A Review of the Development of Glucocorticoid Testing Methods in Doping Control

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Abstract: Glucocorticoids (GCs) are naturally occurring hormones in the human body, known for their anti-inflammatory and immune-regulating functions. However, they have also been misused as performance-enhancing drugs in sports for an extended period. This comprehensive review delves into existing literature to gain insights into the metabolic pathways and negative feedback mechanisms of glucocorticoids in living organisms. One key area of focus is the performance of GC-C-IRMS assays in identifying glucocorticoids. The review critically examines the effectiveness of these assays and sheds light on their potential impact on detecting glucocorticoid abuse in sports. Furthermore, the limitations of current GC assays are thoroughly evaluated, providing a comprehensive understanding of the challenges associated with accurately detecting glucocorticoids. In addition to the present shortcomings, the review also explores future trends in the detection of glucocorticoids. It offers a forward-looking analysis of potential advancements in assay techniques and other innovative approaches that could enhance the detection capabilities in this field. By addressing the current limitations and outlining future possibilities, this review contributes to the ongoing discussion on mitigating the misuse of glucocorticoids in sports through improved detection methods.

Keywords: Glucocorticoids; Detection Techniques; Doping Detection; Gas-phase Combustion Isotope Mass Spectrometry

1. Introduction

Glucocorticoids (GC) are steroid hormones secreted by the adrenal cortex, with functions such as maintaining metabolic homeostasis, anti-inflammatory, and immune suppression [1]. The secretion of GC is regulated by the hypothalamic-pituitary-adrenal axis (HPA axis). Corticotropin-releasing hormone (CRH) secreted by the hypothalamus enters the anterior pituitary, promoting the secretion of adrenocorticotropic hormone (ACTH), which in turn stimulates the secretion of cortisol. Conversely, the increase in blood concentration of GC can inhibit the secretion of CRH and ACTH by the hypothalamus and anterior pituitary, thereby reducing the secretion of GC. The increase in ACTH content also inhibits the secretion of CRH by the hypothalamus, which is a negative feedback regulation process that ensures the balance of GC levels in the body [1]. The local level of GC is controlled by 11β-hydroxysteroid dehydrogenase (11β-HSD).

GC is one of the most widely used and effective drug categories in clinical practice, with anti-inflammatory and immunosuppressive properties that can be used for local and systemic treatment. Oral GCs are usually the preferred treatment drug for certain infectious diseases. The earliest exogenous GC used was cortisone, which was used to treat rheumatism [2]; Dexamethasone and prednisolone are widely used as anti-inflammatory and immunosuppressive agents. High dose or long-term use of GC will cause some damage to the health of the body, which is prone to bacterial fungal infection, steroid diabetes, osteoporosis [3], hyperglycemia, muscle atrophy, Cushing's syndrome and other adverse reactions. Meanwhile, the secretion of GC is beneficial for maintaining the body's homeostasis and maintaining the stability of the internal

environment, thus maintaining normal physiological functions. The administration routes of GC have also greatly developed, including application, inhalation, oral administration, and injection.

GC administration for clinical treatment is usually performed in the early morning. The half-life of naturally secreted GC in the human body is relatively short, and the secretion of endogenous GC in the body under stress conditions can surge to about 10 times the usual level [1]. At the same time, studies have shown that artificial GC synthesized by chemical methods has a relatively long half-life, which to some extent exacerbates the use of such banned substances by athletes.

2. GC and Sports

2.1 The Utilize History of GC in Sports

Since the 1960s, athletes have begun to use GC as a stimulant, which can help enhance oxidative metabolism, increase exercise load, relieve physical pain, accelerate recovery from exercise fatigue, and improve athletic performance. Short-term intake of GC can help store energy [4] and improve attention and endurance. In 1986, GC drugs were included in the "Prohibited List" as a prohibited substance in category S9. WADA prohibits athletes from using GC through oral, intravenous, intramuscular injection, or rectal routes. However, there are situations where GC drugs must be used for sports therapy. WADA is developing the range of metabolite concentrations in urine after local injection of GC at legitimate doses, aiming to distinguish between the use of GC for sports therapy purposes and the use of sports stimulants [5]. By detecting metabolites in urine and establishing threshold concentrations, it is possible to detect and monitor changes in GC concentration during exercise [6].

2.2 The Impact of GC in Sports

During exercise, cortisol increases the utilization rate of metabolic substrates and regulates energy balance and metabolism in skeletal muscle. Studies have shown that high-dose oral GC taken by athletes for seven days can improve maximum exercise performance. A study by Collomp K et al. demonstrated that continuous administration of prednisolone for seven days improved the endurance performance of male athletes. WADA has conducted research on the effects of GC on muscle function, exercise performance, and health, and found that there was no difference in exercise performance between the two groups of subjects who were observed under resting conditions and high-intensity exercise conditions after seven days of prednisolone administration. Therefore, the relationship between short-term systemic use of GC and improved exercise performance requires further scientific research to confirm.

3. The Testing Method of GC

3.1 Common Testing Matrices

Urine is an ideal choice for the analysis of drug metabolites. Urine sampling is convenient, cost-effective, and non-invasive, and most of the samples collected for doping tests are urine samples. However, there are some disadvantages in using urine samples for testing. The detection window for metabolites in urine is relatively short, ranging from 2 to 6 days. GC exists in urine as a large molecule, and enzymatic hydrolysis and extraction are required during preprocessing, which can increase the detection time and cost. The use of diuretics can dilute urine and reduce the concentration of target analytes in urine, making it difficult for doping tests. Urine collection needs to consider the secretion and metabolic patterns of the substances being tested, as well as the metabolic differences between individual athletes. Through dry urine spot sampling (DUS) and volume absorptive microsampling (VAMS) [7], cortisol, hydrocortisone, dexamethasone, methylprednisolone, and fludrocortisone can be quantified in 30uL of urine, with analyte loss less than 15% after three months of storage, meeting the criteria for analytical performance validation (Figure 1).

Figure 1. Urine Sample Collection Device

Whole blood sampling requires venous puncture, which is a more complex collection

method and has higher requirements for the sampling environment and personnel. Transportation and storage are difficult and costly [8]. WADA recommends the use of dried blood spot, which is similar to dried urine spot sampling (Figure 2). This method only requires a small amount of blood from the fingertip, offering advantages such as simple collection, convenient storage, and small storage space. It has great potential for future detection applications [9]. The concentration of substances in urine and blood fluctuates daily, and the metabolic rate is relatively fast, resulting in a relatively short detection window. Currently, research is ongoing to explore the use of more stable matrices as alternatives to blood and urine testing.

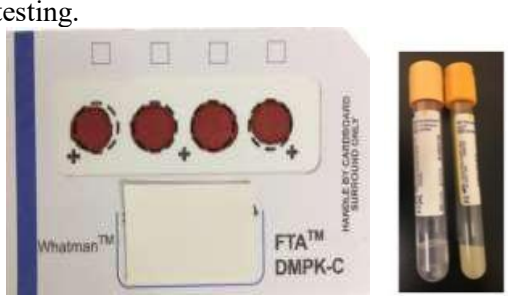

Figure 2. Blood Sample Collection Device

Hair can provide information about substances that the organism has been exposed to over a long period of time. Analysis of blood, urine, and saliva can reveal short-term drug abuse, while hair can provide information about long-term exposure and has the advantages of being non-invasive, easy to collect, and store. Methods have been established to test GC in the hair of racehorses, and the results are specific.

Breath exhalation (EB) has the potential to be used as a matrix for doping control. Using LC-ESI-MS/MS, prednisolone can be detected in half of the samples collected two hours after oral administration of 10mg of prednisolone. Compared to urine collection, it is simpler and easier to operate and collect.

Currently, there are studies exploring the potential of sweat as an alternative matrix to urine. To better achieve detection, researchers are conducting more scientific investigations into alternative testing matrices.

3.2 Advancements in Detection Technology

3.2.1 Gas chromatography-mass spectrometry

Analysis of urine samples from healthy volunteers before and after the use of dexamethasone using GC-MS revealed a decrease in 5β-glucocorticoid reductase activity [10]. A 24-hour urinary steroid metabolome analysis was conducted using GC-MS on 35 young adults with maturity-onset diabetes of the young, examining 5α- and 5β-glucocorticoid reductase activities [11].

GC-MS analysis of urine samples from 85 obese children for the metabolomic characteristics of 31 steroids provided a new method for understanding and diagnosing pediatric fatty liver disease [12]. GC-MS was also used to measure cortisol excretion rates in 36 preterm infants with low birth weight. Analysis of urine samples collected over five years from breast cancer using GC-MS revealed a positive correlation between glucocorticoid levels in urine and the risk of breast cancer development.

A method combining GC-MS/MS-dMRM with SPE and MAD was developed to determine 20 endogenous steroids in human plasma samples. This method provided sufficient detection sensitivity and reliable quantitative results, revealing potential biomarkers for the diagnosis of gastric cancer.

GC-MS demonstrates good sensitivity and robust results in the detection of glucocorticoids. However, the pretreatment process requires derivatization, which is cumbersome, time-consuming, and prone to errors. LC-MS/MS technology is easy to operate and can detect a wide range of substances with accurate and sensitive results. LC-MS/MS has been successfully applied to the detection of steroids and their metabolites in different matrices. However, when exogenous doping agents have similar structures to endogenous substances, gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) is required for discrimination.

3.2.2 Liquid chromatography-tandem mass spectrometry

The LC-MS/MS method for detecting and quantifying the content of cortisol in adipose tissue has demonstrated good accuracy and precision, making it suitable for measuring endogenous steroid concentrations in human plasma.

A high-throughput, rapid, and sensitive LC-MS quantitative analysis method for GCs in serum has been developed. This method

performs well when analyzing serum samples from patients with Cushing's syndrome.

Auriola et al. established an LC-MS/MS method capable of detecting 23 types of steroids in 150 µl of plasma, serum, and prostate tissue homogenates in a single assay. This method demonstrated good specificity during validation.

The LC-MS method has been used to detect the metabolic concentration of tazarotene cream in skin tissue at multiple time points, showing sensitive detection within the concentration range of 2-200 ug/mL.

A UPLC-MS/MS method has been developed for the quantitative analysis of free and conjugated steroids in 23 types of urine samples from healthy individuals and patients with CAH, establishing concentration ranges and steroid profiles. This method also focuses on the potential of tetrahydro metabolites as clinical biomarkers. Twelve healthy volunteers were administered 4 mg of triamcinolone acetonide, and 24-hour urine samples were collected. Using LC-MS, it was determined that 27.76% of the dose was excreted through urine within 24 hours. A method for the simultaneous quantitation of melatonin, cortisol, and corticosterone in human hair has been developed. Cortisol in hair is an ideal biomarker for monitoring sleep status. Additionally, an LC-MS/MS method for determining cortisol and corticosterone in 20µL saliva samples has been established.

Using liquid chromatography-high-resolution mass spectrometry (HRMS, Orbitrap), two new GCs named betamethasone dibutyrate and betamethasone tributyrate were discovered and reported for the first time. Their mass spectrometry profiles were also provided for drug detection reference [13].

LC-IM-MS (liquid chromatography-ion mobility mass spectrometry) offers a robust, multidimensional separation and analysis technique for 16 GCs, showing promise as a clinical detection method for complex biological compounds [14].

3.2.3 Gas chromatography-combustion-isotope ratio mass spectrometry

Isotope ratio mass spectrometry (IRMS) can be traced back to the origin of a substance. The external intake of endogenous steroid doping agents requires confirmation using IRMS. GC-C-IRMS is a reliable method for determining whether an athlete has taken exogenous substances. If there is a significant difference in the carbon isotope ratio between the athlete's metabolites and endogenous reference compounds, it is determined that the athlete has indeed ingested exogenous substances. GC-C-IRMS has high precision, but before IRMS detection, the samples need to be purified meticulously, which is a cumbersome and time-consuming process with low analytical throughput and relatively high detection costs [15].

Eight drugs containing prednisone and prednisolone on the market were analyzed using the GC-C-IRMS method. The average $\delta^{13}C$ (‰) value of the eight drugs in 25 mL urine samples was -28.96±0.39‰, which was well distinguished from the average $\delta^{13}C$ value of endogenous steroids in urine.

A GC-C-IRMS method was used to analyze 22 pharmaceutical preparations containing prednisone and prednisolone available on the market. The $\delta^{13}C$ values in 36-hour urine samples showed significant differences from endogenous glucocorticoids. The study also investigated the potential masking effect on IRMS results when multiple GCs are administered simultaneously for combined therapy in a single individual [23].

3.2.4 Other methods

Development of Liquid Phase Detection Methods. To address the challenges in GC testing, a novel column—MOF-74—was synthesized and utilized, which exhibits hydrophilic and reversed-phase mixed-mode properties. Compared to commonly used C8 columns or silica columns, MOF-74 exhibits superior high-throughput and rapid separation capabilities. Experimental verification has shown that the developed column can successfully detect and separate illegally added GCs in facial creams.

Wang Xuemei and her team synthesized a novel ZIF-8-GOS adsorbent material and combined it with liquid chromatography to analyze the feasibility of steroid doping agent detection. The data presented in the article indicates that this material exhibits satisfactory recovery rates for estrone (E1), estriol (E3), diethylstilbestrol (DES), nandrolone (NDL), and testosterone (TTR), making it suitable for use in dairy product testing.

ESI-MS/MS spectrometry (MS) was used to

determine differences in steroid concentrations in feces. GCs in feces have already been digested and decomposed by the body, which can eliminate the need for enzymatic hydrolysis during preprocessing.

4 Conclusion

The use of GCs in enhancing athletic performance remains controversial due to limited relevant research and the complexity of their mechanism of action. GCs in the human body have a complex secretory feedback mechanism, and their beneficial effects on athletic performance are not guaranteed after administration. When athletes take exogenous stimulants, it is difficult to distinguish the metabolites of endogenous and exogenous GCs. Therefore, the development of rapid and accurate quantitative methods for detecting endogenous GCs is one of the current research hotspots.

To detect and analyze substances on the WADA prohibited list, two MS-based methods are preferred, making them considered as reference methods. Currently, the testing of GCs mainly focuses on qualitative and quantitative detection, but it is difficult to distinguish between endogenous production and exogenous intake through testing, indicating limitations in current methods. However, IRMS has a low throughput. Future plans aim to develop a high-throughput, sensitive, rapid, widely applicable, low detection limit, and easy-to-operate IRMS method.

To address the continuously evolving illegal glucocorticoid doping agents, researchers will further update initial screening and confirmation strategies in the future. Additionally, optimizing pretreatment steps and conducting preliminary experiments will improve sample stability and maximize the reliability of results.

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