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Unraveling the complexity of anti-doping analysis: reassessing meldonium detection and doping verdicts in a case study

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Abstract. – BACKGROUND: The precision and accuracy of mass spectrometry (MS) made it a fundamental tool in anti-doping analysis. High-resolution (HR) mass spectrometers significantly improved compound identification. This study systematically analyzes data from an athlete (Subject 1) who tested positive for meldonium and compares it with data from a healthy volunteer (Subject 2) to examine the correctness of the doping verdict.

CASE PRESENTATION: The documentation related to Subject 1 was thoroughly processed and analyzed. A study involving a volunteer (Subject 2) replicated Subject 1 regimen and urine sample collection for data alignment with anti-doping results, with Subject 2 reporting not using meldonium.

The anti-doping agency's analysis of Subject 1 showed the presence of meldonium at a concentration close to the established cut-off level. However, a closer examination revealed that one specific ion, crucial for meldonium identification, was absent from the mass spectra. Analyzing Subject 2 data, using the same methodology, the absence of the specific ion was confirmed, even though the volunteer did not consume meldonium. The European directive and the method that was validated and cited by the anti-doping agency identified meldonium on at least four specific ions, whereas the anti-doping analysis used only three ions. This discrepancy compromises the specificity of meldonium identification.

CONCLUSIONS: To enhance the analytical methodology, two strategic interventions are suggested: adjusting the meldonium cut-off value and expanding the analysis to include meldonium metabolites. By addressing these avenues, the precision of meldonium detection and doping verdicts can be improved. In conclusion, this study challenges the anti-doping agency's verdict and prompts a reevaluation of meldonium detection methodologies in anti-doping measures.

Key Words:
Mass spectrometry, Meldonium, False positive, Doping verdict, Anti-doping analysis, SANIST.

Background

The anti-doping analysis relies extensively on Mass spectrometry (MS) due to its precision, selectivity, and quantifying accuracy[1]. The high-resolution (HR) and accurate mass spectrometers progressively increased the selectivity in compound identification within the field: high-resolution (HR) mass spectrometers such as ORBITRAP[2] (ThermoFisher, San Jose, CA, USA) and time-of-flight[3] mass analyzers (I.S.B. - Ion Source & Biotechnologies, Bresso, Milan, Italy) can provide compound identification in both full scan mode[4] - owing to their capacity to determi-

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ne the elemental composition - and tandem mass spectrometry (MS/MS)[4] (I.S.B. - Ion Source & Biotechnologies, Bresso, Milan, Italy) by comparing the relative abundance obtained with the standards' analytical signal, according to the criteria set by the EU directive[5] and anti-doping regulations6. So far, several methods[7-9] have been developed by combining high-performance liquid chromatography with high-resolution mass spectrometry (LC-HRMS) for anti-doping purposes. These methods have been employed to confirm the presence of different classes of anti-doping small molecules, such as steroids[7], hormones[8], and certain classes of pharmacologically active drugs[9,10].

The high sensitivity of LC-HR-MS allows a reduction in the analytical cut-off levels. However, in some cases, this could lead to concerns. At such low levels, prohibited compounds, as in the case of meldonium, must adhere to the stipulated number of points for recognition, as outlined by the European directive[5] and the methodology established by the group that validated its doping detection[11]. If any of the specific recognition points for that substance are absent, anti-doping analyses should be expanded to encompass the identification of metabolites of said substance[12].

The aim of this work is to systematically compare the data from a sample collected from an athlete resulted positive for meldonium (Subject 1) at the anti-doping agency analysis with the data provided by the analysis in our laboratories of a sample collected from a healthy volunteer (Subject 2) treated with the same food supplements to reach conclusions drawn from the processed reports.

Case Report

The received documentation pertaining to the case of Subject 1 (analysis carried out by the anti-doping agency, analysis carried out following the method described by Görgens et al[11]) underwent comprehensive processing and analysis. The received documentation encompassed the following components:

- Report of urine analysis for Subject 1, Sample A (utilized for the initial screening test).
- Report of urine analysis for Subject 1, Sample B (utilized for the confirmatory test).
- E-mail correspondence detailing the concentration detected in Sample A.

Subsequent to receipt, each element was subjected to meticulous examination to extract valuable insights. Moreover, Subject 1 reported taking food supplements containing beta-alanine and acetyl-L-carnitine at recommended dosages.

In an enrolled volunteer participant (Subject 2), the food supplements containing 3 g of beta-alanine and 1,500 mg of acetyl-L-carnitine, divided into 3 daily intakes for six days, were administered in order to replicate the conditions of Subject 1. On the seventh day, a urine sample was collected from Subject 2 and analyzed with the same method used for Subject 1 to align the data with those obtained from the anti-doping center. Subject 2 reported not taking meldonium.

Outcomes

Sample A and B (screening and confirming samples, respectively) of Subject 1 were found positive for meldonium by the anti-doping agency, according to the method published[11]. Although the report was declared as an "Adverse Analytical Finding", thus positive, the concentration detected was not present in the Report of analysis on Sample A and B of Subject 1. Only in the e-mail that Subject 1 received from the anti-doping agency, the detected meldonium concentration was 155 ng/mL, a value near the cut-off established by the anti-doping guidelines (100 ng/mL)[6].

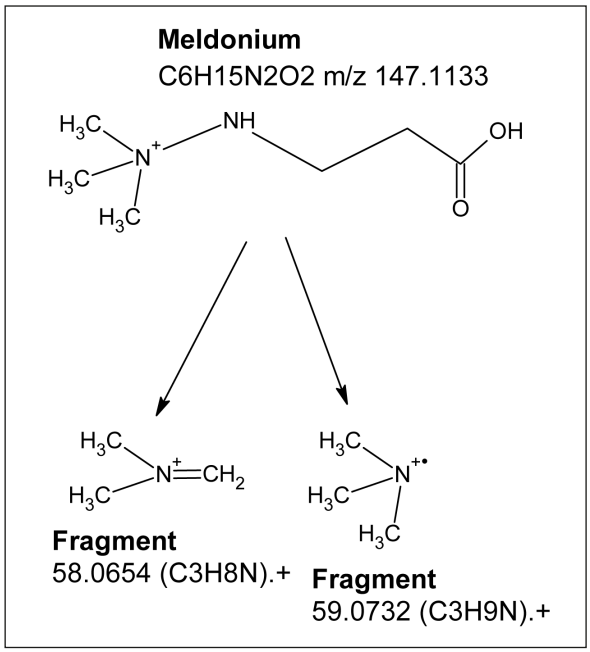

Figure 1. Representation of medoniums' fragmentation.

Reviewing the documentation, we observed that the analysis carried out by the anti-doping agency was performed by monitoring the ion at m/z 58.0654, the ion at m/z 59.0732, and the molecular ion at m/z 147.1133 (Figure 1). Görgens et al[11], who validated the analysis of meldonium in anti-doping, pointed out in their work that four specific ions are needed to determine the presence of meldonium in a urine sample: the ions at m/z 58.0654, m/z 59.0732, m/z 132.0894, and m/z 147.1133. The fourth specific ion of meldonium fragmentation, that was not considered during the analysis of Subject 1, was the one at m/z 132.0894. In fact, this ion was not present in the mass spectra of Samples A and B, consequently making the attribution to meldonium not in line with the validated method[11] and the European directive[6].

To align the data with those obtained from the anti-doping agency, we analyzed the urine sample of Subject 2 (the volunteer subject who reported that he did not take meldonium but only beta-alanine and acetyl-L-carnitine), using the same methodology as Subject 1. The clinical data of Subject 2 are reported in Table I. The meldonium chromatographic peaks of the analytical standard and of the analyte present in the urine sample were detected at a retention time of 10.60 min, and MS/MS spectra were reported in Figure 2a and 2b, respectively. In both cases, the same fragmentation was detected, as well as the ratio of the fragment ion at m/z 58.0654 and m/z 59.0732. The MS/MS spectra acquired by analyzing the analytical standard (Figure 2a) also showed the presence of the ion at m/z 132.0896, as previously observed by the group of Görgens et al[11]. This ion (m/z 132.0896) is not present in the MS/MS spectrum obtained from the urine of Subject 2, as observed for Subject 1.

Table I. Clinical data of Subject 2.

Characteristics	Values
Sex	Male
Age	27
Height	73 kg
Weight	186 cm

Discussion

For substances belonging to Group A (Substances having an anabolic effect and unauthorized substances), such as meldonium, the EU directive 2002/657/EC[5] clearly states that the substance must be identified at least on four points, as well as Görgens et al[11], contrary to the methodology applied in the anti-doping analysis that makes use of 3 points (the ions at m/z 58.0654, m/z 59.0732, and m/z 147.1133).

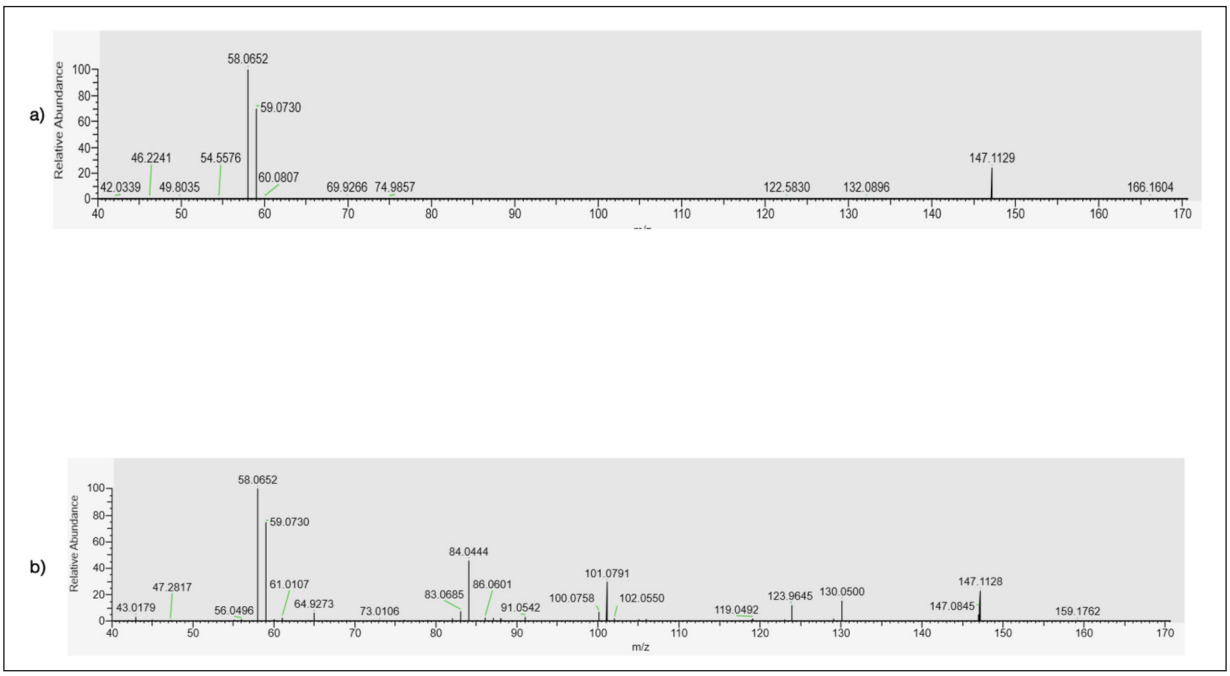

Figure 2. MS/MS spectra. **a**, analytical standard; **b**, the analyte in the urine. Retention time of detection: 10.60 Min.

The concentration of meldonium, as determined by the anti-doping agency in the analysis of Subject 1, measuring 155 ng/ml, stands in close proximity to the established cut-off of 100 ng/ml[6]. Furthermore, the mass spectra of Subject 1 analyses exhibited only the peaks at m/z 58.0654, m/z 59.0732, and m/z 147.1133, conspicuously lacking the distinct peak at m/z 132.0894, which is crucial for unequivocal meldonium identification. Similarly, the mass spectrum pertaining to Subject 2 analyses (Figure 2b) lacks the m/z 132.0894 peak, although the peaks at m/z 58.0654, m/z 59.0732, and m/z 147.1133 are discernible. As demonstrated with our test in Subject 2, which did not take meldonium, the mere lack of the peak at m/z 132.0896 makes the analysis nonspecific.

The European Directive[5] and the validated methodology employed by the anti-doping agency[6] elucidate that meldonium recognition mandates all four points (m/z 58.0654, m/z 59.0732, m/z 132.0894, and m/z 147.1133).

In cases where the m/z 132.0894 peak is absent, the specificity of meldonium identification can be deemed compromised due to the absence of a pivotal marker, as demonstrated by the analysis of Subject 2.

It is reasonable to surmise that the relatively faint at m/z 132.0894 peak's absence may stem from the meldonium concentration's proximity to the cut-off value. In scenarios involving notably low concentrations or the non-detection of all four specific meldonium peaks, our suggestion leans towards refining the analytical methodology. Several avenues for enhancement emerge:

1. Adjustment of cut-off value – One approach involves raising the meldonium cut-off level to ensure recognition across all four key points.
2. Metabolite analysis – In cases where the m/z 132.0894 peak or any other specific peak is absent, an expansion of meldonium analysis to encompass its metabolites[12] becomes imperative for achieving specificity.

By addressing these avenues, the analytical methodology can be fortified, bolstering the reliability of meldonium detection and subsequently enhancing the precision of doping verdicts.

Conclusions

Our investigation has prompted a reassessment of meldonium detection methodologies within the context of anti-doping measures. The absence of a specific ion, notably observed in the mass spectra of Subject 1 analyses, and the absence of the meldonium concentration within the report raises concerns regarding the reliability of meldonium identification.

Our study advocates for two strategic interventions. Firstly, an adjustment of the meldonium cut-off value could enhance the discriminatory power of the analytical framework. Secondly, the unexplored domain of metabolite analysis presents a promising avenue for enhancing specificity and minimizing false positive outcomes.

In parallel, we advocate for the expansion of forensic chemistry and toxicology paradigms. Our findings not only prompt the evolution of anti-doping methodologies but also underscore the intrinsic role of rigorous scientific scrutiny in sustaining the integrity of athletic competition.

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Authors' Contributions

Conceptualization, S.C., and M. Bertelli; Methodology, S.C.; Investigation, S.C., M.L. and L.C.; Writing- original draft preparation, S.C. and K.D.; Writing- review and editing, F.V., S.S., M. Brambilla, C.B., A.M.M., M.C., G.P., T.B., and V.L.; Project administration, S.C., and M. Bertelli; Funding acquisition, S.C., and M. Bertelli. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Ethics Approval

The data were taken from the examination of a subject upon spontaneous request. The Ethical Committee approval was not applicable because the examination falls within the category of metabolomic screenings. Data were not originally generated for research purposes but were subsequently used anonymously after the screening.

Informed Consent

The subject provided consent for the publication of the data in anonymized form.

Data Availability

The analyses and conclusions presented in this article are based solely and exclusively on the data provided by Subject 1 regarding the anti-doping analyses, and Subject 2 regarding the data. We do not have access to any additional or independent data beyond what was shared with us.

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