Research on the Applications of AICAR in Clinical Medicine and Anti-Doping

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Abstract. Objectives: The objective of this review is to examine the role of AICAR as an AMP-activated protein kinase (AMPK) activator and its potential applications in clinical conditions, as well as its significance in anti-doping research. Methods: This review analyzes the current literature on AICAR, focusing on its mechanisms of action as a cell-permeable AMPK activator and its impact on cellular energy homeostasis. Furthermore, it explores the extensive research regarding AICAR's potential applications in cardiovascular diseases, diabetes, organ transplantation, neurodegenerative diseases, and cancer, as well as its implications for athletic performance and muscle glucose uptake. Results: AICAR has emerged as a crucial regulator of cellular energy homeostasis through its activation of AMPK, with implications for various clinical conditions. Additionally, its potential to enhance athletic performance and stimulate muscle glucose uptake has garnered considerable attention in the realm of anti-doping research. Conclusions: This review highlights the multifaceted potential of AICAR in clinical applications and anti-doping research, emphasizing its role as an AMPK activator and its impact on cellular energy homeostasis. The findings underscore the significance of ongoing research and development of AICAR in medicine and anti-doping efforts.

Keywords: AICAR, AMPK activators, metabolic regulators, doping testing.

1. Introduction

Acadesine (5-amino-1-β-D-ribofuranosyl-1H-imidazole-4-carboxamide, AICAR) (Fig. 1) is an endogenous adenosine monophosphate-activated protein kinase (AMPK) activator present in various mammals, including humans [1]. It stimulates fatty acid oxidation and promotes muscle remodeling [2-6], improving hyperglycemia in diabetic patients and reducing obesity and inflammation [7-9]. It also plays a significant role in exercise metabolism [10-12]. AICAR can reprogram atrophic muscle fibers [13], preventing them from experiencing fatigue even after prolonged work periods [14].

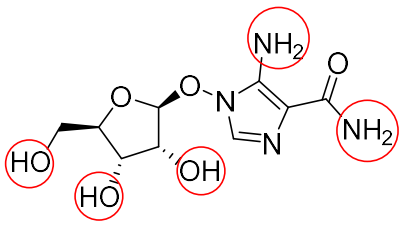

Fig. 1 Chemical structure and active site of AICAR

AICAR, as a pharmacological AMPK activator, has been investigated for the treatment of acute lymphoblastic leukemia. It is an adenosine regulator developed by PeriCor Therapeutics. Due to its potential as a first-in-class drug for preventing reperfusion injury during CABG surgery, the drug was licensed to Schering-Plough for Phase III trials in 2007. However, after failing to demonstrate efficacy, the trials were terminated in 2010 (http://www.sec.gov). Given that AICAR is the most favored AMPK activator, it serves as an intermediate metabolite in purine synthesis and is an endogenous substance. It activates adenosine monophosphate-activated protein kinase (AMPK),

thereby promoting cellular oxidative metabolism and mitochondrial biogenesis. Upon activation by adenosine kinase, AICAR rapidly generates ZMP, further activating AMPK. Additionally, AICAR inhibits cancer cell growth by activating the upstream kinase LKB1 of AMPK [15]. Doxorubicin is a commonly used drug for cancer treatment but can be cardiotoxic. AICAR can ameliorate purine nucleotide synthesis in the heart and skeletal muscle, effectively mitigating the side effects associated with doxorubicin treatment. Over the past decade, various applications of AICAR have been tested, primarily focusing on metabolic disorders, sudden death induction, diabetes, cancer, and other pathologies related to muscle atrophy. In this process, researchers discovered that most diseases related to purine metabolism cause AICAR to accumulate in patients' cells. [16-20](Fig. 2).

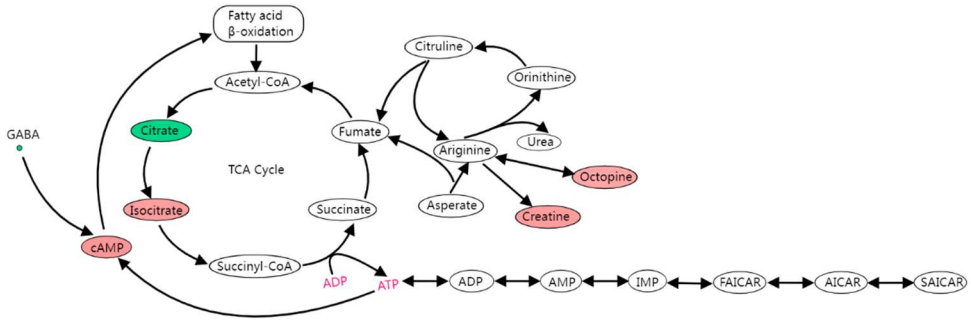

Fig. 2 Chemical structure and active site of AICAR

2. The Role of AICAR in Disease Treatment and Prevention

AMPK initiates catabolic pathways, such as glycolysis and fatty acid β-oxidation, facilitating the generation of ATP. Simultaneously, it inhibits ATP-consuming processes such as those that promote biosynthesis, cell growth, and proliferation. and proliferation. AMPK comprises three subunits: the α subunit acts as the catalytic component, the β subunit features a glycogen-sensitive domain, and the γ subunit possesses two regulatory sites that bind to activating nucleotide AMP and inhibitory nucleotide ATP, respectively.

It plays a crucial role in maintaining overall energy balance. Meanwhile, AICAR plays a critical role in glucose metabolism, cancer treatment, and post-organ transplant immunosuppression (Fig. 3).

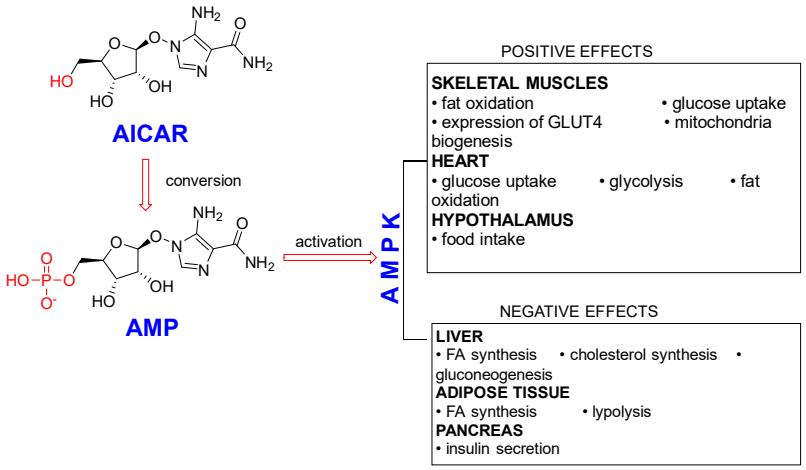

Fig. 3 Effects of AICAR activating AMPK

2.1 Application in Breast Cancer Treatment

Breast cancer ranks among the most prevalent malignancies affecting women on a global scale. Despite various preventive measures, it still causes numerous deaths annually, ranking second only to lung cancer in terms of mortality among all cancer diseases. In February 2021, experts from the

World Health Organization stated that breast cancer had become the most common cancer globally among those diagnosed in 2020. Currently, the treatment approach typically involves surgical removal of tumor tissue along with chemotherapy [21].

Cancer cells are characterized by metabolic alterations, wherein under aerobic conditions, they produce large amounts of lactate, inhibiting mitochondrial oxidation, enhancing glycolysis, and increasing the flux of pentose phosphate pathway to support rapid cell growth. This phenomenon is known as the Warburg effect [22] and can also be understood as mitochondrial dysfunction. The indispensable role of mitochondria in the sustenance of cancer cells lies in the fact that their proliferation and tumorigenic potential are significantly curtailed upon the removal of mitochondrial DNA (mtDNA). The mitochondrial genome encompasses numerous copies of mtDNA along with over 1000 nuclear-encoded genes (nDNA). Mutations in mtDNA have been identified across diverse cancer types, perturbing mitochondrial metabolism, fostering tumorigenesis, and facilitating cancer cell adaptation to fluctuating environments.

Cellular metabolism adaptation is regulated through a complex stress sensor network, where AMPK is activated by an increase in the cellular ATP/AMP ratio. The elevated ATP/AMP ratio reflects decreased cellular energy charge, prompting AMPK to induce catabolic metabolism while reducing synthetic processes [23]. The characteristic feature of breast cancer cells is the Warburg effect. therefore, attempts are made to assist chemotherapy by reversing the Warburg metabolic shift [24]. AMPK activation leads to enhanced mitochondrial oxidation and biogenesis. Reports indicate anti-Warburg metabolic reprogramming and anti-proliferative effects in lymphomas [25]. AICAR is a pharmacological AMPK activator. In 2016, Bai et al. [26] reported the use of AICAR as an activator, with MCF7 cells as the model, employing a combination therapy of AICAR and MTX. Prolonged co-administration of AICAR and MTX induced AMPK activation, reduced cell proliferation, thereby enhancing mitochondrial oxidation and lowering glycolysis rates. The reversal of the Warburg effect primarily results in the inhibition of G1/S and G2/M transitions, thereby slowing down the cell cycle. Experimental data also indicate that in human breast cancer, AICAR and MTX exhibit similar cumulative anti-proliferative effects on other breast cancer cell lines, such as SKBR and 4T1 cells.

2.2 Application in Prostate Cancer Treatment.

In the male population of the United States, prostate cancer is the most common cancer and the second leading cause of cancer-related death in men [27]. Current treatment modalities for prostate cancer include surgery, hormone therapy, chemotherapy, radiation therapy, radiofrequency ablation, high-intensity focused ultrasound, cryotherapy, and cancer vaccines [28]. Over the past few decades, androgen deprivation therapy (ADT) through surgical or chemical castration has been the primary treatment approach for advanced prostate cancer [29]. However, the majority of prostate cancer patients sensitive to androgens develop resistance to ADT within 1 to 3 years, and effective therapies for recurrent prostate cancer have not yet been discovered.

In addition to the mentioned cancer treatment effects, it also includes the anti-tumor effect of AICAR agonists on triple-negative breast cancer in vitro, activation of AMPK via AICAR sensitizes prostate cancer cells to radiotherapy, the effect of thyroid cancer agonists on tumor cell secretion and induction of tumor cell migration, AICAR inhibits cancer cell growth and triggers cell type-specific anaerobic photobiogenesis, significant effects on oxidative stress and antibody activation, AICAR triggers mitochondrial apoptosis in human osteosarcoma cells via a pathway reliant on peroxisomes, and clinical evidence shows that the combined therapy of rapamycin and AICAR reduces the effectiveness of renal cancer.[30-32].

2.3 Application of AICAR in Acute Kidney Injury (AKI)

AKI denotes a rapid deterioration in kidney function, leading to reduced urine output and accumulation of nitrogenous (such as creatinine and urea) and non-nitrogenous waste products in the bloodstream. While the pathophysiology of AKI is intricate and its causes varied, mounting evidence indicates that hypotension, hypoperfusion, hypoxia, oxidative stress, and renal vasoconstriction are

among the primary mechanisms underlying its development. Currently, there are no specific pharmacological treatment methods for AKI. AMPK serves a crucial role in regulating renal ion transport, podocyte function, and renal hypertrophy induced by diabetic nephropathy. Moreover, AMPK acts as a pivotal regulatory factor in lipid and glucose metabolism.

2.4 AICAR Can Promote the survival Rate of Corneal Transplantation

Corneal transplantation stands out as the most successful form of tissue and organ transplantation. However, immune rejection represents a significant complication, often leading to graft failure following corneal transplantation. Despite the utilization of conventional immunosuppressive therapies like corticosteroids, cyclosporine A, and tacrolimus (FK506), allograft rejection persists as a significant complication, frequently culminating in transplant failure. Therefore, new methods for treating allograft rejection are still needed. AMPK, functioning as an energy sensor, exerts regulatory control over multiple facets of cellular functions, encompassing survival, metabolism, and proliferation. AICAR, serving as a pharmacological activator of AMPK, stands as the initial identified AMPK activator, proficient in augmenting AMPK phosphorylation (p-AMPK). Subsequently, it can inhibit inflammation, oxidative stress, and angiogenesis in various cell types. In recent years, there has been extensive attention to the potential role of AICAR as an inhibitor of post-transplantation immune rejection. It has been reported that in a model of corneal transplantation, AICAR can inhibit graft opacity, edema, and vascularization, thereby promoting corneal transplant survival.

2.5 AICAR Treatment's Effect on Skeletal Muscle Glycogen Metabolism

There exists compelling evidence indicating that AMPK plays a pivotal role in augmenting skeletal muscle glucose uptake during contraction. This increased glucose uptake not only contributes to ATP regeneration but also provides substrates for skeletal muscle glycogen synthesis. In order to clarify the physiological role of AMPK in skeletal muscle, multiple studies have employed the AMPK activator AICAR. Treatment with AICAR induces various adaptive changes in skeletal muscle, similar to those occurring during exercise and training periods. AICAR acute incubation in isolated rat skeletal muscle enhances the rate of glucose transport, while long-term administration of AICAR to rats in daily routine treatment (5 to 28 days) increases mitochondrial enzyme activity, expression of GLUT-4 and hexokinase II, and skeletal muscle glycogen content.

Following intraperitoneal injection of AICAR, a rapid decrease in blood glucose levels was induced. reaching a peak within 60 to 90 minutes, with a negative pattern of blood lactate response, increasing to maximal concentration at 60 minutes and remaining unchanged throughout the entire 2-hour period. We hypothesize that in vivo, AICAR facilitates the accumulation of glycogen in skeletal muscle by enhancing glucose uptake, consequently resulting in the enrichment of intracellular G-6-P. Following this, the accumulated G-6-P may function as a feedback inhibitor of glycogen phosphorylase, an activator of glycogen synthase, and a significant substrate for glycogen synthesis, or it may potentially enter the glycolytic pathway.

2.6 AICAR Can Prevent High Blood Pressure Caused by a High Saturated Fat Diet

AICAR can prevent high blood pressure caused by a high-saturated fat diet. Hypertension represents a prevalent condition that can exert a substantial impact on the overall burden of cardiovascular diseases worldwide. Imbalance in nutrient regulation signaling and impaired functioning of the asymmetric dimethylarginine (ADMA, an endogenous inhibitor of nitric oxide synthase) - nitric oxide (NO) pathway could potentially serve as mechanisms leading to hypertension. Among these, AMPK is recognized as a serine/threonine protein kinase that can function as a central hub. AMPK is formed by a catalytic α subunit and a dimeric β and γ regulatory subunit. [42] Research results have shown that AMPK is involved in BP regulation and renal physiological function. In addition, reports show that AMPK exhibits strong antioxidant properties and the ability to regulate nitric oxide production. [43] It has been reported that a high-fat diet can reduce the activity

of AMPK, and maternal metformin (an indirect AMPK activator) can protect adult offspring from HFD-induced hypertension, and AICAR can also lower hypertension.

3. AICAR in Anti-Doping Research

AICAR acts as a principal activator and regulator involved in metabolic processes in vivo (Fig. 4). AMPK serves as a critical energy balance sensor, regulating cellular metabolism in response to external stimuli. It plays a pivotal role as a regulator of cellular metabolism, orchestrating adaptive responses to maintain energy homeostasis. AMPK is a widely expressed heterotrimeric kinase, serves as a highly conserved and ultra-sensitive energy sensor. It plays a crucial role in regulating energy production and expenditure pathways in animals. Additionally, adenylosuccinate is an intermediate in the interconversion of purine nucleotides, inosine monophosphate (IMP), and adenosine monophosphate (AMP). Adenylosuccinate synthase catalyzes the reaction by adding aspartate to IMP and requires the input of energy in the form of a phosphoryl group from guanosine triphosphate (GTP).

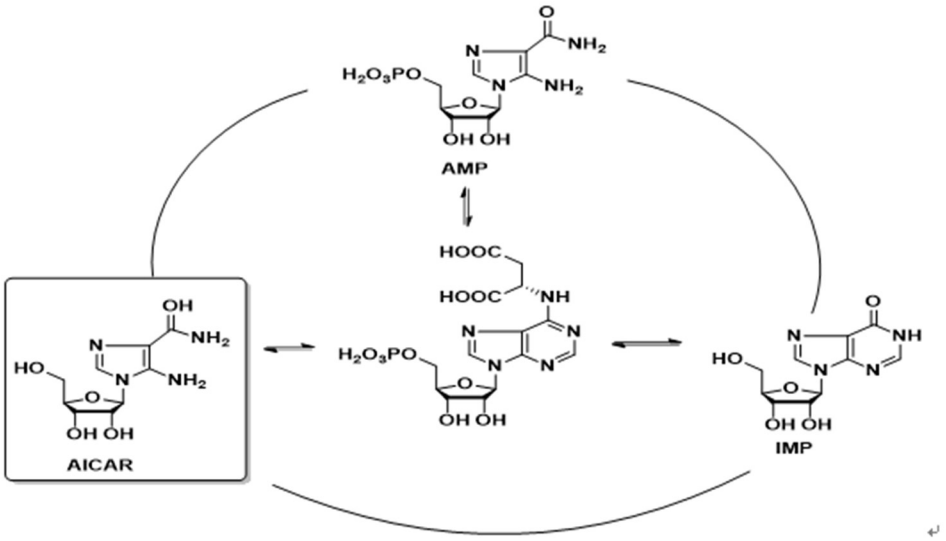

Fig. 4 AICAR involvement in metabolic pathways

3.1 The Impact of AICAR on Enhancing Endurance

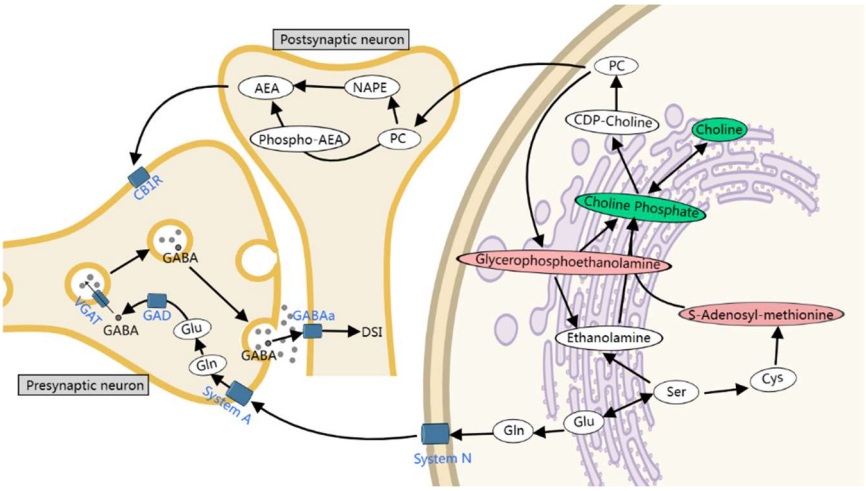

Fig. 5 AICAR involvement in metabolic pathways

Research has shown that injection of AICAR enhances endurance performance in sedentary or untrained mice, leading to the term "exercise pill" [50] (Fig. 5). Studies indicate that approximately 21-25% of breast and colon cancers, 27% of diabetes, and 30% of ischemic heart disease

developments in humans are associated with lack of exercise, and sedentary individuals have a life expectancy 8-10 years shorter than those who exercise regularly. The positive effects of the "exercise pill" on endurance primarily stem from studies on mice, and there is no such data available for healthy humans [51]. Although AICAR can significantly enhance endurance in sedentary individuals, it cannot be denied that it may cause unpredictable harm to the body. However, research has shown that AICAR alleviates acute lung injury by phosphorylating AMPK and upregulating heme oxygenase-1 [52-53]. Nevertheless, athletes still use this drug to improve their performance in numerous major sporting events [54-56].

Since January 2009, the World Anti-Doping Agency (WADA) has prohibited the use of AICAR in sports. Initially classified as a gene doping agent by WADA [57-60], it was later reclassified as a class S4 metabolic modulator based on its usage. Given that AICAR can be endogenously produced, the most effective approach to regulating the exogenous administration of such endogenous substances is to establish suitable thresholds derived from population data. Reports have suggested potential thresholds to manage AICAR abuse in humans, but as of yet, there is no formally confirmed detection standard established. Recently, the International Federation of Horseracing Authorities (IFHA), adhering to the guidelines outlined in the International Agreement on Breeding, Racing, and Wagering (IABRW), classified AICAR as a prohibited substance. This designation prohibits its use at any point during a racehorse's professional career, resulting in it being dubbed the "new EPO." Due to the lack of foundational data across various populations, WADA has not yet established a positive determination standard.

3.2 Research on the Detection Methods of AICAR

3.2.1 Liquid Chromatography Mass Spectrometry (LC-MS) Detection of AICAR

Currently, the detection method for AICAR primarily involves quantitative analysis of its content in urine [65]. In 2010, Mario Thevis [66] reported a quantitative analysis method utilizing isotope liquid chromatography-tandem mass spectrometry (LC-MS/MS) after diluting native urine under mild conditions. The results showed that the average concentration of AICAR in the samples was 2186 ng/mL, with a standard deviation of 1655 ng/mL. The study found variations in AICAR concentration in urine based on gender, type of exercise, and timing of sample collection (pre- or post-competition). Since AICAR naturally occurs in human blood and urine, it cannot be identified for abuse through direct qualitative analysis. After entering the human circulation, AICAR are immediately incorporated into red blood cells (RBCs) and converted into the corresponding ribonucleotides (5'-monophosphate esters) form. In 2011, W. Schänzer reported a novel multi-target method based on liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), which allows for the screening of various prohibited substances by directly injecting urine specimens. This method employs highly sensitive next-generation tandem mass spectrometers, capable of detecting multiple stimulants at concentrations below the required threshold levels. Additionally, the method also explored the detection of a more challenging new target compound, AICAR metabolites. Analysis of post-administration samples against conventional doping control samples demonstrated the applicability of this method in doping testing. This simple and reliable approach combines different screening procedures to form a high-throughput detection method, thus improving laboratory efficiency. In 2013, Mario Thevis reported the determination of AICAR-5'-monophosphate ester concentration in red blood cell (RBC) concentrates using liquid chromatography-tandem mass spectrometry (LC-MS/MS). This method completed methodological validation by investigating precision (intra-day and inter-day), linearity, recovery, accuracy (LOD/LOQ), and stability. Simultaneously, the physiological levels of AICAR-5'-monophosphate ester were determined in samples from 99 young athletes by analyzing the red blood cell (RBC) content, ranging from 10 to 500 ng/mL, with a maximum of not exceeding 921 ng/mL, and it was found that these levels remained stable over several consecutive days. In vitro incubation experiments using fresh blood samples with AICAR-5'-monophosphate ester showed a significant increase in ribonucleotide concentration from baseline to 1-10 μg/mL within 30 minutes. These results indicate

that the method can be used for stimulant detection work. In the field of LC-MS, reverse-phase liquid chromatography is the primary choice for separating prohibited substances from various categories in doping testing. In 2015, Wilhelm Schänzer proposed a novel method based on HILIC high-resolution/high-accuracy mass spectrometry for screening various polar drugs, such as AICAR. This method, which involves direct injection of diluted urine specimens, utilizes a zwitterionic HILIC analytical column in conjunction with a new generation Hybrid Quadrupole-Orbitrap® mass spectrometer. It can detect highly polar analytes without the need for any time-consuming hydrolysis or purification steps, with detection limits far below conventional detection standards. Through qualitative and quantitative analysis of AICAR, the method was validated to meet daily testing requirements. However, A promising method for the separation of hydrophilic compounds is hydrophilic interaction chromatography (HILIC). Despite the great potential and extensive advantages of HILIC in the separation of highly polar compounds, and the availability of various HILIC columns in most manufacturers' product portfolios, HILIC has not been very common in doping analysis so far.

In 2016, Terence SM Wan reported a method [70] that utilizes the combination of ultra-high performance liquid chromatography and high-resolution mass spectrometry (UHPLC-HRMS) to identify numerous prohibited substances without the need for preconcentration of samples. Traditional methods such as liquid-liquid or solid-phase extraction struggle to separate polar drugs in urine and plasma due to their low extraction efficiency, whereas this method is feasible. This method was used to detect 46 polar drugs, including AICAR, in equine urine and plasma, demonstrating that the approach of using the UHPLC-HRMS system in full-scan ESI mode to analyze diluted plasma or urine samples is suitable for qualitative analysis of samples. This simple and reliable method, when combined with other detection procedures, enhances the efficiency of doping testing in the laboratory. In 2016, Xu Youxuan reported a LC-MS/MS detection method for AICAR [71]. This method initially enzymatically hydrolyzes urine samples, followed by solid-phase extraction (SPE) using a C18 column, and finally analyzes the samples using LC-MS/MS. With this method, the endogenous AICAR concentrations were detected in 290 athletes, demonstrating that the method exhibited high sensitivity, strong selectivity, and good reproducibility, thus meeting the requirements for routine stimulant detection. Additionally, this study established a database of endogenous AICAR levels in Chinese athletes, providing reference data for WADA to determine positive criteria for stimulants.

3.2.2 Gas Chromatography Mass Spectrometry (GC-MS) Detection of AICAR

GC-MS has been applied in doping control for many years [72]. However, due to the high polarity of AICAR, GC-MS is not commonly used for its detection. In 2014, Mario Thevis reported a method where concentrated urine samples were first separated and purified by liquid chromatography, followed by derivatization of AICAR using silylation reagents, and finally determined by gas chromatography column separation. The authors tested the repeatability and stability of this method, and by analyzing urine samples from 63 male and female volunteers, demonstrated that the method met all requirements for doping control. Quantitative analysis reference values were provided, proving its direct applicability in doping testing. In 2017, Buisson.C reported [73] a novel gas-phase pretreatment method for identifying AICAR. The author optimized the derivatization steps of AICAR by testing various derivatization conditions to enhance the stability of derivatives, ultimately determining that 3-TMS was the most reproducible derivative. Additionally, the author introduced a "purification step" before the HPLC purification of samples to reduce background noise in detection. Furthermore, additional HPLC purification steps were added to the endogenous reference compound (ERC) fractions to obtain better detection results, albeit with a more cumbersome detection process. In 2019, in an article by Brian Ahrens [74], quantitative detection was conducted on a large population, resulting in the determination of the content of mannitol and AICAR in the urine of 12,377 American athletes. This provided a reference for the International Anti-Doping Agency to establish unified detection threshold standards.

4. Summary

In conclusion, our exploration of the uses of AICAR in medical and anti-doping fields has revealed its extensive potential. Through the examination of its pharmacological effects, clinical trials, and animal experiments, AICAR has demonstrated promise in various areas. In the realm of clinical medicine, AICAR, as an activator of AMPK, exhibits significant potential in addressing cardiovascular diseases, obesity, metabolic syndrome, and cancer, among other conditions. Its anti-inflammatory, antioxidant, and cell metabolism-regulating properties position AICAR as a compelling candidate for innovative therapeutic interventions. In the field of anti-doping, AICAR's capacity to mimic the effects of exercise, bolster muscle endurance, and enhance athletic performance has garnered attention. While conclusive evidence regarding AICAR's stimulant effects is currently lacking, its potential applications in athlete recovery and muscle injury repair warrant further exploration.

Despite the progress made in understanding AICAR's applications in clinical medicine and anti-doping, numerous challenges and uncertainties persist. Additional clinical trials and fundamental research are imperative to substantiate its safety and efficacy across various domains, thereby furnishing more robust scientific evidence to support its expanded utilization in clinical practice and doping regulation.

Acknowledgement

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