Development and validation of an UHPLC-Orbitrap-HRMS method for rapid determination of endogenous L-carnitine in patients on hemodialysis/peritoneal dialysis and its application to promote rational drug use

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Background: L-carnitine is an endogenous vitamin-like amino acid derivate which plays an essential role in energy metabolism and can be easily lost via dialysis. Deficiency of L-carnitine has great effects on many aspects of bodily functions. To determine the deficiency degree and adjust the supplementation dose, a rapid, sensitive, and specific method for the detection of endogenous L-carnitine in the plasma of dialysis patients using ultra-high performance liquid chromatography-Orbitrap high resolution mass spectrometry (UHPLC-Orbitrap-HRMS) was developed and validated.

Methods: The plasma samples were processed by protein precipitation and centrifugation before analysis using UHPLC-Orbitrap-HRMS. Sample separation was achieved with a hydrophilic interaction liquid chromatography (HILIC) column, using an isocratic elution with a runtime of 5 min. The separated analytes were detected by positive ionization mode in full scan mode and targeted-single ion monitoring (t-SIM) mode. Mildronate was used as the internal standard (IS).

Results: All the plasma could be detected in the range of 6.169 to 197.394 μM, with adequate accuracy, precision, and recovery. The method was validated in fortified validation with relative standard deviations (RSD) 5.15–8.74%. This method was applied to the analysis of 105 dialysis patients and 39 healthy participants, the results revealed that peritoneal dialysis patients without L-carnitine supplementation should pay more attention to L-carnitine monitoring, meanwhile, all the hemodialysis patients were advised to be routinely given a full dose of L-carnitine, no matter whether they had taken L-carnitine or not.

Conclusions: This study developed a simple and rapid UHPLC-Orbitrap-HRMS method for detection of endogenous L-carnitine in dialysis patients, which could be useful to promote rational drug use.

Keywords: L-carnitine; dialysis; UHPLC-Orbitrap-HRMS; fortified validation; rational drug use

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Introduction

L-carnitine (*Figure 1A*) is an endogenous vitamin-like amino acid derivate, and it plays an essential role in oxidative metabolism and ketogenesis by decreasing the intramitochondrial acetyl-CoA/CoA ratio through the trapping of acetyl groups (1,2). In addition, L-carnitine is a low molecular weight hydrophilic substance which can be excreted by kidneys. Kidney disease can disturb L-carnitine homeostasis, while L-carnitine can be easily lost via dialysis (3). It has been illustrated that about 95% of dialysis patients experience carnitine deficiency (4). Insufficient carnitine synthesis is another reason for the lack of L-carnitine in patients on hemodialysis and peritoneal dialysis. The effect of L-carnitine deficiency on patient health is profound, impacting on serum lipid, red blood cells, and cardiac and skeletal muscle (5). Because of the recent significant advances in dialysis therapy, the purpose of dialysis therapy is not only to prolong the patients' life, but also to restore the pre-dialysis state of health as much as possible. Furthermore, L-carnitine supplementation improves the complications of patients with kidney disease, such as erythropoietin-resistant anemia, cardiac symptoms, muscle weakness, impaired protein metabolism, and insulin resistance (6). Several recent studies have demonstrated that L-carnitine supplementation could suppresses inflammation by reducing levels of interleukin-6 (IL-6), C-reactive protein (CRP), tumor necrosis factor-α (TNF-α) (7,8). Besides, L-carnitine supplementation could attenuate oxidative stress responses by increasing superoxide dismutase (SOD) or glutathione (7,9).

In order to provide personalized pharmacy services to dialysis patients, promote rational drug use, and reduce the waste of social resources and the economic burden of patients, we need to know the degree of individual L-carnitine deficiency. Currently, the common method for detecting carnitine utilizes solid-phase extraction column, derivatization, and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS) (10,11). For the purpose of obtaining more reliable results, higher sample throughput, and simplifying the operation process, there is an increasing demand for the development of an ultra-high performance liquid chromatography (UHPLC) coupled to mass spectrometry (MS) analytical method (10,12-15).

However, owing to its design, it has a drawback that could be seriously affected by matrix effects. Matrix effects may reduce the sensitivity, precision, and accuracy of the method (15,16). In addition, there is no target-free matrix for the detection of endogenous compounds, which leads to quantification difficulties. A number of approaches have been developed to solve the problem, including background subtraction (17,18), standard addition (19), artificial matrix (11,20), analyte-depleted biological matrix (21), and stable-isotope labeled surrogate analytes (22,23). Although these approaches have promoted the development of quantitative methodologies for endogenous substances, they still have various respective shortcomings, including limited sensitivity, the matrix effect, and recovery inconsistency of biological and the corresponding surrogate matrix (24).

Herein, we have described a simple, rapid, selective and highly sensitive ultra-high performance liquid chromatography tandem Orbitrap high resolution mass spectrometry (UHPLC-Orbitrap-HRMS) method for the determination of L-carnitine in human plasma using a hydrophilic interaction liquid chromatography (HILIC) column. Its method run time is 5 min, and 50 µL plasma is needed for analysis. In this study, mildronate (MILD; *Figure 1B*) was used as the internal standard (IS), because of its similarity in structure, retention time, and ionization to the molecular target (25). UHPLC offers the possibility of rapid and efficient sample separation. Modern mass spectrometry is usually equipped with mass analyzer with higher resolution and accuracy, such as time of flight (TOF) and Orbitrap (26). Most of the interfering ions are effectively eliminated by the narrow window of mass defect filtering (MDF) template, which enable the researchers to accurately capture the target molecular (27). The assay developed here incorporated matrix effects from different sources into quantitation for the first time. In addition, fortified validation was emphasized to ensure the reliability and accuracy of this assay. It is worth mentioning that there is no need to prepare analyte-depleted biological matrix or artificial matrix, which simplifies the analysis procedure and guaranteed the authenticity of the results. Plasma concentrations of L-carnitine were accurately measured using one calibration curve constructed with anyone's untreated plasma.

We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-21-6784/rc).

Methods

Study participants and sample collection

This study was approved by the Ethics Committee of The Second Affiliated Hospital of Soochow University (No. JD-
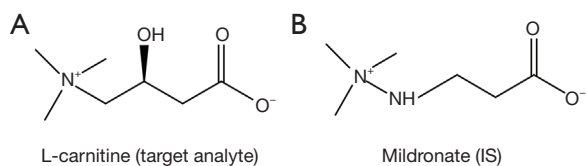
Figure 1 Chemical structures of analyte L-carnitine and mildronate. IS, internal standard.

LK-2020-038-01). All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013), and informed consent was taken from all the patients and healthy controls. Dialysis patients were recruited from the department of nephrology, while healthy controls were recruited from the medical examination department.

The inclusion criteria were as follows: aged 18–70 years, balanced age and gender distribution, no underlying diseases except kidney disease, and never having taken drugs that affect L-carnitine metabolism. The healthy control group was recruited from the physical examination department, and the inclusion criteria were based on the inclusion of the nephropathy group. The proportion of cases included in each age group was similar, as was the basic physical condition. The exclusion criteria were as follows: liver disease, cardiovascular disease, anemia, history of benign and malignant neoplasms, history of drug or alcohol abuse, vegetarian diet, or past use of meldonium.

There were 105 human participants recruited who had not taken L-carnitine, including 33 patients on hemodialysis, 33 patients on peritoneal dialysis, and 39 healthy people. Additionally, 39 patients on hemodialysis who had taken L-carnitine, were recruited. Blood samples were obtained before and after dialysis in the hemodialysis patients, while only blood samples after peritoneal dialysis were obtained from patients on peritoneal dialysis. At the time of sample collection, the proportion of stable hemodialysis patients meeting inclusion criteria was higher in males than in females. The clinical characteristics of the participants are shown in *Table 1*.

Materials

L-carnitine (purity >99.5%) and MILD (purity >99.6%) were purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). Acetonitrile [(ACN); purity >99.9%] and formic acid [(FA); purity >99.0%] of liquid chromatography grade were obtained from Merck KGaA

(Darmstadt, Germany) and Anaqua Chemical Supply (Wilmington, DE, USA), respectively. Ammonium formate (purity >99.0%) was obtained from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Water was purified and deionized with a Milli-Q system manufactured by Millipore (Bedford, MA, USA).

Instruments and conditions

Chromatographic separation of the samples was carried out using a Dionex UltiMate 3000 Series UHPLC (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an analytical column Hypersil GOLD HILIC column (1.9 μm, 100 mm × 2.1 mm; Thermo Fisher Scientific, USA). The mobile phase consisted of 0.1% (v/v) formic acid and 10 mM ammonium formate in water (solvent A) and ACN (solvent B). An isocratic elution with a flow rate of 0.2 mL/min was performed in 50% of solvent A at room temperature (25 ℃) and the total running time was 5 min. Aliquots of 5 μL of the sample extract were injected into the chromatographic system.

For the targeted analysis of plasma L-carnitine, the UHPLC system coupled to an Orbitrap mass spectrometer (Q Exactive™, Thermo Fisher Scientific, USA) were used. Operating with a heated electrospray interface (HESIII, Thermo Fisher Scientific, USA), in positive (ESI+) ionization mode was performed according to the following operational parameters: spray voltage, 3.8 kV; sheath gas (N2, >95%) flow rate, 40; auxiliary gas (N2, >95%) flow rate, 10; resolution, 70,000; S-lens RF level, 50; auxiliary gas heater temperature, 350 ℃; and capillary temperature, 320 ℃. The automatic gain control (AGC) was set at a target value of 1×10^6. The mass spectra were acquired in full scan mode and targeted-single ion monitoring (t-SIM) mode. Mass range in the full scan mode was set at m/z 50–500. In the t-SIM mode, the targeted mass of L-carnitine and mildronate were set at m/z 162.11247 and 147.11280, respectively. All the analyses were performed without lock mass. The data were collected and processed using Xcalibur™ version 2.2.1 (Thermo Fisher Scientific, Les Ulis, France) with Processing Setup, Qualitative and Quantitative browser. Genesis peak detection was applied.

Preparation of stock and calibration standard solutions

Totals of 10.58 mg of L-carnitine and 10.10 mg of MILD were accurately weighed using a one hundred thousandth scale, and then dissolved in 10 mL volumetric flask with

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Table 1 Clinical characteristics of patients on dialysis and healthy control

Parameters	Healthy control (N=39)	Peritoneal dialysis (N=33)	Hemodialysis (N=72)	
			Before	After
Age (Y)	54.3±13.9	54.6±17.5	58.4±15.5	
Gender (M/F)	20/19	17/16	45/27	
Hb (g/L)	143.2±10.4	101.7±13.1	116.6±15.9	–
Cre (µmol/L)	67.3±13.1	875.6±275.2	911.0±312.3	396.8±227.8
TRIG (mmol/L)	0.9±0.4	1.6±1.5	1.7±1.1	–
TC (mmol/L)	4.7±0.6	4.2±1.2	3.7±1.0	–
HDL-C (mmol/L)	1.6±0.4	0.9±0.4	1.0±0.3	–
LDL-C (mmol/L)	2.7±0.4	2.3±1.1	2.0±0.7	–
UA (µmol/L)	286.4±61.3	403.1±108.5	448.0±97.5	137.1±60.6
FPG (mmol/L)	4.9±0.4	5.9±2.2	7.4±3.8	–
HbA1c (%)	5.2±0.3	6.3±1.4	6.2±1.2	–
Urea (mmol/L)	4.8±1.1	19.6±8.1	25.0±7.4	8.4±3.7

Values are mean ± standard deviation. "–" represents that no corresponding examination after hemodialysis. Hb, hemoglobin; Cre, creatinine; TRIG, triglycerides; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; UA, uric acid; FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin.

deionized water, respectively. Afterwards, the stock solutions of L-carnitine and MILD (1 mg/mL) were prepared in deionized water. The calibration standards of L-carnitine were prepared by appropriate dilution of the stock solution with deionized water, and the concentrations of standards were set at 6.169, 12.337, 24.674, 49.348, 74.023, 98.697, and 197.394 µM, while a final concentration of 10 µg/mL MILD was prepared by diluting the MILD stock solution with deionized water and used as the IS working solution. Correspondingly, quality control (QC) samples for lower limit of quantification (LLOQ), low QC (QL), mid QC (QM), and high QC (QH) were 6.169, 18.506, 49.349, and 154.216 µM. All solutions were frozen at –80 ℃ prior to use. Calibration and QC samples were prepared by 50 µL of raw human plasma spiked with an appropriate amount of the analytes (5 µL) and IS (5 µL) solutions.

Sample preparation

For the extraction of plasma L-carnitine, 5 µL IS working solution and 5 µL deionized water were added to 50 µL plasma sample and mixed thoroughly. After adding 450 µL ACN, the mixture was vortexed thoroughly and then centrifuged at 13,000 ×g for 10 min at 4 ℃. The obtained supernatant was further centrifuged and the second supernatant was collected and diluted 10 times with ACN, then analyzed using UHPLC-Orbitrap-HRMS. Blood samples from each participant were processed in the same way 3 times.

Method validation

Selectivity and residual effect

We prepared 6 batches of double blank plasma (no L-carnitine or IS was added to the plasma, 10 µL H₂O was added instead), single blank plasma (no L-carnitine was added to the plasma, 5 µL H₂O and 5 µL IS was added instead), LLOQ, and upper limit of quantification (ULOQ) were prepared to investigate the selectivity and residual effect. Those plasma samples were processed following the sample procedure described above. Blank samples were injected before LLOQ and right after ULOQ.

Accuracy and precision

To assess the accuracy and precision of this method, QC samples were tested at 4 different concentrations in 3 separate validation runs. The precision was expressed as the relative standard deviation (RSD%), within-run and between-run precisions were calculated with the following equation:

$$RSD\% = \frac{SD}{Mean} \times 100 \qquad [1]$$

where Mean is the mean of the actual measured concentrations and SD is the standard deviation of the measured concentrations.

Accuracy was defined as the percent relative error (RE%) and was calculated as follows:

$$RE\% = \frac{M - T}{T} \times 100 \qquad [2]$$

where M is the measured concentration and T is the theoretical concentration.

Matrix effect and extraction recovery

For the determination of matrix effect, firstly, the normalized area ratios of 3 L-carnitine levels (QH, QM, QL) spiked in 6 different human plasma and that of the same amounts dissolved in the same volume of H_2O were compared, and both of them followed the same procedure described above. The normalized area ratio was calculated using the peak area of the molecular target divided by that of IS. Secondly, the calibration curves of spiked plasma and spiked water samples were created (15). The extent of the matrix effect was assessed by comparing slopes of calibration curves prepared in spiked plasma and spiked water samples, which was calculated as follows:

$$MF\% = 100 - \left(k_{spiked} / k_{solvent} \times 100\right) \qquad [3]$$

where MF is the matrix effect, k is the slope of calibration curve.

The extraction recovery from plasma was calculated by the difference between normalized area ratios of pre-spiked plasma (adding standards to the plasma before extraction) and post-spiked plasma (adding standards to the ACN extract of plasma). Similarly, the experiment was conducted with 6 batches of human plasma, each pre- or post-spiked matrix was spiked with 3 levels of L-carnitine.

Fortified validation

The experiment was conducted on six separate occasions using different matrices, the calibration curve and the corresponding single blank plasma samples and QC samples were prepared each time. The normalized area ratio of each single blank plasma sample was then substituted into the calibrations conducted with 6 matrices to acquire the calculated concentrations and then compare the difference between these results. The difference was measured by precision.

Stability testing

To investigate the stability, 5 batches of QL and QH samples were stored at room temperature, 4 ℃, and –80 ℃ for 1 week and thawed in 3 freeze-thaw cycles. The corresponding measured concentrations were compared with that of samples which analyzed directly after extraction.

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the differences of plasma L-carnitine levels among groups, and the results were expressed as mean ± standard deviation ($\bar{x} \pm SD$). Statistical analyses were conducted by GraphPad Prism 6.0 statistical software (GraphPad Software, La Jolla, CA, USA), all the analyses were 2-sided, and P<0.05 was considered statistically significant.

Results

*Selectivity and residual effect*

The typical chromatograms and spectra of L-carnitine and MILD in plasma samples are shown in *Figure 2*. The typical mass spectrum of L-carnitine is shown in *Figure 2A* and that of IS is shown in *Figure 2B*. The chromatograms of L-carnitine and IS in the LLOQ and 2 blank plasma samples, are shown in *Figure 2C* and *Figure 2D*, respectively. There were no interfering peaks observed near the retention time of interest, which meant that the developed method had sufficient selectivity and specificity to detect plasma L-carnitine. The result also showed the response of LLOQ was slightly higher than that of endogenous L-carnitine in the blank plasma, and there was no residue of L-carnitine and IS after the injection of ULOQ.

Linearity, accuracy, and precision

Normal content of plasma endogenous L-carnitine is approximately 40–50 µM (28). Thus, the final concentration range of standard solutions in plasma was set at the level of 6.200–198.386 µM. Besides, the result of the corresponding blank plasma was taken into consideration when constructing the calibration function. The calibration curves were constructed by a weighted linear regression model with a weighting factor of $1/\chi^2$. The relative peak area ratio of the target analyte and the IS was plotted against the L-carnitine concentration. The lowest concentration for the calibration curve was considered to

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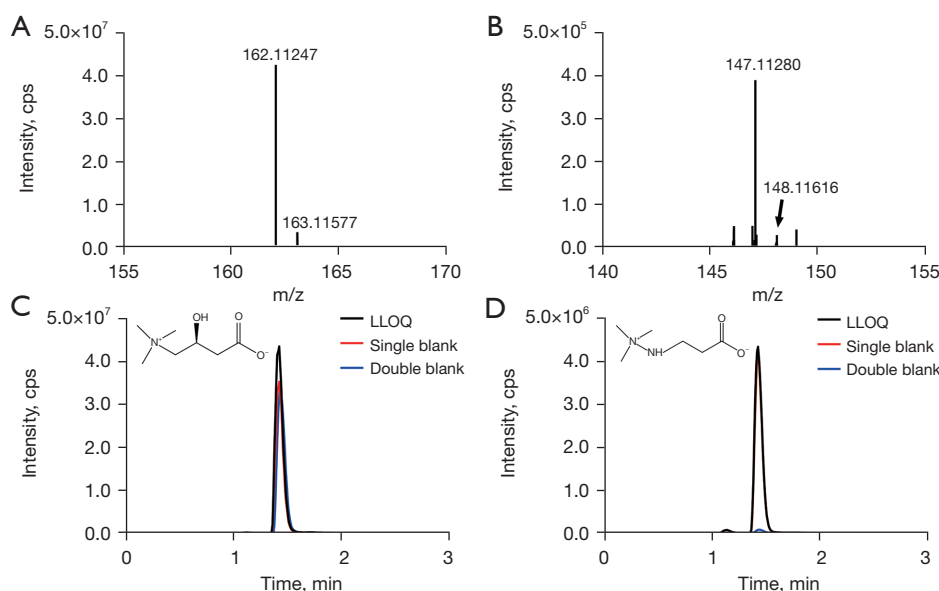
Figure 2 The chromatograms and spectra of LC and MILD in plasma sample. Exact characteristic mass-to-charge ratio of LC (A) and MILD (B) and target-SIM spectra of LC (C) and MILD (D) in plasma. Single blank: no LC was added to the plasma, 5 µL H$_2$O and 5 µL IS was added instead; double blank: no LC or IS was added to the plasma, 10 µL H$_2$O was added instead. LC, L-carnitine; MILD, mildronate; cps, counts per second; IS, internal standard; LLOQ, lower limit of quantification.
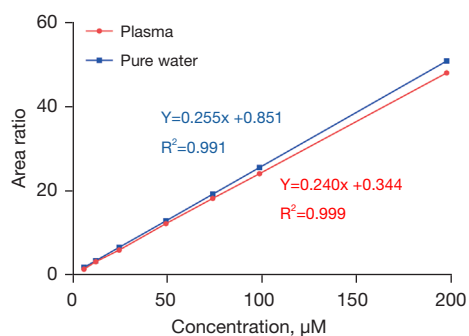
Figure 3 Calibration curves prepared in spiked plasma and spiked water samples. The plasma endogenous L-carnitine was subtracted when constructed the curve.

be the low limit of quantitation (LLOQ) (*Figure 2*), and good linearity was observed (R^2=0.9913, Figure S1). The within-run and between-run precisions and the accuracy obtained in the study were better than prescribed by the acceptance criteria (FDA/CDER, 2003), both accuracy and precision were ≤±15% (LLOQ, ≤±20%) (Table S1).

Matrix effect and recovery

The matrix factor (MF) was calculated by the ratio of

the L-carnitine peak area in the presence of matrix and that in deionized water. The IS normalized MF of QC samples in 6 matrices ranged from 91.803% to 99.862% compared to non-matrix ones. The result illustrated that the ion suppression in different plasma is stable and mild, with RSD ranging from 0.715% to 9.867%. From the slopes comparison, it was observed that the plasma matrices diminished the response of the analytes by 5.882% (*Figure 3*).

Unexpectedly high extraction recoveries were found following the above procedure, that of QL, QM, and QH were 154.416%, 123.053%, and 102.945%, with RSDs of 3.593%, 5.745%, and 6.057%, respectively (Table S2). Besides, this paper lists four sample processing methods, and compares the recovery values of different processing methods (Figure S2 and Tables S3,S4). It can be seen that the high recoveries of this method were caused by the different adding time of IS.

Fortified validation

To further validate the developed method, we carried out a fortified validation assay, which contained 6 calibration curves constructed with different matrix (plasma A – plasma F). The precision of plasma A–F detection in this assay
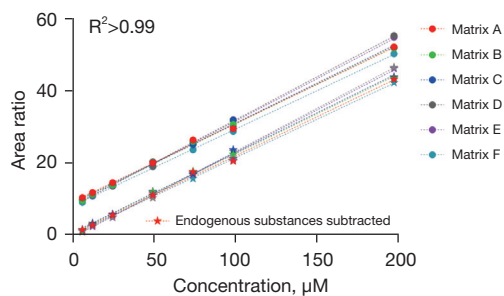
Figure 4 Six calibration curves of L-carnitine using six different plasma matrix. Dots represent initial standard curves containing endogenous level of L-carnitine, while stars represent the endogenous substances subtracted calibration curves of L-carnitine in the plasma.

was 5.145–8.737%, which reflected good precision and confirmed the reliability of the assay developed here. It was further shown that the interference with results was caused by the matrix effect is minimal by this method. The results are listed in detail in *Figure 4* and Table S5.

Stability

L-carnitine in plasma remained stable for 1 week at room temperature. Storage of plasma samples at 4 and –80 ℃ also showed a high stability for 1 week. For further validation of the stability of L-carnitine, thawing in 3 freeze-thaw cycles had a slight impact on the response of analytes (Figure S3).

Quantification of plasma endogenous L-carnitine in patients on dialysis

Using the method developed above, the content of L-carnitine in plasma samples was detected (*Figures 5,6*, and Figure S4). The average value of each participant's 3 tests was taken as the final measured value. As *Figure 5A* shows, the amount of L-carnitine in peritoneal dialysis patients (34.35±3.065 μM, n=33) was lower than that in the normal control group (40.88±1.238 μM, n=39), and it was statistically different (P=0.0402). It is worth noting that 20 out of 33 patients on peritoneal dialysis had different degrees of L-carnitine deficiency (*Figure 6*). Although 48.5% of these patients faced an extreme lack of L-carnitine (lower than 30 μM), the remainder still had acceptable levels of L-carnitine.

Furthermore, comparison between healthy participants and different groups of hemodialysis patients showed a

significant difference (*Figure 5B* and Figure S4). The plasma L-carnitine level of pre-hemodialysis patients without L-carnitine supplementation (29.16±2.243 μM, n=33) was notably lower than that of pre-hemodialysis patients with L-carnitine supplementation (131.1±13.90 μM, n=39) and healthy participants. Although the average L-carnitine level of pre-hemodialysis patients who had taken the L-carnitine supplementation as a routine was notably higher than normal level, a quarter of those patients (9/39) still had different degrees of L-carnitine deficiency. In addition, the plasma L-carnitine concentrations of post-hemodialysis patients with or without L-carnitine supplementation were significantly lower than normal level, which were 31.41±3.409 and 7.856±0.6442 μM, respectively. The average filtration rate of L-carnitine in hemodialysis was approximately 74% here. The results in hemodialysis patients were consistent with previous studies (3,29). Compared with the 100% extreme L-carnitine deficiency after dialysis without medication, the rate of L-carnitine deficiency after dialysis with medication was reduced to 81.8% (27/33) and 2/3 of them experienced extreme deficiency (21/33).

Discussion

L-carnitine is a low molecular weight hydrophilic substance which can be excreted by kidneys and easily lost via dialysis. In healthy people, L-carnitine deficiency is rare because it can be adequately supplied through diet and biosynthesis. However, deficiency is often observed in dialysis patients.

Several researches have declared that L-carnitine plays an important role in improving a variety of dialysis-related symptoms. Recent studies showed that low concentrations of L-carnitine would increase plasma B-type natriuretic peptide (BNP) levels in hemodialysis dialysis patients by affecting the activity of carnitine palmitoyltransferase 2 (CPT2) in cardiomyocytes (30). Additionally, low L-carnitine levels were associated with peripheral arterial stiffness in patients undergoing kidney transplantation (31). L-carnitine supplementation may improve erythropoietin-resistant anemia, reduce muscle cramping, improve hemoglobin levels and cardiac function in patients on hemodialysis/peritoneal dialysis (32,33).

Taken the clinical impacts of absolute L-carnitine deficiency and the huge difference in the deficiency degree of different dialysis situations into consideration, there is an eagerness to seek a means to promote precision treatment and rational drug use, reduce the waste of social resources

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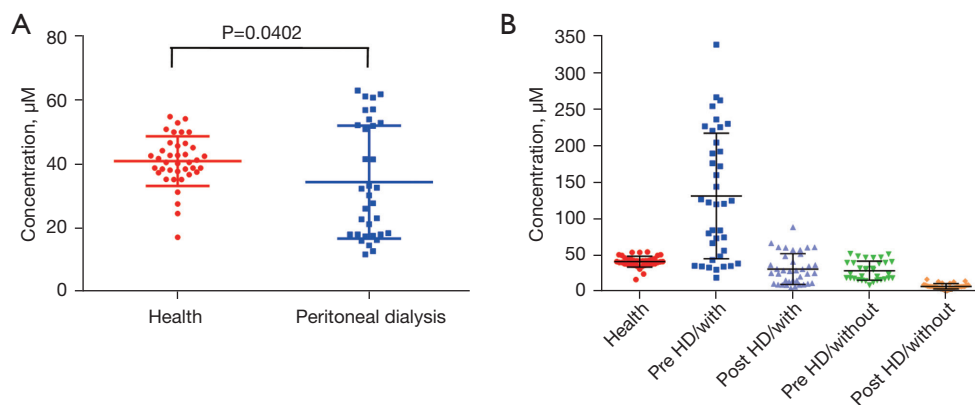
Figure 5 Plasma L-carnitine concentrations of healthy subjects and patients on dialysis. (A) Comparison between healthy subjects and patients with peritoneal dialysis, all of them had never taken L-carnitine, (B) comparison between healthy subjects and different groups of patients with hemodialysis. Patients with HD were divided into four groups according to pre/post HD and whether they were treated with L-carnitine or not; P<0.05 is considered statistically different. HD, hemodialysis.
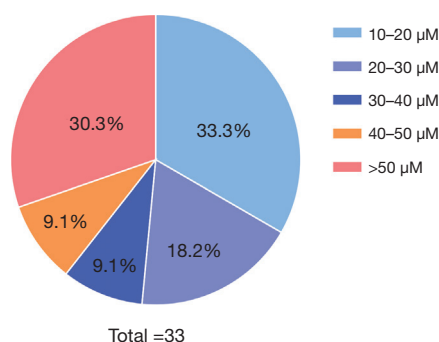
Figure 6 Distribution of plasma L-carnitine concentrations in peritoneal dialysis patients.

and the economic burden of patients.

Thus, this study developed and validated an UHPLC-Orbitrap-MS method for rapid determination of plasma endogenous L-carnitine in dialysis patients. There was no need to prepare analyte-depleted biological matrix or artificial matrix, which simplified the analysis procedure and guaranteed the authenticity of the results. The results of the fortified validation showed that the L-carnitine level in each plasma sample could be accurately quantified using the ameliorated standard curve made from anyone's untreated plasma. Taken the cost of the annual warranty of the instrument, mobile phase and standard solutions into account, the total cost is then divided by the number of tests per year, which is the cost of testing per sample. In our hospital, the cost of using UHPLC-Orbitrap-HRMS to detect L-carnitine was about ¥50–100 per sample.

To our knowledge, there are few published reports on the supplementation of L-carnitine in patients undergoing peritoneal dialysis (5,32). Nevertheless, the results above declared that peritoneal dialysis patients without L-carnitine supplementation should pay more attention to the monitoring of L-carnitine concentration, due to the wide concentration range in the plasma of these patients. The majority of them were facing different degrees of L-carnitine deficiency, and half of them had an extreme lack of L-carnitine. Meanwhile, 39.4% of peritoneal dialysis patients had adequate plasma L-carnitine without additional supplementation. Drug abuse in these patients should be avoided in clinical treatment to reduce their medical burden. Additionally, all hemodialysis patients were advised to be routinely given a full dose of L-carnitine, no matter whether they had ever taken L-carnitine or not. All the hemodialysis patients without L-carnitine supplementation faced extreme L-carnitine deficiency, while the ones with L-carnitine supplementation still had a high risk of L-carnitine deficiency.

A number of limitations need to be further discussed in this study. First of all, the sample size of different groups was small, and these results require further verification through an expanded sample size. Secondly, the basic conditions of population included in the study have not been completely consistent between the groups, and it is unknown whether the inconsistency of these parameters will lead to the deviation of the results. Thirdly, the dialysis duration has not been stratified. The deficiency of L-carnitine may become more serious with the increase

of dialysis years, which makes routine supplement of L-carnitine for peritoneal dialysis patients reasonable and necessary after reaching a certain number of dialysis years. Hence, a more detailed study on the rationality of L-carnitine supplementation in peritoneal dialysis patients according to various parameters is worthwhile.

Conclusions

A simple, rapid and precise UHPLC-Orbitrap-HRMS method for detection of endogenous L-carnitine was developed in this study, and the present results of plasma endogenous L-carnitine in dialysis patients indicated that the peritoneal dialysis patients were more deserving of a content test before the use of L-carnitine, which could be useful to promote rational drug use in future clinical practice.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-21-6784/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-21-6784/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-21-6784/coif). All authors report funding from the Soochow Science and Technology Project (SYSD2019182 and SYSD2020184), the Youth Project of Soochow Scientific Research Project (KJXW2019017), the pre-research project of the Second Affiliated Hospital of Soochow University (SDFEYQN1917), Tianqing Pharmaceutical Foundation of Jiangsu Pharmaceutical Association (TQ2021058), Hengrui Pharmaceutical Foundation of Jiangsu Pharmaceutical Association (H202145), National Natural Science Foundation of China (82070838), and Lift Project for Discipline Construction of The Second Affiliated Hospital of Soochow University (XKTJ-XK202010). The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Ethics Committee of The Second Affiliated Hospital of Soochow University (No. JD-LK-2020-038-01). Informed consent was taken from all the patients and healthy controls. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

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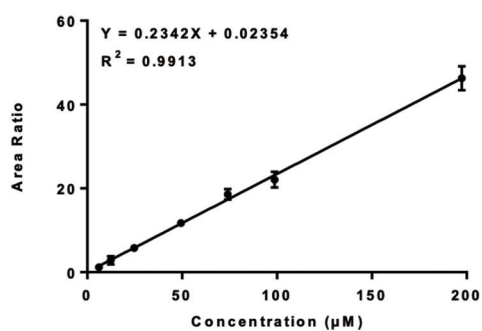
$$Y = 0.2342X + 0.02354$$
$$R^2 = 0.9913$$

Figure S1 Endogenous substances subtracted calibration curve of L-carnitine in the plasma.
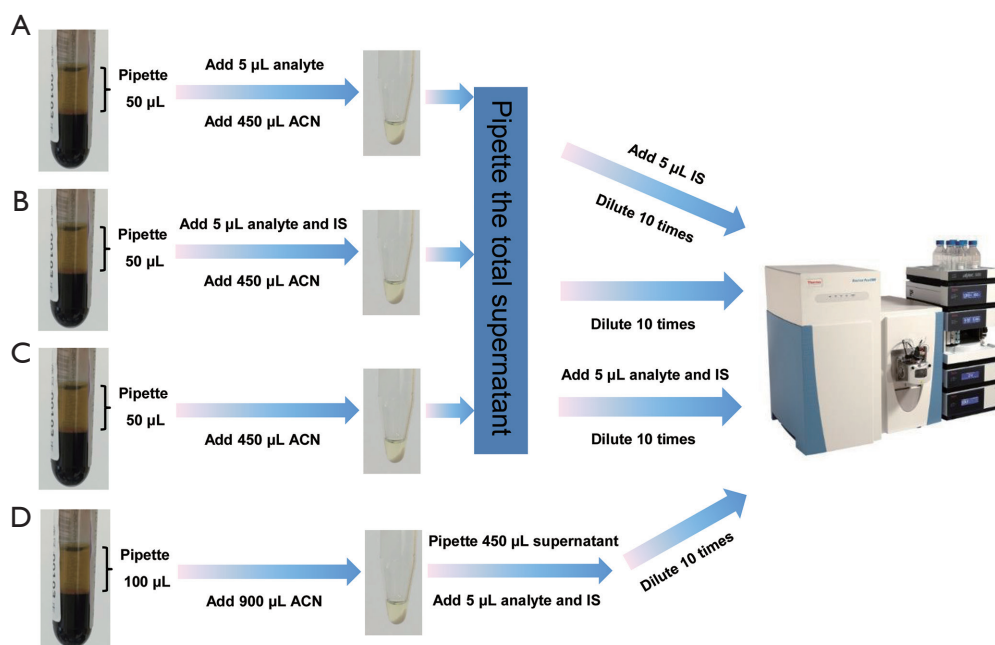
Figure S2 Different sample processing methods. IS, internal standard; ACN, Acetonitrile.

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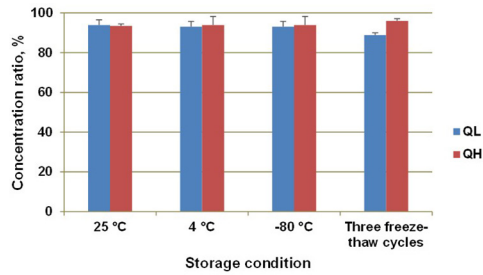
Figure S3 Assessment of stability of the analyte L-carnitine. QL, low concentration quality control sample; QH, high concentration quality control sample.
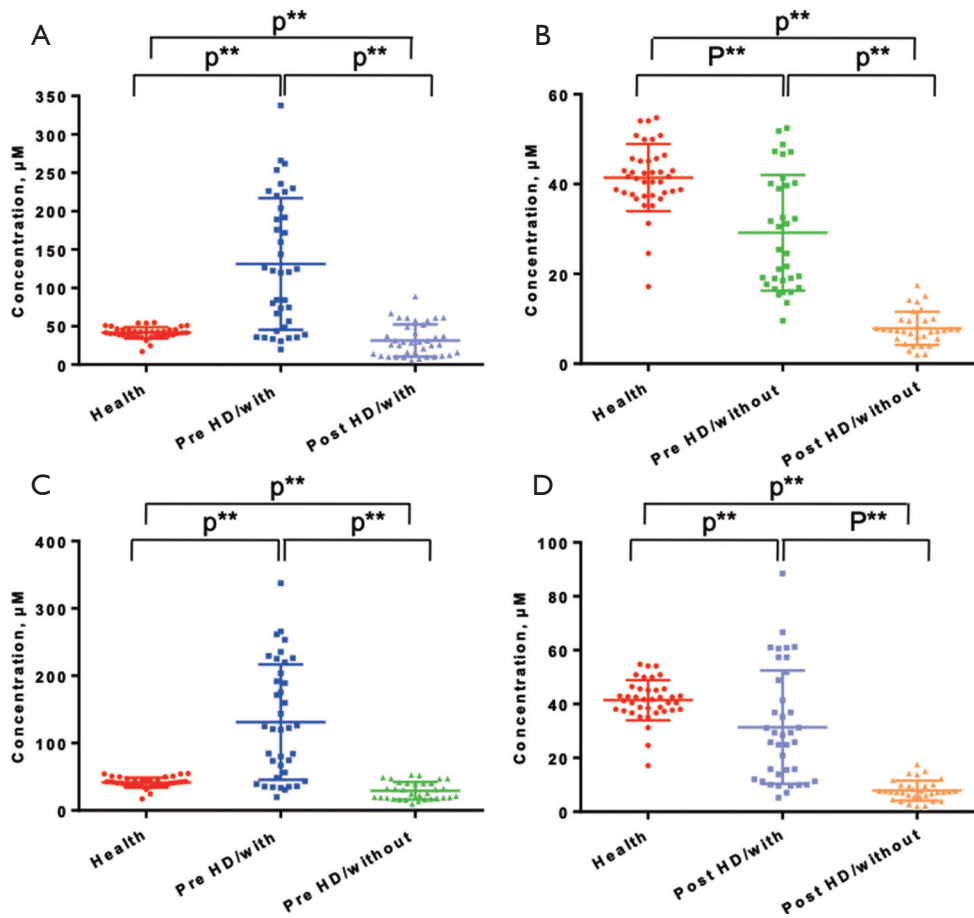
Figure S4 Statistical differences between healthy subjects and different HD groups. Patients with HD were divided into 4 groups according to pre/post HD and whether they were treated with L-carnitine or not. **, P<0.01 is considered to indicate a statistically significant difference. HD, hemodialysis.

Table S1 Intra-day and inter-day precision and accuracy for QC samples

Nominal concentration		Intra-day			Inter-day		
		Mean (µM)	Accuracy (RE%)	Precision (RSD%)	Mean (µM)	Accuracy (RE%)	Precision (RSD%)
LLOQ (6.169 µM)	Day 1	7.424	20.000	7.600	6.688	8.400	12.500
	Day 2	6.909	12.000	6.000			
	Day 3	5.584	-9.500	5.300			
QL (18.506 µM)	Day 1	18.528	0.100	7.800	18.342	-0.900	6.200
	Day 2	17.810	-3.800	5.900			
	Day 3	18.724	1.200	5.100			
QM (49.349 µM)	Day 1	47.617	-3.500	5.900	47.457	-3.800	5.800
	Day 2	45.044	-8.700	5.100			
	Day 3	49.709	0.700	0.900			
QH (154.216 µM)	Day 1	140.867	-8.700	-3.600	143.121	-7.200	4.900
	Day 2	137.223	-11.000	2.200			
	Day 3	151.273	-1.900	1.500			

QC samples, quality control samples; LLOQ, lower limit of quantification; QL, low concentration QC; QM, middle concentration QC; QH, high concentration QC; RE, relative error; RSD, relative standard deviation.

Table S2 The matrix factor and recovery of the method developed

QC samples	Matrix factor (%)		Recovery (%)	
	Mean	RSD	Mean	RSD
QL	91.803	9.867	154.416	3.593
QM	96.229	3.510	123.053	5.745
QH	99.862	0.715	102.945	6.057

QC samples, quality control samples; LLOQ, lower limit of quantification; QL, low concentration QC; QM, middle concentration QC; QH, high concentration QC; RSD, relative standard deviation.

Table S3 The area ratio of different sample processing methods

Sample ID	Response intensity		Area ratio (AR)
	L-carnitine	MILD	
QM-1[a]	31437052.889	3666389.647	8.574
QM-2[b]	34716499.506	1662983.625	20.876
QM-3[c]	58391600.575	3357444.474	17.392
QM-4[d]	55451867.434	3221367.498	17.214

[a]QM-1 is defined as the middle concentration quality control (QM) sample processed according to method A; [b]QM-2 is defined as the QM sample processed according to method B; [c]QM-3 is defined as the QM sample processed according to method C; [d]QM-4 is defined as the QM sample processed according to method D; MILD, mildronate.

Table S4 The recovery comparison of different sample processing methods

Recovery of QMs (H/V*)	AR_{QM-3}	AR_{QM-4}
AR_{QM-1}	49.302%	49.811%
AR_{QM-2}	*120.035%*	121.275%

*H/V is defined as the ratio of the horizontal values and the vertical values and the italic fonts represents the recovery of the method developed here; AR, area ratio; QM-1 is defined as the QM middle concentration quality control (QM) sample processed according to method A; QM-2 is defined as the QM sample processed according to method B; QM-3 is defined as the QM sample processed according to method C; QM-4 is defined as the QM sample processed according to method D.

Table S5 The fortified validation of calculated concentrations of the endogenous L-carnitine with 6 different calibration curves

Calculated concentration (µM)	Calibration curves with corresponding matrix						Mean	RSD (%)
	A	B	C	D	E	F		
Blank-plasma A	40.587	39.055	38.165	44.166	44.955	45.960	42.148	7.822
Blank-plasma B	37.258	35.773	34.800	37.677	39.142	39.848	37.417	5.145
Blank-plasma C	36.947	35.467	34.461	37.542	39.805	40.577	37.466	6.347
Blank-plasma D	36.184	34.714	33.716	39.520	40.702	41.564	37.733	8.737
Blank-plasma E	35.734	34.270	33.278	37.375	39.517	40.260	36.739	7.660
Blank-plasma F	34.415	32.970	31.945	36.716	36.436	36.872	34.892	6.044

RSD, relative standard deviation.