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SFC-MS/MS for orthogonal separation of hydroxylated 17α-methyltestosterone isomers

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Abstract

Because of their performance-enhancing effect, anabolic androgenic steroids (AAS) are often misused in sports. Nearly half of the adverse analytical findings (AAF) in 2022 doping controls are correlated to AAS misuse. Metabolites play a crucial role in the bioanalysis of endogenous and exogenous steroids. Therefore, one important field in antidoping research is the investigation on drug metabolizing and steroidogenic enzymes. The introduction of a hydroxy group is the most common reaction, which is catalyzed by cytochrome P450 (CYP) enzymes in phase-I metabolism. Analysis of AAS metabolites is commonly performed using gas chromatography mass spectrometry (GC-MS) systems. Laborious sample preparation and extended run times compared to liquid chromatography (tandem) mass spectrometry (LC-MS/MS) methods are usually correlated with this type of analysis. On the other hand, liquid chromatography (tandem) mass spectrometry (LC-MS[/MS]) methods have a lower separation efficiency than GC-MS methods. Both techniques lack selectivity for hydroxylated 17α-methyltestosterone metabolites. Therefore, as an orthogonal analytical approach, a supercritical fluid chromatography tandem mass spectrometry method was developed to separate four hydroxy metabolites of 17α-methyltestosterone (2α-/2β-/4-/6β-hydroxy-17α-methyltestosterone). This project aimed to get a more in-depth look at the metabolization and analysis of 17α-methyltestosterone and its hydroxylated metabolites. The developed method revealed lower limits of quantitation between 0.6 and 6 ng/ml at an accuracy of 85–115% using a matrix matched calibration. An in vitro study with human liver microsomes shows 6β-hydroxy-17α-methyltestosterone as main metabolite (15.9%) as well as the metabolite 2β-hydroxy-17α-methyltestosterone (0.5%). The results show that the developed method is sensitive and robust. In addition, the method allows a previously missing discrimination of the hydroxylated metabolites in a short analysis time without prior, complex derivatizations.

KEYWORDS
metabolism, methyltestosterone, SFC, tandem mass spectrometry

1 | INTRODUCTION

Anabolic androgenic steroids (AASs) are a group of substances widely misused in sports and therefore prohibited by the World Anti-Doping Agency (WADA).[1] Such substances can be divided into two groups, endogenous substances like testosterone and exogenous substances like 17α-methyltestosterone (MT). As their character is nonpolar, AASs are extensively metabolized in humans.[2–8] The main phase-I-metabolite of MT is tetrahydro-17α-methyltestosterone (THMT) generated by reduction of the 3-oxo-4-ene structure.[9,10] Additionally, hydroxylation reactions are described in steroid metabolism.[3–5,7,10–12] They do not only allow for improved excretion of steroids but also lead to aromatization process of AAS.[2,13–15] GC-MS or LC-MS methods are typically used to detect AAS and their hydroxylated metabolites. However, both techniques have problems in selectivity, sample preparation, and run time.[10,16] GC-MS methods usually require prior derivatization of steroids, which in the case of MT leads to the formation of 6β-hydroxy-17a-methyltestosterone as artifact, which makes quantitation almost impossible.[10] In addition, such GC-MS methods have relatively long run times. LC-MS methods often have the problem that the isomers are not separated well enough and thus the selectivity of the method is missing.[15]

To fully understand the hydroxylation pathways in MT metabolism, it is necessary to have a suitable method for the detection of hydroxylated metabolites. Therefore, the goal was to develop and validate a SFC-MS/MS method, orthogonal to established GC- and LC-MS(/MS) methods, that is able to separate and quantify 4-hydroxy-17α-methyltestosterone (4OHMT), 6β-hydroxy-17α-methyltestosterone (6βOHMT), and the diastereomers 2α-hydroxy-17α-methyltestosterone (2αOHMT) and 2β-hydroxy-17α-methyltestosterone (2βOHMT) together with their parent compound MT (structures displayed in Figure 1).

2 | MATERIALS AND METHODS

2.1 | Chemicals

17α-Methyltestosterone and metandienone were obtained from Sigma Aldrich (Taufkirchen, Germany), 4-hydroxy-17α-methyltestosterone was purchased from TRC (North York, United States), and 6β-hydroxy-17α-methyltestosterone was obtained from Steraloids (Newport, United States). The enantiomers 2α- and 2β-hydroxy-17α-methyltestosterone were synthesized and characterized in house[15] (supporting information).

Human liver microsomes (HLMs) were obtained from BD Bioscience (Milan, Italy). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) generating system (solution A and B) was purchased from Corning GmbH (Amsterdam, Netherlands). Acetonitrile and methanol in analytical and LC-MS grade were purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a LaboStar 2-DI/UV from SG Wasseraufbereitung und Regenerierstation GmbH (Barsbüttel, Germany) equipped with LC-Pak Polisher and a 0.22-μm membrane point-of-use cartridge (Millipak®, Th Geyer, Berlin, Germany). Carbon dioxide was purchased from Air Liquide (Düsseldorf, Germany). Ammonium fluoride and formic acid in MS quality were obtained from Sigma Aldrich (Taufkirchen, Germany). Sodium bicarbonate, potassium carbonate, and sodium dihydrogen phosphate were purchased from Sigma Aldrich (Taufkirchen, Germany). Dipotassium phosphate was obtained from Carl Roth (Karlsruhe, Germany). t-Butyl methyl ether (TBME) was purchased from AppliChem (Darmstadt, Germany).

2.2 | Solutions

2.2.1 | Internal standard solution

Metandienone solution (50 μl, 1 mg/ml) was quantitatively transferred to a 5 ml volumetric flask and diluted with acetonitrile to result in the final concentration of 10,000 ng/ml. An aliquot of 500 μl of this solution was diluted with acetonitrile in a 5 ml volumetric flask to result in the final concentration of 1,000 ng/ml.

2.2.2 | Stock solutions

Stock solutions of the analytes were prepared by dissolving of 2.0 mg of analyte in 2.0 ml methanol to result in a stock concentration of 1,000 μg/ml. Aliquots (50 μl) of each compound were transferred into a 5.0 ml volumetric flask and diluted with methanol to yield a stock
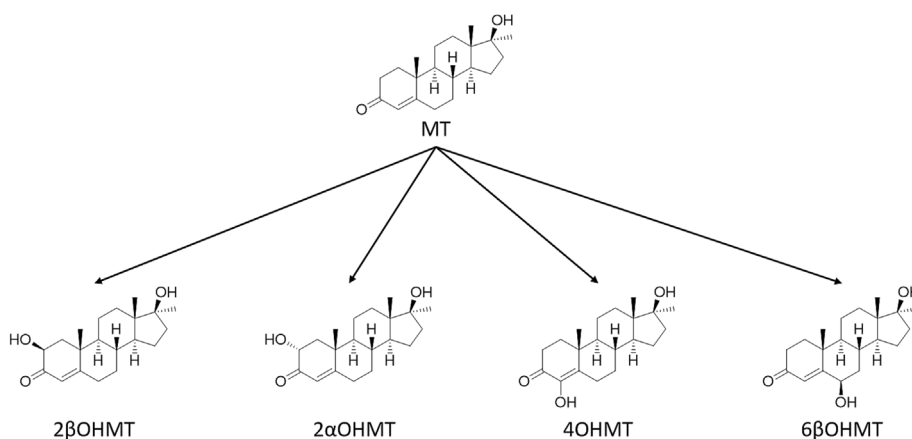

FIGURE 1 Structures of the parent compound 17α-methyltestosterone (MT) and its hydroxylated metabolites 2β-hydroxy-17α-methyltestosterone (2βOHMT), 2α-hydroxy-17α-methyltestosterone (2αOHMT), 4-hydroxy-17α-methyltestosterone (4OHMT), and 6β-hydroxy-17α-methyltestosterone (6βOHMT).

solution of 10 µg/ml. Working solutions were prepared by diluting the stock solution with methanol (6, 9, 10, 20, 60, 100, 450, 500, 1,000, 2,500, and 4,000 ng/ml).

2.3 | In vitro samples

2.3.1 | Samples for matrix effect evaluation

The protocol for in vitro studies was adapted from Kuuranne et al.[17] Phosphate buffer system (226 µl, 0.1 M, pH 7.4) was mixed with 12.5 µl solution A, 2.5 µl solution B (NADPH regenerating system), and 2.5 µl methanol to a total volume of 243.5 µl. The mixture was incubated for 4 h at 37°C while shaking at 850 rpm (Eppendorf Thermomixer comfort). Ice cold acetonitrile (225 µl), internal standard (25 µl; 10 µg/ml), and solid carbonate/bicarbonate ($NaHCO_3/K_2CO_3$, 61:100, w:w) buffer (pH 9–10) were added to the mixture. After centrifugation at 4,500 *rcf* for 5 min, the supernatant was transferred and extracted twice with 2 ml TBME each. The organic phases were combined, and the organic solvent was evaporated at 40°C under nitrogen flow. The resulting residue was dissolved in 225 µl aliquots of reference substance working solutions (either 10 ng/ml or 500 ng/ml) and 25 µl pure methanol (resulting concentrations: 9 ng/ml and 450 ng/ml). Neat methanolic standard solutions with the same concentrations (9 ng/ml and 450 ng/ml) were also prepared for comparison (Section 2.2.2).

2.3.2 | Sample for matrix matched calibration

Samples for matrix matched calibration were prepared following the same protocol as described in Section 2.3.1. However, samples were spiked with 25 µl of reference substance working solutions (6, 20, 60, 100, 500, 1,000, and 4,000 ng/ml) prior to centrifugation and extraction. The residue after solvent evaporation was dissolved in 250 µl methanol.

2.3.3 | Human liver microsome incubation

The protocol for in vitro studies was adapted from Kuuranne et al.[17] Phosphate buffer system (226 µl, 0.1 M, pH 7.4) was mixed with 12.5 µl solution A, 2.5 µl solution B (NADPH regenerating system), and 2.5 µl of methanolic solution of MT (1,000 ng/ml) to a total volume of 243.5 µl. The mixture was pre-incubated for 5 min at 37°C while shaking with 850 rpm (Eppendorf Thermomixer comfort). After this, 2.5 µl of HLM was added to the mixture and incubated for 4 h while shaking with 850 rpm (Eppendorf mixer). To stop the reaction, ice cold acetonitrile (225 µl), internal standard solution (25 µl, 10 µg/ml metandienone), and solid carbonate/bicarbonate ($NaHCO_3/K_2CO_3$, 61:100, w:w) buffer (pH 9–10) were added to the mixture. After centrifugation at 4,500 *rcf* for 5 min, the supernatant was transferred and extracted twice with 2 ml TBME each. The organic phases were combined, and solvent was evaporated at 40°C under nitrogen flow. The residue was dissolved in 250 µl methanol.

Two samples and one negative control sample (without HLM) were prepared two times on different days.

2.4 | Method development

The method development was based on the already established method.[15] Since the mass spectrometry parameters (source parameters, MRM transitions of analytes) were already optimized in the earlier investigation, they were adopted (depicted in Table 2).

2.4.1 | Selection of stationary phase

The already used vancomycin-based stationary phase (Agilent Poroshell Chiral-V) was tested against a cyclodextrin based (Agilent Poroshell Chiral-CD) and a cyclofructan based (Agilent Poroshell Chiral-CF) stationary phase (all 100 × 4.6 mm, 2.7 µm). Column dimensions were kept equal to minimize this influence on the separation and system pressure. The gradient of the method was slightly changed prior to the selection of the stationary phase to decrease the amount of organic modifier and increase the critical resolution on the Chiral-V column. Compressed carbon dioxide with pure methanol as modifier were used as eluents. Segmented linear gradients with different slopes were applied starting at 7% methanol increasing to 12% methanol in 10 min, to 25% after 12 min, and a subsequent plateau at 25% methanol for 2.5 min. After 15 min, mobile phase composition returned to starting conditions. Flow rate was set to 1.2 ml/min. Make-up solvent consisted of methanol, water (2.5), formic acid (0.1%), and ammonium fluoride (1mM). Flow of the make-up solvent was set to 0.150 ml/min.

2.4.2 | Gradient optimization

From the point of green chemistry, an attempt was made to keep the amount of organic solvent as low as possible while maintaining a reasonable run time. Starting conditions and slope of the gradient were changed for optimization as depicted in Table 1. Retention times and resolution were used to compare the results.

2.4.3 | SFC backpressure

Two different SFC backpressures of 120 and 150 bar were tested.

TABLE 1 Starting conditions and gradient for method optimization.

Starting conditions (modifier)	Gradient (modifier)
6%	11% at 10 min
	25% at 12 min
	25% at 13 min
	6% at 14 min

2.5 | Validation

Validation of the method was conducted based on regulations of the European Medicines Agency (EMA) on the validation of on bioanalytical methods,[18] the validation of analytical procedures,[19] and the United States Pharmacopeia.[20]

2.5.1 | Matrix effect

To investigate the matrix effect, three samples with two concentrations (9 ng/ml, 450 ng/ml) were prepared and compared with the results of neat standard solutions (in pure methanol). The equation of Matuszewski et al.[21] was used for the evaluation of the matrix effect.

$$Matrix\ \text{Effect}\ [\%] = \frac{peak\ \text{area matrix sample}}{mean\ \text{peak area neat solvent sample}} \times 100$$

2.5.2 | Calibration, linearity, limit of detection, and limit of quantitation

Calibration was performed using matrix matched calibration series. For each calibration level, all five analytes (4OHMT, MT, 2βOHMT, 2αOHMT, and 6βOHMT) were used at seven different concentration levels (0.6, 2, 6, 10, 50, 100, and 400 ng/ml), for the evaluation of the linear range as well as the lower limit of quantitation (LLOQ) and limit of detection (LOD). The linear range of the regression was estimated by point-to-point slope (tolerance 20%). LLOQ and LOD were determined by signal-to-noise ratio (LLOQ ≥ 10, LOD ≥ 3).[19]

2.5.3 | Accuracy

Three different concentrations (10, 100, and 250 ng/ml) were used to determine the accuracy of the method. Three technical replicates were produced for each concentration level to result in nine accuracy samples (protocol described in Section 2.3.2, reference substance working solutions 100, 1,000, and 2,500 ng/ml). The determined amount of analyte was compared with the theoretical value.[19]

2.5.4 | Precision

To calculate the precision, one homogenous sample with the concentration of 100 ng/ml (reference substance working solutions 1,000 ng/ml) was prepared according to the protocol described in Section 2.3.2 and measured six times. Experiments were repeated on two consecutive days with independently prepared samples to calculate intra-day precision. The relative standard deviation of the measurements was then calculated (%RSD).[19]

2.5.5 | Peak resolution and symmetry

Peak resolution and symmetry were calculated for sample cal3 (50 ng/ml). The critical resolution pairs were 2βOHMT/2αOHMT and 2αOHMT/6βOHMT because their MRM transitions display cross-talk. Peak resolution was calculated with the following equation:

$$R = 1.18 \times \left(\frac{t_{R2} - t_{R1}}{W_{0.5h1} + W_{0.5h2}} \right).$$

t_{R2} and t_{R1} are the retention times of the analytes, and $W_{0.5h1}$ and $W_{0.5h2}$ are the peak width at half of the height of each peak.[20]

Peak symmetry was calculated with the following equation:

$$A_s = \frac{W_{0.05}}{2d}.$$

$W_{0.05}$ is the peak width at 1/20 of the peak height, and d is the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 1/20 of the peak height.[20]

2.5.6 | Carryover

To evaluate the carryover effect of the method, a blank sample was run after measurement of a high concentrated sample of all analytes (1,000 ng/ml).

2.5.7 | Robustness

Robustness of the method was tested by changing key parameters of the method: column temperature (20°C, 23°C, and 26°C), flow rate (1.1, 1.2, and 1.3 ml/min), gradient (% modifier: 5-10-25-25-5, 6-11-25-25-5, and 7-12-25-25-5), and backpressure (130, 150, and 170 bar) were individually changed while keeping the remainders constant.

2.5.8 | Sample stability

To test for sample stability, three samples were stored at different temperatures for 120 h. One sample was stored at −20°C, one sample was stored in the autosampler (5°C), and one sample was stored at room temperature (benchtop stability).

2.6 | Quality control samples (QCs)

Six QC samples were prepared by spiking a blank matrix sample (protocol Section 2.3.2) with 25 μl of the methanolic solutions (1,000 ng/ml) of the compounds resulting in a concentration of 100 ng/ml.

2.7 | Analytical instrumentation

Chromatographic method development was performed on a 1260 Infinity I SFC System with a low dispersion nozzle in the SFC module (Agilent Technologies, Waldbronn, Germany) using an Agilent 6130 Single Quadrupole MS Detector coupled to an Agilent ESI source (Agilent Technologies, Santa Clara, United States).

Method validation was performed on the same SFC System coupled to an Agilent 6495B Triple Quadrupole MS system equipped with an Agilent Jetstream ESI source (Agilent Technologies, Santa Clara, United States). Columns used for method development and validation were Agilent Poroshell Chiral-V, Chiral-CD, and Chiral-CF (100 × 4.6 mm, 2.7 μm; Agilent Technologies, Waldbronn, Germany). Final mass spectrometric and chromatographic conditions are presented in Tables 2 and 3.

3 | RESULTS AND DISCUSSION

3.1 | Method development

The critical resolution pair was used to select the stationary phase. For all three phases, this was the analyte pair 2βOHMT/2αOHMT.

TABLE 2 Chromatographic and mass spectrometric parameters.

Device	Agilent 1260 Infinity I SFC System, equipped with low dispersion nozzle Agilent 6495B Triple Quadrupole with AJS ESI (+)
Column	Agilent Poroshell 120 Chrial-CD (2.7 μm, 4.6 mm × 100 mm)
Temperature	23°C
Injection volume	5 μl (double loop overfill)
Solvent A	CO_2 (compressed)
Modifier	Methanol
Backpressure regulator	150 bar; 60°C
Gradient	6% modifier at 0 min 11% modifier at 10 min 25% modifier at 12 min 25% modifier at 13 min 6% modifier at 14 min
Flow rate	1.200 ml/min
Makeup solvent	MeOH/H_2O/formic acid (97.4:2.5:0.1; v:v:v) + 1 mM ammonium fluoride (NH_4F)
Makeup flow rate	0.150 ml/min
MS parameters	Gas temperature 210°C Gas flow 17 L/min Nebulizer pressure 40 psi Sheath gas temperature 350°C Sheath gas flow 11 L/min Capillary voltage 4,000 V Nozzle voltage 500 V iFunnel high pressure RF 210 V; low pressure RF 60 V

Compared with the vancomycin (Chiral-V)-based phase (R = 1.3), the resolution worsened on the cyclofructan (Chiral-CF) based phase (R = 0.6). The cyclodextrin (Chiral-CD)-based phase provided the best resolution of 3.3 resulting in baseline separation. Therefore, the cyclodextrin based phase was used for further optimization of the method. The change from a vancomycin based to a cyclodextrin-based chiral column together with the change of starting conditions and gradient gave a significant improvement in resolution and overall performance of the method.

Decreasing the starting conditions to 6% methanol and running a gradient to 11% methanol in 10 min further increased the resolution slightly (R = 3.4) while not influencing the complete run time of the method. Furthermore, two different SFC backpressure values were tested. The backpressure on the system is influencing the viscosity and density of the supercritical carbon dioxide. To not harm the column chemistry (max. 400 bar), only 120 and 150 bar backpressures were tested. Lower backpressure yielded an even better resolution of the critical peak pair (R = 3.8) but also increased the runtime. The conditions providing the best compromise between run time, resolution, and reduction of organic solvent consumption are shown in Table 2. Figure 2 shows the comparison of the resulting method using the Chiral-CD column to the already established method with a Chiral-V column by Bredendiek.[15]

3.2 | Method validation

The matrix effect was found between 72% and 86% for most of the compounds (including metandienone as internal standard), except for 4OHMT (23% and 25%) and low concentration for 2αOHMT (9 ng/ml, 51%). Results for all compounds and concentrations are depicted in Supporting Information S1. The high matrix effect for 4OHMT may be explained by a peak splitting in matrix samples, which introduces an additional factor (integration) and may influence the determination of the matrix effect. 4OHMT shows a poor peak shape in neat standards (peak symmetry >3.5), becoming better in matrix samples (peak symmetry 1.5–2.1), but shows a peak splitting without baseline separation (RT 4.48 and 4.85 min). Figure 3 shows the comparison of 4OHMT peak shapes in neat and matrix samples. This phenomenon makes it very difficult to achieve consistent integration and thus quantitation, which leads to a biased result for the matrix effect of 4OHMT.

Because of the high variety of the matrix effect, a matrix-matched calibration is used for further method application. A typical MRM chromatogram is shown in Figure 4.

As described, seven calibration levels using matrix matched sample preparation are used to evaluate the regression type. Linearity is determined by evaluating the point to point slope. The results are plotted as the difference from the median of the values, with tolerance set to 20% (Supporting Information S3). A linear relationship between concentration and response is found for all analytes except of 4OHMT. Because of the high matrix effect and the missing linearity, a quantitation of 4OHMT is not performed in the following. The limit of detection (LOD) is determined via signal-to-noise ratio

Analytes	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)
17α-methyltestosterone	303.2	109.1	30
		97.0	30
2β-hydroxy-17α-methyltestosterone	319.2	283.1	20
		107.1	30
		95.0	30
2α-hydroxy-17α-methyltestosterone	319.2	283.1	20
		107.1	30
		95.0	30
6β-hydroxy-17α-methyltestosterone	319.2	283.2	15
		225.1	25
		173.1	25
4-hydroxy-17α-methyltestosterone	319.2	189.0	20
		113.0	30
Metandienone (ISTD)	301.2	149.0	15
		121.0	35
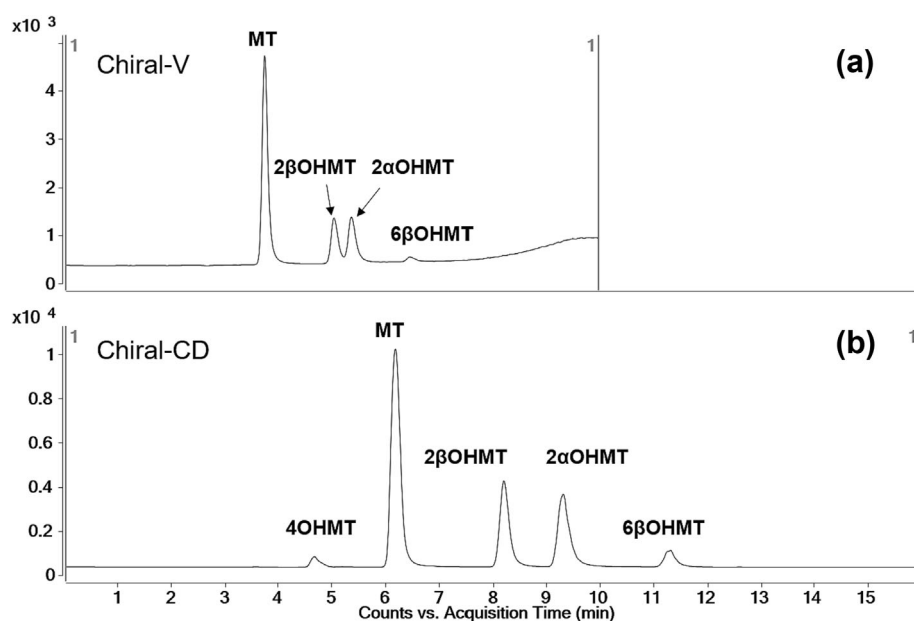
FIGURE 2 Selected ion chromatogram (SFC-SQ-MS, [M + H]$^+$ = 303 and [M + H]$^+$ = 319). (a; sample concentration 500 ng/ml), Applying the already established method using a vancomycin based stationary phase; (b; sample concentration 1,000 ng/ml), applying the final method using a cyclodextrin-based stationary phase; 4OHMT and 6βOHMT are very poorly ionized at the single quadrupole and are therefore not visible or only slightly visible.
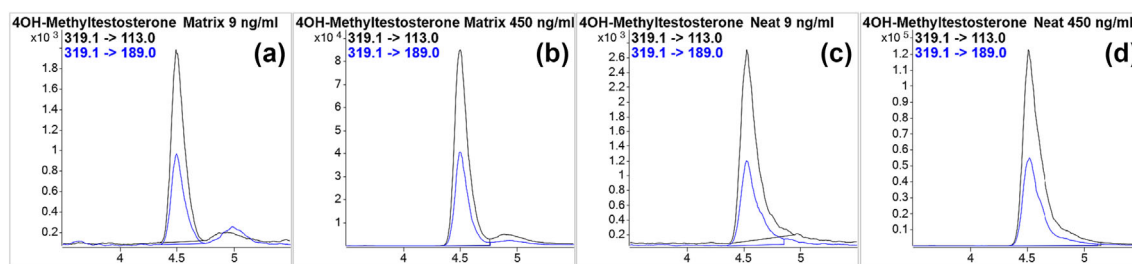

FIGURE 3 MRM-chromatograms of 4OHMT (SFC-QQQ-MS) in matrix samples (a and b) and neat standard solution (c and d) showing the change of the peak shape in matrix samples (concentration levels 9 and 450 ng/ml).

(S/N > 3) and is 0.6 ng/ml for 4OHMT. The linear range for the other four analytes is 0.6–400 ng/ml for 2β- and 6βOHMT, 2–400 ng/ml for MT, and 6–400 ng/ml for 2αOHMT. The calibration curves are weighted $1/x^2$ or $1/x$,[22] yielding a regression coefficient of >0.995 for

all four analytes. The details of regression equations, regression coefficients, weighting of the linear regressions, and the linear ranges are listed in Table 4. To determine the LOD and LLOQ, calibration levels 4–7 are used. All analytes show a signal-to-noise ratio of >10 at
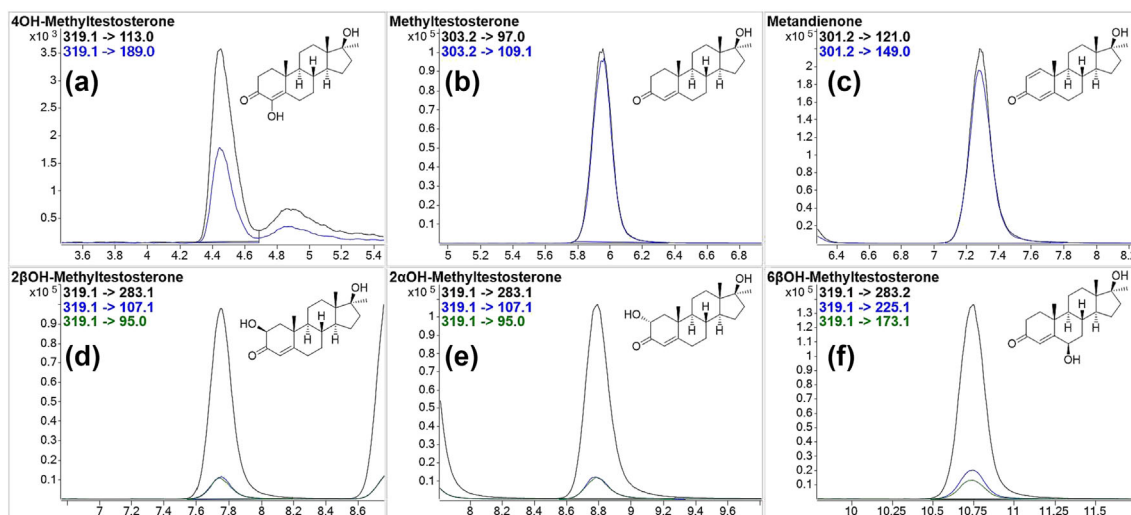
FIGURE 4 MRM-chromatograms (SFC-QQQ-MS) of 4OHMT (a), MT (b), 2βOHMT (d), 2αOHMT (e), and 6βOHMT (f) including the internal standard (c), showing baseline separation and the chemical structure.

TABLE 4 Regression equations, R^2 values, weighting of the calibration curve, and linear range for all tested analytes.

Compound	Regression	R^2	Weighting	Linear range
4OHMT	-	-	-	-
MT	$y = 18{,}060.06x + 1{,}173.56$	0.997	$1/x^2$	2–400 ng/ml
2βOHMT	$y = 18{,}652.05x + 105.29$	0.995	$1/x^2$	0.6–400 ng/ml
2αOHMT	$y = 20{,}375.67x + 2{,}546.29$	0.997	$1/x$	6–400 ng/ml
6βOHMT	$y = 29{,}376.77x + 875.41$	0.995	$1/x^2$	0.6–400 ng/ml

0.6 ng/ml. LLOQ based on S/N ratio is 0.6 ng/ml for all analytes based on signal to noise ratio (LLOQ for 4OHMT is 2 ng/ml). As for MT and 2αOHMT, the LLOQ is not in the linear range, the LLOQ is set to the lower end of the linear range (2 ng/ml for MT and 6 ng/ml for 2αOHMT). LOD is ≤0.6 ng/ml based on signal-to-noise ratio for all analytes. Retention time shifts are ≤0.05 min within the validation procedure. The overall accuracy of all analytes using MRM is between 85% and 115%. Inter- and intra-day precision is ≤2.6% for all analytes.

The critical resolution (2βOHMT/2αOHMT) is R = 4.0, which indicates a baseline separation of all metabolites (R ≥ 1.5).

Calculation of the peak symmetry shows nearly perfect shapes for MT and 6βOHMT (0.99 and 1.05). 2βOHMT and 2αOHMT show a small peak tailing (both 1.28), while 4OHMT has the highest tailing factor (2.09) of all analytes.

Carryover is only found for MT with 0.03%, and no carryover is found for its hydroxylated metabolites. It might be helpful to run a solvent blank prior to very low concentrated samples.

The variation of backpressure and column temperature have only a minor impact on RT, and all compounds are identified based on RT ± 5% (standard for data evaluation method). Flow rate and gradient have a bigger influence on the RT, but all compounds are still identified by increasing the RT window to ± 10%. Quantitation is not influenced by the applied changes in the method. All in all, it was concluded that the method is robust.

Storage stability was evaluated with three samples as described in Section 2.5.8. Concentration of analytes were between 90% and 103% of the nominal value. After storage at −20°C, the concentration varied between 90% and 108%, 88% and 108% in the autosampler, and 87% and 110% at room temperature. The results show that even at room temperature for 120 h, the samples are stable, although storage in an autosampler or at −20°C is preferable.

## 3.3 | In vitro study with human liver microsomes

For proof of concept of the method, in vitro incubations with human liver microsomes were performed. Other steroids have already been characterized in HLM incubations. Ten different CYP450 isoforms are specified for the used HLM. The most abundant cytochrome P450 isoenzyme in HLM is 3A4 (CYP3A4).[4,23] Previous literature presents that androstenedione, testosterone, progesterone, and estradiol showed C-6, C-2 and C-4 as the main positions for the hydroxylation reaction.[2,3,12]

Results of the in vitro transformation of MT with HLM are summarized in Table 5 and illustrated in Figure 5. As expected from the literature, 6βOHMT was found as the main hydroxy metabolite after incubation for 4 h (114–170 ng/ml). This concentration corresponds to a conversion of 11.6–15.9% of the parent compound (results of the negative control samples are subtracted). Conversion to 2βOHMT

TABLE 5 Results of HLM incubation studies for all analytes in ng/ml.

Analytes	HLM_1_1 Conc. (ng/ml)	HLM_1_2 Conc. (ng/ml)	HLM_1_neg Conc. (ng/ml)	HLM_2_1 Conc. (ng/ml)	HLM_2_2 Conc. (ng/ml)	HLM_2_neg Conc. (ng/ml)
4OHMT	-	-	-	-	-	-
MT	2.96	7.66	1003.79[a]	9.65	5.37	926.39[a]
2βOHMT	5.67	7.59	1.95	3.69	2.72	0.03[a]
2αOHMT	1.11[a]	1.21[a]	0.61[a]	0.43[a]	0.44[a]	0.86[a]
6βOHMT	142.6	170.21	0.80	143.59	114.08	0.46[a]

[a]Results that are outside the linear range.
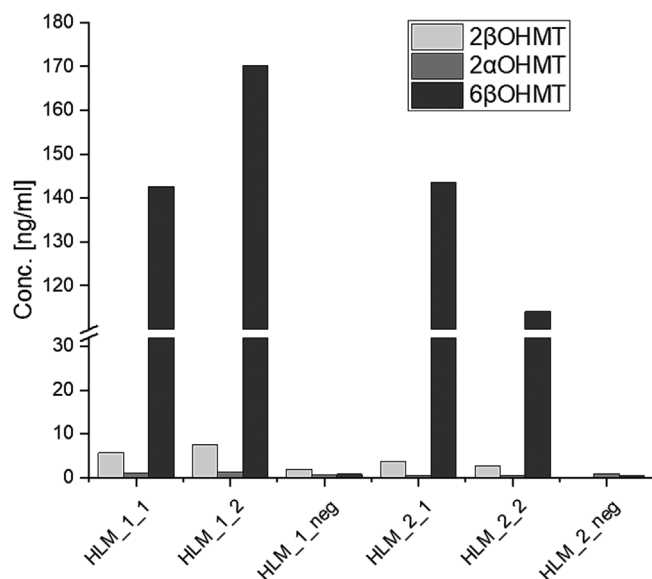
FIGURE 5 Results of HLM incubation against the negative control samples showing 2βOHMT, 2αOHMT, and 6βOHMT as hydroxylated metabolites.

yields 0.3–0.5% (2.7–7.6 ng/ml). 2βOHMT shows the highest conversion rate of the analytes in the negative control sample with up to 0.2%. 2αOHMT was found in the first study between 1.1 and 1.2 ng/ml (0.05–0.06%). In the repetition experiment, the amount of 2αOHMT found in the negative control sample (0.9 ng/ml) is higher than in the samples itself (0.4 ng/ml). All results of 2αOHMT are under the LLOQ, and therefore, it can only be assumed that 2αOHMT is not a metabolite of MT after HLM incubations. 4OHMT is not detected after incubation with HLM. Based on the structures of interest in this study, up to 16.5% of the parent compound become hydroxylated. Nearly all MT is biotransformated after 4 h of incubation, as residual MT is found between 0.3–1.0% after incubation. These results show that besides the focused hydroxylation reactions in positions 2, 6, and 4, also, other (phase-I) metabolization reactions are observed in this study. Further, mono-hydroxylated metabolites may be excluded by the MRM transition m/z 319 → 283 ([M + H-2H$_2$O]$^+$), as no other peak was found in the extracted ion chromatogram. Kuurane et al.[24] predicted that HLM does not show glucuronidation activity for MT. Therefore, phase-II metabolites of MT can almost be excluded, as

no sulfotransferase activity in HLM is described. The other major phase-I pathways for anabolic steroids are catalyzed by 3α-hydroxysteroid dehydrogenase (HSD3B2) and 5α-reductase (SRD5A1/2) resulting in dihydro- (DHMT) or tetrahydro-metabolites (THMT) of the parent compound.[25] The A-ring reduction of steroids in HLM incubation is already described in literature.[26,27] Both DHMT and THMT are poorly ionized with ESI and are therefore not included in the method. Further investigation of this behavior using isolated enzymes, semi-targeted approaches, and orthogonal techniques will be the next steps to gain further insights in the metabolization of MT in the liver.

4 | CONCLUSIONS

A robust method for the detection and quantitation of different hydroxylated metabolites and the parent compound MT was developed and validated. All analytes are baseline separated (R ≥ 4). LLOQ is 0.6 ng/ml (MT, 2βOHMT, 2αOHMT, and 6βOHMT), and linear range is between 0.6 and 400 ng/ml (2βOHMT, 6βOHMT), 2 and 400 ng/ml (MT), and 6 and 400 ng/ml (2αOHMT). 4OHMT showed a LLOQ of 2 ng/ml but has no linear relationship. LOD was ≤0.6 ng/ml for all analytes. Accuracy of the method is between 85% and 115% with an inter- and intra-day precision of ≤2.6%.

The ability of the method is demonstrated by incubation of MT with HLM. The results of HLM incubations are similar to previous results of incubations with other AAS. Hydroxylation in the beta positions (C6 or C2) after 4 h of incubation yield the major hydroxylated metabolites after HLM incubation (6βOHMT 11.6–15.9% and 2βOHMT 0.3–0.5%).

DATA AVAILABILITY STATEMENT

Data are available online (at https://doi.org/10.17169/refubium-39646).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.