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Detection of DHCMT long-term metabolite glucuronides with LC-MSMS as an alternative approach to conventional GC-MSMS analysis

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ABSTRACT

Dehydrochloromethyltestosterone (DHCMT) is one of the most detected illicit used anabolic–androgenic steroids in professional sports. Therefore, a fast and accurate analysis of this substance is of great importance for a constructive fight against doping abuse. The conventional method for the analysis of this drug, GC-MSMS, is very sensitive and selective but also very time- and resource-consuming. With the presented work, a new approach for simple detection with LC-HRMSMS without any sample preparation is introduced. The method is based on the direct analysis of two newly described phase-II metabolites of the DHCMT long-term metabolite 4-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol (M3). LC-HRMSMS, GC-MSMS, fractionation and derivatization experiments are combined to identify and characterize for the first time two different glucuronide-acid conjugates of this metabolite in positive human urine samples. In addition, a third glucuronide metabolite was identified, however without isomeric structure determination. The detection of these metabolites is particularly interesting for confirmation analyses, as the method is rapid and requires little sample material.

1. Introduction

An integral part of professional sports today is regular testing of athletes for doping abuse. Anti-doping measures, as we know them today, began in the early 1960 s and have been subject to a constant process of research and improvement ever since [1]. In the field of anti-doping analysis, the long-term detection of prohibited substances, which are defined by the World Anti-Doping Agency (WADA) [2], is one of the most critical issues. The primary sample type in this field is urine, as the sample collection is non-invasive and quick to collect. In this matrix, it is often not the doping substance itself that is detected but its metabolites. Metabolites that are excreted from the human body over a more extended period are called long-term metabolites (LTMs), even though no unequivocal definition for this term exists. The discovery and characterization of new LTMs have always been of high interest in anti-doping research, as previous studies show [3]. The class of anabolic–androgenic steroids (AAS) is particularly focused as it represents the most widely detected family of illicitly used drugs in professional sport [3–5]. The gold standard procedure for routine AAS analysis in doping control is based on enzymatic cleavage to separate parent substances and phase-I metabolites from their phase-II conjugates, followed by liquid–liquid extraction, trimethylsilyl-derivatization and analysis by gas chromatography coupled to tandem mass spectrometry (GC-MSMS) [6]. Only a few steroids can also be measured with liquid chromatography-tandem mass spectrometry (LC-MSMS) directly after extraction due to their higher polarity or capability of ionization at electrospray conditions [7]. However, with the deployment of more powerful LC-MSMS instruments, new approaches for steroid analysis have been developed. Several studies have shown that the direct detection of steroid phase-II conjugates with LC-MSMS is a suitable approach for the detection of steroids [8,9,18–21,10–17]. All of these studies are based on the analysis of the highly polar phase-II metabolites, glucuronide- and sulfate-conjugates [22–25]. The significant advantage of these techniques is that time- and resource-consuming steps of enzymatic hydrolysis and derivatization can be omitted. In many cases, the liquid/liquid extraction is replaced by solid-phase extraction (SPE) or, in some approaches, even the complete sample preparation is skipped. These so-called dilute-and-shoot methods are extremely resource-efficient, but have also disadvantages in terms of sensitivity, specificity and robustness. Another advanced approach combines SPE with direct measurement, so-called online-SPE, where the separation step is fully automated before the LC separation process. This type of method

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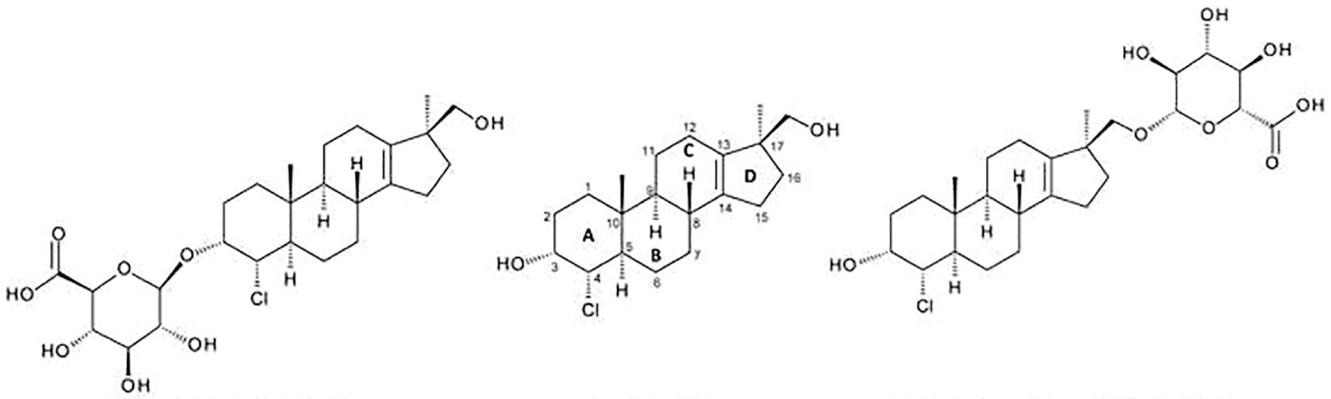

Fig. 1. Chemical structure, formula and exact atomic mass of the long-term metabolite DHCMT-M3 (m) and its two possible glucuronide conjugates DHCMT-M3-3-glucuronide (l) and DHCMT-M3-17-methyl-glucuronide (r).

was also used in the presented work.

Dehydrochloromethyltestosterone – DHCMT (4-chloro-17β-hydroxy-17α-methylandrosta-1,4-dien-3-on), also known as 4-chlorometandienone or oral-turinabol, is one of the most commonly used illicit anabolic steroids in professional sports according to WADA statistics [4]. Its chemical structure is based on the endogenous steroid testosterone and results from dehydrogenation of positions 1 and 2 and halogenation on position 4. This substance was already used as a doping substance in the early 1970 s in East Germany and is still available on the black market today. The detection of DHCMT abuse has an equally long history. The first investigations on DHCMT analysis were published in 1970 by Schubert et al [26,27]. The analysis of the parent compound and three different hydroxyl-metabolites (6β-OH, 16β-OH, 6β,16-di-OH) in human urine samples after application of DHCMT was reported. Subsequently, in 1983, the presence of these metabolites was confirmed, another di-hydroxylated metabolite (6β,12-di-OH) and the epimer of the parent substance (17-*epi*-DHCMT) were described [28]. Several years later, in 1996, the team around W. Schänzer identified a new metabolite, 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one, which is detectable up to 14 days after ingestion of DHCMT [29]. As another important step, more and increasingly complex metabolites with detection windows of up to 22 days were discovered in 2010 [30]. The two newly discovered metabolites 4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one and 4-chloro-18-nor-17β-hydroxymethyl,17α-methylandrosta-1,4,13-trien-3-one, similar to the 18-nor-17-hydroxymethyl metabolite of the well-described steroid metandienone, analyzed with GC–MS/MS, exhibited the largest detection windows. In 2012, based on this knowledge and further investigations, Sobolevsky et al. suggested three structures for new 18-nor-17-hydroxymethyl LTMs, containing a partly or fully reduced A-ring in the steroidal backbone. For the most abundant metabolite, 4-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol (M3) and its 17α-epimer, they estimated a detection window of 40–50 days after ingestion of a single dose of 40 mg of DHCMT [31]. However, the correct conformational isomerism of this metabolite was still unknown. In the presented work, we adopted the acronym “M3” for this long-term metabolite. In 2018, Forsdahl et al. analyzed eight different, synthesized isomeric variants of metabolite M3 and compared them with DHCMT positive urine samples [32]. One of these metabolites matched, so the study concluded that the correct structure of the DHCMT long-term metabolite M3 is 4α-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5α-androst-13-en-3α-ol (Fig. 1). With the knowledge of the correct structure and access to high-quality synthesized reference standards, the analysis of DHCMT metabolite M3 has become the most crucial tool for the detection of DHCMT abuse. The fact that M3 is detectable for quite a long time and its analysis by GC–MSMS offers high sensitivity and great selectivity makes this approach currently the most widely used technique to expose DHCMT abuse. However, as mentioned above, the analysis by GC–MSMS with enzymatic hydrolysis, liquid–liquid extraction and derivatization is very time and resource consuming.

The goal of our study was to shift the detection of the important DHCMT metabolite M3 from GC-MSMS to LC-MSMS analysis. In 2014, Fernandez-Alvarez M. et al. had already undertaken studies in this direction, but no long-term metabolites were investigated [33]. In the presented work, we aimed to identify usable phase-II metabolites of the DHCMT metabolite M3 with a focus on glucuronide conjugates. Theoretically, there are two possible sites for glucuronide conjugation, the 17-hydroxymethyl- and the 3-hydroxy-group, as shown in Fig. 1. If this assumption is true and conjugation takes place at these two sites, two positional isomers DHCMT-M3-3-glucuronide and DHCMT-M3-17-methyl-glucuronide have to be expected. We combined different analytical techniques such as LC-MSMS, GC-MSMS, fractionation and derivatization experiments to verify the presence of these two DHCTM-M3 glucuronides in positive human urine samples and to tentatively identify the correct sites of the glucuronic acid conjugates.

2. Experimental

2.1. Chemicals, reagents and solutions

Methanol (MeOH) and water used for HPLC analysis (HPLC grade) were purchased from Biosolve Chimie (Dieuze, France). Formic acid (FA) for HPLC, potassium dihydrogen phosphate, disodium hydrogen phosphate dehydrate, potassium hydrogen carbonate and potassium carbonate were bought from Merck (Darmstadt, Germany). Water (MQ) for sample dilution was obtained by a Milli-Q water purification system (Millipore, Reference A +). Trityl chloride, methyl-t-butyl-ether (TBME), ammonium iodide (NH4I), ethanthiol (97%) and dimethylformamide (DMF) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Triethyl amine (TEA) was purchased from Acros Organics (Fair Lawn, New Jersey, USA). MeOH for standard solutions was supplied by Chem-Lab (Zedelgem, Belgien). β-Glucuronidase (*E. coli*) for enzymatic hydrolysis was supplied by Roche (Mannheim, Germany). N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Macherey-Nagel (Düren, Germany).

For silylation, a derivatization stock solution was prepared by dissolving 200 mg of NH_4I in a mixture of 10 ml MSTFA and 600 µl

ethanthiol. A derivatization working solution was prepared by mixing 3 ml of the stock solution with 9 ml of MSTFA directly before sample preparation.

The internal standard (IS) 16,16,17α-d3-testosterone-glucuronide was purchased from the National Measurement Institute Australia (Sydney). IS solution was prepared by dissolving 1 μg standard substance in 1 ml MeOH (1 μg/ml). Solution was stored at −20 °C.

2.2. Urine samples

All positive urine samples used in this work were collected by accredited sample collection authorities in compliance with WADA's collection guidelines [34]. The samples were received, analyzed and subsequently provided by the WADA accredited anti-doping laboratory Seibersdorf Labor GmbH (Seibersdorf, Austria). Before the analysis, athletes gave permission to use urine samples for research purposes. This is in accordance with the International Standard for Laboratories (ISL) [35]. Additionally, a quality assurance program sample provided by the World Association of Anti-doping Scientists (WAADS) was used. This sample, which contains pooled DHCMT excretion study samples, had already been used in a previous study to confirm the structure of the unconjugated DHCMT metabolite M3 [32]. Blank urine samples were collected from healthy female and male volunteers. All urine samples were stored frozen at −20 °C until analysis.

2.3. Online – SPE-LC-HRMSMS

A previously established online solid-phase extraction (SPE) method that showed excellent results for the analysis of steroid glucuronides was used in the present work [18]. This automated approach required only straightforward sample preparation: 250 μl of urine was diluted with 250 μl MQ and 15 μl IS solution was added. Afterwards, samples were vortexed for 10 s.

The measurements were performed on a Vanquish Horizon UHPLC$^+$ System coupled to a Q-Exactive Orbitrap high-resolution mass spectrometer (Thermo Fisher, Austin, Texas, USA). Analytes extraction was carried out fully automatically using an Accucore Phenyl-Hexyl, 10 × 3 mm column with 2.6 μm particle- and 80 Å pore size (Fischer Scientific, Loughborough, UK) as extraction column. As an analytical column, a Kinetex EVO C-18, 100 × 2.1 mm column with 2.6 μm particle- and 100 Å pore size was used (Phenomenex, Aschaffenburg, Germany). Chromatography was carried out with mobile phase containing water with 0.2% v/v FA (solvent A) and methanol with 0.1% v/v FA (solvent B). The separation was performed with a constant flow of 0.4 ml/min and constant temperature at 25 °C. After loading and washing the pre-column with 10% solvent B for 2 min, the solvent gradient continues as follows: start with 10% solvent B up to 100% over 7 min, hold 100% B for 2 min and again 10% B for 2 min to flush and re-equilibrate the system. The sample injection volume was 25 μl.

High-resolution mass spectrometry was carried out in positive and negative electrospray ionization mode (ESI+/-) using the following settings. The spray voltage was 3.8 kV and the capillary temperature was set to 320 °C. Nitrogen was used as sheath gas (pressure 25 units) as well as auxiliary gas (pressure 8 units) and the auxiliary gas heater temperature was set to 310 °C. The mass resolution was set to 70 000 at m/z 200 and automatic gain control (AGC) to 2×10^5 ions. Internal calibration with the lock-mass m/z 391.28429 (di-isooctyl phthalate) was used. Full scanning in the range of m/z 300 – 600 and parallel reaction monitoring (PRM) were performed. Collision energies (CE) were optimized to get the most abundant signal intensities. Extracted ion chromatograms (XIC) with an extraction range of 5 ppm and isolation windows of ± 1 m/z were generated by choosing the most specific product ions. All systems were controlled with the software Xcalibur 4.0 (Thermo Fischer). Data procession and calculation of monoisotopic masses was performed with the software Xcalibur Qual Browser 4.1.45 (Thermo Fischer).

2.4. Sample concentration

In order to increase yields, urine samples were alternatively concentrated using the following protocol. An Oasis HLB, 6 ml, 500 mg cartridge and a vacuum ejector-driven glass chamber were utilized for sample extraction (Waters Corporation, Milford, MA, USA). The cartridge was conditioned with 5 ml MeOH and washed with 5 ml MQ. After loading 5 ml of urine, the sample was washed with 2 × 5 ml MQ, dried for 5 min and eluted with 2 ml MeOH. Subsequently, samples were evaporated to dryness using nitrogen stream and reconstituted in 500 μl MQ.

2.5. Fractionation

After identifying potential metabolites by LC-HRMSMS, the next step was to confirm whether the signals found actually correspond to the DHCMT metabolite M3. The idea was to fractionate each peak, collect the separated molecules and confirm their structure doing a standard GC-MSMS analysis. However, since the concentration levels of these metabolites are quite low, in the lower ng/ml range, and the peaks to be separated are quite narrow to each other, conventional HPLC fractionation via UV/VIS detection was not possible. In order to solve this problem we used the above described online – SPE-LC-HRMS system and installed a fractionation arrangement positioned between the analytical column and the ESI-source. This system consisted of a simple *T*-piece and three HPLC capillaries: one leading from the analytical column to the *T*-piece, one from the *T*-piece to the ion source and one capillary leading from the *T*-piece to an open-end used to collect the fractions. Consequently the stream was divided into two flows, one went to the mass spectrometer and the other was used for fractionation. In order to get the same retention times for both measurement and sample collection, both capillary pathways had the same length. With this approach, real-time monitoring of the fractionation procedure was possible. Ten runs of 50 μl injected sample each were performed and three fractions were collected at three different retention time windows, 7.75 – 8.10 min, 8.10 – 8.45 min and 8.45 – 8.80 min. Afterwards, collected and pooled samples were evaporated, dried and subsequently reconstituted in 500 μl MQ. In order to check the successful separation, samples were analyzed with the above described LC-HRMSMS method. Concentration and fractionation were performed twice and the fractions were pooled to obtain the necessary sample volume for the following GC-MSMS analysis and derivatization experiment.

2.6. GC-MSMs

GC-MSMS analysis was carried out according to the standard protocol as accredited by WADA [36]. In brief, 500 μl of each fractionated sample, 1 ml of blank urine sample, 1 ml of the original WAADS sample and 1 ml of blank urine spiked with 1 ng/ml DHCMT M3 reference standard were diluted with 1 ml 0.8 M phosphate buffer (pH 7), 25 μl β-glucuronidase and 50 μl IS solution were added and then samples were heated 2 h at 50 °C to perform enzymatic hydrolysis. Subsequently, 1 ml of 20% potassium carbonate buffer (pH 9.0) and 5 ml of MTBE were added to perform a liquid–liquid (l/l) extraction by shaking samples for 10 min. After centrifugation for 5 min at 2100 rpm, the organic layer was separated by freezing samples in a cooled ethanol bath at −30 °C. Subsequently, samples were evaporated to dryness and dried for 15 min in a heated vacuum chamber. As the last step of sample preparation, 80 μl derivatization working solution was added and samples were heated for 20 min at 60 °C to perform silylation.

GC-MSMS analysis was carried out on a Trace-1300 gas chromatograph coupled to a TSQ-8000 Evo triple quadrupole mass spectrometer and a TriPlus-100 autosampler (Thermo Fisher, Austin, TX, USA) using an optimized method designed for metabolite M3 confirmation purposes. For separation, a RTX-1MS fused silica capillary column, 15 m × 0.25 mm ID, 0.11 μl film thickness (Restek, CP-Analytica, Mistelbach,

Austria) was used. Injections with 2 µl volume were performed in splitless mode at 270 °C injector temperature. The following temperature program for the GC was carried out: 150 °C initial temperature, 25 °C/min to 310 °C, final temperature held for 2 min. High-purity helium with a constant pressure of 90 kPa was used as carrier gas. Transfer line and ion source were heated to 270 °C. Electron ionization (EI) mode with electron energy of 70 eV was carried out and data were acquired with selected reaction monitoring (SRM) mode. Following ion transitions were selected for the DHCMT metabolite M3: *m/z* 379 → 253 (8 eV), *m/z* 381 → 253 (8 eV), and *m/z* 381 → 343 (8 eV) and IS d3-testosterone: 435 → 209 (12 eV).

2.7. Derivatization

The aim of this derivatization experiment was to determine the respective positions of the glucuronic acids on three potential glucuronide metabolites. Two variants of glucuronic acid conjugation, shown in Fig. 1, differ in the steric hindrance of the underlying hydroxyl group. The hydroxyl group on position 3 is a secondary alcohol while the 17-hydroxymethyl group is a primary alcohol. The property of trityl chloride to selectively protect primary alcohols in the presence of secondary alcohols was used to distinguish between these two groups [37]. Only the metabolite with the glucuronide conjugation on position 3 is expected to be etherified with trityl chloride (Fig. 2), as the primary

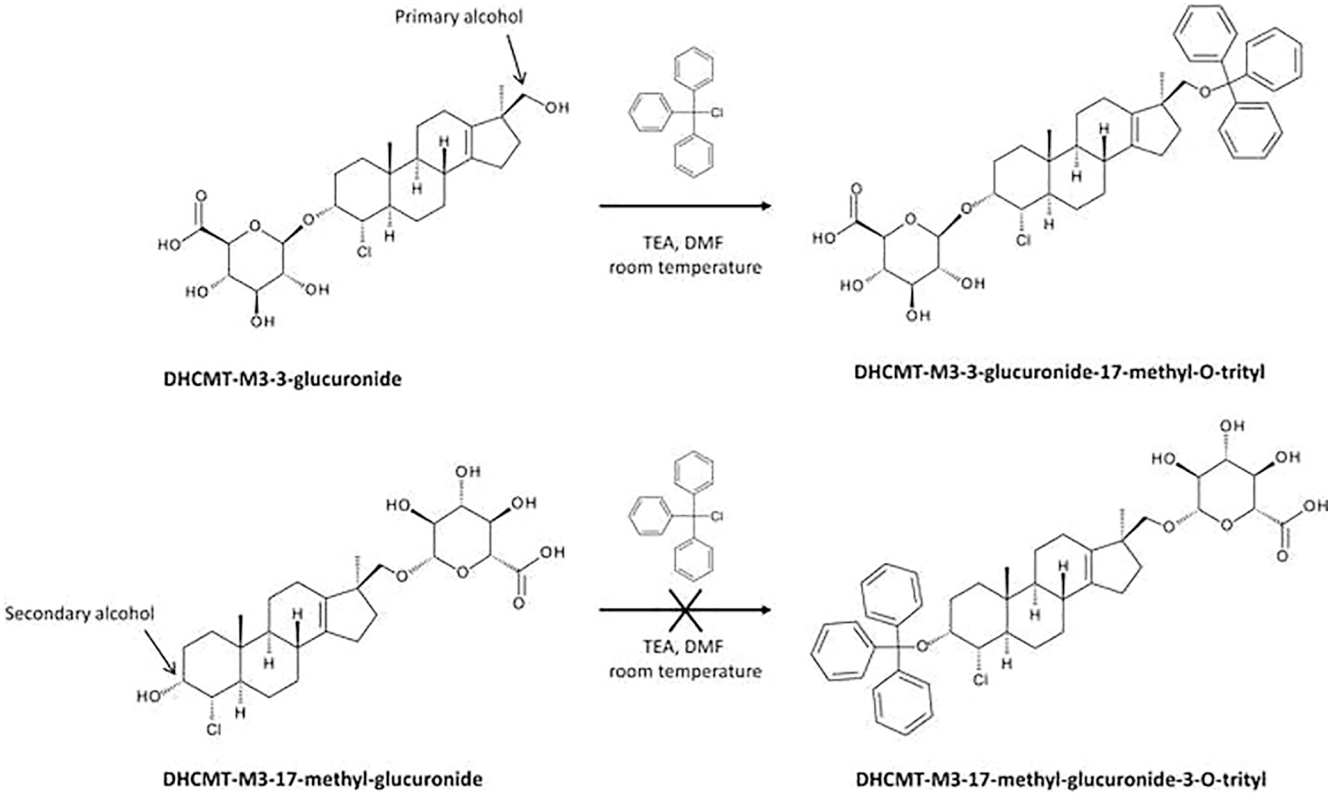

Fig. 2. Reaction scheme of the derivatization experiment of DHCMT-M3-3-glucuronide and DHCMT-M3-17-methyl-glucuronide with trityl chloride.

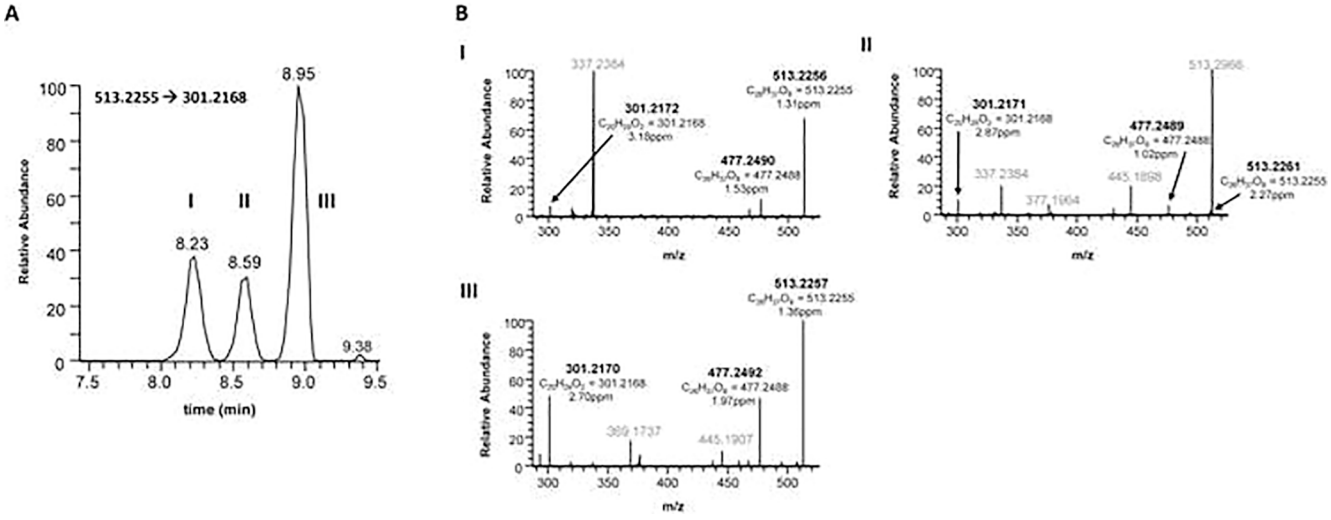

Fig. 3. Results of PRM measurements; **A:** XIC of excretion urine sample; *m/z* 513.2255 -> 301.2168 (35 eV), ESI-, 5 ppm mass tolerance; **B:** corresponding PRM spectra of 3 potential peaks **I-III**; Two most specific fragment and parent substance signals are highlighted.

alcohol on positions 17-methyl remains free, and the metabolite with the 17-methyl glucuronide conjugation is expected to remain unchanged. The reaction should therefore exclusively lead to the formation of DHCMT-M3-3-glucuronide-17-methyl-O-trityl, as illustrated in Fig. 2.

The derivatization with trityl chloride was performed as follows: chemicals were used without further purification as received from the suppliers. All reactions were performed under argon atmosphere. The tritylation agent was prepared by dissolving trityl chloride (1400 mg, 5 mmol) in 5 ml dry DMF to give a 1 N solution. Triethylamine (0.5 ml, 1.3 eq) was added and the solution stirred for 5 min. Portions of 500 µl of the three sample fractionations and of the concentrated WAADS sample were evaporated and subsequently set under argon atmosphere, 5 ml tritylation agent was added and samples were stirred at room temperature. Samples of 500 µl were taken in regular intervals (up to 6 days reaction time) and quenched by the addition of 250 µl of sat. aq. $NaHCO_3$. After stirring for 1 h, water and DMF were removed under high vacuum to give a brown-yellowish solid residue. These residues were then dissolved in 500 µl MQ by vortexing for 5 min and subsequently centrifuged for 8 min at 8000 rpm. The supernatant (≈250 µl) was transferred into an LC-vial and 15 µl IS solution was added. The samples were analyzed with the above described LC-HRMS method.

3. Results and discussion

3.1. Identification of M3 glucuronides

The first step of identifying new phase-II metabolites was to analyze several positive urine samples with LC-HRMSMS using different analytical settings. It turned out that for the analysis of DHCMT M3 glucuronides, the negative ionization mode (ESI-) is most sensitive mode to find potential signals. After identifying some promising peaks with negative full-scans analysis, negative PRM experiments were performed. Precursor ion was set to m/z = 513.2255, which correspond to theoretical species [DHCMT-M3-mono-glucuronide – H] ¯. After optimizing collision energies and chromatographic conditions, XICs (m/z = 513.2255 → 301.2168, 35 eV) as shown in Fig. 3A were achieved for most positive samples. In all samples, 1 to 3 prominent peaks (I-III) were visible, with varying intensity, probably depending on the metabolic status. In blank urine samples, no signals at all have been observed by using this mass transition. The WAADS-excretion sample shown in this figure showed the best signals for all three peaks and was therefore used for further investigations.

As shown in 3B, all three peaks yielded different mass spectrometric patterns. Several fragments were formed during the collision-induced dissociation with 35 eV. However, the two most specific product ions, m/z = 477.2488, which is created by the loss of hydrogen chloride (HCl) and m/z = 301.2168, which is formed by the cleavage of the glucuronic acid and the loss of HCl, were generated in all three peaks. The parent molecule with a mass of m/z = 513.2255 was also detectable in all three signals. The deviations between theoretical mass and experimental mass were below 3.2 ppm for both fragments and the parent molecule in all three peaks. These data gave us the first indication of the existence of DHCMT M3 glucuronides and prompted us to proceed with follow-up experiments.

3.2. Fractionation

In Fig. 4, the results of the separation and collection procedure of the three individual peaks are illustrated. At the top the XIC with transition m/z = 513.2255 → 301.2168 (35 eV) of the concentrated excretion urine sample before HPLC separation is shown and below the XICs of the pooled fractionated and reconstituted signals. As can be seen in the figure, all signals were adequately separated. As a positive side effect of this approach, the analytes were additionally purified. By comparing signal intensities a recovery of about 50 % was roughly estimated.

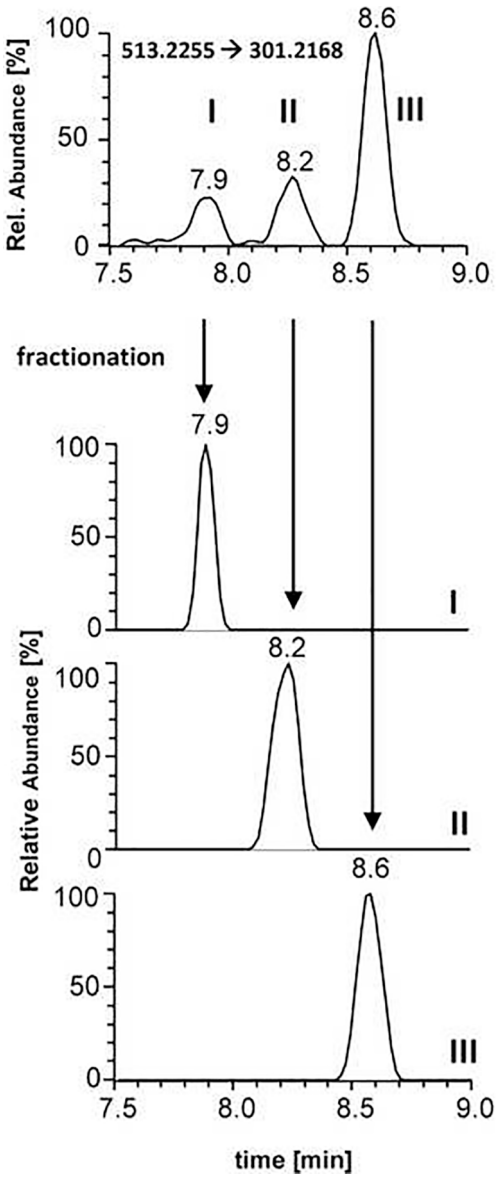

Fig. 4. Results of fractionation; XIC m/z 513.2255 -> 301.2168 (35 eV), ESI-, 5 ppm mass tolerance; **On top:** Concentrated urine sample before fractionation; **Below:** Individual measurements of signals **I-III** after fractionation.

3.3. Confirmation of M3 glucuronides

In Fig. 5, the results of the GC-MSMS analysis of the three fractionation samples, the original WAADS excretion samples and DHCMT M3 reference standard is shown. On the left side the LC-HRMS spectra of the fractionated peaks is illustrated and on the right the corresponding GC-MSMS results for the most abundant transition m/z = 379 → 253 is shown. Below, the original WAADS excretion sample and a blank urine sample spiked with the standard substance of DHCMT M3 (1 ng/ml) are presented as reference samples.

All fractions showed signals after enzymatic cleavage of the glucuronic acid. In order to fulfill WADA identification criteria, a comparison of retention times and at least two MS/MS transitions of the targeted

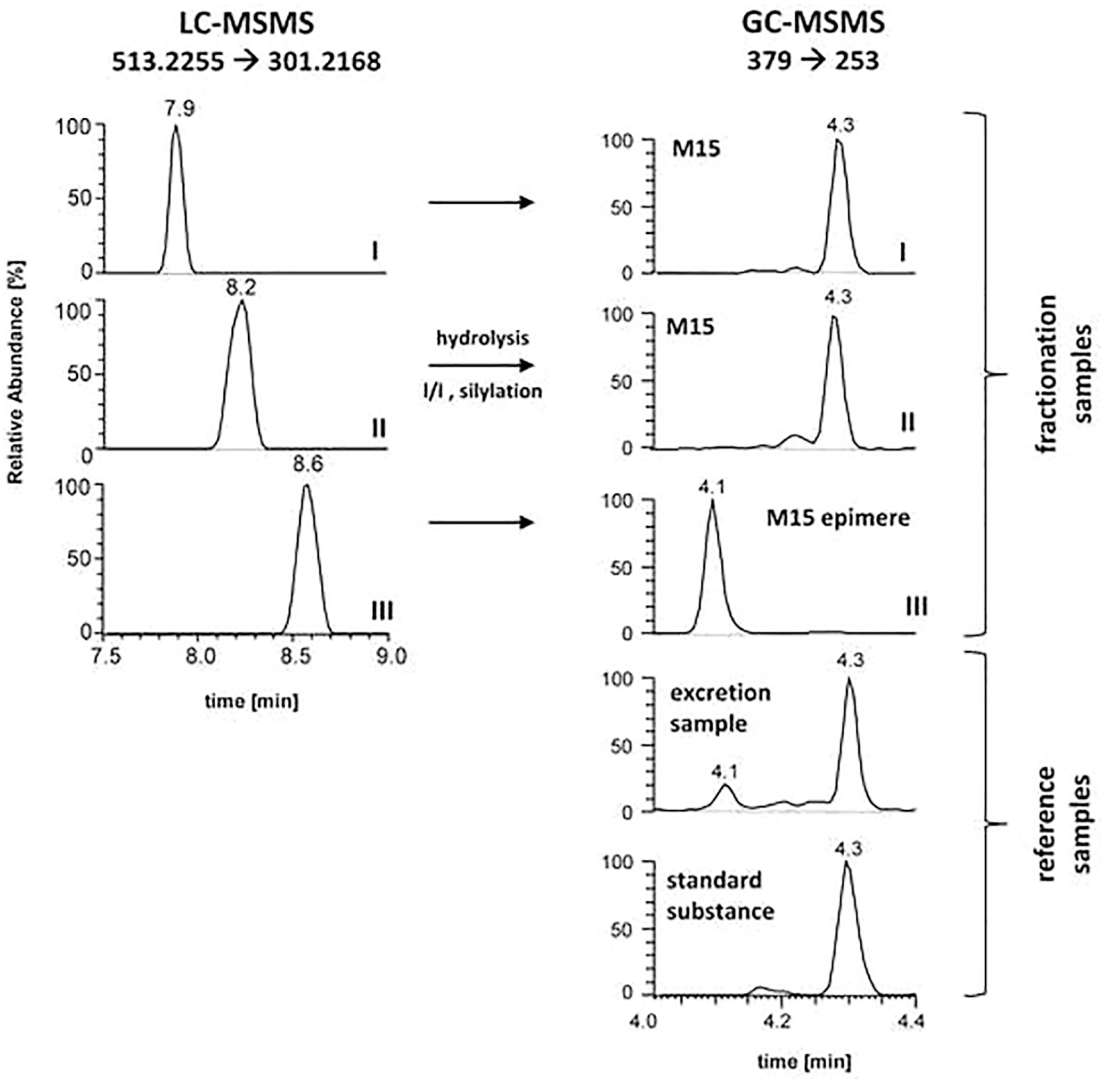

Fig. 5. Confirmation of fractionated peaks with GC-MSMS; **Left:** LC-MSMS XIC of fractions **I - III**, *m/z* 513.2255 -> 301.2168 (35 eV), ESI-, 5 ppm mass tolerance; **Right:** GC-MSMS SRM chromatogram; On top: Fraction **I – III**, below: positive urine sample and blank urine sample spiked synthesized reference standard (1 ng/ml); *m/z* 379 → 253 (8 eV), EI.

Table 1
Comparison of relative abundances of three mass transitions for fraction I and II with a reference standard for DHCMT M3 and relative abundances of fragment III; * Maximum tolerance windows were calculated according to WADA Technical Document – TD2021IDCR[36].

Substance	Transitions	Relative abundance		Difference	Maximum tolerance window*
	[m/z]	sample	reference		
Fraction I	379 –> 253	100%	100%	0.00%	90 - 110%
	381 –> 253	34.30%	33.50%	0.90%	27.5 - 41.2%
	381 –> 343	10.90%	8.50%	2.50%	5.9 -15.9%
Fraction II	379 –> 253	100%	100%	0.00%	90 - 110%
	381 –> 253	30.00%	33.50%	4.00%	24.0 - 36.0%
	381 –> 343	6.00%	8.50%	2.50%	1.0 -10%
Fraction III	379 –> 253	100%	-	-	-
	381 –> 253	34.10%	-	-	-
	381 –> 343	10.30%	-	-	-

analyte in a positive sample and a reference sample is requested [38]. In this work, the relative abundance of three diagnostic ions determined from peak areas was used. Both fraction I and II showed perfect matching retention times (4.3) with the unconjugated reference standard of DHCMT M3, as illustrated in Fig. 5. Transition abundance ratios for three product ions compared with the reference standard are shown in Table 1.

All differences between sample and reference abundance of all fragments for both fractions were quite low and within the maximum tolerance ranges. These data confirm unambiguously that both peak I and peak II correspond to glucuronic acid conjugates of the DHCMT long-term metabolite M3. These findings are supported by the fact that M3 has two different hydroxyl sites where conjugation can occur (Fig. 1). In order to complete the characterization of peaks I and II, the position of the glucuronic acid on each of these molecules was determined by the derivatization experiment as discussed below.

Fraction III matches with a different signal with the retention time 4.1 min measured in the WAADS excretion samples. This finding and the very similar fragmentation pattern of **III** compared to metabolite M15 (Table 1) suggest that peak III is an isomeric variation of metabolite M15. Peak **III** thus appears to be a glucuronic acid conjugate of an M3-epimer of unknown structure. It is conceivable that it is the 17α-epimer of M3 that Sobolevsky already mentioned in his work from 2012 [31]. However, there are no reference materials currently available for this metabolite to confirm this assumption.

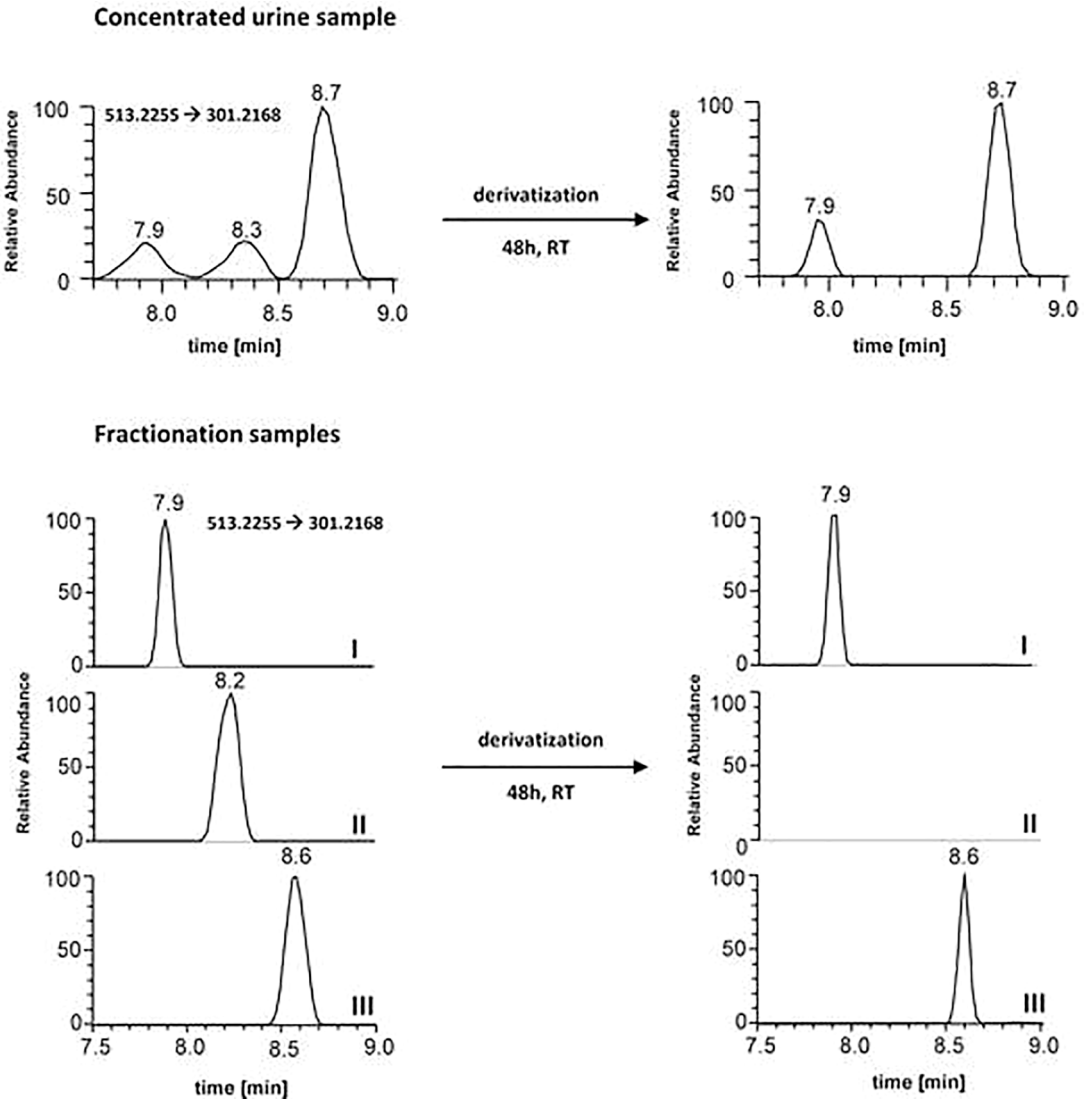

Fig. 6. Results of derivatization experiment with trityl chloride; LC-MSMS XIC, *m/z* 513.2255 -> 301.2168 (35 eV), ESI-, 5 ppm mass tolerance; **Left:** Chromatograms of the concentrated urine sample and fractionation samples (**I** – **III**) before derivatization; **Right:** Chromatograms of the concentrated urine sample and fractionation samples after derivatization; RT = room temperature.

3.4. Determination of glucuronic acid conjugation sites

To visualize the successful derivatization, the three sample fractions and the concentrated WAADS sample were measured before and after the derivatization reaction with trityl chloride with the LC-HRMSMS method described above. In Fig. 6 the results are summarized. Best results were obtained after a reaction time of 48 h. Again XICs with the transition $m/z = 513.2250 \rightarrow 301.2162$ (35 eV) are presented before and after the derivatization reaction. On top of Fig. 6 the concentrated excretion study sample and below the three fractionated samples are illustrated .

In both cases, peak II disappeared entirely after the derivatization. Peak **I** and **III**, on the other hand, remained utterly unharmed. If our assumption is correct, this is a clear sign that selective derivatization of signal II has occurred and conversely, I and III remained unchanged in this reaction. Considering these findings and the theoretical structures of these metabolites allows the conclusion to be drawn that peak I represents DHCMT-M3-17-hydroxymethyl-glucuronide and peak **II** represents DHCMT-M3-3-glucuronide (Fig. 1). Peak III also appears to have a glucuronic acid conjugation at the 17-hydroxymethyl position, but the correct isomerism of the phase-I metabolite is not known.

4. Conclusion

In the presented work we introduced a new approach for the detection of DHCMT abuse. Instead of the comparably time and resource consuming GC-MSMS approach, a simple previously developed LC-HRMSMS method is applied for the direct analysis of DHCMT phase-II glucuronides. Using a combination of LC-MSMS analysis, fractionation and GC-MSMS analysis, we found strong evidence for the presence of two distinct glucuronide conjugates of the important DHCMT long-term metabolite M3 (4-chloro-18-nor-17β-hydroxymethyl-17α-methyl-5β-androst-13-en-3α-ol) in positive human urine samples. The presented approach for the analysis of these metabolites is particularly interesting for routine confirmation analysis due to the small sample volume required and the very short analysis time. Even though the established GC-MSMS method still seems to be more sensitive, the approach presented in this work provides satisfying detection limits. Simple dilution experiments showed that detection of the M3-glucuronides up to approximately 100 pg/ml was possible. Nevertheless, a suitable reference sample for comparison is needed for unambiguous detection as long as no synthesized standards are available.

With the derivatization experiment we demonstrated a

comparatively simple method for distinguishing between a 3- and a 17-hydroxymethyl-conjugation site of glucuronic acid on a steroid molecule. However, the synthesis of high-quality reference standards of the different DHCMT M3 glucuronides is highly recommended to confirm the presented results.

This work is another step on the path of shifting AAS analysis more and more from GC-MSMS to LC-MSMS, by direct analysis of steroidal phase-II metabolites, leading to more resource and time-saving fight against doping abuse.

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