Heliyon 6 (2020) e04771

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Meldonium determination in milk and meat through UHPLC-HRMS

A. Temerdashev *, A. Azaryan, E. Dmitrieva

Kuban State University, Analytical Chemistry Department, Krasnodar, 350040, Russia

ARTICLE INFO

Keywords:
Analytical chemistry
Food analysis
Meldonium
UHPLC-HRMS
Milk
Meat
Liver
Doping
Food safety
Quantification
Emidonol

ABSTRACT

A simple and sensitive procedure for the quantification of meldonium in milk and meat by UHPLC-HRMS is presented. Some products were investigated to contain this substance due to using a veterinary drug called "Emidonol". According to the instruction for this drug, it can be used for injection (for cows) and as an additive in drinking water for chickens. Although meldonium is not a threat for human health, it is strictly prohibited in professional sports according to WADA Prohibited List. Sample preparation conditions were optimized for both matrices that allowed to eliminate matrix effects and achieve reproducible and accurate results. Protein precipitation with dilution were applied for milk samples, while chicken meat and liver were homogenized with quartz sand to achieve satisfactory meldonium recovery. The results of milk and meat samples analysis purchased at the farmers' fair are presented in this article. Meldonium concentration in raw milk was investigated to be up to 880 ng/mL. However, pasteurization can be used for partial cleanup from meldonium (up to 2 times). The same research was conducted for chicken meat and liver. Thermal treatment shows a good result for a meat cleanup. The proposed method was partially validated, limits of detection and quantification were established for each matrix.

1. Introduction

Meldonium, 3-(2,2,2-trimethylhydrazinium)propionate dihydrate, was first synthesized at the Institute of Organic Synthesis in Latvia in 1970 for veterinary medicine (Eremeev et al., 1984). However, it found its first application in Eastern European countries as an anti-ischemic agent (Sobolevsky et al., 2015; Ivanovskaya and Anisimova, 1992). The pharmacokinetics and detection time for meldonium were previously reported for humans and rats (Peng et al., 2010; Zhang et al., 2013; Yoshisue et al., 2000). Meldonium elimination from urine was noticed to be nonlinear, and it could be detected more than a week after a single administration of meldonium at a dose of 250 mg (Zhang et al., 2013). Currently, it is widely used in clinical practice in the CIS countries for the treatment of chronic heart failure, acute disorders of cerebral circulation, or athletic overexertion. It is still used for veterinary purposes in "Emidonol" drug in the form of its stable complex: 3-(2,2,2-trimethylhydrazinium)propionate-2-ethyl-6-methyl-3-hydroxypyridine disuccinate (Figure 1), which becomes a native meldonium in the metabolism process that almost fully excretes from the body in the native form.

One of the purposes of this drug usage is its antihypoxic and low sedative effects for animals, which are especially important for farms. In addition, it is not prohibited as a veterinary drug and has no limitations for use. As a result, it is possible to find meldonium traces in human biological fluids after consuming products contaminated with meldonium which may be dangerous for professional athletes – since January 1 of 2016 it has been included in the WADA Prohibited List (2020 WADA Prohibited list, 2020; Notice – Meldonium, 2016).

Nowadays, a few methods for meldonium determination in biological fluids (such as blood plasma and urine) mostly by liquid chromatography coupled to mass spectrometry have already been described (Table 1).

However, to the best of our knowledge, there have not been any methods of its quantification in products, therefore, the aims of the present research were to develop a procedure for the quantification of meldonium in such objects as cow milk and chicken meat, as well as to study the influence of thermal treatment on the analyte content in the studied matrices.

2. Experimental

2.1. Material and methods

Standard samples of meldonium (≥98%) and gabapentin (≥75%) were purchased from Grindex and Pfizer, respectively. Gradient-grade acetonitrile (Biosolve, Israel), 18.2 MΩ·cm water (Milli-Q, Millipore,

* Corresponding author.
E-mail address: TemerdashevAZ@gmail.com (A. Temerdashev).

https://doi.org/10.1016/j.heliyon.2020.e04771
Received 26 February 2020; Received in revised form 27 June 2020; Accepted 19 August 2020

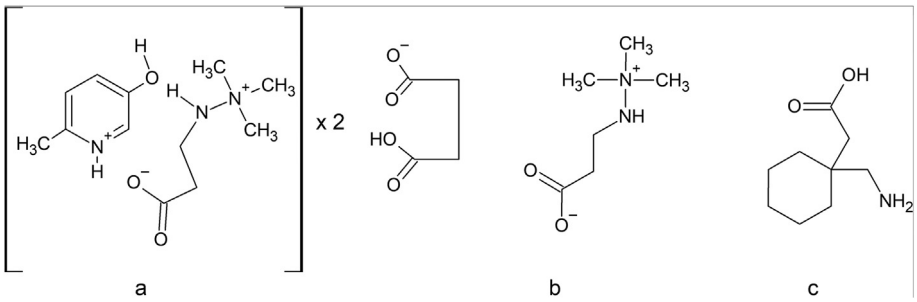

Figure 1. Emidonol (a), meldonium (b) and gabapentin (c) structures.

Table 1. Comparison of techniques for quantitative determination of meldonium.

Matrix	Sample preparation	Column	Detector	LOQ (LOD), ng/mL	References
Plasma	Dilution and centrifugation	Shim-pack VP-ODS C_{18} (150 × 4.6mm, 5μm)	HPLC-ESI(+)-MS/MS (QqQ)	10	Peng et al. (2010)
Plasma	Dilution and centrifugation	ACQUITY UPLC BEH HILIC (2.1 × 50 mm, 1.7μm)	UHPLC-ESI(+)-MS/MS (QqQ)	100	Zhang et al. (2013)
Urine	Dilution	Nucleodur C_{18} Pyramid (2 × 50 mm, 3 μm)	UHPLC-ESI(+)-MS/MS (QqQ)	500 (200)	Görgens et al., 2015
		Nucleodur HILIC (20 × 2 mm, 3μm)	UHPLC-ESI(+)-MS/MS (Orbitrap)	1000 (10)	
Urine	Dilution	Nucleodur HILIC (20 × 2 mm, 3μm)	UHPLC-ESI(+)-MS/MS (Orbitrap)	10	Görgens et al., 2017
Blood, dried blood spot, urine	flow-through desorption (DBS)	Hypersil Gold C8 (2.1 × 30mm, 1.9 μm)	UHPLC-ESI(+)-MS/MS (Orbitrap)	(20)	Tretzel et al. (2016)
Urine	Dilution	Fused-silica separation capillaries (50/375 μm, 55 cm and 25/375 μm, 65 cm)	CE-CD	20 (15)	Slampová and Kuban, 2017
Plasma	Deproteinization (1% TFA), p-bromophenacyl bromide derivatization	Silasorb 600 silica (4.6 × 150 mm)	HPLC-VWD	1000	Sahartova et al., 1993
Pharmaceuticals	Dilution	Inertsil CN - 3 column (4.6 mm × 250 mm, 5 μm)	HPLC-ELSD	10300	Cao et al. (2005)
Urine, blood	Dilution and centrifugation	Hypersil GOLD C_{18} (2.1 × 100 mm, 1.9 μm),	UHPLC-ESI(+)-MS/MS (Orbitrap)	10	Rabin et al. (2019)
Urine	Dilution and centrifugation	ACQUTY UPLC BEH HILIC (2.1 × 50 mm, 1.7 μm)	UHPLC-ESI(+)-MS/MS (QqQ)	500	Cai et al. (2011)
Plasma				100	
Urine	Dilution	Atlantis HILIC Silica (50 × 4.6 mm,3 μm)	UHPLC-ESI(+)-MS/MS (QqQ)	10 (3)	Forsdahl et al. (2018)

QqQ – triple quadrupole, CE-CD – capillary electrophoresis coupled with conductivity detector, VWD – variable wavelength detector, ELSD – Evaporative light scattering detector.

France) and ammonium acetate (reagent grade, Vecton, Russia) were used for mobile phase preparation. "Emidonol 20%" (20% 3-(2,2,2-trimethylhydrazinium)propionate-2-ethyl-6-methyl-3-hydroxypyridine disuccinate water solution) veterinary drug ("Agrovet", Russia) was purchased via Internet.

2.2. Samples for analysis

Seven cow milk samples (1 L) as well as chicken meat and liver were purchased on farmers' fair and stored no more than one day at 4 °C before analysis. Each sample was analyzed three times under the described conditions.

2.3. Milk pasteurization

Milk pasteurization process was conducted by its heating up to 90 °C with subsequent cooling without any continuous thermal treatment.

2.4. Sample preparation

To avoid any losses of analytes, "dilute-and-shoot" procedure was used to prepare milk samples for analysis. Acetonitrile was selected for sample dilution owing to its ability to precipitate proteins and chromatographic separation in HILIC mode, where acetonitrile acts as a weak solvent. According to the procedure, 200 μL of milk sample was diluted with 800 μL of acetonitrile containing internal standard (gabapentin, **ISTD**) in a 1.5 mL Eppendorf tube to achieve its final concentration, vortex mixed for 1 min followed by centrifugation at 10000 rpm for 10 min. 300 μL of collected supernatant was transferred into the glass vial with insert.

To achieve maximum extraction efficiency, 1 g of meat or liver sample was homogenized with quartz sand in glass tube using glass rod and 5 mL of water:acetonitrile mixture (10:90, v/v) containing 50 ng/mL ISTD was added. After 2 min of vortex mixing, 1 mL of extract was collected, centrifuged at 10000 rpm for 10 min and 300 μL of supernatant was transferred into the glass vial with insert.

Prepared milk samples can be stored in the autosampler tray at 4 °C no more than 36 h, while the analysis of meat and liver samples should be performed within 24 h after their preparation.

Dilution was required due to several reasons. Firstly, in hydrophilic liquid chromatography (**HILIC**) water is used as a strong eluent, as a consequence, the lack of dilution can result in peak tailing, retention time shifting and decreased sensitivity and selectivity of analysis. Secondly, dilution is required to decrease concentration of target analyte to avoid detector saturation in case of high concentrations. Lastly, it can be used to decrease matrix effects.

2.5. Instrumentation

A Bruker MaXis Impact quadrupole time-of-flight mass spectrometer equipped with an electrospray ionization source (ESI) and coupled with a Bruker Elute UHPLC system under a Bruker Compass HyStar 4.1 software

Table 2. Gradient elution conditions (mobile flow rate – 0.35 mL/min).

Time, min	A (acetonitrile), %	B (10 mM ammonium acetate), %
0	95	5
2	83	17
4	62	38
6	42	58
8	35	65
10	35	65
12	35	65
14	35	65
16	95	5

were used. Data analysis was performed with a Bruker Data Analysis 4.4 software. Separation was carried out on a Phenomenex Kinetex HILIC (100 × 2.1 mm, 2.6 μm) analytical column with the respective guard column. Samples were stored at an autosampler tray at 5 °C. To avoid autosampler needle contamination and blockage with fat during the sample storage, needle stroke height was set to 5 mm.

2.6. UHPLC-HRMS conditions

The mobile phase consisted of acetonitrile (mobile phase A) and 10 mM ammonium acetate in water (mobile phase B). Elution was performed in gradient mode with a total run time of 20 min (Table 2), which included column equilibration before next analysis. The flow rate was 0.35 mL/min, column temperature – 30 °C and injection volume was 10 μL.

Ionization was carried out by electrospray ionization in the positive mode with the mass spectrometer settings presented in Table 3. Nitrogen was obtained from a Peak NM32LA nitrogen generator (Peak Scientific, UK).

The analyte was detected in positive ionization mode due to ease of its protonation on an ESI source yielding abundant $[M + H]^+$ ion with further confirmation of its presence using product ion scan mode. Theoretical and observed m/z values are presented in Table 4.

Hydrophilic liquid chromatography (**HILIC**) is the method of choice for meldomium determination owing to its high polarity and low molecular mass. It also allows to achieve higher sensitivity during mass spectrometric quantification because of high acetonitrile content in the mobile phase.

To achieve high mass accuracy, mass spectrometer was calibrated using mixture, containing ammonium formate water solution and isopropyl alcohol in the beginning of each analysis.

3. Results

The assay aimed to quantify relatively high meldonium concentrations in the studied objects to prevent positive doping control results after consuming contaminated products.

Calibration curves were constructed in six replicates by spiking blank milk and meat (parts of leg and breast) samples obtained from farmers' fair with standard meldonium solutions at different concentrations (10, 12.5, 25, 50, 100, 125 and 250 ng/mL) and the internal standard at fixed concentration of 50 ng/mL. Gabapentin was chosen as an internal standard owing to its high polarity, small molecular weight and lack of its applicability for veterinary purposes. Calibration curves were constructed as a dependence of meldonium to gabapentin peak area ratio to meldonium concentration. As a result, linear calibration curves in the range from 10 to 250 ng/mL for milk and from 25 to 250 ng/mL for meat were constructed with the coefficients of determination (R^2) of 0.997 and 0.988, respectively. Slopes of six calibration curves obtained from different samples were in a good agreement. All calibration curves should be constructed using blank materials (milk, liver and meat) not originally containing peaks of target analyte and internal standard.

Selectivity tests were conducted by analysis of different blank samples by the proposed procedures. No peaks corresponding to the analyte or ISTD were observed at chromatograms at their retention times. It should be noted that meldonium has only one abundant product peak in MS/MS spectra with very low m/z (58), therefore, it does not make any sense to use this peak for qualitative analysis, but it can be used to confirm the presence of analyte in the sample by using ratio between precursor and product ions.

The *detection limits* (**LOD**), expressed as the signal-to-noise ratio of 3, were observed at 5 and 12.5 ng/mL for milk and meat, respectively. The lower limits of quantification (**LLOQ**), corresponding to the concentration determined with a 20% error, were 10 and 25 ng/mL for milk and meat, respectively.

Chromatograms of milk samples spiked at QC low are given in Figure 2. It should be also noticed that qualitative analysis was performed using precursor ion scans to achieve better sensitivity with further confirmation with MS/MS spectra registration.

Precision, expressed as the percentage relative standard deviation, and *accuracy* were evaluated by analyzing quality control samples of low, medium and high concentrations.

Accuracy was calculated according to the following formula:

$$percentage\ bias = \left(\frac{C_{observed} - C_{nominal}}{C_{nominal}}\right) \times 100$$

The results obtained are presented in Table 5. According to FDA criteria, the results of accuracy assessment should be within ±15% of nominal concentration of low, medium and high QCs.

Matrix effects were estimated by comparison of results obtained with the use of blank samples and aqueous matrices spiked with the analyte at different concentration levels that passed through the sample preparation procedure (Table 4).

Short-term stability of the analytes spiked into the milk and meat was tested after 4, 8, 12 and 24 h at 20 °C. According to this experiment, collected samples could be used for analysis not longer than after 4 h for meat and 12 h for milk (observed concentration deviation should be <15%).

Freeze-thaw stability tests were conducted within two weeks by performing three freeze (–20 °C) and thaw cycles of milk and meat samples spiked with the studied analyte. Differences in results between freshly prepared samples and thawed samples were compared. It was noticed that studied objects could be used only after two freeze-thaw cycles.

To exclude the possibility of false-positive results, blank samples were analyzed after the high QC with no *cross-contamination* observed.

3.1. Analysis of the products purchased at the farmers' fair

Validated method was used for analysis of real samples purchased on the farmers' fair from different vendors. 7 samples of raw and pasteurized milk and meat were analyzed (Table 6).

Only one vendor confirmed the usage of "Emidonol" for cows and chickens. As it can be seen from Table 5, positive samples were found in both types of products. According to the presented data, even thermal milk treatment does not guarantee full milk cleanup from meldonium.

It should be noted that the use of this veterinary drug should be as a 10-day course consisting of 6 days with daily injections and the rest 4 days without any injections. For better pharmacokinetics understanding, we asked the farmer to collect a few milk samples from one cow before, in the process and after the course. As it can be seen from Figure 3,

Table 3. Mass-spectrometric detection conditions.

Parameter	Value
Ion source temperature, °C	250
Capillary voltage, V	4000
End plate offset, V	500
Nebulizing gas pressure (nitrogen), kPa	100
Drying gas flow, L/min	5
Scan speed, Hz	3
Mass range, m/z	100–800
Collision gas pressure (nitrogen), mTorr	1.5

4. Conclusions

The use of the veterinary drugs for animals is a normal practice for modern agriculture and can help to prevent some illnesses, but it should be conducted with serious cautions – drinking of the raw or pasteurized milk or eating chicken meat may cause meldonium presence in urine, resulting in an athlete's disqualification.

Meldonium excretion kinetics in milk samples was shown. As a result, meldonium traces can be found in milk even few days after ending the course, therefore, it should not be consumed by professional athletes to avoid appearance of positive test for meldonium.

In the present paper, a simple, sensitive and fast method for meldonium quantification in some products by UHPLC-HRMS has been described for the first time. What is more, high sensitivity of this procedure allows to use it for preventive products control in routine analytical laboratories and makes possible additional samples dilution in case of extremely high concentrations of the analyte in various samples.

Table 4. Retention times, observed and theoretical masses and mass errors for analyte and ISTD.

Compound	Retention time, min	$[M + H]^+$, theoretical	$[M + H]^+$, observed	Mass error (MS), ppm	Product ion (observed), *m/z*
Meldonium	7.75	147.1128	147.1126	–0.8	58.0652
Gabapentin	5.16	172.1332	172.1331	0.6	137.0960 95.0855 67.0543

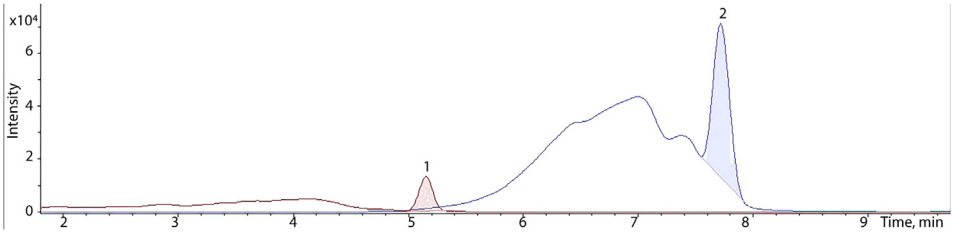

Figure 2. Correlation between meldonium concentration and sampling day during "Emidonol" course.

Table 5. Validation of the procedure (n = 6).

Object	QC concentration, ng/mL	Interday		Intraday		Matrix effect, %
		Accuracy, %	Precision, %	Accuracy, %	Precision, %	
Milk	25	–4.8	13.5	–5.7	11.8	9.4
	100	–2.1	9.2	3.4	10.4	6.2
	250	1.9	5.1	2.7	7.2	2.8
Meat/Liver	50	–12.1	11.3	–14.1	12.9	11.6
	100	6.2	9.5	10.5	11.2	7.4
	250	4.7	8.4	6.6	9.0	3.9

Table 6. Testing results for the products purchased on the farmers' fair.

Vendor	Meldomium concentration in product				
	Raw milk, ng/mL	Pasteurized milk, ng/mL	Chicken meat, ng/g	Chicken liver, ng/g	Roasted chicken liver, ng/g
Farmer 1	–	–	–	–	–
Farmer 2	–	–	230 ± 30	920 ± 75	650 ± 82
Farmer 3	–	–	–	–	–
Farmer 4	–	–	–	–	–
Farmer 5	650 ± 51	343 ± 31	–	–	–
Farmer 6	–	–	–	–	–
Farmer 7	–	–	–	–	–

meldonium concentration dramatically increases after beginning of the injections followed by a decrease after 7 days. According to the data, collected cow milk could be a source of meldonium up to one week after the ending of veterinary drug injections and should not be consumed by professional athletes to avoid appearance of positive test for meldonium.

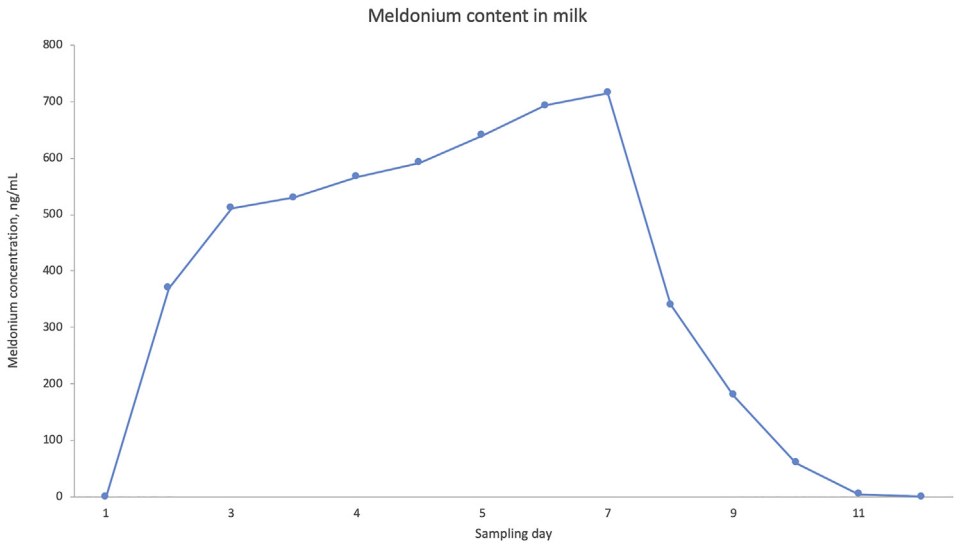

Figure 3. Extracted ion chromatograms of milk sample spiked at QC low: 1 – gabapentin, 2 – meldonium.

The following quantification limits can be achieved using the proposed procedure: 10 ng/mL for milk and 25 ng/mL for chicken meat and liver extracts.

Declarations

Author contribution statement

Alice Azaryan: Performed the experiments.

Ekaterina Dmitrieva: Analyzed and interpreted the data; Wrote the paper.

Azamat Temerdashev: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Funding statement

Dr. Azamat Temerdashev was supported by Российский Фонд ФундаМентальных Исследований (РФФИ) (18-33-20009 mol_a_ved). Dr. Azamat Temerdashev was supported by Russian Ministry of Science and Higher Education (FZEN-2020-0022).

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

Cai, L.J., et al., 2011. Determination of Mildronate in human plasma and urine by UPLC–positive ion electrospray tandem mass spectrometry. Chromatographia 73, 659–665.

Cao, G.-Y., et al., 2005. Determination of mildronate and related substances by HPLC-ELSD. Chin. Pharmaceut. J. 40, 864–866.

Eremeev, A., et al., 1984. 3-(2,2,2-trimethylhydraziniumdpropionate and method for the preparation and use thereof. US Patent No. 4, 481, 218.

Forsdahl, G., et al., 2018. Urinary excretion studies of meldonium after multidose parenteral application. J. Pharm. Biomed. Anal. 161, 289–295.

Görgens, C., et al., 2015. Mildronate (meldonium) in professional sports–monitoring doping control urine samples using hydrophilic interaction liquid chromatography-high resolution/high accuracy mass spectrometry. Drug Test. Anal. 7, 973–979.

Görgens, C., et al., 2017. The atypical excretion profile of meldonium: comparison of urinary detection windows after single- and multiple-dose application in healthy volunteers. J. Pharm. Biomed. Anal. 138, 175–179.

Ivanovskaya, E.A., Anisimova, L.S., 1992. Inversion voltammetric determination of dihydrate-3-(2,2,2-trimethyl hydrazinium) propionate. J. Anal. Chem. 47, 1228–1231.

Notice - Meldonium, 2016. World Anti-doping Agency. https://wada-main-prod.s3.amazonaws.com/resources/files/wada-2016-04-12-meldonium-notice-en.pdf.

Peng, Y., et al., 2010. Determination of mildronate by LC-MS/MS and its application to a pharmacokinetic study in healthy Chinese volunteers. J. Chromatogr. B Analyt. Technol. Biomed Life Sci. 878, 551–556.

Rabin, O., et al., 2019. Meldonium long–term excretion period and pharmacokinetics in blood and urine of healthy athlete volunteers. Drug Test. Anal. 11, 554-566.

Sahartova, O., Shatz, V., Kalvins, I., 1993. HPLC analysis of mildronate and its analogues in plasma. J. Pharm. Biomed. Anal. 11, 1045–1047.

Slampová, A., Kuban, P., 2017. Rapid determination of meldonium in urine samples by capillary electrophoresis with capacitively coupled contactless conductivity detection. J. Chromatogr. A. 1468, 236–240.

Sobolevsky, T., Dikunets, M., Rodchenkov, G., 2015. Use of meldonium (Mildronate) and emoxypine (Mexidol) by Russian athletes: a prevalence study. In: Manfred Donike Workshop, 33rd Cologne Workshop on Dope Analysis, pp. 210–213.

Tretzel, L., et al., 2016. Analyses of meldonium (mildronate) from blood driedblood spots (DBS), and urine suggest drug incorporation into erythrocytes. Int. J. Sports Med. 37, 500–502.

2020 WADA Prohibited List, 2020. https://www.wada-ama.org/sites/default/files/wada_2020_english_prohibited_list_0.pdf.

Yoshisue, K., 2000. Pharmacokinetics and biological fate of 3-(2,2,2-trimethylhydrazinium) proionate (MET-88), a novel cardioprotective agent, in rats. Drug Metab. Dispos. 28, 687–694.

Zhang, J., et al., 2013. Nonlinear pharmacokinetic properties of mildronate capsules: a randomized, open-label, single- and multipledose study in healthy volunteers. Fundam. Clin. Pharmacol. 27, 120–128.