Metabolite Profiling of Drugs using Mass Spectrometry

Identification of analytical targets for doping control and improvements of the metabolite search process

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Abstract

Doping is defined as the use of prohibited substances or methods by the World Anti-Doping Agency and the aim with doping control analysis is to detect the use of these illicit substances or methods. Substances that are prohibited in human or equine sports have either a positive or negative impact on the performance. Since administered drugs generally are metabolized to a varying degree and thereby not only excreted in their original form, their metabolite profiles are of high interest because drug metabolites may be present in the body for a longer time than the administered drug itself. Thereby detection of metabolites can improve the window of detection. Unfortunately, the metabolite profiles of non-approved drugs that are mainly available on the Internet, such as Selective Androgen Receptor Modulators (SARMs) are often unknown.

This thesis consists of four papers that all encompass drug metabolite profiling either in vivo, in vitro or in a combination, utilizing separation with liquid chromatography and detection with high resolution mass spectrometry. In paper I and II, the equine in vivo metabolite profiles of the two SARMs ACP-105 and LGD-3303 were investigated and the results showed that using drug metabolites as analytical targets can prolong the detection time. For ACP-105, the in vivo metabolite profile was compared with different incubation models such as liver microsomes, S9 fractions and the fungus Cunninghamella elegans. The in vivo and in vitro metabolite profiles showed an interesting overlap for several metabolites, demonstrating the importance and usefulness for in vitro methods in doping control, especially since microsome incubates are allowed as reference material. An optimization of microsome incubation conditions utilizing experimental design was presented in paper III and IV, showing that the optimized conditions greatly impacted the yield of drug metabolites, but also that the optimal conditions are substance dependent. In paper III, a multivariate data analysis search tool utilizing OPLS-DA was presented, which greatly simplified the in vitro drug metabolite identification process of ACP-105 and the results showed relevance in comparison with human in vivo metabolites.

In conclusion, several new analytical targets with improved detectability for equine and human doping control have been presented, where the drug metabolite profile showed to be of great importance. All together, these new analytical targets, the optimized microsome incubation conditions for improved metabolite yield and the search tool that aids the metabolite investigation through multivariate data analysis, have made a positive contribution to the doping control area.

Keywords: Mass spectrometry, UHPLC-HRMS, Doping control, Metabolite profile, SARM, Selective Androgen Receptor Modulator, MVDA, Microsomes, Experimental design

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This is what happens when you put a turtle in a church.
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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<tr>
<td>DOE</td>
<td>Design of experiments</td>
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<tr>
<td>EIC</td>
<td>Extracted ion chromatogram</td>
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<td>ELM</td>
<td>Equine liver microsomes</td>
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<td>ESI</td>
<td>Electrospray ionization</td>
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<td>FEI</td>
<td>Fédération Equestre Internationale</td>
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<td>HESI</td>
<td>Heated electrospray ionization</td>
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<td>HLM</td>
<td>Human liver microsomes</td>
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<td>HRMS</td>
<td>High resolution mass spectrometry</td>
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<td>IFHA</td>
<td>International Federation of Horseracing Authorities</td>
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<td>LC-MS</td>
<td>Liquid chromatography – mass spectrometry</td>
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<td>MVDA</td>
<td>Multivariate data analysis</td>
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<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>OPLS-DA</td>
<td>Orthogonal projection to latent structures discriminant analysis</td>
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<td>PCA</td>
<td>Principal component analysis</td>
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<td>QC</td>
<td>Quality control</td>
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<td>RPLC</td>
<td>Reversed phase liquid chromatography</td>
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<td>SARM</td>
<td>Selective androgen receptor modulator</td>
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<td>SPE</td>
<td>Solid phase extraction</td>
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<td>UHPLC</td>
<td>Ultra high-performance liquid chromatography</td>
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<td>WADA</td>
<td>World Anti-Doping Agency</td>
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Introduction

Doping control

Doping is classified as the use of prohibited substances or methods by the World Anti-Doping Agency (WADA). The aim with the classification is to have fair competitions and keep the integrity of sport [1]. Some substances and methods are prohibited at all times and others only in competition. Doping and doping control is important not only for human sports, but also for equine sports such as horse racing and other competitions where animals such as dogs, camels and pigeons are involved [2–5]. Regarding animal sports, the doping aspect may be even more important since often large sums of money are involved, both through winnings and betting, making doping control an important ethical tool with respect of animal welfare [6].

For human sports, WADA is responsible for these rules and regulations, for equine sports, there are international collaboration organizations such as the International Federation of Horseracing Authorities (IFHA) and the Fédération Equestre Internationale (FEI) that sets the regulations for doping control, which then are interpreted in the national regulations [7–10].

Doping classified compounds

There are several different classes of substances that are prohibited in both equine and human sports. Substances that are prohibited in human or equine sports have either a positive or negative impact on the performance. The substances prohibited in human doping are generally for performance enhancement, but for equine doping, performance impairment is also of importance, where for example sedative substances can be used to both calm a horse down prior to racing, but also to slow it down [2,11].

There are some substances that are only prohibited in-competition, generally those with short-term enhancing effects such as stimulants and narcotics. Some substances, such as β-blockers, are prohibited in particular sports, such as archery, darts, billiards, golf and shooting, where stability is of importance [8,12].

Many of the prohibited compounds are known and registered in pharmaceutical products and have thereby been studied to a great extent. Some substances are not as thoroughly studied as others, for example Selective Androgen Receptor Modulators (SARMs). SARMs have been under development
towards a registered pharmaceutical compound and have been in clinical trials, but are not yet registered in a pharmaceutical product, and due to that, their metabolite profiles have often not been fully investigated [13–29].

SARMs are prohibited at all times, in both equine and human sports and are important to include in doping control analysis since they have a similar effect as anabolic androgen steroids but generally with less side effects since they are more tissue and bone selective than anabolic steroids [24]. There have been several cases where SARMs have been identified as adverse analytical findings in doping control [30–35], making them important to investigate further.

To increase chances of a positive identification of illicit use of substances, knowledge about how they are metabolized and how long the parent compound and the drug metabolites are present in blood or urine is of importance both for human and equine doping control. Several studies have been performed on different SARMs where the metabolite profile have been investigated both in vivo and in vitro, and the identified drug metabolites together with the parent compound have been structurally elucidated and studied for suggestions of suitable analytical targets for doping control [36–48].

Biological passports

Long term use of for example anabolic androgen steroids may also have long term effects on performance. To try and combat the use of substances with long term effects, the use of the biological passport is important [49].

There are both synthetic exogenous and endogenous steroids used for doping purposes. Due to large individual differences, a generic threshold for endogenous steroids is not always suitable, that is where the use of the Biological Passport comes in. By measuring the individual’s own levels over time, the levels of endogenous metabolites can be monitored and increase the chances to reveal use of illicit substances or methods. Biological passports for doping control are today in use for humans [50] and at an ongoing research stage for horses [51].

Blood, gene and cell doping

Regarding blood doping, the prohibited methods involve manipulation of blood and blood components, including not only administration or reintroduction of blood or red blood cell products, but also any artificial enhancement of uptake transport and delivery of oxygen with for example haemoglobin products [8,52]. Manipulation of the blood is generally to increase the uptake and delivery of oxygen and thereby increase capacity and physical performance [53].

Gene doping is at this stage a developing area, not only regarding the doping parts, but also regarding detection methods. It is complicated to detect due
to the fact that the changed gene expression then affects the phenotype. Gene and cell doping are prohibited if they have the potential to enhance sport performance. This includes use of normal or genetically modified cells and also any use of nucleic acids or nucleic acid analogues that can alter the genome sequence or gene expression. It also includes gene editing, gene silencing and gene transfer technologies. With the recent advances in gene editing, including techniques such as CRISPR/Cas9, gene and cell doping greatly needs to be taken into consideration ahead [8,52,54].

Drug metabolism

Administered drugs are generally metabolized to a varying degree and thereby not only excreted in their original form, causing the drug metabolite profile to be of high interest. The metabolism consists of both phase I metabolic transformations such as hydroxylation, demethylation, carboxylation, oxidation, reduction and dealkylation, and phase II metabolic transformations such as glucuronic acid conjugation and sulfate conjugation [55,56]. It is also possible that combination of several different transformations takes place on the same analyte to create more complex metabolites. There are several enzymatic processes that are crucial for the metabolic transformations of drug metabolites, but the cytochrome P450 (CYP) enzymes are the most important regarding phase I metabolic transformations [57].

Investigation of the drug metabolite profile can be performed either through in vivo or in vitro studies. Previous studies have shown that metabolites derived from a drug often are detected at a higher intensity and are present for a longer time in biological samples than the parent compound, making drug metabolites suitable analytical targets for doping control [40].

Laboratory guidelines and analytical techniques

Within the analytical guidelines as to what can be used as an analytical standard in doping control, a synthesized and characterized reference standard is preferable if available. But in addition to this, samples from administration studies, liver cells and microsomal incubates are allowed as reference material and can be used in doping analysis, presenting an advantage with in vivo and in vitro drug metabolism studies [58].

There are several analytical techniques that can be used for doping analysis such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) where the most common one is LC-MS [59–62]. When analyzing and searching for suitable analytical targets, it is important that they are detectable by the selected method.
In routine doping control analysis, there are several types of high resolution instruments such as the Quadrupole Time-of-Flight (Q-ToF), Fourier-transform ion cyclotron resonance (FT-ICR) and Orbitrap instruments. The high resolution instruments are often used for screening in general routine doping analysis, but low resolution instruments such as triple quadrupoles can be useful and even more suitable for some purposes [62]. For research purposes to investigate the drug metabolite profile, high resolution mass spectrometry is very useful, especially since the ongoing development greatly has improved the mass accuracy and thereby the possibility to more precise perform identifications and structural elucidations [63,64].

**Multivariate data analysis techniques**

Multivariate data analysis (MVDA) techniques can be very useful for doping analysis research due to the high resolution instruments that creates large amounts of data. Techniques such as Principal Component Analysis (PCA) makes it possible to get an overview of your data, and with an Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) it is possible to compare for example two groups and examine what differences there are between the two. The MVDA approach can thereby be useful for the metabolite search process and assist in the metabolite profiling of drugs and decrease the risk of missing metabolites of interest [65–67].

There have been several studies describing and utilizing multivariate techniques for identification of drug metabolites previously, that have mainly focused on the interpretation of metabolic pathways, but they have also been used for metabolite identification [68]. Schneider et al. [69] performed a study where a PCA model was used to display metabolites produced from a drug with a microsomal incubation model.

Furthermore, there are some multivariate applications for identification of analytical targets in doping control research that have been used in previous studies, but mainly examining the differences in the endogenous metabolite profile and affected pathways [51]. However, there is a need for the development of a more untargeted screening in doping control, to increase the possibility to detect illicit use of substances or methods [70].
Aims

The overall aim of the PhD project has been to improve the area of doping analysis by method development and metabolite profiling of doping classified drugs to be able to present improved analytical targets for LC-MS-based doping control.

The specific aims of the thesis are as follows:

- To identify drug metabolites of selected SARMs through in vivo and in vitro methods, with improved window of detection in comparison to the parent compound, which can be utilized as analytical targets for equine and human doping analysis with high resolution mass spectrometry (Paper I-IV).
- To evaluate the relevance of in vitro incubation models in comparison to in vivo administration studies regarding the drug metabolite profile (Paper I & Paper III).
- To improve the in vitro drug metabolite detection process by adapting a protocol utilizing multivariate data analysis methods (Paper III).
- To optimize and increase the yield of formed drug metabolites from in vitro incubation methods in a systematic way utilizing Design of experiments to improve target identification (Paper III & Paper IV).
Metabolite profiling of drugs

Overview

The development of the projects included in this thesis have been a quite organic process. Performing the analysis and answering the research questions that were set up in the first projects, not only provided scientific results, but also taught me a lot and made me curious of additional research questions. I think that looking at your own research process a bit critically can be the most helpful way of improving your future research by applying all the gained knowledge when tackling new projects.

This thesis consists of several different research projects, each with its specific aims – but independent of the aim, they have all involved investigation of the drug metabolite profile in some way. The overall process for each project has quite a similar setup that can be viewed in Figure 1, but the emphasis of each project has been on different parts of the shown workflow.

A number of different in vivo studies have been performed, both equine (Paper I and Paper II) and human (Paper III) administration studies. Along with that, several different incubation models (Paper I, Paper III and Paper IV) and their ability to produce drug metabolites have been investigated and optimized to some extent.

Depending on the type of biological sample or incubate, they have all been put through some type of sample preparation. Some have just had a protein precipitation while others have also undergone further clean-up by solid phase extraction (SPE). The overall aim with the sample preparation was to reduce the impact of the background, but at the same time, minimize the risk of losing drug metabolites of interest, since the metabolite profiles of these substances has not previously been investigated to any great extent. The analysis has been performed with ultra high-performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS). The raw data from the analysis has thereafter been further analyzed either univariately or with multivariate data analysis methods.
In vivo administration studies

The purpose of administration studies can vary a lot depending on the research field, especially if studying the effect is of interest and there is a need to reach therapeutic levels of the drug in question or if the general aim is something else. The administration studies included in this thesis have only been aimed at investigating the drug metabolite profile and, if possible, also the time profile. The search for drug metabolites was mainly based on the theoretical knowledge of potential metabolic transformations that could take place.

The equine administration parts described in Paper I and Paper II have been performed by our collaborators at University of California, Davis, USA.
The administration consisted of a single oral dose of 0.05 mg·kg\(^{-1}\) of either ACP-105 or LGD-3303. The selected dose can be considered quite low, but was suitable for the purpose of these studies.

In Paper I, a total of 21 metabolites of ACP-105 were tentatively identified from the equine administration study. They consisted of different types of phase I and phase II metabolites and several of these metabolites were detected at a higher intensity and for a longer time than the administered substance.

![Figure 2.](image-url) The equine metabolite profile of LGD-3303 in A, unhydrolyzed urine, B, hydrolyzed urine and C, plasma. The figure shows overlapping extracted ion chromatograms (EIC) from samples taken six hours after oral administration of LGD-3303. Analysis was performed with UHPLC-HRMS and positive heated electrospray ionization (HESI). Figure from Paper II [37].
When the equine metabolite profile of LGD-3303 was investigated, as described in Paper II, a total of eight metabolites were tentatively identified as seen in Figure 2. The identified metabolites differed quite a lot between the different sample types. In plasma, only the administered substance LGD-3303 and a monohydroxylated metabolite could be detected. In untreated urine, phase II metabolites that had gone through glucuronic acid conjugations showed the highest intensity, but several phase I metabolites and LGD-3303 could also be seen. In hydrolyzed urine, mainly phase I metabolites were detected together with LGD-3303 and one monohydroxylated glucuronidated metabolite.

![Figure 3](image)

**Figure 3.** Overlapping extracted ion chromatograms (EIC) of drug metabolites of ACP-105 in **A**, human hydrolyzed urine, **B**, human unhydrolyzed urine, **C**, human phase I microsome incubates and **D**, human phase II microsome incubates. Figure from Paper III [71].

The human administration study of ACP-105 described in Paper III was an oral administration of 100 µg ACP-105 to one individual and was performed by our collaborators at the German Sport University, Cologne, Germany. For this study, a single urine sample was collected after 8.5 hours. Before this study was performed, the metabolite profile of ACP-105 had already been investigated both in vivo through the equine administration study, but also through several different in vitro models. Therefore, the identified drug metabolites in the human urine were compared and positively matched with the metabolites already previously identified.
This is the first time the human drug metabolite profile of ACP-105 has been investigated and a total of seven metabolites of ACP-105 were tentatively identified as seen in Figure 3, the metabolites consisted of both hydroxylated and glucuronidated metabolites.

**Incubations with Cunninghamella elegans**

For some of the investigation of drug metabolites, incubations have been performed with the fungus *C. elegans*, which is a filamentous fungus with similar metabolism to mammals [72–74]. The fungus can be grown on agar plates, but also in sabouraud dextrose broth as seen in Figure 4. The incubation was carried out at 27 °C and by addition of a drug substance, the incubations can be used as a drug metabolite production method.

![Figure 4](image)

**Figure 4.** The fungus *Cunninghamella elegans* grown on an agar plate (left) and in sabouraud dextrose broth (right). When utilizing *C. elegans* for its ability to transform drugs, the substance in question is added to the sabouraud dextrose broth together with fungal suspension and incubated at 27 °C for five days.

The ability of *C. elegans* to produce drug metabolite was investigated by performing incubations with ACP-105 which is described in Paper 1. As seen in Figure 5, the metabolite profile from *C. elegans* incubates does not perfectly match the equine in vivo monohydroxylated metabolites, but two out of three have matching retention times and can be produced using this method. Since the monohydroxylated metabolite M1a also is suggested as a new analytical target for doping control, this is extra useful, especially since the incubations can be scaled up and the incubate thereafter purified and characterized to produce reference material [75].
**Figure 5.** Extracted ion chromatograms (EIC) showing the comparison between three monohydroxylated metabolites of ACP-105, M1a, M1b and M1c in hydrolyzed equine urine, *Cunninghamella elegans* incubates and equine liver microsome incubates after analysis with UHPLC-HRMS. Figure adapted from Paper I [46].

In vitro incubation models

In vitro incubation models are a great complement to the in vivo administration studies, not only for the possibility to gain information regarding the drug metabolite profile, but also for economical and ethical reasons. Working both with equine and human liver models such as microsomes have already proved to be a very useful way to investigate drug metabolite profiles in previous studies [41,76,77]. The possibility to use microsome incubates as reference material in doping control, make these incubation models of extra interest [58].

The use of in vitro incubations models as a tool to investigate the drug metabolite profile was first presented in Paper I, where incubations were performed with equine and human liver microsomes and S9 fractions. The in vitro incubations showed some overlap with the in vivo metabolite profile, especially regarding the phase I metabolites as seen in Figure 5, where all the monohydroxylated metabolites of ACP-105 that were detected in equine hydrolyzed urine, could also be detected in the equine microsome incubates.

Some limitations with the used in vitro incubations models are that the time profiles have not been investigated. In vivo, the drug metabolites are generally excreted over time, in comparison with an incubation in a vial or flask, where the metabolites are not transported away and more complex metabolites with several metabolic transformations are formed over time in a way that might not always be comparable to the in vivo behaviour.
Optimization utilizing experimental design

After working with the metabolite profiling of ACP-105 in Paper I and seeing the overlap between the in vivo and in vitro metabolite profile, especially for the microsome incubates, I realized the great potential they could have. This led us into the optimizations of microsome incubation conditions that were described in Paper III and Paper IV. During a systematic optimization utilizing experimental design, multiple factors are changed at the same time and a wider experimental area is investigated, which aids finding a more optimal setpoint compared to changing one factor at a time [78–80].

**Figure 6.** Following the systematic optimization of human liver microsome incubation conditions, these figures represent the response contour plots of **A**, the monohydroxylated metabolite M4 and **B**, the monohydroxylated metabolite M5 of ACP-105 from an experimental design optimization of human liver microsome incubation settings. The response is defined as the chromatographic peak area for each metabolite and the plot was set at the optimal set point for ACP-105 concentration (33 µM) in the microsome incubates. The color gradient and peak area notations shows how the yield of each metabolite was affected by the different settings. Figure from Paper III [71].

The optimization was performed utilizing a design of experiments approach with the software MODDE® Pro 13 (Sartorius Stedim Data Analytics AB, Umeå, Sweden) and aimed towards increasing the drug metabolite yield. From these optimizations performed, several adjustments from the initial incubation method were made and the yield of the drug metabolites were increased a lot in both Paper III and Paper IV.

For the optimization in Paper III, the incubation parameters with the largest impact were the incubation time, concentration of the substrate ACP-105 and the concentration of microsomes. As seen in Figure 6, an increased incubation time had a positive impact on both metabolites, where as the change in microsome concentration had the opposite effect on the yield of the two metabolites. The lowered yield of the monohydroxylated metabolite M4 that was seen when the microsome concentration was increased, can be
explained by a simultaneously increased yield of two dihydroxylated metabolites. The higher microsome concentration seems to have stimulated further hydroxylation of especially one of the monohydroxylated metabolites (M4). Following the systematic optimization, the yield of the two monohydroxylated metabolites of ACP-105 were increased by 24 and 10 times.

The experimental objective in the two different optimization projects was both towards increased yield of drug metabolites, but for different types and number of metabolites. In comparison, in Paper III, the targets were two monohydroxylated metabolites and in Paper IV, the targets consisted of a wider variety and large number of drug metabolites for each substance, also including transformation products such as dihydroxylated and dephenylated metabolites. The reasoning for this, was to also take in to consideration more complex metabolites and optimize based on a large portion of the drug metabolite profile.

Paper IV describes the systematic optimization of microsome incubation conditions that was performed for a total of six substances from different doping classification groups. The effect different incubation settings have on the yield of drug metabolites of roxadustat, a prolyl hydroxylase inhibitor that affects the erythropoiesis, can be viewed in Figure 7.

**Figure 7.** Response contour plot for A, the monohydroxylated metabolite (M1) and B, the dihydroxylated metabolite (M2) of roxadustat. The x-axis displays the change in response due to the concentration of β-NADPH and the change in response due to the difference in incubation time is shown on the y-axis. The response is defined as the chromatographic peak area for the respective metabolites. The color gradient shows how the yield of each metabolite was affected by the different incubation settings. Figure from Paper IV.

In total, the drug metabolite yield for the different substances investigated in the study described in Paper IV were 3-18 times higher after the optimization. The main conclusion from this study is that the optimal incubation conditions for improved drug metabolite yield are generally individual for different
substances, showing the need for investigation of the incubation parameter settings to get an optimal yield when analyzing new compounds.

Sample preparation

The sample preparation is of great importance, but it is also crucial to think about what the aim with the sample preparation is. Generally, for the studies that have been performed in Paper I-IV, the aim has been to investigate the metabolite profile of a certain drug. With this in mind, it is important to think about the fact that these metabolites will have different physicochemical properties due to their differences in structure. Thereby the general aim is to do a clean-up of the sample and preferably maybe also some up-concentration, but without the risk of making the sample preparation too specific where you risk excluding metabolites of interest.

If possible, the samples have been put through several different types of sample preparation to get as much information out as possible. Since we do not know exactly what we are looking for, there is always a risk of losing analytes of interest during the sample preparation. With urine, a dilute-and-shoot approach has often been used as a minimum sample preparation method. With that approach the sample is generally centrifuged to remove any particles and diluted at a ratio of 1:1 with aqueous formic acid (0.1 %). For plasma and in vitro incubates, a protein precipitation with ice cold acetonitrile at a ratio of 1:4 has been used.

In doping control, it is common to mainly search for phase I metabolites, so these types of drug metabolites are of great importance. Therefore, the urine samples have also been put through hydrolysis with β-glucuronidase. For further sample preparation a SPE has been performed on both unhydrolyzed urine and hydrolyzed urine. The SPE cartridges used were either Oasis HLB or Oasis Prime HLB from Waters (Milford, MA, USA), this type of cartridge has a hydrophilic lipophilic balance (HLB) making in suitable also for more polar compounds [81].

LC-MS analysis

At the start of my PhD studies, prior to starting with the analysis of biological samples from administration studies, the main LC-MS method was selected and, in some parts, developed as described below. The separation was performed with a Vanquish UHPLC system from Thermo Fisher Scientific (Waltham, MA, USA). The selection of analytical column was made based on the fact that a wide range of analytes were planned to be analyzed, especially analytes of a more polar type of range, since the drug metabolite profiles were of interest.
The HSS T3 column from Waters (Milford, MA, USA) was therefore selected since it has a reversed phase C\textsubscript{18} type of stationary phase with a trifunctional end-capping for increased retention of polar compounds [82,83]. The selection of mobile phase composition and gradient were based on several aspects, but since this method was not planned to be used in routine analysis, time efficiency was not of the essence. Of course, there is no need for an unnecessarily time-consuming method, but the choice of a linear gradient and a total analysis time over 20 minutes were made so that the separation method would be suitable for a wide range of analytes. By using a similar separation method for the different research projects, this has simplified the comparison of samples and results over time.

The UHPLC system was coupled to a Q Exactive Orbitrap benchtop mass spectrometer with a heated electrospray ionization probe (HESI-II) from Thermo Fisher Scientific (Waltham, MA, USA). Utilizing the high resolution mass spectrometry technology with an instrument such as an Orbitrap is a key element for the drug metabolite investigation process [84]. The instrument was used in both positive and negative electrospray ionization mode and several different methods such as full scan methods and MS/MS methods were used.

**Structural elucidation using mass spectrometry**

The structural elucidation of drug metabolites is an important step to be able to determine if the detected analyte is a drug metabolite originating from the parent compound of interest. The structural elucidation process has really benefited from the development of the high resolution mass spectrometry instruments and the possibility for very accurate mass measurements [85–87].

The level of identification of drug metabolites when working with mass spectrometry and MS/MS spectra generally reaches a tentative identification level, as presented by Schymanski et al. [88]. For the tentative identification level, the evidence that a drug metabolite is for example a monohydroxylated version of a parent compound is quite strong, but that the exact position of the new hydroxy group is not fully determined. For a fully confirmed identification, comparison with a synthesized and characterized reference standard is needed, something that is often not available at the time when novel drug metabolites are presented. To be able to elucidate the exact position of the metabolic transformations, analysis with nuclear magnetic resonance (NMR) spectroscopy is often needed for a full identification.

Regarding the structural elucidation process of LGD-3303 and drug metabolites thereof described in **Paper II**, a comparison between the fragmentation of the parent compound LGD-3303 and one of its metabolites, a monohydroxylated phase I metabolite is shown in Figure 8. The fragmentation supports the fact that the metabolite originates from the parent compound since the
fragments m/z for the metabolite matches the ones from LGD-3303 but with a +15.995 difference, indicating the presence of an extra oxygen in the structure. The exact position where the hydroxylation has taken place is though not as straightforward to identify for the more rigid type of structures with larger ring systems, such as LGD-3303, where the exact position of metabolic transformations is difficult to place just based on the fragmentation pattern. This is mainly due to the fact that the ring system structure stays quite intact when the fragmentation energy is applied, and if a higher level of fragmentation energy is applied, the ring system completely reforms or break down into very small fragmental parts instead.

**Figure 8.** The suggested fragmentation showing the chemical structures of A, LGD-3303 with the precursor ion C_{16}H_{15}ClF_3N_2O^+ at m/z 343.0823 and B, the monohydroxylated metabolite M1 of LGD-3303 with the precursor ion C_{16}H_{15}ClF_3N_2O_2^+ at m/z 359.0772, C, the MS/MS spectrum of LGD-3303 and D, the MS/MS spectrum of the monohydroxylated metabolite M1. Figure from Paper II [37].

The drug metabolite profile of ACP-105 was first investigated in vivo in horses as described in Paper I. The structural elucidation of the metabolites gave a bit stronger indication towards the location of the metabolic transformations on the structures than what could be seen for LGD-3303. The suggested fragmentation patterns for ACP-105 and one monohydroxylated metabolite of ACP-105 is presented in Figure 9.

The fragmentation pattern firstly indicates that the hydroxylation has taken place on the aliphatic moiety of the structure and not on the aromatic moiety, which also has been previously suggested by Thevis et al. [47]. This is explained by the identification of several fragments; m/z 167, m/z 179, m/z 193 and m/z 233 that can be found in the MS/MS spectra of both LGD-3303 and
the monohydroxylated metabolite, all indicating that the aromatic moiety and the aliphatic parts closest to the aromatic moiety remains unchanged. For the remaining fragments, there is generally a difference of $-2 \text{ m/z}$ for the fragment from the metabolite analysis compared to the fragmentation of the parent compound. This can be explained by loss of an additional hydroxy group, but in the form of $\text{H}_2\text{O}$, for these fragments.

With all the fragmentation information together, it indicates that the hydroxylation on the metabolite has taken place in close proximity to the aliphatic hydroxy and methyl group. So, in this case, the location of the metabolic transformation can be more closely located that what could be done for the LGD-3303 metabolite.

**Figure 9.** Suggested fragmentation pattern of **A**, ACP-105 with the precursor ion $\text{C}_{16}\text{H}_{20}\text{ClN}_2\text{O}^+$ at $m/z$ 291.1262 and **B**, a monohydroxylated metabolite of ACP-105 with the precursor ion $\text{C}_{16}\text{H}_{20}\text{ClN}_2\text{O}_2^+$ at $m/z$ 307.1209. The fragmentation is based on MS/MS spectra from UHPLC-HRMS analysis with positive ionization of equine hydrolyzed urine. Figure adapted from Paper I [46].

When investigating the equine phase II metabolites of LGD-3303 in **Paper II**, a total of five glucuronidated metabolites, where both the parent compound and phase I metabolites had gone through glucuronic acid conjugations, were detected in unhydrolyzed urine. As seen in **Figure 2**, one of the
monohydroxylated glucuronide metabolites (M5c) was detected in both unhydrolyzed and hydrolyzed urine which is interesting since none of the other phase II metabolites were detected in urine after hydrolysis with β-glucuronidase.

We have suggested that the reasoning for the insensitivity towards β-glucuronidase may be due to the presence of the trifluoromethyl group and potentially that the hydroxy group is located nearby the trifluoromethyl group. Something that supports this, is the fact that a similar effect was seen by Hansson et al. [36] where a glucuronidated metabolite of the SARM LGD-4033, that also contains trifluoromethyl groups, as seen in Figure 10, also was mainly unaffected by β-glucuronidase. This would be very interesting to investigate further, but for that, the exact position of both the hydroxylation of LGD-3303 and the glucuronide conjugation position of both LGD-3303 and LGD-4033 needs to be further investigated.

To find the exact positioning of these transformations there is a need for enough purified material of the drug metabolites, so it is enough to be able to analyze with NMR spectroscopy. This can either be done by organic synthesis, or through purification from either administration samples or incubation models. Potentially C. elegans could be used for this, since it has been shown to produce glycosylated metabolites that after oxidation can yield relevant glucuronidated metabolites [72].

**Figure 10.** Comparison of the chemical structures of the two SARMs LGD-3303 (left) with the elemental composition C_{16}H_{14}ClF_{3}N_{2}O and LGD-4033 (right) with elemental composition C_{14}H_{12}F_{6}N_{2}O. Figure from Paper II [37].

**Multivariate data analysis applications**

After investigation of the drug metabolite profiles, as described in Paper I and Paper II, that were mainly achieved with a manual approach by searching for known and probable metabolic transformation products in combination with comparisons with reference and control samples, a more systematic search approach piqued my interest.
When working with high resolution mass spectrometry, it creates a large amount of data and utilizing MVDA methods such as PCA and OPLS-DA can be most useful when analyzing the raw data [68,69,89–92]. It has not been very common to utilize MVDA techniques in doping control research, especially regarding drug metabolite profiling, but the possibility to incorporate these methods in a similar way as is done within the metabolomics research field, could greatly improve the drug metabolite search process, both by saving time and minimizing the risk of missing analytes of interest due to a more untargeted approach [93].

For the study described in Paper III, MVDA was used as a search tool for aiding the identification of the in vitro metabolite profile of ACP-105. Four different in vitro incubation models were analyzed, which consisted of human liver microsomes and S9 fractions with or without phase II co-factors. For each model, two sample groups with eight replicates in each were set up. ACP-105 was added to one of the groups while the other one was used as a control group without ACP-105. For each model, a quality control (QC) sample was created by transferring an aliquot from each of the 16 samples to the same vial prior to analysis. The samples were thereafter analyzed with UHPLC-HRMS, the corresponding QC sample was injected repeatedly prior to analysis of each batch to gain system stability, thereafter the analysis order of the samples was randomized for each model and the QC sample was also analyzed repeatedly during each batch to be able to assess the instrumental stability.

As a part of the MVDA process, the raw data files need to be converted and pre-processed [94]. The main part of the pre-processing was performed with mzMine 2 where the aim is to identify features from the raw MS data. Each feature will consist of a chromatographic peak at a specific retention time and with a specific $m/z$ value [95,96]. The pre-processing goes through several different steps such as peak detection, chromatogram building, peak alignment and gap-filling [97].
Figure 11. Principal component analysis (PCA) of the two in vitro incubation models of ACP-105 with A, phase I human liver microsome incubations and B, phase II human liver microsome incubations. QC represents the quality control sample, T are samples where ACP-105 has been added to the incubates and C are samples where no substance has been added to the incubates. Figure adapted from Paper III [71].

After the identification of features, the data was further analyzed in SIMCA 17 (Sartorius Stedim Data Analytics AB, Umeå, Sweden). In SIMCA, the data was Pareto scaled and a PCA with two components was created. The PCA is an unsupervised method and thereby a good start to get an overview of the data. As seen in Figure 11, the biggest difference between the samples with and without ACP-105 is seen in the first principal component for both displayed models.
Figure 12. Multivariate data analysis process of results from incubations of ACP-105 with human liver microsomes and phase II co-factors showing A, the orthogonal projection to latent structures discriminant analysis (OPLS-DA) and B, the corresponding S-plot. The rectangular marking in the S-plot represent the features of interest that were selected for further investigation. Figure adapted from Paper III [71].

An OPLS-DA was thereafter performed for each model and mainly the S-plot was used for investigation of features of interest as seen in Figure 12. OPLS-DA is a supervised method and can be prone to overfitting. It is always important to make sure you validate your findings prior to drawing too big conclusions from the results. In this case, features with a confidence ≥ 0.90 in the S-plot were selected as features of interest and the drug metabolite search was based on this selection.

For all the four models, a large portion of the selected features could be identified as drug metabolites of ACP-105. The metabolite profile of ACP-105 in human urine was also investigated and after comparison of the in vivo metabolite profile with the metabolites identified in vitro with the MVDA approach, all in vivo metabolites could be found in vitro with the MVDA search tool as seen in Figure 3, clearly showing the relevance and usefulness.

Analytical targets

The purpose of analytical targets in doping control is to be able to detect illicit use of drug substances. There are several important parts when suggesting new analytical targets, for one, the addition of a new target analyte does not stop or hinder the use of the current target analytes.

Drug metabolites can be used as improved analytical targets for doping control, but to be useful, they need to improve the window of detection, either
by being detected at a higher intensity or for a longer time after administration as seen for several equine drug metabolites of ACP-105 in Figure 13.

Figure 13. Time profile of ACP-105 and drug metabolites thereof in equine hydrolyzed urine. Samples taken 6-96 hours after administration of ACP-105. Figure adapted from Paper I [46].

Apart from the improved window of detection, it is of great importance that there is access to reference material, which is needed to be able to make a positive identification. A synthesized and characterized standard is preferable [98]. But what is also allowed is the use of samples from administration studies and also liver cells or microsome incubates [58]. This means that if a substance has been used in an administration study and drug metabolites thereof have been identified, samples from that study may be used. In the same way, if a drug metabolite has been identified in a microsome incubate after incubation with the parent compound, the incubate may be used as reference material.

To be able to fully utilize and implement drug metabolites as new analytical targets I believe that it is very important to value and catalogize the research material and methods that have been used so that the information can be more readily available. For the projects included in this thesis, there are new analytical targets suggested for equine doping control, as described in Paper I where several metabolites of ACP-105 are recommended as analytical targets. From this study there is both equine urine and plasma from administration sample available, together with the knowledge that the monohydroxylated target metabolites can be produced through microsome incubations.

In Paper II, new targets for equine doping control of LGD-3303 is presented and urine and plasma from administration studies are available as reference material. In Paper III, several human drug metabolites of ACP-105 were identified and there is access to both human urine from the administration study and the knowledge that all drug metabolites identified in vivo could
be produced with microsome incubations. And in Paper IV, where we described the drug metabolite profile for several drugs, it is shown that they can be produced with microsome incubations.

If there is no access to reference material for the investigated drug metabolites, it is still possible to add them as screening targets in doping control, and if a positive hit is made, a more targeted analysis focusing on the parent compound could be performed.
Concluding remarks and future perspective

Several new analytical targets for equine doping control analysis have been identified and suggested in Paper I and Paper II. The suggested analytical targets for ACP-105 and LGD-3303 are drug metabolites of each substance that are present for a longer time than the administered substances, prolonging the detection window for equine doping analysis.

In Paper III, a MVDA approach for metabolite identification was developed, which simplifies the search for drug metabolites. In the same study, the human in vivo metabolite profile of ACP-105 was investigated for the first time and new improved analytical targets were suggested.

In Paper III and Paper IV, the systematic optimization of microsome incubation conditions utilizing Design of experiments resulted in increased yield of drug metabolites, and the results also show that the optimal conditions are substance dependent.

The studies performed in this PhD project have together had a positive impact on the area of doping analysis since all have in some way showed either improved analytical targets or improved the search process to identify them.

For further studies, since new drugs are regularly emerging, especially substances such as SARMs that are not approved and often available in supplements that can be purchased on the Internet, there is a great need to continue investigating the metabolite profile of these substances [99]. Looking ahead, working with and implementing MVDA techniques for more in vivo studies and also to implement a more untargeted approach looking for changes in doping control would be very interesting.

Dopingkontroll inom sport är viktigt av flera anledningar, både för att idrotts- tävlingar ska vara rättvisa, men det finns även en viktig etisk aspekt, speciellt vad gäller djurskydd vid tävlingar som involverar djur.


Generellt ska alla läkemedel som tagits upp lämna kroppen och för att det ska ske så bryts ämnen ofta ner eller omvandlas till något som är enklare att uttömmas, ofta en mer vattenlös varianta. Dessa nedbrytningsprodukter eller så kallade metaboliter kan då alltså finnas kvar i kroppen längre, eller i en högre halt än den intagna substansen. Det vanligaste vid dopinganalys är att man undersöker urin och blod och om man då inte bara söker efter den substansen som är förbjuden, utan även söker efter dessa metaboliter, så är det möjligt att öka sannolikheten för att identifiera om någon har tillfört ett olagligt preparat.

För att kunna identifiera vad som finns i ett biologiskt prov så krävs det någon typ av analysmetod eftersom det inte går att se med blotta ögat. I detta fall så är den metod som har använts vätskechromatografi kopplad till högupplösende masspektrometri (LC-HRMS). Vätskechromatografi används för att separera beståndsdelarna i provet. Separationen sker genom att provet injiceras i ett vätskeflöde som sedan passerar vad som kallas en analytisk kolonn där dom olika beståndsdelarna i provet kommer att fastna olika mycket på denna kolonn och därför ta olika lång tid på sig att ta sig fram. Flödet går sedan vidare med provets beståndsdelar som blivit lite mer separerade till en detektor.
som kallas masspektrometer där man med hjälp av ett elektriskt fält skapar laddade joner. Jonerna tar sig sedan vidare in i detektorn och beroende på jonernas egenskaper, alltså storlek, så kommer dessa att bete sig olika samt oscillera med olika frekvens. Detta kan sedan översättas till ett m/z-värde som då motsvarar jokens massa dividerat på dess laddning. För dom mindre analytter som jag studerat är dessa joner så pass små att det är vanligast att dom bara har en laddning och inte flera. Med hjälp av detta m/z-värde kan jag då få reda på hur mycket varje analyt väger på ett väldigt exakt sätt, då dom teoretiskt möjliga beståndsdelarna är inte speciellt många när man har så noggranna värden.

Förutom möjligheten att detektera hur mycket dom väger så kan man även med hjälp av energi som appliceras slå sönder analytterna i mindre bitar och de bildar då typiska fragment, alltså mindre beståndsdelar och man kan då ta reda på ytterligare hur det som är intressant faktiskt ser ut.

I mina forskningsprojekt har jag främst arbetat med substanser som tillhör gruppen Selektiva androgena receptor-modulerare (SARM). Dom har en liknande effekt som klassiska anabola steroider, alltså muskelbyggande etc., men uppvisar ofta mindre biverkningar vilket gör dessa intressanta ur ett dopingperspektiv.


Flera av dom intressanta metaboliterna som hittades i proverna från häst, kunde även ses i proverna från labb-modellerna, vilket visar att det finns en tydlig överensstämmelse mellan dessa. Totalt sett så kunde ACP-105 detekteras fram till 48 timmar efter att substansen hade intagits, medan flertalet metaboliter kunde detekteras även vid den sista provtagningen som skedde efter 96 timmar och därigenom visar hur viktigt det är att även leta efter metaboliter
vid dopingkontroll. I Artikel II presenteras en liknande administrationsstudie på häst, men av en annan SARM som heter LGD-3303 och även där kunde det påvisas att sökningen efter nedbrytningsprodukter förlängde detektionstiden efter att substansen hade intagits.


Sammanfattningsvis har detta arbete resulterat i identifiering av flera nya analytiska måltavlor i form av metaboliter, som ökar möjligheten att detektera illegal användning av substanser i dopingsammanhang. Optimeringen av mikrosom-inkuberingar är även en stor hjälp då dessa prover är godkända att använda som referensmaterial vid dopinganalys och det utvecklade sökverktyget underlättar identifieringsprocessen av metaboliter. Alla dessa bitar tillsammans, ger möjligheten till en positiv inverkan på dopinganalys-området.
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