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# Detection and mass spectrometric characterization of novel long-term dehydrochloromethyltestosterone metabolites in human urine

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ABSTRACT

The biotransformation of dehydrochloromethyltestosterone (DHCMT, 4-chloro-17β-hydroxy,17α-methylandrosta-1,4-dien-3-one) in man was studied with the aim to discover long-term metabolites valuable for the antidoping analysis. Having applied a high performance liquid chromatography for the fractionation of urinary extract obtained from the pool of several DHCMT positive urines, about 50 metabolites were found. Most of these metabolites were included in the GC–MS/MS screening method, which was subsequently applied to analyze the post-administration and routine doping control samples. As a result of this study, 6 new long-term metabolites were identified tentatively characterized using GC–MS and GC–MS/MS as 4-chloro-17α-methyl-5β-androstan-3α,16,17β-triol (**M1**), 4-chloro-18-nor-17β-hydroxymethyl,17α-methyl-5β-androsta-1,13-dien-3α-ol (**M2**), 4-chloro-18-nor-17β-hydroxymethyl,17α-methyl-5β-androst-13-en-3α-ol (**M3**), its epimer 4-chloro-18-nor-17α-hydroxymethyl,17β-methyl-5β-androst-13-en-3α-ol, 4-chloro-18-nor-17β-hydroxymethyl,17α-methylandrosta-4,13-dien-3α-ol (**M4**) and its epimer 4-chloro-18-nor-17α-hydroxymethyl,17β-methylandrosta-4,13-dien-3α-ol. The most long-term metabolite **M3** was shown to be superior in the majority of cases to the other known DHCMT metabolites, such as 4-chloro-18-nor-17β-hydroxymethyl,17α-methylandrosta-1,4,13-trien-3-one and 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one.



## 1. Introduction

The anabolic androgenic steroids (AAS) are the class of performance enhancing substances prohibited in sports by the World Anti-Doping Agency (WADA) at all times [1]. Due to their availability on the black market, some AAS are more likely to be abused than the others. One of such substances is dehydrochloromethyltestosterone (DHCMT), also known as 4-chloromethandienone or oral-turinabol, a steroid which is remarkable, *inter alia*, for its relatively fast elimination rate from the body [2,3].

The data on the metabolism of DHCMT are limited. In 1970 Schubert et al. reported the detection of parent steroid, as well as 6β-hydroxy-, 16β-hydroxy- and 6β,16-dihydroxy-DHCMT in post administration urine [4,5]. Afterward, Dürbeck et al. identified 6β-hydroxy-, 6β,12-dihydroxy-, 6β,16-dihydroxy-DHCMT and minor amounts of epi-DHCMT, but did not detect parent drug nor 16β-hydroxy-DHCMT [2]. The presence of 6β-hydroxy-DHCMT in human urine was also confirmed [6]. However, the detection window was quite small (5 days or less). Later, an in-depth study was performed that revealed a high complexity of the DHCMT metabolism and resulted in the identification and characterization of the other important metabolites [3]. The most long-term metabolite identified was 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one, detectable in urine for up to 14 days using gas chromatography/high resolution mass spectrometry. The authors used high performance liquid chromatography (HPLC) for isolation of the metabolites from human urine after a single oral dose of 40 mg of DHCMT administered to a male volunteer. In vitro metabolic experiments were also attempted which have shown that the incubation of DHCMT with human cytochrome P450 enzymes resulted in the formation of 6β-hydroxy metabolite only [7]. And it was not until 2010 when Parr et al. demonstrated the existence of 18-nor-17-hydroxymethyl metabolite of DHCMT, similar to that of methandienone [8]. This metabolite, 4-chloro-18-nor-17β-hydroxymethyl,17α-methylandrosta-1,4,13-trien-3-one, was found to be as valuable as 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one, both being detectable 22 days after administration by gas chromatography/triple quadrupole mass spectrometry (GC–MS/MS) [9].

The aim of the present study was to identify and tentatively characterize by GC–MS/MS the other long-term metabolites of

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DHCMT isolated from the pooled human urine that could make the detection window even longer, and incorporate them into the GC–MS/MS method for anabolic steroids to select the most relevant metabolites for the doping control analysis.

## 2. Materials and methods

### 2.1. Reagents

The solvents (acetonitrile, water, 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 *Escherichia 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).

### 2.2. Urine samples

For the isolation of metabolites the urine samples from the laboratory reference collection were used. Ten samples were selected for which a validated GC–MS method has confirmed the presence of well-characterized DHCMT metabolites (6β-hydroxy-DHCMT, 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one, 4-chloro-3α,6β,16ξ,17β-tetrahydroxy-17α-methyl-5β-androst-1-en). Aliquots of 10 ml were taken from each sample and pooled together to simulate "average" metabolism. Further, this pooled urine was processed for the HPLC fractionation as described below.

Alternatively, after the inclusion of novel DHCMT metabolites into the GC–MS/MS screening method, all reference collection urine samples available at the laboratory, as well as the routine doping control samples from risky sports were analyzed for the monitoring purposes. The laboratory reference collection included both the excretion studies ($n = 27$) and real positive samples collected before 2008 and retained at the laboratory ($n = 7$). The risky sport samples were represented by weightlifting ($n = 52$), powerlifting ($n = 37$) and athletics ($n = 44$). Only those samples were selected where the athlete agreed to use the remainder of his/her sample for the anti-doping research.

### 2.3. Preparation of urine samples

For the HPLC fractionation, 20 ml of the pooled urine was loaded onto the preconditioned SPE cartridge (BondElut C18, 500 mg, Varian, 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 brief vortexing the urinary extract was transferred into a polypropylene vial with a 0.1 ml conical insert.

Following the 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/$NH_4I$/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 were 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/$NH_4I$/dithiotreitol reagent under the same conditions as described above and transferred to a vial for the GC–MS/MS analysis.

### 2.4. 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 and to decrease backpressure. 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, 26 fractions were collected in slices of 1 min within the time range 4–30 min.

### 2.5. 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, USA) with electron ionization at 70 eV. A HP-Ultra 1 column, 17 m × 0.2 mm × 0.11 μm (Agilent J&W, USA), was used for separation. The temperature program was as follows: 180 to 236 °C at 4 °C/min, then to 310 °C at 20 °C/min (held 4.3 min). One microliter 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 99.995%). The temperature of the transfer line, ion source and quadrupole were 300, 230 and 150 °C, respectively.

In 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 (ThermoFisher Scientific, San Jose, CA, USA). The separation was done on the identical GC column with a slightly modified temperature program: 177 to 233 °C at 4 °C/min, then to 310 °C at 20 °C/min (held 4.15 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 13.7 min. One microliter 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 SRM and fullscan MS/MS modes. The collision

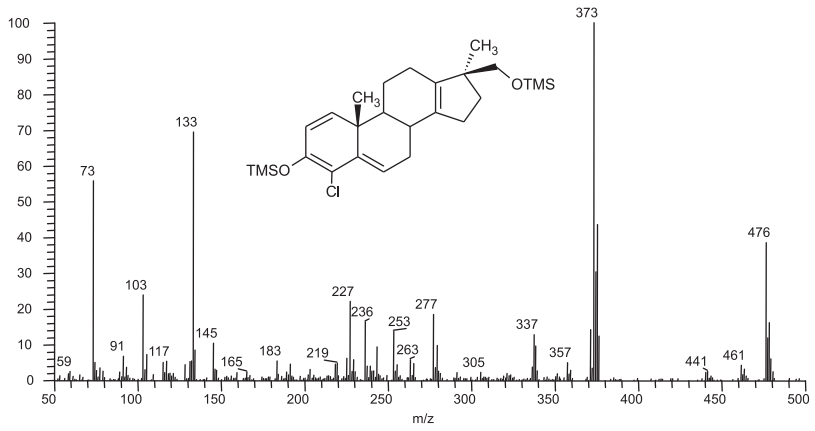


**Fig. 1.** Structure and mass spectrum of the pertrimethylsilyl derivative of **I** obtained after HPLC clean-up.

gas pressure was 0.13 Pa (or $1.0 \times 10^{-3}$ Torr, argon 99.998%). In the MS/MS experiments the collision energy (CE) was ramped from 5 to 25 eV to select the optimal value for every transition.

## 3. Results and discussion

### 3.1. Metabolite detection

Initially, this study was triggered by the necessity of mass spectrometric characterization of 4-chloro-18-nor-17β-hydroxymethyl,17α-methylandrosta-1,4,13-trien-3-one (**I**). In MS/MS experiments such characterization assumes the selection of proper parent – product ion transitions and optimal collision potentials. For this purpose one would normally analyze the pure substance; however, in metabolic studies it is not uncommon that the pure substance is not available. This may significantly complicate or make it impossible to acquire the product ion mass spectra due to the coelution with concomitant compounds present in the sample and/or the low concentration of the metabolite in question.

Our preliminary experiments have shown that the trimethylsilyl derivative (TMS) of **I** elutes very close, with a difference in the retention time of *ca.* 0.02 min, to that of 17α-epi-DHCMT, another DHCMT metabolite which is generally abundant in urines collected during the administration of this steroid or soon after the cessation. Since some of the ion *m*/*z* ratios in the electron ionization mass spectra of both metabolites are isobaric, e.g., *m*/*z* 373 for the TMS derivative of **I** corresponds to $[M-CH_2OTMS]^+$, whereas in case of epi-DHCMT this fragment is produced upon sequential loss of the methyl radical and trimethylsilanol, the reliable mass spectral characterization of **I** is complicated.

Having applied the HPLC clean-up to the pooled human urine, we were able to separate **I** and epi-DHCMT recovered from the fractions corresponding to 15–16 and 16–17 min, respectively. The separation enabled to acquire the mass spectrum of **I** without any interference and establish the selective SRM transitions for its detection by GC–MS/MS. The EI mass spectrum of **I** as bis-TMS derivative obtained after the HPLC clean-up is given in Fig. 1. It is important to note that the loss of the $CH_2OTMS$ group revealed by the presence of the ion $[M-103]^+$ in the mass spectrum is a distinctive feature of the trimethylsilylated 18-nor-17-hydroxymethyl steroids [8,9].

Further, our research was focused on the identification of the new potentially valuable metabolites that may be not clearly visible in the fullscan mode after a routine sample preparation procedure. It is worth noting that the presence of chlorine facilitates the recognition of DHCMT metabolic products against the biological background due to the characteristic isotopic pattern observable in mass spectra of the metabolites retaining chlorine in their steroidal backbone. After careful evaluation of the chromatographic and mass spectral data obtained upon the GC–MS analysis of every HPLC fraction (following the evaporation and derivatization) *ca.* 50 metabolites were found in pooled post administration urine with approximately 100-fold difference in the abundance (data not shown). Many of the metabolites were isomeric compounds with almost identical mass spectra; what is interesting that plenty of them were lacking the ions at *m*/*z* 143 or *m*/*z* 218, 231 typical of 17-methylsteroids or their respective 16-hydroxylated products [3]. This finding supports the fact that during biotransformation the D-ring of DHCMT undergoes extensive metabolism mediated via 17-epimerization [10] followed by the formation of 18-nor steroids, as well as their hydroxylated (**I**) and/or reduced counterparts. It should be emphasized that only the glucuroconjugated metabolites were considered in the present study, as its main goal was to find the best DHCMT administration target to include into the routine screening method for anabolic steroids, which currently focuses on the steroid glucuronides [11].

Of 50 metabolites found after initial evaluation of the GC–MS data, we have chosen about 30 for which the concentration in pooled post administration urine and HPLC fraction purity allowed for acquiring the product ion mass spectra. Having established the dedicated GC–MS/MS screening method incorporating the SRM transitions for these 30 metabolites, we started to analyze the laboratory reference collection samples positive for DHCMT, as well as the routine doping control samples from risky sports such as weightlifting, athletics and powerlifting for the monitoring purposes. To probe the significance of each metabolite for the doping control, the screening method also included **I** and 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one (**II**), which are presently considered as the most long-term metabolites. The only commercially available DHCMT metabolite, 6β-hydroxy-DHCMT, was not included into this screening method as it is detectable in urine only for a short period of time [3].

Based on the results obtained for more than 150 samples with DHCMT findings and the routine doping control samples, 6 of 30 metabolites (including isomeric pairs) were eventually selected. It should be noted that the metabolism of DHCMT was found to demonstrate a considerable inter-individual variability, and the excretion profiles of the same metabolites are difficult to compare from person to person. In particular, the metabolites **I** and **II** were shown to have a noticeable variation in the abundance and are excreted in urine not as long as some novel metabolites reported below. Moreover, in some urines the metabolite **II** was well-detectable while the metabolite **I** was not, and vice versa. Nevertheless, without having the synthetic reference materials for **I**, **II**

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

| Metabolite | $MW_1$[a] | $MW_2$ | RT, min | SRM (CE, V) | HPLC window, min |
|---|---|---|---|---|---|
| **I** | 332 | 476 | 14.76 | 476 > 133 (16)<br>476 > 373 (14) | 15–16 |
| **II** | 368 | 656 | 16.03 | 656 > 244 (17)<br>658 > 244 (15) | 6–7 |
| **M1** | 356 | 572 | 15.48 | 572 > 231 (16)<br>572 > 143 (22) | 14–15 |
| **M2** | 336 | 480 | 12.70 | 377 > 185 (22)<br>287 > 185 (12) | 15–16 |
| **M3** | 338 | 482 | 13.63 | 379 > 343 (7) | 16–18 |
| epi-**M3** | | | 12.64 | 379 > 253 (10) | 19–20 |
| **M4** | 336 | 480 | 13.45 | 377 > 149 (13) | 15–16 |
| epi-**M4** | | | 12.52 | 287 > 125 (22) | 18–19 |

[a] $MW_1$ – molecular weight of native steroids, $MW_2$ – molecular weight of TMS derivative, SRM – parent ion to product ion transition, CE – collision energy.

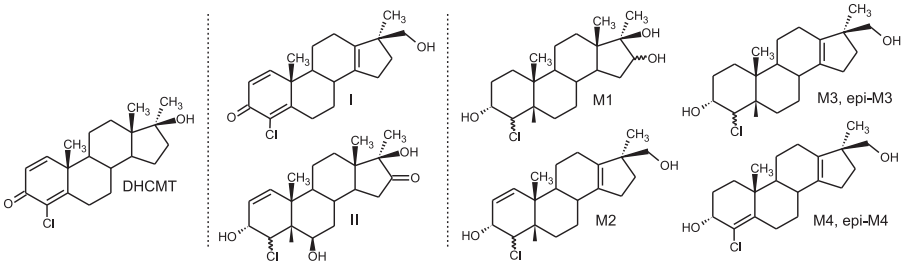


**Fig. 2.** Structures of DHCMT, its known long-term metabolites (**I** and **II**) and proposed structures of DHCMT metabolites **M1**–**M4** identified after HPLC fractionation of the pooled urine.

and novel metabolites it is impossible to determine their concentration in urine samples and only relative comparison could be made at the moment.

### 3.2. Proposed structure of important DHCMT metabolites

Table 1 summarizes the important chromatographic and mass spectrometric data obtained for the TMS derivatives of the DHCMT metabolites; the postulated structures are given in Fig. 2. The metabolite **M1**, which is fully reduced 16-hydroxylated DHCMT as confirmed by the presence of the ions at *m*/*z* 218, 231 in the mass spectrum of its TMS derivative [3] (Fig. 3), is detectable longer that **I** and **II** in most cases. The other long-term metabolites are 18-nor-17-hydroxymethyl steroids with the A-ring being partly (**M2** and **M4**) or fully (**M3**) reduced. Though the stereochemistry at C-3 and C-5 is not known, it may be assumed that **M1**–**M3** are the substituted 5β-androst-13-en-3α-ols (androsta-4,13-dien-3α-ol in case of **M4**) considering that, first, 3-oxo-androsta-1,4-dienes like DHCMT normally do not produce 5α-reduced metabolites and, second, 3β-hydroxylated steroids are excreted mostly as sulfates [12]. Interestingly, **M2** is a single metabolite, while both **M3** and **M4** have their isomeric counterparts, being most likely 17α-epimers which are less abundant. This is supported by the fact that the difference in the retention times for the respective TMS derivatives is *ca*. 1 min, and that the early eluting metabolites in GC fall into the later HPLC fractions (as holds true for testosterone and epitestosterone). The metabolites **M1**–**M4** are excreted conjugated.

The mass spectra of pertrimethylsilylated **M2**–**M4** are presented in Fig. 4. As follows from the data, all these metabolites have the abundant ion corresponding to the loss of 103 Da at *m*/*z* 379 (**M3**) and 377 (**M2** and **M4**). In case of **M3**, the ion at *m*/*z* 379 sequentially eliminates the molecule of hydrogen chloride (36 Da) and trimethylsilanol (90 Da) to give the ions at *m*/*z* 343 and 253, respectively. The absence of 3-oxo function in the metabolites **M2** and **M4** was supported by the fact that trimethylsilylation with MSTFA containing 1% trimethylchlorosilane resulted in the same mass spectra as obtained using MSTFA/$NH_4I$/dithiotreitol reagent (data not shown). Therefore, the dissimilarity between the mass spectra of the pertrimethylsilylated **M2** and **M4** could be explained by different position of the double bond making the elimination of

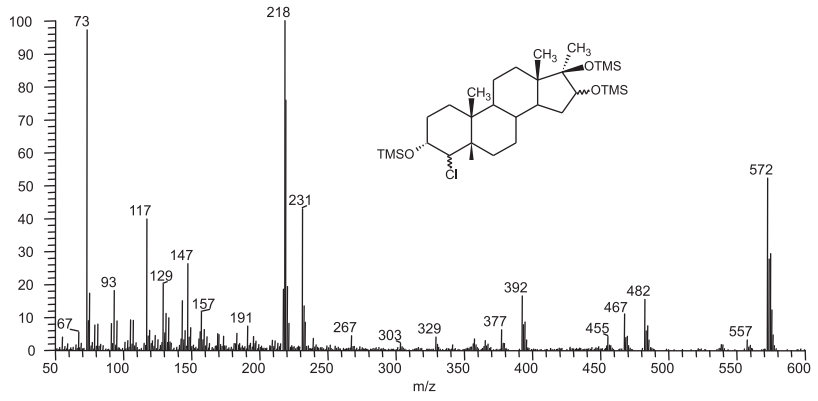


**Fig. 3.** Structure and mass spectrum of the pertrimethylsilyl derivative of **M1**.

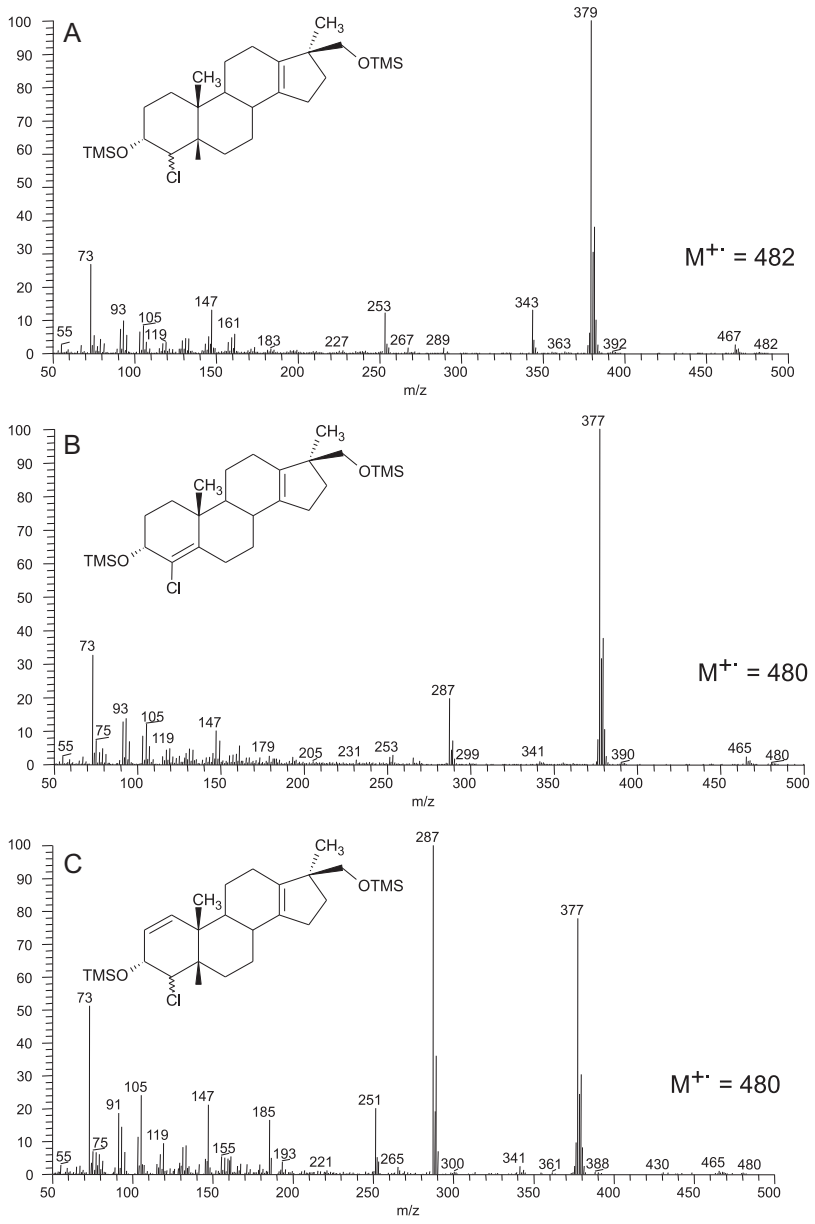


**Fig. 4.** Structures and mass spectra of the pertrimethylsilylated metabolites **M3** (A), **M4** (B), **M2** (C).

hydrogen chloride unfavorable in case of **M4**. At the same time for **M2**, the ion at *m*/*z* 377 sequentially eliminates trimethylsilanol and hydrogen chloride followed by the cleavage of the A- and B-rings between C-2/C-3, C-5/C-10 and C-7/C-8 to form the fragments at *m*/*z* 287, 251 and 185, respectively.

### 3.3. Evaluation for long-term DHCMT detection in doping control analysis

Our study has shown that the metabolite **M3** and, to a lesser extent, its epimer and **M4** are the most long-term metabolites of DHCMT. Taking into account that **I** and **II** are reportedly detectable up to 22 days post administration [3,9] and that the relative concentration of **M3** in DHCMT post administration urines is normally higher compared to **I** and **II**, the detection window of **M3** could be estimated as 40–50 days, while **M1**, **M2** and **M4** are at least as valuable as **I** and **II**. An additional controlled excretion study is needed to fully evaluate the time at which novel metabolites can be detected.

Table 2 summarizes the results of this study demonstrating the significance of novel DHCMT metabolites (exemplified by **M3**). As seen from the data, inclusion of **M3** into the screening procedure resulted in 15 positive findings in addition to 5 revealed by the

**Table 2**
Urine samples analyzed for the presence of the DHCMT metabolites.

| Sample type | Number of samples | | |
|---|---|---|---|
| | Total | Positive findings: **I** and **II** | Positive findings: **I** and **II** + **M3** |
| Excretion urine | 27 | 27 | 27 |
| Real positive sample | 7 | 7 | 7 |
| Routine doping control samples | 133 | 5 | 20 |
| *Weightlifting* | 52 | 3 | 8 |
| *Powerlifting* | 37 | 1 | 7 |
| *Athletics* | 44 | 1 | 5 |

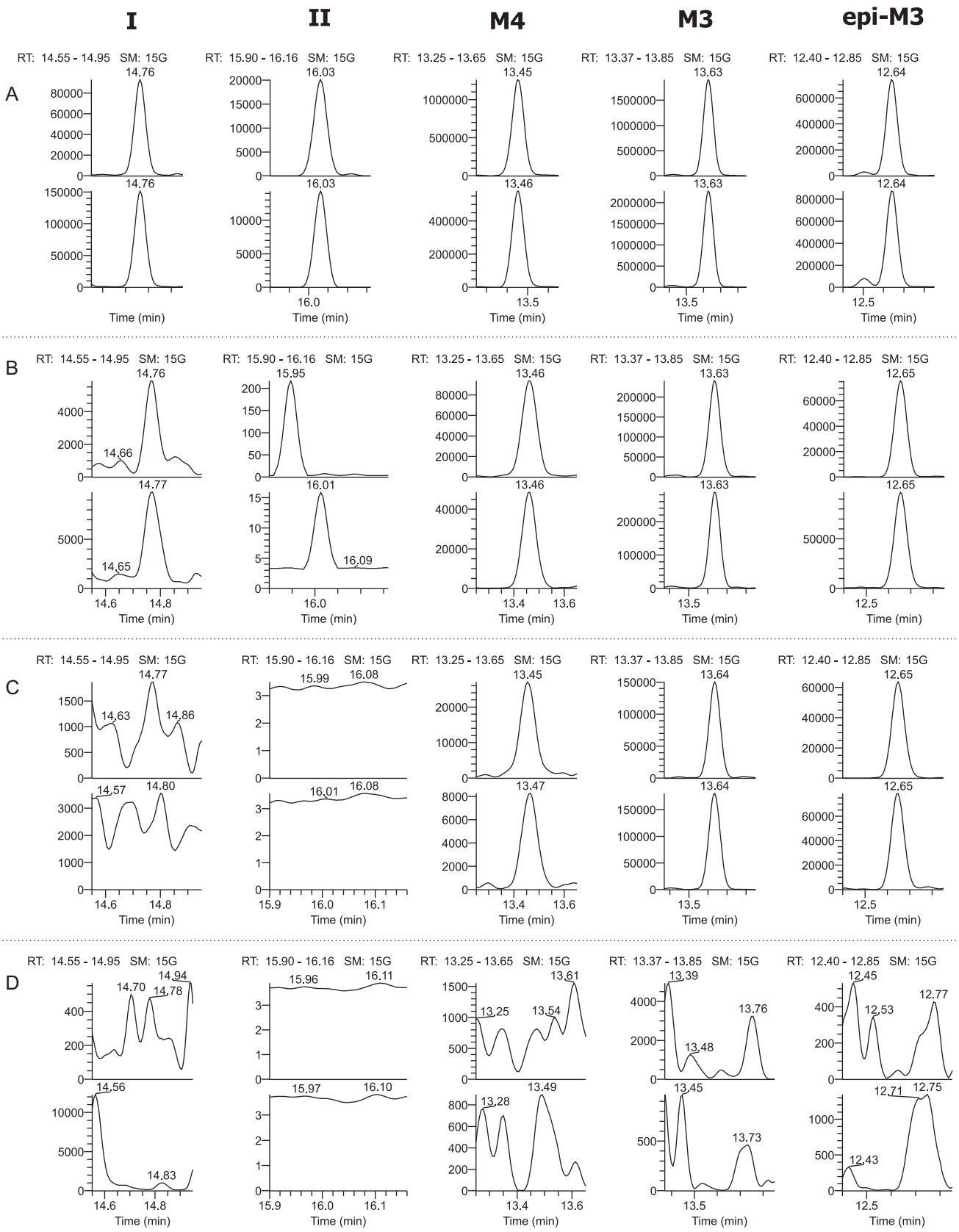


**Fig. 5.** SRM chromatograms for the DHCMT positive urines (A–C) and negative urine (D).

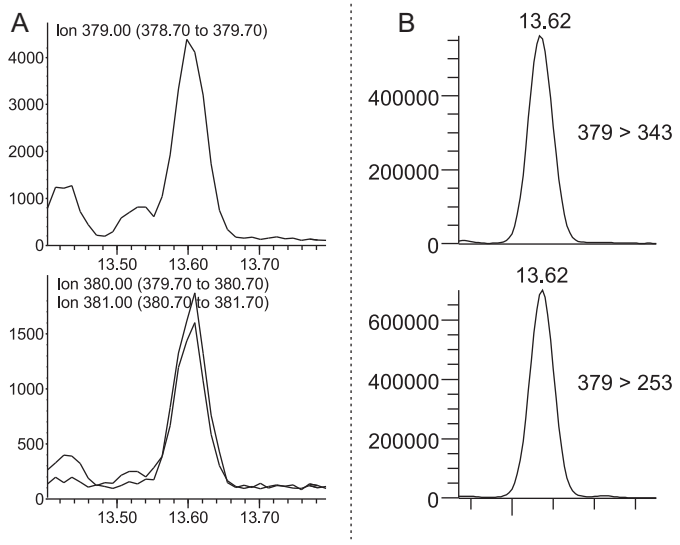


**Fig. 6.** The mass chromatograms from a single-quad (A) and a triple-quad (B) mass spectrometer for the metabolite **M3** obtained for the same sample under equivalent chromatographic conditions.

presence of metabolites **I** and **II** in routine doping control samples. Fig. 5 shows some real life examples when the detection of DHCMT administration is easy (*A*), challenging (*B*) and impossible (*C*), if only metabolites **I** and **II** are monitored. As is seen from this data, the abundance of **M3** is much higher than that of **I** and **II**. Interestingly, only in one sample metabolite **II** was found to be more abundant (by factor of 3–5) than **M3**. It means that antidoping laboratories should keep both **I** and **II** included in their screening procedures for anabolic steroids. In the confirmatory analysis, it is recommended to extract the urine samples with pentane as the metabolites **M2**–**M4** have acceptable recoveries while the biological background is considerably lower.

Another important illustration is given in Fig. 6, where the GC–MS and GC–MS/MS instruments are compared by their ability to detect **M3**. While a single-quad GC–MS can be used for this purpose, and moreover, if **M3** was not included into the screening method the sample would be reported as negative, the intensity obtained even on a well-tuned instrument is quite low compared to a triple-quad mass spectrometer. It is worth mentioning that in this particular sample the abundance of **I** is about 50 times lower than that of **M3**.

## 4. Conclusion

New long-term dehydrochloromethyltestosterone metabolites were found in human urine after HPLC fractionation of a pooled urinary concentrate. Using GC–MS and GC–MS/MS the most important metabolite for the antidoping analysis was tentatively characterized as 4-chloro-18-nor-17β-hydroxymethyl,17α-methyl-5β-androst-13-en-3α-ol. This metabolite together with its 17α-epimer was shown to provide much better detectability of dehydrochloromethyltestosterone abuse for extended period of time compared to the other known metabolites. The synthesis of reference steroids is needed to confirm the proposed structure of the identified metabolites.

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