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Research Paper

Mildronate treatment alters γ-butyrobetaine and L-carnitine concentrations in healthy volunteers

Edgars Liepinsh[a], Ilze Konrade[b], Elina Skapare[a,b], Osvalds Pugovics[a], Solveiga Grinberga[a], Janis Kuka[a], Ivars Kalvinsh[a] and Maija Dambrova[a]

[a]Latvian Institute of Organic Synthesis and [b]Riga Stradins University, Riga, Latvia

Abstract

Objectives In this study, we aimed to investigate the effects of long-term administration of the cardioprotective drug mildronate on the concentrations of L-carnitine and γ-butyrobetaine in healthy volunteers.

Methods Mildronate was administered perorally, at a dosage of 500 mg, twice daily. Plasma and urine samples were collected weekly. Daily meat consumption within an average, non-vegetarian diet was monitored. L-Carnitine, γ-butyrobetaine and mildronate concentrations were measured using the UPLC/MS/MS method.

Key findings After 4 weeks, the average concentrations of L-carnitine in plasma significantly decreased by 18%. The plasma concentrations of γ-butyrobetaine increased about two-fold, and this effect was statistically significant in both the male and female groups. In urine samples, a significant increase in L-carnitine and γ-butyrobetaine levels was observed, which provides evidence for increased excretion of both substances during the mildronate treatment. At the end of the treatment period, the plasma concentration of mildronate was 20 μM on average. There were no significant differences between the effects observed in female and male volunteers. Meat consumption partially reduced the L-carnitine-lowering effects induced by mildronate.

Conclusions Long-term administration of mildronate significantly lowers L-carnitine plasma concentrations in non-vegetarian, healthy volunteers.

Keywords L-carnitine; mildronate; γ (gamma)-butyrobetaine; UPLC/MS/MS

Introduction

The cardioprotective and anti-ischemic drug mildronate [3-(2,2,2-trimethylhydrazinium) propionate dihydrate] is an inhibitor of the biosynthesis of L-carnitine[1] and its reabsorption in the kidneys.[2] In experimental rat models, the administration of mildronate induces a decrease in L-carnitine content and an increase in the concentration of γ-butyrobetaine (GBB), a bioprecursor of L-carnitine, in plasma and various tissues.[3,4] Because L-carnitine is an essential molecule for free fatty acid oxidation and other metabolism pathways,[5,6] its physiological concentration in body tissues is highly regulated by networks of biosynthesis and transport.[7,8] L-carnitine is absorbed from dietary products, particularly meat and dairy items.[9] It is also biosynthesised from lysine and methionine, and its excretion is efficiently maintained by renal reabsorption.[9,10]

The effects of long-term mildronate administration on L-carnitine concentrations have not been investigated in human subjects, even though the pharmacological effects of mildronate are expected to occur via its regulatory effects on L-carnitine concentration, with subsequent changes in downstream pathways of energy metabolism.[11,12] It is known that mildronate treatment also affects the mitochondrial transport of L-carnitine and acylcarnitine.[13,14] Analytical procedures for the measurement of carnitine and its derivatives in biological samples have been widely studied.[15–17] However, only scarce information is available on the methods for simultaneous determination of L-carnitine and GBB.[15,18–20] The pharmacokinetic investigations of mildronate in human subjects have been published recently,[21,22] but simultaneous assessment of L-carnitine, GBB and mildronate in human subjects has not been conducted before.

Correspondence: Edgars Liepinsh, Latvian Institute of Organic Synthesis, Aizkraukles Str. 21, Riga LV1006, Latvia.
E-mail: ledgars@biomed.lu.lv

This study was performed to measure for the first time in human subjects the L-carnitine concentration-lowering effect of mildronate, which was administrated at a cardioprotective dose of 500 mg, twice a day as suggested by manufacturer. We therefore applied a modification of a previously described UPLC/MS/MS procedure[23] that yields good separation of L-carnitine, GBB and mildronate in one analytical run on a HILIC-type column. The effects of long-term mildronate administration were evaluated in 17 healthy volunteers. Each subject received 500 mg of mildronate twice daily for 4 weeks. Meat consumption was monitored to investigate the dietary effects on mildronate-induced changes in L-carnitine and GBB concentrations.

Materials and Methods

Subjects

This study was carried out after approval by the Central Medical Ethics Committee of Latvia (20-05-2010) and informed consent was obtained from all subjects. The study population consisted of 17 healthy volunteers, 9 female and 8 male. The criteria for volunteers to participate in the study were as follows: good health as assessed by clinical examination, non-smoking, not pregnant and no history of drug or alcohol abuse. Data collected at the study inception included age, medical history, anthropometric indices, total cholesterol levels, triglyceride levels, creatinine levels and alanine transaminase (ALAT) activity as a measure of liver function. Blood plasma biochemical markers were measured using clinically accepted methods. Mildronate was administered perorally, at a dosage of 500 mg, twice daily between meals. The subjects came to the trial unit at 8:30 am after fasting. Urine aliquots and plasma samples were stored at −20°C prior to analysis.

Materials

L-Carnitine was purchased from Lonza Ltd (Switzerland). GBB and 3-(2,2-dimethyl-2-prop-1-yl-hydrazinium) propionate (internal standard) were prepared by in-house methods. Mildronate was a gift from J.S.C. Grindeks (Latvia). Acetonitrile, methanol, ammonium acetate, formic acid, picric acid and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich Co. (USA). Creatinine was obtained from Acros Organics (Belgium). Water was purified by reverse osmosis followed by purification with a MilliQ system. All solvents and chemical reagents used were of the analytical grade.

UPLC/MS/MS assay

The concentrations of L-carnitine, GBB and mildronate in human plasma and urine samples were measured using the UPLC/MS/MS method, as previously described,[23] but with minor modifications. Sample preparation consisted of simple 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-fold with water before analysis. Briefly, 100 µl of an acetonitrile-methanol mixture (3 : 1, v/v) containing internal standard was added to 25 µl of plasma or diluted urine sample. Samples were centrifuged at 11 000*g* for 10 min to precipitate proteins. The cleared supernatants were removed and 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 × 100 mm) column (Waters Corp., 120 Manchester, UK) at a flow rate of 0.25 ml/min. The composition of the mobile phase –acetonitrile, 10 mM aqueous ammonium acetate (pH 4) – varied linearly from 75 to 55% of acetonitrile. L-Carnitine, GBB and mildronate were quantified by monitoring the specific transitions for each compound on the Micromass Quattro Micro instrument (Waters Corp., 120 Manchester, UK). Applied analytical procedures provided fair separation of all analytes of interest in one run. The representative UPLC/MS/MS plots of MRM-channels corresponding to internal standard, L-carnitine, mildronate and GBB are presented in Figure 1.

Assay of creatinine in urine samples

Creatinine concentrations were determined according to the Jaffe method,[24] with some modifications. Briefly, 135 µl of a reaction mixture containing one part of 0.6% of picric acid/water solution and five parts of 1 M NaOH (mixed just before the measurement) was added to 15 µl of diluted urine samples or standard creatinine solutions (0–0.2 mg/ml). After a 10-min incubation at room temperature, absorption was measured spectrophotometrically in a 96-well plate using a µQuant™ Microplate Spectrophotometer (BioTek) at 492 nm.

Statistical analyses and calculations

Results are expressed as the mean ± standard deviation (SD) or standard error mean (SEM). Statistically significant differences in the mean values between genders were tested by Student's *t*-test, and paired *t*-tests were used to compare the differences from baseline. The differences were considered significant when $P < 0.05$. The data were analysed using GraphPad Prism 3.0 statistical software (GraphPad Inc., USA). Total body L-carnitine content was calculated based on published data.[25–27] The amount of L-carnitine excreted in the urine was calculated as the area under the curve, after taking into account the average creatinine excretion rate. L-carnitine consumed in the diet was calculated from meat consumption data provided in the diet diaries of each study subject. Correlation analysis was performed using Pearson's correlation test.

Results

Characteristics of study subjects

A summary of subject demographics and plasma biochemical parameters is shown in Table 1. As shown in the table, the average age, weight and body-mass index of the male and female volunteers did not differ significantly. After the 4-week administration of mildronate there were no significant changes in serum ALAT activity, triglyceride levels or total cholesterol concentrations. Average meat consumption did not differ significantly between male and female subjects, but in females a larger deviation in meat consumption was observed (Table 1). Mildronate treatment caused a decrease in plasma

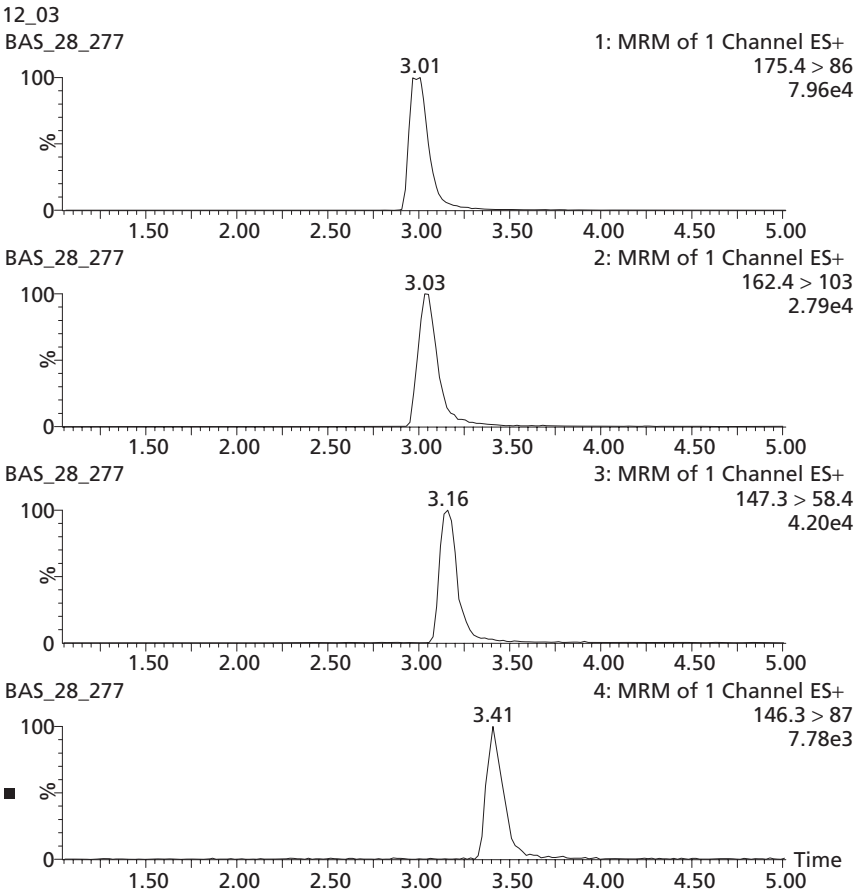

Figure 1 Representative UPLC/MS/MS plots of MRM channels corresponding to internal standard, L-carnitine, mildronate and GBB (from top to bottom).

Table 1 A summary of demographic and plasma biochemical parameters of volunteers

	Male ($n = 8$)		**Female ($n = 9$)**	
	Mean ± SD	Range	Mean ± SD	Range
Age, years	37 ± 14	22–58	37 ± 9	25–56
Height, cm	182 ± 7	174–192	170 ± 7**	157–180
Weight, kg	83 ± 13	65–99	71 ± 13	48–87
BMI, kg/m^2	25 ± 3	20–28	24 ± 3	19–28
Meat consumption, g/day	156 ± 26	125–200	156 ± 54	100–275
Baseline				
ALAT, U/l	31 ± 20	11–74	24 ± 8	15–38
Creatinine, μmol/l	83 ± 12	73–98	72 ± 9	59–91
Total cholesterol, mmol/l	5.1 ± 1.3	3.7–8.0	5.1 ± 0.8	3.6–6.0
Triglycerides, mmol/l	1.4 ± 0.6	0.5–2.5	1.0 ± 0.4	0.4–1.6
4 weeks				
ALAT, U/l	31 ± 12	10–47	20 ± 8	13–37
Creatinine, μmol/l	71 ± 10	58–87	64 ± 10*	53–86
Total cholesterol, mmol/l	5.1 ± 1.1	4.1–7.1	5.2 ± 1.0	3.1–6.3
Triglycerides, mmol/l	1.4 ± 0.5	0.9–2.3	0.9 ± 0.4**	0.45–1.7

Biochemical parameters were determined in blood plasma samples before and after 4 weeks of mildronate administration. Values are represented as the average ± SD of eight to nine subjects. *Significantly different from baseline (paired *t*-test, $P < 0.05$). **Significantly different between genders (Student's *t*-test, $P < 0.05$).

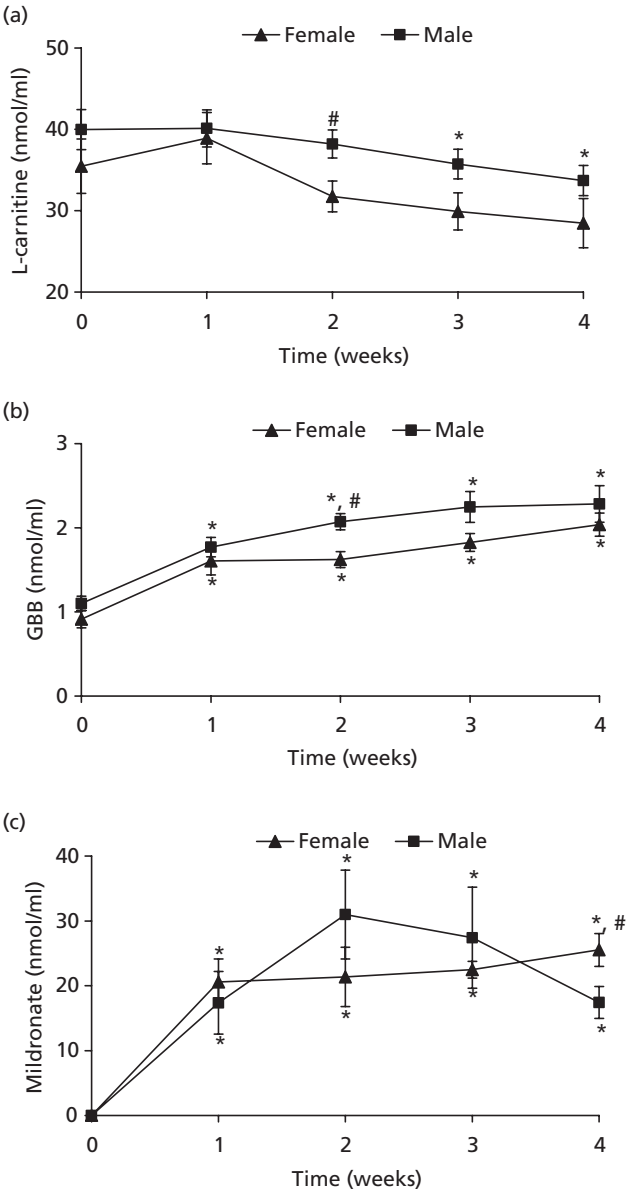

Figure 2 Concentrations in human plasma of (a) L-carnitine, (b) GBB and (c) mildronate. L-carnitine, GBB and mildronate concentrations were determined in blood plasma samples. Values are represented as the average ± SEM of eight to nine subjects. *Significantly different from baseline (paired *t*-test, $P < 0.05$). #Significantly different between genders (Student's *t*-test, $P < 0.05$).

creatinine concentrations in male and female samples by 15 and 11%, respectively, but the effect was statistically significant only for female volunteers (Table 1).

Analysis of plasma samples

Even at baseline, the L-carnitine concentrations in female subjects were about 10% lower than in male subjects (Figure 2a). As shown in Figure 2, mildronate induced time-dependent changes in L-carnitine and GBB concentrations in both female and male plasma samples. However, after 3 weeks of treatment, the statistically significant 11% reduction

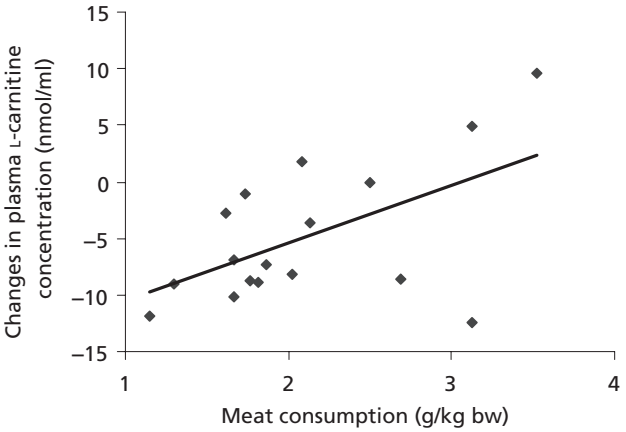

Figure 3 Correlation between the changes in blood plasma L-carnitine concentration with meat consumption per kilogram of body weight.

in L-carnitine was found only in male samples (Figure 2a). Notably, after 1 week of treatment the concentration of L-carnitine in female plasma was slightly increased. At the end of the 4-week treatment, the plasma concentrations of L-carnitine were 16 and 20% lower than at baseline in male and female samples, respectively. On average, mildronate treatment induced a significant decrease in L-carnitine concentration of 18%.

In addition, plasma GBB concentrations changed after mildronate treatment. As shown in Figure 2b, after only 1 week of treatment, plasma GBB concentrations increased 61 and 76% in male and female samples, respectively. At the end of the study, the plasma GBB levels increased 2.2-fold on average, and this effect was statistically significant in both the male and female groups.

Mildronate concentration increased gradually, approaching a plateau in the female group after 1 week of treatment (Figure 2c). In males, the increase was observed until week 2, but then the plasma concentration of mildronate decreased, reaching an average concentration of 20 nmol/ml (Figure 2c).

The average decrease in plasma L-carnitine concentration in study subjects consuming less than 2 g of meat per kg of body weight daily was 6.8 ± 1.2 nmol/ml. In subjects consuming more than 2 g/kg daily, the average decrease in L-carnitine concentration in blood plasma was 2.4 ± 3.4 nmol/ml. As shown in Figure 3, the decrease in blood plasma L-carnitine concentration significantly correlated with meat consumption per kg of body weight ($R = -0.54$; $P = 0.03$).

Analysis of urine samples

The concentrations of L-carnitine, GBB and mildronate in the urine samples mirrored the effects measured in plasma samples. As shown in Figure 4a, in both female and male samples, the excretion and concentration of L-carnitine significantly increased after 1 week of mildronate treatment. After 3 weeks, it levelled off until the end of the study at around 330 nmol/mg creatinine (Figure 4a). The concentration of GBB increased 45-fold on average after 1 week, and stayed at a significantly high level for the remaining period of the study (Figure 4b). The concentration of mildronate increased gradually for 2 weeks, at a similar speed in both

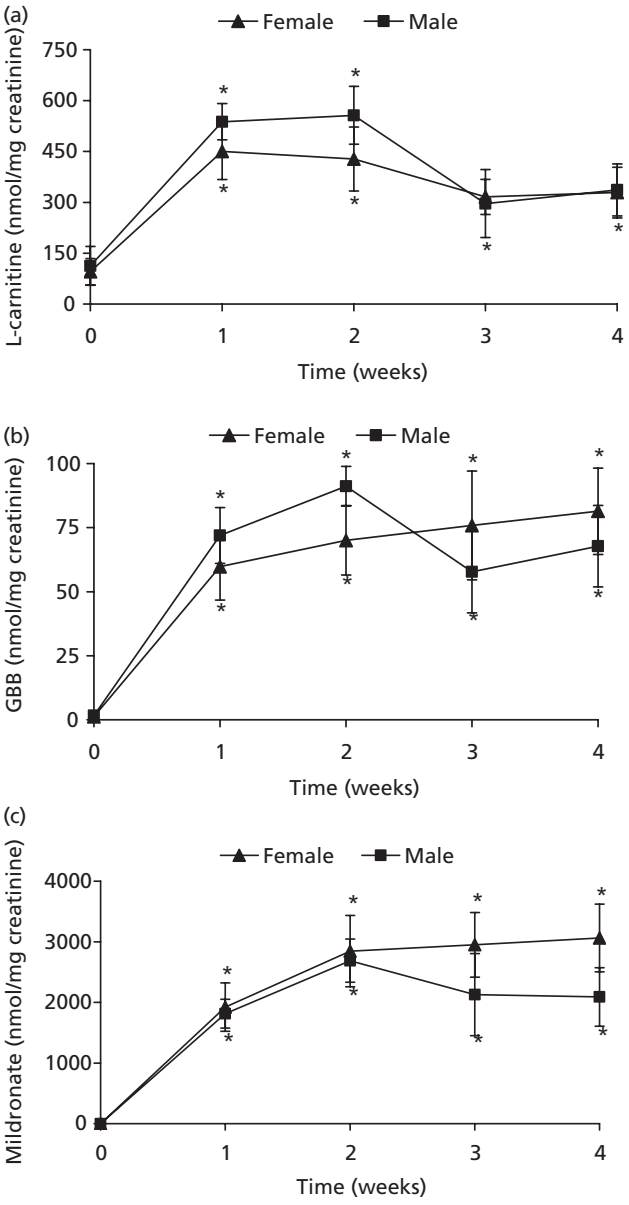

Figure 4 Concentrations in human urine of (a) L-carnitine, (b) GBB and (c) mildronate. L-carnitine, GBB and mildronate concentrations were determined in urine samples. Values are represented as the average ± SEM of eight to nine subjects. *Significantly different from baseline (paired *t*-test, $P < 0.05$). #Significantly different between genders (Student's *t*-test, $P < 0.05$).

male and female samples (Figure 4c). After 3 to 4 weeks, a slight decrease of 21% was observed in mildronate levels in male samples. However, the difference was not significant between male and female groups (Figure 4c).

Discussion

This is the first study to show that mildronate treatment induces a significant decrease in L-carnitine levels and an increase in GBB concentration in the blood plasma of healthy non-vegetarian volunteers (Figure 2). However, the effect is much lower compared to that observed in experimental rat and mouse models, where 4-week treatment with a cardioprotective dose of mildronate (100 mg/kg) at the same plasma concentration (20 nmol/ml) induces up to a three-fold lowering of L-carnitine levels and up to a six-fold increase in GBB levels.[4,28] In experimental animals, a mildronate-induced decrease in tissue L-carnitine content has been detected if the plasma concentration of L-carnitine was several-fold lower than in control animals.[4,23] Thus the recommended daily dose of mildronate of 1 g/day is too low to achieve decreases in L-carnitine concentration similar to those observed in animal studies. A higher dose of mildronate might increase the efficacy of mildronate treatment. Another finding of the present study is the observation that mildronate treatment at a daily dose of 1 g did not influence blood plasma triglycerides and total cholesterol concentrations, as well as ALAT levels, which confirms the safety of mildronate therapy.

In the volunteers of the present study, whole-body carnitine content was approximately 13.8 g. During the 28-day mildronate treatment, the study subjects consumed 1.2 g of L-carnitine in their diets (as calculated from the meat consumption analysis), and they excreted by urine 3.5 g of L-carnitine. Thus, the total overall body L-carnitine content was reduced by 23%, and this effect is similar to that found in blood plasma, where the L-carnitine concentration was reduced by 18%. Urine sample analysis showed up to a three-fold increase in L-carnitine excretion. It can therefore be concluded that in non-vegetarian volunteers, long-term mildronate treatment alters the plasma concentrations of L-carnitine and GBB through the inhibition of renal re-uptake of these molecules.

Mildronate is known to be an inhibitor of the biosynthesis of L-carnitine[1] and its reabsorption in the kidney.[2] At the end of the 4-week treatment, mildronate concentrations in blood plasma reached 20 nmol/ml. According to animal studies, as a result of active transport by OCTN2, the mildronate concentration in tissues should be 30–50 times higher.[28,29] The K_i for GBB hydroxylase enzyme inhibition by mildronate has been determined to be 19 μM (enzyme prepared as described by Tars *et al.*[30]), and the EC_{50} for OCTN2 inhibition by mildronate was calculated to be 21 μM.[31] Thus, after long-term treatment, the plasma concentration of mildronate reaches a level that is able to induce changes in the homeostasis of L-carnitine.

The pronounced L-carnitine concentration-lowering effect of mildronate in experimental animals could also be a result of low L-carnitine content in the animals' diets, which could not compensate for mildronate-induced excretion of L-carnitine. The average non-vegetarian adult diet provides about 75% of daily L-carnitine requirements.[32] In this study, our subjects consumed about 40 mg (156 g meat) of L-carnitine daily, and the urinary excretion of L-carnitine before mildronate treatment was 30 mg/day. Thus, the relative L-carnitine absorption from the diet in the subjects in the present study was 75% and dietary L-carnitine provided all necessary L-carnitine. L-carnitine concentrations in female blood plasma are significantly lower than in males,[33] and L-carnitine-restricted diets have little impact on total body carnitine because of efficient renal reabsorption of carnitine.[27] The results of this study provide evidence that the L-carnitine concentration in female

plasma at baseline was 10% lower than those in male samples, even if meat consumption is the same for the two groups (Figure 2a). However, in study subjects consuming less meat, the mildronate treatment decreased plasma L-carnitine concentrations up to three times more effectively. This result indicates that meat consumption can provide amounts of L-carnitine sufficiently high to mask the effects of mildronate. In addition, the higher L-carnitine content in non-vegetarian human diets compared to animal chow could explain the weaker L-carnitine-lowering effect of mildronate observed in the human volunteers compared to animal studies.

The use of the UPLC system instead of HPLC makes our procedure particularly versatile for pharmacological studies because of the very short (6 min) runtime. The monitoring of mildronate-induced changes in L-carnitine and GBB concentrations could therefore serve as biochemical markers of therapeutic efficiency.

Conclusion

Long-term administration of mildronate significantly lowered L-carnitine plasma concentrations in non-vegetarian, healthy volunteers.

Declarations

Conflicts of interest

The author(s) declare(s) that they have no conflicts of interest to disclose.

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