Research article

Meldonium determination in milk and meat through UHPLC-HRMS

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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, USA) were used in the experiment.

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France) and ammonium acetate (reagent grade, Vecton, Russia) were used for mobile phase preparation. ‘Emidonol 20%’ (20% 3-(2,2,2-tri-
methylhydrnzinium)propionate-2-ethyl-6-methyl-3-hydroxypyridine

dissucinate 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’ farm 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 chromo-

tagraphic 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,

was homogenized with quartz sand in glass tube using glass rod and 5 mL

of collected 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

literature. According to the procedure, 200 μL of milk sample was diluted

with 800 μL of acetonitrile containing internal standard (gabapentin,
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 meldonium 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 isopropl alcohol in the beginning of each analysis.

| 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                             |

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:

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

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,
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.

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 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   |

### 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  |

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

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

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

| Vendor             | Meldonium 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           | –               | –                        | –                   | –                     | –                       |

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

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|--------|-------------------------|----------------------|----------------------|-----------------------|----------------------|-----------------|
| Milk   | 25                      | -4.8                 | -5.7                 | 13.5                  | 11.8                 | 9.4             |
|        | 100                     | -2.1                 | 3.4                  | 9.2                   | 10.4                 | 6.2             |
|        | 250                     | 1.9                  | 2.7                  | 5.1                   | 7.2                  | 2.8             |
| Meat/Liver | 50                  | -12.1                | -14.1                | 11.3                  | 12.9                 | 11.6            |
|        | 100                     | 6.2                  | 10.5                 | 9.5                   | 11.2                 | 7.4             |
|        | 250                     | 4.7                  | 6.6                  | 8.4                   | 9.0                  | 3.9             |

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| Farmer 3 | –               | –                        | –                   | –                     | –                       |
| Farmer 4 | –               | –                        | –                   | –                     | –                       |
| Farmer 5 | 650 ± 51        | 343 ± 31                 | –                   | –                     | –                       |
| Farmer 6 | –               | –                        | –                   | –                     | –                       |
| Farmer 7 | –               | –                        | –                   | –                     | –                       |
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.

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**Competing interest statement**

The authors declare no conflict of interest.

**Additional information**

No additional information is available for this paper.

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