</td>
<td>triplet</td>
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<tr>
<td>TMS</td>
<td>trimethylsilyl</td>
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<td>TMCS</td>
<td>trimethylchlorosilane</td>
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<tr>
<td>TMSCI</td>
<td>trimethylsilyl chloride</td>
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<tr>
<td>TMSI</td>
<td>trimethylsilyl iodide</td>
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<tr>
<td>t-t</td>
<td>triplet of triplets</td>
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CHAPTER 1

INTRODUCTION
Introduction

The first use of androgenic-anabolic steroids (anabolic steroid) to enhance athletic performance was reported in the 1950’s [1]. Since then, especially in the last decade, the misuse and abuse of anabolic steroids in most sporting disciplines have soared to epidemic proportions [2-6]. In order to maintain equality and fairplay in sports and protect athletes from severe adverse effects caused by these drugs, the International Olympic Committee (IOC) and other sport organizations have prohibited the use of anabolic steroids in athletics [7]. The battle against their misuse has fostered the fundamental researches on the chemistry and biochemistry of anabolic steroids, because the detection and confirmation of unlawful use of anabolic steroids (usually by analyzing athletes’ urine samples) require a thorough understanding of their metabolic fate and the development of efficient analytical methods.

Historical development of anabolic 17α-methyl steroids

Testosterone, the endogenous androgen, was first isolated in crystalline form from steer testis tissue by Lacqueur and his associates in 1935 [8]. It was then synthesized from cholesterol and identified by Butenandt and Hanisch [9-10], and confirmed by Ruzicka et al. shortly afterwards [11-13]. Almost at the same time Kochakian reported for the first time that androgens extracted from male urine could induce an anabolic effect (muscle building or myotrophic effect) on castrated dogs [14]. This was also observed
by Papanicolaou [15]. This anabolic effect was confirmed afterwards with testosterone propionate in human [16-17]. After its synthesis, testosterone was soon introduced into clinical use, mainly for the treatment of hypogonadism (testicular deficiency) and certain types of anemia (due to its stimulation of erythropoiesis) [18]. But it was then found that testosterone can not be administered orally because of its prompt degradation by the liver (first pass effect)[18]. The first orally active anabolic steroid, 17α-methyltestosterone (methyltestosterone) was synthesized by Ruzicka et al. [19]. The original thought was to introduce a functional group at the 17α position of testosterone to block the bio-oxidation which transforms testosterone to the much less active androstenedione. The synthesis started from dehydroepiandrosterone (DHEA) (Fig. 1-1.), which was reacted with ten-fold excess of methyl Grignard reagent (because of the steric hindrance, the Grignard reagent could only attack from the α-face [20]). The resulting diol was then oxidized at C-3 with concomitant isomerization of the 5-ene group to give 17α-methyltestosterone.

Fig. 1-1. First synthesis of 17α-methyltestosterone by Ruzicka et al.
This steroid was orally active [21] and had a somewhat stronger anabolic effect than testosterone [22]. The therapeutic success of 17α-methyltestosterone has fostered the development of other orally active 17α-methyl steroids, which account for about 50% of the commonly used anabolic steroids [23].

In 1944, Kochakian reported a partial dichotomy in the anabolic effect and the androgenic activity of 5α-androstane-3α,17β-diol, which increases the weight of the kidney of mice and rats as did testosterone, but had a much weaker effect on accessory sex organs. On the other hand androstenedione was found to be preferentially androgenic [24]. Such results indicated that specific structural modifications of testosterone might lead to the dissociation of anabolic and androgenic effects. This fostered the hope of finding anabolic steroids with little or no androgenic activity, so that clinical patients, especially women and children, would not be affected by androgenic side effects. Although significant strides were made in the next two to three decades toward separation of anabolic and androgenic effects, no specific anabolic steroid has yet been developed [25-27], because anabolic and androgenic effects are not due to different actions of the hormone but result from interaction of these molecules with the same steroid receptors in different tissues [2].

Stimulated by the recognition of the anabolic effects of testosterone and increased oral activity of 17α-methyltestosterone, years of synthesis and screening resulted in development of a series of anabolic 17α-methyl steroids which have been introduced into
Fig. 1-2. Chemical structures of testosterone and several anabolic 17α-methyl steroids
clinical use. Much of this synthetic work was accomplished in the 1950’s. Fig 1-2 lists the structures of several anabolic 17α-methyl steroids, which are the subjects of my research program. Among them, mestanolone, which is the 17α-methyl analogue of 5α-dihydrotestosterone (an important intermediate metabolite of testosterone), was synthesized in 1935 [19]. Methandienone (danabol) with an 1,4-diene group in its A ring was first synthesized in 1955 by microbiological dehydrogenation of 17α-methyltestosterone [28]. It was until the late 1970’s the most prevalent anabolic steroid in the athletic population [1,29]. Unlike other synthetic anabolic steroids, oxymetholone [30] bears a 2-hydroxymethylene function which accounts for its unique biological activity and metabolic fate. Formebolone, an analog of oxymetholone was later synthesized through a modified method [31]. Treatment of oxymetholone with hydrazine introduced another important steroid, stanozolol [32], for which Canadian sprinter Ben Johnson was tested positive at the 1988 Seoul Olympic games [4]. The 2 oxa-dihydro analog of 17α-methyltestosterone, oxandrolone, was synthesized in 1962 [33]. It has a very high anabolic/androgenic index value of 13 with respect to that of testosterone (strong anabolic effect and minor androgenic activity) [34,35].

Due to their oral efficacy, anabolic 17α-methyl steroids have been applied to the treatment of a variety of clinical conditions. Their main therapeutic uses include [18]: (1) replacement androgen therapy for hypogonadal adult men, (2) treatment of catabolic states after major surgery and debilitating states of elderly, (3) stimulation of erythropoiesis in cases of certain types of anemia and (4) treatment of breast cancer. But a series of side effects are also associated with these 17α-methyl steroids, especially
when they are given to the athletes at large doses for quite a long period of time. The primary side effect related to these steroids is liver toxicity. Jaundice and cholestatic hepatitis are the commonly encountered symptoms [18,25,36]. Further development of liver disorder can lead to peliosis hepatitis (blood filled cysts in the liver) or hepatoma [2,25,37]. Liver tumors and cancer are two fatal malignancies related to prolonged use of these steroids [25,37,38]. Other side effects such as: (1) virilization in women and feminization in men, (2) increased cholesterol level and high blood pressure and (3) risk of prostatic cancer are often encountered among users of anabolic 17α-methyl steroids [2,25,39].

Metabolic studies of anabolic 17α-methyl steroids and the development of analytical methods

The metabolism of testosterone has been elucidated in a very detailed manner [40,41]. Fig. 1-3 presents the main metabolic pathways of testosterone. The oxidation of the 17β-hydroxyl to a 17-keto function by 17β-hydroxy steroid dehydrogenase gives androstenedione; then the 4-ene-5α or -5β reductase transform the latter to androstane dione and 5β-androstanedione; the major urinary metabolites androsterone and etiocholanolone are produced through reduction of the 3-keto group by 3α-hydroxy steroid dehydrogenase. Aromatization of the A-ring of testosterone and androstenedione forms estradiol and estrone and constitutes the major biosynthesis route for estrogens. In various androgen targeted organs, testosterone is transformed to 5α-dihydrotestosterone. Before final renal excretion, most metabolites need to be conjugated with
glucuronic acid or sulfure acid to increase their water solubility so as to facilitate their elimination from the body.

Fig. 1-3. Major metabolic routes of testosterone
In contrast to testosterone, very little information about the biotransformation routes of synthetic anabolic 17α-methyl steroids in human was available in the literature when we initiated this research program in 1988. The human metabolism of these compounds cannot be adequately mimicked in animals mainly because of metabolic variability between species. As far as human studies are concerned, only small doses of anabolic steroid can be administered to human volunteers, due to their particular biological activities and strong side effect. Consequently the concentration of the metabolites of interest in biological samples (usually urine) is often in the low nanogram range. As a consequence, traditional extraction and isolation methods, which are normally used to obtain large amount of pure metabolites for further chemical identification, cannot be efficiently employed. Furthermore, the lack of sensitive and specific analytical methods makes it very difficult to identify a particular metabolite, especially when stereochemical assignments must be performed. For all these reasons, only the major and abundant metabolites have been reported in most previously published studies about anabolic 17α-methyl steroid metabolism in humans. For example, in the metabolism studies of 17α-methyltestosterone and methandienone (Fig. 1-2) [42-44], breast cancer patients under androgen therapy were used as subjects, so that high doses of steroids could be applied (1 g daily for four days compared with normal and therapeutic 10 - 50 mg single doses used in our current studies). Even with such high doses, only two urinary metabolites were isolated in each of the studies. Because spectroscopic methods, such as NMR and MS, were then not routinely applied for structure elucidation, the identification of certain metabolites was very difficult if not impossible to achieve. For example, the structure of
17-epimethandienone (Chapter 6), an important metabolite of methandienone, was not confirmed until its synthesis was accomplished eight years later [45]. Before Brooks et al. introduced the radiommmunoassay (RIA) method in the mid 1970's [46] (which was used at 1976 Montreal Olympic Games for anabolic steroids screening [47,48]), there was no practical method available for the routine analysis of urinary anabolic steroids and their metabolites. But because of the relatively poor specificity of the RIA technique, all the positive results must be confirmed with a more specific method, namely gas chromatography/mass spectrometry (GC/MS) [49]. However, packed column GC/MS did not meet the analytical requirement needed for the sensitive detection of minute amounts of metabolites and rapid screening of large number of samples. Although some authors reported packed column GC/MS methods for the detection of anabolic steroids in biological samples [49-52], this technique was not of great significance to comprehensive metabolic studies. In the late 1970's, the introduction of capillary column GC directly interfaced to a bench-top mass spectrometer, and the use of sophisticated data systems, have greatly contributed in improving resolving power, sensitivity, specificity and throughput of the GC/MS technique. This permitted the achievement of comprehensive metabolic studies of anabolic steroids. With the aid of this new technique, Dürbeck et al. reported new and more extensive metabolic studies about methandienone and 4-chloromethandienone [53-55]. The authors identified four urinary metabolites of methandienone and three metabolites of 4-chloromethandienone, which were all isolated from the unconjugated steroid fraction. Their structures were confirmed either by comparison with authentic samples and/or by analysis of the mass spectral features of
their TMS derivatives [54]. This was the first time that HRGC/MS was applied to comprehensive metabolic studies of anabolic steroids. Due to some unreported reasons, the authors did not detect any conjugated metabolites of methandienone in their studies, which interestingly, have been lately reported by several authors[56-58]. Some of the conjugated metabolites of methandienone will be discussed in Chapter 3. In addition to the advances in GC/MS technique, solid phase extraction method with reverse phase \( (C_{18}) \) cartridge (e.g. Sep-Pak\textsuperscript{TM} C\textsubscript{18}) provided a useful alternative to the time-consuming traditional methods used to isolate steroids from biological samples [59-61]. These \( C_{18} \) cartridges have a very high capacity, and rapid flow rates can be used. This enables rapid sample preparation prior to GC/MS analysis. In 1984, solid phase sample preparation and capillary GC/MS were successfully applied in routine doping control analysis at the Los Angeles Olympic Games [62,63]. Although several authors described different screening methods for routine analysis of anabolic steroids [62-66], no comprehensive studies on anabolic steroid metabolism in human was reported in the literature after Dürbeck's reports, until our studies about the metabolism of oxandrolone was published in 1989 (Chapter 2). In addition, Massé and his coworkers reported integrated analytical methods for the detection of urinary anabolic steroids and their metabolites [56,67]. In these reports preliminary results about the metabolism of several 17α-methyl steroids were presented. They have been used as a basis for the comprehensive studies presented hereinafter.
Research initiatives

My research project on the metabolism of several 17α-methyl anabolic steroids, the structures of which are illustrated in Fig. 1-2, has been conducted at the Canadian Center for Doping Control, INRS-Santé, Université du Québec, a research center that is accredited by the IOC and performs drug testing for national and international sport organizations. The whole project encompasses three major parts.

1. Development of new analytical methods (including sample preparation, derivatization methods and GC/MS analysis) to efficiently detect particular anabolic steroids and their metabolites in human urine. Special efforts have been focused on some chemically unstable metabolites whose recoveries are greatly affected by the extraction methods (e.g. oxandrolone metabolites, Chapter 2). Some metabolites with special functional groups require specific types of derivatization to permit detection with high sensitivity (e.g. acidic formebolone metabolite, Chapter 4; and acidic oxymetholone metabolites, Chapter 5). Therefore different derivatization methods were used to achieve maximal sensitivity, and different derivatives also provided additional structural information for certain metabolites.

2. Identification of urinary metabolites by GC/MS and synthesis of reference steroids. The preliminary structural information obtained for particular metabolites were based on GC properties and MS spectral features of different derivatives. In some cases, the MS
spectral comparison of different derivatives, such as TMS, methoxime-TMS, methyl ester-TMS and perdeuterated (d₉) TMS, provided sufficient evidence for the structure elucidation of certain metabolites (e.g. oxandrolone metabolites in Chapter 2, formebolone metabolites in Chapter 4). However, mass spectral data were not sufficient to permit total structure elucidation of metabolites bearing asymmetric carbon atoms resulting from stereoselective metabolic reactions. Therefore, stereoselective partial synthesis of reference steroids had to be conducted in order to confirm the structures of these metabolites. Also, new metabolic reactions that were observed in the course of our investigation were studied using model compounds synthesized from commercially available steroids. The corresponding synthetic reference steroids were used to determine the structure of new metabolites using various spectroscopic methods, such as NMR, IR and HRMS (e.g. acidic oxymetholone metabolites in Chapter 5, and 17β-sulfates, 17-epimers of some 17α-methyl steroids in Chapter 6).

3. Characterization of common and new biotransformation routes of 17α-methyl steroids. Comparative analysis of the various metabolic reactions affecting the studied steroids revealed interesting and new structure-metabolism relationships. For example, due to the presence of a 17α-methyl group, 5β- and 5α- dihydro metabolites arising from the reduction of the Δ⁴-functions showed a 4:1 ratio instead of 1:1 ratio as previously reported for testosterone metabolites (Chapter 3). The extent of 17-epimerization, which is an important metabolic reaction in 17α-methyl steroid metabolism in human, was greatly affected by the presence of other functional groups (Chapter 6). Our studies
showed that some functional groups undergo specific metabolic fate, that lead to the discovery of new metabolic reactions. For example, the 2-formyl and 2-hydroxy-methylene functions of formebolone and oxymetholone are oxidised to yield the corresponding carboxylic acid metabolites (Chapter 4 and 5). We also demonstrated that oxymetholone oxidative metabolism gives rise to the formation of acidic 2,3-seco metabolites through oxidative cleavage of C2-C3 bond. This newly discovered metabolic pathway in androgenic-anabolic steroid metabolism has never been reported in the literature.

**Summary of results**

All the results are presented in the form of manuscript since they have been or will be published in different scientific journals. The original manuscripts have been modified for editorial purposes.

Chapter 2 discusses the GC/MS characterization oxandrolone urinary metabolites and related analytical methodologies, which has been published in *Biomedical Environmental Mass spectrometry* 1989, 18: 429 - 438.

Chapter 3 presents a comparative study investigating the stereoselectivity of the in vivo reduction Δ⁴-3-one functions of several 17α-methyl steroids. These data have been published in *Journal of Chromatography* 1991, 562: 323 - 340.
Chapter 4 discusses the GC/MS analysis of urinary metabolites of formebolone, for which an acidic metabolite of an anabolic steroid was reported for the first time (structure confirmed by synthesis of the reference steroid). These data have been published in *Analytica Chimica Acta* 1991, 247: 211 - 221.

Chapter 5 presents studies on acidic metabolites of oxymetholone. The oxidation of the 2-hydroxymethylene function and further reactions give rise to a series of acidic metabolites, most of which are seco acids. GC/MS analysis and confirmatory synthetic approaches are reported. Also the potential pharmacological and toxicological implication of the occurrence of these unique metabolites are discussed. These two parts will be published in *Journal of Steroid Biochemistry & Molecular biology* (accepted for publication) and *Steroids*, respectively.

Chapter 6 presents studies about a metabolic reaction which is common to anabolic 17α-methyl steroids, namely 17β-sulfation and degradation of the corresponding 17β-sulfates to form 17-epimers and 18-nor-13(14)-ene steroids. A convenient synthetic method for the preparation of 17β-sulfate derivatives of these steroids and the structure elucidation of the degradation products are reported (accepted for publication in *Steroids*). Both qualitative and quantitative analysis of urinary 17-epimers and 18-nor-13(14)-ene steroids produced from the metabolism of several anabolic 17α-methyl steroids have been conducted (accepted for publication in *Journal of Steroid Biochemistry & Molecular Biology*).
About co-authors

Professor Robert Massé, who is my research director, is the associate director of the Canadian Center for Doping Control, INRS-Santé, Université du Québec. Dr. Robert Dugal is the director of this center. Professor George Just, my thesis director, is from Department of Chemistry, McGill University.

Dr. Christiane Ayotte was Dr. Massé's research associate, and mainly involved in routine doping control analysis. She did some preliminary investigation on the metabolism of some anabolic steroids. Miss Huguette Gélinas did master degree research work on methandienone metabolism under the guidance of Professor Massé. Ms. Ping Du was Dr. Massé's research assistant who performed preliminary GC/MS investigations on the urinary metabolites of formebolone and oxymetholone in humans.

Manuscripts and authorship

The candidate has the option, subject to the approval of their Department, of including as part of the thesis the text, or duplicated published text, of an original paper or papers.

- Manuscript-style thesis must still conform to all other requirements explained in the Guidelines concerning Thesis Preparation.

- Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (eg. in appendices) to allow clear and precise
judgement to be made of the importance and originality of the research reported.

- The thesis should be more than mere collection of manuscripts published or to be published.

It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interest of cohesion.

It is acceptable for theses to include, as chapters, authentic copies of papers already published, provided these are duplicated clearly and bound as an integral part of the thesis. In such instances, connecting texts are mandatory and supplementary explanatory material is always necessary.

- While the inclusion of manuscripts co-authored by the candidate and others is acceptable, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims at the Ph.D. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear.
References*


* Refer to each chapter for separate references.
† Translated from German.


† Translated from German.


† Translated from German.


† Translated from Italian.


45. MacDonald B.S., Sykes P.J., Adhikary P.M., Harkness R.A.: 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. *Steroids*


CHAPTER 2†

STUDIES ON ANABOLIC STEROIDS. 2.

GAS CHROMATOGRAPHIC/MASS SPECTROMETRIC CHARACTERIZATION
OF OXANDROLONE URINARY METABOLITES IN MAN

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Summary

The metabolism of 17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one (oxandrolone) in man has been investigated by gas chromatography/mass spectrometry. After oral administration of a 10 mg dose to a male volunteer, five metabolites were detected in the unconjugated fraction of the urinary samples. Oxandrolone, the major compound excreted in urine, was detected up to 72 h after administration. During this period 35.8% and 3.0% of the administered dose was excreted as unchanged oxandrolone and 17-epioxandrolone* respectively. In addition minute amounts of 16α- and 16β-hydroxyoxandrolone and a δ-hydroxy acid resulting from the hydrolysis of the lactone group of oxandrolone were detected in the urine samples from 8 to 60 h after administration. Furthermore, the susceptibility of oxandrolone to hydrolysis was investigated under several pH conditions. Extraction and fractionation of steroidal metabolites was achieved by using C18 and silica Sep Pak™ chromatography. The mass spectra of the metabolites are presented and major fragmentation pathways discussed.

* 17-Epioxandrolone was later synthesized via the 17β-sulfate derivative of oxandrolone. (Chapter 6).
Introduction

The misuse of synthetic anabolic steroids in sports has become so widespread in the last two decades that the International Olympic Committee and other international governing bodies have firmly prohibited their use in sport. In order to be effective, the control of anabolic steroid abuse requires specific and sensitive assays. Gas chromatography/mass spectrometry (GC/MS) has been selected as the technique of choice to achieve this objective. An integrated screening method was recently reported for the detection in urine of anabolic steroid metabolites [1,2]. The development of such a method implies that the major routes of steroids biotransformation had been previously elucidated, so that the most abundant and/or characteristic urinary metabolites can be used as chemical probes for evidence of the misuse of the corresponding anabolic agent.

In previous publications on the excretion and metabolism of some anabolic steroids, several authors reported the isolation and identification of a number of metabolites from human urine [1,3-19]. Several of these studies were reported before the introduction of glass capillary columns whose advent has greatly improved the performance of GC/MS systems and triggered remarkable progress in the area of steroid analysis [20]. As part of a program on the investigation of anabolic steroids metabolism in man, we have been interested in the study of their urinary excretion profiles and the development of GC/MS methods for detection and structural characterization purposes.
Oxandrolone, (17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one) is a synthetic steroid [21] which can be regarded as a 2-oxa-dihydro analog of 17α-methyltestosterone [22]. Oxandrolone has been shown to be a potent anabolic agent in man [23,24], However, little is known about its metabolism in man. Using 14C-labelled oxandrolone, Karim et al. [25] reported that unchanged oxandrolone was the major metabolite excreted in urine, along with minor amounts of a steroid tentatively identified as 16β-hydroxyoxandrolone. Unlike natural steroids and other synthetic anabolic steroids, oxandrolone bears a lactone function which is susceptible to hydrolysis under both acidic and basic conditions [26]. This particular chemical property of oxandrolone is of analytical importance since the method used to extract and hydrolyse urinary steroids prior to GC/MS screening includes the treatment of the urinary sample under acidic [1,2] or basic conditions [27].

This aspect of the analytical chemistry of oxandrolone was investigated and will be presented herein. This paper will also deal with the GC/MS characterization of oxandrolone and four of its urinary metabolites. The mass spectra of the biotransformation products will be discussed and a metabolic pathway accounting for their formation will be proposed.

Experimental

Chemical and reagents

Oxandrolone was obtained from G.D. Searle & Co. (Chicago, IL). Its purity (99.9%)
was assessed by GC/MS analysis. 5α-Androstan-17-one and β-glucuronidase type H-2 from *Helix pomatia* were obtained from Sigma Chemical Co. (St. Louis, MO). Dithioerythritol and trimethylsilyl iodide (TMSI) were purchased from Aldrich Chemical Co. (Milwaukee, WI). N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) was obtained from Regis Chemical Co. (Morton Grove, IL). Silica and C<sub>18</sub> Sep Pak™ cartridges were from Waters Associates (Milford, MA). Inorganic salts were of analytical grade (J.T. Baker Chemical Co., Phillipsburg, NJ). Anhydrous diethyl ether (reagent grade) was redistilled in glass before use. Other solvents (HPLC grade, Caledon Laboratories Ltd., Georgetown, Ontario) were used as such.

**Stock solutions**

Stock solutions of oxandrolone (0.1 and 1.0 mg/ml) and 5α-androstan-17-one (0.1 mg/ml) were prepared in anhydrous methanol. Storage of these solutions at 4 °C in the dark for 2 months did not result in any detectable decomposition.

**Standard curves**

A standard curve for the determination of oxandrolone in the range of 1 to 5000 ng/ml was prepared as follows: stock solution aliquots were transferred to 200 μl vials so as to obtain 0.04 to 200 ng/μl of oxandrolone and 10 ng/μl of 5α-androstan-17-one. After evaporation of the solvent, under a stream of nitrogen at 40 °C, the residue was dissolved in 50 μl of a mixture of MSTFA-TMSI and derivatized as described below. Each sample was prepared in triplicate and 1 μl was injected twice in the gas
chromatograph. Selective ion monitoring (SIM) was performed and peak area ratios of ions of m/z 143 (oxandrolone) and m/z 331 (5α-androstan-17-one) were measured. Linearity was obtained in the range of 0.04 to 200 ng/μl. The data can be fitted by the regression equation $C_o = 80 A_r - 1.60$ ($\gamma = 0.9985$) where $C_o$ is the concentration of oxandrolone in ng/μl and $A_r$ is the peak area ratio of oxandrolone to 5α-androstan-17-one. Since no reference 17-epioxandrolone was available, and because both epimeric steroids provided mass spectra with identical fragmentation pattern, and diagnostic ion ratios, it was assumed for quantitation purposes that the response factor of 17-epioxandrolone to the internal standard was identical to that of oxandrolone.

In order to determine the effect of pH on oxandrolone quantitation, two standard curves were prepared. Solutions of 0.2 M sodium acetate and 0.8 M sodium carbonate-sodium bicarbonate (1:10, w/w) were used to adjust the pH of urine aliquots at 5.2 and 8.0. To a 2 ml aliquot of buffer solutions was added 1 μl of 5α-androstan-17-one and increasing amounts of oxandrolone so as to obtain concentration levels of 1 to 250 ng/ml. The solutions (prepared in triplicate) were extracted with 5 ml of diethyl ether. The organic phase was dried over anhydrous sodium sulfate, evaporated to dryness and the residue derivatized in 50 μl of a mixture of MSTFA-TMSI as described below. SIM GC/MS was performed as described above. The data are fitted by the following regression equations: pH 5.2 curve, $C_o = 232.6 A_r + 0.23$ ($\gamma = 0.9995$) and pH 8.0 curve, $C_o = 416.7 A_r - 4.17$ ($\gamma = 0.9983$). See above for $C_o$ and $A_r$ identification.
Hydrolysis of oxandrolone

The susceptibility of the lactone group of oxandrolone to hydrolysis was investigated in aqueous buffer solutions at pH values ranging from 5.2 to 11. In each experiment, a methanolic solution containing 25 µg of oxandrolone was evaporated to dryness. The residue was dissolved in 2 ml of an aqueous solution buffered with solutions or mixtures of the following solutions: 0.2 M sodium acetate, 0.8 M sodium carbonate and 0.8 M sodium bicarbonate.

The mixtures were vortexed, left at room temperature for 30 min. and extracted with 5 ml of diethyl ether. The organic phase was dried over anhydrous sodium sulfate and evaporated to dryness. Internal standard (1 µg) was added. The steroids were derivatized in 100 µl of a mixture of MSTFA-TMSI as described below. Area peak ratios of ions of m/z 143 (oxandrolone) and m/z 331 (internal standard) were measured.

Extraction of unconjugated metabolites

Following the collection of control urine specimens, a 10 mg therapeutic dose of oxandrolone was administered orally to a 30-year-old male volunteer (60 kg). Urine was then collected for 96 h in sterile plastic containers and stored at -20 °C. Unconjugated steroids were extracted as follows: urine (2 ml) was passed through a Sep Pak™ C18 cartridge (previously washed with 5 ml of methanol and 5 ml of water) and washed successively with 5 ml of water and 2 ml of hexane to remove excess water in the cartridge. Unconjugated steroids were eluted with 5 ml of dichloromethane. The solvent was evaporated to dryness and the residue was derivatized as described below, prior to
GC/MS analysis. The unconjugated steroids were also extracted using a method we reported previously [1,2]. Oxandrolone recoveries obtained from both extraction methods and measured as peak area ratios of oxandrolone to internal standard are given in Table 2-1.

Table 2-1. Relative recovery for oxandrolone using two different extraction methods

<table>
<thead>
<tr>
<th>Urine sample</th>
<th>Sep Pak™ - CH₂Cl₂</th>
<th>Sep Pak™ - CH₃OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.6928</td>
<td>0.5216</td>
</tr>
<tr>
<td>2</td>
<td>0.6663</td>
<td>0.4989</td>
</tr>
<tr>
<td>3</td>
<td>0.6248</td>
<td>0.5643</td>
</tr>
<tr>
<td>Mean value</td>
<td>0.6613±0.034</td>
<td>0.5283±0.033</td>
</tr>
<tr>
<td>c.v.</td>
<td>5.2%</td>
<td>6.3%</td>
</tr>
</tbody>
</table>

1. The urine sample was collected 12 h after oxandrolone administration and analyzed in triplicate. Numbers correspond to peak area ratios of ion of m/z 143 (oxandrolone) and m/z 331 (5α-androstan-17-one) measured by SIM-GC/MS. In both methods, urine was first passed through a Sep Pak™ C₁₈ cartridge.
2. Unconjugated steroids were extracted by elution of the Sep Pak™ cartridge with dichloromethane.
3. Steroids were eluted with methanol. Solvent was evaporated and the residue dissolved in 0.2 M sodium acetate buffer. Unconjugated steroids were extracted with diethyl ether [1,2].

**Fractionation of oxandrolone metabolites**

The crude unconjugated steroid extract obtained from Sep Pak™ C₁₈ chromatography was fractionated on a Sep Pak™ silica cartridge as follows: the unconjugated steroid extract was dissolved in 1 ml of dichloromethane and the resulting solution was slowly passed through the cartridge (previously washed with 5 ml of hexane and 5 ml of dichloromethane). The cartridge was then washed with 5 ml of dichloromethane and neutral steroids were eluted with 5 ml of a mixture of dichloromethane-methanol (7:1, v/v). Oxandrolone hydrolysis products were finally eluted with 5 ml of methanol. The
organic fractions were processed as usual prior to GC/MS analysis.

**Derivatization**

A portion of each extract (either aqueous or urinary) and the steroids from the stock solutions were transformed into their corresponding TMS derivatives prior to GC/MS analysis [1] by treatment with 50 or 100 μl of a mixture of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA)/trimethylsilyl iodide (TMSI) (100:2, v/v) containing 0.5 to 1 mg of dithioerythritol. The mixture was heated at 70 °C for 30 min and 1 μl was injected in the gas chromatograph.

**Gas chromatography/mass spectrometry**

GC/MS analysis were carried out using an HP-5970 mass selective detector (MSD) linked to an HP-5890 gas chromatograph equipped with a 25 m HP Ultra-2 (5% phenyl, methyl silicone) fused silica capillary column (0.2 mm i.d., 0.33 μm film thickness). The oven temperature was programmed from 100 °C (1 min hold) to 220 °C at 16 °C/min and at 3.8 °C/min up to 300 °C (10 min hold). The ion source, injector and transfer line temperatures were set at 220 °C, 270 °C and 310 °C respectively. Injections were performed in the splitless mode (purge off time 30 sec) and helium was the carrier gas at a flow rate of 0.6 ml/min. Mass spectra were recorded in the full scan mode at an ionizing electron energy of 70 eV. SIM experiments for oxandrolone and epioxandrolone quantitation were performed by monitoring their characteristic ions at m/z 143.1, 363.3 and 378.3 and those of 5α-androstan-17-one at m/z 346.3 and 331.3. Dwell times were
100 ms for all ions.

**Accuracy, Precision and Detection limits**

The accuracy of the method used to determine oxandrolone was calculated by adding known amounts of the steroid (50, 250 and 500 ng/ml) to blank urine samples extracted as described above.

Precision was calculated by repeatedly measuring oxandrolone urinary levels on the same and on different days. The resulting data are presented in Table 2-2.

An approximate detection limit of 2.5 ng/ml or 100 pg injected (signal/noise ratio 3:1) was measured for blank urine samples spiked with known amounts of oxandrolone.

<table>
<thead>
<tr>
<th>Oxandrolone (ng/ml)</th>
<th>Intra assays</th>
<th>Inter assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/ml ± s.d., c.v., Recovery</td>
<td>ng/ml ± s.d., c.v., Recovery</td>
</tr>
<tr>
<td>50</td>
<td>48.3 ± 0.6, 1.2% 96.6%</td>
<td>45.7 ± 0.6, 1.2% 91.4%</td>
</tr>
<tr>
<td></td>
<td>47.0 ± 1.0, 2.1% 94.0%</td>
<td>47.0 ± 1.3, 2.8% 94.0%</td>
</tr>
<tr>
<td>250</td>
<td>244.9 ± 2.1, 0.8% 98.0%</td>
<td>246.8 ± 0.6, 0.2% 98.7%</td>
</tr>
<tr>
<td></td>
<td>244.8 ± 3.8, 1.5% 97.9%</td>
<td>245.5 ± 5.6, 2.2% 98.2%</td>
</tr>
<tr>
<td>500</td>
<td>495.8 ± 6.6, 1.3% 99.2%</td>
<td>493.4 ± 3.3, 0.7% 98.7%</td>
</tr>
<tr>
<td></td>
<td>494.0 ± 4.8, 1.0% 98.8%</td>
<td>494.3 ± 8.9, 1.8% 98.9%</td>
</tr>
</tbody>
</table>

1. Prepared by supplementing 2 ml urine aliquots with known amounts of oxandrolone. For each concentration, three different aliquots were prepared and analyzed three times by SIM GC/MS.
Results and discussion

In methods currently used, solid-phase extraction with Sep Pak C_{18} cartridge [2] or Amberlite XAD-2 resin [27] has become the method of choice for recovery of both unconjugated and conjugated anabolic steroids. However, unconjugated anabolic steroids are still isolated by solvent extraction of the combined unconjugated-conjugated steroid fraction obtained following solid-phase extraction either under acidic [1,2] or basic [27] conditions. However, there is a problem regarding the extraction of oxandrolone which is excreted mainly unconjugated in urine. Indeed this steroidal \( \delta \)-lactone is susceptible to hydrolysis in acidic and basic conditions [26], even at pH conditions encountered in normal urine specimens (range 4.8 to 7.8) where the presence of electrolytes, proteins and organic acids could promote this reaction [28]. Before investigating the urinary profile of oxandrolone metabolites this problem was assessed.

![Graph showing pH recovery profile of oxandrolone](image)

**Fig. 2-1.** The pH recovery profile of oxandrolone at room temperature. Aliquots of 2 ml of aqueous solutions buffered in the pH range 5.2 to 11 were fortified with 25 \( \mu \)g of oxandrolone. The steroid was quantified by measurement of the peak area ratios of ion of \( m/z \) 143 (oxandrolone) and \( m/z \) 331 (internal standard) in the SIM mode (see Experimental for further details).
Oxandrolone recovery versus pH

As shown in Fig. 2-1, oxandrolone, which was relatively stable in the pH range 5.2 to 8.0, was rapidly degraded at higher pH values. The degradation curve showed that at pH 9.0 the recovery of oxandrolone was reduced by about 40%, and at pH 9.8 the recovery was dramatically lowered to 1.6%. Finally, no trace of oxandrolone was detected at pH higher than 10.0. To determine the mechanism of degradation of oxandrolone, the basic solutions were acidified with 1.0 M HCl to pH 5.2 and re-extracted. As expected, oxandrolone was extracted with high recoveries from samples where it had been previously completely degraded (e.g. pH 10.0 and over). Thus, the data indicated that under basic conditions, oxandrolone was hydrolysed to the sodium salt of the resulting \( \delta \)-hydroxy acid 2, which relactonized upon acidification to pH 5.2. As shown below, isolation of the hydrolysis product and characterization of its structural features provided further analytical evidence for this equilibrium reaction.

The hydrolysis of oxandrolone is of concern inasmuch as it occurs in a pH range currently used to extract unconjugated anabolic steroids, and that it seems to take place also in the pH range 5.2 to 8.0, but to a much smaller extent. Hydrolysis of oxandrolone in the latter pH range may not be of concern at high oxandrolone levels (e.g. 0.2 \( \mu \)g/ml), but can become of importance when the steroid is present in the low nanograms range as often encountered in doping control situations. To verify this preliminary observation, standard curves of oxandrolone were prepared using aqueous solutions buffered at pH 5.2 and 8.0 and spiked at several concentration levels covering the range 1 to 250 ng/ml.
(Fig. 2-2). Linearity was obtained and the curves were fitted by the regression equations mentioned above. As expected, increasing pH from 5.2 to 8.0 resulted in lower recovery of oxandrolone in the concentration range studied, probably due to increased hydrolysis of the steroid at pH 8.0. Note that for both pHs, oxandrolone could not be detected when aqueous solutions were spiked at concentration levels lower than 5 ng/ml. Trace amounts of the δ-hydroxy acid 2 were detected in some of the extracts from solutions of pH 5.2 and were slightly more abundant in those of pH 8.0, when spiked at concentration levels higher than 50 ng/ml. These data provided evidence suggesting that, although hydrolysis of oxandrolone in the pH range 5.2 to 8.0 is relatively slow, it could impede its detection in the low nanogram range. Consequently, unconjugated steroid should be preferentially extracted at pH 5.0 when solvent extraction has to be used.

![Graph](image)

Fig. 2-2. Standard curves for oxandrolone obtained from 2 ml aqueous solutions buffered at pH 5.2 and 8.0 and fortified with increasing amounts of oxandrolone (1 - 250 ng/ml). The steroid was analyzed and quantified as in Fig. 2-1 (see Experimental for further details).
Solid phase extraction of oxandrolone

To circumvent this problem, a solid-phase extraction method was developed to extract the unconjugated steroids without solvent extraction as in methods previously reported [1,2,27]. Table 2-1 compares the recoveries for oxandrolone extracted using this method and that reported previously [1,2]. Note that the mean recovery for oxandrolone was improved by about 25% when extraction was performed with dichloromethane. Furthermore, the latter method has also the advantage to speed up the extraction procedure and extract the unconjugated steroids from the Sep Pak™ C18 cartridge by elution with dichloromethane, an aprotic solvent which prevents coextraction of steroid conjugates while providing a relatively pure unconjugated steroid extract. This methodology meets the major concerns in anabolic steroid analysis in sports which relate to sensitivity, specificity and time required to report results.

The accuracy of measurement was determined for oxandrolone added to 2 ml aliquots of urine at concentrations which are representative of those which were measured in urine following the administration of one therapeutic dose of 10 mg. As shown in Table 2-2, the amounts of oxandrolone measured were in good agreement with the amounts of oxandrolone added. The intra-assay coefficients of variation (c.v.) were lower than 2.0% (n = 6) for urine spiked with 50, 250 and 500 ng/ml. Conversely, the inter-assay coefficients of variation were 2.8, 2.2 and 1.8% (n = 9) for urine spiked with 50, 250 and 500 ng/ml respectively.
Urinary excretion of oxandrolone metabolites

The cumulative urinary excretion and excretion profile of unconjugated oxandrolone from the administration of a 10 mg oral dose are shown in Fig. 2-3. Oxandrolone was first detected 2 h after administration and remained detectable for a period of 72 h. During this period, 3.58 mg of oxandrolone 1 (35.8% of the administered dose) and 0.30 mg (3.0%) of 17-epioxandrolone 5 (Fig. 2-4) were excreted. 17-epioxandrolone 5 was detected in urine from 4 h to 72 h after oxandrolone administration. Two minor hydroxylated metabolites identified as 16α- and 16β-hydroxyoxandrolone 3 and 4 (Fig. 2-4) were detected only between 8 and 60 h after administration. The levels of both metabolites were fairly low and their cumulative excretion accounted for about 0.3 ± 0.05% of the administered dose by comparison of metabolite 1, 2, 3 and 4 peak areas obtained by repetitive scanning GC/MS. Interestingly, the 5-hydroxy acid 2 was detected in only four samples collected 4, 12, 27 and 56 h after administration, at concentration varying between 0.1 and 0.5 μg/ml as estimated by GC/MS analysis. No relation whatsoever could be established between the presence of the steroidal acid, and pH and specific gravity of the corresponding urine samples. It is likely that the presence in urine of electrolytes, proteins or other endogenous compounds could promote hydrolysis of oxandrolone [28] to yield the corresponding 5-hydroxy acid 2.

The maximum urinary level (4.2 μg/ml) of oxandrolone was attained 8 h after administration (Fig. 2-3), declining afterwards in two distinct phases. First, a rapid elimination phase was observed between 2 and 8 h, followed by a slow phase between
8 and 72 h. These two phases of elimination probably indicate a two-compartment open model which reflects oxandrolone distribution (α-phase) and elimination (β-phase) [25]. The conjugated steroid fraction [1] was also investigated and trace amounts of oxandrolone and metabolites 2, 3 and 4 accounting for about 0.5% of the administered dose were detected.

Fig. 2-3. Cumulative urinary excretion and excretion profile (inset) of unconjugated oxandrolone following administration of a 10 mg oral dose. Oxandrolone was extracted by elution through Sep Pak™ C18 cartridges with dichloromethane.

Fig 2-4. Proposed pathway accounting for oxandrolone metabolites identified in man.
The sensitivity of the SIM GC/MS assay was 0.100 ng per injection with a signal-to-noise ratio of about 3:1.

Identification of metabolites by GC/MS

Fractionation of metabolites With the objective of isolating and characterizing hydroxy acid 2 and other urinary metabolites of oxandrolone, the unconjugated steroids were fractionated on a Sep Pak™ silica cartridge by elution with solvents of increasing polarity. Two fractions "A" and "B" were collected and the presence of compounds 1 to 5 in the steroid extracts was demonstrated by GC/MS analysis (Figs. 2-5 and 2-6). The metabolites identified from urine and their partial GC/MS features are summarized in Table 2-3.

Table 2-3. Partial GC/MS properties of oxandrolone urinary metabolites as TMS derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>RRT¹</th>
<th>RRT²</th>
<th>M⁺³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxandrolone</td>
<td>1</td>
<td>1.00</td>
<td>1.558</td>
</tr>
<tr>
<td>3-hydroxy acid</td>
<td>2</td>
<td>0.898</td>
<td>1.400</td>
</tr>
<tr>
<td>16α-hydroxy oxandrolone</td>
<td>3</td>
<td>1.134</td>
<td>1.767</td>
</tr>
<tr>
<td>16β-hydroxy oxandrolone</td>
<td>4</td>
<td>1.160</td>
<td>1.808</td>
</tr>
<tr>
<td>17-epiandrolone</td>
<td>5</td>
<td>0.926</td>
<td>1.444</td>
</tr>
</tbody>
</table>

1. Retention time relative to that of oxandrolone TMS derivative. (RT, 27.97 min).
2. Retention time relative to that of 5α-androstan-17-one TMS-enol derivative (RT, 17.95 min).
3. Molecular ion (m/z) of the TMS derivatives

As shown in Figs. 2-5A and 2-6, fraction A (dichloromethane-methanol, 7:1 v/v, eluate) contained oxandrolone 1, 17-epiandrolone 5 and 16α- and 16β- hydroxyoxandrolone
3 and 4, whereas the δ-hydroxyacid 2 was isolated from the methanolic fraction B (Fig. 2-5B).

Fig. 2-5. Reconstructed ion fragmentograms of ion m/z 143 from repetitive scanning GC/MS analysis of a free steroid urinary extract obtained after administration of oxandrolone and fractionated on a Sep Pak silica cartridge and obtained from (A) dichloromethane-methanol (7:1 v/v) eluate and (B) methanol eluate (see Experimental for further details).

Fig. 2-6. Reconstructed ion chromatograms of m/z 218, 231, 376 and 466 for the dichloromethane-methanol (7:1 v/v) eluate obtained from a free steroid extract fractionated as in Fig. 2-5. Peaks at 31.72 and 32.45 min correspond to 16α- and 16β-hydroxyoxandrolone 3 and 4 respectively. Analysis conditions were as in Fig. 2-5 (see Experimental for further details).
Mass spectral characterization of the metabolites: The mass spectrum of the TMS derivative of oxandrolone obtained from GC/MS data shown in Fig. 2-5A is illustrated in Fig. 2-7. Interestingly, the corresponding mass spectrum of 17-epi-oxandrolone 5 is very similar to that of its epimer 1. They both exhibit identical fragmentation pathways and ion relative intensities are almost identical. The presence of a lactone function in A-ring orients the fragmentation on D-ring, which upon electron-impact gives rise to the prominent fragment ion of m/z 143 which results from D-ring cleavage as shown in Scheme 2-1 [29].

Fig 2-7. Electron impact mass spectrum of oxandrolone 1 as TMS derivative (see Fig 2-5A)

Scheme 2-1. D-ring cleavage in oxandrolone and its metabolites as TMS derivatives.
The formation of ions at m/z 321 [M-57]$^+$ and 308 [M-70]$^{++}$ also proceeds by cleavage of the D-ring. Mechanisms accounting for their formation in steroids bearing silylated 17-hydroxy-17-methyl groups were proposed by Durbec and Buker [14]. The mass spectra corresponding to metabolites 3 and 4 TMS derivatives (Fig. 2-6) are shown in Fig. 2-8. The molecular ion at m/z 466 indicated mono-hydroxylation of the oxandrolone skeleton, whereas the structurally informative ions at m/z 218 and 231 (Scheme 2-1) indicated that C-16 was the hydroxylation site of both metabolites. Indeed, the ion at m/z 231 is the 16-OTMS analog of ion m/z 143 in the mass spectrum of oxandrolone (Fig. 2-7) whereas the abundant ion of m/z 218 which is generated by cleavage of the C-13—C-17 and C-15—C-16 bonds has the structure of [TMSO=CH₂CH₃—H—OTMS]$^{++}$.

![Fig. 2-8. Electron impact mass spectrum of (A) 16α-hydroxy oxandrolone 3 and (B) 16β-hydroxy oxandrolone 4 as TMS derivatives. (See Fig. 2-6)](image-url)
The mass spectrum of the TMS derivative of compound 2 (Fig. 2-9) is consistent with the structure of the δ-hydroxy acid (Fig. 2-4) resulting from hydrolysis of oxandrolone lactone group. Hydroxylation at C-1 and carboxyl group formation at C-3 resulted in a shift of the molecular ion of oxandrolone from m/z 378 to m/z 540 in compound 2. The consecutive losses of the CH₃OTMS radical and TMSOH molecules give structurally informative ions at m/z 437, [M-103]⁺, m/z 360 [M-2×90]⁺⁺, m/z 347 [M-90-103]⁺ and m/z 257 [M-2×90-103]⁺. As shown in Fig. 2-10, the loss of one molecule of CH₃COOTMS (132 a.m.u) probably occurs from ions of m/z 450, 360 and 347 through a McLafferty rearrangement involving the carbonyl group of the TMS ester function and one hydrogen atom at C-6 to yield the corresponding ions at m/z 318 [M-90-132]⁺⁺, 228 [M-2×90-132]⁺⁺ and 215 [M-90-103-132]⁺. Finally, the prominent ion of m/z 143 and fragment ions of m/z 483 [M-57]⁺ and m/z 470 [M-70]⁺⁺ confirmed that D-ring of the parent steroid 1 was not affected by the hydrolysis reaction [14].
Fig. 2-10. Proposed fragmentation routes of δ-hydroxy acid 2 TMS derivatives accounting for the formation of ions of m/z 215, 228, 318, 347 and 450.

**Metabolism of oxandrolone** Owing to the presence of a lactone group and the absence of a 4-ene function in the A-ring, hydroxylation reactions which normally occur with anabolic steroids bearing a 3-keto group and C-4 double bond [2,10] seem to be suppressed during oxandrolone metabolism in man. Thus, oxandrolone is excreted mainly unchanged and unconjugated in urine. Epimerization at C-17 appears to be the predominant transformation since 17-epiöxandrolone 5, aside of the parent steroid, is the most abundant metabolite identified in urine. To our knowledge, the mechanism of the epimerization of 17β-hydroxy-17α-methyl steroids is still unknown*. Also there is

* See Chapter 6 for detail discussion on 17-epimerization.
remarkably little information about the biological rationales underlying this reaction and its role in steroid excretion. Hydroxylation is a marginal metabolic route for oxandrolone and only low amounts of its 16α- and 16β-hydroxylated analogues 3 and 4 were excreted in urine. Although hydroxylation at C-16 is mainly associated with hepatic tissue activity, evidence has accumulated regarding gonadal hormone control of this reaction in several species [30]. Exogenous testosterone was shown to produce an increase in hepatic dehydroepiandrosterone 16α-hydroxylase in prepubertal males [31]. Thus, the masculine imprinted character of C-16 hydroxylation is likely promoted by the anabolic and masculinizing oxandrolone which induces the hepatic activity of both 16α- and 16β-hydroxylases to yield 3 and 4. Although a considerable number of sites of the oxandrolone molecule could have been hydroxylated as is the case for other anabolic steroids [2,10,14], no other hydroxylated metabolite was detected in urine after administration of the drug. The absence of a C-4 double bond in A-ring, which promotes β-allylic hydroxylation of several anabolic steroids at C-6 [2], may account for the low hydroxylation level of oxandrolone.

Finally, we still do not know whether the formation of δ-hydroxy acid 2 results solely from chemical hydrolysis of oxandrolone when excreted in urine and/or enzymatic hydrolysis before urinary excretion. Since hydrolysis and refactonization are both dependent upon the relative acidity and electrolyte level in urine [28], it is likely that these equilibrium reactions occur in this biological fluid rather than in the hepatic or other body tissues.
Conclusion

We have presented qualitative and quantitative data regarding the urinary excretion profile of the anabolic steroid oxandrolone in man. GC/MS data have shown that oxandrolone was excreted mainly unchanged and unconjugated in urine and accounted for 35.8% of the administered dose. The steroid could be detected 72 h after administration of a 10 mg dose. The other metabolites excreted in urine were 17-epioxandrolone (3.0%) and 16α- and 16β-hydroxyoxandrolone (0.3%). Oxandrolone susceptibility to hydrolysis in the pH range of 5.2 to 11.0 was also demonstrated. An alternative extraction method based on solid-phase extraction and elution with dichloromethane was proposed to improve recovery and minimize the potential hydrolysis of oxandrolone. In this regard, the analytical approach presented herein has provided a better understanding of oxandrolone metabolism in man in comparison with previously reported information while improving sensitivity and specificity of its GC/MS detection and characterization in urine.

Acknowledgements

The financial support of the Sports Medicine Council of Canada, the National Collegiate Athletic Association and the National Science and Engineering Research Council of Canada is gratefully acknowledged.
References


CHAPTER 3†

STUDIES ON ANABOLIC STEROIDS.5.

THE SEQUENTIAL REDUCTION OF METHANDIENONE AND STRUCTURALLY RELATED STEROID A-RING SUBSTITUENTS IN HUMAN:
A GC/MS STUDY OF THE CORRESPONDING URINARY METABOLITES

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Summary

The biotransformation of methandienone (17β-hydroxy-17α-methyl-androsta-1,4-dien-3-one) in human adults, more particularly the sequential reduction of its A-ring substituents has been investigated by gas chromatography-mass spectrometry. Two pairs of 17-epimeric tetrahydro diols resulting from the stereoselective reduction of the Δ4- and 3-oxo groups and of the Δ1-function were characterized. The major diols were 17α-methyl-5α-androstane-3α,17β-diol and 17α-methyl-5β-androstane-3α,17β-diol which were both excreted in the conjugate fraction in a 1:3.8 ratio. The immediate metabolic precursors of the 5β-diol, namely 17β-hydroxy-17α-methyl-5β-androst-1-en-3-one and 17α-methyl-5β-androst-1-en-3α,17β-diol and their corresponding 17-epimers were also identified in post-administration urine samples. These data indicated that reduction of methandienone A-ring substituents proceeds according to the following sequence: Δ4-, 3-oxo and Δ1-. The A-ring reduction products of the structurally related steroids, mestanolone, 17α-methyltestosterone and oxymetholone were also characterized and provided further analytical and metabolic evidence supporting the proposed route of methandienone A-ring reduction. It was demonstrated using synthetic 17β-sulfate conjugates of methandienone and 17α-methyltestosterone that their corresponding 17-epimers are formed by nucleophilic substitution by water of the labile sulfate moiety. The steroidal metabolites were identified on the basis of their characteristic mass spectral features and by comparison with authentic reference standards. Metabolic pathways accounting for the occurrence of the metabolites of interest in post-administration urine samples are proposed.
Introduction

The sequential reduction of the Δ⁴- and 3-oxo functions are key steps in the biotransformation of several endogenous and exogenous steroids in human [1-6]. It is well known that the stereoselective 5α- reduction of testosterone to yield 5α-dihydrotestosterone is of major importance in the expression of androgenic activity in tissue of the male reproductive tract [4]. Among the four tetrahydro urinary metabolites which are produced in the course of testosterone catabolism through the concerted action of 5α- and 5β- reductases and 3α- and 3β-hydroxysteroid dehydrogenases (3-OHSDH), androsterone (3α, 5α) and etiocholanolone (3α, 5β) are the most abundant and excreted in urine in a mean ratio of about 1:0.9 in healthy men [6].

It is of interest to note that the relative affinity of 5α- and 5β- reductases towards steroidal substrates can be significantly modulated by slight but specific chemical modifications at positions either close to or remote from the Δ⁴- function. Typical example of the enzymatic selectivity of these enzymes is given by the reductive metabolism of 19-nortestosterone (19-NT) and 17α-methyltestosterone (17α-MT), which are catabolized into tetrahydro metabolites analogous to androsterone and etiocholanolone. Thus, we previously reported that the metabolic profile of 5α- and 5β-tetrahydro metabolites of 19-NT was markedly influenced by the absence of the 19-methyl group, as 19-norandro-sterone (19-NA) and 19-noretiocholanolone (19-NE) were shown to be always excreted in urine in a ratio larger than 2:1 [7]. The preponderance
of 19-NA was observed not only after 19-NT esters administration, but also in positive urine samples obtained from athletes in the context of drug-testing activities [8, 9].

These findings corroborated those of Engel et al. [10] and Floch et al. [11] who reported that 19-NA was the predominant urinary metabolite of 19-NT in human. Further evidence for the preponderance of $5\alpha$-reduction in 19-NT metabolism was recently obtained in man [12] in the course of the oxidative de-ethynylation of norethisterone into 19-NT, which then undergoes reduction to yield 19-NA and 19-NE. Interestingly, the latter isomeric steroids were also excreted in urine in a ratio larger than 2:1.

Conversely, Rongone and Segaloff [13] showed that 17α-MT was mainly transformed into two isomeric tetrahydro metabolites with $3\alpha$, $5\alpha$-, and $3\alpha$, $5\beta$- configurations, which were excreted in urine in a 1:10 ratio after oral administration of 1 g of 17α-MT to a female patient with advanced breast cancer. These data indicated that the introduction of a 17α-methyl group in testosterone seems to promote $5\beta$-reduction, whereas the absence of a 19-angular methyl group favors $5\alpha$-reduction of the $\Delta^4$-function.

Several 17α-methyl anabolic steroids bearing $\Delta^4$- and/or 3-oxo functions are likely to be metabolized into similar if not identical tetrahydro metabolites [2]. This could be the case of methandienone ($\Delta^1$-17α-MT), 17α-MT, mestanolone and oxymetholone (2-hydroxymethylene-mestanolone) which are differentiated by the structural features of their A-ring.
Therefore, we have undertaken a comprehensive investigation of the urinary metabolites which are likely to arise from the reductive metabolism of their A-ring substituents in order to elucidate the sequence of reaction through which methandienone is partially transformed into tetrahydro compounds, determine the ratio of the corresponding tetrahydro 5α- and 5β- metabolites in urine and characterize the structure of the metabolites of interest. Here, mestanolone, 17α-MT and oxymetholone were used as model steroids so as to obtain reference steroids with known 5α-configuration and 5α- and 5β- steroids with no Δ1- function that we subsequently used to characterize and assess the identity of the metabolites produced from methandienone.

In the present paper we report the GC/MS characterization of two isomeric tetrahydro metabolites and their corresponding 17-epimers which are excreted in urine following methandienone and 17α-MT ingestion. In the case of methandienone, the metabolic intermediates of the tetrahydro metabolites were also isolated and characterized. Reference tetrahydro 17-epimers with a 3α, 5α- configuration were obtained from mestanolone and oxymetholone human studies. 17α-MT sulfate pyridinium salt was synthesized and used as a model substrate to demonstrate that the 17α-hydroxy-17β-methyl-epimeric metabolites are produced from the 17β-sulfate aglycone of the parent steroid or any other metabolic intermediate. Mass spectral data supporting the proposed identity of the urinary metabolites are presented and metabolic pathways accounting for their formation are proposed.
Experimental

Steroids

Authentic 17α-methyl-5α-androstan-3α,17β-diol, 17β-hydroxy-17α-methyl-5β-androstan-3-one, 17α-methyltestosterone, mestanolone (17β-hydroxy-17α-methyl-5α-androstan-3-one), methandienone and oxymetholone (2-hydroxymethylene-17β-hydroxy-17α-methyl-5α-androstan-3-one) were purchased from Steraloids Inc (Wilton, NH) and Sigma Chemical Co. (St. Louis, MO); tablets of methandienone and 17α-methyltestosterone were obtained from Ciba-Geigy Canada Ltd (Dorval, Québec, Canada); tablets of oxymetholone were from Syntex Ltd. (Montréal, Québec, Canada). Reference 17α-methyl-5β-androstan-3α,17β-diol was prepared by NaBH₄ reduction of 17β-hydroxy-17α-methyl-5β-androstan-3-one.

Chemicals

N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) was purchased from Regis Chemical Co. (Morton Grove, IL); sodium borohydride (NaBH₄), dithioerithritol and trimethylsilyl iodide (TMSI) were supplied by Aldrich Chemical Co. (Milwaukee, WI); Sep-Pak™ C₁₈ cartridges were purchased from Waters Associates (Milford, MA). Inorganic salts (J.T. Baker Chemical Co., Philipsburg, NJ) were of analytical grade. All the solvents were of HPLC grade (Caledon Laboratories Ltd., Georgetown, Ontario, Canada) and used as received.
**Urine samples**

Blank urine samples were collected before administrations of anabolic steroids to healthy male volunteers (24 to 40 years of age). The volunteers were given one oral dose of mestanolone (10 mg), methandienone (25 mg), 17α-methyltestosterone (10 mg) or oxymetholone (50 mg). Urine samples were then collected for seven days in order to obtain comprehensive excretion profiles of the parent steroids and their metabolites and were immediately frozen at -20°C after collection until analyzed.

**Extraction of urinary steroids and their metabolites**

Urine sample (3-5 ml) was passed through a Sep-Pak™ C₁₈ cartridge (prewashed with 5 ml of methanol and 5 ml of water). The cartridge was then washed with 5 ml of water and 2 ml of hexane (to eliminate residual water in the cartridge). Steroids and their metabolites were finally eluted with 5 ml of methanol and the solvent was evaporated under a stream of nitrogen below 60°C. The resulting dried residue was dissolved in 1 ml of 0.2 M acetate buffer (pH 5.2) and 100 μl of β-glucuronidase-sulfatase (type H-2) from Helix pomatia (Sigma Chemical Co., St-Louis, MO) was added to the solution. The mixture was incubated for 16 hours at 37°C, or 3 hours at 55°C. After hydrolysis, 100 mg of solid buffer (KHCO₃-K₂CO₃, 9:1, w/w) was added, and the aqueous phase was extracted with 5 ml of diethyl ether. After evaporation of the organic solvent under nitrogen, the residue was derivatized as described below. The crude preparation of Helix pomatia used was tested for potential side activities (e.g. oxidation of steroids) prior to use as reported previously [12].
Derivatization and GC/MS analysis

Derivatization method† using N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) and N,O-bis-(d₉)trimethylsilyl acetamide (d₉-BSA) as reagents and trimethylsilyl iodide (TMSI), trimethylchlorosilane (TMCS) and d₉-TMCS as catalysts, and GC/MS conditions‡ were as reported previously [12,14,15]. The repetitive scan mode was used to obtain full mass spectra of relevant GC peaks for identification of the metabolites of interest, whereas selective ion monitoring (SIM) GC/MS was used to measure relative intensities of particular ions. Capillary columns (30 m) with methylsilicone (HP-1 and DB-1) and methylphenylsilicone (HP-5 and DB-5) stationary phases were used in order to achieve the separation of the isomeric tetrahydrodihols 1 and 2. Unfortunately, all attempts to resolve these steroids by GC were unsuccessful.

Synthesis of model steroids

17α-MT sulfate pyridinium salt (17α-MTSPS) and Fast atom bombardment MS analysis. This steroid conjugate was prepared by the reaction of 17α-MT with chlorosulfonic acid in pyridine. 17α-MSTSPS was characterized by ¹³C NMR and Fast atom bombardment (FAB) mass spectrometry in the negative ion mode. A detailed synthetic procedure and spectral characterization of 17α-MTSPS and of a series of other 17α-methyl anabolic steroids sulfate salts, as well as epimerization conditions will be reported elsewhere*. 17α-MTSPS was characterized by negative-ion FAB/MS using a

† See Chapter 2 and 4 for detail procedures.
‡ See Chapter 2 for GC/MS conditions.
* See Chapter 6-1 for detail discussion.
Kratos MS50TC mass spectrometer. The bombarding atom beam was 6 kV xenon atoms of 1 mA current. Glycerol was used as the sample-supporting matrix and xenon flow rate was 0.5 ml/min. 17α-MTSPS FAB spectrum was measured at a resolution of 10000 and showed a base-peak at m/z 381.1736 (C₂₀H₂₉O₃S) corresponding to the 17α-MTSPSO₃⁻ ion.

**Synthesis of 17β-methyl-5β-androstan-3α,17α-diol 4.** 17α-MTSPS was epimerized in aqueous potassium carbonate and 17-epimethyltestosterone was recovered in about 50% yield by extraction with diethyl ether and characterized by GC/MS analysis. This steroid was identical to 17-epiMT isolated from 17α-MT urine samples [9]. Reduction of the latter 17-epimeric steroid with sodium borohydride in isopropanol using a method adapted from that of Barton [16] preponderantly afforded 4 and trace amounts of 17β-methyl-5α-androstan-3β,17α-diol. Synthetic 4 was shown to be identical to metabolite 4 isolated from 17α-MT and methandienone urine samples. Epimerization of 17α-MTSPS also gave several rearrangement products, namely 18-nor-17,17-dimethyl-4,13(14)-androstan-3-one (M⁺ 284) and 17-methyl-androsta-4,16-dien-3-one (M⁺ 284). These compounds are homologous to those reported by Edlund et al. [17] from the decomposition of methandienone sulfate.

**Synthesis of 17α-methyl-5β-androstan-3α,17β-diol 2.** This steroid was prepared by sodium borohydride reduction of 17β-hydroxy-17α-methyl-5β-androstan-3-one in isopropanol according to a method adapted from that of Barton [16].
Table 3-1. Identity and methylene unit values of the tetrahydro diols detected in human urine

<table>
<thead>
<tr>
<th>Urinary metabolites</th>
<th>No.</th>
<th>Steroids&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MSTL</td>
</tr>
<tr>
<td>17α-methyl-5α-androstan-3α,17β-diol</td>
<td>1</td>
<td>26.65</td>
</tr>
<tr>
<td>17α-methyl-5β-androstan-3α,17β-diol</td>
<td>2</td>
<td>nd &lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>17β-methyl-5α-androstan-3α,17α-diol</td>
<td>3</td>
<td>25.06</td>
</tr>
<tr>
<td>17β-methyl-5β-androstan-3α,17α-diol</td>
<td>4</td>
<td>nd</td>
</tr>
</tbody>
</table>

1. Steroid abbreviations are as follows: MSTL, mestanolone; MHDN, methandienone, 17α-MT, 17α-methyltestosterone; OXYM, oxymetholone. Methylene unit values were determined by linear interpolation of the retention time of the steroid TMS ether derivative between the retention time of C<sub>14</sub>, C<sub>23</sub>, C<sub>38</sub>, and C<sub>47</sub> alkanes.
2. Not detected

Fig. 3-1. Reconstructed ion chromatograms (m/z 143) from conjugated steroid fractions illustrating the detection of the tetrahydro metabolites produced from (A) mestanolone, (B) oxymetholone, (C) 17α-methyltestosterone and (D) methandienone. Note that compounds 1 and 2 (peak 1 and 2) are not chromatographically resolved. The identity of Compounds 1 - 4 (peak 1 - 4) is given in Table 3-1. Other labelled peaks: 5 = 17β-hydroxy-17α-methyl-5β-androst-1-en-3-one (5) and 6 = its 17-epimer (6), 7 = 17α-methyl-5β-androst-1-en-3α-17β-diol (7) and 8 = its 17-epimer (8). See Experimental for GC/MS conditions.
Results and discussion

Mestanolone and oxymetholone

Reconstructed ion chromatograms illustrating typical urinary profiles of the tetrahydro metabolites resulting from the stereoselective reduction of the Δ⁴- and/or 3-oxo functions of mestanolone, methandienone, 17α-MT and oxymetholone are shown in Fig. 3-1. The identity and methylene unit values of the tetrahydro metabolites are given in Table 3-1. There is a striking similarity between the ion profiles of mestanolone, oxymetholone and methyltestosterone urinary extracts in that they are dominated by a prominent peak at 23.35 min. Peak 1 in Figs. 3-1A and 3-1B was identified as 17α-methyl 5α-androstane-3α,17β-diol by comparison of the mass spectrum of its TMS ether derivative (Fig. 3-2A) with that of an authentic reference compound. Further examination of the GC/MS data showed the presence of a minor compound 3 at 20.45 min and the mass spectrum of which (Fig. 3-3A) was virtually identical to that of 1. On the basis of its GC/MS features and comparison with those of other epimeric 17-methyl steroids [14, 15, 17-19], 3 was identified as 17β-methyl-5α-androstan-3α,17α-diol. A scheme illustrating the route of formation of compounds 1 and 3 from mestanolone is proposed in Fig. 3-4. It is of interest to note that compounds 1 and 3 were also detected in human urine after administration of oxymetholone (2-hydroxymethylene mestanolone) as shown by the characteristic ion chromatogram in Fig. 3-1B. Here, the catabolic transformation was probably initiated by oxidation of the 2-hydroxymethylene group to yield the corresponding β-keto acid intermediate, which then undergoes decarboxylation to give...
mestanolone (Fig. 3.4, Chapter 5) Further reduction of mestanolone by 3α OHDH with concomitant epimerization at the C-17 afforded 1 and 3. It is worth noting that, in addition to decarboxylation, the above mentioned β-keto acid intermediate (17β hydroxy 17α-methyl-5α-androstan-3-oxo-2-carboxylic acid) was shown to be also further oxidized to 17β-hydroxy-17α-methyl-2,3-seco-5α-androstan-2,3-dione acid through a pathway reminiscent to β-keto acid oxidation [20] (see Chapter 5). The formation of the epimeric steroid 3 will be discussed below.

Fig. 3-2. Mass spectra of (A) 17α-methyl-5α-androstan-3α,17β-diol 1 isolated in human urine after administration of mestanolone and oxymetholone, (B) a mixture of compounds 1 and 2 isolated from 17α-MT post administration urine samples and (C) authentic 17α-methyl-5β-androstan-3α,17β-diol 2 as TMS ether derivatives.
Fig. 3-3. Mass spectra of (A) 17β-methyl-5α-androstan-3α,17α-diol 3 isolated in human urine after administration of mestanolone and oxymetholone; and (B) 17β-methyl-5β-androstan-3α,17α-diol 4 isolated from 17α-MT and methandienone urine samples and synthesized from 17α-MT sulfate pyridinium salt as the TMS ether derivatives.

17α-methyltestosterone

From the previous work of Rongone and Segaloff [13] it was expected that compound 2 (Fig. 3-4) would be the major tetrahydro metabolite arising from 17α-MT metabolism. Surprisingly, a prominent peak with the same retention time as that of compound 1 was first detected by GC/MS analysis (Fig. 3-1C). However, comparison of the TMS ether derivatives of authentic reference steroids 1 and 2 showed that both compounds coeluted in the GC/MS conditions used (Table 3-1). Attempts to resolve these isomers chromatographically by modifying GC conditions and using various stationary phases were
unsuccessful. Indirect evidence for the presence of both compounds 1 and 2 in this prominent peak was provided by the presence of trace amounts of epimeric steroid 3 (Fig. 3-1C and Table 3-1), and of compound 4, the TMS ether of which exhibited a mass spectrum (Fig. 3-3B) virtually identical to that of authentic 17α-methyl-5β-androstan-3α,17β-diol 2 (Fig. 3-2C). Thus, compound 4 was identified as 17β-methyl-5β-androstan-3α,17α-diol on the basis of the GC/MS rationales mentioned above.

Further examination of the mass spectral data from authentic steroids 1 and 2 showed characteristic differences in the relative intensities of several of their common fragment ions (Table 3-2). Indeed, ions of m/z 345 and 270 are about 2 and 3 times more intense respectively in the mass spectrum of the 5β-steroid 2 than in its 5α-isomer 1. Conversely, ions of m/z 435 and 360 are about 2 and 3 times more prominent respectively in the mass spectrum of 1. It was then possible to use these mass spectral features not only to differentiate 1 from its isomer 2, but also to determine their respective amounts in the chromatographic peak in which they coelute. This was achieved by analyzing the TMS ether derivative of authentic steroids 1 and 2 and the corresponding metabolites isolated from post-administration urine samples using SIM GC/MS. The resulting data are presented in Table 3-2. From the general equations $ax + by = c$ and $x + y = 100\%$ ($a$, $b$, and $c$ are the relative intensities of particular ions of 1, 2 and coeluting [1 + 2], $x$ and $y$ are the percentage of steroid 1 and 2, respectively, in the coeluting peak), we calculated, using the relative intensities of ions of m/z 270, 345, 360 and 435, that metabolites 1 (17%) and 2 (83%) were excreted in urine in a 1:4.9 ratio (c.v. = 1.36%, n = 3). This ratio is half of that previously
reported by Rongone and Segaloff [13] in a woman with advanced breast cancer. In addition to analytical and methodological differences, the lower 5α/5β ratio which they observed can be rationalized by the fact that 1) 5β-reductases are generally more active in female than in male [5], 2) the disease state of the subject could have hindered or reduced the number of steroid receptors associated with microsomal fractions where the 5α-reductases are located [5,21,22], thus promoting the activity of 5β-reductases which are found in the soluble fraction of liver homogenates [5], and 3) the large doses (1g daily for 4 days) ingested could have saturated the 5α-reductase receptors, thus promoting 5β-reduction. However, the marked affinity of some 17α-methyl-Δ4-3-one steroids for 5β-reductases [23] appears to be the key factor accounting for the predominance of compound 2 in 17α-MT catabolism.

Fig. 3-4 Proposed pathways accounting for the formation of tetrahydro diols 1-4 from oxymetholone, mesterolone, 17α-MT and methandienone metabolism in human.
Methandienone

It was then of interest to study the effect that the introduction of a $\Delta^1$-function in 17$\alpha$-MT would have on the stereoselectivity of the $\Delta^4$-group reduction. Preliminary evidence obtained by GC/MS analysis of urine specimens collected after oral administration of methandienone (Fig. 3-1D) indicated that the biotransformation of methandienone A-ring was substantially different from that of 17$\alpha$-MT (Fig. 3-1C).

Table 3-2. Relative intensities of characteristic fragment ions of urinary metabolites 1 and 2 and of corresponding authentic steroids as TMS ether derivatives

<table>
<thead>
<tr>
<th>Metabolite 3</th>
<th>m/z 143</th>
<th>m/z 255</th>
<th>m/z 270</th>
<th>m/z 345</th>
<th>m/z 360</th>
<th>m/z 435</th>
<th>m/z 450</th>
<th>$5\alpha/5\beta^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (MSTL)</td>
<td>100.0</td>
<td>15.4</td>
<td>8.9</td>
<td>7.0</td>
<td>9.1</td>
<td>35.4</td>
<td>5.2</td>
<td>-</td>
</tr>
<tr>
<td>1 (OXYM)</td>
<td>100.0</td>
<td>15.3</td>
<td>8.9</td>
<td>6.9</td>
<td>9.2</td>
<td>34.9</td>
<td>5.2</td>
<td>-</td>
</tr>
<tr>
<td>1 + 2(17$\alpha$-MT)</td>
<td>100.0</td>
<td>14.4</td>
<td>25.7</td>
<td>11.1</td>
<td>4.4</td>
<td>23.0</td>
<td>3.9</td>
<td>1:4.9</td>
</tr>
<tr>
<td>1 + 2 (MHDN)</td>
<td>100.0</td>
<td>14.3</td>
<td>24.4</td>
<td>10.9</td>
<td>4.5</td>
<td>23.6</td>
<td>3.9</td>
<td>1:3.8</td>
</tr>
<tr>
<td>Authentic 1 4</td>
<td>100.0</td>
<td>14.9</td>
<td>9.3</td>
<td>6.7</td>
<td>9.4</td>
<td>36.4</td>
<td>5.4</td>
<td>-</td>
</tr>
<tr>
<td>Authentic 2 5</td>
<td>100.0</td>
<td>14.0</td>
<td>28.9</td>
<td>12.1</td>
<td>3.5</td>
<td>20.3</td>
<td>3.5</td>
<td>-</td>
</tr>
</tbody>
</table>

1. Relative intensities were measured by SIM GC/MS analysis of the ions listed
2. Compounds 1 and 2 refer to the urinary metabolites detected in mestanalone (MSTL), oxymetholone (OXYM), 17$\alpha$-methyltestosterone (17$\alpha$-MT) and methandienone (MHDN) post-administration urine samples.
3. These values refer to the ratio of compounds 1 and 2 as measured by SIM GC/MS of 17$\alpha$-MT and methandienone urine samples
4. Authentic 17$\alpha$-methyl-5$\alpha$-androstan-3$\alpha$,17$\beta$-diol.
5. Authentic 17$\alpha$-methyl-5$\beta$-androstan-3$\alpha$,17$\beta$-diol.

The relative intensities of diagnostic ions (Table 3-2) determined by SIM GC/MS analysis of the prominent peak eluting at 23.35 min. indicated the presence of both compounds 1 (21%) and 2 (79%) in a ratio of 1:3.8 (c.v. = 2.7%, n = 3). This ratio is very similar to that measured for the corresponding metabolites of 17$\alpha$-MT, thus suggesting...
that the presence of a Δ^1- function in methandienone does not significantly influence its relative affinities for 5α- and 5β-reductases with respect to 17α-MT affinities for the same enzymes.

Further examination of the ion chromatogram (Fig. 3-1D) shows a minor compound 5 the TMS ether mass spectrum of which (Fig. 3-5A) exhibited a molecular ion at m/z 374 (m/z 383, d₉-TMS), diagnostic ions at m/z 317 ([M-57]^+; m/z 326, d₉-TMS), m/z 304 ([M-70]^+; m/z 313, d₉-TMS) which are formed according to mechanisms reported
previously [24], and m/z 143 (m/z 152, d₄-TMS) which are consistent with the proposed structure. The mass spectrum of the corresponding TMS enol-TMS ether derivative (Fig. 3-5B) confirmed the presence of a Δ¹-3-oxo moiety in compound 5 by its molecular ion at m/z 446 (m/z 455, d₀-TMS enol-d₄-TMS ether). Structurally informative ions at m/z 194 and 206 arising from B-ring cleavage provided further evidence for the identity of 5. As evidenced by selective dₓ-TMS labelling, (Fig. 3-6) the formation of an ion at m/z 194 in the mass spectrum of the TMS ether derivative of 5 (Fig. 3-5A) was likely initiated by cleavage of the B-ring [25] with concomitant elimination of 2 hydrogen atoms and long-range migration of the C-17 TMS group to the 3-oxo function. Similar long range TMS group migration has been previously described by Gaskell et al. [26]. We also observed a similar long-range TMS group migration in the mass spectrum of the TMS ether derivative of 6β-hydroxy methandienone [9]. Here diagnostic ions at m/z 209 (cleavage of the C-9—C-10 and C-6—C-7 bonds) and m/z 281 (cleavage of the C-9—C-10 and C-6—C-7 bonds with concomitant migration of the 17-O-TMS group to the 3-oxo function), were shifted by 9 and 18 a.m.u. respectively to m/z 218 and 299 in the mass spectrum of the corresponding dₓ-TMS ether, thus confirming TMS group migration from C-17 to C-3. Durbeck and Buker [24] previously proposed that the ion at m/z 281 was arising from the consecutive losses of trimethylsilanol and a TMSO⁻ radical from the molecular ion [M-90-89]+ without supporting their hypothesis by dₓ-labelling experiment.

In the mass spectrum of TMS enol-TMS ether derivative of 5 (Fig. 3-5B), the formation of a similar ion was strongly favored as well as that of ion at m/z 206 which arises from the cleavage of the C-9—C-10 and C-7—C-8 bonds. Here selective dₓ-TMS labelling
demonstrated that ions of m/z 194 and 206 comprise the A-ring and the C-6 and C-7 atoms, respectively (Fig. 3-6) and not the C-ring and carbon atoms C-7 and C-17 as proposed by Durbeck and Bunker [24]. Compound 5 was thus identified as 17β-hydroxy-17α-methyl 5β-androst-1-en-3-one. This structural assignment was also in accordance with that of the prominent tetrahydro diol 2 resulting from further reductive metabolism of 5 as shown below.

![Diagram with arrows indicating the structural relationships between compounds 5, 6, 7, and 8, and their TMS derivatives.]

Fig. 3-6 Proposed structure of diagnostically important ions of m/z 194, 196 and 206 observed in the mass spectra of 5, 6, 7 and 8 as TMS derivatives.

The faster eluting compound 6 (20.70 min.) which exhibited mass spectral features identical to those of 5 was assigned the structure of 17α-hydroxy-17β-methyl-5β-androst-1-en-3-one (17-epimer of 5).
Finally, the prominent peaks 7 and 8 (Fig. 3-1D) provided mass spectra which were virtually identical (Fig. 3-7A and 3-7B), thus suggesting that 8 was the 17 epimer of 7. Their TMS ether derivative showed a molecular ion at m/z 448 (m/z 466, d₄-TMS) indicating that the Δ⁴- and 3-oxo groups were probably reduced. This hypothesis was supported by the fact that attempt to prepare their corresponding Me-TMS derivatives afforded two compounds with mass spectra identical to those shown in Figs. 3-7A and 7B. Interestingly, the low intensity ion at m/z 196 (m/z 205, d₄-TMS) is analogous to that observed at m/z 194 in the mass spectra of compound 5 and 6 (Figs. 3.5A and 3.5B), and probably arises, as shown by d₄-TMS labelling, from B ring cleavage (Fig. 3-6). Thus, 7 was assigned the structure of 17α-methyl-5β-androst-1-en-3α,17β-diol and 8 was identified as 17β-methyl-5β-androst-1-en-3α,17α-diol.

Fig. 3-7. Mass spectra of (A) compound 7 and (B) compound 8 as TMS ether derivatives isolated in human urine after administration of methandienone.
The data presented above provided strong evidence indicating that the sequential reduction of methandienone A-ring functional groups could proceed as proposed in Fig. 3-8. Firstly, methandienone is converted into 5 and its 17-epimer 6 by the action of 5β-reductases with concomitant epimerization at C-17. Compounds 5 and 6 did not accumulate in large amounts in urine because their 3-oxo group can be rapidly reduced by 3α-OHSDH to yield the epimeric Δ^1-steroids 7 and 8, respectively. This observation is supported by the fact that the rate of 3α-OHSDH is generally faster than that of 4-ene-5α- and 5β-reductases in several tissues [5]. Higher rates of 3α-OHSDH could also account for the absence in urine of 17β-hydroxy-17α-methyl-5β-androstan-3-one (Fig. 3-8), the intermediate metabolite resulting from the 5β-reduction of 17α-MT. In all probability, this metabolite was gradually converted by 3α-OHSDH into 2, as it was produced from the parent steroid. The proposed sequence of methandienone A-ring reduction is also in accordance with the reduction patterns of endogenous Δ^4-3-oxo steroids previously reported in the literature [1,4,6]. Although data presented above showed that low amounts of the 5α-steroids 1 and 3 were generated by methandienone biotransformation, no trace of their corresponding metabolic precursors, analogous to 5 and 7 were detected in urine. This probably reflects the low production rate of 1 and 3 (Fig. 3-1), and consequently that of their Δ^1-3-oxo and Δ^1-3-hydroxy precursors. The metabolic route shown in Fig. 3-8 was proposed to account for the presence in urine of 1, 2, 5, 7 and their corresponding epimers 3, 4, 6 and 8. However, there could be another metabolic route whereby the Δ^1-group of methandienone would first be reduced to give 17α-MT, as the intermediate metabolite, instead of 5 (Fig. 3-8). Although 17α-
MT was not detected in urine after methandienone administration, its probable formation could have been demonstrated by GC/MS analysis of plasma samples, which unfortunately were not available in this study.

Fig 3-8. Proposed metabolic pathways accounting for the formation of methandienone and 17\(\alpha\)-methyltestosterone urinary metabolites resulting from the sequential reduction of their A-ring substituents

**Epimerization of 17\(\alpha\)-methyl-17\(\beta\)-sulfate conjugate**

The formation of 17-epimethandienone from methandienone has been first reported by MacDonald et al. [18] and characterized by Durbeck et al. in human urine by GC/MS [19]. The mechanism underlying this reaction remained highly speculative until Edlund et al. [17] recently reported that epimethandienone was readily formed by nucleophilic attack of water on the sulfate conjugate of methandienone. To further investigate this reaction and identify some of the 17-epimeric metabolites reported above, we synthesized the 17\(\beta\)-sulfate conjugates of several 17\(\alpha\)-methyl-17\(\beta\)-hydroxy steroids, more particularly those of methandienone, 17\(\alpha\)-MT and 17\(\beta\)-hydroxy-17\(\alpha\)-methyl-5\(\beta\)-androstan-3-one. The
latter sulfates were characterized by $^1$H and $^{13}$C NMR and FAB-MS (to be reported separately). Epimerization reactions were carried out with the sulfate pyridinium salts in aqueous solutions at 55°C for 60 min. In these conditions the corresponding 17α-hydroxy 17β-methyl epimers were produced in about 50% yield along with dehydration by-products. Fig. 3-9 illustrates the epimerization reaction of a steroidal sulfate pyridinium salt into its corresponding 17-epimer. The reduction of 17-epimethyltestosterone thus prepared with NaBH$_4$ preponderantly afforded the 17-epimer 4. GC/MS analysis of the synthetic 17-epimeric steroids demonstrated that they were identical to the 17-epimeric steroids previously isolated from post-administration urine samples. These results are in accordance with those reported by Edlund et al.

\[ \text{Fig. 3-9. Epimerization of the 17β-sulfate pyridinium salt of 17α-methyltestosterone (17α-MTSPS) into the corresponding 17α hydroxy-17β-methyl-epimers (17-epi MT) and rearrangement products through a transient carbocation} \]

It should be noted here that epimethandienone is recovered only from the free steroid
steroid fraction [17,19] of human urine. This indicates that this steroid is not reconjugated after epimerization, thus suggesting that epimerization could have occurred in the bladder, after excretion from the kidneys. However, it is also possible that epimerization could have occurred in the liver, the gut and/or in the course of the enterohepatic circulation of the 17-sulfate conjugate, since 17-epimethandienone does not bear chemical features favoring conjugation reactions. This hypothesis was strengthened by the fact that the 17-epimeric metabolites 3, 4, 6 and 8 were solely isolated from the glucuronide conjugate fraction. This suggests that sulfation, and subsequently epimerization, could occur in the hepatic tissue concomitantly with A-ring reduction, where the resulting 3α-hydroxyl group could be readily conjugated with glucuronic acid prior to excretion in urine. Since the biliary route of excretion of neutral steroids is of quantitative importance in human [27], biliary excretion and enterohepatic circulation of methandienone and/or its metabolites could partially account for the presence of the epimeric metabolites in the glucuronide fraction. This hypothesis is supported by the fact that sulfate conjugation favors excretion via this route [28]. Thus, it is probable that epimerization of sulfate conjugates and reconjugation with glucuronic acid could have also occurred in the intestine, where the resulting glucuronides can be partly reabsorbed. However, reabsorption of glucuronides involve passive ionic diffusion which is a slow process. Reabsorption of the unconjugated steroid, formed by bacterial enzymes, followed by glucuronidation in the intestinal wall or liver seems more likely. The reabsorbed metabolites could also undergo further metabolism in the intestine wall and/or at hepatic sites and the products may again be excreted in bile or eliminated in urine.
An excretion route involving biliary excretion and enterohepatic circulation and the apparent low rate of \( \Delta^1 \)-reductases could account for the presence of 8 in urine for more than four days after methandienone ingestion, even when its epimer 7 and the tetrahydrodiols 1 and 2 become barely detectable. Retention of methandienone in certain cells or tissues could also account for this phenomenon but is less likely since it does not provide any rationale for the progressive slowing down and stopping of the urinary excretion of the prominent metabolites 1 and 2 (Fig. 3-1D), while that of compound 8 was still observed. This hypothesis was further supported by the fact that metabolic precursors 5 and 7 (Fig. 3-8) of the tetrahydrodiol 2 were first detected in urine 3.5 h after methandienone ingestion whereas the epimeric steroid 8 and compound 2 were first observed 6.5 and 10.0 h after steroid ingestion. Although the urinary level of compound 2 progressively increased during the next 48 h, those of its metabolic precursors 5 and 7 rapidly decreased, thus suggesting that the rates of the 5\( \beta \), 3\( \alpha \) and \( \Delta^1 \)-reductions were concomitantly increased so that 5 and 7 were rapidly metabolized into 2 and were consequently excreted in urine only in minute amounts. On the other hand, the urinary excretion of compound 8 progressively increased during that period of time and was maintained for more than 4 days after steroid ingestion. This suggests that epimerization at C-17 is an important reaction in methandienone metabolism which appears to decrease the rate of \( \Delta^1 \)-reductases, as its corresponding epimer 7 did not accumulate in urine for a long period of time after steroid ingestion.

The epimerization of 17\( \beta \)-sulfate-17\( \alpha \)-methyl steroids could be of toxicological
importance and related to some of the liver disfunctions known to be induced by these steroids [30-32]. Mechanistically, the 17β-sulfate group reacts through a transient carbocation, which could further react in vivo with phenol, amine, and thiol group of endogenous compounds, including biopolymers, to yield covalent adducts. This sequence of chemical reactions is compatible with the hypothesis that metabolic activation into electrophilic species and their interactions with macromolecular targets are critical events in the induction of toxic processes in living organisms [33].

CONCLUSION

Investigation of the sequential reduction of methandienone A-ring substrates demonstrated that they were reduced according to the sequence Δ4-group, 3-oxo group and Δ1-group. The corresponding Δ1-3-oxo, Δ1-3-hydroxyl metabolic intermediates and the resulting tetrahydrodiols were characterized by GC/MS analysis, whereas the structures of their diagnostically important ions were assessed by d6-TMS labelling. Further analytical and metabolic evidence was obtained using mestanolone, 17α-MT and oxymetholone as model steroids. For each metabolite of interest, we detected and identified the corresponding 17-epimer. We have shown that these epimers were produced upon nucleophilic substitution by water of the labile sulfate conjugates. This finding is in agreement with the study of Edlund et al. [17]. Epimerization of 17β-sulfate conjugates could be of toxicological importance owing to the formation of transient electrophilic species that could ultimately promote toxic effects.
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References


9. R. Massé et al., unpublished results.


CHAPTER 4†

STUDIES ON ANABOLIC STEROIDS. 7.

ANALYSIS OF URINARY METABOLITES OF FORMEBOLONE IN MAN

BY GAS CHROMATOGRAPHY - MASS SPECTROMETRY

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Summary

The metabolism of formebolone in man is described. After Sep Pak™ C₁₈ extraction, the unconjugated, conjugated and acidic steroids were separated, and conjugates were hydrolysed. The resulting neutral steroids were identified by GC/MS analysis. Two metabolites identified as 2-hydroxymethyl-11α,17β-dihydroxy-17α-methyl-androsta-1,4-diene-3-one and 11α-hydroxy-17α-methyltestosterone were isolated from both the unconjugated and conjugated steroid fractions. An acidic metabolite identified as 11α,17β-dihydroxy-17α-methyl-androsta-1,4-diene-3-one-2-carboxylic acid was isolated from the unconjugated steroid fraction. This acidic steroid was stable when derivatized as the methyl ester TMS ether derivative but its TMS ester-TMS ether derivative progressively degraded to yield the 17-mono and 11,17-di-O-TMS ether derivatives of 11α-hydroxymethandienone. GC/MS features of formebolone metabolites are presented and the routes of biotransformation are discussed.
Introduction

The problem of misuse of anabolic steroids in sports and their ban by the International Olympic Committee [1] have fostered the development of a new approach for the determination of these compounds and their urinary metabolites in human [2]. This methodology is based on the use of solid supports for the isolation of unconjugated and conjugated steroids, enzymatic hydrolysis of steroid conjugates, derivatization of the neutral steroids and selective determination of the resulting derivatives using high resolution gas chromatography/mass spectrometry (HRGC/MS). A thorough knowledge of the biotransformation routes and urinary excretion profiles of the metabolites of anabolic steroids is an essential prerequisite to their GC/MS monitoring. The unique structural features in many of these substances can significantly alter their biotransformation routes with respect to those of testosterone, after which anabolic steroids were originally synthesized. In that context, a research program on the metabolism of anabolic steroids in man has been undertaken in this laboratory [3-6].

This paper deals with the GC/MS analysis of the urinary metabolites of formebolone (2-formyl-11α,17β-dihydroxy-17α-methyl-androsta-1,4-dien-3-one) [7]. The anabolic properties of this steroid have been investigated in human [8,9] and its biotransformation was investigated in the rat [10]. To the best of our knowledge, the metabolism of formebolone in man and the determination of its urinary metabolites by GC/MS have not been reported in the literature. In the present paper, a detailed study of the urinary
excretion profile of formebolone metabolites in man is described. Three metabolites were characterized by GC/MS analysis and their identity was assessed either by chemical synthesis or comparison with authentic reference steroids. Trimethylsilylation of formebolone 2-carboxylic acid metabolite afforded two major by-products which were characterized by GC/MS analysis.

Experimental

Steroids, chemicals and reagents

Formebolone was obtained from the LPB Instituto Farmaceutico (Milan, Italy). 11α,17β-Dihydroxy-17α-methyl-androst-4-en-3-one (11α-hydroxy-17α-methyltestosterone) and 11α, 17β-dihydroxy-17α-methyl-androsta-1,4-diene-3-one (11α-hydroxy methandienone) and 5α-androstan-17-one were purchased from Steraloids (Wilton, N.H.).

Inorganic salts (J.T. Baker Chemical Co., Philipsburg, NJ) were of analytical grade. All the solvents were of HPLC grade (Caledon Laboratories Ltd., Georgetown, Ontario, Canada) and were used as provided. Sodium chlorite (tech. 80%), dithioerithritol, trimethylsilyl iodide (TMSI), trimethylchlorosilane (TMCS), sulfamic acid (99+ %) and 1-methyl-3-nitro-1-nitrosoguanidine (MNNG)-diazomethane kits were supplied by Aldrich Chemical Company, Inc. (Milwaukee, WI); Sep-Pak™ C18 cartridges were purchased from Waters Associates (Milford, MA). N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) was purchased from Regis Chemical Co. (Morton Grove, IL). (N,O)-bis-d5-
(trimethylsilyl)-acetamide (d₅-BSA) and d₅-trimethylchlorosilane (d₅-TMCS) were obtained from MSD Isotope, Pointe-Claire, Canada.

**Urine samples and extraction of metabolites**

Blank urine samples were collected during a period of 24 h before the administration of a single dose of 40 mg of formebolone to a healthy male volunteer. Urine samples were then collected for the next 7 days and were frozen in the dark at -20°C immediately after collection.

The extraction of steroid metabolites from urinary samples (3-5 ml aliquots), their fractionation into unconjugated and conjugated fractions and the hydrolysis of the conjugated steroids were carried out as described previously*[2,3,6].

For the extraction of acidic metabolites, 10 ml aliquots of urine were passed through a Sep-Pak™ C₁₈ cartridge (prewashed with 5 ml of methanol and 5 ml of water). The cartridge was then washed with 2 ml of hexane and steroids were eluted with 5 ml of MeOH. The solvent was evaporated to dryness under a stream of nitrogen at 40°C and the residue was dissolved in 1 ml of water, the pH of which was set to 1 with 10% (v/v) HCl. The acidic compounds were then extracted with diethyl ether (2 x 5 ml). After evaporation of the solvent, the residue was derivatized and analyzed by GC/MS as described below.

* See Chapter 2 and 3 for details.
Preparation of derivatives

TMS enol-TMS ether derivatives. The steroidal extract was transferred to a 0.3 ml Reactivial and 500 ng of 5α-androstan-17-one (internal standard) was added. After evaporation of the solvent at 40°C under a nitrogen stream, 0.5-1 mg of dithioerythritol was added and the vial stoppered under nitrogen. After the addition of 50 μl of MSTFA: TMSI (100:2, v/v), the mixture was heated at 70°C for 30 min, and 1 μl was injected into the gas chromatograph.

TMS ether and d₅-TMS ether derivatives. The urinary extract was treated with 50 μl of a mixture of MSTFA/pyridine/TMCS (10:10:1, v/v/v) as described above and the resulting mixture heated at 70°C for 30 min. The sample was then cooled to room temperature and the solvent removed at 60°C under a nitrogen stream. The dry residue was dissolved in 50 μl of hexane and 1 μl was injected into the gas chromatograph. The corresponding perdeuterated d₅-TMS derivatives were prepared using a mixture of d₅-BSA/pyridine/d₅-TMCS (10:10:1, v/v/v) under the conditions mentioned above.

TMS ester-TMS ether and d₅-TMS ester-d₅-TMS ether derivatives. These mixed derivatives of the acidic metabolite 3 were prepared as follows: the urinary extract was dissolved in 50 μl of a mixture of pyridine/MSTFA/TMCS (90:10:1, v/v/v) and heated at 70°C for 30 min. The solution was then evaporated to dryness and the residue dissolved in 50 μl of hexane. The d₅-TMS derivative was prepared using pyridine/d₅-BSA/d₅-TMCS (90:10:1, v/v/v) under the same experimental conditions. Note that when the relative amount of MSTFA was increased, the degradation of the TMS ester-TMS ether derivatives.
ether derivative of 3 proceeded at a faster rate.

**O-methoxime TMS ether (MO-TMS) and MO-d₄-TMS derivatives.** The dry steroidal extract was dissolved in 50 μl of a 5% (w/v) solution of methoxylamine hydrochloride in pyridine and heated at 70°C for 30 min. After evaporation of the solvent, the TMS and d₄-TMS ether derivatives were then prepared by adding 25 μl of a mixture of MSTFA/TMCS (10:1, v/v) or a mixture of d₄-BSA/d₄-TMCS (10:1, v/v). The resulting mixture was heated at 70°C for 30 min. The solvent was evaporated at 60°C under a stream of nitrogen and hexane (50 μl) was added to the dry residue. One μl of the resulting solution was injected in the gas chromatograph for GC/MS analysis.

**Methyl ester TMS ether derivatives.** The methyl ester derivatives of the acidic metabolites were prepared using 1-methyl-3-nitro-1-nitrosoguanidine (MNNG)-diazomethane kits. The steroid containing residue was dissolved in 2 ml of ether and the solution was placed in the outside tube of the glass apparatus. Then, 50 mg of MNNG was placed in the inside tube along with 0.5 ml of water. The lower part of the apparatus was immersed in an ice bath and ca. 0.5 ml of 5 N KOH was slowly added dropwise with a syringe through a silicone rubber septum, until the reaction was completed. The reaction mixture was left for 20 min. at room temperature and the solvent was evaporated to dryness. The residue was then reacted with a mixture of pyridine/MSTFA/TMCS as described above.
Gas chromatography/mass spectrometry

The steroidal extracts were analyzed using an HP-5970 mass-selective detector (Hewlett-Packard, Palo Alto, Calif., USA) linked to an HP-5890 gas chromatograph equipped with HP-5 (cross linked 5% phenylmethylsilicone) fused silica capillary column (25 m x 0.2 mm i.d., 0.33 μm film thickness). The injections were made in the splitless mode (purge off time, 30 sec) using helium as a carrier gas at a rate of 0.8 ml/min. The oven temperature was maintained at 100°C for 1 min and programmed at 16°C/min to 220°C and then 3.8°C/min to 300°C and maintained for 10 min.

Synthesis of 11α,17β-dihydroxy-17α-methyl-δandrosta-1,4-dien-3-one-2-carboxylic acid 3

Compound 3 was synthesized according to a method adapted from that reported by Bal et al. [11]. To a solution of formebolone (104 mg, 0.3 mmole) in 4 ml of acetone was added 232 mg (2.4 mmole) of sulfamic acid dissolved in 3 ml of water. The resulting solution was stirred at room temperature while a solution of sodium chlorite (216 mg, 2.4 mmole) in 1 ml of water was added dropwise over a period of 2 min. The resulting yellowish mixture was then stirred for 5 h. The solvent was partly evaporated under vacuum at room temperature until a gelatinous mass was obtained, 3 ml of water were added and pH was adjusted to 9.5 with an aqueous 2 M solution of KHCO₃/K₂CO₃ (1:1, w/w). The resulting solution was extracted three times with 10-ml portions of diethyl ether to remove non acidic compounds. The aqueous phase was acidified to pH 2 with 2 M HCl and extracted as above. The combined organic phases were successively
washed twice with 2 ml of water, dried over anhydrous sodium sulfate and evaporated to dryness under vacuum. The resulting amorphous white solid was recrystallized from diethyl ether to give 25.7 mg (23.7% yield) of pure 3 as white crystalline needles. MP, 86-87°C. IR, \( \nu_{\text{max}} \) 3545, 1745, 1725, 1660, 1650, 1580 and 1460 cm\(^{-1}\). \( ^1\)H NMR (400 MHz, DMSO-\( d_6 \)), \( \delta \) 0.77 (s, 3H, 18-H\(_3\)), 0.99 (s, 3H, 20-H\(_1\)), 1.30 (s, 3H, 19-H\(_1\)), 3.9 (t-d, 1H, 11\( \beta \)-H), 6.17 (s, 1H, 4-H), 8.75 (s, 1H, 1-H). \( ^1\)C NMR, \( \delta \) 153 (C-18), 17.7 (C-19), 26.2 (C-20), 66.4 (C-11), 79.2 (C-17), 122.9 (C-4), 123.9 (C-2), 165.2 (C-5), 169.1 (C-1), 173.0 (2-COOH), 185.5 (C-3).

Results and discussion

Identification of metabolites

Given the structural features of formebolone, it was expected that in vivo biotransformations would be directed by A-ring substituents, particularly at the formyl group. Two metabolites were isolated from the unconjugated fraction (Fig. 4-1A) as well as from the conjugated fractions. We determined by selective ion monitoring GC/MS that 44.2% of compound 2 and 85.5% of compound 5 were excreted in the unconjugated steroid fraction. These data suggested that 2 was probably more hydrophillic than 5.

Metabolite 2. The molecular ion at \( m/z \ 562 \ (m/z \ 589, \text{d}_4\text{-TMS}) \) in the mass spectrum of TMS ether derivative of 2 (Fig. 4-2A) was indicative of the presence of three hydroxyl groups. Structurally informative ions at \( m/z \ 222 \ (m/z \ 231, \text{d}_5\text{-TMS}) \) and 235
(m/z 244, d₄-TMS) arising from B-ring cleavages provided strong evidence for the presence of a 2-hydroxymethyl group. As evidenced by d₄-TMS labelling, ion at m/z 207 (m/z 213, d₄-TMS) is apparently formed by elimination of a CH₄ radical from the TMS group of the fragment ion of m/z 222. Two fragment ions, originating from the same B-ring cleavages and accompanied by long-range migration of a TMS group on the 3-keto function were observed at m/z 281 (m/z 299, d₄-TMS) and 294 (m/z 312, d₄-TMS).
The formation of such ions in the mass spectra of the TMS derivatives of steroids bearing Δ^1,4-3-keto, Δ^1-3-keto and Δ^1-3-hydroxy groups has been reported [6]. Long-range trimethylsilyl group migration under electron impact has been previously reported in hydroxylated Δ^4-3-keto cholestene [12]. The proposed structural assignment for compound 2 was ascertained by the mass spectral features of its MO-TMS derivative.

Fig. 4-2. Electron-impact mass spectra of metabolite 2 as (A) TMS ether, (B) MO-d₅-TMS and (C) MO-TMS derivatives.
(Fig. 4-2C). The molecular ion at m/z 591 (m/z 618, d₉-TMS) and ion fragments at m/z 560 (m/z 587, d₉-TMS), 470 (m/z 488, d₉-TMS), 380 (m/z 389, d₉-TMS) and 143 (m/z 152, d₉-TMS) were consistent with the proposed structure. Interestingly, d₉-TMS labelling (Fig. 4-2B) showed that ion at m/z 290 originates from two different fragmentation pathways. As illustrated in Figs. 4-2B and 4-2C, the successive elimination of a MeO' radical and three molecules of trimethylsilanol gave rise to an ion at m/z 290 which was not shifted in the mass spectrum of the d₉-TMS derivative. Conversely, specific cleavage of the C-10—C-11 and C-8—C-14 bonds also give rise to a fragment ion of m/z 290 comprising A and B-rings which was shifted to m/z 299 in the d₉-TMS derivative. On the basis of the mass spectral properties of the TMS ether and MO-TMS derivatives compound 2 was assigned the structure of 11α,17β-dihydroxy-2-hydroxymethyl-17α-methyl-androsta-1,4-diene-3-one.

Metabolite 3. This metabolite was isolated from the unconjugated and acidic steroid fraction (Fig. 4-1B). This suggested that it was probably arising from the oxidation of the 2-formyl group into the corresponding carboxylic acid. This hypothesis was ascertained by the preparation of the corresponding TMS ester-TMS ether and methyl ester-TMS ether derivatives, the mass spectra of which were consistent with the proposed structure (Fig. 4-3). Furthermore, these mass spectral features were identical to those of the corresponding derivatives of 11α,17β-dihydroxy-17α-methyl-androsta-1,4-dien-3-one-2-carboxylic acid synthesized by selective oxidation of 2-formyl group of formebolone.
Fig. 4-3. Electron-impact mass spectra of metabolite 3 as (A) TMS ester-TMS ether and (B) methyl ester-TMS ether derivative.

The TMS ester-TMS ether derivative (Fig. 4-3A) exhibited a molecular ion at m/z 576 (m/z 603, d<sub>6</sub>-TMS) and fragment ions at m/z 220 (m/z 229, d<sub>6</sub>-TMS), 235 (m/z 244, d<sub>6</sub>-TMS) and 249 (m/z 258, d<sub>6</sub>-TMS) which were analogous of those formed by identical cleavage of B-ring bonds in the mass spectrum of compound 2 TMS ether derivative (Fig. 4-2B). The mass spectrum of methyl ester-TMS ether derivative of 3 (Fig. 4-3B) was also consistent with the proposed structure. Interestingly, the formation of fragment ions of m/z 220, 235 and 249 suggested that intramolecular transesterification of the methyl ester function, most probably by the 11α-OTMS group, seems to occur upon electron-impact ionization prior to cleavage of the B-ring. This hypothesis was supported
by the shift of these fragment ions to m/z 229, 244 and 258 as in the mass spectrum of the \( \text{d}_9\)-TMS ester derivative (Fig. 4-3A). Other fragmentation routes gave rise to ions at m/z 338 (m/z 338, \( \text{d}_9\)-TMS; M-2TMSOH) and 396 (m/z 405, \( \text{d}_9\)-TMS; M-MeOH-TMSOH) and were consistent with the proposed structure.

**Metabolite 5.** A prominent peak eluting at 28.3 min was observed in the selected ion chromatogram (Fig. 4-1A) obtained from repetitive scanning GC/MS of the unconjugated steroid excreted following administration of formebolone. The mass spectrum of its TMS ether derivative (Fig. 4-4A) showed a molecular ion at m/z 462 (m/z 480, \( \text{d}_9\)-TMS) and fragment ions at m/z 143 (m/z 152, \( \text{d}_9\)-TMS) m/z 405 (m/z 423, \( \text{d}_9\)-TMS; M-57), m/z 392 (m/z 410, \( \text{d}_9\)-TMS; M-70) which are characteristics of D-ring cleavage and rearrangements of 17\( \beta \)-hydroxy-17\( \alpha \)-methyl steroids [13-15]. The molecular ion at m/z 462 and fragment ions at m/z 372 and 282 were suggestive of a \( \Delta^4 \)-3-keto steroid with no substituent at C-2. This hypothesis was supported by the formation of the corresponding TMS enol-TMS ether derivative, the mass spectrum (Fig. 4-4B) of which showed a molecular ion at m/z 534 (m/z 552, \( \text{d}_9\)-TMS-enol-\( \text{d}_9\)-TMS ether mixed derivative) and fragment ions at m/z 445 [M-TMSO]\(^+\), 444 [M-TMSOH]\(^+\) and 389 [M-145]\(^+\). The latter ion comprises the A-, B- and C-rings and probably arises from the D-ring fragmentation with concomitant elimination of a hydrogen radical from the C-ring [13,16]. Compound 5 was identified as 11\( \alpha \),17\( \beta \)-dihydroxy-17\( \alpha \)-methylandrost-4-en-3-one. Its identity was ascertained by comparison of the GC/MS properties of TMS ether and MO-TMS derivatives of an authentic standard.
Degradation of compound 3 on trimethylsilylation

Decarboxylation of β-keto acid in acidic or basic conditions is a well known reaction in the chemistry of organic compounds. The characterization of 17β-hydroxy 17α-methyl-5α-androstan-3-one (mestanolone), a urinary metabolite of oxymetholone [17] in man was reported [6]. The formation of this latter metabolite was rationalized according to a metabolic pathway involving the oxidation of oxymetholone 2-formyl group to the corresponding β-keto acid which was then readily decarboxylated to mestanolone. No trace of the intermediate 3-keto-2-carboxylic acid was detected in urine after oxymetholone administration, probably because of the great ability of this steroid to undergo decarboxylation. Interestingly, its homologous β-keto acid 3 produced from formebolone metabolism was much more stable, most likely because of the presence of a Δ1,4 function in this metabolite. The methyl ester-TMS ether derivative was highly
stable and no by-product was detected by GC/MS analysis over a period of 24 h after derivatization. The corresponding TMS ester-TMS ether derivative was kept in hexane after evaporation of the solvent and was apparently stable under the GC/MS conditions used as shown in Fig. 4-1B. Surprisingly, upon prolonged standing at room temperature, this derivative progressively degraded to give derivatives 6 and 7 as shown in Fig. 4-5. These by-products were identified as the 11, 17-di-O-TMS (M** 460) and 11-hydroxy-17-O-TMS (M** 388) derivatives of 11α,17β-dihydroxy-17α-methyl-androsta-1,4-dien-3-one (11α-hydroxymethandienone). Their identity was ascertained by comparison with the authentic steroid and their structures are illustrated in Fig. 4-6. GC/MS monitoring of the degradation reaction of 3 (Fig. 4-5) showed that 6 was the first by-product resulting from decarboxylation of the TMS ester function (Fig. 4-6). Upon prolonged standing at room temperature, compound 7 was then formed. Using 11α-hydroxymethandienone as substrate, we showed that derivative 6 was stable for more than 24 h under the experimental conditions used, demonstrating that derivative 7 was not produced by degradation of derivative 6. This strongly suggested that the loss of the TMS group at C-11 in derivative 3 occurs because of the presence of a TMS ester group at C-2. The loss of the TMS group at C-11 appears to be concerted with the elimination of the carboxyl group since no trace of a by-product, without loss of the COOTMS group, that could have resulted from the selective elimination of the TMS group at C-11, was detected neither in the urinary extracts nor in the derivatization mixture of authentic 3. Examination of a molecular model of 3 (Fig. 4-6) shows a favorable coordination between the TMS group at C-11 and the carboxyl function at C-2 that could lead to the formation of derivative 7.
Fig. 4-5. Reconstructed ion chromatograms from GC/MS analysis of a urine sample collected 6 h after formebolone administration. Free and acidic steroid fractions were isolated and derivatized with a mixture of MSTFA and TMCS in pyridine. The mixture was evaporated to dryness and the residue dissolved in hexane. The progressive degradation of the TMS derivative 3 and the formation of the resulting by-products 6 and 7 were monitored (A) 1.0 h, (B) 8.5 h and (C) 19.0 h after derivatization was initiated. See experimental for details.

Fig. 4-6. Degradation route of the TMS ester-TMS ether derivative of compound 3 upon standing at room temperature in hexane.
Interestingly, the degradation of the TMS ester derivative of 3 proceeded at a slower rate when derivatization was carried out with an urinary extract, with respect to the degradation rate observed with authentic 3. This suggest that derivative 3 is apparently stabilized by interaction with some urinary components, thus decreasing its intrinsic ability to undergo degradation into derivatives 6 and 7. The mechanistic aspects of these reactions were not further investigated.

**Formebolone metabolic routes**

The biotransformation routes of formebolone 1 are summarized in Fig. 4-7. Contrary to several anabolic 17α-methyl steroids [2-6,14,18] no metabolite resulting from hydroxylation of formebolone were detected in urine. Metabolism was solely directed at the A-ring, where the reactive formyl group was reduced to the corresponding 2-hydroxymethyl metabolite 2 which was isolated in the unconjugated and conjugated steroid fractions. Conversely, oxidation of the 2-formyl group gave the β-keto acid 3. The occurrence of 11α-hydroxy-17α-methyltestosterone 5 in urine samples collected after formebolone ingestion suggested that compound 3 was probably metabolized into the intermediate β-keto acid 4 (not detected in this study) by regioselective reduction of the Δ^1^-function. Then, this acidic steroid would undergo decarboxylation to give compound 5. An alternative metabolic route could involve decarboxylation of 3 into 11α-hydroxymethandienone which would then undergo reduction of its Δ^1^-function to yield 5. However, this metabolic route seems unlikely since no trace of 11α-hydroxymethandienone was detected in urine. Instead, this steroid was shown to be a
degradation product of the TMS ester-TMS ether derivative of the carboxylic acid 3 and not a compound resulting from the biotransformation of formebolone. Finally, no metabolite that could have resulted from epimerization at C-17 were detected. This observation was in accordance with the fact that no sulphoconjugate of the metabolites reported in this study were isolated from urine [2,3,6].

Fig. 4-7. Proposed metabolic routes accounting for the occurrence of formebolone urinary metabolites 2, 3 and 5.

In conclusion, it has been shown that formebolone is metabolized in man mainly by oxido-reduction reactions of its 2-formyl group. The 3-keto-2-carboxylic metabolite was further transformed by decarboxylation and reduction of its $\Delta^1$-function to give 11$\alpha$-hydroxy-17$\alpha$-methyltestosterone. No metabolite, the formation of which could have resulted from reduction of the $\Delta^4$- and/or 3-keto group, epimerization at C-17 or hydroxylation at other positions of the steroid, was detected in this study. Unchanged formebolone was not detected in urine, thus indicating extensive first-pass metabolism and/or excretion in bile and faeces and poor absorption of the steroid. These observations
are in accordance with those of De Marchi et al. who reported similar findings about formebolone absorption and excretion in rat [19]. The data presented above also indicated that the methyl ester rather than TMS ester derivative should be prepared to detect the acidic metabolite 3 so as to prevent the formation of the by-products which resulted from the degradation of its TMS ester derivative.

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References


CHAPTER 5

5.1. STUDIES ON ANABOLIC STEROIDS.8.

GC/MS CHARACTERIZATION OF UNUSUAL ACIDIC SECO METABOLITES
OF OXYMETHOLONE IN HUMAN URINE†

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Summary

One of the biotransformation routes of oxymetholone (17β-hydroxy-2-hydroxymethylene-17α-methyl-5α-androstan-3-one) in man leads to the formation of 17β-hydroxy-17α-methyl-5α-androstan-3-one (mestanolone). To demonstrate that this latter steroid may be formed by decarboxylation of an intermediate metabolite of oxymetholone bearing a 2-carboxylic group, we studied the urinary excretion of acidic oxymetholone metabolites. Five new acidic metabolites are reported here for the first time, among which four are unusual seco steroids resulting from the oxidative cleavage of A-ring. The most abundant compound is 17β-hydroxy-17α-methyl-2,3-seco-5α-androstene-2,3-dioic acid 1, the cumulative excretion of which accounted for 1.52% of the dose. Three other seco diacids were produced in smaller amounts, namely 17β-hydroxy-17α-methyl-4α-homo-3,4-seco-5α-androstane-3,4-dioic acid 3, 17β-hydroxy-17α-methyl-4-nor-1,2-seco-5α-androstane-1,2-dioic acid 4 and 17β-hydroxy-17α-methyl-4-nor-2,3-seco-5α-androstane-2,3-dioic acid 5. The fifth acidic metabolite was identified as 3α, 17β-dihydroxy-17α-methyl-5α-androstane-2β-carboxylic acid 2. The excretion in urine of these acidic metabolites suggests that the 2-hydroxymethylene group in oxymetholone is readily oxidized to yield the corresponding β-keto acid which can be 1) decarboxylated to form mestanolone; 2) reduced at C-3 to give compound 2; and 3) further oxidized to afford the unexpected seco diacids 1, 3, 4 and 5. The identity of compounds 1 and 2 was ascertained by GC/MS and 1H and 13C-NMR analysis of reference compounds. The other metabolites were characterized by GC/MS analysis.
Introduction

Despite the widespread use of oxymetholone (17β-hydroxy-2-hydroxymethylene-17α-methyl-5α-androstan-3-one) in therapeutics [1-7], relatively little is known about the biotransformation pathways of this androgenic-anabolic steroid in human. MacDonald et al. reported the isolation and identification of two metabolites of oxymetholone arising from the reduction of the 2-hydroxymethylene and 3-keto groups, the urinary excretion of which accounted for 5% of the administered dose [8]. In a previous communication [9], we have reported that mestanolone (17β-hydroxy-17α-methyl-5α-androstan-3-one) is excreted in urine after oral administration of oxymetholone to man. This finding indicates that one of the plausible metabolic routes accounting for the formation of this steroid from oxymetholone could proceed via the oxidation of oxymetholone 2-hydroxymethylene group to the corresponding 2-carboxylic acid (not detected in urine) with subsequent decarboxylation of the latter β-keto acid to yield mestanolone.

Using formebolone (2-formyl-11α,17β-dihydroxy-17α-methyl-androsta-1,4-dien-3-one) as a model substrate, we provided lately further evidence for the occurrence of these reactions in the metabolism of steroids bearing a 2-hydroxymethylene or a formyl group. Indeed we isolated and characterized from human urine the corresponding 2-carboxylic acid of formebolone and 11α-hydroxy-17α-methyltestosterone, which probably arise from the decarboxylation of the acidic metabolite and concomitant reduction of its 1-ene group [10].
In that perspective, we undertook a specific investigation of oxymetholone biotransformation in order to characterize potential acidic metabolites which are likely to be excreted in human urine. This paper describes the characterization of five acidic metabolites of oxymetholone among which four are unusual seco steroids arising from the oxidative cleavage of the A-ring. To the best of our knowledge, such steroidal metabolites have never been reported in the literature pertaining to the metabolism of androgenic-anabolic steroids in man and animal.

Experimental

Materials

Oxymetholone (Anapolon 50) was obtained from Syntex Research, Palo Alto, California. Other steroids were purchased from Sigma Chemical Co., St-Louis, Missouri, and Steraloids, Wilton, New Hampshire. Their purity was assessed by GC/MS analysis of their TMS derivatives. Biochemicals, chemicals, derivatization reagents, solvents and materials for solid phase extraction of urinary steroids used in this study have been previously described [10,11]. N,O-bis-([d$_5$]-trimethylsilyl)-acetamide (d$_5$-BSA) and d$_5$-trimethylsilyl chloride (d$_5$-TMSCl) were purchased from MSD-Isotopes, Pointe-Claire, Québec. Potassium permanganate, sodium periodate and potassium carbonate were from J.T. Baker Chemical Co., Phillipsburg, New Jersey. Diethylaluminum cyanide (1 M solution in toluene); 3-chloroperbenzoic acid (50-60%) (mCPBA), 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) and the MNNG-diazomethane kits were purchased from
Aldrich Chemical Co., Milwaukee, Wisconsin. Prior to use, mCPBA was treated with an aqueous phosphate buffer (pH 7.5) and extracted with dichloromethane [12]. The organic layer was evaporated under vacuum and the residue was used as such. Aluminum oxide (neutral) was from Terochem Scientific, Edmonton, Alberta. Basic alumina was prepared as follows: 50 g of neutral alumina was mixed with 5 ml of a 10% sodium methoxide solution in methanol. The resulting solid was dried in vacuo and activated at 125°C for 4 h.

**Steroid administration**

Several blank urine samples were collected during a 24 h period prior to the administration of oxymetholone from two male volunteers aged between 30 and 35 years. A single 50 mg oral dose of the steroid was administered and urine samples were collected over a period of six days and immediately frozen at -20°C until analyzed.

**Extraction of acidic metabolites**

In a typical experiment, 5-10 ml of a blank or post-administration urine sample was applied on a Sep-Pak C₁₈ Cartridge (prewashed with 5 ml of methanol and 5 ml of water). The cartridge was then successively washed with 5 ml of water and 2 ml of hexane. The steroidal metabolites were eluted with 5 ml of methanol and the eluate was evaporated to dryness under a nitrogen stream at 40°C. The residue was dissolved in 1 ml of water and pH adjusted to 8 with KHCO₃. The solution was extracted with 2 x 5 ml of diethyl ether. The ether layer was decanted, and evaporated to dryness. The
residue was derivatized as described below and analyzed by GC/MS for unconjugated and neutral steroidal metabolites. The remaining aqueous phase was acidified with 1 M HCl to pH 1 - 2, and extracted with 2 x 5 ml of ether. The organic phase was decanted and 500 ng of internal standard (ISTD, oxandrolone) was added. The resulting final solution was evaporated to dryness under nitrogen and the residue was derivatized as described below prior to GC/MS analysis.

**Trimethylsilyl (TMS) ester and TMS ether derivatives**

The steroid containing residue was dissolved in 50 µl of pyridine, 50 µl of N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) and 5 µl of trimethylsilyl chloride (TMSCl). The mixture was heated at 70°C for 30 min. The excess reagent was then evaporated at 60°C under a nitrogen flow. The residue was redissolved in 50 to 100 µl of hexane and 1 µl of this solution was injected for GC/MS analysis. The deuterated TMS analogues (d9-TMS) were prepared using d9-BSA/pyridine/d9-TMSCl (10:10:1, v/v) under the same experimental conditions.

**Methyl ester-TMS ether derivatives**

Methyl ester derivatives of the acidic metabolites were prepared using 1-methyl-3-nitro-1-nitrosoguanidine (MNNG)-diazomethane kits. The steroid containing residue was dissolved in 2 ml of ether and the solution placed in the outside tube of the glass apparatus. Then, 100 mg of MNNG was added in the inside tube along with 0.5 ml of water. The lower part of the apparatus was immersed in an ice bath and ca. 0.6 ml of
5 N KOH was added dropwise with a syringe through a silicone rubber septum. When the reaction was completed, the reaction mixture was left for 20 min at room temperature and the solvent was evaporated to dryness. The residue was then reacted with a mixture of MSTFA/TMSCl as described above.

**GC/MS and NMR analysis**

The derivatized steroid extracts were analyzed using an HP-5970 mass-selective detector (MSD) (Hewlett-Packard, Palo Alto, California), interfaced to an HP-5890 gas chromatograph equipped with an HP-5 (cross-linked 5% phenylmethylsiline) fused silica capillary column (25 m x 0.2 mm i.d., 0.33 μm film thickness). Splitless injection of 1-2 μl of derivatized urinary extracts was performed with a purge off time of 30 sec. The initial oven temperature was maintained at 100°C for 1 min and programmed at 16°C/min to 220°C and then at 3.8°C/min to 300°C. The GC injection port and GC/MS interface were heated at 270 and 310°C, respectively. Helium was used as carrier gas at a rate of 0.8 ml/min. The MSD was used in the selective ion monitoring (SIM) and repetitive scanning modes for the specific detection of the steroids of interest and recording of their full mass spectra. Negative ion fast atom bombardment mass spectrometry (FAB/MS) was carried out using a Kratos MS50TC mass spectrometer. The bombarding atom beam was 6-kV Xenon atoms of 1 mA current. Glycerol was used as the sample supporting matrix and the xenon flow-rate was 0.5 ml/min. The FAB mass spectra were recorded at a resolution of 10,000 (10% valley).
$^1$H and $^{13}$C NMR spectra of the synthesized steroids were recorded on a Varian VXA 300 and a Bruker WH 400 spectrometer. Structures were assigned by 2D COSY $^1$H NMR and $^{13}$C/$^1$H heteronuclear correlated 2D NMR.

**Quantitation of metabolite 1 in urine**

A standard curve for the quantitation of 1 in the 10-700 ng/ml range was prepared as follows: aliquots of stock solutions of 1 (0.1 and 1 mg/ml) in methanol were transferred into 200 μl vials, along with 500 ng of oxandrolone (ISTD) in methanol so as to obtain 30-3500 ng of the diacid 1 per vial. The solvent was evaporated and the residue treated with MSTFA and TMSCl to prepare the corresponding TMS ester-TMS ether derivatives. Duplicate samples were prepared for each concentration point and each sample was analyzed twice by GC/MS in the selective ion monitoring (SIM) mode. Peak area ratios of ion m/z 143 (a prominent ion in the mass spectra of 1 and oxandrolone TMS derivatives) were measured. The data can be fitted by the regression equation $C_1 = 76.7 A_r + 11.8$ ($r^2 = 0.998$) where $C_1$ is the concentration of compound 1 in ng/ml and $A_r$ the peak area ratio of compound 1 to oxandrolone. Recovery tests were carried out for concentrations of 100 and 300 ng/ml of compound 1 using 5 ml aliquots of blank urine samples collected from volunteers prior to oxymetholone administration. Recoveries were 74.2 $\pm$ 2.7% (c.v. = 3.6%, n=6) and 76.0 $\pm$ 2.8% (c.v. = 3.6%, n = 6), respectively.
Synthesis of reference steroids

17β-Hydroxy-17α-methyl-2,3-seco-5α-androstane-2,3-dioic acid 1.

Oxymetholone (200 mg, 0.6 mmol) was suspended in a 10 ml aqueous solution of NaIO₄ (770 mg), KMnO₄ (20 mg) and K₂CO₃ (900 mg) [12]. The mixture was stirred for 3 hours at room temperature and filtrated. The filtrate was extracted with diethylether (2 x 10 ml). The water phase was then acidified with 1 M HCl to pH 2 and extracted with diethylether (2 x 30 ml). The combined organic extracts were decanted and evaporated to dryness. Recrystallization of the residue in a mixture of acetone and ethyl acetate afforded 17β-hydroxy-17α-methyl-2,3-seco-5α-androstane-2,3-dioic acid 1 (68 mg, 32%) as crystalline needles: m.p. 255-257°C; FAB/MS, m/z 351.2157 for [M-H]⁻ (calculated value, 351.2171 a.m.u.). The structure of 1 was further characterized by nuclear magnetic resonance (NMR) analysis. A two-dimensional spectrum 2D-resolved, COSY and carbon-proton heteronuclear shift correlations were recorded. NMR signals: H-1α (d, δ 2.42-2.45), H-1β (d, δ 2.28-2.31), H-4α (d-d, δ 2.66-2.70), H-4β (d-d, δ 1.91-1.97), H₃-18 (s, δ 0.83), H₃-19 (s, δ 0.84), H₃-20 (s, δ 1.17); ¹³C-2 (δ 175.1), ¹³C-3 (δ 177.4). Detailed ¹³C and ¹H nuclear magnetic resonance (NMR) spectral feature will be reported separately.

3α,17β-Dihydroxy-17α-methyl-5α-androstane-2α-carboxylic acid 2a.

The synthesis of this compound was performed according to the scheme depicted in Fig. 5.1-5. The epoxidation of 17β-hydroxy-17α-methyl-5α-androst-2-ene 7 was carried out
according to a method adapted from that of Fürst and Plattner [13]. To an ice cold solution of 7 (57.6 mg, 0.2 mmol), was added dropwise a 1 ml chloroform solution of mCPBA (54 mg, 0.3 mmol) over a period of 5 min. The reaction mixture was stirred at room temperature for 5 h and diluted with 10 ml of dichloromethane. The resulting solution was successively washed with 5 ml of 10% Na$_2$S$_2$O$_3$, 5 ml 0.5 N KHCO$_3$ and 5 ml of water. The organic phase was dried over Na$_2$SO$_4$ and evaporated to dryness to afford 58 mg (95%) of 17α-methyl-2α,3α-epoxy-5α-androstan-17β-ol 8 as a white solid. This compound was characterized by GC/MS analysis of its TMS ether derivative: M$^{+*}$ 376, (8%), m/z 361 (50%), m/z 286 (7%), m/z 229 (10%) and m/z 143 (100%).

Treatment of compound 8 with diethyl aluminum cyanide (DAC)
The epoxide 8 was reacted with DAC according to a method previously described by Nagata et al. [14,15]. The crude epoxide 8 (58 mg, 0.19 mmol) was dissolved in 3 ml of toluene to which a solution of 1 ml (1.0 mmol) of DAC in 1 ml of toluene was added. The mixture was stirred at room temperature for 4 h and poured into a 2 N NaOH-ice mixture (25 ml). The mixture was extracted with diethyl ether (2 x 20 ml) and the organic phase was decanted, washed with water, dried over Na$_2$SO$_4$ and evaporated to dryness to afford 62 mg (98%) of 2β-cyano 17α-methyl-5α-androstane-3α,17β-diol 9 as a white solid. GC/MS analysis of its TMS ether derivative showed compound 9 as a single peak: M$^{+*}$ 475 (3%), m/z 460 (31%), m/z 418 (16%), m/z 405 (35%), m/z 390 (10%), m/z 385 (10%), m/z 370 (9%), m/z 280 (6%) and m/z 143 (100%).
3α,17β-Dihydroxy-17α-methyl-5α-androstane-2α-carboxylic acid 2a and
3α,17β-Dihydroxy-17α-methyl-5α-androstane-2β-D-2α-carboxylic acid 2b

The crude 9 (60 mg) was dissolved in 2 ml of acetone. To 1 ml of this solution was added 1 ml of an aqueous solution containing 160 mg of barium hydroxide, while the remaining 1 ml acetone solution of 9 was evaporated to dryness. The resulting residue was dissolved in 1 ml of deuterated methanol (CH₃-OD) and then added to a 1 ml solution of a D₂O-barium hydroxide solution (160 mg). Both the aqueous and D₂O-barium hydroxide solutions were heated at 80°C for 48 h and then cooled at room temperature. The aqueous solutions were extracted with diethyl ether (2 x 5 ml) and acidified to pH 1-2 with 20% HCl (v/v). The resulting aqueous phases were extracted with diethyl ether (2 x 10 ml). The ethereal phases were dried over Na₂SO₄ and evaporated to dryness to give 4.2 mg of 2a and 4.0 mg of 2b respectively.

Isomerization of the urinary metabolite 2 into 2a

The urinary metabolite 2 which bears a 2β-carboxylic group was isomerized to its 2α-analog as follows: five 10-ml aliquots of a urine sample collected 5 h after oxymetholone administration were separately applied onto Sep-Pak C₁₈ cartridges and the acidic urinary metabolites were extracted as described above. The combined acidic ethereal extracts were combined and evaporated to dryness. The resulting residue was treated with diazomethane to prepare the methyl ester derivative of 2. The ethereal solution was mixed with 1.5 g of basic alumina. The mixture was placed in a capped test tube at room temperature for 24 h. Then the urinary steroids were eluted from the
alumina with 5 ml of methanol. The solvent was evaporated to dryness and the residue was treated with a mixture of MSTFA and TMSCl as described above to prepare the TMS ether derivatives. Ion chromatograms from GC/MS analysis of the resulting mixture are illustrated in Fig. 5.1-6.

Results

A typical ion chromatogram obtained from GC/MS analysis of the acidic fraction of urine specimens collected 2 h after oxymetholone administration is shown in Fig. 5.1-1A. Five unidentified metabolites, which were not found in blank urine samples (Fig. 5.1-1B) collected prior to steroid administration were detected as the TMS ester-TMS ether derivatives. Their mass spectral features led us to prepare the corresponding methyl ester - TMS ether derivative so as to determine the exact number of carboxylic and hydroxy groups that each metabolite bears. The resulting mass spectral information is summarized in Table 5-1.

Metabolite 1
This compound was the most abundant acidic metabolite detected in urine. As shown in Table 5-2, this metabolite was excreted in relatively large amounts during the first 2 h following oxymetholone administration. Then its urinary excretion rapidly decreased in the next 5 h from a concentration of 8.65 to 0.36 µg/ml. The cumulative urinary excretion of this metabolite over a period of 32 h accounted for 1.52% of the dose.
Table 5.1-1. Identity and partial GC/MS data of ovymetholone acidic urinary metabolites in human

<table>
<thead>
<tr>
<th>Steroid *</th>
<th>M.U.</th>
<th>Derivative</th>
<th>M⁺</th>
<th>Characteristic ions d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 17β-hydroxy-17α-methyl 2,3-seco-5α-androstane-2,3-dioic acid</td>
<td>29.49</td>
<td>TMS</td>
<td>568(1)</td>
<td>553(6), 511(5), 498(2), 437(3), 436(4), 436(19), 347(19), 346(16), 217(27), 214(21), 143(100)</td>
</tr>
<tr>
<td></td>
<td>28.38</td>
<td>Me-TMS</td>
<td>452(3)</td>
<td>437(9), 395(9), 382(6), 378(4), 320(3), 289(21), 288(15), 215(9), 214(6), 143(100)</td>
</tr>
<tr>
<td>2. 3α,17β-dihydroxy-17α-methyl-5α-androstane-2β-carboxylic acid</td>
<td>29.87</td>
<td>TMS</td>
<td>566(1)</td>
<td>551(7), 509(2), 496(1), 476(2), 461(3), 448(4), 386(5), 371(4), 269(6), 268(7), 215(13), 143(100)</td>
</tr>
<tr>
<td></td>
<td>29.54</td>
<td>Me-TMS</td>
<td>508(3)</td>
<td>493(29), 451(3), 438(3), 418(4), 403(3), 376(3), 328(9), 313(7), 269(2), 143(100)</td>
</tr>
<tr>
<td>3. 17β-hydroxy-17α-methyl-4α-homo-3,4-seco-5α-androstane-3,4-dioic acid</td>
<td>30.44</td>
<td>TMS</td>
<td>582(1)</td>
<td>567(16), 525(10), 512(3), 492(3), 374(10), 348(8), 347(5), 217(15), 216(17), 143(100)</td>
</tr>
<tr>
<td></td>
<td>29.82</td>
<td>Me-TMS</td>
<td>466(2)</td>
<td>451(11), 409(12), 396(7), 344(4), 334(5), 302(6), 289(16), 215(4), 143(100)</td>
</tr>
<tr>
<td>4. 17β-hydroxy-17α-methyl-4-nor-1,2-seco-5α-androstane-1,2-dioic acid</td>
<td>28.02</td>
<td>TMS</td>
<td>554(1)</td>
<td>539(17), 497(10), 482(5), 464(5), 436(9), 374(22), 332(25), 215(23), 143(100)</td>
</tr>
<tr>
<td></td>
<td>26.96</td>
<td>Me-TMS</td>
<td>438(2)</td>
<td>423(6), 381(8), 368(6), 316(8), 306(5), 275(12), 274(18), 215(10), 143(100)</td>
</tr>
<tr>
<td>5. 17β-hydroxy-17α-methyl-4-nor-2,3-seco-5α-androstane-2,3-dioic acid</td>
<td>28.95</td>
<td>TMS</td>
<td>554(1)</td>
<td>539(8), 497(2), 464(3), 375(33), 304(32), 257(8), 216(23), 143(100)</td>
</tr>
<tr>
<td></td>
<td>27.46</td>
<td>Me-TMS</td>
<td>438(3)</td>
<td>423(20), 381(8), 368(10), 289(15), 288(10), 216(12), 143(100)</td>
</tr>
</tbody>
</table>

* Urinary metabolites extracted from the free steroid fraction

b Methylene unit (M U) values were calculated by linear interpolation of the retention time of the steroid derivative relatively to that of C₂, C₅, C₈, C₁₀, and C₁₂: linear hydrocarbons

c Trimethylsilyl ester-trimethylsilyl ether (TMS) and methyl ester-trimethylsilyl ether (Me-TMS) derivatives

d Ion relative intensity is given in parenthesis
Fig. 5.1-1. Reconstructed ion current chromatogram (m/z 143) from GC/MS analysis of the acidic fraction from (A) a urine sample collected 2 h after oxymetholone administration and (B) a blank urine sample. Urinary metabolites were analyzed as the TMS ester-TMS ether derivatives and their identity is given in Table 5-1. Oxandrolone was used as internal standard.

Table 5-2. Urinary excretion of metabolite 1 after oral administration of a single 50 mg dose of oxymetholone

<table>
<thead>
<tr>
<th>Time of collection (h)</th>
<th>Urinary concentration (µg/ml)</th>
<th>Total amount excreted (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>8.650</td>
<td>519.2</td>
</tr>
<tr>
<td>5</td>
<td>0.820</td>
<td>164.4</td>
</tr>
<tr>
<td>8</td>
<td>0.360</td>
<td>36.0</td>
</tr>
<tr>
<td>12</td>
<td>0.150</td>
<td>17.9</td>
</tr>
<tr>
<td>16</td>
<td>0.070</td>
<td>14.0</td>
</tr>
<tr>
<td>24</td>
<td>0.021</td>
<td>5.5</td>
</tr>
<tr>
<td>32</td>
<td>0.011</td>
<td>2.3</td>
</tr>
<tr>
<td>36</td>
<td>n.d.</td>
<td>-</td>
</tr>
</tbody>
</table>

1. Metabolite 1 was detected in urine only between 2 and 32 h after oxymetholone administration.
2. Total amount of 1 excreted in urine was 759.3 µg or 1.52% of the dose.
3. Not detected.
The mass spectral features of its methyl ester-TMS ether, TMS ester-TMS ether and corresponding d₆-TMS derivatives indicated the presence of two carboxylic groups and one C₁₇-hydroxyl function. Owing to the chemical features of oxymetholone, these data suggested that the 2-hydroxymethylene group was oxidized to the corresponding 2-carboxylic acid. However, the presence of a second carboxylic function was puzzling and suggested that metabolite 1 probably resulted from oxidative cleavage of the C₇-C₁ bond to yield a 2,3-seco diacid. This hypothesis was consistent with mass spectral features of TMS and Me-TMS derivatives of 1 (Fig. 5.1-2). Indeed, the molecular ion at m/z 568 (m/z 595, d₆-TMS), the prominent ion at m/z 143 (m/z 152, d₆-TMS) [16, 17]
and other structurally informative ions at m/z 511 (M-57; m/z 538, d₇-TMS) and m/z 498 (M-70; m/z 525, d₇-TMS) in the mass spectrum of the TMS derivative (Fig. 5.1-2B) indicated that no metabolic transformation occurred in the C- and D- rings. This hypothesis was further demonstrated in the mass spectrum of the Me-TMS derivative (Fig. 5.1-2A) by the presence of the corresponding ions at m/z 395 (M-57; m/z 404, d₇-TMS) and m/z 382 (M-70; m/z 391, d₇-TMS) [17]. The presence of 2,3-seco-2,3-dioic functions in compound 1 was specifically demonstrated in the mass spectrum of its TMS derivative (Fig. 5.1-2B) by the formation of diagnostic ions at m/z 436 (m/z 454, d₇-TMS) originating from the loss of one molecule of CH₃COOTMS from the molecular ion, probably through a McLafferty rearrangement involving the carboxyl group at C-3 and a γ-hydrogen transfer from C-6 with concomitant cleavage of the C4-C5 bond [18]. The subsequent elimination of a TMSO⁺ radical and one molecule of TMSOH gave rise to the prominent ions at m/z 347 and 346 (m/z 356 and 355, d₇-TMS) respectively. This fragmentation cascade was then terminated by the consecutive losses of °CH₂COOTMS and °H radicals to yield ions of m/z 215 and 214. A similar sequence of fragmentations giving rise to ions of m/z 378 (M-CH₃COOMe; m/z 387, d₇-TMS), m/z 289 and 288 (m/z 289 and 288, d₇-TMS) and m/z 215 and 214 (m/z 215 and 214, d₇-TMS) was observed in the mass spectrum of the methyl ester-TMS ether derivative (Fig. 5.1-2A).

These mass spectral data led us to synthesize compound 1 by treating oxymetholone with a mixture of sodium periodate and potassium permanganate as described above. The GC/MS properties of synthetic 1 were identical to those of the urinary diacid 1 and its
\(^1\)H- and \(^13\)C-NMR features were consistent with the proposed structure. Thus, compound 1 was identified as 17β-hydroxy-17α-methyl-2,3-seco-5α-androstane-2,3-dion acid.

**Metabolite 3**

Molecular ions of the methyl ester-TMS ether and TMS ester-TMS ether derivatives of metabolite 3 were shifted by 14 a.m.u. to m/z 466 (m/z 475, \(d_4\)-TMS) and m/z 582 (m/z 609, \(d_6\)-TMS) respectively with respect to those of compound 1 (Table 5-1). These data suggested that compound 3 was also a seco diacid bearing an additional methylene group, the presence of which being consistent with the increase of the methylene unit (M U.) value of the corresponding derivatives. The structural assignment was supported by the mass spectral data from the Me-TMS derivative (Table 5-1) which showed a prominent ion of diagnostic importance at m/z 289 (m/z 289, \(d_4\)-TMS) arising from the consecutive losses of one molecule of TMSOH and a \(\text{CH}_2\text{CH}_2\text{COOMe}\) radical from the molecular ion \([M-90-87]^+\). A second ion at m/z 302 (m/z 302, \(d_6\)-TMS) also provided useful information about the structure of 3. This ion resulted from the elimination of one molecule of TMSOH and \(\text{CH}_2\text{COOMe}\) (McLafferty rearrangement of the \(\text{CH}_2\text{COOMe}\) group located at C-5 as in compound 1) from the molecular ion \([M-90-74]^+\). These data provided evidence that compound 3 bears ethanoic and propionic acid moieties at C-5 and C-10 respectively. This structural assignment was supported by mass spectral data from the TMS derivative which showed ions of diagnostic importance at m/z 348 ([M-\('\text{CH}_2\text{CH}_2\text{COOTMS-TMSO}')^+]^+; m/z 357, \(d_4\)-TMS), m/z 216 [m/z 348-\(\text{CH}_2\text{COOTMS}\)]^+ and m/z 374 [M-HCOOTMS-TMSOH]^+ (m/z 383, \(d_6\)-TMS). These mass spectral data also
indicated that the propionic acid moiety was located at C-10. Indeed, the hypothetical presence of this group at C-5 would promote McLafferty rearrangements involving the C-5 hydrogen atom, that would lead to the formation of ions bearing a methylene group at C-5 and which were not observed in the mass spectra of its Me-TMS and TMS derivatives.

On the contrary, the mass spectra data were consistent with the presence of an ethanoic acid moiety at C-5. In that respect, the mass spectral features of Me-TMS and TMS derivatives of both 1 and 3 showed similar fragmentation patterns involving McLafferty rearrangement of the C-5 ethanoic acid group. On the basis of these mass spectral evidences, 3 was assigned the structure of 17β-hydroxy-17α-methyl-4a-homo-3,4-seco-5α-androstan-3,4-dioic acid.

**Metabolites 4 and 5**

The Me-TMS and TMS derivatives of these minor metabolites showed identical molecular ions, both shifted by 14 a.m.u. to m/z 438 (m/z 447, δ5-TMS) and 554 (m/z 581, δ6-TMS) with respect to those of compound 1 (Table 5-1). These data and comparison with GC/MS data from compounds 1 and 3 indicated that 4 and 5 were position isomers bearing formic and ethanoic acid groups at C-5 and/or C-10. The mass spectrum of Me-TMS derivative of 4 (Fig. 5.1-3) retained of a characteristic fragmentation route observed in mass spectra of 1 and 3, which involved the concomitant losses of molecules of TMSOH and CH3-COOME (McLafferty rearrangement of the C-5
ethanoate group) to give the prominent ion of m/z 274 [M-90-74]+. The corresponding ions were observed at m/z 288 and m/z 302 in compound 1 and 3 Me-TMS derivatives.

An analogous fragmentation route in the mass spectrum of TMS derivative of 4 gave rise to an intense ion at m/z 332 (M-TMSOH-CH₃COOTMS; m/z 341, d₇-TMS). These data indicated that 4 bore an ethanoic acid group at C-5 and a formic acid group at C-10. This hypothesis was corroborated by the mass spectral features of the corresponding derivatives of its isomeric analog 5 which show the absence of any McLafferty rearrangement ion of the ethanoate group, thus indicating that the latter moiety was located at C-10, where such a rearrangement cannot occur. The mass spectrum of the Me-TMS derivative of 5 showed fragment ions at m/z 289 (M-TMSOH-‘COOMe)+, 288 (M-TMSOH-HCOOMe)+ and 216 (m/z 289-‘CH₃COOMe)+. Thus, the mass spectral features and M.U. values presented in Fig. 5.1-3 and Table 5-1 were consistent with the structure of 17β-hydroxy-17α-methyl-4-nor-1,2-seco-5α-androstan-1,2-diol α-acid for compound 4 and 17β-hydroxy-17α-methyl-4-nor-2,3-seco-5α-androstan-2,3-diol α-acid for compound 5.
Metabolite 2

The mass spectrum of compound 2 Me-TMS derivative (Fig. 5.1-4A) showed a molecular ion at m/z 508 (m/z 526, d₉-TMS) and fragment ions at m/z 493 (M-Me: m/z 508, d₉-TMS), 451 (M-57; m/z 469, d₉-TMS), 438 (M-70; m/z 456, d₉-TMS), m/z 328 (M-2TMSOH; m/z 328, d₉-TMS) and m/z 143 (m/z 152, d₉-TMS) which indicated the
presence of one carboxylic acid and two hydroxyl groups in compound 2. The mass spectrum of its TMS derivative (Fig. 5.1-4B) showed a molecular ion at m/z 566 (m/z 593, d₇-TMS) and structurally informative ions at m/z 509 (M-57; m/z 536, d₇-TMS), 496 (M-70; m/z 523, d₇-TMS), 448 (M-HCOOTMS; m/z 466, d₇-TMS) 386 (M-2TMSOH; m/z 395, d₇-TMS), m/z 269 (M-2TMSOH-·COOTMS; m/z 269, d₇-TMS) and m/z 268 (M-2TMSOH-HCOOTMS; m/z 268, d₇-TMS). These data were in accordance with the structure of 3α,17β-dihydroxy-17α-methyl-5α-androstane-2α-carboxylic acid for compound 2. The synthesis of this metabolite was attempted as depicted in Fig. 5.1-5 in order to determine the stereochemistry of the 2-carboxylic and 3-hydroxyl groups. This synthetic approach afforded the precursor 9 with 2β-cyano and 3α-hydroxy groups. Hydrolysis of 9 with barium hydroxide did not gave the expected 3α-hydroxy-2β-carboxylic acid 2, but the thermodynamically more stable 3α-hydroxy-2α-carboxylic acid 2a. Isomerization of the 2β-carboxylic group upon hydrolysis of 9 was demonstrated by carrying out the reaction in a mixture of deuterated methanol and deuterated water. The incorporation of one deuterium atom in 2b was demonstrated by the characteristic one a.m.u. shift of the molecular and other diagnostic ions of its TMS derivative with respect to the corresponding ions of the TMS derivative of metabolite 2 (Figs 5.1-4B and 5.1-4C). Under the basic hydrolysis condition, 2β-cyanide tends to form the more stable 2α-isomer via a 2-carbanion intermediate. The presence of 2β-carboxy-3α-hydroxy groups in the urinary metabolite 2 was also demonstrated by isomerization. The treatment with basic alumina of an urinary extract containing 2 afforded a mixture of 2 and 2a as illustrated in Fig. 5.1-6. Conversely, treatment of 2a with basic alumina under identical
conditions gave unchanged 2a. This series of experiments demonstrated that compounds 2 and 2a bear a \(2\beta\)- and \(2\alpha\)-carboxylic group respectively.

Finally the stereochemistry of the 2-carboxy and 3-hydroxy groups in compounds 2 and 2a was ascertained by comparison of compounds 2 and 2a GC/MS properties with those from four isomeric 3-hydroxy-2-carboxylic acids (including 2 and 2b) prepared from other synthetic approaches and by proton and \(^{13}\)C-NMR analysis (to be reported separately, see Chapter 5.2) of the corresponding 2\(\alpha\)-carboxy-3\(\alpha\)-hydroxy and 2\(\alpha\)-carboxy-3\(\beta\)-hydroxy steroids which were the sole isomers we could synthesized in sufficiently large amounts.

Fig. 5.1-5. Synthesis of 3\(\alpha\),17\(\beta\)-dihydroxy-17\(\alpha\)-methyl-5\(\alpha\)-androstane-2\(\alpha\)-carboxylic acid 2a and 3\(\alpha\),17\(\beta\)-dihydroxy-17\(\alpha\)-methyl-5\(\alpha\)-androstane-2\(\beta\)-D-2\(\alpha\)-carboxylic acid 2b.
Fig. 5.1-6. Reconstructed ion current chromatograms (m/z 143) from GC/MS analysis of (A) an acidic fraction from a urine sample collected 2 h after oxymetholone administration, (B) after treatment of the previous urinary extract on basic alumina and (C) synthetic 2a. Steroids were analyzed as the methyl ester-TMS ether derivatives. See experimental for further details.

Discussion

This study showed for the first time that oxymetholone is metabolized in man into a series of acidic metabolites. The structural determination of four novel seco seco acids and $3\alpha,17\beta$-dihydroxy-$17\alpha$-methyl-$5\alpha$-androstane-$2\beta$-carboxylic acid 2 was described. Metabolic routes accounting for their formation are proposed in Fig. 5.1-7.
Fig. 5.1-7. Proposed metabolic routes for the formation of oxymetholone acidic metabolites.

Metabolite 2

The formation of this metabolite may be initiated preferentially by the oxidation of two of the three tautomeric forms of oxymetholone [19] bearing a 2-formyl group to give the corresponding 3-keto-2-carboxylic acid A (Fig. 5.1-7). The subsequent reduction of its 3-keto group by 3α-hydroxysteroid dehydrogenase (3α-OHSDH) give rise to metabolite 2. The selective formation of 2 which bears a 2β-carboxylic group and the absence in urine of its 2α-isomer 2a (Fig. 5.1-5) suggest that the β-configuration at C-2 was set in the course of the first oxidation reaction. Then, reduction of the 3-keto and any subsequent oxidative process (Fig. 5.1-7) occurred rapidly, apparently without enolization
of the 3-keto group, which could have promoted the formation of the thermodynamically more stable 2α-isomer 2a. To the best of our knowledge, this is the first time that such an acidic metabolite of an androgenic-anabolic steroid is reported in man. A similar biotransformation route has been reported by Templeton and Michiels [20] who identified 3α,16α,17β-trihydroxy-5α-androstane-2α-carboxylic acid in the urine of rabbits dosed with 17β-hydroxy-2-hydroxymethylene-5α-androstan-3-one, a synthetic analog of oxymetholone. However, there is a possibility that the original stereochemistry at C-2 was not α- but β- because this acidic steroid was isolated from a sodium hydroxide soluble fraction of the crude urinary extract. Under these conditions, it is likely that any 2β-carboxylic group could have isomerized into the corresponding 2α-analog.

Although not detected in urine, compound A (Fig. 5.1-7) is likely a key intermediate in the biotransformation of oxymetholone. Indeed, this β-keto acid can be readily decarboxylated to give 17β-hydroxy-17α-methyl-5α-androstan-3-one 6 (mestanolone) a urinary metabolite of oxymetholone we identified in a previous study [9].

The seco diacids

The acidic seco steroids reported here are the result of oxidative reactions which are encompassed into the general biotransformation scheme of oxymetholone in human. Indeed, these steroids were not detected in blank urine samples spiked with oxymetholone and left at room temperature for several hours or treated under the same storage and experimental conditions as the post-administration samples.
Figure 5.1-7 presents the metabolic routes leading to the interesting biotransformation of oxymetholone into the seco diacids 1, 3, 4 and 5. The proposed biotransformation routes have some similarities to the reaction sequence in the degradation of fatty acids, which involves the formation of a β-keto acid intermediate and its subsequent thiolysis [21]. By analogy to this reaction sequence, it seems reasonable to propose that the initial step of oxymetholone conversion to compounds 1 and 2 is the selective oxidation of the hydroxymethylene group to the corresponding β-keto acid A, which then reacts with coenzyme A (CoA) to yield the corresponding intermediate thioester. The formation of the latter steroid acyl-CoA ester enables the acidic steroid to enter the mitochondrial matrix where further oxidation occurs [21].

The further oxidation of intermediate A or direct oxidation of oxymetholone, most probably via the epoxidation of their enolic forms of the 3-keto functions, could give rise to seco diacid 1 (Fig. 5.1-7). Precedent for such a mechanism is provided by the facile microbial oxidation by electrophilic oxygen of steroidal enols to α-hydroxyketosteroids [22]. A similar process which has been implicated in the oxidative cleavage of the furan ring of 8-methoxysporalen resulted in the formation of a metabolite bearing an ethanoic side chain and a hydroxy group [23].

The alternative oxidative reaction of intermediate A is reminiscent of the last step of the fatty acid oxidation cycle which is the cleavage of 3-ketoacyl-CoA by the thiol group of a second molecule of CoASH upon thiolase catalysis. In fatty acid β-oxidation such a reaction splits off the carboxyl-terminal 2-carbon fragment of the original fatty acid as
acetyl-CoA leaving as the other product, and the CoA ester of the original fatty acid now shortened by two carbon atoms. In the case of the intermediate β-keto acid A derived from oxymetholone, where both the carboxylic and keto groups are situated on the same six-membered nucleus, this retro-Claisen type of thiolysis cleaves the C2-C3 bond of A to form dithio ester of metabolite 3 (Fig. 5.1-7). Further β-oxidation of the propionic acid group of 3 provided metabolite 4, which is two carbons shorter than 3.

As a minor pathway, the α-oxidation of seco diacid 1, with α-keto acids as intermediates [24], could be one of the possible mechanism accounting for the presence of metabolite 4 and 5 in urine.

Perusal of literature on steroid oxidative metabolism indicates that acidic metabolites originating from the metabolism of corticosteroids have been characterized in human [25] and animals [26]. However, none of the acidic steroids reported so far were produced from oxidative ring-cleavage reactions, but rather from oxidation of the C20, C21 carbons and angular methyl groups. On the other hand, several authors have shown that some microorganisms have the capacity to degrade steroids by oxidative cleavage, mainly of the A, B and/or D rings to yield acidic seco steroids [27,28]. Interestingly, these oxidative reactions can be integrated in a general metabolic scheme which encompasses regioselective cleavage of the C4-C5 and C9-C10 bonds [27,28]. From biochemical and mechanistic points of view, there is no relationship between these microbial oxidative reactions and the retro-Claisen cleavage of the intermediate A described above. Data from a metabolic study carried out with formebolone [10] (2-formyl-11α,17β-dihydroxy-
17α-methyl-androsta-1,4-dien-3-one), a model compound of oxymetholone [29], suggest that the presence of a saturated A-ring as in the latter steroid is an essential structural prerequisite that enables the formation of the observed retro-Claisen cleavage products. This hypothesis is supported by the fact that no seco acidic metabolite analogous to metabolites 1, 3, 4 and 5 was detected in formebolone post-administration urine [10]. Examination of the structural features of the intermediate β-keto acid arising from oxymetholone oxidation (Fig. 5.1-7) indicates structural homology of the steroid moiety comprising carbons 2, 3 and 4 with saturated β-keto acids, which are substrates for β-oxidation in living organisms. On the other hand, such a homology does not exist with the unsaturated A-ring of formebolone.

The metabolism of a non-polar steroid such as oxymetholone is generally oriented toward the formation of water-soluble compounds that are easily excreted in urine. This is normally accomplished, according to the classical view, by a sequence of hydroxylation and reductive steps [8,20]. In that respect, the retro-Claisen cleavage of oxymetholone A-ring cannot be considered as a classical biotransformation. One would have expected that the polycyclic structure of this steroid would have prevented this reaction which is, in general, characteristic of linear fatty acids. We could also speculate as to whether the acidic seco steroids 1, 3, 4 and 5 are insignificant end products of oxymetholone or possess some unknown biological properties. The fact that β-oxidation is of importance in regulating fatty acid composition of membrane lipids, suggests that oxymetholone could be incorporated into the lipidic pool of membrane precursors prior to undergo β-oxidative cleavage of its A-ring.
Since oxidative metabolic transformation of steroids take place in largest measure in the liver, the data reported above raise a possibility as to whether the acidic seco steroids described herein are associated to the molecular events leading to the hepatic toxicity that may result from oxymetholone administration [6, 30-32], since several lipophilic acids have been shown to be teratogenic in human [33]. However, such a hypothesis is highly speculative, not only because anabolic 17α-methyl steroids in general are hepatotoxic, which is also true for steroids with an unsaturated A-ring, but also because the latter steroids do not form acidic seco metabolites. This indicates that the formation of acidic seco steroids from oxymetholone is likely not related to its hepatotoxicity in human.

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References


17. Dürbeck, H.W. and Büker, I.: Studies on anabolic steroids. The mass spectra of 17α-methyl-17β-hydroxy-1,4-androstadien-3-one (Dianabol) and its metabolites.


5.2. STUDIES ON ANABOLIC STEROIDS.  
SYNTHESIS AND IDENTIFICATION OF ACIDIC  
URINARY METABOLITES OF OXYMETHOLONINE IN HUMAN

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Summary

Two major unconjugated acidic metabolites of oxymetholone (17β-hydroxy-2-hydroxymethylene-17α-methyl-5α-androstan-3-one), namely 17β-hydroxy-17α-methyl-2,3-seco-5α-androstane-2,3-dioic acid 2* and 3α,17β-dihydroxy-17α-methyl-5α-androstane-2β-carboxylic acid 6a*, were detected by gas chromatography - mass spectrometry (GC/MS) in urine samples collected after oral administration of 1 to a human volunteer. Reference steroid 2 was synthesized and identified. The identification of urinary metabolite 6a was based on the synthesis of its stereoisomers and the isomerization of the methyl ester 6b to its 2-epimer, 3α,17β-dihydroxy-17α-methyl-5α-androstane-2α-carboxylic acid methyl ester 9b. The mechanisms accounting for the formation of these acidic metabolites are discussed.

* Compounds 2 and 6a are the same as compounds 1 and 2a respectively in Chapter 5.1.
Introduction

Oxymetholone (17β-hydroxy-2-hydroxymethylene-17α-methyl-5α-androstan-3-one 1), was first synthesized by Ringold et al. in 1959 [1] and introduced into clinical usage a few years later [2]. Oxymetholone has been widely used in the treatment of anemia [3-5] and other androgen therapies [6,7]. Liver toxicities and carcinogenicity of oxymetholone have been noticed in clinical practice [8-13]. Little information was available in the literature about the metabolism of oxymetholone in man until Adhikary et al. [14] and MacDonald et al. [15] reported the isolation of two neutral urinary metabolites produced from the reduction of the 2-hydroxymethylene and 3-keto groups. Previous studies from this laboratory [16] showed that 17α-methyl-5α-androstan-3α,17β-diol 4 was present in the post-administration urine sample of oxymetholone. This implied that the 2-hydroxymethylene group of the parent steroid was eliminated in the course of its biotransformation. It was then presumed (Scheme 5.2-1) that oxidation of the 2-hydroxymethylene group leads to the intermediate β-keto acid 5a and/or 8a which are sensitive to decarboxylation to form metabolite 3 (17β-hydroxy-17α-methyl-5α-androstan-3-one). The latter keto steroid is then readily reduced by 3α-hydroxydehydrogenase to give 4. The intermediate steroids 5a and 8a may also be further metabolized to form stable acidic metabolites. In order to further investigate the oxidative metabolic pathway of oxymetholone, and to shed some light on the relationship between its biotransformation routes and hepatic toxic side effects, series of experiments have been conducted to identify the acidic urinary metabolites of oxymetholone. This report describes the
syntheses of these reference steroids and assignments of their stereochemistry. The mechanism accounting for the formation of acidic metabolites 2 and 6a will also be discussed.

Scheme 5.2-1. Biotransformation and synthetic routes accounting for metabolites 2 and 6a. Single arrows → indicate synthetic reactions whereas double arrows ↔ indicate biotransformation routes.
Experimental

Materials and methods

Oxymetholone and its tablets (Anapolon 50) were obtained from Syntex Research, Palo Alto, California. Other steroids were purchased from Sigma Chemical Co., St-Louis, Missouri, and Steraloids, Wilton, New Hampshire. Their purities were assessed by GC/MS analysis of their TMS-derivatives. Biochemicals and materials for extraction of urinary metabolites used in this study have been previously described [17,18]. Sodium metal in kerosine, dimethyl carbonate, sodium borohydride (NaBH₄), magnesium methyl carbonate (MMC) in DMF (2.0 M), lithium diisopropylamide (LDA) in heptane/tetrahydrofuran/ethylbenzene (2.0 M), 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) and the MNNG-diazomethane kit (1 mmol scale), 3-chloroperbenzoic acid (mCPBA) (50-60%), and diethylaluminum cyanide in toluene (1.0 M) were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Aluminum oxide (neutral) was from Camag, Switzerland (distributed by Terochem Scientific, Edmonton, Alberta, Canada). Inorganic salts (analytical grade) were from J.T. Baker Chemical Co., Phillipsburg, New Jersey. Organic solvents were purchased from Caledon Laboratories Ltd, Georgetown, Ontario, Canada. Deuterium oxide (D₂O, minimum isotope purity, 99.9 atom % D) and methyl alcohol-OD (CH₃OD, minimum isotope purity, 99 atom % D) were from MSD-Isotopes, Merck Frosst Canada Inc., Montreal, Canada. All solvents were redistilled from calcium hydride. Prior to use, mCPBA was treated with phosphate buffer (pH 7.5) and then extracted with CH₂Cl₂. The organic layer was washed with water, dried with Na₂SO₄ and
then evaporated in vacuo. The residue is ready for use [19].

Melting points were measured on an electrothermal® melting point apparatus and are reported without correction. IR spectra were recorded in Nujol on a Perkin Elmer 267 infrared spectrophotometer. $^1$H and $^{13}$C NMR spectra were recorded on a Varian VXA 300 or a Bruker WH 400 spectrometer. Low resolution mass spectra (LRMS, EI) were recorded on a Hewlett Packard 5890-5970 GC-MSD instrument. High resolution mass spectra in both positive or negative ion mode [HRMS, Cl(NH$_3$) as reagent gas] or FAB (glycerol as matrix) were recorded on a Kratos MS50TC mass spectrometer.

**Steroid administration**

After collection of blank urine samples, Anapolon 50 (50 mg, single dose) was orally administered to one healthy male volunteer (age 33, 60 kg). The post-administration urine samples were collected for at least 6 days and immediately stored at -20 °C until analyzed.

**Extraction of acidic metabolites and GC/MS analysis**

A 10 ml urine sample was applied on a Sep-Pak $C_{18}$ Cartridge (pre-washed with 5 ml of methanol and 5 ml of water). The cartridge was then successively washed with 5 ml of water and 2 ml of hexane. The steroids were eluted with 5 ml of methanol and the eluate was then evaporated to dryness under a slow stream of nitrogen at 40 °C. The residue was dissolved in 1 ml of water and pH was adjusted to 8.0 with aqueous KHCO$_3$. The
solution was then extracted with 2 x 5 ml of diethyl ether and the combined ethereal phases were analyzed for unconjugated neutral steroid metabolites. The remaining aqueous phase was acidified with 1 M HCl to pH 1-2, and extracted with ether (2 x 5 ml). The organic phases were combined, dried over Na₂SO₄ and evaporated to dryness under nitrogen. TMS-ester-TMS-ether and methyl ester-TMS-ether derivatives of the steroids of interest were prepared prior to GC/MS analysis according to methods previously described [18] (see Chapter 4 and 5.1). A typical GC chromatogram of an urinary extract showing the presence of major metabolites 2 and 6a is presented in Fig. 5.2-1. The corresponding mass spectra of these two metabolites (as two different derivatives) are presented in Figs 5.2-2 and 5.2-3.

Syntheses of reference steroids.

*Metabolite 2 (17β-hydroxy-17α-methyl-2,3-seco-5α-androstane-2,3-dioic acid).*

$\text{NaIO}_4/\text{KMnO}_4/\text{K}_2\text{CO}_3$ oxidation [20].

Oxymetholone 1 (200 mg, 0.6 mmol) was suspended in a 10 ml aqueous solution of $\text{NaIO}_4$ (770 mg), $\text{KMnO}_4$ (20 mg) and $\text{K}_2\text{CO}_3$ (900 mg). The mixture was stirred for 3 hours at room temperature. The resulting precipitate was filtered and the aqueous phase was extracted with diethyl ether (2 x 10 ml). The aqueous phase was then acidified to pH 1-2 with HCl and extracted with ether (2 x 30 ml). The combined ethereal extracts were washed with water, dried over Na₂SO₄ and evaporated in vacuo. Crystallization of the residue in acetone and ethyl acetate afforded 68 mg of 2 (yield 32%).
**Peracid (mCPBA) oxidation** [21].

To a 2 ml solution of 1 (33 mg, 0.1 mmol) in CHCl₃ cooled with an ice bath was added dropwise mCPBA (30 mg in 2 ml of CHCl₃). The mixture was then stirred for 5 hours at room temperature. The mixture was then diluted with 10 ml of CH₂Cl₂, washed with water, dried over Na₂SO₄ and evaporated to dryness. GC/MS analysis of the residue showed the presence of both 2 and unreacted 1 in a ratio of ca 1:2.

17β-Hydroxy-17α-methyl-2,3-seco-5α-androstane-2,3-dioic acid 2.

MP, 255 - 257 °C. IR, ν₅₅₃ 3300-2500 (br, H bonded OH); 1716, 1695 (2,3-seco diacid) cm⁻¹. HRMS (FAB, negative ion), 351.2157 ([M-1]⁻⁻, 100%) for C₁₀H₁₅O₅ (theoretical value, 351.2171). LRMS (EI, as two derivatives), see Fig. 5.2-2. ¹H NMR (400 MHz, CD₃OD), δ 0.83 (s, 3H, 18-H₃), 0.84 (s, 3H, 19-H₃), 1.17 (s, 3H, 20-H₃), 1.92, 1.96 (d-d, 1H, J₁ 15.1 Hz, J₂ 11.1 Hz, 4-H₄), 2.11 (t-t, 1H, 5-H), 2.30, 2.44 (AB d-d, 2H, 1-H₂, J 14.2 Hz), 2.66, 2.70 (d-d, 1H, J₁ 15.1 Hz, J₂ 2.4 Hz, 4-H₅). ¹³C NMR, δ 14.6 (C-18), 16.1 (C-19), 26.1 (C-20), 36.8 (C-4), 41.5 (C-5), 42.0 (C-1), 82.2 (C-17), 175.1, 177.4 (C-2 or C-3).

Isomers of metabolite 6a (3α,17β-dihydroxy-17α-methyl-5α-androstane-2β-carboxylic acid)

**Carboxylation of 3 with magnesium methyl carbonate** [22,23].

A magnesium methyl carbonate solution (2.0 M, 5 ml) was added dropwise to a 20 ml
solution of 3 (304 mg, 1 mmol) in DMF under argon. The mixture was then heated at 140 °C overnight. Evaporation of the solvent in vacuo gave a yellowish residue, to which 50 ml of diethyl ether was added. Concentrated HCl was then added to hydrolyse the chelated magnesium salt of the β-keto acid [22]. After hydrolysis the mixture was extracted with 2 x 50 ml of ether. The combined ether phases were back-extracted with 0.5 M aqueous KHCO₃ (2 x 30 ml). The aqueous phase was then reacidified and extracted with ether. Evaporation of the solvent in vacuo afforded a pale yellow residue (175 mg, yield 50%). GC/MS analysis (TMS derivative) showed a single broad peak indicating the presence of a mixture of the 2α- and 2β-carboxylic acids 5a and 8a. LRMS (tri-TMS derivative): m/z 549 ([M-15]+, 86%), 459 ([M-TMSOH]+, 7%), 143 (D-ring fragment [24], 100%). The residue was submitted to the reactions described hereinafter without further purification.

Reduction of 5a and 8a with NaBH₄.

NaBH₄ (0.1 M in isopropanol, 10 ml) was added with stirring into a 10 ml solution of the above mixture of 5a and 8a (120 mg) in isopropanol. The mixture was stirred for 3 hours at room temperature. After the reaction was terminated by adding 2 M HCl, the products were extracted with diethyl ether. The solvent was evaporated and the residue was methylated with diazomethane [25]. GC/MS analysis showed that the reaction afforded a mixture of the isomeric diols 6b, 9b, 7b and 10b (Fig. 5.2-4A). Only 6b had a retention time identical to that of the corresponding urinary metabolite. Attempts to isolate these four isomers by HPLC (Perkin Elmer series 3 liquid chromatograph,
Whatman Partisil 10 semi-preparative silica column, hexane/ethyl acetate (10:4 v/v) as mobile phase) only gave 9b and 10b as chromatographically pure compounds. No fraction containing 6b and 7b as chromatographically homogeneous compounds could be obtained, presumably because 6b and 7b were isomerized under the chromatographic conditions. After crystallization from aqueous methanol 8 mg of 9b and 35 mg of 10b were obtained.

Methylation and isomerization of 5a and 8a.

The methylation of a mixture of 5a and 8a (50 mg) with diazomethane provided 5b and 8b which were then dissolved in 5 ml tetrahydrofuran. To this solution, 100 µl of a 2.0 M LDA solution was added dropwise under argon and temperature was maintained at -5 °C. The mixture was stirred at -5 °C for 2 hours and then quenched with aqueous NH₄Cl. The 2α-carboxylic methyl ester 8b obtained was reduced with NaBH₄ as described above. GC/MS analysis of the reduction products showed that 9b and 10b were the major products and only traces of 6b and 7b were detected, probably due to incomplete isomerization at C-2 (Fig. 5.2-4B).

Carboxylation of 3 with methyl carbonate [26].

A 10 ml dioxane solution of 3 (304 mg, 1 mmol) kept under argon was reacted with dimethyl carbonate (0.6 ml) and sodium methoxide in methanol (50 mg of sodium in 0.7 ml absolute methanol). The reaction mixture was refluxed for 6 hours, then allowed to cool to room temperature and poured into 30 ml of 10% aqueous acetic acid. The
mixture was extracted with diethyl ether (2 x 50 ml) and the ether layer was dried with Na₂SO₄ and evaporated in vacuo. The keto ester 8b [26], contaminated with large amount of unreacted 3 (70%), was then reduced with NaBH₄. GC/MS analysis of the reduction mixture gave a chromatogram similar to that shown in Fig. 5.2-4B illustrating that besides 70% of reduced 3, compounds 9b and 10b were the main reduction products.

Epoxidation of 11 with mCPBA [21].

A 3 ml CHCl₃ solution of mCPBA (54 mg) was slowly added to a 3 ml ice cold CHCl₃ solution of 17β-hydroxy-17α-methyl-5α-androst-2-ene 11 (58 mg, 0.2 mmol). The mixture was stirred at room temperature for 5 hours and then diluted with 10 ml of CH₂Cl₂. The resulting solution was successively washed with 10% aqueous Na₂S₂O₃, 0.5 M aqueous KHCO₃ and water. Evaporation of the organic phase in vacuo afforded crude 12 (58 mg, yield 95%) as a white solid which was characterized by GC/MS (TMS-ether derivative). LRMS: m/z 376 ([M]+, 8%), 361 ([M-15]+, 50%), 286 ([M-TMSOH]+, 7%), 229 (10%), 143 (D-ring fragment [24], 100%).

Opening of epoxide 12 with cyanide [27,28].

To the preceding epoxide 12 (58 mg in 3 ml of toluene) was added 1 ml of diethyl aluminum cyanide (1 M in toluene). The reaction mixture was stirred for 4 hours at room temperature and then poured into a 2 M NaOH-ice mixture and extracted with diethyl ether (2 x 20 ml). The combined ether phase was then washed with water and dried over
Evaporation of ether in vacuo gave 62 mg of crude 13 (yield 98%) which was characterized by GC/MS. LRMS (TMS ether derivative): m/z 475 ([M]+, 3%), 460 ([M-15]+, 31%), 418 (D-ring fragment [24], 16%), 405 (D-ring fragment [24], 35%), 385 ([M-TMSOH]+, 10%), 370 (9%), 280 (6%), 143 (100%).

*Hydrolysis of nitrile 13 with H₂O or D₂O [29].*

Nitrile 13 (30 mg) was dissolved in 1 ml of acetone and 160 mg of Ba(OH)₂ in 5 ml of H₂O was added. Alternatively, 13 (30 mg) was dissolved in 1 ml of methyl alcohol-OD (CH₃OD) and 160 mg of Ba(OH)₂ in 5 ml of D₂O was added. The two mixtures were heated at 80 °C for 48 hours. After work-up (acidification with HCl and extraction with ether), 4.2 mg of 9a (yield 13%) and 4.0 mg (yield 10.6%) of the deuterated hydroxy acid 9c were obtained. GC/MS analysis of 9a and 9c confirmed 100% deuterium incorporation, presumably at C-2 of 9c. LRMS of 9a (TMS derivative) 566 ([M]+, 1%), 551 ([M-15]+, 8%), 386 ([M-2TMSOH]+, 7%), 269 ([M-2TMSOH-COOTMS]+, 10%), 268 ([M-2TMSOH-HCOOTMS]+, 8%), 143 (100%). LRMS of 9c (TMS derivative): m/z 567 ([M]+, 1%), 552 ([M-15]+, 8%), 387 (7%), 270 (10%), 269 (8%), 143 (100%).

3α,17β-Dihydroxy-17α-methyl-5α-androstane-2α-carboxylic acid methyl ester 9b.

MP, 120 - 122 °C. IR. ʋ_max 3100 - 3600 (br, OH), 1720 (2-COOMe) cm⁻¹. HRMS (Cl), m/z 365.2678 ([M+H]+, 44%) for C₂₂H₃₇O₄ (theoretical value, 365 2692). 346 ([M-18]+, 83%), 331 ([M-18-15]+, 100%), 328 ([M-2x18]+, 70%). ¹H NMR (400 MHz, CDCl₃), δ 0.82 (s, 3H, 18-H₃), 0.83 (s, 3H, 19-H₃), 1.18 (s, 3H, 20-H₁), 2.58 (br d, 1H,
$J$ 12.4, 2β-H), 3.67 (s, 3H, 2-COOCH$_3$), 4.29 (br s, 1H, 3β-H). $^{13}$C NMR, δ 12.1 (C-19), 14.7 (C-18), 26.1 (C-20), 75.1 (C-3), 82.3 (C-17), 173.2 (2α-COOMe).

3β,17β-Dihydroxy-17α-methyl-5α-androstane-2α-carboxylic acid methyl ester 10b. MP, 142 - 144 C. IR, $\nu_{max}$ 3100 - 3600 (br, OH), 1720 (2-COOMe) cm$^{-1}$. HRMS (Cl), m/z 365.2708 ([M+H]$^+$, 45%) for C$_{22}$H$_{37}$O$_4$ (theoretical value, 365.2692), 346 ([M-18]$^+$, 82%), 331 ([M-18-15]$^+$, 100%), 328 ([M-2x18]$^+$, 68%). $^1$H NMR (400 MHz, CDCl$_3$), δ 0.83 (s, 3H, 18-CH$_3$), 0.88 (s, 3H, 19-CH$_3$), 1.17 (s, 3H, 20-CH$_3$), 2.47 (m, 1H, 2β-H), 3.67 (s, 3H, 2-COOCH$_3$), 3.72 (m, 1H, 3α-H). Spin decoupling, irradiation at 3.72 ppm gave a broad doublet at 2.47 ppm with virtual coupling constant $J$ 11.2 Hz; while irradiation at 2.47 ppm gave a broad doublet at 3.72 ($J$ 8.9 Hz). $^{13}$C NMR, δ 13.0 (C-19), 14.7 (C-18), 26.1 (C-20), 72.3 (C-3), 82.2 (C-17), 172.9 (2α-COOMe).

Isomerization of urinary metabolite 6a.

Five 10 ml aliquots of post-administration urine were applied on five Sep-Pak C$_{18}$ cartridges and the acidic urinary metabolites were extracted as described above. The combined extracts were then methylated with diazomethane. GC/MS analysis showed the presence of 6b. The ethereal solution of this methylated urinary extract was then mixed with 1.5 g of basic aluminum oxide (prepared from 50 g of neutral aluminum oxide mixed with 5 ml of 10% sodium methoxide, then dried and activated at 125 °C for 4 hours). The resulting mixture was left in a sealed tube at room temperature for 24 hours and was then extracted with methanol. GC/MS analysis of the methanolic extracts
showed that about 50% of 6b has been isomerized to 9b.

Fig. 5.2-1. Reconstructed ion current chromatograms (m/z 143) from GC/MS analysis of (A) Unconjugated acidic metabolites as TMS-ester-TMS-ether derivative from a urine sample collected 2 hours after the oral administration of oxymetholone, (B) Extract from a blank urine sample as same derivative. IS is the internal standard (oxandrolone) The mass spectra corresponding to labelled GC peaks (2 and 6a) are given in Fig 5.2-2 and 5.2-3. Unlabelled metabolite peaks will be discussed in a separate paper. GC/MS conditions are the same as that described in reference 18.

Results and discussion

Identification of urinary metabolites.

A typical GC/MS profile of the major acidic urinary metabolites of oxymetholone 1 is shown in Fig. 5.2-1. Comparison with blank urine samples indicates the presence of several peaks, two of which corresponding to the major acidic metabolites 2 and 6a.
Their respective chemical structure have been elucidated by various synthetic approaches. Other minor acidic metabolites which are also produced from oxymetholone biotransformation in man will be reported elsewhere.

Fig. 5.2-2. Electron-impact(El) mass Spectra of metabolite 2 as (A) TMS-ester-TMS-ether and (B) methyl-ester-TMS-ether derivatives.

**Metabolite 2.**

The mass spectra of methyl and TMS ester derivatives of metabolite 2 are presented in Fig. 5.2-2. The characteristic ions at m/z 143, 395 and 511 ([M-57]⁺), 382 and 498 ([M-70]⁺) arising from the fragmentation of the D-ring [24] indicate that this moiety of oxymetholone skeleton has not been subjected to any biotransformation. A difference
of 116 a.m.u. between the molecular ions of these derivatives indicates the presence of two carboxyl groups. These data suggested that 2 is a 2,3-seco-2,3-dioic steroid formed by the oxidative degradation of oxymetholone C2-C3 bond. Although the occurrence of such a steroidal metabolite has never been reported in the literature, a 2,3-seco-2,3-dioic acid has been previously prepared in the cholestane series to characterize the A-ring of cholestanol [30]. Two different oxidation methods [20,21] were used to prepare seco diacid 2 from oxymetholone 1. Treatment of 1 with a mixture of NaIO₄/KMnO₄/K₂CO₃ was proven to be the most successful approach and afforded 2 in reasonable yield. GC and MS features of synthetic diacid 2 were identical to those of the urinary metabolite 2. HRMS and NMR data were also in agreement with the structure of 17β-hydroxy-17α-methyl-2,3-seco-5α-androstane-2,3-dioic acid proposed for compound 2. This metabolite could be detected in the post-administration urine sample until 32 hours after administration of oxymetholone. The overall excretion of 2 accounts for about 1.52% of the oral dose (detail quantitation data will be reported separately).

**Metabolite 6a.**

The structure elucidation of metabolite 6a has been more difficult to achieve. As in the case of metabolite 2, the mass spectra of 6a and 6b as their TMS derivatives (Fig. 5.2-3) indicated the presence of two hydroxyl groups and one carboxylic acid function and that the D-ring was unchanged. It was also noticed that there was a two mass unit difference between this metabolite and the presumed intermediate 5a or 8a. This indicates that 6a was probably produced by the reduction of the 3-keto function of 5a or 8a. However, these data did not provided any indication about the stereochemistry at C-2 and C-3. We
knew from our previous studies [31] and the literature [32] that the reduction of the 3-keto function of 5α-androstanes by 3α-hydroxy steroid dehydrogenase (3α-OHSDH) mainly yield 3α hydroxy steroids in man. Templeton and Michie [33] reported a similar biotransformation in the rabbit whereby the 2-hydroxymethylene function of 17β-hydroxy-2-hydroxymethylene-5α-androstan-3-one, a model steroid of oxymetholone, was transformed into a 2α-carboxylic acid group. According to the above information from the literature, it seemed likely to us that metabolite 6a had 2α-carboxy and 3α-hydroxy functions. In order to assess this proposed structural assignment, a series of syntheses were carried out.

Fig 5 2-3 El mass spectra of (A) metabolite 6a and (B) 6b as TMS derivative.
The first strategy involved the use of readily available mestanolone 3 as starting material so that carboxylation could provide the keto acids 5a and/or 8a, since functionalization of 5α-androstan-3-ones A-ring is usually oriented at C-2 [34]. Treatment of 3 with magnesium ethyl carbonate (MMC) gave a mixture of the β-keto acids 5a and 8a which upon reduction with NaBH₄ afforded a mixture of the four isomers 6a, 9a, 7a, and 10a which, upon methylation, gave the mixture of 6b, 9b, 7b and 10b (Fig. 5.2-4A). Among the four isomers only the methyl and TMS ester derivatives of 6a exhibited retention times identical to those of the corresponding derivatives of the urinary metabolite 6a.
Isolation of the methyl ester derivatives of these individual isomers by HPLC only gave the 2α-isomers 9b and 10b. This result may be rationalized by the fact that the axial 2β-carboxy function is sensitive to isomerization during the chromatographic separation of the original mixture of isomers, probably because of the steric strain [35] which is induced at C-2 from the angular methyl group at C-10. This hypothesis was further supported by data from the isomerization reaction of a mixture of 5b and 8b with LDA and subsequent reduction of the 3-keto group which, as expected, predominantly afforded the 2α-isomers 9b and 10b (Fig. 5.2-4B).

In a second synthetic approach to 6b, methyl carbonate was used to stereoselectively introduce a 2α-carboxylic group [26] to give the keto ester 8b as sole product. The reduction of 8b with NaBH₄ provided 9b and 10b in a ratio similar to that illustrated in Fig. 5.2-4B. Proton NMR data showed that the 2β-H in both 9b and 10b was axial (2α-carboxy) because of the strong spin-spin couplings with the neighboring C-1 axial protons (J₀,α 11.2 to 12.4 Hz)[36]. Data from these synthetic approaches indicated that both 9a and 10a bear a 2α-carboxy function. Therefore, one may infer that the urinary metabolite 6a bears a 2β-carboxy group (axial) since its methyl and TMS ester derivatives possess GC retention features which are different from those of the corresponding derivatives of 9 and 10.

Attention was then turned to a synthetic approach that provided more chiral control at C-2. Epoxidation of the 2,3-unsaturated steroid 11 with mCPBA gave the α-epoxide 12
as sole product [21]. Treatment of the 12 with diethylaluminum cyanide gave 3α-hydroxy-2β-nitride 13 [27,28] through trans diaxial opening of the oxirane function. Because of the isomerization of the 2β-functional groups, basic hydrolysis of the 2β-nitrile 13 solely afforded the thermodynamically stable 2α-carboxylic acid 9α. Isomerization of the 2β-substituent during basic hydrolysis of 13 was demonstrated by deuterium incorporation at C-2 when the hydrolysis was carried out in D2O which afforded compound 9c. Although this approach to prepare 6α was not successful, it demonstrated that steroid 9α bears 2α-carboxy and 3α-hydroxy functions. Proton NMR data also indicated that 9b has a 3β-H (equatonal) because of the weak spin-spin couplings with neighboring protons (J_a,a or J_a,e were less than 2 Hz)[36], whereas 10b has a 3α-H (axial J_a,a 8.9 Hz). At this stage, we could propose that the urinary metabolite 6a (Fig. 5.2-1A) bears 2β-carboxy and 3α-hydroxy functions.

Definitive evidence for the proposed structure of 6α was obtained by isomerization of the corresponding methyl ester of the urinary metabolite 6b to 9b. The isomerization of urinary 6b was achieved under mild conditions using basic alumina as reagent. The isomer thus obtained exhibited identical chromatographic and mass spectral properties as those of compound 9b prepared from other synthetic routes. Thus, compound 6a was identified as 3α,17β-dihydroxy-17α-methyl-5α-androstan-2β-carboxylic acid.

It is of interest to note that metabolite 6a bears 2β-carboxy and 3α-hydroxy function which are both axially oriented and, theoretically, thermodynamically less stable than the
corresponding 2α,3α and 2α,3β isomers. The formation of 6a can be rationalized by the fact that steroid hormones are frequently metabolized with a high degree of specificity to yield unique metabolites [37]. A possible mechanism accounting for the formation of 6a could involve the formation of the β-keto acid 5a from 1 in which the β-orientation of the 2-carboxylic group would be set and maintained by the specificity and stereoselectivity of the binding of oxymetholone with the oxidative enzyme. Then, the 3-keto group would be reduced by 3α-hydroxysteroid dehydrogenase (3α-OHSDH) to give 6a. This proposed biosynthetic route is further supported by the occurrence of the neutral metabolite 3 which arises from the decarboxylation of its immediate precursor 5a [38]. This indicates that the oxidation of the hydroxymethylene group and the reduction at C-3 probably occur in a sequential manner (Scheme 5.2-1).

The 2-aldehyde functions in both formebolone [18] and oxymetholone appear to be quite labile and can easily degrade to acidic breakdown products. This lability is not surprising, particularly for oxymetholone, given the presence of a unique conjugated β-dicarbonyl moiety which is prone to decarboxylation and to biological and chemical oxidation. The 2,3-seco-2,3-dioic acid 2 can be formed either from direct oxidation of oxymetholone, or from oxidation of the intermediate β-keto acid 5a. Data from the synthetic work described above indicate that both reactions could probably proceed via a sequence that utilized the enol form of oxymetholone 3-keto group [39] as substrate for epoxidation to give an oxirane which can subsequently rearranged and/or be oxidized to yield 2. Similar oxidative reactions involving the double bond of enol functions have
been reported [40].

In conclusion, the structures of the two major acidic urinary metabolites of oxymetholone have been elucidated. The stereochemistry of the A-ring substituents of compound 6a was determined using several synthetic approaches. The isomeric $\beta$-hydroxy acids 6a, 7a, 9a and 10a synthesized in this study can be regarded as useful synthetic intermediates and model compounds for comparative spectrometric studies for structure elucidation of other metabolite of oxymetholone in man. Finally, it is worth mentioning that the occurrence of a 2,3-seco-2,3-dioic steroid such as compound 2 as a metabolite of an endogenous or synthetic steroid has never been reported in the literature.

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References


metabolites of formebolone in man by gas chromatography/mass spectrometry.


CHAPTER 6

6.1. STUDIES ON ANABOLIC STEROIDS.

TERTIARY SULFATES OF ANABOLIC 17α-METHYL STEROIDS:
SYNTHESIS AND REARRANGEMENT*

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Summary

A simple and convenient method has been developed to prepare sulfates of anabolic 17β-hydroxy-17α-methyl steroids. The sulfates of methandienone, 17α-methyltestosterone, mestanolone, oxandrolone and stanozolol were prepared. Different A-ring functions were not affected under the sulfation condition. The buffered hydrolyses of these sulfates provided the 17-epimers of the original steroids and 17,17-dimethyl-18-nor-13(14)-ene steroids, presumably via the 17-carbocations.
Introduction

Sulfates are important steroid metabolites in living organisms. Steroid sulfates are not merely end products of metabolism which are excreted in urine, they are also found in high concentrations in human plasma, and they can serve as intermediates in steroid metabolism and as precursor pools of biologically active androgens and estrogens [1]. In a previous paper of this series on the study of anabolic steroid metabolism in man [2], we reported preliminary data about the role of sulfate conjugates of 17β-hydroxy-17α-methyl steroids in the in vivo formation of their corresponding 17α-hydroxy-17β-methyl epimers. Rongone and Segaloff [3] were the first researchers to report the occurrence of 17-epimethandienone 3 (Scheme 6.1-1) as a probable urinary metabolite of methandienone 1. It was synthesized and identified by MacDonald and co-workers [4] in 1971. More recently, Dürbeck and co-workers [5] reported the detection of the 18-nor-13(14)-ene steroid 4 as an artifact resulting from the dehydration and rearrangement of 1 under the acidic conditions prevailing in the gut. A mechanism accounting for the formation of the epimer 3 was recently proposed by Edlund and co-workers [6]. These authors showed that 3 was produced from the 17β-sulfate conjugate of 1 through the formation of a tertiary carbocation, which upon nucleophilic attack by one molecule of water afforded the corresponding 17-epimer 3 (Scheme 6.1-1). This reaction is apparently governed by the intrinsic lability of the tertiary 17β-sulfate moiety of the steroid in aqueous media. In addition, the presence of a methyl group at the vicinal C-13 position leads to further rearrangement products through migration of the methyl group from C-13 to C-17.
Anabolic 17β-hydroxy-17α-methyl steroids are well known for their strong liver toxicities and carcinogenicities. This may perhaps be due to conjugation with sulfuric acid, leading to highly reactive alkylating agents (17-carbocation) and their probable ability to alkylate nucleic acids and/or other biological macromolecules. Therefore, the preparation of these sulfates is of great importance to further studies about their covalent binding characteristics, which may be crucial to initiation of events leading to hepatic side effects.

The chemical synthesis of steroid sulfates have been extensively reviewed [7-9]. The synthesis of tertiary alcohol sulfates usually requires higher temperature and/or longer reaction times than that of secondary sulfates [7,10,11]. This may cause side reactions complicating the purification steps and lowering the yield of the desired sulfates.

In our previous metabolism studies of several 17β-hydroxy-17α-methyl anabolic steroids, 17-epimers were often encountered as important urinary metabolites [2,12,13]. 17-Epimerization is a common metabolic route for these anabolic steroids. For certain steroids, 17,17-dimethyl-18-nor-13(14)-enes, proposed as artifacts [5], can be detected in the post administration urine. The confirmation of these urinary metabolites and artifacts relies on the synthesis of authentic substances. However, the synthetic method used by MacDonald et al [4] to prepare the 17-epimer 3 was lengthy and the overall yield was only 3%. It seemed to us that 17β-sulfates of these anabolic steroids could be very good precursors to these 17-epimers and rearrangement products. In order to investigate the transformation of 17β-hydroxy sulfates to the 17-epimers and other
rearrangement products, we have developed a simple and convenient synthetic method to prepare 17β-hydroxy sulfates and their rearrangement products. Literature methods were modified to prepare 17,17-dimethyl-18-nor-13(14)-ene steroids 4, 8, 12, 16 and 20, in better yield, directly from anabolic steroids 1, 5, 9, 13 and 17 (the structures of all the steroids being discussed are presented in Fig. 6.1-1).

Experimental

Steroids were purchased from Sigma Chemical Co.(St. Louis, MO) or Steraloids Inc.(Wilton NH); chlorosulfonic acid (CISO$_2$H, 99%) was supplied by Aldrich Chemical Co.(Milwaukee, WI); pyridine (distilled in glass) and other organic solvents (HPLC grade) were from Caledon Laboratories Ltd.(Georgetown, Ontario); inorganic salts were purchased from Caledon or J.T. Baker(Philipsburg, NJ); pyridine was distilled from KOH before use.

Melting points were measured on an electrothermal melting points apparatus and are reported without correction. IR spectra were taken in Nujol on a Perkin-Elmer 267 infrared spectrophotometer. $^1$H and $^{13}$C NMR spectra were recorded in CDCl$_3$ (unless otherwise indicated) on a Varian XL-200, Varian XL-300 or Bruker WH-400 spectrometer with tetramethylsilane as internal standard. Low resolution mass spectra (LRMS, EI) were recorded on Hewlett Packard 5890-5970 GC-MSD instruments. Positive or negative high resolution mass spectra [HRMS, CI (NH$_3$ as reagent gas) or FAB (glycerol as matrix)] were taken on a Kratos MS50TC mass spectrometer.
Fig. 6.1-1. Chemical structures of steroids 1 - 20.
General method for the preparation of steroid 17β-tertiary sulfates 2, 6, 10, 14 and 18 [14]

To 5 ml of cooled pyridine (-5 °C) under N₂ was added slowly with stirring via a narrow-gauge syringe (No. 22) 160 µl of ClSO₂H (2.4 mmol), leading to the formation of a white precipitate. (Caution: addition of ClSO₂H must be very slow to prevent a violent explosion!). A solution of 2 mmol of the steroid in 5 ml pyridine was added. After stirring for 5 min, the cold bath was removed and the reaction was allowed to continue at room temperature for 2 to 3 hours. At the end of the reaction, as established by the disappearance of the starting steroid (TLC condition: hexane:EtOAc, 1:1, v/v), 10 - 15 ml of hexane was added to the gelatinous mixture to completely precipitate the sulfate. After stirring for 10 - 15 min, the precipitate was filtered and washed with hexane. The resulting white solid was further dried *in vacuo*. The resulting pyridinium salt of the sulfate was kept under dry and cold (-20 °C) conditions to prevent decomposition.

General procedure for the preparation of 17-epimers (3, 7, 11, 15 and 19) from 17β-sulfates

A solution of 0.1 mmol of sulfate in 2 ml of 0.2 M phosphate buffer (pH 7.0) was left at room temperature overnight. The precipitated steroids were extracted with 2 x 10 ml of Et₂O, the Et₂O phase was washed with H₂O, dried with Na₂SO₄ and evaporated *in vacuo*. The products were separated on a Sep-Pak™ Silica cartridge (Millipore Co., Milford, MA). The cartridge was first washed with 5 ml hexane, and then a solution of
the above reaction product in 5 ml of hexane/CHCl₃ (10:1, v/v) was applied on the cartridge. The rearrangement products (4, 8, 12, 16 and other isomeric steroids) were eluted with a mixture of hexane/EtOAc (10:2, v/v), and then the 17-epimers (3, 7, 11 and 15) were eluted with EtOAc. For stanozolol, the separation of the 17-epimer and rearrangement products was performed on a Sep-Pak™ NH₂ cartridge (Millipore Co.), rearrangement products (20 and other isomeric steroids) were eluted with hexane/CHCl₃/EtOAc (10:2:2,v/v/v), and 17-epistanozolol (19) was eluted with EtOAc.

**General method for the preparation of 17,17-dimethyl-18-nor-13(14)-ene steroids (4, 8, 12, 16 and 20)**

**Method (1)**

For methandienone 1 and methyltestosterone 5: 0.1 mmol of steroid was suspended in 3 ml of 2 M HCl and allowed to react for 30 min at 60 °C. The reaction mixture was extracted with 2 x 10 ml of Et₂O. The ether layer was washed with aqueous K₂CO₃ and H₂O, and dried over Na₂SO₄. After evaporation of the solvent, the extract was purified by silica cartridges as described above.

**Method (2)**

For mestanolone 9, oxandrolone 13 and stanozolol 17 [15]: 0.1 mmol of steroid was treated with 2 ml of concentrated HCl at room temperature for 15 min. The resulting mixture was diluted with H₂O and extracted with Et₂O. The ethereal phase was washed with aqueous K₂CO₃, H₂O and dried over Na₂SO₄. After removing the solvent in vacuo, the residue was crystallized.
Methandienone 17β-sulfate pyridinium salt 2.

Following the general synthetic method, evaporation of the solvent gave 960 mg of 2 (quantitative yield). MP, 140-145 °C. IR, \( \nu_{\text{max}} \) 1220 (SO), 1180 (SO), 1150 (SO), 1040 (SO) cm\(^{-1}\). HRMS (FAB, negative ion), m/z 379.1563 ([M-H], 100%) for \( \text{C}_{10}\text{H}_{12}\text{O}_{4}\text{S} \) (theoretical value, 379.1579). \(^1\)H NMR (200 MHz, CD\(_2\)OD), \( \delta \) 0.92 (s, 3H, 18-H), 1.17 (s, 3H, 19-H), 1.23 (s, 3H, 17α-CH\(_3\)), 6.05 (s, 1H, 4-H), 6.21 (d, 1H, \( J \) 10 Hz, 2-H), 7.05 (d, 1H, \( J \) 10 Hz, 1-H). \(^{13}\)C NMR, see Table 6.1-1.

17α-Hydroxy-17β-methylandrosta-1,4-diene-3-one 3 (17-epimethandienone).

Separation on silica cartridge and crystallization from CHCl\(_3\)/hexane afforded 5.1 mg of 3 (yield 17%). MP, 224-225 °C (reported [4] 221 °C). IR, \( \nu_{\text{max}} \) 3430 (OH), 1656 (C=C=C=O), 1620 (C=C), 1600 (C=C) cm\(^{-1}\). HRMS(CI), m/z 301.2149 ([M+H]\(^+\), 78%) for \( \text{C}_{20}\text{H}_{29}\text{O}_2 \) (theoretical value, 301.2167), 282 (28%), 161 (34%), 122 (78%). \(^1\)H NMR (400 MHz), \( \delta \) 0.76 (s, 3H, 18-H), 1.21 (s, 3H, 17β-CH\(_3\)), 1.25 (s, 3H, 19-H), 6.07 (s, 1H, 4-H), 6.23 (d-d, 1H, 2-H, \( J_1 \) 9 Hz, \( J_2 \) 10.2 Hz), 7.07 (d, 1H, 1-H, \( J \) 10.2 Hz). \(^{13}\)C NMR, see Table 6.1-1.

17,17-Dimethyl-18-norandrosta-1,4,13(14)-triene-3-one 4.

Treatment of methandienone 1 by the acidic rearrangement method (1) afforded 18 mg of 4 as a light yellow oil (yield 60%). Repeated attempts failed to give a crystalline product. The purity was assessed by GC/MS analysis (>99%). IR, \( \nu_{\text{max}} \) 1660 (C=C=C=O), 1620 (C=C), 1600 (C=C) cm\(^{-1}\). HRMS(CI), m/z 283.2049 ([M+H]\(^+\), 100%)
for C<sub>20</sub>H<sub>27</sub>O (theoretical value, 283.2062), 267 (30%), 161 (36%), 159 (24%), 122 (83%). <sup>1</sup>H NMR (300 MHz), δ 0.92 (s, 3H, 17-CH<sub>3</sub>), 0.96 (s, 3H, 17-CH<sub>3</sub>), 1.21 (s, 3H, 19-H<sub>j</sub>), 6.08 (s, 1H, 4-H), 6.25 (d, 1H, J 10.1 Hz, 2-H), 7.14 (d, 1H, J 10.1 Hz, 1-H) <sup>13</sup>C NMR, see Table 6.1-1.

Methyltestosterone 17β-sulfate pyridinium salt 6.

Evaporation of solvent afforded 966 mg of 6 (quantitative yield). MP, 125 - 130 °C. IR, δmax 1250 (SO), 1180 (SO), 1150 (SO), 1040 (SO) cm<sup>-1</sup>. HRMS (FAB, negative ion), m/z 381.1736 ([M-H], 100%) for C<sub>21</sub>H<sub>26</sub>NO<sub>3</sub>S (theoretical value, 381.1746). <sup>1</sup>H NMR (200 MHz, pyridine-d<sub>5</sub>), δ 0.93 (s, 3H, 18-H<sub>3</sub>), 1.16 (s, 3H, 19-H<sub>3</sub>), 1.89 (s, 3H, 17α-CH<sub>3</sub>), 5.87 (s, 1H, 4-H) <sup>13</sup>C NMR, see Table 6.1-1.

17α-Hydroxy-17β-methylandrost-4-ene-3-one 7 (17-epimethyltestosterone).

Separation on silica cartridge and crystallization from CHCl<sub>3</sub>/hexane gave 6.2 mg of 7 as needles (yield 21%). MP, 180 - 181 °C (reported [16] 182 °C). IR, δmax 3430 (OH), 1665 (C=C-C = O), 1600 (C=C) cm<sup>-1</sup>. HRMS (Cl), m/z 303.2328 ([M+H]<sup>+</sup>, 100%) for C<sub>20</sub>H<sub>21</sub>O<sub>2</sub> (theoretical value, 303.2324), 284 (21%), 269 (18%), 161 (9%), 124 (23%). <sup>1</sup>H NMR (400 MHz), δ 0.73 (s, 3H, 18-H<sub>3</sub>), 1.20 (s, 3H, 19-H<sub>j</sub>), 1.21 (s, 3H, 17β-CH<sub>3</sub>), 5.73 (s, 1H, 4-H). <sup>13</sup>C NMR, see Table 6.1-1.

17,17-Dimethyl-18-norandrosta-4,13(14)-diene-3-one 8.

Treatment of 17α-methyltestosterone 5 by the acid rearrangement method (1) afforded
19.5 mg of 8 as light yellow oil (yield 65%). Its purity was assessed by GC/MS analysis (>98%). IR, \( \nu_{\text{max}} \) 1665 (C=C-C=O), 1620 (C=C) cm\(^{-1}\). HRMS (Cl), m/z 285.2235 ([M+H]\(^+\), 87%) for \( \text{C}_{20}\text{H}_{29}\text{O} \) (theoretical value, 285.2218), 269 (100%), 161 (7%). \( ^1\)H NMR (400 MHz), \( \delta \) 0.94 (s, 3H, 17-CH\(_3\)), 0.96 (s, 3H, 17-CH\(_3\)), 1.15 (s, 3H, 19-H\(_3\)), 5.73 (s, 1H, 4-H). \( ^{13}\)C NMR, see Table 6.1-1.

Mestanolone 17\( \beta \)-sulfate pyridinium salt 10.

Evaporation of solvent afforded 970 mg of 10 (quantitative yield). MP, 140-146 °C. IR, \( \nu_{\text{max}} \) 1250 (SO), 1180-1150 (br, SO), 1050-1030 (br, SO) cm\(^{-1}\). HRMS (ClAB, negative ion), m/z 383.1934 ([M-H], 100%) for \( \text{C}_{20}\text{H}_{41}\text{O},\text{S} \) (theoretical value, 383.1892). \( ^1\)H NMR (400 MHz), \( \delta \) 0.86 (s, 3H, 18-H\(_3\)), 0.95 (s, 3H, 19-H\(_3\)), 1.50 (s, 3H, 17\( \alpha \)-CH\(_3\)). \( ^{13}\)C NMR, see Table 6.1-1.

17\( \alpha \)-Hydroxy-17\( \beta \)-methyl-5\( \alpha \)-androstan-3-one 11 (17-epimestanolone).

Separation on silica cartridge and crystallization from CHCl\(_3\)/hexane afforded 11 as needles (7.0 mg, yield 23.3%). MP, 234 - 235 °C. IR, \( \nu_{\text{max}} \) 3480 (OH), 1705 (C=O) cm\(^{-1}\). HRMS (Cl): m/z 307.2485 ([M+H]\(^+\), 18%) for \( \text{C}_{20}\text{H}_{33}\text{O}_2 \) (theoretical value, 305.2480), 289 (31%), 286 (52%), 271 (100%), 161 (9%). \( ^1\)H NMR (400 MHz), \( \delta \) 0.70 (s, 3H, 18-H\(_3\)), 1.03 (s, 3H, 19-H\(_3\)), 1.20 (s, 3H, 17\( \beta \)-CH\(_3\)). \( ^{13}\)C NMR, see Table 6.1-1.

17,17-Dimethyl-18-nor-5\( \alpha \)-androstan-13(14)-ene-3-one 12.

Treatment of mestanolone 9 by the acidic rearrangement method (2) and crystallization
from aqueous ethanol gave 20 mg of 12 (yield 65%). MP, 140 - 143 °C (reported [15] 132 - 134 °C). IR, $\nu_{\text{max}}$ 1705 (C=O), 1650 (C=C) cm$^{-1}$. HRMS (Cl), m/z 287.2362 ([M+H]$^+$, 27%) for C$_{20}$H$_{31}$O (theoretical value, 287.2375), 271 (100%), 161 (17%).

$^1$H NMR (400 MHz), $\delta$ 0.96 (s, 6H, 17-2CH$_3$), 0.98 (s, 3H, 19-H$_3$). $^{13}$C NMR, see Table 6.1-1.

Oxandrolone 17β-sulfate pyridinium salt 14.

Evaporation of solvent afforded 970 mg of 14 (quantitative yield). MP, 150 - 155 °C. IR, $\nu_{\text{max}}$ 1250 (SO), 1180 (SO), 1040 (SO) cm$^{-1}$. HRMS (FAB, negative ion), m/z 385.1685 ([M-H], 100%) for C$_{19}$H$_{29}$O$_6$S (theoretical value, 385.1594). $^1$H NMR (200 MHz), $\delta$ 0.90 (s, 3H, 18-H$_3$), 0.95 (s, 3H, 19-H$_3$), 1.53 (s, 3H, 17α-CH$_3$). 2.20 (d-d, 1H, 4β-H, $J_{4\beta, 5}$ 13 Hz, $J_{\alpha, \beta}$ 18.7 Hz), 2.50 (d-d, 1H, 4α-H, $J_{4\alpha, 5}$ 6 Hz, $J_{\alpha, \beta}$ 18.7 Hz), 3.89 (d, 1H, 1β-H, $J_{\alpha, \beta}$ 10.7 Hz), 4.20 (d, 1H, 1α-H, $J_{\alpha, \beta}$ 10.7 Hz). $^{13}$C NMR, see Table 6.1-1.

17α-Hydroxy-17β-methyl-2-oxa-5α-androstan-3-one 15 (17-epioxandrolone).

Separation on silica cartridge and crystallization from CHCl$_3$/hexane afforded 7.3 mg of 15 (yield 24%). MP, 205-206 °C. IR, $\nu_{\text{max}}$ 3500 (OH), 1735 (lactone) cm$^{-1}$. HRMS (Cl), m/z 307.2263 ([M+H]$^+$, 10%) for C$_{19}$H$_{31}$O$_3$ (theoretical value, 307.2273), 291 (12%), 288 (28%), 273 (100%). $^1$H NMR (400 MHz), $\delta$ 0.69 (s, 3H, 18-H$_3$), 1.00 (s, 3H, 19-H$_3$), 1.20 (s, 3H, 17β-CH$_3$), 2.22 (d-d, 1H, 4β-H, $J_{4\beta, 5}$ 13 Hz, $J_{\alpha, \beta}$ 18.5 Hz), 2.51 (d-d, 1H, 4α-H, $J_{4\alpha, 5}$ 6 Hz, $J_{\alpha, \beta}$ 18.5 Hz), 3.94 (d, 1H, 1β-H, $J_{\alpha, \beta}$ 10.7 Hz), 4.25 (d, 1H, 1α-H, $J_{\alpha, \beta}$ 10.7 Hz). $^{13}$C NMR, see Table 6.1-1.
17,17-Dimethyl-18-nor-2-oxa-5α-androstan-13(14)-ene-3-one 16.

Treatment of oxandrolone 13 by the acidic rearrangement method (2) and crystallization from CHCl₃/hexane gave 22.5 mg (yield 73%) of 16. MP, 106-107 °C. IR, νmax 1740 (lactone), 1650 (C=C) cm⁻¹. HRMS (Cl), m/z 289.2155 ([M + H]⁺, 23%) for C₁₉H₂₉O₂ (theoretical value, 289.2167), 273 (100%), 161 (3%). ¹H NMR (400 MHz), δ 0.96 (s, 6H, 17-2CH₃), 0.97 (s, 3H, 19-H₃), 2.23 (d-d, 1H, 4β-H, J₄β-5 13.2 Hz, J₁αβ 18.6 Hz), 2.54 (d-d, 1H, 4α-H, J₄α-5 8 Hz, J₁αβ 18.6 Hz), 3.97 (d, 1H, 1α-H, J¹α-2 10.7 Hz), 4.32 (d, 1H, 1α-H, J₁αβ 10.7 Hz). ¹³C NMR, see Table 6.1-1.

Stanozolol 17β-sulfate pyridinium salt 18.

Stanozolol 17 was reacted with 2.4 Equivalents of CISO₃H to complete the sulfation. Complete evaporation of solvent afforded 1390 mg of 18 (quantitative yield). The final sulfate salt contained about 30% of SO₃-Py as impurities (net weight of the sulfate should be 975 mg), which did not affect the preparation of the 17-epimer MP, 205 - 215 °C. IR, νmax 1260 (SO), 1230 (SO), 1180 (SO), 1055 (SO) cm⁻¹. HRMS (I-AB, negative ion), m/z 407.1925 ([M - H], 100%) for C₂₁H₂₁N₄O₄S (theoretical value, 407.2004). ¹H NMR (200 MHz, CD₃OD), δ 0.78 (s, 3H 18-H₁), 0.93 (s, 3H, 19-H₃), 1.51 (s, 3H, 17α-CH₃), 7.79 (br s, 1H, 3'-H). ¹³C NMR, see Table 6.1-1.

17α-Hydroxy-17β-methyl-5α-androstano[3,2-c]pyrazole 19 (17-epistanozolol).

Separation on Sep-Pak³M NH₂ cartridges and crystallization from CHCl₃/hexane afforded 6.6 mg (yield 20%) of 19. MP, 175 - 180 °C. IR, νmax 3150 (br, s, H-bonded OH and
NH), 1600 (C=N) cm\(^{-1}\). HRMS (Cl), m/z 329.2571 ([M+H]\(^+\), 15%) for C\(_{21}\)H\(_{33}\)N\(_2\)O (theoretical value, 329.2593), 310 (46%), 295 (100%), 270 (40%), 258 (28%). \(^1\)H NMR (400 MHz), \(\delta\) 0.71 (s, 3H, 18-H\(_3\)), 0.76 (s, 3H, 19-H\(_3\)), 1.21 (s, 3H, 17\(\beta\)-CH\(_3\)), 7.36 (br s, 1H, 3\(\beta\)-H). \(^1\)C NMR, see Table 6.1-1.


Treatment of stanozolol 17 by the acidic rearrangement method (2) and crystallization from CHCl\(_3\)/hexane afforded 20.5 mg (yield 66%) of 20. MP, 230 -235 °C. IR, \(v\)\(_{\text{max}}\) 3160 (br, H-bonded NH), 1600 (C=N) cm\(^{-1}\). HRMS (Cl), m/z 311.2477 ([M+H]\(^+\), 26%) for C\(_{21}\)H\(_{41}\)N\(_2\) (theoretical value, 311.2487), 295 (100%), 161 (3%). \(^1\)H NMR (400 MHz), \(\delta\) 0.72 (s, 3H, 19-H\(_3\)), 0.98 (s, 6H, 17-2CH\(_3\)), 7.41 (br s, 1H, 3\(\beta\)-H). \(^1\)C NMR, see Table 6.1-1.

Results and discussion

Synthesis of tertiary sulfates

Because the five anabolic steroids studied (methandienone 1, methyltestosterone 5, mestanolone 9, oxandrolone 13 and stanozolol 17) bear different functions in their respective A-ring, mild reagents such as commercial SO\(_3\)-pyridine (SO\(_3\)-Py) complex and SO\(_3\)-Et,N complex were first used so as not to affect the A-ring functions. However, the sulfation yields were very low (< 15%), even if elevated temperatures and prolonged reaction times were used. Under such conditions, reaction mixtures became yellow or
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* The resonances of C3's of stanozolol derivatives are 145.1 ppm for 18, and 141.0 for 17, 19, and 20.
† The δc in parenthesis may be interchangeable.
brown, indicating decomposition of the starting material. It became obvious that the sulfation of these tertiary hydroxy groups requires stronger sulfation reagents. A modified sulfation method [14] by which SO_2-Py was prepared *in situ* by adding ClSO_2H to pyridine was then used. The reaction was convenient and very simple to carry out and allowed for the rapid transformation of the tertiary alcohol function into the corresponding sulfate pyridinium salt. The reaction temperature was kept below 0 °C for the first 10 min to minimize side reactions since with higher temperatures, the reaction mixture darkened and yields were lower. The reaction was then allowed to proceed at room temperature for 2 to 3 hours. At the end of reaction, hexane was added to precipitate the sulfate salt. The yield was almost quantitative. The tertiary sulfate is very labile in the presence of water. The pyridinium salts, which may contain some unreacted SO_2-Py, were stable for more than 14 months when kept as such in dry and cold (-20 °C) conditions. In the case of stanozolol 17, an additional equivalents of ClSO_2H was needed for completion of the reaction. Although there was no evidence to indicate the formation of a N-sulfate, less ClSO_2H lead to incomplete sulfation.

**Preparation of 17-epimers**

Tertiary sulfates are very labile in aqueous systems. Edlund *et al.* [6] reported that the half-lives of 2 in different aqueous buffers and equine urine are in the order of minutes. Under our reaction conditions, the major decomposition products of sulfate 2 were the 17-epimers 3 and 17,17-dimethyl-18-norandrosta-1,4,13(14)-triene-3-one 4. The formation of 17-epimers indicates that a carbocation at carbon 17 is the most likely intermediate (Scheme 6.1-1). However, no detailed kinetic studies were carried out to
Scheme 6 1-1. Synthesis and rearrangement of methandienone 17β-sulfate that is solvolysed to carbonium A. Addition of one molecule of water from the α-site gives the 17-epimer and rearrangement of A to carbonium ion B with concomitant elimination of a proton affords the 17,17-dimethyl-18-nor-13(14)-ene 4.

assess the proposed mechanism. Nucleophilic attack of H₂O on the planar cation at C-17 occurs, as expected [17], from the α-side of the D-ring because steric hindrance is expected from the angular methyl group at C-13 which appears to prevent any nucleophilic attack from the β-side. Alternatively, the carbocation A (Scheme 6.1-1) rearranges to carbocation B, which then, eliminates a proton to give 17,17-dimethyl-18-nor-13(14)-enes. GC/MS analysis showed that along with the major rearrangement products, three other by-products, the molecular ion of which was identical to that of the corresponding major rearrangement product, were also formed upon degradation of the 17-sulfate derivatives. The separation of the 17-epimers from the major rearrangement product was done using Sep-Pak™ silica cartridges, with the exception of stanozolol derivatives. In that specific case, 17-epistanozolol could not be separated from the corresponding rearrangement products, probably because the polar pyrazole moiety
determines their chromatographic retention on silica gel. In order to circumvent this problem, a bonded silica ion-exchange phase (NH$_2$) was used and the epimer 19 was isolated as a homogeneous compound.

**Preparation of 17,17-dimethyl-18-nor-13(14)-ene steroids**

The Wagner-Meerwein rearrangement of tertiary sulfates gave 17,17-dimethyl-18-nor-13(14)-enes and other isomeric by-products. Because of their similar chromatographic properties, these unsaturated steroids could not be efficiently separated by column chromatography. An alternative method, based on an acid catalyzed rearrangement, was therefore used to prepare pure 17,17-dimethyl-18-nor-13(14)-ene steroids. Methods from the literature in which strong acidic conditions (e.g. concentrated HCl or HOAc-HCl) [15,18] are used were found suitable to prepare steroids 12, 16 and 20. However, under these strong acidic conditions, steroids 1 and 5, which bear one and two double bonds respectively in their A-ring, afforded a brown oily residue containing several unidentified products. In order to prepare pure steroid 4 and 8, much milder reaction conditions (2 M HCl) were used. Besides 5 - 10% of the starting steroids and traces of other by-products, the latter reaction afforded 4 and 8 as the major products resulting from the parent steroids 1 and 5 respectively.

**General $^{13}$C NMR spectral features of sulfates, 17-epimers and 18-nor-13(14)-enes**

The $^{13}$C NMR spectral assignments presented in Table 6.1-1 are based on those of the parent steroids 1, 5, 9, 13, and 17 and data from the literature [19,20].
17β-Sulfates (2, 6, 10, 14 and 18)

Examination of the $^{13}$C NMR data reveals, as expected [8], downfield shifts of 10.9 to 11.7 ppm for the tertiary 17-carbon atoms of the sulfate derivatives with respect to the corresponding signals from the parent steroids, due to the strong electronegativity and polarity of the sulfate group. The chemical shifts of neighboring carbons were much less affected. For example, the C-13s and the C-20s showed downfield shifts of 1.3 to 1.6 ppm and upfield shifts of 2.7 to 2.9 ppm, respectively. A small $\beta$- effect from the 17β-sulfate group was noticed for signals associated with C-12 and C-14 and was reflected by upfield shifts of 0.9 to 3.2 ppm and 1.6 ppm respectively relative to parent steroids, except for stanozolol for which the corresponding signals were shielded by 0.7 and 0.4 ppm respectively. No $\beta$- effect was observed at the C-15 methylene group and no long range effect was noticed on the A- and B- ring carbons. These spectral data indicate that the carbon atom bearing the $\beta$-sulfate group exhibit the largest deviation whereas $\alpha$- and $\beta$- effects were negligible, indicating that the introduction of the latter group at C-17 did not induce any significant conformational change in any of the studied steroids with respect to those of the parent steroids.

17-Epimers (3, 7, 11, 15 and 19)

Although epimerization at C-17 brings about new short range interactions between the $\alpha$- and $\beta$- substituents at this position and neighboring carbon atoms, shifts of $^{13}$C resonances were relatively small. The steric interaction between the C-18 and C-20 induced a shielding effect on the C-20s, the resonance of which was shifted upfield by
3.0 to 3.6 ppm. Likewise, resonances of C-12s, C-14s and C-16s were shifted upfield by 0.2 to 0.5 ppm, 0.5 to 0.8 ppm and 1.5 to 2.2 ppm respectively. Conversely, carbon nuclei at the 13, 15, 17 and 18 positions were slightly deshielded by 1.1 to 1.3 ppm, 0.6 to 0.8 ppm, 0.4 to 0.5 ppm and 1.8 to 2.0 ppm respectively. The upfield shifts of the C-16 and C-20 resonances combined to the downfield shift exhibited by C-18 constituted an adequate spectral probe to differentiate 17β-hydroxy-17α-methyl steroids from their 17-epimers.

17,17-dimethyl-18-nor-13(14)-enes (4, 8, 12, 16, and 20)

The introduction of a 13(14) double bond in these steroids brings about the transformation of the chair conformation of the C-ring to a half-chair structure. The two olefinic signals at 134.9 ± 0.7 ppm and 141.7 ± 0.2 ppm were associated to the C-13s and C-14s. Because of the absence of olefinic proton at these positions, $^{13}$C/$^1$H heteronuclear correlated experiments which separate the proton chemical shifts relative to the carbon resonances could not be performed for C-13 and C-14 and definitive assignment of their respective resonances could not be made. However, given that the more highly substituted carbons normally absorb at lower field than the less substituted carbons [19], it seems reasonable to propose that the resonances at 134 and 141 ppm are characteristic of C-14 and C-13 respectively. The two methyl groups at C-17 showed similar resonances of 26.3 ± 0.1 ppm and 26.6 ± 0.1 ppm, whereas that of C-17 was shielded from 81.4 ± 0.2 ppm to 45.4 ±0.1 ppm relative to their corresponding parent 17β-hydroxy-17α-methyl steroids. It is of interest to note that the 13(14)-ene function
induced notable downfield shifts of the C-15s and C-16s resonances by 6.4 ± 0.2 ppm and 8.1 ppm, respectively. Because of the presence of a 13(14)-ene function, the C-ring adopted a half-chair conformation that brings the C-12 methylene group in a closer proximity with the 17β-methyl group [20]. As a result, the signals associated with the C-12s are shielded by 16.6 to 16.9 ppm relative to the parent steroids. Resonances of other neighboring carbon atoms in the C-ring were also shifted but to a lesser extent. For example, there are small upfield shifts of 2.1 to 2.9 ppm and downfield shifts of 1.5 to 2.1 ppm of the signals associated with the C-9s and C-11s respectively compared to their corresponding parent steroids. Finally, resonances of carbons remote from the 13(14)-ene function in the A- and B-rings were not noticeably affected. The specific resonances exhibited by the olefinic carbons in compounds 4, 8, 12, 16 and 20 do not overlap and provide for the unambiguous identification of steroids bearing a 13(14)-ene group and their differentiation from other unsaturated analogs [19].

Acknowledgements

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References


6.2. STUDIES ON ANABOLIC STEROIDS.

EPIMERIZATION AND DEGRADATION OF ANABOLIC
17β-SULFATE-17α-METHYL STEROIDS IN HUMAN:
QUALITATIVE AND QUANTITATIVE GC/MS ANALYSIS*

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Summary

The epimerization and dehydration reactions of the 17β-hydroxy group of anabolic 17β-hydroxy-17α-methyl steroids have been investigated using the pyridinium salts of 17β-sulfate derivatives of methandienone 1, methyltestosterone 4, oxandrolone 7, mesterolone 10 and stanozolol 11 as model compounds. Rearrangement of the sulfate conjugates in buffered urine (pH 5.2) afforded the corresponding 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes in a ratio of 0.8:1. This observation indicated that both epimerization and rearrangement reactions of the 17β-sulfate derivatives were not dependant upon the respective chemical features of the studied steroids, but were instead inherent to the chemistry of the tertiary 17β-hydroxy group. Interestingly, in vivo studies carried out with human male volunteers showed that only methandienone 1, methyltestosterone 4 and oxandrolone 7 yielded the corresponding 17-epimers 2, 5 and 8 and 18-nor-17,17-dimethyl-13(14)-enes 3, 6 and 9 in ratios of 0.5:1, 2:0:1 and 2:7:1, respectively. No trace of the corresponding 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes derivatives of mesterolone 10 and stanozolol 11 was detected in urine samples collected after administration. These data suggested that the in vivo formation of the 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes derivatives of 17β-hydroxy-17α-methyl steroids is also dependant upon phase I and phase II metabolic reactions, which are probably modulated by the respective chemical features of the steroidal substrates, other than sulfation of the tertiary 17β-hydroxy group. The data reported in this study demonstrate that the 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes are not
artifacts resulting from the acidic or microbial degradation of the parent steroids in the gut as previously suggested by other authors, but are arising from the rearrangement of their 17β-sulfate derivatives. Unchanged oxandrolone 7 was solely detected in the unconjugated steroid fraction whereas unchanged steroids 1, 4 and 11 were recovered from the glucuronide fraction. These data are indirect evidences suggesting that the glucuronide conjugates of compounds 1 and 4 are probably enol glucuronides and that compound 11 is likely excreted in urine as a N-glucuronide involving its pyrazole moiety. The urinary excretion profiles of the epimergic and 18-nor-17,17-dimethyl-13(14)-ene steroids are presented and discussed on the basis of their structural features.
Introduction

The epimerization of the tertiary 17β-hydroxy group appears to be an important reaction in the biotransformation of several anabolic 17β-hydroxy-17α-methyl steroids in human. The occurrence of this reaction was first reported by Rongone and Segaloff [1] who isolated 17-epimethandienone 2 from the urine of a cancer patient who had been administered methandienone 1. However, their proposed structural assignment of 2 was confirmed only in 1971 when MacDonald et al. reported its chemical synthesis, and isolation from human urine [2]. This reaction was also reported by Durbeck et al. who performed the GC/MS analysis of methandienone urinary metabolites [3-4] and reported the characterization of 17-epi-4-chloro-methandienone, an abundant metabolite of 4-chloro-methandienone in human [5]. In previous metabolic studies from this laboratory, we reported the characterization of the 17-epimers of methandienone, oxandrolone and stanozolol following administration of the parent steroids to human male volunteers [6-8]. More recently, Harrison and Fennessey also reported the presence of 2 as a metabolite of 1 in urine samples collected from body builders who were given a relatively large dose of 1 [9]. Epimerization at C-17 is not a reaction which is specific to the parent 17β-hydroxy-17α-methyl steroids. Indeed, some authors reported lately the characterization of the 17-epimers of other urinary metabolites of methandienone 1, methyltestosterone 4, mesterolone 10 and stanozolol 11 [8,10-12].

Other authors reported that 18-nor-17,17-dimethyl-13(14)-ene steroids are the major
products resulting from the retropinacol rearrangement of 17-hydroxy-17-methyl steroids in acidic conditions [13,14]. The mechanism of the epimerization of 17β-hydroxy-17α-methyl anabolic steroids in human remained unclear until Edlund et al. [15] reported the formation of 17-epimethandienone 2 and 18-nor-17,17-dimethyl-androsta-1,4,13(14)-trien-3-one 3 as major degradation products of the 17β-sulfate conjugate of 1 in equine urine. These authors showed that the 17-sulfate derivative of methandienone was rapidly solvolyzed in equine urine to give 2 and 3. Although sulfation of steroids is much more active in horse than in human [15], this conjugation reaction is of prime importance in the biosynthesis and/or metabolism of endogenous and exogenous steroids in human.

Biological glucuronidation and sulfation of steroids are effected by the transfer of glucuronic acid and sulfate moieties from uridine diphosphoglucuronic acid (UDP-glucuronic acid) and 3-phosphoadenosine-5-phosphosulfate (PAPS), respectively [16]. The steric hindrance at both the tertiary 17β-hydroxy group and the anomeric carbon of the glucuronic acid moiety appears to prevent the formation of glucuronic acid conjugates at the C-17 position. This hypothesis is supported by previous data from our laboratory about the urinary excretion of methandienone, oxandrolone and stanozolol [7,8,10]. On the other hand, the sulfate moiety in PAPS is much less hindered and relatively vulnerable to nucleophilic attack by the tertiary 17β-hydroxyl group, so that sulfation at that position is more likely to occur than glucuronidation. Conversely, this also suggest that glucuronidation in steroids 1, 4 and 11 may occur with other functional groups, namely the enol function of the 3-keto-4-ene steroids 1 and 4 and the pyrazole moiety of
The objective of this study was to investigate the mechanistic aspects of the epimerization reaction which is characteristic to several anabolic 17\(\beta\)-hydroxy-17\(\alpha\)-methyl steroids in human. A secondary goal was to establish relationships between the occurrence and the relative amounts of the 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes in urine, and the chemical structures of the studied parent steroids. The formation of 17-epimeric and 18-nor-17,17-dimethyl-13(14)-ene steroids was investigated by GC/MS analysis of urine specimens collected after administration of compounds 1, 4, 7, 10 and 11. Excretion profiles of the parent, 17-epimeric steroids and 18-nor-17,17-dimethyl-13(14)-enes are presented as well as their mass spectral features. Mechanistic aspects related to their \textit{in vitro} and \textit{in vivo} formation are discussed.

**Experimental**

**Steroids and materials**

Methandienone 1, methyltestosterone 4, oxandrolone 7, mesterolone 10, 5\(\alpha\)-androstan-17-one (internal standard 1, IS1) and 17\(\alpha\)-methyl-5\(\alpha\)-androstan-3\(\beta\),17\(\beta\)-diol (internal standard 2, IS2) were purchased from Steraloids (Wilton, NH); stanozolol 11 was obtained from Winthrop Labs (Aurora, Canada). The 17\(\beta\)-sulfate derivatives of 1, 4, 7, 10 and 11, the corresponding 17\(\alpha\)-hydroxy-17\(\beta\)-methyl and 18-nor-17,17-dimethyl-13-ene steroids were synthesized in our laboratory according to methods recently reported [17].
Chemical structures of the parent steroids and related urinary 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes are presented in Fig. 6.2-1.

Fig. 6.2-1. The chemical structures of five 17β-hydroxy-17α-methyl anabolic steroids (1, 4, 7, 10 and 11) and corresponding urinary 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes. See Table 6.2-1 for steroid identity.
Sep-Pak C\textsubscript{18} cartridgess were supplied by Waters Assoc. (Milford, MA); β-glucuronidase (from \textit{E. Coli}) and arylsulfatase (from \textit{Helix pomatia}) were purchased from Boehringer Mannheim (Laval, Canada); N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) was obtained from Regis Chemical Co. (Morton Grove, IL); dithioerythritol and trimethylsilyl iodide (TMSI) were purchased from Aldrich Chemical Co. (Milwaukee, WI); N,O-bis (\textsuperscript{6}H\textsubscript{5}) trimethylsilyl)-acetamide (BSA-d\textsubscript{9}) and [\textsuperscript{2}H\textsubscript{9}] trimethylsilyl chloride (TMSCl-d\textsubscript{9}) were obtained from MSD-Isotope (Pointe-Claire, Canada); inorganic salts were of analytical grade (J.T. Baker, Phillipsburg, NJ or Caledon Labs, Georgetown, Canada); all the organic solvents (HPLC grade, Caledon Labs.) were used as provided.

\textbf{Steroid administration and collection of urine samples}

Blank urine samples were collected prior to steroid administration to healthy male volunteers (aged 24 to 35 years old). A single oral dose of methandienone 1 (25 mg), 17α-methyltestosterone 4 (10 mg), oxandrolone 7 (10 mg), mestanolone 10 (10 mg) and stanozolol 11 (20 mg) was administered. Each volunteer received only one steroid. The post-administration urine samples were collected at regular time intervals up to 72 hours after administration. Urine samples were stored at -20 °C immediately after voiding until analyzed.

\textbf{Quantitation of steroids 1 to 9 in urine samples}

\textit{Stock solution and standard curves}

Stock solutions of each steroids (1 to 9, 50 μg/ml and 1.0 mg/ml), IS1 (50 μg/ml) and IS2 (25 μg/ml) were prepared in absolute methanol and stored at 4 °C until used.
All standard curves (5 to 500 ng/ml) were prepared as follows: aliquots of stock solutions were transferred to 300 µl conical vials so as to obtain 25 ng to 2500 ng of the corresponding steroid in each vial, to which 500 ng of IS1 and 250 ng of IS2 were added. The solvent was evaporated to dryness under a N₂ flow at 40 °C and the residue was treated with 50 µl of a mixture of MSTFA/TMSI to prepare the TMS enol-TMS ether derivatives. For each concentration point, samples were prepared in triplicate and 1 µl was injected for GC/MS analysis (duplicate injections for each sample). Quantitation of the steroids was performed by selective ion monitoring (SIM) using the most abundant and characteristic ions of each steroid TMS derivatives, except for compound 9 which cannot be derivatized. Peak area ratios of the selected ions to those of the two internal standards (m/z 331 and m/z 143 for IS1 and IS2 respectively) were measured for each concentration point. Table 6.2-1 lists the major ions of each steroids as TMS ether and TMS enol-TMS ether derivatives and selected ions used for quantitation are indicated. Standard curves were linear for each steroid in the 5-500 ng/ml range. The following regression equations were obtained:

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Equation</th>
<th>ISTD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C = 87.0 A - 0.61</td>
<td>IS1</td>
</tr>
<tr>
<td>2</td>
<td>C = 80.0 A - 0.07</td>
<td>IS1</td>
</tr>
<tr>
<td>3</td>
<td>C = 990.0 A + 2.63</td>
<td>IS1</td>
</tr>
<tr>
<td>4</td>
<td>C = 111.0 A - 2.35</td>
<td>IS1</td>
</tr>
<tr>
<td>5</td>
<td>C = 114.0 A - 1.74</td>
<td>IS1</td>
</tr>
<tr>
<td>6</td>
<td>C = 62.5 A - 2.53</td>
<td>IS1</td>
</tr>
<tr>
<td>7</td>
<td>C = 239.0 A + 4.16</td>
<td>IS2</td>
</tr>
<tr>
<td>8</td>
<td>C = 227.0 A + 1.07</td>
<td>IS2</td>
</tr>
<tr>
<td>9</td>
<td>C = 79.4 A - 6.39</td>
<td>IS1</td>
</tr>
</tbody>
</table>
In these equations, \( C \) is the concentration of the steroid in ng/ml and \( A \) is the peak area ratio of selected ions from the measured steroid and the selected internal standard (ISTD).

**Quantitation of unconjugated steroids**

In a typical experiment, a urine sample (2-5 ml) was passed through a Sep-Pak C\(_{18}\) cartridge (prewashed successively with 5 ml of MeOH and water). The cartridge was then washed with 5 ml of water and 2 ml of hexane to remove residual water in the cartridge. Unconjugated and conjugated steroids were eluted with 5 ml of MeOH. The methanolic solution was evaporated under a stream of \( N_2 \) at 40°C. The residue was dissolved in 1 ml of pH 5.2 acetate buffer (0.2 M) and extracted with 2 x 5 ml of diethyl ether. The organic phase was decanted, dried over Na\(_2\)SO\(_4\) and evaporated to dryness under \( N_2 \) (the aqueous solution was used to quantitate conjugated steroids). The residue was dissolved in 200 \( \mu l \) of MeOH and transferred to a 300 \( \mu l \) vial. Internal standards (500 ng of IS1 and 250 ng of IS2) were added and the solvent was evaporated under \( N_2 \). The residue was then derivatized with 50 \( \mu l \) of a mixture of MSTFA and TMSI and 1 \( \mu l \) of the resulting solution was analyzed by GC/MS.

**Quantitation of conjugated steroids**

Residual diethyl ether were removed from the above aqueous solution with a stream of nitrogen and 100 \( \mu l \) of a \( \beta \)-glucuronidase solution was added and the resulting mixture was incubated at 37°C for 16 h. The hydrolysate was then cooled to room temperature
and extracted with 2 x 5 ml of ether. The organic phase was decanted, dried over Na$_2$SO$_4$ and evaporated to dryness. The residue was transferred to a 300μl vial and solutions of both IS1 and IS2 standards were added. The solvent was evaporated. The dried residue was derivatized and analyzed as above.

Residual diethyl ether was removed from the remaining aqueous phase with a stream of nitrogen and 100μl of an arylsulfatase solution was added. The mixture was incubated for 16 h at 37 °C and the hydrolysate extracted with 2 x 5 ml of ether. The ether layer was analyzed as above.

**Recoveries and detection limits**

Aliquots (5 ml) of blank urine samples were fortified with steroids 1 to 9 so as to obtain 10, 50 and 250 ng/ml. Three aliquots were prepared for each concentration. The resulting mixtures were equilibrated at 37 °C for 4 h, and successively extracted, derivatized and analyzed as described above. Duplicate analyses of each aliquots were carried out. Recoveries are listed in Table 6.2-2.

Detection limits were also determined using the selective ion monitoring and repetitive scanning modes. These experiments were performed as follows: extracts from a blank urine specimen were fortified with increasing amounts of each steroid so as to obtain concentration ranging from 0.25 to 20 ng/ml. TMS enol-TMS ether derivatives were prepared to achieve a higher sensitivity. Selected ions used to determined the detection
limits were also those used for quantitative analysis. Table 6.2-3 lists the detection limits obtained and gives the corresponding lowest urinary concentrations detectable for each of the studied steroids.

**In vitro tests of degradation of the 17β-sulfates in human urine**

Five aliquots of blank urine (5 ml) were buffered to pH 5.2 and fortified with various amounts of the synthetic 17β-sulfates equivalent to 200 ng/ml of the parent steroids 1, 4, 7, 10 and 11. The resulting solutions were incubated at 37 °C for 16 hours. Each sample was then extracted with diethyl ether (2 x 5 ml). The ether extract was processed as described above and the derivatized sample was analyzed by GC/MS. The solvolysis reactions afforded the corresponding 17-epimers and dehydration products (18-nor-17,17-dimethyl-13[14]-enes) in a 0.8:1.0 ratio from each of the studied sulfate derivatives.

**Derivatization and GC/MS analysis**

TMS enol-TMS ether derivatives were prepared using a method previously described [7,8,18]. Perdeuterated(d₉-) TMS derivatives were prepared to obtain further information about fragmentation routes and structural features of the steroids of interest and were prepared as follows: a mixture containing 50 μl of BSA-d₉ and TMSI mixture (100:1; v/v) was added to dried reference steroids or urinary extracts (containing 0.5 mg of dithioerythritol) and the resulting mixture was heated at 70 °C for 30 min. The methods used to prepare the TMS-ether (MSTFA:TMSCl) and the corresponding d₉-TMS-ether (BSA-d₉:TMSCl-d₉) derivatives were also reported in previous papers [18,19]. GC/MS
conditions were as previously reported [7,8,18].

**Results**

**Derivatization and mass spectral analysis of compounds 1-9**

The analytical approach used for the selective detection and quantitation of the 17-epimeric and 18-nor-17,17-dimethyl-13(14)-ene steroids in human urine was based on the preparation of their TMS enol-TMS ether or TMS ether derivatives and the monitoring of their characteristic and intense fragment ions which are produced upon electron-impact (EI) ionization (Table 6.2-1). Their chromatographic and mass spectrometric properties were determined using reference compounds previously synthesized in our laboratory [17]. The TMS enol-TMS ether or simple TMS enol derivatives were selected for the detection and quantitation of the 3-keto steroids 1-6 because they had higher response in the mass selective detector and afforded mass spectra with intense and structurally informative high-mass ions. In addition, the derivatization methods provide for complete enolization of the 3-keto group, which is an essential prerequisite for quantitation purposes. As shown in Table 6.2-1, the corresponding TMS ether derivatives do not provide similar advantages because they exhibit low intensity molecular ions and prominent ions at low m/z, except for compounds 3 and 6, the mass spectra of which showing intense molecular and [M-15]⁺ ions. On the other hand, compounds 7 and 8 which are not keto steroids were quantitated as TMS ether derivatives, and 9 was analyzed underivatized.
Table 6.2-1. Identity and partial GC/MS data of urinary steroids 1 to 9

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Deriv.</th>
<th>R.T.</th>
<th>M.U.</th>
<th>M⁺ + d</th>
<th>Characteristic ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Methandienone</td>
<td>t</td>
<td>26.90</td>
<td>28.72</td>
<td>372(1)</td>
<td>282(28), 219(14), 194(11), 161(18), 143(100).</td>
</tr>
<tr>
<td></td>
<td>e</td>
<td>25.79</td>
<td>28.09</td>
<td>444(54)</td>
<td>339(25), 229(14), 206(100)*, 191(16), 143(25).</td>
</tr>
<tr>
<td>2. 17-epimethandienone</td>
<td>t</td>
<td>24.58</td>
<td>27.41</td>
<td>372(1)</td>
<td>282(39), 219(15), 194(11), 161(24), 143(100).</td>
</tr>
<tr>
<td></td>
<td>e</td>
<td>23.81</td>
<td>26.98</td>
<td>444(42)</td>
<td>339(56), 229(10), 206(100)*, 191(17), 143(14).</td>
</tr>
<tr>
<td>3. 18-nor-17,17-dimethyl-androsta-1,4,13(14)-trien-3-one</td>
<td>un</td>
<td>19.94</td>
<td>24.80</td>
<td>282(71)</td>
<td>267(55), 171(31), 161(95), 122(72), 91(100).</td>
</tr>
<tr>
<td></td>
<td>e</td>
<td>19.06</td>
<td>24.29</td>
<td>354(40)</td>
<td>339(62)*, 206(21), 194(19), 148(56), 133(100).</td>
</tr>
<tr>
<td>4. Methyltestosterone</td>
<td>t</td>
<td>26.30</td>
<td>28.38</td>
<td>374(2)</td>
<td>359(7), 317(15), 304(16), 284(60), 143(100)</td>
</tr>
<tr>
<td></td>
<td>e</td>
<td>26.15</td>
<td>28.29</td>
<td>446(67)</td>
<td>356(11), 314(11), 301(100)*, 208(5), 143(8).</td>
</tr>
<tr>
<td>5. 17-epimethyltestosterone</td>
<td>t</td>
<td>24.11</td>
<td>27.14</td>
<td>374(2)</td>
<td>359(8), 317(15), 304(18), 284(68), 143(100).</td>
</tr>
<tr>
<td></td>
<td>e</td>
<td>24.28</td>
<td>27.24</td>
<td>446(77)</td>
<td>356(12), 314(11), 301(100)*, 208(7), 143(7).</td>
</tr>
<tr>
<td>6. 18-nor-17,17-dimethyl-androsta-4,13(14)-dien-3-one</td>
<td>un</td>
<td>19.38</td>
<td>24.48</td>
<td>284(38)</td>
<td>269(100), 251(7), 161(11), 105(16), 91(27).</td>
</tr>
<tr>
<td></td>
<td>e</td>
<td>19.37</td>
<td>24.47</td>
<td>356(79)</td>
<td>341(11), 208(100)*, 194(13), 193(18), 133(15)</td>
</tr>
<tr>
<td>7. oxandroline</td>
<td>t</td>
<td>27.86</td>
<td>29.27</td>
<td>378(3)</td>
<td>363(18), 321(12), 308(21), 176(5), 143(100)*</td>
</tr>
<tr>
<td>8. 17-epioxandroline</td>
<td>t</td>
<td>25.75</td>
<td>28.07</td>
<td>378(6)</td>
<td>363(21), 321(16), 308(23), 176(8), 143(190)*</td>
</tr>
<tr>
<td>9. 18-nor-17,17-dimethyl-2-oxa-5α-androstan-13(14)-en-3-one</td>
<td>un</td>
<td>20.80</td>
<td>25.28</td>
<td>288(9)</td>
<td>273(100)*, 161(61), 148(3), 133(51), 105(9), 91(12)</td>
</tr>
</tbody>
</table>

a. Derivatives are t TMS-ether, e TMS-enol-TMS-ether and un undervatized steroid
b. Retention time
c. Methylene units were determined by linear interpolation of the retention time of the steroid derivatives between the retention time of C₂₄ to C₃₃ n-alkanes
d. Molecular ions
* Selected ions monitored for quantitation and determination of detection limits
Table 6.2-2. Recovery of steroids 1 to 9 from human urine

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Concentration b (ng/ml)</th>
<th>Recovery c</th>
<th>C.V. d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>63.2 ± 3.3%</td>
<td>5.2%</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>73.9 ± 2.5%</td>
<td>3.5%</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>79.8 ± 2.9%</td>
<td>3.6%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>65.7 ± 3.4%</td>
<td>5.2%</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>71.5 ± 3.0%</td>
<td>4.2%</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>79.1 ± 2.5%</td>
<td>3.2%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>67.9 ± 3.9%</td>
<td>5.7%</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>71.8 ± 3.7%</td>
<td>5.2%</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>77.1 ± 4.2%</td>
<td>5.4%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>62.5 ± 3.4%</td>
<td>5.4%</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>68.7 ± 3.2%</td>
<td>4.7%</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>74.8 ± 2.8%</td>
<td>3.7%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>63.9 ± 3.4%</td>
<td>5.3%</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>69.3 ± 2.5%</td>
<td>3.6%</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>75.1 ± 2.8%</td>
<td>3.7%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>63.6 ± 3.8%</td>
<td>6.0%</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>66.0 ± 3.1%</td>
<td>4.7%</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>68.8 ± 3.1%</td>
<td>4.5%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>71.1 ± 3.3%</td>
<td>4.6%</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>76.8 ± 3.1%</td>
<td>4.0%</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>79.4 ± 2.5%</td>
<td>3.1%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>71.4 ± 2.7%</td>
<td>3.8%</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>74.9 ± 3.4%</td>
<td>4.5%</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>79.2 ± 1.8%</td>
<td>2.3%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>68.1 ± 3.9%</td>
<td>5.7%</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>72.6 ± 4.0%</td>
<td>5.5%</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>79.4 ± 3.4%</td>
<td>4.3%</td>
</tr>
</tbody>
</table>

a. See Table 6.2-1 for steroid identity.
b. 5 ml aliquots of urine were spiked with specific amounts of the steroid.
c. Samples were prepared in triplicates for each concentration and each sample was analyzed in duplicate so that the determined recoveries are the mean of 6 analysis.
d. Coefficient of variation
Recovery and detection limit of compounds 1-9

Recoveries for each pair of the 17-epimeric steroids 1 and 2, 4 and 5, and 7 and 8 were virtually identical and progressively increased from 63 to 79% with increasing concentration of the steroids. Recoveries for the 18-nor-17,17-dimethyl-13(14)-enes 3, 6 and 9 were of the same order of magnitude and varied accordingly (Table 6.2.2). It was also of interest to determine the detection limit of these steroids in urine both in the SIM and repetitive scanning modes (Table 6.2-3). The epimeric steroids 1, 2, 4 and 5 were analyzed as the TMS enol-TMS ether derivatives and detected at concentrations as low as 25 pg and 500 pg (amounts injected on column) in the SIM and repetitive scanning mode respectively. On the other hand, steroids devoided of a 17-hydroxyl or 3-keto group such as compounds 3, 6, 7, 8 and 9 were detected with less sensitivity, since the lowest concentrations detected with a signal-to-noise ratio of 3.5 1 ranged from 50 to 100 pg in SIM and 1 to 2 ng in the repetitive scanning mode.

Table 6.2-3. Detection limits for Steroids 1 to 9

<table>
<thead>
<tr>
<th>Steroids</th>
<th>on column (pg)</th>
<th>SIM&lt;sup&gt;a&lt;/sup&gt; in urine (ng/ml)</th>
<th>on column (ng)</th>
<th>Scan&lt;sup&gt;b&lt;/sup&gt; in urine (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>0.25</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>0.25</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>0.50</td>
<td>1.0</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>0.25</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>0.25</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>0.50</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>1.00</td>
<td>2.0</td>
<td>20</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
<td>1.00</td>
<td>2.0</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>100</td>
<td>1.00</td>
<td>2.0</td>
<td>20</td>
</tr>
</tbody>
</table>

a. TMS-enol-TMS-ether derivatives were used to determine detection limits for compounds 1, 2, 4 and 5, TMS-enol derivatives were used for 3 and 6, TMS-ether derivatives were used for 7 and 8; and 9 was analyzed without derivatization.

b. See Table 6.2-1 for the selected ions for each steroid. Detection limits were measured at a signal to noise (S/N) ratio of 3.5.

c. At the detection limit, for each steroid, reasonable mass spectrum were obtained and proper ratios were maintained for the major ions listed in Table 6.2-1.
Fig. 6.2-2. Reconstructed total ion current chromatograms obtained from SIM GC/MS analysis of (A) unconjugated and (B) glucuronide fractions of an urine sample collected 6.5 hours after the oral administration of methandienone 1. Chromatograms (C) and (D) were obtained from analysis of the unconjugated and glucuronide fractions of a blank urine. See Table 6.2-1 for identification of labelled peaks and experimental for analytical conditions. IS1 and IS2 are the internal standards.

Fig. 6.2-3. Excretion curves of 1 (glucuronide fraction) and of metabolites 2 and 3 (unconjugated fraction) after oral administration of 25 mg of 1.

Methandienone 1

Figure 6.2-2 presents typical ion chromatograms illustrating the detection of unchanged
methandienone 1 in the glucuronide fraction and that of epimethandienone 2 and dehydration product 3 in the unconjugated steroid fraction. Their urinary excretion curves (Fig. 6.2-3) indicate that they can be detected for about 24 to 41 h after methandienone administration. Excretion rates of these steroids in urine were maximum between 2 and 6 h after administration. The above excretion profile of epimethandienone 2 was in agreement with the data recently reported by Schänzer et al. [12] The cumulative urinary excretion of compound 3 which has been previously reported as an artifact by Dürbeck et al. [3,4], corresponded to 0.63% of methandienone dose (Table 6.2-4). It is of interest to note that 2 and 3 were excreted in a 0.5:1 ratio whereas they were produced in a 0.8:1 ratio by hydrolysis of methandienone 17-sulfate derivative. This suggests that the in vivo formation of 2 and 3 is not only dependant upon the intrinsic reactivity of the tertiary 17β-sulfate group but also on biochemical factors as further discussed below.

The identity of compounds 1-3 was determined by comparison with reference steroids. The mass spectrum of the TMS enol-TMS ether derivative of compound 2 (Fig. 6.2-4A) which is dominated by a prominent ion at m/z 206 arising from the cleavage of the C₅-C₁₀ and C₇-C₈ bonds, comprises the A-ring and the C₆ and C₇ carbons [10]. The mass spectrum of compound 3 TMS enol derivative show an intense ion at m/z 148 which results from the cleavage of the same chemical bonds and comprises the C₆- and D-rings. Subsequent elimination of a methyl radical gives rise to the ion of m/z 133 (Fig. 6.2-4B). These mass spectral data are in accordance with those of the corresponding reference steroids.
Fig. 6.2-4. Mass spectra of the TMS enol-TMS ether derivatives of (A) 17-epi-methandienone 2 and (b) 18-nor-17,17-dimethyl-androstan-1,4-13(14)-tren-3-one 3.

Table 6.2-4. Total excretion of steroids 1 - 9

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Total excretion (µg)</th>
<th>% dose</th>
<th>Ratio 1</th>
<th>Ratio 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.15</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>85.68</td>
<td>0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>157.7</td>
<td>0.63</td>
<td>2/3 = 0.54±0.06 : 1</td>
<td>0.8:1</td>
</tr>
<tr>
<td>4</td>
<td>9.31</td>
<td>0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>9.53</td>
<td>0.10</td>
<td>5/6 = 2.0±0.3 : 1</td>
<td>0.8:1</td>
</tr>
<tr>
<td>6</td>
<td>4.64</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2646</td>
<td>26.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>385.9</td>
<td>3.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>157.3</td>
<td>1.60</td>
<td>8/9 = 2.7±0.8 : 1</td>
<td>0.8:1</td>
</tr>
</tbody>
</table>

1. Relative ratios of corresponding 17-epimers and 18-nor-17,17-dimethyl-13(14)-enones.
2. Relative ratios obtained from the solvolysis of the 17β-sulfate derivatives of Steroids 1, 4 and 7 [18].
Methyltestosterone 4

This steroid which is structurally similar to methandienone (Fig. 6.2-1) was also partially excreted unchanged in the glucuronide fraction (Fig. 6.2-5B), whereas its epimer 5 and dehydration product 6 were isolated from the unconjugated steroid fraction (Fig. 6.2-5A). Contrary to methandienone, compounds 4-6 were excreted in urine only over a period of 8 hr, with maximum excretion rates observed 3 hr after methyltestosterone administration. An interesting feature of the excretion profile of compound 4 and 5 is that they were steadily excreted in a 2.0:1.0 ratio throughout their 8 hr excretion period (Table 6.2-4). In addition, the cumulative excretion of compounds 4-6 was relatively small with respect to that of methandienone metabolites 1-3 (Fig. 6.2-6).

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Fig. 6.2-5. Reconstructed total ion current chromatograms obtained from SIM GC/MS analysis of (A) unconjugated and (B) glucuronide fraction of an urine sample collected 3 hours after the oral administration of methyltestosterone 4. Chromatograms (C) and (D) were obtained from the analysis of the unconjugated and glucuronide fractions of a blank urine. See Table 6.2-1 for peak identification. Analytical conditions are as in Fig. 6.2-2.
Fig. 6.2-6. Excretion curves of 4 (glucuronide fraction) and of metabolites 5 and 6 (unconjugated fraction) after oral administration of 10 mg of 4.

Fig. 6.2-7. Mass spectra of the TMS-enol-TMS-ether derivatives of (A) 17-epi-methyltestosterone 5 and (B) 18-nor-17,17-dimethyl-androstan-4,13(14)-dien-3-one 6.
The mass spectrum of the TMS enol-TMS ether and TMS enol derivative of compounds 5 and 6 are presented in Figs. 6.2-7A and 6.2-7B respectively. The mass spectrum of 5 is characterized by a prominent ion at m/z 301 produced from the cleavage of the D-ring with concomitant elimination of a hydrogen radical, most probably from C8. The resulting radical can be readily stabilized by migration to C7 and resonance with the 3,5-diene group of the TMS enol moiety to yield an even-numbered TMS oxonium ion. The high degree of conjugation between this ion at C3 and the resulting 4,6-diene function may account for its intensity and absence of other prominent ions in the mass spectrum of 5 (Fig. 6.2-7A).

The mass spectrum of compound 6 showed an abundant ion at m/z 208 which is produced according to a fragmentation route similar to that giving rise to the ion of m/z 206 in the mass spectrum of the TMS derivatives of 1 and 2 (Fig. 6.2-4A and Table 6.2-1). A low intensity ion characteristic of the 13(14)-ene group is observed at m/z 133. These data are in accordance with the mass spectral features of the reference steroids.

**Oxandrolone 7**

The excretion profiles of oxandrolone 7 and its metabolites 8 and 9 were different from those of methandienone and methyltestosterone in that all three steroids were isolated from the unconjugated steroids fraction (Fig. 6.2-8). Their excretion curves indicate that they can be detected until 48 h after oxandrolone administration (Fig. 6.2-9). Maximum excretion rates were observed 7 h after administration, which is consistent with the fact that these steroids are not conjugated prior to excretion in urine. Because it is barely metabolized, the total excretion of unchanged oxandrolone accounted for 26.5% of the dose, whereas that of epioxandrolone 8 and compound 9 was also relatively abundant with respect to that of their methandienone and methyltestosterone analogs 2, 3, 5 and
6. The mass spectrum of compound 9 (Fig. 6.2-10) was identical to that of the reference steroid. This spectrum which is characterized by a prominent ion at m/z 273 (M-Me)⁺ shows low intensity and structurally informative ions at m/z 133 and 148 (C- and D-rings). The mass spectral data of 7 and 8 have been reported previously [7].

Fig. 6.2-8. Reconstructed total ion current chromatograms obtained from SIM GC/MS analysis of unconjugated fraction of (A) an urine sample collected 7 hours after the oral administration of oxandrolone and (B) a blank urine. See Table 6.2-1 for peak identification. Analytical conditions are as in Fig. 6.2-2

Fig. 6.2-9. Excretion curves of compounds 7, 8 and 9 (unconjugated fraction) after oral administration of 10 mg of 7.
Fig. 6.2-10. Mass spectrum of 18-nor-17,17-dimethyl-2-oxa-5α-androst-13(14)-en-3-one 9.

**Mestanolone 10 and stanozolol 11**

No trace of the 17-epimers and dehydration products of both mestanolone 10 and stanozolol 11 was detected in urine specimens collected after the administration of single oral doses of these steroids to human volunteers. Data about stanozolol were not in complete agreement with previous studies from this laboratory [8] and that of Schanzer et al. [11] reporting the isolation and characterization of 17-epistanozolol, 3'-hydroxy-17-epistanozolol and 16α-hydroxy-17-epistanozolol from human urine. It is of interest to note that in the previous study [11], 17-epistanozolol was detected in the urine samples after administration of an oral 40 mg dose of stanozolol 11. The metabolic differences observed between the present and the above studies may be due to various factors such as the administration dose of stanozolol, which was greater in the above studies than in the present investigation, and the qualitative and quantitative characteristics of exogenous steroids metabolism that vary from one individual to another.
Discussion

Epimerization at the C-17 position

Quantitative results from our previous *in vitro* study [17] on the solvolysis of sulfate derivatives of 17β-hydroxy-17α-methyl steroids are consistent with the data from the *in vivo* study presented above. Our data are also in accordance with those of Edlund *et al.* [15], who used methandienone sulfate as a model substrate. These authors originally suggested that epimerization at the C-17 position occurs through the formation of a tertiary carbonium ion resulting from the elimination of the sulfate group which is labile in aqueous media. Then, nucleophilic attack by water affords 17-epimethandienone. The present study and previous *in vitro* investigations from our laboratory [17], provided further evidence for the occurrence of the above mechanism in the epimerization reaction of sulfate conjugates of steroids 1, 4 and 7. Thus, it is reasonable to assume that the *in vivo* formation of the 17-epimers of 17β-hydroxy-17α-methyl steroids is dependant upon the previous biosynthesis of the corresponding 17β-sulfate conjugates.

However, sulfation, as glucuronidation, is a phase II metabolic reaction which is affected by the phase I reactions such as hydroxylation and reduction. From a mechanistic point of view, there appears to be some selectivity in the sulfation and glucuronidation reactions of 17β-hydroxy-17α-methyl steroids which are probably subjected to "steric hindrance". Examination of molecular models of oxandrolone 7 and uridine diphosphoglucuronic acid (UDP-glucuronic acid), the glucuronic acid donor in the
enzymatic formation glucuronides, shows that there is a great deal of interference between the UDP-glucuronic acid and the steroid molecule when the tertiary 17-hydroxy group draws near to the anomeric center of the glucuronic acid moiety [16]. This interference appears to be strong enough to prevent glucuronidation at C-17. Conversely, the molecular model of 3-phosphoadenosine-5-phosphosulfate (PAPS), the sulfuric acid donor in the enzymatic formation of sulfate conjugates, shows that there is much less interference between the steroid molecule and the 3-phosphoadenosine moiety so that the tertiary 17β-hydroxy group can easily come within bonding distance to the sulfate group. On the basis of these observations, it is reasonable to postulate that the 17β-hydroxyl group of anabolic 17β-hydroxy-17α-methyl steroids is preferentially sulfated in the course of phase II biotransformation reactions in human. This hypothesis is supported by the fact that oxandrolone 7, which bear one tertiary hydroxyl group at C-17 is not excreted in urine as a glucuronic acid conjugate. Furthermore, the occurrence in urine of 17-epioxandrolone 8 and compound 9 demonstrates that oxandrolone was sulfated prior to excretion.

Metabolic factors can also affect the production of tertiary 17-sulfate conjugates. Quantitative data presented in Table 6.2-4 indicate that the relative abundance of the 17-epimers produced parallels the overall rate of biodegradation of their respective precursors. Indeed, relatively low amounts of epitethyltestosterone 4 and compound 5 are produced from methyltestosterone because the latter steroid is rapidly metabolized by reduction of its 3-keto and 4-ene functions [10,20] and the resulting metabolites are
readily glucuronidated. In such metabolic conditions, glucuronidation is generally the major phase II reaction which account for the rapid elimination of the parent steroid and its metabolites in urine and in that case, sulfation at C-17 becomes a minor route of elimination.

The absence of the 17α-epimers and 18-nor dehydration products of mestanolone 10 and stanozolol 11 in urine samples collected after administration of 10 and 20 mg single and oral dose respectively could presumably be the result of their rapid and extensive biodegradation, primarily through reduction of mestanolone 3-keto group and hydroxylation at several sites of the stanozolol nucleus [8,10,11]. In addition, their hydroxylated metabolites are excreted mainly as the glucuronide. When substantial amounts of steroids are used, as it is the case with chronic users, sulfation may then become an important complementary phase II reaction that accelerate the elimination of the parent steroid and its metabolites. In such case, low amounts of 17α-epistanozolol and of 17-epimers of some hydroxylated metabolites can be detected in urine [8].

On the other hand, if the structure of the parent steroid is such that phase I reactions are hampered or their rates decreased, sulfation may then become a complementary and important elimination route of the parent steroid and/or its metabolites. This hypothesis is supported by quantitative data from the excretion of methandienone and oxandrolone presented in Table 6.2-4. The presence of an additional double bond at C1 in methandienone has an important effect on its metabolic routes with respect to those of
methyltestosterone, so that methandienone is metabolized and eliminated at a slower rate. This in turn appears to promote the sulfation of methandienone at C-17 which is reflected by the excretion of larger amounts of its 17-epimer and compound 3. In the case of oxandrolone which is barely degraded via phase I reactions [7] and which is not a substrate for glucuronidation, sulfation at C-17 is apparently a major phase II reaction, the importance of which is demonstrated by the relatively high amounts of 17-epioxandrolone 8 and compound 9 excreted in urine. In conclusion, the 17β-sulfation of 17β-hydroxy-17α-methyl steroids appears to become an important metabolic route when phase I reactions and glucuronidation are hampered due to particular structural features of these steroids.

The *in vivo* formation of 18-nor-17,17-dimethyl-13(14)-enes.

The occurrence in human urine of 18-nor-17,17-dimethyl-13(14)-ene steroids after administration of the parent 17β-hydroxy-17α-methyl steroids has been originally related to their retropinacol rearrangement under acidic conditions [9,13,14]. Other authors reported that compound 3 was an artifact produced by the decomposition of methandienone by gastric acid [4]. According to this hypothesis, all 17β-hydroxy-17α-methyl steroids should give the corresponding 13(14)-unsaturated steroids after ingestion. However, the data presented above showed that no trace of the expected 13(14)-unsaturated dehydration products of mestanolone 10 and stanozolol 11 was detected in urine. Our data are also in accordance with a study from Edlund *et al.* who recently proposed that compound 3 arises from the elimination of the sulfate group of compound
1 sulfate derivative through rearrangement of the resulting 17-carbonium ion [15]. This study provided clear evidence that the 17-epimers 2, 5 and 8 and their corresponding 18-nor-17,17-dimethyl-13(14)-ene analogs are not artifacts, but metabolites arising from the labile 17β-sulfate conjugates of the parent steroids 1, 4 and 6.

**In vivo ratio of the 17-epimers to 18-nor-17,17-dimethyl-13(14)-enes.**

The results presented in Table 6.2-4 clearly show that the *in vivo* rearrangement of the 17β-sulfate conjugates of compounds 1, 4 and 6 leads to the formation of their corresponding 17-epimers and dehydration products in ratio of 0.54:1.0, 2.0:1.0 and 2.7:1.0 respectively. These results were somewhat surprising since the *in vitro* degradation of all three 17-sulfates afforded the same reaction products in a 0.8:1.0 ratio. This leads us to postulate that the *in vivo* formation of the 17-epimeric steroids and their corresponding dehydration products is not solely determined by the chemistry of the tertiary 17β-sulfate group, but also by biochemical factors such as binding or non-covalent interaction of the sulfate conjugates with albumin, sex-hormone-binding globulin or other steroid-binding proteins.

Data shown in Figs. 6.2-3, 6.2-6 and 6.2-9 and in Table 6.2-4 suggest that the degradation of the sulfate conjugate does not occur in urine. Indeed, we demonstrated that the epimerization and dehydration products are formed in a 0.8:1.0 ratio in urine at 37°C. This implies that *in vivo*, these reactions probably occur after sulfation of the 17β-hydroxyl group and are likely modulated by interaction or binding with plasma
proteins which could sterically hindered the C-17 position and prevent, to a certain extent, nucleophilic attack of water which ultimately gives the corresponding 17α-epimers. This, in turn, would favor the formation of dehydration products as observed in the case of methandienone. The formation of dehydration products can also be promoted if the carbonium ion interact with a basic or polar site of the binding protein that would promote the migration of the latter ion at C-13 while assisting or catalyzing the subsequent elimination of the C-14 proton the give the 13(14)-unsaturated steroids. Conversely, interaction with proteins could favor the SN1 nucleophilic attack of water at the carbonium ion to give the 17-epimeric steroids as observed in the case of methyltestosterone and oxandrolone. Although speculative, this hypothesis is in agreement with the underlying mechanistic aspects of the unimolecular SN1 substitution and E1 elimination reactions which lead to the formation of 17-epimers and dehydration products respectively. Moreover, this hypothesis is supported by quantitative data (Table 6.2-4) indicating that these steroids are produced in uneven ratios ranging from 0.54:1 to 2.7:1.

**Glucuronidation of steroids 1, 4, and 11.**

Data presented above indicate that the 17β-hydroxy-17α-methyl steroids studied are not glucuronidated at the C-17 position. This is supported by the fact that oxandrolone, which only bears a tertiary 17β-hydroxyl group, was not found in the glucuronide fraction but in the unconjugated steroid fraction. Although they also have an identical hydroxyl group at C-17, unchanged methandienone 1 and methyltestosterone 4 were
isolated from the glucuronide fraction (Figs. 6.2-2 and 6.2-5). This indicates that 1 and 4 are probably excreted as enol glucuronides. The formation of enol glucuronide of some endogenous 3-keto-4-ene steroids, particularly androstendione and testosterone, has been previously reported [7]. Kjeld et al [22] also provided analytical evidence demonstrating the occurrence of the 3-enol glucuronide of testosterone in human urine. Given the structural similarities of compounds 1 and 4 with those of testosterone, the occurrence of their 3-enol glucuronides is not surprising since their formation is mainly dependant upon the chemistry of the 3-keto-4-ene group. The detection of unchanged stanozolol 11 in the glucuronide fraction indicates that this nitrogen-containing steroid was excreted as a N-glucuronide involving its pyrazole moiety. Further investigation will be carried out to characterize these unusual glucuronides.

**Conclusion**

In conclusion, this study showed that 17β-sulfation is an important phase II reaction in the metabolism of some 17β-hydroxy-17α-methyl steroids in human, particularly when their biotransformation is hampered because of the presence of specific functional groups at positions of metabolic importance. The *in vivo* degradation of these sulfates gives rise to mixtures of the corresponding 17-epimers and 18-nor-17,17-dimethyl-13(14)-enes, which were excreted in the unconjugated fraction. The sulfation reaction at C-17 appears to be affected by competitive phase I metabolic reactions which, as in the case of mestanolone 10 and stanozolol 11, favor the elimination of the parent steroids and their
metabolites through routes other than sulfation at C-17. Quantitative data indicate that the in vivo degradation of 17β-sulfates could be affected by interaction with plasmatic proteins, the demonstration of which would require further investigations. The formation of enol glucuronides of steroids 1 and 4 and that of a N-glucuronide of stanozolol 11 was also proposed.

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References


2. MacDonald B.S., Sykes P.J., Adhikary P.M., Harkness R. A. The identification of 17$\alpha$-hydroxy-17-methyl-1,4-androstadien-3-one as a metabolite of the anabolic steroid drug 17$\beta$-hydroxy-17-methyl-1,4-androstadien-3-one in man. *Steroids*, 18 (1971) 753 - 766.


7. Massé R., Bi H., Ayotte C., Dugal R. Studies on anabolic steroids II - Gas chromatographic/mass spectrometric characterization of oxandrolone urinary


CONTRIBUTION TO ORIGINAL KNOWLEDGE

Chapter 2. 17-Epimerization is the major biotransformation route of oxandrolone in man. The acidic metabolite of oxandrolone was isolated and identified. A simple and reliable method for extracting oxandrolone and its metabolites from human urine was developed to avoid possible hydrolysis of the A-ring lactone of these steroids.

Chapter 3. Stereoselective reduction products of four different 17α-methyl steroids in vivo were identified. The presence of a 17α-methyl group seems to orient the reduction of Δ4 functions to give 5β-androstanes as major metabolites. The 3-keto function is mainly reduced to the 3α-hydroxy group. The sequence of reduction of Δ14-3-one function has been clarified. The mechanism of 17-epimerization of these steroids was explored.

Chapter 4. The metabolism of formebolone showed that the major biotransformation of formebolone occurred on the 2-formyl group. An acidic metabolite due to the oxidation of the 2-formyl group was reported for the first time.

Chapter 5. The oxidative metabolic pathway of oxymetholone has been discovered. Four seco acids and one hydroxy acid have been detected in human urine after oxymetholone administration. The major seco acid was synthesized and the minor ones were characterized by GC/MS. The stereochemical assignments of the 3α-hydroxy-2β-
carboxylic acid was confirmed by different synthetic approaches.

**Chapter 6.** A simple method for preparing 17-tertiary sulfates of 17β-hydroxy-17α-methyl steroids was developed. 17-Epimers of five steroids were prepared through the hydrolysis of the corresponding 17β-sulfates. The mechanism of 17-epimerization of these steroids was further investigated including quantitative GC/MS analysis. Different functional groups have been shown to influence the production of 17-epimeric metabolites.