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Long-term detection of dehydrochloromethyltestosterone: one-year experience

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Abstract

After more than one year experience in the screening for recently reported dehydrochloromethyltestosterone (DHCMT) metabolites in our laboratory, it was demonstrated that the most long-term is a steroid tentatively characterized as 4-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol (M3) and its 17-epimer, which is less abundant but normally always detectable alongside with M3. In the majority of cases, M3 was shown to be superior to the other known DHCMT metabolites, such as 4-chloro-18-nor-17β-hydroxymethyl-17α-methylandrosta-1,4,13-trien-3-one (“night watch” analog) and 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one (metabolite “656”). M3 is best detected by gas chromatography – tandem mass spectrometry (GC-MS/MS), whereas GC-MS could still be used but with much less confidence due to both sensitivity and selectivity issues.
In 2011 Moscow Antidoping Centre reported twenty five adverse analytical findings (AAF) for DHCMT, of which only 4 would be declared positive based on the presence of 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one, and only one – if 6β-hydroxy-DHCMT would be used as the target. In 2012 twenty eight AAFs for DHCMT have already been reported by Moscow Antidoping Centre as of September 1, 2012, with most of them being solely relied on the detection of M3. Therefore, all antidoping laboratories that are capable of running GC-MS/MS technology are advised to include M3 in their screening methods.

Introduction

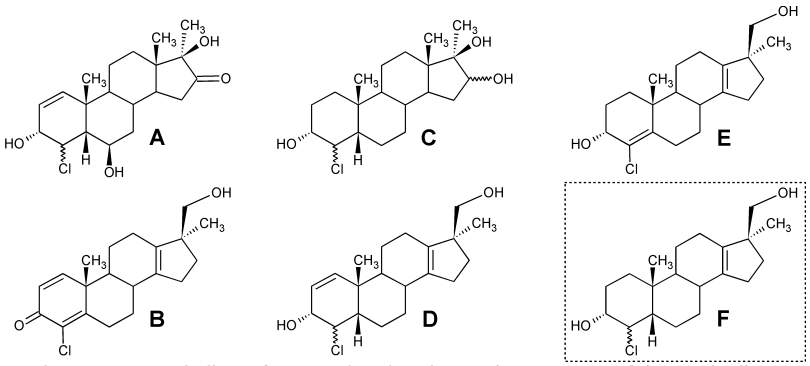

Fig. 1. Structure of known long-term metabolites of DHCMT (A, B) and tentative structure of the newly discovered ones (C-F). Steroid F corresponds to M3.

Since the identification of several new long-term metabolites of dehydrochloromethyltestosterone (oral turinabol) in 2011 [1], we started their detection in routine urine samples to have a more “large-scale” evaluation and select a metabolite that would be most suitable for screening by GC-MS/MS. The previously reported and fully characterized metabolites, such as 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one [2] (**Fig. 1A**) and 4-chloro-18-nor-17β-hydroxymethyl-17α-methyl- androsta-1,4,13-trien-3-one [3] (**Fig. 1B**), were also monitored for comparative purposes (novel metabolites:

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Fig. 1C-F). The aim of the present study is therefore to share the results that demonstrate, by the example of DHCMT, the importance of having a proper target included in screening method.

Experimental

Reagents: 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). *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Macherey-Nagel (Düren, Germany). All other chemicals (potassium carbonate, potassium hydrogen carbonate, potassium phosphate monobasic, sodium phosphate dibasic, sodium sulfate, ammonium iodide, dithiotreitol) were obtained from Sigma-Aldrich (St. Louis, MO, USA).
Sample preparation: urine samples were processed according to a protocol used in Moscow laboratory for the non-volatile conjugated compounds by GC-MS [1]. Briefly, to 3 mL of urine were added 1 mL of phosphate buffer (0.8 M, pH 6.3) containing 30 µL of β-glucuronidase and 1.5 µg of methyltestosterone. After the incubation and addition of carbonate buffer (3 M, pH 10.1), samples were extracted with 5 mL of diethyl ether or pentane (in case of confirmation analysis) in the presence of sodium sulfate. Following the evaporation of organic layer at 70°C, the dry residue was treated with 50 µL of MSTFA/ammonium iodide/dithiotreitol (1000/2/1.5 v/w/w) at 70°C for 30 min.
Instrumentation: GC-MS/MS system comprised a Trace GC Ultra gas chromatograph (Thermo Scientific, Rodano, Italy) coupled to a TSQ Quantum XLS triple quadrupole mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA). An 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: 178°C to 234°C at 4°C/min, then to 310°C at 20°C/min (held 4.2 min). Under these chromatographic conditions the internal standard methyltestosterone elutes at 13.77 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 and ion source temperature was 300°C and 250°C, respectively. The collision gas pressure was 1 mTorr (argon 99.9995%).
Excretion study: once received an approval from the local ethical committee at the Institute of Sport, a controlled excretion study was performed after administration of 1 capsule of P-Mag (Competitive Edge Labs, USA) by a male volunteer (age 34). Urine was collected for 30 days. The composition of the supplement was checked using GC-MS and found consistent with the label ("4-chloro-17α-methyl-androst-4-en-3β,17β-diol"). This is a steroid structurally related to DHCMT, which seems to be available over-the-counter under different trade names in many countries.

Results and Discussion

As illustrated in **Fig. 2**, in the last two years the number of AAFs reported by Moscow Antidoping Centre for DHCMT increased considerably. It has become possible not only due to the use of GC-MS/MS technology which itself is a great step ahead, but also because of the research that resulted in the identification of novel long-term metabolites of this steroid [1].
It was eventually found that a compound tentatively characterized as 4-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol (M3, **Fig. 1F**) is the optimal screening and confirmation target. It is also suggested that screening method also include 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one (**Fig. 1A**), as in some rare cases this metabolite could be more abundant than M3.
It is worth mentioning that under chromatographic conditions described in the *Experimental* section, M3 elutes approximately 0.1 min before the internal standard (methyltestosterone). Except for the cases when M3 is present at the trace level, it is always accompanied with the respective 17α-epimer which elutes 1 min earlier, close to 11β-hydroxy-androsterone. The epimer normally has *ca.* 20% of the abundance of M3.
The EI mass spectrum of the pertrimethylsilylated derivative of M3, as well as the product ion mass spectrum obtained upon fragmenting the ion at *m/z* 379, are given in **Fig. 3A** and **3B**, respectively. **Fig. 3B** also lists the SRM transitions recommended for screening and confirmatory analysis of this metabolite by GC-MS/MS.
These transitions are of exceptional selectivity as they originate from the sequential loss of hydrogen chloride and trimethylsilanol by a steroidal fragment containing either ^{35}Cl or ^{37}Cl, which is not expected to occur in endogenous species. Nevertheless, it is recommended to extract suspicious urine samples with *n*-pentane when performing a confirmatory analysis. Without having a synthetic reference material, the confirmatory analysis can be done against a reference collection sample obtained after a controlled administration of DHCMT. This, in fact, is in compliance with clause 5.4.4.2.1 of the

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International Standard for Laboratories [4]. However, a routine EQAS sample distributed by WADA in 2008 has only trace amount of M3 and cannot be used as a positive control for confirmation.

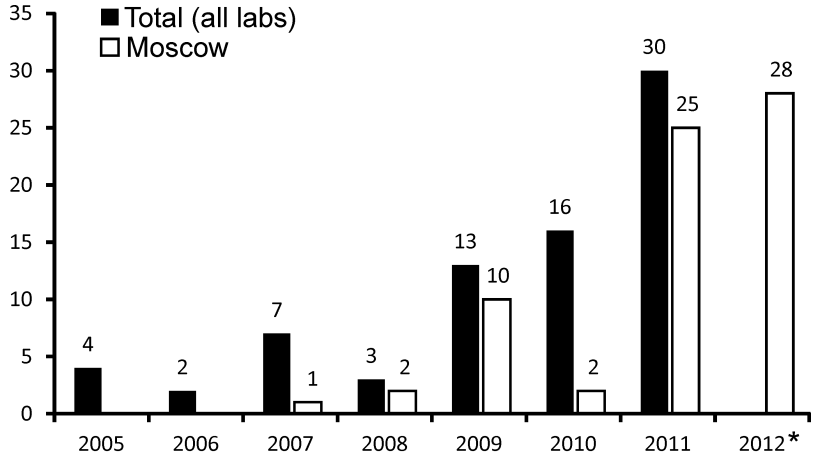

Fig. 2. Adverse analytical findings (AAF) for DHCMT in 2005-2012 reported worldwide and by Moscow Lab (the number of AAF in 2012 is given as of September 1, 2012).

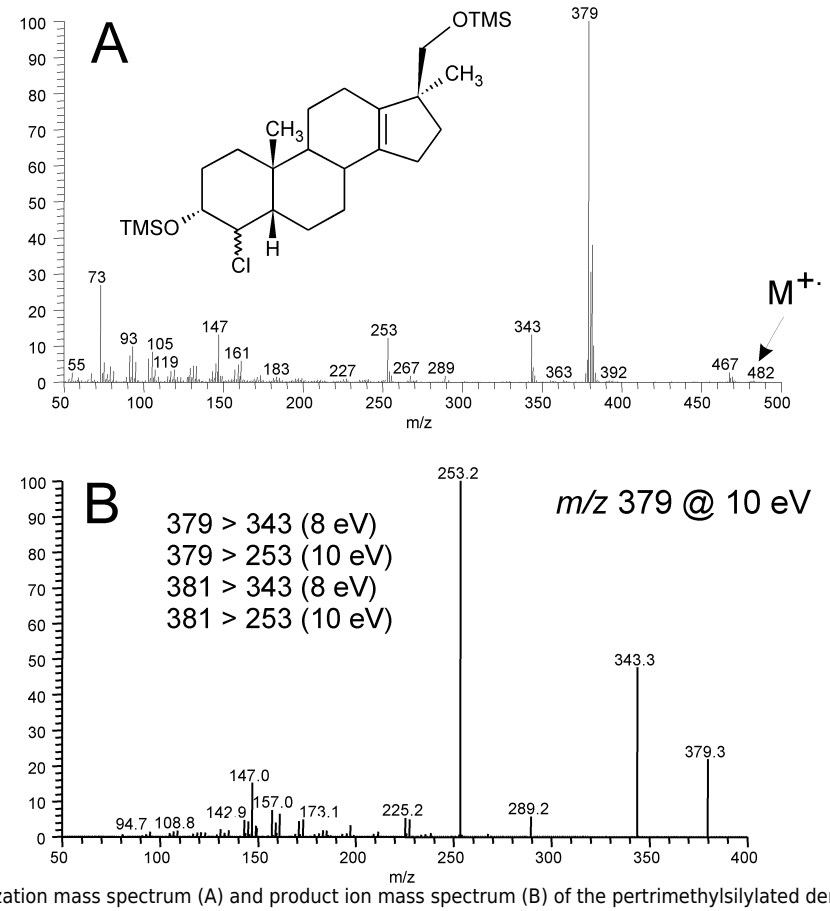

Fig. 3. Electron ionization mass spectrum (A) and product ion mass spectrum (B) of the pertrimethylsilylated derivative of M3. SRM transitions for M3 (B).

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We have recently found that after the administration of a nutritional supplement declared to contain 25 mg of 4-chloro-17α-methyl-androst-4-en-3β,17β-diol, both the metabolite "656" and M3 were well detectable, that is, it produced the same metabolites as DHCMT itself. Moreover, the abundance of M3 had a maximum at 48 h with no sign of decrease up to five days, suggesting a slow kinetics of its formation. The M3 was still detectable 30 days after administration. At present, this nutritional supplement (along with some others) is available over-the-counter in Russia. The EI mass spectrum of the trimethylsilyl derivative of a steroid isolated from this supplement was consistent with the declared structure (data not shown), although the stereochemistry at C3/C17 and a double bond position were not verified.

Conclusions

Once identified in DHCMT post administration urines, a steroid with tentative structure 4-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol greatly extended the detection time window of DHCMT. This resulted in numerous adverse analytical findings reported by our laboratory in 2011 and 2012. While this drug might only be popular in Russia and Eastern Europe, other antidoping laboratories are encouraged to include this metabolite in their screening procedures, as some of nutritional supplements may contain ingredients that would metabolize to 4-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol as the most long-term metabolite as well. The synthesis of this metabolite is still required to fully confirm its structure.

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