Equine Urine Screening for SARMs Using Solid Phase Extraction

Followed by Triple Quadrupole LC-MS

BY

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THESIS

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF FIGURES

LIST OF ABBREVIATIONS

SARMs	Selective Androgen Receptor Modulators
AAS	Anabolic Androgenic Steroids
WADA	World Anti-Doping Agency
IFHA	International Federation of Horseracing Authorities
FDA	Food and Drug Administration
LC-MS/MS	Liquid Chromatography-(Tandem) Mass Spectrometry
SPE	Solid Phase Extraction
LC-MS QQQ	Triple Quadrupole Liquid Chromatography-Mass Spectrometry
DHT	Dihydrotestosterone
HPG	Hypothalamic-Pituitary-Gonadal Axis
AR	Androgen Receptor
EIC	Extracted Ion Chromatogram
LOD	Limit of Detection
QC	Quality Control

SUMMARY

Historically, testosterone and other anabolic androgens have been tried for potential therapeutic use in diseases causing loss of muscle and bone mass. However, steroidal androgens have considerable limitations due to their undesirable physicochemical and pharmacokinetic properties including negative effects on the prostate, serum lipids, and cardiovascular system. The recent discovery of nonsteroidal selective androgen receptor modulators (SARMs) presents an encouraging substitute for testosterone therapies with the ability to selectively stimulate anabolic tissues such as muscle and bone with minimal effects in the prostate.

With the beneficial anabolic effects and lesser pharmacologic side effects, SARMs have a high potential for abuse in the sports and horse racing industry for those who want to gain an unfair advantage. Many SARM clinical candidates including Andarine, LGD-4033, and MK-2866 are easily accessible and illicitly used via the black market. Although there has only been one reported case of a SARM candidate in an equine doping control sample, the number of cases is expected to be much higher due to the lack of validated screening methods included in routine urine analysis and the belief that these compounds will go undetected by anti-doping laboratories. The use of liquid chromatography tandem mass spectrometry triple quadrupole (LC-MS QQQ), a technique implemented by several equine regulatory agencies, allows laboratories to screen for many different classes of drugs with concentrations at or below thresholds limits.

In this study, a sensitive and robust triple quadrupole LC-MS method was successfully validated according to the Analytical Forensic Testing Laboratory *'Validation Requirements for Methods Using Instrumental Analysis'* Standard Operating Procedure #AFTL GE 005-03, allowing for the rapid screening and confirmation of selective androgen receptor modulators Andarine, LGD-4033, and MK-2866 in equine urine. High flow mixed-mode solid phase

extraction (SPE) columns from United Chemical Technologies followed by Agilent 6400 series LC-MS QQQ analysis were employed to screen for these emerging therapeutics and illicitly used drug candidates.

Drug-free equine urine aliquots were fortified with the compounds of interest. After overnight enzyme hydrolysis (beta-glucuronidase), the pH of the samples was adjusted to 6. The SPE columns were conditioned and the samples were added, followed by a wash with 100 mM phosphate buffer (pH 6). After acidification, elution of acidic drugs using ethyl acetate was performed. Basic compound elution was accomplished by using 78:20:2 of dichloromethane: isopropyl alcohol: ammonium hydroxide. Both elutions were dried down and reconstituted in 1:1 0.2% formic acid in water: methanol. Twenty five µL were injected onto the LC-MS QQQ for analysis.

Results showed that the use of the UCT mixed mode column allows successful extraction recovery of all compounds of interest. Extraction recoveries ranged from 47% to 63%. Matrix effect was also investigated and suppression of signal for all compounds was found, ranging from -85% to -99%. Limit of detection (LOD) was approximately 1 ng/µL for all compounds. Quantitative requirements were met for most compounds, with all precision values less than 20%. Bias requirements were met for two of the three compounds, with accuracy values lower than 25%, excluding LGD-4033. Expanded uncertainties ranged from 11% to 19%.

In conclusion, the use of SPE followed by LC-MS QQQ for instrumental screening of Andarine, LGD-4033, and MK-2866 in equine urine produces satisfactory results and is recommended. Adding these emerging SARM therapeutic candidates to routine urine screening will maintain integrity in animal and human sports and deter handlers who are tempted

to administer these readily available drugs without sufficient research on the adverse health effects to the animal.

I. INTRODUCTION

A. Background

Androgens play a significant role in male development and maintenance of male secondary characteristics including muscle and bone mass, body fat, and spermatogenesis *(1)*. Since the discovery of testosterone, an endogenous androgen, and its therapeutic benefits in the 1930's, steroidal androgens have been involved in numerous clinical studies to treat hypogonadism (reduction of testosterone secretion in testes or ovaries), muscle wasting, sarcopenia (age-related decline in lean body mass), osteoporosis, cancer cachexia (ongoing loss of skeletal muscle mass), male contraception, and hormone replacement therapy *(2-7)*. Antiandrogens have also been used to treat acne, alopecia (male-pattern baldness), hirsutism (male-pattern hair growth in women), benign prostatic hyperplasia (BPH, also called prostate gland enlargement), breast cancer, and prostate cancer *(8-10)*. Anabolic steroids have also been used to treat horses with various diseases causing low body mass or strength in equine veterinary medicine *(70)*. In summary, synthetic steroids and testosterone have been used clinically to treat similar diseases in both human and equine models, and therefore have similar effects on humans and horses.

A major drawback of anabolic androgenic steroids (AAS) is their low bioavailability due to extensive metabolism. The majority of testosterone is metabolized after oral administration before it reaches the systemic circulation *(11)*. As a result, testosterone is commonly administered through transdermal patch or intramuscular injections. In addition, AAS were synthesized by altering the structure of testosterone with an attempt to provide greater therapeutic benefit, but risk of hepatotoxicity only increased. AAS administration also led to various adverse side effects especially in the prostate *(12)*. Consequently, steroidal androgens

have restricted clinical applications due to low bioavailability, absence of tissue selectivity, inability to significantly alter the steroid skeleton due to possible hepatotoxicity, cardiovascular risk, and occasional steroid receptor cross reactivity.

The recent discovery of nonsteroidal selective androgen receptor modulators (SARMs) presents an encouraging substitute for testosterone therapies with the ability to selectively stimulate anabolic tissues such as muscle and bone with minimal effects in the prostate *(13)*. SARMs advantages over steroidal androgens include greater oral bioavailability, structural flexibility, androgen receptor specificity, tissue selectivity, and diminished steroidal side effects making them excellent clinical candidates *(14)*.

B. **Statement of the Problem**

Due to the beneficial anabolic effects combined with lesser pharmacologic side effects and ease of accessibility, SARMs have a high potential for abuse in the sports and horse racing industry for those who want to gain an unfair advantage. Handlers illicitly administer performance enhancers to their horses with hopes to improve speed and strength to win more races, and as a result, increase their monetary gain. Many SARM clinical candidates including Andarine, LGD-4033, and MK-2866 are easily accessible and illicitly used via the black market *(15-18)*. SARMs abuse was first recognized by the World Anti-Doping Agency (WADA), with their inclusion of the entire group of compounds on their prohibited list in 2008 *(19)*. Shortly after, SARMs were also prohibited by the International Federation of Horseracing Authorities (IFHA) *(20)*. Since the initial discovery of SARMs abuse, positive cases involving the detection of LGD-4033 and Andarine in human athletes *(21-23)* as well as the detection of Andarine in a racehorse have been published *(20)*. In 2013 alone, 13 positive cases were reported in human

athletes *(24)*. Based on the numerous recently published case reports, it is evident that SARMs have a high potential for abuse.

Often times it is common to find that doping agents abused in human sports are also abused in equine sports. The detection of designer steroids has been observed in human and subsequent equine doping control samples. Many equine racing laboratories have initiated the detection of anabolic steroids in animal sports by utilizing various studies on the metabolism of these unapproved substances. It is essential to study the metabolism of these compounds in order to target appropriate metabolites to detect AAS abuse in horse racing. However, metabolism studies on designer steroids and unapproved substances like SARMs can be challenging with the lack of research and difficulty obtaining ethical approval for *in vivo* studies *(70)*. Metabolism studies focusing on SARMs in equine models have been very recently initiated (2015-2018) *(24, 32)*, but there are no current validated methods to screen for SARMs in equine urine.

In the past, many illegal steroidal substances found their way from doping for performance enhancement in the sports industry to livestock production. Steroids and other hormonally active substances including stilbenes, resorcylic acid lactones, and corticosteroids have historically been used in livestock fattening as they enhance the weight gain of animals raised for food sources *(25-27)*. In addition to sports abuse, SARMs have a high potential for misuse in livestock due to their availability on the black market and tissue selectivity. SARMs can enhance the size and weight of animal products without the unwanted side effects.

C. **Significance of the Problem**

These findings raise major concerns considering these drug candidates have not been approved by the US Food and Drug Administration (FDA). Sufficient research on the health

effects and purity of these illicitly produced products is lacking. There may be unknown health risks associated with the illicit use of these compounds, and athletes and handlers must be cautious when administering SARMs *(16-18)*.

Currently, to the best of the author's knowledge, anti-doping laboratories including the University of Illinois at Chicago Analytical Forensic Testing Laboratory (UIC AFTL) do not have validated methods to screen for SARMs in equine urine. Due to the potential for continued illicit use in racehorses, there is a need for screening methods to detect SARMs in equine samples to prevent handlers from undermining horse racing regulations and fair competition.

In addition to equine screening, the absence of SARMs in screening methods for livestock doping control allows food producers to administer these readily available compounds without sufficient research on the unknown effects to the consumer and/or animal. This further inhibits fair trade between food producers. Therefore, there is a need for validated methods not only to detect these substances in human and equine samples, but livestock samples as well *(26, 28)*. There have been some validation studies performed for the detection of SARMs by liquid chromatography-(tandem) mass spectrometry (LC-MS/MS), including a recent study published in April 2018 involving the successful identification of SARMs in bovine urine *(25)*.

D. **Purpose of the Study**

The purpose of this study was to successfully validate a sensitive and robust method using solid phase extraction (SPE) followed by triple quadrupole liquid chromatography-mass spectrometry (LC-MS QQQ) allowing for the rapid screening and confirmation of selective androgen receptor modulators Andarine, LGD-4033, and MK-2866 in equine urine. The method was developed and validated according to the Analytical Forensic Testing Laboratory,

'*Validation Requirements for Methods Using Instrumental Analysis*' Standard Operating Procedure #AFTL GE 005-03, an accredited anti-doping laboratory for horse racing entities.

E. **Significance of the Study**

Although there has only been one reported case, the number of equine SARM doping cases is expected to be much higher due to the lack of validated screening methods included in routine urine analysis and the belief that these compounds will go undetected by anti-doping laboratories *(29)*. Popularly abused SARMs including Andarine, LGD-4033, and MK-2866 should be implemented in routine screenings in anti-doping laboratories. This will maintain integrity in animal and human sports and deter handlers who are tempted to administer these readily available drugs without enough research on the adverse health effects to the animal *(30).*

Although there has been some analytical method development with these emerging therapeutics for human doping control *(31)*, there is a critical need for validated methods to detect the illicit use of SARMs in horse racing *(32)*. A major challenge in the toxicology field is staying ahead of the black market and those attempting to get around the law. There is an increasing number of recently developed and therefore unapproved substances available on the black market, as observed with SARMs, that go undetected due to lack of knowledge and research. With a validated method to detect these newly developed compounds, suspected and future cheaters can be prevented, and anti-doping laboratories can advance in the fight to control substance abuse *(17).*

II. CONCEPTUAL FRAMEWORK AND RELATED LITERATURE

A. Conceptual Framework

The innovative and detailed nature of this study required a conceptual framework that would expand rather than limit the knowledge of equine urine screening methodology and selective androgen receptor modulators. Their desirable pharmacokinetic and pharmacodynamic properties have significantly motivated their clinical advancement as well as their widespread abuse in sports, horse racing, and even livestock. Understanding these properties is critical and necessary when considering the purpose and significance of this study.

1. Androgens

As previously mentioned, androgens play a crucial role in several biological processes including the development and maintenance of male characteristics such as bone and muscle mass as well as spermatogenesis. These biological effects are typically classified as androgenic which involves secondary sexual organs or anabolic which involves peripheral effects on muscle and bone. Dissolution of these two effects is recommended for optimal therapeutic benefit *(5).*

The predominant endogenous androgens circulating throughout the body are testosterone and its active metabolite, 5α-dihydrotestosterone (DHT). Testosterone is synthesized primarily from Leydig cells of the testes in males and the adrenal cortex in females. Testosterone synthesis is controlled by the hypothalamic-pituitary-gonadal (HPG) axis *(12, 33).* The anterior pituitary gland secretes luteinizing hormone (LH) resulting in the natural production of testosterone. Administration of high levels of exogenous androgens (AAS) causes feedback inhibition within the central nervous system and HPG resulting in decreased synthesis of intratesticular testosterone. Essential initiation and maintenance of spermatogenesis, as

evidenced by the infertility of hypogonadal men, is established by elevated concentrations of naturally produced testosterone *(30).* Therefore, tissue-selective androgens are desired to prevent suppression of the HPG axis (LH suppression) for optimal therapeutic benefit *(12).*

2. **Androgen Receptor**

Much like testosterone, SARMs act on the androgen receptor. The androgen receptor (AR), a member of the nuclear and steroid receptors, is a protein that functions as an intracellular transcriptional factor with its primary function regulated by the binding of androgen ligands. Binding of androgens results in a conformational change to the protein structure affecting receptor-protein-DNA communications. AR is highly expressed in androgen target tissues including the prostate, adrenal gland, and epididymis, and it is also observed at moderate levels in the central nervous system, skeletal muscle, and liver *(33).*

The AR gene is located on the human X chromosome. AR structure is composed of three major domains observed in all steroid receptors; the N-terminal domain (NTD), which is responsible for modulatory function, the DNA-binding domain (DBD), and C-terminal ligand-binding domain (LBD). The ligand-binding domain is responsible for steroid specificity for individual hormones. The similar three domain structure observed between steroid receptors is the basis for the cross reactivity frequently seen with synthetic steroids *(33-35).*

3. **Selective Androgen Receptor Modulators**

Selective androgen receptor modulators (SARMs) can be defined as, "A compound that is an antagonist or weak agonist in the prostate but agonist in the pituitary and muscle and orally available with low hepatotoxicity" *(33).* SARMs can be categorized as steroidal or nonsteroidal, although the main topic of this paper will cover nonsteroidal. Nonsteroidal SARMs provide superior therapeutic benefit and clinical application which is

mainly due to their tissue selectivity resulting in decreased adverse side effects *(36, 37)*. No SARM therapeutic candidate has yet received full clinical approval by the FDA, and most drug candidates are currently undergoing numerous preclinical and clinical studies for androgen replacement therapy to address one or some actions of typical steroidal androgens *(30, 38, 39)*. SARMs in development are provided in Table I below *(36)*.

TABLE I: SARMS IN DEVELOPMENT

Chemotype	**SARM**	**Current Status**	**Company**
Aryl propionamide	Andarine	Phase I	GTx & Janssen
Aryl propionamide	Ostarine	Phase III (cancer cachexia)	GTx
Steroid	MK-0773	Phase II (sarcopenia)	Merck
Quinolinone	LGD-2226	Phase I (dis.)	Ligand
Quinolinone	LGD-2941	Phase I (frailty and osteoporosis)	Ligand & TAP
Quinolinone	LGD-3303	Preparing Phase I	Ligand
Quinolinone	LGD-4033	Preparing Phase II	Ligand
Bicyclic hydantoin	BMS-564929	Phase I (age-related functional decline)	BMS & Pharmacopia
Unknown	GSK-971086	Phase I (safety and tolerability)	GSK
Aniline	ACP-105	Preparing trial	Acadia
Aniline	RAD140	Preparing trial	Radius

One concern raised with the first-generation non-steroidal SARMs was that testosterone and steroidal agents exhibit stronger anabolic effects on lean body mass, strength, skeletal muscle rebuilding, and functional performance. As we have observed in the past 80 years with athlete steroid use, these now regulated steroidal androgens have definitively and continuously exhibited a substantial increase in athletic performance and appearance, which can be hard to match with the newly developed non-steroidal compounds *(3).*

a. **Structures**

Nonsteroidal SARMs were subject to a series of structural modifications to explore structure-activity relationships (SAR) and obtain optimal AR binding efficiency and pharmacologic effect *(40, 41).* Different classes of SARMs were developed by evaluating their desirable pharmacokinetic properties including efficacy and potency *(42, 43).* Major SARM classes include aryl propionamides (Andarine and Ostarine), quinolinones, tetrahydroquinolinones, and pyrrolidinyl-benzonitriles (LGD-4033) *(30).* Structure characteristics of selected SARMs are listed in Table II *(38).* Chemical structure and class of compounds in this study are listed in Table III *(16).*

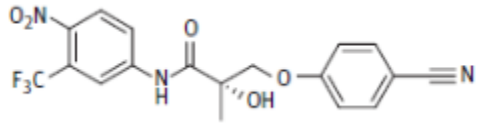
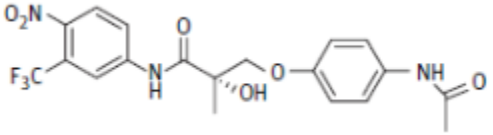
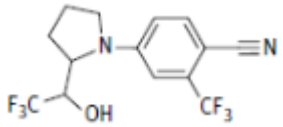
4. **Pharmacodynamics of SARMs**

The pharmacodynamics of SARMs, with their preferential ability to separate anabolic and androgenic effects, has been heavily debated since the discovery of the first nonsteroidal SARM in 1998. First developed were selective estrogen receptor modulators (SERMs). SERMs act as estrogen receptor (ER) antagonists in tissues like breast and uterus, but as agonists in anabolic tissues such as muscle and bone *(11, 44).* Our knowledge of SERMs and ER pharmacology has significantly shaped the hypothesized molecular mechanisms for SARMs *(5).* Although the mechanism of action of these nonsteroidal ligands is still not fully understood,

TABLE II: STRUCTURE CHARACTERISTICS OF SELECTED SARMS

NO.	SARM	Elemental Composition	Pharmacophore	Molecular Mass (Da)
1	S-1	$C_{17}H_{14}F_4N_2O_5$	aryl propionamide	402.08
2	S-4 (Andarine)	$C_{19}H_{18}F_3N_3O_6$	aryl propionamide	441.11
3	S-9	$C_{17}H_{14}ClF_3N_2O_5$	aryl propionamide	418.05
4	S-22 (Ostarine)	$C_{19}H_{14}F_3N_3O_3$	aryl propionamide	389.10
5	LGD-2941	$C_{17}H_{16}F_6N_2O_2$	quinolinone	394.11
6	LGD-3303	$C_{16}H_{14}ClF_3N_2O$	quinolinone	342.07
7	LG-121071	$C_{15}H_{15}F_3N_2O$	quinolinone	296.11
8	S-40503	$C_{15}H_{23}N_3O_3$	tetrahydroquinoline	293.17
9	S-101479	$C_{26}H_{24}F_2N_4O_3$	tetrahydroquinoline	478.18
10	BMS-564929	$C_{14}H_{12}ClN_3O_3$	hydantoin	305.06
11	JNJ-37654032	$C_{11}H_7Cl_2F_3N_2O$	benzoimidazole	309.10
12	RAD140	$C_{20}H_{16}ClN_5O_2$	phenyl-oxadiazole	393.10
13	AC262536	$C_{18}H_{18}N_2O$	tropanol	278.14
14	ACP-105	$C_{16}H_{19}ClN_2O$	tropanol	290.12
15	LGD-4033/ VK 5211	$C_{14}H_{12}F_6N_2O$	pyrrolidinyl-benzonitrile (quinolinone)	338.09
16	Compound 17m	$C_{16}H_{16}N_2O$	pyrrolidinyl-naphthonitrile	252.13
17	GLPG0634	$C_{19}H_{14}F_3N_3O_3$	diarylhydantoin	389.10
18	MK-3984	$C_{17}H_{12}F_7NO_2$	phenylmethanamide	395.08
19	YK-11	$C_{25}H_{34}O_6$	steroidal	430.24

TABLE III: CHEMICAL STRUCTURE AND CLASS OF DRUGS IN THIS STUDY

Compound	Chemical Structure	Class
Ostarine $C_{19}H_{14}F_3N_3O_3$		aryl propionamide
Andarine $C_{19}H_{18}F_3N_3O_6$		aryl propionamide
LGD-4033 $C_{14}H_{12}F_6N_2O$		pyrrolidinyl-benzonitrile (quinolinone)

there are a few hypothesized mechanistic models dealing with genomic and nongenomic pathways and enzyme specificity *(13, 45, 46)*.

a. **Genomic and Nongenomic Pathways**

Androgen binding causes conformational changes to the receptor structure, modifying the surface topology and subsequent protein-protein interactions. Interactions involve cystolic proteins mediating signal transduction pathways (nongenomic pathway) and coregulators involved with transcriptional activation and inhibition (genomic pathway). Some studies have reported that SARMs induce a conformational change distinct from DHT. It has been hypothesized that DHT and SARMs exhibit similar rates of transcriptional initiation in anabolic tissues and variable rates in androgenic tissues leading to differential gene expression *(11, 35)*.

SARMs have also been shown to activate distinct nongenomic effects in different tissue cell lines. In recent studies, both SARMs and DHT initiated similar signaling pathways in bone cells, however, the two androgens activated two completely different signaling pathways in prostate cells. These findings suggest that conformational changes to the AR are ligand specific, mediating different intracellular signaling pathway combinations resulting in distinct biological effects in AR target tissues such as the prostate *(11).*

b. **Other Mechanisms**

Other mechanisms refer to SARMs' lack of specificity for enzymes 5α-reductase and aromatase as well as preferential ligand tissue distribution. As previously mentioned, testosterone is one of the major endogenous androgens circulating throughout the body. Testosterone is converted to its active metabolite DHT by 5α-reductase, an enzyme that is highly expressed in the prostate. Therefore, testosterone is extremely potent in androgenic tissues due to its rapid conversion to DHT in the prostate, with DHT having a much higher binding affinity to the AR. However, nonsteroidal SARMs are not substrates for 5α-reductase, so they do not produce an amplified effect in androgenic tissues like DHT. Similarly, testosterone is converted to estrogen by aromatase. Nonsteroidal SARMs cannot be aromatized, so they are not sources of estrogen production in the body. This further contributes to the tissue selectivity seen with SARMs and lack of side effects like feminization in men or virilization in women *(11).* Regarding compound tissue distribution, recent studies have shown that nonsteroidal ligands do not preferentially accumulate in anabolic tissues, which refutes this hypothesis as a possible mechanism of action *(12, 33).*

In summary, there are many hypothesized mechanisms to explain the tissue selectivity observed with SARMs. Some and/or all of these explained molecular models potentially

contribute to the increased anabolic effects and diminished androgenic effects demonstrated by these compounds.

5. **Pharmacokinetics of SARMs**

a. **Animal and Human Models**

Many nonsteroidal AR ligands demonstrated similar binding affinity and *in vitro* functional activity to that of testosterone, however these compounds were rapidly metabolized yielding them inactive. Slight structural modification significantly decreased the hepatic metabolism allowing for the identification of a SARM candidate, S-4, in a rat model. Major metabolism pathways were identified including oxidation, hydrolysis, and sulfate conjugation. After IV administration, S-4 demonstrated linear pharmacokinetics. The lack of parent drug detected in the urine suggests S-4 and other SARM compounds are rapidly metabolized. After oral dosing, S-4 was immediately absorbed and completely bioavailable, with an average half-life of four hours in a dog and rat model *(47*). *In vivo* studies also exhibited extensive metabolism of SARMs Andarine and Ostarine in humans *(48-50).*

b. **Equine Models**

Metabolite patterns are often distinct in animals of different species. According to the first *in vivo* metabolic study of SARMs in horses published in 2015 *(51),* urine samples collected 3 hours post administration contained high metabolite response with minimal parent compound response. This indicates that SARMs are quickly and extensively metabolized in horses.

These findings suggest that metabolites should be used as target compounds in screening methods which could be beneficial for many reasons. One reason is sample contaminants can be excluded as a possibility because SARM-derived metabolites must have been exposed to the

metabolic system of a horse. Another reason to target metabolites instead of the parent compound is the detection window is significantly extended *(29)*. However, the parent compound of SARM drugs is much easier to obtain commercially, and because these compounds are relatively new, no reference standards are readily available for the metabolites. Most studies considering SARM metabolites as analytical targets in screening methods have synthesized the standards themselves or referenced previously reported literature *(32, 52-54)*. Therefore, this study uses SARM parent compounds and subsequent product ions as analytical targets for method validation.

6. **Equine Drug Screening**

a. **Instrumentation**

Extensive drug screening has been observed in horse racing for a very long time *(55, 56)*. Previous methods used for equine drug screening included enzyme-linked immunosorbent assay (ELISA) and gas chromatography-mass spectrometry (GC-MS). Mass spectrometry has historically been the method of choice for doping control due to high sensitivity and high-throughput analysis *(29)*. While GC-MS is still used, it has several limitations such as extensive labor and maintenance commitments as well as reduced sensitivity. Recent advances in liquid chromatography-(tandem) mass spectrometry have made this technique the gold standard in clinical and forensic laboratories especially for doping control analysis *(57-60)*. Advanced LC-MS technology offers greater sensitivity and the ability to screen for hundreds of drugs in a single run *(61-63)*.

b. **Threshold Limits**

With the increasing sensitivity of these instruments, many drugs can be

detected up to weeks after administration to the horse. The greatest concern with this is whether the drug detected actually influenced the horse. As a result, many regulatory agencies including the IFHA have implemented screening thresholds for therapeutic compounds legally administered to horses to establish there is no pharmacologic effect on the horse during competition *(55)*. However, because no SARM drug candidate has been approved yet by the FDA, threshold limits need not be considered since the drugs follow a zero-tolerance policy at race time, meaning it is prohibited to have any trace of drug present in the system.

c. **Urine Screening**

Urine has traditionally been the favored biological matrix for drug screening because it is non-invasively obtained, generally available in relatively large volumes, and compounds and metabolites are excreted at comparatively higher concentrations *(57)*. Although the parent compound is more likely to be found in the blood, hydrolysis of samples as well as the sensitivity of the instrumentation allows for the detection of parent compounds in urine *(24)*. As a result, urine was the biological matrix chosen for drug identification in the method presented.

B. **Review of Related Literature**

Most recent studies with analytical methods to detect SARMs as illicitly used substances for performance enhancement have used state-of-the-art analytical procedures by liquid chromatography, high resolution/high accuracy (tandem) mass spectrometry using both positive and negative electrospray ionization (ESI) *(17, 20, 64-66)*. LC-MS/MS methodology is also implemented by WADA for detecting the use of banned substances by competing athletes *(67)*.

1. **SARMs and the Black Market**

With the first case report of SARM compound S-4 (Andarine) detected in

a black-market product in 2009 *(17)*, the misuse of these non-approved drugs has only increased with SARMs therapeutic development and ease of accessibility. A black-market product, advertised as oily liquids containing green tea extracts and face moisturizer, was analyzed for illicit substance Andarine using advanced LC-MS/MS technology. The intended drug candidate S-4 was detected at a concentration of 150 mg/mL, along with a byproduct at a concentration of 15 mg/mL, which was most likely the result of an insufficient purification and separation of an intermediate product from the intended active ingredient.

Another study targeting the chemical composition and purity of SARM substances sold via the internet was published in 2017. Of the 44 products advertised as selective androgen receptor modulators, only 23 (52%) contained the intended compounds Ostarine, LGD-4033, and Andarine, and a substantial amount of these products were inaccurately labeled regarding the quantity of active ingredient present in the product *(16).*

Black market products were confiscated and obtained from various sources in a study conducted between 2010 and 2013 in Germany. Sources included police, customs, national anti-doping authorities, and online shops. Further analysis of these products revealed some samples were properly labeled while others were delivered in glass vials, plastic bags, or ampoules without proper specifications. The diverse formulations of products obtained made extraction methods very complex and diverse, depending on the substance. Many of the analyzed products in this study were incorrectly labeled and contained unapproved substances. This further stresses the health risks associated with illicit drug use among athletes and horse racing. Although AAS still account for the majority of illicit substances detected in black market products, other unapproved substances including SARMs are on the rise *(18)*

These findings raise concerns about the availability of non-approved compounds without sufficient research on the adverse effects and the presence of drug impurities with unknown and potentially harmful effects. The production of black market substances often yields drug products of low quality due to the limited technical knowledge and necessary equipment available in clandestine laboratories *(18).* Ease of accessibility of non-approved SARMs on the black market combined with their desirable anabolic properties significantly increases their potential for illicit use to enhance performance and gain an unfair advantage in the athletic and horse racing communities.

2. **Andarine**

Andarine ((2S)-3-(4-acetamidophenoxy)-2-hydroxy-2-methyl-N-[4-nitro-3-(trifluoromethyl)phenyl]propanamide), also known as S-4 and GTX-007, is an aryl propionamide-derived nonsteroidal SARM. Andarine is very potent with reports claiming the drug close to that of DHT, the most potent endogenous androgen *(20).* This non-approved drug demonstrates exceptional pharmacokinetic properties, including rapid and complete oral absorption with reasonable elimination half-life. In addition, Andarine lacks the unwanted side-effects often associated with AAS, making it an excellent drug candidate for clinical development. In animal models, it exhibits the ability to prevent bone loss, reduce body fat, and increase skeletal muscle strength and lean body mass. Andarine has also demonstrated encouraging pharmacodynamic activity to treat benign prostatic hypertrophy and male fertility in animal models *(2).*

Unfortunately, the therapeutic benefits provide this compound with a high potential for misuse in sports where athletes and/or handlers look for a way to surpass their competitors with the expectation that these drugs will go undetected by anti-doping laboratories. Sensitive and

robust analytical methods using LC-MS/MS to detect Andarine have been reported since 2006 *(20, 66)*. In 2009, Andarine was detected in a black-market product, and more recently in 2011 and 2013, S-4 was detected in human doping control samples analyzed by the Lausanne and Los Angeles anti-doping laboratories *(17, 22, 68)*.

More importantly, S-4 was detected for the first time in a routine equine blood doping control sample by the Australian Racing Forensic Laboratory in 2016. On review of the data, deprotonated S-4 precursor ion ($[M-H]^-$) with a mass to charge (*m/z*) ratio of 440.1 produced a peak response at a retention time (RT) of 12.29 minutes. The estimated concentration of S-4 was extrapolated to between 0.2 ng/mL and 0.3 ng/mL by comparison to a 1 ng/mL QC equine plasma spike *(20)*.

In another study conducted by Hansson et al. *(24)*, parent compound S-4 was detected in equine urine samples post administration, and the highest responses were obtained after sample preparation with solid phase extraction. Additionally, a case report detected S-4 parent drug at a concentration of 50 ng/mL in a human athlete urine sample *(68)*. As previously mentioned, a method was validated using SPE followed by LC-MS/MS negative ESI for the detection of S-4 in bovine urine with a limit of detection (LOD) of 0.2 ng/mL *(25)*.

These observations suggest a significant potential for abuse, and Andarine should be included in routine screenings in accredited anti-doping laboratories to prevent abusers from undermining the doping control systems *(20)*.

3. **LGD-4033**

LGD-4033 (4-[(2R)-2-[(1R)-2,2,2-trifluoro-1-hydroxyethyl]pyrrolidin-1-yl]-2-(trifluoromethyl)benzonitrile), also referred to as Ligandrol, Anabolicum or VK5211, is a nonsteroidal SARM recently developed by Ligand Pharmaceuticals *(21)*. Preclinical models

demonstrated an increase in bone mineral density and bone formation and strength. Additional clinical trials conducted on healthy young men showed increasing doses were well tolerated and resulted in significant dose-proportional gains in lean body mass and leg press strength *(3).* Although studies on the pharmacokinetics and effects of LGD-4033 were just recently published in 2013 *(38),* this compound has clearly found its way into illicit distribution and use among athletes. This non-approved drug candidate has been found on multiple websites claiming to sell the compound, and it was detected in six human athlete samples in the USA and Canada in a single year *(69).*

In a recent study conducted by Hansson et al. *(32),* LGD-4033 was administered to horses and detected via LC-MS methodology. The main ion detected was the deprotonated formate adduct ($[M + HCOO]^-$) detected with a mass of 383 at a RT of 10.77 minutes. The longest detection time observed for parent compound LGD-4033 was in hydrolyzed urine samples, which could be detected for up to 96 hours after solid phase extraction. The parent compound was only detected in hydrolyzed samples, and the LOD was extrapolated to 2.6 ng/mL for the deprotonated species and 0.5 ng/mL for the formate adduct. Therefore, it is recommended that the formate adduct be used as the target ion rather than the deprotonated species, and all samples should be hydrolyzed prior to analysis when developing analysis methods for LGD-4033.

These findings suggest LGD-4033 has a significant potential for abuse by athletes and has the potential for abuse in horse racing due to its availability. Anti-doping laboratories should include the drug in their routine screenings for optimal doping control *(21).*

4. **MK-2866 (Ostarine, S-22)**

Ostarine ((2S)-3-(4-cyanophenoxy)-N-(4-cyano-3-(trifluoromethyl)phenyl)-

2-hydroxy-2-methylpropanamide), also known as MK-2866, enobosarm, S-22, and GTx-024, is another aryl propionamide-derived SARM *(49)*. Similar to Andarine, Ostarine exhibits exceptional therapeutic potential and is currently the most advanced SARM clinical candidate. Phase I clinical studies showed Ostarine having rapid absorption after oral administration and a reasonable half-life (4-6 hours). This SARM candidate also exhibited low cardiovascular risk, decreasing LDL and HDL cholesterol levels. Phase II clinical trials with Ostarine treatment demonstrated an increase in lean body mass and decrease in fat mass, improvement in functional performance specifically speed and power, and an interesting reduction in insulin levels and insulin resistance suggesting therapeutic potential in diabetics *(2).* Adverse effects included malignant neoplasm progression, pneumonia, and febrile neutropenia, however these events were not thought to be treatment-related. A study involving enobosarm to treat patients with non-small cell lung cancer (NSCLC) exhibited an increase in lean body mass, improved strength, and improved survival. This treatment has been nominated for the FDA Fast Track development program *(3).*

S-22 was found to be readily available and distributed via the internet. A study conducted by Thevis et al. *(23)* analyzed two human athlete urine samples using LC-MS/MS and detected S-22. In a study conducted by Hansson et al. *(24)*, the parent compound was detected in equine urine samples post-administration, and the highest responses were obtained after sample preparation with solid phase extraction.

Unfortunately, there are a lack of studies providing LOD and expected concentrations of S-22 in equine urine, but there are a few human and animal studies. A study conducting LC-MS/MS to detect enobosarm administration in bovines was able to detect the parent compound in urine samples for up to 9 days post administration when samples underwent phase II hydrolysis

with an LOD of 0.25 ng/mL *(26)*. As seen with Andarine, a method was also validated using SPE followed by LC-MS/MS negative ESI for the detection of S-22 in bovine urine with an LOD of 0.2 ng/mL *(25)*.

Similar studies with comparable findings of Ostarine, Andarine, LGD-4033, and other SARM drug candidates in doping control samples and products sold illicitly via the internet were recently published. This further stresses the need to implement these drugs in routine doping control procedures to allow for the detection of these emerging therapeutics *(49)*.

III. METHODS AND MATERIALS

A. Scope

The objective of method validation is to provide evidence that the method is suitable for its intended purpose and demonstrate that the method is accurate, reliable, and reproducible. This procedure applies to both qualitative and multi-point calibration quantifying assays.

B. Reagents and Materials

All solvents, including ethanol, acetic acid, hexanes, ethyl acetate, dichloromethane, water, acetonitrile, ammonium hydroxide, and formic acid, were purchased from Fisher Scientific and were HPLC grade or better. Sodium phosphate monobasic and sodium acetate were purchased from Fisher Scientific. β-glucuronidase from abalone was purchased from Campbell Scientific. Mixed mode solid phase extraction columns (part #XRDAH13Z) from United Chemical Technologies were used for all extractions. Analytical standards, Andarine, LGD-4033, and MK-2866 (Ostarine), were purchased from Caymen Chemical (Ann Arbor, MI). Butalbital-d3 was bought from Sigma Aldrich (St. Louis, MO) and was used as an internal standard for Andarine, LGD-4033, and MK-2866. The stock standards (5 mg) were prepared in methanol at a concentration of 1 mg/mL. For all analytes, two working mixed standard solutions were prepared at a concentration of 100 ng/mL and 10 ng/mL by dilution with methanol.

C. Sample Preparation

Samples were prepared according to the protocol '*X34-01 Instrumental SPE QQQ Procedure*' provided by the UIC AFTL. Two milliliters of equine urine were fortified with required concentration of analytes and aliquoted into screw cap glass tubes. Five hundred microliters of 0.9 M sodium acetate buffer was added to each tube for qualitative methods. For samples undergoing quantitation, internal standard butalbital-d3 was prepared in sodium acetate

buffer at a level of 500 ng/mL, and five hundred microliters of 0.9 M sodium acetate buffer containing internal standard was added to each tube. Fifty microliters of β-glucuronidase was added to each tube. The tubes were capped loosely and incubated overnight at 37°C or for two hours at 60°C.

After incubation was complete, the samples were removed from the incubator and allowed to cool down to room temperature. Two milliliters of 100 mM phosphate buffer (pH 6) was added to each sample and the pH was adjusted to 6.0 $\pm$ 0.5 using ammonium hydroxide. The samples were then centrifuged at 4000 revolutions per minute (rpm) for 10 minutes.

High flow mixed-mode solid phase extraction columns from United Chemical Technologies were conditioned with 1 mL of methanol and 1 mL of 100 mM phosphate buffer (pH 6). The samples were then added to the columns at a flow rate of 1 to 2 mL/min. The columns were then washed with 1 mL of 100 mM phosphate buffer pH 6 and 1 mL of 0.5 M acetic acid at a flow rate of 1 to 2 mL/min. The columns were allowed to dry for 5 minutes under a flow of nitrogen at 40 pounds per square inch (psi). Samples were then washed with 1 mL hexanes.

Acidic/neutral compounds were eluted using 1 mL of ethyl acetate with 1% formic acid, and basic compounds were eluted using 1 mL of 78:20:2 dichloromethane:isopropanol: ammonium hydroxide. Both elutions were collected into the same glass tube. The eluents were evaporated to dryness under a flow of nitrogen in a water bath at 40°C. The samples were then reconstituted in 100 µL of 95:5 0.2% formic acid in water:acetonitrile. Finally, samples were transferred to a well plate with insert and cap vials and placed on the autosampler for injection on LC-MS QQQ.

D. **Instrumentation**

An Agilent 1200 Series HPLC coupled with an Agilent 6000 Series Mass Spectrometry Triple Quadrupole operating in negative ionization mode was used for all analyses. An Agilent Poroshell 120 EC-C_{18} column 2.1 x 100 mm, 2.7 µm pore size column was used. The temperature of the column was set at 40 ºC and the mobile phase flow was set at a rate of 500 µL/min.

E. **LC Parameters**

For both acidic and basic LC methods, the solvents used were (A) 0.2% formic acid in water and (B) 10% water in acetonitrile. For compounds undergoing negative ionization, the initial conditions were 1% B held for 2 min. Between 2 and 5 minutes, a gradient was employed from 1% to 100% B and held at 100% B for 1 minute.

F. **MS-QQQ Parameters**

The MS-QQQ gas and temperature parameters were the same for both acidic and basic injections. The drying gas temperature was set at 350ºC. The gas flow was set at 12 L/min and the nebulizer was set at 50 psi. Electrospray ionization was employed for all drugs. All compound data was collected in Dynamic Multiple Reaction Monitoring Mode (DMRM). Data for the precursor and product ions for each drug was collected. Data analysis was done using Agilent Masshunter Quantitative software.

G. **Procedure**

This method was validated according to the University of Illinois at Chicago Analytical Forensic Testing Laboratory (UIC AFTL) Standard Operating Procedure #AFTL GE 005-03 '*Validation Requirements for Methods Using Instrumental Analysis*.'

1. **Validation Requirements for Qualitative Methods**

a. **Specificity**

Three blank urine samples were run along with three urine samples spiked with Andarine, LGD-4033, and MK-2866 (all structurally similar) at a concentration of 100 ng/mL. Samples were then analyzed, and determination of specificity was made if the compound of interest could not be identified in the blank urine samples and is positively identified alone and in the presence of other compounds.

b. **Ion Suppression and Enhancement**

Three sets of samples at 100 ng/mL were prepared containing Andarine, LGD-4033, and MK-2866. One set of samples contained the neat standards (Set A). The second and third set of samples contained blank urine spiked with the compounds of interest before (Set C) and after (Set B) performing the extraction. Peak areas were used to calculate matrix effect, extraction recovery, and process efficiency using the following equations:

$$\text{Matrix Effect (\%)} = (\text{Set B}/\text{Set A}) \times 100 - 100 \quad (1)$$

$$\text{Extraction Recovery (\%)} = (\text{Set C}/\text{Set B}) \times 100 \quad (2)$$

$$\text{Process Efficiency (\%)} = (\text{Set C}/\text{Set A}) \times 100 \quad (3)$$

c. **Limit of Detection**

Blank urine samples were spiked with decreasing concentrations of Andarine, LGD-4033, and MK-2866 by 1:2 serial dilutions at 300 ng/mL, 150 ng/mL, 75 ng/mL, 37.5 ng/mL, 19 ng/mL, 9.4 ng/mL, 4.7 ng/mL, 2.3 ng/mL and 1.2 ng/mL. All samples were extracted and analyzed. Limit of detection was determined when the signal to noise ratio of any of the ions used for identification fell below 5:1.

d. **Carryover and Contamination**

Three blank urine samples were spiked with Andarine, LGD-4033, and MK-2866 at a concentration higher than what would normally be expected in an unknown sample (300 ng/mL). Spiked samples were extracted alongside three blank urine samples and analyzed. The three blank urine samples were injected directly after each spiked sample and analyzed for the compounds of interest.

e. **Stability**

i. **Freeze Thaw**

Two sets of blank urine samples were spiked with Andarine, LGD-4033, and MK-2866 at 100 ng/mL in triplicate. One set of samples was stored at -20°C for 48 hours. The other set of samples was stored in the refrigerator for the entire time. Frozen samples were removed, thawed, and placed back in the freezer. These steps were repeated for frozen samples twice. After the final thaw, samples were extracted and analyzed alongside the refrigerated samples, and peak areas of the two sets of samples were compared.

ii. **Long Term**

Two sets of blank urine samples were spiked with Andarine, LGD-4033, and MK-2866 at 100 ng/mL in triplicate. One set of samples was stored at -20°C for 60 days. The other set of samples was stored in the refrigerator for the entire time. After 60 days, the frozen samples were thawed. Frozen samples were then extracted and analyzed alongside the refrigerated samples, and peak areas of the two sets of samples were compared.

iii. **Bench Top**

Two sets of blank urine samples were spiked with Andarine,

LGD-4033, and MK-2866 at 100 ng/mL in triplicate. One set of samples was stored on the bench top at room temperature for 24 hours. Bench top samples were then extracted and analyzed alongside the refrigerated samples, and peak areas of the two sets of samples were compared.

iv. **Processed Samples**

Two sets of blank urine samples were spiked with Andarine, LGD-4033, and MK-2866 at 100 ng/mL in triplicate. Samples were extracted, and one set of samples was immediately analyzed. The second set of samples was stored on the instrument (as though prepared for injection) for 48 hours. The second set of samples was then analyzed, and peak areas of the two sets of samples were compared.

2. **Validation Requirements for Quantitative Methods**

Selectivity, ion suppression and enhancement, and stability testing for the internal standard butalbital-d3 were previously performed at AFTL.

a. **Lower Limit of Quantitation**

Blank urine samples were spiked with Andarine, LGD-4033, and MK-2855. The limit of quantitation was determined when the signal-to-noise ratio of any of the ions used for quantitation fell below 10:1.

b. **Calibration Model (Linearity)**

The range of the calibration curve depends on the purpose of the method but should cover the majority of concentrations to be expected in unknown samples, and the curve should contain at least 6 points. The calibration curve was established by analyzing spiked samples at decreasing concentrations (300 ng/mL, 150 ng/mL, 75 ng/mL, 37.5 ng/mL, 19 ng/mL, 9.4 ng/mL, 4.7 ng/mL, 2.3 ng/mL and 1.2 ng/mL) and plotting the resulting responses versus the

corresponding concentrations. The curve was obtained by simple linear regression and evaluated by residual plots (R^2 values). The R^2 value should be 0.95 or higher and should be based on curves run at least three different times.

c. **Precision**

Blank urine samples were spiked with Andarine, LGD-4033, MK-2866, and butalbital-d3 at three different levels: low (5 ng/mL), mid (10 ng/mL) and high (50 ng/mL). These concentrations must fall within the lowest (1.2 ng/mL) and highest (300 ng/mL) concentrations of the calibration curve. There were 6 replicates per concentration. The quantitative results were analyzed to create one-way ANOVA tables for each compound at each concentration level. Those numbers were then used to calculate the following precision values and expressed in percentage:

i. **Intra-day Precision**

$$RSD_r(\%) = \frac{\sqrt{MS_{wg}}}{\bar{x}} x\ 100 \qquad (4)$$

Where RSD_r = intra-day precision (expressed as a percentage), MS_{wg}= the mean square within groups (determined by one-way ANOVA), and $\bar{x}$ = the grand mean.

ii. **Inter-day Precision**

$$RSD_{bg}(\%) = \frac{\sqrt{\frac{MS_{bg} - MS_{wg}}{n}}}{\bar{x}} x\ 100 \qquad (5)$$

Where RSD_{bg}= inter-day precision (expressed as a percentage), MS_{bg}= the mean square between groups (determined by one-way ANOVA), MS_{wg}= the mean square within groups (determined by one-way ANOVA), n = the number of observations in each group, and $\bar{x}$ = the grand mean.

iii. **Total Precision**

$$RSD_{I(F)}(\%) = \frac{\frac{\sqrt{MS_{bg} + (n+1)MS_{wg}}}{n}}{\bar{x}} x\ 100 \qquad (6)$$

Where $RSD_{I(F)}$= total precision, MS_{bg}= the mean square between groups (determined by one-way ANOVA), MS_{wg}= the mean square within groups (determined by one-way ANOVA), n = the number of observations in each group, $\bar{x}$ = the grand mean. Each of these values should be less than 20% at each concentration.

d. **Accuracy**

The same controls used for precision were used for accuracy. The quantitative results were used to calculate the total accuracy of the method at each concentration. The results are shown as a percentage, and the calculation was as follows:

$$\text{Bias} = \frac{\bar{x} - X}{X}\ x\ 100 \qquad (7)$$

Where $\bar{x}$ = the grand mean and X = the theoretical value. Bias at each concentration should be less than 25%.

e. **Uncertainty**

Uncertainty was calculated using the same data used to calculate precision and accuracy. The Simplified *Guide to the Expression of Uncertainty in Measurement* (GUM) approach was used.

IV. RESULTS

A. Qualitative Requirements

Qualitative results include specificity, ion suppression/enhancement, LOD, carryover and contamination, and stability. Examples of extracted ion chromatograms (EIC) showing Andarine and internal standard butalbital-d3 are demonstrated in Figure 1 below.

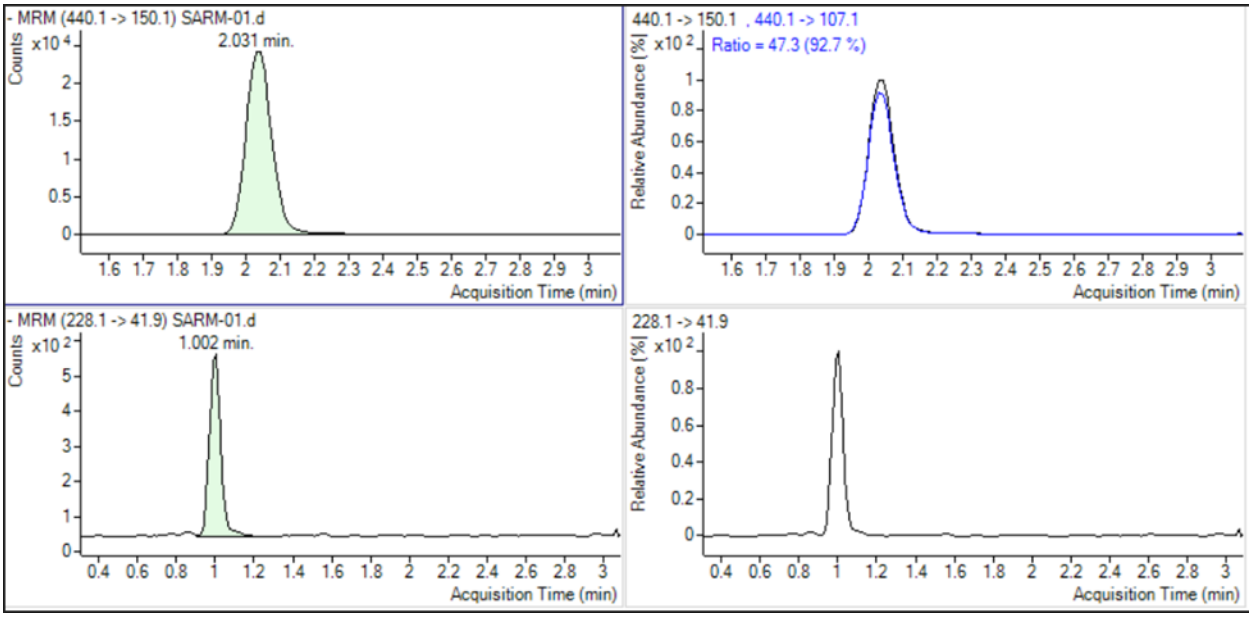


Figure 1. EIC for Andarine (top) and internal standard butalbital-d3 (bottom).

The retention times were 2.0 and 1.0 minutes, respectively. The method allowed for selective monitoring of precursor ions at a mass of 440.1 (parent ion) and product ions at a mass of 107.1. Figure 2 illustrates an example of an extracted ion chromatogram exhibiting LGD-4033 (and internal standard), with a retention time of 2.5 minutes. The method selectively scanned for precursor and product ions at masses 383.1 (formate adduct) and 45.0. Figure 3 is an example of a chromatogram demonstrating MK-2866 (and internal standard) with a peak at 2.4 minutes, and

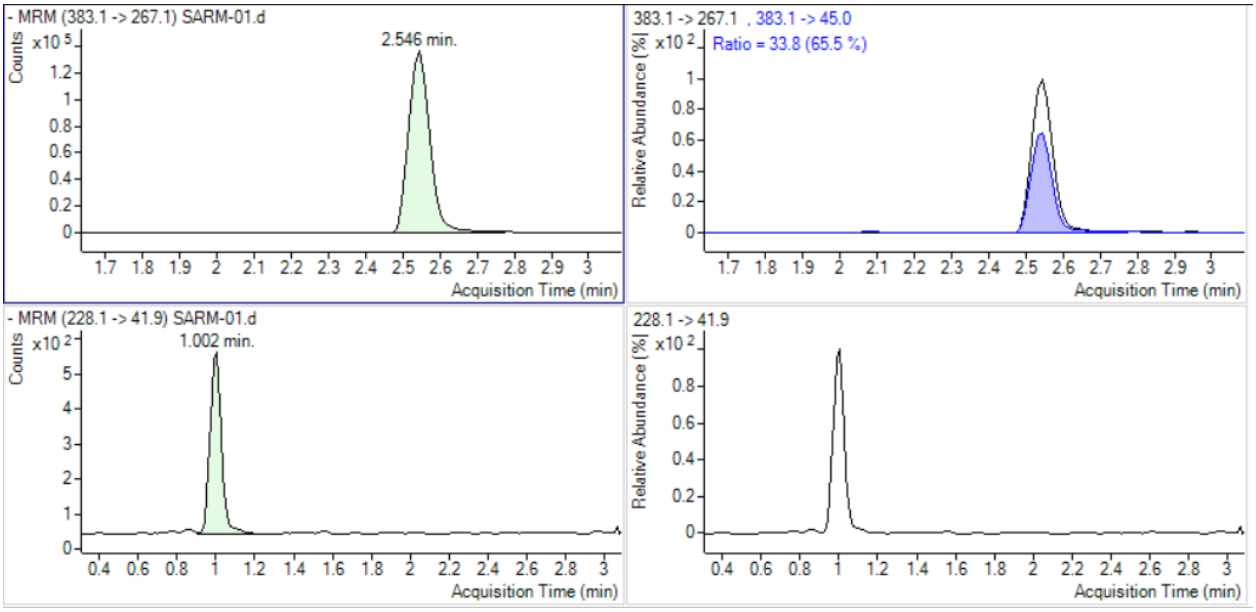


Figure 2. EIC for LGD-4033 (top) and internal standard butalbital-d3 (bottom).

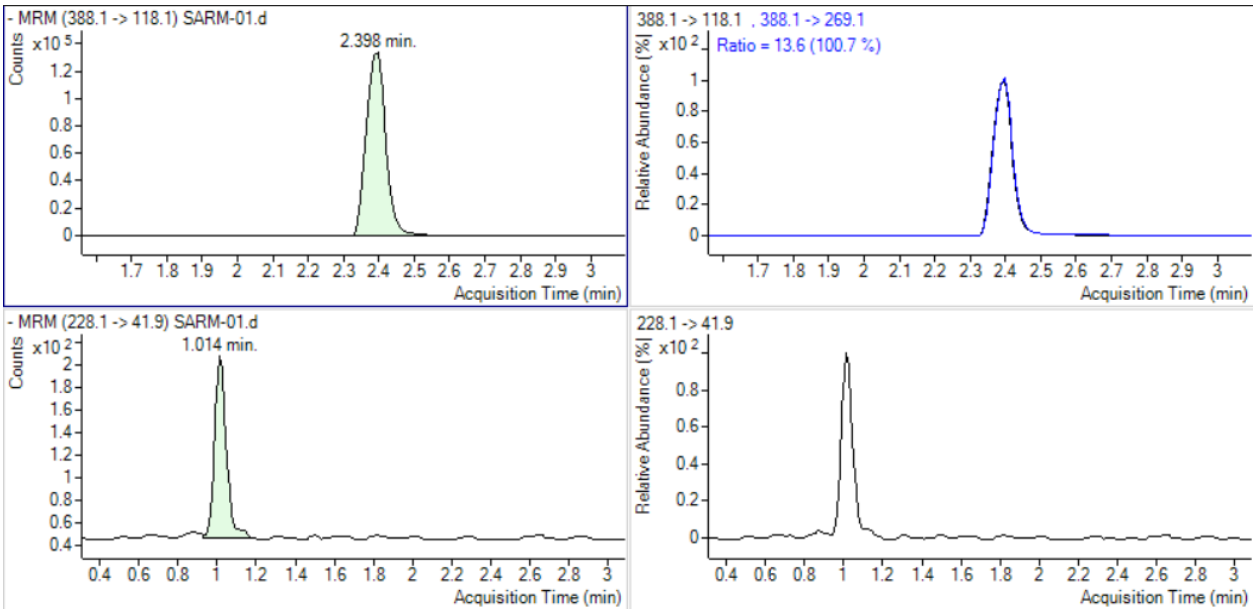


Figure 3. EIC for MK-2866 (top) and internal standard butalbital-d3 (bottom).

targeted ions at masses 388.1 (parent ion) and 269.1. The peaks for all three compounds, as observed in Figures 1-3, are narrow with normal distribution and very minimal tailing. Specificity was determined for all three drugs; each drug was positively identified alone and in the working mixed standard solution, and compounds of interest were not identified in blank urine samples.

Calculated matrix effect and extraction efficiencies for each compound are listed in Table IV. One set of samples spiked pre-extraction was not included in extraction recovery and process efficiency calculations because the peak responses were off from the other two sets of samples undergoing the same conditions. All compounds showed matrix suppression ranging from -85% to -99% (Equation 1). The amount of drug recovered following SPE ranged from 47% to 63% (Equation 2), with LGD-4033 having the highest extraction recovery rate. Process efficiencies for LGD-4033 and MK-2866 were 9.3% and 1.3% (Equation 3), meaning responses were greater after samples underwent SPE. Table V illustrates the elution efficiencies for each drug under acidic and basic conditions. For Andarine and MK-2866, the majority of drug was recovered in the basic elution whereas the majority of LGD-4033 was recovered in the acidic elution. The presence of all three drugs in both the basic and acidic elutions indicates both conditions are necessary for extracting these compounds.

The LOD for all three compounds was determined around 1 ng/mL. Table VI exhibits raw data providing signal to noise ratios (S/N) at decreasing concentrations. Because LOD is determined when the signal to noise ratio falls below 5:1, and ratios were greater than 5 for compounds around 1 ng/mL, it is suggested that the LOD for each compound may be even lower than 1 ng/mL (~0.5 ng/mL).

TABLE IV: MATRIX EFFECT AND EXTRACTION EFFICIENCY

RAW DATA			Area Counts (Peak Responses)								
			Pre-Extraction QC High C			Post Extraction QC High B			QC High Std A		
Extraction Efficiencies	Precursor (*m/z*)	Transition (*m/z*)	Replicate 1[a]	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
Andarine	440	107	379	2815	2438	4441	5608	6649	625443	622075	625910
LGD-4033	383	45	33613	68905	56516	89957	89971	115494	714148	668357	639370
MK-2866	388	269	6180	28601	16951	41176	43140	50645	1787153	1663628	1589621

[a] Replicate 1 peak responses not included in calculations.

Calculations			
Extraction Efficiencies	Matrix Effect (%)	Extraction Recovery (%)	Process Efficiency (%)
Andarine	-99.11	47.19	0.42
LGD-4033	-85.39	63.68	9.30
MK-2866	-97.32	50.63	1.36

TABLE V: ELUTION EFFICIENCY

Elution Efficiencies	Acid (%)	Base (%)
Andarine	21	79
LGD-4033	60	40
MK-2866	23	77

TABLE VI: RAW DATA USED FOR LOD DETERMINATION

	Andarine Results			Qualifier (440.1 -> 107.1) Results	LGD-4033 Results			Qualifier (383.1 -> 45.0) Results	MK-2866 Results			Qualifier (388.1 -> 269.1) Results
Sample (ng/ mL)	RT (mins)	Response (Area Counts)	S/ N[a]	Ratio	RT (mins)	Response (Area Counts)	S/ N[a]	Ratio	RT (mins)	Response (Area Counts)	S/ N[a]	Ratio
blank												
300	2.07	180840.38	135173.73	52.68	2.55	471892.40	26190.83	35.60	2.40	507666.80	35053.24	13.63
150	2.06	114745.79	3948.37	53.21	2.55	327819.39	2656.48	35.44	2.39	302077.99	2727.14	13.66
75	2.04	64450.65	1049.72	53.76	2.55	197020.98	663.86	35.54	2.39	153478.99	907.06	13.66
37.5	2.04	30707.72	1578.98	53.46	2.55	99315.94	2300.02	35.60	2.39	71764.42	3337.15	13.80
19	2.03	17743.31	1430.79	57.81	2.53	60470.90	687.70	36.26	2.39	38528.65	800.60	14.64
9.5	2.03	7542.25	415.83	57.56	2.53	22636.20	282.83	40.36	2.39	17094.66	1320.35	16.03
4.75	2.03	4195.73	260.54	55.16	2.55	12428.10	181.26	39.96	2.39	7260.31	340.86	18.04
2.25	2.03	2195.60	149.00	56.34	2.53	5605.25	162.07	40.91	2.37	3607.21	166.09	21.44
1.1	2.03	1213.06	47.74	53.86	2.55	2836.74	28.90	43.38	2.39	1891.60	94.04	29.29

[a] Signal to noise ratios (S/N) were used to determine LOD.

Carryover and contamination was determined by analyzing blank samples immediately after spiked samples at high concentrations. Compounds were detected in blank samples, however responses were very low with signal to noise ratios below 5:1. Therefore, carryover and contamination can generally be ignored when analyzing the SARM compounds in this study.

Average peak responses of samples undergoing a variety of processes were compared to controls to determine the stability of each compound in this study (Tables VII, VIII, and IX). If peak responses were significantly off from other responses in the same set of samples, they were excluded from average response calculations. Sample responses were unaffected when stored on the bench top at room temperature for 24 hours prior to analysis for all three compounds. After undergoing freeze thaw repetitions, sample responses were unaffected for Andarine, however LGD-4033 and MK-2866 were minimally affected. After samples were stored on the instrument for 48 hours prior to analysis, peak responses were significantly lower for all three compounds. Peak responses also decreased after samples were stored in the freezer for 60 days prior to analysis for all three compounds. These results suggest that the SARM compounds in this study should be immediately analyzed once prepared and placed on the instrument. Also, these drugs should not be stored in the freezer for a long period of time prior to analysis as compounds appeared to degrade over 60 days.

All necessary qualitative requirements were already performed for internal standard butalbital-d3 by UIC AFTL. The qualitative results for butalbital-d3 are listed in Table X.

TABLE VII: ANDARINE STABILITY

	Area Counts (Peak Responses)		Date Run	Average Response[b]
Freeze Thaw	Control Samples	Samples undergoing Freeze Thaw		
Replicate 1	78480	251539	10/9/2018	3.20
Replicate 2	63779	191022		
Replicate 3	152547[a]	240598		

	Area Counts (Peak Responses)		Date Run	Average Response[b]
Long Term	Control Samples	Samples undergoing Long Term		
Replicate 1	66997	9616	8/28/2018	0.18
Replicate 2	63688	13594		
Replicate 3	21142[a]	11516		

	Area Counts (Peak Responses)		Date Run	Average Response[b]
Processed	Control Samples	Samples undergoing Processed		
Replicate 1	767950	178322	9/26/2018 (Controls)	0.25
Replicate 2	359183[a]	183892	9/28/2018 (Processed)	
Replicate 3	735329	191799		

	Area Counts (Peak Responses)		Date Run	Average Response[b]
Bench Top	Control Samples	Samples undergoing Bench Top		
Replicate 1	331049	342326	9/26/2018	1.21
Replicate 2	327321	278162		
Replicate 3	No Response[a]	571111		

[a] Peak responses not included in calculations.

[b] Average response was determined by dividing the average peak response of processed samples by the average peak response of control samples.

TABLE VIII: LGD-4033 STABILITY

	Area Counts (Peak Responses)		Date Run	Average Response[b]
Freeze Thaw	Control Samples	Samples undergoing Freeze Thaw		
Replicate 1	604038	501104	10/9/2018	0.77
Replicate 2	529580	427309		
Replicate 3	679729	475127		
	Area Counts (Peak Responses)		Date Run	Average Response[b]
Long Term	Control Samples	Samples undergoing Long Term		
Replicate 1	344916	131069	8/28/2018	0.46
Replicate 2	310981	169424		
Replicate 3	117378[a]	149636		
	Area Counts (Peak Responses)		Date Run	Average Response[b]
Processed	Control Samples	Samples undergoing Processed		
Replicate 1	1322133	513030	9/26/2018 (Controls)	0.45
Replicate 2	763138[a]	596584	9/28/2018 (Processed)	
Replicate 3	1243126	604269		
	Area Counts (Peak Responses)		Date Run	Average Response[b]
Bench Top	Control Samples	Samples undergoing Bench Top		
Replicate 1	961916	1014380	9/26/2018	1.24
Replicate 2	913200	1058795		
Replicate 3	No Response[a]	1409428		

[a] Peak responses not included in calculations.

[b] Average response was determined by dividing the average peak response of processed samples by the average peak response of control samples.

TABLE IX: MK-2866 STABILITY

	Area Counts (Peak Responses)		Date Run	Average Response[b]
Freeze Thaw	Control Samples	Samples undergoing Freeze Thaw		
Replicate 1	734639	492849	10/9/2018	0.62
Replicate 2	607392	403641		
Replicate 3	993767	555603		

	Area Counts (Peak Responses)		Date Run	Average Response[b]
Long Term	Control Samples	Samples undergoing Long Term		
Replicate 1	146126	28818	8/28/2018	0.28
Replicate 2	139327	58028		
Replicate 3	37024[a]	33929		

	Area Counts (Peak Responses)		Date Run	Average Response[b]
Processed	Control Samples	Samples undergoing Processed		
Replicate 1	1809155	297039	9/26/2018 (Controls)	0.19
Replicate 2	819324[a]	338849	9/28/2018 (Processed)	
Replicate 3	1676221	346441		

	Area Counts (Peak Responses)		Date Run	Average Response[b]
Bench Top	Control Samples	Samples undergoing Bench Top		
Replicate 1	790636	1107563	9/26/2018	1.77
Replicate 2	715341	1100016		
Replicate 3	No Response[a]	1789422		

[a] Peak responses not included in calculations.

[b] Average response was determined by dividing the average peak response of processed samples by the average peak response of control samples.

TABLE X: QUALITATIVE RESULTS OF INTERNAL STANDARD

Butalbital-d3[a]	**Result**
Extraction Efficiency	75%
Matrix Effects	-90.80%
Bench Top	106%
Processed	75%
Freeze Thaw	90%
Long Term	85%
LOD	6 ng/mL

[a] Qualitative requirements for butalbital-d3 were already performed by AFTL.

B. **Quantitative Requirements**

The linear range of the calibration curves generated for Andarine, LGD-4033, and MK-2866 was determined below 300 ng/mL. Therefore, 1:2 serial dilutions of spiked urine samples from 300 ng/mL to 1 ng/mL were prepared and analyzed, and the standard curves were obtained by simple linear regression. Examples of generated calibration curves for compounds Andarine, LGD-4033, and MK-2866 are provided in Figures 4-6. Nine total calibration points were used, with R^2 values higher than 0.95 (most were 0.98 or higher). Each curve was also weighted to 1/x, meaning when the curve was generated, more weight was given towards the lower concentrations rather than the high concentrations because most of the calibrators fell on the lower half of the curve.

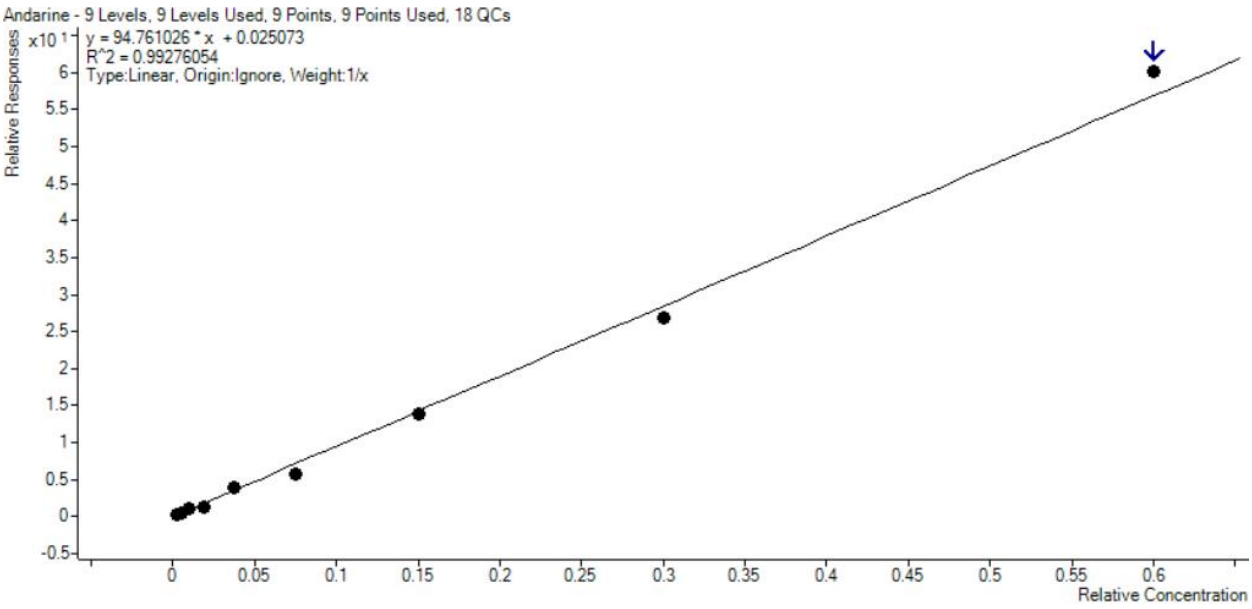


Figure 4. Example of a calibration curve generated for Andarine. Relative responses are expressed in area counts and relative concentration is expressed in ng/mL.

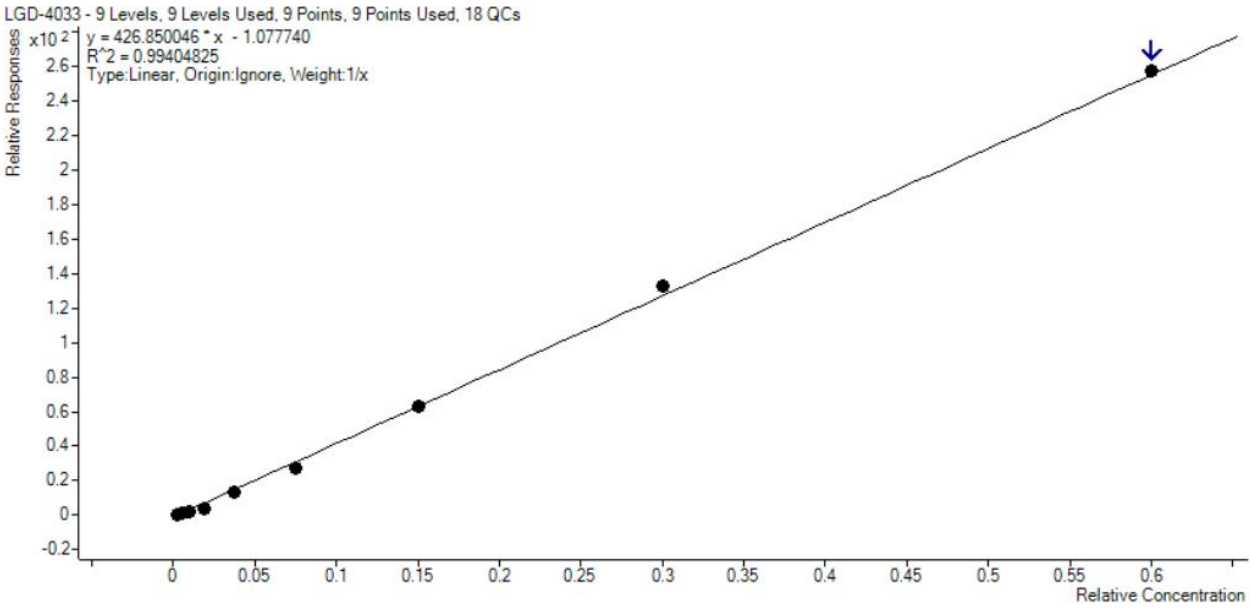


Figure 5. Example of a calibration curve generated for LGD-4033. Relative responses are expressed in area counts and relative concentration is expressed in ng/mL.

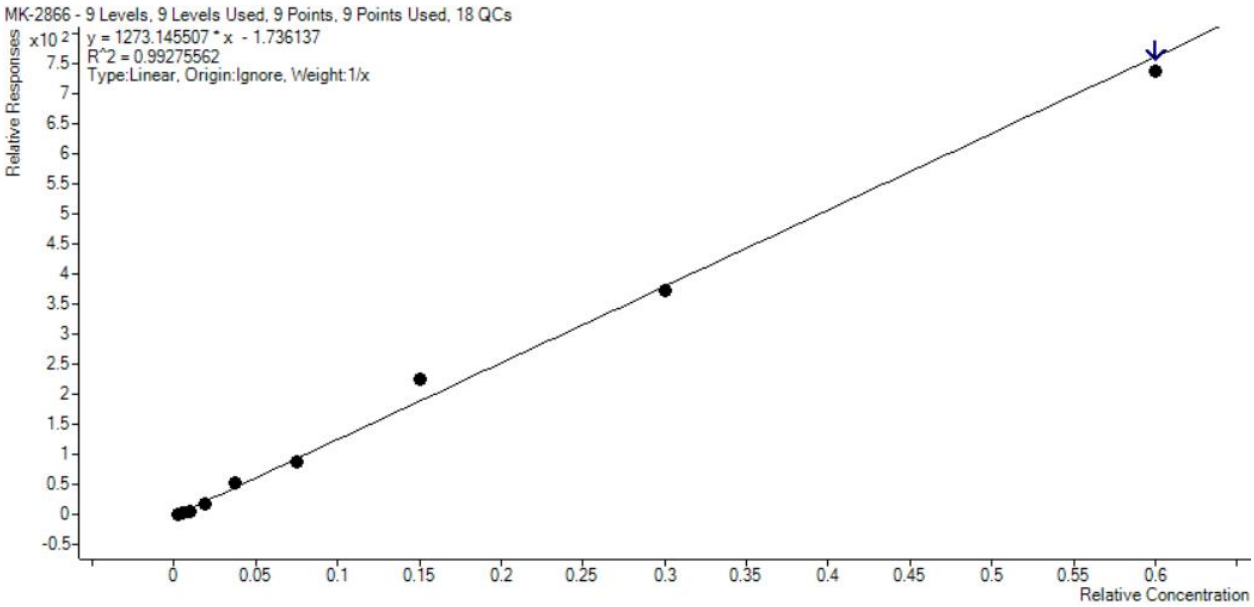


Figure 6. Example of a calibration curve generated for MK-2866. Relative responses are expressed in area counts and relative concentration is expressed in ng/mL.

Quality control (QC) samples were run along with the calibrators at high (50 ng/mL), mid (10 ng/mL), and low (5 ng/mL) concentrations which were within the linear range of the calibration curve and well above the LOD. The raw data of the five separate runs used to calculate precision, accuracy, and uncertainty is provided in Table XI. The sum, mean, and range for each group have been provided. The most extreme outliers in each data set, which were outside three standard deviations from the grand mean, were eliminated prior to any calculations (highlighted in yellow). The adjusted range is also provided, excluding the outliers.

The statistics for high, mid, and low QCs are provided in Table XII. Average standard uncertainty for the compounds ranged from 10.2% to 15.3%. Quantitative results from Table XI were analyzed to create one-way ANOVA tables at each QC concentration for the compounds of interest. ANOVA tables created using the high QC data at 50 ng/mL for Andarine, LGD-4033,

and MK-2866 are provided in Tables XIII, XIV, and XV. Generated ANOVA tables using mid QC data at 10 ng/mL for each compound are provided in Tables XVI, XVII, and XVIII. Low QC results at 5 ng/mL were used to create ANOVA Tables XIX, XX, and XXI for each compound. The numbers generated in the nine ANOVA tables listed were used to calculate intra-day precision (Equation 4), inter-day precision (Equation 5), and total precision (Equation 6) for all compounds at each concentration. These values are listed under the provided ANOVA tables and are expressed as a percentage. Precision values were less than 20% for each compound at all three concentrations.

Quality control statistics (listed in Table XII) for compounds Andarine, LGD-4033, and MK-2866 were used to calculate the total accuracy (Equation 7) of the method at each concentration. The results are expressed as a percentage and are listed under ANOVA tables. Bias for Andarine and MK-2866 was determined less than 25%. However, LGD-4033 had a calculated bias greater than 25% at mid and low concentrations.

Uncertainties were calculated using the same controls used to calculate precision and accuracy and are provided in the Uncertainty Budget Form (Table XXII). Uncertainty values were mainly dependent on QC data because all other sources of uncertainty were very minimal and could be ignored. The concentration yielding the highest total imprecision for each compound was used to calculate uncertainty. The highest imprecision values were found at mid concentration for all three compounds, with total precision values of 16.20%, 11.10%, and 19.20% for Andarine, LGD-4033, and MK-2866. Expanded uncertainties were determined using total precision values and ranged from 11.12% to 19.23%, with MK-2866 having the highest expanded uncertainty.

TABLE XI: RAW DATA USED TO CALCULATE PRECISION AND ACCURACY

QC High Data (50 ng/mL)	8/10/18			8/14/18			8/17/18			8/22/18			8/28/18		
	Andarine	LGD-4033	MK-2866	Andarine	LGD-4033	MK-2866	Andarine	LGD-4033	MK-2866	Andarine	LGD-4033	MK-2866	Andarine	LGD-4033	MK-2866
	56.80	63.54	63.42	58.67	64.50	56.97	53.98	64.98	49.79	53.75	68.26	56.99	61.27	70.92	47.42
	52.01	59.43	57.43	53.26	62.64	45.74	54.64	65.93	46.05	52.94	65.78	57.00	72.73	72.44	59.24
	50.08	57.57	51.59	62.79	62.76	61.87	62.72	69.38	57.47	58.19	59.50	57.51	54.33	59.85	34.46
	49.83	56.17	55.59	46.24	51.60	44.38	51.35	62.61	40.13	36.54	63.85	41.66	46.26	66.43	36.89
	43.97	55.72	47.81	44.35	53.97	36.21	46.94	57.41	33.19	38.17	60.95	39.63	44.43	57.33	31.94
	56.71	68.46	68.21	48.93	51.20	45.00	63.35	71.37	53.57	49.50	84.37	72.03	48.28	71.40	49.70
Sum	309.40	360.89	344.04	314.25	346.67	290.16	332.98	391.68	280.20	289.09	402.71	324.83	327.29	398.37	259.65
Mean	51.57	60.15	57.34	52.37	57.78	48.36	55.50	65.28	46.70	48.18	67.12	54.14	54.55	66.39	43.28
Range	12.82	12.74	20.40	18.44	13.30	25.66	16.41	13.96	24.28	21.65	24.87	32.40	28.30	15.11	27.30
Adjust Range[a]			9.62	14.32	10.54	17.48	7.71	11.97	17.34	8.69	8.76	17.88	16.84	13.59	11.81
QC Mid Data (10 ng/mL)															
	10.62	13.17	10.44	9.87	8.93	8.42	15.94	17.44	19.04	6.76	11.53	7.15	11.89	13.60	11.20
	15.73	14.99	15.59	11.76	10.17	11.64	10.82	13.52	10.69	8.62	11.67	7.51	12.80	14.92	10.60
	12.52	13.73	12.88	2.83	8.34	3.44	12.43	14.28	12.16	22.10	27.89	32.19	9.77	12.39	7.72
	8.27	11.06	8.34	4.35	9.90	4.50	9.04	12.84	8.16	8.27	12.95	8.23	11.02	15.87	10.33
	11.70	13.05	12.11	9.68	9.56	9.58	9.87	11.84	8.29	10.36	13.57	8.84	13.33	16.10	12.01
	11.37	13.61	11.09	9.71	8.73	5.41	7.09	11.79	7.90	10.26	16.94	11.64	13.59	16.24	13.67
Sum	70.22	79.60	70.47	48.21	55.63	42.99	65.19	81.71	66.24	66.37	94.55	75.57	72.39	89.12	65.53
Mean	11.70	13.27	11.74	8.03	9.27	7.16	10.86	13.62	11.04	11.06	15.76	8.68	12.07	14.85	10.92
Range	7.47	3.93	7.25	8.93	1.84	8.20	8.85	5.65	11.14	15.34	16.36	25.03	3.82	3.85	5.95
Adjust Range[a]	4.25		4.54	2.09	0.61	3.22	5.34	2.50	4.26	3.61	2.05	4.49			
QC Low Data (5 ng/mL)															
	6.73	6.71	6.63	5.96	5.53	5.26	3.92	5.66	5.24	6.80	7.26	6.30	6.06	7.29	5.55
	6.02	6.47	5.64	3.99	5.88	3.37	4.17	6.30	5.48	6.77	8.23	7.70	6.27	6.91	6.21
	6.14	6.21	6.24	4.73	4.99	4.29	4.18	5.44	5.44	5.19	6.21	4.76	5.96	6.96	6.11
	6.47	6.18	6.19	2.83	5.50	2.96	4.01	5.98	5.44	8.53	10.46	11.41	3.68	5.63	4.93
	4.83	5.85	4.54	5.83	8.39	7.58	4.17	6.12	5.36	5.11	6.58	4.69	4.92	6.67	4.73
	5.95	6.68	5.45	3.62	5.59	3.37	6.84	6.95	8.50	5.57	6.55	5.30	5.34	6.70	5.11
Sum	36.13	38.10	34.70	26.96	35.88	26.84	27.28	36.45	35.47	37.97	45.29	40.16	32.24	40.16	32.63
Mean	6.02	6.35	5.78	4.49	5.98	4.47	4.55	6.07	5.91	6.33	7.55	6.69	5.37	6.69	5.44
Range	1.90	0.86	2.09	3.13	3.40	4.62	2.92	1.52	3.25	3.41	4.25	6.71	2.59	1.66	1.49
Adjust Range[a]	1.64			1.98	0.38	3.29	0.17		0.24	1.66	1.05	1.60	1.35		

[a] Adjusted range was calculated after outliers (highlighted in yellow) were eliminated from each data set.

TABLE XII: HIGH, MID, AND LOW QC STATISTICS

QC High Data 50 ng/mL[a]

	Andarine	LGD-4033	MK-2866			Andarine	LGD-4033	MK-2866
Subgroup Size	6.00	6.00	6.00		Grand Mean	51.53	62.41	51.02
Grand Average	51.53	62.41	51.02		Grand StDev	4.85	4.67	6.69
Average Range	12.07	11.52	14.83		Std Unc.	0.09	0.07	0.13
rxa2	5.83	5.56	7.16					
D4	2.00	2.00	2.00					
UCLX	57.36	67.98	58.19		UCLR	24.20	23.09	29.71
CLX	51.53	62.41	51.02		CLR	12.07	11.52	14.83
LCLX	45.70	56.85	43.86		LCLR	0.00	0.00	0.00

QC Mid Data 10 ng/mL[a]

	Andarine	LGD-4033	MK-2866			Andarine	LGD-4033	MK-2866
Subgroup Size	6.00	6.00	6.00		Grand Mean	10.46	13.01	10.03
Grand Average	10.46	13.01	10.03		Grand StDev	1.83	1.86	1.91
Average Range	3.82	2.59	4.49		Std Unc.	0.18	0.14	0.19
rxa2	1.85	1.25	2.17					
D4	2.00	2.00	2.00					
UCLX	12.30	14.26	12.20		UCLR	7.66	5.18	9.00
CLX	10.46	13.01	10.03		CLR	3.82	2.59	4.49
LCLX	8.61	11.77	7.86		LCLR	0.00	0.00	0.00

QC Low Data 5 ng/mL[a]

	Andarine	LGD-4033	MK-2866			Andarine	LGD-4033	MK-2866
Subgroup Size	6.00	6.00	6.00		Grand Mean	5.35	6.30	5.52
Grand Average	5.35	6.30	5.52		Grand StDev	0.87	0.56	0.75
Average Range	1.36	1.09	1.74		Std Unc.	0.16	0.09	0.14
rxa2	0.66	0.53	0.84					
D4	2.00	2.00	2.00					
UCLX	6.00	6.83	6.36		UCLR	2.72	2.19	3.49
CLX	5.35	6.30	5.52		CLR	1.36	1.09	1.74
LCLX	4.69	5.77	4.68		LCLR	0.00	0.00	0.00

	Andarine	LGD-4033	MK-2866
Average Std Uncertainty (%)[b]	14.39	10.23	15.28

[a] Statistics for high, mid, and low QC data were calculated after outliers were eliminated from each data set. UCLX and LCLX (outside 3 standard deviations from the mean) were used to determine outliers.

[b] Average standard uncertainty was determined by averaging the standard uncertainties calculated at each QC level.

TABLE XIII: ANOVA TABLE FOR ANDARINE AT 50 NG/ML

ANDARINE

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	309.40	51.57	23.38
Column 2	5	251.46	50.29	33.14
Column 3	4	206.91	51.73	12.22
Column 4	4	214.38	53.59	12.77
Column 5	5	254.56	50.91	47.37

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	26.78	4.00	6.70	0.25	0.91	2.90
Within Groups	513.95	19.00	27.05			
Total	540.74	23.00				

Grand Mean (ng/mL)[a]	51.53
Intra-day Precision (%)[b]	10.09
Inter-Day Precision (%)[c]	1.79
Total Precision (%)[d]	10.35
Accuracy (%)[e]	3.06

[a] Grand mean was taken from Table XII for Andarine at 50 ng/mL and was used to calculate accuracy.

[b] Intra-day precision was calculated using Equation 4 and numbers generated in one-way ANOVA table.

[c] Inter-day precision was calculated using Equation 5 and numbers generated in one-way ANOVA table.

[d] Total precision was calculated using Equation 6 and numbers generated in one-way ANOVA table.

[e] Accuracy was calculated using Equation 7 with the theoretical value at 50 ng/mL.

TABLE XIV: ANOVA TABLE FOR LGD-4033 AT 50 NG/ML

LGD-4033						
Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	6	360.89	60.15	24.65		
Column 2	4	243.87	60.97	22.52		
Column 3	5	320.31	64.06	19.75		
Column 4	5	318.34	63.67	12.57		
Column 5	4	254.52	63.63	38.31		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	66.53	4.00	16.63	0.73	0.58	2.90
Within Groups	435.06	19.00	22.90			
Total	501.59	23.00				
Grand Mean (ng/mL)[a]	62.41					
Intra-day Precision (%)[b]	7.67					
Inter-Day Precision (%)[c]	0.82					
Total Precision (%)[d]	7.94					
Accuracy (%)[e]	24.83					

[a] Grand mean was taken from Table XII for LGD-4033 at 50 ng/mL and was used to calculate accuracy.

[b] Intra-day precision was calculated using Equation 4 and numbers generated in one-way ANOVA table.

[c] Inter-day precision was calculated using Equation 5 and numbers generated in one-way ANOVA table.

[d] Total precision was calculated using Equation 6 and numbers generated in one-way ANOVA table.

[e] Accuracy was calculated using Equation 7 with the theoretical value at 50 ng/mL.

TABLE XV: ANOVA TABLE FOR MK-2866 AT 50 NG/ML

MK-2866						
Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	4	212.41	53.10	18.40		
Column 2	5	253.95	50.79	65.24		
Column 3	5	247.01	49.40	44.98		
Column 4	5	252.80	50.56	82.45		
Column 5	3	156.36	52.12	39.29		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	35.40	4.00	8.85	0.17	0.95	2.96
Within Groups	904.46	17.00	53.20			
Total	939.86	21.00				
Grand Mean (ng/mL)[a]	51.02					
Intra-day Precision (%)[b]	14.30					
Inter-Day Precision (%)[c]	2.78					
Total Precision (%)[d]	14.67					
Accuracy (%)[e]	2.05					

[a] Grand mean was taken from Table XII for MK-2866 at 50 ng/mL and was used to calculate accuracy.

[b] Intra-day precision was calculated using Equation 4 and numbers generated in one-way ANOVA table.

[c] Inter-day precision was calculated using Equation 5 and numbers generated in one-way ANOVA table.

[d] Total precision was calculated using Equation 6 and numbers generated in one-way ANOVA table.

[e] Accuracy was calculated using Equation 7 with the theoretical value at 50 ng/mL.

TABLE XVI: ANOVA TABLE FOR ANDARINE AT 10 NG/ML

ANDARINE						
Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	5	54.49	10.90	2.63		
Column 2	4	41.02	10.26	1.02		
Column 3	5	49.25	9.85	3.96		
Column 4	5	44.27	8.85	2.26		
Column 5	6	72.39	12.07	2.18		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	31.35	4.00	7.84	3.18	0.04	2.87
Within Groups	49.35	20.00	2.47			
Total	80.69	24.00				
Grand Mean (ng/mL)[a]	10.46					
Intra-day Precision (%)[b]	15.02					
Inter-Day Precision (%)[c]	4.43					
Total Precision (%)[d]	16.23					
Accuracy (%)[e]	4.57					

[a] Grand mean was taken from Table XII for Andarine at 10 ng/mL and was used to calculate accuracy.

[b] Intra-day precision was calculated using Equation 4 and numbers generated in one-way ANOVA table.

[c] Inter-day precision was calculated using Equation 5 and numbers generated in one-way ANOVA table.

[d] Total precision was calculated using Equation 6 and numbers generated in one-way ANOVA table.

[e] Accuracy was calculated using Equation 7 with the theoretical value at 10 ng/mL.

TABLE XVII: ANOVA TABLE FOR LGD-4033 AT 10 NG/ML

LGD-4033						
Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	6	79.60	13.27	1.65		
Column 2	3	29.63	9.88	0.09		
Column 3	5	64.27	12.85	1.16		
Column 4	4	49.72	12.43	0.99		
Column 5	6	89.12	14.85	2.43		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	51.69	4.00	12.92	8.71	0.00	2.90
Within Groups	28.20	19.00	1.48			
Total	79.89	23.00				
Grand Mean (ng/mL)[a]	13.01					
Intra-day Precision (%)[b]	9.36					
Inter-Day Precision (%)[c]	5.30					
Total Precision (%)[d]	11.09					
Accuracy (%)[e]	30.15					

[a] Grand mean was taken from Table XII for LGD-4033 at 10 ng/mL and was used to calculate accuracy.

[b] Intra-day precision was calculated using Equation 4 and numbers generated in one-way ANOVA table.

[c] Inter-day precision was calculated using Equation 5 and numbers generated in one-way ANOVA table.

[d] Total precision was calculated using Equation 6 and numbers generated in one-way ANOVA table.

[e] Accuracy was calculated using Equation 7 with the theoretical value at 10 ng/mL.

TABLE XVIII: ANOVA TABLE FOR MK-2866 AT 10 NG/ML

MK-2866

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	54.88	10.98	3.04
Column 2	3	29.64	9.88	2.67
Column 3	5	47.20	9.44	3.58
Column 4	5	43.38	8.68	3.17
Column 5	6	65.53	10.92	3.91

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	20.22	4.00	5.05	1.50	0.24	2.90
Within Groups	64.05	19.00	3.37			
Total	84.27	23.00				

Grand Mean (ng/mL)[a]	10.03
Intra-day Precision (%)[b]	18.31
Inter-Day Precision (%)[c]	2.64
Total Precision (%)[d]	19.24
Accuracy (%)[e]	0.26

[a] Grand mean was taken from Table XII for MK-2866 at 10 ng/mL and was used to calculate accuracy.

[b] Intra-day precision was calculated using Equation 4 and numbers generated in one-way ANOVA table.

[c] Inter-day precision was calculated using Equation 5 and numbers generated in one-way ANOVA table.

[d] Total precision was calculated using Equation 6 and numbers generated in one-way ANOVA table.

[e] Accuracy was calculated using Equation 7 with the theoretical value at 10 ng/mL.

TABLE XIX: ANOVA TABLE FOR ANDARINE AT 5 NG/ML

ANDARINE

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	29.40	5.88	0.39
Column 2	4	20.50	5.13	0.88
Column 3	4	16.53	4.13	0.01
Column 4	4	22.64	5.66	0.59
Column 5	5	28.56	5.71	0.31

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8.59	4.00	2.15	5.05	0.01	2.96
Within Groups	7.23	17.00	0.43			
Total	15.82	21.00				

Grand Mean (ng/mL)[a]	5.35
Intra-day Precision (%)[b]	12.20
Inter-Day Precision (%)[c]	5.23
Total Precision (%)[d]	13.77
Accuracy (%)[e]	6.93

[a] Grand mean was taken from Table XII for Andarine at 5 ng/mL and was used to calculate accuracy.

[b] Intra-day precision was calculated using Equation 4 and numbers generated in one-way ANOVA table.

[c] Inter-day precision was calculated using Equation 5 and numbers generated in one-way ANOVA table.

[d] Total precision was calculated using Equation 6 and numbers generated in one-way ANOVA table.

[e] Accuracy was calculated using Equation 7 with the theoretical value at 5 ng/mL.

TABLE XX: ANOVA TABLE FOR LGD-4033 AT 5 NG/ML

LGD-4033						
Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	6	38.10	6.35	0.11		
Column 2	4	22.50	5.62	0.03		
Column 3	6	36.45	6.07	0.28		
Column 4	4	26.60	6.65	0.19		
Column 5	6	40.16	6.69	0.32		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.56	4.00	0.89	4.41	0.01	2.84
Within Groups	4.25	21.00	0.20			
Total	7.81	25.00				
Grand Mean (ng/mL)[a]	6.30					
Intra-day Precision (%)[b]	7.14					
Inter-Day Precision (%)[c]	2.58					
Total Precision (%)[d]	7.85					
Accuracy (%)[e]	26.01					

[a] Grand mean was taken from Table XII for LGD-4033 at 5 ng/mL and was used to calculate accuracy.

[b] Intra-day precision was calculated using Equation 4 and numbers generated in one-way ANOVA table.

[c] Inter-day precision was calculated using Equation 5 and numbers generated in one-way ANOVA table.

[d] Total precision was calculated using Equation 6 and numbers generated in one-way ANOVA table.

[e] Accuracy was calculated using Equation 7 with the theoretical value at 5 ng/mL.

TABLE XXI: ANOVA TABLE FOR MK-2866 AT 5 NG/ML

MK-2866						
Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	6	34.70	5.78	0.56		
Column 2	3	17.14	5.71	2.86		
Column 3	5	26.98	5.40	0.01		
Column 4	4	21.05	5.26	0.55		
Column 5	6	32.63	5.44	0.39		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.91	4.00	0.23	0.36	0.84	2.90
Within Groups	12.11	19.00	0.64			
Total	13.02	23.00				
Grand Mean (ng/mL)[a]	5.52					
Intra-day Precision (%)[b]	14.46					
Inter-Day Precision (%)[c]	2.37					
Total Precision (%)[d]	14.86					
Accuracy (%)[e]	10.41					

[a] Grand mean was taken from Table XII for MK-2866 at 5 ng/mL and was used to calculate accuracy.

[b] Intra-day precision was calculated using Equation 4 and numbers generated in one-way ANOVA table.

[c] Inter-day precision was calculated using Equation 5 and numbers generated in one-way ANOVA table.

[d] Total precision was calculated using Equation 6 and numbers generated in one-way ANOVA table.

[e] Accuracy was calculated using Equation 7 with the theoretical value at 5 ng/mL.

TABLE XXII: UNCERTAINTY BUDGET FORM

UNCERTAINTY BUDGET FORM: QUANTITATION OF SARMS IN EQUINE URINE						
Method: Quantitation of SARMs in Equine Urine						
Analyst: Marina Divine			Date: 9/28/18			
Sources of Uncertainty (Andarine)	Type A or B	Std. Dev or Outside Limits (%)[a]	Distribution Model	Divisor	Standard Uncertainty (%)	Can it be ignored?
Average Repeatability - QC Data	A	16.20	Student's t	1.73	9.36	N
Calibration of Andarine Stock Std	B	0.02	Rectangular	1.73	0.02	Y
10 mL Vol Flask	B	0.10	Rectangular	1.73	0.06	Y
Pipet 50 uL of Butalbital-d3 Stock Std	B	0.50	Rectangular	1.73	0.29	Y
Pipet 15 uL of Andarine Stock Std	B	0.50	Rectangular	1.73	0.29	Y
Combined Uncertainty (%):	9.36					
99.7% Confidence Level k value:	3.00					
Expanded Uncertainty (%):	16.21					
Sources of Uncertainty (LGD-4033)	Type A or B	Std. Dev or Outside Limits (%)[a]	Distribution Model	Divisor	Standard Uncertainty (%)	Can it be ignored?
Average Repeatability - QC Data	A	11.10	Student's t	1.73	6.42	N
Calibration of LGD-4033 Stock Std	B	0.02	Rectangular	1.73	0.02	Y
10 mL Vol Flask	B	0.10	Rectangular	1.73	0.06	Y
Pipet 50 uL of Butalbital-d3 Stock Std	B	0.50	Rectangular	1.73	0.29	Y
Pipet 15 uL of LGD-4033 Stock Std	B	0.50	Rectangular	1.73	0.29	Y
Combined Uncertainty (%):	6.42					
99.7% Confidence Level k value:	3.00					
Expanded Uncertainty (%):	11.12					
Sources of Uncertainty (MK-2866)	Type A or B	Std. Dev or Outside Limits (%)[a]	Distribution Model	Divisor	Standard Uncertainty (%)	Can it be ignored?
Average Repeatability - QC Data	A	19.20	Student's t	1.73	11.10	N
Calibration of MK-2866 Stock Std	B	0.02	Rectangular	1.73	0.02	Y
10 mL Vol Flask	B	0.10	Rectangular	1.73	0.06	Y
Pipet 50 uL of Butalbital-d3 Stock Std	B	0.50	Rectangular	1.73	0.29	Y
Pipet 15 uL of MK-2866 Stock Std	B	0.50	Rectangular	1.73	0.29	Y
Combined Uncertainty (%):	11.10					
99.7% Confidence Level k value:	3.00					
Expanded Uncertainty (%):	19.23					

[a] Standard deviation for QC data was determined from the total precision values calculated from one-way ANOVA tables. Highest total precision values were determined at the mid QC level for all three drugs (Tables XVI, XVII, XVIII), and these precision values were used as the standard deviation to determine uncertainty.

V. DISCUSSION

The method presented in this paper, with the use of solid phase extraction followed by LC-MS QQQ analysis, allowed for the successful detection of selective androgen receptor modulators Andarine, LGD-4033, and MK-2866 in equine urine. The use of this method provided increasing sensitivity compared to GC-MS and ELISA and exceptional peak shape and resolution, which is necessary for detecting SARM parent compounds in urine due to their rapid metabolism in the body. The use of two product ions per compound lowers the number of false positive results from the initial testing. While this study provides an accepted method to detect SARMs, it also presents some limitations that need to be considered.

A. **Limitations**

Matrix effects were observed for each drug and provided in Table IV, with all drugs showing appreciable suppression of signal. In future studies, additional clean up steps should be attempted, however these attempts could potentially lower extraction recovery of some or all compounds and further decrease process efficiency. Additionally, the use of a single extraction allows for lower laboratory costs in both personnel time and consumables. Also, as observed in Table V, the use of two elutions allowed for more compounds to be extracted using a single solid phase column. Another way to reduce ion suppression would be to increase the amount of sample injected. Since this was a qualitative requirement, matrix effect can be largely ignored if detection levels are at acceptable and expected levels.

In addition, this study focuses on the detection of SARM parent compounds. As previously mentioned, studies have shown that these compounds are rapidly metabolized in horses *(32, 51)*. Thus, parent compounds go undetected in non-hydrolyzed samples, and are detected at very low concentrations in hydrolyzed urine samples. Another challenge presented

was the lack of published research on expected concentrations of parent compound in equine urine. Therefore, it was difficult to determine the linear range for the calibration curve, especially at lower concentrations nearing the LOD. Again, lower concentrations may need to be tested for LOD determination since the parent compound is found at low concentrations in the urine. Although LC-MS QQQ is a very sensitive technique, it may be beneficial to consider SARM metabolites as doping control targets. Another option of study is using equine plasma as the biological matrix to detect parent compounds, since they are more likely to be found in blood. Both options should be acknowledged in future studies.

There are several classes of SARM compounds as observed in Table II. This study only considers two classes of drugs (aryl propionamides and pyrrolidinyl-benzonitriles), which have a high potential for abuse in both human athletes and racing horses. However, due to the extensive list of SARM drug candidates available on the black market, the number of SARMs abuse cases is expected to be much higher. Therefore, screening methods need to be able to screen for all classes of SARM candidates, for there is a high possibility that many other SARMs are abused without a method to screen for these compounds. Slightly different parameters may be needed for different classes of SARMs, which is another reason all classes need to be tested for method development. As observed in the data in Tables XVII and XX, LGD-4033 exhibited noticeably higher bias compared to the other drugs in this study, with a bias greater than 25% at mid and low concentrations. It is unclear why the accuracy values were elevated for this compound when the other two compounds were right on target (all drugs were mixed together in the same working standard), but it could be that LGD-4033 is in a different class than the other two compounds and therefore reacted slightly different to the method. Looking forward, method

validation studies need to cover all SARM classes, and it may be beneficial to study each class of compounds separately.

Finally, a negative ionization method was used to screen for the SARM compounds in this study. However, most compounds in routine urine screening methods positively ionize, so most internal standards used for instrumental analysis provided at the AFTL also positively ionize. Therefore, the options for choosing an internal standard that negatively ionize, like the SARM compounds in this study, were minimal. The internal standard used in this study had much lower peak responses compared to the SARM compounds even after increasing concentrations, which most likely means it does not negatively ionize as well as the SARM drugs. Typically, a deuterated internal standard is preferred in method development and analysis due to the structural similarity to the analyte of interest and similar peak response. This aids in the quality of results for quantitation of analyte(s) under study. The use of a more structurally similar internal standard, such as a deuterated SARM compound, may provide even greater quality of precision and accuracy results. As SARM compounds are in the early stages of development and research, deuterated standards are not readily available and would be a more expensive option. However, future studies should consider a more structurally similar internal standard for method development and validation.

B. **Conclusion**

The method presented in this paper allows for the successful detection of emerging therapeutics and illicitly used selective androgen receptor modulators at or below concentrations needed for effective drug monitoring. This is, to the best of the author's knowledge, the first method validation study to detect LGD-4033, Andarine, and MK-2866 in equine urine using SPE

followed by LC-MS QQQ. Advanced LC-MS QQQ technology is cost effective and offers greater sensitivity and the ability to screen for hundreds of drugs in a single run.

Future studies should look at the addition of more SARM compounds as well as the possible use of metabolites as doping control targets. If parent compounds are used as the analytical target, plasma may be a wiser option as the biological matrix of choice with higher concentrations of parent compound. It may be beneficial to test different classes of compounds separately and mixed to observe the effect they have on one another. Further, deuterated SARM internal standards should be used if available to obtain optimal quality of quantitative results. Implementing these drugs in routine equine screening methods is recommended. This will deter handlers who are tempted to administer these readily available drugs and maintain integrity in the horse racing industry.

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