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Method for Screening and Confirming Meldonium in Human Urine by High-Resolution Mass Spectrometry and Identification of Endogenous Interferences for Anti-Doping Testing

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Abstract : Meldonium is a drug for treating ischemia by expanding the arteries but it can also enhance the performance of sports players. The World Anti-Doping Agency (WADA) has included it in the list of prohibited substances since 2016. Meldonium is one of the challenging substances for anti-doping testing because it is difficult to recover by general liquid-liquid or solid phase extraction due to its permanent charge and high polarity. Therefore, high-performance liquid chromatography (HPLC) is currently used by injecting a diluted urine sample (known as the "dilute-and-shoot" strategy). There is no loss of target compounds in the extraction/cleanup procedure but its high matrix effect could interfere in their separation or detection from the endogenous urinary compounds. We report a single method using high-resolution mass spectrometry that can be used for both screening and confirmation, which follows the "dilute-and-shoot" strategy. In this method, the endogenous compounds' interfering peaks in the mass spectrum are separated at a high resolution of FWHM 140,000, and the results are suitable for substance detection following the WADA guidelines. The interferences in the obtained mass spectrum of the urine matrix are identified as acetylcholine, lysine, and glutamine by further analysis and database searching. Validation of the method is performed in routine anti-doping testing, and the limit of detection is 50 ng/mL. This method uses simple sample preparation and a general reverse phase HPLC column, and it can be easily applied to other substances.

Key words : Meldonium, Doping control, Dilute-and-shoot, High-resolution mass spectrometry, Method validation

Introduction

Meldonium (mildronate; 3-(2,2,2-trimethylhydrazinium) propionate) was synthesized as a growth promoting agent in the Institute for Organic Synthesis of the Latvian Soviet Socialist Republic Academy of Sciences.[1,2] Meldonium is a competitive inhibitor of the enzyme γ-butyrobetaine hydroxylase, which is involved in synthesizing carnitine. Carnitine plays an essential role in cellular energy metabolism, and it also controls the acetyl-CoA/CoA ratio and decreases the accumulation of toxic acetylCoA.[3] Meldonium also inhibits the function of the sodium-coupled carnitine transporter Organic Cation/Carnitine Transporter 2 (OCTN2), which constrains the carnitine level in the cell membrane of the liver and kidneys.[4] Therefore, meldnoium is an effective anti-ischemic drug because it can be used to reduce both the synthesis and the biological functions of carnitine. Owing to its anti-ischemic effects, meldonium shows several positive advantages in the elite and professional sports fields. Meldonium increases the peroxisomal utilization of fatty acids, energy storage, and endurance work capabilities by decreasing the production of lactic acid.[5] Therefore, meldonium has been included in the list of prohibited substances since 2016, and its minimum required performance level (MRPL) was limited to 200 ng/mL by the World Anti-Doping Agency (WADA).[6] It has especially attracted wide attention since the announcement of an elite tennis player Maria Sharapova's positive result.

Several approaches have been developed for the detection of meldonium in human urine and plasma. Most methods are based on liquid chromatography–mass

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spectrometry (LC–MS),[7-9] and a few approaches utilize capillary electrophoresis[10] or dried blood spot analysis.[11] However, meldonium is known as a challenging substance to analyze due to its high polarity and low molecular mass. Due to its chemical properties, it is hardly extracted by liquid-liquid extraction or solid phase extractions, sample preparation methods that were widely used prior to analysis by LC-MS. Furthermore, its separation efficiency is modest on reverse phase columns, and therefore the analysis of meldonium in blood was performed in hydrophilic interaction liquid chromatography (HILIC) or amino columns.[12] For anti-doping testing, a screening method using a C18 reverse phase column was previously reported; however, confirmation testing was accomplished using a HILIC column due to high matrix effects.[13] The limit of detection (LOD) of the confirmation method was remarkably low (1 ng/mL) but the screening LOD was relatively high (~100 ng/mL). Moreover, it required an additional LC-MS system using a different gradient and an additional sample preparation procedure.

In this study, we develop a new method, which can be applied for both screening and confirmation and is based on the "dilute-and-shoot" strategy and high-resolution mass spectrometry (HRMS). In this method, we obtain a product ion spectrum from a urine sample, which can fulfill the WADA guidelines for a confirmation procedure. Interfering ion peaks are also observed in the same spectrum but they are sufficiently separated by high-resolution mass spectrometry and attributed to acetylcholine, lysine, and glutamine by database searching and further experiment. Method validation is also performed for routine analysis.

Experimental

Chemicals and reagents

Meldonium was obtained from TRC (Toronto, Canada). Acetylcholine, L-Lysine monocholoride, L-Glutamine, and methaqualone were purchased from Sigma (St. Louis, USA). Methanol was purchased from J.T. Baker (Center Valley, USA) and formic acid was obtained from Kanto Chemical (Tokyo, Japan). All reagents were of analytical grade. Water was purified using Aqua MAX from Millipore (Darmstadt, Germany) for the mobile phase of chromatography and for sample preparation.

Sample preparation

For the sample preparation, 300 μL pooled urine was centrifuged for 10 minutes at 10,400 g. The supernatant (90 μL) was transferred to an autosampler vial, and 10 μL methaqualone (1 μg/mL) was spiked as an internal standard. ISTD (10 μL) and meldonium working solution (10 μL) were spiked into 80 μL of distilled water containing 0.1% formic acid. The working solution of meldonium, acetylcholine, lysine, and glutamine was prepared from 1 mg/mL of the reference stock solution. Each sample was vortexed, and 5 μL was injected into the LC–MS system.

LC–MS/MS analysis

The conditions of liquid chromatography–mass spectrometry were similar to those applied in the previous study.[14] The target substances were separated using a UFLC XR series high-performance liquid chromatography (HPLC) system (Shimadzu, Japan) with a Kinetex C18 column (100 mm × 2.1 mm I.D., 2.6-μm particle size; Phenomenex, Torrance, USA) connected to a guard column (2.1 mm I.D.). The mobile phase comprised 0.1% aqueous formic acid solution for mobile phase A and 0.1% formic acid in methanol for mobile phase B. Gradient elution was applied at a flow rate of 0.5 mL/min, and 2% mobile phase B was held for 0.5 min, ramped to 95% B over 8.5 min, and then kept until 9.0 min. Subsequently, re-equilibration for 1 min at 2% B was performed, thereby giving an overall runtime of 10 min. Q exactive plus tandem mass spectrometer from Thermo Scientific (San Jose, USA) was used in a positive-ion mode, and the capillary temperature was set at 300°C. The spray voltage was 4000 V, and the mass spectra were acquired via the product ion-scan mode, which was called parallel-reaction monitoring. The sheath gas, ion sweep gas, and aux gas-flow rates were 53 arb (arbitrary unit), 3 arb, and 14 arb, respectively. The resolution of the mass spectrometer was set at the lowest value (FWHM 17,500) for a fast scan rate, and then increased up to FWHM 140,000 in further analysis for better separation.

Results and Discussion

Isolation of meldonium ions from urinary sample matrix

The product ions of meldonium are isolated from endogenous compounds having similar retention times and *m*/*z* values by HRMS. In the initial screening, a spectrum is obtained at FWHM 17,500 (the lowest resolution). The product ion spectrum of the reference standard and that of the spiked urine sample (100 ng/mL) are shown in Figure 1a. The major product ions at *m*/*z* 58.0661 and 59.0739 resulted from the cleavage of trimethylamine residue and its additional loss of hydrogen.[9,13] The meldonium retention time is extremely short (0.48 min) due to its high polarity and permanent charge but there is no difference in a retention time in the present analyses. However, the *m*/*z* value and the product ion ratio of one of the product ions (*m*/*z* 147.1128, non-dissociated meldonium molecular ion) do not correspond to the reference standard. The differences are 0.0072 (48.9 ppm) for the *m*/*z* value and 57% of the ion ratio; therefore, the result is not sufficient to confirm the presence of meldonium according to the WADA guidelines (20% of tolerance ranges for 25%-50% of relative abundance), and it could be reported as a false

negative. The difference in the ion ratio may be attributed to endogenous compounds, which are not excluded through the quadrupole prior to the MS/MS analysis. The minimum isolation width of the quadrupole is *m/z* 0.4; therefore, other compounds with similar retention times and *m/z* values can be detected in the same spectrum. The meldonium molecular ion is isolated from the interference peak (*m/z* 147.1210) at a resolution of FWHM 140,000. As shown in Figure 1b, many peaks are not completely separated at FWHM 17,500 but the meldonium molecular ion is isolated from the interfering peaks at FWHM 140,000. Moreover, its *m/z* value and ion ratio correspond well to those of the reference standard. Therefore, HRMS should be highly recommended for meldonium screening or confirmation testing using a reverse phase column.

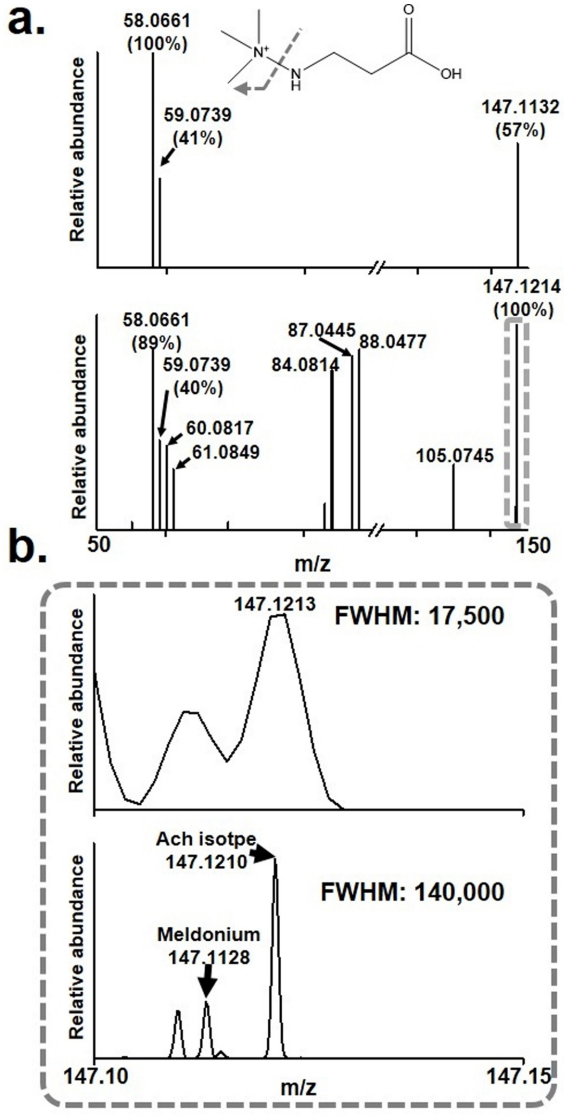

Figure 1. (a) The product ion spectrum of the meldonium standard (top) and the spiked urine sample (100 ng/mL meldonium) at a resolution of FWHM 17,500. The spectrum of the urine sample shows several interfering peaks, and the ratio of the meldonium molecular ion (dashed square) does not correspond to the reference. (b) Enlarged spectrum of the dashed square of (a) at two different resolution of mass spectrometer. At 17,500 resolution, multiple peaks are observed but not completely separated (top). However, these are separated into three peaks at a high resolution of FWHM 140,000 (bottom).

Identification of endogenous compounds in the urine matrix

It is successfully demonstrated here that meldonium analysis based on the "dilute-and-shoot" method and HRMS is efficient; however, various interfering peaks are observed in the spectrum of the urine matrix. We investigate the possible interfering compounds by database searching via mzCloud from Thermo (www.mzcloud.org) and by further analysis of their standards. Then, we compare these to the results of the urine sample for accurate confirmation or clear explanation. Figure 2a shows that the interfering peaks correspond to endogenous acetylcholine isotope, lysine, and glutamate; however, *m/z* 101.0796 cannot be identified. Among them, the peaks of *m/z* 84.0814 and 130.0865 are the product ions of lysine, whereas *m/z* 84.0451 and 130.0501 originate from glutamine. Although product ions from both amino acids are observed at similar *m/z* values, they were separated in HRMS and their spectra correspond to those of the references. The spectra of lysine and glutamine are

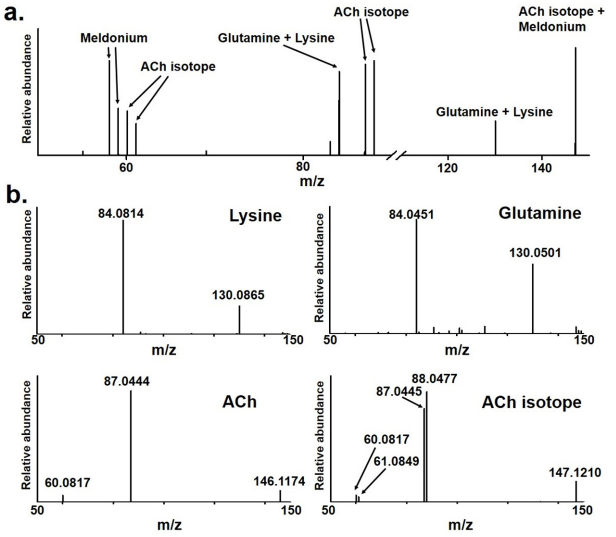

Figure 2. (a) The product ion spectrum of the spiked urine sample with annotation of the identified compounds. Some peaks are annotated together because of their similar *m/z* values but they were separated to a single peak. (b) The spectrum of the standards, which are identified as endogenous compounds in human urine. ACh and ACh isotope spectrum are obtained from a single LC–MS running with two different precursor ions (ACh: *m/z* 146.1174, ACh isotope: *m/z* 147.1210)

presented in Figure 2b.

Among the matrix peaks, two product ions (*m/z* 60.0817 and 87.0445) are attributed to acetylcholine (ACh). However, the ACh precursor ion is observed at *m/z* 146. In addition, the adjacent peaks (*m/z* 61 and 88) have not been found in any previous study or database. Therefore, all five ions, including *m/z* 147.1210, are considered to correspond to the acetylcholine isotope. ACh has a permanent positive charge and forms $[M]^+$ ion through electrospray ionization. It is known to dissociate to a tertiary amine radical, with the other part having an acetyl group.[15] As described in Figure 2, the product ion spectrum of the ACh monoisotope (*m/z* 146.1174) produces only two product ions but its isotope (*m/z* 147.1210) produces additional ion peaks at *m/z* 61.0849 and 88.0477 (Figure 2b, ACh isotope), and these ions are considered to originate from the acetylcholine with a ^{13}C atom. When a ^{13}C atom is present in the tertiary amine group, the precursor produces product ions with *m/z* 61.0849 and 87.0442, and another precursor having a ^{13}C atom in the other part dissociates to form product ions with *m/z* 60.0815 and 88.0483. Therefore, all four product ions can be detected in the MS/MS spectrum considering the random position of the ^{13}C atom in the isotope.

The retention times of the endogenous compounds are similar to that of meldonium, and their structure and chromatograms are shown in Figure 3. Lysine and glutamine show slightly different retention times but these overlap at the peak of meldonium. As a result, we can identify most interfering peaks of the urine matrix, and they are acetylcholine, lysine, and glutamine. Due to the relatively low resolving power of the quadrupole, endogenous compounds are detected in the spectrum of meldonium but its separation is only achieved with the high-resolution mass spectrum. This result offers additional information that helps prevent a potential false negative result in meldonium analysis.

Method validation

The method validation is performed for screening by considering the parameters of selectivity, matrix effect, linearity, intra- and inter-day precision, and LOD, according to the ISO/IEC 17025 and WADA guidelines. The validation result for each parameter is shown in Table 1. Selectivity is validated by analysis of five spiked urine samples (100 ng/mL), and there is no false positive/negative result or significant difference in the retention time. Matrix effect validation is performed by analyzing

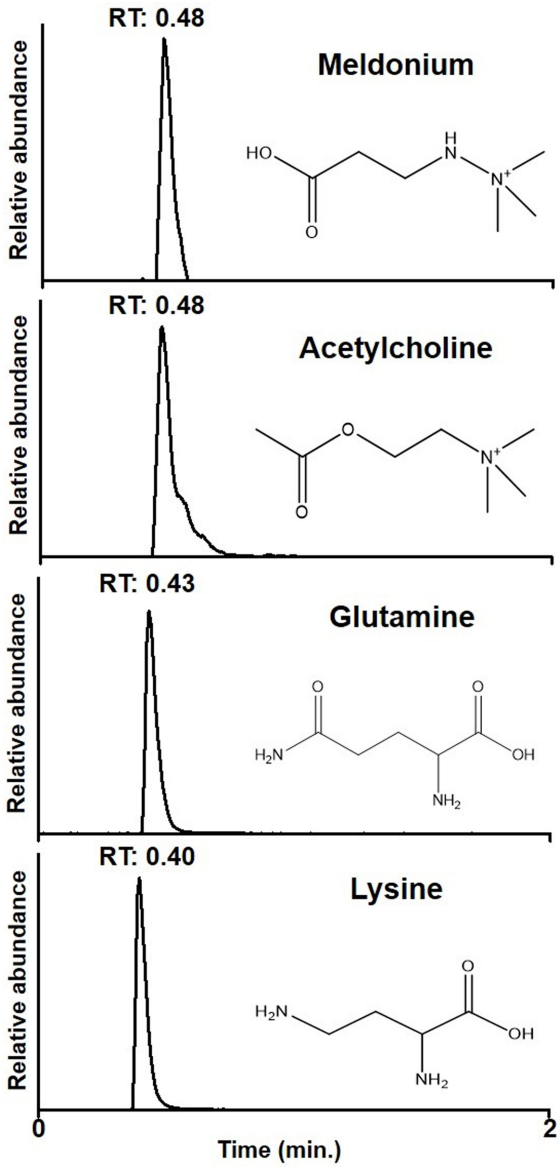

Figure 3. The structures and chromatograms of meldonium and endogenous compounds.

Table 1. Validation result

LOD [ng/mL] (n = 5)	Matrix effect (n = 5)	Calibration curve	Robustness (RT, n = 6)	Intra-day precision (n = 6/6/6)		Inter-day precision (n = 18/18/18)	
				levels [ng/mL]	CV	levels [ng/mL]	CV
~50	5.2%	slope: 0.82009 intercept: 36.978 (R^2 = 0.9941)	1.6%	100	7.7–18.0%	100	16.6%
				500	8.4–15.5%	500	20.4%
				1000	5.0–8.6%	1000	21.0%

five replicates of spiked urine sample (100 ng/mL) and a quality control (QC) sample of equal concentration in mobile phase A. The matrix effect is calculated (peak area from urine sample/QC), and it is 5.2%. This low matrix effect is attributed to the fact that various polar urinary compounds may be eluted without retention in a reverse phase column. Despite the strong ion suppression effect, the method is sufficient for detecting meldonium at a concentration of ~50 ng/mL (LOD), which is 25% of the MRPL from WADA. LOD is determined by the lowest concentration with a signal to noise ratio of 3:1 and showing 25% of CV or less. The linearity was 0.9941 of the R^2 value evaluated in a range of 50-2000 ng/mL. The robustness (variation of retention time) was 1.6% and a precision test was performed by analyzing six replicates at three concentrations (100, 500, and 1000 ng/mL) and repeating this for three days. The CV% values were less than 25% for all inter- and intra-day precision tests. As a result, we confirm that the method is applicable for screening/confirmation.

Conclusions

A successful demonstration for screening or confirming meldonium based on the "dilute-and-shoot" strategy and high-resolution mass spectrometry is performed in this study. We were able to detect low level of meldonium using a general reverse phase C18 column. The endogenous compounds in human urine affected the confirmation results; however, their product ions can be separated in HRMS. Peaks from the urinary matrix were attributed to acetylcholine, lysine, and glutamine by database searching and further experiments. This study enabled screening and confirmation with a single method LC-MS system and could be easily adapted for the screening of other substances as well for anti-doping testing.

Acknowledgments

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