A multivariate data analysis approach for the investigation of in vitro derived metabolites of ACP-105 in comparison with human in vivo metabolites

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ABSTRACT

Selective androgen receptor modulators (SARMs) such as ACP-105 are prohibited in sports due to their anabolic properties. ACP-105 has in previous equine studies shown to undergo extensive metabolism, which makes its metabolite profile important to investigate in humans, since the metabolism is unknown in this species. The aims of the study were to systematically optimize in vitro microsome incubations for improved metabolite yield and to utilize a multivariate data analysis (MVDA) approach to aid the metabolite discovery. Microsomes together with S9 fractions were used at optimal conditions, both with and without phase II additives. Furthermore, the relevance of the in vitro derived metabolites was evaluated as analytical targets in doping control by comparison with results from a human post-administration urine sample collected after a single dose of 100 μg ACP-105. All samples were analyzed with liquid chromatography - Orbitrap mass spectrometry.

The use of the systematical optimization and MVDA greatly simplified the search and a total of 18 in vitro metabolites were tentatively identified. The yield of the two main monohydroxylated isomers increased by 24 and 10 times, respectively. In the human urine sample, a total of seven metabolites of ACP-105, formed by a combination of hydroxylations and glucuronic acid conjugations, were tentatively identified. The main metabolites were two monohydroxylated forms that are suggested as analytical targets for human doping control after hydrolysis. All the in vivo metabolites could be detected with the MVDA approach on the in vitro models, demonstrating its usefulness for prediction of the in vivo metabolite profile.

1. Introduction

Selective Androgen Receptor Modulators (SARMs) are a pharmacological class of substances prohibited in human sports, both in and out of competition, according to the World Anti-Doping Agency (WADA) \cite{1}. Their anabolic properties in combination with milder side effects than anabolic androgenic steroids, make them tempting to use illicitly for performance enhancement \cite{2}. The metabolite profile of the SARM ACP-105 has previously been studied by several different in vitro methods and also in vivo in both rats and horses \cite{2–6}. Several metabolic transformation products were described in these studies, and the most prevalent ones were results of hydroxylations and glucuronic acid conjugations.

Investigation of the metabolite profiles of SARMs is of interest since drug metabolites are often present in biological samples such as blood and urine for a longer time than the parent compounds \cite{5,7}. The search for metabolites in doping control can thereby increase the window of detection. In vitro systems, such as microsomal preparations, are useful for metabolite profiling, however, their relevance generally needs to be evaluated by comparison with in vivo results. There are several different experimental parameters that need to be set in the in vitro methods, and the selected conditions can greatly influence the composition of the products formed \cite{8–10}. There are only a few examples in the literature of systematic optimization (Design of experiments) of in vitro metabolic conditions \cite{11,12}. However, most studies include generic settings described in previous publications without further justification.

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When working with chromatographic separation and high resolution mass spectrometric detection for identifying metabolites, it is generally performed in a targeted way by searching for known metabolic transformations. However, these types of analyses generally generate a large amount of data, and there is a risk of missing out on important results with a targeted evaluation. The use of approaches with MVDA, such as an unsupervised principal component analysis (PCA) and a supervised orthogonal projections to latent structure discriminant analysis (OPLS-DA) can be used to find differences between samples and sample groups [13]. They can thereby simplify the investigation of the drug metabolite profile by an unbiased search and thereby increase the possibility of finding unusual metabolites. There are several examples where MVDA approaches have been used to investigate drug metabolite profiles in vivo an in vitro [13-18], but to the best of our knowledge, there are no such publications within the doping control area. However, there are studies with MVDA that are looking in to the changes in the endogenous metabolite profile with the aim to find potential endogenous biomarkers for detecting administration of illicit substances [19].

The aims of this study were (I) to optimize the metabolic yield of the liver microsome incubations of ACP-105 and (II) to utilize an MVDA approach to aid the discovery of in vitro formed metabolites after analysis with ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) and (III) to investigate the human in vivo metabolite profile for the first time, and to evaluate the relevance of the identified metabolites as analytical targets in doping control.

2. Material and methods

2.1. Chemicals and reagents

ACP-105 (99.33 %, 2-chloro-4-[(3-endo)-3-hydroxy-3-methyl-8-azabicyclo[3.2.1]oct-8-yl]-3-methyl-benzonitrile) was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Formic acid (Optima LC-MS grade) and Pierce™ electrospray ionization (ESI) Positive/ Negative Ion Calibration Solution were obtained from Thermo Fisher Scientific (Waltham, MA, USA). β-glucuronidase from Escherichia coli K12 (80 U mg⁻¹ at 25 °C) was purchased from Roche (Basel, Switzerland). Human pooled liver microsomes and liver S9 fractions at the concentration of 20 mg mL⁻¹ were purchased from Sekisui Xenotech (Kansas City, KS, USA). β-Nicotinamide adenine dinucleotide 2-phosphate reduced tetrasodium salt hydrate (97 %, β-NADPH), alamethicin from Trichoderma viride (≥ 98 %), uridine 5' diphosphoglucuronic acid trisodium salt (≥ 98 %) (UDPGA), acetonitrile (LiChrosolv®, gradient grade, ≥ 99.9 %), methanol (LC-MS grade, ≥ 99.9 %), MgCl₂ (≥ 98 %), potassium phosphate monobasic (≥ 99 %) and potassium phosphate dibasic (≥ 98.0 %), were acquired from Merck (Munich, Germany). Purified water was obtained using Milli-Q® IQ 7000 Ultrapure Water System with a Millipak® 0.22 µm filter from Merck Millipore (Burlington, MA, USA).

2.2. Optimization of phase I human liver microsome incubations

The parameters for the phase I incubations were systematically optimized with human liver microsomes using a Design of experiments approach with the software MODDE® Pro 13 (Sartorius Stedim Data Analytics AB, Umeå, Sweden). A screening was performed with fractional factorial design (FrFD) and a linear model with 20 samples. The following parameters were used for screening within the described range: incubation time (30–330 min) and concentration of potassium phosphate buffer (PPB) pH 7.4 (50, 100 mM), ACP-105 (2–20 µM), microsomes (0.5–2.0 mg mL⁻¹), β-NADPH (1.0–3.0 mM) and MgCl₂ (1.0–3.0 mM). After the screening, the optimization process was performed using circumscribed central composite design (CCC) as a quadratic model with 18 samples. Based on the screening results, the following parameters were further investigated within the described range: incubation time (180–360 min) and the concentration of both ACP-105 (16–30 µM) and microsomes (0.5–1.5 mg mL⁻¹). The remaining parameters were set to the following: PPB pH 7.4 (50 mM), β-NADPH (3.0 mM) and MgCl₂ (3.0 mM).

The incubation order was randomized to minimize systematic errors and included three center points and a replicate of axial points to get an understanding of repeatability. The experimental objective was aimed toward increased yield, i.e. increased chromatographic peak area of two monohydroxylated metabolites (labeled M4 and M5 in this study) of ACP-105 that had been tentatively identified in a previous study with human microsome incubates [5]. The response was logarithmically transformed and the relationship between factors and responses were evaluated by analysis of the raw data and by fitting the data in multiple linear regression (MLR), including model fit (R²), predictability (Q²), model validity and reproducibility. The model fit, R², should be greater than 0.5 and closer to 1.0 for a good fit, Q² should be greater than 0.5 and the difference between R² and Q² should not be bigger than 0.2-0.3. The model validity should always exceed 0.25 and the reproducibility should be greater than 0.5 [20]. The outcome of the optimization, the final setting of parameters for the optimal setpoint, is described in Section 2.3. The final method was performed in triplicates to examine repeatability of the experiment.

In order to have a reference point for comparison after optimization, microsome incubations with ACP-105 was performed with a generic non-optimized method described by Broberg et al. [5] but with the following change; the stock solution of ACP-105 was in methanol and the solvent was evaporated prior to addition of the other components.

2.3. Phase I incubations with human liver microsomes and S9 fractions

For the systematically optimized method, 30 µL MeOH and 45.2 µL ACP-105 solution (44 µM in MeOH, total incubation concentration 33 µM) were added to 1.5 mL Eppendorf tubes and the samples were evaporated for 10 min until dryness using a vacuum concentrator, Christ RVC 2–8 (Martin Christ, Osterode am Harz, Germany) at 50 °C. The samples were re-dissolved with 19.7 µL PPB at pH 7.4 and a 50 mM total concentration of phosphate. Thereafter, 18.0 µL MgCl₂ (10 mM in PPB, total incubation concentration 3.0 mM) were added and the samples were vortexed. After that, 18.0 µL β-NADPH (10 mM in PPB, total incubation concentration 3.0 mM) and 4.3 µL of thawed human liver microsomes (total incubation concentration 1.4 mg mL⁻¹) were added. The samples with a total volume 60.0 µL each were incubated in a Thermomixer Compact from Eppendorf (Hamburg, Germany) at 37 °C and 300 rpm for 250 min.

The incubation was terminated by addition of 200 µL ice-cold acetonitrile to each sample, which was then vortex-mixed and stored at 4 °C for 10 min. The samples were thereafter centrifuged at 4 °C, 11,500 g for 10 min in a Micro Star 17R from VWR (Radnor, PA, USA). From the supernatants, 230 µL were transferred to 1.5 mL Eppendorf tubes without disturbing the pellet in the bottom. The samples were thereafter evaporated for 60 min until dryness using a vacuum concentrator at 50 °C. The samples were reconstituted in 100 µL formic acid in water (0.1 %) and transferred to vials for analysis.

The entire procedure described above was also performed for phase I incubations with human liver S9 fractions, the only difference from the description above was the addition of 4.3 µL of thawed S9 fractions instead of human liver microsomes.

2.4. Phase II incubations with human liver microsomes and S9 fractions

For Phase II incubations, aimed toward glucuronic acid conjugations, the procedure was the same as described in Section 2.3, but with the following differences (not optimized using experimental design); 15.3 µL UDPGA (30 mM in PPB), 10.5 µL alamethicin (0.21 mM in 14.6 % AcN in PPB) and 6.0 µL β-NADPH (10 mM in PPB) were added after 130 min of incubation. The samples with a total volume 91.8 µL each were
thereafter incubated for an additional 120 min. The same procedure was also followed for the termination of the incubations, but the volume of supernatant that was transferred prior to evaporation was adjusted to 260 µL instead of 230 µL.

2.5. Replicates and control samples for the incubation methods

In total, four different incubations models were created, phase I incubations with human liver microsomes, phase II incubations with human liver microsomes, phase I incubations with human liver S9 fractions and phase II incubations with human liver S9 fractions. For each of these four models, the following control samples were prepared.

Negative control samples without ACP-105 were created by addition of only MeOH prior to the first evaporation step described in the incubation method described in Section 2.3. Eight replicates of each in vitro method were prepared, i.e., eight incubates with ACP-105, eight incubates without ACP-105, creating a total of 16 samples for each model.

Both the incubation order and analysis order of the 16 samples were randomized separately. A quality control (QC) sample was created directly before analysis of each model by combining 15 µL from each of the 16 vials in the same vial. An enzyme blank control sample without addition of microsomes or S9 fractions was also created. To control the enzymatic activity of the microsomes and S9 fractions, the metabolite formation was compared with previously published results of ACP-105 incubations [5].

2.6. Human administration study

A single dose of 100 µg ACP-105 was orally administered to one individual, where 10 µL of an ethanolic solution containing ACP-105 (10 µg·µL−1) was added to 100 g of drinking yoghurt, shaken, and the entire volume was consumed. Administration via yoghurt was done following an earlier protocol by Walpurgis et al. [21], which proved useful in the context of SARMs for anti-doping research regarding the ingestion of minute amounts of the target compounds. Urine was collected 8.5 h after administration and stored at −20 °C until analysis.

The administration study was approved by the Institutional Animal Care and Use Committee of the University of California, Davis (Protocol #20319, date of approval 11 January 2020).

2.8. UHPLC-HRMS analysis

The analysis was performed using a Vanquish UHPLC+ focused binary pump F from Thermo Fisher Scientific (Waltham, MA, USA) with a Split Sampler FT autosampler kept at 8 °C with an Acquity UPLC® HSS T3 column (2.1 × 100 mm; particle size 1.8 µm) and a 5 mm guard column with a stationary phase of the same composition from Waters (Milford, MA, USA) The flow rate was 450 µL·min−1 with a column temperature at 40 °C and an injection volume of 5 µL. The mobile phase consisted of 0.1 % formic acid in water (mobile phase A) and in acetonitrile (mobile phase B). The LC gradient started at 3 % B for 1 min, thereafter a linear increase to 97 % B over 16 min and thereafter remaining at 97 % B for 4 min. The column was then re-equilibrated by changing back to 3 % B for 3 min. The UHPLC system was coupled to a Q Exactive™ Orbitrap™ benchtop mass spectrometer with a heated electrospray ionization probe (HESI-II), all from Thermo Fisher Scientific used through the software TraceFinder™ 5.1. The spray voltage was 3.5 kV in positive ionization, and −3.0 kV in negative ionization. The capillary temperature was 320 °C and the aux gas heater temperature 400 °C. The sheath gas was 50 µL, auxiliary gas 10, S-lens RF level 60; all in arbitrary units. Nitrogen gas was used as collision gas.

Data analysis was performed using the software FreeStyle™ 1.8 from Thermo Fisher Scientific. The system was operated at a resolution of 70,000 at m/z 200 at full width at half maximum (FWHM), in full scan mode (m/z 100–1000) and the data was collected in profile mode. For initial analysis, a full scan MS combined with data dependent MS/MS analysis (full scan MS/ddMS2) was used both in positive and negative ionization mode by separate injections. For structural investigation of selected metabolites, data independent MS/MS analysis was performed and the identification classification was made in accordance with the Schymanski et al. scale [22].

For samples analyzed for MVDA purposes, full scan MS mode with positive ionization was applied. The four different in vitro models were analyzed separately, for each model, the injection order of the samples was randomized and a QC sample was analyzed every fifth sample to assess system stability. Repeated injections of the QC sample were performed prior to analysis of the samples to minimize drift in both retention time and peak intensity.

2.9. Data processing and analysis

The in vitro raw MS data files were converted to mzXML files using ReAdW (Trans Proteomic Pipeline platform [23]), that utilized Xcalibur 4.2 from Thermo Scientific. Results from each in vitro method was processed separately. The files were thereafter imported to mzMine 2, version 2.53 and the following settings were applied: the noise level was set to 1.0·10−5, the m/z tolerance to 5.0 ppm or 0.001 absolute units and the retention time tolerance was set to 0.1 min. Peak detection was performed by mass detection and the ADAP Chromatogram Builder Module was used for chromatogram building. The local minimum search was used for peak deconvolution, thereafter peak aligning and gap-filling was performed and the data were exported as a csv file [24,25].

The data was then further processed in Excel by removal of features with a coefficient of variation (CV) above 30 % in the QC sample group (n = 5), and sample meta data was added. The data was then imported into SIMCA 17 (Sartorius Stedim Data Analytics AB, Umeå, Sweden), pareto scaled and a PCA with two components were carried out. The loadings and scores plots were analyzed and thereafter an OPLS-DA was used and the S-plot was analyzed. The S-plot was the main tool for metabolite search, where features with a confidence ≥ 0.90 were processed and further investigated. Each feature consisted of a chromatographic peak with information about peak area, m/z value and retention
time which all was used for further metabolite identification. A manual search for metabolites was also performed, where knowledge of known metabolite transformations was used to confirm that these metabolites were not missed by the MVDA approach.

3. Results and discussion

3.1. Optimization of microsome incubations

In the optimization of phase I microsome incubations, the parameters with the largest impact in the screening were the incubation time, concentration of ACP-105 and concentration of microsomes, which is why these parameters were further investigated. During the optimization it could be seen that out of the three selected parameters, the concentration of ACP-105 and the incubation time had the highest influence on the yield of metabolites. A total of 13 phase I metabolites were tentatively identified, denoted M1-M13 (Table 1). An increase in incubation time and ACP-105 concentration increased the yield for the two monohydroxylated metabolites (M4 and M5), but regarding the microsome concentrations in parallel with the decline of M4. This could be explained by further hydroxylation.

With the optimized method, there was a higher yield and variety of metabolites than what could be observed prior to the optimization. The yield of the two monohydroxylated metabolites M4 and M5 were increased by 24 and 10 times respectively, compared to results from incubations using conditions from a previously published method [5]. The repeatability for the chromatographic peak area of the triplicates at the optimal set point showed an %RSD of 2.1 for M4 and 6.0 for M5. The optimized model yielded a reproducibility of 0.99 for both metabolite M4 and M5, a model validity of 0.66 for M4 and 0.41 for M5. The R2/Q2 was 0.99/0.96 for M4 and 0.97/0.93 for M5. Evaluation of the described performance indicators showed a reliable model in accordance with set values.

Table 1

<table>
<thead>
<tr>
<th>Metabolite transformation</th>
<th>Elemental composition</th>
<th>Th. m/z s [M+H]+</th>
<th>Metabolite No.</th>
<th>tR (min)</th>
<th>MPI</th>
<th>MPII</th>
<th>S9 PI</th>
<th>S9 PI</th>
<th>Human in vivo</th>
<th>Equine in vivo</th>
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<td>ACP-105</td>
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<td>M1</td>
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<td>M8a</td>
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</table>

4 The metabolite labels in the column equine in vivo are annotations from the previous publication [5] where the equine metabolite profile of ACP-105 was investigated.

3.2. Multivariate analysis of in vitro data

There were in total four different human liver models analyzed. They either contained microsomes or S9 fractions, and both of them were also incubated with or without phase II additives aimed toward glucuronic acid conjugations. All models showed good separation between the groups with or without ACP-105 in the PCA scores plot and also a tight clustering of the QC sample injections (Fig. 2).

The features of interest could already be seen in the PCA loadings plot (results not shown), but for a simplified selection, an OPLS-DA was performed for each in vitro model, as shown for the microsome phase II model in Fig. 3. Since the OPLS-DA is a supervised method, a comparison with the corresponding PCA loadings and scores plot and permutation tests for each model was performed prior to further evaluation, the results of the permutation tests are visualized in the supplementary material. An S-plot was used to select which features that should undergo further investigation and the features of interest are marked with a rectangle in Fig. 3. For the microsome phase II model, there were 28 features selected by this method and all of those could readily be associated with ACP-105 or metabolites thereof. Four of the features originated from the addition of ACP-105 and could also be found in incubated control samples without the addition of microsomes. The remaining features corresponded to protonated ions of tentatively identified metabolites, formed mainly by combinations of hydroxylations and glucuronic acid conjugations. There were also additional signals corresponding to the same species containing 37Cl isotopes. The procedure was performed for all four in vitro models and several metabolites of interest could be detected from each model as demonstrated in Table 1.

In total, 18 different metabolites of ACP-105 were tentatively identified.
identified, formed by phase I and phase II transformations such as hydroxylations in combination with double bond formation (loss of H₂) and glucuronic acid conjugations. Regarding the identification process, all in vitro metabolites that matched those previously tentatively identified in vivo in an equine administration study of ACP-105 were further investigated [5]. The equine in vivo samples were re-analyzed in the same sequence and the metabolites were compared and linked to each other based on mass difference (m/z within ± 2.0 ppm), retention time (±0.02 min) and matching product ions from the MS/MS spectrum (results not shown).

Fig. 1. The response contour plot with the response defined as chromatographic peak area showing (A) metabolite M4 and (B) metabolite M5. The plot was set at the optimal set point for ACP-105 concentration (33 µM). The color coding and peak area notations in the figures show the impact on the yield with changes in incubation time (y-axis) and concentration of microsomes (x-axis).

Fig. 2. Principle component analysis (PCA) scores plots of the microsome and S9 fractions models with or without phase II additives. There are three different types of samples displayed in each plot: samples with ACP-105 (T), a control group (C) without ACP-105, with eight samples in each of these two groups. Also displayed is the quality control sample (QC) that was analyzed five times evenly distributed over the entire batch. (A) Microsome phase I model (B) Microsome phase II model (C) S9 fraction phase I model (D) S9 fraction phase II model.
Fig. 3. Example of how the OPLS-DA was utilized for metabolite search, this method was applied on all four models. (A) OPLS-DA analysis of phase II microsome model and (B) the corresponding S-plot, where features marked with a rectangle have a confidence level ≥ 0.90 and was selected as features of interest for metabolite investigation.

Fig. 4. Comparison of phase I and phase II metabolites of ACP-105 that were present in both the human in vivo and in vitro samples. Extracted ion chromatograms (EIC) of (A) ACP-105 and phase I metabolites in hydrolyzed urine (B) phase II metabolites in unhydrolyzed urine (C) metabolites from phase I microsome incubates (D) metabolites from microsome incubates with phase II additives (E) metabolites from phase I S9 fraction incubates (F) metabolites from S9 fraction incubates with phase II additives.
A manual search in the microsomal and S9 fraction data was also performed to check for any metabolites not detected by the MVDA approach, but no such metabolites with high intensity were found. This shows that the MVDA approach was a useful tool for metabolite profiling of in vitro models. It has the potential to make the data evaluation more efficient, decrease the risk for false negative results and increase the potential for identification of metabolites formed by rare metabolic transformations. The selection of ACP-105 for this study was made owing to the fact that its metabolite profile had been previously studied in different species, making it possible to in some way validate the relevance of the MVDA findings.

3.3. Human in vivo urine metabolites of ACP-105

In human urine, a total of seven metabolites of ACP-105 were tentatively identified in unhydrolyzed and hydrolyzed urine (Fig. 4 and Table 1). In untreated urine, ACP-105 could not be observed, but several glucuronidated phase I metabolites were detected (M14, M15 and M17). In hydrolyzed urine, both ACP-105 and a combination of mono-hydroxylated and dihydroxylated forms were detected (M4, M5, M9 and M10). It is noteworthy that ACP-105 appeared after hydrolysis, even though neither itself nor its glucuronide could be found in untreated urine. This could be explained by low ionization efficiency for the glucuronide or by a reformation of the parent compound from some other metabolite.

The monohydroxylated metabolites M4 and M5 had the highest intensity in hydrolyzed urine in comparison with ACP-105 and the other metabolites. M4 and M5 are thereby suggested as analytical targets for human doping control after hydrolysis with β-glucuronidase. The results indicate that ACP-105 is metabolized to a high degree in humans, but further studies with sampling over time would be of interest to investigate the presence of long-term metabolites. It is likely that the search for M4 and M5 in doping control could increase the detection window for illicit use of ACP-105, which is in line with what has been demonstrated in a previous equine administration study [5]. The main difference between the in vivo metabolite profiles is that a wider range of metabolites was detected in the horse than in the human urine.

3.4. Comparison of human in vivo and in vitro metabolites of ACP-105

The possibility of reliable predictions of in vivo metabolite profiles is important for several reasons. Firstly, there is an ethical issue when it comes to the use of animals and humans for research. In this work, the human administration was performed with only one individual, which needs to be taken in to consideration when evaluating the metabolite profile. However, administration studies of substances with incompletely evaluated toxicological effects are problematic. Secondly, the in vitro models are also beneficial when it comes to cost and time effectiveness for the ability to find analytical targets for doping control.

Seven metabolites of ACP-105 were tentatively identified for the first time in a human in vivo administration study. All of them were also detected with MVDA on the in vitro models (Fig. 4 and Table 1), demonstrating the usefulness of this approach for in vivo prediction. All four in vitro models showed some overlap with the in vivo results, but the most valuable results originate from the microsome models. In the phase I microsome model, all in vivo phase I metabolites could be tentatively identified, and in the phase II microsome model, all in vivo phase II metabolites and some of the phase I metabolites could be found. This is also important from a doping control perspective, since microsome incubates are allowed as a reference samples in suspected doping cases [26].

4. Conclusion

The optimization of the microsome phase I incubations with ACP-105 improved both the yield and variety of metabolites and by using the OPLS-DA model, the search was greatly simplified. A total of 18 metabolites of ACP-105, mainly formed by combinations of hydroxylations and glucuronic acid conjugations, could readily be found in vitro. A total of seven metabolites were tentatively identified in the human urine sample. To the best of our knowledge, this is the first report of the metabolite profile of ACP-105 in humans. In unhydrolyzed urine, parent ACP-105 could not be observed, but several glucuronides were detected. After hydrolysis, two monohydroxylated (M4 and M5) and two dihydroxylated metabolites were detected together with parent ACP-105. Based on the intensity of all the tentatively identified metabolites, M4 and M5 are suggested as analytical targets for human doping control.

Comparison of the in vivo and in vitro metabolites showed a great overlap and all in vivo-derived metabolites could also be detected in vitro through the MVDA approach, demonstrating its usefulness for in vivo prediction.

CRediT authorship contribution statement

Malin Nilsson Broberg: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. Rebecca Tillgren Ohlsson: Methodology, Formal analysis, Investigation, Writing – review & editing. Ulf Bondesson: Conceptualization, Methodology, Writing – review & editing, Supervision. Curt Pettersson: Conceptualization, Methodology, Writing – review & editing, Supervision. Börje Tidstedt: Resources, Writing – review & editing, Supervision. Mario Thevis: Conceptualization, Methodology, Resources, Writing – review & editing, Supervision. Mikael Hedeland: Conceptualization, Methodology, Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Ethical declaration

The human administration study was approved by the local ethics committee at the German Sport University, Cologne Germany (Approval #040/2020) and written informed consent was obtained from the study participant. The equine administration study was approved by the Institutional Animal Care and Use Committee of the University of California, Davis (Protocol #20319, date of approval 11 January 2020).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2023.123927.
References


