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Carnitine and γ-butyrobetaine Stimulate Elimination of Meldonium due to Competition for OCTN2-mediated Transport

Edgars Liepinsh[1], Elina Makarova[1], Eduards Sevostjanovs[1], Dace Hartmane[1], Helena Cirule[1], Olga Zharkova-Malkova[1], Solveiga Grinberga[1] and Maija Dambrova[1,2]

[1] Latvian Institute of Organic Synthesis, Riga, Latvia

[2] Riga Stradins University, Faculty of Pharmacy, Riga, Latvia

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Author for correspondence: Edgars Liepinsh, Latvian Institute of Organic Synthesis, Aizkraukles Str. 21, Riga LV1006, Latvia (fax: +371 67702408, e-mail: ledgars@farm.osi.lv).

Abstract: Meldonium (3-(2,2,2-trimethylhydrazinium)propionate) is the most potent clinically used inhibitor of organic cation transporter 2 (OCTN2). Inhibition of OCTN2 leads to a decrease in carnitine and acylcarnitine contents in tissues and energy metabolism optimization-related cardioprotective effects. The recent inclusion of meldonium in the World Anti-Doping Agency List of Prohibited Substances and Methods has raised questions about the pharmacokinetics of meldonium and its unusually long elimination time. Therefore, in this study, the rate of meldonium washout after the end of treatment was tested with and without administration of carnitine, γ-butyrobetaine (GBB) and furosemide to evaluate the importance of competition for OCTN2 transport in mice. Here, we show that carnitine and GBB administration during the washout period effectively stimulated the elimination of meldonium. GBB induced a more pronounced effect on meldonium elimination than

carnitine due to the higher affinity of GBB for OCTN2. The diuretic effect of furosemide did not significantly affect the elimination of meldonium, carnitine and GBB.

In conclusion, the competition of meldonium, carnitine and GBB for OCTN2-mediated transport determines the pharmacokinetic properties of meldonium. Thus, due to their affinity for OCTN2, GBB and carnitine but not furosemide stimulated meldonium elimination. During long-term treatment, OCTN2-mediated transport ensures a high muscle content of meldonium, while tissue clearance depends on relatively slow diffusion, thus resulting in the unusually long complete elimination period of meldonium.

Keywords: meldonium, pharmacokinetic, drug transport, drug elimination

Abbreviations: OCTN2, organic cation transporter 2; GBB, γ-butyrobetaine;

Meldonium (3-(2,2,2-trimethylhydrazinium)propionate), a regulator of glucose and fatty acid metabolism, is used clinically as a cardioprotective drug [1-3]. At the beginning of 2016, meldonium was included in the World Anti-Doping Agency List of Prohibited Substances and Methods. Since then, more than 100 adverse analytical findings about meldonium have been recorded [4]. The unusual pharmacokinetics of meldonium was recently outlined [5-7]; however, detailed measurements of meldonium pharmacokinetics in plasma, tissues and urine after the end of treatment have not been performed.

For a long time, meldonium was known as the most potent inhibitor of organic cation transporter 2 (OCTN2) [8], and new and more active inhibitors have been synthesized only recently [9]. The main function of OCTN2 is to ensure high tissue content of organic cations such as carnitine and γ-butyrobetaine (GBB) [10]. Importantly, OCTN2 also ensures the absorption of organic cations in the ileum and reuptake in kidneys. Thus, the mechanism of meldonium action is the inhibition of OCTN2, which leads to carnitine elimination by urine and decreased tissue carnitine content. Recent studies have shown that limited tissue content of carnitine prevents acylcarnitine accumulation during ischaemia-reperfusion and atherosclerosis and protects cardiac and vascular tissue against acylcarnitine-induced damage [11-13]. In addition, decreased acylcarnitine content improves muscle and heart insulin sensitivity and glucose tolerance [14].

Because meldonium is an organic cation that contains an N-trimethyl group, it not only inhibits OCTN2-mediated carnitine transport but is also effectively transported by OCTN2 [15]. This ensures reuptake of meldonium in kidneys and transport into tissues, where meldonium competes with carnitine and GBB for OCTN2-mediated transport. Although the higher affinity of meldonium for OCTN2 might suggest a higher priority for transport, the limited transport capacity of OCTN2 and transporter saturation must be considered [16]. Thus, OCTN2 can ensure kidney reuptake of organic cations such as carnitine to maintain its plasma concentration at up to 60 μM; if compounds exceed this concentration, they are eliminated by urine [17, 18]. The highest tissue content (~1 mM) of carnitine is in the heart, and this could be related to the higher expression of OCTN2 in cardiac tissue

[17]. The administration of meldonium decreases carnitine transport, but carnitine might be expected to compete with meldonium and decrease the transport of meldonium, particularly when meldonium intake is discontinued. In addition, administration of meldonium stimulates a significant, several-fold increase in the plasma GBB concentration [19]. Compared to meldonium (EC_{50} for OCTN2 of 63 μM), the ability of GBB to inhibit OCTN2-mediated carnitine transport is approximately 15 times higher [20]. Therefore, GBB must be considered as an important competitor for meldonium transport. Furosemide does not inhibit OCTN2 and is used as non-specific control compound that influences the clearance of many exogenous and endogenous compounds, including carnitine [21]. Therefore, this study was designed to characterize the competition among meldonium, GBB and carnitine for OCTN2-mediated transport.

MATERIALS AND METHODS

Animals

The experimental procedures were carried out in accordance with the guidelines of the European Community (2010/63/EU), local laws and policies and were approved by the Latvian Animal Protection Ethical Committee, Food and Veterinary Service, Riga, Latvia. All studies involving animals are reported in accordance with the ARRIVE guidelines [22, 23].

Twenty-five male SW mice (6 weeks old, Laboratory Animal Centre, Institute of Biomedicine and Translational Medicine, Tartu, Estonia) were housed under standard conditions (21-23°C, relative humidity 50% ± 10%, 12-hr shifted light-dark cycle) with unlimited access to food and water. The mice were adapted to local conditions for two weeks before the start of treatment. Meldonium (3-[2,2,2-trimethylhydrazinium] propionate dihydrate) was obtained from JSC Grindeks (Riga, Latvia). Carnitine was purchased from Lonza (Basel, Switzerland) and γ-butyrobetaine hydrochloride (GBB) was from obtained from Sigma-Aldrich (Steinheim, Germany). Furosemide solution was purchased from Sopharma (Bulgaria). Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Heparin sodium was purchased from Panpharma (Fougeres, France). All compounds were administered with drinking water. The mice received meldonium at a dose of 400 mg/kg with drinking water for 2 weeks, and then all animals were divided into 5 groups (5 animals per group). One group was killed after 2 weeks of meldonium treatment; the other groups received water or GBB at a dose of 200 mg/kg, carnitine at a dose of 200 mg/kg and furosemide at a dose of 20 mg/kg with drinking water for one week. Urine samples were collected 1 and 7 days after the start of the washout period. At the end of the treatment, the mice were killed in the morning, and blood plasma, heart and muscle tissue samples were collected. Urine, plasma and tissue samples were stored at –20°C prior to analysis.

UPLC/MS/MS assay

The concentrations of carnitine, GBB and meldonium in plasma, tissue and urine samples were measured using the UPLC/MS/MS method, as previously described [24]. Sample preparation involved a simple one-step protein precipitation with acetonitrile-methanol solution. As an internal standard, we used 3-(2,2-dimethyl-2-prop-1-yl-hydrazinium) propionate for all calculations. Urine samples were diluted 20- or 1000-fold with water before analysis. Briefly, 100 μL of an acetonitrile-methanol mixture (3:1, v/v) was added to 25 μL of plasma, tissue homogenate or diluted urine

sample. Samples were centrifuged at 11,000 x g for 10 min. to precipitate proteins. Then, 60 µL of supernatant were diluted with 500 µL of acetonitrile-methanol mixture (3:1, v/v) containing internal standard. Samples were injected into the UPLC system (Acquity, Waters Corp., 120 Manchester, UK). Chromatographic separation was carried out on a BEH HILIC (1.7 µm, 2.1 × 50 mm) column (Waters Corp., 120 Manchester, UK) at a flow rate of 0.25 mL/min. The composition of the mobile phase [acetonitrile - 10mM aqueous ammonium acetate (pH 4)] varied linearly from 75% to 55% of acetonitrile. Carnitine, GBB and meldonium were quantified by monitoring the specific transitions for each compound on the Micromass Quattro Micro instrument (Waters Corp., 120 Manchester, UK). The ion source parameters were as follows: capillary voltage 0.3 kV; source temperature 120°C; desolvation gas temperature 350°C; desolvation and cone gas flow 500 L/h and 50 L/h respectively. The MS/MS transitions (cone voltage; collision energy) were: 162.20 → 102.90 Da (25V; 16eV) for carnitine; 146.20 → 86.90 Da (25V; 17eV) for GBB; 147.20 → 58.30 Da (20V; 15eV) for meldonium; 175.30 → 86.10 Da (22V; 14eV) for IS. The lower limit of quantification was 0.37 nmol/mL for carnitine, 0.18 nmol/mL for GBB and 0.46 nmol/mL for meldonium. Applied analytical procedures provided fair separation of all analytes of interest in one run.

Statistical analyses and calculations

Results are expressed as the mean ± standard error mean (SEM). Statistically significant differences in the mean values were tested by one-way ANOVA with Dunnett's Multiple Comparison test. The differences were considered significant when $p < 0.05$. The data were analysed using GraphPad Prism 5.0 statistical software (GraphPad Inc., USA).

RESULTS

In this study, a high dose of meldonium (400 mg/kg) was used to ensure high plasma and tissue levels of meldonium at the end of treatment. Administration of meldonium for 14 days significantly elevated its concentration in plasma up to 320 µM (Fig. 1A). The 7-day washout resulted in an approximately 100-fold decrease in the meldonium concentration. In contrast to furosemide administration, carnitine and GBB stimulated meldonium elimination and decreased concentrations of meldonium in plasma by 3-fold compared to the water control group (Fig. 1A).

Meldonium administration induced a significant decrease in the carnitine plasma concentration (Fig. 1B). After the 7-day washout period, the carnitine plasma concentration was partially restored in the water and furosemide group. In the GBB group, the carnitine plasma concentration after 1 week was restored to the control level (Table 1), whereas carnitine administration not only restored the carnitine plasma concentration but induced significant elevation above the control level.

Consistent with the results of previous studies, administration of meldonium induced a significant 3-fold increase in the GBB plasma concentration (Table 1). During the washout period, the GBB plasma concentration in the water group decreased to the control level (1.2 ±0.11 µM). A similar decrease was observed in the furosemide group (Fig. 1C). GBB and carnitine administration increased the GBB plasma concentration by approximately 60- and 40-fold compared to the control level, respectively. Overall, administration of GBB and carnitine but not furosemide during the washout period stimulated the elimination of meldonium and restored the carnitine concentration in plasma.

Similar to the results in plasma samples, administration of meldonium for 14 days significantly elevated the content of meldonium in heart tissues up to 5000 nmol/g, which corresponds to an intracellular concentration of approximately 5 mM (Fig. 2A). One week of washout resulted in a 15-fold decrease in the meldonium concentration. Furosemide did not stimulate meldonium elimination. By contrast, both GBB and carnitine almost completely eliminated meldonium from heart tissue. Thus, both compounds in one week reduced meldonium content in the heart from 5000 to 18 nmol/g of tissue.

Two-week administration of meldonium induced a 90-fold decrease in carnitine content in heart tissue, and after the washout period, the carnitine content was restored to control level in all groups (Fig. 2B). Carnitine treatment induced an increase in heart carnitine content to ~45% above the water control level. The increase in the heart carnitine content was much less pronounced than the increase in the plasma carnitine concentration in the carnitine supplementation group.

Meldonium treatment at a dose of 400 mg/kg did not induce an increase in the heart content of GBB. A high dose of meldonium stimulated both GBB production and elimination by urine. In contrast to the minor increase in carnitine content in the heart, GBB and carnitine administration during washout induced a substantial increase in the GBB heart content above the control level (Fig. 2C). Thus, the GBB content was elevated approximately 12-fold after GBB administration compared to the control level, whereas carnitine treatment induced an approximately 6-fold increase in GBB content in the heart.

The effects of meldonium treatment and subsequent treatment with and without furosemide, carnitine and GBB in muscle were similar to those observed in heart tissue. The content of meldonium in muscle after the 2-week treatment was approximately 3-fold lower than that in the heart. After one week of washout, the meldonium content was decreased by 3.4-fold (Fig. 3A). The decrease was significantly less pronounced in muscle than that in heart and plasma (15- and 100-fold, respectively). Similar to the effects observed in the heart, furosemide did not decrease meldonium content in muscles, whereas administration of GBB and carnitine induced similar 12- and 10-fold decreases, respectively.

Meldonium treatment induced a substantial 34-fold decrease in carnitine content in muscles; however, during one week of washout, carnitine content in muscles was restored to control levels in water, furosemide and GBB groups, whereas carnitine administration elevated the carnitine content even 2-fold higher compared to the non-treated control level (Fig. 3B). Meldonium treatment induced a 2-fold increase in GBB content. During washout, the muscle GBB content in all groups were significant increased even more (Fig. 3C).

To evaluate the elimination rate of meldonium, the concentrations of the compounds in urine were measured. Excess administered meldonium was eliminated in urine, and at the end of the 14-day treatment period, the concentration of meldonium in urine reached up to 59 mM (Table 1). During the washout period, the meldonium concentration in urine significantly decreased to 3943±567 μM on day 1 and to 19±3 μM on day 7 after the end of the treatment (Fig. 4A). Furosemide administration did not influence meldonium elimination (Fig. 4A, B). By contrast, GBB administration significantly increased meldonium elimination in urine. The concentration of meldonium in urine was 5- to 10-fold higher in the GBB group than in the water group (Fig. 4A). Similarly, carnitine administration also stimulated the elimination of meldonium in urine; however, the concentration of

meldonium in urine was lower in the carnitine group than in the GBB group. The concentration of meldonium was 1.7- to 7-fold higher in the carnitine group than in the water group, but this difference was only significant at the end of the washout period (day 7) (Fig. 4B).

During meldonium administration, both GBB and carnitine were eliminated in urine. The GBB concentration in urine after meldonium administration increased over time, reaching up to 248±44.6 µM at the end of the 14-day meldonium treatment period. By comparison, in the control mice, the GBB concentration in urine was approximately 4.4 µM (Table 2). Excess GBB and carnitine were also eliminated in urine during the administration of these compounds (Fig. 4C). *In vivo*, GBB was partially metabolized to carnitine, whereas GBB was converted to carnitine. The sums of the concentrations of both eliminated compounds were approximately 30 mM in both the GBB and carnitine groups at the end of the washout period (Fig. 4C).

DISCUSSION

In this study, the competition among compounds with different affinities for OCTN2 for OCTN2-mediated transport was investigated *in vivo*. Here, we show that carnitine and, in particular, GBB administration during washout effectively stimulated the elimination of meldonium, similar to the meldonium-induced elimination of carnitine and GBB. The greater affinity of GBB for OCTN2 resulted in a more pronounced stimulatory effect on meldonium elimination. *In vivo*, GBB was partially converted to carnitine; therefore, GBB, although stimulating carnitine elimination, did not induce a decrease in carnitine content in heart and muscle. In turn, carnitine stimulated GBB elimination and was metabolized to GBB by the microbiota, resulting in a significantly increased GBB concentration. The diuretic effect of furosemide did not result in increased elimination of meldonium, carnitine and GBB.

Recently, it was reported that even 4 weeks after the end of the treatment, the meldonium plasma concentration was 1.15±0.6 µM in healthy volunteers [5]. This 4-week washout period followed 4 weeks of meldonium treatment at the clinically suggested dose (500 mg twice a day) [25]. In addition, the concentrations of meldonium at the end of the treatment correlated with the concentrations measured after the washout period, suggesting that the complete elimination time is longer in individuals who receive higher initial concentrations [25]. In this study, the plasma concentration of meldonium in mice after the end of treatment decreased approximately 6-fold faster than in healthy volunteers. This discrepancy is not surprising because the plasma clearance rate is faster in rodents than in humans [26]. However, mouse muscle clearance was quite similar to that in healthy volunteers. These data suggest that the pharmacokinetic properties of meldonium after long-term treatment in mice are similar to those in humans.

The meldonium-induced decrease in the heart carnitine content can be achieved by increased carnitine elimination by urine [2]. However, carnitine has a higher affinity for OCTN2, and therefore high meldonium doses should be used to ensure an effective decrease in carnitine levels in plasma and tissue. Moreover, meldonium is also transported by OCTN2, and therefore carnitine stimulates meldonium elimination. GBB, a precursor of carnitine, has greater affinity for OCTN2 compared to carnitine, and administration of GBB during the washout period stimulated even faster elimination of meldonium during the first days of treatment. On day 7, however, the meldonium content in

heart and plasma was similar in the GBB and carnitine groups due to the significant interconversion of GBB and carnitine via GBB and carnitine metabolism [19]. Thus, the GBB and carnitine concentrations were similar at day 7, and an unbiased comparison of the effects of GBB and carnitine on meldonium elimination was therefore possible only on day 1. In addition, during treatment, meldonium stimulated the biosynthesis of GBB, which then induced meldonium elimination. Overall, higher doses of meldonium should be used to overcome competition with GBB and carnitine for transport by OCTN2 and achieve a carnitine-lowering effect.

The main compartments in which compounds compete for OCTN2 transport are plasma and primary urine. In the washout period, even without GBB and carnitine administration, increasing plasma concentrations of carnitine stimulate meldonium plasma clearance via elimination in urine. By contrast, the decrease in meldonium content was significantly slower in mouse heart and, in particular, muscle than in plasma. Moreover, carnitine and GBB did not directly stimulate meldonium washout from tissue. In previous studies, the release rate from muscles to plasma was reported to be approximately 100-fold slower than OCTN2-mediated carnitine transport into muscle tissues, and the half-life of release from tissues into plasma is approximately 139 hr [17, 27, 28]. The results of this study confirm that, similar to carnitine, more than 90% of the meldonium accumulates in muscles and that meldonium has prolonged retention within the muscles. Overall, meldonium accumulation in the muscles and slow muscle clearance explain the unusually long complete elimination rate of meldonium.

Due to competition for OCTN2, both endogenous compounds stimulated the elimination of each other; however, at the end of the experiment, carnitine increased the GBB concentration, whereas GBB increased the carnitine concentration only in plasma and heart tissues. These effects can be explained by the following mechanisms. Carnitine conversion to GBB is due to catalysis by the microbiota and enhanced GBB synthesis from trimethyllysine [19, 29]. Both GBB and carnitine can be metabolized to trimethylamine and its N-oxide; these compounds are not transported by OCTN2 and therefore can be quickly eliminated in urine [30]. In this study, we also observed that an increased concentration of the carnitine precursor GBB can effectively increase the carnitine concentration. Based on the high affinity of GBB for OCTN2, it would be expected that similar to meldonium, GBB would induce the elimination of carnitine and subsequent decrease in carnitine tissue content. Indeed, the carnitine concentration in the urine was significantly increased during GBB administration. However, GBB, as a substrate for carnitine synthesis, completely compensated for the increased carnitine elimination. These results also confirm that increases in carnitine and GBB content can be achieved by both GBB and carnitine administration.

Furosemide is a potent and widely used diuretic drug that inhibits the Na^+-K^+-Cl^- co-transporter in the ascending limb of the loop of Henle [31]. Furosemide influences the clearance of many exogenous and endogenous compounds, including carnitine [21]. By contrast, a recent study demonstrated that continuous administration of furosemide increased the renal expression of organic anion transporter 1 and multidrug resistance protein 2 and decreased p-aminohippurate clearance, thus significantly elevating the plasma concentration of p-aminohippurate [32]. In this study, furosemide administration during the washout period did not result in significantly increased washout of meldonium, suggesting that furosemide does not stimulate the elimination of drugs whose reuptake is based on active transport. In addition, the carnitine and GBB concentrations in plasma and heart tissues were not significantly influenced by furosemide administration. These

results indicate that simultaneous administration of meldonium and furosemide does not change the pharmacokinetics of meldonium and that meldonium and furosemide have no significant drug-drug interactions.

In conclusion, the competition among meldonium, carnitine and GBB for OCTN2-mediated transport determines the pharmacodynamics and pharmacokinetics of meldonium. Thus, due to their affinity for OCTN2, GBB and carnitine but not furosemide stimulate the elimination of meldonium. During long-term treatment, OCTN2-mediated transport ensures a high muscle content of meldonium, whereas tissue clearance depends on relatively slow diffusion, thus resulting in the unusually long complete elimination period of meldonium.

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CONFLIC OF INTEREST

Authors declare no conflict of interests.

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Table 1 Concentrations of GBB and carnitine in control mice plasma, muscle and heart tissues.

	GBB	Carnitine
Plasma, nmol/ml	1.1±0.12	27±2.5
Muscle, nmol/g	12±0.5	169±9
Heart, nmol/g	24±6	941±76

All values represent the average of at least 5 measurements ±SEM.

Table 2 Concentrations of meldonium, GBB and carnitine in the urine of non-treated control and meldonium-treated mice.

	Meldonium, µM	GBB, µM	Carnitine, µM
Control	---	4.4±0.3	17±2.3
Meldonium	58562±11700	248±45*	43±8*

Meldonium was administered at a dose of 400 mg/kg for 14 days. All values represent the average of at least 5 measurements ±SEM. *significantly different from the water control (one-way ANOVA, Dunnett's multiple comparison test)

Figure 1

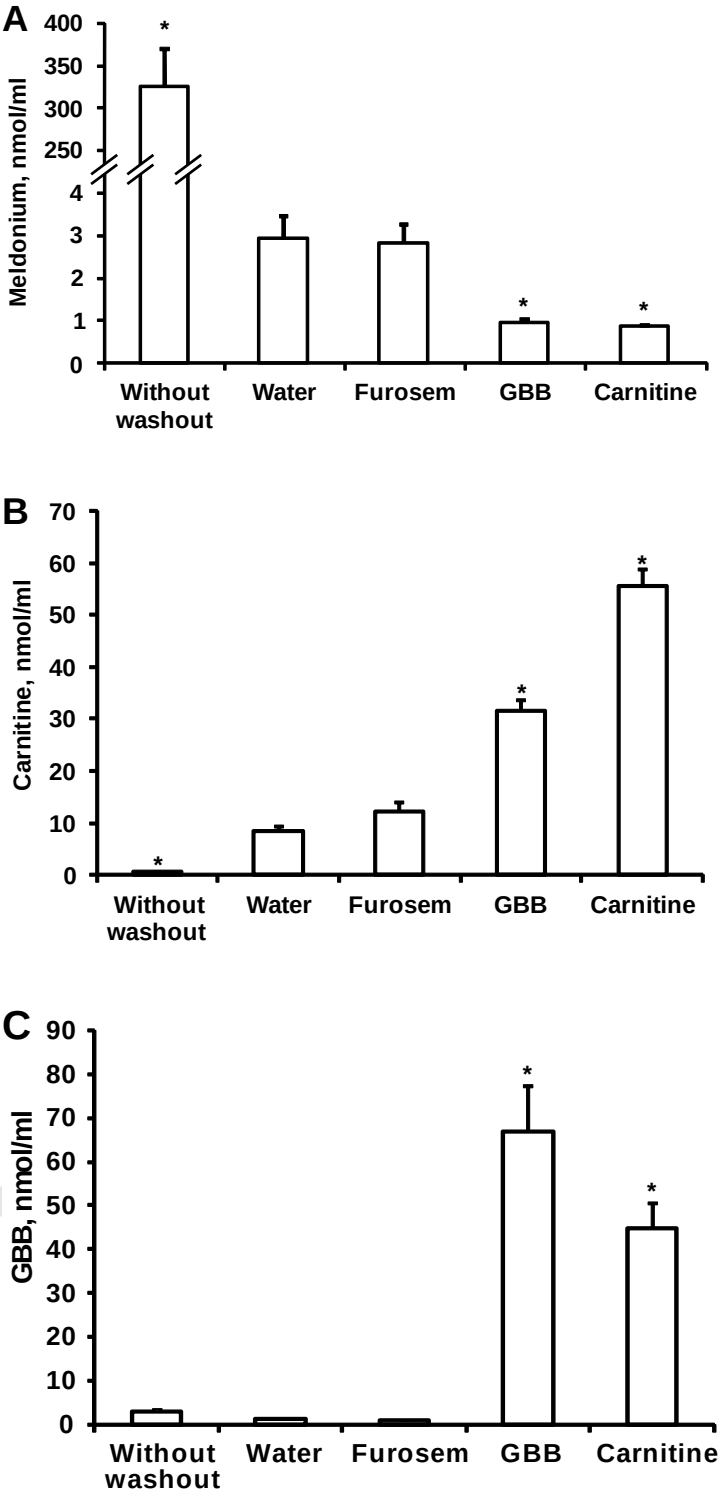

Figure 1 Concentrations of meldonium (A), carnitine (B) and GBB (C) in plasma. Meldonium administration at a dose of 400 mg/kg for 14 days was followed by a 7-day washout period. During the washout period, water, furosemide (Furosem), GBB and carnitine were administered in drinking water. All values represent the average of at least 4 measurements ±SEM. *significantly different from water control (one-way ANOVA, Dunnett's multiple comparison test)

Figure 2

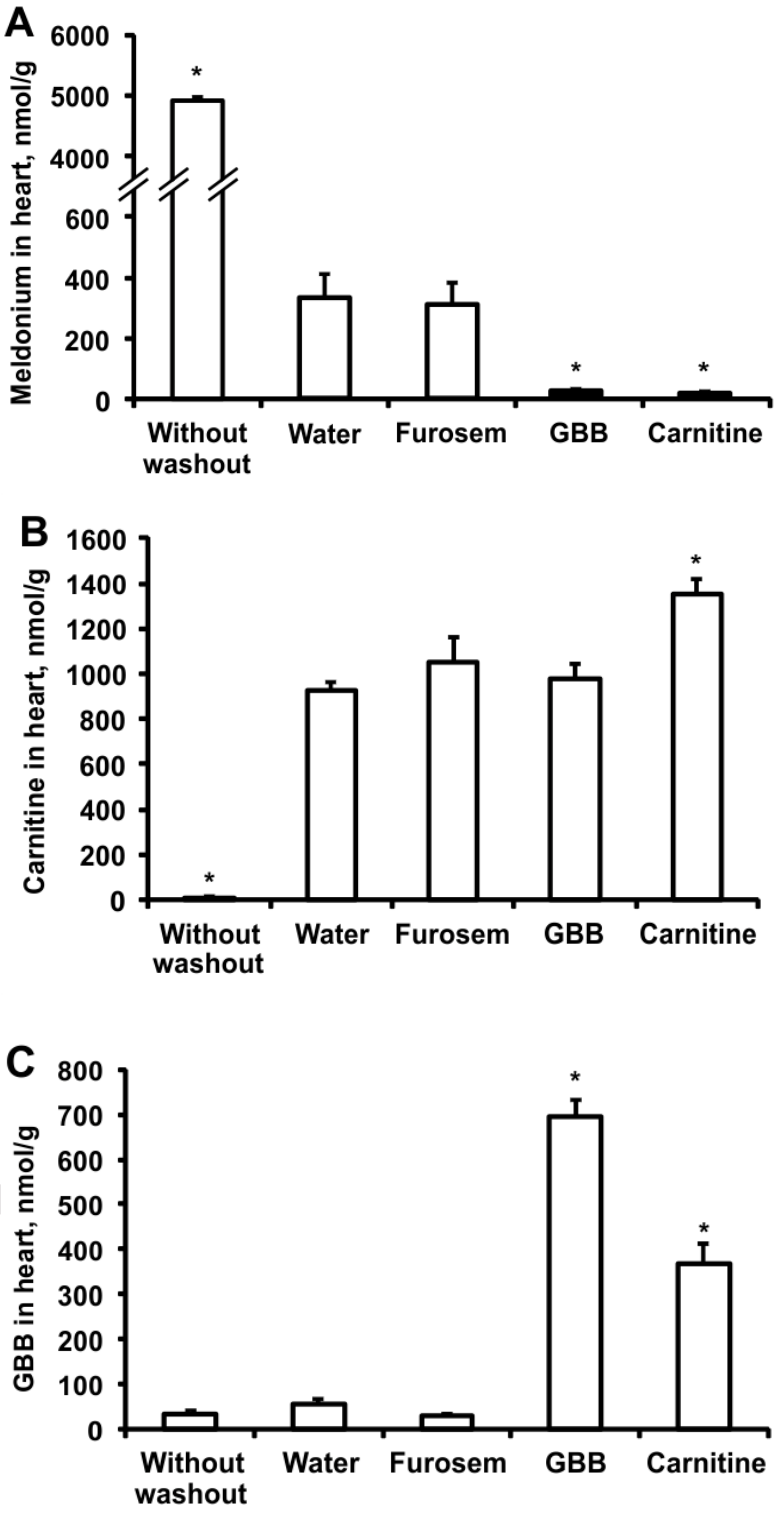

Figure 2 Concentrations of meldonium (A), carnitine (B) and GBB (C) in heart tissues. Meldonium administration at a dose of 400 mg/kg for 14 days was followed by a 7-day washout period. During the washout period, water, furosemide (Furosem), GBB and carnitine were administered in drinking water. All values represent the average of at least 4 measurements ±SEM. *significantly different from the water control (one-way ANOVA, Dunnett's multiple comparison test)

Figure 3

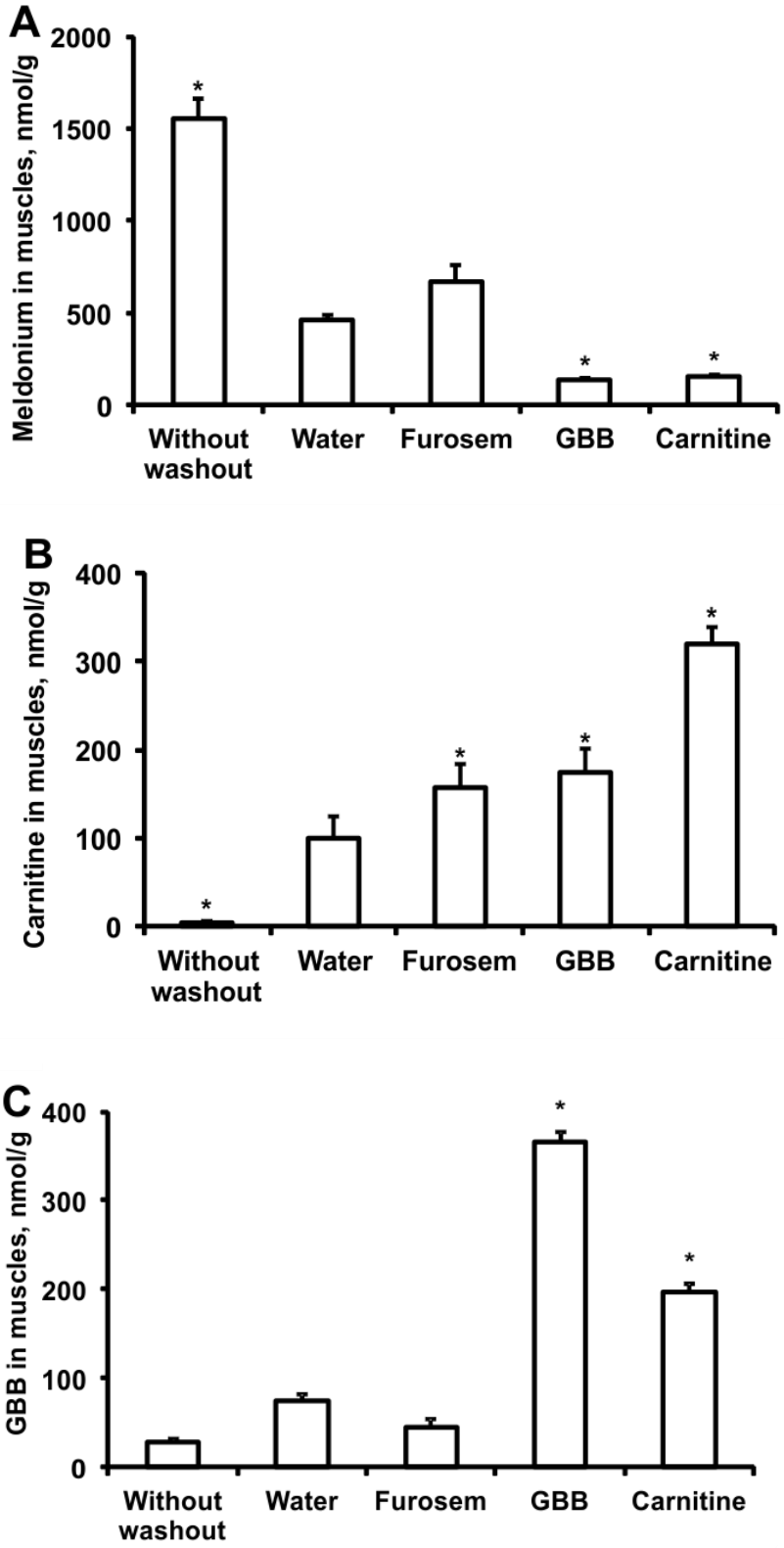

Figure 3 Concentrations of meldonium (A), carnitine (B) and GBB (C) in muscles. Meldonium administration at a dose of 400 mg/kg for 14 days was followed by a 7-day washout period. During the washout period, water, furosemide (Furosem), GBB and carnitine were administered in drinking

water. All values represent the average of at least 4 measurements ±SEM. *significantly different from the water control (one-way ANOVA, Dunnett's multiple comparison test)

Figure 4

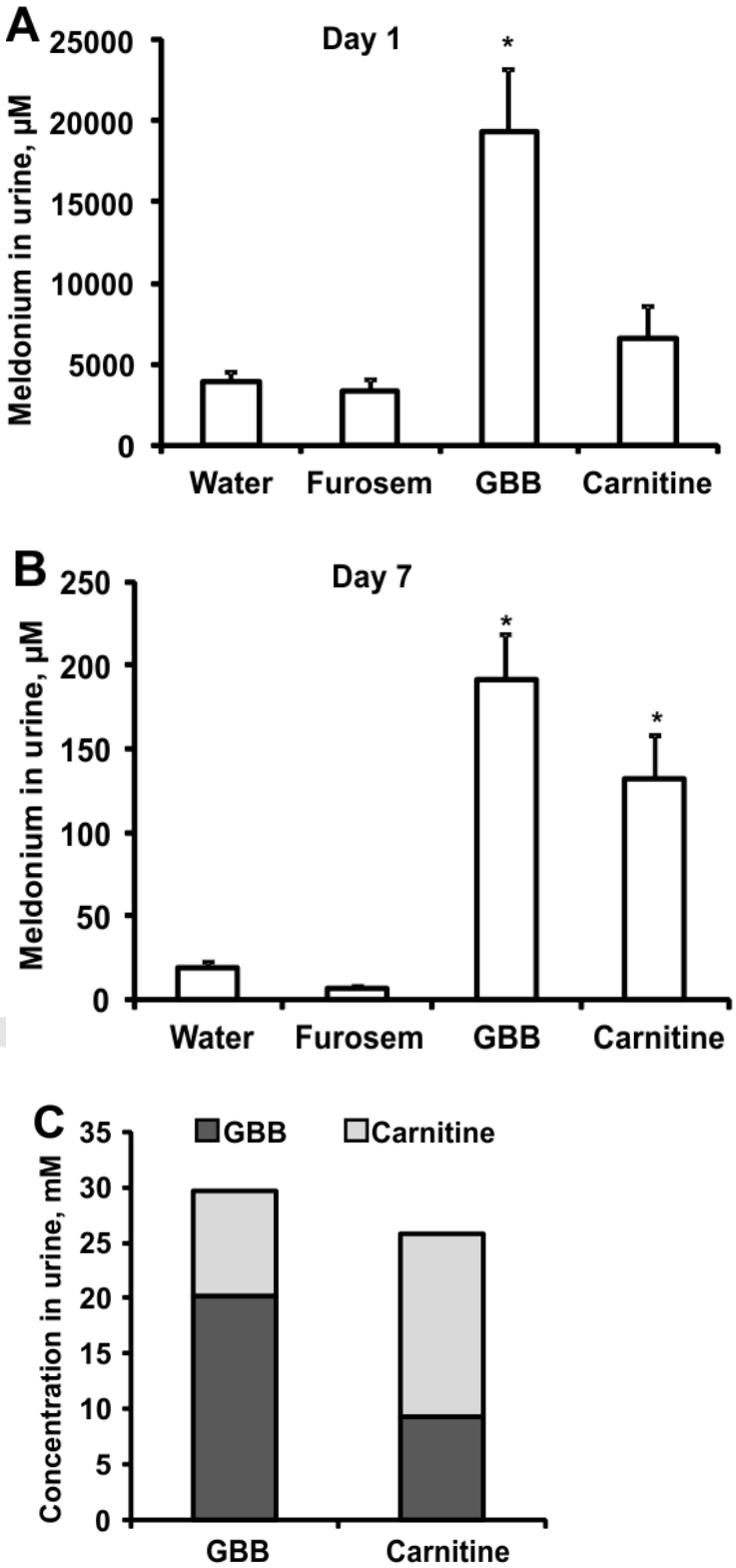

Figure 4 Concentrations of meldonium (A, B), carnitine, and GBB (C) in urine. Meldonium administration at a dose of 400 mg/kg for 14 days was followed by washout periods of 1 (A) and 7 (B) days. During the washout period water, furosemide (Furosem), GBB and carnitine were administered with drinking water. All values represent the average of at least 4 measurements ±SEM. *significantly different from the water control (one-way ANOVA, Dunnett's multiple comparison test)