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OVERVIEW

A review of the analytical techniques for the detection of anabolic–androgenic steroids within biological matrices

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Abstract

Anabolic–androgenic steroids (AASs) and other image and performance enhancing drugs (IPEDs) are controlled by governments and sport institutions such as the World Anti-doping Agency (WADA). Although elite athletes and professional bodybuilders are the most visible AAS abusers, the introduction of the internet has increased the accessibility of AASs, with use being observed among recreational gym goers at increasing prevalence. Despite reported increase in use, routine analysis for these substances is uncommon, with many forensic laboratories opting to outsource AAS analysis. This review collates information regarding the extraction and analysis of AASs from various biological matrices with the considered purpose of providing a reference for the development of AAS methods to allow for routine detection by forensic laboratories.

This article is categorized under:

Toxicology > in Sport

Toxicology > Analytical

KEYWORDS

AAS, steroids, toxicology

1 | INTRODUCTION

Anabolic–androgenic steroids (AASs) covered under the broader term "image and performance enhancing drugs" (IPEDs) are predominantly synthetic derivatives of the biologically synthesized sex hormone testosterone, produced by Leydig cells in the male testes, adrenal glands and peripheral sites (Freeman et al., 2001; Hartgens & Kuipers, 2004). AASs bind to androgen receptors producing both anabolic (body building) and androgenic (masculinizing) effects (Brower, 2002). AASs used for therapeutic or recreational purposes are administered via intramuscular injection, orally (Kimergård & McVeigh, 2014), and transdermally (Sitruk-Ware, 1989).

It has been argued that AAS usage is the youngest of the world's major substance use disorders, with tens of millions of predominately men worldwide believed to have used these substances at least once in their lifetime (Kanayama et al., 2020). There are fears that AAS use has been incorporated within mainstream health and beauty consumerism (Brennan et al., 2017), with Google searches observed between January 2011 and December 2015 exhibiting increased market share for AASs, peaking during the summer months in the United Kingdom (Tay Wee Teck & McCann, 2018).

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Body dissatisfaction, perception of physical inadequacy, lack of genetic physiological response to training, media ideals, a fear of aging, and interpersonal competitiveness are believed to be some of the reasons behind the recreational use of AASs (Cohen et al., 2007; Kimergård, 2014; Mosley, 2009; Parkinson & Evans, 2006; Van Hout & Kean, 2015).

Forensic toxicologists play an important role in the reporting and monitoring of the increasing AAS prevalence within the wider population (Evans-Brown et al., 2012); however, a lack of knowledge regarding the impact of drug–drug interactions (DDIs) on AAS metabolism exists. These DDI risks are compounded by enhanced recreational and IPED polydrug use demonstrated within the AAS administering community (Piatkowski et al., 2021; Sagoe et al., 2015) as well as prescription misuse (Ip et al., 2019).

The aim of this manuscript is to provide a reference regarding the current different analytical methods (strengths and limitations) used for the analysis of AASs in biological matrices to monitor the consumption of AASs within the general population. A literature search was performed on Science Direct, PubMed, as well as internet search browsers using key terms "anabolic–androgenic steroids," "AASs," "doping compounds," "exogenous steroids," and "anabolic steroids." Papers of relevance were selected based on their applicability to the forensic science community, including method development, validation and interpretation of results. Due to the lack of published forensic methods, published methods relating to anti-doping were also reviewed and included where appropriate.

2 | CHEMICAL STRUCTURE AND PHARMACOLOGY

Most AASs derive from three compounds: testosterone, 19-nortestosterone (nandrolone) and dihydrotestosterone (DHT) (Hoffman et al., 2009). The basic testosterone molecule consists of 19 carbon atoms forming three cyclohexane rings and one cyclopentane ring with a methyl group in positions 10 and 13, as shown in Figure 1. Additionally a hydroxy group can be found at position 17, ketone group at position 3 and a double bond at position 4 (Srinivas-Shankar & Wu, 2006). Nandrolone is structurally identical except for the demethylation at the 19th carbon position (Hoffman et al., 2009), which increases its anabolic activity in comparison to testosterone (Bhasin & Jasuja, 2009). DHT is a hydrogenated analogue of testosterone with the reduction of the double bond at position 4. Alteration of the 17α-H of the original base molecule results in varying effects, with the 17α-alkyl substitution (Type B), causing the rate of pre-systemic metabolism to decrease, resulting in the extension of the molecules half-life (Bhasin & Jasuja, 2009). This subsitition also sterically hinders the oxidation of the 17β-hydroxy group (Type A), preventing the deactivation of the steroid by first-pass metabolism, making it orally active (Kicman, 2008). 17α-alkylated androgens are potentially hepatoxic and markedly reduce high-density lipoprotein cholesterol (Bhasin & Jasuja, 2009; Hoffman et al., 2009). Addition to the oxygen (Type A) makes the compound suitable for "depot" injection (a slow-release method of administration involving intramuscular injection, where the drug is slowly released from the muscle to the blood over a relatively long time) (Mottram & George, 2000). 7α-alkyl substitutions make testosterone less susceptible to 5α reduction and increases its prostate tissue selectivity (Bhasin & Jasuja, 2009). Substitution at any point within the three hexane rings (Type C) allow for oral administration with increased potency and slower metabolism (Mottram & George, 2000). Many AASs

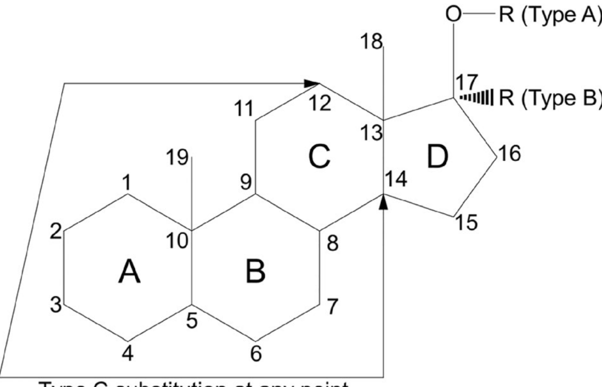

FIGURE 1 General AAS chemical structure, showing possible sites of modification. Modifications at "Type A" site result in compounds suitable for injection preparations; changes made at Type B and C result in compounds suitable for oral preparations.

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have metabolites in common due to the similarities of the parent drugs, posing analytical challenges. Furthermore, the majority of AASs are excreted as conjugates (discussed further in Section 5).

3 | BIOLOGICAL MATRICES USED FOR AAS DETECTION

Urine represents the favored biological matrix for confirming the administration of AASs due to the presence of long-term metabolites (Balcells et al., 2015), longer detection windows when compared to other matrices (Protti et al., 2019), as well as the unspecialized non-invasive collection process and high sample volumes available (see Table 1). Difficulties linked with the use of urine for AAS analysis include: the large number of potential steroids and respective metabolites sought to be identified and the complexity of the urine matrix, which contains structurally similar endogenous steroids at concentrations many times higher than the analytes of interest (Bowers, 1997; de Albuquerque Cavalcanti et al., 2018).

AAS positive urine samples from suspected perpetrators in Swedish drug-related offenses ($N = 1799$) spanning from 2003 to 2009 found the six most commonly identified AASs with mean hydrolysed concentrations to be: testosterone ($n = 696$, 539 μg/L), nandrolone ($n = 392$, 135 μg/L), metandienone ($n = 255$, 650 μg/L), stanozolol ($n = 103$, 243 μg/L), boldenone ($n = 202$, 1246 μg/L) and trenbolone ($n = 118$, 1116 μg/L). Major metabolites produced a mean range of 302 μg/L (3′-Hydroxystanozolol)—2080 μg/L (17β-Trenbolone) (Lood et al., 2012). This provides examples of the typically high urine AAS concentrations found in toxicological cases compared to those detected in anti-doping. Therefore a high degree of method sensitivity is not necessarily required for the detection of AASs in samples from the general population.

Serum is an alternative matrix for the detection of exogenous AASs and has been shown to allow more sensitive measurements of exogenous testosterone provided after initial injection, although it is important to note the time frame between administration to analysis (Savkovic et al., 2018). Testosterone concentrations (3.2 and 2.3 μg/L) detected in serum using LC–MS, were found to indicate doping in two females, which had not surpassed the typical urine testosterone (T) to epitestosterone (E) ratio (T/E ratio) threshold required for further investigation due to potential urine sample tampering and a genetic deletion (Handelsman & Bermon, 2019) (discussed further in Section 7). However, serum concentrations of drostanolone and metenolone were lower than their metabolite concentrations in the corresponding urine (Makvandi et al., 2023). Furthermore, the analysis of blood matrices (plasma and/or serum) can detect intact AAS esters, strongly indicating exogenous AAS administration (de la Torre et al., 2021; Tretzel et al., 2014) (discussed further in Section 8).

The ease and non-invasive nature of oral fluid collection has led to interest for potential use in clinical and forensic toxicology for the detection of endogenous AASs (Lood et al., 2018). There are two main mechanisms AASs can enter saliva; free, non-protein bound unconjugated AASs will pass into saliva through salivary gland acinar intracellular diffusion, and conjugated AASs are restricted to ultrafiltration via the salivary gland acinar cells (Wood, 2009). Oral fluid analysis was found to be relevant in the evaluation of intramuscularly injected testosterone undecanoate and have potential forensic toxicology uses, with maximal testosterone concentrations observed 7–14 days after administration (0.53 ± 0.40 μg/L). Salivary testosterone concentrations significantly corresponded to serum testosterone concentrations (12.1 ± 6.3 μg/L); however, larger inter-individual variation and weaker correlation was found between saliva testosterone and urinary testosterone (47 ± 37 nmol/mmol of creatinine). As well as examining unconjugated testosterone levels, concentrations of free testosterone were also calculated using the Vermeulen equation (Lood et al., 2018). Furthermore, a marked increase in testosterone concentrations after transdermal application can be observed when testing oral fluid, while conventional urinary steroid concentration determination did not exceed critical thresholds during a 72-h post-administration period (Thieme et al., 2013). However, limited detection windows for exogenous AAS detection, low steroid oral fluid concentrations as well as reduced oral fluid volumes compared to urine are major limitations for the implementation of oral fluid for AAS analysis (Anizan & Huestis, 2014).

3.1 | Alternative biological matrices

The use of hair samples for AAS detection has been attempted and documented (Deng et al., 1999; Kintz, 1998; Kintz et al., 1999, 2001, 2020, 2021a; Kintz & Gheddar, 2021); however, the Society of Hair Testing has explicitly stated that negative findings in hair does not overrule a positive urine result (Sachs & Kintz, 1999). The collection of hair samples

TABLE 1 An overview of published analytical methods for the simultaneous detection of unconjugated AASs.

Sample	Sample hydrolysis	Sample extraction	Sample derivatization	Analytical technique	BLA	BOL	CAL	CDM	CLO	DRO	ETH	ETS	FLU	FOR	FUR	MSA	MSE	MTA	MTN	MTS	NAN	NOE	OMS	OMT	OXA	STA	TES	TRE	RF
Urine	β-Glucuronidase from *H. pomatia*	LLE	MSTFA/NH4I/DTE, 1000:2:4 v/w/w	GC-μAPPI-QQQ																+	+						+		(Hintikka et al., 2013)
Hair and urine	β-Glucuronidase from *H. pomatia*	SPE	Methoxyamine hydrochloride (2%, w/v in pyridine)	GC-EI-Q	+				+			+	+			+	+	+	+	+	+			+		+	+	+	(Deng et al., 1999)
Urine	β-Glucuronidase from *E. coli*	LLE	MSTFA:ammonium iodide:2-mercaptoethanol	GC-EI-Q-Orbitrap MS				+		+	+					+						+			+				(de Albuquerque Cavalcanti et al., 2018)
Urine	β-Glucuronidase from *E. coli*	LLE	MSTFA/NH4I/DTE (1000:2:4, v/w/w)	GC-EI-Q	+		+				+								+						+				(Mazzarino et al., 2007)
Urine	β-Glucuronidase from *E. coli*	SPE	MSTFA/NH4I/DTE (500:4:2, v/w/w)	GC-EI-Q or LC-ESI-QQQ	+	+					+	+											+		+		+		(Moon et al., 2008)
Urine	β-Glucuronidase from *E. coli* with nitrophenyl-b-D-glucuronidase as substrate	Reverse-phase SPE	MSTFA/NH4I/DTE (1000:2:4, v/w/w)	GC-ITMS and GC-Q	+		+					+			+				+	+					+		+		(Galesio et al., 2010)
Urine	β-Glucuronidase from *E. coli*	LLE	MSTFA/ethanethiol/NH4I (500:4:2, v/v/w)	GC-EI-QQQ	+	+	+					+	+						+				+		+		+		(Van Eenoo et al., 2011)
Urine	β-Glucuronidase from *E. coli*	LLE	O-TMS derivatives	GC-ITMS		+						+	+		+								+		+		+		(Marcos et al., 2002)
Urine	β-Glucuronidase from *E. coli*	LLE	MSTFA, NH4I and propanethiol	GC-EI-QTOF			+			+																			(Abushareeda et al., 2018)
Urine	β-Glucuronidase from *E. coli*	LLE	None	HPLC-IS-CID-APCI-Orbitrap MS				+															+		+			+	(Virus et al., 2008)
Plasma	None	LLE	None	LC-HESI-Orbitrap MS														+								+		+	(Thomas et al., 2010)
Urine	β-Glucuronidase from *E. coli*	LLE	None	LC-ESI-QQQ								+													+		+		(Pozo, Deventer, et al., 2007)
Urine	None	LLE	None	LC-ESI-QQQ		+							+				+						+		+				(Pozo, Van Eenoo, et al., 2007a)
Urine	β-Glucuronidase from *E. coli*	LLE	1.5 M hydroxyl amine solution (pH 10.0) or picolinic acid—0.52 mmol; 2-methyl-6-nitrobenzoic anhydride—0.52 mmol; and 4-dimethylamino pyridine—0.37 mmol	LC-ESI-QQQ		+										+		+		+							+	+	(Baranov et al., 2010)
Urine	β-Glucuronidase/arylsulfatase from *H. pomatia*	Reverse-phase SPE	None	LC-ESI-QQQ		+				+		+					+	+	+	+	+				+	+	+	+	(Andersen & Linnet, 2014)

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TABLE 1 (Continued)

Sample	Sample hydrolysis	Sample extraction	Sample derivatization	Analytical technique	BLA	BOL	CAL	CDM	CLO	DRO	ETH	ETS	FLU	FOR	FUR	MSA	MSE	MTA	MTN	MTS	NAN	NOE	OMS	OMT	OXA	STA	TES	TRE	RF
Urine	β-Glucuronidase from *E. coli*	SPE	None	LC-ESI-TOFMS		+									+										+				(Kolmonen et al., 2009)
Urine	β-Glucuronidase from *E. coli*	LLE	None	LC-ESI-TOFMS	+	+	+																			+		+	(Vonaparti et al., 2010)
Urine	β-Glucuronidase from *E. coli*	SPME	None	LC-ESI-Q		+														+	+					+	+		(Saito et al., 2010)
Urine	β-Glucuronidase from *E. coli*	SLE	None	UHPSFC-ESI-QQQ	+	+	+						+		+				+				+		+				(Desfontaine et al., 2016)
Urine	β-Glucuronidase from *E. coli*	SLE	None	UHPSFC-ESI-QQQ	+	+	+						+		+				+				+		+	+		+	(Nováková et al., 2016)

Note: It should be noted AASs were not included when used as internal standards within each method.

Abbreviations: APCI, atmospheric pressure chemical ionization; BLA, bolasterone; BOL, boldenone; CAL, calusterone; CDM, oral turinabol (chlorodehydromethyltestosterone); CID, collision-induced dissociation; CLO, clostebol; DRO, drostanolone; EI, electron ionization; ESI, electrospray ionization; ETH, ethylestrenol; ETS, epitestosterone; FLU, fluoxymesterone; FOR, formebolone; FUR, furazabol; GC, gas chromatography; HPLC, high performance liquid chromatography; IS, insource; ITMS, ion trap mass spectrometry; LC, liquid chromatography; LLE, liquid–liquid extraction; MS, mass spectrometry; MSA, mestanolone; MSE, mesterolone; MTA, metandienone; MTN, metenolone; MTS, 17a-methyltestosterone; NAN, nandrolone; NOB, norboletone; NOE, norethandrolone; OMS, oxymesterone; OMT, oxymetholone; OXA, oxandrolone; OXB, oxabolone; Q, singe quadrupole; QQQ, triple quadrupole; QTOF, quadrupole time-of-flight mass spectrometry; SLE, supported liquid extraction; SPE, solid phase extraction; SPME, solid phase microextraction; STA, stanozolol; TES, testosterone; TOFMS, time-of-flight mass spectrometry; TRE, trenbolone; UHPSFC, ultra-high performance supercritical fluid chromatography; μAPPI, microchip atmospheric pressure photoionization.

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BOX 1 The benefit of alternative matrices

Although alternative matrices such as oral fluid and hair are not currently recognized by the IOC and WADA, the detection of AASs in these matrices may still be important despite the limited incorporation, particularly if these compounds are to be monitored as part of workplace drug testing programs or mandatory drug testing within prisons.

is less invasive in comparison to blood or urine, giving a more accurate history of drug use by providing a wider time window for detection (Kintz, 1998; Kintz et al., 2001). Due to their neutral chemical properties, steroids are poorly incorporated into hair making detection challenging. The external contamination of hair with other recreational drugs such as cannabis and cocaine has been observed (Tsanaclis et al., 2014), and therefore should also be considered when interpreting any positive results for these compounds. Additionally, it should be noted that hair is not classified as a valid specimen by the International Olympic Committee (IOC) or WADA, although it is accepted by most judicial systems around the world (Kintz et al., 2020).

When head hair is not available, body hair can be used for the detection of AASs. Body hair, however, has been found to exhibit higher drug concentrations compared to head hair when interpreting quantification results (Gheddar et al., 2020; Kintz et al., 2021a). Differences in hair growth and the incorporation rate of AASs within each type of hair as well as sweat exposure and urine contamination, have been provided as explanations for the varying AAS concentrations observed across all hair origins (Gheddar et al., 2020). Additionally, it is thought body hair provides a wider detection window, as it has been noted that the AAS administrating demographic generally have short hair resulting in a detection window of 1–3 months for head hair, compared to a body hair detection window of 8 months (Kintz et al., 2021a).

Nail clippings have also proven to be a potential specimen for the detection of stanozolol, nandrolone, trenbolone, drostanolone and testosterone enanthate, with quantitative analysis reporting a concentration range of 6–89 pg/mg for both cleaned fingernail and toenail clipping specimens from six cases after repetitive exposures. It has thus been stipulated that due to the low concentrations detected, liquid chromatography–tandem mass spectrometry (LC–MS/MS) and gas chromatography–tandem mass spectrometry (GC–MS/MS) following trimethylsilyl (TMS) derivatization is a requirement for analysis using fingernail and toenail clippings (Kintz et al., 2021b). Similar to hair, these samples are not considered valid specimens by the IOC or WADA (Box 1).

Dried blood spots (DBSs), providing small volumes of capillary blood, is seen as complementary to traditional urine and venous blood samples. A GC–MS/MS method was developed and validated in accordance with International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use guidelines for the quantification of testosterone, nandrolone, boldenone, mesterolone, drostanolone, metenolone, metandienone, oxandrolone and chlorodehydromethyltestosterone following volumetric absorptive microsampling of 20 μL with a limit of detection (LOD) of 0.1–0.78 ng/mL and runtime of 6.4 min (Chang et al., 2020). DBSs were fully introduced into routine doping control in the 2022 Beijing Winter Olympics (Yuan et al., 2022) and are being more frequently used in a forensic toxicology setting (Sadler Simões et al., 2018; Sadones et al., 2014).

4 | METABOLISM

Phase I of AAS metabolism usually involves conversion via enzymatically catalyzed oxidation, reduction and hydroxylation reactions at the A, B, C and D rings (as shown in Figure 1) into more polar compounds inactivating the drug and facilitating its elimination from the body (Schänzer, 1996). Phase II metabolism involves enzyme-controlled reactions resulting in the conjugation of Phase I metabolites, with the production of glucuronide AAS conjugated metabolites, assisted by UDP-glucuronic acid, and conjugated sulfate metabolites, the result of a reaction with 3′phosphoadenosine 5′-phosphosulfate (Schänzer, 1996). Although glucuronidated and sulfated conjugates are the most common, cysteine conjugated testosterone metabolites have been reported, with this metabolic pathway expected to be present in the metabolism of exogenous steroids (Fabregat et al., 2013). Potential evidence of trenbolone cysteine conjugates resulting from exogenous oral consumption supports this notion (Putz et al., 2020). Methasterone and its metabolites have been found not to be significantly excreted as cysteine or N-acetylcysteine conjugates (Magalhães et al., 2019).

Most AASs are completely metabolized with little to no parent steroid excreted (Schänzer & Donike, 1993). Oxandrolone, fluoxymesterone, chlorodehydromethyltestosterone, formebolone and the metabolites of oxandrolone, metandienone and stanozolol are known to be excreted unconjugated (Schänzer, 1996). There have been found to be differences in the metabolic functions of endogenous and exogenous nandrolone resulting in varying AAS metabolite conjugation as exogenously sourced nandrolone produced exclusively glucuro-conjugated 19-norandrosterone, while endogenous nandrolone excreted some sulfoconjugated 19-norandrosterone (Le Bizec et al., 2002), enabling doping tests to determine the origin.

4.1 | AAS metabolite detection windows

Improved metabolic knowledge of AASs has led to the identification of long-term metabolites (LTMs) that can be employed for the detection of AASs several weeks after consumption has ceased. The AASs seen in Table 2 have all benefited from greater examination leading to the identification of new metabolites which have longer detection windows, enabling the identification of previously undetected AASs (Kuuranne & Saugy, 2016). The addition of LTMs (18-nor-17β-hydroxymethyl,17α-methyl-androst-1,4,13-trien-3-one) to a screening method for the detection of metandienone resulted in positive findings increasing by 400%, despite the number and origin of the analyzed samples remaining constant (Geyer et al., 2014). The prementioned metabolite had a reported detection window of 19 days using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), over twice the detection time frame compared with other commonly detected metandienone metabolites via GC–MS (Schänzer et al., 2006).

4.2 | Conjugated metabolite detection

A recent focus on the detection of phase II glucuronide or sulfate conjugates has arisen due to the potential to extend detection time windows for AASs (Davis et al., 2021; Görgens et al., 2016). An example of this would be 3α-glucuronide-6β-hydroxyandrosterone and 3α-glucuronide-6β-hydroxyetiocholanolone which are present after oral testosterone administration and are both resistant to enzymatic hydrolysis. Subsequent analysis via UHPLC–MS/MS has extended the detection window to 96-h, an increase of 84-h compared to traditional methods (Kotronoulas et al., 2017). The detection of AAS sulphated metabolites using LC–MS requires specific sample preparation that is incompatible with other analytes of interest; however, it has been demonstrated that non-hydrolysed sulphated AASs could be incorporated into initial GC–MS testing procedures (Albertsdóttir et al., 2020). Additionally, comparable sensitivities have been demonstrated for the analysis of non-hydrolyzed sulphated AASs; gas chromatography-chemical ionization-triple quadrupole- (GC-CI-QQQ), low energy-electron ionization-gas chromatography-quadrupole time of flight-mass spectrometry and LC-QQQ with the sulfate group cleaved off in the injection port forming two isomers during GC–MS analysis (Polet et al., 2019). A LC–MS/MS method provided LODs for 10 sulfate conjugates ranging from 0.25 to 0.5 ng/mL, 19 glucuronide conjugates were 0.5 to >20 ng/mL and 7 unconjugated steroids were 0.25 to 4 ng/mL, with sulfated conjugates demonstrating lower LODs in comparison to equivalent glucuronide conjugates (Balcells et al., 2015). A metenolone metabolite (1β-methyl-5α-androstan-17-one-3ζ-sulfate) analyzed using gas chromatography-chemical ionization triple quadrupole mass spectrometry (GC-CI-MS/MS) was found to provide a detection window of up to 17 days, twice the detection time frame in comparison to contemporary routine doping control metabolites

TABLE 2 Long-term AASs metabolites increasing the duration of detection.

AAS	Duration of metabolite detection (days)	Reference
Mesterolone	15	(Polet et al., 2017)
Metandienone	26	(Gómez, Pozo, Garrostas, et al., 2013)
Methasterone	9	(Magalhães et al., 2019)
Methylstenbolone	29	(Piper et al., 2019)
Oxymesterone	46	(Polet et al., 2017)
Stanozolol	28	(Schänzer et al., 2013)

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(Albertsdóttir et al., 2020). Ion paired extraction utilizing Sep-Pak® C_{18} SPE cartridges followed by dichloromethane (DCM) liquid liquid extraction (LLE) was found to be the most reliable method for the direct isolation of sulfate steroids from urine samples (Iannone et al., 2020). Following LC–MS/MS analysis, using ESI—a 97 m/z product ion is always present corresponding to HSO_4^-, while an 80 Da neutral loss equating to SO_3 is commonly found, which despite being not very selective does allow for the easy detection of unknown sulfur metabolites in (Balcells et al., 2016; Gómez, Pozo, Marcos, et al., 2013). However, liquid chromatography-ion mobility-high resolution mass spectrometry (LC-IM-HRMS) successfully separated 20 out of 22 AAS intact phase II metabolites including isomers, providing increased selectivity and improved identification in comparison to LC–MS/MS, which can struggle with isomer selectivity (Davis et al., 2021). A WADA validated dilute and shoot LC–MS/MS method for the detection of free, glucuronidated and sulfated endogenous AASs provides a heavily reduced workload alternative to routine GC–MS based methods (De Wilde et al., 2020). An atypical hydrophilic interaction liquid chromatography–field asymmetric waveform ion mobility spectrometry–mass spectrometry method provided rapid analysis (8 min) for the successful qualitative and quantitative determination of glucuronide and sulfate metabolites from seven AASs (Arthur et al., 2017). It should be expected that further research of AAS metabolites and instrumentation development will lead to increased detection time windows for AASs, greatly benefitting anti-doping analyses.

5 | SAMPLE PRETREATMENT AND EXTRACTION METHODS

5.1 | Hydrolysis of phase II AAS metabolites

Phase II metabolites which have undergone conjugation can be subjected to hydrolysis to cleave the conjugate group, reverting the steroid back to its parent form for ease of analysis. Β-glucuronidase and arylsulfatase are the two main enzymes used for the cleavage of steroid conjugates and can be obtained from various sources including bovine liver, *Escherichia coli* and *Helix pomatia*. Arylsulfatase originates from *Aerobacter aerogenes*, *Helix pomatia* and *Patelincludiata* (Ferchaud et al., 2000). *Helix pomatia* has been found to create by-products when incubated with non-conjugated steroids, with data suggesting that variability in *H. pomatia* extracts affect reactivity and selectivity during incubation with free steroids (Massé et al., 1989). Additionally, β-glucuronidase deriving from *H. pomatia* (Pedersen et al., 2017) and bovine liver (Ferchaud et al., 2000) does not contain sulfatase activity, preventing the cleavage of sulfate conjugates and should therefore not be used where the hydrolysis of sulfate conjugates is required. Enzymatic hydrolysis utilizing *E. coli* following pH adjustments are typically carried out at 40–55°C for 1 h (Bulska et al., 2015; de Albuquerque Cavalcanti et al., 2018; Mussell et al., 2007; Tseng et al., 2006).

Chemical hydrolysis offers an alternative to enzymatic hydrolysis, involving either solvolysis (Hauser et al., 2008) or hot acid (typically HCl or H_2SO_4) hydrolysis (Dumasia & Houghton, 1981; Konieczna et al., 2011; Pizzato et al., 2017). Many hydrolyses undergo methanolysis, a variation of solvolysis (Cooper et al., 2001; Tseng et al., 2006), in which the oxygen attached to C-17 undergoes protonation during strong acidic conditions, utilizing acetyl chloride in methanol, resulting in the simultaneous separation of sulfate and glucuronide moieties (Viljanto et al., 2018). Chemical hydrolysis via ethyl acetate/methanol/sulfuric acid 80:20:0,12 (v/v) provided the best hydrolysis response of at least 50% across testosterone, epitestosterone, androsterone, etiocholanolone, 5-androstene-3β,17β-diol, 5α-androstane-3β,17β-diol, DHEA, epiandrosterone, 11-ketoetiocholanolone and cholesterol for the cleaving of the sulfate moiety in comparison to enzymatic hydrolysis (Iannone et al., 2020). Although faster than enzymatic hydrolysis, chemical hydrolysis may cause undesired effects such as the degradation of some analytes, increased levels of co-extractants and increased matrix interference from degradation of macromolecules (Wynne et al., 2004). This coupled with inefficient sulfate hydrolysis has led to enzymatic hydrolysis using *E. coli* being the preferred technique (Gomes et al., 2009). The use of purified β-glucuronidase from *E. coli* is also recommended for hydrolysis of the glucuro-conjugated urinary steroids by WADA (WADA, 2020).

5.2 | Sample extraction methods

In order to detect AASs in biological matrices sample extraction steps are required prior to analysis. Previous publications commonly utilize LLE and solid-phase extraction (SPE), while supported liquid extraction (SLE) methods are less frequently seen as shown in Table 1.

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LLE is the most used sample extraction technique, typically involving tert-butyl methyl ether (MTBE). LLE carried out with dichloromethane (DCM), obtained recoveries for 15 AASs from human urine ranging from 91.2% to 103% using high-performance liquid chromatography (HPLC) (Gonzalo-Lumbreras et al., 2001). While, LLE followed by GC–MS analysis of 10 AASs from dietary supplements using a mixture of n-pentane and diethylether (75:25) obtained recoveries of 88.6% to 111.1% from an energy drink, powder solid, and liquid matrices (Dahmani et al., 2018).

As well as LLE, SPE is commonly used for the extraction of AASs from biological matrices. The extraction of 46 AASs from urine using four commercially available SPE cartridges, Serdolit PAD-1, Sep-pak® C_{18}, amino-propyl and Oasis® HCB, combined with three extraction solvents, diethyl ether, MTBE and n-pentane was conducted by Cho and Choi (Cho & Choi, 2006). Extracted samples were analyzed via GC–MS. Oasis® HLB combined with MTBE extraction consistently provided the highest recoveries for 39 of 46 AASs with a yield range of 72.6%–97.3%. Higher extraction yields of 91.4%–106% were seen for the extraction of 12 AASs from urine using Extra-Sep C_{18} cartridges. These were conditioned with MeOH and dH_2O, washed with 4:1(v/v) dH_2O/acetone and n-hexane, and elution was carried out with diethyl ether. Although this method successful extracted 12 AASs, danazol, dehydroepiandrosterone (DHEA) and oxymetholone experienced difficulties due to substituent polarity. When compared to a LLE method the C_{18} cartridges produced cleaner chromatograms and shorter analysis times (Gonzalo-Lumbreras et al., 2001). A further study utilized reverse-phase SPE using a Strata-X plate for the extraction of 18 exogenous AASs from urine. Cartridges were washed using a dH_2O/MeOH (40:60) mixture with IPA/MeOH (1:1) used for elution. (Andersen & Linnet, 2014).

An instance of SLE with DCM extraction solvent recovered 18 free endogenous and exogenous AASs at recovery efficiencies ranging from 42% (methasterone) to 91% (boldenone) from serum when analyzed using an UHPLC–MS/MS (Langer et al., 2022).

5.3 | Derivatization

AASs can undergo derivatization (targeting their hydroxy and ketonic groups) via various derivatization agents prior to either GC or LC analysis to improve their detectability (Casals et al., 2014). Following the synthesis of N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) silylating agent in the late 1960's (Donike, 1969), it was quickly implemented for the derivatization of AASs (Donike, 1976). MSTFA remains the most common method of trimethylation used for AASs prior to GC–MS analysis (Abushareeda et al., 2018; Bowden et al., 2009; Marcos et al., 2002; Moon et al., 2008) with the addition of various catalysts such as ammonium iodide and dithioerythritol (Galesio et al., 2010; Hintikka et al., 2013) or ammonium iodide and ethanethiol (Van Thuyne et al., 2008; Van Thuyne & Delbeke, 2005). Other derivatization agents used are N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) (Deng et al., 1999). More recently, microwave-accelerated derivatization (MAD) before GC–MS analysis has been shown to provide similar derivatization yields compared to standard thermal derivatization while also significantly reducing the incubation time to under 3 minutes (Bowden et al., 2009; Casals et al., 2014).

Problems can occur in the derivatization step of 19-norsteroids with a 3-keto-4,9,11-trien ring leading to tautomerism, whereby more than one peak is produced from unstable artifacts (de Boer et al., 1991; Kim et al., 2000). A derivatization procedure utilizing O-methylhydroxylamine hydrochloride/pyridine (8:100 (w/v)), followed by evaporation, and reconstitution in MSTFA/TMS-imadazole (100:2 (v/v)) prior to incubation at 60°C for 30 min can produce single peaks for AASs (gestrinone, tetrahydrogestrinone [THG] and trenbolone) that undergo tautomerism during typical derivatization (Marques et al., 2007). This derivatization method with C_{18} SPE produces a LOD of 3 ng/mL and achieves recoveries of $99.4 \pm 14.6\%$ for THG, $104.5 \pm 8.5\%$ for trenbolone and $94.8 \pm 16.9\%$ for gestrinone from spiked urine samples by GC–MS analysis (Marques et al., 2007). Alternatively, to circumvent tautomerism, LC–MS/MS has been used to detect AASs (Marques et al., 2007); however, GC–MS remains a reliable and low-cost technique, indispensable in doping control labs (Marcos & Pozo, 2015; Marques et al., 2007).

Although derivatization is not necessary prior to LC–MS/MS analysis, derivatized steroids demonstrate increased ionization efficiency and so improved sensitivity compared to non-derivatized AASs (Athanasiadou et al., 2013; Higashi & Ogawa, 2016; Marcos & Pozo, 2015). Other derivatizing agents have been used for endogenous AAS esters within DBSs prior to LC–MS/MS analysis. Girard's Reagent P (GP), produced the best yield for the derivatization of all 14 testosterone esters and 2 nandrolone esters within serum when compared to Girard Reagent T (GT) and a hydroxylamine derivatization mixture (hydroxylamine hydrochloride/pyridine [200:5; w/v]), producing responses multiple times better than the non-derivatized equivalent compound (de la Torre et al., 2021). Additionally, GP was used for the derivatization of 20 endogenous anabolic steroid esters in fortified DBSs with analysis via ultra-high performance liquid

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chromatography-quadrupole-orbitrap mass spectrometry (UPLC-Q-Orbitrap-MS) (Yuan et al., 2022). Furthermore, urine containing free and conjugated methenolone and mesterolone metabolites derivatized with GT and analyzed using LC–MS/MS observed significantly increased detection and identification time windows compared to TMS derivatized GC–MS/MS analysis (Angelis et al., 2023).

6 | AAS ANALYTICAL DETECTION METHODS

6.1 | History of AAS detection

Since the first application of GC–MS to detect and identify performance enhancing drugs in sport was developed in 1967 (Beckett et al., 1967), there have been numerous advancements in the analytical detection of AASs overtime as shown in Figure 2. The history of AAS use and detection can be further explored in a variety of literature (Bhasin et al., 2021; Kanayama et al., 2010; Kanayama & Pope, 2018; Shackleton, 2009), but falls beyond the scope of this review. The majority of analytical development has been driven by WADA due to the restrictions placed upon AASs within sports and challenges presented by anti-doping. Outside sport, AAS analysis has been incorporated into the UK Ministry of Defence Compulsory Drug Test for military personnel (Ministry of Defence, 2019). However, despite these advancements, analysis remains low, with AAS testing not typically included within routine drugs of abuse analysis.

6.2 | Immunoassay detection of AASs

Recent advancements in enzyme-linked immunosorbent assay (ELISA) have led to their application for the preliminary detection of AASs, although the majority of published ELISA based screening methods are not for biological matrices (Huml et al., 2020; Jurášek et al., 2017). An Androgen Receptor (AR) BioAssay was developed that enabled the detection of 17 AASs in urine, but its period of effective usage was limited to immediately after AAS administration while intact active AASs were present, as 12 metabolites of 10 commonly abused AASs produced limited or no AR BioAssay activity (Bailey et al., 2016).

6.3 | Comparison of confirmatory techniques for the detection of AASs

AASs and respective metabolites can almost be exclusively detected via GC–MS or GC–MS/MS due to their non-polar nature. However, LC–MS/MS has been utilized in the detection of a subset of AASs that are difficult to volatilize, with stanozolol and its major metabolites typically problematic (Thevis et al., 2011), due to the polarity caused by an

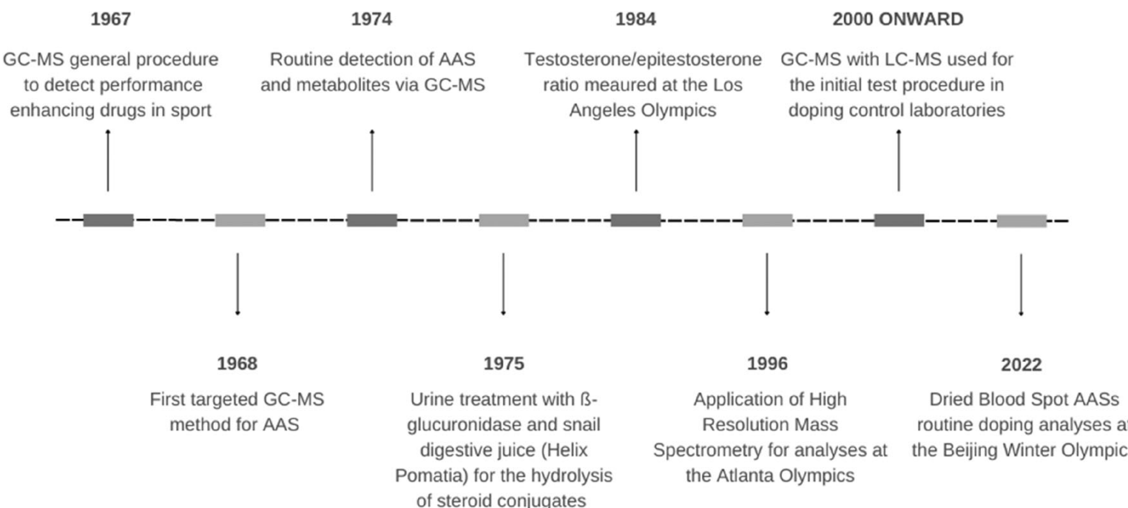

FIGURE 2 A timeline of the key analytical detection developments for AASs.

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included pyrazole ring (Ward et al., 1975). As previously mentioned in Section 5.3, 19-norsteroids with a 3-keto-4,-9,11-trien ring system such as gestrinone, tetrahydrogestrinone and trenbolone are known to experience difficulties in the derivatization step as a result of tautomerism prior to GC–MS analysis and therefore LC–MS/MS is used for detection (Marques et al., 2007). TMS-enol-TMS ether trenbolone derivatives predominately formed during on-column derivatization with an injection port temperature of 275°C and column temperature of 180°C, demonstrate tautomerism leading to several products with an ion at m/z 414 when analyzed by GC–MS, while off-column derivatization at room temperature for 15 min yields TMS ethers (de Boer et al., 1991). Despite these hinderances, GC–MS analysis is the superior instrumentation to separate epimeric steroids, demonstrating inherently better chromatographic resolution over LC–MS/MS due to the LC's need for short run times. LC–MS/MS has the advantage of rapid specific analysis of a limited number of compounds at high sensitivity (Krone et al., 2010). ESI was determined to have the lowest LOD for unconjugated AASs during LC–MS/MS analysis, in comparison to atmospheric pressure photo ionization and atmospheric pressure chemical ionization (Leinonen et al., 2002). LC–MS is not appropriate for AASs containing a hydroxy-group at carbon position 3 that is not conjugated by any double bond such as methandriol. This structure results in a resilience to ESI causing a reduced sensitivity in comparison to other AASs and thus a recommendation to use GC–MS (Pozo, Van Eenoo, et al., 2007b; Van Poucke et al., 2005). This further highlights the importance of understanding the structural chemistry of the AASs to be analyzed.

Deuterated internal standards have been recommended for the detection of AASs in urine when using UHPLC–MS/MS due to the complexity of urine as well as possible suppression of endogenous AASs resulting from exogenous administration (Gosetti et al., 2013). The determination of concentration ratios between conjugate and free fractions is possible using LC–MS, while GC–MS only allows the total prepared AAS to be analyzed (Gosetti et al., 2013).

Typically, for the analysis of AASs and their metabolites, GC column phases are non-polar and contain 5% phenyl (Galesio et al., 2010; Hintikka et al., 2013) or 100% dimethylpolysiloxane (de Albuquerque Cavalcanti et al., 2018; Deng et al., 1999; Marcos et al., 2002; Mazzarino et al., 2007; Moon et al., 2008; Schänzer et al., 2006; Van Eenoo et al., 2011) with an assortment of column internal diameters, film thickness and column lengths.

LC usually utilizes a variation of C18 (octyldecylsilane) phase columns (Andersen & Linnet, 2014; Balcells et al., 2015; Baranov et al., 2010; Deshmukh et al., 2010; Görgens et al., 2016; Guddat et al., 2013; Kolmonen et al., 2009; Kuuranne et al., 2003; Mazzarino et al., 2008, 2010; Moon et al., 2008; Peters et al., 2010; Pozo, Deventer, et al., 2007; Pozo, Van Eenoo, et al., 2007a; Saito et al., 2010; Thomas et al., 2010; Virus et al., 2008; Viryus et al., 2009; Vonaparti et al., 2010), at varying lengths, inner diameters and particle sizes. Some previous C8 (octylsilane) column use can also be seen in the literature for AASs analysis (Samanidou et al., 2009; Schänzer et al., 2006; Thevis et al., 2006).

A comparison of conventional AAS detection instrumentation for 76 exogenous AASs and 3 hormone and metabolic modulators with steroid backbone structures, found liquid chromatography-silver ion coordination ionspray/triple quadrupole mass spectrometry (LC-AG^+CIS-MS/MS) detected 68, LC-ESI-MS/MS detected 54, and following TMS derivatization GC-EI/MS detected 56, GC-EI-MS/MS detected 65, and GC-ESI-MS/MS detected 27 at a LOD of 2.0 ng/L or lower (Cha et al., 2015). This demonstrates the importance of considering the ionization method employed when analyzing AASs as the LOD differences between GC-EI-MS/MS and GC-ESI-MS/MS can be clearly observed.

Table 3 shows a comparison between both GC–MS and LC–MS/MS instrumentation in the context of AAS analysis. Considering the advantages and disadvantages of both techniques it should come as no surprise that current screening procedures see both GC–MS and LC–MS/MS as complementary.

Recently, supercritical fluid chromatography (SFC) has reemerged as a viable alternative to conventionally methods used for AAS detection following instrumentational improvements allowing for enhanced performance and more stable conditions (Parr & Botrè, 2022). Ultra-high performance supercritical fluid chromatography (UHPSFC) has demonstrated excellent performance for the detection of AASs below the WADA minimum required performance levels (MRPLs) with a Diol stationary phase employed (Nováková et al., 2016). UHPSFC coupled with a triple quadrupole detector has been implemented for the detection of 43 anabolic agents including AASs and metabolites; however, at concentrations of 0.1 ng/mL sensitivities were demonstrated to be 73% in comparison to UHPLC–MS/MS (98%) and GC–MS/MS (14%) (Desfontaine et al., 2016).

6.4 | Anti-doping versus forensic AASs method requirements

WADA has set MRPL; concentrations that represent a minimum routine detection and identification capability for test methods. Exogenous AASs have a typical MRPL of 5 ng/mL with exceptions including metandienone,

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TABLE 3 Comparison of the performance of GC/MS and LC–MS/MS for AAS analysis (Krone et al., 2010).

Task	GC/MS	LC–MS/MS
Ease of sample prep	Time consuming	Minimal
Derivatization	Necessary	Generally, not needed
Automation	Injection only	All stages
Speed of Analysis	Long	Short
Chromatographic resolution	Excellent	Poor (short run time)
Steroid conjugate detection	No	Good
Epimer separation	Good	More difficult
Specificity	Excellent	Excellent
Sensitivity 3-oxo-4-ene steroids	Moderate	Excellent
Sensitivity 3-hydroxysteroids	Good	Poor
Non-targeted steroid profiles	Good	Poor
Non-polar compounds (sterols)	Good	Poor, derivatization necessary

BOX 2 A surmountable challenge

Although AASs propose a more complex analytical challenge than more traditional drugs of abuse, the instrumentation required for their detection is commonly available within forensic science laboratories. It should therefore be possible for non-WADA laboratories to incorporate AAS analysis into existing workflows.

17α-methyltestosterone and stanozolol requiring 2 ng/mL (WADA, 2019b). Due to the growing use of AASs within the general public, laboratories not currently accredited by WADA may wish to incorporate AAS screening methods into their routine analysis. Although it would be ideal to adhere to the MRPL detailed by WADA technical documents (WADA, 2021) it has been suggested that toxicological requirements and anti-doping requirements are very different (Kintz et al., 2020). In contrast anti-doping relies upon the monitoring of individuals including indirectly via the Athlete Biological Passport (ABP). The typical concentrations observed in samples from individuals who are not trying to avoid adverse analytical findings are generally much more easily determined in comparison to competitive athletes (Box 2) (Lood et al., 2012).

7 | ENDOGENOUS STEROID ADMINISTRATION AND DETECTION

The production of endogenous AASs may lead to false positive results. As methods of analysis became more sensitive and knowledge of innate AAS production improved, more accurate analytical methods capable of discriminating between endogenous and exogenous AASs were necessary. The detection of endogenous AASs relies upon the urinary steroid profile, assembling part of the ABP. An Atypical Passport Finding (ATPF) is determined by an adaptive model reliant on subject-based longitudinal testing (WADA, 2019a). The longitudinal testing enables the prediction of expected values for steroid doping markers and defines individual limits (Sottas et al., 2010), this is not usually possible in a forensic setting. However, there may be instances when exogenous versus endogenous analysis of testosterone would be required. Suggested methods for discriminating AAS origin included GC-C-IRMS (Ayotte, 2006; Piper et al., 2010), hair analysis (Kintz et al., 1999) and the analysis of conjugated metabolites (Le Bizec et al., 2002). IRMS provides conclusive evidence of origin, endogenous or exogenous, of urinary metabolites, even when found in low quantities (Ayotte, 2006). The origin can be determined as variation exists in the stable carbon isotope ratio ($^{13}C/^{12}C$, reported as $\delta^{13}C$), throughout the geographic environment (Lacombe & Bazinet, 2021). GC-C-IRMS is principally based upon natural AASs having a different carbon isotopic signature from synthetic AASs (Kohler & Lambert, 2002), with exogenous urinary steroids having comparatively lower $^{13}C/^{12}C$ ratios than endogenous (Aguilera et al., 2001; Kohler &

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Lambert, 2002; Piper & Thevis, 2022). However, the use of GC-C-IRMS for determining exogenous/endogenous origin can be difficult when pharmaceutical preparations display a carbon isotopic composition range value similar to endogenous urinary AASs (de la Torre et al., 2021).

Since 2004, WADA required further analysis of samples using GC-C-IRMS to determine $^{13}C/^{12}C$ ratios of suspected administered endogenous AASs. Testosterone, epitestosterone, androsterone, etiocholanolone, androstanediols and DHEA are specifically listed as potential targets of analyses if the urinary T/E ratio is equal or greater than 4.0. (WADA, 2004). The chemical structure is the same for both endogenous and exogenous testosterone, therefore administration cannot be confirmed solely by determining the presence of testosterone.

There have been extraordinary examples of individuals with naturally occurring mean ratios of testosterone and epitestosterone which exceed the set limit (Garle et al., 1996; Oftebro, 1992; Raynaud et al., 1992), with one example ratio reportedly as high as 12.4 (Garle et al., 1996). Moreover, excessive alcohol consumption (>1.5 mg/kg) in four men significantly increased T/E ratios (Große et al., 2009) and low ethanol doses (0.2 and 0.4 g/kg) in some men and women elevated T/E ratios (Albeiroti et al., 2018). Ethylglucuronide may be used as a marker to track alcohol induced T/E ratio elevation which is easily detected by LC–MS/MS (Große et al., 2009; Thieme et al., 2011). The use of T/E ratios is less effective for the determination of exogenous testosterone in females due to the lack of sources producing testosterone endogenously that are impacted by negative feedback in comparison to men, exogenous testosterone reduces the natural testosterone and epitestosterone production enlarging the T/E ratio. Furthermore, false negatives can arise in individuals with UGT2B17 gene deletion as the phenotype does not form testosterone glucuronide, resulting in very low or undetectable urine testosterone concentrations (Anielski et al., 2011; Handelsman & Bermon, 2019). Suspicions of UGT2B17 deletion should arise when T/E ratios do not fall within a normal curve of distribution (median of T/E 0.5) and therefore a PCR-ELISA system (Genotype® UGT test, AmplexDiagnostics) can determine the UGT2B17 phenotype from the same collected urine sample (Anielski et al., 2011). Instances of UGT2B17 deletion are known to be common in individuals of Asiatic decent (66.7%) and less prevalent in Caucasian individuals (9.3%) (Jakobsson et al., 2006).

False positive results for exogenous boldenone administration can also occur, as target compounds used for the determination of illicit use are produced naturally in the body or produced by bacterial activity (Piper et al., 2010; Schänzer, 1996). This is true for nandrolone as well with very low concentrations of endogenous nandrolone metabolites thought to appear in urine; however, these are considered negligible when compared to those obtained form an exogenous source (Björkhem & Ek, 1982; Dehennin et al., 1999; Le Bizec et al., 1999; Reznik et al., 2001). Pregnant women are known to produce increased concentrations of nandrolone (Ayotte, 2006; Mareck-engelke et al., 2002); however, analytes were only detectable after the 14th week of pregnancy with 93% of samples from pregnant women containing concentrations less than 5 ng/mL (Mareck-engelke et al., 2002).

8 | ESTERED AAS DETECTION

The detection of estered AASs in an individual provides strong evidence that AASs have been administered from an exogenous source and would remove the necessity to determine the origin. After 2 intramuscular injections testosterone decanoate, isocaproate and phenylpropionate were detectable <14 days and testosterone propionate was detected <5 days from DBSs via nanoLC-HRMS (Solheim et al., 2022). The length of the ester chain significantly impacts the rate of elimination with blood samples providing a similar pattern to DBSs, testosterone propionate (<5 days), phenylpropionate and isocaproate (<11 days), decanoate (<18 days) and undecanoate (>60 days) when analyzed via LC–MS/MS (Forsdahl et al., 2015). An LC–MS/MS method developed for the detection of testosterone esters as well as 8 AASs was highly applicable to both urine and serum for the detection of exogenous AASs (Makvandi et al., 2023). Sixteen estered AASs observed no significant matrix effects and recovery efficiencies of ≥33% from serum when analyzed via UHPLC–MS/MS, with longer chained esters demonstrating reduced recoverability when extracted via ISOLUTE® SLE+ with DCM. It was suggested heptane be used to improve AAS ester extraction, but to the detriment of free AAS extraction (Langer et al., 2022).

A GC-CI-MS/MS quantitation method was developed for the detection of 9 testosterone esters and 2 nandrolone esters in fortified serum samples. This was validated in agreement with the WADA International Standard for Laboratories and produced a linear range of 100–2000 pg/mL following diethyl ether LLE and trimethylsilyl derivatization. Extraction recoveries ranged from 62.6 ± 11.2% to 118.5 ± 14.3% across 100, 500, and 2000 pg/mL. LOD ranged from 10 to 50 pg/mL for testosterone esters and 200 pg/mL for nandrolone esters (Van Renterghem et al., 2020).

9 | STABILITY OF AAS IN BIOLOGICAL MATRICES

Understanding the stability of illicit compounds is extremely important in forensic toxicology and should be taken into consideration during any interpretation of drug concentrations. The stability of androstenedione, DHEA and testosterone was examined in plasma, stored at room temperature, 4°C and −20°C for a period of 28 days (Kushnir et al., 2010). When stored at room temperature, androstenedione showed a concentration decrease of 15% per week, whereas DHEA concentrations increased approximately 5% per week. Both analytes were deemed stable when stored at 4°C and −20°C. Testosterone was deemed stable for the entirety of the study at all temperatures. Long-term storage of testosterone serum samples stored for 10 years at −20°C were also found to be stable (Fitzgerald et al., 2010). Testosterone ethanoate in contrast has been shown to be less stable, with studies conducted in equine plasma showing an approximate loss of 50% when stored at room temperature within 5 days (You et al., 2010). Higher pH values (pH = 7.9) saw greater testosterone glucuronide and epitestosterone glucuronide deconjugation and degradation in comparison to lower pH values (pH = 5). Elevated temperatures (25°C and 37°C) resulted in rapid deconjugation, which was not observed in urine samples stored at 4°C and −20°C (Mazzarino et al., 2011).

Thirteen AASs were stable for at least 6 months at −20°C in serum and a methanolic stock solution was stable for at least 2.5 months at −20°C. Processed solutions were stable for 24 hours at 8°C in the autosampler (Makvandi et al., 2023). Testosterone esters were found to be highly stable (>18 months) in DBSs when stored in a freezer (−20°C) (Solheim et al., 2022). Esterase inhibitor NaF drastically reduced the enzymatic hydrolysis of short chained esters in blood when stored in a fridge (+4°C) for 2 days or freezer (−20°C) for 50 days (Forsdahl et al., 2015).

As with any sample submitted to the laboratory, its contents are unknown until after testing. It is therefore important to not only consider the stability of AASs, but also the stability of other compounds which may be present. As a result, samples are recommended to be stored at cooler temperatures initially, with long-term storage recommended at −20°C or if available, −80°C.

10 | POLYDRUG USE

As previously mentioned, polydrug use within AASs users is common. A typical method of administration involves the use of different AASs simultaneously known as stacking, in cycles of fluctuating concentrations known as pyramiding (Pope et al., 2014). Alternatively, an administrative regime known as "blast and cruise" is also commonly used whereby individuals alternate between constant periods of high and reduced dose intake (Sagoe et al., 2015; Smit et al., 2019). Stacking has become increasingly prevalent, with biological samples seized by the police and from inmates were found to contain increasing numbers of compounds. In 1999 urine samples typically contained one or two AASs in comparison to urine samples in 2009 where as many as eight different AASs were identified (Lood et al., 2012). The reason for increased compounds in samples could be due to a limited supply route, for example in prison samples, or the availability and use of pre-prepared mixtures which contain many AASs concurrently. A review of 3132 dietary supplement found AASs in 228 products, representing 26.06% of the total number of undeclared substances (Kozhuharov et al., 2022), demonstrating that users themselves may not be aware they are taking AASs.

As well as coadministration of AASs, other IPEDs are also used, in an attempt to counteract negative AAS side effects such as aromatase inhibitors and estrogen receptor agonists. Diuretics, thyroid hormones, and β_2-androgenic receptor agonists are co-administered to enhance fat and water loss, whereas gonadotropins are administered to reactivate endogenous testosterone production post cycle. To reduce the risk of detection, diuretics and probenecid are used whereas, hGH, IGF, and insulin enhance anabolic effects (Pope et al., 2014; Sagoe et al., 2015). This was demonstrated in a study where 58.4% of athlete respondents ($n = 500$) reported the use of clenbuterol alongside AASs, as well as 96% of AAS users reporting non-AAS polydrug usage (Parkinson & Evans, 2006).

AASs abusers partaking in polydrug use are not restricted to IPEDs, with other illicit recreational drugs also commonly administered. Urine samples taken from police cases and inmates testing positive for AASs in 2004 detected a minimum of one narcotic drug in 60.0% of cases with cannabis the most common (Lood et al., 2012). Additionally, 77% of IPED abusing participants ($n = 60$) reported using an illicit drug in the last 6 months, while 27% of participants reported ever injecting another illicit drug (Larance et al., 2008). Data suggests that AAS use may be a gateway/precursor to other illicit drug use with AAS users found to display much higher rates of illicit polydrug use compared to non-users (Sagoe et al., 2015). Furthermore, 28.3% of patients attending substance use disorder treatment reported lifetime use of AASs in Norway, with AAS users found to be significantly more likely to engage in the weekly consumption

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BOX 3 Polydrug unknowns

It should be anticipated that when an AAS is identified in a biological sample, there will infact be multiple drugs present. This can be other AASs and licit or illict drugs, due to the high polydrug use within the AAS administering community, increasing the liklihood of drug-drug interactions. Common recreational AAS dose regimes are associated with an increased risk of cardiovascular incidents and mortaility. This therefore makes the monitoring of these compounds important within traditional forensic cases.

of opioids, benzodiazepines, cocaine, and amphetamines (Havnes et al., 2020). An increased number of analytes should therefore be anticipated in AAS containing samples, due to the high polydrug use demonstrated within the AAS administering community.

The impact of DDIs should be considered as oxandrolone is a known moderate inhibitor of a major CYP isozyme, CYP2C9 (Sychev et al., 2018; Wu et al., 2013). This enzyme inhibition may increase the time required to metabolize molecules that rely on CYP2C9. This could potentially impact anticoagulants, antihypersensitives, NSAIDs, and oral hyperglycaemic agents. Potentially resulting in an unforeseen enhancement in bodily concentrations of other drugs, as well as prolonged effects and reduced therapeutic effects (Daly et al., 2017). In another study Wistar rats, which had been administered cannabis and nandrolone simultaneously, were found to exhibit greater neurotoxic effects with more serious long-term behavioral and cognitive consequences posed to adolescents than either drug alone (El-Shamarka et al., 2020). A potentially more lethal consequence of DDIs may result through the enhancement of effects deriving from traditional anticoagulants (warfarin, bromindione, etc.) and increased chances of hemorrhaging by AASs (Howard et al., 1977; Murakami et al., 1965), with orally active C_{17}-alkylated derivatives thought to produce the largest effect (Howard et al., 1977).

A highly sensitive high resolution accurate mass–liquid chromatography/mass spectrometry (HRAM-LCMS) method was initially deployed for the screening of a wide range of drugs in a cross-sectional study utilizing mandatory prison drug testing. Despite the use of HRAM-LCMS for the detection of traditional illicit compounds, it is less suited for the detection of AAS. As a result, only a limited number of AAS (stanozolol, trenbolone and dienedione) were detectable using this initial method, therefore an additional analytical technique based on GC–MS was deployed (Hudson & Willmott, 2015), demonstrating that AAS are not easily congruent with the detection of other recreational drugs and further demonstrating the complimentary nature of GC–MS and LC–MS for AAS analysis. Additionally, polydrug use may lead to an increased likelihood of toxicologists coming across AAS positive results due to increased mortality associated with polypharmacy (Box 3) (Leelakanok et al., 2017).

11 | CONCLUSION

From the isolation and synthesis of testosterone in 1935 a great number of synthetic AAS analogues have been created for therapeutic and recreational use. The development of analytical techniques for AAS detection has been driven by the need to identify instances of doping within sport with methods of extraction, hydrolysis and instrumentation seeing evolution over the years. However, the abuse of AASs is an increasing issue within recreational gym goers and the more general population. The continuation of increasing prevalence is anticipated with a rise of male body dissatisfaction resulting from media representation of the ideal body.

Although AASs pose challenges not frequently seen with other illicit drugs of abuse, the analytical instrumentation needed for their detection is commonly available within forensic laboratories. It is therefore important that laboratories look to expand their drug testing panels to consider these compounds, as worldwide usage increases.

In consideration of the advantages and disadvantages of GC–MS and LC–MS it should be considered that these two instruments be used ideally in tandem to increase the chances of correct identification of a wide scope of AASs. If GC–MS analysis is being used it is highly recommended that derivatization utilizing MSTFA/NH_4I/DTE is carried out after hydrolysis using *E. coli*.

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Urine is the preferred matrix of choice for routine AAS analysis, despite the variation in phenotypic expression, as seen with UGT2B17 gene deletion needing to be considered when determining testosterone concentrations. Alternative matrices should not be ignored and can provide complimentary sources of exploration.

Although the detection of AASs within the general population does not require the sensitivities demonstrated with elite athlete anti-doping testing, new analysis and interpretation challenges should be expected with the increased likelihood of recreational polydrug administration.

AUTHOR CONTRIBUTIONS

Richard L. Harries: Data curation (lead); investigation (lead); writing – original draft (lead); writing – review and editing (equal). **Giorgia De Paoli:** Project administration (supporting); supervision (equal); visualization (lead); writing – original draft (equal); writing – review and editing (supporting). **Sarah Hall:** Project administration (supporting); supervision (supporting); writing – review and editing (supporting). **Lorna A. Nisbet:** Conceptualization (lead); data curation (supporting); project administration (equal); supervision (lead); writing – original draft (equal); writing – review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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