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Urinary excretion studies of meldonium after multidose parenteral application

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ABSTRACT

Meldonium is a drug exhibiting cardioprotective and anti-ischemic effects. Due to its potential performance-enhancing benefit in sports, meldonium was added to the World Anti-Doping Agency list of prohibited substances in 2016. Since then, a high number of adverse analytical findings reported on meldonium has questioned meldonium's detection time in urine. Hence, the objective of the current study was to characterize the pharmacokinetic urinary excretion pattern of meldonium when administered as multiple intravenous injections. Three injections of 250 mg meldonium were given over a time period of five days to six healthy volunteers and urine samples were collected for eight months after the last injection of the drug. For the quantification of meldonium in urine, a liquid chromatography-tandem mass spectrometry method was fully validated according to the World Anti-Doping Agency guidelines in terms of specificity, matrix interferences, intra- and inter-day precision, accuracy, carry-over, robustness, linearity, limit of detection, and limit of quantification. The assay was successfully applied to the pharmacokinetic study. A three-compartment model was found to best describe the pharmacokinetics of meldonium with average alpha, beta, and gamma half-lives of 1.4 h, 9.4 h, and 655 h, respectively. The detection time in urine varied between 94 and 162 days.

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1. Introduction

Meldonium, also known as mildronate, is manufactured in Latvia and primary distributed in the Baltic countries and Russia. It is mainly used to treat cardiovascular disease including ischemia, as well as neurodegenerative disorders and bronchopulmonary diseases [1,2].

The mechanism of action involves a competitive inhibition of the enzyme λ-butyrobetaine hydroxylase, which converts λ-butyrobetaine in L-carnitine. Furthermore, the absorption and transport of L-carnitine is reduced by inhibition of the carnitine transporter carnitine/organic cation transporter type 2 (OCTN-2) [2,3]. The resulting decreased availability of carnitine in the cell leads to a shift in energy metabolism from fatty acid oxidation to increased glucose consumption, and as a consequence, ATP is generated more efficiently. This is beneficial in low oxygen conditions, like for instance in heart conditions where the cardiac muscle is deprived of oxygen [4]. For the same reason, meldonium might have performance enhancing effects in sports. Suggested benefits are amongst others a decrease in the production of lactic acid, the prevention of oxidative stress, enhanced endurance, and increased physical work capabilities [3]. Due to these effects, meldonium was added to the World Anti-Doping Agency's (WADA) prohibited list in 2016 and is classified as a metabolic modulator under section four [5].

In early 2016, meldonium produced a surprisingly high number of adverse analytical findings in doping control analysis, and in many cases, the athlete claimed that the substance was taken before the time of the ban. At this time point, the minimum required performance level for meldonium as a non-threshold substance was 20 ng/mL urine, based on WADAs technical docu-

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ments [6]. The high number of adverse analytical findings, however, raised the question if there is sufficient knowledge on meldonium's elimination in humans for an optimal interpretation of low urine concentrations. As a consequence, WADA gave instructions not to report cases with urine concentrations below 1 μg/mL as adverse analytical findings [7]. Today, meldonium should only be reported above a level of 100 ng/mL urine [8].

Pharmacokinetic data for meldonium in humans are limited to a few studies and previously reported data are not overall consistent. Available studies suggest both nonlinear pharmacokinetics, as well as linear pharmacokinetic properties. Furthermore, there are considerable variations in the reported half-life of meldonium, both in single and multiple dose studies.

For single dose oral administration, Zhang et al. [9] reported proportionality between dose levels (250–1500 mg) and the area under the curve (AUC), as well as proportionality in maximum plasma concentrations (C_{max}) to the administered dose. The half-life, on the other hand, was dose dependent and ranged between 3.6 and 6.6 h. Furthermore, a multiple oral dose study was conducted, in which volunteers received 500 mg meldonium three times a day for 13 days. Also here, non-linearities, in addition to accumulation, were observed. The half-life reported was 14 ± 2 h. In a different study, Peng et al [10] investigated the meldonium pharmacokinetics for 24 h after single intravenous administrations of 250, 500 and 100 mg meldonium. In contrast to Zhang et al., they reported linear pharmacokinetics with a half-life ranging from 5.6 to 6.6 h. They also monitored pharmacokinetic parameters after multiple intravenous administrations of 500 mg for 6 days, and like Zhang et al, the observed half-life was longer (15 ± 3 h) compared to single-dose administration. Hence, the authors suggested accumulation of the compound. In a further study, Cai et al. [11] carried out analysis of meldonium in plasma – and urine samples subsequent to intravenous administration of 250, 500 and 750 mg of meldonium. The estimated elimination half-life increased with increased administered dose (reported half-lives were 2.7–5.2 h), and consequently, the authors concluded that meldonium may exert non-linear pharmacokinetics in humans. Pidpruzhnykov et al. [12] investigated pharmacokinetic properties in a bioequivalence study. Plasma concentrations were monitored 24 h after single oral administrations of two generic formulations of the drug, and the pharmacokinetic elimination profile was reported to be linear (half-life = 3.6–3.7 h). They also observed a double peak in the plasma concentration-time profile, which they suggested could be associated with two areas of absorption in the gastrointestinal tract.

In the perspective of doping control analysis, one major limitation of the studies above is the relatively short sample collection time subsequent to drug administration. A few available studies, though, deal with longer sample collection times. In 2011, Liepinsh et al. [13,14] conducted a long-term study, in which they monitored plasma, as well as urine concentrations of meldonium. Volunteers were treated orally with 500 mg meldonium twice a day for a period of 4 weeks and samples were collected weekly. In addition to the treatment period, samples were collected during a washout period of 4 weeks after the end of treatment. After the 4 week wash-out period, meldonium was still present in both plasma and urine samples, and the authors concluded that meldonium accumulates after long-term treatment, and that the elimination time is treatment – and dose depended. In a recent investigation, Görgens et al. [15] examined urinary excretion profiles single-dose and multiple-dose oral application. They found that the elimination of meldonium was characterized by two phases, in which the second phase is considerably slower than the first one. The urinary detection windows expanded as much as 64 and 117 days, after single – and multiple dose administration, respectively. A further study performed by Tretzel et al. [16], where meldonium was analysed in dried blood spots, supports the presence of more than one elimination phase.

In recent years, a number of assays have been published on the analysis of meldonium in human plasma and urine [11,12,17–21]. Taking into account the polar characteristics of meldonium, a majority of the methods are based on hydrophilic interaction liquid chromatography – tandem mass spectrometry (HILIC-MS/MS) [11,12,17–19].

In this study, for the first time, the excretion of meldonium after multiple parenteral administration in six healthy volunteers was investigated. Urine samples were collected over a time period of eight months, in order to characterize the long-term excretion pattern of the drug.

2. Experimental

2.1. Design of the clinical study

Six volunteers (three male and three female) aged from 37 to 55 received three parenteral doses of Mildronates® (500 mg meldonium/5 mL), Grindex, Latvia. All volunteers received 250 mg of meldonium in the morning on day 1 and the injection was repeated on day 3 and 5. All urine samples were collected for nine consecutive days after the first administration. For the next five days, urine samples were collected once (in the morning) every day. Furthermore, one urine sample was collected once a week for the next eight months. All participants were non-athletes and on normal diets. They were healthy, not taking any diuretic medications, which could interfere with the excretion of meldonium [22].

The study was conducted in accordance with the International Conference on Harmonization guidelines for Good Clinical Practice, and was compliant with the ethical principles described in the current version of the Declaration of Helsinki. Prior to study initiation, all protocols were approved by the local ethics committee of the Sports Medicine Association of Serbia (Belgrade, Serbia).

2.2. Chemical and reagents

Meldonium dihydrate was purchased from Sigma (St Louis, MO, USA) and meldonium-d3 (internal standard) was provided by TLC Pharmachem (Ontario, Canada). Methanol of HPLC grade was obtained from Chem Lab (Zedelgan, Belgium) and water of HPLC grade was provided by Merck (Darmstadt, Germany). Formic acid (99%, ULC/MS-CC/SFC) was purchased from Bisolve (Valkenswaard, The Netherlands).

2.3. Sample preparation

To an aliquot of 100 μL urine, 50 μL of a meldonium d3 internal standard solution (1 μg mL^{-1}) and 300 μL of solvent were added. The solvent was a mixture of methanol and water in the ratio 90:10 (v/v) with 0.1% of formic acid. The sample was mixed and an aliquot of 10 μL was injected into the LC/MS/MS instrument. Samples with a meldonium concentration exceeding the highest point of the calibration curve were diluted to fall within the calibration range.

2.4. Liquid chromatography – mass spectrometry

The samples were analyzed using a CTC HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland) and an Aria Transcend TLX-1 LC system (Thermo, Austin, TX, USA) interfaced to a TSQ Vantage triple quadrupole (Thermo, Austin, TX, USA). A silica precolumn (4.0×2.0 mm, particle size 5 μm) (Phenomenex, Aschaffenburg, Germany) was used for sample clean-up and the analytical HPLC column was an Atlantis HILIC Silica (50 mm × 4.6 mm, 3 μm particle size) (Waters, Milford, MA, USA). Column selection was performed by a Maylab Mistraswitch column selector (6 column selection system) (Maylab Analytical Instruments, Vienna, Austria). Mobile

phase A consisted of water with 0.2% of formic acid, and mobile phase B consisted of methanol with 0.1% formic acid. A constant flow rate of 0.4 mL min^{-1} was applied with the following gradient: 100% B (0–1 min), 100–40% B (1–5 min), 40% B (5–7 min), 100% B (7–11 min). The column temperature was maintained at 25 °C and the temperature in the autosampler was set to 4 °C.

The mass spectrometer was equipped with a heated electrospray ionization (ESI) source and was operated in positive ionization mode with a spray voltage set at 3300 V. The capillary temperature was adjusted to 270 °C. The sheath and auxiliary gas (nitrogen) flow rate was 35 and 10 arbitrary units, respectively. The system was operated in selected ion monitoring (SRM) mode with argon as the collision gas at a pressure of 1.5 mTorr. Selected transitions for meldonium were *m/z* 147 → *m/z* 42, *m/z* 147 → *m/z* 58 and *m/z* 147 → *m/z* 59 and collision energies were 52 eV, 18 eV, and 17 eV, respectively. For quantitative determination, the transition *m/z* 147 → *m/z* 58 was selected. The selected transition for meldonium d3 (internal standard) was *m/z* 150 → *m/z* 61 with a collision energy of 18 eV.

2.5. Validation

The analytical method was validated according to current WADA guidelines [23,24]. Investigated parameters included specificity, matrix interferences, intra- and inter-day precision, accuracy, carry-over, robustness, linearity, limit of detection (LOD) and limit of quantification (LOQ).

Specificity and matrix interferences were investigated by comparing the chromatograms of blank urine samples from six volunteers (three female, three male) with the corresponding spiked urine samples. The samples were spiked with meldonium at a concentration of 100 ng mL^{-1}.

Carry-over from sample to sample during instrumental analysis was evaluated by injecting a high concentrated spiked urine sample (4000 ng mL^{-1}) prior to the injection of two consecutive blank water samples.

Linearity of the method was tested in the range 10 ng mL^{-1} - 4000 ng mL^{-1} (10 ng mL^{-1}, 50 ng mL^{-1}, 100 ng mL^{-1}, 500 ng mL^{-1}, 1000 ng mL^{-1}, 2000 ng mL^{-1} and 4000 ng mL^{-1}). For all calibration levels, four replicates were prepared. The calibration samples were prepared by spiking blank urine samples.

Limit of quantification (LOQ) was determined as the lowest concentration level with a signal to noise ratio > 10:1 and RSD% for five repetitions less than 20%. Limit of detection (LOD) was defined as the lowest concentration level at which meldonium could be detected and identified with a signal to noise ratio > 3:1 for two ion transitions (limit of identification).

Intra-day precision was evaluated at two different concentration levels (100 ng mL^{-1} and 1000 ng mL^{-1}) with 10 independent spiked urine samples on each level. The analysis was performed on the same day. Inter-day precision was assessed by repeating the experiment on three consecutive days.

Accuracy was determined in spiked urine samples at two different concentration levels (100 ng mL^{-1} and 1000 ng mL^{-1}) The calculated theoretical amount of analyte was compared to the actually quantified value in the intra-assay studies.

To demonstrate robustness of the assay, one qualitative and one quantitative factor were probed. As a qualitative factor, six different urine samples were selected (3 male and 3 female), and as a quantitative factor, the content of water (with 0.1% formic acid) in the solvent for sample preparation was evaluated. Four levels were tested (10%, 15%, 20% and 30% water with 0.1% formic acid). The samples were spiked with 100 ng of meldonium per mL of urine. In total, 24 experiments were performed and RSD values for retention time and the meldonium concentration were calculated.

Table 1
Validation data for meldonium.

Linearity (10–4000 ng/ml)	R^2 = 0.9971
Intra-day precision (RSD%)	
100 ng/ml	4.7%
1000 ng/ml	3.3%
Inter-day precision (RSD%)	
100 ng/ml	1.0%
1000 ng/ml	14.9%
Limit of quantification (LOQ)	10 ng/mL
Limit of detection (LOD)	3 ng/mL
Robustness (RSD%)	
Retention time	0.9%
Area ratio	8.0%
Accuracy (%)	
100 ng/ml	103.6 ± 5.4%
1000 ng/ml	106.2 ± 3.5%

2.6. Pharmacokinetics

For the estimation of elimination half-lives, urinary excretion rate data was plotted against the midpoint of the urine collection interval in a semilogaritmic plot. The elimination rate constant, k, was determined from the slope of the plot, and elimination half-lives were calculated from k. For the last phase of the study, the collection time interval was estimated based on previous collection patterns for each volunteer.

3. Results and discussion

The presented study was conducted in two parts. In the first part, a full validation of the LC/MS/MS procedure was performed according to current WADA guidelines [23,24]. In the second part, the validated method was applied for the determination of the concentration of meldonium in urine samples from six volunteers after multidose parenteral application of meldonium injections. On the basis of the obtained results, pharmacokinetic parameters were calculated.

3.1. Method validation

The results of the method validation are summarized in Table 1. By analysing six different blank urine samples, a satisfactory specificity was demonstrated with no interfering signals observed at the meldonium retention time. Furthermore, meldonium could easily be detected in the same six samples spiked with the analyte. In these samples, stable product ion ratios were observed according to WADA criteria [25]. The method proved to be linear over the concentration range studied with a determination coefficient (R^2) above 0.99. The LOQ was 10 ng mL^{-1} (RSD value lower than 20% for n = 10) and the LOD and limit of identification was 3 ng mL^{-1}, which is considered to be sensitive enough for both doping analysis and the pharmacokinetic study. The calculated values for intra - and inter-assay precision, summarized in Table 1, are also within an acceptable range. Furthermore, the method demonstrated sufficient accuracy, robustness, and no sample carry-over. In Fig. 1, extracted ion chromatograms generated during the validation procedure are shown, representing a spiked urine sample at LOQ, a urine sample from the clinical study, and a blank urine sample. In conclusion, the analytical assay proved to be fit for purpose.

3.2. Clinical study

In this study, all pharmacokinetic parameters were estimated from urine data. Urine samples are highly relevant for doping analysis representing a non-invasive sample collection. For the

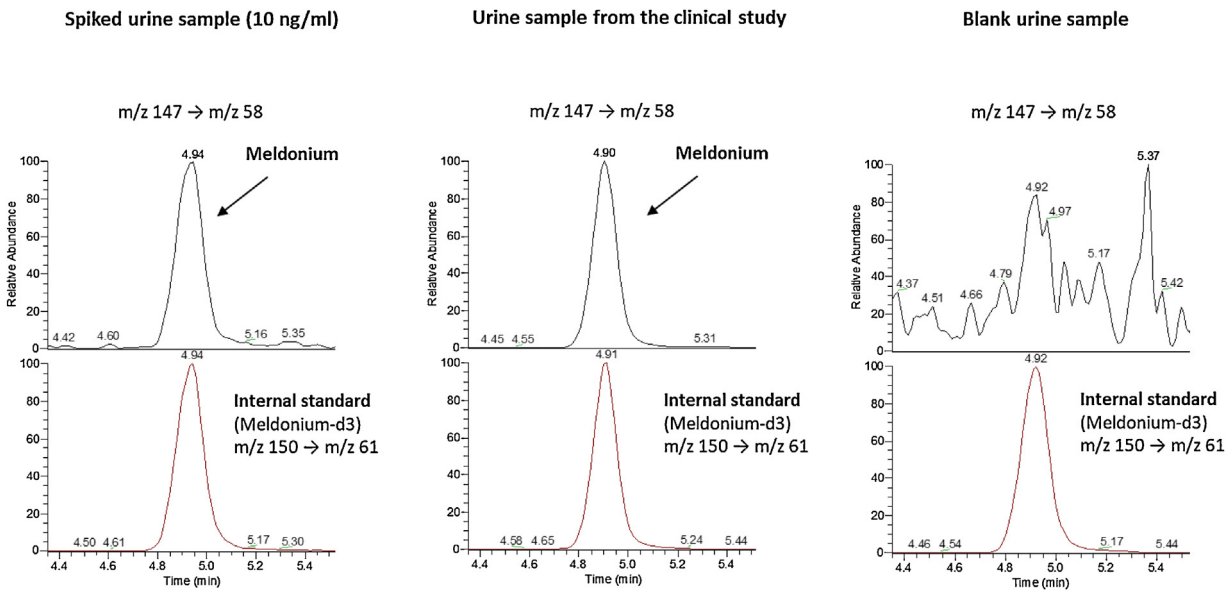

Fig. 1. Extracted ion chromatograms of meldonium (*m/z* 147 → *m/z* 58) and meldonium-d3 (*m/z* 150→ *m/z* 61) of a spiked urine sample at 10 ng/mL (LOQ), a urine sample from the clinical study (963 ng/ml), and a blank urine sample.

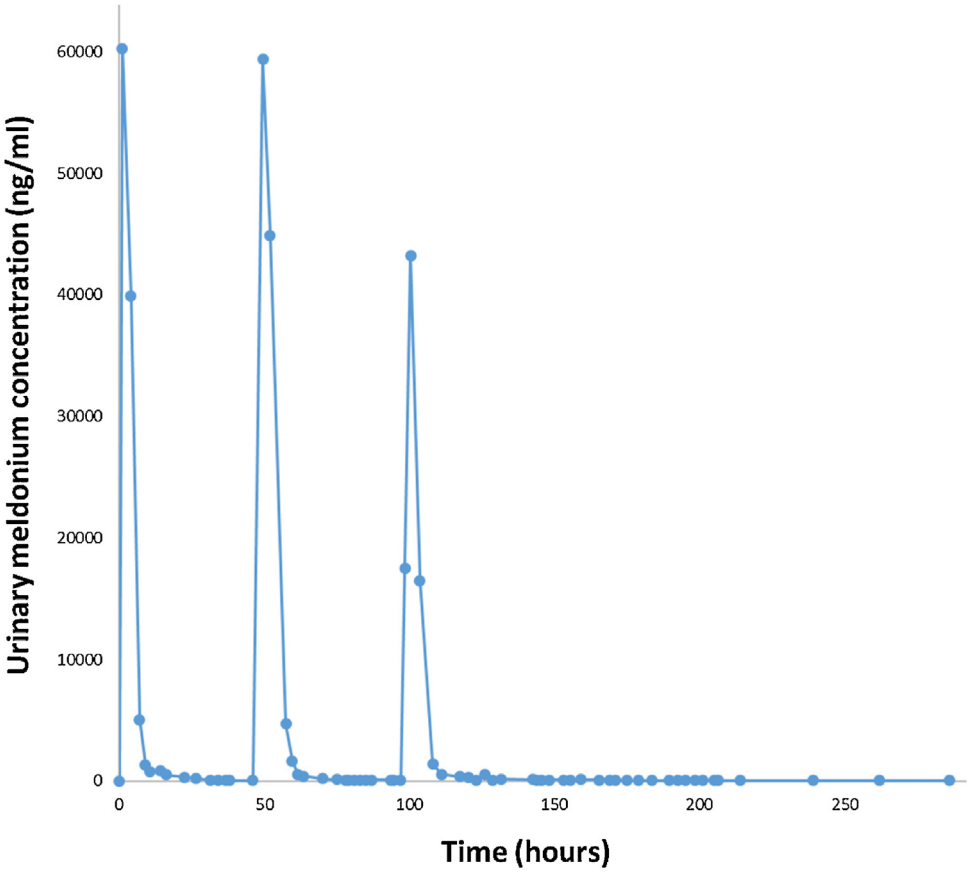

Fig. 2. Urinary meldonium concentration profile for one volunteer obtained after administration of three injections of 250 mg meldonium given over a time period of five days. Data up to 280 h are shown.

calculation of pharmacokinetic data, however, the uncertainty of complete bladder emptying and need to collect urine over short intervals might represent some limitations on the accuracy of the excretion rate data. Nevertheless, such data can provide a good basis for the estimation of half-lives, detection times and identification of excretion patterns.

Obtained urine concentrations of meldonium were corrected for specific gravity and urinary excretion rates were calculated and graphically displayed. Maximum meldonium urine concentrations were observed shortly after injection of the drug. For all volunteers, the highest urine concentrations were observed in the first urine sample collected after administration of meldonium. Max-

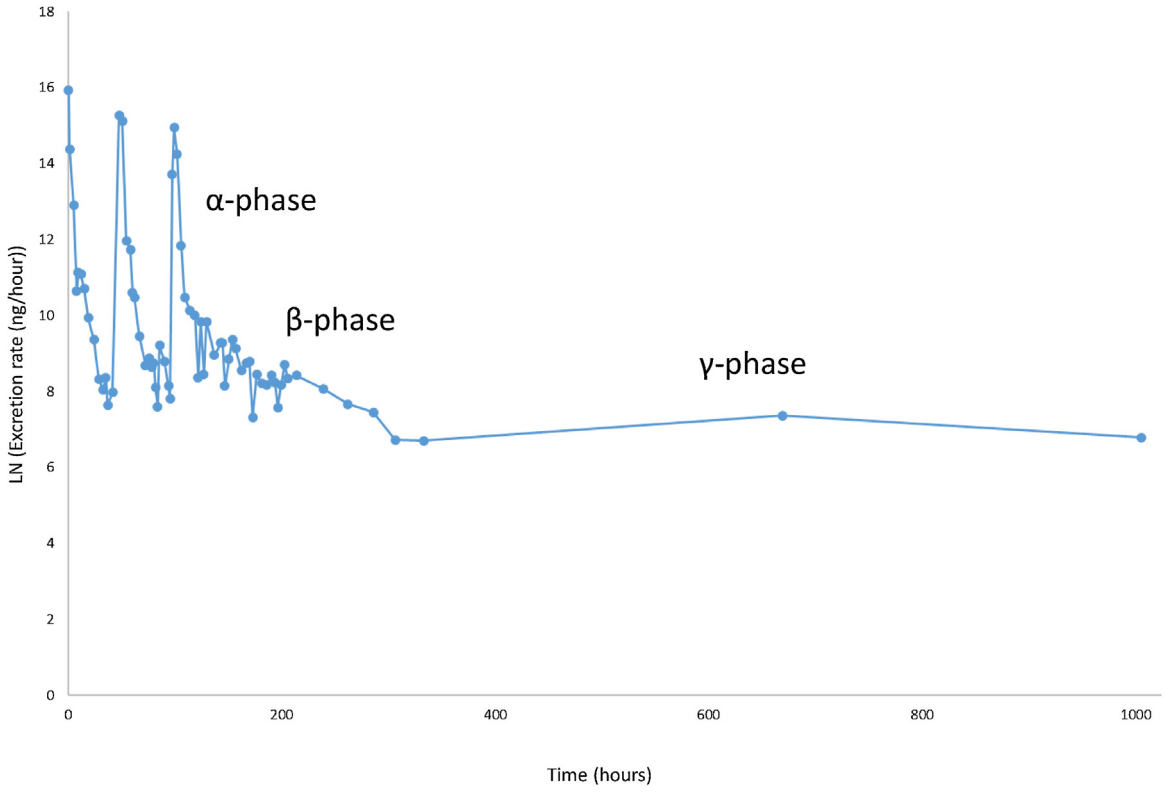

Fig. 3. Urinary excretion rate of meldonium for volunteer 1. Three disposition phases can be identified: alpha (α)-phase, beta (β)-phase and gamma (γ) –phase. Data up to 1000 h are shown.

Table 2
Pharmacokinetic data for meldonium.

		$t_{1/2}$ alpha (hr)	$t_{1/2}$ beta (hr)	$t_{1/2}$ gamma (hr)	Detection time
Volunteer 1	1. administration	1.3	6.3		
	2. administration	1.4	8.6		
	3. administration	1.4	6.4	630	
	Average	**1.3**	**7.1**	**630**	> 94 Days
Volunteer 2	1. administration	1.3	11.6		
	2. administration	0.6	10.9		
	3. administration	1.0	9.2	866	
	Average	**0.9**	**10.6**	**866**	> 142 Days
Volunteer 3	1. administration	1.3	9.6		
	2. administration	1.3	10.3		
	3. administration	1.5	9.2	630	
	Average	**1.4**	**9.7**	**630**	> 149 Days
Volunteer 4	1. administration	1.2	12.7		
	2. administration	1.5	9.6		
	3. administration	1.0	11.1	533	
	Average	**1.2**	**11.1**	**533**	> 130 Days
Volunteer 5	1. administration	1.1	8.2		
	2. administration	1.6	7.9		
	3. administration	1.7	15.4	693	
	Average	**1.4**	**10.5**	**693**	> 157 Days
Volunteer 6	1. administration	2.3	7.4		
	2. administration	2.6	6.8		
	3. administration	1.8	8.0	578	
	Average	**2.2**	**7.4**	**578**	> 162 Days
Average all		**1.4**	**9.4**	**655**	**139 Days**

imum concentrations ranged between 21 and 348 μg ml^{-1} urine and was followed by a rapid decrease within the first hours. As an example, the meldonium concentration profile for volunteer 1 is displayed in Fig. 2. In Fig. 3, the excretion rate for meldonium is shown for volunteer 1 in a semilogaritmic plot. The results clearly suggest a three-compartment model for meldonium with three disposition phases: alpha, beta and gamma. This pattern was seen for all six volunteers.

The three-compartment model has two peripheral compartments; a shallow tissue compartment and a deep tissue compartment. The deep tissue compartment may contain poorly perfused tissue or it may represent the binding of a drug in certain

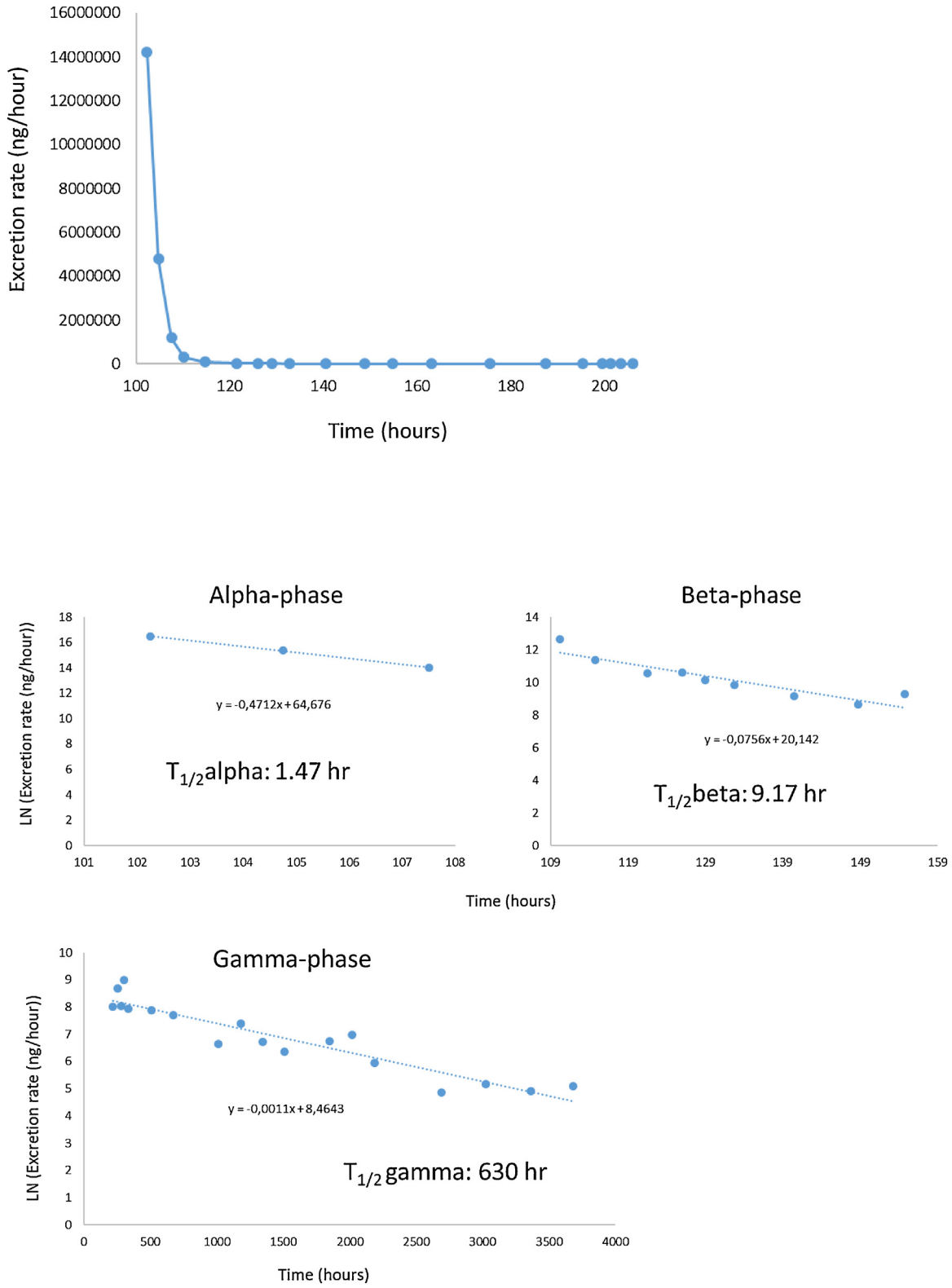

Fig. 4. Urinary excretion of meldonium for volunteer 3 after the 3. injection of meldonium on day 5. The semilogaritmic plots and calculations are illustrated for each disposition phase.

tissues. In the case of meldonium, the drug is an inhibitor of OCTN-2, which main task is to preserve high tissue contents of organic cations like carnitine. By being substrate for the same transporter as carnitine, meldonium is effectively transported and preserved in tissues [2]. Hence, this accumulation of meldonium in tissues may contribute to a tri-exponential disposition and subsequently a long elimination time. An incorporation of meldonium into erythrocytes has also been suggested as explanation for a slow elimination phase [16].

In any case, the three-compartment model is in accordance with the distribution model previously described for L-carnitine, which is substrate for OCTN-2 and structurally resembles meldonium. In the case of L-carnitine, the following three compartments have been suggested: Extracellular fluid including plasma as the central compartment, fast equilibrating tissues such as liver and kidneys, and finally slow equilibrating tissues such as skeletal and cardiac muscle [26].

For meldonium, elimination half-lives were calculated based on urinary excretion rates. In Fig. 4, the meldonium urinary excretions rates and calculation plots are illustrated for volunteer 3 (3. injection). Calculations for all volunteers are summarised in Table 2. The terminal meldonium half-life (representing the gamma-phase of the disposition) was estimated to be as long as 24–36 days in the six volunteers. This explains the long detection time for meldonium in urine. In all volunteers, meldonium was detectable for more than three months, and in some cases, meldonium could be detected for more than 5 months (volunteer 5 and 6). No major differences in pharmacokinetics were observed between male and female volunteers.

Estimates of half-lives of meldonium seem consistent across the six volunteers for the dose levels included in this study, indicating linear pharmacokinetics. However, blood sample values and a broader dosing range should be investigated in order to investigate dose proportionality in derived pharmacokinetic values, as well as consistency in both secondary and primary pharmacokinetic parameters.

4. Conclusion

In this study, the urinary excretion pattern of meldonium in six healthy volunteers was characterized after multiple parenteral application of the drug. A terminal half-life of 22–36 days was demonstrated, allowing meldonium to be detected in urine for several months after administration. It was shown, for the first time, that the observed excretion profiles of meldonium in urine can be adequately described by a three-compartment model.

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