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Detection Methods of Androgenic-Anabolic Steroids in Sports

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Abstract

The use of androgenic-anabolic steroids in sports has grown in popularity and new methods to evade detection have become equally as popular. Athletes are discovering new ways to compete while using these substances, however, agencies like the World Anti-Doping Agency (WADA) are taking a stance on drug testing for competitions. The components of substances used, detection windows, methods of administration, and current procedures all contribute to the detection of these drugs. Understanding these methods aids in the development of new testing and guidance to be implemented to help eliminate steroids in sports. The comparisons of these variables can help researchers to understand the limitations of current testing procedures, while also developing new strategies to help eliminate and detect prohibited steroid usage.

Introduction

The use of anabolic and androgenic steroids in sports has become increasingly popular since athletes began using them as early as the 1940s. Steroids accentuate masculine features and contribute to gains in strength and muscle size, which has contributed to their popularity (National Institute on Drug Abuse, 2020).

Today, these substances are used for enhancing sports performance, sculpting the "ideal" physique, and in some cases, for medical purposes. The use of steroids is controversial as they are not always detected, and the user gains an unfair advantage in a particular sport.

In an attempt to decrease steroid usage, the Anabolic Steroid Act of 1900 was enacted, which aimed to classify anabolic steroids as a Schedule III non-narcotic substance, which are categorized as having low physical dependence and high psychological dependence (United States Congress, 1990). Subsequently, in 2004, another act was passed that aimed to ban over the counter sales of steroid precursors and implemented penalties for possessing, making, and distributing these properties.

This act also outlined in more detail what constitutes an anabolic steroid by defining it as " to mean any drug or hormonal substance, chemically and pharmacologically related to testosterone" (United States Congress, 2004). However, steroids are legal in certain countries and can be smuggled into the United States or purchased online, and in some cases, produced by secret laboratories. Due to this accessibility and widespread use, sanctioned sporting events require drug testing in an attempt to detect an array of substances that are considered banned in competition. To combat the use of illicit substances in athletics and competitions, the World Anti-Doping Agency (WADA) maintains lists of all prohibited substances in various sports, with Anabolic-Androgenic steroids having over 60 variations listed. Along with anabolic steroids, growth factors, Beta-2 agonists, and various hormones also are included (WADA, 2021). Steroids assist with building muscle by increasing skeletal muscle growth, creating faster recovery times after a workout, and training more

frequently and at higher intensities (Mayo Clinic Laboratories, 2020). Due to this, in 2017, bodybuilding, cycling, and weightlifting/powerlifting account for many anti-doping violations each year as shown in figure 1 (National Institute on Drug Abuse, 2020).

Agencies like WADA aim to eliminate steroid usage in sports by testing for substances that are listed on the prohibited list. However, drug testing is not always a reliable and simple process. There are several variables that can impact the results of a drug test, such as the type of test, what drug was ingested, whether the usage is chronic or acute, whether or not masking substances were used, what type of masking substance was used, or if designer steroids were ingested. These influences can alter the results of the given test and allow a participant to pass, even though they are using a prohibited substance.

Mechanism / Mode of Action

Steroids are commonly used in sporting events and contribute to increased muscle growth, faster recovery times, and allow the user to train with increased intensity and frequency. Endogenous or natural testosterone aids in the growth and development of male sex organs and helps to maintain secondary sexual characteristics and is produced mainly by the Leydig cells in the testes. All the androgenic-anabolic steroids are derivatives of the male sex hormone, testosterone, and the effects of testosterone are separated into two classes, anabolic and androgenic (Muscle Physiology, 2000). Anabolic steroids are associated with muscle building, while

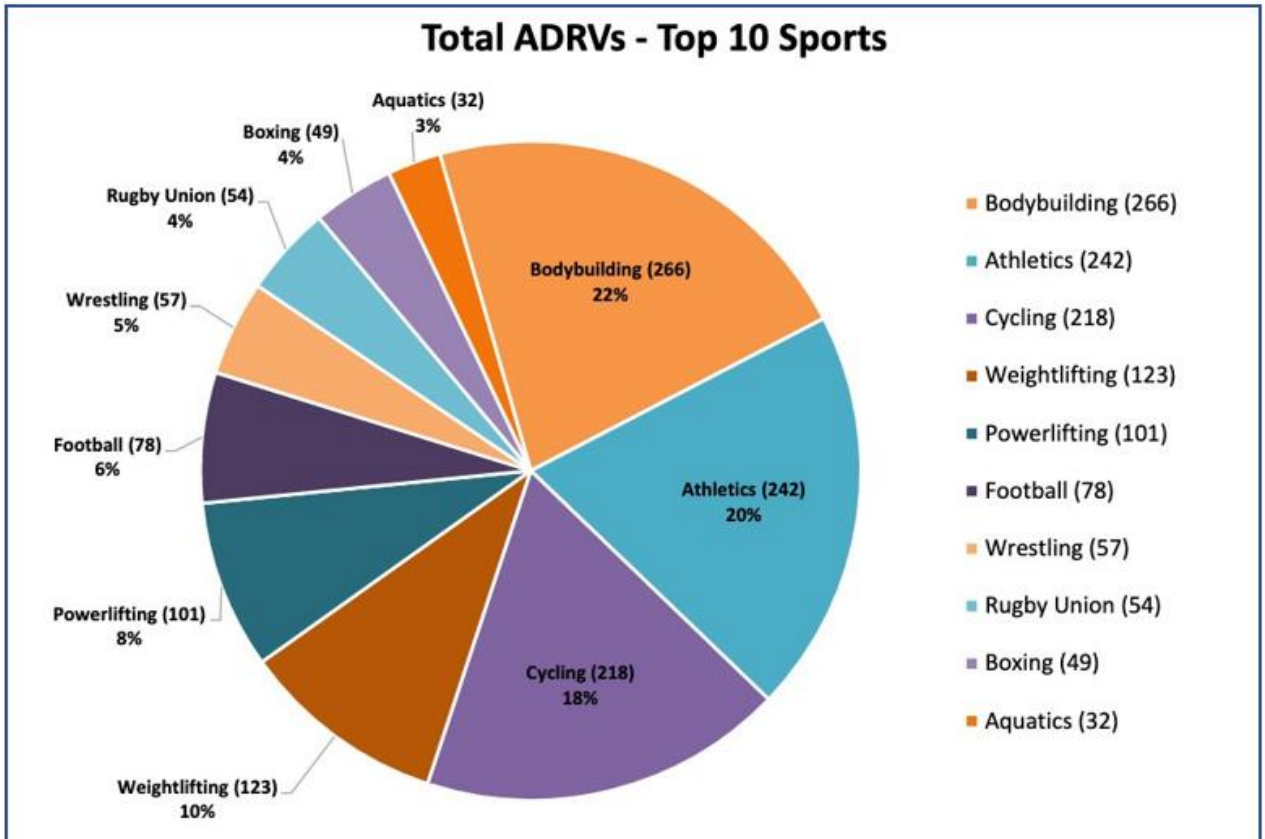

Figure 1. Pie chart displaying breakdown of Anti-Doping violations in sports. The top 10 sports based on violations are displayed. Taken from WADA.

androgens are related to increasing male sex characteristics (Bailey, 2019).

Androgenic-anabolic steroids are fat-soluble and therefore, membrane permeable, and due to these characteristics, they can directly influence the target cells. The steroids will penetrate the target cell membrane and then bind to the androgen receptors that are located in the cytoplasm, and once bound, can alter gene expression or alter processes that send signals throughout the cell (Cheskis, 2004). This description can be seen in figure 2. Additionally, androgenic-anabolic steroids can affect cell differentiation and can influence other cells to be made into muscle cells rather than fat-storage cells (Cheskis, 2004). Androgens can exert their effects on many parts of the body, including reproductive tissues, muscle, bone, hair follicles, liver, kidneys, and the immune and central nervous system (Kicman, 2008). The affinity of the steroid to the receptor is also important as different substances have varying affinities, which can alter this result to a lesser or greater degree (Cheskis, 2004).

The muscle can be affected by steroids in two main ways, which are the increase in protein production and the reduced muscle recovery time due to the blocking of cortisol on muscle tissue. The increase in production of protein is an anabolic effect as the more protein being produced, the greater the size of the muscle, and therefore, the stronger the muscle becomes. Along with the increase in protein production, there is also an increase in bone marrow along with red blood cells (Basaria, Wahlstrom, & Dobs, 2001). Lastly, anabolic steroids can promote the release of growth hormone which can help to increase the muscle size (Bailey, 2019). The decrease in muscle recovery time is related to the blocking of cortisol effects on the muscle tissue and preventing the breakdown of

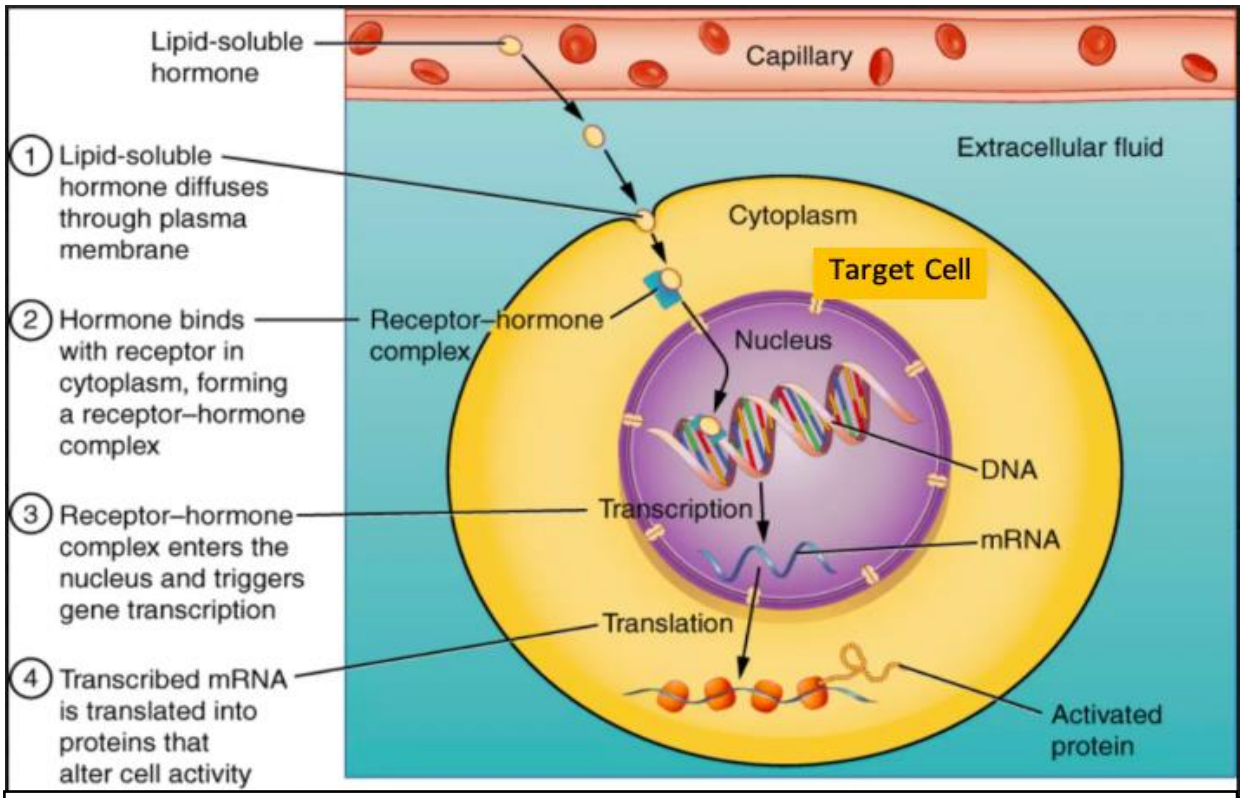

Figure 2. Diagram that shows how the lipid-soluble hormone (or in this case, an anabolic steroid), can penetrate the cell and effect cell activity. Adapted from https://www.thoughtco.com/how-steroid-hormones-work-373393

protein. This creates a decrease in catabolism on the muscle since the steroid will inhibit glucocorticoids that will promote the breakdown of muscles (Fahey, 1998).

Steroid usage is accompanied by several side effects, such as severe acne, breast development, baldness, erectile dysfunction, and infertility in males. In females, issues like unwanted body/facial hair, deepened voice, and irregular periods can occur. Aside from these sex-specific conditions, severe disorders can include increased blood pressure, heart attack, blood clots, and psychological issues, like mood swings and aggression (NHS, 2018). Lastly, steroids affect more than just the muscle, metabolism, and certain brain areas. Other lasting side effects must be considered as steroids can cause damage to other areas of the body. Adverse effects include cardiovascular, endocrine, metabolic, gastrointestinal, genitourinary, hematologic/oncologic, neuromuscular, skeletal, neuropsychiatric, dermatologic, and renal conditions (Ganesan, Rahman, & Zito, 2021). The prevalence of some of these common side effects are compared in figure 3.

Drug Testing Methods

Urinalysis

Testing urine for illicit substances has been a staple for many organizations, including the International Powerlifting Organization, American Drug-Free Powerlifting Federation, the United States of America Powerlifting, and the Olympic games, to name a few. Urinalysis is a preferred method of testing since it is relatively non-invasive and can provide information regarding steroid usage anywhere from days to weeks after the substance has been taken (these times can vary based on the substance used) (Saudan et al., 2006). However, there are some downsides to testing by urinalysis. It is difficult to determine if the substance has been taken chronically or acutely; either way, determining the length of use may be impossible. Additionally, since urinalysis is a standard testing method, it is common for test-takers to find new ways to pass, whether by altering the collection process or using the urine of others (Saudan et al., 2006).

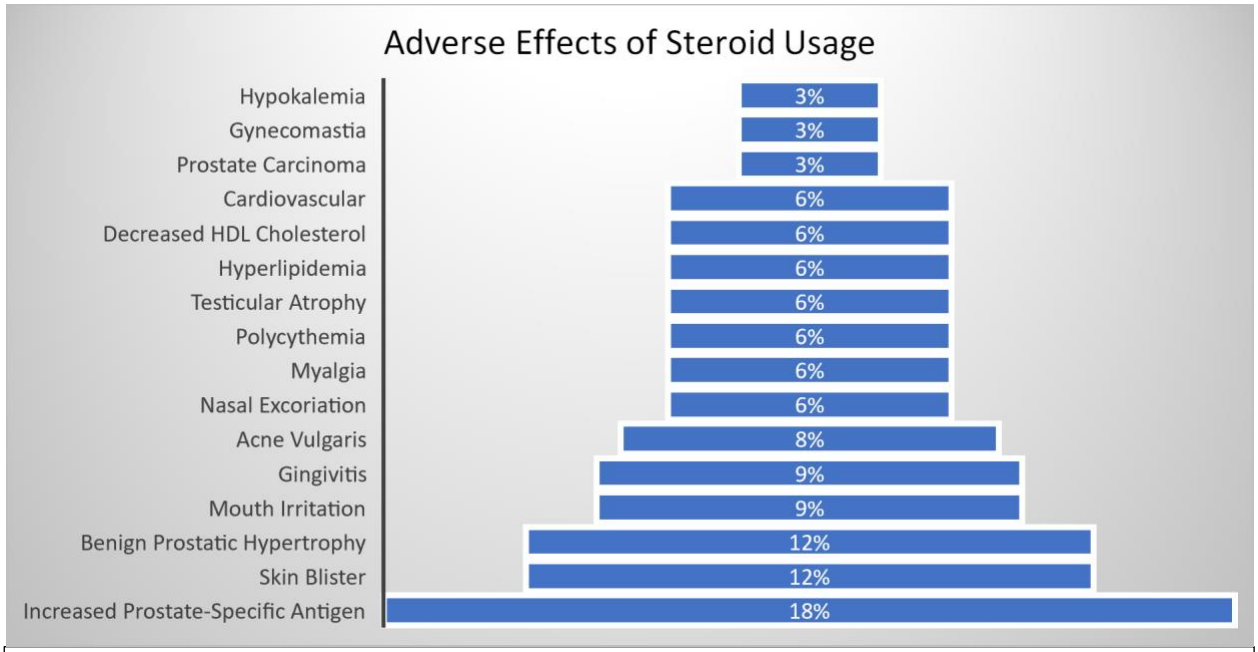

Figure 3. Adverse effects of AAS usage and percentage of occurrence. Adapted from (Ganesan, Rahman, & Zito, 2021).

Methods

Gas chromatography/mass spectrometry resides as a standard and efficient test for urinalysis. The chromatography uses a gas medium, which then separates the compounds present in the sample based on how they interact molecularly based on lipophilicity and polarity. Once separated, these compounds are then sent to the mass spectrometer, which then ionizes the compounds and can detect fragments based on the mass-to-charge ratio. Overall, the presence of steroids can be detected by their molecular characteristics (Raouf, Bettinger, & Fudin, 2018). Finally, there is a component of data analysis in which the components are compared to stored components located in libraries of mass spectra. The goal is to find the highest compatibility score, determining the compound at hand (Stein, 2012). This process is diagramed in figure 4.

Generally speaking, a preliminary urine test is conducted first in which levels of testosterone and epitestosterone will be compared and analyzed as a ratio. It has been shown that in healthy adult males, the ratio of these two substances will be close to 1:1. Additionally, of the two, testosterone contributes more performance-enhancing effects, so those taking exogenous testosterone will have a higher ratio of testosterone than epitestosterone. So, by conducting this first test, the urine is analyzed so this ratio can be determined for the athlete. WADA does allow a ratio of 4:1, meaning that an athlete can have under four times as much testosterone as epitestosterone, and if this level is over, it will be counted as a failed test. For the Olympic games, this level is set at 6:1 (Saudan et al., 2006), and other organizations set limitations along with these parameters. It is worth noting that it is shown that around 5% of the population have levels exceeding the established 4:1 ratio, which would affect the results of the ratio test. If this initial test is failed, WADA regulations state that a confirmatory test will need to be completed, in which carbon isotopes are compared (Lependorf, 2012).

Additionally, the presence of anabolic steroid usage can also be detected by carbon isotope comparisons, in which natural versus unnatural isotope distribution (or abnormal distribution) can be detected via Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) (Cawley & Flenker, 2008). Carbon exists as a combination of isotopes, and nearly all carbon contains six protons and six neutrons inside the atom (carbon-12). In figure 5, the comparison of carbon-12 to carbon-13 is shown. Due to plants selecting this carbon over millions of years, this carbon-12 is seen

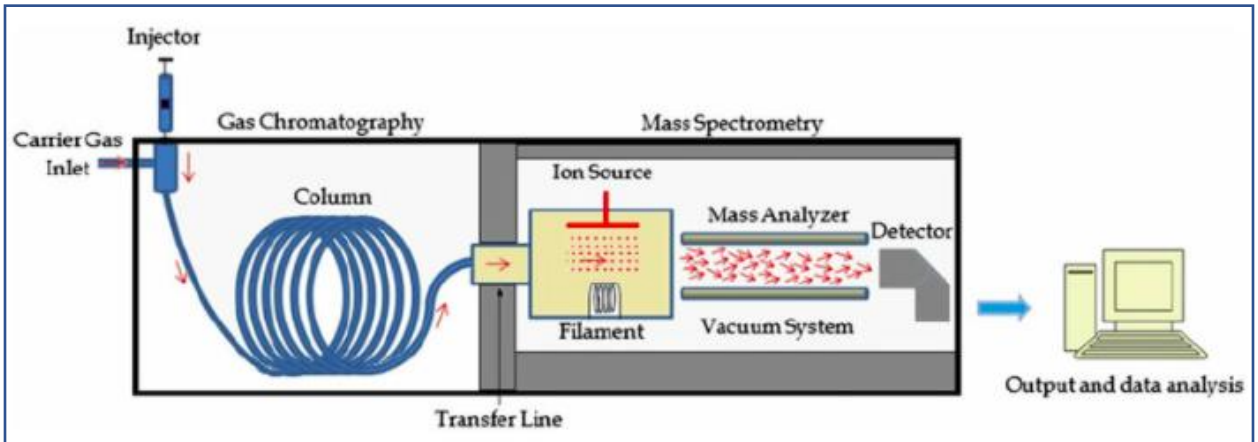

Figure 4. Diagram of main components and process of Gas Chromatography-Mass Spectrometry. The substance is processes through a gas medium and compounds are separated and analyzed based on lipophilicity and polarity. Adapted from ResearchGate.

more often. A small percentage of carbon has an extra neutron (carbon-13), and natural testosterone produced has a distinguishable carbon-13/carbon-12 ratio. Since carbon-13 has not gone through this long selection process through plants, it is easily detectable in synthetic testosterone, thus affecting the natural carbon-13/carbon-12 ratio (where carbon-13 is increased) (Lependorf, 2012). In order to pass the isotope ratio mass spectrometer test, which determines this isotopic ratio in urine, WADA allows a deviation of only three carbon-13 atoms per thousand or less. Anything above this marker indicates the use of exogenous testosterone, and thus, a failed test (WADA, 2021).

Overall, urinalysis to test for anabolic steroids is widely used and considered a standard for athlete testing as it is non-invasive, quick, and reasonably accurate. However, specific measures must be implemented to ensure a reliable and valid test. There are ways to tamper with the sample to conceal steroid usage, including tampering with the sample, diluting the sample, or using the urine of others to pass. To maintain the integrity of the sample, a chaperone may be required to observe the collection to avoid substitution. The collected sample should be at a temperature between 90-100 degrees Fahrenheit (to ensure substitution has not taken place), and visual inspection is required to ensure no additional alterations have been made to the sample (Raouf, Bettinger, & Fudin, 2018).

Hair Testing

Testing hair samples from an athlete has grown in interest, although most research on the subject focuses on substance abuse regarding illegal drugs, such as cannabis and cocaine. Due to this lack of research, there is still much to learn about perfecting the process of collecting and analyzing results from hair tests, especially regarding

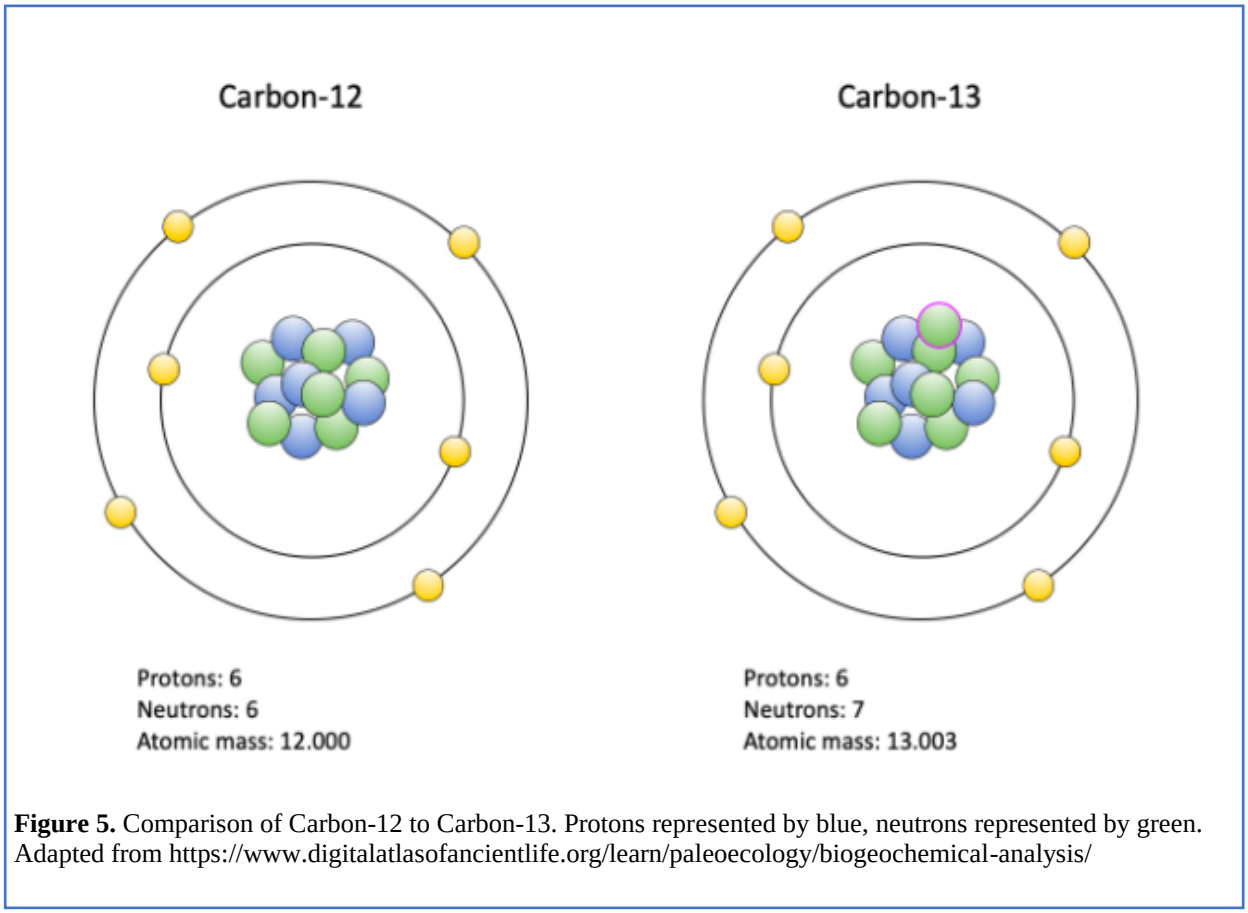

Figure 5. Comparison of Carbon-12 to Carbon-13. Protons represented by blue, neutrons represented by green. Adapted from https://www.digitalatlasofancientlife.org/learn/paleoecology/biogeochemical-analysis/

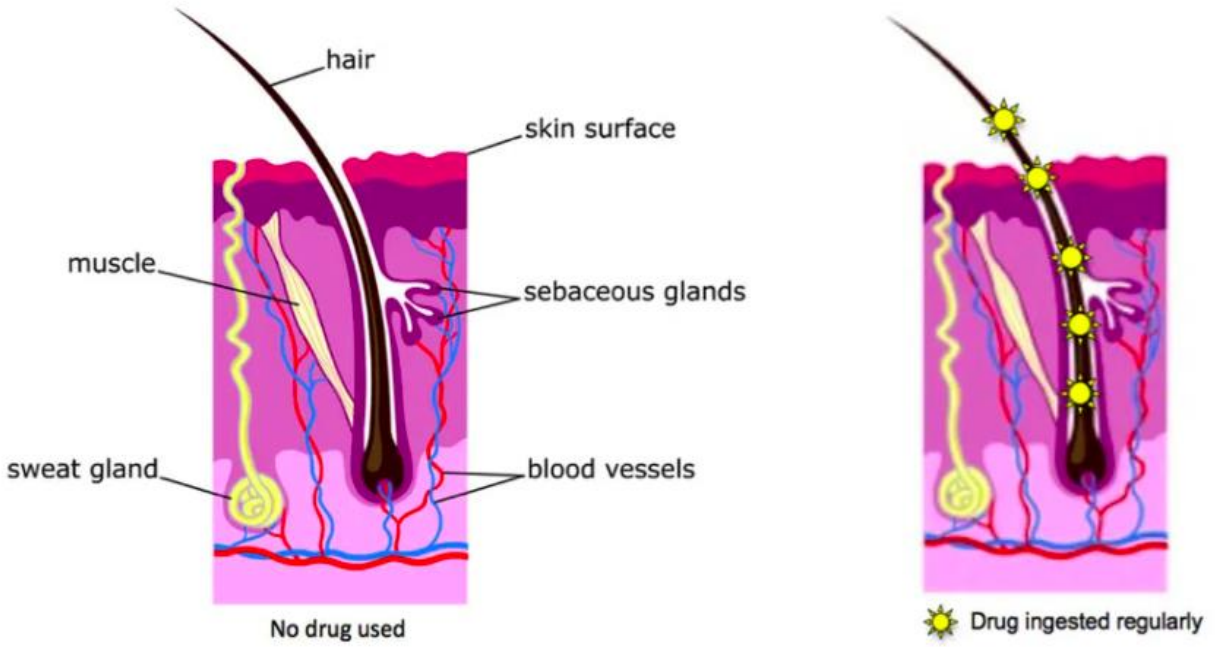

Figure 6. Diagram displaying how drug usage can affect the hair strand. Drug enters bloodstream, transfers to hair follicle, and becomes trapped in the hair strand. Left side shows normal hair strand compared to right side where regular drug usage has occurred. Taken from Crystal Drug & Alcohol testing.

anabolic steroids and other related substances. Hair testing does provide some benefits compared to other methods of drug testing, such as its long window of detection (around 90 days, depending on the length of hair), its sensitivity in detecting substances, its non-intrusive collection, and the inability for the athlete to "beat the test," meaning the patient cannot use the hair of others (Sharma, 2016).

Methods

In order to conduct the hair test, a reasonable sample of hair is required. Hair growth can vary for each individual but averages 0.6 cm to 1.4 cm per month (.24 inches to .55 inches per month). So, in order to obtain a drug history of 90 days, a sample of 3.9 cm (1.5 inches) would need to be taken from the scalp to account for three months of growth (Sharma, 2016). Additionally, other sources of body hair may also be acceptable, including chest, underarm, and even leg hair, but may not yield the same results due to difficulty establishing a timeframe from these samples (Sharma, 2016). It is also essential to understand how it is possible to detect the drug in the sample. When a drug is ingested, the substance is absorbed into the bloodstream, and each hair follicle can grow due to a blood vessel feeding into the hair follicle. Therefore, this blood with substance will inevitably reach the hair follicle and remains there in trace amounts until the hair grows out and is cut or falls out (Sharma, 2016). The process of drug usage affecting the hair strand is shown in figure 6.

To test the sample, a digestion method is used where the hair is liquefied, and the drugs that are present will be released and ready to be analyzed (Sharma, 2016). As with urinalysis, the process is composed of Gas Chromatography and a tandem Mass Spectrometry or GC/MS/MS (Gambelunghe, 2005). During this process, the sample is separated based on the physical and chemical properties. Once it enters the tandem mass spectrometer, there are two scanning mass analyzers, with a collision cell

between the two analyzers. Pieces selected from the first chamber are reacted with inert gas in this middle collision cell to defragment further (EAG Laboratories, 2021). Lastly, the organization or lab will have standards or acceptable values by which the results can be compared and determined to be passed or failed.

While this process is accepted in courts of justice while conducting substance abuse/drug testing, many organizations do not yet adopt it as a stand-alone test for anabolic steroids and other doping substances due to several factors and unanswered questions. These questions include external contaminants and to what extent these can alter results, including cosmetic treatments and hair dying (Kintz, 2006). However, it can serve as a secondary or confirmatory test in some cases after a preliminary test has been failed.

Blood

Testing blood is a common occurrence, and it is a viable option as this process can detect a wide range of entities. Even though this can be considered more invasive than some of the other testing options, it provides valuable information regarding a subject's health. An initial blood test can indicate steroid use by analyzing the clinical data and looking for elevated glucose levels, decreased HDL levels, increased LDL levels, and possible abnormal liver function tests (which would be more likely an indicator of chronic usage) (Kao, 2004).

While a general profile of the subject's health can be outlined, it is more challenging to determine exogenous steroids based solely on a blood test, as a urine test is a more comprehensive choice. Instead, these tests can be used to look at health risks associated with steroid use, including liver toxicity, kidney issues, glucose intolerance, blood disorders, and thyroid issues, to name a few (Kao, 2004). Generally, these issues continue with the steroid usage, taper off, and typically return to a steady state once the steroids have been stopped (Kao, 2004).

Sweat/Saliva

The use of sweat or saliva is an area of anti-doping testing that has an increasing interest within the research community, and best practices in collection and detection are still being developed. Sweat can easily be captured from the individual and collected into a container to be tested using methods previously described, such as Gas Chromatography/Mass Spectrometry (Anizan & Huestis, 2014). Even though sweat testing is an exciting take on specimen collection, saliva testing may prove to be a route that has shown the most promise as more developments have taken place in terms of detection.

Urine has been discussed as being a gold standard in drug testing. However, some of the limitations mentioned are ease of contamination/dilution, issues with obtaining proper sample sizes due to dehydration, and inconsistencies in testing for drugs in a particular window related to competition (Anizan & Huestis, 2014). Given these shortcomings, alternative matrix oral fluid (OF) can address some of these issues, such as being easy to collect, non-invasive, sex-neutral (do not need to differentiate the sex of chaperone), and the patient can even collect their specimen while also being easily observed (Anizan & Huestis, 2014). However, there are limitations to OF collection. These include dry mouth (limiting volume collected), inability to differentiate endogenous or exogenous production, and difficulty detecting certain substances (such as synthetic steroids) within their respective detection window, which to an extent can be an issue with any method mentioned (Anizan & Huestis, 2014).

Saliva is produced chiefly by three salivary glands named the parotid, the submandibular, and the sublingual. Additionally, the buccal glands line the mouth and also aid in production (Lewis, 2006). Saliva is a complex fluid and variable in that

it includes amounts of different fluids, such as gingival crevicular fluid (leaks from tooth and gums), plasma exudates, blood from oral lesions, and hormones (Lewis, 2006).

Testing Summary

Overall, each testing method has pros and cons related to its accuracy, detection windows, substances detected, and methods. Researchers are continually striving to improve testing methods in hopes of detecting and eliminating steroid doping in sporting events. An ideal testing method would include complete accuracy, ease of use for tester and athlete, non-invasive, conclusive, and have few external influences. These parameters may seem impossible to accommodate, but new methods are constantly being researched to one day detect known and unknown substances that are anti-doping violations. Table 1 outlines the various testing methods and provides comparisons for different qualities.

Detection Window

Several variables interact to influence the detection window for anabolic substances, including what type of AAS (androgenic-anabolic steroid) is taken, if it is designer or a known steroid, what test is being conducted, what dose was taken, and how long the athlete has been using the particular drug, method of administration, and the fat solubility (Levy, 2020). Given that urinalysis is the most common and standard test used, other tests will be examined to determine how they differ in detection methods. With most steroids, the time frame of detection can be as little as a few days, up to months

Table 1. Summary of Drug Testing Methods

Method	Can be influenced by	Invasive level	WADA approved	More tests needed	Ability to cheat easily	Benefit
Urinalysis	Alteration of test	Moderate	Yes	No (Unless initial test is positive)	Yes	Detects wide range of substances
Hair	Environment, hair dye	Low	No,but gaining attention	Possibly	No	Longer detection window
Blood	Other physiological factors	High	No	Yes, usually urine	No	Can indicate more general health risks associated with substance
Sweat/saliva	Dehydration, low volume of sample	Low	No	Yes	No	May be a promising way to complement other methods of testing, gaining attention in research community

following usage, depending on the above factors. When steroid drug tests are conducted, they look for metabolites produced by the medication and these drugs would not show up on a standard 12-panel drug test, but rather a more sophisticated process is needed to detect these substances (Levy, 2020). The first consideration is what type of drug is used; while some steroids can clear the body in a few days, others can remain detectable or in the body for months, depending on how the drug metabolizes (Levy, 2020).

Secondly, designer steroids will be discussed more thoroughly in a later section, but taking designer steroids may help mask the steroid during testing. There are various methods in which these steroids can avoid detection, but this makes them more desirable for athletes and will pay higher prices for this result (Levy, 2020). The test used also determines the detection window, and some tests, such as the hair test, can test for chronic usage, while other tests, like the saliva test, can test more acute drug usage.

Dosing, length of use, and method of administration can significantly vary the detection window. The greater a dose that an athlete uses, the more likely it is that this substance will appear on a drug test (Levy, 2020). Additionally, the length of use increases this chance, and tests such as the hair test can help prove chronic usages, up to months longer than a urinalysis could provide. Administration methods include oral, injected, dermal (gel), and transdermal (Ganesan, Rahman, & Zito, 2021). Oral steroids are processed faster by the body that if injected, meaning that injected steroids will have a longer detection window. Lastly, fat solubility is a factor since AAS drugs are lipids, meaning they are fat-soluble. Fat-soluble drugs are pulled into body fat or fat stores in the body and held on to in these areas. These metabolites stored in the body fat for extended periods may be detected in drug tests for months (Levy, 2020). Table 2 summarizes half-life and detection window times for various anabolic-androgenic steroids.

Masking

Masking agents are used to interfere in the chemical analysis of the substance to mask or disguise the drug or steroid of choice. They can be used to alter the results of a

Table 2: Table comparing half-life and detection windows of specific anabolic steroids. Adapted from Addiction Resource.

AAS	Half-life	Detected
Primobolan tablets	5 hours	More than 1 month
Andriol testosterone undecanoate	Up to 8 hours	Up to 7 days
Dianabol Oral	6 to 8 hours	More than 1 month
Halotestin	6 to 8 hours	More than 1 month
Winstrol tablets	8 hours	Up to 1 month
Anavar	8 to 12 hours	Up to 1 month
Anadrol	Up to 16 hours	More than 1 month
Masteron proprionate	2 to 3 days	More than 1 month
Trenbolone	2 to 3 days	More than 1 month
Winstrol Depot (Injected)	48 hours	More than 1 month
Dianabol (Injected)	60 to 72 hours	More than 1 month
Equipoise	7 to 9 days	More than 1 month
Masteron enanthate	8 days	More than 1 month
Primobolan Depot (Injected)	10 to 14 days	More than 1 month
Deca Durabolin	14 to 16 days	More than 1 month

drug test or dilute the substance so it will not show up during testing, which will be explained later in more detail (Mayo Clinic Laboratories, 2021). Essentially, they are used to eliminate the detection of banned substances that an athlete may have used, including anabolic steroids or stimulants (WADA, 2021). A few different kinds of masking agents can be used. Some that will be focused on are diuretics, uricosuric agents, steroid 5-alpha-reductase inhibitors, and anti-fungal drugs, to name a few, as well as some tactics that are used. Lastly, another category of drug masking involves using designer steroids, which are synthetic steroids that have been created specifically to evade drug tests and be undetectable by methods of drug testing (Mayo Clinic Laboratories, 2021).

Diuretics

Diuretics are a common and straightforward way to lessen the likelihood of detection of a banned substance taken by an athlete. In addition to their masking effects, they are also suitable for athletes seeking increases in water loss, which is desirable in sports where weight classes are used, and also in bodybuilding or physique competitions, in that it can help the athlete meet their weight class or appear leaner (WADA, 2021). In terms of masking, the more water lost, the less concentrated the urine becomes, making it challenging to detect substances in the urine while being analyzed. Diuretics can accomplish this by increasing the rate of urine flow and increasing sodium excretion, along with dilution of steroid metabolites in the urine. This dilution can bring these metabolites to a lower or acceptable limit, as set by WADA or the overseeing organization (Alquraini & Auchus, 2018).

Diuretics are used in clinical settings to treat certain diseases or illnesses, such as hypertension, liver cirrhosis, heart failure, renal failure, lung disease, and kidney disease, and reduce salt and water retention (Cadwallader, De La Torre, Tieri, & Botrè, 2010). As with any drugs, their use can be accompanied by adverse effects, even when taken under physician supervision. These effects are mainly related to fluid and electrolyte imbalances and include hyponatremia, extracellular fluid depletion, hypotension, thromboembolic issues, hypokalemia, cardiac arrhythmias, and hypomagnesemia, to name a few (Cadwallader, De La Torre, Tieri, & Botrè, 2010). Additionally, as stated, the main effects of diuretics include enhancing renal excretion of salts and water and also may affect chloride levels, as well as affecting renal absorption and excretion of cations like potassium and magnesium, anions like bicarbonates, dihydrogen phosphate, and uric acid (Cadwallader, De La Torre, Tieri, & Botrè, 2010). Comparison of common diuretics and effects is shown in table 3.

<u>Other Masking Agents</u>

There are many additional substances that athletes take to mask exogenous steroid usage, including uricosuric agents, steroid 5-alpha reductase inhibitors, and anti-fungal medications, to name a few. These types of masking agents are included in the WADA list of prohibited substances, even though they have different modes of action than traditional doping substances (Alquraini & Auchus, 2018). Uricosuric agents interfere with the renal excretion of substances, which include steroid glucuronide conjugates. This drug has been shown to decrease the excretion of conjugates and is sought after due to its masking effects (Alquraini & Auchus, 2018). One example of this category of drug is Probenecid, which is commonly used to treat gout. Probenecid can be detected (along with other diuretics) by GC-MS, and detecting this substance is grounds for establishing a doping violation (Alquraini & Auchus, 2018). Within these categories, examining the biological passport may aid in determining recent changes and help determine any substances used.

Another group of substances that are considered masking agents are the steroid 5-alpha reductase inhibitors. These substances reduce a double bond in testosterone, resulting in the androgen, Dihydrotestosterone (DHT), which is more potent. This androgen contributes to many masculine characteristics, such as body hair (Alquraini & Auchus, 2018). The most well-known example in this class is Finasteride, which is used for prostatic hyperplasia and baldness (Alquraini & Auchus, 2018). These substances can be alluring to athletes as they can prevent the detection of androgenic-anabolic steroids. An example noted with Finasteride is that it can inhibit the development of 19-norandrosterone to nandrolone, and therefore, masks the detection of nandrolone, which is used to increase lean body mass and enhance physical performance (Alquraini & Auchus, 2018).

Anti-fungal medications are substances worth mentioning. As previously discussed, the T/E ratio (testosterone glucuronide to epitestosterone glucuronide) is important in determining doping violations as set by WADA. The anti-fungal drugs inhibit endogenous steroid synthesis, including testosterone glucuronide in the T/E ratio (Alquraini & Auchus, 2018). One such drug, Ketoconazole, lowers the T/E ratio for endogenous levels but not for exogenous

Table 3. Summary of variety of diuretics and effects
Adapted from Cadwallader, De La Torre, Tieri, & Botrè

Diuretic	Acts on:	Example Drug:	Main Effect
Carbonic Anhydrase Inhibitors	Proximal Tubule	Acetazolamide	Used for AMS (high altitude mountain sickness), increases bicarbonate excretion in urine, makes blood more acidic
Inhibitor of Na+/K+/Cl- Symporter	Loop of Henle: Thick Ascending Limb	Furosemide, Bumetanide	Results in significant reduction of ability of kidney to concentrate urine, increase in urinary excretions of Na+ and Cl-, common cause of positive test
Inhibitor of Na+/Cl- Symporter	Distal Tubule	Thiazide	Reduces Na+ reabsorption, low cost, high tolerance, good compliance
Osmotic Diuretics	Distal Nephron/Collecting Duct	Glycerin, Mannitol, Urea	Enhances osmolarity of plasma and tubular fluid, creates increase of urine osmolarity with reduced water reabsorption
Renal Epithelial Na+ Channel inhibitors	Late Distal Tubule/Collecting Duct	Amiloride (usually in combination)	Block epithelial sodium channels and inhibit reabsorption of sodium
Mineralocorticoid Receptor Antagonists	Collecting Duct	Spironolactone	Inhibitor of aldosterone, bind and inhibits cytosolic MRs, effect is based on levels of aldosterone

testosterone (Alquraini & Auchus, 2018). Testing for Ketoconazole was common when T/E levels were elevated to help separate endogenous from exogenous levels but is not used commonly anymore. However, this does demonstrate the variety of effects that different substances can have on the body and how anti-doping agencies respond.

Biological Passport

One strategy to help mitigate instances of masking is using the biological passport as a detection method. The biological passport is the process of obtaining appropriate physiological measures with the idea that these values (serum, urine hormone, and prohormones) will not change dramatically over time (Anawalt, 2019). Certain values like testosterone, DHT, epitestosterone, and precursors are measured at baseline, usually before the first competition that requires testing and enforced doping regulations. These values are followed and analyzed each time the athlete is tested, and significant deviations are identified and require further investigation. Figure 7 displays a typical athlete's Hemoglobin test and shows the results compared over time. The main goal of this process is to detect the usage of exogenous androgenic-anabolic steroids by comparing personal data (Anawalt, 2019).

Even though the idea of the biological passport seems like a simple process, some issues can arise. The assumption is that athletes would not have used an AAS before their first baseline assessment; however, studies have shown that AAS usage can commonly occur as young as 16 through 22, meaning the initial assessments would be skewed from the beginning (Anawalt, 2019).

Additionally, these baseline numbers may be inaccurate as athletes may use designer steroids or other substances that do not have a drastic effect on urinary or blood samples, meaning they could have taken substances that were not identified. Lastly, this process assumes that all points in the collection process were collected, transported, and stored correctly, which for urinary samples, the risk of alteration remains high (Anawalt, 2019). Data entry errors must be considered in this category, and the assumption of

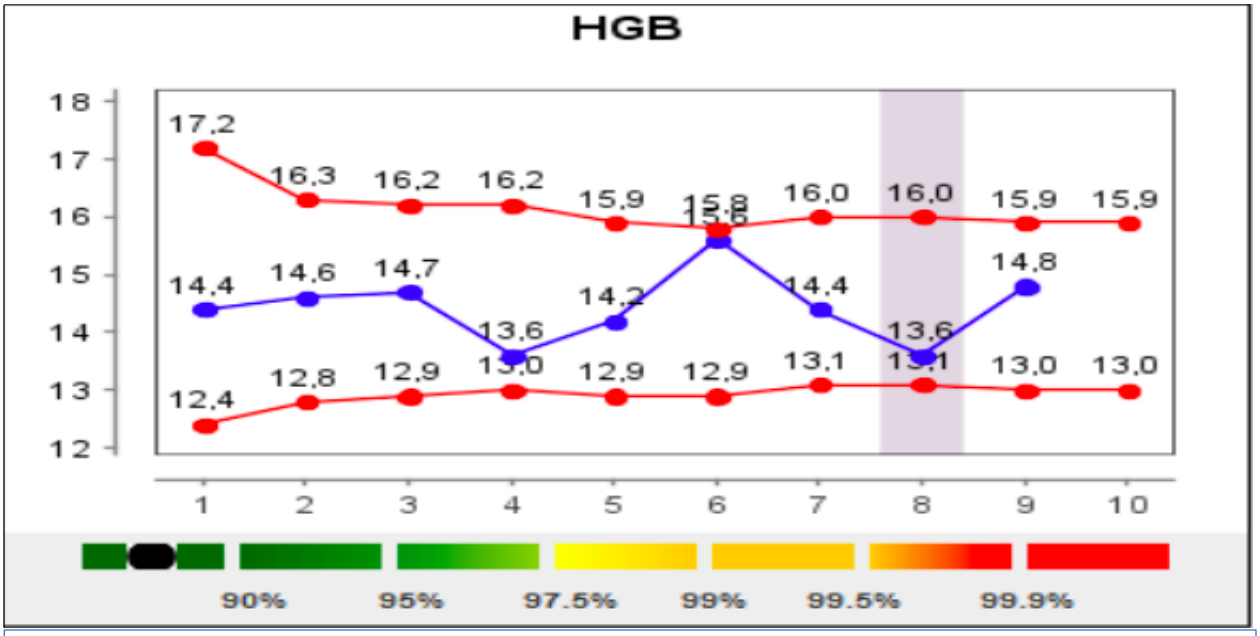

Figure 7. Graph showing Hemoglobin test results over time, comparing athlete levels with minimum and maximum allowed levels. Adapted from Anti-Doping Denmark.

secured databases is another area of concern. When assumptions are made during the process, this can weaken the validity of the procedure and results (Anawalt, 2019).

Medical Use/Exemption

One way to bypass the rules is to become eligible for a TUE (therapeutic use exemption). The athlete can claim a medical condition to take certain medications prescribed by a physician that would otherwise be considered doping (Alquraini & Auchus, 2018). A few examples include beta-agonists (asthma), glucocorticoids (inflammatory diseases), or hormones (endocrine deficiencies) (Alquraini & Auchus, 2018). TUEs for testosterone are sought for obvious reasons and will not be given out solely based on one-time laboratory results but rather a disease or chronic issue. Laboratory tests can be skewed as athletes may attempt to manipulate their testosterone levels by overtraining, taking certain drugs, losing weight, and avoiding sleep, to name a few (Alquraini & Auchus, 2018).

For the TUE to be granted for an athlete requesting testosterone, there must be an organic and irreversible cause unequivocally identified. In addition, women cannot be granted a TUE for testosterone, as the guidance states that only men with low testosterone levels and a medical condition are qualified (Alquraini & Auchus, 2018).

TUE guidelines state that an athlete may be prescribed a particular medication if all pertinent criteria are met, but this is not always an honest process. As athletes may be desperate to obtain a prescription, athletes and physicians may produce stories proclaiming that the athlete needs T-therapy (testosterone therapy), hoping to receive testosterone without having to worry about doping violations (Alquraini & Auchus, 2018). However, sometimes T-therapy may be prescribed falsely but in good faith. These athletes may be diagnosed as hypogonadal (the body does not produce enough male hormones), while the physician does not realize the false diagnosis or implications (Alquraini & Auchus, 2018).

This “false diagnosis” group creates problems for anti-doping agencies and makes it difficult to truly implement fairness. The normal range for testosterone varies by age but can be placed between 250-300 ng/dl. If men are found slightly below this limit but have normal gonadotrophin serum and normal pituitary imaging, the problem would not be considered organic and would result from lifestyle factors, such as weight gain, depression, and sleep apnea (Alquraini & Auchus, 2018). Additional contributors to low testosterone are pictured in figure 8. However, physicians may prescribe T-therapy regardless, which many may consider being unfair, while at the same time, it may or may not be considered within a proper scope of medical practice. The medical exemption may never be resolved since it would also be unfair to exclude those who genuinely have a medical condition.

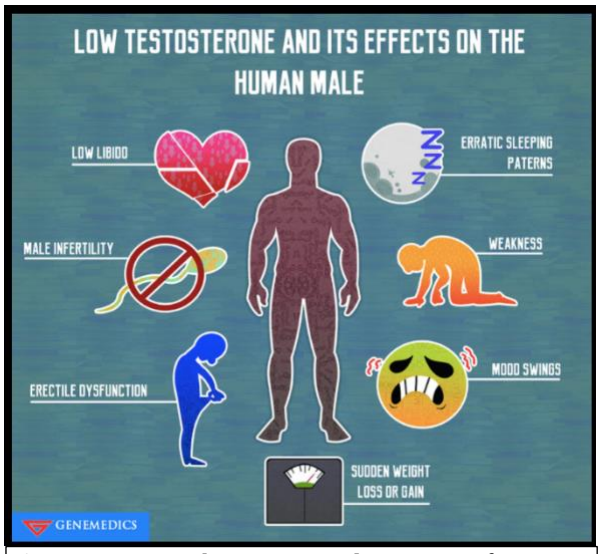

Figure 8. Figure showing typical symptoms for men who suffer from hypogonadism and low testosterone levels. Taken from Genemedics Health Institute.

False Positives

As with any scientific process or method, it is assumed that no process is perfect. False positives/false negatives can still occur within drug testing, creating a gray area for

examiners. A.H. Wu (1995) states gas chromatography/mass spectrometry as “the definitive procedure for confirming positive immunoassay screening results of urine for drugs of abuse,” even though this is the gold standard in testing, there still can be mistakes (Wu, 1995). A false positive occurs when a test is conducted, and the results come back positive, or a substance is detected when in reality, the athlete does not have the drug in their system. The opposite is true of false negatives, where an athlete tests negative for a drug when in reality, they have the drug in their system (Forstmeier, Wagenmakers, & Parker, 2016). These anomalies can make it difficult for examiners to state with confidence that they trust the results they receive. A false negative can result when there are interfering drugs present at a high concentration. This highly concentrated drug competes for the receptor and can produce results for the steroid. This interfering drug may co-elute with the target drug, affect the results, and produce a false negative (Wu, 1995). These can be more difficult to detect in routine testing conditions, as negative results generally do not result in further testing by the examiners unless disputed.

If a false positive is suspected, WADA requires double tests to be conducted on positive samples used from the primary sample (the athlete’s original sample is split in two, and the second sample is used when confirmatory testing is needed) (WADA, 2004). False positives can occur if two substances are optical isomers and produce the same molecular-weight mass fragment ions, which may be indistinguishable by the CG/MS (Wu, 1995). Lastly, there are certain products and foods that may alter the results of a drug test, with a common example of eating poppy seeds can skew a test for opiates or meats from livestock that were given certain drugs can alter the results of an anabolic steroid test (Wu, 1995).

According to the International Standards for Laboratories, a second sample will be tested if a false positive is received by WADA. If confirmed to be a false positive, the laboratory is required to stop testing for the given class of drug and method that was used until the process is examined further (WADA, 2004). If any samples were analyzed before the false positive, they would need to be re-analyzed, and the results of the analysis will need to be reported to WADA within 24 hours. If a false negative is found, the laboratory is required to inspect the root cause, and some samples that were analyzed prior will be re-analyzed to assess the risk of other samples being inaccurately labeled as well (WADA, 2004).

Interestingly, there are many recent examples of both false positives and false negatives in the news. The UFC bantamweight fighter Rob Font was accused of using prohibited drugs in a recent match after a drug test detected 4-chloropenoxyacetic acid, prohibited by the USADA (Raimondi, 2021). The article continued to say WADA required the fighter to take a urinalysis, which came back positive. Font’s manager sent in documents of recent foods eaten to the USADA in hopes of a reexamination. As mentioned, other substances like food and products can contain traces of prohibited substances. Upon examination, the shampoo and lotion that fighter used the week before the fight had threshold levels of 4-CPA, which resulted in his failing the test. Based on these findings, the decision was reversed, and it was declared that a false positive had been found, and Font was cleared of any accusation (Raimondi, 2021).

Another example is a recent instance from Olympic runner Shelby Houlihan, who failed a preliminary drug test for nandrolone (a synthetic, anabolic steroid), claiming this to be a false positive (Martin & Silverman, 2021). The news article states that the runner claimed she did not take any substances and logged her food from the week before the test. The only item on the list that stood out for her was a pork burrito she consumed ten hours before the test from a food truck near her home. Houlihan stated from her research

that some pigs produce nandrolone naturally, and pig meat can have elevated levels. Synthetic nandrolone is used for testosterone replacement therapy and helps to increase nitrogen retention and gain muscle mass with little fat, a desirable composition for athletes (Martin & Silverman, 2021). WADA informed the athlete of her violation, along with a four-year ban from competition, including the upcoming Olympics. Houlihan conducted a hair test and a polygraph test, which she claims WADA confirmed not having a buildup of nandrolone in her body. She also submitted her explanations in hopes of getting a reversal. The official Court of Arbitration did not accept the statements and kept the ban in place (Martin & Silverman, 2021).

Designer Steroids

Designer steroids are steroids that are created to resemble endogenous and known compounds. However, they are different enough from the original compound to be not matched or detected during a drug test (Alquraini & Auchus, 2018). Overall, the goal is to encompass the desired effect of the chosen steroid but make it undetectable using current methods, so official laboratories cannot match it to already known and documented substances. Usually, these substances are created in private laboratories in hopes of selling them to athletes. Since these are kept secret, there is an element of danger with ingesting these compounds as they do not go through official safety or efficacy tests, so the athlete or user must consider whether the risk is worth the reward (Yuan & Forman, 2005). Until someone comes forward and presents samples to authorities to determine the chemical structure, it can be impossible for detection methods to pick up the structure (Yuan & Forman, 2005). Not having the chemical structure is a significant issue for anti-doping authorities since if the structure is not known, how will tests be used for detection? This outlines one of the major problems faced with steroid usage; how can these illicit steroids be detected without knowing the chemical structure?

New Developments

Some innovations and new ways of thinking could help close the gap of detecting unknown substances. As mentioned, without knowing the chemical structure of a substance, it poses an immense challenge for regulators and scientists to know what to look for and adequately detect it in a test. In order to solve this problem, it seems reasonable to think one way to counteract this is to create a test that can identify substances, even though the specific substance is not known.

Currently, athletes can evade test detection by taking designer steroids that are not currently known to authorities. The current approach for testing is CG/MS or ELISA screenings to detect steroids with the methods mentioned earlier. (ELISA tests are enzyme-linked immunosorbent assays, which measure antibodies, proteins, or glycoproteins in the blood). Therefore, laboratories have great demand to create these designer steroids, as athletes are willing to consume them to pass the testing (Yuan & Forman, 2005). These laboratories are successful due to high-throughput AR-ligand screens and additional literature on anabolic steroids, which helps them to create better and more effective steroids (Yuan & Forman, 2005).

Additionally, surveys have shown that athletes are prone to use more than one substance at a time, referred to as polypharmacy or stacking. Frequently, athletes rotate on and off steroids for periods, known as cycling. This stacking introduces even more issues like the safety of these substances is not at the forefront, along with unknown drug interactions and side effects (Yuan & Forman, 2005). This raises the question of whether or not new or old tests can detect more than one substance at a time when an athlete is abusing multiple steroids. The most common

methods that are being continuously developed for new detection are gas chromatography and liquid chromatography attached to various mass spectrometry instruments to be sensitive enough to detect these substances and differentiate between endogenous and exogenous steroids (Yuan & Forman, 2005). Researchers are constantly trying to improve procedures and increase the accuracy of testing by developing these new processes.

One example comes from Otago University in New Zealand. A test is being developed to detect designer anabolic steroids before regulators or authorities are even aware of their existence or structure. Interestingly, it is a non-targeted test, and it does not attempt to locate any particular markers but instead follows cellular pathways and determine changes on that level (Stutchbury, 2020).

The basis of this test is that every anabolic steroid must activate a pathway and leave an impression throughout its course, so the test is based on finding those exploited areas and analyzing them (Stutchbury, 2020). Professor Alison Heather is leading this development and states that any anabolic steroid will activate an associated pathway. The test is aimed at picking up drugs that activate the anabolic pathway. It uses principles of the biological passport, with the test showing any inconsistencies in the passport. This team hopes this testing will become adopted in the near future, which would be a great achievement in the anti-doping community if it proves to be successful (Stutchbury, 2020).

Another innovation comes from a team at the Florida Institute of Technology, where researchers are experimenting with ion mobility-mass spectrometry to help detect current and unknown substance usage. This assay would help distinguish between exogenous and endogenous steroids and help determine the new structure of these unknown substances, two issues that are paramount in the race against doping (American Chemical Society, 2021). Instead of using the standard tandem mass spectrometry and gas/liquid chromatography (which break up molecules in the sample and can be difficult to determine differences between isomers), the team uses the separation technique with ion mobility-mass spectrometry, which helps to distinguish some of those differences (American Chemical Society, 2021). This process is outlined in figure 9.

Additionally, they had even more success with the process when the sample was modified before analysis by exposing them to other compounds, such as ozone or acetone with UV light. This process has helped improve isomer distinctions. They have tested this process with nearly half of the prohibited items on the WADA list successfully in characterization and identification. They are developing computational modeling and machine learning to predict unknown substances, which is a significant roadblock in the current anti-doping world (American Chemical Society, 2021). The downside of these processes is the cost of the ion mobility instruments. However, the assays are quick, simple, and inexpensive, and if successful, they can gain support from major anti-doping agencies.

Conclusion

One of the most important concepts relating to understanding drug testing for anabolic steroids is accepting that there is substantial variability in almost every step of the process, from ingesting the drug, to testing for it, to creating new methods to detect it. This variability is responsible for the challenges that researchers face in creating new tests and contributes to the difficulty that exists in developing new technologies and strategies. As discussed, there is variability with dosing, administration, type of substance used, the test used, masking substances being used, designer steroids being developed, and other changes that lead to unreliable test results like the use of masking agents, and results like false

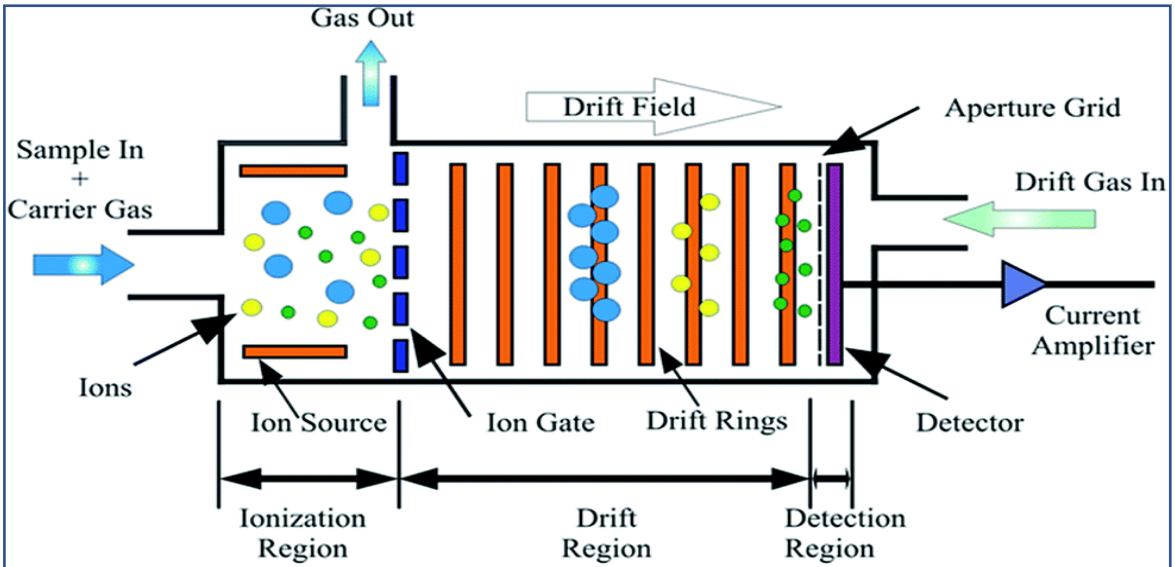

Figure 9. Diagram showing process of Ion Mobility-Mass Spectrometry as a separation technique for detecting unknown compounds during anabolic and androgenic steroid testing. Taken from Cumeras, Figueras, Davis, Baumbach, & Gracia, 2015.

positives and false negatives. Without aiming to limit some of the variability that exists when drug testing, the proposed solutions that arise may not ever be truly reliable.

In all, many components ensure that doping violations are detected and that drug test results are accurate, inexpensive, and easy to conduct. Many of the areas discussed have room for further research to be developed to make this process more seamless and reliable. Many medications double as masking agents and detecting the steroid can be difficult in these cases. Additionally, today's most current and reliable testing measures cannot detect new, designer steroids until their chemical structure is known. However, these unknowns drive researchers to work harder to end the battle on anabolic steroids. Identifying areas where more research is needed is the first step in developing solutions. It appears that analyzing the use of non-targeted tests could be the next breakthrough that researchers need. Additionally, the use of innovations in testing could also help authorities differentiate between chronic and acute usage of steroids, and these efforts are already underway. Overall, the detection issue may not be resolved at this time, but innovations are being created and will be implemented to help authorities detect and eliminate anabolic steroid use.

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