

Review article

Doping in sports: A survey of the analytical techniques applied for unmasking the abuse of performance-enhancing drugs

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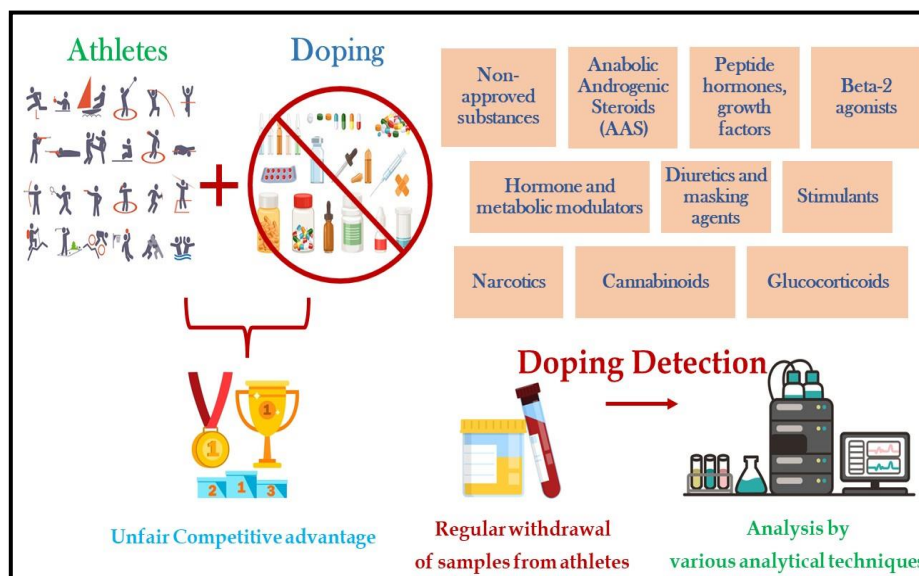
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Abstract:

The use of illegal performance-enhancing drugs (PEDs) in sports, and other tactics by athletes to gain an unfair competitive advantage is becoming a global concern since it negatively impacts the athlete, participants, and the integrity of sports. Annually, a list of drugs and techniques that are prohibited from being used in sports is published by the World Anti-Doping Agency (WADA). The WADA list consists of different classes namely, non-approved substances, anabolic androgenic steroids (AAS), peptide hormones, growth factors, related substances and mimetics, beta-2 agonists, hormone and metabolic modulators, diuretics and masking agents, stimulants, narcotics, cannabinoids, glucocorticoids, and beta blockers. Moreover, athletes tend to use other cheating techniques such as blood doping, urine adulteration, and gene doping to increase their chances of winning. This review encompasses the various classes of PEDs and methods that are most widely used to enhance the performance of athletes. In addition, herein we discuss some of the most commonly used methods that are implemented in the detection of doping.



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1. Introduction

Doping is the use of illicit performance-enhancing drugs (PEDs), artificial enhancements, and other strategies by athletes to obtain an unfair competitive advantage ⁽¹⁾. Doping is currently a global issue as it is considered cheating and intrinsic opposition to the spirit of sport. Prohibited substances can impair an athlete's health or provide them with an unfair competitive advantage. For viewers and players alike, knowing that the competition is fair is fundamental to the essence of sports.

Athletes of all levels are negatively impacted by doping. Future generations might potentially be influenced by the actions of elite athletes. To ensure the safety of athletes, sports` integrity, and the future generation of athletes around the globe, a coordinated and all-encompassing strategy is needed to combat doping.

PED use has been known since the early days of Greek and Roman athletic events ⁽²⁾. Currently, associations like the World Anti-Doping Agency (WADA), Major League Baseball (MLB), and National Football League (NFL) publish lists of drugs and techniques that are prohibited from being used in doping. The most comprehensive list is the WADA list, which is available on the WADA website and is updated yearly ⁽³⁾.

Many of the previously published articles in the literature have covered the use of PEDs in sports ⁽⁴⁻⁷⁾. This work not only provides an overview of the most widely used PEDs that are listed in the WADA 2024 prohibited list but also summarizes and discusses some of the most popular methods that are employed in the unmasking of doping. The WADA 2024 list of prohibited substances and techniques includes the following categories:

2. Prohibited Substances

2.1. Non-approved substances

2.2. Anabolic Androgenic Steroids (AAS)

2.3. Peptide hormones, growth factors, related substances, and mimetics

2.4. Beta-2 agonists

2.5. Hormone and metabolic modulators

2.6. Diuretics and masking agents

2.7. Stimulants

2.8. Narcotics

2.9. Cannabinoids

2.10. Glucocorticoids

3. Substances prohibited in particular sports

3.1. Beta-blockers

4. Prohibited Methods and Manipulations

4.1. Manipulation of blood and blood components

4.2. Chemical and physical manipulation

4.3. Gene and cell doping

2. Prohibited Substances

2.1. Non-approved substances

Non-approved substances include pharmacological substances that are not covered by the list and that are currently disapproved for use as human therapeutics by any regulatory authority (such as medications undergoing pre-clinical or clinical investigations or that have been ceased, designer drugs, or substances that are only approved for veterinary use) are always forbidden.

2.2. Anabolic Androgenic Steroids (AAS)

The natural hormone testosterone as well as its synthetic and endogenous counterparts are examples of anabolic steroids. Anabolic steroids have the ability to stimulate cells to generate protein, which promotes growth and muscle tissue repair ⁽⁸⁾. Sportsmen believe that the intake of anabolic steroids enables them to work out at a higher rate and intensity, which, when paired with a healthy food regime, can increase performance and endurance. The use of steroids can have negative side effects on men including Infertility, testicular atrophy, and

gynecomastia. While in women abuse of AAS may lead to excessive body hair growth, menstrual irregularity, and psychosis. Further side effects include liver damage and changes in cardiovascular function. WADA forbids the use of all anabolic steroids and lists over 70 examples, including androstenedione, androsterone, testosterone, hydroxytestosterone, and gestrinone, among others, along with other drugs having comparable chemical structures or biological effects.

2.2.1. Detection of Anabolic Androgenic Steroids (AAS)

2.2.2. Direct Quantitation of AAS and their metabolites

Since urine samples may be collected non-invasively, it is greatly employed in doping analysis⁽⁹⁾. Phase I and Phase II are the two successive phases of metabolism that can be observed when examining the metabolic pathways of steroids. To render the steroid inactive and aid in its excretion from the body, it undergoes a conversion into more polar molecules.

In cases where the administration of the steroid is in the form of an ester, the primary processes in phase I are hydrolysis, as well as oxidation and reduction that take place at various locations along the steroid ring. Phase II involves the formation of the proper conjugates, sulphates, glucuronides, and these processes are regulated by enzymes⁽⁹⁾. The analysis of metabolites is performed by an indirect technique, which includes hydrolysis of phase II metabolites. Moreover, sample preparation is completed by liquid-liquid extraction and derivatization for GC-MS/MS. If LC-MS/MS is used, no derivatization is carried out. Studies conducted in the last few years have reported using LC-MS/MS to directly analyze Phase II metabolites of AAS without the need for hydrolysis step^(10, 11). This offers a great advantage as it reduces the sample preparation steps and increases the sensitivity

of the method. Long-term metabolites (LTMs) are compounds that linger in the urine for extended time durations and are considered as AAS misuse markers. The development of accurate and powerful GC-MS/MS and LC-MS/MS techniques capable of detecting LTMs represents an excellent tool in the fight against the abuse of PEDs.

Moreover, the use of “designer steroids” was adopted as another strategy to evade doping testing. Designer steroids are chemical compounds that laboratories do not screen for and hence will be undetected. These are mostly synthetic compounds made from alterations in testosterone chemical structure. These alterations include methylation of the 17 α position (crucial for oral administration), alterations of ring A (methylation of position C-1, dehydrogenation on position 1, 2, attaching different groups at C-2, condensation with various rings like pyrazole and hydroxylation or halogenation C-4, etc.), and esterification of the 17 β -hydroxy group, which improves suitability for oral or intramuscular application and produces a depo-like outcome. These alterations in the structure result in an enhancement in anabolic and a decrease in androgenic activity. Since its chromatographic and mass spectral parameters are unknown, it will not be detected by the standard steroid screen⁽¹²⁾.

By using GC-MS/MS, Joumier *et al.* have developed a sensitive technique to quantitate 10 anabolic steroids and 11 anabolic steroid esters in athletes' hair⁽¹³⁾. Some athletes use anabolic steroids for extended periods of time in the winter and quit before competitions, or they use them for four to eighteen weeks at a time, alternating with drug-free months. This is the rationale behind the drug-free status of abusers. Except for ester forms, anabolic steroids can only be found in urine 2-4 days post exposure. Depending on hair length, hair analysis permits an extension of the window of detection (weeks -months). Using the

developed method, the LOD for each of the several steroids ranged from 1 to 5 pg/mg. The collection of body hair presents a substitute in cases where the collection of head hair samples is problematic. The use of body hair has two main benefits. Firstly, anabolic steroids are often more concentrated in body hair than in head hair, allowing for the detection of steroids abuse at lower doses and frequency of abuse. Moreover, a longer detection window is obtained with body hair than with head hair, especially in male athletes with short hair.

Raul *et al.* have developed a method to investigate anabolic steroid misuse of Clostebol, drostanolone, metandienone, 19-norandrostenedione, stanozolol, trenbolone in real cases. The anabolic steroid concentrations in athletes' body hair were found to be consistently greater than those found in the hair present on the head⁽¹⁴⁾.

Gashimova *et al.* have developed an ultra-high performance liquid chromatography (UHPLC) method for quantifying different classes of steroid hormones in human urine (estrogens, progestins, androgens, and corticosteroids). The method involves preparing the sample using dispersive liquid-liquid extraction post enzymatic hydrolysis using *E. Coli*'s β -glucuronidase, and then using UHPLC-high resolution mass spectrometry (quadrupole time-of-flight) for mass fragments measurement. A concentration range of 0.25–500 ng/mL of synthetic urine was used to validate the procedure. Subsequently, the method was utilized to examine actual urine specimens, and the outcomes were compared to a standard liquid-liquid extraction process. The application of this method in human urine has demonstrated sensitive and accurate measurement of steroidal hormones⁽¹⁵⁾.

2.2.3. Determination of Testosterone/Epitestosterone ratio (T/E ratio)

Athletes frequently use testosterone for performance enhancement, despite the

obvious appeal of designer steroids for undetectable use. This is partly because testosterone poses a challenge to testers owing to the difficulty of distinguishing between natural and synthetic testosterone in the urine of an athlete. Since individual levels of testosterone (T) vary greatly, it is not effective to simply measure T.

Rather, testers capitalize on a natural "quirk" in which the human body produces epitestosterone (E), a testosterone epimer. The arrangement of the hydroxyl group at the 17 position of the steroid ring is the only difference between the two epimers (**Fig. 1**). Unlike epitestosterone (E), testosterone (T) has anabolic activity, T and E make a great illustration of how the function is dependent on minute structural variations.

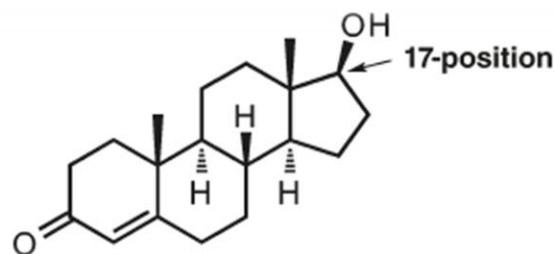


Fig. 1. The structure of testosterone.

In urine, the T/E concentration ratio is typically 1:1 in most healthy males. Less frequent values fall between 0.1 and 3. The T/E concentration ratio can be easily estimated using GC-MS⁽¹⁶⁾. The T/E concentration ratio is used to detect abuse of testosterone since it temporarily increases after T is consumed; findings must be reported by a threshold of 4⁽¹⁷⁾. However, every threshold value has two drawbacks: i. People who abstain from PEDs may naturally have a higher T/E. ii. T users tend to keep a low profile by taking both T and E or by using microdoses of T to maintain a T/E ratio slightly below four. Plotting T/E against time in months/weeks allows the segregation of T dopers from non-

dopers. In cases of non-dopers values should remain constant, contrarily the values rise after T is used, before falling again ⁽¹⁶⁾.

El hawiti *et al.* have described a derivatization procedure for examining testosterone and dehydroepiandrosterone using LC-MS/MS in saliva samples of soccer players ⁽¹⁸⁾. In this work, a polymeric reverse-phase cartridge called Strata-XL was utilized to extract endogenous steroids. 1-methylpyridine (2-hydrazino) was reacted with the separated steroids (HMP). Twenty young soccer players provided saliva samples, and saliva samples were used to measure the testosterone (T) and its precursor, dehydroepiandrosterone (DHEA) levels, using LC-MS/MS operating at a positive mode with multiple reaction monitoring (MRM). The method exhibited limits of quantitation (LOQ) for T and DHEA of 20 pg/mL and 50 pg/mL for epitestosterone (EPI).

Shi *et al.* have developed a novel capillary electrophoresis technique with diode-array detection for estimating the testosterone to epitestosterone concentration ratio in human urine ⁽¹⁹⁾. Solid extraction was employed to first purify urine samples. The ideal experimental conditions were 15.0 mmol/L acetate buffer (pH 4.74), separation voltage 25 kV, temperature 25 °C, and the run time was 10 seconds. The testosterone and epitestosterone linearity ranges were found to be 8–960ng/mL. The testosterone and epitestosterone detection limits were found to be 4.6 and 4.5 ng mL⁻¹ respectively.

The T/E ratio in men's urine indicates when exogenous testosterone has been administered because it suppresses endogenous T synthesis by providing a strong negative feedback loop on endogenous T and E production, as well as because of the excess testosterone excreted in the urine. However, in females, whose circulating T levels are substantially lower, this system might not be fully functional because T

originates from three sources, none of which are strongly negative feedback-driven. Therefore, more techniques are needed to identify T doping in females. A liquid chromatography mass-spectrometry (LC-MS) was developed by Bermon *et al.* to investigate two cases of top-tier female athletes who were found guilty of T doping based on serum T levels ⁽²⁰⁾.

2.2.4. Carbon Isotope Ratio (CIR) testing

Natural and synthetic testosterone differ in their ¹³C content, testers concentrated on the carbon backbone of testosterone in an attempt to identify a better strategy to close T/E gaps. ¹³C makes up about 1.1% of the carbon present in nature, yet because artificial and natural T are produced via separate biochemical routes, natural T has a greater content of ¹³C than artificial T.

Carbon isotope ratio (CIR) testing, also known as gas chromatography-combustion-isotope ratio mass spectrometry (GCC-IRMS), is the method used to investigate the difference. Urine contains anabolic steroids which are isolated and separated using gas chromatography. T passes via the combustion furnace (-C-) after leaving the gas chromatography section, in which pyrolysis converts each carbon atom into ¹²CO₂ or ¹³CO₂.

The isotope ratio mass spectrometry (IRMS) records the frequency of isotopic variations of CO₂ and determines the difference between the sample's ¹³C/¹²C ratio and an international standard, or δ¹³C (delta) value which is measured as ‰. Accordingly, the international standard's δ value is zero ‰.

Examples of natural testosterone values could be -23‰, while for synthetic testosterone, -30‰. Since neither of the compounds has as much ¹³C as the international standard, the values are negative.

Without the need to gather further samples, final decisions based on a single urine sample analyzed using a CIR test showing T/E > 4

can be made. The most potent feature of CIR testing, however, is that its most advanced variant may also identify T precursor and metabolite usage, even in cases when the precise substance ingested is never identified⁽²¹⁾.

Even in the absence of an increased T, steroid use can be detected with CIR testing. For example, even though the urine sampled from Floyd Landis's from several levels of the 2006 Tour de France had T/E ratios of no more than 4, CIR testing on those samples revealed indications of doping⁽²²⁾. Lastly, anti-doping regimes are permitted by WADA regulations to penalize athletes solely based on CIR results. Consequently, CIR is genuinely a revolutionary method for detecting doping in sports^(23, 24). Despite the fact that GC-C-IRMS is a trusted way to prove T misuse, a large amount of urine (20-25mL) must be available for the analysis and obtaining the highly pure steroid fractions required for the assay making it a laborious purification process.

Brito *et al.* have studied the doping of endogenous urine steroids by athletes for doping using carbon isotope ratio⁽²⁵⁾. β -glucuronidase was added to carry out the enzymatic hydrolysis. Following the addition of sodium carbonate buffer to adjust the pH to 9–10, 5 mL of n-pentane was used to extract the analytes. The method covers a linear concentration range from 6 -100 ng/mL.

Since the administration of epitestosterone reduces the urine T/E ratio, an indicator of testosterone abuse, it is prohibited by sport authorities. A gas chromatography–combustion–Isotope ratio mass spectrometry method for quantitating the ^{13}C values for epitestosterone in urine was described by Catlin *et al.* Sample preparation involved solid-phase extraction, semipreparative HPLC, and deconjugation with β -glucuronidase. Using GC-MS, the urinary epitestosterone levels collected from 456

healthy male control subjects were detected. Urine samples from ten athletes with E concentrations >180 g/L and forty-three control urine samples with E concentrations >40 g/L had their E ^{13}C values determined, respectively⁽²⁶⁾.

A method for the determination of the endogenous steroids' origin found in human urine samples was described by Catlin *et al.* by calculating the $^{13}\text{C}/^{12}\text{C}$ carbon isotope ratio. Multiple procedures including liquid-liquid, solid-phase extraction, and semi-preparative liquid chromatography were employed to differentiate fractions containing testosterone, 17β -diols, 5β - and 5β -androstane- 3α , androsterone, ethiocholanolone, 5β -pregnane- 3α , 20S -diol, and $16(5\alpha)$ -androstene- 3α -ol. Gas chromatography was used to analyze more than 100 samples. Isotope mass spectrometry in conjunction with gas chromatography was used to evaluate over 100 samples. After statistical processing of the resulting values of $^{13}\text{C}/^{12}\text{C}$, the ranges needed for the results' interpretation were discovered^(27, 28).

2.2.4. Estimation of testosterone sulfate/testosterone glucuronide ((ES/EG)/(TS/TG)) combined ratios

Only after an intramuscular testosterone injection the combined ratio utilizing sulfate conjugated T and E offers greater sensitivity than T/E glucuronide ratios. As a supplementary biomarker for testosterone doping, the epitestosterone sulfate/epitestosterone glucuronide combined ratios to the testosterone sulfate/testosterone glucuronide ratios ((ES/EG)/(TS/TG)) have previously been studied.

The latter biomarker has been assessed in a reported study by Ekström *et al.* over a broader sample population. Testosterone enanthate was given in a single dose to male volunteers. The findings demonstrate that, across all participants, the combined ratio

increased more than the conventional T/E ratio. A Waters triple-quadrupole was utilized for the LC-MS/MS analysis, and a UPLC BEH RP18 reversed-phase column was used for the separation⁽²⁹⁾.

(Table 1) summarizes the measurement conditions of some of the analytical methods applied in the quantification of Anabolic Androgenic Steroids (AAS).

2.3. Peptide hormones, growth factors, related substances, and mimetics

2.3.1. Erythropoietin (EPO)

Naturally, a protein hormone known as erythropoietin (EPO) is secreted by the kidneys and works on the bone marrow by encouraging the formation of erythrocytes (red blood cells). Abuse of EPO in sports improves endurance and stamina by raising levels of hemoglobin and consequently, the amount of oxygen delivered to muscles. The

drawback of EPO is that it also raises blood viscosity, which increases the risk of heart attacks and cardiovascular disorders.

Human EPO (uEPO) mainly consists of a 30.4 kDa glycoprotein including four carbohydrate chains and 165 amino acids. The size, content, branching pattern, and length of carbohydrate chains may differ. Moreover, these chains differ in the quantity of sialic acid groups. As a result, EPO is made up of a group of "isoforms" with identical proteins but differing in total negative charges as a result of having various amounts of sialic acid residues.

In the 1980s, the development of recombinant human EPO (rEPO) was prompted by the possibility that pharmaceutical EPO would replace

Table 1. Summary of the measurement conditions of some of the analytical methods applied in the quantification of Anabolic Androgenic Steroids (AAS).

Technique	Matrix	Conditions	Linearity range	LOD	Reference
GC-MS/MS	Hair	*Valley OV 30 m x 0.25 mm i.d, 0.25 μ m. *Temperature ramp rate 150 to 295 $^{\circ}$ C (10 min)	1-100 pg/mg	1pg/mg	13
UHPLC with HRMS detection	Urine	*Phenomenex Kinetex C18 column (100 x 2.1mm, 1.7 μ m) *Mobile phase A: 0.1% Formic acid in water *Mobile phase B: 0.1% Formic acid in methanol	0.25-500 ng/mL	0.25 ng/mL	15
Capillary Electrophoresis	Urine	*Running buffer pH = 4.74, 15.0 mmol L ⁻¹ HAc- NaAc *Separation voltage 25 kV *Temperature 25 $^{\circ}$ C *Sample injection pressure 3.43 \times 10 ³ Pa *Duration 10 s.	8-960ng/mL	Testosterone: 4.6ng/mL Epitestosterone: 4.5 ng/mL	19
LC-MS/MS	Urine	*Waters triple-quadrupole, Reversed-phase column (Acquity UPLC BEH RP18, 50x2.1 mm). *Mobile phase A : 5-mM NH ₄ Ac (pH 9.6) *Mobile phase B: MeOH	-	-	29
LC-MS/MS	Saliva	*ACE Ultracore 2.5 Superphenylhexyl column (150 \times 3.0 mm id) *Mobile phase: Water: Acetonitrile (75:25), with 0.01% Formic acid *Flow rate :0.4 mL/min.	T and DHEA: 20-800 pg/mL EPI: 50-1200 pg/mL	T and DHEA : 20 pg/mL EPI : 50 pg/mL	18
LC-MS/MS	Hair	Isolute C18 columns	1-200 pg/mg	1pg/mg	14

blood than urine. While it is easy to differentiate Epoetin R, NESP, and CERA from uEPO using IEF, this may not be feasible for other EPO biosimilars. In developing nations, it is estimated that 80 rEPO biosimilars could be offered for sale⁽³⁴⁾.

Not all of these EPO variants meet the WADA requirements for announcing a positive EPO abuse test from IEF patterns. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was recently approved by the WADA for supplementary identification in an effort to close this potential loophole. Similar to IEF, analytes are driven across a polyacrylamide gel using an electric field in the SDS-PAGE procedure. Nevertheless, SDS-PAGE separates material based on size (mass) rather than charge. The protein mixture is denatured by being subjected to sodium dodecyl sulfate (SDS), followed by coating of the protein with dodecyl sulfate anions. This results in a net negative charge that is roughly correlated with the size of the protein. Larger molecules are retarded more than smaller ones during the electrophoresis process, which separates the mixture. The WADA Technical Document's Figure 2 illustrates how to separate uEPO from biosimilars using SDS-PAGE⁽³⁵⁾. Naturally, this technique is only effective in cases where the mass difference between uEPO and a particular biosimilar is large enough.⁽³³⁾

HIF stabilizers, a rising class of kidney-disease medications, have been used by athletes, particularly cyclists, in recent years. The body is encouraged to produce EPO by the intake of these medications as they work via EPO-expressing genes activation. Blood tests may identify several HIF stabilizers, including argon and xenon, however, cobalt chloride is more difficult to identify while doing the same function⁽³⁵⁾.

2.3.2. Growth hormone (GH)

The pituitary gland produces the 191-amino acid protein hormone known as human growth hormone (hGH). The release of insulin-like growth factor (IGF-1) by the liver in response to human growth hormone (hGH) promotes the growth of bones, muscles, and organs⁽³⁶⁾. Additionally, it controls the body's proportion of fat to lean muscle mass. There are acceptable medicinal applications for recombinant human growth hormone (rhGH), such as the management of growth abnormalities and the AIDS-related muscle-wasting syndrome. The most mysterious PED is rhGH owing to its suspected widespread use and the difficulty in its detection⁽³⁷⁾.

The research of Meinhardt *et al.* now casts doubt on the scientific literature's previous conclusion that the use of human growth hormone (hGH) could improve athletic performance⁽³⁸⁾.

These researchers conducted a randomized study on athletes, by giving them both hGH alone and hGH coupled with testosterone. According to their findings, hGH boosted sprint capacity by 3.9% in men and increased to 8.3% in those who also took testosterone. Furthermore, athletes may benefit from a notable "boost" from mixing rhGH with low testosterone levels as long as the T/E ratio remains below the 4-point threshold⁽³⁹⁾.

Less than 1% of the hormone's blood levels are found in urine, which is a very low amount. As a result, it is currently unlikely to detect rhGH in urine, and the only technique accepted by the WADA for rhGH detection requires a blood sample. Like erythropoietin, normal human growth hormone is composed of many isoforms. These vary in bulk because of differences in length. rhGH solely comprises the 22 kDa isoform, which is similar to its natural complement, while hGH mostly consists of three isoforms with weights of 17, 20, and 22 kDa. The detection method uses two assays synchronically; the

first to quantify the 22 kDa isoform and the second to quantify the rest of the isoforms in order to examine for any differences in isoform quantities caused by the usage of rhGH.

It is anticipated that athletes utilizing rhGH will exhibit a typically elevated "isoform ratio" between the 22 kDa isoform and the rest of hGH isoforms⁽⁴⁰⁾. Antibodies, which are proteins made by the immune system in reaction to a foreign material (antigen), are the basis of the test procedure. Animals that have human growth hormone (hGH) as the antigen develop anti-hGH antibodies, which are potent detection tools as they attach to the antigen (hGH) robustly and selectively. This serves as the foundation for the immunoassay method. The limited detection window of the isoform technique is one of its drawbacks. Even when given subcutaneously, recombinant growth hormone is quickly eliminated, and blood samples obtained in the morning following an injection often show no measurable levels of GH.

In contrast, spontaneous GH secretion recovers to normal forty-eight hours post the last dose of rhGH. As such, no one will know if an athlete stops taking GH a few days before a competition. Because of this, the best application for this technique is probably "out of competition" testing that is conducted without prior notice. This method's inability to identify the abuse of GH secretagogues or GH of cadaveric origin, which do not trigger an alteration in the profile of isoforms, is another drawback⁽⁴¹⁾.

2.3.2.1. Detection of Growth hormone (GH)

2.3.2.2. Immunoassay

The immunoassay for the detection of human growth hormone is shown in **Fig. 3**. A polystyrene tube is coated with captured antibodies on the inside, which are anti-hGH antibodies that favor to get attached to the 22 kDa isoform. The addition of collected blood serum containing hGH triggers the 22 kDa hGH to bind to it. After washing the tube to

get rid of any free molecules, the bound hGH and another anti-hGH antibody (also known as a "detection antibody") are combined to form a sandwich composite. The second antibody's tail contains a substance that, when chemically activated, emits chemiluminescence that can be detected. To get rid of unattached molecules, the tube is cleaned⁽⁴²⁾.

Chemiluminescence can be used to quantify human growth hormone at 22 kDa since it only happens when human growth hormone is present in the sample to form the sandwich composite. Using capture antibodies that bind preferentially to all other isoforms, the same method is applied to quantify all other isoforms in the same sample⁽⁴³⁾.

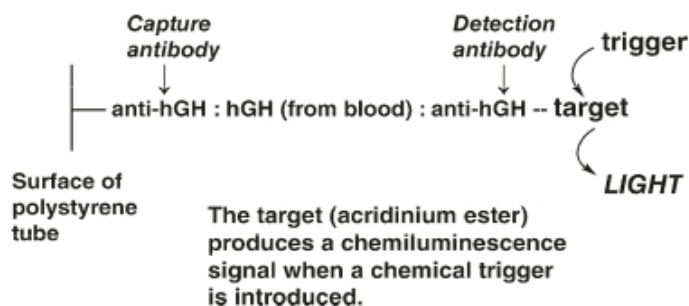


Fig. 3. The WADA -approved detection technique of hGH⁽¹⁾.

One reason why rhGH is hard to detect is that the body excretes it within 24 to 36 hours. There is a lot of interest in enhancing the detection of rhGH because of this test's restriction and the prevalence of rhGH use. By evaluating particular biomarkers that are released when rhGH is administered, alternative, indirect ways examine the "downstream" consequences of rhGH administration. Immunoassays are one method used to assess procollagen III peptide and insulin-like growth factor-1 (IGF-1) hormones, the serum quantities of which are elevated by rhGH⁽⁴⁴⁾.

A urine test would be ideal for rhGH testing for its lower invasiveness than the existing blood-based test. Provided the low concentrations and short half-life of rhGH in urine, developing a urine test is extremely difficult. Using nanotechnology, Botterell *et al.* have adopted an intriguing strategy. It has been demonstrated that all of the hGH in a urine sample (around 30 mL) may be captured by hydrogel nanoparticles operationalized with Cibacron Blue F3G-A dye ⁽⁴⁵⁾. After that, hGH can be released at a concentration appropriate for immunoassay into a considerably smaller sample volume. Insulin-like growth factor-I (IGF-I) and N-terminal propeptide of type III procollagen (P-III-NP) are two serum proteins that are measured via the GH-2000 marker technique. Both proteins rise in a dose-proportional pattern in reaction to GH administration, serving as “indicators” of GH abuse. Peter Sönksen was the original inventor of this technique ^(46, 47). A randomized controlled GH administration study ⁽⁴⁹⁾ yielded the optimum discrimination between individuals taking GH and placebo, hence IGF-I and P-III-NP were chosen for the GH-2000 examination out of 25 possible indicators of GH action ⁽⁴⁸⁾. IGF-I and P-III-NP both exhibit limited variance in response to exercise and little variation in the day-to-day or intraindividual variation ^(46, 47).

2.3.2.3. LC-MS-MS

A second method detects the fragments of IGF-1 and leucine-rich R-2-glycoprotein (LRG), another sign of rhGH usage, using liquid chromatography-tandem mass after enzymatic hydrolysis ⁽⁴⁹⁾. This technique is highly sensitive, and its detection period is longer than that of the isoform ratio technique.

Bonnaire *et al.* were able to quantitate Recombinant Equine Growth Hormone (reGH) in horse plasma using liquid chromatography-tandem mass ⁽⁵⁰⁾. Since 1998, equine growth hormone (eGH)

including recombinant eGH (reGH) has been accessible as an authorized medication (EquiGen-5, Bresagen). It is believed to be given to racehorses illegally in an effort to enhance their physical prowess and hasten the healing of wounds. Therefore, it might be regarded as a doping agent, and its usage in racehorses would need to be controlled by a sensitive and reliable quantitative method. reGH possesses an extra methionine at the N-terminal (met-eGH), which sets it apart from native eGH. At minimal concentrations (1–10 microgram/liter), reGH has never been clearly found in any kind of biological matrix. Ammonium sulfate was added to a plasma sample at the reGH isoelectric point, and solid-phase extraction was employed to purify the pellet. Trypsin digestion produced specific peptides, which were then examined using LC-MS/MS. There was a 1 microg/L detection limit. Additionally, it was effectively used with a linear ion trap mass analyzer to calculate the reGH plasma concentrations over time. Triple quadrupole mass spectrometry was also effective in detecting the reGH abuse up to 48 hours after reGH was given to a horse ⁽⁵⁰⁾.

2.3.3. Agents preventing Activin Receptor II B activation.

International sports drug testing procedures should take into account the ability of some drugs to improve muscle size and function as well as mitochondrial biogenesis since the abuse of these compounds calls for caution and regulation ⁽⁵¹⁾. This includes the following:

2.3.4. Mitochondrial-derived peptide mitochondrial open reading frame of the 12S rRNA-c (MOTS-c)

Thevis *et al.* have reported a method for the determination of (MOTS-c) via liquid chromatography-electrospray ionization tandem mass spectrometry. Sample preparation involves the addition of an isotope-labeled internal standard followed by acetonitrile-protein precipitation. In addition

to the target analyte, other potential metabolites and degradation products are identified using this technique on the concentrated supernatant. The test procedure was described and found to enable detection up to 100 pg/mL, which corresponds to the published ranges of human MOTS-c plasma concentrations⁽⁵²⁾.

2.3.5. Adiponectin receptor agonists (ADP355, ADP399)

Nonpeptidic AdipoR1 agonists and their metabolites have been the subject of test methods for doping control⁽⁵³⁾, and analytical techniques that target a range of peptides with similar molecular mass and composition to ADP355, ADP399, and BHD1028 are now a standard component of most regular sports drug testing programs⁽⁵⁴⁻⁵⁶⁾. Solid-phase extraction or simple dilution of urine spiked with deuterium-labeled analogs to corresponding target analytes are common sample preparation techniques used in these test methods. Subsequently, Liquid chromatography high-resolution mass spectrometry (HRMS) and liquid chromatography-tandem mass spectrometry can determine precise molecular masses and diagnostic precursor/product ion pairs⁽⁵³⁾.

2.3.6. Agents counteracting activin receptor II activation such as bimagrumab and sotatercept.

Cytokines of the transforming growth factor beta (TGF- β) superfamily, that communicate via activin type II transmembrane receptors (ActRII), negatively regulates the skeletal muscle mass⁽⁵⁷⁾. Agents influencing TGF- β function or ActRII activation are thus considered intriguing medicines for muscle wasting illnesses treatment as well as possible performance-enhancing drugs in sports, as recent studies have demonstrated that activin A is a critical regulator of muscle mass and function. Many therapeutic candidates have been developed in the last few years, including multitargeting strategies

and medicines that selectively interact with certain TGF- β cytokines⁽⁶⁰⁾. Antibodies that bind selectively to circulating growth factors, like domagrozumab (antimyoostatin)⁽⁵⁸⁾ and REGN2477 (antiActivin A)⁽⁵⁷⁾ selectively bind to the circulating growth factors, and hence block the binding of ligands to receptors..

The proactive design of selective and sensitive testing technologies is crucial for drug doping detection in sports, even in the absence of clinical approval for these medication candidates. The target analytes were quantitatively determined in preclinical and clinical studies using both enzyme-linked immunosorbent assays⁽⁵⁸⁻⁶¹⁾ and LC-MS/MS^(59, 62). The determination of the protein drugs at the amino acid level is achieved by HRMS. This technique was implemented in the quantitation of anti-ActRII antibody, bimagrumab, and the antimyoostatin antibody domagrozumab combined with affinity purification, tryptic digestion, and liquid chromatography in doping control serum samples^(63,64). The obtained detection limits by the doping control testing techniques are as follows; (bimagrumab: 20 ng/mL⁽⁶³⁾; domagrozumab: 50 ng/mL⁽⁶⁴⁾) in serum samples. The aforementioned limits are comparable to the majority of techniques utilized in clinical settings⁽⁵⁸⁻⁶¹⁾. The predicted limits of detection ought to allow for detection windows ranging from a few weeks to several months.

Table 2 summarizes the measurement conditions of some of the analytical methods employed in the quantification of peptide hormones, growth factors, related substances, and mimetics.

2.4. Beta2-agonists

In adults, the prevalence of asthma is approximately 5%, making it one of the most common chronic disorders. But among athletes, it's thought to be between 10% and 20%. It is commonly known that engaging in

Table 2. Summary of the measurement conditions of some of the analytical methods applied in the quantification of peptide hormones, growth factors, related substances, and mimetics.

Technique	Matrix	Conditions	Linearity range	LOD	Reference
Isoelectric focusing with chemiluminescent detection of EPO	Urine	*Multiphor II Electrophoresis system at 1W/cm of the gel length, *Migration width 9 cm , *Run stopped at 4000 Vh.	---	---	32
Chemiluminescence Immunoassay	Human serum	*Tracer mAb: Purified mAb AK569 labeled with Acridinium-NHS- Ester *Polystyrene tubes (Greiner) coated with AK568 (recA) and AK567 (pitA) or AK566 (recB) and AK565 (pitB)	hgH : 1.3-9.6 µg/L	0.05 µg/L	40
Immunoassay using Cibacron Blue F3G-A	Urine	*Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-P AGE) using 18% Tris-Glycine gel in the presence of Tris-Glycine SDS running Buffer *Novex X-Cell IITM Mini- Cell at 200 V. *hGH concentration was measured using the Immulite 1000 Growth Hormone System	0.05 to 40 ng/mL	0.05 pg/mL	45
LC-MS/MS	Plasma	*Linear ion trap (LTQ XL) or on a triple quadrupole (TSQ Quantum Ultra), with an electrospray ionization (ESI) source operated in the positive mode. * UP5WC4 column (300 Å, 2.0 mm i.d. × 250 mm, 5 µm particle size. *Mobile phase: water/acetonitrile + 0.2% formic acid. *Flow rate: 300 µL/ min.	1-10 µg/L	1 µg/L	50
LC-MS	Plasma	Xevo® TQ-XS triple quadrupole mass spectrometer equipped with an UniSpray™ (US) ion source operated in activated soft ionization positive mode	0.1-10 ng/mL	100 pg/mL	52
LC-Q-HRMS	Urine	*Reversed-phase column (Syncronis – Thermo, USAC18, 1.7 µm, 50 mm × 2.1 mm) *Mobile phase A: H ₂ O with 5.0 mM ammonium formate and 0.1% formic acid, * Mobile phase B: MeOH and 0.1% formic acid, *Flow rate: 400 µL.min ⁻¹ .	--	1 ng/mL	56

intense physical activity can trigger asthmatic symptoms, which are referred to as "exercise-induced asthma" (EIA). Winter sports athletes are more likely to be impacted than athletes in other sports due to the possibility of inhaling significant volumes of cold, dry air, which can lead to the development of bronchial edema. Compared to other sports people, endurance athletes are more likely to have EIA. The incidence of EIA is notably high in swimming despite the generally favorable humid conditions, most likely because of the intake of chlorine gas, a known provocative agent ⁽⁶⁵⁾.

When used in large doses, beta-2 agonists have both an anabolic (building muscle) and lipogenic (reducing fat) impact because they stimulate protein synthesis. They have no negative, sex-specific (androgenic) side effects, which is why doping athletes use them instead of anabolics.

Abuse of beta-2 agonists can have negative effects that increase oxygen consumption when the heart rate is elevated, which can result in the heart muscle cells receiving insufficient oxygen. This could result in angina pectoris and a drop in blood potassium levels, which could occasionally result in arrhythmias, or abnormal heartbeats ⁽⁶⁶⁾.

The latest reported prohibited substances list provided by the World Anti-Doping Agency (WADA) forbids non-asthmatic athletes from using inhaled β_2 agonists, which are commonly used to treat asthma. This means that before beginning β_2 agonist treatment, an athlete with asthma or EIA must wait to receive a therapeutic use exemption (TUE) from a medical committee proving the existence of the illness ⁽⁶⁷⁾.

2.4.1. Therapeutic use exemptions (TUE)

A Therapeutic Use Exemption (TUE), as specified by the WADA World Anti-Doping Program TUE Guidelines, is permission to use a Prohibited Substance under specific, limited circumstances (medical condition). The international standard for TUE should be followed when applying for a TUE ⁽⁶⁸⁾.

Before using any substance mentioned in the prohibited list, a TUE must be obtained. One may be issued a conventional TUE or an abbreviated TUE (ATUE), based on the drug in question and the mode of administration. A TUE must be obtained from the appropriate Anti-Doping Organization (ADO) for any athlete who requires medical attention that involves the use of a prohibited substance or method and is subject to testing. Athletes must have a medical issue that is well-documented and backed by pertinent and trustworthy medical data in order to be approved for a TUE ⁽⁶⁸⁾.

The primary rationale behind the ban on inhaled β_2 agonist use in athletes who do not have asthma is the substance's reported ergogenic potential ⁽⁶⁹⁾. As of right now, β_2 agonists are entirely banned both in and after competition, except for terbutaline, formoterol, salmeterol, and salbutamol when inhaled to treat or prevent EIA and asthma as long as a threshold is not crossed ⁽⁶⁸⁾.

2.4.2. Detection of β_2 - agonists

The WADA has set daily use levels for a wide range of substances in each class; nevertheless, the limits are quite high, and it can be challenging to discern between the

drug's inhaled and systemic versions. Because this concentration cannot be reached by inhalation alone, salbutamol concentration greater than 1000 ng/ml or a formoterol concentration greater than 40 ng/mL in urine is regarded as a positive PED test. These results are inconsistent with the substance's therapeutic use ⁽⁷⁰⁾.

It is possible to identify β_2 -adrenergic agonists in urine, hair, and serum utilizing gas chromatography(GC-MS) ^(71, 72) and/or liquid chromatography ⁽⁷³⁾, as well as to purify the analytes using conventional isolation techniques for instance; liquid-liquid extraction (LLE) and/or solid-phase extraction (SPE) ⁽⁷⁴⁾.

2.5. Hormone and metabolic modulators

Indirect androgen doping techniques have been designed to generate a prolonged increase in endogenous testosterone. These include aromatase inhibitors (4-Androstene-3,6,17-trione (6-oxo); aminoglutethimide; and anastrozole. Other methods include estrogen-blocking by medications that function as estrogen receptor antagonists or antiestrogens (Raloxifene, Tamoxifen, Toremifene) ⁽⁷⁵⁾.

2.5.1. Detection of hormone and metabolic modulators

Numerous techniques were created to identify aromatase inhibitors and estrogen antagonists, such as liquid chromatography with fluorescence detection ⁽⁷⁶⁾, and gas chromatography with mass spectrometric detection ^(77, 78).

2.6. Diuretics and masking agents

Athletes can employ drugs and/or systems known as "masking methods" to conceal the usage of illicit substances under surveillance by anti-doping organizations. The prohibited substance in the athlete's urine samples can be concealed by the masking agents. Diuretics and other masking agents are forbidden in sports both in and after competition in line with the WADA prohibited list. The 2024 list includes

desmopressin, diuretics, plasma expanders, probenecid, and drugs that have comparable biological effects⁽⁷⁹⁾.

By postponing their elimination, probenecid is used to conceal the use of doping chemicals, or masking agents, particularly anabolic steroids. Probenecid is used in medicine to treat gout rheumatism. Nonetheless, out of all the masking agents, finasteride is most commonly used(15.2%), followed by epitestosterone (5.2%)⁽⁸⁰⁾.

Since diuretics can be used by sportsmen as masking agents or to lose weight, they were originally outlawed in sports in 1988. Because of their powerful action in water elimination from the body, they can help people lose weight quickly, which may be employed to reach certain weight requirements for sports like boxing and judo. Additionally, they can be employed as mask because doing so causes the body to excrete less of the prohibited substance. A masking effect can also be produced by certain diuretics, which alter the urine pH and prevent the excretion of basic and acidic medications⁽⁸¹⁾.

With a total of 436 cases, diuretics accounted for about eight percent of all adverse analysis results reported by WADA's laboratories in 2008⁽⁸²⁾, of which hydrochlorothiazide was most commonly used⁽⁸³⁾.

Due to the drug's ability to mask hematic indicators helpful for blood doping detection, WADA has added plasma volume expanders to the prohibited list. This includes desmopressin, a synthetic analogue of the endogenous peptide hormone arginine vasopressin. The way desmopressin functions is by reducing the volume of water excreted in the urine⁽⁸⁴⁾.

2.6.1. Detection of diuretics and masking agents

Reviewing the analytical techniques used to identify these illegal substances, high-performance liquid chromatography with photo-diode array detection was first utilized

in diuretics identification⁽⁸⁵⁾. However, the most popular analytical method for their quantitation has been gas chromatography/mass spectrometry^(83, 86).

2.7. Stimulants

Since the 19th century, stimulants including cocaine, strychnine, and caffeine have been utilized by athletes⁽⁸⁷⁾. The 2024 WADA list identifies roughly 60 stimulants and forbids their use in competition. The most well-known is amphetamine, which acts on the central nervous system by causing the release of neurotransmitters into the synapses between neurons, including dopamine, and thus amphetamine fights fatigue⁽⁸⁸⁾. Athletes who take stimulants take the risk of suffering from irregular heart rhythms, injuring themselves and their competitors^(89, 90).

2.7.1. Detection of stimulants

It is necessary to separate the target medication from other compounds in the biological sample before recognizing it to screen for it. Many of the anti-doping labs across the world are switching from gas chromatographic-mass spectrometric to highly sensitive liquid chromatographic-mass spectrometric techniques for routine stimulant screening⁽⁹¹⁾, which includes amphetamine.

2.8. Narcotics

Narcotics are drugs that change the mood and relieve pain. Painkillers are often used in sports, particularly by athletes who play violent sports. Furthermore, narcotic analgesics may lessen anxiety, which may improve performance in sports like pistol shooting and archery where high levels of anxiety could negatively impact fine motor coordination.

The following drugs are not allowed: Buprenorphine, Dextromoramide, Diamorphine (heroin), Hydromorphone, Methadone, Fentanyl and its derivatives, and Morphine.

Opioids have several adverse effects such as drowsiness, breathing difficulties, reduced

heart rate, and alterations in blood pressure. Opioids misuse can be fatal owing to respiratory and cardiovascular inadequacy⁽⁹²⁾.

2.8.1. Detection of narcotics

Only 33 laboratories globally have received WADA accreditation to analyze all samples. The World Anti-Doping Code is an international reference that must be used by all WADA-accredited laboratories⁽⁹³⁾. The approved techniques for the detection of narcotics are liquid chromatography (LC) with detection via UV/VIS- or mass spectrometry (MS) and gas chromatography (GC) with mass spectrometry (MS) and tandem mass spectrometry (MS/MS). Several methods were developed for the simultaneous quantitation of narcotics along with the subsequently prohibited substances namely, cannabinoids, glucocorticoids, and beta-blockers⁽⁹⁴⁻⁹⁷⁾.

2.9. Cannabinoids

The most common forms of cannabinoids are found in marijuana, hashish oil, and hashish. Cannabis primarily affects the brain's cannabinoid receptors, which are responsible for feelings of pleasure, relaxation, and enhanced auditory and visual experiences. Tetrahydrocannabinol (THC) also enhances the release of dopamine by counteracting the inhibitory impact of GABA neurons.⁽⁹⁸⁾ Prohibited cannabinoids include; natural cannabinoids, e.g., cannabis, hashish, and marijuana. Moreover, synthetic cannabinoids e.g. Δ^9 -tetrahydrocannabinol (THC) and other cannabimimetics. Excluding cannabidiol, unlike THC, synthetic cannabidiol lacks psychoactive effects. Rather, it is mostly used medicinally to alleviate inflammation, anxiety, and discomfort⁽⁹⁹⁾.

2.9.1. Detection of cannabinoids

The approved techniques for the detection of cannabinoids are gas chromatography (GC) with mass spectrometry (MS) and tandem mass spectrometry (MS/MS)⁽¹⁰⁰⁻¹⁰²⁾.

2.10. Glucocorticoids

It is not permitted to use any glucocorticoids (including cortisone, betesonide, and betamethasone) via any administration route. Glucocorticoids are either naturally occurring steroid hormones or synthetic compounds that block the inflammatory process. The adrenal glands, which are situated right above the kidneys, are the source of natural glucocorticoids. They are not the same as the notorious anabolic steroids that some sportsmen take to bulk up and get stronger. On the contrary, glucocorticoids are catabolic steroids. Owing to its analgesic properties at higher doses and its ability to relax the respiratory tract, Athletes can perform better during training and competition thanks to their open airways and lowered pain threshold⁽¹⁰³⁾.

2.10.1. Detection of glucocorticoids

The approved techniques for the detection of glucocorticoids are liquid chromatography (LC) with detection via UV/VIS- or mass spectrometry (MS) and tandem mass spectrometry (MS/MS)⁽¹⁰⁴⁻¹⁰⁶⁾.

LC-MS/MS can detect many anabolic androgenic steroids, β_2 -agonists, glucocorticosteroids, hormone antagonists and modulators, and beta-blockers. Anti-doping testing facilities frequently use liquid chromatography/tandem mass spectrometry (LC-MS/MS) for this purpose, and a number of quick techniques have been reported to concurrently detect various classes of substances⁽¹⁰⁷⁾.

3. Substances prohibited in particular sports

3.1. Beta-blockers

Beta-blockers are banned in and out-of-competition is specific sports including but not limited to shooting, archery, golf etc⁽¹⁰⁸⁾. Although beta-blockers were originally created as medications to improve heart function in patients with compromised cardiovascular systems, they also have soothing neurological effects that reduce

anxiety and nervousness while stabilizing motor function. Enhancing psychomotor performance could prove advantageous in sports like archery and shooting that demand accuracy and precision.

Beta-blockers are used as PEDs in sports as they protect against social phobia. Beta-blockers as propranolol, have shown an improvement in athletes' physical symptoms⁽¹⁰⁹⁾.

3.2. Detection of beta-blockers

The approved techniques for the detection of beta blockers are gas chromatography (GC) with mass spectrometry (MS) and tandem mass spectrometry (MS/MS)^(96, 110).

Delbeke *et al.* have described the simultaneous quantitation of diuretics and beta-blockers including twenty-one beta-blockers and eight diuretics using LC-MS in human urine. After liquid-liquid extraction using ethyl acetate, analysis was conducted utilizing an ion trap apparatus equipped with electrospray ionization. The diuretics and beta-adrenergic blocking medications have detection limits of 5–100 ng/mL and 5–500 ng/mL, respectively. This technique could potentially be used to detect other doping drugs such as strychnine, norbuprenorphine, and mesocarb hydroxysulfate⁽¹¹¹⁾.

(Table 3) summarizes the measurement conditions of some of the analytical methods applied in the simultaneous quantification of prohibited substances 6,7,8,9, 10 and Beta blockers.

4. Prohibited Methods and Manipulations

4.1. Manipulation of blood and blood components

According to the WADA 2024 list⁽¹¹²⁾, the following are banned:

1. The process of introducing or reintroducing any amount of red blood cell products or autologous, allogenic (homologous), or heterologous blood into the circulatory system.

2. Manipulating the uptake, transport, or distribution of oxygen, including perfluorochemicals; efaproxiral (RSR13) and modified hemoglobin products (which do not include inhaled oxygen supplements) such as hemoglobin-based blood replacements and microencapsulated hemoglobin products.
3. Any type of physical or chemical intravascular manipulation of blood or blood components.

The WADA has adopted a way to discover blood doping known as the "biological passport". It works by tracking an athlete's blood parameters over time in search of changes suggestive of potential doping. The ratio of hemoglobin to reticulocytes is known as the "off-score," and it is particularly informative. When blood is extracted and reinfused, the ratio rises, making it simpler to detect blood doping covertly⁽¹¹³⁾.

Krafft *et al.* have developed a novel technique for identifying perfluorocarbon compounds (PFCs) in blood samples. Gas chromatography-mass spectrometry (GC/MS) using an ion trap mass spectrometer in MS and MS/MS modes is used to identify PFCs following an extraction and preconcentration step carried out by headspace solid-phase microextraction (HS-SPME)⁽¹¹⁴⁾.

4.2. Chemical and physical manipulation

According to the WADA 2024 list⁽¹¹²⁾, the following are banned:

1. Tampering, or attempting to tamper, to change the validity and integrity of samples obtained during doping control. Including, but not restricted to: Adulteration or substitution of urine (e.g., proteases).
2. More than 100 mL of intravenous infusions or injections in 12 hours, unless those are lawfully administered as part of hospital therapy, surgeries, or clinical diagnostic investigations.

Table 3. summarizes the measurement conditions of some of the analytical methods applied in the simultaneous quantification of prohibited substances from 2.6 - 3.0 and Beta blockers.

Technique	Matrix	Conditions	Linearity range	LOD	Reference
LC-ESI-MS/MS	Determination of desmopressin in urine	Zorbax 300SB-C18 reverse-phase column (1.0 mm × 50 mm, 3.5 µm) protected with a Zorbax 300SB-C8 guard column. Mobile phase A: H ₂ O, 0.1% HOAc, 0.01% TFA; Mobile phase B consisted of ACN, 0.1% HOAc, 0.01% TFA.	25-100 pg/mL	25 pg/mL	35
LC-MS/MS	Diuretics and beta-blocking agents in urine	A Nucleosil C18 column (3 mm x100 mm, 5 mm; and a guard column (Chromsep, SS 10 mm x 2 mm; Chrompack). Mobile phase: 1% acetic acid in water and acetonitrile. Flow rate 0.3 mL/min	--	Diuretics : 5 -100 ng/mL Beta- adrenergic blocking agents: 5 -500 ng/mL.	103
GC/MS	Detection of perfluorocarbons in Blood	HP-624 column (30 m, 0.25 mm i.d., 1.4 mm film thickness); in splitless mode. Carrier gas: Helium	F-decaline (FDC) : 0.65 -32.5 µg/mL; F-methyl-cycloheyl-piperidine (FMCHP) :0.325- 16.25 µg/mL.	FDC 350 µg/mL; FMCHP :175 µg/mL	107
HPLC method with electrochemical detection	Detection of beta-2 agonist in human and calf urine	Linear ion trap (LTQ XL) or on a triple quadrupole (TSQ Quantum Ultra), with an electrospray ionization (ESI) source operated in the positive mode. Separations were performed on a wide-pore UP5WC4 column (300 Å, 2.0 mm i.d. × 250 mm, 5 µm particle size). The LC flow was 300 µL/ min. Mobile phase: water/acetonitrile + 0.2% formic acid.	1-10 µg/L	1 µg/L	74
GC-MS-MS-MS	Urine	HP5 column (cross-linked phenylmethylsilicone, 30 m) in splitless mode. Carrier gas: Helium	0.5 -5 µg /L	0.2 µg /L	72

4.2.1. Detection of chemical manipulation agents

A method using high-performance liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) has been established for the identification of desmopressin in human urine ⁽¹¹⁵⁾ with a lower detection limit of 25 pg/mL. The method's suitability was demonstrated through the examination of actual urine samples that were taken following the intravenous, oral, and intranasal injection of

desmopressin. In every instance, the peptide was detected.

4.3. Gene and cell doping

A gene is the basic unit of heredity that codes for the synthesis of a single protein. Sometimes these genes are defective and contain incorrect information, which results in either increased protein synthesis or vice versa. Hence, gene therapy attempts to fix the broken gene. However, abuse should be considered if increasing for instance; muscle proteins is conceivable ⁽¹¹⁶⁾.

As a result, WADA has included gene doping in the prohibited list due to the potential for gene therapy to be abused in sports. It is defined as: "the non-therapeutic use of cells, genes, genetic elements, or of the modulation of gene expression, having the capacity to enhance athletic performance" (WADA, 2024) ⁽¹¹²⁾. However, manipulating these genes is more difficult than it first appears. Several genes contain information that can be used to produce proteins that increase muscle mass or endurance when engaging in physical exercise. A single "sports" gene does not exist.

Four intriguing endurance genes have been identified ⁽¹¹⁷⁾; these are angiotensin converting enzyme (ACE), which can either dilate or constrict blood vessels, erythropoietin (EPO), which stimulates erythropoiesis, peroxisome proliferator-activated receptor (PPAR), which encodes enzymes involved in fatty acid oxidation, and hypoxia-inducible factors (HIF), which regulates the amount of oxygen available. Conversely, three intriguing muscle genes ⁽¹¹⁷⁾; First, growth hormone (GH) controls muscle mass; next, mechano growth factor (MGF), insulin-like growth factor-1 (IGF-1), and insulin-like growth factor binding protein (IGFBP) controls muscle growth; and last, myostatin/growth differentiation factor (gdf-8) or transforming growth factor-b (tgf-b) acts as a negative regulator of muscle growth. The following are forbidden as they might improve athletic performance:

1. The utilization of nucleic acid polymers or their equivalents.
2. The utilization of gene editing tools intended to modify the transcriptional or epigenetic control of gene expression, as well as genome sequences.
3. Using either regular or genetically altered cells.

Even though most gene therapies are still in their early stages, it is quite likely that

athletes will take advantage of these innovative methods to improve their performance before they wait for clinical approval.

Gene doping can cause leukemia-like illnesses or extremely strong immune responses that can result in mortality in the short term. Secondly, the absence of regulation over gene expression has long-term consequences and may raise the danger of tumors. If drug abuse of EPO is halted, it is usually reversible; however, if EPO is abused by integrating extra EPO genes, the number of erythrocytes is raised throughout life. As a result, there is a rise in cardiovascular thromboembolic issues ⁽¹¹⁷⁾.

There are two different ways to transport DNA into an organism: ex vivo transfer and in vivo transfer. DNA direct injection into the target tissue is implied by in vivo gene therapy. The transgene can be inserted by physical (direct injection), chemical (cationic liposomes), or biological (viral vectors, plasmids) techniques ⁽¹¹⁸⁾.

4.3.1. Detection of Gene doping

While the mode of administration is only a matter of conjecture, the most plausible approach would include injecting transgenes as viral constructs into the skeletal muscle, whereupon the cell's biochemical machinery would be recruited to produce the transgene ^(4, 119). A muscle biopsy would be necessary to accurately identify this type of gene doping, but it is not the right kind of invasive treatment. Transgenes in small quantities, would, however, seep into the blood in this condition, and these can be separated from a massive surplus of genomic DNA (gDNA).

4.3.1.1. Real-time Polymerase chain reaction (rPCR)

The detection of gene doping is mainly achieved through real-time Polymerase chain reaction (rPCR). Currently, exon-exon junctions in the intron-less transgene correlate to distinct sequences in a doping gene that are targeted by PCR-based

techniques for the detection of gene doping. This strategy is based on the observation that a "doping" transgene's sequences differ slightly from those of its endogenous equivalent in genomic DNA (gDNA). Exons and introns are present in most human genes, including gene doping candidates ⁽¹²⁰⁾.

Transgenes are often generated from the complementary DNA (cDNA) of the gene to be transferred, and cDNA is introns-free (Fig. 4). This is relevant to gene therapy as well as possible gene doping. It suggests that target regions inside the transgene that correspond to exon/exon junctions in cDNA but are lacking in gDNA because of their interruption by introns can be specifically found using molecular testing based on real-time PCR. This makes it possible to identify a low quantity of transgenic in a huge background of the endogenous gene in gDNA, particularly when combined with the high sensitivity of PCR owing to its exponential amplification of the target sequence.

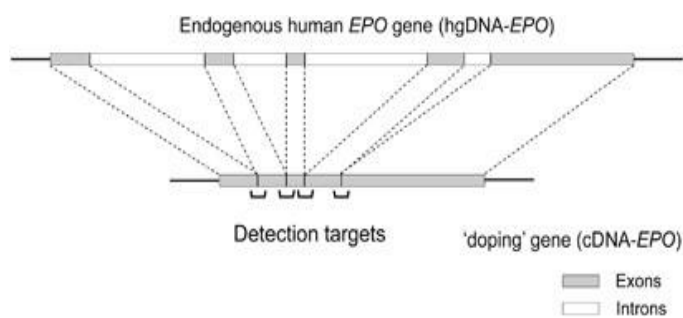


Fig. 4. Scheme representing the strategy employed for the direct detection of EPO transgene ⁽¹²⁰⁾.

To further reinforce the applicability of this technique as the foundation for a gene doping test, real-time PCR, which permits a one-step analysis in a closed system, helps to prevent sample contamination that could result in a false-positive test ⁽¹²⁰⁾. Assays that are well-designed have a 100% specificity for

transgene detection and a sensitivity of 80–130 copies/mL for plasma or blood.

Nevertheless, as the short PCR primers are even interrupted by the smallest sequence change and the exon-exon junctions of doping genes are known, it is feasible to avoid detection using the current PCR-based methods by altering the doping gene, for example with synonymous alterations, which will consequently trip a false-negative result ⁽¹²¹⁾.

Reviewing the literature, several articles have covered the use of PEDs in sports from different perspectives considering doping prevalence in different societies, regulations that restrict their use in competitions, ethical considerations, athletes' awareness and knowledge of PEDs, etc ⁽¹²²⁻¹²⁵⁾. Consequently, the significance and novelty of this article as the first work to include a comprehensive summary of the most common analytical techniques implemented in the detection of the different PED classes is noteworthy.

5. Conclusions

The misuse of illegal performance-enhancing drugs (PEDs), and other strategies by athletes to obtain an unfair competitive advantage is a worldwide issue. The abuse of PEDs has detrimental impacts on athletes, competitors, and the integrity of sports. The World Anti-Doping Agency (WADA) releases a list of substances and methods that are forbidden from use in athletic competition each year. To enhance their athletic performance, athletes also turn to specific doping techniques like gene doping, blood doping, and urine adulteration. This article encapsulates the different classes of PEDs most popular techniques used to improve athletes' performance. Furthermore, this work summarizes the most common techniques applied for the detection of doping.

Highlights

- The use of illegal performance-enhancing drugs (PEDs), and other tactics by athletes to gain an unfair competitive advantage is becoming a global concern.
- A list of drugs and techniques that are prohibited from being used in sports is annually published by the World Anti-Doping Agency (WADA).
- This work reviews the different classes of PEDs and methods and summarizes the analytical techniques that are implemented in the detection of doping.

Conflicts of interest

The authors of the article declare that they have no conflicts of interest.

Authors' contributions

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Eman I. El-Kimary: Writing - review & editing, Supervision.

Tarek S. Belal: Writing - review & editing, Supervision.

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