



UPPSALA
UNIVERSITET

*Digital Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Pharmacy 251*

Structural Determination of Drug Metabolites from Doping Classed Compounds Using Mass Spectrometry

ANNELIE HANSSON



ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2018

ISSN 1651-6192
ISBN 978-91-513-0276-8
urn:nbn:se:uu:diva-344310

Dissertation presented at Uppsala University to be publicly examined in B:42, BMC, Husargatan 3, Uppsala, Friday, 4 May 2018 at 09:15 for the degree of Doctor of Philosophy (Faculty of Pharmacy). The examination will be conducted in English. Faculty examiner: Professor Peter Hemmersbach (School of Pharmacy, University of Oslo).

Abstract

Hansson, A. 2018. Structural Determination of Drug Metabolites from Doping Classified Compounds Using Mass Spectrometry. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy* 251. 58 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-0276-8.

Doping control in equine sports is important for a fair competition, but also to ensure the integrity of the betting system, as well as for animal welfare reasons. To detect the use of illicit compounds, screening for the parent compound is common. However, by using a metabolite as the analytical target instead, the detection time can be prolonged. For some compounds, the use of a metabolite is a necessity since the parent drug may not be detected at all.

The metabolites of the selective androgen receptor modulators (SARM) S1, S4 and S22 were investigated in horse urine and plasma. The unchanged parent compounds had the longest detection time in plasma, but were not detected at all in urine. Instead, the longest detection time was measured for the metabolites 2-amino-5-nitro-4-(trifluoromethyl)phenyl hydrogen sulfate (SARMs S1 and S4) and 2-amino-5-cyano-4-(trifluoromethyl)phenyl hydrogen sulfate (SARM S22). These metabolites were thus suggested as analytical targets for doping control in urine while the parent compounds were suggested for plasma samples. 2-amino-5-nitro-4-(trifluoromethyl)phenyl hydrogen sulfate could also be produced in large quantities by the fungus *Cunninghamella elegans* to potentially be used as reference compound.

The horse metabolites of the SARM LGD-4033 were also studied in urine and plasma. The formate adduct of LGD-4033 had the longest detection time in plasma and in urine after hydrolysis with β -glucuronidase. In non-hydrolyzed urine, the glucuronidated LGD-4033 was detected instead.

Different *in vitro* models were used to predict *in vivo* metabolites of roxadustat, a hypoxia-inducible factor stabilizer. *Cunninghamella elegans* was successful in producing more metabolites compared to human and equine liver microsomes and human hepatocytes.

The metabolite detection and identification in all experiments were accomplished using a UHPLC-Q-TOF MS instrument, where the high-resolution MS data was vital in determining which metabolites were formed.

The thesis shows the benefits of investigating the metabolites of doping substances to allow for a successful doping control method in horse urine and plasma by prolonging the detection time. It also highlights the usefulness of *Cunninghamella elegans* as an alternative to the more commonly used *in vitro* models for both predicting and producing metabolites.

Keywords: mass spectrometry, UHPLC-MS/MS, doping control, *Cunninghamella elegans*, selective androgen receptor modulator, SARM, andarine, ostarine, LGD-4033, roxadustat, HIF stabilizer

Annelie Hansson, Department of Medicinal Chemistry, Analytical Science, Box 574, Uppsala University, SE-751 23 Uppsala, Sweden.

© Annelie Hansson 2018

ISSN 1651-6192

ISBN 978-91-513-0276-8

urn:nbn:se:uu:diva-344310 (<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-344310>)

*It is a mistake to think you can solve any
major problems just with potatoes.*

— Douglas Adams

List of Papers

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

- I **Hansson, A.**, Knych, H., Stanley, S., Thevis, M., Bondesson, U., Hedeland, M. (2015) Characterization of equine urinary metabolites of selective androgen receptor modulators (SARMs) S1, S4 and S22 for doping control purposes. *Drug Testing and Analysis*, 7:673-683.
- II **Hansson, A.**, Knych, H., Stanley, S., Thevis, M., Bondesson, U., Hedeland, M. (2016) Investigation of the selective androgen receptor modulators S1, S4 and S22 and their metabolites in equine plasma using high-resolution mass spectrometry. *Rapid Communications in Mass Spectrometry*, 30:833-842.
- III **Hansson, A.**, Knych, H., Stanley, S., Berndtson E., Jackson, L., Bondesson, U., Thevis, M., Hedeland, M. (2018) Equine *in vivo*-derived metabolites of the SARM LGD-4033 and comparison with human and fungal metabolites. *Journal of Chromatography B*, 1074-1075:91-98.
- IV **Hansson, A.**, Thevis, M., Cox, H., Miller, G., Eichner, D., Bondesson, U., Hedeland, M. (2017) Investigation of the metabolites of the HIF stabilizer FG-4592 (roxadustat) in five different *in vitro* models and in a human doping control sample using high resolution mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, 134:228-236.

Reprints were made with permission from the respective publishers.

Additional papers not included in this thesis

Following papers are not included in this thesis. They are referred to in the text by their Arabic numerals.

1. Salomonsson M.L., **Hansson A.**, Bondesson U. (2013) Development and in-house validation of a method for quantification of BMAA in mussels using dansyl chloride derivatization and ultra performance liquid chromatography tandem mass spectrometry. *Analytical Methods*, 5:4865–4874.
2. Rydevik, A., **Hansson, A.**, Hellqvist, A., Bondesson, U., Hedeland, M. (2015) A novel trapping system for the detection of reactive drug metabolites using the fungus *Cunninghamella elegans* and high resolution mass spectrometry. *Drug Testing and Analysis*, 7:626-633.
3. Thevis, M., Lagojda, A., Kuehne, D., Thomas, A., Dib, J., **Hansson, A.**, Hedeland, M., Bondesson, U., Wigger, T., Karst, U., Schänzer, W. (2015) Characterization of a non-approved selective androgen receptor modulator drug candidate sold via the Internet and identification of *in vitro* generated phase-I metabolites for human sports drug testing. *Rapid Communications in Mass Spectrometry*, 29:991–999.
4. Garg, N., **Hansson, A.**, Knych, H.K., Stanley, S.D., Thevis, M., Bondesson, U., Hedeland, M., Globisch, D. (2018) Structural elucidation of major selective androgen receptor modulator (SARM) metabolites for doping control. *Organic & Biomolecular Chemistry*, 16:698-702.

Contents

Introduction.....	9
Drug metabolism.....	11
<i>In vivo</i> and <i>in vitro</i> metabolism.....	12
<i>Cunninghamella elegans</i>	13
Mass spectrometry and mass analyzers	15
The investigated compounds.....	18
Selective androgen receptor modulators	18
Roxadustat.....	20
Aim	21
Sample preparation	22
Sample cleanup	22
Enzymatic hydrolysis.....	23
Chromatographic separation	24
High-resolution mass spectrometry in metabolite identification	26
Administration studies in horses.....	33
Metabolites from SARMs of the aryl propionamide class	33
Quantification of SARM S1 in plasma.....	36
<i>In vivo</i> and <i>in vitro</i> metabolites of LGD-4033	37
Metabolite detection in urine compared to plasma.....	41
<i>In vitro</i> metabolites of roxadustat	42
Metabolite production using <i>Cunninghamella elegans</i>	45
Summary and conclusion.....	47
Populärvetenskaplig sammanfattning	49
Acknowledgement	52
References.....	54

Abbreviations

<i>C. elegans</i>	<i>Cunninghamella elegans</i>
CYP	Cytochrome P450
ELM	Equine liver microsomes, horse liver microsomes
EMA	European Medicines Agency
ESI	Electrospray, electrospray ionization
FDA	U.S. Food & Drug Administration
HIF	Hypoxia-inducible factor
HLM	Human liver microsomes
IFHA	International Federation of Horseracing Authorities
LC	Liquid chromatography
LLE	Liquid-liquid extraction
MS/MS	Tandem mass spectrometry
MS	Mass spectrometry
MS ^E	A mass spectrometric analysis mode
<i>m/z</i>	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
PHD	Prolyl hydroxylase domain
Q	Quadrupole
QC	Quality control
SARM	Selective androgen receptor modulator
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
TOF	Time-of-flight
UDP	Uridine 5'-diphosphate
UHPLC	Ultra-high performance liquid chromatography
WADA	World Anti-Doping Agency
XIC	Extracted ion chromatogram

Introduction

Doping is described as a way to illicitly improve the performance of athletes by the use of a technique or administration of compounds with a pharmacological effect. As early as 1666, a regulation prohibited administration of stimulating substances to horses racing in Worksop.^[1] But even so, the practice of doping horses, and humans, has been around for longer.

In the beginning of the 20th century, the doping of horses was becoming a major issue that threatened the faith in the wagering system, and to combat this, doping control of the animals was initiated.^[1] One of the first doping control methods entailed sampling of horse saliva. Both the sampling and the method itself were very convoluted, but it was successful in curbing the use of stimulants in horses.^[1,2] So historically, it was the doping control in animals, and horses in particular, that prompted the development of anti-doping methods to move forward.

Nowadays, focus in the media has shifted towards human doping control and doping in sports has gained much more publicity, especially after the systematic doping of athletes in Russia was exposed. Now, questions have been raised if sports were ever clean, and if it will be possible to stop those who want to cheat.

Every year, the World Anti-Doping Agency (WADA) issues a list of prohibited methods and compounds, including analogs, prohibited in human sports.^[3] For non-human athletes like horses, there is no worldwide organization like WADA. Instead, it is up to the authorities in each country to decide. However, many national racing authorities have adopted the same principles as the International Federation of Horseracing Authorities (IFHA).^[4] This guideline states that the horses are not allowed to compete with any of the prohibited substances in their system, and that all compounds with a pharmacological effect are prohibited.^[5]

Having a blanket statement that covers all compounds that have a pharmacological effect on the horse is necessary since they, unlike humans, are doped not only to run faster, but also to run slower.^[4,6] Whether the doping is deliberate to improve the horse's performance or due to sabotage from a competitor, it skews the odds and undermines the trust in the betting system. Animal welfare is another reason for this type of regulation. Injured animals are not allowed to race, so this also discourages the trainers and owners from masking lameness to let the horse race.^[6]

The number of compounds to test for in both horse and human doping control increase each year due to the continuous development of new drugs. Even though the drugs are mainly developed to treat diseases, some of them may have performance enhancing effects on healthy people. This makes them interesting from a doping control perspective.

Many of these compounds are available for purchase on the internet long before they were approved as pharmaceutical drug by regulatory authorities, such as the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA), or even after development was cancelled. They can thus be obtained, and used, by athletes that want to gain an edge over their competitors, albeit an unfair one. Because of the increasing number of new substances available, the analytical methods of doping laboratories need to be developed and improved constantly.

In doping control, the detection of a substance not only entails the parent compound itself, but can also include its metabolites. Using metabolites as targets can reduce the risk of outside contamination of the samples. Furthermore, it can potentially prolong the detection time. One example is from 2012, when the samples of athletes competing in the Olympic Games in Athens in 2004 were reanalyzed. The analysis methods had improved over time, and a new long-term metabolite from the androgenic anabolic steroid oxandrolone had been identified. This metabolite was detected in the samples from two medalists, which disqualified them from the competition, meaning that they also lost their medals.^[7,8] But in order to observe the metabolites, you first need to know what to look for.

Drug metabolism

When a drug is introduced to an organism, it undergoes metabolism so it can more easily be removed from the system. The metabolism entails making the compound more hydrophilic as to facilitate excretion, most often through urine, although it also occurs through other routes such as biliary or pulmonary excretion.^[9] In many cases, the metabolites can be detected for a longer period of time compared to the parent compound, since the biotransformation can occur at a faster rate than the excretion. This is something that can be utilized in doping control.

The metabolism can render a substance pharmacologically inactive and aid in detoxifying exogenous compounds.^[9-11] However, the pharmacological activity can also be increased, as with prodrugs, or retained after metabolism. There are also cases when the metabolite holds toxic abilities.^[9] Because of this, the metabolism of new drugs is investigated extensively, not only in humans but in other metabolic models as well, before being approved by the EMA or FDA.

Not only pharmaceutical drugs are subjected to metabolism. Most endogenous compounds and xenobiotics are metabolized to some extent. This includes pesticides and pollutants from the environment as well.

The enzymatic transformations can be divided into two groups, phase I and phase II.^[9,10,12] These transformations can either occur separately or be a combination of both phase I and phase II reactions. The major phase I modifications are hydroxylation, reduction, and hydrolysis. These are ways to introduce or reveal a functional group to the molecule, like -OH, -NH₂, or -COOH.^[9-11] Cytochrome P450 (CYP) is the most important group of enzymes for the phase I biotransformation of drugs. This group of enzymes is present in several different species including mammals like humans and horses.^[10,13]

In phase II metabolism, an enzyme-driven conjugation reaction occurs that adds a larger, hydrophilic structure, such as glucuronide, glucoside or sulfate, to the molecule.^[9,11] Different enzymes are responsible for the different transformations, for example the glucuronidation reaction is performed by the enzyme uridine 5'-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferase) while the sulfonation is performed by sulfotransferase enzymes.^[9]

Compared to the phase I reactions, which are similar between species, the phase II metabolic transformations show different patterns between different

species.^[10] One example is that horses are more prone to sulfonation of androgen anabolic steroids, while humans tend to form more glucuronides.^[13]

In vivo and *in vitro* metabolism

There are different ways to investigate the metabolism of a drug. For the purpose of equine doping control, the most accurate results will be achieved by investigating the metabolites in the horse, as was done for paper **I-III**, rather than using an alternative metabolic model. However, it is not always possible to perform *in vivo* experiments. The metabolism can thus be studied in metabolic models *in vitro*.

The liver is considered to be the primary organ for metabolic transformations, although other tissues like the kidneys, play an important role too.^[9,10,12,14] Due to its importance, liver tissue is often used as *in vitro* models for human metabolism. The most popular *in vitro* system is human liver microsomes (HLM) and is a well-established method for investigating the biotransformation of drugs. Both human and equine liver microsomes were used in paper **IV** to generate drug metabolites. HLM are easy to use and commercially available at a low cost, which adds to its popularity.

Liver microsomes are vesicles formed by pieces of the endoplasmic reticulum from hepatocytes and separated from other components by differential centrifugation. Most of the enzymes present are from the CYP family, but they also contain UDP-glucuronosyltransferase enzymes, which are involved in the phase II metabolism.^[12] But due to the lack of other phase II enzymes, liver microsomes are mainly useful for predicting phase I metabolites. Since it is an incomplete metabolic system, the results cannot be directly translated to the *in vivo* situation and the differences are both of qualitative and quantitative nature.^[12,14]

The liver can be used to prepare other metabolic models as well, such as hepatocytes or liver slices. Since these systems are more similar to the whole liver, they are supposed to give a result more like the situation *in vivo*. The drawback is that these models are more complicated to use.^[12]

Laboratory animals are also used as *in vivo* models to predict which transformations will occur in humans. In many cases, they give a more accurate representation compared to *in vitro* models.^[10,12,14] Still, there are species differences and no animal model replicate the metabolic pattern of humans perfectly.

The use of metabolic models in doping control can have multiple purposes. The *in vitro* models could be used to predict potential *in vivo* metabolites that could be used as analytical targets. If that is the case, then it is important to choose a model that has qualitative results similar to those *in vivo*.

Another use of metabolic models is for metabolite production. The metabolites could be used to aid in the characterization of *in vivo* metabolites, or as

reference compounds. Then the model has to be able to produce large amounts of the desired metabolite(s).

There are other metabolism models that do not require live animals or parts of organs. One example is electrochemical oxidation. By applying a voltage over a solution containing the compound of interest, the compound can be oxidized. But where enzymes are region-selective, this oxidation instead occurs on the least stable site of the molecule.^[15] A comparison of several pharmaceutical compounds showed that electrochemical oxidation has the ability to mimic CYP enzyme-catalyzed oxidation in some cases.^[16] Another example of an alternative metabolic model is incubation with the fungus *Cunninghamella elegans* (*C. elegans*).

Cunninghamella elegans

In 1974, Smith and Rosazza suggested that the metabolism of drugs could be investigated using different microbial models, both bacteria and fungi.^[17] The advantages include the possibility of producing larger amounts to be used for structural elucidation, as reference standards or in toxicity studies during drug development. It could also reduce the need for animal testing.^[14,17,18]

Fungi of the different *Cunninghamella* species have the ability to produce many of the same metabolites as those found in mammals. These fungi contain both CYP enzymes that control the phase I metabolism, as well as enzymes responsible for the phase II transformations, such as sulfotransferase and UDP-glucosyltransferase.^[17,19,20] It is this ability that has made these fungi useful as metabolic models for several different drugs, including roxadustat, which was investigated in paper **IV**.

The *Cunninghamella* species are filamentous fungi that are easily cultivated on broth. The fungus grows to cover the entire surface of the growth medium. The part in contact with the broth is similar to a cotton pad while the aerial mycelium forms a cotton-candy-like fuzz on top (*Figure 1*). After incubating the fungus in a spiked growth medium for a few days, the broth can be analyzed for metabolites.^[21] In this project, smaller batches were used (papers **III** and **IV**), but this can be scaled up for an increased metabolite production.^[22]

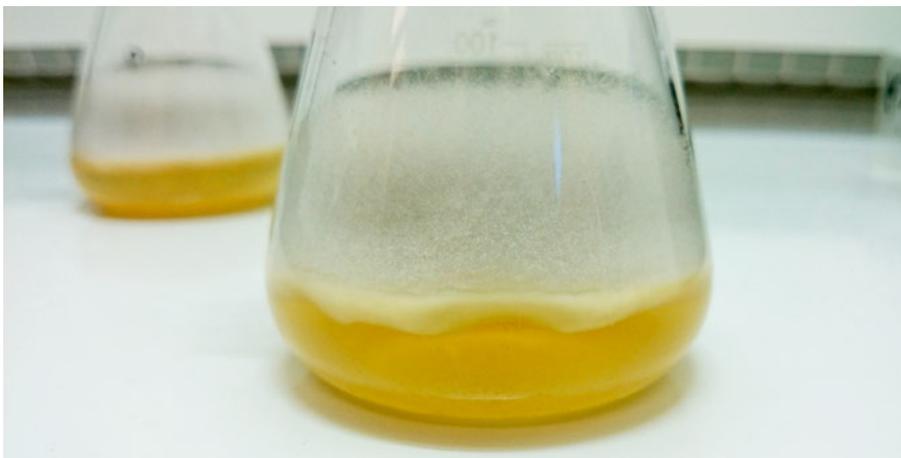


Figure 1. *Cunninghamella elegans* growing on top of broth in an Erlenmeyer flask.

Fungi of the *Cunninghamella* species have been used for a variety of purposes, such as an alternative *in vitro* model for predicting potential *in vivo* metabolites for compounds used illicitly by athletes like LGD-4033 (paper 3). Other drugs with different structures have also been investigated, like flutamide, clemastine, meloxicam, bupivacaine, and oxandrolone.^[7,21,23–25] It has been proven that the fungi can produce the same metabolites as those observed in mammals and it has also aided in the structural characterization of metabolites. In the case of oxandrolone, *C. elegans* was able to produce a long-term metabolite found in humans in large enough quantities to use as reference standard in doping control.^[7] Furthermore, this fungus has been suggested as a model to predict reactive metabolites (paper 2).

Mass spectrometry and mass analyzers

Mass spectrometry (MS) can be used to determine the structure of unknown compounds, such as drug metabolites. The MS instrument separates compounds based on their mass-to-charge ratio (m/z). There are different types of mass analyzers and depending on the principle by which the m/z is measured, different resolutions are achieved.

A high-resolution instrument facilitates accurate mass measurements, which makes it possible to determine the elemental composition of the ions, whereas this is not possible using a low-resolution instrument. This means that it can be measured if a shift in m/z of +16 is due to a hydroxylation reaction (exact m/z shift +15.9949) or the transformation of an ethyl group to a carboxyl acid (exact m/z shift +15.9585). This type of information is very valuable for characterization of unknown compounds.^[11]

The qualitative analyses included in this thesis (papers **I-IV**) were performed using high-resolution mass spectrometry. Separation of the samples was achieved using ultra-high performance liquid chromatography (UHPLC) and the mass spectrometers were hybrid instruments with a quadrupole (Q) mass filter followed by a time-of-flight (TOF) mass analyzer. The instrument consists of a quadrupole for mass filtration, a collision cell for fragmenting the ions, a pusher that accelerates the ions into the flight tube, a reflectron that compensates for the difference in kinetic energy between ions of the same mass, and a detector that detects the ions (*Figure 2*).^[26]

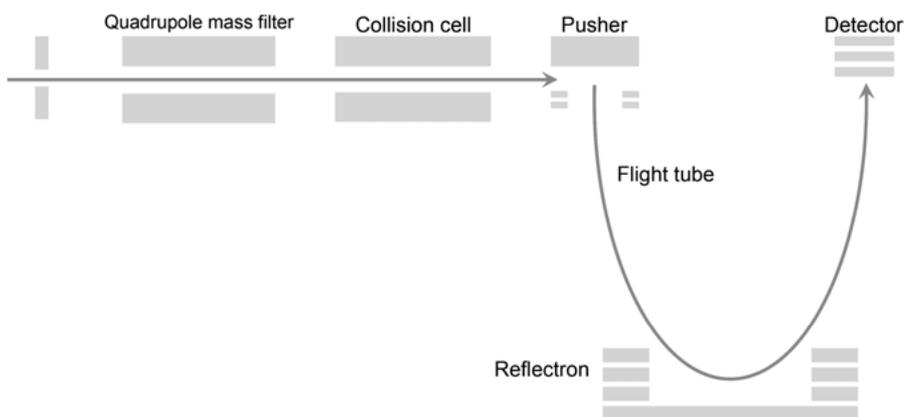


Figure 2. A schematic drawing of a Q-TOF instrument. Illustration by A. Hansson.

In TOF instruments, all ions in one push are analyzed at the same time. The m/z is measured based on the time it takes for the ions to reach the detector, the different flight times. The ions are accelerated to the same kinetic energy in an electric field before they enter the flight tube^[26]. Since the ions have the same kinetic energy, their different masses will lead to different velocities. This means that the ions with a lower mass will have a higher velocity and thus reach the detector before ions with a high mass (*Figure 3*).

Time-of-flight instruments are high-resolution mass analyzers and by using a longer flight tube, a higher resolution between the different m/z can be achieved. A longer flight tube gives a longer flight time, so that the ions have more time to separate from each other and smaller differences in m/z can thus be measured.

There is a vacuum in the flight tube, but it is not completely devoid of molecules other than the ions of interest. Some ions will thus collide with the gas molecules which prohibits them from reaching the detector. So even if a longer flight time gives a higher resolution, it is at the expense of a lower sensitivity.

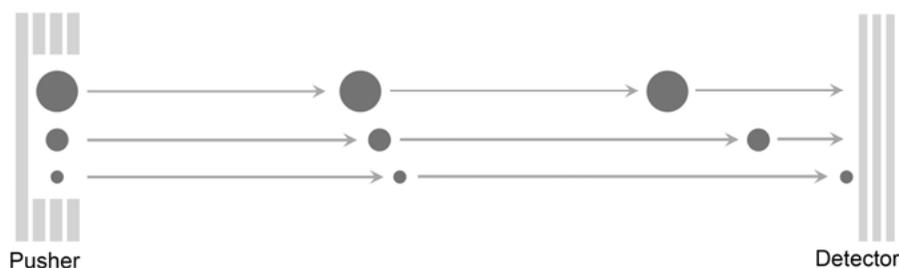


Figure 3. Illustration of the separation of ions in a TOF instrument based on their different m/z . Illustration by A. Hansson.

The quadrupole, present in both Q-TOF instruments and tandem quadrupole instruments, consists of four parallel rods and the ions travel in the space between them. The rods have a positive or negative potential applied to them, and the opposing ones have the same potential. The ions are travelling in the oscillating electric field between the rods and only ions with a certain m/z will have a stable trajectory that allows them to pass through. Ions of other m/z will collide with the rods (*Figure 4*).^[26]

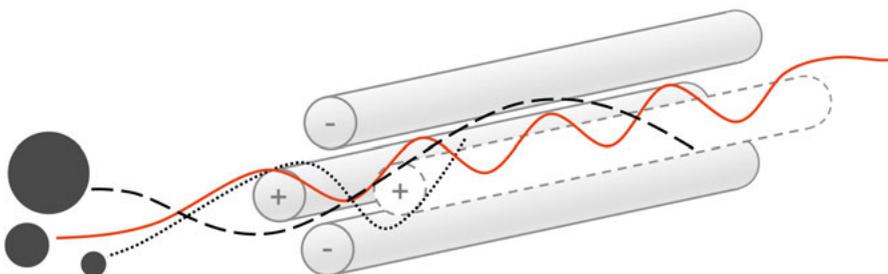


Figure 4. Illustration of a quadrupole mass analyzer. Illustration by A. Hansson.

Quadrupole instruments have low resolution and are not suited for structural elucidation of unknown compounds. They are, however, very good for quantitative work.^[11] Operating a tandem quadrupole instrument in selected reaction monitoring (SRM) mode gives both a high sensitivity and a high selectivity. In SRM, the first quadrupole is locked on a specific m/z corresponding to the analyte and the second quadrupole is set on an m/z corresponding to a product ion. The collision cell in between the quadrupoles fragments the ions. This means that only compounds with the correct m/z for both parent ion and product ion can pass. Since most background compounds are removed, this can reduce the noise significantly and allow for a low detection limit. This type of instrument was used for the quantification of SARM S1 in plasma (paper II).

The investigated compounds

In this thesis, five substances belonging to two different pharmacological classes were studied. The first class was selective androgen receptor modulators (SARMs) where the compounds SARM S1, S4, S22 were investigated in horses (papers **I** and **II**). The SARM LGD-4033 is of a different structural class and was also studied in the horse (paper **III**). The final compound is a hypoxia-inducible factor (HIF) prolyl hydroxylase domain (PHD) inhibitor called roxadustat and it was investigated *in vitro* (paper **IV**).

All five compounds are of interest from a doping control perspective. They are prohibited by WADA and human athletes have tested positive for both the SARMs and roxadustat.^[27–30] If these compounds are used in human sports, it is likely that they will be used on racehorses as well.

For the SARMs S1, S4, S22, and LGD-4033, there was little to no information regarding the metabolites in horses even though metabolites from human urine and *in vitro* models had been published. For roxadustat, there was no data on its metabolites published in any species prior to our work. At the time of writing this thesis, none of these compounds has been approved as a pharmaceutical drug by any regulatory authorities.

Selective androgen receptor modulators

Muscle atrophy and reduced bone density can be debilitating and severely reduce the quality of life for those affected. These symptoms can occur due to cancer cachexia, chronic illness or they can be age-related. Selective androgen receptor modulators are a pharmacological class of compounds that are under development as a medical treatment for both osteoporosis and muscle wasting disease.^[31–33] They are also investigated as a treatment for breast cancer^[34] and as a potential male contraceptive.^[31,32,35] These compounds are partial receptor agonists that stimulate muscle growth as well as increase bone density. However, they are tissue-selective and many of the side-effects normally associated with androgen therapy, such as prostate events, virilization, and cardiovascular disease, are reduced.^[31,33,34,36]

The combination of increased muscle mass, but reduced side-effects are also making SARMs interesting to athletes who want to improve their performance. Despite being banned from use both in and out of competition by WADA since 2008,^[37] several positive cases are reported each year.^[27–30]

As mentioned, there are several different structural classes of SARMs, for example aryl propionamides, quinolines, and bicyclic hydantoin. The investigated SARMs S1, S4 and S22 (Figure 5 A-C, papers I and II) belong to the aryl propionamide class, while LGD-4033 has a pyrrolidine-benzonitrile structure (Figure 5 D, paper III).^[31,38]

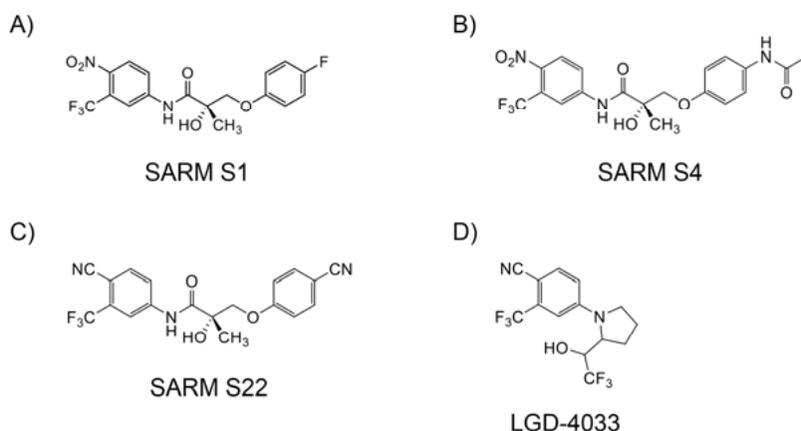


Figure 5. The chemical structures of the four selective androgen receptor modulators studied A) SARM S1, B) SARM S4, C) SARM S22, and D) LGD-4033.

The metabolism of the aryl propionamide class, including SARMs S1, S4 and S22, had previously been investigated both *in vivo* and in different *in vitro* models. The wide range of metabolic systems investigated comprised human liver microsomes (HLM) and S9 fraction^[39], horse liver microsomes (ELM)^[40], bovine liver S9 fraction^[41], the fungus *C. elegans*^[42], dog^[43], calf^[41], and human^[44,45]. Positive cases of SARM S4, sometimes referred to as andarine, had also been reported in human urine doping samples^[46,47] and the parent compound was also identified in a plasma sample from a racehorse^[48]. However, no metabolic study had been performed in the horse.

Of these three SARMs, only S22 is still in clinical trials. It is being developed under the name enobosarm and is currently in phase II trials as a treatment for breast cancer and stress urinary incontinence.^[49]

The SARM LGD-4033 is also referred to as VK5211, and in online web shops, it is sold under the names Ligandrol and Anabolicum. It is currently in phase II clinical trials for improving recovery after hip fracture.^[50] Its metabolites had also been investigated in a urine sample from a human athlete^[51], in HLM^[38,52], after electrochemical conversion, and after incubation with *C. elegans* (paper 3). Also in this case, no administration study with the purpose of identifying horse metabolites had been performed.

Roxadustat

Erythrocytes transport the oxygen in mammals and the production of these cells is regulated by the concentration of O₂. If the level is too low, the kidneys release erythropoietin, which in turn stimulates the erythropoiesis. This makes the kidneys important for an adequate oxygenation of the body.^[53]

Patients with chronic kidney disease often suffer from anemia due to erythropoietin deficiency. The standard treatment for these patients are intravenous or subcutaneous injections with erythropoietin stimulating agents.^[54] Not only has the safety of erythropoietin stimulating agents been questioned, the administration route is also inconvenient.^[54–57] An alternative oral treatment would thus be welcome for this patient group.

The compound roxadustat, or FG-4592 (*Figure 6*), is currently in phase III clinical trials as an oral treatment for anemia in patients with chronic kidney disease.^[58] It stimulates the erythropoiesis by targeting the hypoxia-sensing pathway.

The transcriptional factors called hypoxia-inducible factors (HIFs) regulate the gene expression of genes responsible for the erythropoiesis. At a normal oxygen level, the HIFs are degraded by HIF-propyl hydroxylase domain (PHD) enzymes. However, when the oxygen level is low, the activity of the PHD enzymes is reduced which leads to an elevated HIF level. The HIF level regulates the gene expression resulting in an increased erythropoiesis.^[55,59] Roxadustat inhibits the HIF-PHD which mimics hypoxic conditions and stimulate the erythropoiesis.^[55–57]

Despite not being approved as a pharmaceutical drug, it is already available online and used as doping in sports to enhance the performance of athletes.^[29,60] However, no investigation regarding the metabolism of roxadustat had been published prior to paper **IV**.

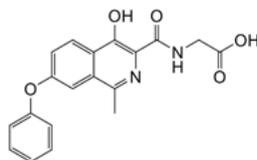


Figure 6. The chemical structure of the HIF-PHD inhibitor roxadustat.

Aim

The aim of the research presented in this thesis was to structurally characterize the metabolites formed from compounds with a high relevance to doping control in general, and equine doping control in particular. To achieve this, liquid chromatography coupled to high-resolution mass spectrometry was used.

Specific aims for the papers were:

- To structurally characterize the metabolites from the SARMs S1, S4 and S22 in horse urine (paper **I**) and plasma (paper **II**), and to propose potential targets for equine doping control.
- To validate a quantitative method for SARM S1 in equine plasma and apply the method to administration samples (paper **II**).
- To structurally characterize metabolites from SARM LGD-4033 in horse urine and plasma, and to propose potential targets for equine doping control (paper **III**).
- To compare the metabolites from SARM LGD-4033 in the horse to metabolites formed in other metabolic systems (paper **III**).
- To use different *in vitro* models to investigate the metabolites of FG-4592 to predict potential human and equine metabolites (paper **IV**).

Sample preparation

Biological matrices are in general complex, and each matrix has its own difficulties. Therefore, sample preparation is often required. For the projects included in this thesis, different types of samples were analyzed. Blank samples from in the *in vitro* experiments with liver microsomes and fungal incubates show less interferences, compared to blank urine and blood samples, which required more cleanup.

In bioanalysis, a sample preparation method that removes as much of the unwanted compounds as possible while still retaining the analyte(s) of interest is desired. One problem is that compounds that are likely to interfere with the analyte during analysis, i.e. chromatographic separation, often have similar properties. These interferences are thus more difficult to remove during cleanup. Sample preparation for metabolite detection adds another layer of difficulty. Since the structures of the metabolites are unknown, the sample cleanup is difficult to optimize. The commercial availability of drug metabolites as reference material is limited, and even more so for non-approved drugs. Therefore, testing the method on a spiked matrix is, in many cases, not an option.

To avoid losing metabolites because of discrimination during sample preparation, non-selective methods have been used. In addition, different methods have been applied to the same set of samples in trying to detect a majority of the metabolites.

Sample cleanup

One way to reduce the loss of metabolites during cleanup is to avoid it altogether. But when analyzing samples using LC-MS, omitting cleanup will make the detection of the metabolites more difficult. There is a risk of ion suppression occurring which can reduce the signal. Over time, the signal can also decrease due to contamination of the instrument.

By diluting the sample, the sensitivity can increase. The compound of interest will be diluted, but so will the interferences. The absolute signal of the compound of interest will be reduced, but the signal to noise ratio can be improved, leading to a higher sensitivity. This method was applied in paper **I**, **III** and **IV** on both urine samples and broth from fungal incubations.

Samples of horse urine were analyzed for the projects presented in papers **I** and **III**. This matrix contains mucus that forms in the urinal tract of the horse making it a much dirtier matrix compared to human urine.^[61] Because of this, it can be advantageous to perform an extraction on the horse urine and not only dilute the samples prior to injection.

In liquid-liquid extraction (LLE), the conditions such as the pH and type of organic phase can be changed to influence the distribution of analyte between the phases. In papers **I**, **III** and **IV**, LLE with *tert*-butyl methyl ether was used for sample cleanup to facilitate the detection of low intensity metabolites that could not be detected using direct injection. To avoid missing metabolites, different pH was used.

In solid-phase extraction (SPE), different stationary phases and washing protocols can be used for a higher selectivity. This technique was applied in papers **I**, **III** and **IV**.

Plasma samples contain protein that can interfere with the analysis. Protein precipitation was thus used to remove the proteins from the sample without losing the metabolites. In paper **II** and **III**, protein precipitation with cold acetonitrile was performed.

Enzymatic hydrolysis

It is common to have a deconjugation step during the sample preparation in horse doping control.^[13,62] By using enzymatic hydrolysis, the conjugate from the phase II metabolites can be removed and the metabolites return to their unconjugated form. For directly conjugated metabolites, this step enables detection of the parent compound, which in turn facilitates for easier detection due to the availability of reference standards. This is not only useful in the detection of illicit drug use, it can also aid in confirming the identity of a phase II metabolite.

Enzymatic hydrolysis with β -glucuronidase or a combination of β -glucuronidase and arylsulfatase was used during the work leading up to paper **I** and **III**. The glucuronidase removes glucuronic acid from the metabolite while arylsulfatase removes sulfate conjugates from arylsulfates. The samples are more complex after hydrolysis, so it was followed by an extraction step. This added complexity of the sample is something that must be considered in developing effective doping control methods since the sensitivity, and thus also the detection time, can be affected.

Chromatographic separation

Liquid chromatography (LC) was used in all projects included in this thesis. It was utilized to achieve separation between the different components in the samples to facilitate the identification of metabolites. In all experiments, C18 columns were used and the mobile phases consisted of 0.1% formic acid in water and methanol (papers **I**, **II** and **IV**), or 0.1% formic acid in water and 0.1% formic acid in acetonitrile (paper **III**). The mobile phase was delivered as a gradient with an increasing amount of organic solvent over time.

To ensure that the separation was stable over the time period the analyses of the samples in one project were performed, a standard solution containing two or three known compounds was analyzed. This standard solution was analyzed before and after every sample set. A drift in retention time for these compounds would indicate if the column was worn-out or damaged.

The metabolites are often similar, sharing structural elements with each other, so to determine which metabolites are formed without using chromatographic separation would be difficult, if not impossible. One example is phase II metabolites; the conjugate is often lost for a small fraction of the metabolites during the ionization process because of in-source fragmentation. The m/z of the in-source fragment would then be exactly the same as for the unconjugated phase I metabolite or parent. Were these not separated in time by a chromatographic method, it would be impossible to determine the origin of the observed m/z . This would also apply to isomers since they too have the same m/z .

Most metabolites are more polar than their respective parent compound; analysis in a reversed phase chromatographic system will give a shorter retention time for the metabolites compared to their respective parent. This was useful for all the qualitative analyses, but even more so when analyzing the metabolites of roxadustat (paper **IV**). Roxadustat formed a degradation product with the same m/z , but a shorter retention time. This behavior of the metabolites aided in identifying which metabolites originated from roxadustat, and which came from the degradation product.

Although chromatographic separation was necessary in these projects, complete separation was not achieved for all metabolites with the separation methods reported. It would have been desirable to fully separate the metabolites from each other, but that would have meant sacrificing analysis time and signal intensity. However, in combination with MS, the attained separation was sufficient for the purposes.

The samples contain different matrix components as well. These components can interfere with MS detection and cause a suppression of the signal from the analyte.

A simple separation method, such as the ones reported here, is beneficial. Since the analytical targets suggested can be analyzed using a common separation column and mobile phases, they should be easier to incorporate into pre-existing screening methods of horse doping control laboratories.

High-resolution mass spectrometry in metabolite identification

In each of the four papers that make up this thesis, the identification of unknown metabolites was central. Using mass spectrometry for this purpose can be advantageous. The high sensitivity of mass spectrometers makes it possible to detect metabolites that are present at low levels. Since the parent structure of the drug is known, the use of both high-resolution MS data and tandem mass spectrometry (MS/MS) data, makes it possible to characterize the metabolites.

The ability to measure the m/z with a high accuracy was integral in the qualitative experiments. To ensure that the mass accuracy was high enough, it was measured on two or three known compounds in a standard solution before and after each batch of samples. The limit for an accepted deviation from the theoretical mass was set to < 5 ppm.

To cover the potential biotransformations that may occur, all qualitative analyses were performed using a full scan mode. This means that all m/z in a predefined interval were recorded. The m/z corresponding to specific biotransformations were then isolated for further investigation using digital filtration. Using a full scan mode allows for a non-selective data collection where the potential metabolites are not lost because of a mass filter setting.

The primary analysis mode utilized for the qualitative analyses (paper **I-IV**) was MS^E. This is a data-independent scan mode that scans both parent ions and product ions in one run. The voltage in the collision cell alternates between a low value, giving the parent ions, and high value, fragmenting the ions. The data is then stored in two separate traces, so that the parent ions and the fragmentation data can be studied separately. The advantage is that only one injection per sample is needed to give both full scan data as well as information about the fragmentation pattern. However, the detected metabolites were also analyzed using MS/MS, to confirm the findings of the MS^E data.

How the sample is introduced to the mass spectrometer is also important to consider. In this thesis, electrospray ionization (ESI) was used. ESI is a soft ionization technique, meaning that little energy is transferred during the ionization, leaving an intact parent ion. If an ionization technique that introduces more energy is used, the ion will fragment.^[11,26] For the projects included in this thesis, the masses of the intact metabolites were of interest. Therefore ESI

was a suitable ionization technique. The metabolites were also expected to be polar compounds, for which ESI is also an appropriate choice.^[11]

The choice of ionization mode can affect which metabolites are detected as their ability to ionize differs. Previous metabolite studies for the SARMS S1, S4 and S22 had used negative ESI, which was the reason for choosing that in the study reported in papers **I** and **II** as well.^[39–46]

There were fewer metabolite studies available on LGD-4033 and in those studies both negative and positive ESI were utilized.^[38,51,52] Therefore, both were tested on the samples from the administration study with LGD-4033 (paper **III**). Negative ESI gave a higher intensity for the detected metabolites and no additional information was gained with positive ESI. Because of this, only the results from negative ESI were reported.

The same procedure was applied on the *in vitro* generated samples with FG-4592 (paper **IV**), as there was no information available on its metabolism in any biological model.^[60] The results showed that most metabolites were detected in both positive and negative ESI, although the intensity varied between the different modes. However, one of the minor metabolites that had undergone hydroxylation and glucosylation was only detected in positive ESI, and another minor metabolite that was dihydroxylated and sulfonated was only detected in negative ESI. For this reason, both positive and negative ESI were used.

Different methods were applied in order to find the metabolites. One way was to compare the samples containing the drug and its metabolites to a blank sample. The degree of difficulty in finding the metabolites by comparison to a blank depends largely on the matrix. The samples from *in vitro* incubation with *C. elegans* (Figure 7 A) have very few matrix components that co-elute within the time that metabolites were expected. This can be compared to horse plasma (Figure 7 B) or horse urine (Figure 7 C) where several more components are visible in the chromatogram.

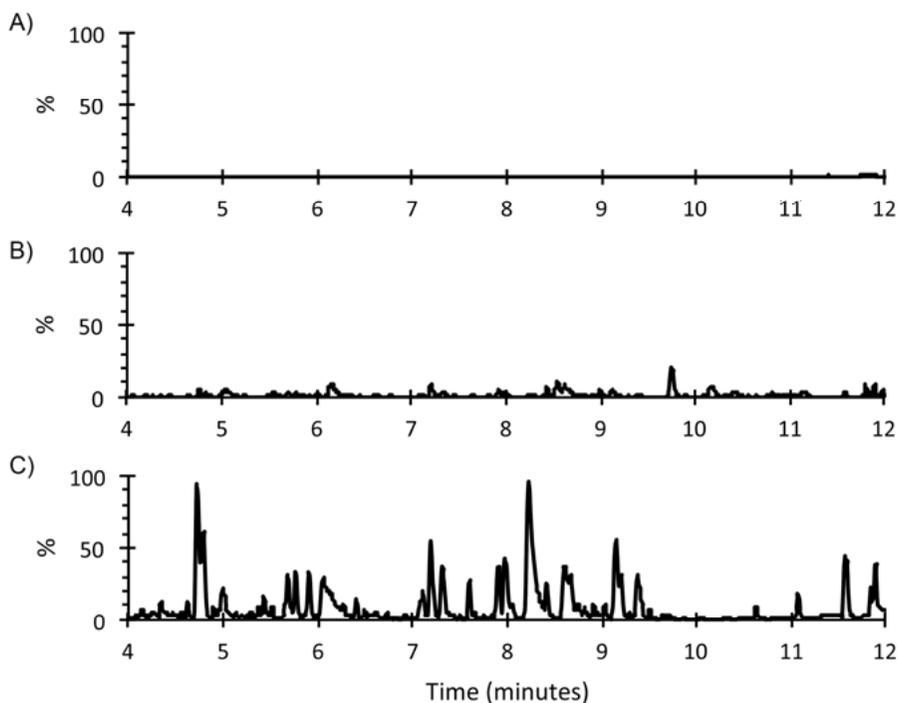


Figure 7. Base peak intensity chromatograms of the blank matrices A) broth incubated with *C. elegans*, B) horse plasma and C) horse urine. The plasma sample was subjected to protein precipitation while the incubate and the urine were diluted 1:10 with 0.1% formic acid in MilliQ water.

For less complex matrices such as the growth medium for *C. elegans*, the sample containing the added compound and its metabolites can be visually compared to the blank sample (*Figure 8*). This allows for detection of the major metabolites. This is, however, not an option in more complex matrices or for the minor metabolites.

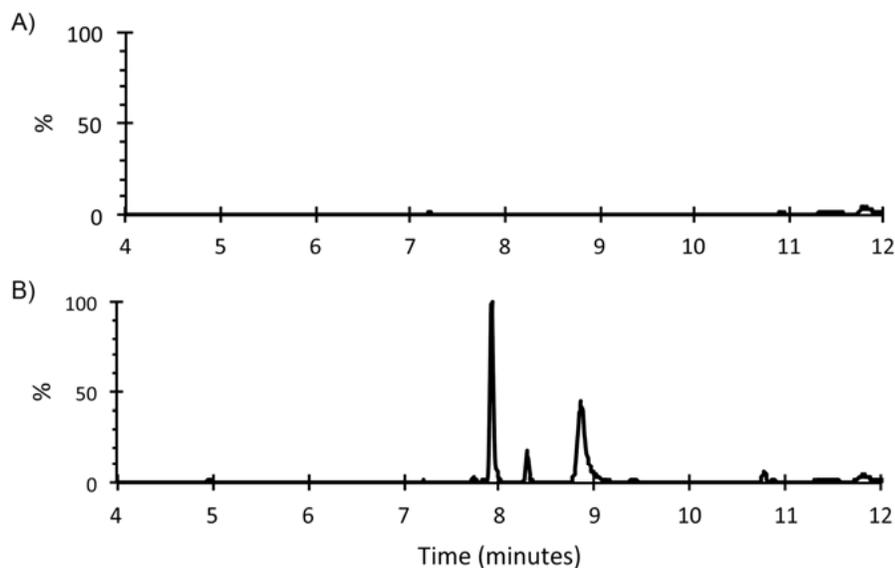


Figure 8. A comparison of base peak intensity chromatograms of A) a blank sample containing broth and *C. elegans*, and B) broth and *C. elegans* incubated with LGD-4033. The major metabolites and the parent compound can be seen as peaks in chromatogram B.

To detect metabolites in a complex matrix, the m/z corresponding to different biotransformations are investigated. This investigation can occur either automatically using a specialized software (papers **I**, **II**, and **IV**) or it can be performed manually (papers **I-IV**). To compile the list over possible biotransformations and their exact m/z , the already reported metabolites for the parent drug(s) of interest are a good starting point, independent of the metabolic system used.

By only extracting the masses of interest from the full scan chromatogram, i.e. those corresponding to possible biotransformations, the majority of the background noise can be reduced. This can then be compared to an extracted ion chromatogram (XIC) of the same m/z for the blank. This process can be performed manually or automatically by a software. When a potential metabolite is identified, the exact mass and the product ions are investigated further to determine if the finding is a metabolite or not.

The type of phase II metabolites can be determined by characteristic losses of the conjugate which is helpful in identifying metabolites. The conjugates often dissociate at low collision energies. If this occurs before the parent structure fragments, the position of the conjugate can be difficult to determine. For phase I metabolites, such as hydroxylated metabolites, the change in mass for some fragments can indicate the site of hydroxylation. This is illustrated in Figure 9 where the spectrum from a monohydroxylated LGD-4033 is compared to the spectrum of the parent.

The monohydroxylated metabolite have a fragment at m/z 227, which corresponds to the fragments at m/z 211 for the parent compound but, with a shift of +16. The change in m/z agrees with the addition of one oxygen and suggest that hydroxyl group remains on this product ion. The fragment m/z 170 correspond to the benzonitrile group and is the same for both structures. The added mass from the oxygen is no longer present, which indicates that the hydroxyl group is places on the pyrrolidine moiety.

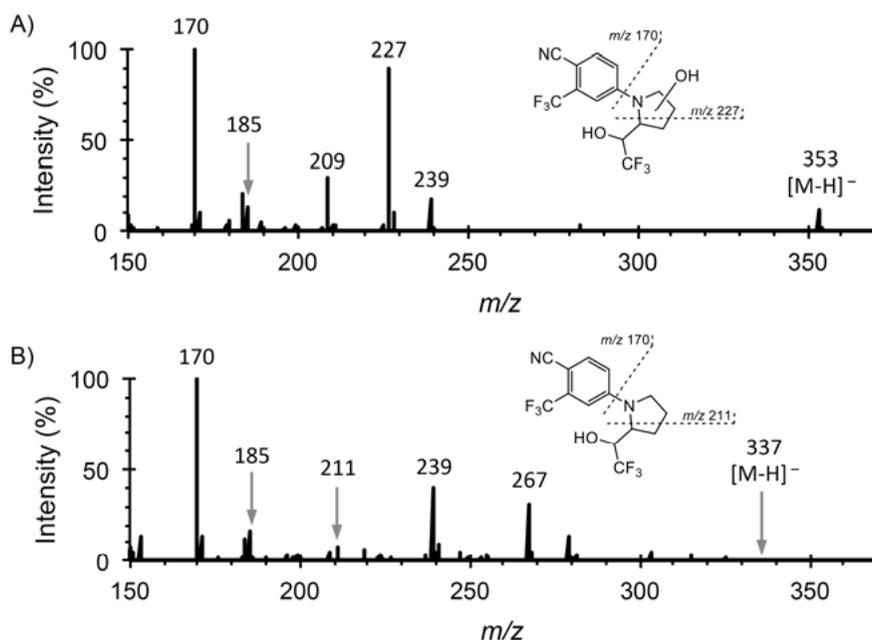


Figure 9. Spectrum of A) monohydroxylated LGD-4033 and B) LGD-4033.

For a fully identified structure, it is not enough to have the elemental composition or to know what metabolic reactions have occurred, the exact position of each atom must also be determined. In a publication by Schymanski *et al.*^[63], it was suggested that the degree of identification was divided into five different levels (**Table 1**). For a complete identification, the LC-MS data must be compared to a known reference standard. This level of identification is difficult to achieve for drug metabolites, since they rarely are commercially available.

Table 1. Different levels of characterizing an unknown structure, adapted from Schymanski *et al.*^[63]

Level of identification	Requirements
Confirmed structure	LC-MS/MS data compared to a reference standard.
Probable structure	MS/MS data matched to library data, or MS/MS data that fits only one structure.
Tentative candidate(s)	MS/MS data that fits more than one structure.
Unequivocal molecular formula	MS(/MS) data that support only one molecular formula, but no structure can be suggested.
Exact mass	An exact mass of interest can be measured, but no molecular formula can be proposed.

For some of the metabolites identified in papers **I** and **II**, a probable structure could be confirmed based on MS and MS/MS data. This means that the mass data supports one possible structure, as after an amide hydrolysis of SARM S1 (*Figure 10 A*). However, it cannot be classified as a confirmed structure since it has not been compared to a reference compound or analyzed using an analysis method that provides the exact structure, such as nuclear magnetic resonance (NMR).^[64]

In many cases, only a tentative identification can be achieved with MS, meaning that one single structure cannot be determined, but rather a few structural isomers, as illustrated in *Figure 10 B* where the exact position of the sulfate conjugate cannot be determined after SARM S1 has undergone both an amide hydrolysis, a hydroxylation, and a sulfonation.

In papers **I-IV**, most of the metabolites detected were tentatively identified due to a lack of commercially available references. It is generally difficult to purify urine or HLM samples to yield an amount both large enough, and pure enough, to be analyzed with NMR.^[64,65] The option to investigate the metabolites further using NMR was thus not pursued.

Another way to completely identify the metabolites is to synthesize the different isomers, confirm their structure with NMR, and compare to the metabolite using MS/MS. Essentially, to make your own reference compound. This method was later applied to the main metabolites observed in papers **I** and **II** (*Figure 10 C*, paper 4).

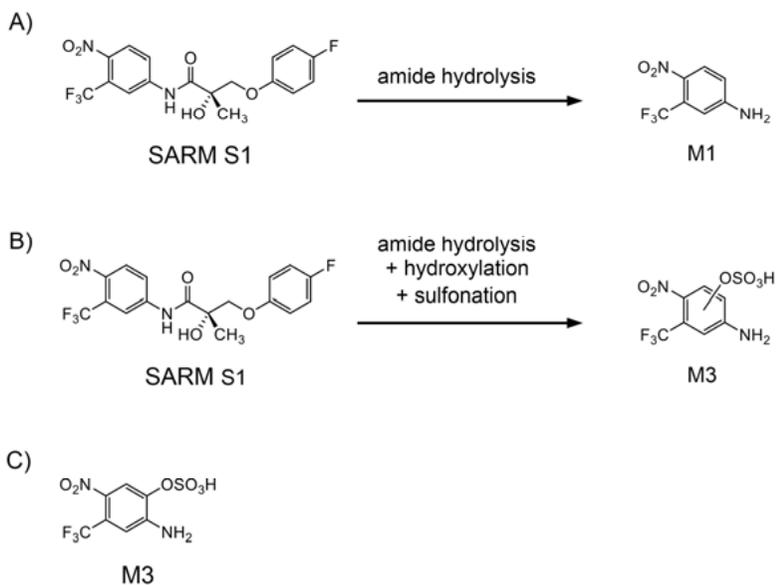


Figure 10. Different degrees of identification are illustrated. A) shows a probable structure of metabolite M1 after an amide hydrolysis of SARM S1, B) shows a tentative structure of metabolite M3 after amide hydrolysis, hydroxylation, and sulfonation of SARM S1, and C) shows a confirmed structure of metabolite M3 after comparison to a known standard.

Administration studies in horses

The *in vivo* studies performed and included in this thesis resulted in three published papers. Two where the metabolites of the SARMS S1, S4, and S22 were investigated in horse urine (paper **I**) and plasma (paper **II**) to find a suitable analytical target for horse doping control. A quantification of SARM S1 in plasma was also performed (paper **II**). Another study investigated the metabolites from LGD-4033 in horse urine and plasma with the same purpose. These metabolites were also compared to a positive human urine doping control sample and metabolites generated by *C. elegans* (paper **III**).

In the *in vivo* studies, the compounds were administered as an intravenous bolus dose to three horses each. Urine and plasma were collected for up to 96 h post administration with the first time point being at 3 h after administration for urine, and 5 minutes for plasma. The full description of the administration and sample collection can be found in papers **I-III**.

Metabolites from SARMS of the aryl propionamide class

The investigation showed that the SARMS S1, S4 and S22 underwent similar biotransformations in the horse (*Figure 11*). In the plasma samples, the parent compounds had the longest detection time, or the same detection time as the major metabolite. Since it is easier to obtain reference compounds for the parent, it was thus suggested as the analytical targets for doping control in horse plasma.

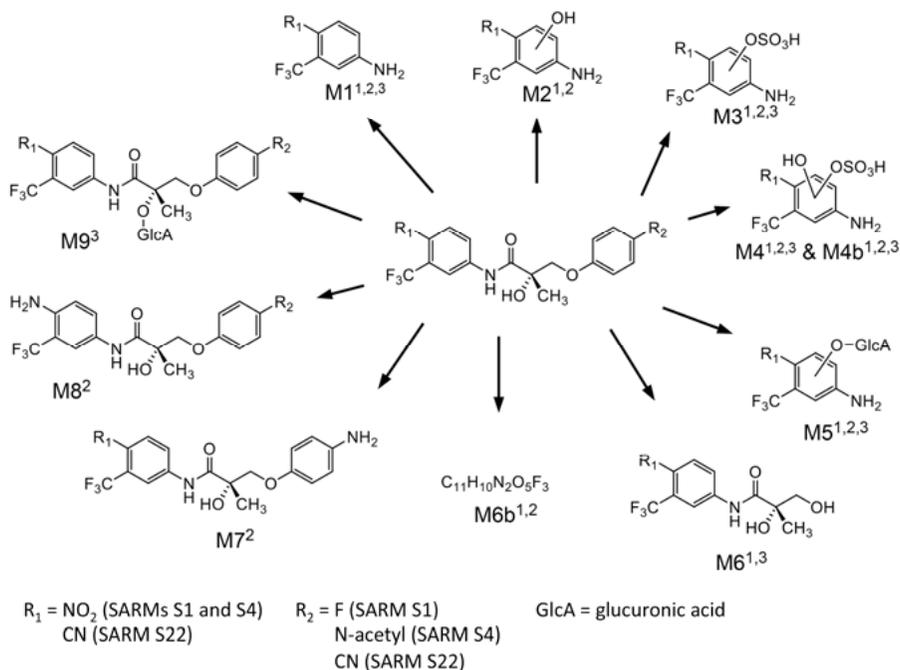


Figure 11. Metabolite map showing all observed transformations of SARMs S1, S4 and S22 in horse urine and plasma. The metabolites marked with (1) were formed from SARM S1, metabolites marked with (2) were formed from SARM S4 and those marked with (3) were formed from SARM S22.

In the urine samples, the parent compounds could not be detected. The metabolites with the highest intensities for each of the three SARMs had undergone the same transformation reactions. These reactions were amide hydrolysis, hydroxylation, and sulfonation and the metabolites were denoted M3 (*Figure 12*). This metabolite could be detected in urine for up to 96 h for SARM S1 and up to 48 h for SARMs S4 and S22. The highest intensity of M3 in the plasma samples were observed within 1 h post administration for all three SARMs, which indicates a very fast metabolism in the horse.

No metabolite corresponding to M3 was reported in the *in vivo* experiments performed in human^[44,45], dog^[43], or calf^[41] for either S4 or S22, and no *in vivo* metabolites of S1 were published. The *in vitro* experiments performed on S4 and/or S22 included HLM^[39,43], ELM^[40], bovine S9 fraction^[41], and *C. elegans*^[42], but no metabolite corresponding to M3 was detected there either. However, incubation of SARM S1 with HLM^[39] and *C. elegans*^[42] both produced the M3 metabolite. So it is possible to generate this metabolite for SARM S1 and S4 using *in vitro* models, but based on the data reported, the amounts were small.

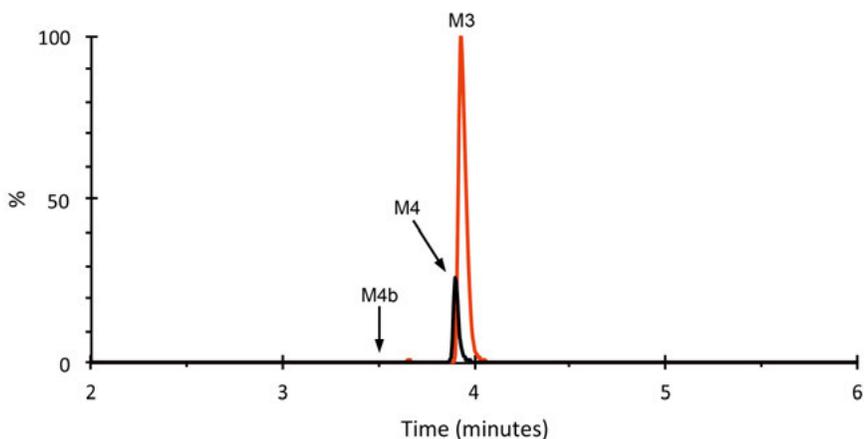


Figure 12. XIC of the three metabolites with the highest intensity observed in horse urine 3 h after administration of SARM S1. Similar results were obtained for SARM S4 and S22 as well.

Based on the MS/MS data obtained in papers I and II, the exact structures of the metabolites M3 could not be deduced. But they were later confirmed in a study by Garg *et al.* (paper 4) who synthesized M3, the major metabolites for SARMs S1, S4 and S22 and confirmed the exact structure with NMR. The fragmentation patterns of the synthesized M3 metabolites were compared to urine samples from the administration study presented in paper I. The retention times were also compared using both a C18 column and a biphenyl column. The results concluded that the structure of the major metabolites were 2-amino-5-nitro-4-(trifluoromethyl)phenyl hydrogen sulfate (Figure 13 A) for SARMs S1 and S4, and 2-amino-5-cyano-4-(trifluoromethyl)phenyl hydrogen sulfate (Figure 13 B) for SARM S22.



Figure 13. The structures for the main urinary metabolites from horses administered with the SARMs A) S1, S4 and B) S22 as described by Garg *et al.* (paper 4).

A publication by Perrenoud *et al.* discussed the potential of misidentifying the approved drug flutamide as SARM S4 in human doping control.^[66] Since flutamide and SARM S4 can form the same metabolites, it is important to consider which metabolites are used as targets to avoid false positives. And due to the structural similarities, this would also apply to SARM S1.

This could potentially also be a problem in horse doping control. In humans, flutamide was shown to form a metabolite that corresponds to M3.^[67] No administration study of flutamide in horses has investigated the metabolite formation, only the pharmacokinetics^[68]. If M3 is formed from flutamide in

the horse, it can be mistaken for SARM S1 or S4 in a doping control analysis. The IFHA guidelines state that all compounds with a pharmacological effect are prohibited during a race^[5]. So either way, detection of M3 would be considered doping. However, it is possible that since flutamide and SARMS have different pharmacological effects, the severity of the penalty can be affected.

Overall, there was little difference between the metabolites the three SARMS formed. Only three of the biotransformation products (denoted M7-M9) observed could only be detected for one of the three compounds. The metabolite M7 had undergone a deacetylation and only appeared for SARM S4. The other SARMS did not have an acetyl group as a substituent and this metabolic pathway was thus not an option. For metabolite M8, a nitro-reduction had occurred. Again, this was only observed in the samples from SARM S4 administered horses, even though SARM S1 also had the structural prerequisite needed.

The metabolite M9 was only detected for SARM S22 and had undergone a direct glucuronidation. Both SARM S1 and S4 have the structure required to undergo a direct glucuronidation, but the corresponding metabolite was not detected for either of them. This metabolite had previously been observed in human urine.^[45]

As discussed here, the metabolism of the three different SARMS was very similar and it can be suspected that other SARMS of the aryl propionamide structure class will also metabolize like this. Investigating the presence of metabolites corresponding to M3 should thus be considered. This must, of course, be investigated for each new compound, but it is likely that the amide hydrolysis reaction is an important biotransformation pathway in the metabolism of SARMS or the aryl propionamide class in the horse.

Quantification of SARM S1 in plasma

A method to quantify SARM S1 in plasma was developed and validated on relevant parts of the EMA guidelines for bioanalysis methods.^[69] It was then applied to the plasma samples of the administration study (paper II). An isotope labelled version of SARM S1, SARM S1-d4, was used as the internal standard. The quantification was performed on a tandem mass spectrometer operated in SRM mode. There was no isotope labelled standard available for the SARMS S4 or S22, which is why no validation was performed for these compounds. The calibration curve for SARM S1 was prepared between 1-150 ng/mL with four quality control (QC) levels. Both the calibration curve and the QC samples were prepared in plasma that had sodium citrate as the anticoagulant, while the administration samples were prepared in K₂EDTA plasma. To evaluate the differences between matrices, one extra set of QC samples were prepared in K₂EDTA plasma to compare to the calibration

curve. All tested validation criteria were fulfilled and the method was used on the administration samples.

The results showed that the highest concentration was reached after 5 minutes, in the first sample taken after administration. Since the compound was administered intravenously, this was expected. The concentrations at that point for two of the horses were measured to 97 and 142 ng/mL. The maximum concentration for the third horse was outside of the calibration curve, but was extrapolated to 170 ng/mL (*Figure 14*). With a limit of quantification of 1 ng/mL, SARM S1 was quantified for up to 8 h, but it could be detected for up to 96 h post administration.

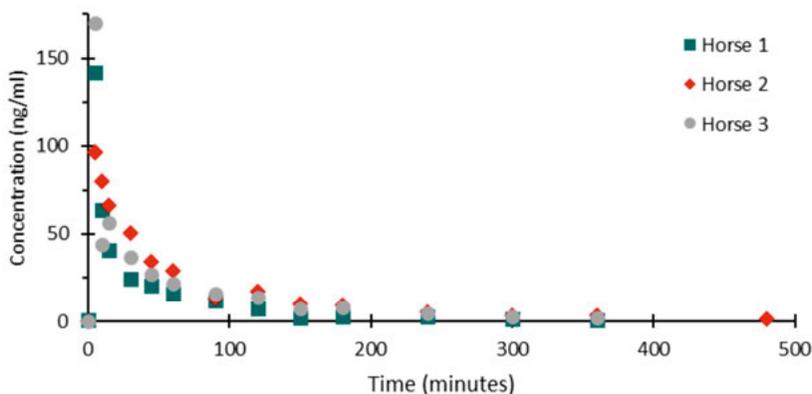


Figure 14. Plasma concentration for SARM S1 in the three horses for up to 8 h (480 minutes) post administration.

For the SARMS S4 and S22, the concentration was estimated by analyzing the samples together with a calibration curve ranging from 6-200 ng/mL. As no isotope labelled compounds were available, a standard of S22 was used as internal standard for the S4 administration samples and vice versa. As for SARM S1, the highest concentrations were reached for the samples collected 5 minutes post administration, and the mean value of the concentrations for the three horses administered with the same compound was 103 ng/mL for SARM S4 and 120 ng/mL for SARM S22. The detection times for SARM S4 and S22 were up to 12 h and 18 h respectively.

In vivo and *in vitro* metabolites of LGD-4033

Eight metabolites of LGD-4033 were detected in the urine and plasma samples from horses (*Figure 15*, paper III). Six of the metabolites were detected in both plasma and urine, but the two with the lowest intensity were only observed in the urine samples. Two of these were phase I metabolites, one dihydroxylated and one trihydroxylated metabolite. The MS/MS data suggested that the hydroxylation had all occurred on the pyrrolidine moiety. The parent

compound was detected in the plasma samples and could only be observed in urine after hydrolysis.

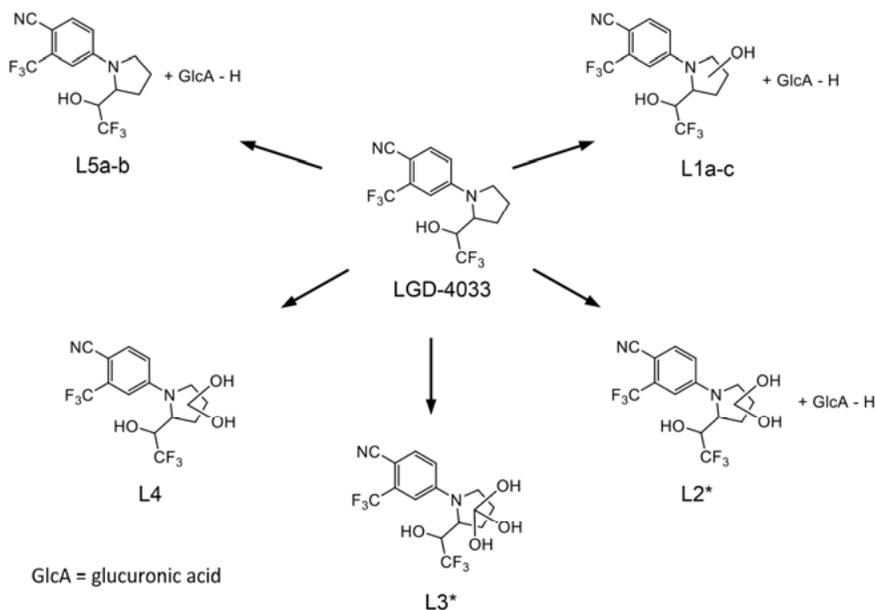


Figure 15. LGD-4033 and the metabolites observed in the horse. Metabolites marked with an asterisk (*) were only detected in urine samples.

The remaining six metabolites were all conjugated with glucuronic acid. Three of them had undergone a hydroxylation and glucuronidation, one was dihydroxylated and conjugated with glucuronic acid and two were directly conjugated with glucuronic acid. After the urine samples were treated with β -glucuronidase, none of these metabolites were detected, with one exception. One of the directly glucuronidated metabolites was unaffected by the enzymatic hydrolysis (Figure 16). The reason for this could not be determined with the data at hand, since the fragmentation patterns of the two metabolites were similar. However, one suggestion was that the metabolite eluting at 8.54 minutes was an *N*-glucuronide. This would mean that the metabolite is a quaternary ammonium compound and those have a permanent positive charge. To be detected in negative ESI, two protons must therefore be removed during the ionization process. If the pyrrolidine ring opened during the biotransformation to form a tertiary amine, this metabolite would not have a permanent positive charge. But for this compound to then have the same fragmentation pattern as the *O*-glucuronide, rearrangements must occur during the formation of the product ions.

Another suggestion is that both metabolites are *O*-glucuronides, but with different stereochemistry. The fragmentation pattern would still be the same, but this could explain why the enzyme only hydrolyzes M5a.

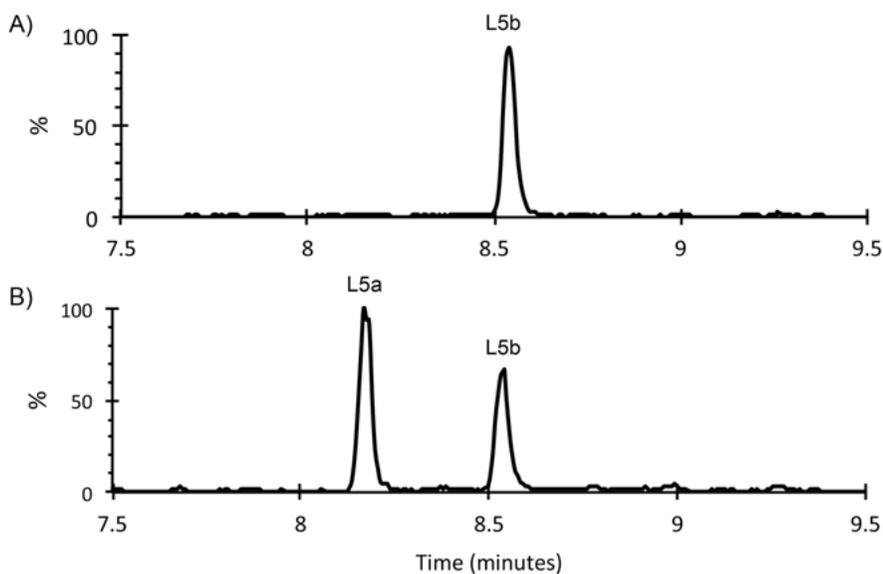


Figure 16. XIC of directly glucuronidated LGD-4033 (m/z 513) in A) horse urine hydrolyzed with β -glucuronidase and B) non-hydrolyzed horse urine. Both samples have been extracted using SPE.

The metabolite with the highest intensity and the longest detection time was one of the directly glucuronidated metabolites. However, after treating the samples with β -glucuronidase, the parent compound could be detected for longer, up to 96 h post administration if the formate adduct was analyzed.

The formate adduct of LGD-4033 had the longest detection time in plasma as well. It was thus suggested as the analytical target for doping control in both horse urine and plasma. This requires the urine to be hydrolyzed, but this is already a common step during sample preparation in horse doping control^[4], so it should be possible to incorporate this compound into already existing methods.

The comparison between human and horse urinary metabolites in paper **III** showed three common metabolites, one dihydroxylated and two glucuronidated metabolites (*Figure 17*). The human urine also contained one additional dihydroxylated metabolite.

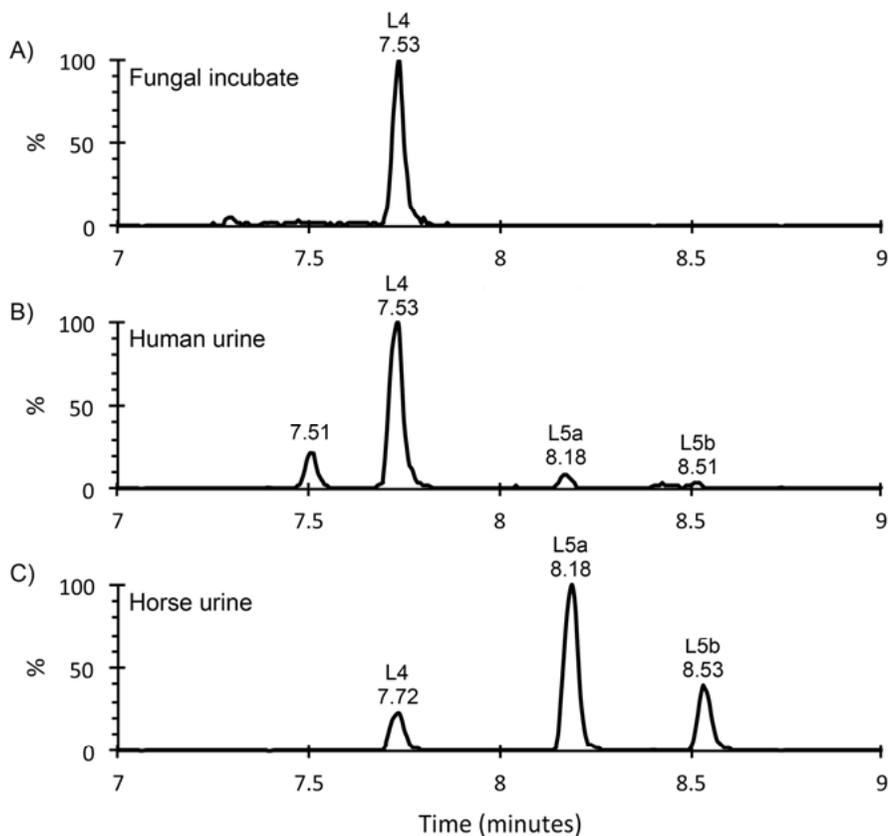


Figure 17. XIC of the metabolites L4 (m/z 369) and L5a-b (m/z 513) observed in A) fungal incubate with *C. elegans*, B) human urine, and C) horse urine.

The two dihydroxylated metabolites were in agreement with an earlier study in of the human urinary metabolites of LGD-4033, but in that study no glucuronides were reported.^[51] Since the sample preparation performed followed a standard protocol for doping samples, which included hydrolysis with β -glucuronidase, the possibility for metabolites still conjugated with glucuronic acid may not have been investigated.

The horse metabolites of LGD-4033 were also compared to those formed from the fungal incubate (paper III). As six of the eight metabolites detected in the horse samples were conjugated with glucuronic acid it was expected that the metabolic pattern from the fungus would not match. Although it has been suggested that *C. elegans* expresses UDP-glucuronosyltransferase and thus can form glucuronides^[20,70], it is rarely observed for pharmaceutical compounds. Instead *C. elegans* seems to prefer phase II conjugation with glucosides.^[42,71,72]

Previous *in vitro* studies had investigated the phase I metabolites of LGD-4033 produced by electrochemical conversion, HLM, and *C. elegans*.^[38,52] The

investigation by Thevis *et al.* (paper 3) only detected two monohydroxylated metabolites formed by the fungus, but four additional metabolites were observed in the experiment with *C. elegans*, reported in paper **III**. These were a dihydroxylated metabolite, a monohydroxylated metabolite where a ring opening of the pyrrolidine moiety had occurred, a monohydroxylated metabolite with double bond and a phase II metabolite with a sulfo conjugate.

All of the phase I metabolites detected in the incubate from *C. elegans* were described for LGD-4033 before, in HLM incubates^[38] and in human urine^[51]. This suggests that *C. elegans* metabolizes LGD-4033 in a similar way as humans. However, further analysis of the samples side by side is required before it can be asserted that LGD-4033 forms the same metabolites in both *C. elegans* and humans.

Metabolite detection in urine compared to plasma

Something that both the studies on the SARMS S1, S4 and S22 (papers **I** and **II**) and the SARM LGD-4033 (paper **III**) showed was the difference between the metabolite concentration in the urine and plasma matrices.

The parent compound was not detected in the urine samples for any of the four compounds. This means that metabolism was necessary before urinary excretion was possible. For LGD-4033, detection of the parent compound in urine was only possible after treatment with β -glucuronidase. A directly glucuronidated metabolite was observed for SARM S22; however, this metabolite was minor and the aglycone could not be detected after hydrolysis. So in the urine samples from the administration study with SARMS S1, S4 and S22, the parent compounds were not detected.

The SARMS in the two *in vivo* studies were administered intravenously. It was thus expected for the compounds to be observed in the plasma and for the concentrations to be highest at the first time point after administration. It was also expected for fewer metabolites to be found in plasma compared to urine.^[13] Only for SARMS S1 and S4 were all metabolites observed in both urine and plasma. For SARM S22 and LGD-4033, fewer metabolites were detected in plasma. There were no metabolites observed for any of the four compounds that were detected in plasma samples only.

In addition to the fewer number of metabolites in plasma, the response was also lower in general. This leads to a shorter detection time in all four cases. So although both plasma and urine should be investigated for metabolites in a metabolism study, these results show that when it comes to doping control, urine is the preferred matrix for these compounds.

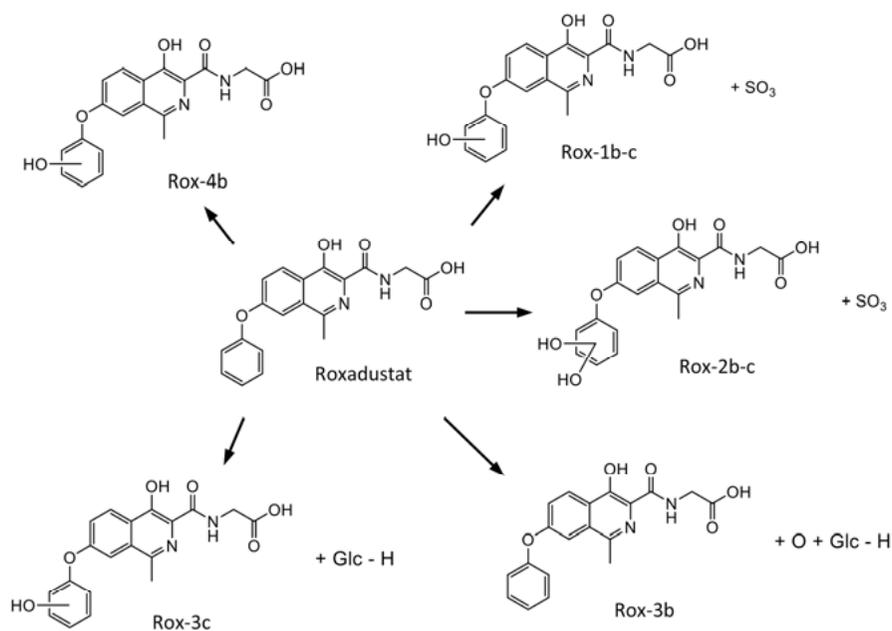
In vitro metabolites of roxadustat

Very little information regarding the metabolites formed by roxadustat was available prior to the *in vitro* results published in paper **IV**. In a report of a human urine doping sample that tested positive for roxadustat (FG-4592), the need for metabolite studies was stressed as a potential way to prolong the detection time.^[60] Because of this, different *in vitro* models were used to investigate potential *in vivo* metabolites.

The *in vitro* models tested were human hepatocytes, HLM, ELM and S9 fraction, and incubation with *C. elegans*. Incubation with *C. elegans* generated most metabolites while HLM and ELM only produced one metabolite. No metabolites were detected from the human hepatocytes or the equine liver S9 fraction.

Eleven metabolites were identified in the fungal incubate. Two monohydroxylated metabolites, three dihydroxylated and sulfonated metabolites, three hydroxylated and sulfonated metabolites, and three hydroxylated and glucosylated metabolites. Out of these eleven metabolites, only seven of them originated from roxadustat (*Figure 18*). The other four, one of each kind of metabolite, appeared to come from a degradation product of the parent compound.

The chromatogram showed a significant difference in retention time between roxadustat and its degradation product, but the structure of the degradation product could not be determined based on the mass spectral data. Since the compounds had the same exact mass it was concluded that the degradation product was the result of an internal rearrangement. This was confirmed in a later publication by Eichner *et al.* where the degradation product was described as a photoisomer.^[73]



Glc = glucose

Figure 18. Roxadustat and the metabolites observed in fungal incubates with *C. elegans*.

The samples were analyzed using both positive and negative ESI since all metabolites could not be detected with either of the ionization modes. However, the negative mode aided in the characterization of most of the hydroxylated metabolites. The fragments suggested that the hydroxylation had occurred on the benzene moiety, and that structure was confirmed by the presence of the product ion at m/z 108 (*Figure 19*). No such fragment was observed when the samples were ionized using positive ESI.

For the dihydroxylated and sulfonated metabolites, a product ion at m/z 124 was detected, which corresponds to the m/z 108 but with a shift of +16 Da. This confirmed that both hydroxyl groups were situated on the benzene part of the metabolites.

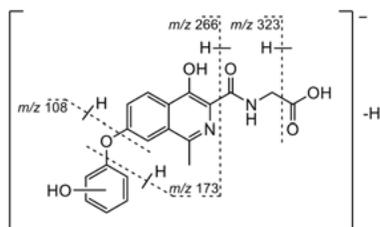


Figure 19. The fragmentation of the monohydroxylated metabolites Rox-4b in negative ESI. The fragment m/z 108 shows the position of the hydroxyl group added during the metabolism.

The HLM and ELM, only produced a monohydroxylated metabolite, here denoted Rox-4b, which was found to be formed by *C. elegans* too. In the fungal incubate, this was one of the major metabolites. Due to the lower intensity achieved for the HLM and ELM samples, the position of the added hydroxyl group could not be determined. But since the fungal incubate contained a higher concentration, it was determined that the hydroxyl group was situated on the benzene moiety. The fungus ability to produce larger amounts is thus useful in structural characterization. A corresponding metabolite to Rox-4b was later detected in human urine and plasma from a phase I clinical trial.^[73]

In addition to this metabolite, a hydroxylated and sulfonated metabolite was also detected in the human administration samples^[73] which corresponds to observed metabolites in the fungal incubate. But since the fungus produced two isomers of this metabolite, the retention times must be compared in the same chromatographic system to determine if the fungal metabolites are the same as the human one and if so which ones. But this is still a strong indication that *C. elegans* can produce human metabolites.

Metabolite production using *Cunninghamella elegans*

To identify a doping sample as positive, the sample needs to be compared to a reference compound. Unfortunately, metabolites are not as available as references as the parent compounds. However, the fungus *C. elegans* has previously produced a metabolite of oxandrolone, which was purified and used as a reference standard.^[7] Further experiments were therefore conducted with the aim of using *C. elegans* for producing a larger amount of the metabolite M3, the main metabolite of SARMs S1 and S4 detected in horse urine.

In a publication by Rydevik *et al.*, it was shown that the fungus was able to produce M3, but it was one of the minor metabolites with a fraction of only 4% of the total chromatographic peak area for all metabolites.^[42] It appeared like the amide hydrolysis was not a favored metabolic pathway, which is necessary for the M3 metabolite to form from SARM S1 or S4. So instead of spiking the growth medium with the SARMs, the compound 4-nitro-3-(trifluoromethyl)aniline was added instead. It corresponds to SARM S1 or S4 after an amide hydrolysis has occurred.

The results show that a hydroxylated and sulfonated version was formed, and it had the second highest response of the metabolites (*Figure 20*). It was compared to the horse urine samples and it was concluded to be the same structure as the equine M3, since both the fragment pattern and retention times matched.

The major metabolite in this experiment was directly sulfonated. Two minor metabolites with exact masses corresponding to an acetylation were also observed as well as a dihydroxylated and sulfonated metabolite.

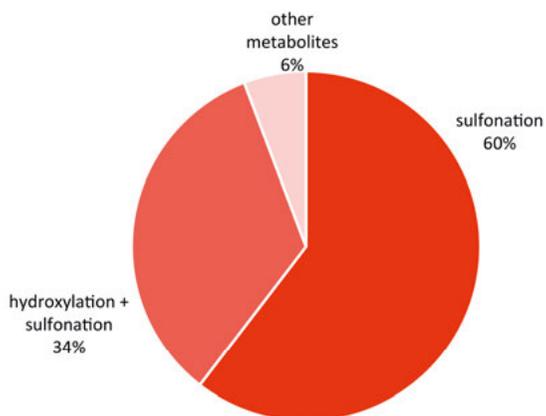


Figure 20. Relative peak area for the metabolites found in the fungal incubate of 4-nitro-3-(trifluoromethyl)aniline.

Compared to the amount of M3 produced by *C. elegans* when adding SARM S1, this approach generated a larger amount of the desired metabolite. The starting material used is also commercially available at a lower price compared to SARM S1. Further experiments to purify and characterize the metabolite must be performed before it can be used as a reference standard in equine doping control.

Summary and conclusion

Doping control is a field that requires a constant development of new methods in order to catch those who use prohibited substances or methods to increase their chances of winning. During development, it is important that new compounds are not just added to pre-existing screening methods; the metabolites of these compounds should be investigated as well. By studying and possibly incorporating metabolites in the analysis method, detection can be improved. In this thesis, the metabolites of five compounds that are banned from use in both human and equine sports were investigated. Both *in vivo* administration to horses (papers I-III) and *in vitro* models (papers III and IV) were used to produce metabolites for this purpose.

The results presented in paper I show that one of the standard procedures in doping control analysis of horse urine that include a deconjugation step with enzymatic hydrolysis and analysis of the parent compound does not always work.^[4] For the SARMs S1, S4 and S22, no parent compound was detected in any urine sample even after hydrolysis with arylsulfatase and/or β -glucuronidase. Instead, the major metabolite for the three SARMs had undergone an amide hydrolysis, a hydroxylation and sulfonation. The highest measured intensities were reached after only 3 h post administration, which suggests a very fast transformation. These metabolites were suggested as the analytical targets in urine samples, while the parent compounds were suggested as targets in plasma (paper II).

In the urine samples, most metabolites had undergone an amide hydrolysis suggesting that it is a significant route of metabolism for this class of SARMs in the horse, which makes these results stand out in comparison to *in vivo* studies in other species. This could be useful information if new SARMs of this structural class become relevant for horse doping control.

The main metabolites observed in the horse for the SARMs S1, S4 and S22 were not commercially available as reference compounds, so only a tentative identification could be made at the time of publication. However, they were later fully identified and their synthetic route was described (paper 4), so it is possible to synthesize the metabolites for use as references in horse doping control. Another option could be to utilize the metabolizing ability of *C. elegans* for producing larger amounts of these metabolites. This would require developing a method for purification of the compound.

For LGD-4033 (paper **III**), the aforementioned procedure that included enzymatic hydrolysis would have worked out well since one of the major metabolites was directly glucuronidated, and a deconjugation process would thus have returned it to its original form. The longest detection time was observed for the formate adduct of LGD-4033 in plasma samples after protein precipitation and in urine samples after hydrolysis and SPE.

Comparing the use of *C. elegans* as a metabolic model for LGD-4033 (paper **III**) to results in the horse showed that the fungus was able to successfully produce the two phase I metabolites found in horse urine. *C. elegans* was also able to generate a range of metabolites for roxadustat, where the human and horse liver microsomes were only able to produce one, a monohydroxylated roxadustat (paper **IV**). This metabolite was also one of the major ones produced by *C. elegans*. A comparison to subsequently published data on the metabolites in human urine showed that the *C. elegans* was successful in predicting more *in vivo* metabolites in humans compared to both HLM and human hepatocytes. This shows that *C. elegans* is not only useful for producing metabolites as reference compounds, but it can also be an alternative to liver microsomes and hepatocytes as an *in vitro* model.

Populärvetenskaplig sammanfattning

Under 2016–2017 har det rapporterats flitigt om olika dopingskandaler i medierna. Framförallt har det diskuterats mycket kring den organiserade dopingen som förekommit i Ryssland. Men det är inte enbart människor som dopas, även hästars prestation kan förbättras med hjälp av olika substanser.

Då det finns många fler människor än hästar som utövar sport så är även dopingkontroll för människor större. Där finns den världsomspännande organisationen, WADA (World Anti-Doping Agency), som bestämmer vilka substanser som är förbjudna att använda. Där ingår bland annat alla substanser som inte blivit godkända som läkemedel av någon myndighet. För hästar finns inte någon motsvarande lista. Däremot har organisationen IFHA (International Federation of Horseracing Authorities) ett dokument med riktlinjer där i princip alla ämnen som ger en farmakologisk effekt räknas som doping, och många av de nationella hästsportsförbunden följer dessa riktlinjer.

Vid analys av dopingprover så söks det inte enbart efter själva dopingsubstansen, utan även efter dess nedbrytningsprodukter, så kallade metaboliter. Fördelen med att söka efter metaboliter är att de kan finnas kvar längre i kroppen än dopingsubstansen, vilket kan ge en förlängd detektionstid. Dessa metaboliter är ofta mer vattenlösliga än ursprungssubstansen och bildas för att kroppen lättare ska kunna göra sig av med ämnet. Men för att veta vilka metaboliter som man bör söka efter så måste man först ta reda på vilka som bildas.

Det finns olika sätt att undersöka metaboliter på. Ett sätt är att låta leverpreparat metabolisera substansen. Detta är en vanlig modell för att skapa metaboliter utan att behöva ge substansen till ett människor eller djur. Eftersom olika arter bryter ner substanser till olika saker så är det viktigt att undersöka metabolismen i den art som är av intresse. Därför måste det studeras vilka metaboliter som bildas även i hästar så att dessa kan användas inom dopingkontroll.

I de fyra artiklar som ingår i denna avhandling har metaboliterna från fyra olika substanser studerats i hästurin och plasma. Metaboliter från en femte substans studerades också, men de framställdes med hjälp av metabolismmodeller på laboratorium. Syftet var att med hjälp av vätskekromatografi kopplat till högupplösande masspektrometri undersöka vilka metaboliter som bildades, samt föreslå metaboliter som kan användas inom hästdopingkontroll.

Ingen av de totalt fem substanser som undersöktes har blivit godkända som läkemedel, men forskning pågår för tre av dem. Däremot kan alla köpas över

internet. Flera av dem påträffas varje år i de dopingkontroller som WADA genomför, och om det förekommer i humandoping så är det mycket troligt att det även används på hästar. Därför är det viktigt att analysmetoderna för hästdoping förbättras och utvecklas, och denna avhandling är en del i det arbetet.

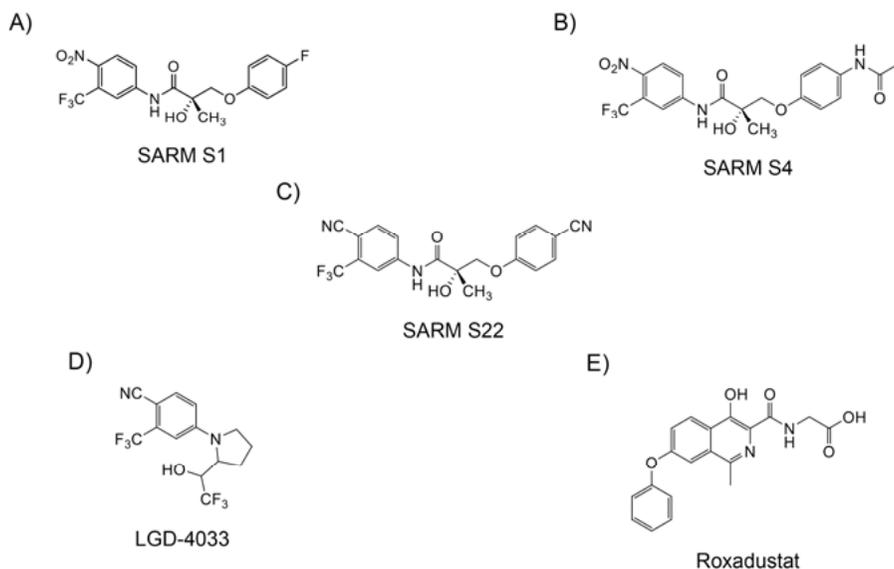
Analysmetoden som har använts i alla fyra arbeten är vätskekromatografi kopplat till högupplösande masspektrometri. I vätskekromatografi finns ett rör packat med små partiklar (den fasta fasen), samt en vätska (den mobila fasen) som pumpas igenom röret. Ämnen i provet separeras sedan baserat på hur ofta de fastnar på den fasta fasen jämfört med hur gärna de följer med den mobila fasen. Substanser som fastnar mer på partiklarna kommer därför att komma ut senare.

Provet sprayas därefter in i en masspektrometer och där joniseras substanserna man vill titta på, det vill säga de blir laddade. Sedan mäts massa över laddning för dessa joner. Lite förenklat kan man säga att jonerna ”vägs”. I högupplösande masspektrometri kan jonerna vägas så noga att det går att avgöra vilka sorters atomer jonen består av. Just detta är väldigt bra när strukturen för okända metaboliter ska bestämmas.

I artikel **I** och **II** undersöktes tre substanser från substansklassen SARM (selective androgen receptor modulator). SARMar är ämnen som, i människa, ger muskeltillväxt likt anabola steroider, men som har betydligt mindre biverkningar. De har därför hög potential att användas som dopingpreparat. I dessa studier gavs SARMarna S1, S4 och S22 (*Figur 1 A-C*) intravenöst till tre hästar vardera, varefter urin och plasma samlades in under 96 timmar.

För artikel **I** visade resultaten att redan tre timmar efter administration så kunde ingen av de tre SARMar längre detekteras i urinproverna, varför det är meningslöst att söka efter dessa. Istället föreslogs att den metaboliten som gav högst utslag på instrumenten användes istället då den också hade längst detektionstid. Denna metabolit hade genomgått samma transformationer (hydrolyt, hydroxylering och sulfonering) för alla tre ursprungssubstanserna. De övriga metaboliterna som detekterades (åtta för S1, nio för S4 och sju för S22) var till stor del gemensamma för de tre ämnena.

För artikel **II** undersöktes både vilka metaboliter som bildades och vilken koncentration av modersubstansen som fanns i plasman vid olika tidpunkter. Metabolitprofilen i plasman återspeglade den i urinen, med undantag för S22, där färre metaboliter hittades i plasman. För plasma hade modersubstansen lika lång eller längre detektionstid jämfört med huvudmetaboliten vilket ledde till att modersubstanserna rekommenderades som den lämpligaste strukturen att söka efter i dopingkontroll i plasma. De högsta uppmätta koncentrationerna i plasma återfanns i första mätpunkten efter administration (5 min) och var i genomsnitt 136 ng/ml för S1, 103 ng/ml för S4 och 125 ng/ml för S22.



Figur 1. Strukturformler för A) SARM S1, B) SARM S4, C) SARM S22, D) LGD-4033 och E) roxadustat.

I artikel **III** beskrivs metaboliterna för SARMen LGD-4033. Den har samma effekt som de tidigare nämnda SARMarna, men med en helt annan struktur (Figur 1 D). Även här hade tre hästar fått substansen intravenöst, urin- och plasmaprov samlades in och analyserades. Totalt hittades åtta metaboliter i urinen, varav sex även återfanns i plasman. LGD-4033 gick att finna plasma och i urinen efter en enzymatisk behandling. Då den hade längre detektionstid än metaboliterna både i behandlad urin (upp till 96 timmar) och i plasma (upp till 8 timmar) föreslogs det att den används i analys av dopingprov för hästar.

I projektet som ledde till artikel **IV** så undersöktes vilka metaboliter som bildas av substansen roxadustat (Figur 1 E) i olika laboriemetoder. Roxadustat är ett ämne som ökar bildandet av röda blodkroppar så att förmågan att transportera syra förbättras hos anemiska patienter. Detta är någon som kan komma att utnyttjas av atleter inom uthållighetsidrott då det i teorin kan ge samma effekt som höghöjdst träning. Dels användes en vanlig metod, att låta leverenzymen från häst och människa bryta ner ämnet, dels en ovanligare metod där en svamp får metabolisera substansen. Totalt hittades åtta olika metaboliter.

Tillsammans bidrar denna avhandling med kunskaper om vilka metaboliter som bildas i hästar för fyra nya substanser samt vilken struktur som bör analyseras vid dopingkontroll av hästar (artikel **I-III**). Avhandlingen har också visat möjliga metaboliter för en substans vars metaboliter inte studerats så mycket tidigare (artikel **IV**). Förhoppningsvis kommer dessa resultat att kunna implementeras inom hästdopingkontroll och därmed, på sikt, kunna minska användandet av otillåtna substanser inom hästsport.

Acknowledgement

The research presented in this thesis was carried out at the Department of Medicinal Chemistry at Uppsala University and at the Department of Chemistry, Environment and Feed Hygiene at the National Veterinary Institute (SVA). During my time at both these places, I've met several people that I would like to thank.

First adjunct professor *Mikael Hedeland*, my main supervisor. Thank you for always being encouraging and taking the time to discuss the projects with me. Your questions have made me think further. And my co-supervisor professor *Ulf Bondesson*, for being supportive and enthusiastic about my work.

I would also like to thank the co-authors for their contributions to the papers that are included in this thesis.

I am grateful to Apotekarsocieten for their generous travel grants that allowed me to attend international conferences.

I would take this opportunity to thank the people that I met at the (former) Division of Analytical Pharmaceutical Chemistry:

Professor emeritus *Curt Pettersson*, for the support over the years. *Ylva*, *Jakob* and *Micke* for taking the time to answer my teaching related questions. *Alfred* and *Albert*, I have had so much fun as a part of the A-team, both at work and at our conference trips. Discussions with you were not always helpful, but at least always entertaining. My time at BMC would not have been the same without you. *Axel*, for introducing me to the art of fungus cultivation. *Ida* and *Kristian* for fun conversations and for sharing your insights on various topics, scientific or not (mostly not). *Malin*, for always caring and being such a star. *Caroline*, *Mário* and *Louis* for the good times both inside and outside of BMC. *Daniel* and *Neeraj*, for a fruitful collaboration. And to everyone else at the department who have lifted the spirit both in the old and in the new lunch room over the past years.

A special merci beaucoup to *Elisabet* and *Alfred*, for being the best part of the spectacular trip to Paris. And *Ilse*, *Erik*, *Carro*, and *Rebecka*. Organizing the YSC conference with you was one of the best choices I've made during my time as a PhD student. Not only was it quite an experience, but I also got to know you guys (and learn about the proper use of emojis).

Och tack till er på SVA:

Gabor, för all hjälp med struliga instrument. Utan dig hade jag nog aldrig blivit klar! *Matilda*, *Annica* och *Lena E* för alla tips och tricks ni delat med er av. *Lotta*, för all hjälp med olika beställningar. *Emelie*, för att du alltid tänker på mig när du bakar. Och tack till alla andra på KMF för att ni bidragit till att jag har trivts så bra på SVA de här åren!

Joachim, tack för att du alltid finns där för mig.

Mamma och *pappa*, tack för att ni har stöttat mig oavsett vad jag har tagit mig för. Tack för att ni alltid tror på mig även när jag själv tvivlar. Tack för allt.

References

- [1] E. G. Clarke, M. S. Moss. A brief history of dope detection in racehorses. *Br. J. Sports Med.*, **1976**, *10*, 100–102.
- [2] M. Thevis. *Mass Spectrometry in Sports Drug Testing: Characterization of Prohibited Substances and Doping Control Analytical Assays*, John Wiley & Sons, In, Hoboken, New Jersey, **2010**.
- [3] World Anti-Doping Agency. Standard Prohibited List 2018. **2017**, 1–9.
- [4] J. K. Y. Wong, T. S. M. Wan. Doping control analyses in horseracing: A clinician 's guide. *Vet. J.*, **2014**, *200*, 8–16.
- [5] International Federation of Horseracing Authorities. International agreement on breeding, racing and wagering. **2016**, <http://www.horseracingintfed.com/Default.asp?secti>.
- [6] J. Sanford. Doping of Racehorses. *Br. J. Sports Med.*, **1974**, *8*, 176–180.
- [7] S. Guddat, G. Fußhöller, S. Beuck, a Thomas, H. Geyer, a Rydevik, U. Bondesson, M. Hedeland, a Lagojda, W. Schänzer, M. Thevis. Synthesis, characterization, and detection of new oxandrolone metabolites as long-term markers in sports drug testing. *Anal. Bioanal. Chem.*, **2013**, *405*, 8285–94.
- [8] Olympic.org. “IOC disqualifies four medallists from Athens 2004 following further analysis of stored samples,” Available at: <http://www.olympic.org/news/ioc-disqualifies-four-medallists-from-athens-2004-following-further-analysis-of-stored-samples/184931>, **2012**.
- [9] I. Corina, C. Mino R. *Drug Metabolism*, Springer-Verlag, Berlin/Heidelberg, **2005**.
- [10] L. B. Mellett. Comparative Drug Metabolism, in *Prog. Drug Res.*, **1969**, pp. 136–169.
- [11] M. Holcapek, L. Kolárová, M. Nobilis. High-performance liquid chromatography-tandem mass spectrometry in the identification and determination of phase I and phase II drug metabolites. *Anal. Bioanal. Chem.*, **2008**, *391*, 59–78.
- [12] E. F. A. Brandon, C. D. Raap, I. Meijerman, J. H. Beijnen, J. H. M. Schellens. An update on in vitro test methods in human hepatic drug biotransformation research: Pros and cons. *Toxicol. Appl. Pharmacol.*, **2003**, *189*, 233–246.
- [13] J. P. Scarth, P. Teale, T. Kuuranne. Drug metabolism in the horse: A review. *Drug Test. Anal.*, **2011**, *3*, 19–53.
- [14] R. V. Smith, D. Acosta, J. P. Rosazza. Cellular and microbial models in the investigation of mammalian metabolism of xenobiotics, in *Adv. Biochem. Eng. Vol. 5*, Springer-Verlag, Berlin/Heidelberg, **1977**, pp. 69–100.
- [15] H. Faber, M. Vogel, U. Karst. Electrochemistry/mass spectrometry as a tool in metabolism studies-A review. *Anal. Chim. Acta*, **2014**, *834*, 9–21.
- [16] U. Jurva, H. V. Wikström, L. Weidolf, A. P. Bruins. Comparison between electrochemistry/mass spectrometry and cytochrome P450 catalyzed oxidation reactions. *Rapid Commun. Mass Spectrom.*, **2003**, *17*, 800–810.

- [17] R. V. Smith, J. P. Rosazza. Microbial models of mammalian metabolism. Aromatic hydroxylation. *Arch. Biochem. Biophys.*, **1974**, *161*, 551–558.
- [18] C. D. Murphy. Drug metabolism in microorganisms. *Biotechnol. Lett.*, **2015**, *37*, 19–28.
- [19] J. Ferris, M. Fasco, F. L. Stylianopoulou, D. M. Jerina, J. W. Daly, A. M. Jeffrey. Monooxygenase activity in *Cunninghamella bainieri*: Evidence for a fungal system similar to liver microsomes. *Arch. Biochem. Biophys.*, **1973**, *156*, 97–103.
- [20] D. Zhang, Y. Yang, J. E. Leakey, C. E. Cerniglia. Phase I and phase II enzymes produced by *Cunninghamella elegans* for the metabolism of xenobiotics. *FEMS Microbiol. Lett.*, **1996**, *138*, 221–6.
- [21] A. Tevell Aberg, C. Olsson, U. Bondesson, M. Hedeland. A mass spectrometric study on meloxicam metabolism in horses and the fungus *Cunninghamella elegans*, and the relevance of this microbial system as a model of drug metabolism in the horse. *J. Mass Spectrom.*, **2009**, *44*, 1026–37.
- [22] L. Quinn, R. Dempsey, E. Casey, A. Kane, C. D. Murphy. Production of drug metabolites by immobilised *Cunninghamella elegans*: from screening to scale up. *J. Ind. Microbiol. Biotechnol.*, **2015**, *42*, 799–806.
- [23] J. Amadio, C. D. Murphy. Production of human metabolites of the anti-cancer drug flutamide via biotransformation in *Cunninghamella* species. *Biotechnol. Lett.*, **2011**, *33*, 321–326.
- [24] A. T. Åberg, H. Löfgren, U. Bondesson, M. Hedeland. Structural elucidation of N-oxidized clemastine metabolites by liquid chromatography/tandem mass spectrometry and the use of *Cunninghamella elegans* to facilitate drug metabolite identification. *Rapid Commun. Mass Spectrom.*, **2010**, *24*, 1447–1456.
- [25] A. Rydevik, U. Bondesson, M. Hedeland. Structural elucidation of phase I and II metabolites of bupivacaine in horse urine and fungi of the *Cunninghamella* species using liquid chromatography/multi-stage mass spectrometry. *Rapid Commun. Mass Spectrom.*, **2012**, *26*, 1338–46.
- [26] E. de Hoffmann, V. Stroobant. *Mass Spectrometry Principles and Applications*, John Wiley & Sons, Ltd, Chichester, **2007**.
- [27] World Anti-Doping Agency. 2013 Anti-Doping Testing Figures - Laboratory Report. **2014**, <https://wada-main-s3.amazonaws.com/resources/>.
- [28] World Anti-Doping Agency. 2014 Anti-doping testing figures report. **2014**.
- [29] World Anti-Doping Agency. 2015 Anti-Doping Testing Figures Anti-Doping Testing Figures by Laboratory. **2015**, *22*, 117–239.
- [30] World Anti-Doping Agency. 2016 Anti-doping testing figures report. **2016**, *9*.
- [31] J. Chen, J. Kim, J. T. Dalton. Discovery and therapeutic promise of selective androgen receptor modulators. *Mol. Interv.*, **2005**, *5*, 173–88.
- [32] S. Bhasin, R. Jasuja. Selective androgen receptor modulators as function promoting therapies. *Curr. Opin. Clin. Nutr. Metab. Care*, **2009**, *12*, 232–240.
- [33] J. T. Dalton, R. P. Taylor, M. L. Mohler, M. S. Steiner. Selective androgen receptor modulators for the prevention and treatment of muscle wasting associated with cancer. *Curr. Opin. Support. Palliat. Care*, **2013**, *7*, 345–351.
- [34] R. Narayanan, C. C. Coss, J. T. Dalton. Development of selective androgen receptor modulators (SARMs). *Mol. Cell. Endocrinol.*, **2017**, 1–9.
- [35] A. Jones, J. Chen, D. J. Hwang, D. D. Miller, J. T. Dalton. Preclinical characterization of a (S)-N-(4-Cyano-3-trifluoromethyl-phenyl)- 3-(3-fluoro, 4-chlorophenoxy)-2-hydroxy-2-methyl-propanamide: A selective androgen receptor modulator for hormonal male contraception. *Endocrinology*, **2009**, *150*, 385–395.

- [36] M. L. Mohler, V. a Nair, D. J. Hwang, I. M. Rakov, R. Patil, D. D. Miller. Nonsteroidal tissue selective androgen receptor modulators: a promising class of clinical candidates. *Expert Opin. Ther. Pat.*, **2005**, *15*, 1565–1585.
- [37] World Anti-Doping Agency. “The 2008 Prohibited List,” Available at: https://www.wada-ama.org/sites/default/files/resources/files/WADA_Prohibited_List_2008_EN.pdf, **2008**.
- [38] L. Geldof, O. J. Pozo, L. Lootens, W. Morthier, P. Van Eenoo, K. Deventer. In vitro metabolism study of a black market product containing SARM LGD-4033. *Drug Test. Anal.*, **2017**, *9*, 168–178.
- [39] T. Kuuranne, A. Leinonen, W. Schänzer, M. Kamber, R. Kostianen, M. Thevis. Aryl-propionamide-derived selective androgen receptor modulators: liquid chromatography-tandem mass spectrometry characterization of the in vitro synthesized metabolites for doping control purposes. *Drug Metab. Dispos.*, **2008**, *36*, 571–81.
- [40] O. Krug, A. Thomas, S. Beuck, I. Schenk, M. Machnik, W. Schänzer, U. Bondesson, M. Hedeland, M. Thevis. Characterization of In Vitro Synthesized Equine Metabolites of the Selective Androgen Receptor Modulators S24 and S4. *J. Equine Vet. Sci.*, **2012**, *32*, 562–568.
- [41] E. de Rijke, M. L. Essers, J. C. W. Rijk, M. Thevis, T. F. H. Bovee, L. a. van Ginkel, S. S. Sterk. Selective androgen receptor modulators: in vitro and in vivo metabolism and analysis. *Food Addit. Contam. Part A*, **2013**, *30*, 1517–1526.
- [42] A. Rydevik, M. Thevis, O. Krug, U. Bondesson, M. Hedeland. The fungus *Cunninghamella elegans* can produce human and equine metabolites of selective androgen receptor modulators (SARMs). *Xenobiotica.*, **2013**, *43*, 409–20.
- [43] M. Thevis, E. Gerace, A. Thomas, S. Beuck, H. Geyer, N. Schlörer, J. D. Kearbey, J. T. Dalton, W. Schänzer. Characterization of in vitro generated metabolites of the selective androgen receptor modulators S-22 and S-23 and in vivo comparison to post-administration canine urine specimens. *Drug Test. Anal.*, **2010**, *2*, 589–598.
- [44] M. Thevis, A. Thomas, G. Fushöller, S. Beuck, H. Geyer, W. Schänzer. Mass spectrometric characterization of urinary metabolites of the selective androgen receptor modulator andarine (S-4) for routine doping control purposes. *Rapid Commun. Mass Spectrom.*, **2010**, *24*, 2245–54.
- [45] M. Thevis, A. Thomas, I. Möller, H. Geyer, J. T. Dalton, W. Schänzer. Mass spectrometric characterization of urinary metabolites of the selective androgen receptor modulator S-22 to identify potential targets for routine doping controls. *Rapid Commun. Mass Spectrom.*, **2011**, *25*, 2187–95.
- [46] E. Grata, L. Perrenoud, M. Saugy, N. Baume. SARM-S4 and metabolites detection in sports drug testing: a case report. *Forensic Sci. Int.*, **2011**, *213*, 104–8.
- [47] B. Starcevic, B. D. Ahrens, A. W. Butch. Detection of the selective androgen receptor modulator S-4 (Andarine) in a doping control sample. *Drug Test. Anal.*, **2013**, *5*, 377–9.
- [48] A. T. Cawley, C. Smart, C. Greer, M. Liu Lau, J. Keledjian. Detection of the selective androgen receptor modulator andarine (S-4) in a routine equine blood doping control sample. *Drug Test. Anal.*, **2015**, DOI 10.1002/dta.1867.
- [49] “GTx, Inc - Pipeline,” Available at: <http://www.gtxinc.com/pipeline/>, **2018**.
- [50] “Viking Therapeutics - Pipeline Overview,” Available at: <http://www.vikingtherapeutics.com/pipeline/overview/>, **2018**.

- [51] H. D. Cox, D. Eichner. Detection of LGD-4033 and its metabolites in athlete urine samples. *Drug Test. Anal.*, **2017**, *9*, 127–134.
- [52] M. Thevis, A. Lagojda, D. Kuehne, A. Thomas, J. Dib, A. Hansson, M. Hedeland, U. Bondesson, T. Wigger, U. Karst, W. Schänzer. Characterization of a non-approved selective androgen receptor modulator drug candidate sold via the Internet and identification of in vitro generated phase-I metabolites for human sports drug testing. *Rapid Commun. Mass Spectrom.*, **2015**, *29*, 991–9.
- [53] J. D. Webb, M. L. Coleman, C. W. Pugh. Hypoxia, hypoxia-inducible factors (HIF), HIF hydroxylases and oxygen sensing. *Cell. Mol. Life Sci.*, **2009**, *66*, 3539–3554.
- [54] A. Hayat, D. Haria, M. O. Salifu. Erythropoietin stimulating agents in the management of anemia of chronic kidney disease. *Patient Prefer. Adherence*, **2008**, *2*, 195–200.
- [55] A. Besarab, R. Provenzano, J. Hertel, R. Zabaneh, S. J. Klaus, T. Lee, R. Leong, S. Hemmerich, K.-H. P. Yu, T. B. Neff. Randomized placebo-controlled dose-ranging and pharmacodynamics study of roxadustat (FG-4592) to treat anemia in nondialysis-dependent chronic kidney disease (NDD-CKD) patients. *Nephrol. Dial. Transplant.*, **2015**, *30*, 1665–1673.
- [56] A. Besarab, E. Chernyavskaya, I. Motylev, E. Shutov, L. M. Kumbar, K. Gurevich, D. T. M. Chan, R. Leong, L. Poole, M. Zhong, K. G. Saikali, M. Franco, S. Hemmerich, K.-H. P. Yu, T. B. Neff. Roxadustat (FG-4592): Correction of Anemia in Incident Dialysis Patients. *J. Am. Soc. Nephrol.*, **2016**, *27*, 1225–1233.
- [57] R. Provenzano, A. Besarab, S. Wright, S. Dua, S. Zeig, P. Nguyen, L. Poole, K. G. Saikali, G. Saha, S. Hemmerich, L. Szczech, K. H. P. Yu, T. B. Neff. Roxadustat (FG-4592) Versus Epoetin Alfa for Anemia in Patients Receiving Maintenance Hemodialysis: A Phase 2, Randomized, 6- to 19-Week, Open-Label, Active-Comparator, Dose-Ranging, Safety and Exploratory Efficacy Study. *Am. J. Kidney Dis.*, **2016**, *67*, 912–924.
- [58] FibroGen. “FibroGen - Pipeline,” Available at: http://www.fibrogen.com/clinical_pipeline, **2017**.
- [59] S. Nagel, N. P. Talbot, J. Mecinović, T. G. Smith, A. M. Buchan, C. J. Schofield. Therapeutic Manipulation of the HIF Hydroxylases. *Antioxid. Redox Signal.*, **2010**, *12*, 481–501.
- [60] C. Buisson, A. Marchand, I. Bailloux, A. Lahaussais, L. Martin, A. Molina. Detection by LC–MS/MS of HIF stabilizer FG-4592 used as a new doping agent: Investigation on a positive case. *J. Pharm. Biomed. Anal.*, **2016**, *121*, 181–187.
- [61] N. Stojiljkovic, A. Paris, P. Garcia, M.-A. Popot, Y. Bonnaire, J.-C. Tabet, C. Junot. Evaluation of horse urine sample preparation methods for metabolomics using LC coupled to HRMS. *Bioanalysis*, **2014**, *6*, 785–803.
- [62] A. G. Fragkaki, N. Kioukia-Fougia, P. Kioussi, M. Kioussi, M. Tsivou. Challenges in detecting substances for equine anti-doping. *Drug Test. Anal.*, **2017**, DOI 10.1002/dta.2162.
- [63] E. L. Schymanski, J. Jeon, R. Gulde, K. Fenner, M. Ruff, H. P. Singer, J. Hollender. Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. *Environ. Sci. Technol.*, **2014**, *48*, 2097–2098.
- [64] K. Levsen, H.-M. Schiebel, B. Behnke, R. Dötzer, W. Dreher, M. Elend, H. Thiele. Structure elucidation of phase II metabolites by tandem mass spectrometry: an overview. *J. Chromatogr. A*, **2005**, *1067*, 55–72.

- [65] X. Huang, R. Powers, A. Tymiak, R. Espina, V. Roongta. Introduction to NMR and Its Application in Metabolite Structure Determination, in *Drug Metab. Drug Des. Dev. Basic Concepts Pract.*, (Eds: D. Zhang, M. Zhu, W.G. Humphreys), **2007**, pp. 369–409.
- [66] L. Perrenoud, C. Schweizer Grundisch, N. Baume, M. Saugy, R. Nicoli. Risk of false positive results to SARM S-4 in case of therapeutic use of antineoplastic/antiandrogen drug containing flutamide: a case study. *Drug Test. Anal.*, **2016**, *8*, 1109–1113.
- [67] A. Tevell, H. Lennernäs, M. Jönsson, M. Norlin, B. Lennernäs, U. Bondesson, M. Hedeland. Flutamide metabolism in four different species in vitro and identification of flutamide metabolites in human patient urine by high performance liquid chromatography/tandem mass spectrometry. *Drug Metab. Dispos.*, **2006**, *34*, 984–92.
- [68] F. J. Mendoza, J. M. Serrano-Rodriguez, A. Buzon-Cuevas, A. Perez-Ecija. Pharmacokinetics of the anti-androgenic drug flutamide in healthy stallions. *Vet. J.*, **2017**, *224*, 50–54.
- [69] European Medicines Agency. “Guideline on bioanalytical method validation,” **2011**.
- [70] C. E. Cerniglia, J. P. Freeman, R. K. Mitchum. Glucuronide and Sulfate Conjugation in the Fungal Metabolism of Aromatic Hydrocarbons. **1982**, *43*, 1070–1075.
- [71] S. Watanabe, U. Kuzhiumparambil, Z. Winiarski, S. Fu. Data on individual metabolites of synthetic cannabinoids JWH-018, JWH-073 and AM2201 by *Cunninghamella elegans*. *Data Br.*, **2016**, *7*, 332–340.
- [72] B. Ma, H. H. Huang, X. Y. Chen, Y. M. Sun, L. H. Lin, D. F. Zhong. Biotransformation of metoprolol by the fungus *Cunninghamella blakesleeana*. *Acta Pharmacol. Sin.*, **2007**, *28*, 1067–1074.
- [73] D. Eichner, R. M. Van Wagoner, M. Brenner, J. Chou, S. Leigh, L. R. Wright, L. A. Flippin, M. Martinelli, O. Krug, W. Schänzer, M. Thevis. Implementation of the prolyl hydroxylase inhibitor Roxadustat (FG-4592) and its main metabolites into routine doping controls. *Drug Test. Anal.*, **2017**, DOI 10.1002/dta.2202.

Acta Universitatis Upsaliensis

*Digital Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Pharmacy 251*

Editor: The Dean of the Faculty of Pharmacy

A doctoral dissertation from the Faculty of Pharmacy, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy".)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-344310



ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2018