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# Mass spectrometric description of novel oxymetholone and desoxymethyltestosterone metabolites identified in human urine and their importance for doping control

**Tim Sobolevsky* and Grigory Rodchenkov**

**The metabolism of two anabolic steroids – oxymetholone and desoxymethyltestosterone – was reinvestigated to identify new targets potentially valuable for the antidoping analysis. Excretion urine samples from the laboratory reference collection were used in this study. Following fractionation of the urinary extract by means of high performance liquid chromatography (HPLC), each fraction was subjected to gas chromatography–mass spectrometry (GC-MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis after trimethylsilylation. About 20 metabolites were found for desoxymethyltestosterone and more than 40 for oxymetholone, with many of them being isomeric compounds. In addition to the well-known reduced and hydroxylated metabolites, 18-nor-17,17-dimethyl and 18-nor-17-hydroxymethyl-17-methyl steroids were also identified. Having evaluated all the metabolites in terms of how long they could be detected, we suggest that 18-nor-2ξ,17β-hydroxymethyl-17α-methyl-5α-androst-13-en-3α-ol is an important marker of oxymetholone abuse. In case of desoxymethyltestosterone, better detectability could be achieved if 18-nor-17,17-dimethyl-5α-androst-13-en-2ξ,3α-diol is monitored. These novel metabolites could be detected using GC-MS/MS at least for 14 days after administration of these anabolic steroids compared to 5–7 days for previously reported metabolites. Copyright © 2012 John Wiley & Sons, Ltd.**

**Keywords:** oxymetholone; desoxymethyltestosterone; long-term metabolite; GC-MS/MS; antidoping analysis

## Introduction

The World Anti-Doping Agency (WADA) prohibits the use of anabolic androgenic steroids (AAS) by professional athletes.[1] AAS – performance-enhancing drugs – are the most frequently abused and constitute about 60% of the adverse analytical findings worldwide.[2] Nevertheless, for the whole antidoping system to work efficiently, antidoping laboratories are encouraged to constantly update and improve their analytical methods. In other words, identification of the long-term and characteristic metabolites that would enable a reliable detection of prohibited substances is of utmost importance.

Oxymetholone (2-hydroxymethylene-17α-methyl-17β-hydroxy-5α-androstan-3-one) and desoxymethyltestosterone (17α-methyl-17β-hydroxy-5α-androst-2-en) are the examples of AAS featuring 17α-methyl group, which makes the steroids orally active.[3] While oxymetholone, known since 1960s,[4] is a prescription drug currently approved in several countries for the treatment of anemia and HIV-related muscle wasting,[5] desoxymethyltestosterone was not marketed until 2005 when it was identified in a clandestine product by Sekera *et al.*[6] and independently by a group of Canadian scientists at the *Institut national de la recherche scientifique* (INRS).[7,8] Soon after, desoxymethyltestosterone shown to be a potent agonist of anabolic receptors[9] became available as a component of sport nutritional supplements under the non-systematic names 17α-methyl-etioallocholan-2-en-17β-ol or 17-methyl-delta-2-etioallocholan. Though apparently discontinued at the moment, products containing desoxymethyltestosterone are still advertised over the Internet despite the fact that the Federal Drug Administration (FDA) issued an advisory warning to avoid taking these supplements[10] and that desoxymethyltestosterone is now classified as a Drug Enforcement Agency (DEA) Schedule III Controlled Substance.[11]

The metabolism of oxymetholone in humans was first studied by Adhikary and Harkness[12] and MacDonald *et al.*[13] They identified 2ξ-hydroxymethyl-17α-methyl-17β-hydroxy-5α-androstan-3-one and 2ξ-hydroxymethyl-17α-methyl-5α-androstan-3α,17β-diol as the metabolites formed upon sequential reduction of the A-ring. Later, Masse *et al.*[14] reported also 6β-hydroxylation product, 2ξ-hydroxymethyl-17α-methyl-5α-androstan-3α,6β,17β-triol, and 17α-methyl-5α-androstan-3α,17β-diol, a non-specific metabolite being also common for methyltestosterone and mestanolone.[15] The same laboratory demonstrated that after reduction of 3-oxo function and oxidation of 2-hydroxymethylene group to the corresponding β-keto acid, the unusual seco acidic metabolites are produced.[16,17] However, their isolation required extraction at the low pH values, whereas a typical procedure of the steroid analysis in doping control assumes extraction at neutral to basic pH.[18] Vladimirova *et al.*[19] identified 19 oxymetholone metabolites and studied the excretion kinetics for 6 of them. Two major metabolites – 2ξ-hydroxymethyl-17α-methyl-5α-androstan-3α,17β-diol and 2ξ-hydroxymethyl-17α-methyl-5α-androstan-3α,6β,17β-triol – provided

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a detection window of up to 5 days and were recommended for the screening of oxymetholone abuse by gas chromatography–mass spectrometry (GC-MS). An interesting finding was that the 2-hydroxymethyl group may undergo dehydration to give the series of 2-methylene steroids, which were not evaluated at that time. Another report also confirmed tetrahydro-oxymetholone and tetrahydro-6-hydroxy-oxymetholone as two major oxymetholone metabolites.[20]

Much less is known about the metabolism of desoxymethyltestosterone. At this point it is worth mentioning that most desoxymethyltestosterone preparations available were shown to contain a mixture of 2-en and 3-en isomers in a ratio of *ca*. 5:1,[6,7] and of course both would metabolize. In the first report which described the identification of this designer steroid and its metabolite after incubation with human liver microsomes and a baboon excretion study, it was suggested that desoxymethyltestosterone itself, detectable up to 60 h after administration, should be monitored for doping control purposes. The metabolite with two more oxygen atoms and one fewer unsaturation than the parent compound (MW 538 as the pertrimethylsilylated derivative) was considered less valuable.[6] Later in our laboratory we showed that desoxymethyltestosterone is subject to extensive metabolism via hydroxylation and reduction of the double bond, and is difficult to detect since the pertrimethylsilylated derivatives of its metabolites have non-informative electron ionization mass spectra (*i.e*. the low intensity of characteristic ions) and strongly interfere with the urinary matrix. No parent drug was detected while the metabolites were only tentatively identified as 3 isomeric mono hydroxy-, one 3-oxo dihydro hydroxy-, 2 isomeric dihydro dihydroxy- and 2 isomeric dihydro trihydroxy-steroids.[21] Very recently, Gauthier *et al*. fully characterized the main metabolite of desoxymethyltestosterone as 17α-methyl-2β,3α,17β-trihydroxy-5α-androstan, which was produced from cultures of human fresh hepatocytes and then chemically synthesized to confirm its structure.[22]

Based on the above, it is clear that the detection time window of the known metabolites of oxymetholone and especially desoxymethyltestosterone is currently too short. At the same time, novel metabolites recently identified for the other 17α-methylated steroids like methandienone and dehydrochloromethyltestosterone greatly increased their detectability in human urine,[23,24] and are the good examples of the importance of selecting a proper target for the analysis. The aim of the present study was to find the metabolites of oxymetholone and desoxymethyltestosterone other than those currently known, which could provide a longer detection time window of these steroids.

## Materials and methods

### Reagents

Acetonitrile, water and methanol were of gradient grade or better and were purchased from Mallinckrodt Baker (Leuven, Belgium), Biosolve (Valkenswaard, the Netherlands) and Merck (Darmstadt, Germany), respectively. Diethyl ether was obtained from Medkhimprom (Moscow, Russia). *n*-Pentane was purchased from Acros (Geel, Belgium). β-Glucuronidase from *E. Coli* K12 (solution in 50% glycerol) was purchased from Roche Diagnostics (Mannheim, Germany) and used as supplied. *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Macherey-Nagel (Düren, Germany). All other chemicals (potassium carbonate, potassium hydrocarbonate, potassium phosphate monobasic, sodium phosphate dibasic, sodium sulfate, ammonium iodide, dithiotreitol) were obtained from Sigma-Aldrich (St Louis, MO, USA).

### Urine samples

In case of oxymetholone, three sets of excretion study urines were available which were collected from three healthy male volunteers up to three weeks after administration. A pharmaceutical preparation – Anapolon – containing 50 mg oxymetholone per tablet (Balkan Pharmaceuticals, Moldova) was administered in this study: 25 mg twice a day during a period of 5 days. For desoxymethyltestosterone, only one set of excretion study samples was at our disposal which was collected from another healthy male volunteer during a controlled administration of the nutritional supplement Phera-Plex (Anabolic Xtreme, Tempe, AZ, USA) declared to contain 10 mg desoxymethyltestosterone per capsule. The protocol assumed administration of two capsules per day during a period of five days and collection of urine samples up to three weeks after the last capsule was taken. Both administration studies were approved by local Ethics Committee at the Institute of Sport. The steroid preparations were checked for purity by GC-MS before the administration studies, and the composition was shown to be consistent with the label. In case of desoxymethyltestosterone, 2-en/3-en isomers were present in the ratio of *ca*. 5:1, as anticipated.

For the isolation of oxymetholone metabolites, selected urine samples from three volunteers were pooled together to simulate average metabolism, while in the case of desoxymethyltestosterone, the urine sample with the highest concentration of known metabolites was chosen and subjected to high performance liquid chromatography (HPLC) fractionation as is. After the inclusion of novel oxymetholone and desoxymethyltestosterone metabolites into the gas chromatography-tandem mass spectrometry (GC-MS/MS) screening method, all reference collection urine samples available at the laboratory were reanalyzed, which included the excretion studies, real positive samples collected before 2010 and retained at the laboratory (oxymetholone, $n = 2$), and miscellaneous spot urines (oxymetholone, $n = 3$; desoxymethyltestosterone, $n = 2$).

### Preparation of urine samples

For the HPLC fractionation, 20 ml of the urine was loaded onto the preconditioned solid phase extraction (SPE) cartridge (BondElut C18, 500 mg, Agilent, Walnut Creek, CA, USA). The cartridge was washed out with 5 ml of water followed by the elution of free and conjugated steroids with 4 ml of methanol. The eluate was evaporated to dryness at 50 °C in vacuum prior to the reconstitution in 1 ml of phosphate buffer (0.8 M, pH 6.3). After the addition of 100 µl of β-glucuronidase, the mixture was vortexed briefly and placed in an incubator where enzymolysis was allowed to proceed at 55 °C for 60 min. After that, 1 ml of carbonate buffer (3 M, pH 10.1) was added and the sample was extracted with 5 ml of diethyl ether by rigorous vortexing in the presence of $Na_2SO_4$ as a salting out agent. After centrifugation at 3200 rpm for 4 min the aqueous layer was frozen in a low-temperature bath (−30 °C) and the ethereal extract was poured out into the other test tube followed by evaporation at 70 °C in a solid-state heater. The dry residue was dissolved in 60 µl of methanol containing 3 µg of dehydropregnenolone acetate as the retention time marker. Next, 40 µl of water was added and after a brief vortexing the urinary extract was transferred into a polypropylene vial with a 0.3 ml conical insert.



  

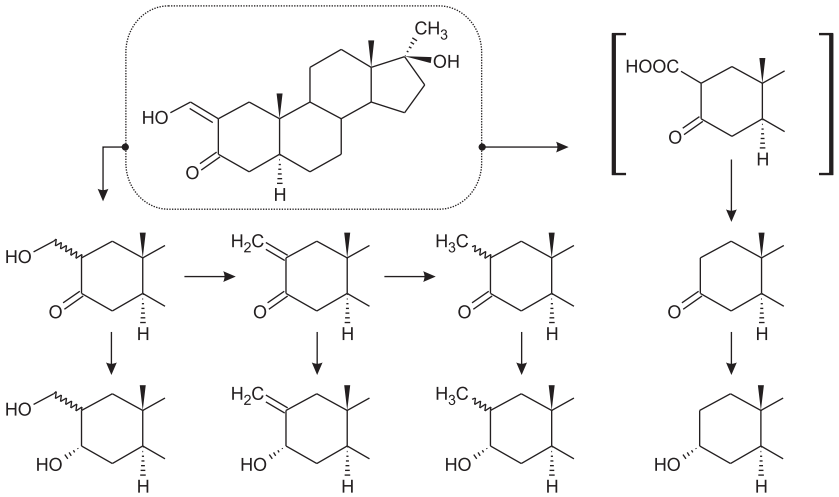


**Figure 1.** Oxymetholone and its A-ring biotransformation routes.

Following HPLC fractionation carried out as described below, each collected fraction was spiked with 10 μl of methyltestosterone solution in methanol (300 ng per fraction, external standard), evaporated to dryness at 50 °C in vacuum and treated with 50 μl of MSTFA/$NH_4$I/dithiotreitol (1000/2/1.5 v/w/w) or alternatively with 50 μl of MSTFA/trimethylchlorosilane (100/1 v/v) at 70 °C for 30 min. Finally, the reaction mixture was transferred into a vial for the GC-MS analysis.

When urine samples were prepared according to the routine procedure for anabolic steroids used in our laboratory, to 3 ml of urine was added 1 ml of phosphate buffer containing 30 μl of β-glucuronidase and 1.5 μg of methyltestosterone. After the incubation and addition of carbonate buffer, the samples were extracted with 5 ml of diethyl ether or pentane in the presence of $Na_2SO_4$. Following the evaporation of organic layer at 70 °C, the dry residue was treated with 50 μl of MSTFA/$NH_4$I/dithiotreitol reagent under the same conditions as described above and transferred to a vial for the GC-MS/MS analysis.

### HPLC fractionation

Agilent 1100 HPLC system comprising a degasser unit, binary pump, autosampler, column compartment, diode array detector and preparative scale fraction collector was used to collect fractions. For the analysis of urinary extracts a Waters SunFire C18 column (250 mm × 4.6 mm, 5 μm) protected by a guard column (20 mm × 4.0 mm) was used. The column was maintained at 35 °C for better retention time stability. Gradient elution was applied as follows: 70% A (water) to 0% A within 0–20 min, then 100% B (acetonitrile) for 10 min, then 0% A to 70% A within 5 min, and equilibration at 70% A for 5 min. The eluent flow rate was 1 ml/min, injection volume – 90 μl, detector wavelength – 197 nm. In total, 16 fractions were collected in slices of 1.5 min within the time range 5–29 min.

### GC-MS and GC-MS/MS

The GC-MS analyses were performed in fullscan mode (50–750 amu) on the system comprising a 6890 gas chromatograph coupled to a 5973 mass spectrometer (Agilent, Palo Alto, CA, USA) with electron ionization at 70 eV. A HP-Ultra 1 column, 17 m × 0.2 mm 0.11 μm (Agilent J&W, Walnut Creek, CA, USA), was used for separation. The temperature program was as follows: 178 °C to 234 °C at 4 °C/min, then to 310 °C at 20 °C/min (held 4.2 min). One μl injections were done at 300 °C in the split mode (1:15) with a carrier gas flow rate set to 0.6 ml/min (helium 99.9999%). The temperature of the transfer line, ion source and quadrupole were 300, 230 and 150 °C, respectively.

In the case of GC-MS/MS analyses, the system comprised a Trace GC Ultra gas chromatograph (Thermo Scientific, Rodano, Italy) coupled to a TSQ Quantum GC triple quadrupole mass spectrometer upgraded to XLS version (ThermoFisher Scientific, San José, CA, USA). The separation was done on the identical GC column with a slightly modified temperature program: 179 °C to 235 °C at 4 °C/min, then to 310 °C at 20 °C/min (held 4.25 min). This was necessary to make the retention times on the two systems as close as possible. Under these chromatographic conditions, the internal standard (methyltestosterone) elutes at 13.78 min. One μl injections were done at 250 °C in the split mode (1:20) with a carrier gas flow rate set to 0.6 ml/min (helium 99.9999%). Transfer line temperature was 300 °C, the ion source was held at 250 °C. The mass spectrometer was operated in selected reaction monitoring (SRM) and fullscan MS/MS modes. The collision gas pressure was 0.13 Pa (or $1.0 \times 10^{-3}$ Torr, argon 99.9995%). In the MS/MS experiments the collision energy (CE) was ramped from 5 to 40 eV to select the optimal value for every transition.

**Table 1.** Chromatographic and mass spectrometric data for the oxymetholone metabolites

| Metabolite | MW*$_1$ | $MW_2$ | RT, min | SRM (CE, V) | HPLC window, min |
|---|---|---|---|---|---|
| OXM-I | 336 | 552 | 15.38 | 462 > 143 (16)<br>537 > 267 (14) | 10-11 |
| OXM-II | 352 | 640 | 16.22 | 405 > 225 (17)<br>495 > 315 (15) | 8-9 |
| OXM-III | 366 | 450 | 11.53 | 435 > 255 (14)<br>255 > 199 (11) | 15-16 |
| OXM-M1 | 334 | 550 | 14.47 | 447 > 267 (10)<br>267 > 173 (10) | 11-12 |
| OXM-M2 | 316 | 460 | 12.60 | 357 > 193 (14)<br>357 > 161 (14) | 17-19 |

* $MW_1$ – molecular weight of native steroids, $MW_2$ – molecular weight of per-TMS derivative, SRM – parent ion to product ion transition, CE – collision energy.

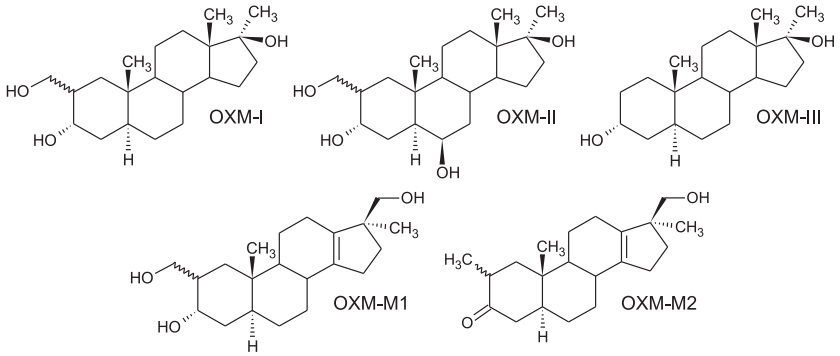


**Figure 2.** Structure of known oxymetholone metabolites (OXM-I, II, III) and proposed structures of novel metabolites OXM-M1, M2 identified after HPLC fractionation of the pooled urine.

## Results and discussion

Successful identification of the long-term metabolite of methandienone[23] and dehydrochloromethyltestosterone[24] demonstrated that transformation of the D-ring in 17α-methylated steroids mediated via 17-epimerization[25] represent a minor but very important metabolic pathway. Therefore, the main goal of this study was to find 18-nor-17,17-dimethyl- and 18-nor-17-hydroxymethyl-17-methyl-steroids in urine samples collected after administration of oxymetholone and desoxymethyltestosterone. Only glucuronide fraction was processed as steroid sulfates are currently neglected in routine doping control analysis.[18]

### Oxymetholone

More than 40 metabolites were tentatively identified after HPLC clean-up, with many of them being isomeric compounds. It should be noted that the seco acidic metabolites were outside the scope of this study since their isolation requires an additional extraction step which is not normally performed by the antidoping laboratories. In general, oxymetholone metabolism in the A-ring occurred via concomitant reduction of 3-oxo and 2-hydroxymethylene groups followed by dehydration of 2-hydroxymethyl function to give the series of 2-methylene steroids (Figure 1). Interesting to mention that at early post-administration periods, methasterone (2α,17α-dimethyl-17β-hydroxy-5α-androstan-3-one) and 3α-hydroxy-methasterone (2α,17α-dimethyl-3α,17β-dihydroxy-5α-androstan) were identified as minor metabolites indicating further reduction of 2-methylene group. The other metabolic pathway mediated via decarboxylation of an acidic intermediate lead to mestanolone (17α-methyl-17β-hydroxy-5α-androstan-3-one), also detectable for a short period of time, and 17α-methyl-5α-androstan-3α,17β-diol, a non-specific but abundant oxymetholone metabolite. The electron ionization mass spectra of pertrimethylsilylated derivatives of all these metabolites feature an intense ion at *m/z* 143 which is typical of intact D-ring of 17-methyl steroids. It is worth mentioning that when searching for the metabolites of 17-methyl steroids in post-administration urines one would normally check for the presence of ions at *m/z* 143, or at *m/z* 218, 231 showing hydroxylation at C16.[26] It means that formation of 18-nor-17,17-dimethyl and 18-nor-17-hydroxymethyl-17-methyl steroids may easily be overlooked due to the unpredictable mass spectra. The only distinctive feature in the latter case is the loss of trimethylsilyloxymethyl group from the molecular ion[27] resulting in the prominent peak $[M–103]^+$.

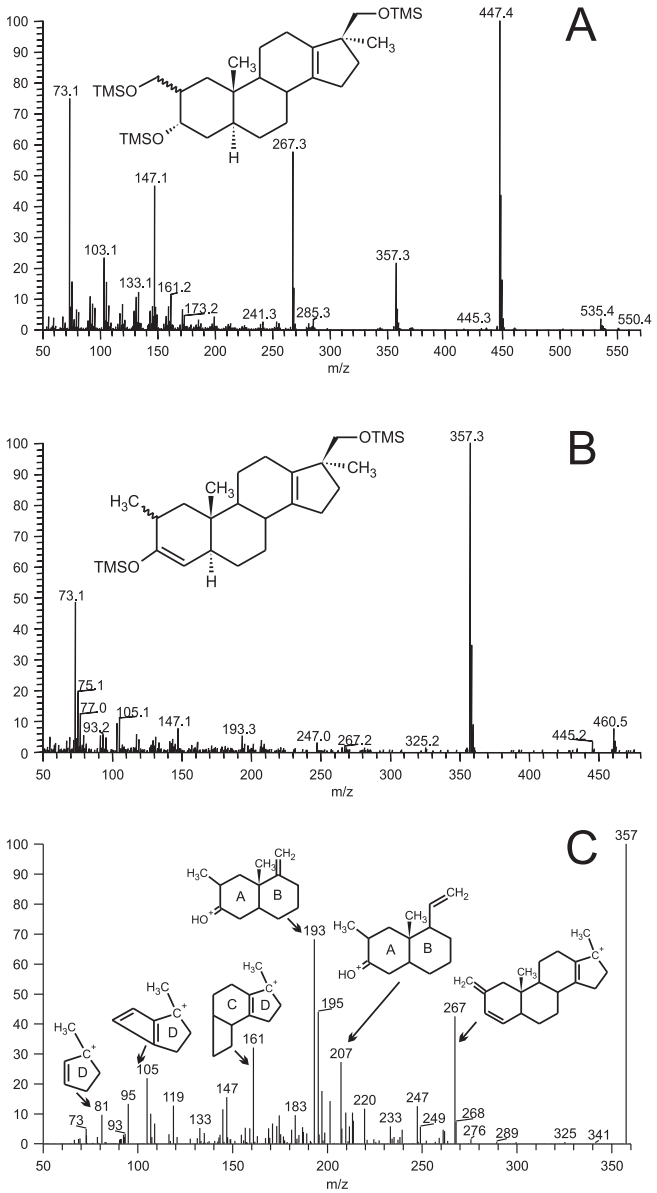


**Figure 3.** Structure and mass spectra of the pertrimethylsilyl derivatives of OXM-M1 (*A*) and M2 (*B*), as well as product ion mass spectrum of *m/z* 357 of M2 recorded at 14 eV (*C*).

For the most of metabolites identified after HPLC clean-up, the product ion mass spectra were obtained to select the SRM transitions necessary for their detection by GC-MS/MS as trimethylsilyl (TMS) derivatives. Then, the excretion urine samples were reanalyzed using a routine procedure to evaluate the detectability of each metabolite during the wash-out period. Most of them, particularly 2ξ-hydroxymethyl-17α-methyl-5α-androstan-3α,17β-diol (OXM-I) and 2ξ-hydroxymethyl-17α-methyl-5α-androstan-3α,6β,17β-triol (OXM-II) being very abundant during the administration or soon after the cessation, were found to have fast elimination rate from the body and are difficult to detect five days after, even though such a sensitive method as GC-MS/MS is used. However, this is not surprising because, as a rule of thumb, the higher the polarity of a steroid metabolite (*e.g.* the number of hydroxy groups) the faster its elimination rate is. The other known metabolite, 17α-methyl-5α-androstan-3α,17β-diol (OXM-III) was detectable a little longer but still not long enough to efficiently reveal the oxymetholone abuse. What is also important that the detection of OXM-III alone does not allow an analyst to unambiguously conclude which steroid was administered, oxymetholone, mestanolone or some $\Delta^1$-methylandrostenediol.

As a result of our study, two novel metabolites both featuring 18-nor-17-hydroxymethyl-17-methyl structure were eventually selected which were detectable in urine longer than OXM-I, OXM-II and, in most cases, than OXM-III. The important chromatographic and mass spectrometric data for all these steroids are summarized in Table 1. The metabolite OXM-M1 was tentatively identified as 18-nor-2ξ,17β-hydroxymethyl-17α-methyl-5α-androst-13-en-3α-ol, while OXM-M2 is most probably 18-nor-17β-hydroxymethyl-17α-methyl-2ξ-methyl-5α-androst-13-en-3-one (Figure 2). The structure assignment was done based on the electron ionization mass spectra produced for the TMS-enol (Figures 3A and 3B) and TMS-ether derivatives (data not shown). Initially we have suggested

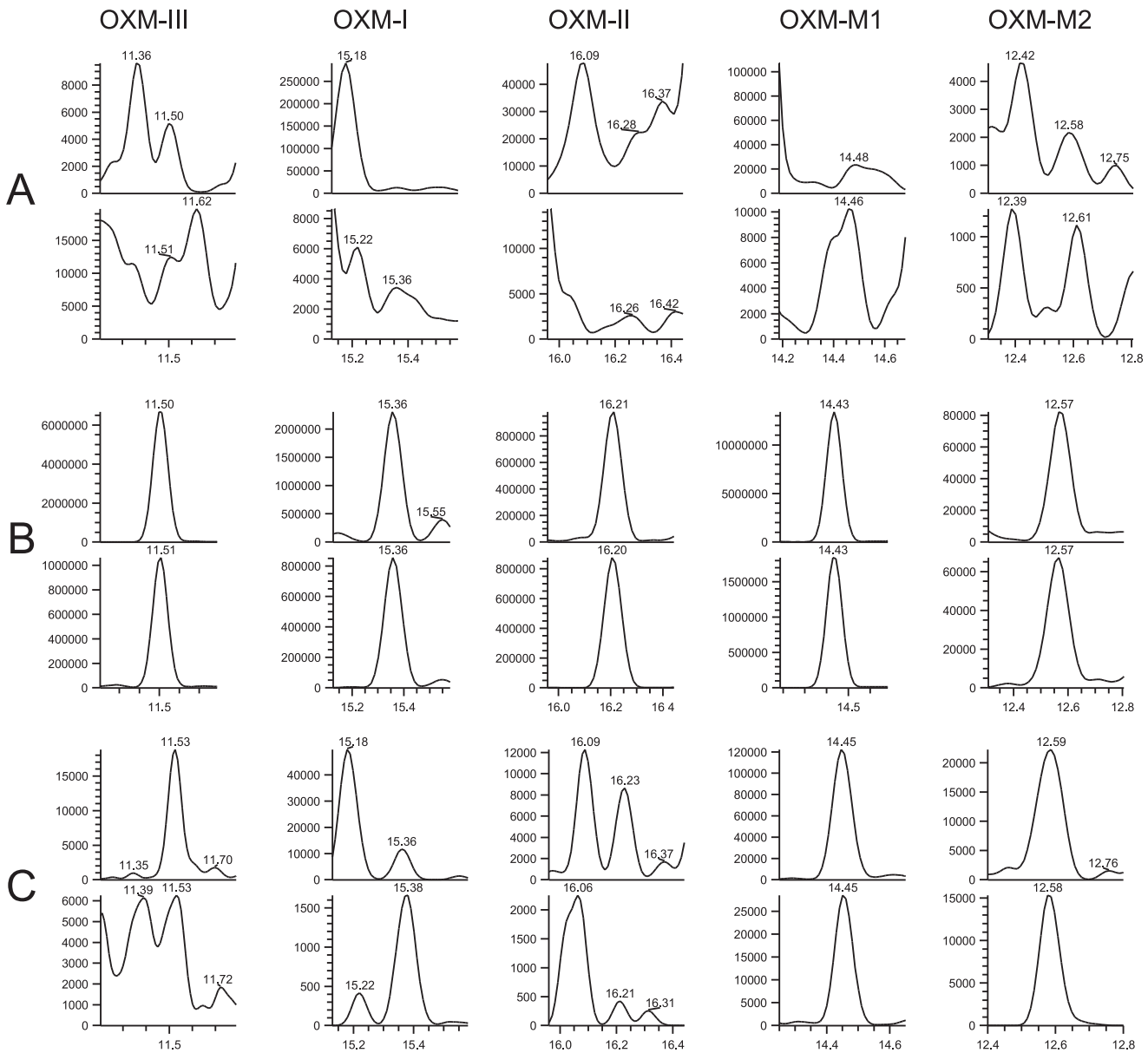


**Figure 4.** SRM chromatograms for negative urine (A), oxymetholone positive urine (*B*), and washout urine collected 14 days after administration (*C*). SRM transitions according to Table 1.

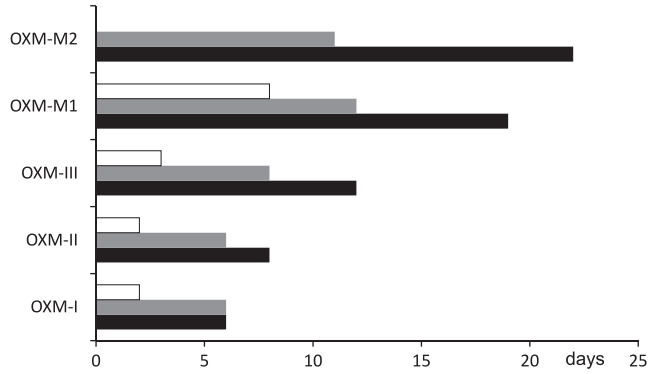


**Figure 5.** Detection window of oxymetholone metabolites in three volunteers. OXM-M2 was not produced in a volunteer shown in white.

that OXM-M2 is *in vivo* dehydration counterpart of OXM-M1, *i.e.* 18-nor-17β-hydroxymethyl-17α-methyl-2-methylene-5α-androst-13-en-3α-ol, but the absence of respective peak in the chromatogram obtained after TMS-ether derivatization of the urinary extract has argued against this assumption. In the case of OXM-M1, the TMS-ether derivatization yielded the same product with an identical retention time and mass spectrum as the TMS-enol derivatization.

The OXM-M1, featuring the low-intensity molecular ion at *m/z* 550 as per-TMS derivative, eliminates trimethylsilyloxymethyl group from C17 to give the abundant ion at *m/z* 447, which sequentially loses trimethylsilanol (90 *Da*) resulting in the ions at *m/z* 357 and 267. The ion at *m/z* 147 normally (but not necessarily) indicates the presence of two adjacent TMS groups, while ions at *m/z* 133 and 161 most likely correspond to the fused C/D- or B/C/D-rings (without C5 and C10), respectively, after the loss of $TMSOCH_2$. On the contrary, the OXM-M2 produces only one abundant ion at *m/z* 357 corresponding to the loss of trimethylsilyloxymethyl group from C17, therefore its detection should exclusively rely on the MS/MS technology. By fragmenting the latter ion, a more informative mass spectrum is produced. Besides common loss of trimethylsilanol to give the ion at *m/z* 267 and sequential cleavage of B/C/D-rings resulting in a series of ions separated by a methylene unit (*m/z* 81, 95 and *m/z* 105, 119, 133, 147, 161), one may observe less obvious fragments at *m/z* 193 and 207 which probably come from an alternative fragmentation pathway initiated by the elimination of trimethylsilyl group from carbonyl oxygen at C3, as shown in Figure 3C. It is worth noting that upon collision induced dissociation of a minor ion $[M\text{-}CH_3]^+$ of pertrimethylsilyl methasterone (*m/z* 447), the ion at *m/z* 357 is formed which further cleaves to *m/z* 193 and 207 (data not shown). If we postulate that *m/z* 447 is formed after loss of the angular methyl at C13, and *m/z* 357 is then produced by eliminating trimethylsilanol at C17, then we would come to the same substructure as *m/z* 357 of OXM-M2. This may be considered as indirect evidence that the A-ring of OXM-M2 is the same as in methasterone.

To demonstrate the usefulness of novel metabolites for detection of oxymetholone abuse, Figure 4 illustrates blank urine (A), post-administration urine (B), and wash-out urine collected 14 days after cessation (C). It is seen that OXM-M1 and OXM-M2 are well-detectable in urine even two weeks after oxymetholone administration while other known metabolites including non-specific OXM-III are not. To further support the advantage that new metabolites provide, one may compare the detectability of oxymetholone OXM-I, II, III, as well as M1 and M2 in three excretion studies performed. As seen from Figure 5, in all cases OXM-M1 was detectable longer. However, OXM-M2 being *per se* a minor metabolite was found to be subject-dependent as in one of three volunteers it was barely visible. Our recent study revealed that OXM-M2 is also a long-term metabolite of methasterone (T. Sobolevsky, unpublished data). It could mean that OXM-M2 is produced in the human body from methasterone, which in turn is a minor metabolite of oxymetholone. Therefore, more data need to be accumulated to fully evaluate OXM-M2.

The other important issue to consider is that under chromatographic conditions used in this study, OXM-M1 elutes very close to pregnanetriol (PT) which is normally abundant in human urine. The TMS derivative of PT has the molecular weight of 552 that means the presence in its mass spectrum of the ion at *m/z* 537 (loss of methyl group). Though being negligible, this fragmentation pathway generates the series of ions at *m/z* 447, 357 and 267 due to sequential cleavage of trimethylsilanol. As a result, PT contributes to the SRM transitions of OXM-M1 that could impede its detection when concentration of OXM-M1 is low. For this reason it is recommended to monitor one SRM transition for PT (*e.g.* 435 > 255) to avoid confusion (Figure 6). Good point is that

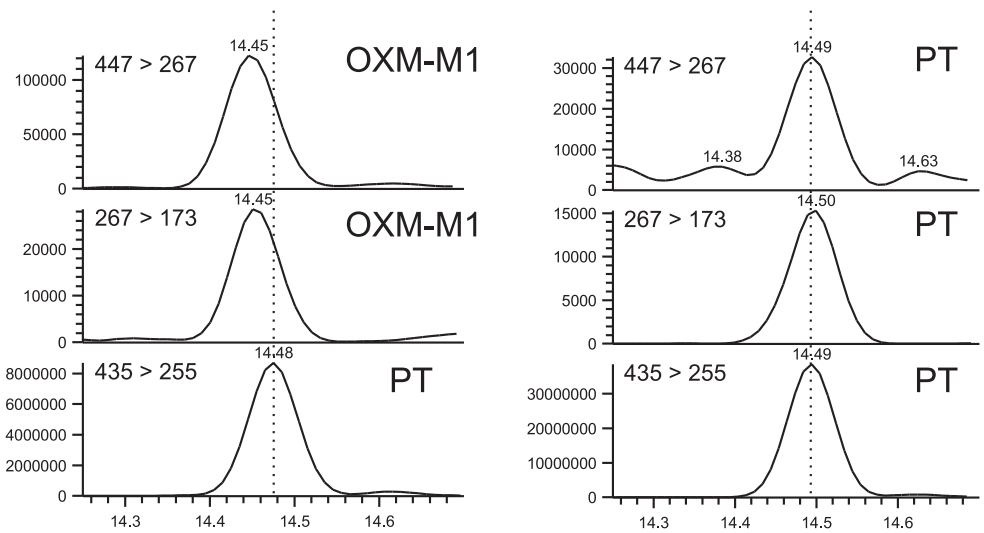


**Figure 6.** The pertrimethylsilyl derivatives of pregnanetriol *vs* OXM-M1.

PT and OXM-M1 are separable during HPLC clean-up as the former falls into the later fraction (14–15 min), and therefore OXM-M1 could be easily isolated from urine for confirmation purposes.

### Desoxymethyltestosterone

The reinvestigation of desoxymethyltestosterone metabolism was triggered by the necessity to identify new targets which could provide a more reliable disclosure of abuse of this steroid in sports. Our previous findings[21] demonstrated the complexity of its determination by GC-MS related to interferences with urinary matrix and the low-informative mass spectra of the TMS derivatives of metabolites. In this study, HPLC fractionation allowed us to isolate several novel metabolites and also the isomers of previously reported ones. About 20 metabolites were tentatively identified which included mono-, di- and trihydroxylated steroids and their reduced counterparts, one compound having 14 *Da* shift relative to others which could indicate the presence of non-derivatizable 2,3-epoxy group, and a series of 18-nor-17,17-dimethyl steroids. It should be noted that no steroids to which 18-nor-17-hydroxymethyl-17-methyl structure could be unambiguously attributed were found.

By applying the same strategy as described above, each metabolite was evaluated in terms of its clearance rate in the excretion urine samples. For comparative purposes the screening method also included 17α-methyl-5α-androstan-2ξ,3α,16ξ,17β-tetrol (DMT-I) and 17α-methyl-5α-androstan-2ξ,3α,17β-triol (DMT-II), which were earlier selected for detection of desoxymethyltestosterone administration in our laboratory.[21] It is important to mention that DMT-II almost coelutes with pregnanediol (PD) under the chromatographic conditions used in present study.

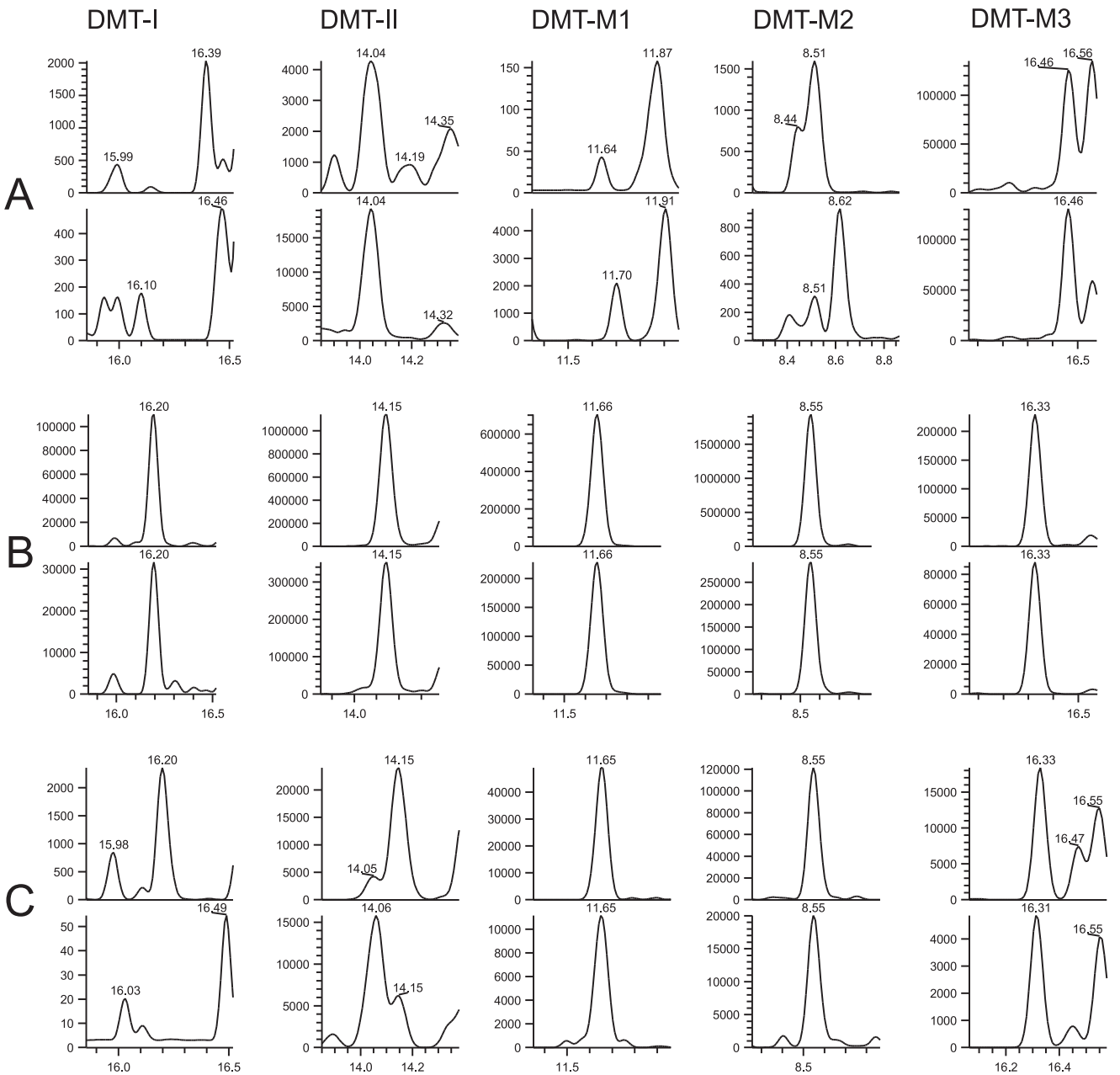


**Figure 7.** SRM chromatograms for negative urine (*A*), desoxymethyltestosterone positive urine (*B*), and washout urine collected 14 days after administration (*C*). SRM transitions according to Table 2.

According to,[22] the major desoxymethyltestosterone metabolite is 17α-methyl-5α-androstan-2β,3α,17β-triol; probably, DMT-II is exactly this compound.

As a result of analysis of the excretion urine samples, two novel metabolites (DMT-M1 and DMT-M2) well detectable two weeks after administration were chosen (Figure 7), which were present in the two spot urines available at the laboratory as well. Despite being also detectable, the epoxy metabolite (DMT-M3) was deselected because of a higher background from the urinary matrix and potential of artifact formation during the derivatization process that could make its detection unreliable. The histogram in Figure 8 helps visually compare how long the metabolites may be determined in urine. The novel metabolites clearly extend the detection window for desoxymethyltestosterone abuse. The SRM transitions and other relevant data for the known and new metabolites are reported in Table 2 and Figure 9.

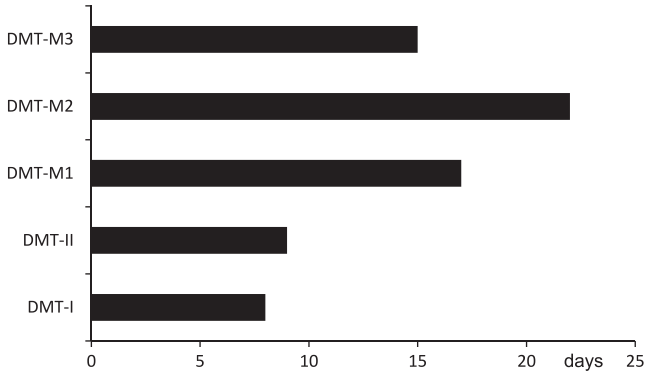


**Figure 8.** Detection window of desoxymethyltestosterone metabolites.

**Table 2.** Chromatographic and mass spectrometric data for the desoxymethyltestosterone metabolites

| Metabolite | $MW_1$ | $MW_2$ | RT, min | SRM (CE, V) | HPLC window, min |
|---|---|---|---|---|---|
| DMT-I | 338 | 626 | 16.20 | 626 > 231 (15)<br>626 > 143 (20) | 5-6.5 |
| DMT-II | 322 | 538 | 14.15 | 523 > 343 (10)<br>523 > 253 (15) | 8-9.5 |
| DMT-M1 | 322 | 538 | 11.66 | 523 > 343 (10)<br>523 > 253 (15) | 11-12.5 |
| DMT-M2 | 304 | 448 | 8.55 | 433 > 253 (12)<br>433 > 147 (22) | 20-21.5 |
| DMT-M3 | 336 | 552 | 16.33 | 537 > 357 (10)<br>537 > 267 (10) | 8-9.5 |

Interestingly, DMT-M1 is the isomer of DMT-II which elutes *ca*. 3 min earlier, close to epitestosterone, in a much cleaner part of chromatogram. Gauthier *et al*. suggested that the early eluting isomer, if the same metabolite was assumed, could be 3,4-diol arising from 3-en DMT[22] but this was not elucidated by synthesis. Considering that concentration of DMT-M1 in urine was found comparable to that of DMT-II while theoretically it should be lower by factor of the ratio of desoxymethyltestosterone 2-en/3-en isomers, it could be postulated that DMT-M1 is the isomeric 2,3-diol rather than 3,4-diol. It is worth mentioning that when single quadrupole GC-MS method is used for analysis, DMT-M1 manifests itself in the window of methyltestosterone metabolites (methylandrostanediols), and, if judged superficially, this may lead to a wrong conclusion that 17α-methyl-5β-androstan-3α,17β-diol is also a metabolite of desoxymethyltestosterone. The other important point is that both DMT-M1 and DMT-M2 were present in urine collected after administration of the related steroid, 2α,3α-epithio-17α-methyl-17β-hydroxy-5α-androstan (T. Sobolevsky, unpublished data), which is currently available as a component of over-the-counter nutritional supplements, and this also makes these metabolites valuable for the antidoping analysis.

Based on the mass spectra of the TMS derivatives and the fact that the TMS-ether derivatization did not change the retention time nor the mass spectra, it was suggested that DMT-M2 is 18-nor-17,17-dimethyl-5α-androst-13-en-2ξ,3α-diol (Figure 10B), while DMT-M1 – 17α-methyl-5α-androstan-2α,3α,17β-triol (Figure 10A) or, less probable, 17α-methyl-5α-androstan-3α,4ξ,17β-triol. The TMS derivative of DMT-M2 has a rich mass spectrum with the abundant molecular ion at *m/z* 448 which eliminates methyl group and trimethylsilanols giving rise to the series of ions at *m/z* 433, 358, 343, 268, 253. The absence of the ions at *m/z* 143 or *m/z* 218, 231 clearly demonstrates that the D-ring has been metabolized. In case of DMT-M1 the mass spectrum of its TMS derivative is essentially the same as reported in Rodchenkov *et al*.[21] and Gauthier *et al*.[22] and is fully consistent with the structure proposed. The metabolite DMT-M3 was hypothesized to be 17α-methyl-2,3-epoxy-5α-androstan-4ξ,6ξ,17β-triol (Figure 10C); the mass spectrum of its pertrimethylsilyl derivative has prominent ion at *m/z* 143 indicating the intact D-ring, and shows successive loss of three trimethylsilanol molecules (*m/z* 447, 357, 267) from the ion at *m/z* 537 which

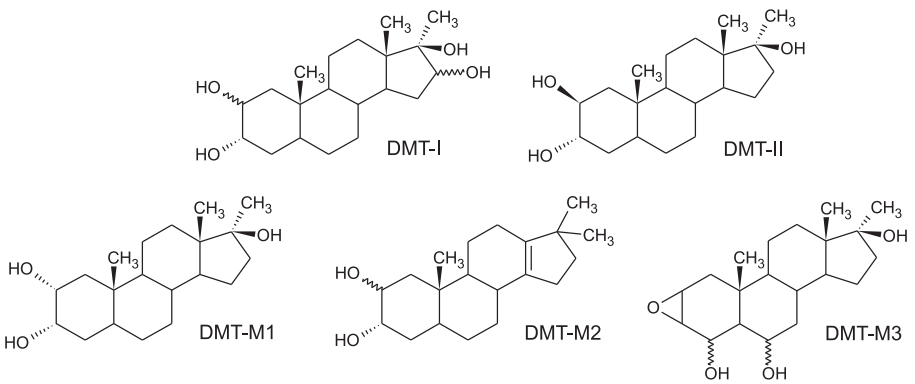


**Figure 9.** Structure of known desoxymethyltestosterone metabolites (DMT-I and II) and proposed structures of novel metabolites DMT-M1, M2 and M3 identified after HPLC fractionation of the pooled urine.

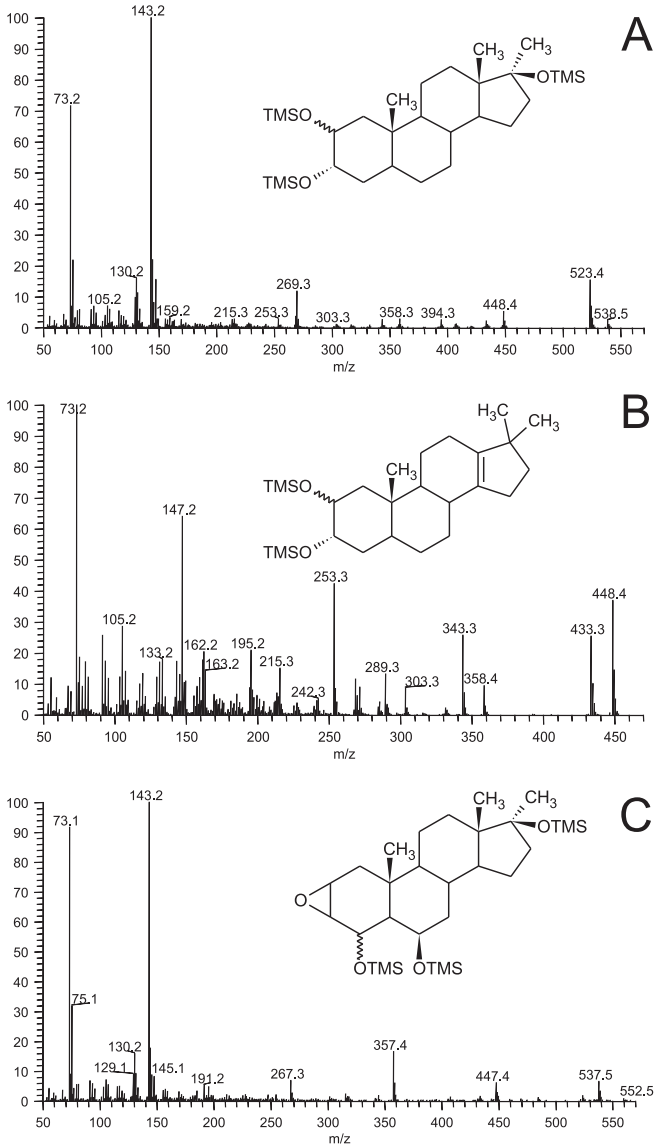


**Figure 10.** Structure and mass spectra of the pertrimethylsilyl derivatives of DMT-M1 (*A*), M2 (*B*) and M3 (*C*).

is $[M\text{-}CH_3]^+$. While the formation of 16α,17α-epoxy-androst-4-en-3-one from androsta-4,16-dien-3-one upon incubation with rat liver microsomes has been previously reported,[28] nothing is known about the possibility of such a process in human.

## Conclusion

As a result of this study, the novel metabolites of oxymetholone and desoxymethyltestosterone with the so-called nightwatch and 18-nor-17,17-dimethyl structure were described. Though not fully evaluated due to the limited number of excretion studies available, steroids with tentative structures 18-nor-2ξ,17β-hydroxymethyl-17α-methyl-5α-androst-13-en-3α-ol and 18-nor-17,17-dimethyl-5α-androst-13-en-2ξ,3α-diol were shown to be promising targets for the long-term detection of oxymetholone and desoxymethyltestosterone, respectively. Synthesis of the reference material is needed to confirm the proposed structure of these metabolites.

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