

Mildronate (Meldonium) in professional sports – monitoring doping control urine samples using hydrophilic interaction liquid chromatography – high resolution/high accuracy mass spectrometry

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To date, substances such as Mildronate (Meldonium) are not on the radar of anti-doping laboratories as the compound is not explicitly classified as prohibited. However, the anti-ischemic drug Mildronate demonstrates an increase in endurance performance of athletes, improved rehabilitation after exercise, protection against stress, and enhanced activations of central nervous system (CNS) functions.

In the present study, the existing evidence of Mildronate's usage in sport, which is arguably not (exclusively) based on medicinal reasons, is corroborated by unequivocal analytical data allowing the estimation of the prevalence and extent of misuse in professional sports. Such data are vital to support decision-making processes, particularly regarding the ban on drugs in sport. Due to the growing body of evidence (black market products and athlete statements) concerning its misuse in sport, adequate test methods for the reliable identification of Mildronate are required, especially since the substance has been added to the 2015 World Anti-Doping Agency (WADA) monitoring program.

In the present study, two approaches were established using an in-house synthesized labelled internal standard (Mildronate-D₃). One aimed at the implementation of the analyte into routine doping control screening methods to enable its monitoring at the lowest possible additional workload for the laboratory, and another that is appropriate for the peculiar specifics of the analyte, allowing the unequivocal confirmation of findings using hydrophilic interaction liquid chromatography-high resolution/high accuracy mass spectrometry (HILIC-HRMS). Here, according to applicable regulations in sports drug testing, a full qualitative validation was conducted. The assay demonstrated good specificity, robustness (rRT=0.3%), precision (intra-day: 7.0–8.4%; inter-day: 9.9–12.9%), excellent linearity (R>0.99) and an adequate lower limit of detection (<10 ng/mL). Copyright © 2015 John Wiley & Sons, Ltd.

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Introduction

New drug entities in preclinical and clinical trials as well as traditional drugs, genuinely studied and developed to treat serious diseases, occasionally possess great potential for misuse in elite sports. Some of these compounds are subject of active discussions on various Internet platforms and, moreover, are commercially available but not (yet) prohibited in sports. In the last few years, a prominent example for that scenario was xenon, a hypoxia-inducible factor (HIF) activator, which was included in the World Anti-Doping Agency (WADA) Prohibited List in September 2014.^[1–3]

In context of the extended method validation of a novel multi-target screening assay based on high resolution/high accuracy mass spectrometry, an interfering signal at m/z 147.1128 repeatedly appeared in full-scan acquisition mode of selected authentic sports drug testing specimens. As it was not observed in all urine samples, an exogenous origin was suspected. The calculated molecular formula of the unknown compound matched that of Mildronate (Meldonium),

an approved drug in several countries of Eastern Europe with anti-ischemic properties, which was further in accordance with several athlete statements on the sample control form. The use of Mildronate was eventually confirmed by comparison of high resolution/high accuracy mass spectrometric data of the suspicious urine samples with a Mildronate reference standard.

Mildronate [3-(2,2,2-trimethylhydrazinium)propionate] was originally developed in the late 1970s as a growth-promoting agent for animals in the Latvian Institute of Organic Synthesis.^[4–6] In

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recent years, several studies and clinical trials identified Mildronate as an effective anti-ischemic drug with multiple indications besides its cardioprotective properties, including the treatment of neurodegenerative disorders, bronchopulmonary diseases and application as an immunomodulator.^[7,8] Most of Mildronate's clinical benefits are mediated through its modulation of the carnitine metabolism, which is the essential factor in regulating the cellular energy metabolism through fatty acid β -oxidation and glycolysis in the myocardium, as carnitine is the key molecule in fatty acid metabolism in mitochondria. As an analogue of carnitine, Mildronate inhibits the last step of carnitine biosynthesis by inhibition of γ -butyrobetaine hydroxylase, which catalyzes the formation of L-carnitine from γ -butyrobetaine (GBB). Furthermore, Mildronate inhibits the transport of carnitine through the cell membranes of liver and kidneys and reduces carnitine palmitoyl transferase-I (CPT-I) activity in the outer mitochondrial membrane.^[9]

Under aerobic conditions, carnitine improves myocardial functioning through enhancement of fatty acid β -oxidation that supplies about 80% of myocardial ATP generation.^[8] However, under oxygen deficiency, cytotoxic intermediates can accumulate in the cell due to insufficient oxygen supply. A reduced intracellular concentration of free carnitine leads to suppression of fatty acid metabolism and therefore enhances glycolysis during ischemia, which has a cytoprotective effect and increases the effectiveness of ATP-generation, as carbohydrate oxidation requires less oxygen per ATP molecule than β -oxidation of free fatty acids.^[10–14] Moreover, glycolysis is stimulated directly via Mildronate by increasing the expression of hexokinase type 1, which catalyzes the formation of glucose-6-phosphate from glucose.^[9]

Under sport-physiological aspects, reports on positive effects on the physical working capacity of elite athletes were published and dosages of Mildronate (per os between 0.25 and 1.0 g twice a day over 2–3 weeks during the training period and 10–14 days before competition) were discussed. Further studies demonstrated an increase in endurance performance of athletes, improved rehabilitation after exercise, protection against stress, and enhanced activations of central nervous system (CNS) functions.^[15,16] Moreover, Mildronate shows mood-improving effects as well as an increased learning and memory performance, which are properties athletes may also benefit from.^[8,17,18] In dubious online shops, the performance-enhancing effects of Mildronate are advertised overtly.^[19] Additionally, Mildronate-containing products can also be obtained from well-known online auction platforms as an over-the-counter (OTC) drug, which give easy access to the drug worldwide.

Since January 2015, Mildronate is subject of WADA's Monitoring Program to assess its prevalence and misuse in sport, necessitating methodologies capable of measuring and confirming the presence and absence of this drug in human urine.^[20] In recent years, different assays were presented for the identification of Mildronate in human plasma and urine, while most of them were based on hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS).^[21–24]

In the present study, two approaches were established. One aimed at the implementation of the analyte into an existing initial test method to enable the detection at lowest possible additional workload for the laboratory and another allowing the unequivocal confirmation of findings using isotope-dilution hydrophilic interaction liquid chromatography high resolution/high accuracy mass spectrometry (HILIC-HRMS).

Experimental

Chemicals and reagents

1,1-Dimethylhydrazine, tert-butylhydroquinone, methyl-acrylate, dimethylsulphate-D₆, calcium hydroxide and the reference compound of Mildronate dihydrate were obtained from Sigma-Aldrich (Deisendorf, Germany), while Mildronate-D₃ was synthesized in house for the use as internal standard (IS). Acetonitrile and acetone were bought from VWR International (Darmstadt, Germany). Acetic acid, ammonium acetate, and ethanol were supplied by Merck (Darmstadt, Germany). Deionized water was obtained from a water purification system (Sartorius Stedim Biotech S.A., Aubagne, France).

Synthesis of labelled IS

For in-house synthesis of Mildronate-D₃ (3-(2,2,2-trimethylhydrazinium)propionate-D₃) as IS, 37.9 g (0.63 mol) of 1,1-dimethylhydrazine was added to 0.19 g (1.1 mmol) of stirred and heated (50±5°C) tert-butylhydroquinone. To form the intermediate product of 3-(2,2-dimethylhydrazino)methylpropionate, 51.6 g (0.6 mol) of methyl acrylate was added and the mixture was heated up to 80±5°C for 2.5 hs. To stop the reaction process, the mixture was cooled in ice bath, and subsequently, 100 mL of acetone and hexadeuterated dimethylsulphate was added. Thereafter, the reaction mixture was maintained at 50–60°C for 5 h. After removing the solvent by distillation, the emulsive product of hexadeuterated 3-(2,2,2-trimethylhydrazinium)methylpropionate methylsulphate was obtained. To remove the methylsulphate-D₃ residue, 500 mL of distilled water, 200 mL of ethanol (96%), and calcium hydroxide (54.2 g, 0.73 mol) was added to the reaction mixture. After heating at 50–60°C for 2 h the formed calcium sulphate dehydrate precipitated and was removed by filtration. The filtrate was concentrated under reduced pressure and the 3-(2,2,2-trimethylhydrazinium)propionate-D₃ dihydrate was obtained (Figure 1).^[25] The synthesized internal standard, Mildronate-D₃ was characterized using high resolution/high accuracy mass spectrometry (Figure 2).

Sample preparation

For the initial testing assay, two aliquots of urine samples each with 45 μ L were pooled and fortified with 10 μ L of Mildronate-D₃ (IS, conc. 1 μ g/mL). After shaking, the mixture was injected into the instrument (reversed phase liquid chromatography-tandem mass spectrometry; RPLC-MS/MS).

For confirmatory analysis, suspicious urine samples were diluted appropriately using deionized water. Subsequently, an aliquot of 270 μ L of the diluted urine sample was fortified with 30 μ L internal standard (Mildronate-D₃, conc. 1 μ g/mL). The mixture was further diluted with 700 μ L of acetonitrile and 100 μ L of a 100 mM ammonium acetate solution. The samples were mixed and an aliquot of 20 μ L was injected into the instrument. Mildronate concentrations were estimated semi-quantitatively using a single-point calibrator (Mildronate reference standard).

LC-MS/MS

Initial testing

For initial testing, an ion transition diagnostic for Mildronate was implemented in a frequently used multi-target LC-MS/MS method for the determination of diuretics, stimulants, masking agents,

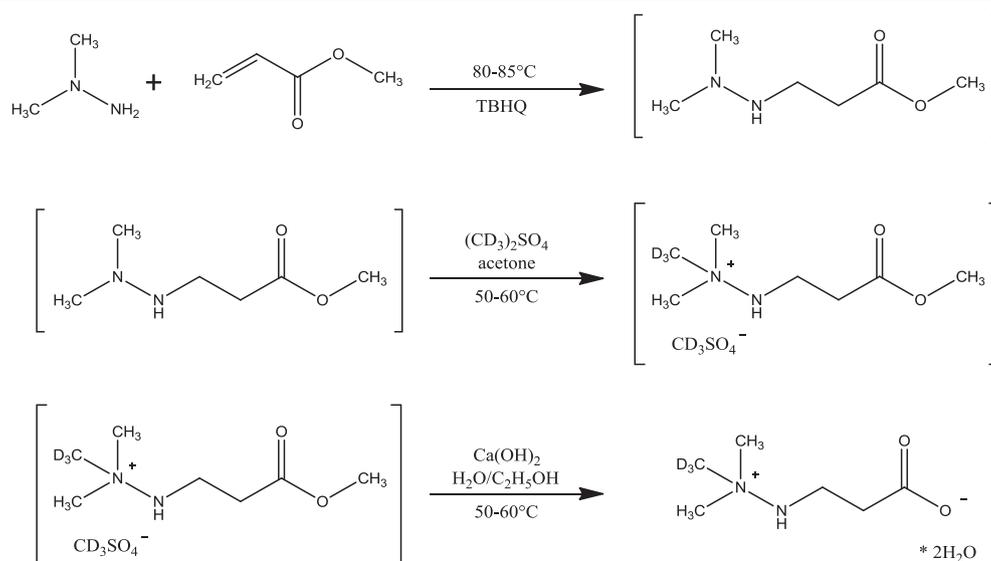


Figure 1. Synthesis of Mildronate-D₃ (3-(2,2,2-trimethylhydrazinium)propionate-D₃) as internal standard.

SARMs, and others, which is based on direct injection of urine samples.^[26] In brief, a linear gradient with 5 mM ammonium acetate buffer containing 0.1% glacial acetic acid (pH = 3.5, solvent A) and acetonitrile (solvent B) on a Nucleodur C18 Pyramid analytical column (2 x 50 mm, 3 mm particle size; Macherey-Nagel, Düren, Germany) was used. For mass spectrometric detection, a hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex 5500 QTrap; Darmstadt, Germany) interfaced by an electrospray ionization ion-source operating in both positive and negative ionization mode was applied. The analyte and the internal standard Mildronate-D₃ were detected utilizing multiple reaction monitoring (MRM) of the diagnostic ion transitions at m/z 147→58 (Mildronate) and at m/z 150→61 (Mildronate-D₃) (CE = 30eV, dwell time = 10 ms).

Confirmatory analysis

To confirm suspicious initial testing results, a HILIC-HRMS approach was developed. The method is based on direct injection of diluted urine specimens followed by an effective online sample-clean up, accomplished by a dual pump setup in combination with a HILIC trapping column (Nucleodur HILIC, 20 x 2 mm, particle size 3 μm, Macherey-Nagel, Düren, Germany) and a HILIC analytical column (Nucleodur HILIC, 100 x 2 mm, particle size 1.8 μm, Macherey-Nagel, Düren, Germany). The LC dual pump system consisted of an Agilent 1100 Series binary pump operating in isocratic mode and an Accela 1250 quaternary pump (Thermo Fisher Scientific, Bremen, Germany) for gradient elution. For sample injection a Thermo PAL autosampler (Thermo Fisher Scientific, Bremen, Germany) was utilized. Both pumps operated at a flow rate of 250 μL/min. Mobile phase was composed of deionized water (solvent A), acetonitrile (solvent B) and a 200 mM ammonium acetate buffer containing 0.15% glacial acetic acid (pH = 5.5, solvent C). The Initial conditions of 0% A, 95% B and 5% C were isocratically held for 1 min before switching valve positions to backflush from trapping to analytical column. The content of 5% C was maintained stable throughout the gradient, while the content of solvent B was decreased linear from 95% to 40% within 10 min. After 2 min of isocratic elution, re-equilibration started for 5 min at initial mobile phase conditions. The overall runtime was 18 min (injection-to-injection).

For the detection of Mildronate a Q Exactive hybrid quadrupole-orbitrap® mass spectrometer (Thermo Fisher Scientific, Bremen,

Germany) interfaced by an electrospray ionization ion-source (ESI) operating in positive mode (+4 kV, source temperature 300 °C, capillary temperature 350 °C) was utilized. The positively charged precursor ion at m/z 147.1128 was detected using full-scan mode, while the diagnostic product ions (m/z 58.0651, m/z 59.0730 and m/z 132.0893) were detected using targeted higher energy collision dissociation (t-HCD) acquisition mode with a resolution of 17,500 FWHM and an isolation window for the quadrupole of 1 Da. Automated gain control value was set to 2×10^5 with a maximum allowed fill time of 100 ms. The applied normalized collision energy was set to 50%. The collision gas utilized was nitrogen provided by a CMC nitrogen generator (CMC Instruments, Eschborn, Germany).

Method validation

Method validation of the analytical approaches were carried out according to WADA guidelines considering the parameters specificity, intra- and inter-day precision at low, medium and high concentration levels (*initial testing*: 1 μg/mL, 10 μg/mL, 100 μg/mL; *confirmation*: 1 μg/mL, 5 μg/mL, 10 μg/mL) linearity (*initial testing*: 0.5, 1.0, 5.0, 10.0, 50.0, 100.0 μg/mL; *confirmation*: 1.0, 2.5, 5.0, 7.5, 10.0, 12.5 μg/mL), lower limit of detection (LLOD), ion suppression / enhancement effects and robustness.^[27]

Routine doping control samples

Over a period of six months, analysis of a total of 8320 routine doping control samples (female/male/unknown: 2455/5846/19) from elite athletes covering different classes of sport as well as in- and out-of-competition samples (IC/OOC: 4459/3861) were analyzed for the presence of Mildronate.

Results and discussion

Mass spectrometry

The MS behaviour of Mildronate and the IS Mildronate-D₃ was studied using high resolution/high accuracy mass spectrometry. In Figure 2, the t-HCD product ion spectra of the positively charged precursor ions at m/z 147.1129 (C₆H₁₅N₂O₂, [M⁺]) and m/z

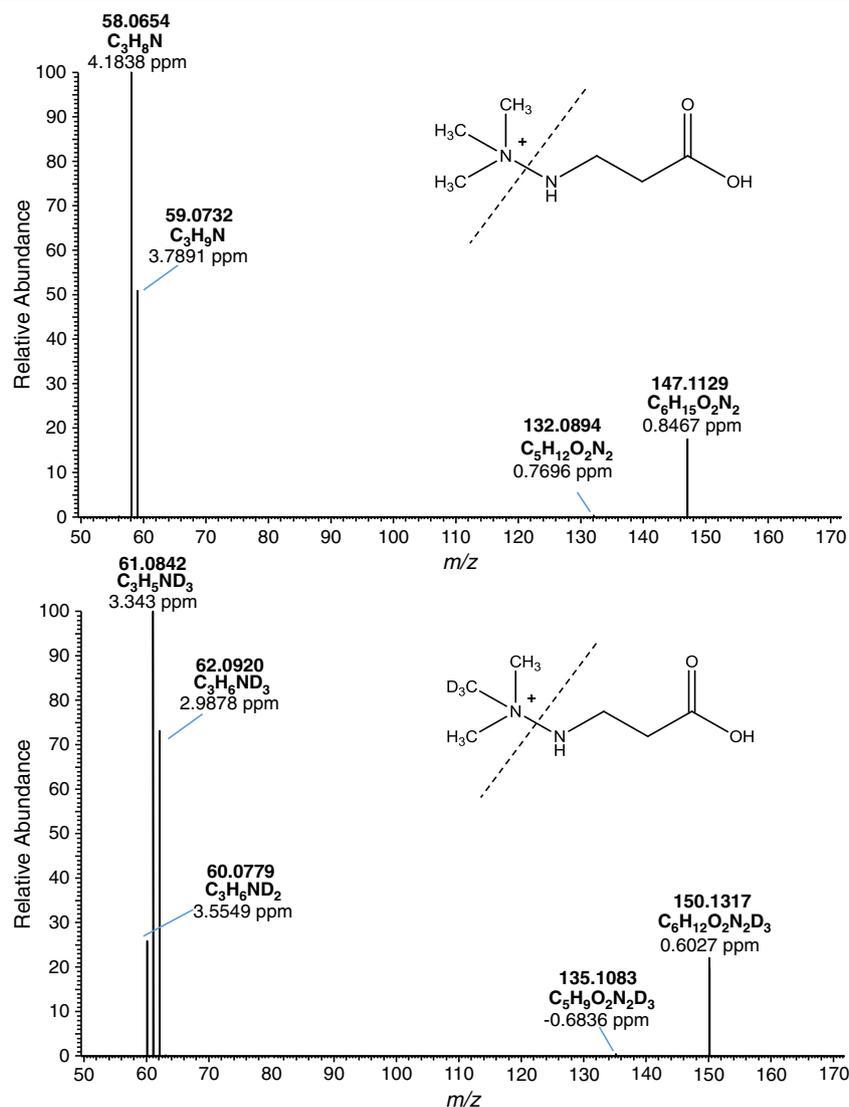


Figure 2. Product ion t-HCD mass spectra of the permanent positively charged Mildronate and Mildronate-D₃ (IS) precursor ions at m/z 147.1129 and m/z 150.1317 measured in a spiked urine sample by means of high resolution/high accuracy mass spectrometry using a Q Exactive hybrid quadrupole-orbitrap® mass spectrometer (17,500 FWHM, NCE: 50%).

150.1317 ($C_6H_{12}N_2O_2D_3$, $[M]^+$) are depicted. When subjected to a normalized collision energy (NCE) of 50% the Mildronate precursor ion dissociates leading to at least 3 product ions: m/z 58.0654 (C_3H_9N), m/z 59.0732 (C_3H_9N) and m/z 132.0894 ($C_5H_{12}N_2O_2$). The most abundant product ions at m/z 58.0654 and m/z 59.0732 resulted from the loss of the trimethylamine residue, while the only additional fragment at m/z 132.0894 is formed by elimination of a methyl group (-15 Da) from the precursor ion. The proposed collision-induced fragmentation pathway of Mildronate could be further confirmed by HRMS data of the IS Mildronate-D₃. Here, for the precursor ion as well as for the described product ions a 3 Da shift of the *mass-to-charge* ratio was observed due to the substitution with a deuterated methyl group at the amino residue (m/z 150.1317 ($C_6H_{12}N_2O_2D_3$, $[M]^+$), m/z 61.0842 ($C_3H_6ND_3$), m/z 62.0920 ($C_3H_6ND_3$), m/z 135.1083 ($C_5H_{12}N_2O_2D_3$). Moreover, for the IS an additional fragment at m/z 60.0779 ($C_3H_6ND_2$) was detected, according to proton/deuteron substitution. Our results, based on accurate mass measurements, are in accordance with proposed collision-induced fragmentation pathways of Mildronate in recent publications.^[28,29]

Liquid chromatography

Since Mildronate is of comparably low molecular mass and comprises a permanent charge, its retention on conventional reversed-phase materials is modest. However, the sensitivity and specificity of the applied initial analytical approach was sufficient to allow screening for Mildronate-containing urine samples, especially in consideration of the recommended daily dose of a few hundred to thousand mg per day. Due to Mildronate's highly polar chemical structure (Figure 2), the analyte elutes at the very beginning of the chromatographic run (RT: 0.52 min). Here, a deuterated internal standard is necessarily recommended to compensate for matrix effects. For confirmatory analysis, a more sophisticated chromatography using a HILIC stationary phase was established. In Figure 3, the extracted ion chromatograms generated with the confirmatory HILIC-HRMS approach are shown, representing a blank urine sample, a spiked urine sample containing Mildronate at 1 μ g/mL, and an authentic doping control urine sample found to contain Mildronate. The data illustrate that the chosen methodology provides the required chromatographic retention of the target

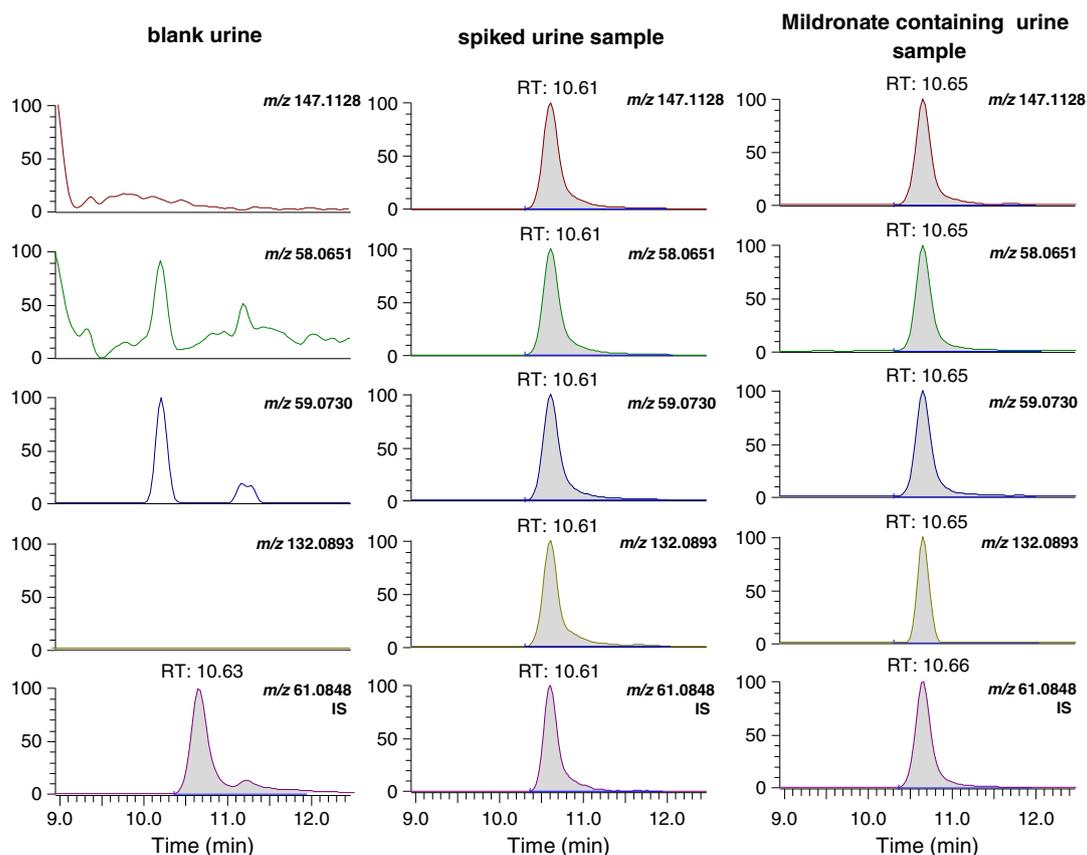


Figure 3. Extracted ion chromatograms of Mildronate (m/z 147.1128; resolution: 17,500 FWHM, NCE: 50%) of a blank urine sample, a spiked urine sample at 1 $\mu\text{g/mL}$ and a diluted urine sample (142 $\mu\text{g/mL}$).

analyte and unambiguous mass spectrometric data for the identification of the xenobiotic.

Method validation

The fitness-for-purpose of the employed analytical approaches for the initial testing and confirmation of Mildronate was determined according to WADA guidelines.^[27] The results of the method validation are summarized in Table 1. Analysis of 10 different blank urine specimens (5 female and 5 male) demonstrated good specificity without any interfering signals for both assays and almost zero biological noise using high resolution/high accuracy mass spectrometric detection. Furthermore, both assays allowed the reliable detection of Mildronate in 10 different spiked urine specimens at

a concentration of 1 $\mu\text{g/mL}$ with reproducible retention times. Moreover, the confirmation assay provides stable product ion ratios according to relevant WADA criteria.^[30] Both approaches are characterized by good intra- and inter-day precisions at low, medium and high concentration levels (*intra-day*: initial testing: 5.9 – 12.3%; confirmation: 7.0 – 8.4%; *inter-day*: initial testing: n.d.; confirmation: 9.9 – 12.9%).

The lower limit of detection (LLOD) was estimated either by measuring the respective signal to noise ratio ($S/N > 3$; initial testing) or by estimation of the lowest concentration that could reliably be detected in urine samples with reproducible RT and mass accuracy of less than 5 ppm (confirmation assay). Regarding the administered amount of up to 2000 mg per day and the half-life of around 6.5 h, the estimated LLOD at 200 ng/mL for the initial testing assay

Table 1. Validation results

| | LLOD [ng/mL] ($n = 6$) | ion suppression ($n = 6$) | calibration curve | robustness (r_{RT} , $n = 6$) | intra-day precision ($n = 6/6/6$) | | inter-day precision ($n = 18/18/18$) | |
|-----------------|-----------------------------|--------------------------------|---|--------------------------------------|--|---------------------|---|---------------------|
| | | | | | levels [$\mu\text{g/mL}$] | CV [%] | levels [$\mu\text{g/mL}$] | CV [%] |
| initial testing | <200 | 71 – 93% | slope: 1.0034 intercept: 3.5106 ($R^2 = 0.9901$) | 1.7% | 1.0 10.0 100.0 | 12.3 5.9 10.9 | — | — |
| confirmation | <10 | 73 – 97% | slope: 0.4554 intercept: 0.0440 ($R^2 = 0.9971$) | 0.3% | 1.0 5.0 10.0 | 8.4 7.0 7.0 | 1.0 5.0 10.0 | 9.9 12.9 12.1 |

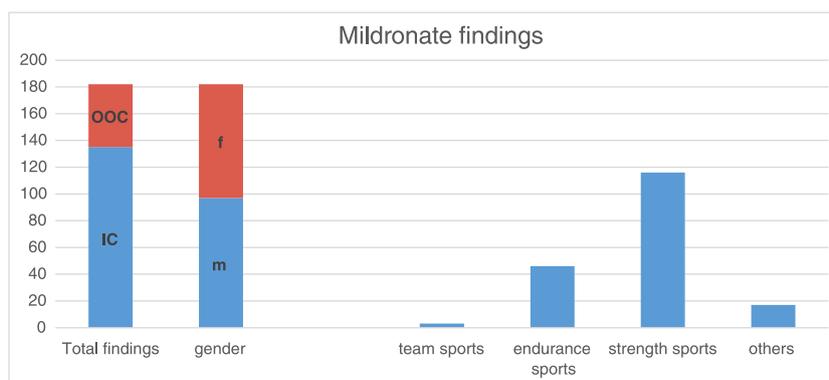


Figure 4. Mildronate findings in official doping control samples ($n = 8320$) and distribution between in- and out-of-competition samples (IC/OOC), gender (f = female; m = male) and type of sports (team sports, endurance sports, strength sports, others)

and 10 ng/mL for the confirmation assay is suggested to be sufficient for a reliable Mildronate detection over a couple of days after administration.^[29] Linearity was tested in the range between 0.5 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ (initial testing) as well as 1.0 $\mu\text{g/mL}$ and 12.5 $\mu\text{g/mL}$ (confirmation), providing correlation coefficients > 0.99 between Mildronate concentration and signal response. Ion suppression / enhancement effects were determined by comparing six different urine samples spiked with 1 $\mu\text{g/mL}$ of Mildronate with matrix-free standard solutions at corresponding concentrations. The matrix effect ranged from 71 to 93% (initial testing) and from 73 to 97% (confirmation). Although the method demonstrated sufficient robustness under these conditions, the usage of the in-house synthesized Mildronate-D₃ as internal standard is recommended.

Routine doping control samples

A total of 8320 random doping control urine samples covering different classes of sport either from in- or out-of-competition were analyzed for the presence of Mildronate. In total 182 positive Mildronate findings (2.2%) in a concentration range between 0.1 and 1428 $\mu\text{g/mL}$ were detected and confirmed using the employed HILIC-HRMS assay. Mildronate was found in both in- and out-of-competition samples (IC: 135 (74%); OOC: 47 (26%)), and no considerable gender specific differences regarding the number of findings (female: 85 (47%); male: 97 (53%)) (Figure 4) or the mean Mildronate concentrations (female: 120.9 $\mu\text{g/mL}$; male: 136.0 $\mu\text{g/mL}$) were observed. Within the investigated classes of sport Mildronate findings were found to a greater extent in samples from strength sports (67%) and endurance sports (25%). However, the misuse of Mildronate is not limited to a particular sport or to a group of sports but is rather used in a wide range of sports. In the absence of further information as to why the drug was (mis)used, the findings of high Mildronate concentrations in samples originating not from so-called 'high-risk' sport disciplines are alarming.

Conclusion

Mildronate, an approved drug with multiple indications besides its anti-ischemic properties, is known to have a positive effect on the endurance performance of athletes, improves rehabilitation after exercise, protect against stress and activates CNS functions. So far, Mildronate is not part of WADA's Prohibited List. Effects on the human organism similar to those exhibited by the prohibited

substance Trimetazidine are given, as both substances cause a significant inhibition of the β -oxidation of free fatty acids.^[1,31] Originally included in Section S6.b of the WADA Prohibited List, Trimetazidine was categorized under sub-section S4.5.3 in January 2015 due to its function of a metabolic modulator of the cardiac metabolism.

Based on a growing body of evidence concerning Mildronate misuse in sport and the inclusion of the substance into the 2015 WADA monitoring program, adequate test methods for both initial testing and confirmation of the analyte were established. The molecule's specific physico-chemical properties and the fact that the drug is mostly excreted unchanged via renal route makes Mildronate an ideal analyte for the presented 'dilute-and-inject' HILIC-HRMS approach. The reduced sample preparation, the flexibility of the present approach and the compatibility of the initial testing assay with existing analytical protocols enables the simple implementation of Mildronate into screening assays of fellow anti-doping laboratories for utmost comprehensive monitoring of the substance's misuse.

Furthermore, the present study indicates the wide prevalence of Mildronate in international elite sports and demonstrates the alarming extent of administered dosages, finding more than 180 cases of Mildronate use within numerous different sport disciplines and urinary concentration levels of more than 1 mg/mL. Additionally, the easy access to Mildronate from numerous online shops certainly plays an important role for the widespread use in international elite sports. Under medical and pharmacological aspects as well as to preserve the integrity of sport the ban of Mildronate from sport is deemed indicated.

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