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Analyses of Meldonium (Mildronate) from Blood, Dried Blood Spots (DBS), and Urine Suggest Drug Incorporation into Erythrocytes

Initially developed in the late 1970s for veterinary applications due to proposed growth-promoting effects in animals [5], meldonium has become an approved drug in selected Eastern European countries and is the subject of ongoing clinical trials focusing the compound's anti-ischemic and cardioprotective properties [2, 3, 12, 15] as well as potential applications regarding diabetes, neurodegenerative disorders, and bronchopulmonary diseases. In the context of athletic performance, beneficial effects on the individuals' physical working capacity, increased endurance performance, and accelerated recovery after physical activity were discussed [4, 10, 11], mentioning oral doses of meldonium of up to 2.0g per day over 2–3 weeks in the course of pre-competition preparation phases [4]. In 2015, the World Anti-Doping Agency (WADA) initiated a one-year monitoring program [22] regarding the prevalence of meldonium (mildronate) in doping controls. Obtained data demonstrated a considerable extent of meldonium use by athletes [8, 16], which was further corroborated by a significant number of declarations of use and analytical findings at the Baku 2015 European Games [18]. Subsequently, the WADA Prohibited List that became effective in January 2016 [24] classified meldonium as banned under S4 (Hormone and Metabolic Modulators).

Pharmacokinetic properties of meldonium were reported for single- and multiple-dose administration studies with healthy volunteers [25], where the drug's elimination was monitored in plasma over 24 h post-administration and characterized by non-linear pharmacokinetics. To date, doping controls are based on urine and blood as test matrices, and a variety of alternative matrices including amongst others dried blood spots (DBS) and dried plasma spots (DPS) have been considered lately [20]. Consequently, the knowledge about factors that potentially influence the elimination of target analytes is of particular importance to sports drug testing and, to the best of our knowledge, the role of erythrocytes and their ability to affect detection windows of meldonium in doping controls (e.g., by incorporation) has not been investigated.

Therefore, in the context of a pilot study, DBS, whole blood (Na₂-EDTA), and urine samples were collected from 2 healthy male volunteers who orally administered meldonium either as single dose (500 mg) or as multi-dose (3×500 mg/day over a period of 6 consecutive days). The study protocol was approved by the local ethics committee of the National Institute for Sports Research (Bucharest, Romania, approval number #162/2016), written consent was obtained from the study participants, and the study was conducted in accordance with ethical standards in sports medicine and exercise science [9].

DBS samples were collected prior to and post-administration up to 16 days using standard DBS collection cards (Whatman DMPK-C, GE Healthcare Europe, Freiburg, Germany), dried at room temperature, and stored at +4 °C in a plastic bag with desiccant until analysis. Na₂-EDTA-stabilized whole blood specimens (3.5 mL) were sampled within the multi-dose study on day 4 and day 28 post-administration, and aliquots (4×20 µL) were

immediately spotted on DBS cards. Further, following centrifugation of the blood samples at 1000×g for 15 min at 10 °C, the plasma was separated from the red blood cell (RBC) fraction, and 200 µL of the RBCs (retained for deposit onto DBS cards) was subsequently washed twice with 600 µL of phosphate-buffered saline (pH 7.4). The obtained plasma and washed erythrocytes were spotted onto DBS cards (four 20 µL aliquots each) and were also stored at +4 °C in a plastic bag with desiccant until analysis. Online sample preparation of DBS was performed using a DBS card autosampler (DBSA) directly coupled to an automated solid-phase extraction (SPE) cartridge exchange module (SPE^{XOS}) (Gerstel GmbH, Mülheim a.d.R., Germany). The sample preparation protocol was adapted from a previous application and was optimized to meet the current requirements [21]. In brief, the spots were extracted by means of flow-through desorption technology using 1200 µL of acetonitrile/water (70:30, v/v), which included the online-addition of stable isotope-labeled meldonium (triple deuterated, TRC Toronto, Canada) as internal standard. Sample purification was performed by means of online-SPE using hydrophilic interaction liquid chromatography (HILIC) SPE cartridges. The target compounds were eluted onto the analytical column (Hypersil Gold C8, 2.1 mm×30 mm, 1.9 µm particle size) via the LC mobile phase applying a gradient program with A: 5 mM ammonium acetate buffer (pH 3.5) and B: acetonitrile. LC-HR-MS/MS analysis was performed with a Thermo Dionex Ultimate 3000 liquid chromatograph interfaced to a Q Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany). Data were acquired in full scan mode with concomitant targeted higher energy collisional dissociation (HCD) experiments (precursor ion: *m/z* 147.1126, normalized collision energy: 40). The total sample-to-sample cycle time was 13 min.

In addition to blood sampling, post-administration urine specimens were collected over a period of up to 49 days. These samples were subjected to analysis using a hydrophilic interaction liquid chromatography-high resolution high accuracy mass spectrometry approach (HILIC-HR-MS) published previously [8]. The analytical method for DBS measurements was validated for qualitative result evaluation purposes according to current guidelines of the International Standard for Laboratories (ISL) of the World Anti-Doping Code (WADC) [23]. Investigated parameters included specificity, carry-over, LOD (20 ng/mL), robustness, matrix interferences, and linearity (0–2000 ng/mL), which allowed for estimating meldonium concentration levels in DBS by means of calibration curves prepared and analyzed with each batch of cards. Based on the method validation results (Table 1), the fitness-for-purpose of the assay was demonstrated.

A total of 8 DBS samples collected prior to and up to 16 days post-administration of a single-dose (500 mg) of meldonium were analyzed using the automated isotope-dilution mass spectrometric approach. Maximum concentration levels were

Table 1 Main validation results.

Specificity	(n = 10)	✓
Linearity	0–2000 ng/mL	r ² = 0.9985 slope: 13 623.2 intercept: 23 291.9
LLOD	(n = 6) 20 ng/mL	CV = 2.9%
Carry Over		Not observed
Ion suppression		23 %
Robustness		✓

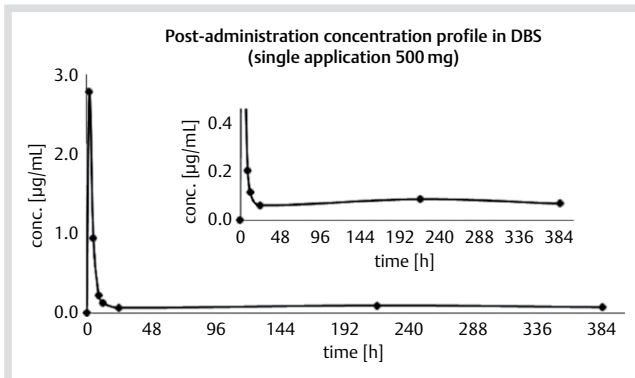


Fig. 1 DBS-derived concentration profile of meldonium measured from post-administration samples collected in a pilot study with a single oral dose of 500 mg of meldonium. Maximum concentrations were observed 2 h post-administration with approximately 2.8 µg/mL. After 24 h concentrations of ca. 80 ng/mL were observed up to 16 days (384 h) post-application.

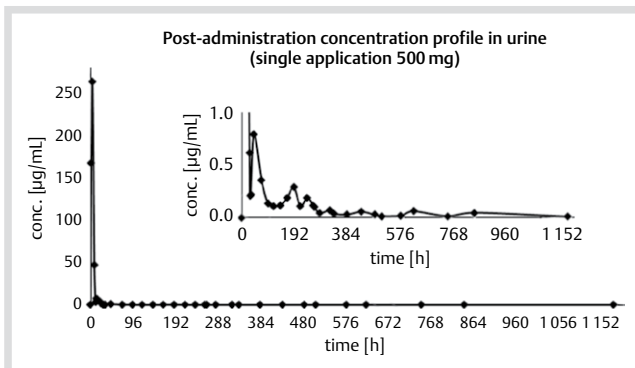


Fig. 2 Urinary meldonium concentration profile obtained from post-administration samples (single application, 500 mg of meldonium). An initial phase with a rapid clearance of the active substance within the first 12–24 h is followed by a second elimination phase, where concentrations of ca. 10–200 ng/mL were observed for up to 49 days (1 176 h).

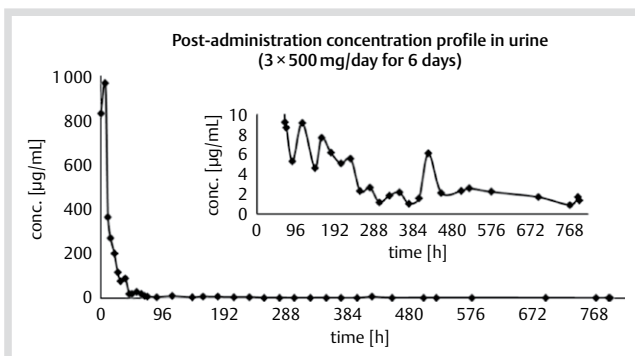


Fig. 3 Urinary concentrations of meldonium in post-administration samples collected after multiple applications (3 × 500 mg/day for 6 days) of the drug. Peak values of ca. 960 µg/mL were observed 5 h after cessation of the drug intake. After 72 h, concentrations ranged between 1 and 9 µg/mL until day 33.

observed with ca. 2.8 µg/mL 2 h post-administration, followed by a rapid decrease to ca. 130 ng/mL within the first 12 h. Although this suggested a rather rapid elimination of meldonium, the analyses of DBS samples collected subsequently at 24 h, 9 and 16 days, still yielded concentrations of ca. 80 ng/mL as illustrated in **Fig. 1**. This translated into an elimination profile of meldonium in urine (not specific gravity-adjusted) as shown in **Fig. 2**, which continued with urine samples until day 49 post-application. Peak urinary meldonium concentrations were observed 5 h post-administration with ca. 260 µg/mL, and urine samples still contained ca. 10–60 ng/mL of the drug in the pilot study test days 18–49 (432–1 176 h). Noteworthy, within the first 24 h, ca. 190 mg (38%) of the administered meldonium were excreted, while only ca. 1.5 mg were renally eliminated during the period from 24 to 48 h. The concentration profile of urine samples obtained from the multi-dose study, for which pre-, inter-, and post-administration samples up to 33 days were available (**Fig. 3**), was in accordance with that of the single-dose administration study results. While maximum urinary meldonium concentrations of more than 1.5 mg/mL were observed during the drug administration period, urine concentrations rapidly decreased in a first elimination phase after cessation of the drug from ca. 960 µg/mL to ca. 9 µg/mL within 72 h. Subsequently, meldonium was detected at concentrations between ca. 1 and 9 µg/mL until day 33, corroborating the aforementioned slow excretion process of the drug.

This atypical elimination behavior of meldonium suggested an incorporation of the substance into the cellular fraction of blood, which was further investigated by means of whole blood samples collected from the multi-dose administration study on day 4 and day 28. Here, plasma was separated from the RBC fraction, and dried plasma spots (DPS) as well as dried spots of washed RBC fraction (20 µL each) were subjected to the same analytical protocol as the DBS. The analyses revealed that meldonium was, despite intense washing of the intact erythrocytes, still abundantly present in the RBC fraction with ca. 1 800 ng/mL on day 28. Conversely, a ca. 30-fold lower concentration was found in the corresponding plasma spot, which corroborated the hypothesis that meldonium can be incorporated into RBCs. To date, the reversibility of the suggested incorporation and underlying (active or passive) transport mechanisms are unknown and further investigations are deemed warranted, also in consideration of data suggesting a slow but distinct partitioning of the structurally similar l-carnitine between plasma and RBCs [1,6] as well as muscle cells [14, 17, 19]. The successive and slow release of erythrocyte-entrapped meldonium into the circulation would plausibly explain the observed and beforehand unexpected detection window for meldonium, particularly in the light of an erythrocyte lifespan of approximately 70–140 days [7]. According to literature data, the estimated half-life ($t_{1/2}$) of meldonium during the initial rapid elimination phase is 5–15 h [13, 25]. The results of this pilot study suggest the existence of a subsequent second and substantially slower elimination phase, attributed to a proposed incorporation of meldonium into erythrocytes. The administration of a single oral dose of meldonium was detected in human urine for up to 49 days using established doping control analytical approaches and in DBS for at least 16 days. In consideration of the multi-dose administration data and reported accumulation effects regarding meldonium [25] and the aforementioned results that suggest the incorporation of the drug into erythrocytes allowing for sustained liberation during eryptosis, detection windows after long-term administration of

high but yet therapeutic amounts of meldonium span over several weeks and might even extend to months.

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