Meldonium: Pharmacological, toxicological, and analytical aspects

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Abstract
Meldonium is the active molecule from Mildronate® with similar chemical structure to an amino acid, and it is known as (3-(2,2,2-trimethylhydrazine) propionate) (CAS 76144-81-5). This pharmaceutical substance is approved in Eastern Europe for cerebral and myocardial ischemia and has been on the World Doping Association’s banned substances list since January 2016. The goal of this review is to relate the use of meldonium as a doping agent, considering its pharmacological, toxicological, and analytical aspects. This review is based on the scientific literature from digital platforms. The main mechanism of action of meldonium is based on a decrease in L-carnitine levels and increase of peroxisomes activity in the cytosol. Females were more susceptible to the substance in animal experiments for toxicological tests. There is currently no report in the scientific literature about acute or chronic intoxication cases by meldonium in humans. Based on the literature findings, meldonium showed ergogenic effect in animals and human volunteers. For anti-doping analysis, urine is the biological matrix of choice, and dilute-and-shoot is the most common sample treatment in addition to liquid chromatography–mass spectrometry analysis. Other approaches could be used to determine meldonium levels, mainly for screening tests, such as L-carnitine or gamma-butyrobetaine levels.

Keywords
Meldonium, doping, review, pharmacological, toxicological and analytical aspects

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Introduction
The World Anti-Doping Agency (WADA) is an independent organization created by the International Olympic Committee to protect the rights of athletes to participate in doping-free competitions and to ensure athlete health, fairness, and equality among participants. According to WADA,1 doping is defined as the use of substances and/or methods that improve athletic performance, and it can be harmful to the athlete himself/herself as well as to other competitors. Therefore, anti-doping programs seek to preserve “the spirit of sport,” the pursuit of human excellence through the dedicated perfection of an individual’s natural talents.

Many reasons lead to doping such as status, fame, money, pressure from sponsors, and/or club or even the state or country which athletes represent. On some occasions, the technical team or state officials are responsible for the athlete’s doping, as occurred with the Soviet Union during the Cold War,2 Finland in 2001,3 and recently with Russia.4 These issues make doping a complex subject.

According to WADA,1 the list of monitored substances and methods is classified as (1) substances and methods prohibited at all time, (2) prohibited substances in competition, and (3) prohibited in particular sports. Despite the efforts of WADA and other sectors of society, there are
new substances and/or methods that can be used for doping purposes, such as meldonium.\textsuperscript{1}

Meldonium, also known as MET-88, is a substance present in the medicinal product Mildronate\textsuperscript{6}, which is marketed in Eastern Europe, specifically in Azerbaijan, Belarus, Georgia, Kazakhstan, Kyrgyzstan, Moldova, Ukraine, Uzbekistan, and Russia. It is used for the treatment of cerebral and cardiac ischemic disorders.\textsuperscript{5,6}

Since January 2016, meldonium has been classified as a WADA type I substance. Indeed, this substance is included in “Metabolic and Hormonal Modulators,” which also includes insulin and its mimetics, activators of adenosine monophosphate-activated protein kinase (AMPK), peroxisome proliferator-activated receptor agonist gamma (PPAR\textsubscript{\gamma}), and trimetazidine.\textsuperscript{1}

Since its prohibition from sporting events, there have been several doping cases that involve meldonium, especially for Eastern European athletes.\textsuperscript{7,8} According to the WADA,\textsuperscript{9} meldonium was added (to the Prohibited List) because of evidence of its use by athletes with the intention of enhancing performance.\textsuperscript{9} Therefore, there are studies that may indicate its ergogenic effects.\textsuperscript{10–13} However, according to Schobersberger et al.,\textsuperscript{6} there is insufficient scientific evidence on performance enhancement in athletes caused by meldonium.\textsuperscript{6}

Given this relatively new meldonium status, the goal of this review is to relate the use of meldonium as a doping agent, considering its pharmacological, toxicological, and analytical aspects.

**Methods**

**Study design**

The review was based on the following platforms: MEDLINE (Pubmed), LILACS (Scielo), EMBASE (Elsevier), Scopus (Science Direct), and Academic Google. The keywords employed for this review were Meldonium; Doping; Review; Pharmacology; Pharmacokinetics; Pharmacodynamics; Toxicology; Sports; Risk; Determination; Analyze; Biological Matrices; Urine; Blood; Chromatography; Mass spectrometry; Physicochemical properties and their Portuguese, Spanish and English versions.

**Criteria for inclusion and exclusion**

All published studies in the scientific literature on meldonium are according to the digital platforms. Absence of ISSN indexing, references without authors, journalistic notes without scientific nature, and impossibility of accessing the full article were used as criteria for exclusion.

**Meldonium**

**Physicochemical properties**

Meldonium is the active molecule from Mildronate, and it is known as (3-(2,2,2-trimethylhydrazine) propionate) (CAS 76144-81-5). Originally synthesized by the Latvian Institute for Organic Synthesis in the 1970s with anti-ischemic action, Mildronate is not approved by the Food and Drug Administration (FDA). This substance is used only in Eastern Europe and Russia.\textsuperscript{5,6} Meldonium has a chemical structure similar to an amino acid. This molecule exhibits ionized and non-ionized fraction and its physicochemical properties are described in Table 1.\textsuperscript{14–17}

![Chemical structure](image)

**Table 1. Physicochemical properties of meldonium.\textsuperscript{16}**

| Parameters                      | Characteristics |
|---------------------------------|-----------------|
| pKa                             | 4.14            |
| log \( P \)                     | -1.37           |
| Molecular weight (g/mol)        | 146.19          |
| Melting point (°C)              | 87              |
| Solubility (mol/L)              | 0.31            |
| Molecular formula               | C\textsubscript{6}H\textsubscript{14}N\textsubscript{2}O\textsubscript{2} |

**Pharmacokinetics**

Meldonium is a relatively small, highly hydrophilic molecule that has no affinity for plasma proteins.\textsuperscript{14–15} The maximum plasma concentration occurs at approximately 1 h, and its half-life varies depending on the posology, treatment time, and pharmaceutical form.\textsuperscript{6} For the ingestion of meldonium capsules, \( t_{1/2} \) values were 3.61–15.34 h,\textsuperscript{18,19} while for meldonium injections, \( t_{1/2} \) ranges from 1.76 h to 6.12 h.\textsuperscript{20}

Meldonium excretion and maximum urine concentrations vary according to the type of administration; the single dose injectable form has a linear excretion profile, whereas administration of multiple doses shows nonlinear elimination.\textsuperscript{20} Single or multiple oral dose administration also exhibits nonlinear excretion.\textsuperscript{18} Physical exercise may also influence meldonium excretion.\textsuperscript{21} However, Forsdahl et al.\textsuperscript{22} reported that the maximum urine meldonium concentration is observed shortly after injection of the substance.\textsuperscript{22}
In the study by Rabin et al., 21 32 volunteers received meldonium treatment for 21 days, who were divided into two groups. The first group of 14 volunteers (7 men and 7 women) received 1.0 g of orally daily meldonium, while the second group of 18 volunteers (9 men and 9 women) received 2.0 g of meldonium orally daily. Six volunteers were selected on the basis of the lowest meldonium urinary concentration to perform single functional load testing (physical exercise). The volunteers underwent the Wingate test on a veloergometer performed by the Ramp-30 protocol, which caused a decrease of meldonium urinary concentration in the six volunteers (3 men and 3 women). The changes in meldonium urinary concentrations were 0.38–0.13; 0.12–0; 0.04–0.09; 0.19–0.08; 0.085–0.068; and 0.015–0 μg/mL.21

Meldonium can be metabolized mainly in the liver by gamma-butyrobetaine hydroxylase (BBOX) to form dimethylamine, 2-hydroxymethyl-2(hydroxymethylamino)-propane-1,3-diol, 3-amino-4-(hydroxymethyl-methyl-amino)-butyric acid, and 3-hydroxypropionic acid.14,20,23 This last metabolite can be converted into succinic acid, the main metabolite found in plasma, as illustrated in Figure 1.14,20 The main meldonium excretion route is in urine; approximately 34–60% of this substance is eliminated unchanged15,20 as described in Figure 1.

Blood circulation of meldonium forms hydrophobic ion pairs with plasma lipids based on non-covalent complexes with polar molecules and with neutral weakly hydrophobic molecules. This particular ability of meldonium allows its easy penetration into membrane cells, including the blood–brain barrier.21

Excretion is reduced approximately by 70% when multiple doses are administrated. These nonlinear excretion

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**Figure 1.** Main route of elimination and its route of metabolization by BBOX. BBOX: gamma butyrobetaine hydroxylase.
parameters may be due to BBOX, a key enzyme of meldonium metabolism.\textsuperscript{18,20,25} Based on the study of Forsdahl et al.,\textsuperscript{22} meldonium has an excretion profile by a three-compartment model: alpha ($t_{1/2}: 1.47$ h), beta ($t_{1/2}: 9.17$ h), and gamma ($t_{1/2}: 630$ h); $\ell$-carnitine follows the same distribution model.\textsuperscript{22} The three compartments have been described as follows: extracellular fluid, in which plasma is considered as the central compartment, representing the initial volume of distribution; fast balancing tissues such as the liver and kidneys; and slow balancing tissues such as the skeletal and cardiac muscles.\textsuperscript{22}

According to Zhao et al.,\textsuperscript{20} meldonium BBOX saturation occurred in healthy Chinese volunteers after a single dose (750–1500 mg) or multiple daily doses (500 mg) for 5 days.\textsuperscript{20} Another explanation for the nonlinear kinetics is the absorption phase. Since meldonium is highly soluble in water, active transport in the gastrointestinal tract becomes very important, and saturation of these transporters could lead to this kinetics.\textsuperscript{24}

Tretzel et al.\textsuperscript{26} orally administered a single dose (500 mg) or multiple doses (3 × 500 mg/day for 6 consecutive days) of meldonium and evaluated the substance concentration by dried blood spot (DBS). DBS method is a form of biosampling where blood samples are blotted and dried on a specific filter paper, with positive results after 16 days for a single dose or 28 days for multiple doses. In urine samples, this substance was detected after 49 days from oral administration of a single dose (limit of detection: 20 ng/mL). Considering these results, the authors suggest that meldonium has the ability to bind erythrocytes, a phenomenon that would also explain the lack of linearity for urinary excretion.\textsuperscript{26}

**Pharmacodynamics**

Meldonium has the capacity to interfere in $\ell$-carnitine-mediated mechanisms.\textsuperscript{6} $\ell$-Carnitine action is based on the transfer of fatty acids from the cytosol to the mitochondrial matrix to form adenosine triphosphate (ATP).\textsuperscript{6,14} Meldonium has a similar structure to $\ell$-carnitine and its precursor, gamma-butyrobetaine (GBB), which are present in the liver, kidney, and brain. Due to this similarity, meldonium becomes a competitive inhibitor of some fatty acid oxidation enzymes and carnitine transporters.\textsuperscript{27}

The main meldonium mechanism of action is inhibition of $\ell$-carnitine biosynthesis. It is a competitive inhibitor of BBOX, which converts GBB to $\ell$-carnitine, and thus decreases $\ell$-carnitine and the levels of its metabolites.\textsuperscript{28} Meldonium can also inhibit OCTN2-mediated $\ell$-carnitine reabsorption in the liver and brain.\textsuperscript{29} OCTN2 is expressed in proximal tubule renal cells, and it prevents transport from the lumen to the bloodstream by an unknown carrier.\textsuperscript{30} Competitive inhibition prevents $\ell$-carnitine from entering the cells and, in the kidney, it returns to the circulation and is then eliminated in the urine; meldonium is reabsorbed. This substance also prevents $\ell$-carnitine synthesized in the kidney from entering systemic circulation,\textsuperscript{14,23,31} an action that explains the half-life.\textsuperscript{15}

In the intestine, $\ell$-carnitine is degraded by intestinal microbiota to generate trimethylamine. Posteriorly, trimethylamine is rapidly metabolized by hepatic flavin monoxygenase 3 (FMO3) to become $\text{N}$-oxide-trimethylamine (TMAO).\textsuperscript{32,33} High TMAO plasma concentrations are related to arteriosclerosis in some studies, and it was demonstrated that TMAO induces expression of pro-inflammatory cytokines and adhesion molecules through activation of nuclear factor-\kappaB, such as tumor necrosis factor alpha (TNF-\alpha) and interleukin 6 (IL-6). However, this mechanism is not fully understood.\textsuperscript{32,34–36} Thus, meldonium can reduce TMAO levels by reducing $\ell$-carnitine and/or increasing its urinary excretion.\textsuperscript{32} This mechanism is demonstrated in Figure 2.

Another meldonium mechanism of action includes the inhibition of carnitine palmitoyltransferase-1 (CPT1). This enzyme uses $\ell$-carnitine as a substrate, together with acyl-CoA from fatty acids, to generate acylcarnitines, which are transported from the intermembrane space of mitochondrial into the mitochondrial matrix by carnitine/acylcarnitine transferase (CACT). Despite the low affinity of meldonium for CACT, meldonium can enter the mitochondrial matrix because its chemical structure is similar to acylcarnitine.\textsuperscript{14,29,32} In the mitochondrial matrix, meldonium interacts with carnitine palmitoyltransferase-2 (CPT2), the enzyme responsible for conversion of acylcarnitine to $\ell$-carnitine and acyl-CoA. Consequently, low levels of acyl-CoA promote a decrease in beta-oxidation in the mitochondrial matrix. However, there is a rise of cytosolic peroxisome activity that promotes beta-oxidation.\textsuperscript{37}

Meldonium inhibition of CPT1 leads to an accumulation of acyl-CoA and fatty acids in the cytosol. This behavior enhances the gene expression of peroxisome proliferator-activated receptor alpha (PPAR\textalpha) and peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC1\textalpha). Due to these nuclear receptor activations, cytosolic peroxisome activity to promote beta-oxidation of fatty acids and acyl-CoA increases.\textsuperscript{37} Acetyl-CoA derived from carnitine acetyltransferase (CrAT), an acetyl-CoA/CoA regulatory enzyme within the mitochondria, are weakly inhibited by meldonium.\textsuperscript{14,27}

In diabetes, there are high acylcarnitine concentrations that induce insulin resistance.\textsuperscript{13,38,39} Dambrova et al.\textsuperscript{14} showed that meldonium treatment induces GLUT4 gene expression and insulin receptor proteins in rat hearts. This meldonium-mediated regulation decreases lactate levels, an action that indicates an increase in aerobic glucose oxidation. These authors indicated changes in glucose metabolism caused by meldonium through the inhibition of CPT1 and decreased acylcarnitines.\textsuperscript{14,40}

Type 2 diabetic rats treated with meldonium exhibited decreased glycated hemoglobin and normalized GLUT1 gene expression levels in the heart, liver, kidney, and skeletal muscle. In normal rats treated with meldonium, there
was a small increase in GLUT1 expression in these tissues. The most probable hypothesis proposed by the authors was increased glucose metabolism in tissues and consequently reduced blood glucose. Similar findings were described in studies by Liepinsh et al. in previous years. These mechanisms are shown in Figure 3.

Carnitine transport in cardiomyocytes occurs via Na-dependent channels. Pathological acylcarnitine dysregulation in myocardial cells is bypassed with meldonium treatment. A reduction in acylcarnitine affects the endoplasmic reticulum and sarcoma, which regulates the activity of Ca ATPase and Na+-K ATPase, respectively. Thus, meldonium regulates myocardial contractility and increases hexokinase I activity. Peroxisome-mediated fatty acid metabolism increases the AMP/ATP ratio, which activates AMPK, an enzyme responsible for restoring ATP levels, and increases cellular glucose oxidation.

In the blood–brain barrier, OCTN2 is apparently the main mechanism that allows meldonium entry into the central nervous system (CNS). Meldonium in the brain improves hemodynamics, electrolyte balance, and oxygenation in cerebrovascular disorders. Meldonium normalizes caspase 3 and cellular apoptosis susceptibility protein (CAS) levels and induces nitric oxide synthase (iNOS) expression that is activated under ischemic conditions. It also promotes improved glucose absorption in the CNS, probably due to increased GLUT4 expression. However, these mechanisms have not yet been fully elucidated.

In the brain, BBOX inhibition displaces GBB into an alternative route of esterification by an unknown enzyme, and this process generates GBB methyl ester (at low levels) and GBB ethyl ester. The GBB ethyl ester structure is similar to acetylcholine, and it displays cholinergic activity, as demonstrated by Dambrova et al. based on decreases in diastolic and systolic pressure. GBB esters are hydrolyzed by GBB esterase (acting as an acetylcholinesterase). This hydrolysis product acts as a secondary signal to activate NO synthesis. This reaction may occur in
other tissues, including the cerebral cortex, cerebellum, liver, heart, and kidneys, considering that endothelial nitric oxide synthase (eNOS) is activated by GBB esters in the endothelium via muscarinic receptors \(^29\) (Figure 4).

In addition to all the above-mentioned effects, meldonium acts as an immunomodulator, since it supports humoral immunity. It also acts as a bronchodilator in asthmatics; however, this mechanism has yet to be elucidated. \(^49\)

**Therapeutic use and acute and chronic clinical manifestations**

Mildronate GX, which contains meldonium as the active substance, is marketed in capsule form with a therapeutic dose of 500 mg orally twice daily, mainly for the treatment of cardiac and neurological disorders such as ischemia. The maximum administrated dose in humans is 2000 mg by oral form per day. \(^6\) However, with appropriate posology, this substance can be applied for other clinical disorders such as asthma, bronchoconstriction, gastric ulcers, cocleovestibular dysfunction, glaucoma, diabetes, and vaccine adjuncts. \(^14,29,49\)

There may be differences in meldonium dosage and treatment time according to the symptoms, but the usual regimen is 500 mg twice daily for 4–6 weeks. Glucose levels and cardiac parameters change after 4 weeks of treatment (100 or 200 mg/kg orally daily). \(^41,42\) Two weeks of meldonium treatment reduced L-carnitine by 60% in rat heart (100 mg/kg orally daily). \(^44\) However, humans treated with the therapeutic dose for 4 weeks exhibited only an 18% decrease in plasma L-carnitine. \(^50\)
Another study conducted by Liepinsh et al.\textsuperscript{40} administered 200 mg/kg meldonium intraperitoneally in mice once a day for 20 days. Ten days of treatment decreased blood glucose levels and increased glycemic metabolism related to gene expression in the heart.\textsuperscript{40} Kūka\textsuperscript{51} administered 100 mg/kg meldonium daily for 4 weeks to Wistar rats. This treatment reduced arrhythmia duration and ventricular fibrillation frequency after 14 days of treatment compared to the control. After 4 weeks, blood pressure was not significantly different in the animals. However, after 8 weeks of treatment, there was a decreased heart rate in treated Dahl rats compared to rats given a high salt diet.\textsuperscript{51}

Other studies showed several positive effects from meldonium treatment, mainly against chronic heart failure, systolic function disturbances,\textsuperscript{10} and left ventricle dysfunctions.\textsuperscript{43,44} Thus, it reduces acute myocardial infarction Casilde\textsuperscript{52} and prevents atherosclerosis.\textsuperscript{14} This substance also combats peripheral vascular resistance.\textsuperscript{11} In the CNS, concomitant intraperitoneal meldonium (120 mg/kg) and GBB (30 mg/kg) treatment increased brain NO levels within 15 min of administration.\textsuperscript{29}

Preliminary meldonium administration (10 mg/kg orally) reduced hemodynamic disturbance and improved oxygenation.\textsuperscript{29} Considering meldonium effects in the brain,
daily intraperitoneal administration of this substance (250 mg/kg) during 26 days was able to significantly reduce intracerebral tumor growth in orthotopic glioblastoma (GBM) mouse model. GBM is related to OCTN2 overexpression and L-carnitine accumulation.53

In the study of Di Cristo et al. (2019),54 transgenic Huntington’s disease (HD) Drosophila model were treated with meldonium, added to the surface of assay fly food. The study demonstrated that meldonium increased PGC-1α expression, which showed reduced symptoms of HD, alleviating motor dysfunction.54 Meldonium also demonstrated reduction of oxidative stress levels in brain tissues,29 while Lindquist et al.55 showed an increase in the expression of genes linked with the reduction of reactive oxygen species.55

Based on the literature findings (Table 2), beta-oxidation inhibition, lipid metabolism modulation, and consequently the beneficial cardiac effects require at least 10 days of meldonium administration in ICR mice.29,40 However, cerebral and vasodilatory effects are pronounced less than 1 h after intraperitoneal administration in Wistar rats.29,56

### Table 2. A resume of the studies of meldonium treatment and its impact.

| Dose/route | Period of treatment | Animal | Induced pathology | Effects | Reference |
|------------|---------------------|--------|-------------------|---------|-----------|
| (M) 1000 mg + 20 mg (L) (M) 1000 mg + 5 mg (L) | 3 months/daily | Men and women | Chronic heart failure | Decreased symptoms caused by chronic heart failure and increased exercise time | 10 |
| 100 mg/kg oral | 14 days/daily | Wistar rats | Infarction | Reduce about 60% L-carnitine in rat heart | 37 |
| 200 mg/kg IP | 20 days/daily | Male ICR mice | type 1 diabetes | Decreased glucose blood levels and increased glycemic metabolism related gene expression in mouse hearts | 40 |
| 100 mg/kg oral | 6 weeks/daily | Wistar rats | Type 1 diabetes | Decreased glucose blood levels and GLUT1 over expression in several organs and this, together with reduction of HbA1c% | 41 |
| 200 mg/kg oral | 4 and 8 weeks/daily | Goto-Kakizaki mouse | Type 2 diabetes | Reduced plasma concentrations of glucose | 42 |
| 100 mg/kg oral | 8 weeks/daily | Goto-Kakizaki mouse | Type 2 diabetes | Reduction of myocardial infarction size | 42 |
| 500 mg oral | 4 weeks/twice a day | Healthy volunteers (men and women) | type 1 diabetes | Reduce 18% L-carnitine plasma concentration | 50 |
| 200 mg/kg oral | 14 days/daily | Wistar rats | Arrhythmia | Reduction in the duration of arrhythmias and frequency of ventricular fibrillation | 51 |
| 200 mg/kg oral | 4 week/daily | Dahl rats | Hypertension | Reduction of heartbeat | 51 |
| 200 mg/kg subcutaneously | 10 days/daily | Sprague-Dawley | Acute myocardial ischemia | Reduction of the acute myocardial infarction | 52 |
| 250 mg/kg IP | 26 days/daily | Orthotopic GBM mouse model | Glioblastoma | Reduce intracerebral tumor growth | 53 |
| 200 mg/kg IP | Single administration | Wistar rats | Convulsions | Improved blood flow and oxygen saturation in the brain | 56 |

(M): meldonium; (L): Lisinopril; IP: intraperitoneally.

*There was no induced pathology for this experiment.

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### Adverse effects and toxicity

According to the manufacturer of Mildronate GX, the commercial pharmaceutical product of meldonium, there are some rare adverse effects, including allergic reactions (redness and itchy skin, urticaria, rash, and/or angioedema), dyspepsia, tachycardia, and change (increase or decrease) in blood pressure. There are no reported toxicological symptoms in healthy patients in human clinical studies, and the overall substance toxicity is low.6,56

A study of 40 Chinese volunteers that consumed Mildronate GX (500 mg/day three times daily for 13 days) revealed no toxicity, and the only adverse effects, insomnia, allergic reactions, dizziness, and nausea, disappeared
Bioequivalence, and biochemical studies. Daily oral administration over 6 months to rats did not alter any tissues.

Another study conducted in male Sprague-Dawley rats (200 mg/kg orally for 3 or 6 weeks) demonstrated hepatic steatosis, increased acyl-CoA long chain and elevated very low-density lipoproteins (VLDL) for both treatment periods. Oral administration of 400 mg/kg daily for 60 days increased triglyceride levels in the liver for male Sprague-Dawley rats; however, the liver functions and heart triglyceride levels remained unchanged.

A 13-week daily oral administration of 1600 mg/kg experiment was performed on Sprague-Dawley rats to obtain nontoxic data (unpublished observations). The nontoxic meldonium dose was estimated for female rats at 25 mg/kg/day, while it was 100 mg/kg/day for male rats (n = 10 per sex). The same treatment regimen was performed with male and female Beagle dogs (n = 4 per sex), and the nontoxic dose was estimated at 100 mg/kg/day. A chronic toxicity study in Beagles performed over 52 weeks (daily oral dose of 400 mg/kg) indicated that the nontoxic dose is 25 mg/kg/day. For acute toxicity, Kirimoto et al. reported an approximate lethal single meldonium dose of over 5000 mg/kg for rats and male dogs; both species presented similar behavior, and there were no sex-related differences. However, the number of animals and administration route were not described in this communication.

According to TOXNET, the responsible dose in which 50% of the animals must die (median lethal dose) was 7850 and 4430 mg/kg by intraperitoneal and intravenous administration in mice, respectively; 18,500 mg/kg by oral application in rats; and 8000 mg/kg by intravenous administration in rabbits.

Analytical methods for biological matrices in vivo

Biological matrices in vivo. Urine, blood, and plasma are the traditional biological matrices to determine meldonium concentrations. Urine is the biological matrix of choice for anti-doping analysis in athletes because of noninvasive collection, amount of sample, and a reasonable window of detection. Total blood, plasma, and serum samples for meldonium determination were used for other approaches, mainly pharmacokinetics, pharmacodynamics, bioequivalence, and biochemical studies. There are no scientific papers that have used alternative biological matrices, such as hair, nails, sweat, or oral fluid, despite the viability of these matrices, especially oral fluid (because meldonium lacks affinity for plasma proteins).

Tretzel et al. investigated the possibility of using DBS for analysis; it is considered an alternative matrix. Recently, a study published by Thevis et al. determined meldonium in exhaled breath using breath sampling units from SensAbues® (Huddinge, Sweden). This methodology allows the determination of this substance without invasive approach and great facility to collect the biological sample.

Sample treatment. Sample treatment varies according to the biological matrix and available equipment. Most studies use urine samples to determine meldonium concentration. For this specific biological matrix, dilutions with different solvents and combinations were evaluated in specific proportions, including acetonitrile:ammonium acetate, acetonitrile:ammonium formate, deionized water or deionized water followed by an acetonitrile:methanol mixture. Lv et al. did not dilute urine samples, but rather mixed them with an internal standard solution and centrifuged the mixture to remove large particles. Subsequently, the supernatant was injected into a liquid chromatography–mass spectrometry (LC-MS) system.

For serum and plasma samples, deproteinization followed by centrifugation is necessary to obtain an appropriate supernatant. Some studies added acetonitrile:methanol, acetonitrile:acetic acid or methanol. There are variations among the proportion of the solvent(s) and their combinations as well as the amount of sample. Liepinsh et al. used solid phase extraction (SPE) with a SAX cartridge (silica gel with N,N,N-trimethyl-3-(trimethoxysilyl)-1-propanaminium iodide (1:1)) to obtain free meldonium for a subsequent derivatization step.

DBS samples require different steps for adequate analysis. Ethylenediaminetetraacetic acid (EDTA)-stabilized whole blood is spotted on DBS cards followed by centrifugation. Plasma is separated from the erythrocyte fraction, and red blood cells retained on DBS cards are washed twice with phosphate-buffered saline (pH 7.4). Plasma and washed erythrocytes are spotted onto DBS cards and stored at 4°C in a plastic bag with desiccant until analysis. An online DBS sample preparation is performed using a DBS card autosampler directly coupled to an automated solid-phase extraction that employed an exchange module cartridge. The spots are extracted by flow-through desorption technology using acetonitrile:water (70:30, v/v).

Thevis et al. developed a method to determine meldonium in exhaled breaths. The samples were collected from volunteers according to the SensAbues guidelines in an approximate volume of 20 L. Methanol was incorporated into the filter membrane that contained a cartridge. This device was gently agitated for 5 min, followed by elution of the solvent into a glass tube. Subsequently, the cartridge outlet was placed into the ground orifice of the tube and centrifuged at 660 × g for 2 min. The extract was evaporated until dry under nitrogen flow and resuspended with 100 μL of a methanol:water (1:1, v/v) mixture before injection into the equipment.

Equipment. Due to its structural and physicochemical characteristics, LC-MS is the most viable option for meldonium determination. It is also possible to analyze this substance with liquid chromatography with
a refractive index detector (LC-RID)\(^6^2\) and capillary electrophoresis with capacitive detection of coupled conductivity (EC-C\(^4\)D) using urine samples as the biological matrix.\(^5^9\)

There are no chromophore groups in meldonium; however, it is possible to identify this substance after a derivatization step. This procedure was employed for liquid chromatography with an ultraviolet detector ($\lambda = 262$ nm) using $p$-bromophenacyl bromide solution associated with a catalyst agent for plasma samples.\(^6^7\) Yoshishue et al.\(^6^2\) developed a two-dimensional thin-layer chromatography technique using autoradioluminography with $p$-bromophenacyl bromide solution as the derivative agent in the same sample.\(^6^2\) Liepinsh et al.\(^6^6\) evaluated meldonium with liquid chromatography with a fluorescence detector (excitation $\lambda = 248$ nm, emission $\lambda = 418$ nm) using 1-aminoanthracene (1-AA) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) solutions that were added to the sample as derivatizing agents. After agitation and incubation, the excess reagent was removed with diethyl ether. After pH adjustment and dilution with KH\(_2\)PO\(_4\) buffer, this solution was injected into the equipment.\(^6^6\)

A description of meldonium determination in biological matrices in vivo is presented in Table 3.

**Discussion**

Recently, Russia has been involved in doping, a reality that makes the doping issue very complex.\(^6^8\) Substances such as meldonium were being used indiscriminately by athletes, a large majority of whom were from Eastern Europe, and this situation came to a head at the 2015 European Games in Baku, Azerbaijan. In this competition, 66 athletes tested positive for meldonium (0.7–273 mg/mL); 23 athletes declared their meldonium use during the games and 13 athletes who used the substance won medals. Notably, of the 21 sports, meldonium use was detected in 15.\(^6^9\) In 2016, athletes were caught and banned for using meldonium,\(^3\) and most recently at the 2018 Winter Olympics in Pyeong-Chang, South Korea.\(^8\) Table 4 presents the sports for which athletes tested positive for meldonium at the last European Games and Winter Olympics.\(^8^,6^9\)

Following the addition of meldonium to the Prohibited List, WADA stated that meldonium was banned for evidence that athletes would be using for the purpose of increasing the sporting performance.\(^3\) In the current literature, there are few studies that link meldonium to sports performance in humans, although studies indicate its ergogenic effect.\(^6^,1^0^–1^2^,6^9\)

A study conducted with seven judokas from the Georgia National team who used 250 mg meldonium orally four times daily for 20 days concluded that meldonium could increase physical labor capacity. However, the methodology was of a relatively poor quality, due to the small sample size, the utilized dose, and other factors.\(^6\)

Male and female patients with chronic coronary disease and decreased physical tolerance ($n = 512$) were divided into four groups with oral administration of meldonium (2 $\times$ 50 mg/day, 2 $\times$ 150 mg/day, 2 $\times$ 500 mg/day, or 2 $\times$ 1500 mg/day) plus standard therapy (beta blocker, acetylsalicylic acid, statin, and angiotensin-converting enzyme inhibitor); there was also a placebo group with standard therapy for at least 12 h prior to the test. All subjects underwent a treadmill test; the initial load was 50 W, which was increased by 25 W within 3 min until exhaustion or onset of any symptoms related to chronic coronary disease. Treatment results were compared with baseline results, which were the last two scores in the 4-week period prior to treatment. After 12 weeks, there was an increase in exercise time, and the 2 $\times$ 500 mg/day group presented the best results with an average exercise increase of 35 s on the ergonomic bicycle compared to baseline.\(^1^1\)

Another study evaluated 119 male and female patients aged 30–80 years with heart failure and coronary atherosclerosis. They were separated into three groups: ML20 received meldonium (M) 1000 mg and lisinopril (L) 20 mg daily; ML5 received M 1000 mg and L 5 mg, and L20 (control) received L 20 mg daily. The groups were referred for the ergonomic bicycle test following the standard protocol: the initial load was 25 W, which was increased by 25 W within 3 min. Before treatment, the final load was approximately 70 W, and at the end of the treatment, it was approximately 80 W. The 6-min walk test was also performed, which evaluates the distance traveled over 6 min. At the end of treatment, the ML20 group covered approximately 25 m, an increase compared to the 14 m walked by the control group.\(^1^0\)

In animals, Baulin et al.\(^1^2\) evaluated 10 adult albino rats in the forced swimming test that simulated a physical load of 7% of the rat body weight in a cylindrical pool. The test protocol was initial swimming, 5 min of rest followed by the second swimming session until exhaustion, and finally 10.7 mg/kg of meldonium (via oral gavage) followed by 40 min rest and the last swimming session. Meldonium improved recovery and increased performance by 65.02 and 49.4%, respectively, compared to the control.\(^1^2\)

Another approach to determine the ergogenic effects from meldonium could be by examining AMPK activation, as described by Liepinsh et al.\(^3^7\) Although this study did not directly test the relationship between physical exercise and meldonium, the authors reported increased AMPK activity.\(^3^7\) This enzyme plays a key metabolic role in exercise, since it acts directly as a metabolic fuel gauge in skeletal muscle as the AMP/ATP and creatine/phosphocreatine ratios increase. Consequently, there is an inhibition of ATP-consuming pathways and activation of other pathways from carbohydrate and fatty acid metabolism to reestablish ATP levels. Thus, meldonium may increase skeletal muscle response to exercise training due to an ability to alter muscle fuel reserves and exercise-responsive gene expression based on AMPK activity.\(^1^3,7^0,7^1\)
| Biological matrix | Sample treatment | Equipment | Stationary phase | Mobile phase | Detector conditions | Sensitivity | Reference |
|-------------------|------------------|-----------|-----------------|--------------|--------------------|------------|----------|
| Plasma (300 µL)   | Sample is diluted in 750 µL ACN. After vortex mixing for 2 min, centrifuged at 12,000 × g at 4°C for 5 min, and 50 µL of the upper layer was removed from the mixture into another tube and diluted with 950 µL mobile phase; after a thorough vortex mixing for another 2 min, 150 µL of the upper layer was removed into injection bottle, and 1 µL of the solution was injected into the UPLC-MS/MS system. | LC-MS/MS | ACQUITY UPLC BEH HILIC (50 mm × 2.1 mm × 1.7 µm) | Aqueous solution (0.08% formic acid: 30 mM ammonium acetate): ACN (73:27: v/v) | ESI-positive mode, capillary 0.5 kV; Cone voltage 17 V; flow rate 750 L/h; ionization sources 120 C; desolvation 400 C; analyzed ions 147.2 and 58.0 m/z | 100 ng/mL | 18 |
| Plasma (200 µL)   | Sample was mixed with 50 µL 1st (10 µg/mL) and 20 µL methanol in a 1.5 mL tube. The mixture was vortexed for 30 s. Posteriy it is added with 700 µL methanol, vortexed for 3 min and then centrifuged at 10,000 × g for 10 min. It was injected an aliquot of 10 µL from supernatant into the equipment | LC-MS/MS | Shim-pack VP-ODS C18 (150 mm × 4.6 mm × 5 µm) | Methanol: 10 mM ammonium acetate solution (55:45: v/v). | ESI-positive mode; Dwell time 200 ms; gas flow 10 L/min; gas T: 350°C; nebulizer pressure 50 psi; fragmentor voltage 60 V (mildronate) and 90 V (IS); collision energy 36 V (mildronate) and 14 eV (IS); Analyzed ions 147.2 and 58.3 m/z | 10 ng/mL | 19 |
| Serum (500 µL)    | Sample diluted with 500 µl of CAN: 1 M acetic acid (9:1, v/v), mixed by 2 min, refrigerated by 30 min. Posteriy it was mixed again for 1.5 min, centrifuged at 3300 rpm for 10 min at 4°C. It was injected 10 µL into the equipment | LC-MS/MS | Atlantis HILIC silica (100 mm × 2 mm × 3 µm) | CAN: aqueous solution (0.2% formic acid: 10 mM ammonium formate). Linear gradient mode started with 95% CAN for 0.5 min, ramped to 10% over 6 min and held for 0.17 min before re-equilibration | ESI-positive mode; spray voltage 3500 V; analyzed ion 147.1 m/z | 0.5 ng/mL | 24 |
| Urine (100 µL)    | 800 µL of ACN and 100 µL containing 3-meld degradation IS at 20 ng/mL aqueous 10 mM ammonium formate. After, the sample was mixed for 1 min and centrifuged at 3,800 rpm for 5 min at 4°C. 10 µL was injected into the equipment | LC-MS/MS | Atlantis HILIC silica (100 mm × 2 mm × 3 µm) | ACN: aqueous solution (0.2% formic acid: 10 mM ammonium formate). Linear gradient mode started with 95% ACN for 0.5 min, ramped to 10% over 6 min and held for 0.17 min before re-equilibration | ESI-positive mode; Spray voltage 3500 V; analyzed ion 147.1 m/z | 2.5 ng/mL | 24 |
| Dried blood spots (DBS) | Total blood samples with EDTA (3.5 mL) are centrifuged at 1000 × g for 15 min at 10°C. Plasma was separated from the RBC fraction, and 200 µL of the RBCs (retained for deposit onto DBS cards) was subsequently washed twice with 600 µL of phosphate-buffered saline (pH 7.4). The obtained plasma and washed erythrocytes were spotted onto DBS cards (four 20 µL aliquots each) and were also stored at +4°C in a plastic bag with desiccant until analysis. Spots were extracted by flow-through desorption technology with 1.2 mL of ACN: water (70:30) | LC-HR-MS/MS | SPE™, Hypersil Gold C8 (30 mm × 2.1 mm × 19 µm) | Solvent A: 5 mM ammonium acetate buffer (pH 3.5). Solvent B: ACN. Linear gradient mode | Full scan mode; normalized collision energy: 40 eV; analyzed ion: m/z 147,1126 | 20 ng/mL | 26 |
| Urine (5 µL)      | Sample was diluted in 50 µL of deionized water. The homogenates were vortexed for 30 s and injected into a separation capillary without any further treatment | CE-C™D | Fused-silica capillaries (50/375 and 25/375 µm i.d./o.d.). Total lengths (Ltot) and effective lengths (Leff) were 55 or 65 cm and 42 or 52 cm, respectively | Constant voltage = 30 kV; 2 M acetic acid (pH 2.3) at 25°C; injections were performed at 50 nl for 10 s (0.5 µm i.d.) | 50 Vpp; 1.84 MHz | 15 ng/mL | 59 |

(continued)
| Biological matrix | Sample treatment | Equipment | Stationary phase | Mobile phase | Detector conditions | Sensitivity | Reference |
|-------------------|------------------|-----------|------------------|--------------|---------------------|-------------|-----------|
| Urine (270 μL)    | Sample was fortified with 30 μL of the IS working solution; the mixture was further diluted with 100 μL of a 100 mM ammonium acetate solution and 700 μL of ACN. The sample was mixed and an aliquot of 10 μL was injected. | LC-HR-MS/MS | Nucleodur C18 HILIC (100 mm × 2 mm × 1.8 μm) for analytical approach | Deionized water (A): ACN (B): aqueous solution 200 mM ammonium acetate buffer containing 0.15% acetic acid (pH 5.0) (C). Gradient mode. Initial conditions of 0% (A), 95% (B) and 5% (C) in isocratic mode for 1 min. The content of 5% (C) was maintained during chromatographic analysis. Solvent B decreased linearly from 95% to 40% within 10 min. After 2 min of isocratic elution, re-equilibration started for 5 min at initial mobile phase conditions. | Normalized collision energy: 50%; Resolution: 35 000 FWHM; Analyzed ion 147.1126 m/z | 10 ng/mL | 60 |
| Exhaled breaths (v.u.) | The exhaled breath is collected in the SensAbues® EB; add 4 mL of methanol with the standard references to the filter membrane containing cartridge; agitate for 5 min, and subsequent elution of the solvent into a glass test tube; The outlet of the cartridge is placed into the ground orifice of the test tube and centrifuged at 660 g for 2 min. The obtained extract was evaporated to dryness in N2 flow, the residue was dissolved in 100 μL of a mixture of methanol-water (1:1, v/v), and 10 μL is injected | LC-MS/MS | Poroshell C-8 analytical column (50 mm × 3 mm × 2.7 μm) | Solvent A (10 mM aqueous ammonium acetate): Solvent B (ACN). Gradient elution with 95% solvent A decreasing to 0% in 10 min for 2 min before equilibration | ESI-positive mode; Impactor voltage: 1 kV; CE: 12/12 eV; N2 as collision gas; analyzed ions 147, 59/58 m/z | 5 pg/filter | 61 |
| Plasma (v.u.) | Total blood samples (100–200 μL) was centrifuged and plasma were deproteinized by methanol and applied to TLC plates; Leave in contact with IP overnight and detect by photo stimulated luminescence using the RLG system (10 μCi/mg radiochemical reagent) using n-butanol/acetic acid/water (4:1:2; v/v/v) as solvent system | Two-dimensional TLC-RLG | Kieselgel 60F254, 0.25 mm thick (20 cm × 40 cm) | First solvent system: ethanol/water (3:2, v/v); second solvent system: 2-butanol/acetic acid/water (4:1:2; v/v/v) | 14C RLG equipment | Uninformed | 62 |
| Plasma (v.u.) | Total blood samples (100–200 μL) was centrifuged and plasma were deproteinized by methanol and applied to chromatographic system | LC-RID | NH2-P-50 column (250 mm × 4.6 mm × 5 μm) | ACN: distilled water (70:30) | | Uninformed | 62 |
| Plasma (300 μL) | Sample was mixed with 50 μL IS solution and 2 mL methanol; the mixture oscillated for 3 min and centrifuged at 3000 r/min for 10 min; the supernatant was evaporated to dryness under nitrogen under 37°C; the dried sample was reconstituted in 300 μL mobile phase, oscillated for 3 min and centrifuged at 12,000 r/min for 10 min; the supernatant was separated and 10 μL injected | LC-MS/MS | Inersil NH2 (250 mm × 4.6 mm × 5 μm) | Water: methanol (60:40; v/v) | ESI-positive mode; ion spray voltage: 4 kV; capillary temperature: 300°C; pressure of the nebulizing gas (N2): 80 ps; the collision gas (N2) pressure: 40 ps, DP voltage was 20.0 V; CID voltage: mildronate (40 V), IS (23 V); analyzed ions: mildronate (147 and 58 m/z), IS (162 and 103 m/z) | 1 ng/mL | 63 |
| Urine (100 μL) | Sample was fortified with 10 μL of IS solution. Samples were briefly vortex-mixed and centrifuged to remove large particles; 10 μL of supernatant was injected | LC-MS/MS | Inersil NH2 (250 mm × 4.6 mm × 5 μm) | Water: methanol (60:40; v/v) | ESI-positive mode; ion spray voltage: 4 kV; capillary temperature: 300°C; pressure of the nebulizing gas (N2): 80 ps; The collision gas (N2) pressure: 40 ps, DP voltage was 20.0 V; CID voltage: mildronate (40 V), IS (23 V); Analyzed ions: mildronate (147 and 58 m/z), IS (162 and 103 m/z) | 1 ng/mL | 63 |
| Biological matrix | Sample treatment                                                                 | Equipment | Stationary phase | Mobile phase | Detector conditions | Sensitivity | Reference |
|-------------------|-----------------------------------------------------------------------------------|-----------|------------------|--------------|--------------------|-------------|-----------|
| Plasma (100 μL)   | Sample was mixed with 10 μL IS stock solution (equivalent to 40 μg TEA) and 10 μL methanol, and vortexed. After the addition of 900 μL of methanol, the mixture was vortexed again following by centrifugation for 15 min at 20,000 r/min. Supernatant (200 μL) was transferred to the glass autosampler vials containing 600 μL of the mobile phase. The vials were capped and vortexed, and 5 μL was injected. | LC-MS/MS  | BEH HILIC column (50 mm × 2.1 mm × 1.7 μm) | Water: ACN: 200 mM formic acid (adjusted to pH 3.0 with 12.5% NH4OH) (25:70:5; v/v/v) | ESI-positive mode; capillary voltage: 300 V; source T°C: 150°C; desolvation T°C: 500°C; collision gas pressure (argon): 2.8 μbar; 147.15 and 58.1 m/z | 10 ng/mL | 64 |
| Plasma (100 μL)   | Load the sample into an SPE SAX cartridge (conditioned with 2 mL of methanol and 2 mL of water); eluate with 3 mL of 0.01 mol/L KH2PO4 buffer (pH = 3.5); Collect the eluate into a 5 mL volumetric flask, and add 0.01 mol/L KH2PO4 (pH = 3.5) up to the mark; 20 mL of 1 mol/L HCl, 100 mL of 1-AAA solution, and 100 mL of EDC solution were added to 1 mL of sample (eluate); Incubated the mixture at room temperature for 20 min; Remove the excess reagent by washing the sample with 5 mL of diethylether; Transfer 300 μL aliquot of the aqueous phase into a test tube and add 700 μL of 0.01 mol/L KH2PO4 buffer (pH = 9.1); mix; wash the mixture with 5 mL of CHCl3; transfer 500 μL aliquot of the final aqueous phase into a chromatographic vial and dilute with 500 μL of 0.01 mol/L KH2PO4 buffer (pH = 3.5); inject 30 μL of this solution | LC-FD     | ZORBAX bonus-reverse phase (250 mm × 4.6 mm × 5 μm) | Aqueous solution 0.1 mol/L ammonium acetate buffer (pH 3.5): 20% ACN (80:20, v/v) | Exciation λ = 248 nm, emission λ = 418 nm | Uninformed | 66 |
| Plasma (v.u.)     | A 400 mg mass of Dowex 50W×5 resin (20–50 mesh) were placed in a plastic column (8 mm i.d.) and flushed with 1% of TFA until pH reached 2-3, then with 2 mL of water. Deproteinized plasma (1 mL) was filtered through the prepared column and the filtrate was neutralized with 10 μL of 25% NH4OH until mixture pH reached 6-8. The catalyst solution was prepared from 600 μL of 18-crown-6 solution (1 mg/mL in MeCN), 150 μL of K2HPO4 (0.15 mg/mL in water), and 150 μL of KH2PO4 (0.1 mg/mL in water). The purified plasma sample (100 μL) was mixed with 450 μL of p-bromophenacyl bromide solution (3 mg/mL in MeCN) and 75 μL of catalyst solution in an amber glass vial. The vial was heated for 2 h at 70°C and solvents evaporated under vacuum. The residue was dissolved in 400 μL of water and solid impurities were removed by centrifugation. The solution was used for HPLC analysis | LC-UV/Vis | Silasorb 600 silica (150 mm × 4.6 mm × 10 μm) | ACN: 0.025 M phosphate buffer (pH 5.7) (10:90, v/v). | λ = 262 nm | 1000 ng/mL | 67 |

ACN: acetonitrile; 1-AA: 1-aminoanthracene; ECD: capillary electrophoresis with capacitively coupled contactless conductivity detection; ESI: electrospray ionization; HILIC: hydrophilic interaction liquid chromatography; LC-UV/Vis: liquid chromatography with ultraviolet and visible detector; LC-RID: liquid chromatography with refractive index detector; LC-MS/MS: liquid chromatography–mass spectrometry tandem; LC-EC: liquid chromatography-electrochemical detector; LC-FD: liquid chromatography-fluorescence detector; i.d.: internal diameter; IS: internal standard; MeCN: methyl cyanide; TEA: triethanolamine; CID: collision-induced dissociation; TFA: trifluoroacetic acid; TLC: thin-layer chromatography; RLG: radioluminography; v.u.: volume unspecified; RBC: red blood cell.
Meldonium could also enhance physical performance by elevating testosterone levels. Bruveris et al. \(^{72}\) administrated 2 g of meldonium (diluted with 2–3 mL of water) with food to wild boars. This study revealed a significant increase in testosterone levels compared to before treatment, namely, an increase from 2.0 ng/mL (±0.6) to 3.4 ng/mL (±0.7). However, this mechanism remains unclear. Although the authors used wild boars, testosterone level increase is a very important factor for improving physical performance. If these conditions could be repeated in humans, it may explain the ergogenic effects of meldonium. Therefore, further studies should be performed. \(^{72}\)

In addition to effects related to exercise, a study demonstrated the beneficial effects of meldonium on the vasodilatory capacity of atrial conduction vessels and resistance vessels and during static muscle loading with the static load tolerance test. This study reported that the combination of meldonium and lisinopril improves quality of life, exercise capacity, and peripheral circulation mechanisms compared to lisinopril. \(^{10}\) Therefore, as demonstrated by meldonium pharmacodynamics, it benefits sports performance and we must relate its use in doping to its effects.

Among the meldonium benefits (Table 5), its cardioprotective action is considered as the main positive effect. The increase of PPARα and PGC1α activity shifts beta oxidation from the mitochondrial matrix to peroxisomes in the cell cytosol. Therefore, PPARα activity is also related with rise of pyruvate dehydrogenase kinase 4 (PDK4) enzyme mRNA expression in all tissues, however, it was not shown to increase PDK4 protein expression. \(^{73}\) Probably the increase of PDK4 enzyme mRNA expression may be related in an attempt to regulate glucose metabolism.

Studies have shown that meldonium increases glucose metabolism, decreases lactate production, and increases liver glycogen. \(^{14,40,50}\) Therefore, the meldonium metabolic effect that decreases insulin resistance and increases glucose receptor expression (e.g. GLUT1 and GLUT4) provide higher glucose concentrations in tissues without hypoglycemia. \(^{14,40,41}\) These effects decrease lactic acid production during exercise, which consequently reduces fatigue and improves physical recovery. \(^{14,28,69,73}\)

Activation of eNOS causes vasodilatation and increases blood flow in several organs, increasing the availability of substrates necessary for exercise. Since meldonium increases cerebral oxidation and hemodynamics, during long physical exercise there are several benefits. With optimal brain oxygenation during long periods of physical exercise, movement control, decision-making, and cognitive function are preserved. \(^{74}\)

BBOX inhibition by meldonium decreases L-carnitine, an action that elevates plasma, heart, and brain GBB levels. \(^{14,66}\) GBB accumulation in the brain may explain GBB levels in the CNS; it is metabolized by an unknown enzyme to form GBB esters (Figure 4). GBB esters have high affinity for muscarinic receptors, and this affinity produces cardiac effects and NO activation. \(^{29,48}\) However, if GBB esters interact with muscarinic receptors in other systems, it may cause undesirable effects on sports performance, including bronchoconstriction and increased secretions. If they interact with CNS muscarinic receptors, where the effects are excitation and memory gain, \(^{75}\) these could benefit sports practice.

Meldonium decreases methemoglobin concentrations and consequently allows greater O₂ transport by erythrocytes. \(^{49}\) The structural similarity of meldonium to L-carnitine probably allows it to bind to erythrocytes. According to Mescka, \(^{76}\) L-carnitine supplementation increases

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### Table 4. Sport modalities had positive tests for meldonium in European Games at Baku (Azerbaijan) in 2015 and Winter Olympics at PyeongChang (South Korea) in 2018. \(^{8,69}\)

| Sports modes          | Meldonium detected | Medalists who declared use of meldonium |
|-----------------------|--------------------|-----------------------------------------|
| Aquatic               | Positive           | Yes                                     |
| Archery               | Positive           | —                                       |
| Athletics             | Positive           | Yes                                     |
| Badminton             | Negative           | —                                       |
| Basketball            | Positive           | —                                       |
| Boxing                | Positive           | Yes                                     |
| Canoeing/Kayaking     | Positive           | Yes                                     |
| Cycling               | Positive           | Yes                                     |
| Fencing               | Negative           | —                                       |
| Soccer                | Negative           | —                                       |
| Olympic gymnastics    | Positive           | Yes                                     |
| Judo                  | Negative           | —                                       |
| Karate                | Positive           | —                                       |
| Sambo                 | Positive           | —                                       |
| Target shooting       | Negative           | —                                       |
| Climbing              | Positive           | —                                       |
| Table tennis          | Negative           | —                                       |
| Taekwondo             | Positive           | Yes                                     |
| Triathlon             | Positive           | —                                       |
| Volleyball            | Positive           | Yes                                     |
| Wrestling             | Positive           | Yes                                     |
| Curling               | Positive           | —                                       |

### Table 5. Possible ergogenic effects of meldonium.

| Possible ergogenic effects of meldonium                                      |
|---------------------------------------------------------------------------|
| Oxidation of fatty acids by peroxisomes                                   |
| Decreased production of lactic acid                                      |
| Increased storage and use of glycogen                                     |
| Decreases oxidative stress                                               |
| Improvement in cardiac activity                                          |
| Improves circulation and oxygenation at the CNS level                     |
| Activations of CNS enhancement functions (cognitive enhancement)         |
| Improves recovery after exercise                                          |
| Improves aerobic resistance                                              |

CNS: central nervous system.
glutathione levels in erythrocytes. This effect was previously only demonstrated with elevated L-carnitine levels.76 Therefore, reduced L-carnitine synthesis mediated by meldonium likely plays a prominent role in determining glutathione levels, namely, by increasing the ability to reduce Fe(II) to Fe(II) and thus maximizing oxygen transport capacity. This effect could improve aerobic resistance, however, there is no convincing evidence to prove these effects.28

Another important point that must be considered is dosage (Table 2). The dose and administration time should correlate with the desired effect. For metabolic effects, changes in plasma and cardiac glucose levels and heart failure symptom reduction, at least 2 weeks of meldonium treatment is necessary.40 For CNS effects and eNOS activation, a single administration promotes biological actions.29,56 However, Tretzel et al.26 showed ergogenic performance improvement in athletes based on oral doses of meldonium of up to 2.0 g per day over 2–3 weeks during pre-competitions period.26

With regard to meldonium toxicity, Kirimoto et al.44 demonstrated non-lethal meldonium doses of 25 and 100 mg/kg for female and male Sprague-Dawley rats, respectively. Thus, the non-lethal dose in female rats may be four times lower compared to male rats, a result that suggests females may have greater susceptibility to toxicity.44 A hypothesis for this particular behavior may be the excretion rate. Forsdahl et al.22 demonstrated differences between women and men volunteers, although it is not the purpose of the study.22 It was verified by Rabin et al.,21 showing differences on meldonium excretion in male versus female human volunteers, where male volunteers showed a higher excretion ratio of this substance than female volunteers. In contrast, plasma concentrations in female volunteers were higher compared to male volunteers,21 demonstrating greater susceptibility of female volunteers to meldonium toxicity.

This fact makes the use of meldonium even more dangerous, since athletes often use ergogenic substances without adequate knowledge about them. However, Zhang et al.18 administered meldonium orally in 40 Chinese volunteers (20 men and 20 women), specifically a single dose of 250, 500, 1000, 1250, or 1500 mg, and volunteers who received the 500 mg dose continued treatment for 13 days three times daily. The study revealed no meldonium sex difference;18 however, it is possible that this dose does not reveal potential differences. Furthermore, the administration route utilized which was not reported by Kirimoto et al.44 may influence sex differences.

Considering the collateral effects of meldonium, one point that should be explored is the use of this substance in the medium and long periods for athletes and/or healthy people and their respective negative effects. In fact, at this moment, it was not finding any data addressing chronic exposure to meldonium in these populations.

Meldonium determination is another important point (Table 2); a medalist detailed the use of meldonium as a doping agent, but this substance was not detected in the athlete’s sample. There is no official method for analysis by anti-doping agencies; however, high-resolution MS-MS or MS equipment is necessary for confirmatory purposes. For WADA, this agency establishes the minimum concentration to detect the Reliable Substance called Minimum Required Performance Levels (MRPL) at 200 ng/mL in urine samples.6,65,77 There are many methods to identify meldonium in biological matrices, and most of them are based on dilute-and-shoot after sample preparation (blood and/or urine) and LC-MS determination. Given meldonium toxicokinetics, it is possible to determine this molecule in the body for several days, even after use cessation.

According to Knych et al.,24 meldonium accumulates in tissues due to its affinity for OCTN2, which leads to reabsorption in the renal tubules and uptake in various tissues that express this transporter.24 However, Peng et al.19 suggest that meldonium accumulates in plasma while Rabin et al.21 showed higher levels of plasmatic meldonium in female volunteers compared to male volunteers based on the excretion capacity of this substance.23 Forsdahl et al.22 noted that meldonium has a three-compartment model excretion profile, which these authors verified as meldonium detection in urine in a period between 94 days and 162 days.22 Another explanation for meldonium accumulation is the ability of this molecule to bind to erythrocytes (16–28 days), even after substance consumption ends.26 In this sense, DBS samples seem to have great potential for examining meldonium accumulation, as demonstrated by Tretzel et al.26

For screening tests, other approaches could be used to determine meldonium use. One possibility is assessing urine and/or blood L-carnitine levels. Since meldonium competitively inhibits BBOX, L-carnitine levels would be low in urine and blood. GBB could also be evaluated for anti-doping analysis because its plasma levels would increase.14,19 For this approach, the reference value and excretion of GBB and L-carnitine should be determined, and possible interferences and methodologies for detection with screening bias(es) should be known. However, there are few studies that consider alternative biological matrices to determine meldonium for anti-doping analysis for both screening and substance confirmation.

**Conclusion**

The use of meldonium for sports competitions is considered to violate sports ethics according to WADA. In this review, it was verified that meldonium showed ergogenic effect in animals and humans volunteers. However, this drug has therapeutic applications in patients with cardiovascular diseases. Also, meldonium can be used for the treatment of other pathologies, and it has no pronounced adverse effects, demonstrating a certain safety and effectiveness. In fact, this molecule shows high lethal dose and its positive effects are pronounced at doses much lower than its lethal dose,
which decreases problems due to overdose. Therefore, there are few studies that demonstrate the ergogenic effect of meldonium and its mechanisms of action, mainly in athletes. The same aspect occurs with toxicological effects of meldonium, for which there are limited toxicological data. Furthermore, studies with meldonium are required mainly in healthy individuals, and new analytical methodologies should be developed for the detection of this molecule in anti-doping tests.

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