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ARTICLES

Determination of Meldonium in Human Urine by HPLC with Tandem Mass Spectrometric Detection

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Abstract—A procedure is proposed for determining Meldonium in human urine, including sample preparation to analysis and analyte determination by HPLC with tandem mass spectrometric detection. For sample preparation, the procedure of "dilute-and-shoot" was used. The lower limit of the analytical range is 10 ng/mL; the limit of detection is 7.5 ng/mL; and the linearity range is 10–250 ng/mL. The proposed procedure is tested on real samples obtained from volunteers. A possibility of the direct analysis of urine samples after dilution is demonstrated; the limit of detection is 20 ng/mL. The high sensitivity of the procedure ensures its use for the determination of Meldonium in clinical diagnosis and doping control.

Keywords: Meldonium, HPLC–MS/MS, doping control

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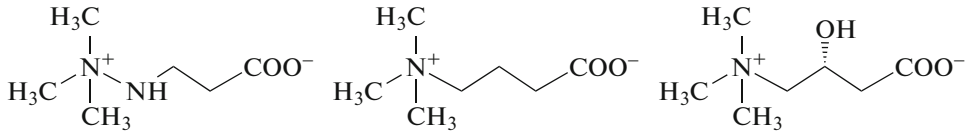
Meldonium, 3-(2,2,2-trimethylhydrazinium)propionate dihydrate, was first synthesized at the Institute of Organic Synthesis in Latvia in 1970 for veterinary medicine [1]. However, it found its first application in Eastern European countries as an anti-ischemic agent [2]. Currently, it is widely used in clinical practice in the CIS countries for the treatment of chronic heart failure, acute disorders of cerebral circulation, or athletic overexertion.

Meldonium is a complete structural analogue of natural γ-butyrobetaine, a precursor of carnitine (diagram). Because of its high polarity and small molecular weight, the determination of Meldonium in biological objects can be hampered by strong matrix effects and a lack of retention in reversed-phase HPLC, which necessitates the use of hydrophilic chromatography. It is seen from the diagram that there are no chromophore groups in the Meldonium structure; therefore, UV or fluorescence detection is inapplicable without preliminary derivatization. At the same time, this compound is easily ionized, which in combination with hydrophilic chromatography ensures the achievement of sufficiently low limits of detection.



(I) **(II)** **(III)**

Chemical structures of Meldonium (**I**), γ-butyrobetaine (**II**), and carnitine (**III**).

To date, a number of chromatography and chromatography–mass spectrometry procedures are known for determining Meldonium in urine and plasma [3–7], in which carbachol [3], acetaminophen [5], mildronate-d5 [7], and levocarnitine [4] are used as internal standards. The use of levocarnitine, which is an endogenous compound, as an internal standard is not useful, as the error of quantification significantly increases. In addition, the use of Meldonium can affect the production of free levocarnitine in the body [8].

Since January 1, 2016, Meldonium has been included in the list of banned substances by the World Anti-Doping Agency (WADA) [9] because of its trimetazidine-like effects on the body [10], which makes it urgent to develop a method for determining

Table 1. Conditions of gradient elution; the eluent flow rate, 0.3 mL/min

Time, min	A (acetonitrile), %	B (a 10 mM ammonium acetate solution), %
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

trace amounts of Meldonium in urine by HPLC with tandem mass spectrometric detection (HPLC–MS/MS).

EXPERIMENTAL

Materials and methods. We used standard samples of Meldonium (≥98%, Grindex) and gabapentin (≥75%, Pfizer). Acetonitrile for HPLC–MS (Biosolve, Israel), 18.2 MΩ cm (Milli-Q, Millipore, France), and ammonium acetate (cp grade, Vecton, Russia) were used as the mobile phase.

Urine samples. To construct the calibration curves and to examine the procedure, we used urine samples of volunteers (men and women) of 20 to 45 years old. Samples were preserved with sodium azide and then stored at –20°C before analysis.

Instruments and equipment. An ultra-HPLC system with a tandem mass spectrometer (UHPLC–MS/MS) was used consisting of a Thermo TSQ Access Max triple quadrupole mass spectrometer (San Jose, United States) with a heated electrospray ionization source and a Dionex Ultimate-3000 liquid chromatograph equipped with a degasser, a binary gradient pump, an automatic sampler, and a thermostat. The components were separated in a Phenomenex Kinetex HILIC column (100 mm × 2.1 mm, 2.6 μm) at a column thermostat temperature of 30°C. To ensure reproducibility of the results and to minimize cross-contamination of samples, the autosampler needle was washed five times before and after the injection of sample with a water–acetonitrile–methanol mixture (70 : 20 : 10 vol).

RESULTS AND DISCUSSION

In the absence of isotopically labeled standards, gabapentin was used as an internal standard. This substance is close in structure to Meldonium and is not an endogenous compound, which prevents significant distortion of the results. Alpha glycerol phosphoryl choline was also tested as an internal standard, but it was abandoned because of the low reproducibility of the results.

As a mobile phase, the systems of acetonitrile–0.1% formic acid and acetonitrile–10 mM ammonium acetate solution were studied. The highest separation efficiency was achieved by using an acetonitrile–10 mM ammonium acetate solution. The most reproducible results and the best shape of peaks were obtained at a flow rate of the mobile phase of 0.3 mL/min and the temperature of the column thermostat of 30°C. The conditions of gradient elution are given in Table 1. The injected sample volume was 10 μL.

Because of the high polarity of the analyte, the use of electrospray ionization is optimal. The following optimal conditions for the detection of analytes by UHPLC–MS/MS were selected: the evaporator temperature of the ionization source of 350°C; the transfer capillary temperature of 320°C; the voltage on the ionization source of 3000 V; positive ion mode; the nebulizer gas (nitrogen) pressure of 60 arb. units; the auxiliary gas (nitrogen) pressure of 10 arb. units; and the target gas (argon) pressure in the collision cell of 1.5 mTorr. The MS/MS detection conditions were optimized by inflowing the analyte substances into the source chamber using a syringe.

Under these conditions, there are no peaks with the retention times and the intensity ratios between the MRM-transitions similar to the analytes and interfering with their determination (Fig. 1) This indicates that the selected conditions meet the requirements of selectivity, and endogenous compounds do not interfere significantly in the application of "dilute-and-shoot" procedure.

Samples were prepared as follows: 200 μL of test urine and 1800 μL of acetonitrile containing the internal standard were placed into 2-mL Eppendorf tubes, mixed, and then centrifuged at 10 000 rpm for 10 min. The supernatant was transferred into glass vials and analyzed under the conditions described above. Urine samples prepared in this way can also be used in the direct injection of the sample into a mass spectrometric detection system without preliminary chromatographic separation, but in this case, the detection of Meldonium at concentrations below 20 ng/mL is difficult due to matrix effects. In addition, there is a significant contamination of the ion optics and the transfer capillary: these systems require purification after only 100 sample injections.

For quantitative determinations, a series of calibration solutions were prepared. A stock solution with the analyte concentration of 1 mg/mL was prepared by dissolving a weighed portion of the substance in a water–acetonitrile mixture (20 : 80 vol). Working

solutions were prepared by successive diluting of the stock solution with acetonitrile. For calibration, the analytes were introduced into a control urine sample at concentrations of 10, 12.5, 25, 50, 100, 125, and 250 ng/mL. Gabapentin with the concentration in the final extract of 50 ng/mL was used as an internal standard. The samples were stored for no more than 48 h at room temperature.

The calibration solutions were analyzed as described above to find out a linear range ($n = 3$). The calibration curves were plotted as the dependence of the peak area ratio (the analyte peak area to the internal standard peak area) on the concentration and processed as linear dependences to determine the correlation coefficient, which was 0.994.

To determine the accuracy of the analysis, several quality control (QC) solutions were prepared with high (250 ng/mL), medium (100 ng/mL), and low (25 ng/mL) concentrations; the QC solutions were analyzed five times for 1 day. The accuracy of the results obtained on different days was also controlled by the analysis of the QC solutions. The concentrations of solutions were calculated from the calibration curves obtained on the day of analysis. The result was considered satisfactory if the error of determination did not exceed 15% [11] (Table 2).

The limit of detection of the analyte was found experimentally by decreasing the concentration of reference solutions until the signal-to-noise ratio was 3 : 1 at a concentration of Meldonium of 7.5 ng/mL. The lower limit of the analytical range was also determined experimentally; it was 10 ng/mL.

In the qualitative and quantitative analysis of the samples, the WADA criteria [12] were taken into account, according to which the retention times of the analyte and the standard substance should not differ by more than 0.1 min with the reproducibility of the absolute ratios of the relative intensities of the ions.

It should be noted that, because of its low molecular weight, Meldonium has only one characteristic transition, which significantly complicates the reliability of its determination and requires careful selection of the separation conditions (Table 3).

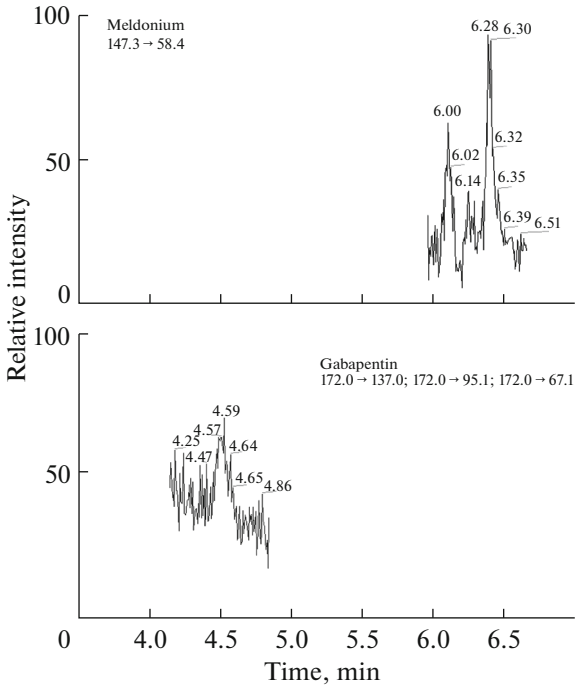


Fig. 1. HPLC–MS/MS chromatogram of a control urine sample obtained by the "dilute-and-shoot procedure.

The proposed procedure was used for the analysis of urine samples obtained from volunteers, 12 and 24 h after administration of Meldonium at a dose of 500 mg (Fig. 2).

CONCLUSIONS

A procedure is proposed for the quantitative determination of Meldonium in urine by HPLC with tan-

Table 2. Determination of Meldonium in urine by the "dilute-and-shoot" procedure

Added, ng/mL	On the same day		On different days	
	found, ng/mL	error, rel %	found, ng/mL	error, rel %
25	27.9 ± 0.2	11.97	24.30 ± 0.05	–2.79
100	117.3 ± 0.2	17.73*	111.4 ± 0.2	11.42
250	249.2 ± 0.2	–0.30	246.78 ± 0.03	–1.29

* Unsatisfactory result.

Table 3. Retention times, MRM-transitions, and collision energy for analytes in their determination by HPLC with tandem mass spectrometric detection (electrospray ionization)

Substance	Retention time, min	Precursor ion, *m/z*	Product ion, *m/z*	Collision energy, eV	Extraction lens voltage, V
Meldonium	6.20	147.1	58.3	32	57
Gabapentin	4.35	172.3	137.2	14	75
			95.3	20	
			67.3	28	

dem mass spectrometric detection. It meets the requirements of accuracy, speed, reliability, and sensitivity. The possibility of using this procedure in the practice of doping control and clinical diagnostics is demonstrated. The high sensitivity of the procedure ensures its use for the determination of Meldonium after some time after its use, as well as in the study of its pharmacokinetic properties.

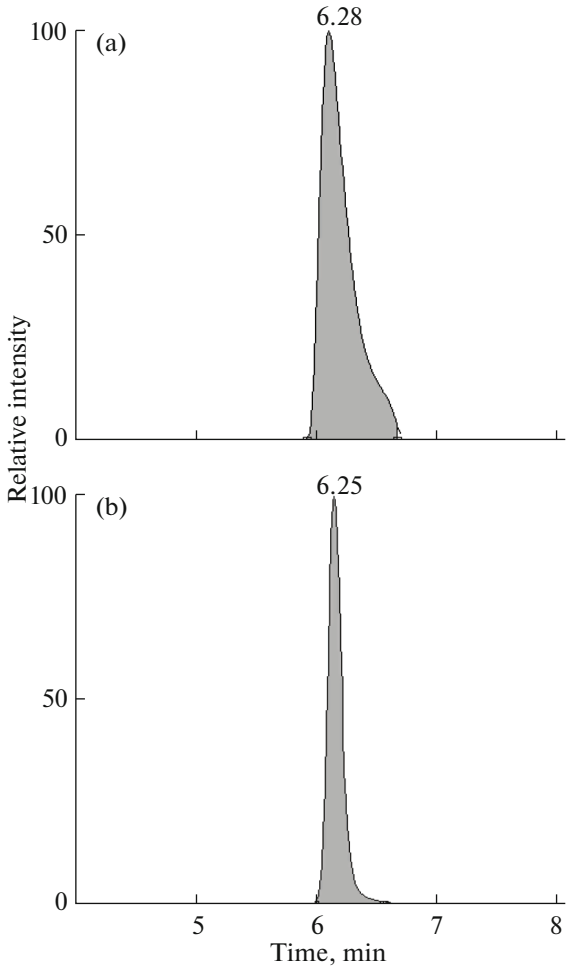


Fig. 2. HPLC–MS/MS chromatograms of urine samples after (a) 12 and (b) 24 h after administering 500 mg of Meldonium.

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