DOPING CONTROL IN HORSERACING: PHARMACOKINETICS AND METABOLIC STUDIES OF PROHIBITED SUBSTANCES/DRUGS IN HORSES

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Abstract

Regulatory authorities have been employing advanced technologies to tackle the doping trends observed in human and animal sports. In both equestrian sports and the horseracing industry, the fight against the misuse of prohibited substances (PS) and/or drugs is crucial, as these could distort horse performance and impact competition outcomes. Furthermore, certain medications may be misused to mask illness or injury, posing serious risks to the health and welfare of horses. To effectively manage this issue, it is essential to have a comprehensive understanding of drug metabolism and pharmacokinetics (DMPK) in horses, which is critical for identifying the proper targets for effective control. Unfortunately, the metabolic fate of many drugs in horses remains unclear, highlighting the need for extensive DMPK studies on substances with potential doping effects. Biotransformations of several potential doping agents, including estra-4,9-diene-3,17-dione, 2-hydroxyethyl salicylate and ranitidine, were studied and reported in this thesis.

Anabolic androgenic steroids (AASs) are one of the prevalent classes of drug with high doping potential in equine sports. Estra-4,9-diene-3,17-dione (dienedione), an AAS marketed as a dietary supplement for bodybuilding, is prohibited in both human and equine sports due to its potential performance enhancing effect. With the rare presence of 4,9-diene configuration in endogenous steroids, dienedione has been considered as a synthetic AAS. However, the recurring detection of dienedione in entire male horse urine samples has led to the investigation of its possible endogenous nature and conjugation in entire male horses. Whilst dienedione remains exogenous in castrated horses, it could be reported with zero-tolerance once detected. To control the illicit use of dienedione in castrated horses, it is essential to study its *in vivo* metabolism and

elimination. In chapter 2, the potential endogenous nature of dienedione in entire male horses was studied and confirmed. Dienedione-3-glucuronide was further proposed to be the major form of dienedione in entire male horse. An in-house threshold at 30 ng/mL of free and glucuronide-conjugated dienedione in entire male horse urine was established, with a risk factor of 1 in 14,269 (with a degree of freedom of 173). In chapter 3, the *in vivo* metabolites detected in castrated horse after dienedione administration included 17-hydroxyestra-4,9-dien-3-one (M1a and M1b), hydroxylated dienedione (M2a, M2b, M3a, M3b, M4, M5) and hydroxylated M1 (M6a, M6b, M7a, M7b, M8a and M8b), formed from hydroxylation and reduction of dienedione were presented. Metabolite M3a and M3b were identified as the appropriate target to monitor misuse of dienedione, as it gave the longest detection time. They could be detected for up to 2–5 days in urine and 0.4–4 days in plasma after the horses were given the substance.

2-Hydroxyethyl salicylate (2HES), a non-steroidal anti-inflammatory drug (NSAID), is commonly used to treat musculoskeletal injuries and inflammation in both humans and horses. Its misuse could affect the performance of horses and mask injuries. In horseracing, its use is considered an adverse finding when detected in competition. The metabolism of 2HES has not been reported in either humans or horses, leaving its metabolic fate unknown. In chapter 4, the proposed *in vivo* metabolites including glucuronide-conjugated 2HES (2HES-Glu) and sulfate-conjugated 2HES (2HES-SO4) resulting from phase II conjugation, likely at the hydroxyethyl group, and salicylic acid (SA) formed by the hydrolysis of 2HES were described. The parent drug, 2HES, was identified as the most suitable target for monitoring its potential misuse, as it was detectable in hydrolysed urine for up to 10 days and in plasma for up to 16 hours. Since the concentration of SA in post-administration urine and plasma samples did not exceed

the corresponding international thresholds, monitoring SA levels is not an effective indicator of 2HES exposure.

Ranitidine, a histamine H2-receptor antagonist, was widely used to treat gastric ulcers in horses. Therapeutic substances like ranitidine are also required to be controlled under racing regulations to ensure a level playing field.

Hence, in chapter 5, the *in vivo* metabolism and elimination of ranitidine in horses were studied to support its effective control, with the potential to establish a screening limit that enables laboratories to report this therapeutic agent consistently. The *in vivo* metabolites, namely ranitidine-S-oxide (M1), ranitidine-N-oxide (M2), desmethylranitidine (M3a/b), and a furoic acid analogue of ranitidine (M4), resulting from oxidation, demethylation, and oxidative deamination, were identified. To monitor the potential misuse of ranitidine in horses, elimination profiles for urinary and plasma ranitidine were established. Free ranitidine was detectable for up to 8 days in urine and up to 72 hours in plasma. Additionally, ranitidine-S-oxide and ranitidine-N-oxide were also detectable for 8 days, making them potential screening targets alongside the parent drug to confirm that ranitidine has gone through the horse's body. With the intended detection window, the authorities could adopt a suitable screening cut-off for its control.

Research Publications

Journal papers

Ho, H. S. M., Ho, E. N. M., & Wong, W. T. (2024). Endogenous nature of estra-4,9-diene-3,17-dione in entire male horses. *Drug Test Anal*. https://doi.org/10.1002/dta.3685

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Lists of Abbreviations

Abbreviation	Full form
EHS	2-Ethylhexyl salicylate
2HES	2-Hydroxyethyl salicylate
2HES-Glu	2-Hydroxyethyl salicylate glucuronide
2HES-SO_4	2-Hydroxyethyl salicylate sulfate
amu	Atomic mass unit
AAS	Anabolic androgenic steroid
AR	Androgen receptor
AX	Anion exchange
APCI	Atmospheric pressure chemical ionisation
API	Atmospheric pressure ionisation
APPI	Atmospheric pressure photoionisation
AORC	Association of Official Racing Chemists
AGC™	Automatic gain control
β-glucuronidase	Beta-glucuronidase
CI	Chemical ionisation
CX	Cation exchange
CID	Collision-induced dissociation
r	Correlation coefficient
Da	Dalton
DNA	Deoxyribonucleic acid
DMPK	Drug metabolism and pharmacokinetics
E.coli	*Escherichia coli*

EI	Election impact ionisation
eV	Electron volt
ESI	Electrospray ionisation
Dienedione	Estra-4,9-diene-3,17-dione
FEI	Fédération Equestre Internationale
FWHM	Full-width at half maximum
GC-MS	Gas chromatography-mass spectrometry
r^2	Goodness of fit
HESI-II	Heated-electrospray ionisation
HCD	Higher collision energy dissociation
H_2-receptor antagonist	Histamine type 2 receptor antagonist
IGF-1	Insulin-like growth factor-1
IS	Internal standard
IABRW	International Agreement on Breeding, Racing and Wagering
IFHA	International Federation of Horseracing Authorities
ISLs	International screening limits
IUPAC	International Union of Pure and Applied Chemistry
kV	Kilovolt
LoD	Limit of detection
LoQ	Limit of quantification
LC-HRMS	Liquid chromatography-high resolution mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid-chromatography-tandem mass spectrometry technique
LLE	Liquid-liquid extraction

m/z	Mass-to-charge ratio
mTorr	Millitorr
NSAIDs	Non-steroidal anti-inflammatory drugs
NCE	Normalised collision energy
NMR	Nuclear magnetic resonance
PAR	Peak area ratio
PPARδ	Peroxisome proliferator activated receptor δ
ppm	Parts per Million
PS	Prohibited substance
QC	Quality control
RF	Radio frequency
RRT	Relative retention times
RSD	Relative standard deviation
SA	Salicylic acid
SRM	Selected-reaction monitoring
SARMs	Selective androgen receptor modulators
SERMS	Selective estrogen receptor modulators
SIM	Single ion monitoring
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
SPE	Solid-phase extraction
SD	Standard deviation
SAX	Strong anion exchange
SCX	Strong cation exchange
SLE	Supported-liquid extraction

MS/MS	Tandem quadrupole system
TBME	*tert*-butyl methyl ether
UPLC	Ultra-performance liquid chromatography
UPLC-MS/MS	Ultra-performance liquid chromatography tandem mass spectrometry
WAX	Weak anion exchange
WCX	Weak cation exchange
WADA	World Anti-Doping Agency
y/o	Years old

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Chapter 1. Introduction

1.1 Regulation on drugs in equine sports – horseracing

1.1.1 Doping control rules on equine sports

Sport authorities have been dedicated to controlling doping both in and out of competition by using advanced combative screening methods over the past decades. Doping refers to the administration of prohibited substances or the use of prohibited methods to cause an action or effect or both on the performance of athletes and/or animals in sports. The World Anti-Doping Agency (WADA) has developed a universal code of standards to ensure regulation consistency across all sports in categories such as testing and investigations, prohibited list, and results management. The fairness of international equestrian sports, as well as the welfare and safety of horses, is safeguarded by authorities including The Fédération Equestre Internationale (FEI) and the International Federation of Horseracing Authorities (IFHA).

The framework for doping-free horseracing activities is set out by the IFHA and adopted by horseracing authorities including The Hong Kong Jockey Club Racing Laboratory in Hong Kong for enforcement. IFHA coordinates and harmonizes rules on horse breeding, racing and wagering which are stated in the *International Agreement on Breeding, Racing and Wagering (IABRW)* (IFHA, 2024a), which i) bans the presence of drugs and/or their metabolites, and ii) controls medications that have legitimate therapeutic use at the time of racing. According to Article 6A in the *IABRW*, a PS is defined as any substance that may trigger an action or an effect or both within one or more than one mammalian body systems: nervous system, cardiovascular system, respiratory system, digestive system, urinary system, reproductive system, musculoskeletal system, blood system, immune system and endocrine system. Both

equestrian sport and the horseracing industry demand effective drug screening methods for anti-doping, as prohibited substances (PS) that either enhance or impair a horse's performance can significantly impact the integrity of the competition and public confidence in the sport.

1.1.2 Exogenous drugs including therapeutic substances

Prohibited substances can be classified into exogenous and endogenous substances. Exogenous drugs are defined as synthetic drugs and medications that are not naturally present in the mammalian body. According to Article 6E of the *IABRW*, certain classes of exogenous drugs including i) non-approved substances, ii) peptide hormones, growth factors and related substances and iii) hormones and metabolic modulators, which were listed in **Table 1.1**, should not be given to racehorses at any time in their career (IFHA, 2024b).

Table 1.1. Banned substances at all times for racehorses

Non-approved substances
Anabolic agents (a) anabolic androgenic steroids (AAS) (b) selective androgen receptor modulators (SARMs) (c) beta-2 agonists
Peptide hormones, growth factors and related substances (a) erythropoiesis-stimulating agents (b) growth hormones and growth hormones releasing factors, insulin-like growth

factor-1 (IGF-1), and other growth factors (c) synthetic proteins and peptides and synthetic analogues of endogenous proteins and peptides not registered for medical or veterinary use
Hormones and metabolic modulators (a) aromatase inhibitors (b) selective estrogen receptor modulators (SERMS) and other anti-estrogenic substances (c) agents modifying myostatin function (d) insulins (e) peroxisome proliferator activated receptor δ (PPARδ) agonists (f) AMPK activators

Some therapeutic substances are permitted for the treatment of racehorses but are controlled under International Screening Limits (ISLs), for instance meloxicam, flunixin, omeprazole, etc. The concentration of any detected therapeutic substance should not exceed its respective ISL in urine or plasma; otherwise, it will be considered as a violation.

1.1.3 Endogenous substances

Endogenous substances are present naturally in the mammalian body, for example testosterone, nandrolone and estrone. The misuse of endogenous substances is controlled by threshold values as described in Article 6A of the *IABRW* (**Table 1.2**), by which the level determined in a sample exceeds the respective threshold may constitute a violation and be considered as positive (IFHA, 2024c).

Table 1.2. Threshold list on *IABRW*

Arsenic	0.3 µg total arsenic/mL in urine
Boldenone	0.015 µg free and conjugated boldenone/mL urine from male horses (other than castrated horses)
Carbon dioxide	36 mmol available carbon dioxide/L in plasma
Cobalt	- 0.1 µg total cobalt/mL in urine - 0.025 µg total cobalt (free and protein bound)/mL in plasma
Dimethyl sulfoxide	- 15 µg dimethyl sulfoxide/mL in urine, or - 1 µg dimethyl sulfoxide/mL in plasma
Estranediol in male horses (other than castrated horses)	0.045 µg free and glucuroconjugated 5α-estrane-3β,17α-diol/mL in urine when, at screening stage, the free and glucuroconjugated 5α-estrane-3β,17α-diol exceeds the free and glucuroconjugated 5(10)-estrene-3β,17α-diol in urine
Hydrocortisone	1 µg hydrocortisone/mL in urine
Methoxytyramine	4 µg free and conjugated 3-methoxytyramine/mL in urine
Salicylic acid (SA)	- 750 µg salicylic acid/mL in urine, or - 6.5 µg salicylic acid/mL in plasma
Testosterone	- 0.02 µg free and conjugated testosterone/mL in urine from castrated horses, when at screening stage, free and conjugated testosterone > 5 times the free and conjugated epi-testosterone, or

	- 100 pg free testosterone/mL in plasma from castrated horses, fillies and mares, or - 0.055 µg free and conjugated testosterone/mL in urine from fillies and mares
Prednisolone	0.01 µg free prednisolone/mL in urine

1.2 Approaches in studying DMPK in equine

The study of DMPK provides extensive details on the overall disposition rate and form of a drug candidate. Similar to humans, drugs would be metabolised and excreted after administration in horses. The aim of drug metabolism is to facilitate its elimination in the body, by undergoing different biotransformations such as oxidation, reduction, hydrolysis, hydroxylation, conjugation, condensation, or isomerisation. Despite being studied in humans, metabolism and pharmacokinetics of drugs between horses and humans could considerably vary due to factors such as different diet (Budiansky, 1998) and capacity in altering blood volume (Stewart and McKenzie, 2002). In comparison to human spleen, the horse spleen is larger in size which can alter the red blood cell content by up to 50 %. Therefore, the blood volume in horses can have massive changes depending on their strength of exercise, which may potentially influence the pharmacokinetics of drugs (Scarth et al., 2011).

1.2.1 *In vitro* and *in vivo* metabolic studies

Metabolism of drugs, such as AASs and NSAIDs, occurs in two phases. Phase I metabolism involves chemical reactions such as oxidation, reduction and hydroxylation (Le, 2022) to form phase I metabolites, whereas phase II metabolism involves

conjugations of parent drug or its phase I metabolites to different endogenous polar compounds, namely glucuronic acid, sulfate, glutathione and/or amino acid, to increase the water solubility of drugs for better excretion (Iyanagi, 2007). The direct detection of phase I and II metabolites is crucial in doping control analyses as they serve as the proof of the PS being administered and passed through the horse body (Wong et al., 2016). Alternatively, an indirect approach by using deconjugation techniques may be taken to study phase II conjugation of the drug. The two major conjugation pathways existed in equine were determined to be glucuronidation and sulfation, which have been extensively studied in steroids (Dumasia, 2003; Mckinney et al., 2004; Salomonsson et al., 2006; Teale and Houghton, 2010). Deconjugation of glucuronide conjugates can be accomplished by enzyme hydrolysis using β-glucuronidase as shown in **Figure 1.1** (McCarter and Withers, 1994), whereas deconjugation of sulfate conjugates can be achieved by solvolysis using concentrated sulfuric acid or methanolysis using acidified methanol (Gomes et al., 2009; Tang and Crone, 1989).

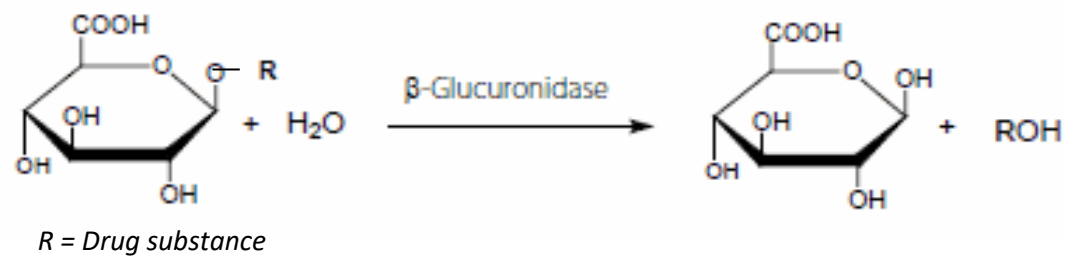

Figure 1.1. General beta-glucuronidase hydrolysis reaction

The use of *in vitro* models with homogenised horse liver tissues, such as S9 fractions and microsomes, is common in studying phase I and II metabolisms in a wide range of drugs (Choi et al., 2018; Leung et al., 2013; Scarth et al., 2010a; So et al., 2021; Wong et al., 2011; Wong et al., 2016). It is particularly useful to identify possible drug metabolites without animal administration studies which demand much more time and resources. In addition, the relatively simple and clean matrix from *in vitro* studies facilitates the identification of metabolites and these metabolites can serve as reference materials for further *in vivo* studies as well as for future doping control monitoring. Despite the advantages of *in vitro* metabolism studies, it could not provide the detection windows and relative quantities for the metabolites. Therefore, *in vivo* studies remained important in DMPK studies. During the *in vivo* drug administration study, horses are administered with the drug or medication of interest for a certain period. The duration, route and frequency of administration will be decided with reference to the recommended dosage from manufacturers, drug effect and potential way of doping.

During *in vivo* administration study, the actual biotransformation of the drug can be elucidated by direct detection of metabolites in horse urine and plasma with verification against the respective *in vitro* study. Since metabolism can occur in other part of the body (i.e. other than liver), certain *in vivo* metabolites may only be present and detected from administration studies. In this case, the identification of these *in vivo* metabolites will be based on the comparison of chromatograms acquired from liquid chromatography-mass spectrometry (LC-MS) analyses of pre- and post-administration samples and followed by mass spectral interpretation. Structure of drug metabolites were proposed with reference to the reported biotransformations in other species and metabolism studies of drugs with similar structure. The identified metabolites would be

verified with accurate mass measurement and should not deviate by more than 5 ppm from the postulated structures.

1.2.2 Drug elimination studies

Drug elimination profiles in horse biological matrices are essential to determine detection times of the drug in the relevant matrix and could serve as references to suggest withdrawal times for medications. To study elimination profiles, the concentrations of the drug and its metabolites in horse urine and plasma will be quantified by internal standard (IS) calibration. Such calibration method provides better precision and accuracy of results as the variations caused by evaporation of solvents, injection and sample extractions can be compensated by the internal standard. A known concentration of deuterated form of the interested drug or a substance with similar properties to the drug is spiked in every sample. Multiple levels of calibrators and quality controls are also prepared and analysed in parallel with the test samples. To obtain the calibration curve, the peak area ratios (PARs) of analyte to IS *versus* the concentration of the calibrators were fitted using linear regression.

1.3 Sample preparation and analytical techniques for studying DMPK

In drug surveillance research, drug targets in different matrices are screened in forensic laboratory settings. Matrices including urine, blood, hair, faeces and saliva can be choices for doping control testing. Among the published equine pharmacokinetics and metabolic studies, equine urine and blood are most commonly studied due to the ease and convenience of sample collection. Despite collection of blood is rather invasive, blood remains as one of preferred matrices for drug testing since its analyses required

simpler extraction procedures (generally without deconjugation step) and have better relevance of the drug concentrations to the pharmacological effect (Ho et al., 2013; Kwok et al., 2010). Nevertheless, equine urine is another most common biological fluid in horseracing drug testing as it is non-invasive and has prolonged excretion for various classes of analytes. Urinary elimination is the predominant pathway to excrete drugs from the body. Thus, this research focuses on investigating the pharmacokinetics and metabolism of selected compounds in horses based on their urine and blood samples.

1.3.1 Sample preparation

Sample pretreatment is an essential procedure that ensures the drug target being detected with the least matrix interference for qualitative and/or quantitative analyses purposes.

1.3.1.1 Dilute-and-shoot

A sample dilution approach, also known as **dilute-and-shoot**, is a quick and highly productive method to prepare samples. Such a method is applicable to drug targets with relatively high concentrations in urine or plasma for drug testing, where the matrix components do not significantly interfere with the ionisation efficiency of the analytes (Henion et al., 1998). It is noteworthy that the dilute-and-shoot method is particularly suitable for polar drugs such as angiotensin-converting enzyme inhibitors, anti-epileptics, or haemostatics due to their hydrophilic properties that result in poor recovery by liquid-liquid extraction (LLE) or solid-phase extraction (SPE) (Kwok et al., 2016). In equine anti-doping, the dilute-and-shoot method coupled with LC-MS was employed in screening of multiple analytes (Goktas et al., 2025; Kwok et al., 2016;

Vonaparti et al., 2009a; Vonaparti et al., 2009b; Vonaparti et al., 2009c). The method was also proven applicable to human sport doping control (Beck and Ericsson, 2014; Goergens et al., 2015; Kim et al., 2015; Kim et al., 2016). However, the dilute-and-shoot method has various shortcomings that could result in poor analyses. Diluted samples contain considerably more matrix components than post-LLE or post-SPE extracts. Sample matrix in diluted specimens could be co-eluting and cause ion suppression at the ionisation source of LC-MS, resulting in depreciation of analyte peak response as analyte and co-eluted matrix components may compete for charging at the ionisation source (Peters and Remane, 2012; Taylor, 2005). Despite the fact that the matrix components will be diluted, the level of drug analyte will also be reduced accordingly during the dilution procedure. Consequently, the detection capability of analytes might be significantly impaired by dilution and matrix effect (Kiontke et al., 2016; Tamama, 2023). In view of the drawbacks of dilute-and-shoot method, biological samples are generally cleaned up prior to targeted analysis. Targets of interest may be extracted by various techniques such as LLE, SLE, or SPE.

1.3.1.2 Liquid-liquid extraction

Liquid-liquid extraction (LLE), a traditional and straightforward extraction method, is a selective partitioning of drug targets based on their solubilities, by mixing the biological aqueous liquid and immiscible organic solvent homogeneously (Schmidt and Strube, 2018). After vigorous shaking, the solvent breaks into small droplets which allow the analyte to contact and migrate away from the aqueous sample to the solvent. It is a common separation technique to extract non-polar analytes such as AASs. However, LLE has limited efficiency in extraction of polar compounds and drug metabolites as the immiscible organic solvents are hydrophobic in nature (Hamad et al.,

2020; Mansour et al., 2013; Mansour and Danielson, 2012). The formation of tiny solvent droplets can create emulsions that could also lower the extraction efficiency. Moreover, regarding safety and environmental impact, large amounts of potentially toxic or explosive solvent are disposed after LLE which could be hazardous to humans and non-eco-friendly (Chen et al., 2008). To address some of the aforementioned drawbacks, LLE has evolved into supported-liquid extraction (SLE) in the past decade.

1.3.1.3 Supported-liquid extraction

Supported-liquid extraction (SLE) cartridges consist of a porous support bed packed with chemically inert diatoms, which allows the targets from aqueous biological samples such as plasma to adsorb onto the disc surface. Similar to LLE, a water-immiscible organic solvent in SLE interacts with the target analytes by capturing them while passing through the support bed under gravity. No shaking step is involved in SLE; hence no emulsion would occur. Matrix compounds such as phospholipids, salts, and proteins are retained in the support bed. SLE is found to outperform LLE as it generally gives better extraction recovery and reproducibility when isolating drugs from pig plasma and mouse serum (Porvair Sciences Limited, 2022; Wu et al., 2010). A method using SLE coupled with liquid chromatography-high resolution mass spectrometry (LC-HRMS) has been successfully developed recently to simultaneously analyse more than 120 drugs in horse urine (Wong et al., 2020).

1.3.1.4 Solid-phase extraction

In forensic toxicology analyses, **solid-phase extraction (SPE)** also plays an important role in purification of sample and concentration of analytes. Prior to sample loading,

selected SPE cartridges require pre-conditioning and equilibration procedures to maximise retention of analytes. Urine or plasma samples containing target analytes pass through a stationary phase with adsorbent particles, where analytes can be retained on the sorbent by hydrophobic (for reverse-phase SPE), hydrophilic (for normal-phase SPE) or electrostatic (for cation and anion exchanger) interactions. Unwanted interferences are removed by washing steps. Analytes of interest are then eluted with a solvent by breaking the interaction between analyte and sorbent (Augusto et al., 2013; Hennion, 1999). SPE is categorised into three major retention mechanisms, namely reversed-phase, normal phase and ion-exchange. Normal-phase SPE is suitable for extracting polar analytes from non-aqueous matrices, thus it is not applicable to aqueous biological fluids in this study. For aqueous matrices including urine and plasma, SPE using reverse-phase or ion-exchange phase retention mechanisms is recommended. In reverse-phase SPE, mid to non-polar compounds from aqueous sample matrices are extracted based on hydrophobic van der Waals forces, where analytes of interest retain on nonpolar stationary phase that consist of silica-based materials modified with hydrophobic groups such as C_2, C_4, C_8, C_{18}, C_{30}, cyclohexyl, phenyl and cyano (Badawy et al., 2022; Boguslaw and Malgorzara, 2012). The elution of analytes is achieved using nonpolar solvent of suitable strength. For acidic or basic analytes, the ion-exchange retention mechanism can be employed. The mechanism is based on the high-energy electrostatic interaction between charged functional groups of the analyte and the sorbent. Basic compounds such as amines can be extracted by cation exchange (CX) cartridges, whereas acidic drugs such as carboxylic acid and phosphates can be isolated by anion exchange (AX) cartridges (Badawy et al., 2022). AX and CX cartridges are classified into weak (W) and strong (S) ion exchangers corresponding to the ionic group bonded to the sorbent bed, which are summarised in **Table 1.3**. In general, the sample

pH should be two units lower than the analyte pKa for AX mode, while the sample pH should be two units higher than the analyte pKa for cation exchange mode.

Table 1.3. Ion-exchange retention mechanism

Sorbent type		**Bed sorbent**	**Target analyte**	**Sample pH**
AX	SAX	Quaternary ammonium	Negative charge	Analyte pKa + 2
	WAX	Primary, secondary or tertiary amine		
CX	SCX	Ionised sulfonic acid	Positive charge	Analyte pKa - 2
	WCX	Carboxylic acid		

In the past decade, mixed-mode SPE is frequently used as it is effective to recover a wide range of polar and non-polar drugs in horse urine and plasma (Wong et al., 2011; Wong et al., 2012; Karatt et al., 2023). The sorbent can demonstrate two or more interaction mechanisms, including reversed-phase and ion-exchange retention modes. Selective elution of various compounds can be accomplished by adjusting the pH and organic component percentage.

1.3.1.5 Deconjugation of drugs

As mentioned in Section 1.2.1, some drugs need to undergo metabolism to form phase I metabolites and/or phase II conjugates for the ease of excretion from body, particularly non-polar drugs that are heavily conjugated. More than 90 % of AAS are eliminated as

phase II conjugates of sulfate and/or glucuronide (Houghton, 1977; Houghton and Dumasia, 1979; Houghton and Dumasia, 1980; Pranata et al., 2019). Indeed, direct detection of intact phase I metabolites or phase II conjugates is the most ideal way to prove the administration or natural occurrence of a substance. Nevertheless, reference standards for phase II conjugates are normally unavailable on the market. The screening or quantification of the parent drug or its phase I metabolite is therefore preferred, where the parent drug and/or its metabolites are released from their glucuronide and/or sulfate conjugates *via* hydrolysis. When designing a sample preparation protocol for horse urine samples, a suitable hydrolysis step is critical due to the extent of phase II metabolism.

Deconjugation of glucuronide-conjugated compounds can be carried out using enzyme hydrolysis, incubating the sample with a proper biocatalyst β-glucuronidase sourced from *Escherichia coli* (*E.coli*), *Helix pomatia* or *Patella vulgata*. In equine conjugation studies, β-glucuronidase sourced from *E.coli* and *Patella vulgata* is commonly used. Each type of enzyme has its own optimal pH and specific storage temperature as listed in **Table 1.4**. Deconjugation by *Helix pomatia* was not preferred as cases of target conversion and/or decomposition were reported previously (Houghton et al., 1992; Makin et al., 1995).

Table 1.4. Specifications of *Patella vulgata* and *E.coli*

	Patella vulgata **(Molluscan Source)**	***E.coli*** **(Limpets)**
Optimal pH	4.5-5.0	6.0-6.5
Enzyme	- Sulfatase (Can be inhibited by 0.1M phosphate buffer) - Glucuronidase	- Glucuronidase
Storage Temperature	-20 ℃	2-8 ℃

Apart from glucuronidation, sulfation is also a major phase II metabolic pathway of endogenous and exogenous compounds such as testosterone, nandrolone and boldenone. Deconjugation of sulfate conjugates can be achieved by solvolysis or methanolysis. Sulfate conjugates are cleaved by solvolysis using concentrated sulfuric acid. The acid hydrolysis process in solvolysis is promoted by the presence of organic solvent, and a salting-out step is incorporated to promote sulfate conjugates to the organic layer. The ionic strength of aqueous urine is increased by adding excess sodium chloride powder, in which conjugates become less soluble and are pushed to the organic layer. Methanolysis, an alternative method using anhydrous methanolic hydrogen chloride, can also be employed for hydrolysis of sulfate conjugates. This method was first reported by Tang and Crone (1989) to hydrolyse steroid sulfates. However, there are drawbacks to sulfate conjugate hydrolysis under high temperatures and drastic pH. These harsh conditions could potentially cause decomposition of the analyte and the formation of by-products, which consequently lower the recoveries of analyte (Dumasia and Houghton, 1981; Gomes et al., 2009). Therefore, stability studies of interested targets under specific hydrolysis conditions are normally conducted before

applying the method in routine doping control testing. For drugs that exist mainly as phase II conjugates, enzyme hydrolysis and/or acid hydrolysis will be required for better estimation of the total amount of the drug and metabolites excreted.

1.3.2 Analytical techniques – instrumentation for evaluation of DMPK

Drug surveillance in equine sports can be achieved through evaluation of DMPK. Apart from sample preparation and extraction, the use of appropriate instrumentation is also one of the key elements to facilitate effective drug surveillance. Over the past decade, gas chromatography/mass spectrometry (GC-MS) and LC-MS have become the major analytical techniques in equine doping control research, in particular on metabolite identification. Nuclear magnetic resonance (NMR) analysis is also a popular technique to definitively identify drug metabolites (Pearce and Lushnikova, 2006). Nonetheless, it is not always feasible for equine research because a large sample size is required and extensive purification of biological samples is needed prior to NMR analysis (Scarth et al., 2011).

Gas chromatography-mass spectrometry (GC-MS) has remained the gold standard for analysing small, volatile and thermally stable compounds such as AAS in complex matrix backgrounds (McKinney 2009). It can accomplish both targeted screening and non-targeted drug screening by library searching. In recent years, the application of LC-MS has rapidly progressed in the industry. LC-MS is suitable for analytes that are non-volatile, thermally labile and polar. In comparison to GC-MS, sample preparation could be simplified as chemical derivatisation step may not be necessary prior to LC-MS analysis. There are three common ion sources for LC-MS systems, namely atmospheric pressure ionisation (API), electron impact ionisation (EI) and chemical ionisation (CI).

API, a soft ionisation technique, is preferred in metabolite identification and quantification due to good compatibility with reversed phase chromatography and generation of highly sensitive molecule ions (Kamel and Prakash, 2006; Kostiainen et al., 2003). Among the API techniques, ESI-MS technique is the most commonly used for DMPK study as it is a considerably gentle ionisation that could yield intact molecular ions (Roskar and Trdan, 2012). It is ideal for drug metabolites which are polar, ionic and thermally labile, for instance phase II drug metabolites. Coupled with ultra-performance liquid chromatography (UPLC), many compounds can be analysed with high throughput, high sensitivity and selectivity. Considering the advantages of ESI-MS, this study utilised ESI-MS for all qualitative and quantitative molecular analyses.

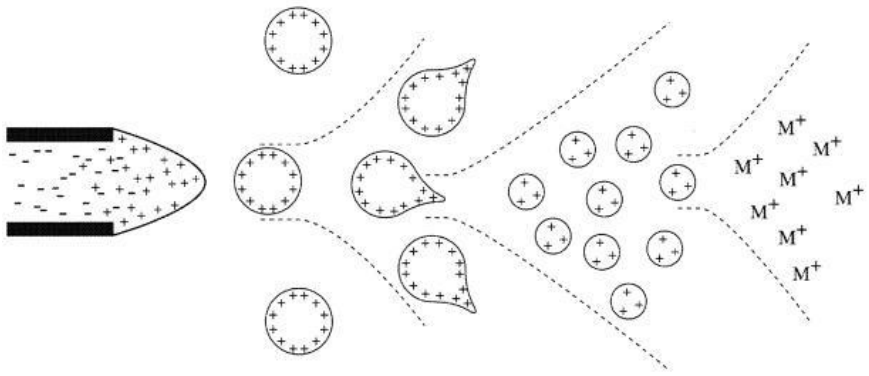

Figure 1.2. Mechanism of ESI at positive polarity (Ho et al., 2003)

The mechanism of ESI involves three main steps that ionising analytes prior to introduction into ion source as shown in **Figure 1.2**. Firstly, the sample solution eluted from the LC column is pumped into the ESI steel capillary tube, where a high voltage of 5 kV is applied across the ion-sampling aperture at atmospheric pressure.

Subsequently, electrospray nebulisation occurs where a fine spray of charged droplets with the same polarity are generated. The nebulised droplets will either be positively or negatively charged depending on the voltage polarity. Next, solvent evaporation takes place with the heated nitrogen gas flow. As the size of the droplet reduces continuously, the electrical charge density increases and hence the electrostatic repulsion in the droplet also increases. The droplet further splits when the electrostatic repulsion exceeds the surface tension of the droplet, forming multiple tiny particles. The explosion of the droplet occurs repeatedly until gas phase ions are ejected (Kebarle, 2000). A sampling skimmer then samples and drives the ejected ions into a mass analyser to measure the molecular mass and ion intensity (Ho et al., 2003).

Ionised analytes are introduced into the mass analyser and separated according to their mass-to-charge (*m/z*) ratios. In this current work, tandem quadrupole system (MS/MS) and high-resolution accurate mass MS (HRMS) systems are employed for the DMPK study of various targets. Tandem quadrupole system (MS/MS), consisting of three quadrupoles, can detect predicted molecular weights and fragmentation patterns of multiple targets (Lee et al., 2021). It involves selected-reaction monitoring (SRM), with the mechanism illustrated in **Figure 1.3**. First, the *m/z* ratio of target precursor ion is selected by the first quadruple (Q1). Next, the precursor ion passes through quadrupole collision cell (Q2) and collides with collision gas argon. The collision process, also known as collision-induced dissociation (CID), results in fragmented daughter ions. The daughter ions can then be monitored by the third quadrupole analyser (Q3) before reaching the mass detector. SRM transitions are especially useful for quantitative analysis; however, they are not appropriate for determining metabolites with unknown molecular weights and fragmentation.

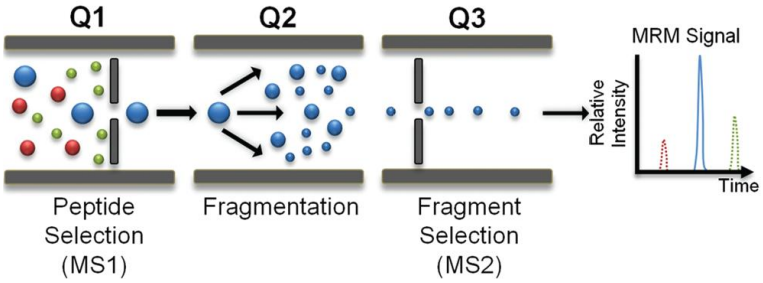

Figure 1.3. A schematic diagram of SRM mass spectrometry (Boja and Rodriguez, 2011)

In the light of the limitations of MRM, HRMS technique is often used in equine doping control to accomplish reliable and accurate structural elucidation of unknown metabolites (Choi et al., 2018, Cutler et al., 2022; Kwok et al., 2015), as it enables determination of the elemental formula of metabolites (Roskar and Trdan, 2012) and differentiation of compounds that differ in mass by as little as 0.01 Da (Lee et al., 2021). Subtle differences between the high resolution full-scan analyses of pre- and post-administration samples could also be differentiated. By coupling collision-activated dissociation with HRMS, structural elucidation of drug metabolites can be achieved through accurate mass assignments of the fragment ions (Scarth et al., 2010b). Orbitrap mass spectrometry is one of the commonly used HRMS techniques in analytical toxicology (Ojanpera et al., 2012), which is ideal for qualitative identification. The full MS and MS/MS detection of compounds with high resolution and mass accuracy can be done simultaneously by the Orbitrap MS, which is not achievable with a triple quadruple mass spectrometer using SRM technique (Hecht et al., 2019).

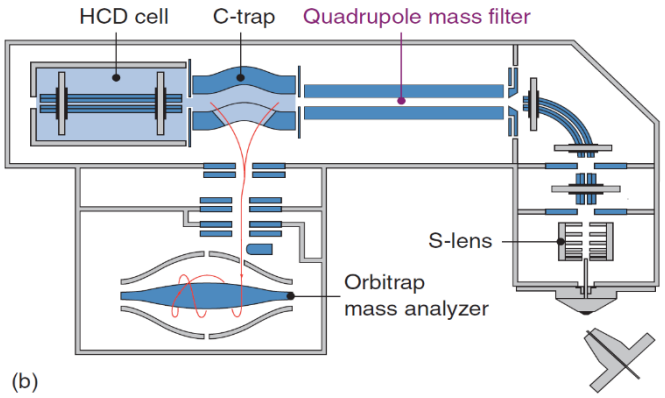

Figure 1.4. A schematic diagram of quadrupole-Orbitrap hybrid mass spectrometer (Hecht et al., 2019)

The Orbitrap MS features several components, including a quadrupole mass filter, C-trap, higher-energy collision dissociation (HCD) cell, and Orbitrap analyser (**Figure 1.4**). The quadrupole mass filter, with fast switching times between different narrow mass ranges, is responsible for precursor ions selection. It allows multiplexed scan modes such as multiplexed single ion monitoring (SIM) mode. Under SIM mode, ions of the selected mass range then pass into and accumulate at C-trap. The ions are analysed jointly in Orbitrap mass analyser. In the Orbitrap mass analyser, ions are trapped in an orbital motion, where a specific frequency of oscillation is translated to a specific *m/z* value and their amplitude into intensity by Fourier transformation (Hecht et al., 2019). In MS/MS mode, only ions of interest are selected by the mass filter and are transferred to the HCD cell for fragmentation to product ions. The fragment ions are collected and stabilised in C-trap, followed by detection at Orbitrap mass analyser.

Biotransformation and pharmacokinetics of drugs can be investigated by conducting *in vitro* or *in vivo* metabolic studies and drug elimination studies. Appropriate LC-MS instrumentation and effective sample preparation are critical to the detection and structural elucidation of drugs and their metabolites. LC-MS/MS gives a high

sensitivity in targeted analyses, which is particularly suitable for quantitative analysis. LC-HRMS allows retrospective data analysis and provides fast and robust metabolite profiling (Roskar and Trdan, 2012).

1.4 Aims and objectives

Drugs and/or prohibited substances with high potential for abuse in equine sports and horseracing are a detrimental threat to the health and welfare of racehorses. A comprehensive understanding of the metabolism and pharmacokinetics of drugs in equines is hence vital to combat their potential misuse. As mentioned earlier, the control of misuse of exogenous drugs and endogenous compounds requires different approaches. To date, the metabolic fate of numerous drugs in equine remains unclear, thus extensive DMPK studies of drugs is crucial to the horseracing industry.

This thesis focuses on various approaches on doping control of three prohibited substances in horseracing utilising LC-MS/MS, LC-HRMS and/or LC-MS/HRMS techniques: namely estra-4,9-diene-3,17-dione (dienedione) (Chapter 2 and 3), 2-hydroxyethyl salicylate (Chapter 4) and ranitidine hydrochloride (Chapter 5). Chapter 2 aims to study the endogenous nature of dienedione in entire male horses and to set up an in-house threshold for regulating its illicit use. Chapter 3 extends the study of dienedione in castrated horses, aiming to determine the DMPK of dienedione in horses after dienedione administration. Chapters 4 and 5 consider the DMPK of 2-hydroxyethyl salicylate and ranitidine hydrochloride in horses, respectively.

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Chapter 2. Endogenous nature of dienedione in entire male horse

2.1 Introduction

2.1.1 Abuse of anabolic androgenic steroids (AASs) in human and equine

Anabolic androgenic steroids (AAS), among a large variety of prohibited substances, are among the most prevalent drugs of abuse in human sports and horseracing due to their ability to enhance muscle mass and strength (Houghton, 1992). AAS accounted for more than 40% of the drug abuse cases from 2018 to 2022 in human sports, which ranked the highest among all reported Adverse Analytical Findings reported by WADA. According to the 2022 Anti-Doping Testing Figures from WADA, anabolic agents including AAS remained the most reported drugs of abuse (1,124 occurrences; 42 % of all reported Adverse Analytical Findings) amongst substance groups. The most frequently misused AASs include stanozolol, metandienone, drostanolone and 19-norandrosterone (WADA, 2024). One of the reasons for frequent AAS intake is its widespread availability in black market. It is reported that drug trafficking organization operated vendor accounts at darknet such as AlphaBay, Dream Market and Wall Street to distribute AAS in person (United States Attorney's Office, 2019).

AASs can be administered through different routes, common ones including oral, injection and topical applications. They are employed in clinical settings for treating hormonal diseases that may result in muscle loss (National Institute on Drug Abuse, 2019). Apart from legitimate applications, AASs are reportedly misused by athletes for illicit performance enhancement, in which the dosage of drug might be increased to 10 or even 100 times of that of the prescribed medicine. Negative psychological effects, such as impaired judgement, mania and delusions, may be provoked. Illicit use of AAS can also lead to irreversible harm to the human body, for example kidney failure, liver damage, blood clotting and heart attack (National Institute on Drug Abuse, 2018). AAS

is classified as Schedule III drugs of the US Controlled Substances Act, Class C in the UK Misuse of Drugs Act 1971 and Schedule 4 Part II of the Misuse of Drugs Regulations 2001, which should only be prescribed by medical practitioners or sold by pharmacists with prescription.

Potential abuse of AAS in equine sports has been recognised since the 1960s, which stimulated racing authorities to develop methods for detecting of AAS misuse (Teale and Houghton, 2010). On one hand, AAS could be employed by veterinarians in several conditions legitimately, such as debilitating condition, hepatic disorders, orthopaedic use, prolonged corticosteroid therapy, anaemia treatment, and post-surgery recovery. On the other hand, steroids may also be applied during training to increase speed and stamina, as a result damaging the fairness of competition. Nevertheless, limited studies have demonstrated advantageous effects of AAS on racing and competition performance. Horse may suffer from behavioural and reproductive side effects when undergoing prolonged AAS treatment. Signs such as increased aggressiveness and erections are observed in castrated horses. Virilisation and suppression of oestrous behaviour have also commonly occurred in fillies (female horses aged three and/or younger) and mares (female horses above the age of three). Reproductive side effects include reduction of testis and suppression or delay of ovulation (Snow, 1993).

2.1.2 Steroid structure, pharmacology and metabolism

All steroid compounds consist of three cyclohexane rings (A-, B- and C-ring) and one cyclopentane ring (D-ring), forming gonane (perhydrocyclopentanophenanthrene nucleus) with A-, B-, C- and D-ring attached to one another. The naming of steroids with different hydrocarbon backbones is adopted, follows the system nomenclature

recommended by the International Union of Pure and Applied Chemistry (IUPAC). Steroids differ in orientation of carbons ranging from 17-carbon gonane nucleus to 27-carbon cholestane nucleus (**Figure 2.1**).

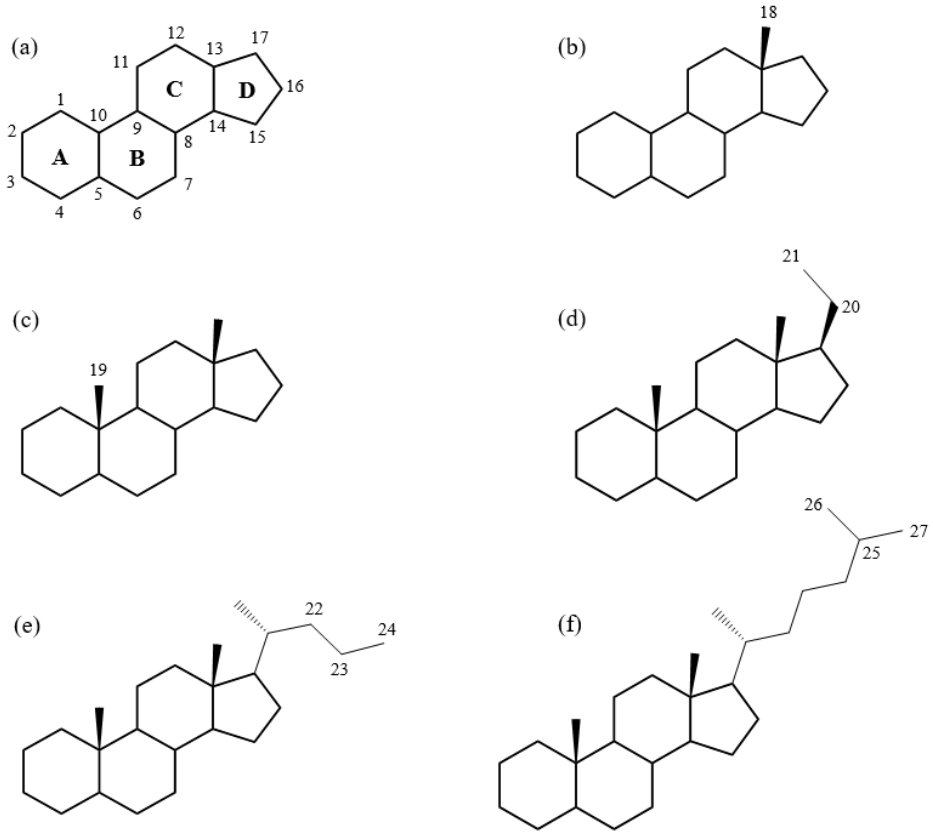


Figure 2.1. Chemical structure of range of hydrocarbon backbones, including (a) 17-carbon gonane nucleus, (b) 18-carbon estrane, (c) 19-carbon androstane, (d) 21-carbon pregnane, (e) 24-carbon cholane and (f) 27-carbon cholestane nucleus (IUPAC, 1989)

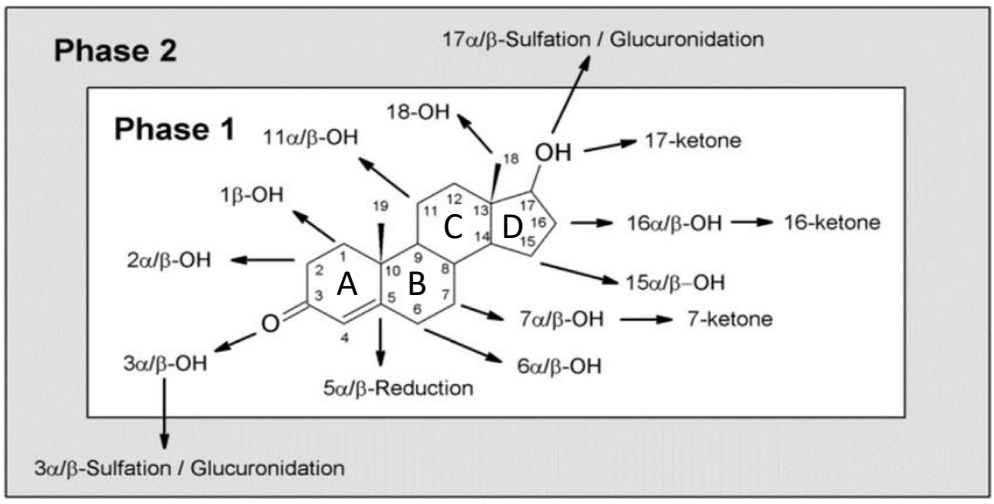


Figure 2.2. Scheme of Phase I and II metabolism of AAS

The two major physiological functions of AAS are muscle building (anabolic) and promotion of secondary male sex characteristics (androgenic). Hydrophobic AAS first penetrates the membrane of the muscle cell and adheres to an androgen receptor (AR) in the cytoplasm. The steroid-AR complex then diffuses into the nucleus and binds to DNA. Subsequently, alteration of gene expression or signal-transmitting processes activation occurs. Different binding of AAS to corresponding sites of AR causes different anabolic effects such as stimulation of muscle cells formation and protein synthesis, resulting in gains in muscle size and strength (Fahey, 1998).

In view of their hydrophobic nature, AASs can be excreted by converting the drug into hydrophilic metabolite at liver *via* phase I and phase II metabolism. Phase I metabolism involves chemical reactions such as oxidation, reduction and hydroxylation, where functional groups are introduced to positions shown in **Figure 2.2** (Schiffer et al., 2018). Polar functional groups, for instance, hydroxyl, thiol and amine group, inactivate AASs and promote their elimination from the body. Common phase I metabolic pathways include reduction of keto groups and sites of unsaturation in A-ring, epimerisation of

17-hydroxyl group and hydroxylation at various sites mainly on A- and D-rings but not limited to B- and C-rings (Scarth, Teale & Kuuranne, 2011; Schänzer, 1996). In horses, AASs tend to undergo reduction of C3 keto group to the 3β isomer and/or hydroxylation at C16. Take testosterone as an example, C3 keto group is reduced during phase I metabolism to form metabolites with 5α,3β-hydroxy configuration (Scarth et al., 2011). Phase II metabolism refers to conjugation of AASs with glucuronic acid, sulfate, glutathione or amino acid. More than 90% of AASs are eliminated from equine as water-soluble conjugates of sulfate and glucuronide. Conjugations normally occur at C3α/β and/or C17α/β. In general, sulfation predominates in horses. Steroids with 17β-hydroxyl group, such as testosterone and boldenone, are mostly sulfate-conjugated whereas steroids with 17α-hydroxyl group are glucuronide-conjugated (Teale & Houghton, 2010).

2.1.3 Dienedione

Estra-4,9-diene-3,17-dione, commonly named dienedione, is a marketed AAS sold as a bodybuilding supplement. It possesses an 18-carbon estrane backbone. It is a structural analogue of another popular steroid trenbolone (estra-4,9,11-trien-17β-ol-3-one), with reduction of the double bond at C11,12 position and oxidation of hydroxyl group at C17 position (**Figure 2.3**). Due to its hydrophobic nature similar to other AASs, dienedione tends to undergo biotransformation to promote urinary excretion by being converted to hydrophilic metabolites, including conjugation with either a glucuronic acid, sulfate or other polar moieties to form water-soluble products.

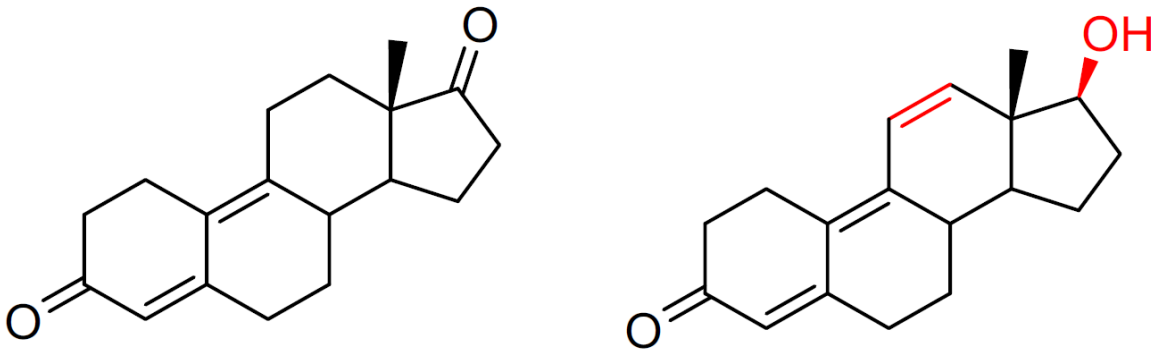


Figure 2.3. Chemical structure of dienedione (left) and trenbolone (right)

To the best of our knowledge, there is no report on the endogenous nature of dienedione nor its conjugation in humans and in equines. In view of its 4,9-diene configuration, which has not been reported in any endogenous steroid, dienedione has long been considered as a synthetic AAS, and its *in vitro* metabolites were reported by Scarth et al. (2010). Nevertheless, the recurring presence of dienedione in entire male horse urine samples indicated that dienedione has possible natural presence in horses. Dienedione was detected in the authors' laboratory from an in-house screen for free basic drugs in urine using GC-MS. The sample was reportedly collected from an entire male horse and the observation was considered as unusual because dienedione has been commonly known as designer steroid at that time. Further investigation was then conducted to investigate any similar observation from other samples.

Gas chromatography-mass spectrometry (GC-MS) has been the gold standard for screening anabolic steroids in equine doping control (Choi et al., 2018; Ho et al., 2007; Leung et al., 2013; Parr and Schänzer, 2010; Revelsky et al., 2011). However, the application of LC-MS in AASs analysis has in fact progressively increased. Many LC-MS methods were reported for equine anti-doping which had higher sensitivity and

rapid turnaround time (Fragkaki et al., 2017; Guan et al., 2010; Kwok et al., 2015; Pozo et al., 2013; Kwok et al., 2017; Wong et al., 2012). Moreover, LC-MS techniques typically involve simpler sample preparation. Unlike GC-MS, derivatisation such as acetylation, acylation or silylation is not necessary prior to LC-MS detection. Therefore, liquid chromatography-tandem mass spectrometry technique (LC-MS/MS) was employed in our further investigation.

This chapter depicts the detection of endogenous dienedione in entire male horses and proposes the possible conjugation of dienedione (if any). The analytical protocols included LLE, enzyme hydrolysis, solvolysis and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analyses on a TSQ Quantum Ultra instrument. A population study of free and glucuronide-conjugated dienedione, was carried out using UPLC-MS/MS after automated SPE to streamline the extraction process for a large number of samples. The basal levels of urinary dienedione of entire male horses were established. An in-house threshold for free and glucuronide-conjugated dienedione in entire male horse urine was set through statistical analysis. The sensitivity and robustness of the quantitative method employed in the population study were validated.

2.2 Methods

2.2.1 Materials

Dienedione was purchased from Steraloids (Newport, RI, USA) and Chromadex (Longmont, USA). Testosterone sulfate, testosterone glucuronide and androsta-4,6-diene-3,17-dione were obtained from Steraloids (Newport, RI, USA). Testosterone was obtained from United States Pharmacopeia (Rockville, USA). d_3-Testosterone was

obtained from Sigma-Aldrich (St. Louis, MO, USA). d_3-Boldenone was obtained from National Measurement Institute (Sydney, Australia). Methanol (LC-MS Chromasolv) was obtained from Riedel-de Haen (Seelze, Germany). Chloroform (GR grade), potassium phosphate, diisopropyl ether (Emsure®), *n*-hexane (GR grade), ethyl acetate (GR grade) and methanol (LiChrosolv®) were purchased from Merck (Darmstadt, Germany). Ammonium formate (extra pure, 97 %) was obtained from International Laboratory Limited (San Bruno, CA, USA). Beta-glucuronidase (β-glucuronidase, *from Patella vulgata*, lyophilised powder) was purchased from Sigma-Aldrich (St. Louis, MO, USA). ABS Elut Nexus cartridges (60 mg, 3 mL) were purchased from Varian (Palo Alto, CA, USA). Deionised water was generated from an in-house water purification system (Milli-Q, Molsheim, France).

2.2.2 Sample Collection

Urine samples from local or overseas entire male horses were analysed, involving 58 overseas samples and 117 local ones. Multiple entire male horse urine samples were gathered from the same local horse on separate occasions (not applicable to overseas horses due to lack of horse identity information). The samples were aliquoted into six individual 5-mL screw cap tubes and preserved at -70 ℃ pending analysis. One of these portions was taken out for the necessary analysis to prevent unnecessary freeze-thaw cycles. Castrated horse samples were used to prepare the calibrators, spiked controls and matrix blanks.

2.2.3 Extraction procedures

2.2.3.1 Detection of free dienedione (Figure 2.4)

Entire male horse urine (3 mL) was portioned to 15-mL corex tube and centrifuged at 2500 rpm for 10 min. Supernatant (2 mL) was transferred to a 15-mL graduated tube. Phosphate buffer (0.1 M, pH 6.0, 1 mL) was added to adjust pH to 7.0. Diisopropyl ether (5 mL) was then added to the urine aliquot (2 mL) for LLE. The mixture was rotated for 10 min and centrifuged at 3600 rpm for an additional 10 min. The organic layer was pipetted to a clean 15-mL corex tube, base-washed with NaOH/NaCl (1 M/0.15 M, 2 mL) and vortexed for 0.5 min. Following a 0.5-min centrifugation at 2500 rpm, the organic fraction was extracted and passed through a Pasteur pipette packed with cotton wool into a 5-mL Reacti-vial to remove the residual water. The fraction was then dried under a stream of nitrogen at 60 °C, reconstituted with ammonium formate (5 mM, pH 3.0)/methanol (70:30, *v/v*) (50 µL) and vortexed. The content was moved to a plastic vial for UPLC-MS/MS analysis.

2.2.3.2 Conjugation study of phase II metabolites of dienedione (Figure 2.4)

Prior to the LLE extraction for free dienedione (as described in ***Section 2.2.3.1***), control samples of free testosterone (20 ng/mL), testosterone glucuronide (equivalent to 20 ng/mL of free testosterone) and testosterone sulfate (equivalent to 20 ng/mL of free testosterone) were spiked in three separate aliquots of negative castrated horse urine. These samples were then analysed concurrently to assess the efficiency of the enzyme hydrolysis and solvolysis. Internal standard d_3-testosterone (final concentration of 20 ng/mL in control samples) was added.

Deconjugation of dienedione glucuronide (if any) – free dienedione was first extracted using the procedures outlined in ***Section 2.2.3.1***, and androsta-4,6-diene-3,17-dione (as an IS, final concentration of 2 ng/mL in sample) was added to the sample prior to LLE. Following the extraction of free dienedione, diisopropyl ether (5 mL) was then added to the remaining aqueous fraction. The mixture was rotated for 10 min and centrifuged at 3600 rpm for another 10 min. The aqueous fraction was transferred to a clean 15-mL corex-tube and subjected to an additional wash with diisopropyl ether (5 mL). Andro-4,6-diene-3,17-dione as IS (final concentration of 2 ng/mL in sample) was added to the remaining aqueous fraction. Phosphate buffer (0.1 M, 1 mL) was added, and the mixture was adjusted the pH to 5.0.

Subsequently, β-glucuronidase (*Patella vulgata*, 18000 units/mL, 330 µL) was introduced for enzyme hydrolysis. The mixture was incubated at 65 ℃ for 2.5 hrs. After incubation, the hydrolysate was cooled to room temperature and centrifuged at 2500 rpm for 5 min. The supernatant was moved into a 15-mL plastic tube. Phosphate buffer (0.1 M, 1 mL) was then added to adjust pH to 7.0. Diisopropyl ether (5 mL) was added, mixed on a rotator for 10 min and centrifuged at 3600 rpm for 10 min. The organic layer was extracted to a clean 15-mL corex tube and base-washed with NaOH/NaCl (1 M/0.15 M, 2 mL). After a centrifugation at 2500 rpm for 0.5 min, the organic portion was pipetted out and passed through a Pasteur pipette packed with cotton wool into a 5-mL Reacti-vial. The fraction was dried under a stream of nitrogen at 60 ℃, reconstituted with ammonium formate (5 mM, pH 3.0)/methanol (70:30, v/v) (50 µL) and vortexed. The contents were then transferred to a plastic vial for UPLC-MS/MS analysis.

Deconjugation of dienedione sulfate (if any) – All pre-wash steps mirrored those for the deconjugation of dienedione glucuronide with the enzyme hydrolysis step replaced

by with solvolysis and salting out to release the potential sulfate conjugates. Sodium chloride powder (3 g), ethyl acetate (5 mL) and concentrated sulfuric acid (70 µL, 3.5% *v/v*) were added to the pre-washed aqueous fraction. The sample was mixed and rotated for 15 min and centrifuged at 3600 rpm for 10 min. The organic fraction was transferred to 15-mL corex tube and incubated at 55 °C for 2 hrs. Finally, the organic extract underwent base-washing, cleanup, drying and reconstitution using identical procedures as depicted for the deconjugation of dienedione glucuronide.

Calibrator and Quality Control (QCs) – A calibrator and a QC sample were prepared at respectively 2 ng/mL and 0.5 ng/mL of dienedione in castrated horse urine with no detectable dienedione to estimate the extent of conjugation of dienedione.

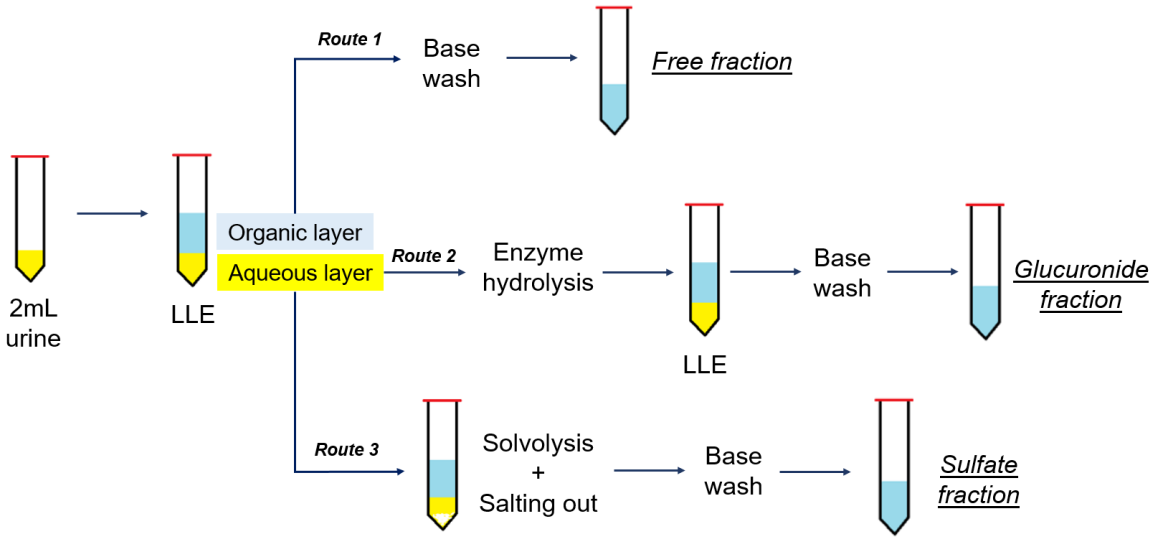


Figure 2.4. Flow diagram of detection of free dienedione and conjugation study of dienedione phase II metabolites

2.2.3.3 Quantification of free and glucuronide-conjugated dienedione

Entire male urine (3 mL) was portioned to 15-mL corex tube and centrifuged at 2500 rpm for 10 min. Subsequently, supernatant (2 mL) was transferred to 15-mL plastic tube. Phosphate buffer (0.1 M, 1 mL) was added, and the mixture was adjusted pH to 5.0. d_3-Boldenone (final concentration of 2 ng/mL in sample) was spiked to entire male horse urine as an IS. β-glucuronidase (*Patella vulgata*, 18000 units/mL, 330 µL) was added for enzyme hydrolysis, and the mixture was incubated at 65 ℃ for 2.5 hrs. After cooling to room temperature, the hydrolysate underwent centrifugation at 2500 rpm for 5 min before being loaded onto an Abs Elut Nexus cartridge. Prior to sample loading, the cartridge was conditioned with methanol (2 mL) and water (2 mL). Washing steps with deionised water (2 mL) and hexane (2 mL) were then performed, followed by 2-min drying. All SPE drying steps were carried out using nitrogen purge at 20 psi. The cartridge was then eluted with chloroform (4 mL). The eluate was evaporated to dryness at 65 ℃ under nitrogen, and the residue was reconstituted with ammonium formate (5 mM, pH 3.0)/methanol (70:30, v/v) (50 µL) for UPLC-MS/MS analysis.

2.2.3.4 Preparation for calibrators and quality controls for population study of free and glucuronide-conjugated dienedione

Calibrators and quality controls were prepared using different sources of dienedione. Calibrators of dienedione were spiked at 0, 0.5, 1, 2, 4, 8 and 12 ng/mL in castrated horse urine where no dienedione was detected. Quality controls were set at three levels: 0.5 ng/mL, 2 ng/mL and 5 ng/mL of dienedione. Each analytical batch comprised calibrators and QCs, which samples including calibrators, QCs and test samples were processed in duplicate during every run.

2.2.4 Instrumentation

2.2.4.1 LC/HRMS analyses (only for structural confirmation of dienedione)

UPLC-HRMS analyses for structure elucidation of dienedione were conducted employing a Thermo Scientific Q Exactive mass spectrometer (Thermo Fischer Scientific, Bremen, Germany), equipped with a heated-electrospray ionisation (HESI-II) source interfaced with a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA). The sample tray of the autosampler was maintained at 15 °C. Separation of target analytes was achieved using a reverse-phase Acquity UPLC BEH C18 column (Acquity, 100 mm length x 2.1 mm ID; 1.7 µm particle size). The mobile phases consisted of ammonium formate in deionised water (pH 3, 5 mM) as mobile phase A and methanol as mobile phase B. A linear gradient was run at a flow rate of 300 µL/min, starting with 90 % solvent A at 0 min, dropping to 58 % at 2.0 min then held at 58 % until 20 min, decreased to 2 % solvent A at 21 min. The gradient returned to 90 % solvent A at 24 min and remained stable until 27 min before the subsequent injection. Each injection volume was set at 5 µL.

Sample ionisation was performed in a positive ionisation mode utilising HESI-II, with a capillary temperature of 350 °C, a sheath gas flow of 50 arbitrary units, and an auxiliary gas flow of 10 arbitrary units. The sweep gas flow was adjusted to 2 arbitrary units with a curtain plate in place. The ion spray voltage was set approximately 3 kV. The S-Lens radio frequency (RF) level was maintained at 40 %. Full-scan mass spectra were acquired using a mass resolution of 35000 (full-width at half maximum, FWHM at *m/z* 200). An internal lock mass solution containing benzyldimethylphenylammonium chloride (*m/z* 212.14338 for positive mode) at a concentration of 0.1 ng/µL in deionised water was infused post column into the system at a rate of 20 µL/min via a T-joint using a LC-20AB Solvent Delivery Unit (Shimadzu

Corporation, Kyoto, Japan). The normalised collision energy (NCE) of the High-energy Collisional Dissociation (HCD) collision gas for dienedione was set at 50 %. The scan range encompassed *m/z* 50 to 295.

2.2.4.2 LC-MS/MS analyses for conjugation and population studies

The UPLC-MS/MS employed in the conjugation and population studies involved of a Waters Acquity UPLC system capable of withstanding pressures up to 15,000 psi, interfaced to a TSQ Quantum Ultra mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA). The mobile phases and running conditions were identical to that detailed in the UPLC-HRMS analyses in the preceding ***Section 2.2.4.1***.

Injection volumes were 10 µL each, and the HESI source operated in the SRM positive ion mode, using a spray voltage of 2 kV. The capillary and vaporizer temperature were set at 320 ℃ and 350 ℃ respectively. The sheath, auxiliary and ion sweep gas pressure were maintained at 50, 10 and 2 arbitrary TSQ unit. The resolution of the quadrupole mass filter was configured with a peak width of 0.7 amu (FWHM) for both Q1 and Q3. The collision gas pressure of Q2 was set at 1.2 mTorr. The monitored ions included protonated dienedione (*m/z* 271 → 97, 133, 159 and 175), testosterone (*m/z* 289 → 97), IS androsta-4,6-diene-3,17-dione (*m/z* 285 → 149; for conjugation study), IS d_3-boldenone (*m/z* 290 → 121; for quantification) and IS d_3-testosterone (*m/z* 292 → 97; experimental control). Details regarding precursor ion and CID energies for UPLC-MS/MS are presented in **Table 2.1**.

Table 2.1. The UPLC-MS/MS parameters of target, experimental control and ISs

	Precursor ion (*m/z*)	**Ion monitored (*m/z*)**	**CE (eV)**	**RF Lens (V)**
Dienedione	271	97	26	85
		133	26	
		159	31	
		175	19	
Testosterone	289	97	23	87
Androsta-4,6-diene-3,17-dione	285	149	21	92
***d*$_3$-Boldenone**	290	121	28	90
***d*$_3$-Testosterone**	292	97	24	87

2.2.5 Method validation of the quantification method for free and glucuronide-conjugated dienedione by UPLC-MS/MS

The validation of the quantification method for free and glucuronide-conjugated dienedione in entire male horse urine for the population study was conducted by assessing the estimated limit of quantification (LoQ), inter-day precision, accuracy and extraction recovery. Statistical analysis of this population data was carried out using IBM® SPSS® Statistics to determine the possible threshold. The validation of this quantification method follows the *Guidelines for deriving thresholds* outlined by the Association of Official Racing Chemists (AORC) (AORC, 2022).

The LoQ was established as 10 times the standard deviation (SD) at the lowest QC (0.5 ng/mL) across 12 analytical batches. Accuracy was evaluated by comparing the measured concentrations of QC samples at different levels with their respective theoretical concentrations (0.5, 2 and 5 ng/mL). The means and standard deviations of each QC level were computed. The acceptable accuracy for the mean concentration is

within ± 15 % of the spiked concentrations as per the guidelines of the AORC (AORC, 2022).

The inter-day precision was assessed by determining the relative standard deviation (% RSD) of the PAR of the target analyte to the IS and the relative retention times (RRT). The evaluation involved spiking six castrated horse urine samples (at the QC levels) over four different days. The acceptable % RSD falls within ± 15 % (± 20 % at LoQ) (AORC, 2022).

Extraction recovery was examined by analysing duplicate spiked castrated horse urine samples at QC levels. One set had dienedione spiked before enzyme hydrolysis, whilst the other set had it spiked after SPE. The IS was added post-SPE in both sets of samples, with the latter set considered to have 100 % recovery.

2.3 Results and discussion

2.3.1 Confirmatory analysis of free dienedione in entire male horse urine

An ultra-sensitive UPLC-MS/MS method was developed to investigate the possible presence of endogenous dienedione in urine samples. A high-resolution mass spectrum was obtained for dienedione, where four diagnostic ions of *m/z* 97, *m/z* 133, *m/z* 159 and *m/z* 175 were identified. The proposed fragments were displayed in **Figure 2.5** and well verified by the accurate mass measurements. The measured masses deviated from the theoretical masses by no more than 0.3 ppm (**Table 2.2**). These four transitions were subsequently utilised as multiple reaction monitoring (MRM) transitions to detect any possible presence of endogenous dienedione (if any) in the horse urine samples.

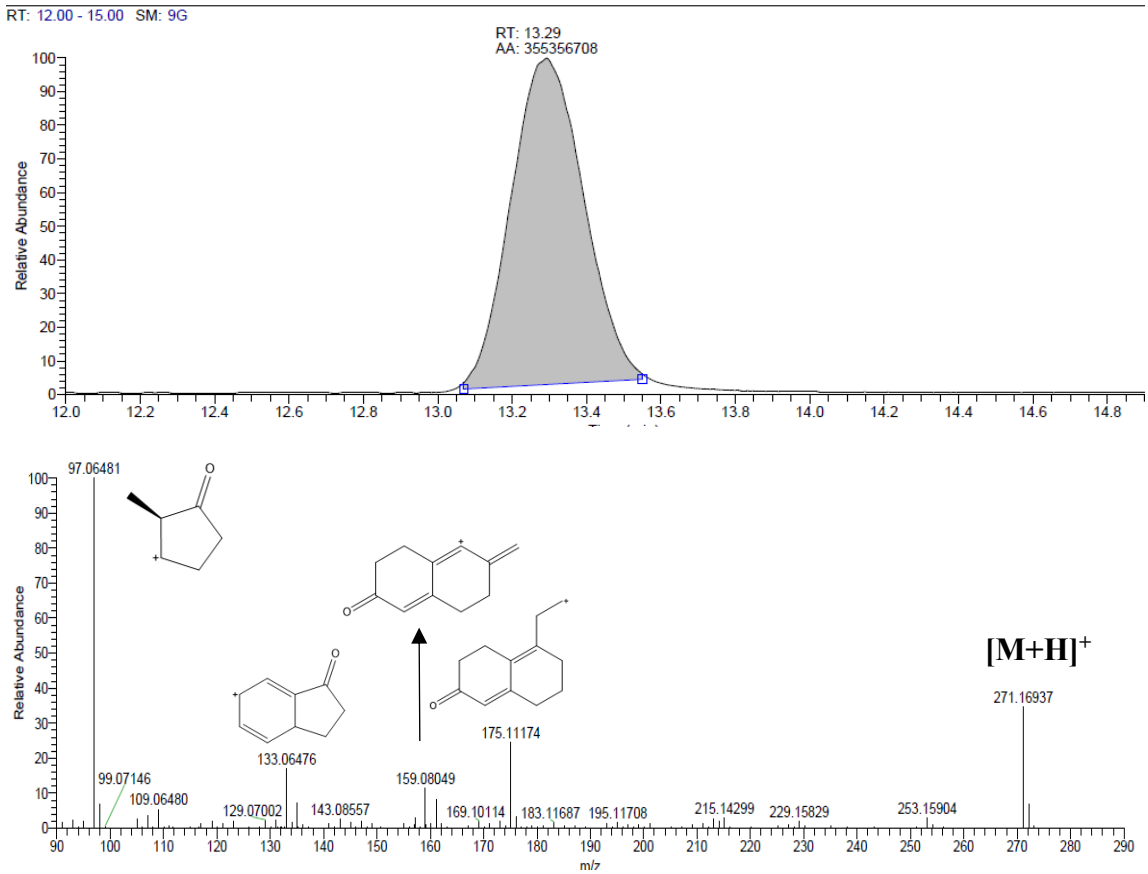


Figure 2.5. (Top panel) Extracted ion chromatogram of dienedione at m/z 271.16937 and (bottom panel) product ion mass spectrum of dienedione with precursor ion at m/z 271.16937

The analysis results of UPLC-MS/MS of an entire male horse urine and a castrated horse urine, depicted in **Figure 2.6**, proved the natural presence of free dienedione in entire male horse urine and no trace of dienedione in castrated horse urine. The relative abundance of the four diagnostic ions identified in the entire male horse sample closely matched those from the reference standard. In addition, the slight difference of 0.02 min in retention times between the chromatographic peaks observed in the standard and the entire male horse urine sample again meets the retention time acceptance criteria

speculated in the *Minimum Criteria for Identification by Chromatography and Mass Spectrometry Criteria* issued by the AORC (AORC, 2024).

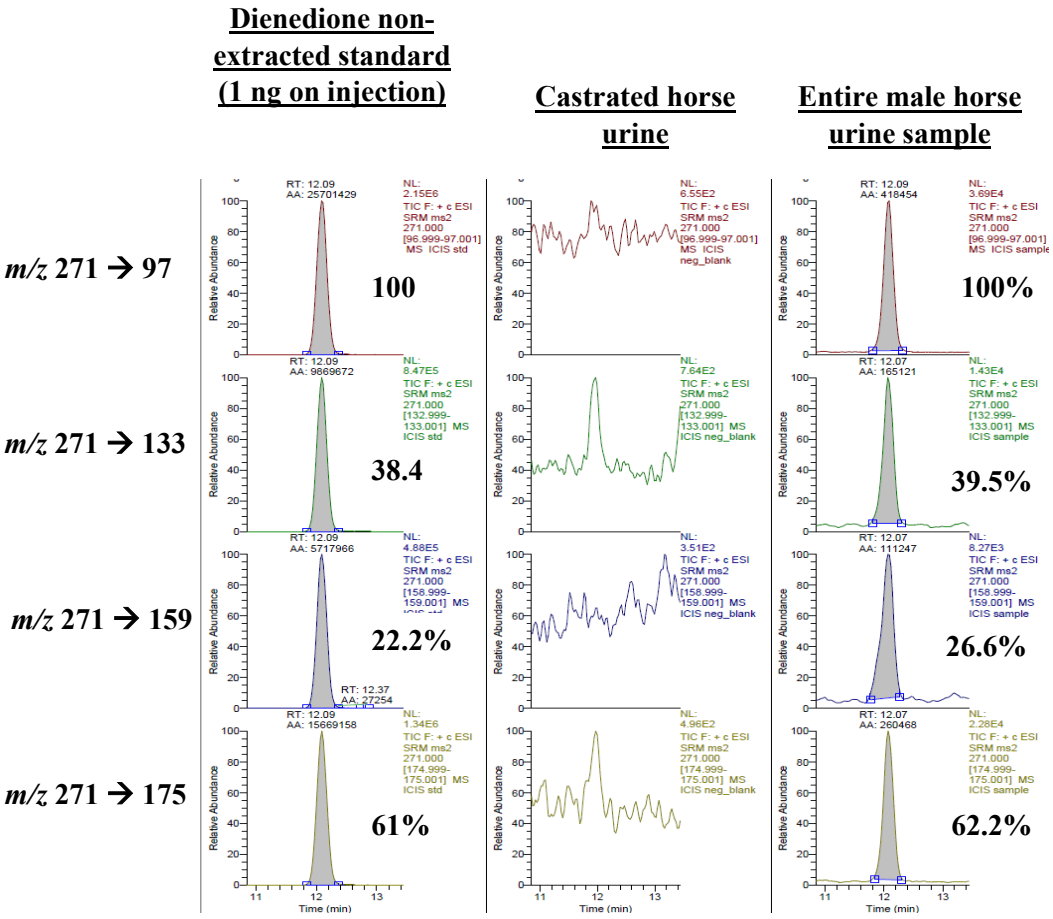
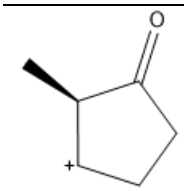
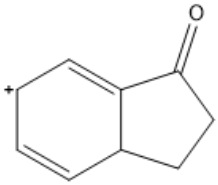
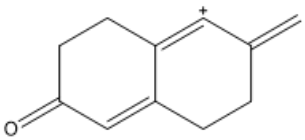
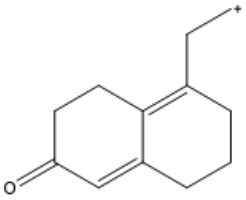


Figure 2.6. Product-ion chromatograms of dienedione using UPLC-MS/MS in (left panel) target standard, (middle panel) castrated horse urine sample and (right panel) entire male horse urine sample

Table 2.2. UPLC-HRMS measurement of dienedione fragment ions

Structure of proposed fragment	Formula	Calculated mass (amu)	Fragments from dienedione	
			Measured mass (amu)	Mass error (ppm)
	C_6H_9O	97.06479	97.06481	0.12716
	C_9H_9O	133.06479	133.06476	-0.22922
	$C_{11}H_{11}O$	159.08045	159.08049	0.02649
	$C_{12}H_{15}O$	175.11174	175.11174	0.01394

2.3.2 Conjugation study of dienedione in entire male horse

In our initial investigation, the amount of dienedione liberated was significantly increased when employing a method involving deconjugation procedures. The major focus was on the evaluation of the form and extent of conjugations. To study the possible conjugations of dienedione, an indirect approach involving fractional deconjugation to release dienedione from various conjugate forms was adopted. Generally, the keto-moiety in the structure of AASs is less prone to conjugation. Given the structure of dienedione, where both C3 and C17 positions are each attached to a keto group, dienedione is theoretically less likely to undergo conjugation. However,

due to the presence of double bonds in the A-ring, possible conjugation at C3 is suggested and depicted in **Figure 2.7**. Enolisation of the 3-keto group is predicted to be favourable due to the stabilisation by the extended conjugation system, allowing glucuronic acid or sulfate to conjugate at the enol group at C3 to form dienedione-3-glucuronide or dienedione-3-sulfate.

Notably, 5-androstene-3,17-dione, another 3-oxo steroid, has been reported to isomerise to 4-androstene-3,17-dione through enolisation of the 3-keto group, a process was characterised by ketosteroid isomerase (Houck and Pollack, 2003; Penning et al., 1981). Therefore, the enolisation of the 3-keto group in dienedione could potentially result in the presence of dienedione-3-glucuronide in the entire male horse. Despite the direct detection of intact glucuronide conjugates of dienedione unsuccessful, the possibility of glucuronide conjugation following enolisation remains plausible, albeit without direct confirmation.

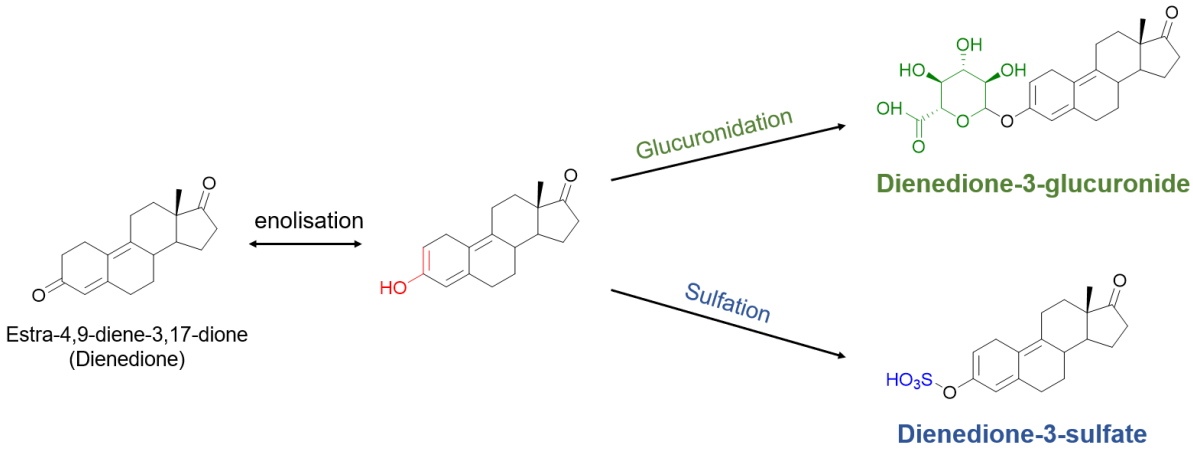


Figure 2.7. A proposed conjugation pathway of dienedione - conjugations at 3-enol moiety of dienedione

Deconjugation of dienedione-3-glucuronide was achieved through enzyme hydrolysis. Beta-glucuronidase sourced from *Patella vulgata* was chosen for enzyme hydrolysis as it catalyses the hydrolysis of glucuronide metabolites to form free dienedione and glucuronic acid. *Patella vulgata* has been noted to exhibit weak sulfatase activity (Gomes et al., 2009). To inhibit the sulfatase activity, potassium phosphate buffer was introduced. Additionally, the activity of *β*-glucuronidase was optimised by adjusting the pH of samples to pH 5.0 (Venturelli et al., 1995). To further assess its activity, the recovery of testosterone from testosterone sulfate following enzyme hydrolysis was investigated. No testosterone was detected from the spikes of testosterone sulfate, while enzyme hydrolysis effectively released testosterone from testosterone glucuronide, achieving over 92 % recovery in all the experiments.

To determine whether dienedione is present as sulfate conjugates in entire male horse urine samples, solvolysis was utilised to release dienedione from dienedione-3-sulfate (if present) by treating it with concentrated sulfuric acid. The solvolysis method followed the procedure described by Kwok *et al* for androsta-1,4,6-triene-3,17-dione and its sulfate conjugates in equine, with slight modifications (Kwok et al., 2015). It was assumed that solvolysis would not release a significant amount of dienedione from dienedione-3-glucuronide. This assumption was confirmed by testing the recovery of testosterone from testosterone glucuronide under solvolysis. No testosterone was detected from the spikes of testosterone glucuronide, whereas solvolysis successfully released over 82 % of testosterone from the testosterone sulfate spikes.

Solvolysis, an acid hydrolysis process, was facilitated by organic solvent (ethyl acetate), along with a salting-out step to drive sulfate conjugates into the organic layer. The ionic strength of the aqueous urine was increased by adding excess sodium chloride powder, which reduced the solubility of the conjugates, forcing them to migrate to the organic

layer. Such a strategy significantly improved the overall efficiency of acid hydrolysis. A similar method was employed by Hauser et al (2008) to hydrolyse sulfate conjugates of 23 endogenous steroids using ethyl acetate under acidic condition with heating at 55 ℃. The optimal solvolysis conditions for dienedione-3-sulfate (if present) were determined by investigating the recovery of testosterone from testosterone sulfate at various temperatures.

The most effective solvolysis condition involved using 3.5 % *v/v* of concentrated sulfuric acid in ethyl acetate with heating at 55 ℃ for 2 hrs, resulting in a recovery rate of 92.4 %, notably higher than that achieved at other temperatures (63.3 % at 65 ℃ and 65.1 % at 75 ℃). The decrease in recovery at higher temperatures was likely due to the degradation of the targeted steroid - testosterone under elevated temperatures (Dumasia and Houghton, 1981). Whilst an alternative acid hydrolysis, methanolysis, could be utilised for deconjugating steroid conjugates after extraction (Tang and Crone, 1989). However, methanolysis caused more severe matrix effects compared to solvolysis. Given the better signal-to-noise ratio obtained, solvolysis was preferred over methanolysis for hydrolysing dienedione sulfate.

Nineteen entire male horse samples were analysed in two batches to assess the form and extent of conjugation of endogenous dienedione. Alongside the test samples, spike controls and calibrators were analysed in parallel. The quantity of dienedione was determined using the most prevalent fragment ion *m/z* 97 of dienedione and *m/z* 149 of the IS (androsta-4,6-diene-3,17-dione). The analysis revealed that glucuronide conjugates constituted at least 60 % of total dienedione (on average 82 %) in each sample. On average, sulfate conjugates and free dienedione contributed approximately 10 % and 8 % of the total dienedione in each sample respectively.

The relative percentages of different forms of dienedione in the 19 samples (**Figure 2.8**), indicated that glucuronide conjugate was the dominant form of dienedione in equine urine from entire male horse. The mean concentrations of free dienedione, dienedione released from glucuronide form, dienedione released from sulfate form and total dienedione were 0.3 ng/mL, 2.5 ng/mL, 0.2 ng/mL and 3.0 ng/mL respectively across the 19 entire male horse urine samples. The concentration of total dienedione varied across the 19 samples, with a standard deviation of 2.7 ng/mL. The concentration of total dienedione ranged from 0.2 to 11 ng/mL. Control experiments involving enzyme hydrolysis and solvolysis of the respective testosterone conjugates showed average recoveries of 93.3 % and 84.9 %, as presented in **Table 2.3**.

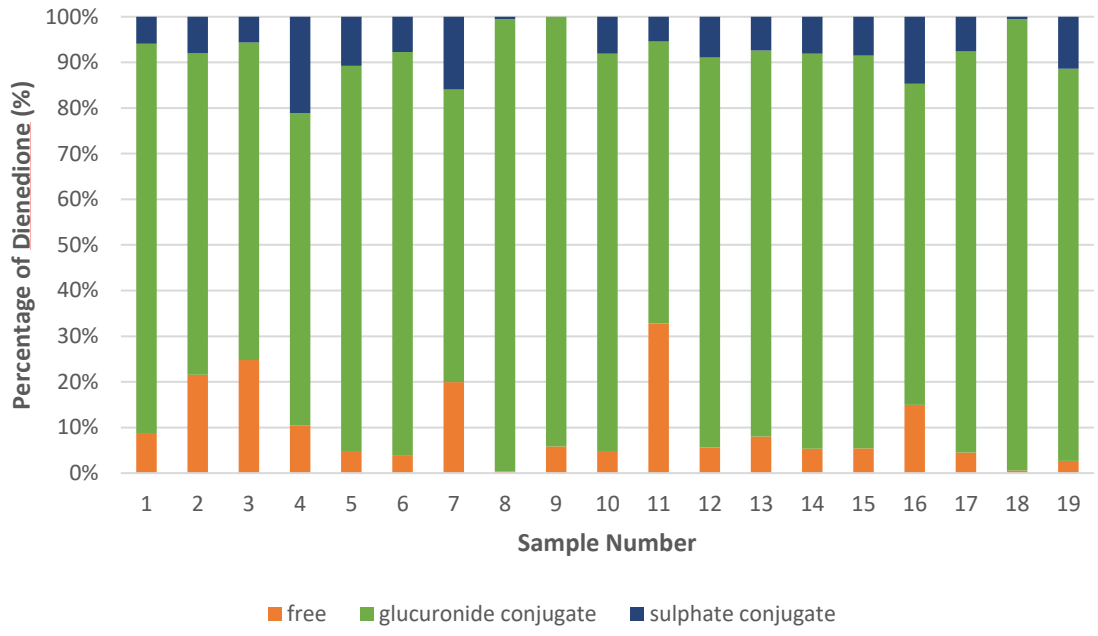


Figure 2.8. Results of conjugation study of possible phase II metabolites of dienedione in entire male horse urine (n =19)

Table 2.3. Recovery of enzyme hydrolysis and solvolysis in conjugation study of dienedione

	Testosterone released from their conjugates (at equivalent 20 ng/mL of free testosterone) (ng/mL)	Average Recovery (%) (n =2)
Enzyme hydrolysis	18.7	93.3
Solvolysis	17.0	84.9

2.3.3 Population study of free and glucuronide-conjugated dienedione in entire male horse urine

It is important to highlight that significant interferences were observed in the extracted-ion chromatograms of dienedione released from its sulfate conjugates, resulting in a relatively poor signal-to-noise ratio for the detected dienedione. These interferences were likely caused by sample degradation under the drastic acid hydrolysis conditions. To ensure a better controlled experiment condition, a quantification method was developed for free and glucuronide forms of dienedione. This measurand (free and glucuronide conjugates of dienedione) was used to establish the proposed threshold and future evaluation regarding whether dienedione in a sample surpasses the threshold must be based on the same measurand, similar to the international threshold set for 5α-estrane-3β,17α-diol in entire male horse urine (IFHA, 2022).

A population study of free and glucuronide-conjugated dienedione in entire male horse urine (n = 175) has been performed. To enhance throughput and streamline the experimental procedures, a fully automated SPE was employed. The 175 post-race

entire male horse urine samples (117 from local runners and 58 from overseas horses) were quantified in 12 batches (from 141 different entire male horses) using UPLC-MS/MS.

Each analytical batch comprised 7 levels of calibrators and 3 levels of QCs. A correlation analysis using linear regression of dienedione concentrations was conducted. The goodness of fit (r^2) and correlation coefficient (r) of every batch exceeded 0.994 and 0.997 respectively, indicating good linearity of the fitted calibration curve. The concentrations of free and glucuronide-conjugated dienedione present in entire male horse ranged from 0.08 to 29.6 ng/mL, with a mean ± SD of 2.5 ± 3.5 ng/mL. Two out of 175 samples had concentrations exceeding the mean ± 3SD. The highest level of free and glucuronide-conjugated dienedione, 29.6 ng/mL, was found in an overseas entire male horse sample, approximately 12 times the mean value. There is a possibility that this sample is either abnormal or has prior exposure to dienedione. Grubb's test identified 29.6 ng/mL as an outlier in the population. Among those horses being sampled, nine local horses were sampled more than one occasion. Results were summarised in **Table 2.4**. Dienedione was present in all samples, with consistent levels of free and glucuronide conjugated dienedione among individual horses ranging within several ng/mL. Such findings further supported the endogenous nature of dienedione in entire male horse urine.

The LoQ of dienedione was estimated to be 0.8 ng/mL (10 times the SD at control level 0.5 ng/mL, n = 12). The accuracy of QCs at 0.5, 2 and 5 ng/mL were 91.5 %, 105 % and 99.3 % (**Table 2.5**). The intra-day method precision for PAR at each QC level ranged from 3.5 % to 17.3 %, while the precision for RRT was between 0.07 % and 0.4 %. The inter-day method precision, expressed as pooled % RSD, for the RRT across QC levels ranged from 0.09 % to 0.3 % and that for the PAR ranged from 5.2 % to

12.4 %, which were considered sufficient for quantitative analysis (**Table 2.5**). The extraction recoveries of dienedione at different QC levels ranged from 75.9 % to 78.3 %. No correction of recovery was required, as ISs were used to compensate for losses during the extraction process.

Table 2.4. Concentration of free and glucuronide-conjugated dienedione in urine samples collected from local horses which being sampled on different occasions

Horse name	**Race date (DD/MM/YY)**	**Concentration (ng/mL)**
Horse 1	06/09/2020	0.8
	06/09/2020	1.3
	22/11/2020	1.5
	01/01/2021	0.9
	13/03/2021	2.3
	25/04/2021	1.4
Horse 2	10/02/2021	2.1
	05/05/2021	2.9
Horse 3	01/11/2020	1.4
	01/01/2021	0.2
	24/02/2021	1.7
	05/04/2021	1.0
	23/05/2021	0.6
Horse 4	02/05/2021	4.1
	23/05/2021	2.3
Horse 5	21/02/2021	1.1
	23/05/2021	1.1
Horse 6	21/03/2021	1.9
	02/05/2021	2.6

Horse 7	11/10/2020	7.0
	01/11/2020	7.1
	06/01/2021	2.0
	10/02/2021	1.8
	10/03/2021	2.6
	04/07/2021	6.6
Horse 8	13/12/2020	6.6
	01/01/2021	5.8
Horse 9	09/12/2020	0.7
	01/01/2021	1.1

Table 2.5. Precision data and extraction recoveries for dienedione at different QC levels

Concentration (ng/mL)	Accuracy (n = 6, %)	Day	Precision (% RSD)			
			PAR		RRT	
			Intra-day	Inter-day	Intra-day	Inter-day
0.5	91.5 ± 12.0	1st	17.3	12.4	0.4	0.3
		2nd	8.0		0.3	
		3rd	10.2		0.2	
		4th	12.3		0.1	
2.0	105 ± 3.6	1st	3.5	5.2	0.07	0.09
		2nd	8.5		0.09	
		3rd	3.5		0.1	
		4th	3.6		0.08	
5.0	99.3 ± 3.3	1st	7.7	6.6	0.1	0.2
		2nd	5.1		0.3	
		3rd	8.9		0.1	
		4th	3.6		0.08	

Statistical analysis was conducted to access the normality of the population data, with one outlier excluded by Grubb's test. Kolmogorov-Smirnov test ($p > 0.05$) was used to determine the skewness of data. The untransformed data exhibited a skewed distribution and did not fit a normal distribution, hence various transformations of the data were performed. These included logarithmic, natural logarithmic, exponential, square root, third root, fourth root and fifth root transformation. Only the data from fifth root transformation fitted a normal distribution. As shown in **Figure 2.9**, the fifth root-transformed data had a p value of 0.073 ($p > 0.05$), indicating it followed a normal distribution. A possible threshold could then be established based on the untransformed 'mean + 3.72 SD' value of 27.1 ng/mL, representing a risk of 1 in 10,000 (assuming the degree of freedom to be infinity) for a normal sample to exceed this threshold. A 'rounded-up' threshold of 30 ng/mL of free and glucuronide-conjugated dienedione in entire male horse urine samples was proposed, following the Guidelines for deriving thresholds published by the AORC and other thresholds defined for equines (AORC, 2016; Bonnaire et al., 2000; Ho et al., 2006; Ho et al., 2010; Ho et al., 2015a; Ho et al., 2015b; Houghton and Crone, 2000). The risk associated with this threshold was approximately 1 in 14,269, with one outlier as per Grubb's test and a degree of freedom of 173.

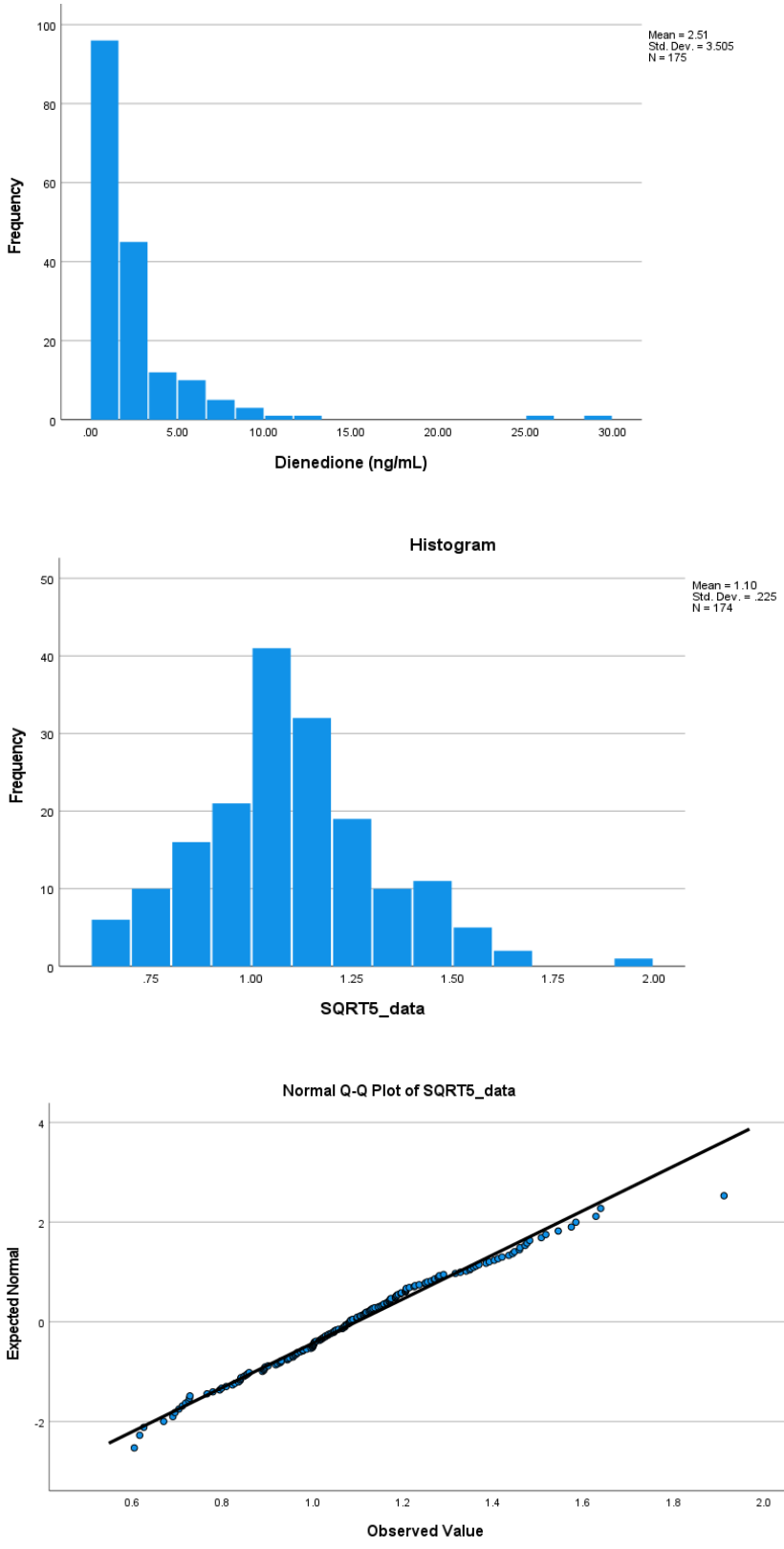


Figure 2.9. Statistical analysis of population study of free and glucuronide-conjugated dienedione: (top panel) histogram of free and glucuronide-conjugated dienedione data in entire male horses, (middle panel) histogram of free and glucuronide-conjugate

Random castrated horse urine samples (n = 20) were also analysed, and no free dienedione or glucuronide-conjugated dienedione was detected. This confirmed that dienedione was detected only in entire male horses but not in castrated horse. Therefore, the endogenous nature of dienedione in entire male horses was confirmed and the potential misuse of dienedione in entire male horses should be controlled by a threshold. To set up an internationally agreed threshold for dienedione, ring tests across laboratories and data from various regions will be sought in the future.

2.4 Conclusion

The natural presence of dienedione was first detected in entire male horse using UPLC-MS/MS. A conjugation study (n = 19) on phase II metabolites of dienedione was conducted, revealing that the extent of dienedione in the glucuronide-conjugated fraction accounted for at least 60 % of total dienedione in each sample. It is predicted that most dienedione present in entire male horse is glucuronide-conjugated at the C3 enol moiety (dienedione-3-glucuronide). While glucuronide conjugation following enolisation is a possible explanation, it could not be confirmed by direct analysis of intact dienedione glucuronide in entire male horse urine samples. A population study on free and glucuronide-conjugated dienedione in entire male horse urine samples (n = 175, from 141 different entire male horses) was conducted to establish an in-house threshold for dienedione. The urinary dienedione levels ranged from 0.08 to 29.6 ng/mL, with a mean ± SD of 2.5 ± 3.5 ng/mL. The estimated LoQ for the quantification method was 0.8 ng/mL.

An in-house threshold of 30 ng/mL of free and glucuronide-conjugated dienedione in entire male horse urine samples was proposed with a risk factor of 1 in 14,269 (with a

degree of freedom of 173 and one outlier excluded by Grubb's test). This threshold could be used to regulate the misuse of dienedione in entire male horses. The quantitative method developed for the population study has good accuracy, precision and extraction recovery (AORC, 2022). The results validated the reliability of the UPLC-MS/MS for quantifying low levels of dienedione, which could be extended to study other endogenous steroids in equine. Endogenous origin of dienedione was further supported by consistent and similar levels of dienedione found in entire male horse urine samples collected on different days post-race from the same horse. To establish an appropriate international threshold for dienedione in entire male horses, a collaborative ring test and additional data on basal dienedione levels from a substantial number of untreated entire male horses across diverse geographical regions would be essential.

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Chapter 3. *In vivo* metabolism of dienedione in horses

3.1 Introduction

In the previous chapter, dienedione was determined to be endogenous in entire male horse urine upon investigation, where an in-house threshold was established to control its misuse. While dienedione remains exogenous in castrated horse, it could still be reported with zero-tolerance if detected. To effectively regulate the illicit use of dienedione in castrated horses, it is essential to identify its *in vivo* metabolites and to study its elimination. Over the years, extensive research has been conducted on the metabolism of exogenous AASs to combat their potential misuse and uphold the integrity of horseracing (Cawley et al., 2016; Choi et al., 2018; Clarke et al., 2011; Harding et al., 2022; Ho et al., 2005; Ho et al., 2007a; Ho et al., 2007b; Kwok et al., 2013; Leung et al., 2013; Viljanto et al., 2024; Waller et al., 2016; Waller et al., 2020; Wong et al., 2015). In an early study by Scarth et al. (2010a), *in vitro* metabolism of dienedione was first reported in equine, human and canine models. A total of 11 metabolites were detected in equine liver S9 tissue employing liquid chromatography-high resolution mass spectrometry (UPLC-HRMS). Dienedione possibly transforms into various metabolites such as 17-hydroxy-estra-4,9-dien-3-one (proposed major metabolite), hydroxylated dienedione, hydroxylated 17-hydroxy-estra-4,9-dien-3-one and estra-4,9-diene-3,17-diol. Nonetheless, conducting an *in vivo* administration study remains the staple method to elucidate the actual biotransformation of dienedione. Along with understanding dienedione metabolism in castrated horses, it is essential to investigate its elimination in plasma and urine.

This chapter described the doping control of dienedione in castrated horses. The *in vivo* metabolism study and elimination of dienedione was conducted using UPLC-HRMS,

with the goal to identify the most appropriate target for regulating the potential misuse of dienedione in castrated horses.

3.2 Methods

3.2.1 Materials

Dienedione was sourced from Steraloids (Newport, RI, USA) and Chromadex (Longmont, USA). Dienedione (65 g) needed for administration trial was procured from Hubei VanzPharm Co. Ltd (Wuhan, China), and was verified with the reference standard from Steraloids. d_3-Boldenone was purchased from National Measurement Institute (Sydney, Australia). Methanol (LC-MS Chromasolv) was acquired from Riedel-de Haen (Seelze, Germany). Chloroform (GR grade), potassium phosphate, diisopropyl ether (Emsure®), n-hexane (GR grade), ethyl acetate (GR grade), sodium chloride (GR grade), *tert*-butyl methyl ether (TBME, LiChrosolv®) and methanol (LiChrosolv®) were purchased from Merck (Darmstadt, Germany). Sodium hydroxide (pellets, analytical grade) was obtained from Sigma Aldrich (St. Louis, MO, USA). Ammonium formate (extra pure, 97 %) was sourced from International Laboratory Limited (San Bruno, CA, USA). Beta-glucuronidase (β-glucuronidase, *from Patella vulgata*, lyophilised powder) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anhydrous methanolic hydrogen chloride used for methanolysis was prepared following the procedures reported previously (Tang and Crone, 1989). Abs Elut Nexus cartridges (60 mg, 3 mL) were obtained from Varian (Palo Alto, CA, USA). Isolute SLE cartridges were supplied by Biotage (Uppsala, Sweden). Deionised water was produced from an in-house water purification system (Milli-Q, Molsheim, France).

3.2.2 Drug administration studies

Three thoroughbred castrated horses were each administered 1500 mg of dienedione (obtained from Hubei VanzPharm) once daily for seven consecutive days. Dienedione powder was resuspended in water and given to the horses through stomach tubing. Urine and blood samples were collected before 1st, 4th and 7th administration, and 1, 2 and 6 hrs after last administration, then once per day on Days 8, 9, 10, 11, 12, 14, 17, 20, 23 and 27. Prior to the commencement of the administration experiments, approval was granted by the Animal Ethics Committee of the Hong Kong Jockey Club.

3.2.3 Extraction procedures

3.2.3.1 Identification of dienedione metabolites in post-administration urine samples

Urine (3 mL) was transferred to 15-mL corex tube and centrifuged at 1500 *g* for 10 min. The supernatant (2 mL) was then pipetted into a 15-mL plastic tube. Phosphate buffer (0.1 M, pH 6.0, 1 mL) was added to adjust the pH to 5.0. d_3-Boldenone (at a final concentration of 5 ng/mL in sample) was spiked in the urine samples as an IS. β-glucuronidase (*Patella vulgata*, 18,000 units/mL, 330 μL) was added for enzyme hydrolysis. The mixture was incubated at 65 ℃ for 2.5 hrs. After incubation, the hydrolysate was allowed to cool to ambient temperature, then centrifuged at 1500 *g* for 5 min before being loaded onto an Abs Elut Nexus cartridge. The cartridge was pre-conditioned with methanol (2 mL) and water (2 mL) prior to sample loading. The sample was then washed with deionised water (2 mL) and hexane (2 mL), followed by a 2-min drying step. All SPE drying steps were carried out using nitrogen purge at 20 psi. The cartridge was eluted with chloroform (4 mL). The eluate was transferred to a 15 mL graduated centrifuge tube containing NaOH/NaCl (1 M/0.15 M, 2 mL) and

vortexed for 0.5 min, then centrifuged at 1500 *g* for 0.5 min. The organic layer was passed through a Pasteur pipette packed with cotton wool. The extract was evaporated to dryness at 65 ℃ under nitrogen and the residue was reconstituted with ammonium formate (5 mM, pH 3.0)/methanol (70:30, *v/v*) (50 μL) for UPLC-HRMS analyses.

3.2.3.2 Dienedione in post-administration blood samples

Plasma (1 mL) was loaded onto an Isolute SLE cartridge with a 5 min holding time before elution. The cartridge was then eluted with methanol/diisopropyl ether (5:95, *v/v*, 3 mL). The eluate was dried under nitrogen at 25 ºC, and the residue was reconstituted with methanol (50 μL) for UPLC-HRMS analyses.

3.2.3.3 Determination of conjugation forms of dienedione and its metabolites in post-administration urine samples

a) Unconjugated metabolites

Urine (2 mL) was adjusted to pH 7.0 by adding phosphate buffer (0.1 M, pH 6.0, 1 mL) and then extracted with TBME (4 mL). The organic layer was transferred to a 15 mL graduated centrifuge tube containing NaOH/NaCl (1 M/0.15 M, 2 mL). The mixture was vortexed for 0.5 min and centrifuged at 1500 *g* for 0.5 min. The organic layer was then passed through a Pasteur pipette packed with cotton wool. The extract was evaporated to dryness at 65 ℃ under nitrogen, and the residue was reconstituted with ammonium formate (5 mM, pH 3.0)/methanol (70:30, *v/v*) (100 μL) for UPLC-HRMS analyses.

b) Glucuronide-conjugated metabolites

The aqueous portion from ***Section 3.2.3.3 (a)*** was subsequently adjusted to pH 5.0 and incubated with β-glucuronidase (*Patella vulgata*, 18,000 units/mL, 330 µL) for 2.5 hrs. The enzyme-hydrolysed metabolites were extracted following the procedures for unconjugated metabolites.

c) Sulfate-conjugated metabolites

Following the enzyme hydrolysis (***Section 3.2.3.3 (b)***), the resulting aqueous layer was treated by loading it on an ABS Elut Nexus SPE cartridge. It underwent a washing step with deionised water (2 mL) and hexane (2 mL), followed by a 2-min drying. Subsequently, the cartridge was eluted with chloroform (2 mL) and methanol/ethyl acetate (5:95, *v/v*, 2 mL). The combined eluate was evaporated to dryness at 65 ºC under nitrogen. Anhydrous methanolic hydrogen chloride (1 M, 0.5 mL) was added, and the solution was heated for 15 min. Next, diisopropyl ether (4 mL) was added and the mixture was transferred to a 15 mL graduated centrifuge tube containing NaOH/NaCl (1 M/0.15 M, 2 mL). The mixture was vortexed for 0.5 min and centrifuged at 1500 *g* for 0.5 min. The organic layer was passed through a Pasteur pipette packed with cotton wool. The extract was then evaporated to dryness at 65 ºC under nitrogen and the residue was reconstituted with ammonium formate (5 mM, pH 3.0)/methanol (70:30, *v/v*) (100 µL) for UPLC-HRMS analyses.

3.2.3.4 Quantification of dienedione in post-administration samples

a) Free and glucuronide-conjugated dienedione in post-administration urine samples

Urinary free and glucuronide-conjugated dienedione was extracted following the methods outlined in ***Section 3.2.3.1***. d_3-Boldenone (final concentration of 5 ng/mL in the sample) was spiked in the urine as an IS.

b) Dienedione in post-administration plasma samples

Plasma (1 mL) was loaded onto an Isolute SLE cartridge and held for 5 min before elution. The cartridge was then eluted with methanol/diisopropyl ether (5:95, *v/v*, 3 mL). The eluate was dried under nitrogen at 25 ºC, and the residue was reconstituted with methanol (50 µL) for UPLC-HRMS analyses. The internal standard androsta-4,6-diene-3,17-dione (final concentration of 5 ng/mL in the sample) was spiked prior to extraction.

3.2.3.5 Preparation for calibrators and quality controls for quantification of free and glucuronide-conjugated dienedione in urine and free dienedione in plasma

Calibrators and quality controls were prepared independently by weighing different amounts of the drug standard. Calibrators were prepared in duplicates at 0, 0.2, 0.5, 1, 2, 5, 10, 20 and 40 ng/mL in castrated horse urine. Additionally, quality control samples were also prepared in duplicates, which were spiked with dienedione at 0.2, 2, 5 and 20 ng/mL in urine. In plasma, calibrators were prepared in duplicates at concentrations of 0, 0.1, 0.2, 0.5, 1, 2 and 5 ng/mL, with quality control samples spiked in duplicates with dienedione at 0.1, 0.5 and 2 ng/mL. The calibrators and QC urine or plasma samples were analysed alongside pre- and post-administration urine or plasma samples. The PARs of the target to IS were plotted against the calibrator concentrations and fitted

using linear regression to generate the calibration curve. The responses from all quantifications (***Section 3.2.3.4***) were linear across the concentration ranges of each study, each with r values exceeding 0.999. The measured concentrations of the QC samples showed a deviation of less than ± 15% (± 20 % for the lowest QC) from their spiked values.

3.2.4 Instrumentation

UPLC-MS/MS analyses were conducted on a Waters Acquity UPLC system capable of withstanding pressures up to 15,000 psi, interfaced to a TSQ Quantum Ultra mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA). In addition, UPLC-HRMS analyses were performed employing a Thermo Scientific Q Exactive mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) equipped with a HESI-II source interfaced with the same UPLC system.

3.2.4.1 UPLC condition

The sample tray of the autosampler was maintained at 15 °C. A reverse-phase Acquity UPLC BEH C18 column (Acquity, 100 mm length x 2.1 mm ID; 1.7 µm particle size) was utilised to separate target analytes. Mobile phase A was ammonium formate in deionised water (pH 3, 5 mM) and mobile phase B was methanol. A linear gradient was run at a flow of 300 µL/min, starting with 90 % solvent A at 0 min, reducing to 58 % at 2.0 min then held at 58 % until 20 min, decreasing to 2 % solvent A at 21 min. The gradient returned to 90 % solvent A at 24 min and held steady until 27 min before the subsequent injection.

3.2.4.2 MS conditions

Injection volumes of 10 µL were used, and the HESI source operated in SRM positive ion mode with a spray voltage of 2 kV. The capillary and vaporizer temperature were set at 320 ℃ and 350 ℃ respectively. The pressures for the sheath, auxiliary and ion sweep gas were set at 50, 10 and 2 arbitrary TSQ units. The resolution of the quadrupole mass filter was set with a peak width of 0.7 amu (FWHM) for both Q1 and Q3. The collision gas pressure in Q2 was set at 1.2 mTorr. The following ion transitions were monitored: protonated dienedione (*m/z* 271 → 97), IS androsta-4,6-diene-3,17-dione (*m/z* 285 → 149; for conjugation study) and IS d_3-boldenone (*m/z* 290 → 121; for quantification) were monitored. The CID energies and radio frequencies were set as follows: 26 eV, 85 V for dienedione; 21 eV, 92 V for androsta-4,6-diene-3,17-dione; and 28 eV, 90 V for d_3-boldenone.

3.2.4.3 HRMS conditions

Sample ionisation was carried out in positive ionisation mode using HESI-II, with a capillary temperature of 350 ℃, a sheath gas flow of 50 arbitrary units, and an auxiliary gas flow of 10 arbitrary units. The sweep gas flow was set to 2 arbitrary units with curtain plate in place. The ion spray voltage was approximately 3 kV, and the S-Lens RF level was set to 40 %. Full-scan mass spectra were obtained with a mass resolution of 35,000 (FWHM at *m/z* 200). The maximum injection time was 100 ms, and the automatic gain control (AGC^{TM}) was configured at 3e6. For the acquisition of product ion mass spectra, a mass resolution of 17,500 (FWHM at *m/z* 200) was employed with an isolation window of 1 amu. The maximum injection time was 100 ms, with AGC^{TM} set at 5e5. Nitrogen (purity > 99.995 %) was used as the Higher-energy Collisional

Dissociation (HCD) collision gas. Data processing was conducted employing the Thermo Finnigan Xcalibur software (Version 2.2) with a mass tolerance window of ± 5 ppm. The measured masses of all identified product ions in this study were within ± 5 ppm of their theoretical values.

An internal lock mass solution containing benzyldimethylphenylammonium chloride (*m/z* 212.14338 for positive mode) at a concentration of 0.1 ng/µL in deionised water was introduced into the system post column at a flow rate of 20 µL/min through a T-joint using a LC-20AB Solvent Delivery Unit (Shimadzu Corporation, Kyoto, Japan). The UPLC-HRMS parameters for precursor ion and NCE can be found in **Table 3.1**.

Table 3.1. The UPLC-MS/HRMS parameters for precursor ion and NCE

Targets	**Precursor ion (*m/z*)**	**NCE (%)**
Dienedione (Parent)	270.16143	55
17-hydroxy-estra-4,9-dien-3-one (M1)	272.17708	25
Hydroxylated dienedione (M2 to M5)	286.15635	45
Hydroxylated 17-hydroxy-estra-4,9-dien-3-one (M6 to M8)	288.17200	45

3.3 Results and discussion

3.3.1 Phase I metabolic studies of dienedione in castrated horses

Possible metabolites of dienedione detected through UPLC-HRMS were inferred by comparing chromatograms acquired from urine samples collected before and after administration. The detected metabolites should not exhibit a deviation of more than 5 ppm from the postulated structures. Ions were extracted based on the theoretical molecular masses of the metabolites derived from potential biotransformations such as reduction, hydroxylation, hydrogenation, or combinations thereof. Ions meeting these criteria were selected for further UPLC-MS/HRMS analyses to generate product-ion mass spectra for structural interpretation. The structures of all metabolites were provisionally detected through mass spectral elucidation due to the absence of corresponding reference standards for conclusive structural confirmation.

Dienedione and fourteen metabolites (M1a, M1b, M2a, M2b, M3a, M3b, M4, M5, M6a, M6b, M7a, M7b, M8a and M8b) were detected in the post-administration urine samples according to the specified criteria. The structures of these metabolites and the postulated biotransformation pathway of dienedione were shown in **Figure 3.1**. The extracted-ion chromatogram for the metabolites detected in a post-administration urine sample was presented in **Figure 3.2**. It included a pair of 17-hydroxy-estra-4,9-dien-3-one epimers (M1a and M1b), hydroxylated 17-hydroxy-estra-4,9-dien-3-one (M6a, M6b, M7a, M7b, M8a and M8b), as well as hydroxylated dienedione (M2a, M2b, M3a, M3b, M4, M5).

3.3.1.1 Metabolite M1

M1a, a mono-reduced metabolite, was detected as the major metabolite of dienedione, showing the most abundant peak intensity in the extracted-ion chromatogram. The reduction site of metabolite M1 was found to occur at position C17, which aligns with findings from a previous *in vitro* study (Scarth et al., 2010a). Characteristic fragments *m/z* 159 and 215, observed in the parent drug spectrum, were also present in the M1a spectrum, suggesting that the A/B/C ring structure of M1a was very likely identical to that of the parent drug (**Figure 3.3**). This provides strong evidence that the reduction did not take place at position C3. Furthermore, four product ions at *m/z* 67, 81, 95 and 119 served as diagnostic fragments, signifying that the reduction occurred at D-ring. M1b, another later eluting mono-reduced form, was also observed (refer to **Figure 3.2**), and due to significant resemblance between the mass spectra of M1a and M1b, they were assigned to be a pair of 17-epimers. Despite indications from a previous *in vitro* study suggesting that dienedione could be reduced to 3-hydroxy-estra-4,9-dien-17-one at position C3 (Scarth et al., 2010a), this specific metabolite was not detected in this *in vivo* study.

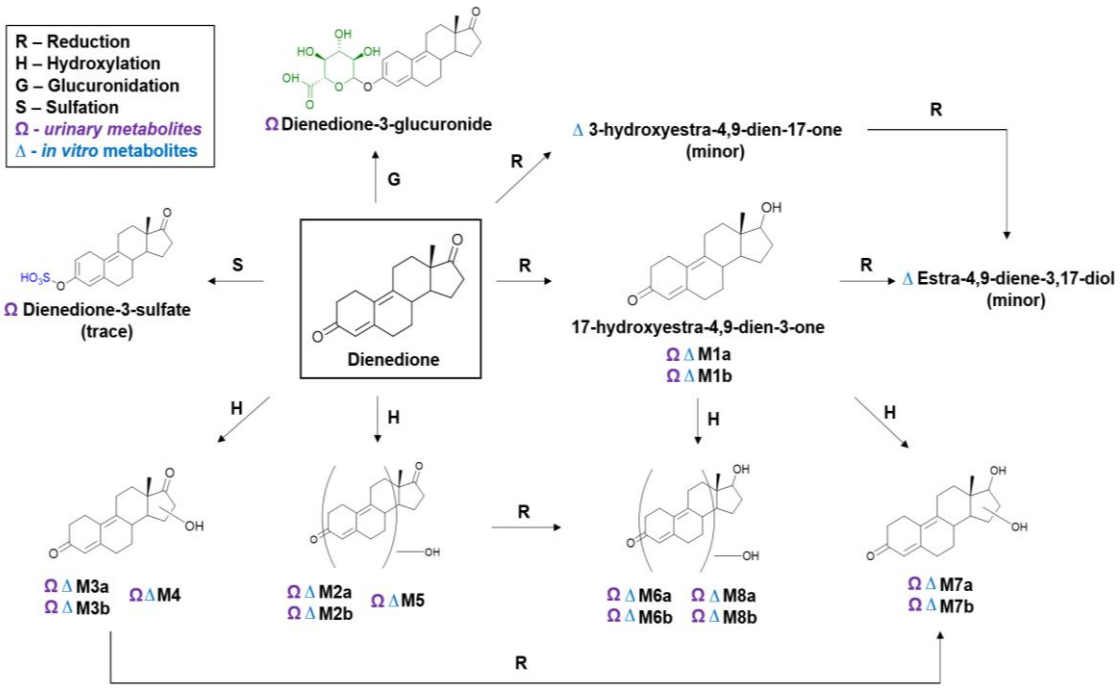

Figure 3.1. Chemical structure of the *in vitro* and *in vivo* metabolites and the postulated metabolic pathway of dienedione in castrated horses

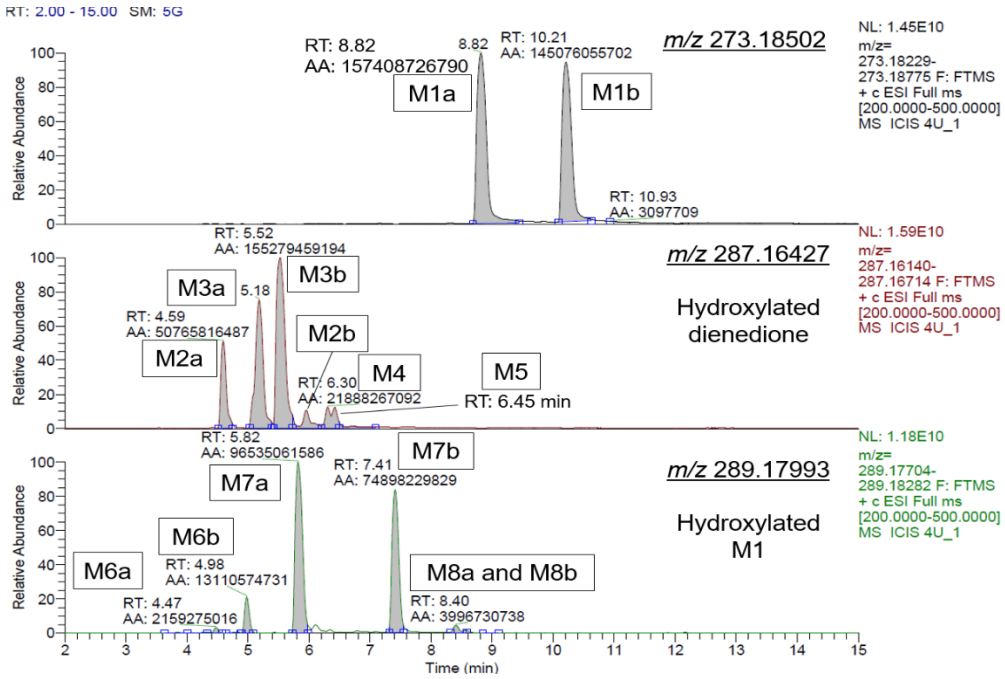

Figure 3.2. Extracted-ion chromatogram of dienedione metabolites M1 to M8 detected in a 1.5-hr post-administration urine sample

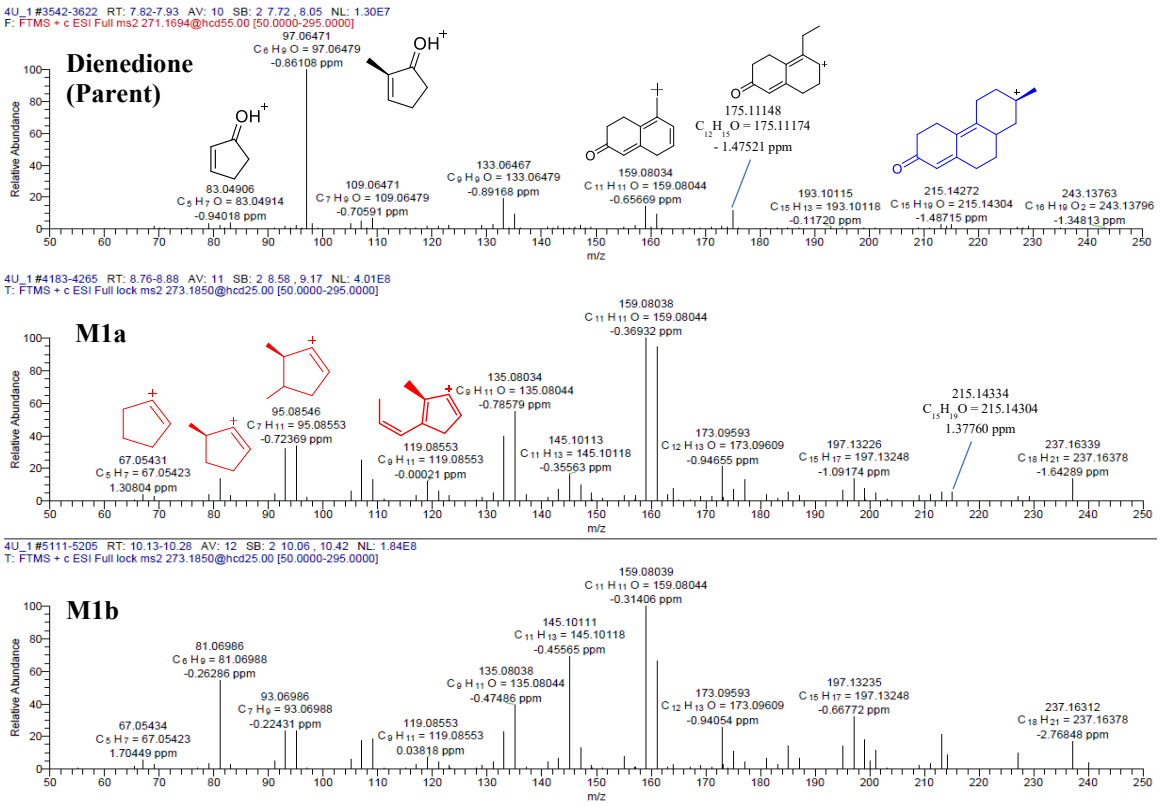

Figure 3.3. Comparison of product-ion mass spectra of parent drug and metabolite M1a and M1b detected in a 1.5-hr post-administration urine sample

Metabolite M1 could undergo hydroxylation to form mono-reduced-hydroxy-metabolites M6a, M6b, M7a, M7b, M8a and M8b, collectively referred to as hydroxylated M1. Metabolites M6a/b, M7a/b and M8a/b were epimer pairs. The site of hydroxylation for M6 and M8 could not be definitively determined from the mass spectral data, while the hydroxylation site for metabolite M7 was likely on the D-ring. The fragment ions *m/z* 175 and 214 observed in the product ion spectrum of M7 (**Figure 3.4**) were characteristic for D-ring hydroxylation (Scarth et al., 2010a). The presence of *m/z* 175 has ruled out the possibility of hydroxylation on A/B/C-ring, as this fragment ion is common in the mass spectra of the parent drug. In addition, product ion *m/z* 214 was diagnostic for cleavage between C14-15 and C13-17. In contrast, the absence of

both diagnostic ion *m/z* 175 and 214 in the spectra of metabolites M6 and M8 (**Figures 3.5 and 3.6)** suggests that hydroxylation in M6 and M8 likely occurred at A/B/C ring. Among all the mono-reduced-hydroxy-metabolites (M6 to M8), metabolites M7a and M7b showed higher abundance, indicating that D-ring hydroxylation was the predominant pathway. Such observation aligns with an *in vitro* study (Scarth et al., 2010a). In fact, D-ring hydroxylation at C15 and C16, is a more common metabolic pathway in comparison to A/B/C-ring hydroxylation in horses (Houghton, 1992; Houghton and Dumasia, 1979; Kwok et al., 2015; McKinney, 2009; McKinney et al., 2004; Scarth et al., 2010a; Scarth et al., 2010b; Scarth et al., 2011). Moreover, other transformations could contribute to the production of M6 to M8. For instance, M6a/b and M8a/b could arise from M2a/b and/or M5, while M7a/b could arise from M3a/b and/or M4.

3.3.1.3 Metabolite M4 and M5

Dienedione, the parent drug, could also undergo hydroxylation to hydroxy metabolites. Six mono-hydroxylated dienedione, namely M2a, M2b, M3a, M3b, M4 and M5, were identified. The mass spectra of metabolites M4 and M5 from this *in vivo* study closely resemble those of the previous *in vitro* study (Scarth et al., 2010a). Diagnostic product-ions *m/z* 175 and 214 representing a C15/16-hydroxylated metabolite were observed in the product-ion scan of metabolite M4. Two other A/B ring fragments *m/z* 133 and 159 found in parent drug mass spectra were also present in M4 mass spectra, which ruled out the possibility of A/B-ring hydroxylation (**Figure 3.7**). Furthermore, the D-ring hydroxylation was further supported by characteristic ion *m/z* 95. On the contrary, the mass spectrum of metabolite M5 lacked the aforementioned characteristic fragments

(**Figure 3.7**), hence suggesting that the hydroxylation occurred somewhere on the A/B/C-ring.

3.3.1.4 Metabolite M2

Metabolite M2a, M2b, M3a and M3b were reported for the first time as metabolites of dienedione. M2a and M2b were proposed to be a pair of epimers based on the similarity of their mass spectra (**Figure 3.8**). The possibility of D-ring hydroxylation was ruled out due to the presence of fragment ion *m/z* 97 (a D-ring fragment also appearing in the parent's spectrum) and the absence of characteristic ions *m/z* 95, 175 and 214, which are indicative of D-ring hydroxylation. This suggests that hydroxylation should have occurred at A/B/C-ring. Theoretically, C-ring hydroxylation was considered a possible pathway, supported by the presence of product ion *m/z* 173 and the absence of *m/z* 175. In comparison to the product-ion mass spectrum of M4 (**Figure 3.7**), fragment ion *m/z* 175 was missing while *m/z* 173 was present in the product-ion mass spectra of M2 (**Figure 3.8**). The mass variance of two amu suggested a potential variation due to a double bond at C11 and C12, possibly caused by the loss of a hydroxyl group at C11 position during fragmentation. It is important to note that C-ring hydroxylation typically occurs at C11 position, according to AAS metabolism studies in equine, while hydroxylation at C12 is very rare (Scarth et al., 2011). However, product ion *m/z* 173 could also result from the elimination of a hydroxyl group at C6 position. In equine steroid metabolism, the formation of a 6-hydroxy metabolite is more common than an 11-hydroxy metabolite (Scarth et al., 2011). Still, further research is necessary to determine the exact site of hydroxylation. Consequently, M2a and M2b were postulated as mono-hydroxylated dienedione with hydroxylation at A/B/C-ring.

3.3.1.5 Metabolite M3

Hydroxy-metabolites M3a and M3b were identified as another pair of epimers with similar mass spectra (**Figure 3.9**). Similar to M4, fragment ions *m/z* 133, 159 and 175 were observed in the spectra of M3, indicating hydroxylation at the D-ring. The presence of diagnostic fragment ions *m/z* 95 and 113 further supports hydroxylation occurring at the C15 or C16 position on the D-ring. As depicted in **Figure 3.2**, metabolite M3 with D-ring hydroxylation exhibited the highest peak intensity among all hydroxy-metabolites (M2 to M5), which was consistent with the fact that this is a common metabolic pathway in horses (Houghton, 1992; Houghton and Dumasia, 1979; McKinney, 2009; Scarth et al., 2010a; Scarth et al., 2010b).

A proposed *in vivo* metabolic pathway for dienedione was shown in **Figure 3.1**. The major metabolic process of dienedione in castrated horse urine involved reduction and/or hydroxylation. Reduction of the C17 keto group of dienedione produced a pair of epimers M1a and M1b, which were the major metabolites. One possible transformation of M1a/b was subsequent hydroxylation on either the A/B/C-ring or D-ring of M1 epimers, resulting in M6 to M8. Additionally, hydroxylation of dienedione on either A/B/C-ring or D-ring yielded M2 to M4.

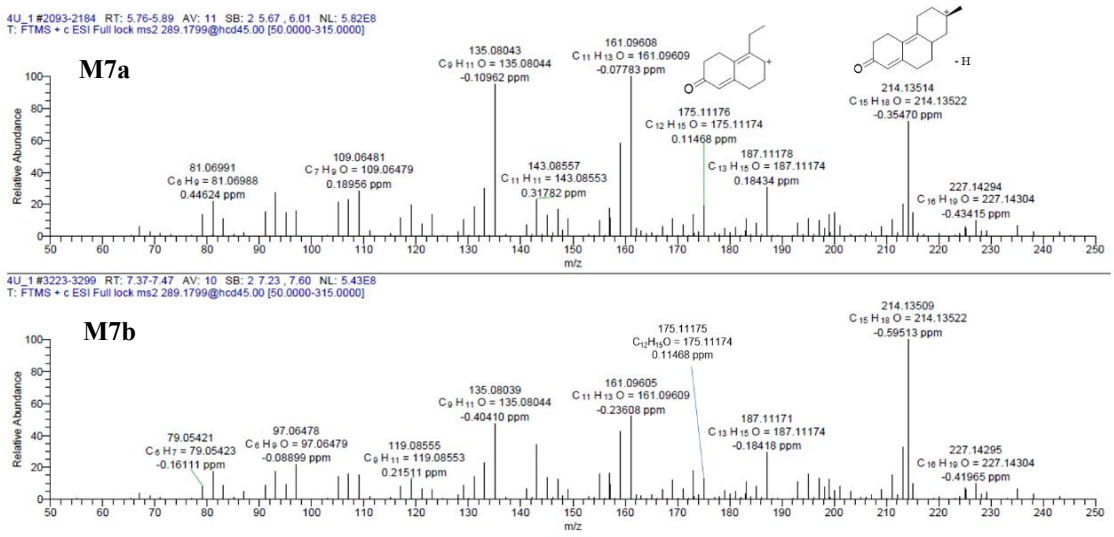

Figure 3.4. Product-ion mass spectra of metabolite M7a (upper panel) and M7b (lower panel) detected in a 1.5-hr post-administration urine sample

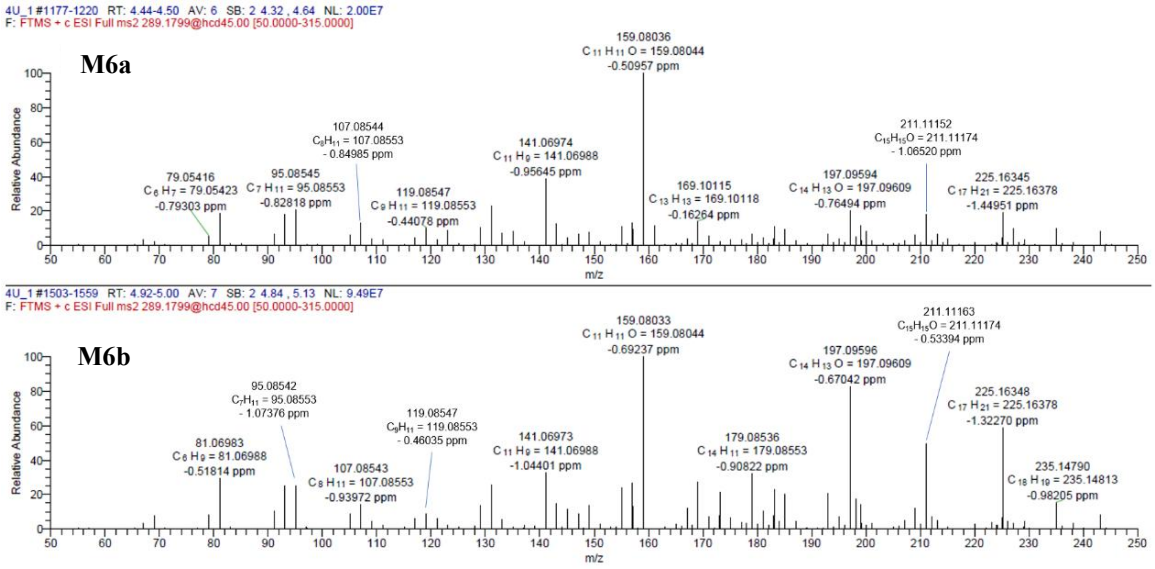

Figure 3.5. Product-ion mass spectra of metabolite M6a (upper panel) and M6b (lower panel) detected in a 1.5-hr post-administration urine sample

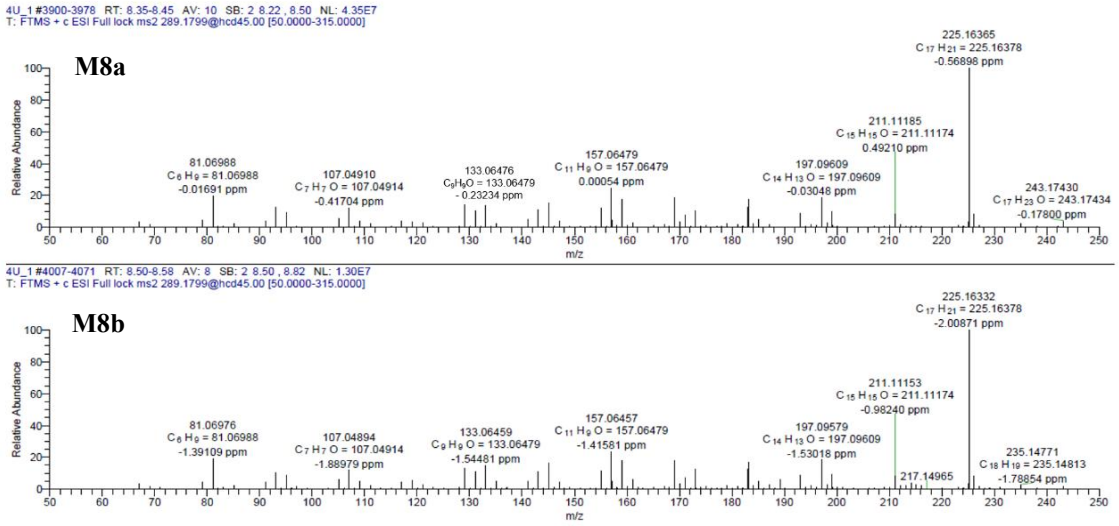

Figure 3.6. Product-ion mass spectra of metabolite M8a (upper panel) and M8b (lower panel) detected in a 1.5-hr post-administration urine sample

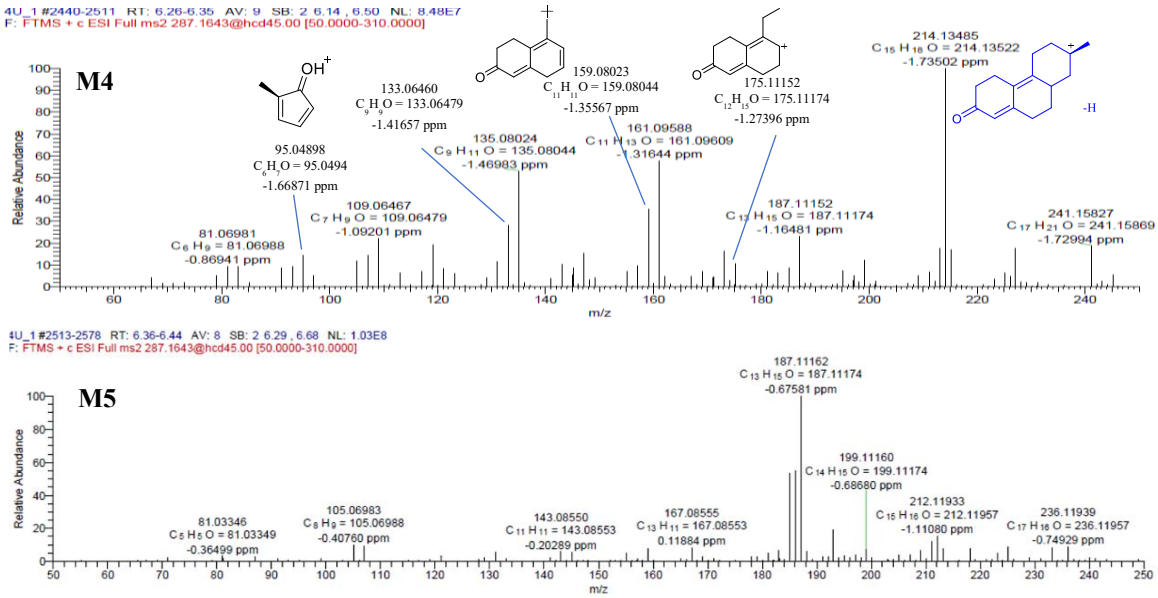

Figure 3.7. Product-ion mass spectra of metabolite M4 (upper panel) and M5 (lower panel) detected in a 1.5-hr post-administration urine sample

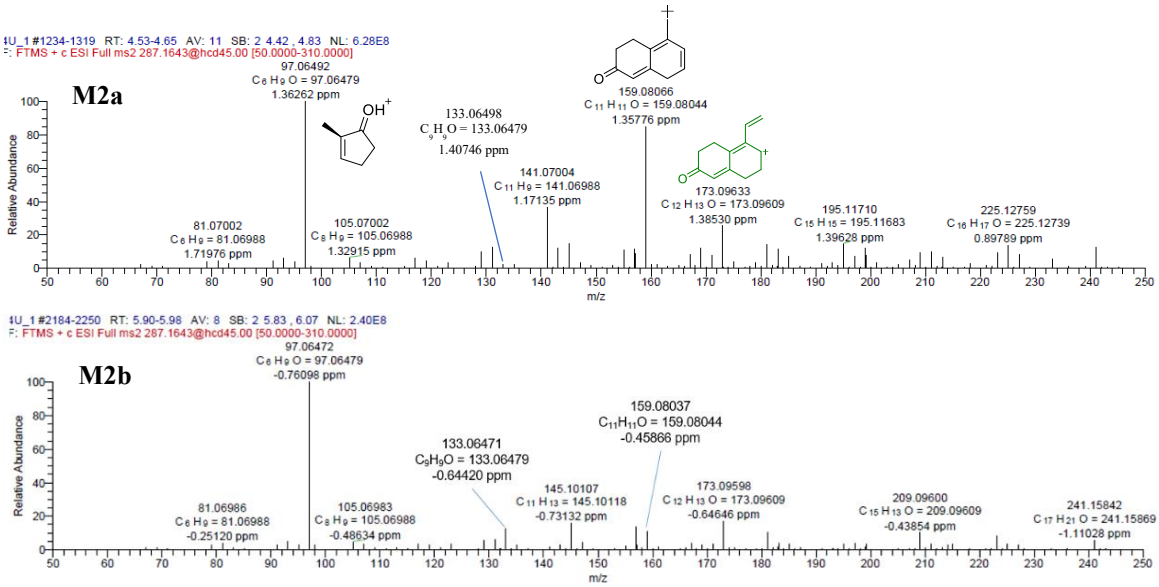

Figure 3.8. Product-ion mass spectra of metabolite M2a (upper panel) and M2b (lower panel) detected in a 1.5-hr post-administration urine sample

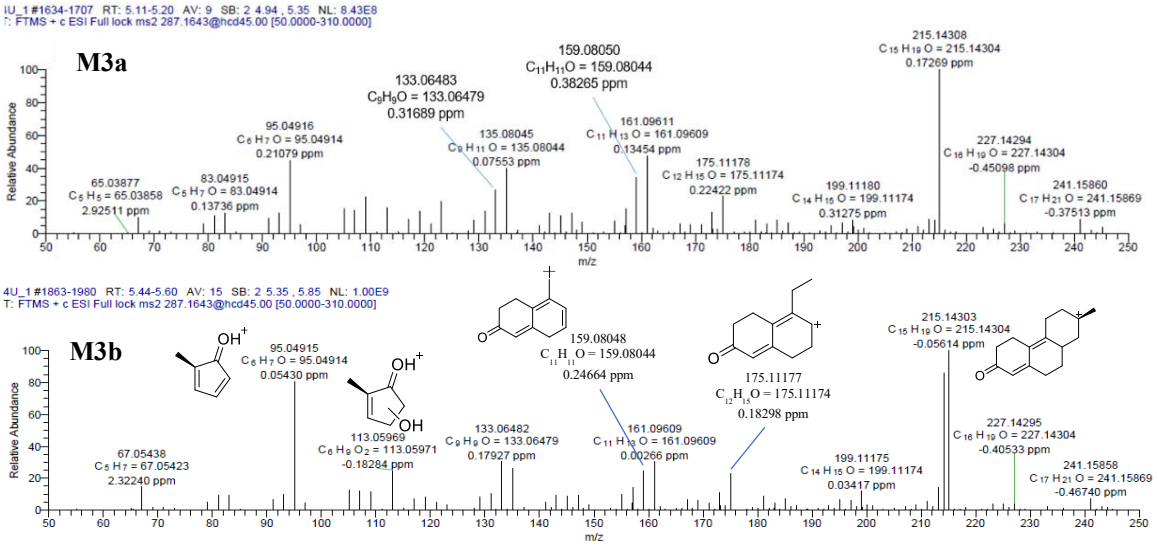

Figure 3.9. Product-ion mass spectra of metabolite M3a (upper panel) and M3b (lower panel) detected in a 1.5-hr post-administration urine sample

3.3.2 Phase II metabolism studies of dienedione in castrated horses by indirect detection of glucuronide and sulfate conjugates of metabolites

The conjugation forms of dienedione metabolites were identified by sequential analysis of post-administration urine samples. The samples underwent three processing steps prior to UPLC-HRMS analysis: (a) LLE for the detection of unconjugated analytes; (b) enzyme hydrolysis using β-glucuronidase (*Patella vulgata*) of the aqueous layer from (a) to detect glucuronide-conjugated analytes; and (c) methanolysis of the remaining aqueous layer to detect sulfate-conjugated analytes. The results from conjugation studies based on an indirect detection method were summarised in **Table 3.2.** Since reference standards for different conjugates were unavailable, the extent of conjugation was determined by comparing the peak intensities of the corresponding molecular ions from the full scan analysis. Metabolites M2a, M2b, M8a and M8b were found to be excreted exclusively in their glucuronide-conjugated fractions. Metabolite M7b, however, was not detected in any of the three fractions from the conjugation studies, although it was identified in the metabolic study employing the extraction method described in ***Section 3.2.3.1***. The same post-administration urine sample was used in the two studies, and the difference in detection may be due to the varying extraction recoveries between SPE (employed in metabolic study) and LLE (employed in the aforementioned conjugation study). Metabolite M7b was better recovered using SPE. Given its relative abundance in **Figure 3.2** as well as the deconjugation (enzyme hydrolysis) used in metabolism study, it is reasonable to believe that a substantial amount of M7b is present as a glucuronide conjugate. In conclusion, dienedione and its metabolites M1a, M1b, M2a, M2b, M3a, M3b, M4, M5, M6a, M6b, M7a, M8a, and M8b were predominantly excreted as glucuronide conjugates.

Table 3.2. Maximum detection periods by UPLC-HRMS/MS and the form of conjugation for dienedione and its metabolites in post-administration samples

Targets				Max. detection time (days)	
	U	G	S	Urine	Plasma
Dienedione	✓	✓✓	✓	5	4
17-hydroxyestra-4,9-dien-3-one (M1a)	✓	✓✓	✓	3	7
17-hydroxyestra-4,9-dien-3-one (M1b)	✓	✓✓	✓	10	9
Hydroxylated dienedione (M2a)	x	✓	x	20	ND
Hydroxylated dienedione (M2b)	x	✓	x	5	ND
Hydroxylated dienedione (M3a, M3b)	✓	✓✓	✓	20	13
Hydroxylated dienedione (M4)	✓	✓✓	✓	10	ND
Hydroxylated dienedione (M5)	✓	✓✓	✓	7	ND
Hydroxylated M1 (M6a)	✓	✓✓	✓	1	ND
Hydroxylated M1 (M6b)	✓	✓✓	✓	3	9
Hydroxylated M1 (M7a)	✓	✓✓	✓	1	10
Hydroxylated M1 (M7b)	Only detected in metabolic study using SPE extraction			20	ND
Hydroxylated M1 (M8a, M8b)	x	✓	x	3	ND

U = Unconjugated; G = Glucuronide-conjugated; S = Sulfate-conjugated.

ND = not detected; ✓✓ Major conjugation

3.3.3 Excretion of metabolites of dienedione in castrated horses

To effectively combat the misuse of dienedione in castrated horses, this study also aimed to identify targets with extended detection windows for screening purposes. The maximum detection time of dienedione and its metabolites in urine and plasma from the three Thoroughbred castrated horses were outlined in **Table 3.2**. In plasma, only

M1a, M1b, M3a, M3b, M6b, M7a as well as dienedione were detectable. In urine, metabolites M2a, M3a, M3b, and M7b could be detected for the longest period (up to 20 days after last administration). Metabolites M3a and M3b were also detectable for the longest period (up to 13 days after the last administration) in plasma. The suggested candidates for monitoring dienedione exposure would be M3a and M3b. Other metabolites could act as supplementary analytes, and the variations in their detection times may offer valuable insights into the timing of drug exposure.

3.3.4 Elimination of dienedione in castrated horse urine and plasma

With the availability of reference material, the urinary and plasma elimination profiles of dienedione following oral administration in the three castrated horses were investigated. The limit of detection (LoD) of dienedione in urine and plasma based on the reported method was approximately 0.05 ng/mL and 0.02 ng/mL respectively. The estimated LoD denotes the lowest spiked concentration evaluated that yielded a signal-to-noise ratio greater than 3:1 in the product ion chromatogram. The LoQ of dienedione in urine and plasma was estimated at 0.2 ng/mL and 0.06 ng/mL respectively. The LoQ was determined as 10 times the SD at the lowest QC (0.2 ng/mL for urine; 0.1 ng/mL for plasma) across three analytical batches. The accuracy of quantification was evaluated by comparing the measured concentrations of QC samples at each level with the corresponding theoretical concentration. The means and standard deviations (SDs) of each QC level were calculated, with acceptable accuracy defined as within ± 15 % of the spiked concentrations. For the quantification method of urinary dienedione, the mean ± SD of QCs at 0.2, 2, 5 and 20 ng/mL (n = 6 each) were 92.2 ± 11.8 %, 108 ± 5.4 %, 111 ± 7.9 % and 110 ± 6.1 % respectively. For the quantification method of

plasma dienedione, the mean ± SD of QCs at 0.1, 0.5 and 2 ng/mL (n = 6 each) were 105 ± 11.0 %, 102 ± 4.6 % and 98.9 ± 5.6 % respectively.

The highest urinary concentration of dienedione, at approximately 23 ng/mL, was observed 2 hours after the last administration (**Figure 3.10**). Dienedione was detectable in the urine of all three horses for a period of 2 to 5 days. The elimination profile of dienedione in plasma was illustrated in **Figure 3.11**. The peak plasma concentration of dienedione, at around 16 ng/mL, was observed at 1 h after drug administration. Dienedione remained detectable for a duration ranging from less than 24 hours to 4 days in the three horses.

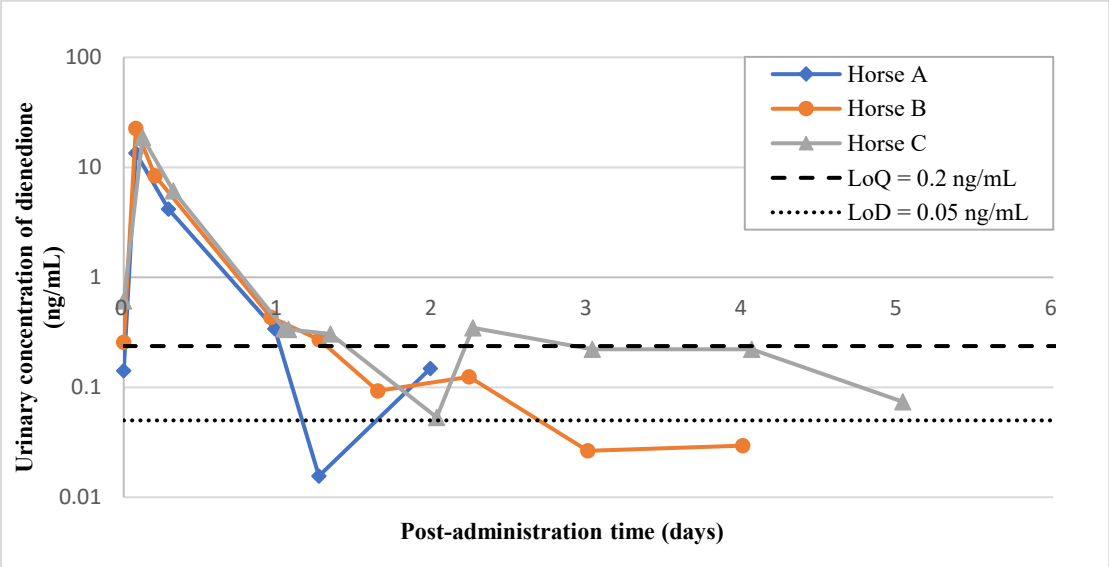

Figure 3.10. Urinary elimination of free and glucuronide-conjugated dienedione in the three castrated horses

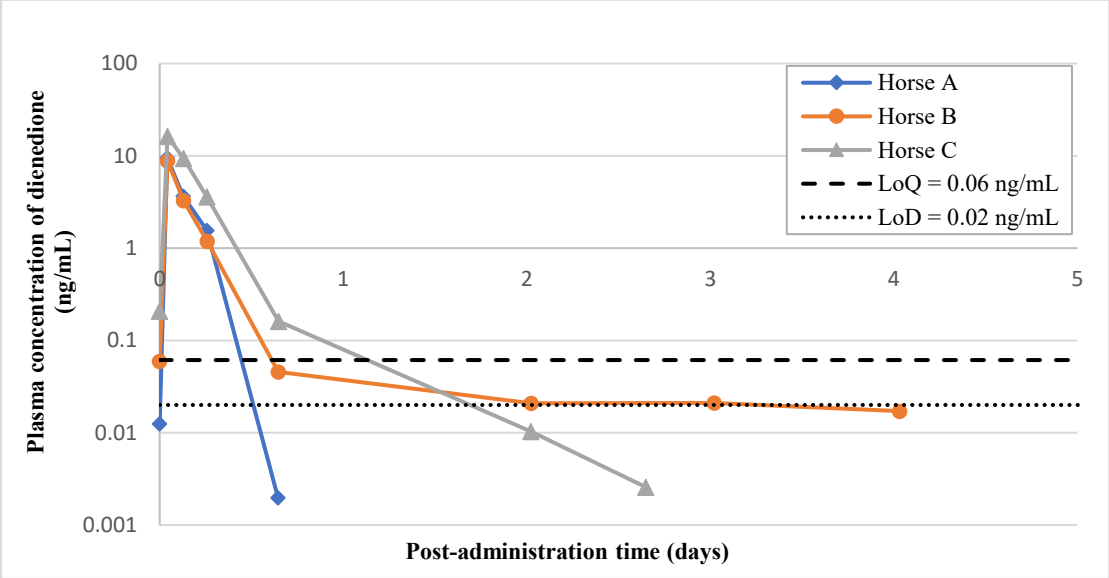

Figure 3.11. Plasma elimination of free dienedione in the three castrated horses

3.4 Conclusion

Dienedione was orally administered to three thoroughbred castrated horses to identify possible dienedione *in vivo* metabolites and to establish the elimination profiles of dienedione in urine and plasma for doping control purposes. The parent drug and a total of fourteen metabolites (M1a, M1b, M2a, M2b, M3a, M3b, M4, M5, M6a, M6b, M7a, M7b, M8a and M8b) were successfully identified. These metabolites resulted from various transformations, including hydroxylation and/or reduction at the C17 position, in combination with sulfate/glucuronide conjugations.

Among the identified metabolites, M1a and M1b were early-excreted metabolites, showing high peak abundance in urine samples collected shortly after the last administration. For detecting dienedione exposure in castrated horses, the most effective screening targets were M3a and M3b (hydroxylated dienedione), as they remained detectable in urine for up to 20 days and in plasma for up to 13 days post-administration. Notably, dienedione metabolites were predominantly excreted as glucuronide-conjugates. The variations in detection times of these metabolites could offer valuable insights into the timing of drug exposure.

An investigation into the elimination patterns of dienedione in urine and plasma indicated that urinary dienedione in its free and glucuronide-conjugated form could be detected for a period ranging from 2 to 5 days, whereas free dienedione in plasma remained detectable for 0.5 to 4 days. By monitoring the parent drug and its metabolites in urine and plasma, the potential misuse of dienedione could be effectively monitored in castrated horses.

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Chapter 4. *In vivo* metabolic studies of 2-hydroxyethyl salicylate in horses

4.1 Introduction

4.1.1 Non-steroidal anti-inflammatory drug in equine

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in both human and veterinary medicine. In addition to their analgesic effects, NSAIDs are well known for their anti-inflammatory and fever-reducing properties. They are frequently used to treat conditions such as soft tissue injuries, musculoskeletal issues, inflammation, swelling and bruising (Göktaş et al., 2020; González et al., 1996; Jedziniak et al., 2012). In equine veterinary practice, salicylic acid, phenylbutazone, flunixin, meloxicam and ketoprofen are some of the common NSAIDs used in horses (Flood and Stewart, 2022).

While offering therapeutic effects, NSAIDs can also be misused in racehorses to mask inflammation and pain, posing a welfare risk to horses (FEI, 2023). Certain NSAIDs are permitted for controlled or authorised use. In horseracing, therapeutic substances are also prohibited during races at any concentration under Article 6A of *IABRW* published by IFHA (IFHA, 2023a), unless specific ISLs have been voluntarily and selectively adopted by each signatory country. Furthermore, Article 6D of the *IABRW* strictly prohibits the use of therapeutic substances or medications to conceal the effects or symptoms of injury or illness during training, as it can be detrimental to horses' health and welfare (IFHA, 2023b).

4.1.2 2-Hydroxyethyl salicylate

Salicylates, such as SA and acetylsalicylic acid (aspirin), are extensively used NSAIDs. Glycol salicylate, also recognised as 2-hydroxyethyl salicylate (2HES), is one such approved for use in horses in Hong Kong. It is chemically related to ethylene glycol and salicylic acid. This compound is an active ingredient in topical analgesics such as patches or gels, marketed under the brand name 'Tensolvet'. Tensolvet is commonly used to alleviate pain and stiffness in the muscles, joints and tendons of racehorses. Racing authorities recommend a withdrawal period for this drug. In the absence of a screening limit, its presence at any level could be considered an adverse finding. To effectively regulate the use of 2HES in horses, it is crucial to identify its *in vivo* metabolites and to study its elimination from horse urine and plasma. To date, there are no reports on the metabolism and elimination profiles of 2HES in humans or horses.

This chapter describes the investigation of *in vivo* metabolism of 2HES using LC-HRMS, aiming to identify the appropriate target(s) for controlling its misuse in horses. The elimination of the parent drug and its metabolite SA in horse plasma (as free 2HES) and urine (as total 2HES) was examined using LC-MS/MS. The elimination profiles of its metabolite SA were also established in both matrices.

4.2 Methods

4.2.1 Materials

2HES standard was acquired from Sigma-Aldrich (St. Louis, MO, USA). Tensolvet gel, which contains 2HES (5 g per 100 g), was obtained from Dechra (Aulendrof, Germany) and was verified against the reference standard from Sigma-Aldrich. Salicylic acid was purchased from British Drug Houses Chemical Limited (London, UK). d_6-Salicylic acid was obtained from Cambridge Isotope Laboratories, Inc (Andover, MA, USA). d_3-

Meloxicam was acquired from WITEGA Laboratorien Berlin – Adlershof GmBH (Berlin, Germany). Various reagents, including acetic acid (96%), acetonitrile (LiChrosolv®), ammonium acetate, chloroform (GR grade), citric acid monohydrate, disodium hydrogen phosphate, potassium phosphate, diisopropyl ether (Emsure®), ethyl acetate (GR grade), n-hexane (GR grade), TBME (LiChrosolv®) and methanol (LiChrosolv®) were purchased from Merck (Darmstadt, Germany). AG1-X8 resin (200-400 mesh; acetate form) was obtained from BIO-RAD (California, USA). Ammonium formate (extra pure, 97 %) was sourced from International Laboratory Limited (San Bruno, CA, USA). Beta-glucuronidase (β-glucuronidase, *from Patella vulgata*, lyophilised powder) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anhydrous methanolic hydrogen chloride used for methanolysis was prepared following procedures outlined in a previous study (Tang and Crone, 1989). Abs Elut Nexus cartridges (60 mg, 3 mL) were purchased from Varian (Palo Alto, CA, USA). C18 Sep-Pak Vac cartridges (3cc, 500 mg) were obtained from Waters (Hertfordshire, UK). Deionised water was generated using an in-house water purification system (Milli-Q, Molsheim, France).

4.2.2 Drug administration studies

Three fine coated thoroughbred castrated horses (Horse 1: 6 y/o, 495 kg; Horse 2: 6 y/o, 532 kg; Horse 3: 5 y/o, 481 kg) were administered a total of 100 g of Tensolvet gel (obtained from Dechra; containing 5 g of 2HES) once evenly on each distal limb and neck. Urine and blood samples were taken just before administration, and 1, 3, 6 and 24h after the final dose, then once per day on Days 3, 4, 5, 6, 8, 11, 14, 17 and 21.

4.2.3 Extraction procedures

4.2.3.1 Identification of 2HES intact metabolites in post-administration urine samples

Urine was transferred to 15-mL corex tube and centrifuged at 1500 *g* for 10 min. The supernatant (0.5 mL) was then loaded to C18 Sep-Pak Vac cartridge, which was pre-conditioned with methanol (4 mL) and water (5 mL) prior to sample loading. The cartridge was washed with deionised water (6 mL) and dried for 2 min. A subsequent wash with hexane (4.5 mL) was performed. All SPE drying steps were accomplished using nitrogen purge at 20 psi. The cartridge was then eluted with methanol (4 mL). The eluate was dried under nitrogen at 65°C and reconstituted with methanol (100 µL) for UPLC-HRMS analyses.

4.2.3.2 Quantification of SA and 2HES in post-administration samples

a) Quantification of SA in post-administration urine samples

To quantify SA in urine samples, a dilute-and-shoot method described by Vonaparti et al (2009) was used with minor modifications.

First, urine (3 mL) was aliquoted to a 15-mL corex tube and centrifuged at 1500 *g* for 10 min. The supernatant (25 µL) was pipetted to 1.5-mL Eppendorf tube. Urine samples collected 1 and 3 hrs after Tensolvet administration were diluted 10-fold with treated blank castrated horse urine. d_6-Salicylic acid (final concentration of 100 µg/mL in urine) was added in the urine samples as an IS. The mixture was brought to a final volume of 250 µL with ammonium acetate (10 mM, pH 6.7). An additional 20-fold dilution was made using ammonium acetate (10 mM, pH 6.7) to reach a total volume of 5 mL,

resulting in a final dilution of 1:200. The diluted extract (100 μL) was then analysed using UPLC-MS/MS.

Prior to the preparation of the calibrators and quality controls, urine collected from castrated horses underwent LLE to remove SA in samples. Urine (5 mL) was aliquoted into a 15-mL corex tube and centrifuged at 1500 *g* for 10 min. The supernatant (2.5 mL x 2) was transferred into two 15-mL graduated centrifuge tubes and adjusted to pH 1.0. The samples were then extracted with diethyl ether (5 mL each) for 10 min and centrifuged at 1500 *g* for 5 min. The organic layer was discarded, and the extraction was repeated twice to remove the remaining SA in urine. The final aqueous layer was then used to prepare the calibrators and quality controls. If urine samples had drug concentrations outside the calibration range, they would be diluted with treated castrated horse urine before sample preparation.

b) Quantification of SA in post-administration plasma samples

Blood samples were centrifuged at 1500 *g* for 10 min. d_6-Salicylic acid was added to the separated plasma (0.5 mL) as an IS. Then, glacial acetic acid (20 μL) and acetonitrile (250 μL) were added. The mixture was vortexed and centrifuged at 14,000 rpm for 10 min. The supernatant (20 μL) was further diluted 10-fold with ammonium acetate (10 mM, pH 3.8) (180 μL). The mixture was centrifuged again at 19,283 *g* for 10 min, and the diluted extract (100 μL) was analysed using UPLC-MS/MS.

Prior to the preparation of the calibrators and quality controls, plasma collected from castrated horses was treated with AG1-X8 resin to remove SA in samples. AG1-X8 resin (BIO-RAD; 200-400 mesh; acetate form) was first washed with deionised water until the washings were clear and then allowed to air-dry. Plasma (10 mL) was mixed with treated AGI-X8 resin (1 g) in a 15-mL polypropylene tube for 2 hrs. The mixture was then centrifuged at 1500 *g* for 10 min. The supernatant was transferred to a clean

15-mL polypropylene tube, serving as the matrix blank for the calibrators and quality controls. If plasma samples had drug concentrations outside of the calibration range, they would be diluted with treated blank plasma before sample preparation.

c) Quantification of total 2HES in post-administration urine samples

A new method was developed to quantify total 2HES in horse urine, where 2HES was released from its conjugates through enzyme hydrolysis and methanolysis. The stability of 2HES under methanolysis was assessed by analysing spiked 2HES in methanolic hydrogen chloride (1M, 0.5 mL) at a concentration of 500 ng/mL, while the other one adding methanol instead of methanolic hydrogen chloride. d_3-Meloxicam (final concentration of 300 ng/mL in the solvent mixture) was added to the solvent as an IS. Both mixtures were incubated at 65 ℃ for 15 min.

Supernatant (2 mL) from the centrifuged urine in ***Section 4.2.3.2 (a)*** was aliquoted to a 15-mL plastic tube. Urine samples collected 1, 3, 6 and 24 hrs after Tensolvet administration were diluted with blank castrated horse urine at 100-fold (Horse 1 and 2), 50-fold (Horse 3), 20-fold and 5-fold respectively. d_3-Meloxicam (final concentration of 300 ng/mL in urine) was added as an IS. The pH was adjusted to 5.0 by adding phosphate buffer (0.1 M, pH 6.0, 1 mL). β-glucuronidase (*Patella vulgata*, 18,000 units/mL, 330 μL) was introduced for enzyme hydrolysis, and the mixture was incubated at 65 ℃ for 2.5 hrs. After incubation, the hydrolysate was cooled to room temperature and centrifuged at 1500 *g* for 5 min before loading onto an Abs Elut Nexus cartridge. The cartridge was washed with deionised water (2 mL) and hexane (2 mL) were performed, followed by 2-min drying. All SPE drying steps were completed employing nitrogen purge at 20 psi. The cartridge was eluted with diisopropyl ether (3 mL), and the eluate was evaporated to dryness at 65 ℃ under nitrogen. Anhydrous methanolic hydrogen chloride (1 M, 0.5 mL) was added, and the solution was heated at

65 ℃ for 15 min. Next, deionised water (3 mL) was added, and the mixture was transferred to a 15 mL graduated centrifuge tube. Liquid-liquid extraction was performed with diisopropyl ether (3 mL). The mixture was vortexed for 0.5 min and centrifuged at 1500 *g* for 0.5 min. The organic layer was passed through a Pasteur pipette packed with cotton wool. The extract was dried at 40 ℃ under nitrogen, and the residue was reconstituted with ammonium acetate (10 mM, pH 6.7)/methanol (50:50, *v/v*) (50 µL) for UPLC-MS/MS analyses.

d) Quantification of total 2HES in post-administration plasma samples

The separated plasma (1 mL), separated from the centrifuged blood sample as described in ***Section 4.2.3.2 (b)***, was added with d_6-salicylic acid as an IS. Citric phosphate buffer (pH 2.8) was added to the sample, which was then mixed on a rotator for 10 min. Plasma samples were extracted using LLE with diisopropyl ether (3 mL). The organic layer was dried at 25 ℃ under nitrogen, and the residue was reconstituted with ammonium acetate (10 mM, pH 6.7)/methanol (50:50, *v/v*) (50 µL) for UPLC-MS/MS analyses.

4.2.3.3 Preparation for calibrators and quality controls for quantification of 2HES and SA in urine and plasma

a) Quantification of SA in urine and plasma

Calibrators were prepared in duplicate at 0, 5, 10, 25, 40, 80 and 120 µg/mL in the treated urine. Quality control samples were also prepared in duplicate, with SA spiked at concentrations of 5, 25 and 80 µg/mL in the treated urine. In plasma, calibrators were prepared in duplicate at 0, 0.2, 0.4, 0.8, 1.6, 3 and 6 µg/mL in treated plasma, with quality control samples prepared in duplicate with SA spiked at concentrations of 0.2, 0.8 and 3 µg/mL in the treated plasma.

b) Quantification of 2HES in urine and plasma

Calibrators and quality controls were prepared independently using different weighing of a drug standard. Calibrators of 2HES were prepared in duplicate at concentrations of 0, 50, 100, 150, 200 and 500 ng/mL in castrated horse urine. Quality control samples were prepared in duplicates, with 2HES spiked at concentrations of 50, 150 and 300 ng/mL in urine. In plasma, calibrators were prepared in duplicate at concentrations of 0, 10, 25, 50, 100, 250, 500 and 800 ng/mL in castrated horse plasma, with quality control samples prepared in duplicate with 2HES spiked at concentrations of 10, 50 and 500 ng/mL in plasma.

These calibrators and QC urine or plasma samples were analysed alongside pre- and post-administration urine or plasma samples. The PARs of the target to IS versus the concentration of the calibrators were fitted using linear regression to obtain the calibration curve. The quantification results in ***Section 4.2.3.2*** showed linear responses across the concentration ranges for each study, with r values exceeding 0.997. The concentrations measured for the QC samples deviated by less than ± 15 % (± 20 % for the lowest QC) from the spiked values.

4.2.4 Instrumentation

UPLC-MS/MS analyses were performed on a Waters Acquity UPLC system that can handle pressure up to 15,000 psi, interfaced to a TSQ Quantum Ultra mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA) and UPLC-HRMS analyses were carried out using a Thermo Scientific Q Exactive mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) with a HESI-II source interfaced with the identical UPLC system.

4.2.4.1 UPLC conditions

a) Identification of 2HES metabolites

The sample tray of the autosampler was maintained at a temperature of 15 ℃. To separate the target analytes, a reverse-phase Acquity UPLC BEH C18 column (Acquity, 100 mm length x 2.1 mm ID; 1.7 µm particle size) was utilised. Mobile phase A was ammonium formate in deionised water (pH 3.0, 5 mM) and mobile phase B was methanol. A linear gradient was employed at a flow rate of 300 µL/min, starting with 98 % solvent A at 0 min, decreasing to 2 % at 8.0 min, held at 2 % solvent A until 10.0 min. Subsequently, the gradient returned to 98 % solvent A at 10.1 min and remained steady to 12 min before the next injection.

b) Quantification of SA and 2HES in plasma and total 2HES in urine

The sample tray of the autosampler was maintained at 15 ℃. A reverse-phase Acquity UPLC BEH C18 column (Acquity, 100 mm length x 2.1 mm ID; 1.7 µm particle size) was employed to separate target analytes. Mobile phase A was ammonium acetate in deionised water (pH 6.7, 10 mM) and mobile phase B was methanol. A linear gradient was applied at a flow rate of 350 µL/min, beginning with 98 % solvent A at 0 min, reducing to 70 % at 3.0 min, then to 30 % at 5.5 min, and further decreasing to 0 % at 6.0 min. This composition was held at 0 % solvent A until 8.0 min. At 8.1 min, the gradient returned to 98 % solvent A and was maintained to 10 min before the next injection.

4.2.4.2 HRMS conditions

Sample ionisation was conducted in a negative ionisation mode employing HESI-II at a capillary temperature set at 350 °C. The sheath gas flow was maintained at 50 arbitrary units, while the auxiliary gas flow was set at 10 arbitrary units. A sweep gas flow of 2 arbitrary units was utilised with curtain plate installed. The ion spray voltage operated at approximately 3 kV, and the S-Lens RF level was set at 40 %. Full-scan mass spectra were captured with a mass resolution of 35,000 (FWHM at *m/z* 200). The maximum injection time was 100 ms, and the AGC^{TM} was adjusted to 3e6. For product ion mass spectra, a mass resolution of 17,500 (FWHM at *m/z* 200) was employed with an isolation window of 1 amu. The maximum injection time was maintained at 100 ms, and the automatic gain control (AGC^{TM}) was set at 5e5. Nitrogen (> 99.995 % purity) was employed as the collision gas for Higher-energy Collisional Dissociation (HCD). Data processing was carried out using the Thermo Finnigan Xcalibur (Version 2.2) software with a mass tolerance window of ± 20 ppm. All identified product ions in this study exhibited observed masses within ± 20 ppm in comparison to their theoretical values.

An internal lock mass solution, containing citric acid (*m/z* 191.01973 for negative mode) at a concentration of 10 ng/µL in deionised water, was infused post-column into the system at a flow rate of 20 µL/min through a T-joint using a LC-20AB Solvent Delivery Unit (Shimadzu Corporation, Kyoto, Japan). The UPLC-HRMS parameters for the precursor ion and NCE were provided in **Table 4.1**.

Table 4.1. The UPLC-MS/HRMS parameters for the precursor ion and NCE

Targets	**Precursor ion (*m/z*)**	**NCE (%)**
2HES	181.08	55
SA	137.02	45
2-hydroxyethyl salicylate glucuronide (2HES-Glu)	357.08	25
2-hydroxyethyl salicylate sulfate (2HES-SO_4)	261.00	25

4.2.4.3 MS/MS conditions

Injection volumes of 10 µL were used, and the HESI source operated in the SRM negative ion mode with a spray voltage of 2 kV. The capillary and vaporizer temperatures were set to 320 ℃ and 350 ℃ respectively. The sheath, auxiliary and ion sweep gas pressures were set to 50, 10 and 2 arbitrary TSQ units. The resolution of the quadrupole mass filter was configured with a peak width of 0.7 amu (FWHM) for both Q1 and Q3. The collision gas pressure of Q2 was set at 1.2 mTorr. Transitions monitored included 2HES (*m/z* 181 → 137, 181 → 93), SA (*m/z* 137 → 93), IS d_6-salicylic acid (*m/z* 141 → 97) and IS d_3-meloxicam (*m/z* 353 → 289.1). The CID energies for 2HES (*m/z* 181 → 137), 2HES (*m/z* 181 → 93), salicylic acid, d_6-salicylic acid and d_3-meloxicam were 14 eV, 26 eV, 30 eV, 30 eV, and 13 eV respectively. The radio frequencies for 2HES, salicylic acid, d_6-salicylic acid and d_3-meloxicam were 82 V, 54 V, 30 V, and 82 V respectively.

4.3 Results and discussion

4.3.1 Metabolic studies of 2HES

One of the objectives of this study was to identify potential screening targets to monitor the potential misuse of 2HES in horses. Possible metabolites of 2HES were identified using UPLC-HRMS by comparing chromatograms from pre- and post-administration samples. Using high resolution full-scan data, ions were extracted based on the theoretical molecular masses of the metabolites, which were predicted from possible and common biotransformations such as reduction, hydroxylation, hydrogenation, hydrolysis or combinations of the above. If the extracted-ion chromatograms were satisfactory, further UPLC-MS/HRMS analyses were conducted to generate product-ion mass spectra, which were then compared with those of the corresponding reference standards.

Parent drug 2HES was not detected in the post-administration urine samples. However, three metabolites were identified: 2HES-Glu, 2HES-SO_4 and SA. The structures of 2HES-Glu and 2HES-SO_4 were tentatively assigned based on mass spectral interpretation, as no reference standards were available for confirmation. These findings suggest that most of the administered 2HES was metabolised before being eliminated. The proposed structures of the metabolites and their biotransformation pathways were shown in **Figure 4.1**. The extracted-ion chromatograms of these metabolites in a post-administration urine sample were presented in **Figure 4.2**. The fragment ion *m/z* 181, corresponding to $[2HES\ -H]^-$, was prominent in the 2HES-SO_4 spectrum, along with supporting fragments such as *m/z* 137 and 93, likely originating from *m/z* 181 (**Figure 4.3**). Similarly, *m/z* 181, 137 and 93 were observed in the 2HES-Glu spectrum. In addition to 2HES conjugates, metabolite SA was also detected in the

post-administration urine sample, as confirmed by the presence of diagnostic product-ions *m/z* 93 and 65 in the SA spectrum (Vonaparti et al., 2009).

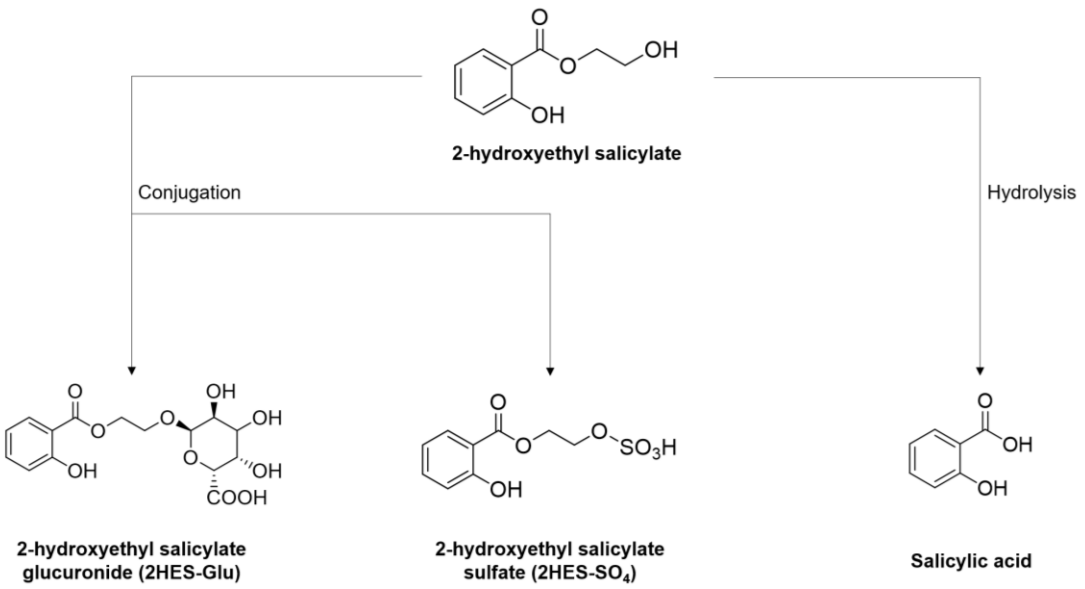

Figure 4.1. Chemical structure of the *in vivo* metabolites and the proposed metabolic pathway of 2HES in horses

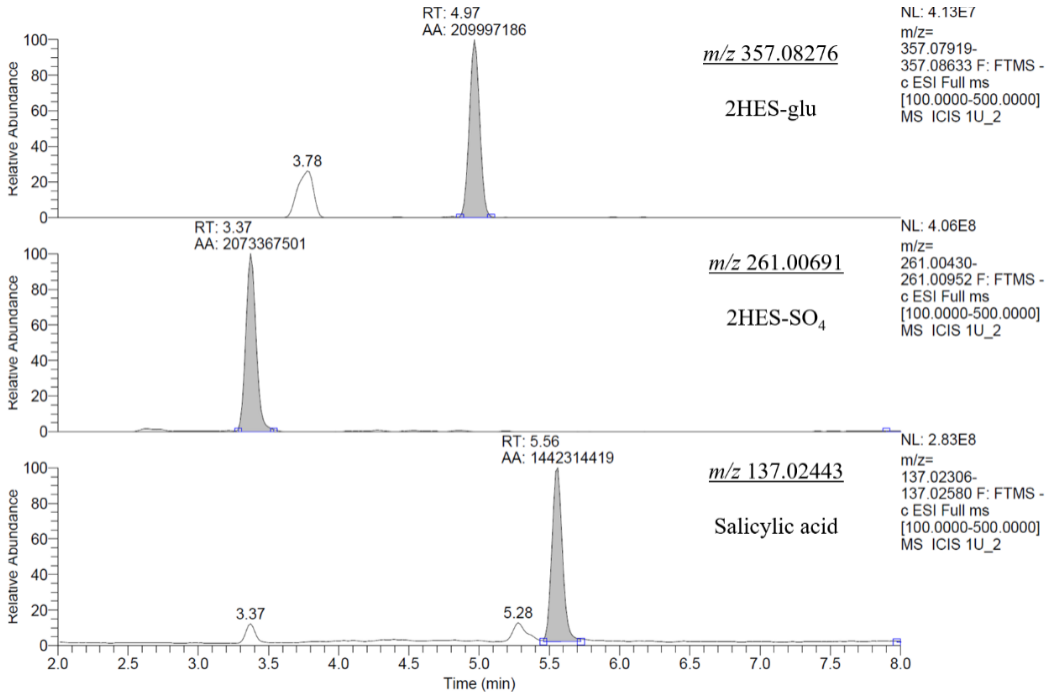

Figure 4.2. Extracted-ion chromatogram of 2HES *in vivo* metabolites detected in a 2.4 hr post-administration urine sample

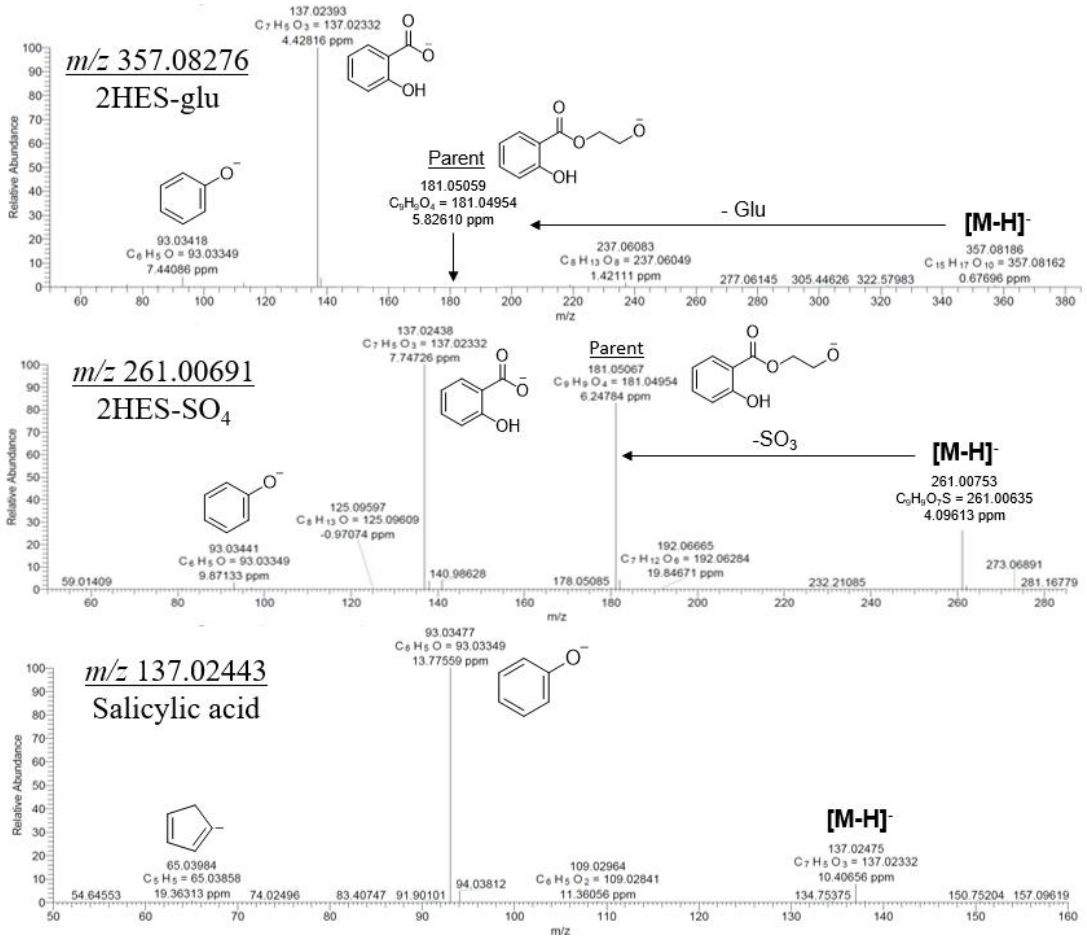

Figure 4.3. Product-ion mass spectra of 2HES-glucuronide (upper panel), 2HES-sulfate (middle panel) and SA (lower panel) detected in a 2.4 hr post-administration urine sample

Despite the absence of prior research on the metabolism of 2HES in humans or horses, the metabolism of similar NSAIDs has been investigated. For example, 2-ethylhexyl salicylate (EHS), which consists of a 2-ethylhexyl moiety instead of a hydroxyethyl moiety, undergoes hydroxylation and oxidation to form 5OH-EHS, 5oxo-EHS and/or carboxyheptyl salicylate in humans after oral administration. SA and its subsequent metabolite salicyluric acid (a glycine conjugate of SA), were also detected (Bury et al.,

2019). Methyl salicylate, containing a methyl moiety, is also metabolised readily to SA and primarily excreted in human urine as glucuronide-conjugated SA or salicyluric acid (Anderson et al., 2017; Chyka et al., 2007; Flomenbaum et al., 2006; Vree et al., 1994). A similar outcome is observed after aspirin administration in humans (Dubovska et al., 1995; Uzzaman et al., 2019). In horses, acetylsalicylic acid mainly metabolises to SA after oral administration (Broome et al., 2003; Friebe et al., 2013). The detection of SA as a metabolite after 2HES administration to horses aligns with these findings from other NSAID metabolism studies. However, neither salicyluric acid nor SA conjugates were found in this study. It is worth noting that different routes of administration in different animal species could result in different metabolites from 2HES.

Furthermore, 2HES did not follow common metabolic pathways such as hydroxylation or oxidation before excretion. Instead, conjugation of the parent compound was the dominant biotransformation route, which has not been observed in the metabolism of other related NSAIDs. This suggests that the hydroxyethyl moiety in 2HES may be a favourable site for conjugation. In fact, phenolic conjugation is a rare metabolic pathway for NSAIDs. Phenolic conjugation (glucuronidation) has only been reported as a minor metabolic pathway in SA, but not in other related NSAIDs such as methyl salicylate or EHS (Anderson et al., 2017; Bury et al., 2019; Vree et al., 1994). Hence, it is hypothesised that glucuronic acid and/or sulfuric acid likely conjugate with the hydroxyethyl moiety of 2HES to form 2HES-Glu and 2HES-SO_4, as shown in **Figure 4.1**.

4.3.2 Elimination of SA in urine and plasma

Since SA is a prevalent metabolite produced from the metabolism of various NSAIDs such as aspirin, methyl salicylate and 2HES (Bury et al., 2019; Dubovska et al., 1995;

Morra et al., 1996), it could potentially serve as an indicator of the illicit use of these drugs.

This study focused on examining the urinary and plasma elimination profiles of SA following the topical administration of Tensolvet to three castrated horses. The LoD of SA in urine and plasma was determined to be 1 µg/mL and 0.1 µg/mL respectively. The estimated LoD represents the lowest spiked concentration evaluated that resulted in a signal-to-noise ratio greater than 3:1 in the product-ion chromatogram. The LoQ of SA in urine and plasma was estimated at 2.6 µg/mL and 0.3 µg/mL respectively. The LoQ was based on 10 times the SD at the lowest QC (5 µg/mL for urine, n = 10; 0.2 µg/mL for plasma, n = 10). The accuracy of quantification was assessed by comparing the measured concentrations of QC samples at each level with theoretical concentrations. An acceptable accuracy range for the mean concentration was set at ± 15% (± 20 % for lowest QC) of the spiked concentrations. For urinary SA quantification, the accuracy of QCs (n = 6 each) at 5, 25 and 80 µg/mL were 93.7 %, 100.4 % and 99.7 % respectively. For plasma SA quantification, the accuracy of QCs (n = 6 each) at 0.2, 0.8 and 3 µg/mL were 101.5 %, 103.6 % and 101 % respectively. In addition, the pooled relative standard deviation (RSD %) was determined to evaluate the precision. The RSD % were determined from the means and SDs of three replicates of the spiked controls. For quantification method of urinary SA, the RSD % for precision in 3 analytical batches at 5, 25 and 80 µg/mL were 5.4 %, 0.76 % and 4.4 %. For quantification method of plasma SA, the RSD% for precision in 3 analytical batches at 0.2, 0.8 and 3 µg/mL were 6.3 %, 5.4 % and 4.7 %.

The highest urinary concentration of SA, approximately 500 µg/mL, was recorded 3 hrs after administration (**Figure 4.4**). The plasma elimination profile of SA is shown in **Figure 4.5**. The peak plasma concentration of SA, around 5.3 µg/mL, also occurred 3

hrs post-administration. SA is naturally detected in horses since it is found in common equine feed such as alfalfa hay or willow bark (Beaumier et al., 1983). As a result, its misuse both in and out of competition is regulated by international urinary and plasma thresholds of SA. The IFHA sets the thresholds for SA in equine urine and plasma at 750 µg/mL and 6.5 µg/mL respectively (IFHA, 2023a). These international threshold values were established based on studies involving horses from various countries with different diets (Beaumier et al., 1983; Lakhani et al., 2004; Moss et al., 1985). The peak concentrations of SA in urine and plasma determined in this administration study did not exceed the corresponding international threshold value, suggesting that monitoring SA levels is ineffective for detecting potential 2HES misuse (Beaumier et al., 1983; Schenk et al., 2012).

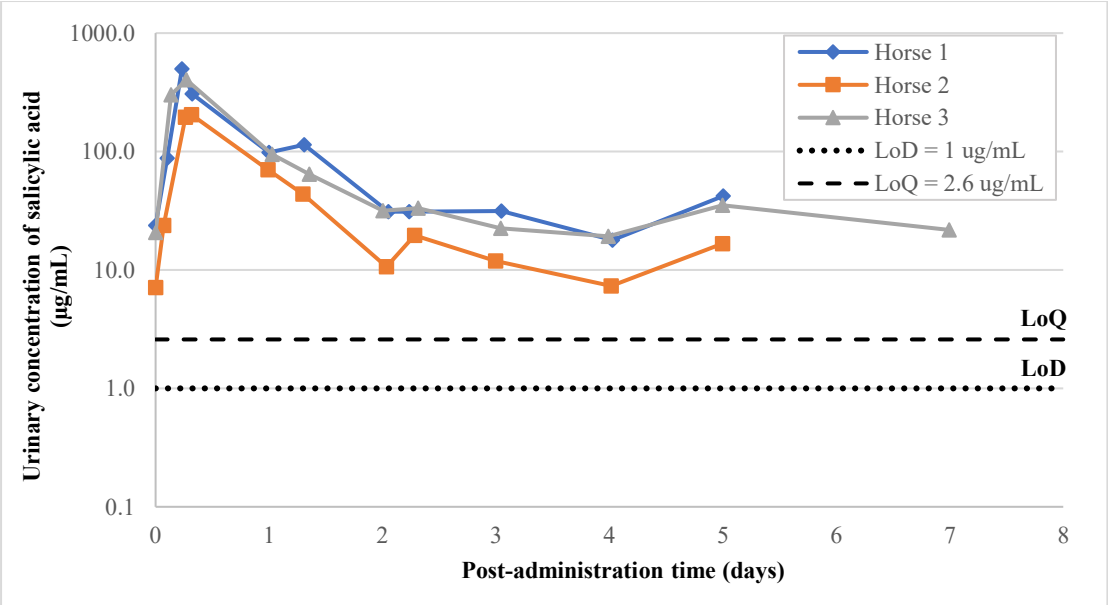

Figure 4.4. Urinary elimination of SA in castrated horses

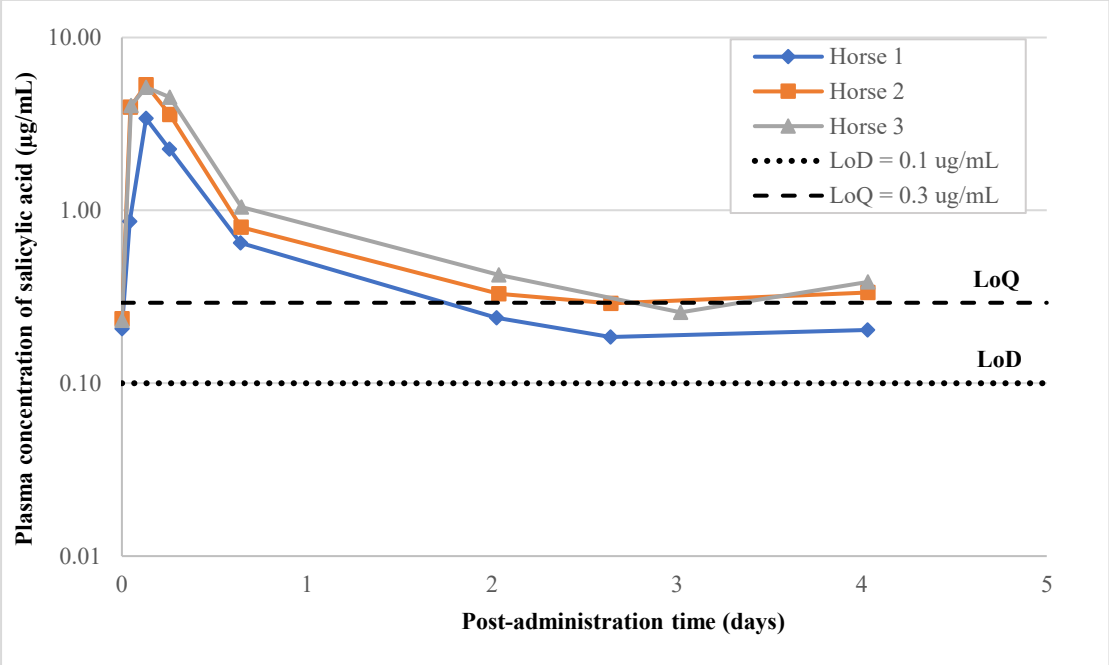

Figure 4.5. Plasma elimination of SA in castrated horses

4.3.3 Elimination of 2HES in urine and plasma

The metabolic study indicated that 2HES mainly existed in its glucuronide- (2HES-Glu) and sulfate-conjugated (2HES-SO_4) forms in urine. To determine the elimination profile of total 2HES following the topical administration of Tensolvet, a method was developed to release 2HES from its conjugates. To the best of our knowledge, no deconjugation method has been reported for 2HES or for similar NSAIDs, such as methyl salicylate and sodium salicylate, in urine.

Deconjugation of 2HES-Glu was achieved through enzyme hydrolysis using beta-glucuronidase sourced from *Patella vulgata*, which catalyses the hydrolysis of glucuronide metabolites to produce 2HES and glucuronic acid. On the other hand, the deconjugation of 2HES-SO_4 was performed via methanolysis. Methanolysis is a common method for cleaving glycosides and has also been applied to desulfate sulfated oligosaccharides and various steroid sulfates (Choi et al., 2018; Ho et al., 2005; Kwok

et al., 2015; Tang et al., 1986; Tang and Williams, 1984). The extraction method for total 2HES was adapted from the protocol for quantifying total androsta-1,4,6-triene-3,17-dione and its sulfate conjugates in equine, as published by Kwok et al. (2015) with modifications. The stability of 2HES under methanolysis was examined by comparing the peak area ratios of 2HES to d_3-meloxicam (IS) of two 2HES spiked samples each at 500 ng/mL: one incubated with methanolic hydrogen chloride (1M, 0.5 mL), the other with methanol. The difference between the peak area ratios of 2HES to IS from the two samples was less than 10 %, depicting that 2HES did not degrade significantly under the drastic acidic conditions of methanolysis.

To accomplish accurate quantification of total 2HES in urine with acceptable accuracy, d_3-meloxicam was selected as the IS due to its good calibration linearity (> 0.996) and high r (> 0.998). Since deuterated 2HES is not commercially available, other compounds with structures similar to 2HES such as d_6-salicylic acid, methyl salicylate and ethyl salicylate, were evaluated as potential IS. Yet none of these compounds could be consistently extracted using the developed method. Consequently, d_3-meloxicam, which could be successfully extracted, was selected as the IS. After determining a suitable IS, the SPE recovery of 2HES was assessed using various elution solvents. The choices of SPE elution solvents of 2HES from urine were examined by analysing spiked castrated horse urine samples at 500 ng/mL, one set with 2HES spiked before SPE while the other spiked after SPE. The IS was added after the SPE process in both sets, with the latter serving as the 100 % recovery reference. The SPE recoveries of 2HES (n = 2) using different solvents - methanol in ethyl acetate (5 %, *v/v*), chloroform, diisopropyl ether and TBME - were 86.2 %, 85.4 %, 93.3 % and 54.1 % respectively. Therefore, diisopropyl ether was selected as the elution solvent for SPE.

The urinary elimination profiles of 2HES following the topical administration of Tensolvet to three castrated horses were examined. The LoD for 2HES in urine was found to be 5 ng/mL. The LoQ for 2HES in urine was established at 32.0 ng/mL. The LoQ was calculated as 10 times of the SD at the lowest QC (50 ng/mL for urine). Accuracy and precision of quantification was assessed using the method described in ***Section 4.3.2***. For urinary 2HES quantification, the accuracy at QC levels of 50, 150 and 300 ng/mL (n = 6 each) was 95.0 %, 88.1 % and 99.4 % respectively. The RSD% for precision in 3 analytical batches at 50, 150 and 300 ng/mL were 12.3 %, 11.7 % and 7.6 %. The highest urinary concentration of 2HES, approximately 25 μg/mL, was observed in the initial urine samples collected from the three horses 1 hr after administration (**Figure 4.6**). The parent drug remained detectable in hydrolysed urine for 10 days. The extraction recovery of total 2HES in urine was 52 %, determined by analysing the spiked castrated horse urine samples at 500 ng/mL. One set of samples spiked with 2HES before enzyme hydrolysis, while the other was spiked after LLE (the final extraction step in the method). The IS was added after the LLE step in both sets. The second set, where the spiking occurred after LLE, was considered to have a 100 % recovery. No correction of recovery was necessary, as the IS was used to account for any loss during the extraction process.

For plasma 2HES quantification, the LoD of 2HES in plasma was determined to be 5 ng/mL. The LoQ (10SD at the lowest QC level at 10 ng/mL, n = 10) of 2HES in plasma was 6.44 ng/mL. The accuracy at QC levels of 10, 50 and 500 ng/mL (n = 6 each) were 95.2 %, 106.9 % and 105.3 % respectively. The RSD% for precision in 3 analytical batches at 10, 50 and 500 ng/mL were 8.0 %, 1.4 %, and 4.7 % respectively. The elimination profile of 2HES in plasma was depicted in **Figure 4.7**. The peak plasma concentration of 2HES, approximately 606 ng/mL, was also detected in the first

samples collected after administration. The parent drug was detectable in plasma for a period of 6 to 16 hr. This short detection window in plasma aligns with the results of the metabolic study, which showed that 2HES was primarily present in its conjugated forms. The extraction recovery of 2HES in plasma was 69.4 %, determined by analysing the spiked castrated horse plasma samples at 500 ng/mL. One set with 2HES spiked before LLE, while the other spiked after LLE. The IS was added after the LLE step in both sets. No correction of recovery was necessary, as the IS was used to account for any loss during the extraction process.

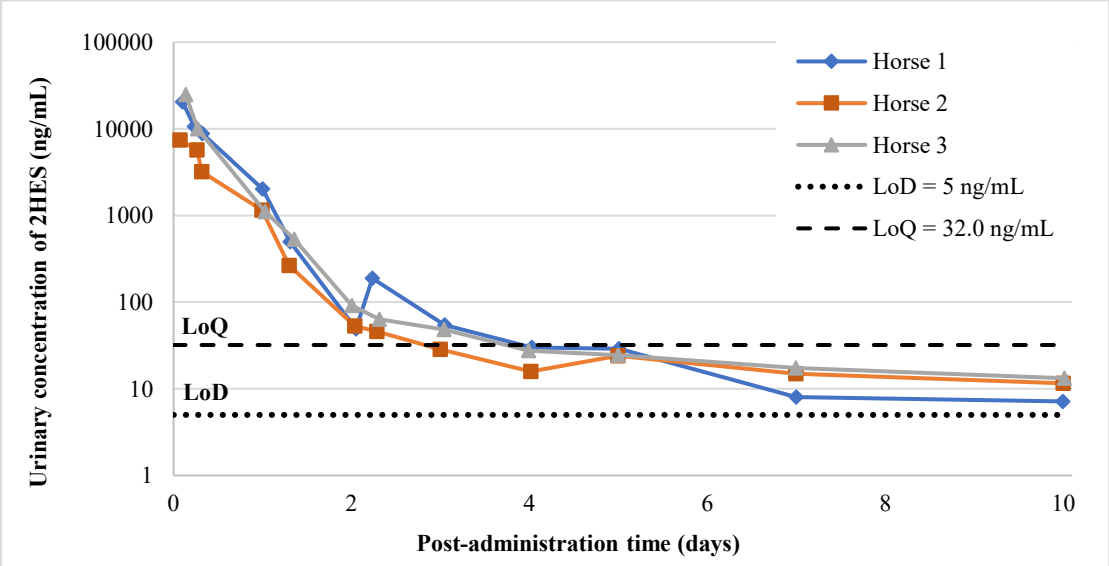

Figure 4.6. Urinary elimination of 2HES in castrated horses

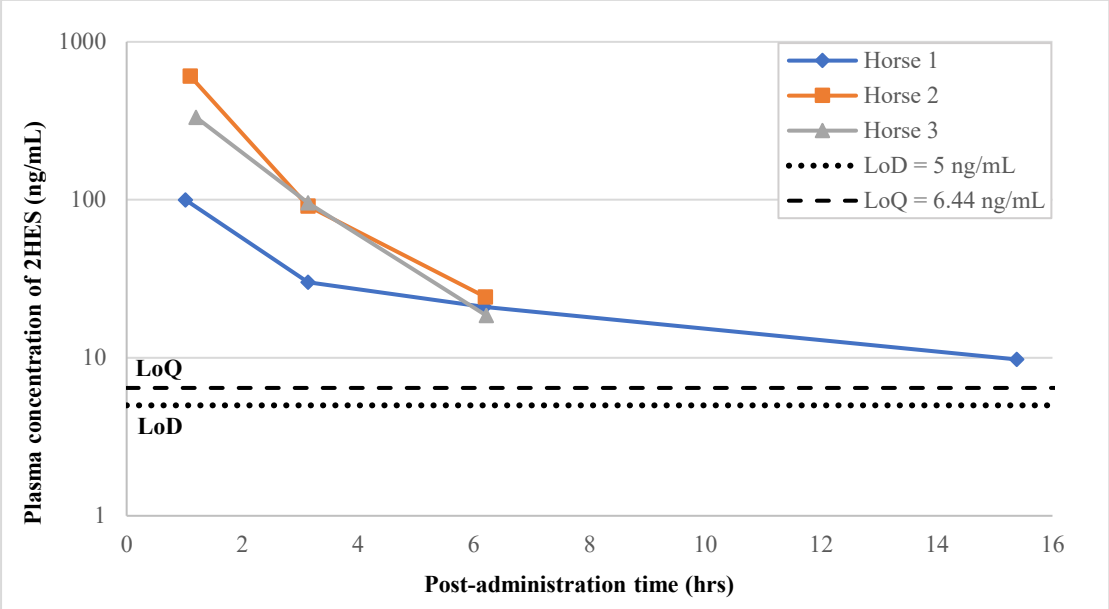

Figure 4.7. Plasma elimination of 2HES in castrated horses

4.4 Conclusion

2-Hydroxyethyl salicylate (2HES), a NSAID, is used to treat musculoskeletal injuries and inflammation in both humans and horses. It has the potential to enhance performance and mask injuries, which could pose detrimental health risk. In horseracing, the detection of 2HES during competition is considered an adverse finding. The metabolism of 2HES in humans or horses has not been studied, thus its metabolic pathways remain largely unknown.

A topical application of Tensolvet was administered to three castrated horses to identify possible *in vivo* metabolites of 2HES and to assess its elimination in horse urine and plasma for doping control purposes. Three metabolites - 2HES-Glu, 2HES-SO_4, and SA - were detected in urine samples collected after administration using SPE method. A metabolic pathway for 2HES in horses was proposed, involving conjugation and hydrolysis. To better regulate the misuse of 2HES, elimination profiles for both the metabolite SA and 2HES were established. However, SA was deemed unsuitable for monitoring the misuse of 2HES, as the peak concentration of SA in urine and plasma samples did not surpass their respective international thresholds in horses. The parent drug, 2HES, was suggested as the preferred screening target, as it was detectable in hydrolysed urine for at least 10 days and in plasma for up to 16 hrs after administration.

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Chapter 5. Doping control of ranitidine in horses

5.1 Introduction

The prevalence of gastric and duodenal ulcers in horses has been reported to range from 55% to 100%. Such prevalence is believed to be influenced by various factors such as feeding practices, type and intensity of activity, etc (Lester et al., 2005; McClure et al., 1999; Murray et al., 1996; Orsini et al., 2003; Vatistas et al., 1999). Horses undergoing race training, particularly Thoroughbreds and Standardbreds, are at the highest risk of developing ulcers, with reported frequencies typically ranging from 80 % to 95 % (Lester et al., 2005; Murray et al., 1996; Orsini et al., 2003; Rabuffo et al., 2002; Vatistas et al., 1994). Hence, the use of antacid medications is a common approach to prevent and treat ulcers.

Histamine type 2 receptor (H_2-receptor) antagonists, one of the antacid medications, are frequently used in equine practice. They block histamine type 2 receptors, as a result inhibiting the secretion of gastric acid (Garg et al., 1983). Ranitidine, a H_2-receptor receptor antagonist, is a common cure for peptic ulcer diseases in performance horses (Knych et al., 2017). Although ranitidine is recognised as common therapeutic for horses, it is prohibited in all concentrations by racing regulations under Article 6A of the *IABRW* issued by the IFHA (IFHA, 2023). Hence, it is crucial to study the metabolism and elimination of ranitidine in horses for effective regulation and control, with the potential to establish a screening limit that allows laboratories to report the substance in a consistent manner.

The metabolism of ranitidine has been studied in various species, with metabolites identified in rats, dogs and humans, including ranitidine-N-oxide, ranitidine-S-oxide, desmethylranitidine and furoic acid analogue of ranitidine (which was not detected in

humans) (Carey et al., 1981; Eddershaw et al., 1996; Roberts, 1984). Three metabolites were also identified in equine, including two hydroxyranitidine metabolites and desmethylranitidine using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the positive atmospheric pressure chemical ionisation (APCI) mode in early 2000s (Chung et al., 2004). However, the detection times of these metabolites were neither studied nor reported. The elimination of plasma ranitidine has been documented in humans (Roberts, 1984; Woodings et al., 1980). A study conducted by Knych et al. (2016) explored the disposition and elimination pharmacokinetics of serum ranitidine in exercised horses following oral administration of ranitidine tablets suspended in water. Another study by Holland et al. (1997) examined the plasma pharmacokinetics of ranitidine HCl in healthy adult horses following intravenous and oral administration. To evaluate the elimination of ranitidine in horses following their standard administration regimen, six castrated horses were administered a bona fide ranitidine product (Ulcerguard™ oral paste) according to the regular treatment protocol.

This chapter presents the study on the *in vivo* metabolism of ranitidine using UPLC-HRMS in positive ESI. The aim was to identify and re-investigate the appropriate target(s) for controlling misuse of ranitidine in horses. In contrast to the APCI method employed in previous research, ESI is also widely used for analysing more polar compounds such as ranitidine. Additionally, the elimination profiles of ranitidine in horse plasma and urine were studied using UPLC-MS/MS for accurate quantitation.

5.2 Methods

5.2.1 Materials

Ranitidine hydrochloride (ranitidine HCl) standard, nizatidine and famotidine were sourced from USP (Rockville, MD, USA). Ulcerguard™ oral paste, used for administration trial and containing ranitidine HCl (220 mg/mL) and ranitidine (197 mg/mL), was purchased from Ranvet (Beaconfield, NSW, Australia). Formic acid (≥ 99%) was obtained from VWR International LLC (Pennsylvania, USA). Potassium dihydrogen phosphate, diisopropyl ether (Emsure®) were obtained from Merck (Darmstadt, Germany). Acetonitrile (Riedel-de Haen™), ethyl acetate (Riedel-de Haen™), TBME (Riedel-de Haen™), dichloromethane (Riedel-de Haen™), potassium carbonate (Fluka™) and methanol (LiChrosolv®) were supplied by Honeywell International Inc (Seelze, Germany). Ammonium formate (extra pure, 97 %) was obtained from International Laboratory Limited (San Bruno, CA, USA). Trichloroacetic acid was purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium sulfite was sourced from Peking Chemical Works (Peking, China). Ultrafree®-MC centrifugal filters (PVDF, 0.1 µm) were purchased from Merck Millipore Ltd (Carrigtwohill, County Cork, Ireland). Oasis® WCX cartridges (60 mg, 30 µm, 3 mL) were obtained from Waters (Milford, USA). Deionised water was produced using an in-house water purification system (Milli-Q, Molsheim, France).

5.2.2 Drug administration studies

Six thoroughbred castrated horses were administered 25 mL of Ulcerguard™ oral paste (equivalent to 9.8 g of ranitidine) in the morning and 20 mL (equivalent to 7.9 g of ranitidine) in the afternoon daily for eight consecutive days. The ages of Horse 1, 2, 3, 4, 5 and 6 were 9, 9, 9, 8, 4, and 11 years old respectively. The weights of Horse 1, 2,

3, 4, 5 and 6 in the morning and afternoon were 476 kg, 478 kg; 535 kg, 535 kg; 519 kg, 518 kg; 550 kg, 550 kg; 495 kg, 497 kg; 551 kg, 557 kg respectively. The dose of ranitidine given to Horse 1, 2, 3, 4, 5 and 6 in the morning was 20.6 mg/kg, 18.3 mg/kg, 18.9 mg/kg, 17.8 mg/kg, 19.8 mg/kg, and 17.8 mg/kg respectively. The dose of ranitidine given to Horse 1, 2, 3, 4, 5 and 6 in the afternoon was 16.5 mg/kg, 14.8 mg/kg, 15.3 mg/kg, 14.4 mg/kg, 15.9 mg/kg, and 14.2 mg/kg respectively. Blood samples were taken just before the first administration, and then at 0.5, 1, 2, 4, 8 and 12 hrs after the last administration. On Day2, samples were collected twice a day, followed by once a day on Days 3, 4, 5, 7 and 9. Urine samples were collected one day before the first administration, and then at least two samples were obtained within 0.5, 1, 2, 4, 8 and 12 hrs on Day 1. On Day 2, urine was sampled twice a day, and once a day on Days 3, 4, 5, 7 and 9.

5.2.3 Extraction procedures

5.2.3.1 Identification of ranitidine intact metabolites in posts-administration urine samples

Urine samples were transferred into 15-mL corex tubes and centrifuged at 1500 *g* for 10 min. The supernatant (20 µL) was then pipetted to 1.5-mL Eppendorf tube. Nizatidine (final concentration of 10 ng/µL in urine) was added to the samples as an IS. The sample solution was adjusted to a final sample volume of 1000 µL with 10% trichloroacetic acid in ammonium formate buffer (5 mM, pH 3.0). The sample was centrifuged at 19,283 *g* for 5 min. The supernatant was transferred into Ultrafree®-MC centrifugal filter (PVDF, 0.1 µm) for further centrifugation at 19,283 *g* for another 5 min. The resulting extract was then analysed using UPLC-HRMS.

5.2.3.2 Quantification of ranitidine in post-administration samples

a) Quantification of ranitidine in post-administration urine samples

Urine samples were transferred into 15-mL corex tubes and centrifuged at 1500 *g* for 10 min. The supernatant (0.5 mL) was then pipetted into a 1.5-mL graduated tube. Famotidine (final concentration of 100 ng/mL in urine) was added in the samples as an IS. Phosphate buffer (0.1 M, pH 6.0, 1 mL) was added to adjust the pH to 5.0. The mixture was then loaded to an Oasis® WCX (weak cation exchange) cartridge, which has been pre-conditioned with methanol (2 mL) and phosphate buffer (0.1 M, pH 6.0, 2 mL). The cartridge was washed with deionised water (2 mL) and acetonitrile: methanol (2:1, *v/v,* freshly prepared), followed by a 5-sec drying step. The cartridge was eluted with formic acid in methanol (20 %, *v/v*, freshly prepared, 3 mL). The eluate was dried at 60 °C under nitrogen, and the residue was reconstituted with ammonium formate (5 mM, pH 3.0)/methanol (95:5, *v/v*) (100 µL) for UPLC-MS/MS analysis.

b) Quantification of ranitidine in post-administration plasma samples

Sample preparation for the quantitative analysis of ranitidine in plasma was carried out according to the in-house protocol for screening basic drugs in plasma at the authors' laboratory.

Blood samples were centrifuged at 1500 *g* for 10 min, and the plasma (1 mL) was separated. Nizatidine (final concentration of 8 ng/mL in sample) was added as an IS. Sodium sulfite (25 %, *w/v*) (50 µL) and potassium carbonate (4 M, 200 µL) were added, and the mixture was vortexed. The samples were then extracted by LLE using dichloromethane (2 mL). The organic layer was dried at 25 ℃ under nitrogen, and the residue was reconstituted with ammonium formate (5 mM, pH 3.0)/methanol (70:30, *v/v*) (100 µL) for UPLC-MS/MS analysis.

5.2.3.3 Preparation for calibrators and quality controls for quantification of ranitidine in urine and plasma

Calibrators and quality controls were prepared independently by weighing different amounts of the drug standard. Calibrators were prepared in duplicate at 0, 5, 10, 20, 40 and 100 ng/mL in castrated horse urine, along with quality control samples prepared in duplicate with ranitidine spiked at concentrations of 5, 20 and 80 ng/mL. In plasma, calibrators were prepared in duplicate at concentrations of 0, 0.5, 1, 2, 4, 8 and 12 ng/mL, with quality control samples prepared in duplicate with ranitidine spiked at concentrations of 0.5, 2 and 8 ng/mL. Samples with drug concentrations outside the calibration range, dilution with their respective blank matrix was performed before sample preparation. The calibrators and QC urine or plasma samples were analysed alongside pre- and post-administration urine or plasma samples. The PARs of the target to IS were plotted against the calibrator concentrations and fitted using linear regression to generate the calibration curve. The responses from all quantifications (***Section 5.2.3.2***) were linear across the concentration ranges of each study, each with r values exceeding 0.995. The measured concentrations of the QC samples showed a deviation of less than ± 15 % (± 20 % for lowest QC) from their spiked values.

5.2.4 Instrumentation

5.2.4.1 UPLC conditions

UPLC-MS/MS analyses were conducted using Waters Acquity UPLC system capable of handling pressure up to 15,000 psi, connected to a TSQ Quantum Ultra mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA). For UPLC-HRMS analyses, a Thermo Scientific Q Exactive mass spectrometer (Thermo Fischer Scientific,

Bremen, Germany) equipped with a HESI-II source was used, interfaced with the same UPLC system.

The sample tray of the autosampler was maintained at 15 °C. A reverse-phase Acquity UPLC BEH C18 column (Acquity, 100 mm length x 2.1 mm ID; 1.7 µm particle size) was employed to separate the target analytes. Mobile phase A was ammonium formate in deionised water (pH 3.0, 5 mM) and mobile phase B was methanol. A linear gradient was applied at a flow rate of 350 µL/min, starting with 98 % solvent A at 0 min, reducing to 70 % at 3.0 min, then to 30 % at 5.5 min, and further dropping to 0 % at 6.0 min. The gradient was held at 0 % solvent A until 8.0 min, after which it returned to 98 % solvent A at 8.1 min and stabilised to 10 min before the next injection.

5.2.4.2 HRMS conditions

Sample ionisation was carried out in positive ionisation mode using HESI-II with a capillary temperature of 350 °C. The sheath gas flow was set at 50 arbitrary units, and the auxiliary gas flow at 10 arbitrary units. The sweep gas flow of 2 arbitrary units was used with curtain plate installed. The ion spray voltage was maintained at around 3 kV. The S-Lens RF level was set at 40 %. Full-scan mass spectra were obtained with a mass resolution of 35,000 (FWHM at *m/z* 200). The maximum injection time was 100 ms, and the AGC^{TM} was set to 3e6. The product ion mass spectra were acquired at a mass resolution of 17,500 (FWHM at *m/z* 200), using an isolation window of 1 amu. The maximum injection time was 100 ms, and the AGC^{TM} was set to 5e5. Nitrogen (> 99.995 % purity) was used the Higher-energy Collisional Dissociation (HCD) collision gas. Data processing was done using the Thermo Finnigan Xcalibur (Version 2.2)

software, with a mass tolerance window of ± 5 ppm. The observed masses of all identified product ions were within ± 5 ppm of their theoretical values.

An internal lock mass solution, containing benzyldimethylphenylammonium chloride (*m/z* 212.14338 for positive mode) at a concentration of 0.1 ng/µL in deionised water, was infused into the system post-column at a rate of 20 µL/min through a T-joint using a LC-20AB Solvent Delivery Unit (Shimadzu Corporation, Kyoto, Japan). The UPLC-HRMS parameters for precursor ion and NCE are provided in **Table 5.1**.

Table 5.1. The UPLC-MS/HRMS parameters for precursor ion and NCE

Targets	**Precursor ion (*m/z*)**	**NCE (%)**
Ranitidine (Parent)	315.15	25
Ranitidine oxide	331.14	25
Desmethylranitidine	301.13	25
Furoic acid analogue of ranitidine	302.08	25

5.2.4.3 MS/MS conditions

Injection volumes were 10 µL were used, and the HESI source was operated in the SRM negative ion mode with a spray voltage of 2 kV. The capillary and vaporizer temperatures were set at 320 ℃ and 350 ℃, respectively. The sheath, auxiliary and ion sweep gas pressures were set to 50, 10 and 2 arbitrary TSQ units. The resolution of the quadrupole mass filter was adjusted to a peak width of 0.7 amu (FWHM) for both Q1 and Q3. The collision gas pressure in Q2 was set to 1.2 mTorr. Transitions for ranitidine (*m/z* 315 → 176, 315 → 130), IS famotidine (*m/z* 338 → 189) and IS nizatidine (*m/z* 332.1 → 155.1) were monitored. The CID energies for ranitidine (*m/z* 315 → 176), ranitidine (*m/z* 315 → 130), famotidine and nizatidine were 30 eV, 26 eV, 20 eV and 20

eV respectively. The radio frequencies for ranitidine, famotidine, and nizatidine were 45 V, 100 V and 56 V respectively.

5.3 Results and discussion

5.3.1 Metabolic studies of ranitidine

Possible metabolites of ranitidine were deduced using UPLC-HRMS by comparing chromatograms from pre- and post-administration samples. The measured masses of the identified metabolites were required to deviate by no more than ± 5 ppm from those predicted on the proposed structures. Ions were selected based on the theoretical molecular masses of metabolites formed through potential biotransformations such as oxidation, hydroxylation, oxidative deamination, dealkylation or combinations of the above. Ions that met these criteria were then further analysed by UPLC-MS/HRMS to generate product-ion mass spectra for structural elucidation.

Parent drug and five metabolites (M1, M2, M3a, M3b and M4) were detected in the post-administration urine samples. The structures of these metabolites and the postulated biotransformation pathway of ranitidine are illustrated in **Figure 5.1**. The extracted-ion chromatogram of ranitidine and its metabolites in a urine sample taken 3.5 hrs post-administration is presented in **Figure 5.2**. These include ranitidine-S-oxide (M1), ranitidine-N-oxide (M2), desmethylranitidine (M3a and M3b), and the furoic acid analogue of ranitidine (M4). No conjugates of ranitidine or its metabolites were detected. Additionally, hydroxyranitidine, which was previously postulated by Chung et al. (2004) using APCI-MS/MS, was not observed in this study.

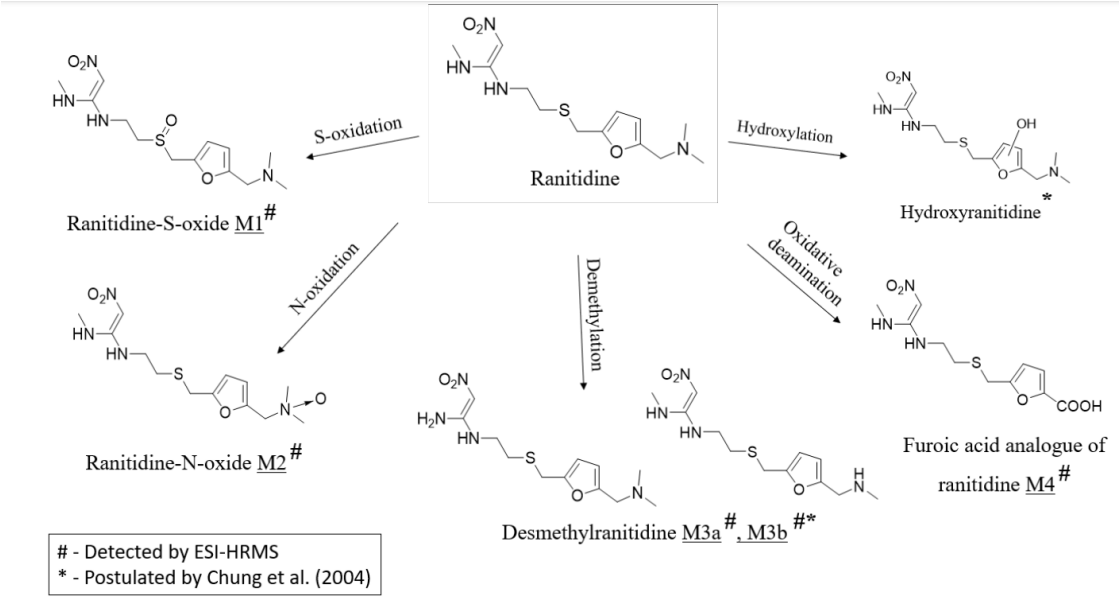

Figure 5.1. Chemical structure of the *in vivo* metabolites detected by ESI-HRMS and the proposed metabolic pathway of ranitidine in horses

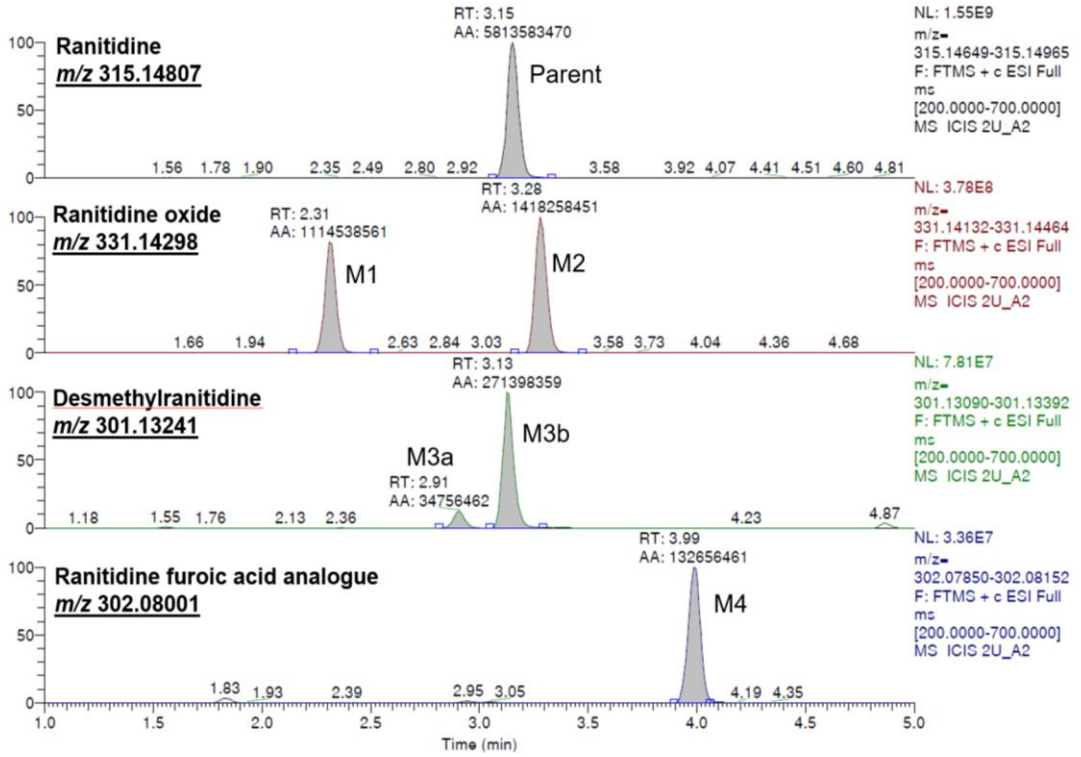

Figure 5.2. Extracted-ion chromatogram of ranitidine and its metabolites M1 to M4 detected in a 3.5-hr post-administration urine sample

Ranitidine was predominantly found in its free form in post-administration horse urine. Fragments *m/z* 270, 224, 176 and 124 were detected in the parent drug spectrum, which matched the previously reported parent mass spectrum as depicted in **Figure 5.3** (Chung et al., 2004). The major metabolites of ranitidine were ranitidine-S-oxide and ranitidine-N-oxide (M1 and M2), which had the most abundant peak intensities in the extracted-ion chromatograms, after the parent compound. Metabolites M1 and M2 were also common metabolites found in urine of various species, including rats, dogs and human (Carey et al., 1981; Eddershaw et al., 1996; Roberts, 1984). In humans, ranitidine-N-oxide is the primary metabolite (Bell et al., 1980). S-oxidation, a common metabolic pathway in H_2-receptor antagonists such as cimetidine and nizatidine, was also observed (Brimblecombe et al., 1985; Morton, 1987). However, previous studies did not present the mass spectra of ranitidine-S-oxide. In this study, S-oxidation of ranitidine in horses was proven by the detection of characteristic fragment *m/z* 192 in the M1 mass spectrum, as shown in **Figure 5.4**. The dominant fragment ion *m/z* 138 and the diagnostic fragment *m/z* 192 were proposed to result from cleavage at either side of the sulfoxide moiety.

In addition to S-oxidation, ranitidine could also be metabolised through N-oxidation and hydroxylation. The M2 mass spectrum (**Figure 5.3**) showed fragment ions *m/z* 270, 224 and 176, which are also present in the ranitidine spectrum, confirming that the oxidation occurred on the dimethylamine moiety.

Protonated desmethylranitidine with *m/z* 301 was detected, identifying M3a as a newly discovered metabolite in this study. The characteristic fragment pair *m/z* 256 → 210 in the M3a mass spectrum indicated the demethylation at methylamine moiety. The product-ion mass spectrum of M3a showed a diagnostic product ion *m/z* 162, which was likely produced by cleavage at either side of the sulfide moiety. Metabolite M3b,

previously identified in horses and other species (Carey et al., 1981; Chung et al., 2004; Eddershaw et al., 1996; Roberts, 1984), also showed a characteristic pair of fragments *m/z* 270 → 224, present in both M3b and parent ranitidine spectrum, suggesting demethylation occurred on dimethylamine group. Fragment ion *m/z* 124 was predicted to be the diagnostic ion for M3b, resulting from the cleavage at either side of the sulfide moiety (**Figure 5.5**).

Furoic acid analogue of ranitidine (M4) has been previously reported in animals other than horses and humans (Bell et al., 1980; Carey et al., 1981; Martin et al., 1981; Martin et al., 1982). In this study, the furoic acid derivative of ranitidine was detected, with the protonated molecular ion of M4 at *m/z* 302. Limited information is available on the fragmentation of the furoic acid analogue. After oxidative deamination of ranitidine, the remaining methylene group was oxidized to form a carboxylic acid. Product ions *m/z* 125 and 185, both consisting of a carboxylic acid group, were observed. A neutral loss of CO_2 from these fragments resulted in the formation of fragment ions *m/z* 81 and 141 respectively, as shown in **Figure 5.4**.

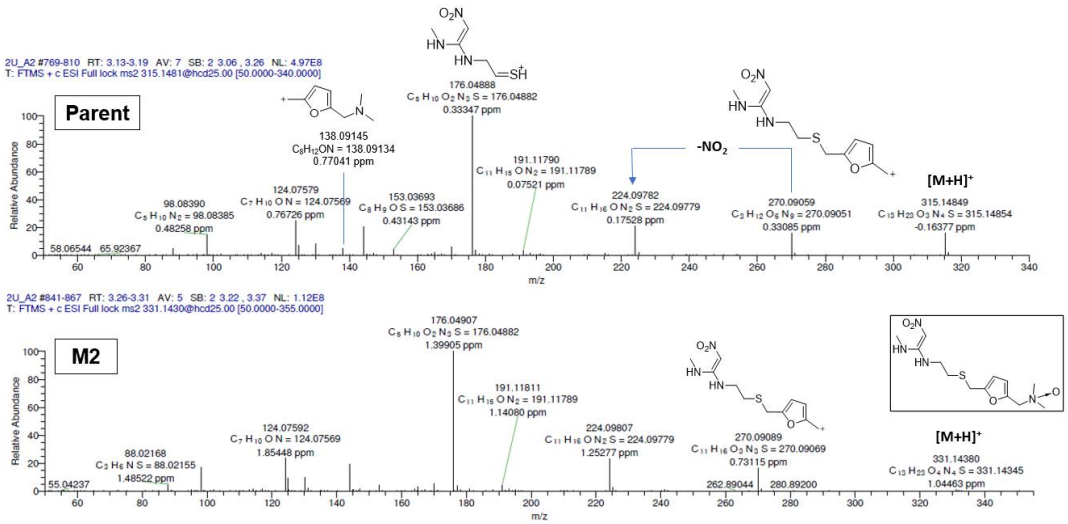

Figure 5.3. Product ion-ion mass spectra of parent drug (upper panel) and metabolite M2 (lower panel) detected in a 3.5-hr post-administration urine sample

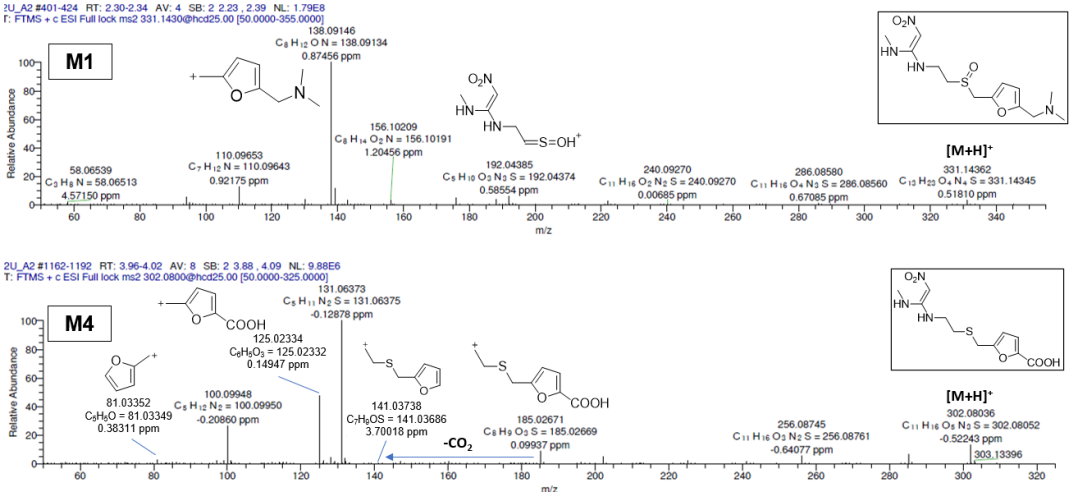

Figure 5.4. Product-ion mass spectra of metabolite M1 (upper panel) and M4 (lower panel) detected in a 3.5-hr post-administration urine sample

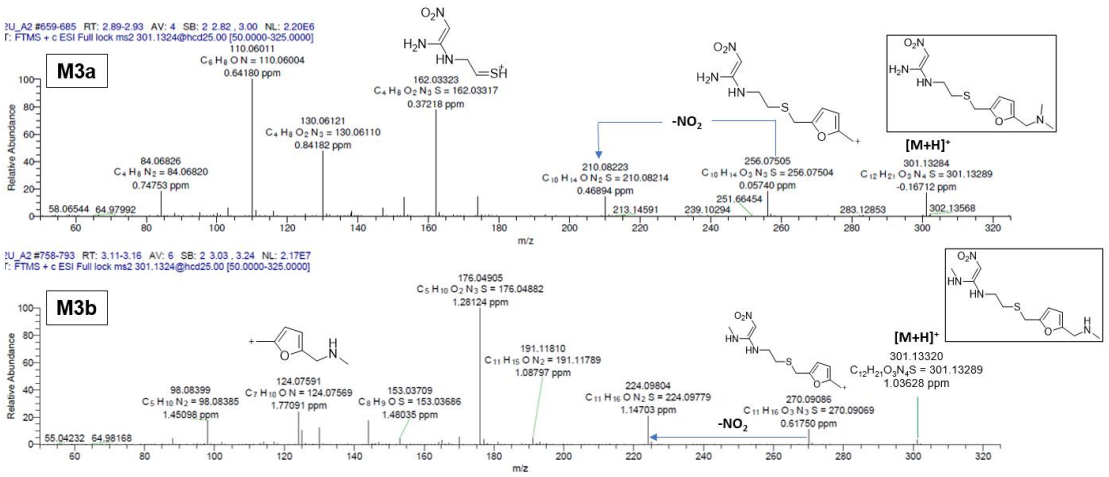

Figure 5.5. Product-ion mass spectra of metabolite M3a (upper panel) and M3b (lower panel) detected in a 3.5-hr post-administration urine sample

One of the goals of this study was to identify potential targets for screening in order to effectively control the misuse of ranitidine in horses. The maximum detection times of ranitidine and its metabolites in urine were established. Parent drug ranitidine was detectable for at least 8 days in horses in the last sample being collected. The primary urinary metabolites, M1 and M2, were also detectable for 8 days, implying that ranitidine-S-oxide and ranitidine-N-oxide could serve as viable alternatives for monitoring ranitidine abuse. The maximum detection times of metabolites M3a, M3b and M4 were 3, 6 and 4 days respectively. Additionally, the co-detection of metabolites provided direct evidence that the substance has passed through the horse's body, ruling out the possibility of contamination during and/or after sampling.

5.3.2 Elimination of ranitidine in post-administration urine and plasma samples

The urinary and plasma elimination profiles of ranitidine were examined following oral administration to six castrated horses to determine how long ranitidine remains

detectable in horses. This study aimed to establish the appropriate detection sensitivity for monitoring the use of ranitidine in racehorses.

An established sample preparation method for basic drugs screening in plasma, as described by Leung et al. (2017), was initially attempted to quantify plasma ranitidine levels. This method involved deproteination with trichloroacetic acid (10 % in deionised water, *w/v*) and SPE using a Bond Elut Certify cartridge. Sample was then eluted with a dichloromethane/ethyl acetate (4:1, *v/v*). However, the accuracy of this method did not fall within an acceptable range, as the measured concentrations of QCs at each level deviated by more than 20 % from the theoretical values. This was likely due to poor recovery of ranitidine from SPE and/or loss of drugs during deproteination. As a result, an alternative method was developed, which involved LLE without deproteination. The LLE recovery of ranitidine was evaluated using different solvents, including diisopropyl ether, TBME, ethyl acetate, and dichloromethane. Recoveries of ranitidine were assessed by analysing spiked castrated horse plasma samples at 50 ng/mL, with one set with ranitidine spiked before LLE, while the other spiked after LLE. The IS was added after the LLE step for both sets, with the second set being taken as 100 % recovery. The LLE recoveries for ranitidine were 0.2 %, 4.5 %, 43.2 % and 69.1 % extracting with diisopropyl ether, TBME, ethyl acetate and dichloromethane respectively. Since ranitidine is a highly polar drug, solvents with higher polarity, such as ethyl acetate and dichloromethane, provided better extraction of ranitidine in plasma, resulting in relatively higher extraction recovery (Carey et al., 1981). Therefore, dichloromethane was selected as the solvent for LLE of plasma ranitidine.

The LoD for ranitidine in urine and plasma was determined to be 1 ng/mL and 0.1 ng/mL respectively. The estimated LoD represents the lowest spiked concentration evaluated that resulted in a signal-to-noise ratio greater than 3:1 in the product ion

chromatogram. The LoQ of ranitidine in urine and plasma was found to be 4.26 ng/mL and 0.520 ng/mL respectively. The LoQ was calculated as 10 times the SD at the lowest QC (5 ng/mL for urine, n = 10; 0.5 ng/mL for plasma, n = 8) across 3 analytical batches. Accuracy of quantification was assessed by comparing the measured concentrations of QC samples at various levels with their theoretical concentrations. The means and standard deviations (SDs) of each QC level were calculated, and the acceptable accuracy for the mean concentration was ± 15 % (± 20 % for the lowest QC) from the spiked concentrations. For the urinary ranitidine quantification in all 4 sample batches, the accuracies of QCs at 5, 20 and 80 ng/mL were 100.3 ± 8.1 %, 90.9 ± 8.2 % and 103.8 ± 8.9 % respectively. For plasma ranitidine quantification in all 3 batches, the accuracies of QCs at 0.5, 2 and 8 ng/mL were 108.6 ± 6.5 %, 106.8 ± 4.0 % and 109.8 ± 3.8 % respectively. The extraction recovery of ranitidine in urine (n = 2) was 45.3 %, assessed by analysing the spiked castrated horse urine samples at 80 ng/mL. The extraction recovery of ranitidine in plasma (n = 2) was studied once further with spiked castrated horse plasma samples at 0.5 ng/mL, which returned an extraction recovery of 63.9 %. No correction of recovery was needed, as the IS was used to account for any loss during the extraction process.

The highest urinary concentration of ranitidine, approximately 138 µg/mL, was recorded 3.5 hrs after the last administration (**Figure 5.6**). Ranitidine was detectable in urine for 8 days (the last sample collected) in all six horses. The plasma elimination profile of ranitidine was depicted in **Figure 5.7**. The peak plasma concentration of ranitidine, around 670 ng/mL, was observed 0.5 hrs after administration. Plasma ranitidine was detectable for a period ranging from 24 to 73 hrs across the six horses. This study demonstrated that the urinary and plasma elimination profiles following oral administration of ranitidine show a strong correlation, with peak concentrations in both

biological samples occurring within the first few hours after the final dose, followed by a gradual decline over time. Based on the desired detection window, screening limits can be set to effectively monitor this commonly used therapeutic in horses.

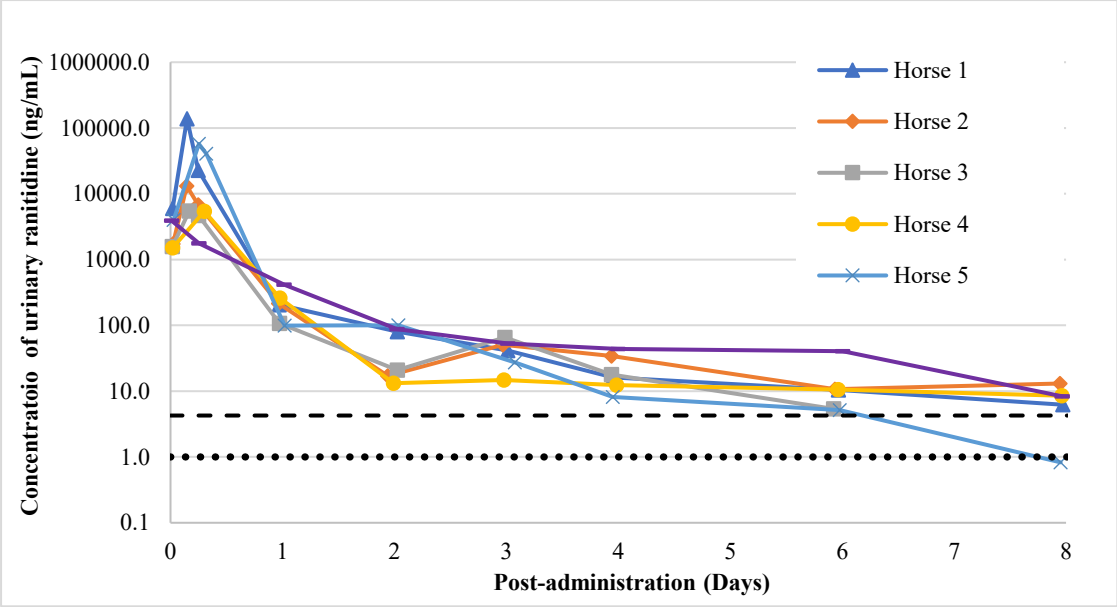

Figure 5.6. Urinary elimination of ranitidine in six castrated horses

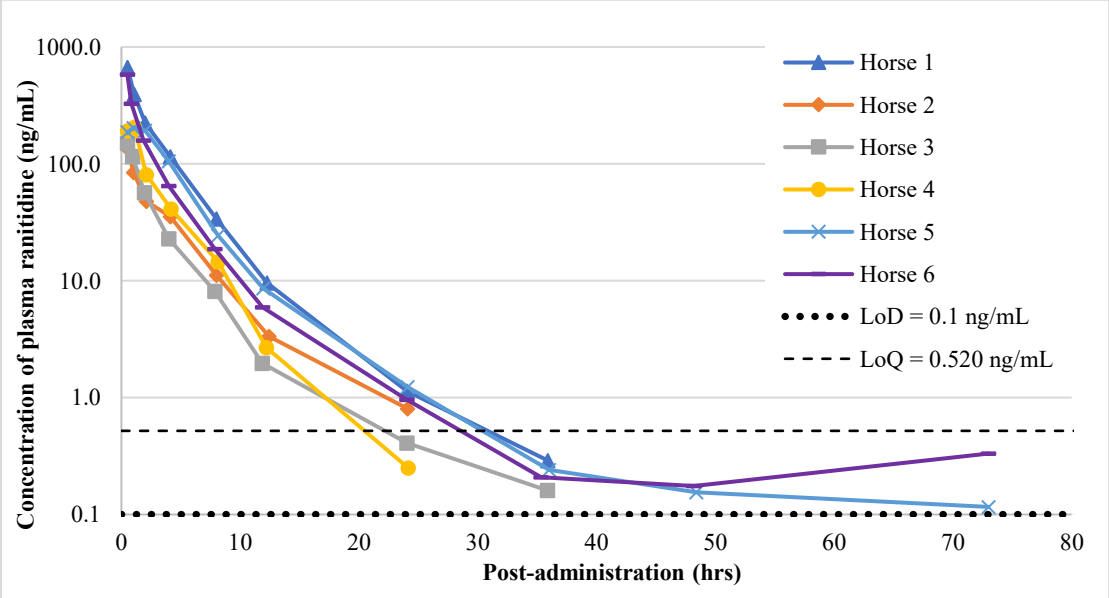

Figure 5.7. Plasma elimination of ranitidine in six castrated horses

5.4 Conclusion

Ranitidine is a histamine H_2-receptor antagonist used to treat gastric ulcers in horses. Nevertheless, therapeutic substances like ranitidine are prohibited in racehorses at any concentration under the rules of racing to ensure medication-free competition. While there was a report on urinary metabolites of ranitidine, there is limited information on their detection times in horses and which metabolite(s) would be most suitable for ranitidine doping control. Additionally, since blood is increasingly used as a matrix in doping control, it is crucial to investigate the elimination of ranitidine in plasma.

An oral administration of ranitidine to six castrated horses was carried out to identify possible ranitidine *in vivo* metabolites and to assess its elimination in horse urine and plasma for doping control purposes. Five metabolites were detected in post-administration urine samples, including ranitidine-S-oxide (M1), ranitidine-N-oxide (M2), desmethylranitidine (M3a/b) and the furoic acid analogue of ranitidine (M4). A proposed metabolic pathway for ranitidine in horses involves oxidation, demethylation and oxidative deamination. Ranitidine was primarily excreted in its free form, with ranitidine-S-oxide and ranitidine-N-oxide being the major metabolites with the highest peak intensities. These metabolites could serve as alternatives to the parent drug for detecting ranitidine administration and provide direct evidence that the substance has been processed by the horse's body. Notably, the furoic acid analogue of ranitidine (M4) was detected in horses for the first time. To effectively control the misuse of ranitidine, elimination profiles of urinary and plasma ranitidine were established. Free ranitidine was detectable for at least 8 days in urine and 72 hrs in plasma. With these elimination profiles, screening limits can be established to effectively monitor this widely used therapeutic in horses based on the desired detection window. As reference materials for

the primary ranitidine metabolites become available, additional studies should investigate their elimination profiles and assess their possible presence in blood.

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Chapter 6. Conclusions and prospects

Over the past decades, sport authorities have made substantial efforts in controlling doping both in and out of competition by developing advanced drug screening methods. Within the horseracing industry, effective drug testing approaches are essential as the misuse of prohibited substances (PS) that either enhance or impair the performance of horses can have significant impact on the integrity of the competition as well as public confidence in the sport. The abuse of drugs and/or PS in equine sports and horseracing also poses a substantial threat to the health and welfare of racehorses. A thorough understanding of the metabolism and pharmacokinetics of drugs in horses is vital to identify the effective approaches for monitoring their potential misuse. However, the metabolic pathways of many PSs remain unclear, Hence, extensive DMPK research is crucial for the industry. As prohibited substances vary significantly in their chemical properties, the evaluation of their structures and development of respective effective detection strategies are required. In view of the aforementioned discussion, the goal of this thesis was to explore and develop various approaches for doping control of three different PSs in horseracing employing various liquid chromatography and mass spectrometry techniques. These substances included dienedione (an AAS), 2HES (a NSAID) and ranitidine (a H_2-receptor receptor antagonist).

Dienedione was initially considered a synthetic AAS, as no 4,9-diene configuration has been reported in endogenous steroids. Nonetheless, its repeated detection in urine samples from entire male horses led to an investigation into its potential endogenous origin. In chapter 2, the detection of endogenous dienedione in entire male horse urine and investigation of possible conjugation of dienedione (if any) were achieved using

UPLC-MS/MS. Results indicated that dienedione is endogenous in entire male horse urine, primarily existing as glucuronide conjugates. The glucuronidation of dienedione is believed to occur at the 3-enol position, forming dienedione-3-glucuronide. A population study of free and glucuronide-conjugated dienedione in entire male horse urine sample was then conducted, revealing a mean concentration of 2.5 ± 3.5 ng/mL. The data followed a normal distribution after a fifth root transformation and excluding one outlier from Grubb's test. An in-house threshold of 30 ng/mL for free and glucuronide-conjugated dienedione in entire male horse was proposed, with a risk factor of 1 in 14,269 (with 173 degrees of freedom). This chapter exemplified the first report of endogenous dienedione in entire male horses and presented an approach for controlling its potential misuse using an in-house threshold. The developed method and approach could be extended to other endogenous steroids in horses. In the future, an appropriate international threshold could be established by creating a database of basal dienedione levels from a wider scale of population study. Data from a substantial number of untreated entire male horses across various geographical regions will be required, which would need further international collaboration. In addition to the establishment of an international threshold, further work may be to clarify the origin of endogenous dienedione in entire male horse urine.

Since dienedione remains exogenous in castrated horse urine, controlling its misuse can be achieved by identification and analysis of the drug and its metabolites within the biological samples, rather than relying on a threshold-based approach. The aim of chapter 3 was to study the elimination of dienedione and identify its *in vivo* metabolites in castrated horse for effective doping control employing UPLC-MS/MS and UPLC-HRMS. Administration experiments were conducted in which three castrated horses

each received a single oral dose of 1500 mg dienedione powder for seven consecutive days. The possible *in vivo* metabolites identified included 17-hydroxyestra-4,9-dien-3-one (M1a and M1b), hydroxylated dienedione (M2a, M2b, M3a, M3b, M4, M5), and hydroxylated M1 (M6a, M6b, M7a, M7b, M8a, and M8b), which resulted from the hydroxylation and reduction of dienedione. Metabolites M3a and M3b were considered potential targets with the longest detection windows, which remained detectable for up to 20 days in urine and 13 days in plasma post administration. Urinary free and glucuronide-conjugated dienedione could be detected for 2 to 5 days, while free dienedione in plasma was detectable for 0.5 to 4 days. Chapter 3 demonstrated that the potential misuse of dienedione in castrated horse can be effectively monitored by tracking both the parent drug and its metabolites in urine and plasma.

In chapter 4, the biotransformation and elimination of 2HES, a NSAID, in horses was studied using UPLC-HRMS and UPLC-MS/MS. The aim was to identify the most appropriate targets for detecting 2HES administration. A topical administration was carried out by applying a total of 100 g of Tensolvet gel (containing 5 g of 2HES) to three castrated horses. The proposed *in vivo* metabolites included glucuronide-conjugated and sulfate-conjugated 2HES, which likely resulted from phase II conjugation at the hydroxyethyl moiety. Another 2HES metabolite, SA, was also detected in post-administration urine samples. Nevertheless, SA was deemed unsuitable for monitoring potential 2HES exposure as the maximum concentration of SA in post-administration urine and plasma samples did not exceed its corresponding international thresholds. To effectively regulate the misuse of 2HES in horses, 2HES was identified as the most appropriate target, which was detectable for 10 days in hydrolysed urine and 16 hours in plasma.

The use of therapeutics is necessary to treat horses according to their physiological conditions. For instance, ranitidine, an H_2-receptor antagonist, is widely employed to cure peptic ulcer diseases in horses. However, it is prohibited by the rules of racing at all concentrations to ensure medication-free competition. Chapter 5 aimed to identify the most suitable *in vivo* metabolites for monitoring ranitidine misuse by employing LC-HRMS and to explore the elimination of the parent compound in horse urine and plasma. To examine the elimination and metabolism of ranitidine, six castrated horses were given daily doses of Ulcerguard™ oral paste (a total of 17.7 g ranitidine) for eight consecutive days. The expected *in vivo* metabolites were ranitidine-S-oxide (M1), ranitidine-N-oxide (M2), desmethylranitidine (M3a/b), and a furoic acid analogue of ranitidine (M4), resulting from oxidation, demethylation, and oxidative deamination. Free ranitidine was detectable for 8 days in urine and 72 hrs in plasma. Ranitidine-S-oxide and ranitidine-N-oxide, in addition to the parent drug, could be detected in urine for 8 days. They could be monitored in case an extended detection window is needed and serve as evidence that the substance has gone through the horse's body. With the elimination profiles, based on the intended detection window, an appropriate screening cut-off could be developed for its effective control. Further studies should be conducted to determine the elimination profiles of major ranitidine metabolites, as well as to study their potential presence in blood.

In summary, the research presented in this thesis offers animal sports drug monitoring laboratories approaches and insights to combat the abuse of various endogenous, exogenous drugs and therapeutics in veterinary species. All research outcomes have been successfully adopted and implemented in the regular testing of official racehorses in Hong Kong by the Racing Laboratory of the Hong Kong Jockey Club; and have been

submitted for publication in the international peer-reviewed journals, with three manuscripts already published (Ho et al., 2024a; Ho et al., 2024b; Ho et al., 2025a; Ho et al., 2025b). In addition, these developed methodologies could be further expanded to detect compounds with similar structures and properties. To further enhance the applicability of the research outcomes, one future direction would be to conduct a ring test between racing laboratories and to collect further data on endogenous levels of dienedione from entire male horses from other geographical regions to establish an international threshold of dienedione.

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