Title

Detection of Anabolic Steroid Metabolites in Equine Urine using Liquid Chromatography - Mass Spectrometry

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Detection of Anabolic Steroid Metabolites in Equine Urine using Liquid Chromatography - Mass Spectrometry

By

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THESIS

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Abstract

Anabolic Androgenic Steroids (AAS) are a group of compounds with high potential for abuse in sports including horse racing. Within the last three decades, both gas chromatography – mass spectrometry (GC-MS) and liquid chromatography – mass spectrometry (LC-MS) methods have been developed for routine qualitative and quantitative detection of endogenous or exogenous AAS in urine and serum to address their misuse in sports. However, most current screening methods for the detection of AAS require using enzymatic or chemical hydrolysis to detect free steroids rather than their phase II conjugates which constitute the majority of excreted drug for most anabolic agents. In recent decades, new long-term phase II metabolites of AAS that were previously not detected by GC-MS have been identified by LC-MS methods, including glucurono-conjugates and sulfo-conjugates that are difficult to cleave using enzymatic hydrolysis. This research project focuses on the development and validation of a method capable of the simultaneous detection of 32 steroid phase II metabolites using LC-MS/MS, with prior clean-up steps including protein precipitation and Solid Phase Extraction (SPE) through weak anion exchange cartridges. All compounds, including isomers and epimers, were chromatographically separated over a 20-minute run using a reversed phase C18 column using methanol and water as the organic and aqueous mobile phases. The mass spectrometer data acquisition was set to use selected reaction monitoring scans following introduction via electrospray ionization in both positive and negative modes. The method was validated, and the following parameters were determined: linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), recovery, matrix effect, and stability. Finally, a population study using gelding, mare, and stallion urine samples from horses actively competing was conducted to evaluate the endogenous production of the targeted compounds.

1.0 Introduction

The use of anabolic steroids as performance-enhancers has been of concern in professional sports including horse racing for over 50 years. Ever since regulatory agencies established regulations and thresholds on the use of doping agents or therapeutic drugs, laboratories have developed testing methods to detect potential doping activities. The gold standard for the detection of anabolic steroids has been gas chromatography - mass spectrometry (GC-MS) for many years, and even until this day it is still the preferred methodology in many laboratories particularly when coupled with the ability to perform MS/MS selection (Wong et al., 2012). However, steroid detection has slowly gravitated towards the use of liquid chromatography - mass spectrometry (LC-MS) in recent decades because of its ability to provide higher sensitivity and throughput (McKinney, 2009; Waller et al., 2016; Hintikka et al., 2008).

In this project, we attempted to develop a method which will differentiate and quantitate 32 glucuronidated and sulfonated AAS in equine urine utilizing LC-MS/MS. The goal was to provide a sufficient separation method for 32 similarly structured AAS to enhance the detection of these compounds in horse urine to help monitor the potential abuse of synthetic versions of the endogenously produced compounds. Currently, methods for the detection and quantitation of AAS only target free steroids (after cleaving the conjugates) and not intact metabolites. Therefore, this project focused on the detection and quantitation of intact conjugated AAS metabolites. The method was validated for its linearity, LOD, LOQ, dilution, recovery, matrix effect, and stability. This thesis describes the development stages and validation of an analytical method capable of the simultaneous detection of 32 steroid metabolites in equine urine following solid phase extraction (SPE) and LC-MS/MS detection using selected reaction monitoring mode.

Following method validation, the analytical approach developed in this project can contribute

to the evaluation of current endogenous ranges of endogenous anabolic androgenic steroids (EAAS) and provide an analytical approach for monitoring AAS drug abuse in the future. Accordingly, a population study using more than 500 equine urine samples from females (mares/fillies), males (stallions) and castrated males (geldings) was conducted with the goal of determining endogenous circulating levels of the various analytes and evaluating the potential feasibility for use in routine screening of samples submitted for analysis.

2.0 Background

Doping in sports has been a common practice among human athletes during the early 20th century. As it became increasingly evident that using performance-enhancing drugs was threatening the integrity of competition and had negative health effects, the World Anti-Doping Agency (WADA) was formed in the 1990s to combat doping in the Olympics (WADA, 2023). Nevertheless, suspicion of both doping and misuse of therapeutic medications in animal sports such as horseracing required regulatory authorities to take action to ensure racing integrity and safety/welfare of the horses competing. There are many classes of compounds that can be used to obtain a competitive advantage including stimulants, sedatives, opiates, β_2-agonists, anabolic steroids, and peptide hormones. Of all the drug classes, anabolic steroids are the most abused drug class in human sports (WADA, 2014). Reports of anabolic steroid doping in equine races date back to 1941, when a Standardbred US trotter named Holloway was injected with testosterone for several months during training, and as a result, gained much of his racing ability (Holt et al., 2009). A more recent case involved the 2008 Kentucky Derby winner Big Brown. His trainer publically admitted on national television to treating the horse with stanozolol (Winstrol) prior to the 2008 Kentucky Derby, although stanozolol was still legal in US racing at the time (Scheinman, 2008). Big Brown went on to win the first two races of the Triple Crown

but had a poor performance at the final leg of the series when his new ownership discontinued the administration of stanozolol prior to the race. In addition to potential performance enhancing effects, there is evidence to show that anabolic steroid administration impacts the closure of epiphyseal growth plates in Standardbreds and thus increases the potential for injury (Van Der Kolk et al., 2014). Additionally, aggressive behavioral changes have been commonly reported which could result in accidents with other horses or human riders and trainers (Van Der Kolk et al., 2014; Snow et al., 1982). In order to protect the fairness of equine sports internationally and to protect the wellness of horses, the International Federation of Horseracing Authorities (IFHA) was formed in 1993 and includes over 60 international members. The IFHA meets annually to update the International Agreement on Breeding, Racing and Wagering, which outlines the protocols for how to effectively regulate doping in equine sports (Waller et al., 2016).

Testosterone, shown in **Figure 1b.,** is a major androgen produced endogenously in humans and animals. Testosterone has both androgenic and anabolic effects on reproductive and non-reproductive target tissues. Androgenic effects refer to the growth of the male reproductive system and development of secondary sexual characteristics, whereas anabolic effects stimulate positive nitrogen balance and increased protein synthesis (Kicman, 2008). Derivatives of testosterone, known as anabolic androgenic steroids, were synthesized to prolong the metabolic half-life of the parent molecule, increase their efficacy, and reduce their androgenic effects (Kuhn, 2002). In sports, the main desired effects of testosterone-derived compounds are their potential to improve physical performance of skeletal muscle and to regulate catabolic condition in the body after stress (Maravelias et al., 2005).

In the International Agreement on Breeding, Racing, and Wagering document published by the IFHA, the majority of the AAS are prohibited internationally with exception of testosterone,

boldenone, and nandrolone, which have established regulatory thresholds in urine and plasma (IFHA, 2021). Boldenone and nandrolone occur naturally in intact male horses, and testosterone occurs naturally in all horses at various concentrations (Soma et.al, 2008; Ho et.al, 2004). Because of this, detecting steroid abuse is an exceedingly difficult task, since they are substances produced naturally in the body and are related to the metabolic pathway of testosterone. Additionally, there is a large inter-individual variation in concentrations of EAAS due to many factors, raising doubts about whether elevated concentrations of unmodified naturally occurring AAS are due to exogenous administrations (Wilde et al., 2020).

A typical steroid structure has a four-membered hydrocarbon ring as its core (**Figure 1a**), and thousands of synthetic and natural steroids derived from that core with the variations of functional groups at positions 3 and 17 positions, changes to the position of hydrogens or functional groups on the ring (α vs β), modification of ring saturation, and esterification being the most common modifications (Kasal, 2010). Many of the synthesized anabolic steroids have had these modifications made to impact the pharmacokinetics and pharmacodynamics of the compounds and more recently to attempt to evade detection by anti-doping testing laboratories (Catlin et al., 2002).

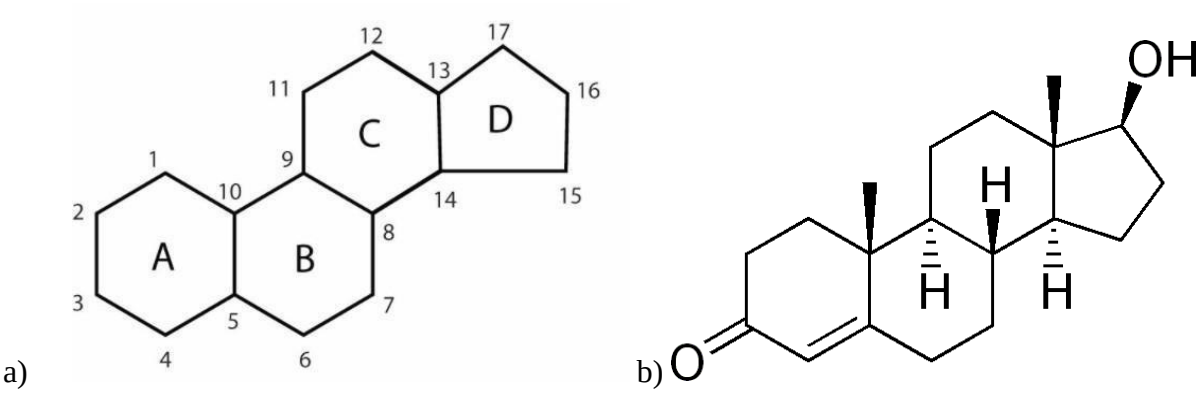
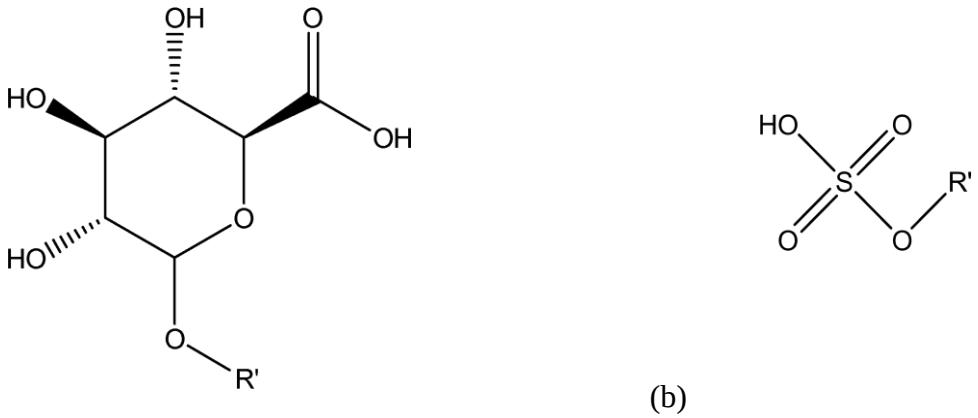


Figure 1. a) steroid skeleton numbering system and b) testosterone structure

In general, following administration, anabolic steroids are extensively metabolized prior to elimination (Schänzer, 1996) . AAS are largely nonpolar compounds that are biotransformed by both Phase I and Phase II metabolic enzymes, ultimately facilitating elimination primarily via the urine. Phase I reactions (oxidation, reduction, and hydroxylation) increase the polarity of the steroid structure and serve as sites for Phase II reactions. Phase II reactions (mainly glucuronidation and sulfonation) are conjugation reactions which increase the polarity of the compound and enhance elimination by either attaching glucuronide(s) or sulfuric acid functional groups (**Figure 2**) to the 3 and/or 17 hydroxyl positions on the steroid skeleton (Waller et al., 2016; Hintikka et al., 2008). Steroid glucuronide metabolites are formed from an enzyme-mediated transfer of glucuronic acid from a uridine diphosphate (UDP) glucuronide acid donor, while steroid sulfate metabolites are formed by an enzyme-mediated transfer of sulfate from a 3'-phosphoadenosine-5'-phosphosulfate (PAPS) donor (Schänzer, 1996).



(a) (b)

Figure 2. (a) glucuronide functional group, (b) sulfate functional group

Conventionally, analysis of AAS and their metabolites in biological matrices such as urine is accomplished using chromatographic separation by GC-MS and detection using mass spectrometry after enzymatic and/or chemical hydrolysis of the phase II conjugates and derivatization of the

resulting compounds (Schänzer, 1996 ; Hintikka et al., 2008). Methods using GC-MS are robust and sensitive, but sample preparation is time-consuming, some compounds have poor recovery, and the overall throughput is relatively low. Moreover, the majority of the GC-MS methods are indirect, only targeting the hydrolyzed products of steroid glucuronides and sulfates, whereas only a few studies focus on conjugated AAS (Hintikka et al., 2008). Thus, a more direct method to detect conjugated metabolites of AAS is desired for anti-doping analysis and therapeutic monitoring. The application of LC-MS allows the direct measurement of AAS glucuronides and sulfates from biological matrices. Along with the increase in the availability of reference materials due to the surge of interest in regulating anabolic steroids in the past decade, method development for the detection of intact Phase II metabolites of AAS employing LC-MS became more popular.

3.0 Material and Methods

3.0.1 Reference Standards with Common Names and Abbreviations

5β-Androstan-3α-ol-17-one glucosiduronate (Etiocholanolone 3-glucuronide, Etio-3G), 5α-androstan-3β-ol-17-one glucosiduronate (Epiandrosterone 3-glucuronide, EpiA-3G), 4-androsten-17β-ol-3one sulfate (Testosterone 17-sulfate, T-17S), 4-androsten-17α-ol-3-one sulfate (Epitestosterone 17-sulfate, EpiT-17S), 5-androsten-3β-ol-17-one glucosiduronate (Dehydroepiandrosterone 3-glucuronide, DHEA-3G), 4-androsten-17β-ol-3-one glucosiduronate (Testosterone 17-glucuronide, T-17G), 4-androsten-17α-ol-3-one glucosiduronate (Epitestosterone 17-glucuronide, EpiT-17G), 4-estren-17α-ol-3-one glucosiduronate (Epinandrolone 17-glucuronide, EpiN-17G), 4-estren-17β-ol-3-one glucosiduronate (Nandrolone 17-glucuronide, N-17G), 5α-Androstan-3β-ol-17-one sulfate (Epiandrosterone 3-sulfate, EpiA-3S), 5β-Androstan-3α-ol-17-one sulfate (Etiocholanolone 3-sulfate, Etio-3S), 5α-Androstan-3α-ol-17-one sulfate (Androsterone 3-sulfate, A-3S),

1,3,5(10)-Estratrien-3-ol-17-one sulfate (Estrone 3-sulfate, estrone-3S), 1,3,5(10),7-Estratetraen-3-ol-17-one glucosidurnate (Equilin 3-glucuronide, equilin-3G), 5α-Androstan-3β,17β-diol-17-sulfate (5αββ-diol 17-sulfate, 5αββ-diol-17S), 4-Estren-17β-ol-3-one sulfate (Nandrolone 17-sulfate, N-17S), 1,4-Androstadien-17β-ol-3-one sulfate (Boldenone 17-sulfate, B-17S), 5α-Androstan-3α,17β-diol 17-glucosiduronate(5ααβ-diol 17-glucuronide, 5ααβ-diol-17G), 5-Pregnen-3β-ol-20-one sulfate (Pregnenolone 3-sulfate, Preg-3S) were purchased from Steraloids (Newport, RI, USA). Dehydroepiandrosterone 3-sulfate sodium salt (DHEA-3S), Dehydroepiandrosterone-d_5-3-sulfate sodium salt (DHEA-d_5-3S) were purchased from Cerilliant Corporation (Round Rock, TX, USA). β-Estradiol 17(β-D-glucuronide) sodium salt (17β-Estradiol-17G), (17β)-Estra-1,3,5(10),7-tetraene-3,17-diol 3-sulfate sodium salt (17β-Dihydroequilin-3S), 3-(sulfooxy)Estra-1,3,5(10),7-tetraen-17-one sodium salt (equilin-3S), (11β)-11,17-Dihydroxy-21-(sulfooxy)pregn-4-ene-3,20-dione potassium salt (cortisol-21S), 3,17β-Dihydroxyestra-1,3,5(10)-triene 17-sulfate (17β-Estradiol-3S), 17β-Hydroxyestra-1,3,5(10)-trien-3-yl β-D-glucupyranosiduronic Acid (17β-Estradiol-3G), 5α-Estrane-3β,17α-diol Bis(β-D-glucuronide) (5αβα-diol-Bis-G), (11β)-11,17-Dihydroxy-21-(sulfooxy) pregn-4-ene-3,20-dione-d_4 potassium salt (Cortisol-d_4-21S), (17β)-17-Hydroxyestra-1,3,5(10)-trien-3-yl-d_3 β-D-glucopyranosiduronic Acid (17β-Estradiol-d_3-3G), 1,3,5(10)-Estratrien-3-ol-17-one-d_5 3-sulfate (estrone-d_5-3S) were purchased from Toronto Research Chemicals (Ontario, Toronto, Canada). Androsterone 3-glucuronide sodium salt (A-3G) was purchased from IsoSciences (Ambler, PA, USA). sodium(3α,5α)-3-hydroxyestran-17-one β-D-glucopyranosiduronate (5α-estran-3α-ol-17-one 3-glucuronide, 19-NorA-3G), sodium(3α,5β)-17-Oxoestran-3-yl β-D-

glucopyranosiduronate (5β-estran-3α-ol-17-one 3-glucuronide, 19-NorEtio-3G), Triethylammonium (3α,5α)-17-oxo-estran-3-yl sulfate (5α-estran-3α-ol-17-one 3-sulfate, 19-NorA-3S), (17)-3-Oxoandrosta-1,4-dien-17-yl--D-glucupyranosiduronic acid potassium salt (Boldenone 17-glucuronide, B-17G), 2,2,4,4-d4-(3α,5α)-17-Oxoestran-3yl β-D-glucopyranosiduronic acid sodium salt (d4-5α-estran-3α-ol-17-one 3-glucuronide, d4-19-NorA-3G), 17β-(Sulfoxy)-Androsta-1,4-dien-3-one-16,16,17-d3 triethylamine salt (d3-Boldenone-17S) were purchased from LGC Standards (Teddington, UK).

3.0.2 Reagents and Raw Materials

HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), sucrose, activated charcoal, and dextran were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid, sodium acetate trihydrate, ammonium hydroxide, ammonium acetate, magnesium chloride, and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Nalgene™ Rapid-Flow™ bottle filters were purchased from Thermo Scientific (Waltham, MA, USA). Deionized nanopure water was obtained from a nanopure system also from Thermo Scientific. Formic acid, acetonitrile, HPLC grade methanol, and HPLC grade water were purchased from Honeywell - Burdick & Jackson (Muskegon, MI, USA)

3.2.1 Preparation of Standard Solutions

All reference standards were prepared with methanol at a 1 mg/mL concentration unless premade by the vendor. Salt and purity correction calculations were applied to certain compounds to calculate the volume necessary for the amount of raw material weighed out. Finally, depending on the solubility, the appropriate volume of methanol or a mixture of water and methanol were added to dissolve each compound to make the desired

concentration. All targeted compounds were mixed together into a working solution at 10 ng/µL except for estrone-3S, which was made at a 10x higher concentration (100 ng/µL). Dilutions from the 10 ng/µL solution was performed in methanol to make a 1 ng/µL and a 0.1 ng/µL mixture using the 10 ng/µL working solution. Most of the internal standards were also prepared at 1 mg/mL using the procedure described above except for DHEA-d5-17S which was from a commercially prepared reference standard vial premade by the supplier company. All six internal standards were mixed together in methanol to make a working solution at 10 ng/µL, which is then diluted to 0.25 ng/µL for use.

3.2.2 Stock Solutions

The following stock solutions were prepared for this experiment: 9:1, Acetonitrile:1 M acetic acid, 1.6 M acetate buffer (pH 5), 2 % formic acid and 5% methanol in water, 10% ammonium hydroxide in methanol, 5 mM ammonium acetate in HPLC grade methanol, 5 mM ammonium acetate in HPLC grade water, 10:90 methanol:water re-dissolve solution.
The following procedure was used to prepare 500 mL of charcoal stripping solution: weighing out 1.3015 g HEPES, 42.788 g sucrose, 71.4 mg $MgCl_2$, 1.25 g of activated charcoal, and 12.5 mg of dextran and adding each to 500 mL of nanopure water with thorough mixing after each addition (Green & Leake, 1987).

3.3 Calibrators and Quality Control Preparation

Negative control urine was prepared from a pool of cleared post-race gelding samples that was charcoal stripped prior to use. The charcoal stripping solution was prepared as described previously (Green & Leake, 1987) and utilizing a fine suspension of dextran coated activated charcoal that was stirred prior to centrifuging 50 mL at 500 x g for 10 minutes to pellet the charcoal. The supernatant was decanted, and 50 mL of gelding urine was added and vortexed

overnight at 4°C. Afterwards, the charcoal infused gelding urine was centrifuged again to pellet the charcoal and the urine was taken out to be used as negative control. Finally, the charcoal stripped urine was filtered by a sterile polyethersulfone (PES) filter to remove any residual charcoal. Negative control urine was stored at 4°C until use.

A 6-point calibration curve was constructed from spiking 0.5 mL of the negative control urine with compound working solutions and internal standard working solution. Spike concentration and amounts are specified in **Table 1.** The range of the calibration curve for 31 out of 32 compounds is from 1 ng/mL to 500 ng/mL. The range for estrone-3S was from 10 ng/mL to 5 mg/mL.

Level	Concentration at Each Level in Urine	Volume of Each AAS Working Solution Spiked Into 0.5mL Urine	Concentration of Internal Standard in Urine	Volume of Internal Standard
C1	1 ng/mL	5 uL of 0.1ng/µL	25 ng/mL	50 uL of 0.25 ng/µL
C2	5 ng/mL	25 uL of 0.1ng/µL	25 ng/mL	50 uL of 0.25 ng/µL
C3	10 ng/mL	5 uL of 1 ng/µL	25 ng/mL	50 uL of 0.25 ng/µL
C4	50 ng/mL	25 uL of 1 ng/µL	25 ng/mL	50 uL of 0.25 ng/µL
C5	100 ng/mL	5 uL of 10 ng/µL	25 ng/mL	50 uL of 0.25 ng/µL
C6	500 ng/mL	25 uL of 10 ng/µL	25 ng/mL	50 uL of 0.25 ng/µL

Table 1. Calibration curve spiking volumes and concentrations. Starting urine volume is 0.5 mL.

Quality control samples were prepared at low, mid, and high levels of 3, 30, and 300 ng/mL, respectively. Similarly, estrone-3S concentration in quality control samples were also 10 times higher than other compounds. For matrix effect evaluation, the neat standards were prepared by adding the same volumes of the AAS working solutions and internal standard working solutions to HPLC autosampler vials and dried down before re-dissolving.

3.4 Sample Preparation

0.5 mL of urine was transferred into 12 x 75 mm glass test tubes followed by the addition of 50 µL of the internal standard to each tube. Samples were vortexed briefly (Fisher Scientific, Pittsburgh, PA, USA) and 1 mL of cold 9:1 acetonitrile:1 M acetic acid solution was added to each urine sample to precipitate proteins. Samples were vortexed again and placed in a 4°C refrigerator for 30 minutes. After incubation, samples were centrifuged at ~1000 x g for 5 minutes. The sample was then carefully transferred into a new 12 x 75 mm glass test tube, leaving the protein pellet undisturbed in the original test tube. The extract was dried down under a nitrogen stream in a 60°C water bath using a TurboVap LV Evaporator (Zymark Corporation, Hopkinton, MA, USA) for approximately 30 minutes. Lastly, the test tubes were reconstituted with 3 mL of the 1.6 M acetate buffer solutions, pH 5.0 and vortexed thoroughly.

3.5 Solid Phase Extraction Using Weak Anion Exchange

Oasis® Weak Anion Exchange (WAX) solid-phase extraction cartridges 3cc, 60 mg, 30µm (Waters Corporation, Milford, MA, USA) were placed in a 48 -well positive pressure manifold (Cera Inc., Baldwin Park, CA, USA). The SPE cartridges were conditioned with 3 mL of methanol followed by 3 mL of the 1.6 M acetate buffer solution, pH 5.0 to adjust the pH of the cartridges. Reconstituted urine samples were then introduced to the cartridges and passed through at a rate of 1 drop per second. Cartridges were then washed with 3 mL of 2% formic acid 5% methanol in water, then 3 mL of water, and lastly with 3 mL of methanol. The final eluate was collected in a 12 x 75 mm test tube by passing through 1.5 mL of the 10% ammonium hydroxide in methanol eluent. The tubes were then placed into a TurboVap LV Evaporator and evaporated for 15 minutes in a 45℃ water bath. The tubes were reconstituted with 200 µL of 5 mM ammonium acetate in 10:90 methanol:water re-dissolve solution and

vortexed. Finally, each re-dissolved sample was pipetted into an additional PES nano filter vial® (Thomson, Oceanside, CA, USA) and filtered to ensure no particles were present in the final sample extract prior to LC-MS/MS analysis.

3.4 Solid Phase Extraction Using Polymeric Sorbent

A secondary SPE method using Agilent Nexus Bond-Elut cartridges was also initially tested and determined to be an interchangeable method to the Oasis WAX cartridge. The SPE steps were: first, condition the column with 3 mL of methanol then 3 mL of water, load samples, then wash with 3 mL of water, and finally elute with 1.5 mL of methanol. The eluate was then dried down, re-constituted, and filtered by a PES vial before instrumental analysis.

3.6 LC-MS/MS Analysis

LC-MS analysis was performed on a Vanquish™ Duo HPLC system coupled to an Altis™ triple quadrupole mass spectrometer (Thermo Scientific, Waltham, Massachusetts, USA). The HPLC column used was a Kinetex EVO C18 Core-Shell column, 2.1 x 100 mm, 1.7 µm (Phenomenex, Torrance, CA, USA) held at 45℃. Extracts were introduced to the HPLC system using a 10 µL injection volume and chromatographic separation was achieved by reversed phase chromatography employing a gradient elution using organic mobile phase A: 5 mM ammonium acetate in methanol and aqueous mobile phase B: 5 mM ammonium acetate in water over a 20-minute run time. Mobile phase composition as follows: at 350 µL/min flow rate: 0.00 min, 10% A hold for 2 minutes; 2.00 min, increase to 45% A over 2 minutes; 4.00 min, slowly increase to 55% A over 8 minutes; 12.00 min, 90% A in 1 minute; 13.00 min flush with 95% A for 2 minutes at 400 µL/min flowrate; then finally 15.00 min, flush column with 10% A and 90% B for 5 minutes at 550 µL/min flow rate. Following chromatographic separation, the compounds were

introduced to the mass spectrometer using heated electrospray ionization (H-ESI) using both positive and negative modes. The mass spectrometer data acquisition time was between 4 min to 13 min post injection and HPLC flow was diverted to waste outside this time period. Argon gas, set at 1.5 mTorr, was used as the collision gas in the collision cell of the mass spectrometer. The mass spectrometer was operated in selected reaction monitoring (SRM) mode with predetermined parent ions and product ions along with their corresponding collision energies shown in **Tables 2, 3, 4.** Compound optimization was done by using the Xcalibur Tune™ software with direct infusion of neat standard solutions at 10 ng/µL concentration. The optimal source parameters were found to be 4800 V for positive spray and 4000 V for negative spray, sheath gas at 50 arbitrary units, auxiliary gas at 10 arbitrary units, sweep gas at 2 arbitrary units, ion transfer tube temperature at 300℃, and vaporizer temperature at 100℃. The MS resolution setting were 0.4 and 0.7 full width at half maximum height (FWHM) for the first quadrupole and third quadrupoles, respectively. The LC-MS/MS system was controlled using the Xcalibur™ (version 4.3.73.11) and Aria MX™ (version 2.6.13) software from Thermo Scientific (Waltham, Massachusetts, USA). Data analysis and review were accomplished by using Quan browser (version 4.3.73.11) also from Thermo Scientific.

3.7 Validation

The following validation parameters were determined: linearity, accuracy, and precision, LOD, LOQ, recovery, matrix effect, carryover, dilutional linearity, and stability. Linearity was assessed by a 6-point calibration curve using spiked negative control urine samples. Due to the endogenous nature of some analytes, DHEA-17S and cortisol-21S were still present in the negative control urine. Accuracy and precision were assessed by quality controls at the low, mid, and high level (n=6 per level) over 3 days. Recovery evaluation was done by comparing pre-

extraction spike samples at each QC level (n=6 per level) and post-extraction spike samples at each QC level (n=6 per level) by adding the same volumes of the AAS working solutions and internal standard working solution**.** to the post-extracted samples, prior to drying down the eluate. Matrix effect was evaluated by comparing post-extraction spike samples at each QC level (n=6 per level) and neat standards at equivalent concentrations to each QC level. Limit of detection was assessed by spiking negative control urine with concentrations below the lowest point of the calibration curve at 0.75 ng/mL, 0.5 ng/mL, 0.25 ng/mL and 0.1 ng/mL. Carryover was checked by running a blank sample (5 mM ammonium acetate in 10:90 methanol:water re-dissolve solution) after the highest calibrator. Dilutional linearity was verified by spiking 1 mL of urine with 1500 ng of analytes (15 µg for estrone-3S) and diluting the sample to 15 mL, then aliquot 0.5 mL of urine (n=6) for analysis. Stability was assessed by preparing negative urine samples spiked at the mid QC level, without adding internal standards. The limit of detection was defined by the lowest concentration detectable with a signal to noise ratio over 3, and the limit of quantitation was defined by the lowest concentration of a substance that was detectable with a S/N greater than 10 and with an accuracy within 20% of the theoretical value. The following storage conditions and durations were tested: room temperature at t =0 and 24 h and 4℃elcius at t = 24h, 4 and 7 days, and -20℃elcius at t = 7 and 14 days, n = 3/time point. Internal standards were added on the day of each analysis time point. Additionally, freeze thaw cycle was also tested by spiking urine samples at the mid QC level (n=3) and stored in a -20℃ freezer until frozen, then thawing the samples at room temperature, and repeated freeze-thaw cycle for a total of 3 times before analysis.

4.0 Results

The chromatographic and mass spectrometry conditions were optimized for the 32 targeted compounds along with their corresponding stable isotope labelled internal standards (**Figure 3, 4 Table 2, 3, 4).**

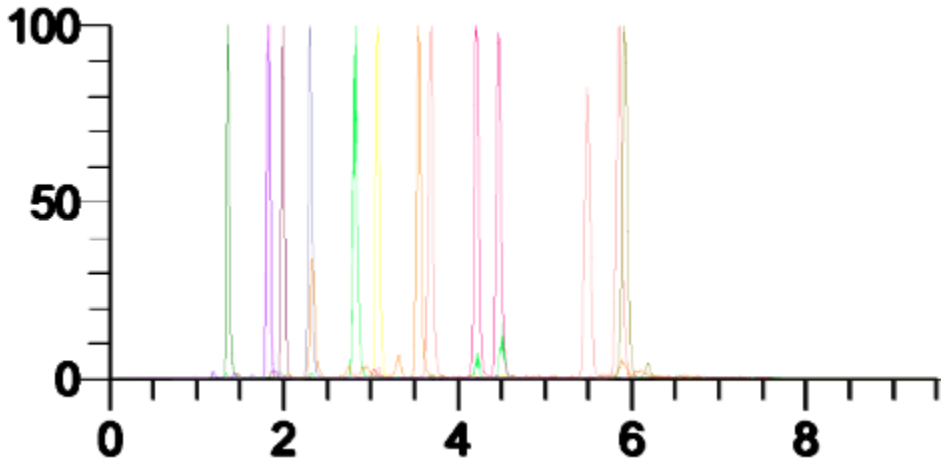


Figure 3. Representative Extracted Ion Chromatograms of 16 AAS Glucuronide Conjugates. y-axis represents relative intensity and x-axis represents time in minutes.

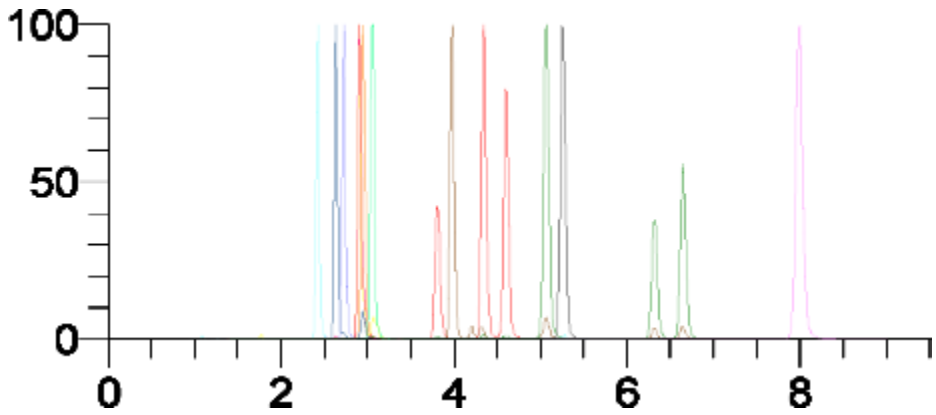
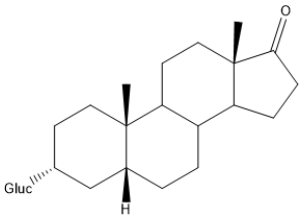
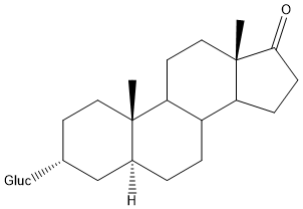
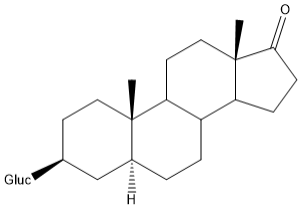
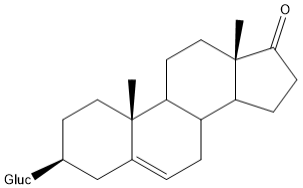
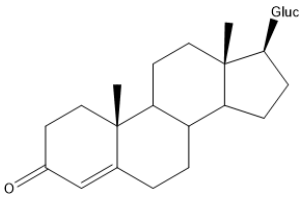
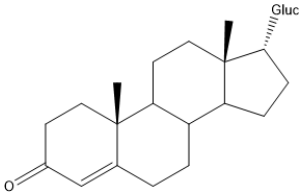
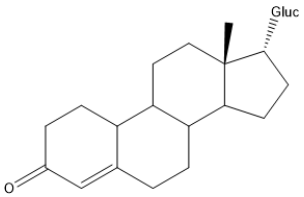
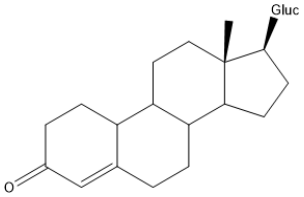
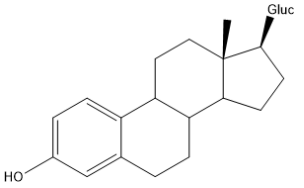
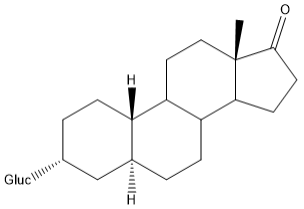
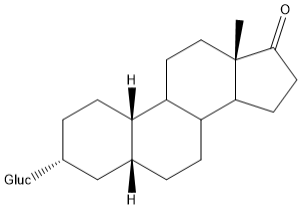
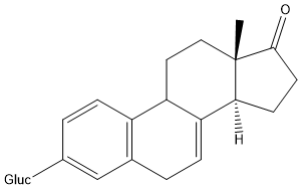
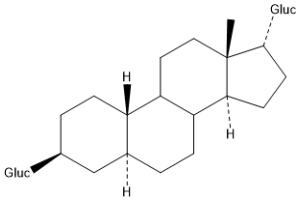


Figure 4. . Representative Extracted Ion Chromatograms of 16 AAS Sulfate Conjugates. y-axis represents relative intensity and x-axis represents time in minutes.

AAS Glucuronides	Structures	ESI mode and Precursor Ion (m/z)	Product Ion (m/z) and Collision Energy (eV)	Retention Time (min)
Etio-3G		(+) 484.2	215(32), 255(20), 273(20), **291(17)**, 431(13)	9.4
A-3G		(+) 484.2	215(32), 255(20), **273(20)**, 291(17), 431(13)	9.9
EpiA-3G		(+) 484.2	141(27), 177(16), 255(23), **273(15)**, 449(10)	7.6
DHEA-3G		(+) 482.2	97(38), 175(30), 253(27), **271(15)**, 447(9)	7.0
T-17G		(+) 465.2	97(30), 109(33), 253(24), 289(20), **271(22)**	6.9
EpiT-17G		(+) 465.2	97(36), 109(39), 253(25), **271(17)**, 289(13)	8.4

EpiN-17G		(+) 451.2	113(16), 145(35), 147(32), 239(26), **257(16)**, **275(12)**	7.5
N-17G		(+) 451.2	**113(16)**, 145(35), 147(32), **239(26)**, 257(16), 275(12)	6.3
17β-Estradiol-17G		(+) 466.2	133(34), 141(26), 159(33), **255(14)**, 431(9)	6.1
19-NorA-3G		(+) 470.2	141(10), 241(26), 259(17), 277(13), 417(12), **435(10)**	8.4
19-NorEtio-3G		(+) 470.2	141(10), **241(26)**, 259(17), 277(13), 417(12), 435(10)	8.2
Eequilin-3G		(+) 462.1	251(29), 269(17), **286(12)**, 445(12)	5.9
5αβα-diol-Bis-G		(+) 648.2	177(22), **243(23)**, 261(20), 419(14)	5.3

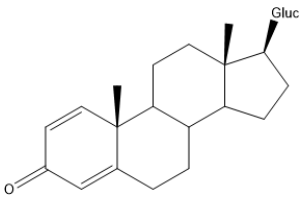
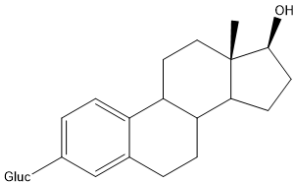
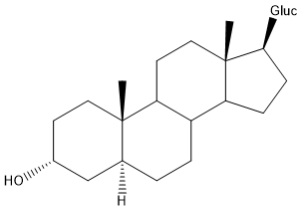
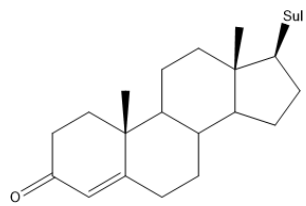
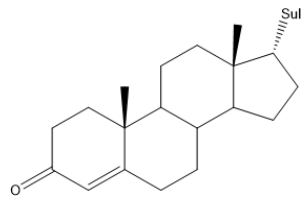
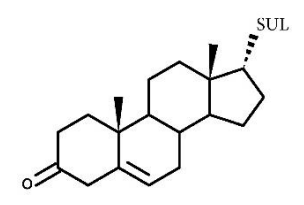
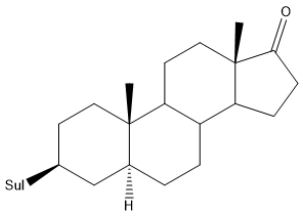
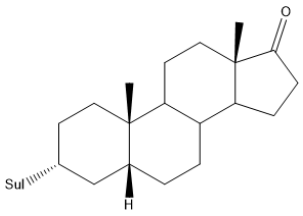
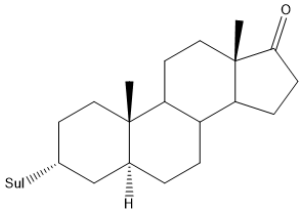
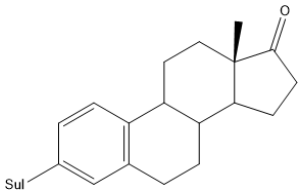
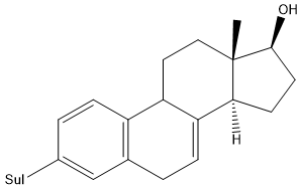
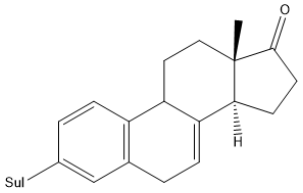
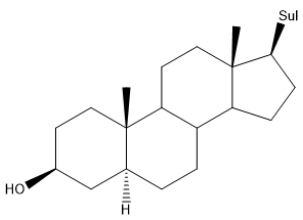
B-17G		(+) 463.2	121(32), **135(21)**, 173(24), 269(14), 287(13)	6.3
17β-Estradiol-3G		(+) 466.2	**194(15),** 273(18), 413(15)	5.8
5ααβ-diol-17G		(+) 486.2	141(28), 177(16), **257(19)**, 275(14), 451(10)	9.8

Table 2. AAS Glucuronides, structures, precursor ion, product ion(s), and retention time. Bolded numbers represent the quantifying ion and its collision energy.

AAS Sulfates	Structure	ESI Mode and Precursor Ion (m/z)	Product Ion (m/z) and Collision Energy (EV)	Retention Time (min)
T-17S		(-) 367.2	80(55), **97(38)**, 177(48), 337(39), **352(34)**	7.7
EpiT-17S		(-) 367.2	80(55), **97(38)**, 177(48), 337(39), 352(34)	8.3
DHEA-3S		(-) 367.2	80(55), **97(38)**, 177(48), 337(39), 352(34)	8.5

EpiA-3S		(-)369.2	80(55), **97(41)**, 173(20), 259(46), 287(49)	10.2
Etio-3S		(-)369.2	80(55), **97(41)**, 173(20), 259(46), 287(49)	10.7
A-3S		(-)369.2	80(55), **97(41)**, 173(20), 259(46), 287(49)	9.0
Estrone-3S		(-)349.1	**145(55)**, 159(54), 183(54), 253(50)	7.0
17β-Dihydroequilin-3S		(-) 349.1	181(55), 209(55), 211(52), **267(42)**	6.7
Equilin-3S		(-) 347.1	223(51), 239(40), **265(39)**, 267(30)	6.8
5αββ-diol-17S		(-) 371.2	80(55), **97(46)**, 285(55), 287(50), 289(47)	7.9

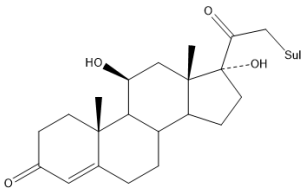
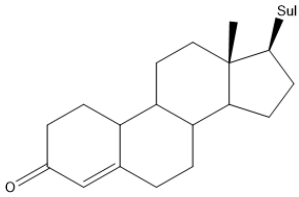
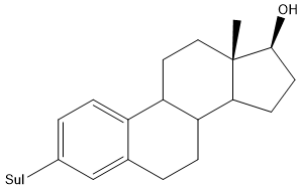
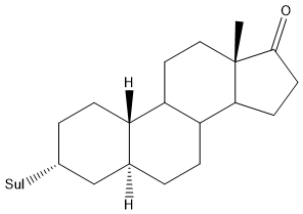
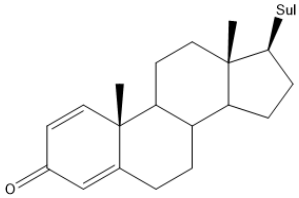
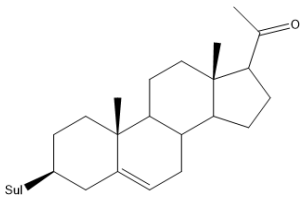
Cortisol-21S		(-) 441.2	81(40), **97(40)**, 282(43), 297(37)	6.4
N-17S		(-) 353.1	80(55), 97(42), **271(43)**	6.8
17β-Estradiol-3S		(-) 351.1	**145(55)**, 183(55), 239(55), 269(50), **271(36)**	6.8
19-NorA-3S		(-) 355.2	80(55), **97(38)**, 231(47), 271(54), 273(48)	9.2
B-17S		(-) 365.1	97(50), 177(42), **350(32)**	7.0
Preg-3S		(-) 395.2	80(55), **97(36)**, 123(51), 253.3(22), 337(35)	12

Table 3. AAS Sulfates, structure, precursor ion, product ion(s), and retention time. Bolded numbers represent the quantifying ion and its collision energy.

Internal Standards	Structure	ESI Mode and Precursor Ion (m/z)	Product Ion (m/z) and Collision Energy (EV)	Retention Time (min)

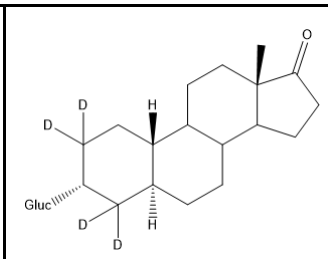
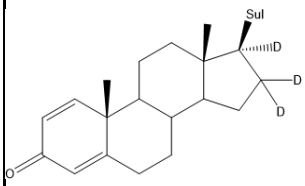
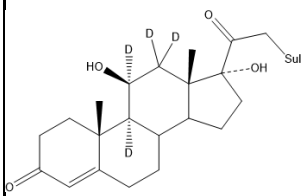
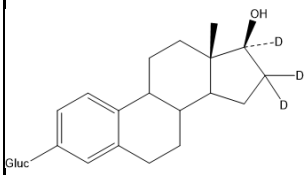
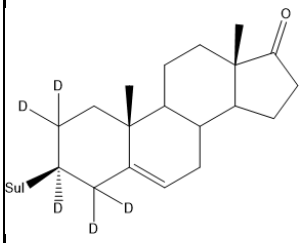
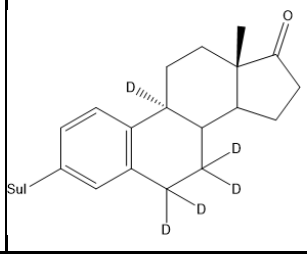
d_4-19-Norandrosterone-3-glucuronide		(+) 474.2	245(28), 263(19), 281(14), 421(13), **439(10)**	8.4
d_3-Boldenone 17-sulfate		(-) 368.1	**98(50)**, 353(32)	7.0
d_4-Cortisol 21-sulfate		(-) 445.2	81(44), **97(25)**, 285(32), 286(40), 301(39)	6.4
d_3-17β-Estradiol 3-glucuronide		(+) 469.3	194(15), **276(18)**, 416(15)	5.8
d_5-DHEA 3-sulfate		(-) 372.2	**98(36)**, 232(48), 356(40)	8.2
d_5-Estrone 3-sulfate		354.2	150(55), 164(54), 187(55), 258(51), **274(34)**	7.0

Table 4. List of Internal Standards, structures, precursor ion, product ion(s), and retention time. Bolded numbers represent the quantifying ion and its collision energy.

The results for the assessment of linearity, LOQ, LOD, and dilution accuracy are shown in **Table 5.** The limit of detection varied depending on the compound, with some detectable as low as 0.1 ng/mL.

Compound	Linearity		LOD (ng/mL)	LOQ (ng/mL)	Dilution 100 ng/mL (% Acc)
	Equation				
Etio-3G	-0.020866+0.0268931x	0.996	1	5	105
A-3G	-0.0274702+0.0789678x	0.999	0.5	1	104
EpiA-3G	-0.18274+0.085463x	0.997	1	5	102
DHEA-3G	-0.0265704+0.0391353x	0.997	0.5	5	97.7
T-17G	-0.0496266+0.012959x	0.995	0.75	5	103
EpiT-17G	0.00354783+0.0028081x	0.999	0.75	5	116
EpiN-17G	-0.0833818+0.0204101x	0.994	4	5	95.5
N-17G	-0.0150359+0.00486883x	0.992	0.5	5	110
17β-Estradiol-17G	0.483661+0.0235493x	0.998	0.1	5	94.7
19-NorA-3G	-0.101283+0.0832994x	0.995	2	5	103
19-NorEtio-3G	-0.096382+0.187752x	0.996	0.5	1	103
equilin-3G	0.00412315+0.0012482x	0.998	5	10	97.5
5αβα-diol-Bis-G	-0.0357677+0.0604039x	0.997	0.5	1	96.2
5ααβ-diol-17G	-0.0402522+0.0548129x	0.999	0.75	5	103
B-17G	-0.0192785+0.0233536x	0.998	0.75	1	107
17β-Estradiol-3G	-0.00035446+0.00644471x	0.997	0.75	5	94.2
T-17S	-0.0203354+0.0201971x	0.998	1	5	96.3
EpiT-17S	-0.00367292+0.0438012x	0.999	0.5	5	86.4
DHEA-3S	0.0185864+0.0387535x	0.998	0.1	5	93.0
EpiA-3S	-0.0183514+0.0276316x	0.999	0.75	5	97.4
Etio-3S	-0.0183059+0.0341989x	0.999	0.5	5	103
A-3S	-0.032017+0.0658617x	0.999	0.5	1	99.0
**estrone-3S	-0.00133624+0.00603555x	0.998	1	10	110
17β-Dihydroequilin-3S	-1.24037e-005+0.01458x	0.998	0.25	1	98.2
equilin-3S	-0.00258923+0.00744869x	0.999	0.5	1	94.2
5αββ-diol-17S	-0.00236816+0.0118343x	0.998	0.75	5	98.0
cortisol-21S	0.870223+0.207748x	0.998	0.1	5	93.3
N-17S	-0.000985015+0.00618255x	0.999	0.5	1	103
17β-Estradiol-3S	-0.00650649+0.056264x	0.999	0.25	1	97.5
19-NorA-3S	-0.00161806+0.0266925x	0.999	0.1	1	106
B-17S	-0.0749046+0.129825x	0.997	0.5	1	102
Preg-3S	-0.00413537+0.0084187x	0.999	0.5	1	108

Table 5. Linearity, LOD, LOQ, and Dilution accuracy of each analyte.
**Estrone-3S was diluted to 1000 ng/mL.

The linear range for each compound was evaluated with regression correlation coefficient, R^2, applying a 1/x weighting across all analytes. All compounds achieved a R^2 value greater than 0.99. The inter- and intra-day accuracy and precision was determined by analyzing 6 QC samples each at the low, mid, and high concentration level as shown in **Table 6**. The accuracy was calculated from the percent nominal from the theoretical value. The precision or CV was calculated by dividing the standard deviation by the mean of the QC samples at each concentration. Carryover was assessed by injecting a solvent blank using the re-dissolve solution after the highest calibrator and no compounds were observed in the solvent blank. As shown in **Table 7,** recovery for every compound was above 40%, determined by dividing the average peak area of 6 QC samples with the average peak area of 6 post-extraction spike samples at the low, mid, and high level. Matrix effects ratio was above 0.6 for all compounds, which was determined by dividing the average peak area of post-extraction spike samples with the average peak area of neat standards at the low, mid, and high QC levels.

Stability assessment as shown in **Table 8** was accomplished over two weeks and most analytes showed no significant degradation under the monitored conditions except cortisol-21S decreased by more than 20% after 3 freeze/thaw cycles**.**

A population study of 178 geldings, 163 mares, and 164 stallions was undertaken, and the results are summarized in **Table 9.** The minimum and the maximum concentration, as well as the number of positive identifications within each horse population are shown.

Compound	Day 1 - Precision			Day 1 - Accuracy			Day 2 - Precision			Day 2 – Accuracy			Day 3 - Precision			Day 3 – Accuracy			Inter-day Precision			Inter-day Accuracy		
	low	mid	high	low	mid	high	low	mid	high	low	mid	high	low	mid	high	low	mid	high	low	mid	high	low	mid	high
5αβα-diol-Bis-G	9.8	10.8	13.2	97.8	95.1	98.4	13	7.1	8.7	101	96	100	6.9	5.7	7.6	105	97.5	101	9.96	7.37	9.48	101	96.2	99.8
Equilin-3G	*	10	6.7	*	105	86.7	*	10.6	3.7	*	113	88.4	*	13.2	6.5	*	95.5	92.6	*	12.5	6.15	*	105	89.2
17β-Estradiol-3G	14.5	3.2	3.9	108	88.7	90.1	10.8	11.2	6.9	136	99.3	100	12.1	3.9	4.4	102	95.4	98.4	17.5	8.41	6.93	115	94.5	96.2
N-17G	*	2.6	4.6	*	90.8	88.2	*	13.8	6.1	*	93.9	94.8	*	6.7	6.5	*	100	103	*	9.43	8.55	*	94.9	95.3
17β-Estradiol-17G	*	8.2	2.6	106	88.1	93.6	*	13.5	6.7	*	88	98.8	*	4.3	9.1	*	100	96	*	10.8	6.74	*	92	96.1
B-17G	9.4	2.8	4.3	118	98.2	94.2	11.9	10.4	6.2	104	93.8	102	5.6	3.8	3.9	106	102	108	10.5	7.06	7.42	109	98	101
cortisol-21S	7.9	9.5	12.4	95.6	90.5	89.7	21.5	14.6	9.6	178	96.3	108	29.8	10.9	11.7	101	103	103	37.9	12.4	13	125	96.6	100
17β-Dihydroequilin-3S	5.7	5.9	7.1	112	101	100	11.1	4.5	4.7	102	102	103	7.2	4.9	8.3	96.9	104	109	9.89	5	7.6	104	102	104
T-17G	*	3.1	4.9	*	110	91.4	*	7.9	6.2	*	98.4	101	*	2.9	8.2	*	102	98.8	*	6.86	7.61	*	104	97.1
Equilin-3S	8.9	6.7	1.8	116	95.6	94	11.5	3.2	5	108	98.9	101	11.6	4.7	5	106	101	94.7	10.8	5.24	5.22	110	98.4	96.6
**estrone-3S	6	5.7	2	117	101	98.7	6.4	5.8	4.4	102	109	111	4.3	2.1	3.2	96	105	103	10.3	5.63	5.99	105	105	104
N-17S	11.5	7.4	8.4	98.2	110	101	14	11.1	5.4	95.7	93	89.2	31.1	11	5.3	88.4	106	107	19.3	11.9	9.76	94.1	103	99
17β-Estradiol-3S	5.6	5.9	0.6	119	96.8	95	4.9	3.1	4.8	97.7	102	102	7.1	1.9	5.1	94.9	104	99.9	12	4.71	4.98	104	101	99
B-17S	9.6	2.5	7.4	96.4	109	103	11.9	3.4	2.5	111	105	105	10.8	3.8	9.2	88.9	100	96.6	14.2	4.68	7.33	98.9	105	101
DHEA-3G	11.3	2.4	2.9	105	89.2	90	9.3	7.1	5.4	118	92.8	97.4	6	4.9	4.8	107	100	102	9.95	6.99	6.89	110	94	96.6
EpiN-17G	*	4	7.4	*	96.9	85.8	*	9	8.2	*	95.6	96.6	*	5.1	4.4	*	95.1	109	*	6.04	11.78	*	95.9	97.1
EpiA-3G	12.9	12	10.4	100	96.1	95.8	3.1	5.8	9.4	141	99.6	103	14.9	3.5	13.7	96.5	94	99.6	20.9	7.85	11.1	113	96.6	99.4
T-17S	0.6	3.3	8.4	95.3	103	96.4	6.9	8.2	4.8	117	92.9	105	8.8	3.6	9.9	115	95.5	91.3	12.3	6.53	9.31	109	97	97.4
19-NorEtio-3G	8.7	9.8	10.7	110	95.3	85.4	8.9	6.1	8.2	101	101	103	4.5	2.1	10.8	103	101	104	8.17	6.77	13	105	98.8	97.6
5αββ-diol-17S	14.4	3.5	4.9	113	95.4	94.1	9.5	8.6	2.5	101	95.7	109	12.8	4.5	8.2	98.8	100	97.9	13.1	6.03	8.31	106	97.1	100
EpiT-17S	12.2	4.6	13.9	99.8	96.4	91.1	11.6	6.3	8.4	102	98.8	107	7.4	3	14.7	107	96.1	84.2	10.4	4.73	15.4	103	97.1	94
19-NorA-3G	13.2	11.4	11.6	105	97.9	95.6	9	6.3	8.9	110	101	103	4	4	10.4	106	99	91.3	9.1	7.48	10.9	107	99.3	96.6
EpiT-17G	*	5.3	0.2	*	102	97.8	*	10.3	7.2	*	99.5	99.2	*	3.8	11	*	106	91.1	*	8.13	12.9	*	102	96
DHEA-3S	13.3	5.2	11.2	94	97.5	97.7	11	4.4	3.8	107	99.6	104	14.3	5	13.7	103	93.8	101	13.3	5.23	10.1	102	97	101
A-3S	7.2	4.9	9.1	97.4	98.2	94.6	3.1	4.6	4.2	105	99.2	107	8.6	2.8	7.7	98.5	94	94.6	6.99	4.66	9.09	100	97.1	98.8
19-NorA-3S	5.1	2.8	7.1	117	102	98.4	6.9	3.9	4.9	104	108	109	5.4	1.4	5.9	104	107	100	8.18	3.61	7.24	108	106	102
Etio-3G	5.7	4.1	8	118	86.3	82.2	9.4	10.1	6.8	98.1	99.4	101	8.8	4.4	13.4	118	112	98	11.5	12.7	13	111	99.3	93.6
5ααβ-diol-17G	5.1	4.1	2.4	113	88.9	91.1	6.7	6.2	7.7	102	94.4	103	6.2	5.5	7.3	93.7	99.5	98.7	9.61	6.93	8.1	103	94.3	97.7
A-3G	11.2	2.4	5.9	109	94.3	95.7	5.5	9.3	7.7	97.9	99.5	98.2	5.7	3.8	6.8	96.5	109	104	9.61	8.42	7.39	101	101	99.3
EpiA-3S	5.4	5.9	14.4	111	96.6	87.4	9	4.1	3.9	117	98.7	109	8	5.3	10.7	103	91.6	86.8	8.93	5.81	14.6	110	95.6	94.3
Etio-3S	7.3	4.4	3.9	101	94.9	105	10.4	7.1	3.1	103	101	112	4.9	4.6	6.3	107	90.5	103	7.76	7.06	5.81	104	95.5	107
Preg-3S	7.5	2.8	8.1	110	91.5	95.8	5.3	7.2	6.1	111	105	117	5.4	4.8	4.8	107	111	104	6.03	9.76	10.2	109	103	106

Table 6. accuracy and precision evaluated at each QC level (low, mid, high at 3 ng/mL, 30 ng/mL, 300 ng/mL, respectively)

*Compounds with limit of quantitation above QC low.

**QC levels for estrone-3S was 30 ng/mL, 300 ng/mL, and 3000 ng/mL.

Compound	Recovery (%)			Matrix Effect		
	QC low	QC mid	QC high	QC low	QC mid	QC high
5αβα-diol-Bis-G	68	64	64	0.603	0.582	0.623
Equilin-3G	*	82	45	*	0.624	0.697
17β-Estradiol-d3-3G	101	107	96	0.814	0.773	0.808
17β-Estradiol-3G	51	55	48	0.839	0.730	0.762
N-17G	*	58	47	*	0.747	0.803
17β-Estradiol-17G	49	68	56	2.40	0.801	0.722
B-17G	62	62	58	0.854	0.788	0.842
Cortisol-d4-21S	99	105	102	0.985	0.923	0.898
Cortisol-21S	93**	67	52	2.02**	0.981	0.943
17β-Dihydroequilin-3S	54	61	57	0.916	0.821	0.876
T-17G	*	54	48	*	0.803	0.835
Equilin-3S	57	57	46	0.881	0.838	0.887
Estrone-3S	53	55	52	0.928	0.876	0.871
Estrone-d5-3S	96	99	94	0.974	0.956	0.919
N-17S	54	58	47	0.845	0.839	0.875
17β-Estradiol-3S	54	60	52	0.938	0.846	0.867
B-17S	60	58	45	0.905	0.834	0.887
B-17S-d3	108	113	82	0.900	0.870	0.898
DHEA-3G	66	59	54	0.932	0.809	0.850
EpiN-17G	76	57	52	0.640	0.770	0.822
EpiA-3G	49	56	48	0.847	0.799	0.857
T-17S	58	57	43	0.901	0.836	0.878
19-NorEtio-3G	55	58	50	0.914	0.821	0.858
5αββ-diol-17S	60	63	48	0.984	0.830	0.867
EpiT-17S	60	59	42	0.992	0.822	0.889
DHEA-3S-d5	104	110	89	0.930	0.854	0.910
19-NorA-3G	64	56	45	0.885	0.855	0.858
19-NorA-3G-d4	110	104	88	0.980	0.903	0.905
EpiT-17G	*	72	50	*	0.818	0.878
DHEA-3S	66	55	45	1.21**	0.854	0.883
A-3S	53	57	47	0.919	0.822	0.881
19-NorA-3S	56	58	47	0.910	0.834	0.873
Etio-3G	59	62	51	0.884	0.805	0.883
5ααβ-diol-17G	50	56	51	0.950	0.832	0.880
A-3G	58	62	57	0.940	0.818	0.868
EpiA-3S	54	57	45	0.926	0.835	0.855
Etio-3S	53	52	49	0.909	0.852	0.868
Preg-3S	50	54	44	0.873	0.781	0.831

Table 7. Recovery and matrix effect at each QC level

*Limit of quantitation is above the QC low level.

**the compound is endogenous at low concentrations in blank urine, therefore adding to the spiked concentration and yields an amount higher than its neat standard.

Compound	Room Temperature (% Accuracy)		2-8°C (% Accuracy)			-20°C (% Accuracy)		
	t = 0h	t = 24h	t = 24h	t = 4 days	t = 7 days	t = 7 days	t = 14 days	Freeze/Thaw Cycle
5αβα-diol-Bis-G	96.3	103	96.1	89.3	108	109	127	131
equilin-3G	70.1	115	136	106	86.1	84.9	97.4	98.5
17β-Estradiol-3G	98.5	100	101	103	97.1	94.3	99.2	97.5
N-17G	101	99.0	104	99.4	110	112	99.9	102
17β-Estradiol-17G	103	101	99.3	111	91.6	98.0	61.7	60.3
B-17G	91.6	98.2	95.9	102	104	106	102	99.6
cortisol-21S	95.6	92.4	92.8	96.8	88.4	94.4	86.6	76.1
17β-Dihydroequilin-3S	90.6	104	108	102	99.9	99.7	99.8	99.7
T-17G	104	90.3	97.5	109	110	110	96.9	97.1
equilin-3S	93.7	101	105	103	101	103	97.1	96.6
**estrone-3S	97.6	103	109	98.9	112	105	103	99.7
N-17S	110	121	129	109	105	108	100	108
17β-Estradiol-3S	88.0	105	112	104	108	106	105	102
B-17S	95.1	94.6	96.6	104	101	102	104	101
DHEA-3G	96.5	98.0	96.7	103	101	105	99.2	97.5
EpiN-17G	91.9	97.9	94.6	101	97.9	100	99.9	98.6
EpiA-3G	87.6	95.1	96.3	99.3	95.1	99.0	97.0	96.4
T-17S	92.6	95.8	100	95.3	95.9	97.7	108	99.2
19NorEtio-3G	95.4	97.2	92.2	90.6	92.4	87.6	90.1	94.8
5αββ-diol-17S	90.4	102	100	95.0	96.0	98.9	97.6	92.2
EpiT-17S	86.2	92.5	94.9	91.3	95.3	95.6	103	98.3
19NorA-3G	94.5	98.5	96.0	95.6	92.3	94.7	92.9	95.7
EpiT-17G	84.8	95.5	99.8	106	83.6	78.7	106	106
DHEA-3S	94.7	94.5	98.9	88.5	91.8	92.3	91.3	89.4
A-3S	90.2	96.9	93.6	93.0	96.3	95.8	105	101
19NorA-3S	93.4	96.5	96.2	102	90.4	88.7	101	97.0
Etio-3G	102	99.8	98.9	96.6	96.3	98.5	94.7	93.2
5ααβ-diol-17G	98.3	100	97.3	110	102	104	100	100
A-3G	100	106	100	113	109	108	107	106
EpiA-3S	85.4	94.3	90.7	91.9	95.3	95.8	104	99.3
Etio-3S	90.0	97.8	95.6	88.8	88.9	92.8	96.3	85.2
Preg-3S	101	85.3	88.9	91.3	82.8	88.0	88.1	81.4

Table 8. Stability assessment over two weeks at various conditions at the mid QC level 30 ng/mL (Room Temperature, 2-8℃ refrigerator, -20℃ freezer).

**estrone-3S was at 300 ng/mL

Equine Urine			
Compound Name	**Gelding (178)**	**Mare (163)**	**Stallion (164)**
17β-Estradiol-3G	<LOQ-40.3 (21)	<LOQ-113 (44)	2.11-652 (162)
cortisol-21S	<LOQ-63.1 (148)	<LOQ-35.6 (145)	<LOQ-13.0 (107)
T-17G	NF	NF	<LOQ-7.91 (3)
equilin-3S	NF	<LOQ-1.09 (8)	<LOQ-14.3 (41)
estrone-3S	<LOQ-3080 (108)	<LOQ-3220 (159)	206-63600 (164)
N-17S	<LOQ (1)	<LOQ (1)	NF
17β-Estradiol-3S	<LOQ-212 (24)	<LOQ-147 (121)	19.0-6430 (160)
B-17S	<LOQ (1)	NF	<LOQ-2.11 (16)
DHEA-3G	<LOQ-278 (155)	<LOQ-394 (134)	<LOQ-88.9 (113)
EpiN-17G	NF	NF	<LOQ-11.9 (4)
EpiA-3G	<LOQ-29.4 (15)	<LOQ-31.0 (22)	<LOQ-76.3 (123)
T-17S	<LOQ-235 (79)	<LOQ-164 (110)	<LOQ-1120 (164)
5αββ-diol-17S	<LOQ-337 (51)	<LOQ-238 (101)	<LOQ-535 (161)
EpiT-17S	<LOQ-52.1 (139)	<LOQ-139 (124)	<LOQ-45.8 (127)
EpiT-17G	<LOQ-21.6 (5)	<LOQ-23.7 (5)	<LOQ-93.5 (15)
DHEA-3S	<LOQ-23.9 (142)	<LOQ-50.4 (143)	<LOQ-8.61 (79)
A-3S	<LOQ-28.4 (73)	<LOQ-17.3 (105)	<LOQ-94.9 (134)
19-NorA-3S	NF	NF	2.61-9.87 (7)
Etio-3G	<LOQ-195 (37)	<LOQ-256 (51)	<LOQ-223 (27)
5αββ-diol-17G	<LOQ-69.8 (17)	<LOQ-12.0 (20)	<LOQ-62.6 (27)
Etio-3S	<LOQ-<LOQ (2)	<LOQ-7.00 (2)	<LOQ-<LOQ (10)
Preg-3S	<LOQ-7.76 (20)	<LOQ-176 (21)	<LOQ-58.1 (20)

Table 9. Steroid profile of 505 urine samples categorized by gender. The minimum and the maximum detected values are shown, as well as the number of positive detections within the population. NF = not found.

5.0 Discussion

Anabolic steroids are one of the most abused classes of drugs in sports (WADA 2014). An anti-doping screening method for anabolic steroids is necessary to ensure the integrity of equine sports and the safety and welfare of horses. The methodology developed in this study can be utilized to enhance the current steroid screening approaches as some metabolites are not effectively hydrolyzed into their free steroid form. For example, while **β**-glucuronidase enzymes are effective at cleaving glucuronidated steroids, there is not a sulfatase currently available on the market that achieves the same effectiveness for hydrolyzing sulfo-conjugates as the **β**-glucuronidase to glucurono-conjugates (Waller et al, 2016; Gomes et al, 2009). Additionally, enzymatic hydrolysis of glucuronide conjugates and sulfate conjugates require extensive incubation periods which limits sample throughput.

Hence, alternative methods such as the direct detection and quantitation of steroid metabolites have the potential to give a more accurate estimate of AAS concentrations. In the last two decades, researchers have shifted their focus towards developing a method with simpler clean-up using LC-MS instead of GC-MS, although a complete steroid metabolite profile has yet to be established due to the lack of reference standards. Conjugated steroid reference standards are costly, not widely available, hard to synthesize in some cases, and are lacking some of the sulfo- or glucurono- position isomers. While the current methodology consists of 32 steroid conjugates, there is certainly room to grow the list of covered analytes as the availability of reference materials increases.

The first challenge to developing this method was achieving chromatographic separation between isomers and epimers. Many compounds used in this study are isomers (a pair of compounds with the same molecular formula but different arrangements) or epimers (a pair of

compounds with the same arrangement except a different configuration at a stereocenter), which have similar physiochemical properties and elute at a similar percentage of organic phase composition due to their similar polarities. Initial experiments adopted mobile phases that were also used for the current method for detection of AAS which consisted of 0.2% formic acid in acetonitrile and in water as its mobile phases. The initial HPLC column tested was a Kinetex EVO C18 Core-shell 2.6 µm (Phenomenex, Torrance, CA, USA). A constant ramping gradient of organic mobile phase was applied but separation under these conditions was poor, with co-eluting and unresolved peaks. Furthermore, most compounds eluted before reaching 50% of acetonitrile composition in the gradient. Methanol provided a better separation because it has a lower elution strength than acetonitrile, which makes it a better mobile phase for chromatographically separating structurally similar compounds with identical polarity. Under the same gradient condition, the run with methanol demonstrated a better separation of the overall total ion chromatogram. Next, the two buffers, ammonium formate and ammonium acetate, were assessed. Twenty mM of each buffer were added to the mobile phases, with the pH of ammonium formate at 3-4 and the pH of ammonium acetate at pH 5-6. Mobile phases using ammonium acetate buffer had better separations between closely eluting compounds and therefore was chosen as the optimal buffer. Ultimately, the concentration was reduced to 5 mM to reduce potential electrospray signal suppression. With a 5 mM ammonium acetate buffer in both methanol and water, it was discovered that most compounds elute between 45%-55% organic phase, with the exception of Preg-3S at above 85% organic. A shallower ramping rate for organic percentage over a longer period of run time was tested. The overall TIC had adequate separation over the entirety of the run (data not shown). However, the isomers and epimers continued to co-elute or not achieve baseline resolution. Accordingly, a HPLC column with smaller particle size (Phenomenex Kinetex EVO C18 Core-shell 2.1 x 100 mm, 1.7 µm) was

then evaluated to aid with the separation of such compounds. The results of using a column with smaller particle size showed a significant improvement in resolving co-eluting and better separation of closely eluted peaks. Furthermore, a series of experiments evaluating the column temperature and mobile phase flow rate was also conducted. The following conditions were tested: 350 µL/min at 45 ℃, 400 µL/min at 35 ℃, 450 µL/min at 45 ℃, 400 µL/min at 50 ℃) The combination that provided the best result was 45℃ at a flow rate of 0.350 mL/min.

During the method development, different HPLC systems and different mass spectrometers were also tested. Initially, a Thermo LTQ linear ion trap MS (Thermo Scientific, Waltham, MA, USA) was used. However, due to the design of a linear ion trap mass spectrometer, the collision induced dissociation (CID) has a ⅓ lower mass cut-off that prevents *m/z* less than a third of the parent ion from being observed on the spectra. The major fragmentation among the sulfoconjugates is *m/z* = 97 and *m/z* = 80. Since most AAS sulfates have *m/z* higher than 345, both qualifying product ions are excluded, making quantification of sulfate conjugates unattainable. On the other hand, the instrument has a pulsed Q collision-induced dissociation (PQD) that has the ability to detect lower *m/z* product ions which allows the product ions of sulfo-conjugates being observed on the PQD spectra. Although all target analytes can be detected using PQD, the single quadrupole instrument has limitations on selectivity especially with urine matrix interference and co-eluting compounds. It was concluded that a MS/MS instrument was necessary to achieve higher selectivity and better S/N ratio. The next instrument tested in this study was an Agilent 1100 series (Agilent, Santa Clara, CA, USA) HPLC coupled to a Thermo Finnigan TSQ Quantum Ultra (Thermo Scientific, Waltham, MA, USA). The Agilent 1100 series HPLC had a maximum pressure at 400 psi which limits the flow rate of the method especially while using a column with smaller diameter and particle size. Additionally, when the gradient reaches approximately 50% organic and 50% aqueous, the pressure of the column

reaches its maximum due to an exothermic reaction that occurs between water and methanol. Equally hindering the process, the MS has limits on the number of scan events possible for the method. Increasing the numbers of pre-set transitions increased the cycle time for MS scans and sacrificed the number of scans per compound. Since the method had 32 compounds plus 6 internal standards, the average scan rate per compound was approximately 4 - 6 over the chromatographic peak. Insufficient scans were obtained to give useful product-precursor ion relationships pertaining to ion ratios that distinguished isomer peaks from each other. An instrument with greater capability was required to further advance the method development. Thereafter, the method was transitioned to a Vanquish LC (Thermo Scientific, Waltham, MA, USA) system coupled with an Altis triple quadrupole mass spectrometer (Thermo Scientific, Waltham, MA, USA) for its rapid scan speed and high sensitivity.

Each compound was optimized for their product ion and collision energy through direct infusion. Sulfo-conjugated AAS were largely only detectable in negative mode, whereas many gluco-conjugates analytes were detectable in both negative and positive mode. In this method, all the AAS glucuronides were detected via positive mode ESI for higher sensitivity and specificity (Jäntti et al., 2010) . Following optimization of the chromatography and mass spectrometry, target analytes were spiked into the urine matrix and extracted with WAX cartridges to examine matrix interferences. The most selective product ion(s) were selected for quantification, which are shown in bold in **Table 2** and **Table 3** for glucuronides and sulfates, respectively**.** Most sulfo-conjugates only produced fragmentation at 97 m/z which comes from the sulfate moiety (Waller et al., 2016). Therefore, chromatographic separation for isomeric compounds became extremely important in distinguishing between analytes. For example, T-17S, EpiT-17S, DHEA-3S all have the same 367 m/z precursor ion and a major 97 m/z product ion. As seen in **Figure 5**, EpiT-17S and DHEA-3S eluted 0.2 minutes apart but were still able to achieve chromatographic separation using the

developed chromatography method. Similarly, A-3S, EpiA-3S, and Etio-3S all share the same m/z = 369 precursor ion as well as m/z = 97 product ion. Few other isomer and epimer pairs also exist in this method, but they were able to be qualitatively distinguished using both relative retention time and/or monitored product ion ratios.

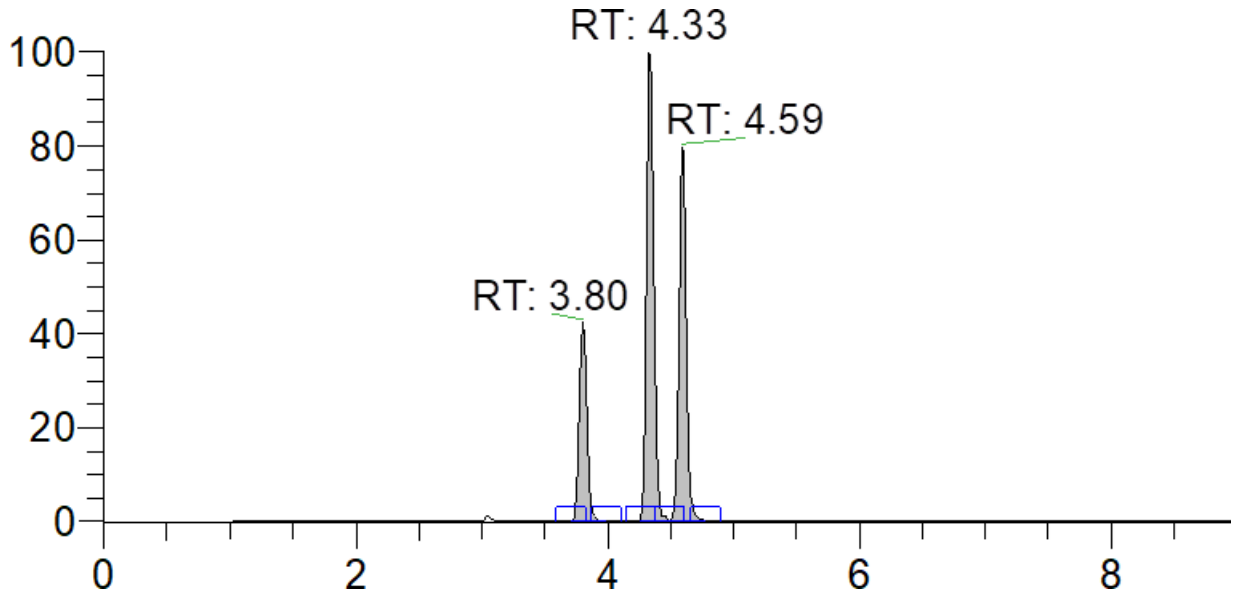


Figure 5. Chromatogram of precursor ion m/z = 367. From left to right: T-17S, EpiT-17S, DHEA-3S

While MS/MS spectra produced by a triple quadrupole mass spectrometer are highly informative, proper identification of specific product ions is key to achieving the highest sensitivity and selectivity in biological samples. Equilin-3G was heavily impacted by the horse urine matrix with its first two product ions, m/z = 269 and m/z = 251, showing interference from the matrix (**Figure 6**). Therefore, 286 m/z was the only specific quantifying ion that is relatively unaffected by the matrix. In comparison, a neat standard of equilin-3G in methanol is shown in **Figure 7.** Unlike in the blank urine, no peak interferences were observed in other transitions of the neat standard. However, 286 m/z is not an abundant product ion which is why the LOD and LOQ for Equilin-3G was much higher than other analytes.

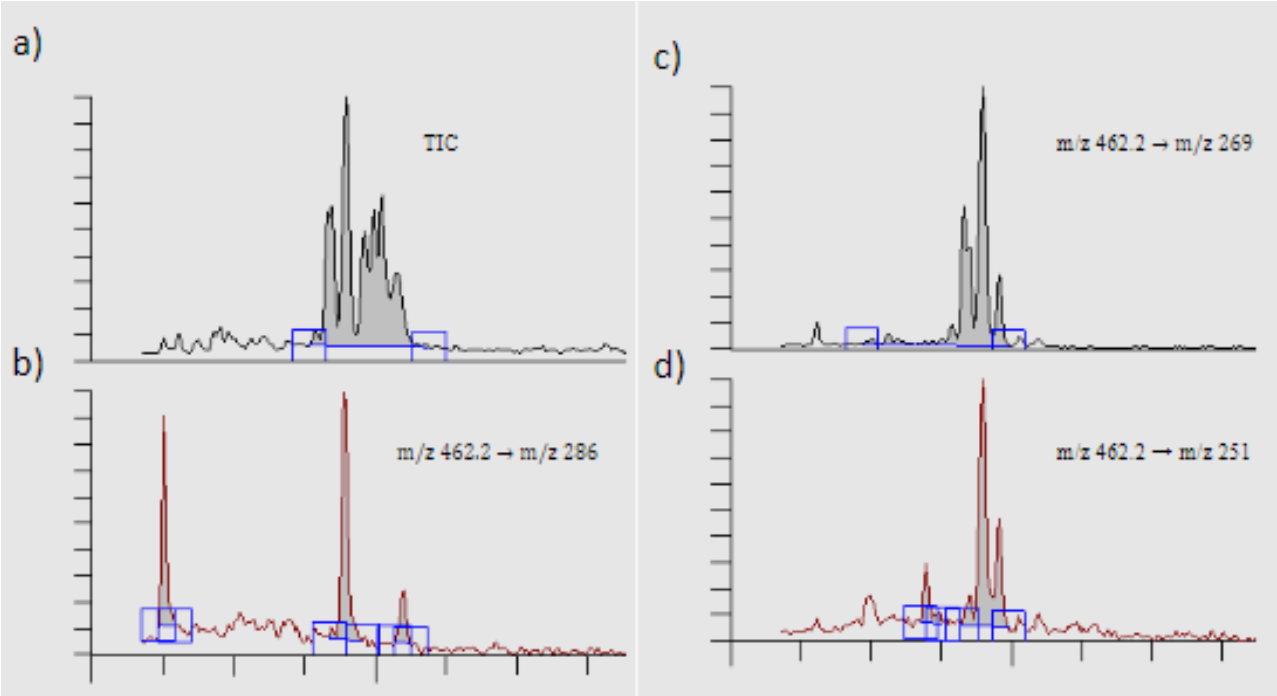


Figure 6. Equilin-3G in blank urine matrix. a) the total ion chromatogram for Equilin-3G. b) the extracted ion chromatogram for product ion m/z = 286, also the quantifying ion. c) the extracted ion chromatogram for product ion m/z = 269. d) the extracted ion chromatogram for product ion m/z = 251.

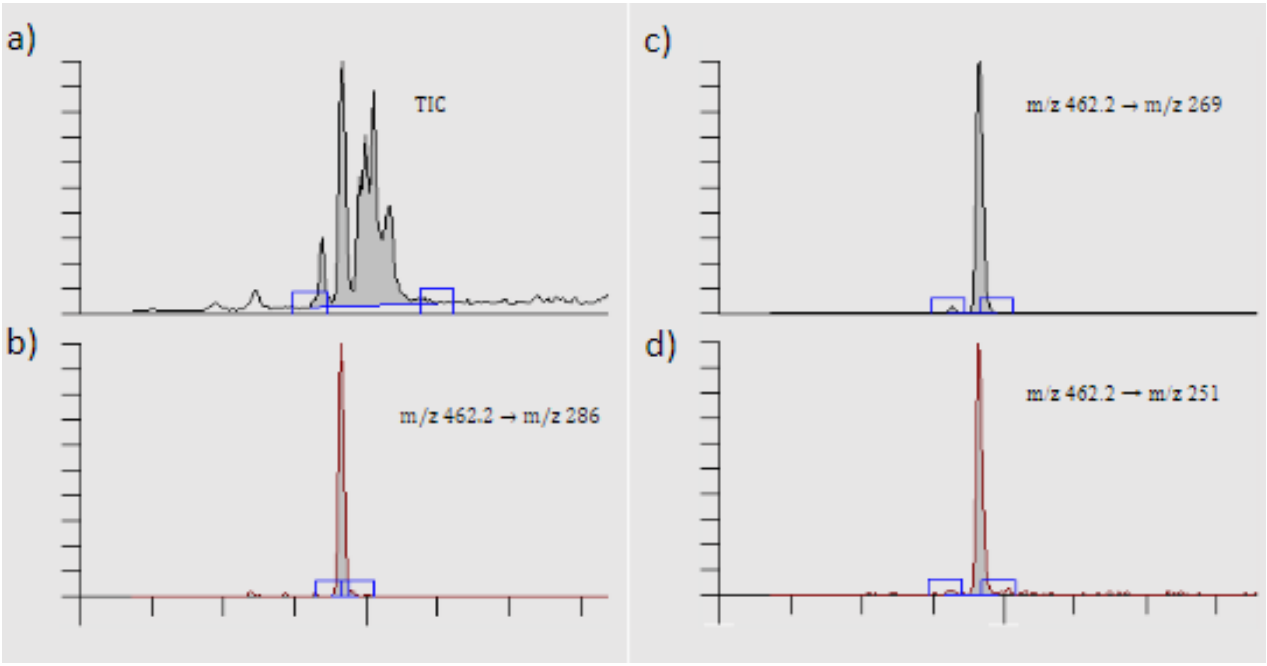


Figure 7. Equilin-3G neat standard in methanol. a) the total ion chromatogram for Equilin-3G. b) the extracted ion chromatogram for product ion m/z = 286, also the quantifying ion. c) the extracted ion chromatogram for product ion m/z = 269. d) the extracted ion chromatogram for product ion m/z = 251.

Per the IFHA International Agreement on Breeding, Racing, and Wagering, there are established thresholds for several compounds monitored: 0.015 μg/mL of free and conjugated boldenone in urine for male horses other than gelding, 0.045 μg/mL for free and conjugated 5α-estrane-3β,17α-diol in urine for male horses other than gelding, and 0.02 μg/mL of free and conjugated testosterone in gelding urine or 0.055 μg/mL in mares [4]. This method was validated for the concentration range from 1 ng/mL to 500 ng/mL for most analytes, which covers the range at which illegal AAS use would have been found. As shown in **Table 5,** the limits of detection for most analytes are at or below 1 ng/mL, with the exception of a few analytes due to matrix interference. The limits of quantitation for all the analytes are below currently established thresholds for detecting AAS abuse. In (Doué et al., 2015), glucuronide and sulfate steroids in bovine urine were detected by using ultra high performance supercritical-fluid chromatography – tandem mass spectrometry (UHPSFC-MS/MS). According to (Douéet al., 2015) , the limit of quantitation for the glucuronide conjugates Etio-3G, DHEA-3G, EpiA-3G, EpiT-17G, T-17G were 0.5 ng/mL and the LOQ for sulfates A-3S, EpiA-3S, DHEA-3S, EpiT-17S, T-17, and 5ααβ-diol-17S were 0.1 ng/mL. Their LOQ values obtained from using UHPSFC-MS/MS were much lower than LOQ established using the method developed here. They noted that at low concentration spiking (1 ng/mL), UHPSFC-MS/MS analysis provided S/N better than 10, whereas UHPLC-MS/MS had poorer performance. They also mentioned that in LC-MS, the chromatographic conditions strongly affected ionization efficiency, whereas the use of make-up solvent in UHPSFC-MS/MS can override that effect and enhance ionization efficiency. In conclusion, choice of instrument can greatly affect parameters such as sensitivity.

The developed method should achieve adequate accuracy quantitating analytes at much higher concentrations that exceed the abuse threshold if dilution of the sample was allowed. A

dilution study was conducted with (n=6) replicates and the results are shown in **Table 5.** All analytes obtained values close to the target value.

The extraction recoveries of the analytes were determined by comparing the ratio of the average area count of samples obtained from a pre-extraction spiked urine to that of a blank urine extract spiked after SPE with the same amount of the analytes. The recoveries at the mid-QC level (30 ng/mL) ranged from 52% to 82% using weak anion exchange SPE cartridges. Matrix effect for most analytes were around 0.7 and 0.8, with the exception of 5αβα-diol-Bis-G at 0.58 and equilin-3G at 0.62. Recovery and matrix effects for each QC level are shown in **Table 7.** It should be noted that cortisol-21S at the lowest QC level had a higher recovery and an observed matrix effect greater than the mid and high QC levels. This is likely attributed to the presence of this compound in our negative control matrix. In a previous study (Pu et al, 2004), an C18 SPE column and diethylaminopropyl (DEA) cartridges were used to extract boldenone glucuronide and sulfate. The authors managed to recover 53%-79% of boldenone sulfate but only 22% of boldenone glucuronide. The DEA cartridges were found suitable for extracting sulfate but were not acceptable for glucuronide conjugates. Oasis HLB and PBA cartridges were also tested, but neither improved boldenone glucuronide recovery, nor did NH_2 or SAX cartridges. In other published methods (Wilde et al, 2020), steroid stripped urine obtained from a child was used for the validation assessment including matrix effect. The average area of internal standards in the urines samples was compared to the area in the water sample for matrix effect calculation. Using a dilute-and-shoot method, the authors were able to record the matrix effect from -0.9%±3.0% to 26.0%±10.7%.

Horse urine is a complex matrix that is both protein rich and high in viscosity, especially following intensive exercise when the protein sparing capacity of the kidneys is reduced (Schott

et al, 1995). Therefore, extensive sample clean-up prior to instrumental analysis is required Protein crash pretreatment was necessary to remove large protein aggregates visibly present in horse urine which helped reduce clogging in SPE elution steps. Many protein precipitation/preparation methods have been tested by researchers working with horse serum or urine. These methods include acetonitrile precipitation, proteinase K hydrolysis, membrane filtration, and sample dilution with water. In an evaluation study of these preparatory methods, (Stojiljkovic et al, 2014) the authors concluded that acetonitrile precipitation ("crash") was the most efficient method when considering number of detected signals, their intensity and their detection repeatability. In this project, organic solvent crash (acetonitrile and methanol), acid crash and ammonium sulfate methods were all evaluated. Similar to (Stojiljkovic et al, 2014), the acetonitrile crash was found to be the most effective at removing excess protein from the urine samples. As a result, multiple sample transferring steps were involved in the pre-treatment which could have been one factor that affected analyte recovery.

Similarly, because of the complex nature of horse urine, the decision was made to use charcoal stripped horse urine as the medium for studying validation parameters so that it can best replicate real sample complications and matrix interferences. Charcoal stripping was used on cleared post-race gelding urine since geldings produced less anabolic steroids endogenously compared to stallions and mares. Charcoal stripping method was able to reduce the amount of endogenous conjugated steroids in the urine samples but did not completely remove some steroids with higher concentrations such as cortisol-21S and DHEA-3S.

During the process of discovering a working sample clean-up method, two types of SPE columns were tested, the Agilent Nexus Bond-Elut column and the Oasis WAX column. Initially, all experiments were conducted using negative control urine and no issues were

encountered with viscosity or high protein content. Therefore, the Nexus column was chosen as the best option as it can capture analytes of interest as well as requiring only a very simple procedure. There were several pros to the Nexus columns. First, it used a polymeric sorbent which had low specificity, allowing both free and conjugated steroids to be captured. This would be beneficial for getting an accurate concentration of both free and conjugated steroids from submitted samples from one extraction. Second, there were minimal steps involved, reducing the time and labor needed to perform the extraction. Lastly, Nexus columns had large particle sizes that allowed viscous samples, such as horse urine, to pass easily. Protein precipitation is unnecessary when using Nexus columns. However, the cons were also very apparent. With the simple wash step and a non-selective sorbent, it does not clean up the samples well enough. Dried down extracts were often dirty: particles could be seen in the re-dissolved extracts. As real post-race samples were introduced, the Nexus column could not clean up the samples sufficiently, leaving particles in the eluate and leaving residue in the extract. Several modifications, such as adding additional wash steps, were attempted to rectify the problem without success. As a result, a pre-extraction clean-up method was developed and, in the process, WAX columns were found to be equally effective for all analytes. The WAX column was ultimately chosen to replace the Nexus column as it provided better clean up even though, the Nexus cartridge overall produced a higher signal intensity for most analytes. Experiments have shown that data obtained from using Oasis WAX cartridge results in lower background interference and a higher S/N ratio. Thus, Oasis WAX cartridge was determined to be the optimal method for sample clean-up. Therefore, all of the validation experiments and population study used the Oasis WAX cartridge as the SPE method.

Selection of appropriate internal standards is important when designing a quantitative method to account for various biases that may occur during sample preparation and instrumental analysis. Stable isotope labeled internal standards used in this method included d_4-19-norandrosterone 3-glucuronide, d_3-boldenone 17-sulfate, d_4-cortisol 21-sulfate, d_3-17β-estradiol 3-glucuronide, d_5-DHEA 3-sulfate, and d_5-estrone 3-sulfate. Since the blank urine still had detectable amounts of DHEA-3S and cortisol-3S, it was crucial to have the internal standards for these compounds for an accurate measurement when constructing the calibration curve as the IS behaves similarly to their non-deuterated standards. For analytes without an identical IS, structural similarity and response intensities were considered when choosing IS.

Compound stability is an important factor to evaluate when determining an analytical approach. Considering turnaround time, storing samples in the right condition and analyzing them before degradation occurs will greatly increase accuracy of the results. Luckily, the anabolic steroid metabolites in this method were stable over a 2-week period under various conditions, with the exception of cortisol-21S which degraded by more than 20% after one week shown in Table 8. In prior studies (Pozo et al., 2007), the stability of free steroids was assessed over the course of one week. The authors reported that extracted samples were stable over one week in refrigerated conditions with deviations lower than 20%. Similarly, freeze-thawed samples were also found to be stable after 3 cycles with less than 20% deviation. Their findings on stability were consistent with what was observed with the method developed here. Overall, no significant degradation was observed for the anabolic steroids metabolites evaluated.

Preliminary data from a population study (**Table 9**) suggests that there are large differences between individual horses regardless of gonadal status. For example, T-17S detection in stallions ranged from below the LOQ (5 ng/mL) to 1120 ng/mL, a difference of more than 220% between

the highest detected amount and the lowest detected amount. With such large inter-individual differences, it would be difficult to establish a threshold value solely on the concentration of one analyte. Perhaps a ratio between analytes could be used to normalize the scale of concentrations, for those phase II metabolites that arise from the same parent compound. For example, estradiol and estrone are metabolized via the same pathways which explains why a horse with high levels of estrone-3S also has high concentrations of 17β-estradiol-3S and 17β-estradiol-3G. The population data also suggests there are sex-specific steroid metabolite production, as some metabolites are only found in one sex. Overall, stallions had the most abundant steroid profile and higher concentration of detectable steroid metabolites.

6.0 Conclusion

A method for the simultaneous detection and quantitation of 32 anabolic androgenic steroid metabolites was developed and validated using liquid chromatography - mass spectrometry. Samples underwent organic protein crash pretreatment to eliminate the majority of the proteins in horse urine and weak anion exchange SPE was used for further purification. This method was validated for the concentration range 1-500 ng/mL for most compounds and 10-5000 ng/mL for estrone-3S, which includes the concentration at which therapeutic thresholds were established by the IFHA. Compound stability suggests that samples can be stored at 2-8℃ (refrigerator) or -20℃ (freezer) for up to a week before degradation occurs. As more and more reference standards become available in the future, there is certainly room to expand the method.

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