CHEMISTRY AND TECHNOLOGY OF MEDICINAL COMPOUNDS AND BIOLOGICALLY ACTIVE SUBSTANCES

ХИМИЯ И ТЕХНОЛОГИЯ ЛЕКАРСТВЕННЫХ ПРЕПАРАТОВ И БИОЛОГИЧЕСКИ АКТИВНЫХ СОЕДИНЕНИЙ

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REVIEW ARTICLE

Biological functions of cobalt and its toxicology and detection in anti-doping control

Irina V. Pronina[1,2,@a], Elena S. Mochalova[1], Yuliya A. Efimova[3], Pavel V. Postnikov[1,@b]

[1]National Anti-Doping Laboratory (Institute), Lomonosov Moscow State University, Moscow, 105005 Russia
[2]Institute of General Pathology and Pathophysiology, Moscow, 125315 Russia
[3]MIREA – Russian Technological University (M.V. Lomonosov Institute of Fine Chemical Technologies), Moscow, 119571 Russia
[@]Corresponding author, e-mail: [a]pronina@dopingtest.ru, [b]postnikov@dopingtest.ru

Abstract

Objectives. *Over the last decade, hematopoietic stimulants have grown increasingly popular in elite sports. This is supported by the growing number of high-profile doping scandals linked to their use. A group of these stimulants includes cobalt salts, which cause an increase in the oxygen capacity of the blood as well as a powerful stimulation of metabolic processes, resulting innoticeable competitive advantages. The use of cobalt salts is regulated according to the Prohibited List of the World Anti-Doping Agency (WADA). Currently, only a few works have been dedicated to solving the problem of detecting the abuse of cobalt salts in anti-doping control. Only a few laboratories have included cobalt salt determination in their methodological bases. The purpose of this review is to attract the attention of the scientific community to the toxicity of cobalt compounds, consequences of their intake, and pharmacokinetics, as well as the problems in their detection methods due to their widespread availability in the modern market and the growing number of abuse cases.*

Results. *The main biological functions of cobalt, cellular levels of exposure, toxicity, and symptoms of cobalt salt poisoning are presented in detail in this review article. The data from the literature on the main methods for detecting cobalt as a doping agent have been generalized and systematized. There is a major focus on the amount of cobalt in dietary supplements that could cause an athlete to test positive for cobalt when they are consumed.*

Conclusions. *After analyzing promising cobalt detection approaches and methods, it was determined that high-performance liquid chromatography in combination with inductively coupled plasma mass spectrometry has an undeniable advantage for detecting cobalt as a doping agent. The lack of explicit WADA requirements for detection methods and the lack of its obligation to determine cobalt make it tempting for unscrupulous athletes to use its salts. Therefore, anti-doping laboratories must implement the abovementioned method as soon as possible.*

Keywords: *hematopoietic stimulants, cobalt, biological functions, dietary supplements, HIF, anti-doping control, toxic effect, mass spectrometry*

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ОБЗОРНАЯ СТАТЬЯ

Биологические функции кобальта, токсикология и обнаружение в антидопинговом контроле

И.В. Пронина[1,2,@a], Е.С. Мочалова[1], Ю.А. Ефимова[3], П.В. Постников[1,@b]

[1]Национальная антидопинговая лаборатория (Институт), Московский государственный университет им. М.В. Ломоносова, Москва, 105005 Россия
[2]Научно-исследовательский институт общей патологии и патофизиологии, Москва, 125315 Россия
[3]МИРЭА – Российский технологический университет (Институт тонких химических технологий им. М.В. Ломоносова), Москва, 119571 Россия
[@]Автор для переписки, e-mail: [a]pronina@dopingtest.ru, [b]postnikov@dopingtest.ru

Аннотация

Цели. *В последнее десятилетие чрезвычайную популярность в спорте высших достижений приобрели стимуляторы кроветворения. Этот факт подтверждают и участившиеся громкие допинговые скандалы, связанные с их употреблением. Соли кобальта относятся к данному классу веществ, их использование приводит к увеличению кислородной емкости крови и к мощной стимуляции обменных процессов, что дает несомненные конкурентные преимущества. Применение солей кобальта регламентировано в соответствии с Запрещенным списком Всемирного антидопингового агентства. В настоящее время проблематике выявления злоупотреблений солями кобальта в антидопинговом контроле посвящено всего несколько работ. Лишь единичные лаборатории вводят определение солей кобальта в свою методологическую базу. Цель данного обзора состоит в том, чтобы обратить внимание научного сообщества на токсичность соединений кобальта, последствия их приема, фармакокинетику, проблематику и способы обнаружения ввиду их доступности на современном рынке и участившихся случаев злоупотребления ими.*

Результаты. *В представленном обзоре рассмотрены основные биологические функции кобальта и клеточные уровни воздействия, токсичность и симптоматика при отравлении его солями. Обобщены и систематизированы литературные данные по основным используемым методам идентификации кобальта как допингового агента. Особое внимание уделено содержанию кобальта в биологически-активных добавках, при приеме которых спортсмен может сдать положительный допинг-тест на кобальт.*

Выводы. *На основе анализа перспективных подходов и методов определения кобальта, сделан вывод о несомненном преимуществе высокоэффективной жидкостной хроматографии в сочетании с масс-спектрометрией с индуктивно-связанной плазмой для детекции кобальта как допингового агента. Отсутствие четких требований к методам идентификации со стороны ВАДА и обязательности определения кобальта, несомненно, делает привлекательным прием его солей недобросовестными спортсменами. Ввиду этого существует необходимость внедрения вышеуказанного метода в практику антидопинговых лабораторий в ближайшем будущем.*

Ключевые слова: *стимуляторы кроветворения, кобальт, БАД, HIF, антидопинговый контроль, масс-спектрометрия*

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INTRODUCTION

For many decades, cobalt(II) chloride has been used effectively to treat various types of anemia. However, cobalt and its compounds are the deadliest inorganic poisons, and they are very toxic in high doses[1]. Cobalt sulfate, e.g., is carcinogenic [1, 2] and mutagenic. Cobalt has been completely excluded from the list of modern clinically significant stimulants of erythropoiesis and its use in experiments has been limited because of the side effects of its preparations that have been discovered over time and the subsequent unambiguous recognition of it as a carcinogen. At the same time, the problem of identifying the use of cobalt as a doping agent arises from the availability of the drug on the pharmaceutical market, a convenient method of oral administration, powerful stimulation of erythropoiesis, and the fact that validated methods for determining soluble cobalt salts in human urine are usually not included in the methodological arsenal of modern anti-doping analysis. The alleged use of cobalt chloride ($CoCl_2$) as a doping agent has already been discussed several times [3–5]. In this case, anti-doping rules promote fair competition in sports and protect athletes from the harmful effects of substances, of which the danger may be underestimated. According to the 2021 World Anti-Doping Agency (WADA) Prohibited List, the use of cobalt salts by athletes is strictly regulated under Article S2 “Peptide hormones, growth factors, related substances, and mimetics,” paragraph 1.2 “hypoxia-inducible factor (HIF) activating agents.”

Biological functions of cobalt as a microelement

Cobalt, a naturally occurring trace mineral with properties similar to those of iron and nickel, induces a noticeable and stable polycythemic response

[1] Normy fiziologicheskikh potrebnostei v energii i pishchevykh veshchestvakh dlya razlichnykh grupp naseleniya Rossiiskoi Federatsii. Metodicheskie rekomendatsii (Norms of physiological needs for energy and nutrients for various groups of the population of the Russian Federation. Guidelines). Moscow: Federal’nyi tsentr gigieny i epidemiologii Rospotrebnadzora; 2009. 36 p. (in Russ).

[6, 7] through a more efficient transcription of the erythropoietin (EPO) gene. When $CoCl_2$ is taken in dosages of 120 or 150 mg/day, it causes a significant increase (up to 20%) in hematocrit and hemoglobin [7]. Cobalt chloride may soon become the most appropriate adjunct or substitute for erythropoiesis-stimulating substances, given the natural propensity of some athletes to experiment with novel, illegal, and potentially harmful doping agents and methods. Nevertheless, $CoCl_2$ administration has unsafe consequences, including toxic effects on the heart, liver, kidneys, and thyroid gland, as well as the development of oncological processes [2, 5].

The main biological function of cobalt is its presence in the vitamin B12 (cyanocobalamin) molecule, where its mass fraction is about 4%. Vitamin B12 is essential for appropriate nervous system function and is involved in the process of hematopoiesis. In humans, cobalt deficiency causes malignant (pernicious) anemia, also known as the Addison–Biermer disease. Cobalt quantification plays an important role in differentiating B12 deficiency anemia from folate deficiency, in which the concentration of cobalt in the blood is within the normal range. However, because cobalt deficiency, according to many scientists, corresponds to vitamin B12 deficiency, the quantitative determination of cobalt in the blood is more typically employed in clinical medicine to detect intoxication rather than deficiency.

To a lesser extent, cobalt is known as a coenzyme that is found in the active center of several vital enzymes in the human body, including ribonucleoside triphosphate reductase (EC 1.4.3.8), methyltransferase (EC 2.1.1.13), methylmalonyl-CoA mutase (EC 5.4.99.2), methylmalonyl-CoAcarboxyltransferase (EC 2.1.3.1), and propionyl-CoAcarboxylase (EC 6.4.1.3) [8]. Cobalt can also act as a coenzyme in some pyrophosphatases, peptidases, and arginases [9]. There is some evidence that cobalt may affect the activity of enzymes, particularly adenylate cyclase and several others [10, 11]. It has a particular effect on heme metabolism enzymes [12].

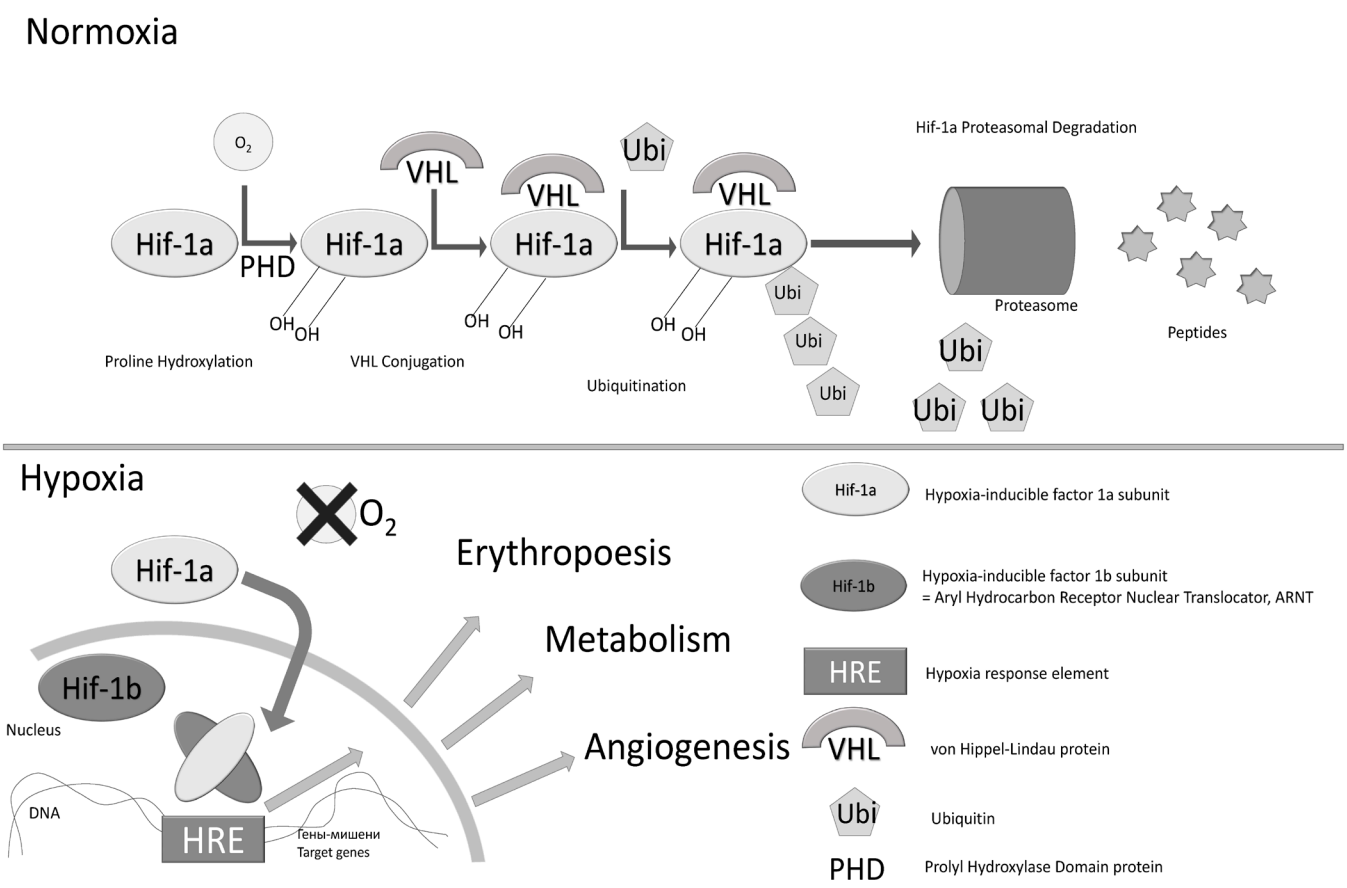
Cobalt has a variety of physiological and pathophysiological effects. There is evidence that it influences carbohydrate and lipid metabolism, thyroid gland function, and the state of the myocardium. Cobalt is on the carcinogenic agent list of the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO); however, some of its complex compounds have antitumor effects [13]. It is toxic, but because it forms strong bonds with cyan ions, it can be used as an antidote for cyanide intoxication [14]. The epileptogenic effect of cobalt has been documented. Several studies from the first half of the twentieth century have demonstrated the effect of cobalt on blood pressure and vascular tone [15, 16].

Inorganic cobalt is obtained from food. Fish and seafood, liver, kidneys, nuts, mushrooms, vegetables, and fruits all contain sufficient amounts of cobalt required for the body's daily needs. According to studies carried out on healthy people, when 1 μg to 1.2 mg of $CoCl_2$ is administered orally, 5–20% of inorganic cobalt supplied with food is absorbed in the gastrointestinal tract. Women absorb more soluble cobalt than men [17]. The half-life has not yet been established.

According to studies on rats whose drinking water had the $^{57}Co^{2+}$ isotope added to it, the Co^{2+} ion accumulates mostly in the liver, lungs, and kidneys [18], as well as in the pancreas and spleen [10, 19, 20]. In the human blood, the cobalt concentration is an average of 0.238 mg/kg, while in erythrocytes it ranges from 0.059 to 0.13 mg/kg, and in serum from 0.0055 to 0.40 mg/kg. The kidneys eliminate 86% of the excess, whereas the intestines excrete 14%. Additionally, the concentration of cobalt in the blood varies based on the season and time of day, which is linked to human nutritional characteristics.

Cellular exposure to cobalt

The presence or absence of oxygen regulates the EPO gene under the control of the HIF-1 transcription factor. It has been shown that after the signal of a decrease in the oxygen concentration in the cell environment is received, a chain of events begins in the cell, which is the key to the binding of HIF-1 to hypoxiaresponse elements (HREs), which enhance transcription of the EPO gene [21–26]. The HIF-1 is a transcription factor that belongs to the basic helix-loop-helix family, and it consists of two subunits, HIF-1a and HIF-1b [14, 26]. The HIF-1a is a major regulator of cellular and systemic oxygen homeostasis due to an increase in the activity of binding to the DNA sequence of the EPO target gene during hypoxia. Under normoxic conditions, the main mediator, HIF-1a, is rapidly cleaved by the proteasome [26] (see figure). The Fe^{2+} ion, which is reversibly bound to the active center of these metalloenzymes, is known to be a required cofactor for prolyl hydrolase activity in the proteasome [27]. Consequently, a decrease in iron availability caused by competitive substitution with Co^{2+} ions of cobalt inhibits enzyme activity [28, 29]. Hypoxia or $CoCl_2$ introduction, which mimics hypoxia, significantly inhibits HIF-1a degradation. As a result, HIF-1a binds to HIF-1b, penetrates the nuclear membrane, and activates the transcription of the EPO gene as part of the HRE [26].



The effect of HIF at normal partial pressure of oxygen is normoxia in the cell and at oxygen deficiency is hypoxia (similar to the effect of cobalt preparations).

The Hep3B and HepG2 human hepatoblastoma cell cultures proved to be appropriate models for *in vitro* examination of the hypoxia induction patterns of EPO gene expression [11]. According to the authors of the hypothesis, a hemoprotein molecule, whose conformation is dependent on the partial pressure of oxygen in the environment where EPO-producing cells are located, serves as an oxygen sensor in cells. When the partial pressure of oxygen is low, the hemoprotein molecule is converted into a deoxy conformation, which triggers a chain of molecular events that eventually result in EPO gene expression. When the partial pressure of oxygenis sufficiently high, the hemoprotein molecule is in an inactive oxy-conformation and does not stimulate the production of EPO. Cobalt chloride, in the context of this hypothesis, appears to work in a similar mechanism; the cobalt atom displaces the iron atom from the heme of the sensory molecule and takes its place, resulting in hemoprotein"locking" in an active deoxyconformation and EPO gene expression, as in hypoxia conditions.

Cobalt is a typical d-element from a chemistry viewpoint, and it has two oxidation states in compounds: +2 (in most compounds) and +3 (mainly in complexes). The characteristic chemical properties of d-elements that are reflected in biology are variable oxidation states, participation in redox reactions, the ability to form complex ions, and catalytic activity. Since the atom and ion of cobalt have similar sizes to those of other micro and macroelements, it can imitate or modify the activity of other elements in addition to providing its biological effects [29, 30–32].

The atomic and electronic structures of metalloporphyrins (MeP) complexes with oxygen ($MeP–O_2$) and water ($MeP–H_2O$) molecules in the presence and absence of imidazole as a second ligand (the imidazole group of histidine is the functional group closest to heme) were studied using quantum chemistry methods [33]. Researchers have evaluated and compared the affinity of iron and cobalt ions for heme under conditions that mimic normoxia ($MeP–O_2$) and hypoxia ($MeP–H_2O$). In summary, the hypothesis is that cobalt ions can displace iron ions

from hemoprotein molecules, including hemoglobin, which changes the heme conformation in the same manner that low oxygen concentrations inside the cell (hypoxia) do. This mechanism explains the universal response of various body cells to excess cobalt [34–36] and the erythropoietic effect of inorganic cobalt action on the body as a whole.

Researchers have determined the energy and characteristics of the chemical bond as well as changes in the spatial configuration of heme under hypoxia conditions and when the iron atom is replaced with a cobalt atom [36]. In complexes of iron and cobalt porphyrins with oxygen (O_2) and water (H_2O) molecules, both molecules occupy the fifth coordination position, whereas the imidazole ring of the amino acid histidine occupies the sixth coordination position. The cobalt ion in a porphyrin complex in the presence of imidazole in the sixth coordination position (imitation of the globin environment) and the addition of oxygen in the fifth coordination position (imitation of normoxia) is displaced in the same manner as the iron ion when water is added in the fifth coordination position (imitation of hypoxia)[2]. These data explain why cells interpret an excess of cobalt in the body as hypoxia and promote the activation of corresponding compensatory processes, such as the activation of EPO gene expression[3].

It is worth noting that the effect of inorganic cobalt on the body is not limited to the stimulation of erythropoiesis. To date, HIF-1 has been demonstrated to cause the expression of diverse proteins in various cells, i.e., the cells and the body universally respond to hypoxia—through the activation of HIF proteins, which are transcription factors for genes. Genes encode proteins that stimulate not only the production of new erythrocytes but also angiogenesis (this may be the basis for the carcinogenic effect of cobalt), i.e., the formation of new blood vessels [2], as well as glycolysis as a method of obtaining energy in the absence or shortage of oxygen [37–42]. The HIF-1 has been demonstrated to stimulate the expression of genes of several glycolysis enzymes [43–46] and regulate angiogenesis and vascular tone [47–49], transferrin and its receptor [50–52], and a protein that binds to insulin-like growth factors [53] under hypoxia conditions. The HIF-1 is estimated to control about 5% of the human genome and genes that regulate cell growth, division, survival, and cell motility in addition to genes that control glycolysis and angiogenesis [37] (see figure).

Some HIF-activated genes encode proteins that can enhance physical performance independent of erythropoiesis (e.g., glycolytic enzymes, glucose transporters, and angiogenic peptides). Currently, reliable data on the effect of cobalt ions on biological processes in the body, which are not associated with the synthesis of erythrocytes but capable of influencing athletic performance, has been accumulated. For ethical reasons, research on this subject is primarily conducted on animals, but the possibility of applying the results to humans is evident even to a layperson. For example, preliminary administration of $CoCl_2$ to rats at a dose of 12.5 mg/kg of body weight has been demonstrated to protect them from high-altitude pulmonary edema that occurs during hypoxia caused by being under high-altitude conditions [54]. In another study, prefeeding rats with $CoCl_2$ supplementation increased mitochondrial biogenesis, glucose uptake, and metabolism by improving aerobic cellular respiration in the skeletal muscle, which improved physical performance [55, 56]. In 2018, a group of scientists from Germany and the United States investigated the effects of low doses of cobalt on the performance of volunteers during aerobic exercises [57]. The scientists discovered that administering 5 mg of cobalt per day to the volunteers for three weeks increased their endurance and maximum strength, even though none of them were involved in professional sports.

Biologically active additives and cobalt

Professional athletes, particularly elite athletes, widely use various nutritional supplements. According to studies in recent years, competitive athletes have been increasingly encountering prohibited compounds in supplements available on the pharmaceutical market to improve training performance, performance level, and overall athletic performance. The problem is that manufacturers of dietary supplements do not always accurately indicate the composition of the product on the packaging, resulting in the detection of prohibited substances in athletes who are sincerely confident that the substances are not present during doping tests. This trend can be found everywhere

[2] Morgulis I.I. Early reaction of the mammalian organism to exposure to cobalt chloride. Cand. Thesis. Krasnoyarsk: Krasnoyarskii nauchnyi tsentr; 2006. 112 p. URL: https://www.dissercat.com/content/rannyaya-reaktsiya-organizma-mlekopitayushchego-na-vozdeistvie-khloridom-kobalta (accessed May 12, 2021) (in Russ.).

[3] Postnikov P.V. Development of a highly sensitive method for the qualitative determination of the hybrid protein erythropoietin fused with the FC-part of human immunoglobulin g (EPO-FC) in blood serum samples for the purpose of anti-doping control. Cand. Thesis. Moscow: Moskovskii tekhnologicheskii universitet; 2017. 152 p. https://www.dissercat.com/content/razrabotka-vysokochuvstvitelnoi-metodiki-kachestvennogo-opredeleniya-gibridnogo-belka-eritro (in Russ.).

[58, 59]. The sensational messages of some anti-doping agencies, including the Russian Anti-Doping Agency and National Anti-Doping Agency of the Republic of Belarus, on the importance of cautiously using the Complivit vitamin complex, which contains inorganic cobalt, are worth remembering. However, erythropoiesis-stimulating agents (e.g., EPO and HIF stabilizers) are not often mentioned as ingredients in such products. Simultaneously, mixtures of a patented and undisclosed composition (including those intended only for veterinary use) have been discovered in the personal environment of elite athletes in recent years [60].

In Geyer's works [61, 62], products that are openly advertised with legitimate properties that assist in improving athletic performance were the subject of research on doping. These mixtures, which were confiscated or purchased for testing from onlinesuppliers by the Center for Preventive Doping Research (Germany) and various anti-doping organizations with allegedly declared properties for improving hematopoiesis, were sent for biochemical analysis to detect EPO and its derivatives; low molecular weight HIF stabilizers or transition metals such as cobalt; growth hormones, which are hormones that stimulate the production of growth hormone-releasing hormones and growth hormone-releasing peptides; low molecular weight organic analytes such as anabolic agents, stimulants, and β2-agonists; and selected trace minerals, including cobalt and nickel, according to established protocols. A total of 19 products were tested using various analytical testing methods, including high-resolution gas and liquid tandem chromatography–mass spectrometry (GC–MS and LC–HRMS–MS), gel electrophoresis, and inductively coupled plasma mass spectrometry (ICP–MS), as described in a previous work [59]. The test results are presented in the table below.

It is worth noting that three of the products had similar labels and the same brand names, but their contents were significantly different. It is assumed that there were fakes, counterfeits, and/or products with different compositions but a quite similar packaging among them [62].

Analysis of the presence of inorganic cobalt in products confiscated and purchased from online suppliers (on data of the Center for Preventive Doping Research, Germany) [62]

Product No.	Advertised effect	Product composition (suggested route of administration)	Components identified in the product relevant to doping control (≥0.1 mg/mL)	Stated on the label (Yes/No)	Note
1	Erythropoiesis stimulation	Water solution (intravenously)	Cobalt (0.1 mg/mL) Nickel (7.5 mg/mL)	No No	Cyanocobalamin is detected (about 2.0 mg/mL), which is about 90 μg/mL of cobalt
2	Erythropoiesis	Water solution (intravenously)	Cobalt (4.8 mg/mL)	No	Cyanocobalamin is detected (about 1.7 mg/mL), which is about 75 μg/mL of cobalt
3	Increases oxygen supply to muscle tissue	Water solution (intravenously)	–	–	Composition is not determined
4	Counteracts fatigue	Water solution (for injection)	–	–	Composition is not determined

Table. Continued

Product No.	Advertised effect	Product composition (suggested route of administration)	Components identified in the product relevant to doping control (≥0.1 mg/mL)	Stated on the label (Yes/No)	Note
5	Anti-inflammatory properties	Gel (intravenously or intramuscularly)	–	–	Composition is not determined
6	–	Water solution	Cobalt (3.4 mg/mL)	No	–
7	–	Water solution	–	–	Composition is not determined
8	Erythropoiesis stimulation	Water Suspension (intravenously)	Cobalt (1.9 mg/mL)	No	Cyanocobalamin is detected (about 2.6 mg/mL), which is about 110 μg/mL of cobalt
9	Erythropoiesis	Water Suspension (intravenously)	Cobalt (2.2 mg/mL)	No	–
10	Erythropoiesis	Water solution (intravenously)	Cobalt (3.3 mg/mL)	No	Cyanocobalamin is detected (about 3.0 mg/mL), which is about 270 μg/mL of cobalt
11	Increased competitiveness	Water solution (intravenously)	–	–	Composition is not determined
12	Counteracts fatigue	Water Suspension (intravenously)	–	–	Composition is not determined
13	Increases oxygen transport	Water solution (intravenously)	–	–	Composition is not determined

Table. Continued

Product No.	Advertised effect	Product composition (suggested route of administration)	Components identified in the product relevant to doping control (≥0.1 mg/mL)	Stated on the label (Yes/No)	Note
14	Increases oxygen transport	Water solution (intravenously)	–	–	Composition is not determined
15	Erythropoiesis	Water Suspension (intravenously or intramuscularly	Cobalt (3.3 mg/mL)	Yes	The label indicates the presence of cobalt gluconate (2.0 mg/mL), which is about 260 μg/mL of cobalt, cyanocobalamin (0.25 mg/mL), which is about 10 μg/mL of cobalt
16	Erythropoiesis stimulation	Water Suspension (intravenously or intramuscularly	Cobalt (0.2 mg/mL)	Yes	The label indicates the presence of cobalt gluconate (0.7 mg/mL), which is about 90 μg/mL of cobalt, cyanocobalamin (0.15 mg/mL), which is about 7 μg/mL of cobal
17	Erythropoiesis	Water solution (intravenously)	Cobalt (0.1 mg/mL)	No	Cyanocobalamin is detected (about 4.0 mg/mL), which is about 175 μg/mL of cobalt
18	Erythropoiesis	Water solution (intravenously)	Cobalt (0.1 mg/mL)	No	Cyanocobalamin is detected (about 3.3 mg/mL), which is about 140 μg/mL of cobalt
19	–	Water solution in confiscated syringe	Cobalt (5.5 mg/mL)	Unknown	Cyanocobalamin is detected (about 5.3 mg/mL), which is about 230 μg/mL of cobalt

In the case of veterinary drugs, interesting studies were conducted jointly by the Hong Kong Jockey Club and the United Arab Emirates Racing Society based on the Racing Laboratory of the Hong Kong Jockey Club [63], as well as independently by the British Racing Society based on the School of Veterinary Medicine and Science of the University of Nottingham [64]. They investigated widely used vitamin preparations, some of which can be purchased without a prescription in veterinary pharmacies in Russia (for example, Hemo-15 or VAM® Injection). Laboratory analysis of blood samples from horses that received these drugs strictly according to the manufacturer's instructions and under the supervision of veterinarians revealed that the cobalt threshold was exceeded within 3–5 days after drug administration.

The results of Geyer's research [61, 62] lead us to believe that additional explanation and educational work on the usage of vitamin–mineral supplements and other biologically active supplements among athletes are necessary. The National Anti-Doping Laboratory of Moscow State University has begun research on developing methods for detecting prohibited substances not only in the blood samples of athletes but also in dietary supplements on the market.

Use of cobalt preparations in clinics and as a doping agent

Over the last ten years, advances in the understanding of the physiological regulation of the erythropoietic system have revealed new anchor points for pharmacological manipulation. The aerobic capacity of the blood correlates with the total hemoglobin mass in erythrocytes and the number of erythrocytes. Since muscle performance is directly dependent on the amount of oxygen supplied through the bloodstream, an increase in red blood cell mass results in an increase in aerobic endurance [65]. This phenomenon prompts dishonest athletes to artificially stimulate erythropoiesis. Recombinant human EPO (rhEPO) and its analogs are commonly used for these purposes, but they can be easily detected through doping tests[4] [66, 67], they are quite expensive, and their use is associated with certain difficulties (e.g., rhEPO exists only in injection forms). In terms of doping control, cobalt(II) chloride is particularly interesting because it is an inexpensive and readily available drug. Cobalt ions activate hypoxia-induced transcription factors that increase EPO expression [68].

The erythropoiesis-stimulating activity of inorganic cobalt was first discovered in the 1940s. Cobalt chloride was used to treat anemic patients from the late 1940s to the late 1970s. The medication drug was normally administered as tablets in divided doses with meals. Cobalt chloride is a reference substance for *in vivo* calibration of the rhEPO drug substance; 5 μmol of $CoCl_2$ has the same erythropoiesis-stimulating activity as one international unit of rhEPO [69].

Jefferson [70] discovered that about 50% of highland residents with excessive erythrocytosis had elevated cobalt levels. The percentage of free cobalt ions is only 5–12% of the total cobalt concentration in the blood plasma, of which the main part is associated with albumin [71]. Cobalt is mainly excreted in the urine, and a small amount is excreted in the bile via the gastrointestinal tract. Normal urinary cobalt concentrations are less than 2 μg/L in individuals who do not use any supplements or are unexposed to a cobalt-rich environment. Women have a higher cobalt concentration in urine (median: 109.7 nmol/mmol creatinine) than men (median: 38.4 nmol/mmol creatinine) when they consume $CoCl_2$. After a single intravenous injection of inorganic cobalt in adult men, 40% of cobalt is excreted from the body within the first 24 h, 70% within one week, and 80% within one month, with approximately 10% remaining after a year.

Cobalt salts are assumed to be used as an alternative to other erythropoiesis-stimulating agents (rhEPO injections) or blood doping drugs in the form of autologous blood transfusion or erythrocyte transfusion by athletes [5]. Indeed, $CoCl_2$ is readily available, inexpensive, easy to administer, and highly effective. It is available as a pill or as an ingredient in sports drinks (electrolytes, protein shakes, and energy drinks). Since the parenteral route prevents the development of unwanted gastrointestinal effects, only a few trials of intravenous or intramuscular administration of $CoCl_2$ have been conducted, but the specific toxicity of intravenous administration is not fully known. Intramuscular administration of $CoCl_2$ can be painful and cause tissue necrosis.

The potential abuse of cobalt deserves special attention from anti-doping organizations. Unscrupulous athletes and trainers who use $CoCl_2$ to improve their athletic performance ignore its cumulative toxic effects and the many side effects

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that have resulted in its medical abandonment. Although $CoCl_2$ therapy has been shown to effectively stimulate erythropoiesis in both extrarenal and renal anemia, septic infection, myeloid hypoplasia, sickle cell anemia, rheumatoid arthritis, and chronic kidney disease, the accumulation of the element resulting from long-term use makes it toxic.

Toxic effects of cobalt

Although cobalt is not a highly toxic metal, it can have undesirable effects and cause serious health problems in high concentrations. Pulmonary edema, nausea, vomiting, bleeding, and renal failure are some of the acute symptoms of cobalt poisoning. Chronic intoxication causes pulmonary pathology, allergic dermatitis, hyperkeratosis, thyroid dysfunction, cardiomyopathy and heart failure (particularly in alcoholics), and neuropathy [72]. The toxic effect of cobalt is amplified by increasing air temperatures. Dust inhalation during cobalt-doped metal processing can cause interstitial lung diseases and asthma.

The first reports on cobalt toxicity appeared in the 19th century; however, researchers focused their attention on the study of this issue when the syndrome of "beer cardiomyopathy" was discovered. In the 1960s, some breweries used cobalt salts (chloride and sulfate at concentrations of 1.2–1.5 mg/L) to stabilize foam. People who consumed more than four liters of beer a day experienced major side effects including heart problems, which might be fatal in some cases. Cardiomegaly, various types of arrhythmias, cyanosis, low cardiac output, pericardial effusion, and hypotensionare all symptoms of the syndrome. From 1964 to 1966, cases of beer-related cobalt cardiomyopathy were reported in Omaha (Nebraska, USA), Quebec (Canada), Leuven (Belgium), and Minneapolis (Minnesota, USA). The disease occurred in humans even though the cobalt dose(up to 10 mg/day) was less than the doses used to treat anemia. Cobalt has not been used for brewing since then, and it is now illegal. This also influenced the early discontinuation of its use for the treatment of anemia.

Cardiomyopathies have also been observed in hard metal workers who inhaled cobalt in concentrations exceeding 100 μg/m^3 air. Intravenous or intramuscular administration of $CoCl_2$ can also cause heart failure. For example, a 17-year-old woman with chronic kidney disease died of rapidly progressing dilated cardiomyopathy after nine months of $CoCl_2$ therapy (25 mg per day). According to the postmortem examination, the cobalt concentration in the myocardium was 8.9 μg/g (dry tissue) (norm 0.2 μg/g). Weißbecker [73, 74] observed an increase in the systolic blood pressure in all patients treated with $CoCl_2$. This is consistent with the findings, which revealed that treatment with recombinant epoetin may also be associated with an increase in blood pressure, although the mechanisms of this increase are not fully understood.

Curtis [75] administered 50 mg of $CoCl_2$ daily for three months to 23 hemodialysis patients with chronic kidney disease. As expected, this treatment resulted in a 10 g/L increase in the hemoglobin concentration in about 50% of the patients. However, one patient died three months after completing the cobalt therapy, and histological examination of the myocardial tissue indicated that he developed cardiomyopathy. In the postmortem period, the cobalt concentration in the myocardium was 1.65 μg/g, which is approximately 25–80 times higher than that in the control samples. Curtis [75] conducted a prospective study on blood cobalt concentrations in healthy individuals and hemodialysis patients after two weeks of administration of $CoCl_2$. In both groups, there was a long-term increase in the blood cobalt concentration, which returned to normal six weeks after discontinuation of $CoCl_2$ administration.

Schirrmacher [76] described the case of a 35-year-old woman who was treated for renal anemia with 100 mg of $CoCl_2$ daily. After six months,the intake of cobalt(II) chloride was discontinued because of the development of neurological disease. On examination, the patient had bilateral sensorineural hearing loss, loss of vibration sensitivity in both legs, and disturbances during the calcaneal-knee test, as well as extensive thyroid gland enlargement. Hearing loss has also been reported as a side effect of $CoCl_2$ therapy. Gardner [15] orally administered 50–150 mg of $CoCl_2$ daily to 17 patients with chronic kidney disease, and four of them complained of tinnitus after 4–16 weeks. The audiogram revealed a hearing loss of over 1000 Hz. When the therapy was discontinued, the hearing returned. Hearing loss caused by $CoCl_2$ therapy and its reversibility when treatment is discontinued has also been confirmed by other researchers. Licht [77] observed optic atrophy in a 32-year-old patient who was treated for pancytopenia with up to 200 mg of cobalt(II) chloride per day in four treatment intervals, each lasting three to four months, for three years. The patient developed choroidal perfusion and optic nerve atrophy, which affected visual acuity, as well as nausea and vomiting, which often accompanied the state of chronic intoxication of the body.

Cobalt prevents the thyroid gland from absorbing iodine. Thus, myxedema and thyroid hyperplasia are relatively common side effects of cobalt salt

treatment. During cobalt therapy, Kriss [78] observed thyroid abnormalities in five patients. Among them were four children with sickle cell disease who received 30–100 mg of $CoCl_2$ daily for 14–30 weeks. A few weeks after the therapy was stopped, goiter and thyroid dysfunction decreased. Since the undesirable effects were associated with cobalt treatment, the author criticized its careless use as a therapeutic agent.

Oral administration of 500 mg of $CoCl_2$ can cause gastrointestinal diseases, nausea, vomiting, and weight loss. Mucklow [37] reported the case of a 6-year-old boy who developed abdominal pain and vomiting after taking a drink containing 2.5 g of $CoCl_2$. The cobalt concentration in his blood plasma was 7.23 μM (normal value <0.02 μM) 7 h after ingestion and 0.09 μM one month later. According to Jacobziner and Raybin [79], the worst case was that of a 19-month-old boy who died about 7 h after swallowing about 30 mL of $CoCl_2$ solution. Autopsy results revealed necrosis of the gastric mucosa, and the liver, kidneys, and spleen were overloaded with cobalt.

Various works have described some aspects of the biochemical properties of inorganic cobalt in relation to its bioavailability and the potential hazard of usage [27, 37, 38]. Later, a staff of the Birmingham Center for the National Poison Information Service of Great Britain compiled a monograph on the toxic effects of $CoCl_2$ [39]. Cobalt and its compounds can enter the human body through various routes (oral, dermal, inhalation, intravenous, and subcutaneous). Cobalt toxicity, body tissue or organ, and the extent of damage will vary depending on the route of ingestion. In addition, the exposure time and the amount of cobalt consumed are critical. There is a risk of intolerance and organ damage at high dosages (>25 mg/day) [40].

Even though the toxic effect of cobalt has been proven many times, the molecular mechanisms of its toxicity remain unidentified. When high concentrations of cobalt are used, it causes harmful effects that are associated with the effect of hypoxia or the "sensation" of a lack of oxygen by the cell. Moreover, oxygen is necessary for the proper functioning of all the cells in any organism. Cobalt is a genotoxic metal [52, 80] that induces oxidative stress [53] and apoptosis [81]. By stabilizing HIF, cobalt ions can activate genes that encode proteins involved in tumor growth (e.g., vascular endothelial growth factor [56] and multidrug-resistant P-glycoprotein transporter). Cobalt salts were discovered to promote the development of carcinoma when introduced in experimental animals. Furthermore, inorganic cobalt was discovered to induce DNA strand breaks, DNA-protein cross-linking, exchanges of sister chromatids, and the formation of micronuclei in mammalian cell cultures. Soluble cobalt(II) salts are classified by the IARC of WHO as group 2B carcinogens (possibly carcinogenic to humans) [22]. Their usage as hypoxia mimetics is currently limited to laboratory experiments because of their toxic side effects [23].

The risk of adverse events increases as the dose and duration of treatment increase. The duration of the therapeutic administration of $CoCl_2$ averages approximately 10 weeks. A daily cobalt dose of 0.03 mg/kg of body weight for oral administration is considered safe in terms of toxic health effects in the general population. This dose is much lower than the therapeutic doses used to stimulate erythropoiesis in clinics. Unfortunately, many athletes are either unaware of or unconcerned about the possible health risks associated with using cobalt as a doping agent.

Determination of cobalt in biological samples

The first attempts to develop strategies for detecting cobalt salt doping in athletes were made earlier. The cobalt content in erythrocytes was proposed as a parameter because the absorption of inorganic cobalt by erythrocytes is practically irreversible and reflects the concentration of cobalt in the plasma [43]. Unice [44] used a biokinetic model to estimate the cobalt levels in the blood and urine as a result of oral ingestion of cobalt amounts that exceeded typical dietary intake. The expected cobalt concentrations after 10 days of ingesting cobalt in a daily dose of 0.4–1.0 mg ranged from 1.7 to 10 μg/L in the blood and 20–120 μg/L in the urine. It was expected that using inorganic cobalt preparations at a rate of 1.0 mg of cobalt per day for a year or more would result in the determination of cobalt concentrations of 5.7–13 μg/L in the blood and 65–150 μg/L in the urine.

The concentration of cobalt was also established using atomic absorption spectroscopy with a graphite cell, and according to the findings of the IARC WHO working group, it was 0.1–0.5 μg/L in the human blood plasma in experimental samples [22].

The GC–MS [82] and a validated quantitative tandem electrospray ionization MS method with a low detection limit of 50 pg/mL [83] are two methods that are compatible with routine doping control procedures for determining cobalt in human urine. Other widely used sensitive methods of quantitative determination include ICP–MS, ICP atomic emission spectrometry, and electrothermal atomic absorption spectroscopy [84]. Since cobalt is a natural and essential trace element [50] that can be found in the urine of healthy people in concentrations ranging

from 40 to 810 pg/mL [85], control and threshold values must be determined to confirm an unfavorable analytical conclusion. The highly sensitive LC–ICP–MS method with detection limits of up to 0.8 pg/mL is the best analytical approach for solving this problem.

The use of only plastic laboratory glassware made of polypropylene is regulated in modern anti-doping methods for determining inorganic cobalt because glassware may contain trace amounts of cobalt as a result of the manufacturing process, which may affect the analysis result [63, 86].

Urine, plasma, or blood serum can be used for analysis. Additionally, a method for determining and comparing the cobalt concentration in erythrocytes to the cobalt concentration in the plasma is being developed to distinguish the source of cobalt ingestion in the body (various forms of vitamin B12 or inorganic cobalt) [76]. Blood is collected in tubes containing Li-heparin (lithium heparin salt) or the potassium or sodium salt of ethylenediaminetetraacetic acid and centrifuged to separate the plasma. The urine is collected in clean plastic containers. Samples are kept frozen at −20°C until analysis.

Screening analysis is usually performed with the ICP–MS method [59, 63, 86–88] using standard solutions of cobalt and germanium with certified values that correspond to standard reference materials 3113 and 3120a of the National Institute of Standards and Technology, which are used to construct a calibration curve (cobalt solutions) and as an internal standard (germanium solutions). Working standard solutions of cobalt and germanium are prepared by diluting the corresponding standard solutions with 3.25% (v/v) nitric acid. For the ICP–MS analysis and construction of calibration curves, European laboratories often use the standard solution of cobalt CertiPUR® (1 mg/mL) from *Merck*, Germany, as an internal standard, the indium plasma emission standard (1 mg/mL) from VWR *International GmbH* (Bruchsal, Germany), and a standard solution containing 10 μg/mL of germanium, cobalt, lithium, titanium, and yttrium in 2% nitric acid from *Agilent Technologies* (Waldbronn, Germany).

Calibration mixtures, quality control (QC) samples, and corresponding blanks are analyzed along with each batch of the test samples. Calibration mixtures and QC samples are prepared using standard working cobalt solutions. To obtain a calibration curve, the ratios of the area of cobalt peaks to the internal standard (germanium) versus the added cobalt concentrations are fitted using linear regression. The total cobalt concentrations in the test samples are interpolated using a calibration dependence. For the QC samples, the recovered cobalt concentration is obtained by subtracting the concentration of the corresponding empty matrix from the total determined concentration. At the discretion of the laboratory, calibration mixtures can be prepared in ultrapure water, plasma, or urine depending on the samples that are being analyzed. At least six points in the range of 0–25 ng/mL or more in the plasma and 0–500 ng/mL or more in the urine are plotted on the calibration curves. Each batch includes one or two QC samples and/or a Certified Reference Material. The QC samples are prepared by adding cobalt standard solutions to the purified plasma and urine.

The internal standard (germanium solution) is added to the urine sample before it is diluted 5–50 times with 3.25% nitric acid for screening analysis. After adding an internal standard, blood plasma (serum) samples are deproteinized with a trichloroacetic acid (TCA) solution (10 g of TCA and 120 mg of NaCl per 100 mL of ultrapure water) followed by the addition of 3.25% nitric acid and centrifugation. The plasma sample should be finally diluted 5–50 times. For analysis, 80–100 μL of the original sample is usually used. The diluted sample is injected into the ICP–MS spectrometer through an autosampler. Argon is used as a plasma-forming gas, and helium is used as a target gas. Detected isotopes: m/z 59 for cobalt and m/z 72 for germanium [55]. Three parallel determinations were used to collect all data. To minimize carryover, the autosampler needle is flushed with deionized water for 5 s in the rinse port and 5 s in the rinse bottle after each sample injection, followed by a 0.07% Triton-X rinse for a maximum of 100 s. Finally, the autosampler probe is flushed again with deionized water for 20 s before the next infusion.

Confirmatory analysis for cobalt doping is usually performed using LC–MS [63, 86, 87]. The internal standard is usually not used in this case. Before plasma or serum analysis, protein precipitation is performed. Inorganic cobalt is determined using diethyl thiocarbamate (DDC), which requires additional sample preparation. The Co-DDC complex is detected in a one-time interval in a positive electrospray ionization mode using the selected reaction monitoring. The selected ion-precursor of the Co-DDC complex has an m/z of 355 [63].

Routine doping tests require fast and simple protocols for preparing samples for analysis. In this regard, Knoop's work [87] is interesting because it describes in detail the method of high-performance LC in combination with ICP–MS (HPLC–ICP–MS), which allows chromatographic peaks to distinguish the cobalt prohibited for use (inorganic cobalt) from endogenous cobalt, which is a component

of cyanocobalamin which is extremely important for doping control. This method also has high sensitivity and the ability to detect one cobalt particle out of 10^{12} other particles and analyze the isotopic composition of the ion of interest, making it the method of choice for modern doping control.

Additional testing strategies based on the identification of indirect biological effects of $CoCl_2$ administration, such as activation of vascular endothelial growth factor gene transcription [45], altered microRNA marker profiles, or enhanced synthesis of delta-aminolevulinate [46], could be reliable alternatives. However, they require a lengthy and complex process of clinical and analytical validation.

CONCLUSIONS

The use of cobalt as a doping agent to improve athletic performance has been and will continue to be a relevant topic of discussion. This article discusses in detail its main biological effects on the body in comparison to the use of erythropoiesis-stimulating agents such as recombinant EPOs or inhibitors of HIF-prolyl hydroxylases. The absence of a clear WADA-approved technical document on the detection of cobalt or the analysis method and the obligation for all anti-doping laboratories in the world to determine it makes its use even more appealing to unscrupulous athletes. More focus is placed on the problem of using cobalt salts for medical purposes and as biologically active supplements, the potential harm of their use due to their toxic effects on the human body, and the possible sanctions for athletes who use such dietary supplements.

The data from the literature on the main methods for detecting cobalt were collected and summarized. The HPLC–ICP–MS method, which distinguishes endogenous cobalt (a part of vitamin B12) from the cobalt prohibited for use (inorganic cobalt), is the most promising and highly sensitive of all the methods considered in the review.

Currently, anti-doping laboratories are tasked with the primary responsibility of introducing the abovementioned method into their methodological base for the unambiguous determination of the abuse of cobalt salts by athletes. This will reduce the number of disqualifications associated with their use and prevent possible cases of potential poisoning by them.

The authors declare no conflicts of interest.

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About the authors:

Irina V. Pronina, Cand. Sci. (Biol.), Main Specialist, Doping Control Department, National Anti-Doping Laboratory (Institute), Lomonosov Moscow State University (10-1, Elizavetinskii per., Moscow, 105005, Russia); Senior Researcher, Pathogenomics and Transcriptomics Laboratory, Institute of General Pathology and Pathophysiology (8, ul. Baltiiskaya, Moscow, 125315, Russia). E-mail: pronina@dopingtest.ru. Scopus Author ID 8161867200, ResearcherID G-3951-2014, https://orcid.org/0000-0002-0423-7801

Elena S. Mochalova, Acting Director, National Anti-Doping Laboratory (Institute), Lomonosov Moscow State University (10-1, Elizavetinskii per., Moscow, 105005, Russia). E-mail: mochalova@dopingtest.ru.

Yuliya A. Efimova, Cand. Sci. (Chem.), Assistant Professor, I.P. Alimarin Department of Analitical Chemistry, M.V. Lomonosov Institute of Fine Chemical Technologies, MIREA – Russian Technological University (86, Vernadskogo pr., Moscow, 119571, Russia). E-mail: efimova_yulia@bk.ru. https://orcid.org/0000-0002-3582-0012

Pavel V. Postnikov, Cand. Sci. (Chem.), Head of the Doping Control Department, National Anti-Doping Laboratory (Institute), Lomonosov Moscow State University (10-1, Elizavetinskii per., Moscow, 105005, Russia). E-mail: postnikov@dopingtest.ru. eLibrary Index 7251-9937, https://orcid.org/0000-0003-3424-0582

Об авторах:

Пронина Ирина Валерьевна, к.б.н., главный специалист отдела допингового контроля Национальной антидопинговой лаборатории (Института) Московского государственного университета им. М.В. Ломоносова (Россия, 105005, Москва, Елизаветинский переулок, дом 10, стр. 1); старший научный сотрудник лаборатории патогеномики и транскриптомики Федерального государственного бюджетного учреждения «Научно-исследовательский институт общей патологии и патофизиологии» (Россия, 125315, Москва, ул. Балтийская, д. 8). E-mail: pronina@dopingtest.ru. Scopus Author ID 8161867200, ResearcherID G-3951-2014, https://orcid.org/0000-0002-0423-7801

Мочалова Елена Сергеевна, исполняющий обязанности директора Национальной антидопинговой лаборатории (Института) Московского государственного университета им. М.В. Ломоносова (Россия, 105005, Москва, Елизаветинский переулок, д. 10, стр. 1). E-mail: mochalova@dopingtest.ru.

Ефимова Юлия Александровна, к.х.н., доцент, кафедра аналитической химии им. Алимарина И.П. Института тонких химических технологий им. М.В. Ломоносова, ФГБОУ ВО «МИРЭА – Российский технологический университет» (119571, Россия, Москва, пр. Вернадского, д. 86). E-mail: efimova_yulia@bk.ru. https://orcid.org/0000-0002-3582-0012

Постников Павел Викторович, к.х.н., начальник отдела допингового контроля Национальной антидопинговой лаборатории (Института) Московского государственного университета им. М.В. Ломоносова (Россия, 105005, Москва, Елизаветинский переулок, д. 10, стр. 1). E-mail: postnikov@dopingtest.ru. eLibrary Index 7251-9937, https://orcid.org/0000-0003-3424-0582

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