Mass Spectrometry for the Detection of Endogenous Steroids and Steroid Abuse in (Race) Horses and Human Athletes

Decloedt Anneleen, Van Landschoot Anita and Vanhaecke Lynn

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.68593

Abstract

The higher the pressure to win, the more athletes are inclined to take steps to improve one’s performance through questionable means. To minimize this, strict anti-doping and medication rules are being enforced. All human and equine athletes are regularly subjected to doping analysis to prevent abuse of forbidden substances from affecting their performance. Anabolic-androgenic steroids (AASs) have been part of the forbidden substances list for years, because of their muscle building and performance-enhancing capacities and possible side effects. For most of the AAS, zero-tolerance is held. However, some AASs can be endogenous to the athletes, such as for example testosterone in males. These endogenous steroids can render it very difficult to reveal steroid abuse. Specific mass spectrometric (MS) methods, including ultra-high performance liquid chromatography-MS (UHPLC-MS/MS), high resolution mass spectrometry (HRMS) and gas chromatography–combustion-isotope ratio MS (GC-C-IRMS), have been put forward to overcome these analytical difficulties. Currently, high-tech metabolomic methods are being used to build athlete specific biological passports. In the near future, these passports might allow putting a stop to abuse, by staying ahead of the cheats. These are bright prospects, leading towards clean and fair sports competitions worldwide.

Keywords: steroids, doping, natural, horses, athletes
1. Introduction

1.1. Steroid structures

Steroids are cyclic, organic compounds with basic skeleton 17 carbon atoms (C17) arranged in a four-ring structure: three C6 rings, followed by a C5 ring and a C8 side chain linked to C-17. The four-ring structure is formed after cyclization of a C30 chain, squalene, into lanosterol or cycloartenol [1]. The three cyclohexane rings are designated as rings A, B and C and the cyclopentane ring as ring D. The three cyclohexane rings form the skeleton of a perhydro-derivative of phenanthrene. The D-ring has a cyclopentane structure; hence, though it is uncommon, IUPAC steroids can also be named as various hydro-derivatives of cyclopentaphenanthrene (Figure 1A). This 17-carbon compound is also called gonane, the simplest steroid and a substructure present in most steroids. When the two methyl groups (C-10 and C-13) and C8 side chain (at C-17) are present, the steroid is said to have a cholestane framework (5α-cholestane, a common steroid core, e.g. cholesterol, Figure 1B). Cholesterol is the precursor of steroids in both humans and animal species [2].

Despite the shared basic steroid skeleton, hundreds of different steroids can be found in animals, plants and even fungi. They include the sex hormones such as 17β-estradiol and testosterone, bile acids, phytosterols, cortisol and drugs such as the anti-inflammatory corticosteroids (e.g. dexamethasone, prednisolone), ergosterols and many more. Individual steroids vary by the oxidation state of the carbon atoms in the rings (single or double bounds) and by the chain and functional groups attached to the four-ring skeleton [3].

Additionally, steroids can vary more markedly via changes to the ring structure (e.g. via ring scissions that produce secosteroids). Secosteroids enhance intestinal absorption of calcium, iron, magnesium, phosphate and zinc. In humans, the most important compounds in this group are vitamin D3 (also known as cholecalciferol) and vitamin D2 (ergocalciferol) [4]. Sterols, including cholesterol and phytosterols, are another particularly important form of steroids, having a cholestane-derived framework and a hydroxyl group at the C-3 position (e.g. cholesterol, Figure 1B) [5]. These closely related chemical structures of the different steroids already illustrate the challenges faced with steroid detection, identification and quantification methods.

Figure 1. (A) Chemical structure of the basic steroid cyclopentaphenanthrene skeleton including IUPAC-approved ring lettering and (B) chemical structure of cholesterol, including all normally seen branches, atom numbering and stereochemistry.
1.2. Natural steroids and their role in the endocrine system

Hormones are chemical compounds that are naturally produced by both animals and human beings and have a number of important functions in life, such as reproduction and growth. They act as signalling molecules between the different parts of the organism and trigger and modulate key reactions to support and promote life [6]. The most well-known hormones are the steroid hormones, e.g. 17β-estradiol, progesterone and testosterone, which are involved in endocrine regulation pathways. Next to influencing reproduction and growth, these steroids play other important roles as well. Testosterone regulates protein synthesis, 17β-estradiol triggers protein disposition and progesterone has an antagonistic role in oestrogens.

In mammals, including horses and humans, steroid hormones are secreted primarily by the testicles of males and the ovaries of females, although smaller amounts are also secreted by the adrenal glands (Figure 2). The adrenal glands are located just in front of the kidneys and consist of two parts, the cortex and the medulla. The adrenal cortex consists of three layers, each of which produces a different set of steroid hormones. The inner layer produces sex hormones such as oestrogen and progesterone [6, 7].

Next to adrenal and gonadal production of steroids, recent papers implied that uterine and oviductal tissues can produce steroids as well [8–10]. Adipose tissue is also no longer considered to be an inert tissue that stores fat. White adipose tissue (WAT) is now being recognized as a major endocrine and secretory organ, releasing a wide range of protein factors and signals termed adipokines, in addition to fatty acids and other lipid moieties [11, 12].

In general, steroid hormone biosynthesis involves a battery of oxidative enzymes located in two distinct cell organelles: mitochondria and the endoplasmic reticulum (ER). The transport of free cholesterol from the cytoplasm into mitochondria is the rate-limiting step in this process. CYP11A1, an enzyme bound to the inner membrane of mitochondria, will initiate the biosynthesis by converting cholesterol into pregnenolone. Pregnenolone (3β-hydroxypregn-5-en-20-one, also known as P5) undergoes further steroid metabolism in one of three ways, making it the immediate precursor for the synthesis of all of the other steroid hormones, including progestogens, mineralocorticoids, glucocorticoids, androgens and oestrogens, as well as the neuroactive steroids [1].

Figure 2. Endocrine system of humans and horses. The adrenal glands and gonads (ovaries in females and testicles in males) are the main organs involved in steroid biosynthesis. Reproduced from https://medlineplus.gov/endocrinesystem.html and the Merck Veterinary Manual (2010).
1.3. Anabolic-androgenic steroids (AAS)

The term anabolic-androgenic steroids (AASs) is used to group the naturally occurring male sex hormone testosterone, testosterone precursors and metabolites, and sometimes also (synthetically) produced testosterone variants [13, 14]. ‘Anabolic’ refers to the muscle-building capacity, ‘androgenic’ refers to increased male sexual characteristics and ‘steroid’ refers to the class of these compounds (Section 1.1).

Valid medicinal use of AAS is limited. It is only allowed to use it in the treatment of patients with a negative nitrogen balance, like weakened horses, or to accelerate healing after trauma or surgery. Anabolic steroids can be given by injection, taken orally or used externally. In humans, AASs are classified as controlled substances, due to the possibility of serious adverse effects and a high potential for abuse, as these hormones increase lean muscle mass and can improve athletic performance.

Nevertheless, non-therapeutic (abuse) of AAS has been a matter of all times. The first widespread usage of steroids and other forbidden substances in horses dates back to the early 1960s and 1970s (Figure 3). In humans, the first documented use of testosterone as a performance-enhancing substance in sport was already reported in the early 1950s. Russian weightlifters out-competed all other athletes and their trainers conceded that they were using testosterone.

Anti-doping policies in horse racing and other horse sports date back to the ’1960s. In May 1968, the first horse, Dancer’s Image, winner in the Kentucky Derby, was disqualified for using a banned substance. Traces of phenylbutazone, a non-steroidal anti-inflammatory drug (NSAID), were found in his urine post-racing (Figure 3).
2. Doping regulations for horses and humans

2.1. Human athletes

In response to the widespread (abuse) of steroids, the American Congress developed the *Anabolic Steroids Control Act in 1990*, placing steroids in the same legal class (class III controlled substances) as amphetamines, methamphetamines, opium and morphine. The World Conference on Doping in Sport held in Lausanne (1999) produced the Lausanne Declaration on Doping in Sport. This document provided for the creation of an independent international anti-doping agency to be fully operational for the Olympic Games in Sydney (2000). Pursuant to these terms set by the Lausanne Declaration, the *World Anti-Doping Agency (WADA)* was established on the 10th of November 1999 (Lausanne, Switzerland). Since then WADA aims to promote and co-ordinate the fight against doping in sport internationally. WADA was founded under the initiative of the International Olympic Committee (IOC), and the IOC still occupies 50% of the positions within the agency. The other 50% of the representatives belong to inter-governmental organizations and governments, public authorities and other public and private bodies that are also involved in the fight against doping in sport [15].

Currently, AASs are still classified as *class III controlled substances (class S1 anabolic agents)*, they are part of the first section of WADA’s List of Prohibited Substances and Methods (2017), which discusses substances and methods that are prohibited at all times, both in-competition and out-of-competition, and any athlete can be tested for these substances at any time. The list of anabolic agents is extensive and even if one is not specifically listed, it is still prohibited if it is a metabolite or has a similar chemical structure or similar biological effect(s) to anabolic agents (WADA, 2017 List of Prohibited Substances and Methods). The list includes both exogenous (S1 1.a.) and endogenous anabolic-androgenic steroids (when administered exogenously) (S1 1.b.).

2.2. Race and sport horses

Race and sport horses are, just like human athletes, frequently subjected to doping controls to guarantee a safe and fair competition. *Fédération Equestre Internationale (FEI)*, responsible for all Olympic disciplines including jumping, dressage, endurance and eventing and International Federation of Horseracing Authorities (IFHA) regulations state that ‘*any use of substances with a potential to affect equine performance, health or welfare and/or with a high potential for misuse is contrary to the integrity of equestrian sport and the welfare of the horses*’.

As strict zero-tolerance has been held for many years, anabolic-androgenic steroids might seem to be an issue of the past as new and possibly more effective ‘*designer*’ drugs have been developed over the years (e.g. AICAR, a metabolic modulator and TB-500, a synthetic peptide stimulating muscle development in horses [16, 17]). However, recent cases of steroid abuse (FEI equine anti-doping decisions, 2013–2016) prove that, although AAS abuse is better under control than it was some decades ago, it will be of all times. The potential of AAS to improve performance remains too tempting to some trainers and riders, and not only in racing and endurance. If Group VII (Middle-East) countries were excluded from the endurance records,
endurance worldwide even has a cleaner AAS abuse record than show jumping. This illustrates that, unfortunately and despite great efforts from the regulatory bodies, AAS abuse is still rooted deeply into different equine sport disciplines at both the amateur and professional level.

Additionally, there is no worldwide restriction to the use of AAS as growth promoting and performance-enhancing agents. Boldenone (Bol), androstadienedione (ADD) and Bol esters, for example, are easily available on the (European) black market as anabolic preparations, imported from the US [18]. In the United States, it also took until 2008 for steroids to be banned from the racing courses. Under this more recent law, a horse may be given steroids only under certain therapeutic conditions, and a horse may not race for at least 60 days afterwards (Press release, Kentucky Horse Racing Commission website, September 5, 2008 and Racing Medication and Testing Consortium (RMTC), 2008). In Australia, anabolic steroids were only prohibited on race day, but a new total ban (November 1st 2013 and effective from May 1st 2014, Australian Racing Board, ARB) applies to all thoroughbreds from the age of 6 months, both in- and out-of-competition. These decisions have been welcomed by the IFHA and FEI, to further two of their key objectives, being to co-ordinate and harmonize the rules of all member countries worldwide.

3. How endogenous AAS complicate anti-doping analysis

Steroids can be classified into three broad classes: exogenous, designer and endogenous steroids. With the present-day AAS abuse issue in mind, the development of new and better detection techniques is needed to detect and to distinguish between steroids belonging to these different classes. Additionally, not only AAS but also oestrogens, gestagens and androgens (EGAs) as well as thyreostats, corticosteroids and β-agonist compounds, are used alone or in growth promoting ‘cocktails’ with low concentrations of several compounds, compromising their detection [6].

Since the first discovery of AAS over 50 years ago, numerous anabolic-androgenic steroids with a variety of functional groups have been produced and/or published. Only a small number of them, the so-called ‘known’ exogenous steroids, have made it to the pharmaceutical market and are still available on the market today. They contain synthetic structures that do not occur in natural steroids (e.g. stanozolol and trenbolone, Figure 4). Confirmation of exogenous steroid abuse is relatively straightforward as qualitative demonstration of the compound in the sample suffices.

Figure 4. Chemical structures of exogenous steroids: (A) trenbolone (also known as Revalor, Parabolan, Hexabolan, Finaplix, trienolone, trienbolone or Tren), and (B) stanozolol (Winstrol, Winny). Stanozolol and trenbolone both contain a synthetic, conjugated system.
**Designer steroids** are similar to exogenous steroids, containing synthetic structures that are thought not to occur naturally, but they have not yet been classified as controlled substances and in many cases, like a pro-hormone, require a chemical reaction or enzymatic alteration once in the body to become active [19]. In most cases, they possess additional minor modifications compared to the well-known marketed exogenous steroids. These minor changes render their detection with **targeted mass spectrometry** more difficult, due to the *lack of standards*. An additional worrying feature is that no data are available on the efficacy and safety of the use of these compounds [19].

**Endogenous steroids**, such as testosterone, are steroids that are known to exist naturally, in one or more animal species (see also **Tables 1 and 2** [20]. Confirmation of endogenous steroids abuse is difficult, as simple qualitative or quantitative detection of the compound does not suffice. In horses, only testosterone and 17β-boldenone are generally regarded as endogenous (respectively in all horses and in stallions only) (Section 3.1). The classification of a steroid as ‘endogenous’ is, however, a *grey area*. The ‘semi’-endogenous presence of 17β-boldenone and related compounds, for example, in mares and geldings, is a complicating factor in doping control. Proper investigation on the origin of these AASs is required to continue to ensure adequate doping policies in the future [20, 21] (Section 4).

### 3.1. Reference ranges for AAS in humans and horses

Over the years, multiple studies have measured *excreted concentrations* of testosterone in humans, both in blood plasma and in urine (**Table 1**). On average-levels of testosterone are up to 10 times as great in adult males as in adult females [22]. The reference ranges for blood test of adult males are between 1.8 and 7.5 ng/mL (>50 years old) and 2.90 and 13 ng/mL (<50 years old), while the reference range for adult females is between 0.2 and 0.85 ng/mL [23, 24]. As the metabolic consumption of testosterone in males is greater too, the daily production was estimated to be about 20 times higher in men [25].

Futterweit et al. were one of the first to set a reference range for testosterone in urine, using thin layer chromatography (TLC) and gas chromatography (GC) [26]. Doberne and New and Tresguerres et al. on the other hand used a, at that time, very new and high-tech isotopically labelled ligand binding assay (Radioimmunoassay, RIA) [27, 28]. Mass spectrometric (MS) methods, coupled to either gas chromatography (GC) or liquid chromatography (LC), were introduced later on in the 1980s and 1990s and used for all types of anti-doping screening and quantitative methods [29–32]. Pesant et al. were an exception, using a competitive immunochemiluminescent assay for the determination of testosterone concentrations [23]. The detailed evolution of analytical methods used for steroid detection will be discussed in detail later on in this chapter (Section 5.1).

Generally, less data are available regarding the normal ranges of excretion of testosterone and its related metabolites in horses. **Testosterone** and its precursors/metabolites are known to be endogenous in males (stallions and geldings) and female horses at varying concentrations [20]. In a recent study, high performance liquid chromatography/mass spectrometry (HPLC-MS) was used to investigate the effect of γ-oryzanol supplementation on endogenous testosterone levels in horses. During that study, urine β-testosterone concentrations were always...
lower than 1.7 ng/mL for mares and geldings, Mösseler [33]. Both Ho et al. and Popot et al. measured β-Bol, ADD as well as testosterone in urine (and faeces) of (male) horses [34, 35]. Ho et al. used an immunoaffinity column (IAC) purification, followed by liquid chromatography/mass spectrometry (LC-MS/MS) analysis on a quadrupole-time of flight (Q-ToF) instrument while Popot’s extraction protocol included diethylether extraction, lipid removal, HPLC purification, derivatization and GC-EI/MS/MS detection. Testosterone levels measured by Popot et al. were between 71 and 214 ng/mL (stallions). If urine samples are being analysed with gas chromatography/mass spectrometry (GC-MS) for the identification of cryptorchidism (presence of an undescended testis in geldings), a cut-off level of 8 ng/mL is held as a marker. Testosterone levels below 8 ng/mL are regarded normal for geldings and, according to these thresholds, no β-Bol should be found in geldings [36]. Bonnaire et al. found that plasma concentrations for cycling mares vary between 20 and 60 pg/mL and can go up to 245–350 pg/mL in bearing mares. Urine concentrations in cycling mares were found to be between 1.4 and 20.1 ng/mL (GC-MS) [37].

| Reference                                | Population | Male  | n   | Mean (ng/mL) | Outliers (ng/mL) |
|------------------------------------------|------------|-------|-----|--------------|------------------|
| Futterweit et al. [26]                   | American   | 10    | 114*|              | 167*             |
| Doberne et al. [27]                      | American   | 10    | 56* |              | n.a.             |
| Tresguerres et al. [28]                  | American   | 26    | 100*|              | 231*             |
| Gonzalo-Lumbreras et al. [29]            | Spanish    | 12    | 125**|             | 191**            |
| Van Renterghem et al. [30]               | Caucasian  | 2027  | 37  |              | >100             |
| Martinez-Brito et al. [31]               | Latin-American | 2454 | 60  |              | >200             |
| Moon et al. [32]                         | Korean     | 337   | 26  |              | >150             |

*Recalculated based upon an expected average daily urine excretion of 1.5 L/day.
**Samples were collected early in the morning, at the maximum of the excretion curves.
n.a. data non available.

Table 1. Reference ranges for total testosterone in urine of humans (male).

| Reference                                | Population | Female | n   | Mean (ng/mL) | Outliers (ng/mL) |
|------------------------------------------|------------|--------|-----|--------------|------------------|
| Futterweit et al. [26]                   | American   | 10    | 4.0*|              | 5.3*             |
| Doberne et al. [27]                      | American   | 10    | 2.8*|              | n.a.             |
| Tresguerres et al. [28]                  | American   | 16    | 16* |              | n.a.             |
| Van Renterghem et al. [30]               | Caucasian  | 1004  | 12  |              | 200              |
| Martinez-Brito et al. [31]               | Latin-American | 1181 | 13  |              | 54               |

*Recalculated based upon an expected average daily urine excretion of 1.5 L/day.
n.a. data non available.

Table 2. Reference ranges for total testosterone in urine of humans (female).
\(\beta\)-Boldenone levels measured by Popot et al. (GC-EI/MS/MS) in stallions varied between 1.0 and 2.9 ng/mL urine \((n = 7)\) [35]. The range of free and conjugated (e.g. boldenone sulphate) determined by Ho et al. (LC-MS/MS, Q-ToF) was between 0.1 and 4.34 ng/mL \((n = 63, \text{from 37 male horses})\), and the mean was 1.27 ± 1.03 ng/mL [34]. Boldenone was not detected in geldings \((n = 8)\), in line with the results of Leung et al. (GC-MS) [36]. The mean \(\beta\)-boldenone concentration measured in male horses by Dehennin et al. (GC-MS) was 0.34 ng/mL \((\text{minimum 0.02, maximum 1.51 ng/mL}) (n = 156)\) [38].

### 3.2. Threshold levels for endogenous steroids

Additionally, as sport horses are frequently subjected to doping analysis, normal levels can be derived from anti-doping regulatory bodies accepted levels. Very strict zero-tolerance policies are held for most steroids, but exceptions have been made for the naturally occurring androgenic steroids: boldenone and testosterone (and stanozolol and nandrolone, according to a limited number of regulatory organs) (Table 3).

Pu et al. were able to directly detect boldenone sulphate and glucuronide conjugates in horse urine by ion trap liquid chromatography-mass spectrometry [39] and Ho et al. [34] and Popot et al. [35] also found endogenous \(\beta\)-boldenone in urine and faeces of entire males. Following these results, IFHA (Article 6, 2017), RMTC (Banned Medication List, 2017) and FEI (2017 Equine Prohibited Substances List) abandoned the zero-tolerance policy for entire male horses; a threshold for free and conjugated boldenone of 15 ng/mL was set. Despite this threshold for stallions, the presence of \(\beta\)-Bol in urine from mares or geldings is still prohibited. Nevertheless, occasionally traces of \(\beta\)-Bol or related metabolites have been found in urine of horses that were not treated with AAS [40].

For testosterone, thresholds were set for mares and fillies (unless in foal), up to 55 ng/mL free and conjugated testosterone in urine and 20 ng/mL urine for geldings are allowed. For entire male horses, amounts in excess of amounts existing naturally in the untreated horse at normal physiological concentrations are considered to be non-naturally occurring physiological concentrations. The international threshold for testosterone in plasma is 100 pg/mL for geldings. Currently, no threshold for mares is set yet, but it is being suggested to introduce the same threshold of 100 pg/mL (IFHA and FEI, 2017).

For nandrolone or nortestosterone (free and conjugated), the RMTC threshold was set at 1 ng/mL in urine \((\text{geldings, fillies and mares})\). In male horses other than geldings, 45 ng/mL of metabolite, 5α-estrane-3β,17α-diol in urine or a ratio in urine of 5α-estrane-3β,17α-diol to 5α-estrane-3β,17σ-diol of > 1:1 is considered to be indicative for abuse. Only a limited number of regulatory organs (e.g. The Canadian Horse Racing Board, CHRB) set a threshold for stanozolol, at 1 ng/mL urine. For FEI and IFHA, for example, stanozolol is listed as a banned substance and therefore strictly forbidden. Estranediol has also been added to the list of threshold substances. Free and conjugated \((5\alpha\text{-estrane}-3\beta,17\alpha\text{-diol})\), at 45 ng/mL in urine of male horses (other than geldings).

All these thresholds and reference ranges are within the low ppm or ppb range, in matrices such as urine, blood and faeces, underlining the need for very sensitive and specific detection methods that are able to determine the exact steroidal status of (race) horses in- and out-of-competition. This explains the extensive use of sensitive, state-of-the-art mass spectrometric methods in this field.
| 17α-Hydroxyprogesterone | Drostanolone | Methandrostenolone | Normethandrolone |
|-------------------------|-------------|-------------------|------------------|
| Androstenediol          | Epitrenbolone | Methasterone | Oxabolone |
| Androstenedione (AED)   | Estranediol | Methenolone | Oxandrolone |
| Bolandiol               | Ethinylestradiol | Methyldienolone | Oxymesterone |
| Bolasterone             | Ethylestrenol | Methylnortestosterone | Oxymetholone |
| **Boldenone**           | Fluoxymesterone | Methyltestosterone | Paramethadione |
| Boldione (ADD)          | Formebolone | Methyltrienolone | Prostanozol |
| Calusterone             | Furazabol | Mibolerone | Quinbolone |
| Clostebol               | Gestrinone | Nandrolone/nortestosterone | Stanozolol |
| Danazol                 | Hydroxytestosterone | Norandrostenediol | Stenbolone |
| Dehydrochloromethyltestosterone | Mestanolone | Norandrostenedione | Testosterone |
| Dehydrochlorotestosterone | Mesterolone | Norbolethone | Tetrahydrogestrinone |
| Desoxymethyltestosterone | Methandienone | Norclostebol | Tibolone |
| Dromostanolone          | Methandriol | Norethandrolone | Trenbolone |

All anabolic steroids listed as banned substances according to the FEI 2017 banned substances list. FEI and IFHA set thresholds for two AAS, boldenone and testosterone, accepting that these compounds can be present as endogenous steroids (black). Other regulatory organs set thresholds for stanozolol and nandrolone (nortestosterone) as well (gray). For **boldenone** a threshold has been set at 15 ng free and conjugated boldenone per millilitre in urine from male horses (other than geldings). Zero-tolerance is held in mares and geldings (FEI, IFHA among others). A for **testosterone** 20 ng free and conjugated testosterone per millilitre in urine or 100 ng free testosterone per millilitre in plasma is acceptable for geldings and 55 ng free and conjugated testosterone per millilitre in urine from fillies and mares (unless in foal) (FEI, IFHA and others). For nandrolone (nortestosterone) a threshold of 1 ng/mL urine has been set for mares and geldings. For stallions the threshold is significantly higher, at 45 ng/mL urine (RMTC). Only a limited number of regulatory organs (e.g. The Canadian Horse Racing Board, CHRB) set a threshold for stanozolol, at 1 ng/mL urine. Estranediol has also been added to the list of threshold substances. Free and conjugated 5α-estrane-3β,17α-diol can be tolerated up to 45 ng/mL, in urine of male horses (other than geldings).

**Table 3.** All anabolic steroids listed as banned substances according to the FEI 2017 banned substances list.
4. Analytical instrumentation

4.1. Historical evolution

Both in food residue and sport drug surveillance laboratories, big progress has been made over the last few decades regarding the detection of residues and forbidden substances (doping, incl. AAS) in different matrices [18, 41] (Figure 5).

In the 1960s and early 1970s, thin layer chromatography (TLC) combined with fluorescence detection (TLC-FL) was the most used technique. Later on in the 1970s, immunoassays such as enzyme linked immunosorbent assays (ELISA) and enzyme immunoassay (EIA) were developed and widely used. Both EIA and ELISA systems [42] are based on the principle of immunoassay linked to an enzyme rather than radioactivity as the reporter label (radioimmunoassay, RIA) [27, 28].

Mass spectrometry (MS) was introduced in the late 1970s but took until the late 1990s to conquer analytical labs worldwide. MS was first coupled to gas and later on to liquid chromatography (GC-MS and LC-MS) [43, 44]. Modern MS instruments are able to perform MS in series. The detection is carried out in the same compartment (MSⁿ) or in different compartments (MS/MS), on both the precursor ion and fragment ions, allowing to reach higher specificity and sensitivity. Over the years, various methods have been designed as screening tools to detect a large number of compounds in different drug classes, including anabolic steroids.

4.2. GC-MS versus LC-MS

GC-MS has been the gold standard for the detection of residues and anabolic steroids (in urine) for many years. In the past decades, however, there has been a general shift from GC-MS towards LC-MS/MS for drug residue and in doping control testing [45, 46]. This is mainly attributed to the rapid improvement of LC-MS/MS in recent years, leading to better sensitivity, faster instrument turnaround time and the ability to handle heat labile and large biomolecules.

Recent work has proven that ultra-high performance liquid chromatography-MS (UHPLC-MS/MS) instrumentation can provide exceptional detection capability of AAS in multiple equine matrices including mane hair [21], plasma [47] and urine [40, 48]. The development of ultra-high performance liquid chromatography (UHPLC), using sub-2 μm particles in the column, allowed higher flow rates and improved separation of compounds with similar or identical masses and retention times (e.g. α- and β-isomers of testosterone), while at the same time shortening the run time. LC-MS/MS is widely used by anti-doping testing laboratories for this purpose, and several rapid methods have been described to simultaneously detect different classes of compounds [20, 49, 50].

High resolution mass spectrometry (HRMS), on the other hand, operating at higher resolutions of 7500 up to more than 140,000 full width at half maximum (FWHM), is being optimized not as a screening technique only, but also for specific ‘omics’ biomarker approaches such as metabolomics, proteomics and transcriptomics [51–55].
Recently, however, this shift to LC has come to a standstill, with even a partial shift back to GC, depending on the type of analysis [45, 46, 56, 57]. As of 2010, GC is again gaining importance as an anti-doping approach, but then coupled to MS/MS, complimentary to LC-MS/MS. GC-MS(/MS) is an important tool for analysing saturated steroid metabolites, as they suffer from poor ionization [58]. Alternatively, many urinary screening procedures include hydrolysis of phase-II metabolites, releasing the free compounds for detection, allowing to determine the overall concentration of the compound (free and conjugated), as used to define the thresholds of both the IFHA and FEI (See earlier, Figure 5). Additionally, comprehensive two-dimensional separation techniques LC × LC or GC × GC are also being developed [59, 60].

Therefore, at the moment, LC and GC techniques can be considered complimentary, as both techniques have their specific advantages and disadvantages, depending on the compound, matrix and goal of the analysis.

4.3. Gas chromatography–combustion-isotope ratio MS (GC-C-IRMS)

As mentioned earlier, the administration of synthetic steroids, especially tackling the exogenous administration of steroids of endogenous origin (e.g. testosterone), is an important obstacle for anti-doping regulatory organs. Therefore, doping control laboratories accredited by the WADA require methods of analysis that allow endogenous steroids to be distinguished from their synthetic analogues in urine. To that extend, GC is used in hyphenation with combustion isotope ratio mass spectrometry (GC-C-IRMS) a highly specialized instrumental confirmatory technique, measuring the carbon isotope ratio ($\Delta^{13}C$) of urinary steroids and confirming their synthetic origin based on the abnormal $^{13}C$ content [18, 61].

**Figure 5.** Evolution of analytical techniques used for steroid detection.
The average isotope ratio of each element (e.g. $^{12}$C/$^{13}$C, $^1$H/$^2$H) was fixed around the time of the earth’s formation. However, variations can occur based on selective enrichment or depletion of the heavier isotopes (such as $^{13}$C), a process known as fractionation. Fractionation can, for example, take place during phase transition; a process also known as equilibrium fractionation. When water vapour condenses, the lighter isotopes ($^{16}$O and $^1$H) tend to remain in the vapour phase, while the heavier isotopes ($^{18}$O and $^2$H) accumulate in the liquid phase [62]. GC-C-IRMS is capable of measuring these differences in relative ratio of light stable isotopes of carbon ($^{13}$C/$^{12}$C), hydrogen ($^2$H/$^1$H), nitrogen ($^{15}$N/$^{14}$N) or oxygen ($^{18}$O/$^{16}$O) in individual compounds, separated from often complex mixtures of components [63, 64].

Already in 1998, Mason et al. showed that when the isotopic composition of 5β-androstane-3α,17α-diol (the main metabolite of testosterone in bile) was normalized with respect to that of an endogenous reference compound (ERC, cholesterol) in the same sample, the metabolite could be used to distinguish between animals treated intramuscularly with testosterone and untreated animals [65]. Throughout the last decade, a variety of different methods have been developed and the number of different steroids under investigation by IRMS has grown considerably. Misuse of norandrosterone, boldenone, corticosteroids or epitestosterone can now be detected with the aid of carbon isotope ratios as well [66]. However, some limitations of GC-C-IRMS need to be kept in mind (Section 4.4.4).

4.4. Matrix and method of choice

The type of matrix used for steroid detection varies according to the specific goal of the analysis. Traditionally, urine and blood samples are being sampled for the detection of forbidden substances in the light of anti-doping controls, but faeces and hair are possible matrices as well [21, 35]. The analysis of equine mane hair, for example, has the potential to greatly extend the time period over which the detection of anabolic steroid abuse can be monitored. Parent steroids (e.g. testosterone esters) are incorporated into the mane hair and can be detectable for months post-treatment. Additionally, the use of segmental analysis can potentially provide additional information on the timing of administration [21]. In residue analysis, meat samples and skin swabs are also used [20, 41, 67].

4.4.1. Direct detection and the use of thresholds or zero-tolerance policy

Depending on the context and the specific class of steroids (endogenous, exogenous or synthetic, Section 3), the method and matrix of choice can be different. Most methods are based upon the direct mass spectrometric detection of the steroid of interest and/or its metabolites in biological matrices, merely urine and/or blood samples. This direct detection is coupled to two different approaches: a threshold concentration and zero-tolerance policy.

For most anabolic steroids in horses (Table 3), zero-tolerance is held: neither the compound itself nor its direct metabolites should be detected to consider the sample as clean. For some (endogenous) steroids, a threshold concentration has been determined. For boldenone, for example, a threshold concentration has been set for stallions (15 ng/mL), while zero-tolerance is held for mares and geldings. For testosterone, a threshold concentration of 20 ng/mL for
geldings and 55 ng/mL for mares is held. For stallions, no threshold has been set as the natural testosterone concentration is under the influence of age, seasonal variations and whether or not the stallion is a breeding stallion [68]. The idea of a threshold concentration relies on the statistical likelihood that a certain concentration can be detected in an untreated horse. The threshold must be based upon populations’ studies and relevant to the concentrations measured post-treatment [49]. If the threshold concentration is set too high, concentrations measured post-treatment could be inadvertently listed as endogenous. If the threshold is set too low, non-treated horses could be unjustly banned from competitions and their owners/trainers sanctioned.

4.4.2. Measuring biological effect

As an alternative to the direct measurement of steroids, a range of assays have been developed measuring the biological effect of the steroids rather than the responsible compounds themselves. Two categories of assays can be distinguished: biosensors and biomarkers.

Biosensors utilize biological techniques to detect steroidal activity in a sample ex vivo, whereas biomarker techniques aim to monitor perturbation of ‘normal’ in vivo physiological parameters. With the rise of high resolution non-targeted approaches, the latter use of specific qualitative biomarkers has gained popularity over the last few years. Biomarkers or biological markers are metabolites that are measurable indicators of some biological state or condition, e.g. (illegal) treatment with a certain compound or compound cocktail. Biomarker monitoring can already be considered a new era in human anti-doping [69] and different biomarker approaches are being developed for equine purposes at the moment. As such, longitudinal monitoring of biomarkers can reveal non-physiological responses independently of the used doping technique or substance and may cause sanctioning of illicit practices [70].

An example hereof is an efficient strategy that has been developed to screen for abuse of nandrolone, a ‘semi’-endogenous steroid in stallions (Table 3), monitoring the endogenous steroid profile disruption in urine and blood upon nandrolone administration [70]. A panel of (endogenous) steroids of interest was extracted from equine urine and plasma samples and quantified by GC-MS/MS. Statistical processing of the collected data permitted to establish statistical models capable of discriminating control samples from those collected post-administration. These statistical models succeeded in predicting the compliance status of routine samples collected from racing horses.

4.4.3. Phase I and II metabolites

A typical problem associated with the direct detection of boldenone and other (related) AAS is that they often do not result in a measurable excretion of the parent steroid in urine and faeces. Instead, these AAS are metabolized, by the liver, and excreted as their more hydrophilic phase I and II metabolites [39]. Phase I metabolites are merely formed through classical oxidative and reductive reactions. Phase-II metabolites arise from the conjugation of these hydroxyl groups as either sulphates or β-glucuronides. These conjugates account for up to 90% of the excreted metabolites, making them an important class for screening [71].
In the case of boldenone metabolism, the 1,4-diene-3-one structure of the A-ring appears to stabilize the steroid for reductive metabolism and boldenone-17β-sulphate is the major (phase II) metabolite [72]. Therefore, extraction of urine and faeces samples includes hydrolysis of both metabolites, releasing the free compounds for detection. Hydrolysing conjugates allow determining the overall concentration (free and conjugated) of the compound, as used to define the thresholds of both the IFHA and FEI (Section 3.2). Ho et al., for example, identified intact boldenone sulphoconjugates as a direct evidence for the endogenous nature of boldenone in entire male horses.

These conjugates can, however, also be used as biomarker in equine anti-doping. They have been used is the detection of \textit{boldenone misuse}. Exogenous boldenone is known to be extensively conjugated in phase-II metabolism. Gomez et al. found that after boldenone treatment, boldenone sulphate and in some cases α-Bol sulphate were present in urine samples, together with low concentrations of exogenous boldenone (the original, active drug) and BM1 (the main boldenone metabolite, 5β-androst-1-en-17β-ol-3-one) [73]. Thus, according to Gomez et al., \textit{BM1, β-Bol and α-Bol sulphates} may be used as markers for the exogenous administration of boldenone, and they can be used to reduce the number of samples to be analysed by GC-IRMS. In samples where boldenone and BM1 are detected at low concentrations and these concentrations thus might be of endogenous origin, further analysis by GC-IRMS will only be needed if boldenone sulphates and α-Bol sulphates are also present. GC-IRMS will then be used to confirm exogenous administration.

\textit{4.4.4. Limitations of GC-C-IRMS}

As described earlier, GC-C-IRMS can be used as a confirmatory tool. However, the limitations of the IRMS approach need to be kept in mind. In equine, anti-doping establishing IRMS as a \textit{confirmatory tool} is not that straightforward, as one of the factors influencing fractionation is genetic. Monocotyledonous plants (C4 plants), such as corn and desert or marine plants, typically have $\Delta^{13}$C values varying from $-8$ to $-20\%$. Most dicotyledons (C3 plants, including up to 95\% of the plants on earth) have $\Delta^{13}$C values varying from $-22$ to $-35\%$. Because animals can only incorporate carbon through the ingestion of plant (or animal matter), the carbon isotope ratios in an animal will reflect the isotope ratios of the food source: ‘you are what you eat’ [63]. For horses, this implies that $\Delta^{13}$C values are very close to the ones of exogenous substances, hampering the ability to differentiate between endogenous and exogenously administered compounds (steroids).

Another problem associated with the use of IRMS in horses is the \textit{‘third sex’}: geldings. If tests are performed to determine if an atypical steroid profile in humans is due to administration of an endogenous steroid androsterone (Andro), etiocholanolone (Etio) and/or the androstane-diols (5α- and 5β-androstane-3α,17β-diol) are typically analysed by IRMS to determine the $\Delta^{13}$C values. The ratios of these target compounds are compared to the $\Delta^{13}$C ratio of an endogenous reference compound (ERC) such as 5β-pregnane-3α,20α-diol (Pdiol) [74]. For geldings, it is very difficult to obtain such a \textit{reliable endogenous reference compound} (ERC). It is possible to find a reliable ERC for stallions and mares, but this ERC is not consistent with geldings (personal communication, Laboratoire des Courses Hippiques, L.C.H.). In this context, Piper et
al. and Cawley and Flenker also described some of the complexities that can be encountered to obtain valid $\Delta^{13}$C measurements from GC-C-IRMS and the need for careful interpretation of all relevant information concerning an individual’s metabolism in order to make an informed decision with respect to a doping violation [66, 75].

Overall, this illustrates that the direct detection approach (relying on threshold concentrations), the biosensor/biomarker approaches and GC-C-IRMS have their limitations, including for requiring large population studies for validation and the fact that statistical outliers can be present at any time. These limitations are a part of the reason that zero-tolerance is still the preferred method to deal with ‘semi’-endogenous steroids, unless irrefutable evidence has been gathered proving the endogenous prevalence of a certain compound, in mares, geldings and/or stallions.

5. (Equine) biological passport

To conquer the latter limitations, WADA introduced the athlete biological passport (ABP) for human athletes in 2009. An APB is an individual, electronic record for professional athletes in which profiles of biological markers of doping and results of doping tests are collated over a period of time. The ABP is considered to be an effective tool in the fight against doping as monitoring selected biological variables over time can indirectly reveal the effects of doping, rather than attempting to detect the doping substance or method itself (Section 4.4.2) [76].

The concept of launching a similar equine biological passport (EBP) is not new, the earlier discussions started back in 2010, but the tone and urgency of these discussion among veterinary and regulatory authorities are changing in the wake of recent doping scandals (e.g. cobalt scandal in Australia, 2015). This sudden public focus might spur funding that helps turn complicated concepts, such as introduction of EBPs as a means to curtail doping, into reality.

With the metabolomics technology invested in the equine passport, the industry can be proactive in identifying those who are engaging in doping practices, without necessarily knowing what the specific substance is, eliminating the strict reliance on targeted analysis of post-racing urine and blood samples. Every horse has a unique metabolic signature that can be identified by monitoring metabolites or markers. This individualized approach facilitates a better understanding of the specific traits of each horse, providing more precise measurement of the biological effect of training, stress and/or drugs (controlled medication or doping) than is available with current technologies. Metabolomics is already recognized as a cutting-edge science in human medicine and anti-doping.

Additionally, the EBP can include monitoring the genes of racehorses, identifying changes throughout their careers, to combat gene doping. Gene doping is defined by the World Anti-Doping Agency (WADA) as ‘the non-therapeutic use of cells, genes, genetic elements or of the modulation of gene expression, having the capacity to improve athletic performance’. Suspected targets for gene doping are erythropoietin (EPO), myostatin and the insulin-like growth factor (ILGF).

Unfortunately, the use of biological passports will be a logistic and analytical challenge to all but the top-flight racing analytical labs around the world. The EBP is a whole-of-organization
initiative requiring high-tech equipment and the co-operation of analysts (scientists), stewards, anti-doping investigators and veterinarians to ensure all of the available information concerning prohibited practices to be integrated in real time. Racing New South Wales (Racings N.S.W., Australia) and leading French racing authorities (including L.C.H.) are currently working together on the development of EBPs.

In the past, doping sinners have always been a step or two ahead of the authorities, but with these new spectrometric techniques changing the way of handling drug abuse—not by finding the drugs but finding changes in the physiology of the horse created by the drugs—the gap may be about to close. A cheerful outlook for the fight against doping abuse.

Author details

Decloedt Anneleen1,2*, Van Landschoot Anita1,2 and Vanhaecke Lynn1

*Address all correspondence to: anneleen.decloedt@ugent.be

1 Laboratory of Chemical Analysis, Department of Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
2 Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

References

[1] Hanukoglu I. Steroidogenic enzymes: Structure, function, and role in regulation of steroid hormone biosynthesis. The Journal of Steroid Biochemistry and Molecular Biology. 1992;43:779–804

[2] Hu J, Zhang Z, Shen WJ, Azhar S. Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. Nutrition and Metabolism (London). 2010;7:47

[3] Shoppee CW. Chemistry of the Steroids. London: Butterworths Scientific Publications; 1958

[4] Holick MF. High prevalence of vitamin D inadequacy and implications for health. Mayo Clinic Proceedings. 2006;81:353–373

[5] Norman AW, Henry HL. Hormones. 3rd ed. Academic Press; China by Elsevier, ISBN: 978-0-12-369444-7

[6] Regal P, Cepeda A, Fente CA. Natural Hormones in Food-Producing Animals: Legal Measurements and Analytical Implications. InTech; 2012. ISBN: 978-953-307-887-8, http://www.intechopen.com/books/food-production-approaches-challenges-and-tasks/natural-hormones-infood-producing-animals-legal-measurements-and-analytical-implications
[7] Becker KL. Principles of Practice of Endocrinology and Metabolism. Lippincott Williams & Wilkins; 2001. p 2477. ISBN 0781717507, 9780781717502

[8] Nelis H, Vanden Bussche J, Wojciechowicz B, Franczak A, Vanhaecke L, Leemans B, Cornillie P, Peelman L, Van Soom A, Smits K. Steroids in the equine oviduct: Synthesis, local concentrations and receptor expression. Reproduction Fertility and Development. 28(9):1390–1404 https://doi.org/10.1071/RD14483

[9] Franczak A, Kotwica G. Secretion of estradiol-17beta by porcine endometrium and myometrium during early pregnancy and luteolysis. Theriogenology. 2008;69:283–289

[10] Franczak A, Kotwica G. Androgens and estradiol-17beta production by porcine uterine cells: In vitro study. Theriogenology. 2010;73:232–241

[11] Trayhurn P. Endocrine and signalling role of adipose tissue: New perspectives on fat. Acta Physiologica Scandinavica. 2005;184:285–293

[12] Coelho M, Oliveira T, Fernandes R. Biochemistry of adipose tissue: An endocrine organ. Archives of Medical Science. 2013;9:191–200

[13] Hoberman JM, Yesalis CE. The history of synthetic testosterone. Scientific American. 1995;272:76–81

[14] Dotson JL, Brown RT. The history of the development of anabolic-androgenic steroids. Pediatric Clinics of North America. 2007;54:761–769

[15] W.A.-D.A. WADA [Internet]. 2017. Available from: https://www.wada-ama.org/en/who-we-are

[16] de Laat MA, Robinson MA, Gruntmeir KJ, Liu Y, Soma LR, Lacombe VA. AICAR administration affects glucose metabolism by upregulating the novel glucose transporter, GLUT8, in equine skeletal muscle. Veterinary Journal. 2015;205:381–386

[17] Ho EN, Kwok WH, Lau MY, Wong AS, Wan TS, Lam KK, Schiff PJ, Stewart BD. Doping control analysis of TB-500, a synthetic version of an active region of thymosin beta(4), in equine urine and plasma by liquid chromatography-mass spectrometry. Journal of Chromatography A. 2012;1265:57–69

[18] McKinney AR. Modern techniques for the determination of anabolic-androgenic steroid doping in the horse. Bioanalysis. 2009;1:785–803

[19] Kazlauskas R. Designer steroids. Handbook of Experimental Pharmacology. 2010;(195):155–85. doi: 10.1007/978-3-540-79088-4_7.

[20] Scarth J, Akre C, van Ginkel L, Le Bizec B, De Brabander H, Korth W, Points J, Teale P, Kay J. Presence and metabolism of endogenous androgenic-anabolic steroid hormones in meat-producing animals: A review. Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment. 2009;26:640–671

[21] Gray BP, Viljanto M, Bright J, Pearce C, Maynard S. Investigations into the feasibility of routine ultra high performance liquid chromatography-tandem mass spectrometry
analysis of equine hair samples for detecting the misuse of anabolic steroids, anabolic steroid esters and related compounds. Analytica Chimica Acta. 2013;787:163–172

[22] Mooradian AD, Morley JE, Korenman SG. Biological actions of androgens. Endocrine Reviews. 1987;8:1–28

[23] Pesant MH, Desmarais G, Fink GD, Baillargeon JP. Reference ranges for total and calculated free and bioavailable testosterone in a young healthy women population with normal menstrual cycles or using oral contraception. Clinical Biochemistry. 2012;45:148–150

[24] MedlinePlus.gov. National Library of Medicine Reference Values; 2016

[25] Southren AL, Gordon GG, Tochimoto S, Pinzon G, Lane DR, Stypulkowski W. Mean plasma concentration, metabolic clearance and basal plasma production rates of testosterone in normal young men and women using a constant infusion procedure: effect of time of day and plasma concentration on the metabolic clearance rate of testosterone. The Journal of Clinical Endocrinology and Metabolism. 1967;27:686–694

[26] Futterweit W, Mcniven NL, Guerragarcia R, Gibree N, Drosdowsky M, Siegel GL, Soffer LJ, Rosenthal I.M, Dorfman RI. Testosterone in human urine. Steroids. 1964;4:137–145.

[27] Doberne Y, Levine MS, New MI. Elevated urinary testosterone and androstanediol in precocious adrenarche. Pediatric Research. 1975;9:794–797

[28] Tresguerres JAF, Lisboa BP, Tamm J. Simple radioimmunoassay for measurement of testosterone glucosiduronate in unextracted urine. Steroids. 1976;28:13–23

[29] Gonzalo-Lumbreras R, Pimentel-Trapero D, Izquierdo-Hornillos R. Development and method validation for testosterone and epitestosterone in human urine samples by liquid chromatography applications. Journal of Chromatographic Science. 2003;41:261–265

[30] Van Renterghem P, Van Eenoo P, Geyer H, Schanzer W, Delbeke FT. Reference ranges for urinary concentrations and ratios of endogenous steroids, which can be used as markers for steroid misuse, in a Caucasian population of athletes. Steroids. 2010;75:154–163

[31] Martinez-Brito D, Correa Vidal MT, de la Torre X, Garcia-Mir V, Ledea Lozano O, Granda Fraga M. Reference ranges for the urinary steroid profile in a Latin-American population. Drug Testing and Analysis. 2013;5:619–626

[32] Moon JY, Kwon W, Suh S, Cheong JC, In MK, Chung BC, Kim JY, Choi MH. Reference ranges for urinary levels of testosterone and epitestosterone, which may reveal gonadal function, in a Korean male population. The Journal of Steroid Biochemistry and Molecular Biology. 2014;140:100–105

[33] Mösseler A, Licht S, Wilhelm L. Kamphues J. Can oral intake of gamma-oryzanol (experimentally given orally as pure substance) result in doping relevant testosterone levels in the urine of mares and geldings? 5th European Workshop on Equine Nutrition; 2010; Cirencester
[34] Ho EN, Yiu KC, Tang FP, Dehennin L, Plou P, Bonnaire Y, Wan TS. Detection of endogenous boldenone in the entire male horses. Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences. 2004;808:287–294.

[35] Popot MA, Boyer S, Menaut L, Garcia P, Bonnaire Y, Lesage D. Boldenone, testosterone and 1,4-androstadiene-3,17-dione determination in faeces from horses, untreated and after administration of androsta-1,4-diene-3,17-dione (boldione). Biomedical Chromatography. 2008;22:662–670.

[36] Leung DKK, Tang FPW, Wan TSM, Wong JKY. Identification of cryptorchidism in horses by analysing urine samples with gas chromatography/mass spectrometry. Veterinary Journal. 2011;187:60–64

[37] Bonnaire Y, Dehennin L, Plou P, Toutain PL. Testosterone administration to mares: Criteria for detection of testosterone abuse by analysis of metabolites in plasma and urine. Journal of Analytical Toxicology. 1995;19:175–181

[38] Dehennin L, Bonnaire Y, Plou P, Ho ENM, Yiu KCH, Wan TSM. Urinary excretion of endogenous boldenone by entire male horses: Identification and quantification by gas chromatography–mass spectrometry. In: Proceedings of the 14th International Conference Racing Analysts Veterinarians; 2002; Orlando, Florida, USA. Newmarket, England; R & W Publications; 2003. pp. 58–64

[39] Pu F, McKinney AR, Stenhouse AM, Suann CJ, McLeod MD. Direct detection of boldenone sulfate and glucuronide conjugates in horse urine by ion trap liquid chromatography-mass spectrometry. Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences. 2004;813:241–246.

[40] Decloedt A, Bailly-Chouriberry L, Vanden Bussche J, Garcia P, Popot MA, Bonnaire Y, Vanhaecke L. A validated UHPLC-MS/MS method to quantify low levels of anabolic-androgenic steroids naturally present in urine of untreated horses. Analytical and Bioanalytical Chemistry. 2015 Jun;407(15):4385–96. doi: 10.1007/s00216-014-8428-x. Epub 2015 Jan 17.

[41] De Brabander HF, Poelmans S, Schilt R, Stephany RW, Le Bizec B, Draisci R, Sterk SS, van Ginkel LA, Courtheyn D, Van Hoof N, Macri A, De Wasch K. Presence and metabolism of the anabolic steroid boldenone in various animal species: A review. Food Additives and Contaminants. 2004;21:515–525

[42] Lequin RM. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). Clinical Chemistry. 2005;51:2415–2418.

[43] Erni F. Liquid chromatography-mass spectrometry in the pharmaceutical industry: Objectives and needs. Journal of Chromatography. 1982;251:141–151

[44] Gohlke RS, McLafferty FW. Early gas chromatography/mass spectrometry. Journal of the American Society for Mass Spectrometry. 1993;4:367–371

[45] Thevis M, Thomas A, Schanzer W. Current role of LC-MS/(MS) in doping control. Analytical and Bioanalytical Chemistry. 2011;401:405–420
[46] Ahrens BD, Starcevic B, Butch AW. Detection of prohibited substances by liquid chromatography tandem mass spectrometry for sports doping control. Methods in Molecular Biology. 2012;902:115–128

[47] Guan F, Uboh CE, Soma LR, Luo Y, Rudy J, Tobin T. Detection, quantification and confirmation of anabolic steroids in equine plasma by liquid chromatography and tandem mass spectrometry. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences. 2005;829:56–68

[48] Wong CH, Leung DK, Tang FP, Wong JK, Yu NH, Wan TS. Rapid screening of anabolic steroids in horse urine with ultra-high-performance liquid chromatography/tandem mass spectrometry after chemical derivatisation. Journal of Chromatography A. 2012;1232:257–265

[49] Scarth J. Approaches to the Detection of Steroid Abuse in Veterinary Species. Ghent: Faculty Veterinary Medicine, Public Health and Food Safety, Ghent University; 2011. p. 331

[50] Scarth JP, Teale P, Kuuranne T. Drug metabolism in the horse: A review. Drug Testing and Analysis. 2011;3:19–53.

[51] Van Meulebroek L, Bussche JV, Steppe K, Vanhaecke L. Ultra-high performance liquid chromatography coupled to high resolution Orbitrap mass spectrometry for metabolomic profiling of the endogenous phytohormonal status of the tomato plant. Journal of Chromatography A. 2012;1260:67–80.

[52] Cho K, Evans BS, Wood BM, Kumar R, Erb TJ, Warlick BP, Gerlt JA, Sweedler JV. Integration of untargeted metabolomics with transcriptomics reveals active metabolic pathways. Metabolomics: Official Journal of the Metabolomic Society. 2015;11(3): 503–517 doi: 10.1007/s11306-014-0713-3

[53] Giron AJ, Deventer K, Roels K, Van Eenoo P. Development and validation of an open screening method for diuretics, stimulants and selected compounds in human urine by UHPLC-HRMS for doping control. Analytica Chimica Acta. 2012;721:137–146

[54] Scarth JP. Modulation of the growth hormone-insulin-like growth factor (GH-IGF) axis by pharmaceutical, nutraceutical and environmental xenobiotics: an emerging role for xenobiotic-metabolizing enzymes and the transcription factors regulating their expression. A review. Xenobiotica; The Fate of Foreign Compounds in Biological Systems. 2006;36:119–218

[55] Granger JH, Baker A, Plumb RS, Perez JC, Wilson ID. Ultra performance liquid chromatography-MS(TOF): New separations technology for high throughput metabonomics. Drug Metabolism Reviews. 2004;36:252

[56] Van Eenoo P, Van Gansbeke W, De Brabanter N, Deventer K, Delbeke FT. A fast, comprehensive screening method for doping agents in urine by gas chromatography-triple quadrupole mass spectrometry. Journal of Chromatography A. 2011;1218:3306–3316
[57] Revelsky AI, Samokhin AS, Virus ED, Rodchenkov GM, Revelsky IA. High sensitive analysis of steroids in doping control using gas chromatography/time-of-flight mass spectrometry. Drug Testing and Analysis. 2011;3:263–267

[58] Le Bizec B, Pinel G, Antignac JP. Options for veterinary drug analysis using mass spectrometry. Journal of Chromatography A. 2009;1216:8016–8034

[59] Stoll DR, Li XP, Wang XO, Carr PW, Porter SEG, Rutan SC. Fast, comprehensive two-dimensional liquid chromatography. Journal of Chromatography A. 2007;1168:3–43

[60] Dalluge J, Beens J, Brinkman UAT. Comprehensive two-dimensional gas chromatography: A powerful and versatile analytical tool. Journal of Chromatography A. 2003;1000:69–108

[61] Teale P, Houghton E. Metabolism of anabolic steroids and their relevance to drug detection in horseracing. Bioanalysis. 2010;2:1085–1107

[62] Faure G, Mensing TM. Isotopes: Principles and Applications. Hoboken N.J. 3rd ed. 2004 ISBN: 978-0-471-38437-3

[63] Muccio Z, Jackson GP. Isotope ratio mass spectrometry. Analyst. 2009;134:213–222

[64] Piper T, Thevis M, Flenker U, Schanzer W. Determination of the deuterium/hydrogen ratio of endogenous urinary steroids for doping control purposes. Rapid Communications in Mass Spectrometry: RCM. 2009;23:1917–1926

[65] Mason PM, Hall SE, Gilmour I, Houghton E, Pillinger C, Seymour MA. The use of stable carbon isotope analysis to detect the abuse of testosterone in cattle. Analyst. 1998;123:2405–2408

[66] Piper T, Emery C, Saugy M. Recent developments in the use of isotope ratio mass spectrometry in sports drug testing. Analytical and Bioanalytical Chemistry. 2011;401:433–447

[67] Bussche JV, Decloedt A, Van Meulebroek L, De Clercq N, Lock S, Stahl-Zeng J, Vanhaecke L. A novel approach to the quantitative detection of anabolic steroids in bovine muscle tissue by means of a hybrid quadrupole time-of-flight-mass spectrometry instrument. Journal of Chromatography. A. 2014;1360:229–239

[68] Irvine CHG, Alexander SL, Turner JE. Seasonal variation in the feedback of sex steroid-hormones on serum LH concentrations in the male horse. Journal of Reproduction and Fertility. 1986;76:221–230

[69] Pottgiesser T, Schumacher YO. Biomarker monitoring in sports doping control. Bioanalysis. 2012;4:1245–1253

[70] Kaabia Z, Dervilly-Pinel G, Popot MA, Bailly-Chouriberry L, Plou P, Bonnaire Y, Le Bizec B. Monitoring the endogenous steroid profile disruption in urine and blood upon nandrolone administration: An efficient and innovative strategy to screen for nandrolone abuse in entire male horses. Drug Testing and Analysis. 2014;6:376–388
[71] Dumasia MC, Houghton E, Bradley CV, Williams DH. Studies related to the metabolism of anabolic steroids in the horse: The metabolism of 1-dehydrotestosterone and the use of fast atom bombardment mass spectrometry in the identification of steroid conjugates. Biomedical Mass Spectrometry. 1983;10:434–440.

[72] Houghton E, Maynard S. Some aspects of doping and medication control in equine sports. Handbook of Experimental Pharmacology; 2010;(195):369-409. doi: 10.1007/978-3-540-79088-4_17.

[73] Gomez C, Pozo OJ, Geyer H, Marcos J, Thevis M, Schanzer W, Segura J, Ventura R. New potential markers for the detection of boldenone misuse. Journal of Steroid Biochemistry and Molecular Biology. 2012;132:239–246.

[74] Van Renterghem P, Polet M, Brooker L, Van Gansbeke W, Van Eenoo P. Development of a GC/C/IRMS method-confirmation of a novel steroid profiling approach in doping control. Steroids. 2012;77:1050–1060.

[75] Cawley AT, Flenker U. The application of carbon isotope ratio mass spectrometry to doping control. Journal of Mass Spectrometry. 2008;43:854–864.

[76] Saugy M, Lundby C, Robinson N. Monitoring of biological markers indicative of doping: The athlete biological passport. British Journal of Sports Medicine. 2014;48:827–832.
