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Simultaneous Determination of Free and Total Carnitine in Human Serum by HPLC with UV Detection

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Abstract

A simple and reliable high-performance liquid chromatographic (HPLC) method for simultaneous determination of free and total carnitine in human serum has been developed, and its clinical significance has been investigated. After proteins in serum were precipitated, carnitine in serum was derivatized to form its ester. HPLC separation of the sample solution was performed on a SiO_2 column and detected by UV absorbance at 260 nm. A mobile phase composed of acetonitrile–citric acid–triethanolamine was found to be the most suitable for this separation. The free and total carnitine levels in serum were studied in 347 subjects. The method proved to be linear in the range of carnitine from 5 μmol/L to 400 μmol/L. The relative standard deviations of within-assay for free and total carnitine analyses were 3.36% and 1.97%, respectively; and between-assay for free and total carnitine analyses were 3.34% and 1.77%, respectively. The average recovery was 98.2% for free carnitine and 96.3% for total carnitine, respectively. The method has been applied to the simultaneous determination of free and total carnitine in serum. Statistics analysis showed that total and free carnitine levels of males were higher than that of females ($p < 0.01$) while acylcarnitine level had no statistically significant difference ($p > 0.05$).

Introduction

Carnitine (gamma-trimethylamino-beta-hydroxy butyric acid), or vitamin B_T, is an essential nutrient in the human body. It is generally accepted that carnitine is the only carrier for the transport of activated long-chain fatty acids from the cytosolic compartment into the mitochondrial matrix, producing the acylcanitines that undergo beta-oxidation and tricarboxylic acid cycle for energy production (1). The deficiency or disorder of carnitine in the body will lead to the disturbance of fatty acid metabolism, which will produce several disorders such as hyperlipemia, hypoglycemia, hyperammonemia, skeletal muscle disease, and myasthenia (2). As more research on biological function and metabolism of carnitine have been carried out in recent years, the studies of carnitine for medical applications are becoming more and more popular (3–5).

Total carnitine in serum includes free carnitine and acylcarnitines. Although carnitine is supplied externally in the diet (animal proteins), it can also be biosynthesized at a low rate in the liver, kidney, and brain. Primary and secondary carnitine deficiencies are usually characterized by poor availability of free carnitine. Numerous disorders have been described leading to disturbances in energy production and in intermediary metabolism in the organism, which are characterized by the production and excretion of unusual acylcarnitines (6). Hence, carnitine deficiency causes several disorders in fatty acid and related metabolism. Free and total carnitine levels in serum are the useful diagnostic indicators of the carnitine metabolic status. Therefore, it is important to determine free carnitine and acylcarnitine in serum for studying the behavior of carnitine in disease and therapy as well as the reasonable uses of carnitine and acylcarnitine as supplements in clinical application. It is the aim of the present study to establish a rapid, simple, and reliable method for simultaneous determination of free and total carnitine levels in human serum and to investigate its clinical significance.

The most widely used assay for the determination of carnitine in biological fluids, including serum and urine, is enzyme-spectrophotometric assay (7,8). The method is based on the free coenzyme A, one of reaction products generated from the acetylation reaction of carnitine, catalyzed by carnitine acetyl transferase with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), to produce a chromogenic substance that is detectable at 412 nm using a simple spectrophotometer. This original assay is subsequently found to suffer from a range of pitfalls, and modifications are required to overcome problems such as interference from endogenous thiols, reversibility of the reaction, and phosphate buffer acetylation, general nonspecificity, and poor sensitivity. In addition, the method is tedious and time-consuming. A radio-enzymatic assay is a significant improvement for the determination of carnitine. The method is more sensitive than the spectrophotometric approach, and it avoids the problems associated with endogenous thiols. However, it still retains many of the problems associated with the spectrophotometric assay. What is worse, the radioactive injury and pollution for the operator and environment are still problems, which are difficult to be overcome. Mass spectrometry, especially tandem mass spectrometry (MS–MS), or high-performance liquid chromatography with mass spectrometer detection (HPLC–MS)

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(9–12) is the recently applied technique for the measurement of carnitine in human fluids. The technique provided increased sensitivity, specificity, and reliability and allowed the direct analysis of the carnitine in complex mixtures of substances without the need for derivatization and chromatographic pre-separation. However, the major limitation of this technique is the high cost of the equipment and the need for dedicated technical personnel, which makes the method unsuitable for routine analysis.

To overcome some of these problems, in recent years the chromatographic techniques have been proposed for the analysis of carnitine in plasma, serum, and urine samples. Among them, the most widely applied is HPLC. Carnitine is a very polar, water-soluble, and relatively small molecule (MW 161.2), and it only has weak UV-absorbing at the range of short UV wavelength. So the chromatographic separation and the detection sensitivity are unsatisfactory when direct UV detection is utilized in combination with a reversed-phase chromatographic separation without pre-treatment of derivatization, and this assay has very limited applications. However, with the introduction of pre-column derivatization, HPLC has become more widely used in research and clinical studies. Nevertheless, it has been found that some problems occur with this assay because with some procedures it is not reactive enough to form the ester in a high enough yield to be analytically useful, sample preparation is time-consuming, and separation requires a long chromatographic run. Each step is modified in order to improve the efficiency and sensitivity of the method. For example, derivatization is carried out with different reagents for UV (13,14) or fluorescence (15–17) detection. Nevertheless, they are considered complicated and costly for routine clinical analysis. Furthermore, some reagents, especially derivatizing reagents, are not only quite expensive but also difficult to be bought in the market.

This report describes a modified HPLC method for simultaneous determination of free and total carnitine in human serum, which combines a pre-column derivative reaction of carnitine with *p*-bromophenacyl bromide (*p*-BPB) and a liquid chromatographic separation. The major advantages of the method developed in our present work are the low cost and simplicity of the technology. In addition, the reagents including the derivatizing reagent are easy to be bought, which is also a considerable benefit. It could, therefore, be more suitable for routine clinical analysis.

Experimental

Principle

In the anhydrous condition, free carnitine is derivatized with *p*-BPB to form a stable *p*-bromophenacyl ester with maximum UV absorption at 260 nm. After the carnitine derivative is well-separated by a liquid chromatographic separation with minimal interference from other bromophenacyl derivatives formed simultaneously, free carnitine level can be determined by detecting the UV absorption at 260 nm. An aliquot of the supernatant is dried by vortexing with anhydrous disodium phosphate. The determination of total carnitine requires the complete saponification of acyl carnitines. At present, the saponification reaction conditions recommended were the concentration of alkali solution (sodium hydroxide) was 2 mol/L, and the saponification reaction time was 8 min at room temperature. When the acyl carnitines in the serum sample were completely saponified into free carnitine, total carnitine level can be obtained by using the application of free carnitine determination.

Apparatus and chromatographic conditions

Chromatographic analysis was performed using a Model 100 Integral LC System (Perkin-Elmer, Norwalk, CT) operated at 260 nm, and a N2000 Chromatographic Workstation was used to record chromatograms and to calculate peak areas. Chromatographic separations were carried out isocratically on a SiO_2 column (200 × 4.6 mm i.d., particle size 5 µm). The mobile phase consisted of acetonitrile and citric acid (1.8×10^{-2} mol/L) in the ratios 90:10 (v/v), and then 250 µL of triethanolamine was added to 1000 mL of this solution, filtered through a 0.45-µm membrane filter (Millipore, Bedford, MA), and degassed by sonication in an ultrasonic bath prior to use. The flow rate of the mobile phase was 1.2 mL/min.

Reference standards and reagents

L-carnitine hydrochloride (purity 98%) and acetylcarnitine were supplied by Sigma (St. Louis, MO). *p*-BPB and tetrabutylammonium hydroxide were supplied by Tokyo Kasei Kogyo (Tokyo, Japan). HPLC-grade acetonitrile and methanol (Tedia, Fairfield, OH) were used. All chemicals, except where otherwise stated, were analytical-grade, and water used in this assay was doubly distilled. The saponification reagent was prepared by mixing 8 g of sodium hydroxide in 100 mL of water.

Enzymatic UV test kit (Cat. No. 11242008001) for determination of carnitine by an enzymatic reaction and spectrophotometric quantitation was supplied by Roche Diagnostics (Boehriger Mannheim, Germany).

Study subjects

The population studied included 182 male subjects (21–89 years of age, mean age 53.0 ± 18.4) and 165 female subjects (20–82 years of age, mean age 48.8 ± 16.3). All were selected by route healthy examination. Total cholesterol, triglyceride, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and apolipoprotein (apo) AI and apo B were determined. The subjects receiving lipid-reducing drug and having deviant results of the laboratory tests were excluded from the study.

Samples collection and analysis

Blood was collected by venipuncture following a 12-h overnight fast. The sample was centrifuged at 4000 r/min for 10 min, and the supernatant was kept at –70°C until analyzed.

Total-cholesterol, triglyceride, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and apolipoprotein (apo) AI and apo B were determined using commercial reagents (Boehriger Mannheim, Germany) on a Model 7600 automatic analyzer (Hitachi, Tokyo, Japan).

The concentration of free carnitine in serum was measured as a modified method (18). Briefly, an aliquot of 100 µL serum was mixed with 1 mL of precipitating reagent (acetonitrile–methanol 9:1), vortexed, and processed twice with 400 mg of a mixture of

9 parts Na_2HPO_4 (i.e., anhydrous), 1 part Ag_2O, and about 400 mg of KH_2PO_4. After centrifugation, an aliquot of 400 µL supernatant sample was mixed with 50 µL of the derivatizing reagent. The mixture was heated at 60°C for 90 min, and then cooled to be injected onto the HPLC (20 µL). For the assay of total carnitine in serum, an aliquot of 100 µL serum sample was mixed with 10 µL volume of NaOH solution (2 mol/L), vortexed, and maintained at room temperature for approximately 15 min. Then, a 0.99 mL volume of the precipitating reagent was poured in the tube, and the following procedure was the same as the assay of free carnitine in serum. Figure 1 shows typical chromatograms of blank (substitution water for the serum sample), reference standard, and human serum sample. Under the chromatographic conditions described, the derivate had a retention time of approximately 10.2 min, and the analysis time was approximately 14 min per injection. It can be seen from Figure 1 that good separation and detectability of the ester was obtained with baseline resolved peaks and chromatograms with minimal interference from the serum sample.

Table I. Recovery of the Determination of Free Carnitine in Serum*

Added (µmol/L)	Found (µmol/L)	Recovery (%)	RSD (%)
25	65.73 ± 9.29	99.04 ± 4.6	4.6
50	88.13 ± 0.51	94.32 ± 1.0	1.1
100	140.85 ± 0.21	99.88 ± 0.2	0.2
200	240.16 ± 0.15	99.6 ± 0.1	0.1

* $\bar{x} \pm s$; $n = 5$, background: 40.97 ± 0.35 µmol/L.

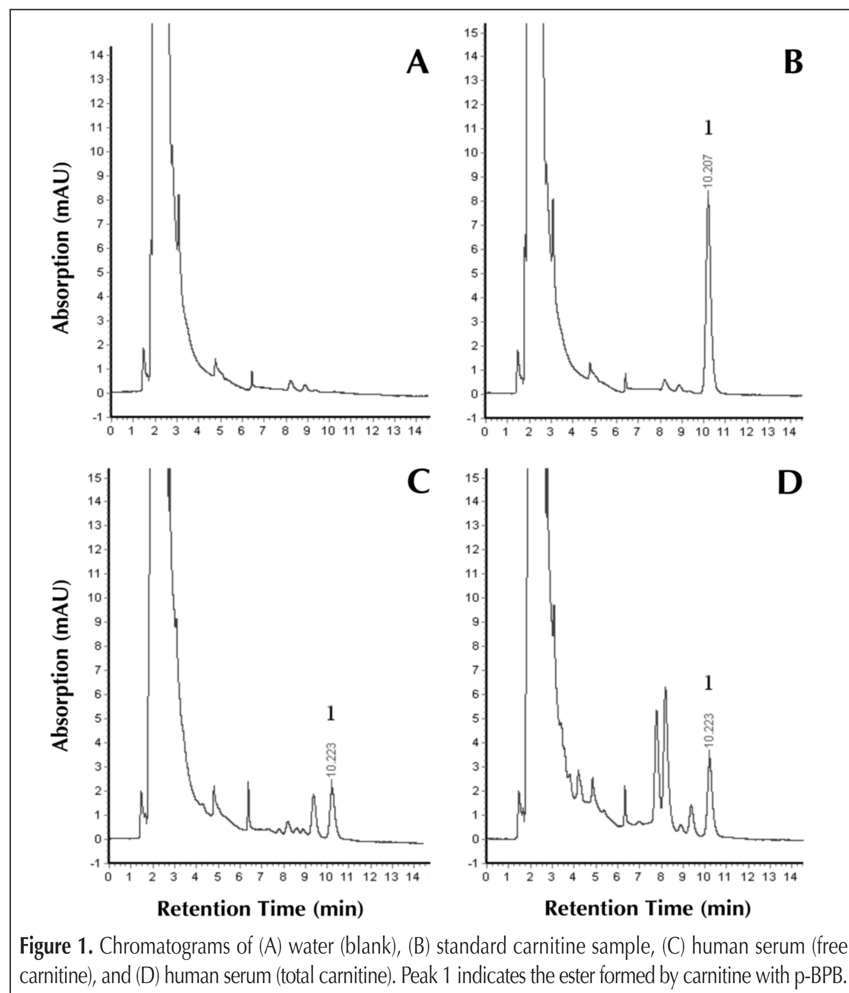

Figure 1. Chromatograms of (A) water (blank), (B) standard carnitine sample, (C) human serum (free carnitine), and (D) human serum (total carnitine). Peak 1 indicates the ester formed by carnitine with p-BPB.

Statistical analysis

Data were statistically evaluated with the Statistics Package for Social Sciences (SPSS) program for Windows version 12.0. All the values are presented as mean ± standard deviation ($\bar{x} \pm s$). Statistical significance between individual groups was analyzed with the independent Student's test. The values were considered statistically significant if the P value was less than 0.05.

Results and Discussion

Precision, reproducibility, and linearity

For assaying human serum sample, the reproducibility of the present method was assessed, and the results showed that the relative standard deviations of within-assay (n = 5) for free and total carnitine analyses were 1.97% and 3.36%, respectively, and between-assay (n = 7) for free and total carnitine analyses were 1.77% and 3.04%, respectively.

Based on the level of carnitine in serum, a series of samples containing 5, 25, 50, 100, 200, and 400 µmol/L of carnitine were prepared to study the relationships between the peak area of carnitine and the concentrations of carnitine under selected conditions. The results showed that the peak area was linearly related to the carnitine concentration for the range of 5–400 µmol/L. The linear equation for the concentration vs. the peak area was $y = 878.1x + 3101$ with a correlation coefficient of 0.9997. The detection limit of detection of free carnitine in serum was approximately 1 µmol/L. Although the detection limit of detection is slightly higher than the HPLC–MS method, it can suffice to meet the requirement of sensitivity for the clinical practical analysis.

Recovery

The water content in the sample is an important factor for the recovery of this method because the derivatization reaction of carnitine with p-bromophenacyl bromide should be waterless (i.e., anhydrous). The experimental results indicate that the mean recovery is approximately 90% when one time dehydration procedures are carried on in the proposed method. When twice dehydration procedures are carried on in the sample preparation so that the sample can be completely dried before the derivatization reaction, the average recovery is over 95%.

To the mixed serum samples in which free carnitine and total carnitine were determined (n = 5), various amounts of standard carnitine were exogenously added into the serum sample to test

the analytical recovery. The samples, after being dealt with previously mentioned analytical procedure, were determined by HPLC. The experimental results showed that the average recovery of free and total carnitine for serum samples was 98.2% and 96.3%, respectively (Table I–II).

Comparison of HPLC method with enzymatic UV method

The most widely used assay for the determination of carnitine in serum is enzyme-spectrophotometric assays. Twenty-eight serum samples were analyzed, and the results obtained by this method were compared with determination using an enzymatic UV test kit by an enzymatic reaction and spectrophotometric quantitation. The relationship found by regression analysis was $y = 1.09x - 2.83$, where y was the concentration obtained by the present HPLC analysis and x was the concentration obtained by the enzymatic UV method. The correlation coefficient $r^2 = 0.9695$.

Applications

Human serum samples were collected and prepared, and the levels of free and total carnitine in serum were determined by the proposed HPLC method. The results for free carnitine, total carnitine, and the acylcarnitine (acylcarnitine = total carnitine – free carnitine) in serum are statistically evaluated and summarized in Table III.

In our study men showed higher mean of the levels of free and total carnitine than women ($P < 0.01$). However, no significant differences between male and female groups ($P > 0.05$) were observed in the level of acylcarnitine. These results agree well with those reported in other laboratories (19,20). These may be induced by the differences between the male and female in diet, metabolism, and sexual hormones. On the other hand, some other laboratories reported the difference of the values for these analytes in serum between male and female groups was not significant (21) probably because of the different numbers of the subjects selected, different physiological characteristics of the subjects, or both.

Table II. Recovery of the Determination of Total Carnitine in Serum*

Added (μmol/L)	Found (μmol/L)	Recovery (%)	RSD (%)
25	70.57 ± 0.79	95.91 ± 1.0	1.0
50	93.43 ± 0.41	93.01 ± 0.8	0.89
100	145.97 ± 0.76	99.04 ± 0.8	0.77
200	241.5 ± 2.36	97.29 ± 1.2	1.2

* $\bar{x} \pm s$; $n = 5$, background: 46.93 ± 0.54 μmol/L.

Table III. Reference Intervals for Free and Total Carnitine in the Normal Population

	Free carnitine (μmol/L)		Total carnitine (μmol/L)		Acylcarnitines (μmol/L)	
	Range	Mean value $\bar{x} \pm s$	Range	Mean value $\bar{x} \pm s$	Range	Mean value $\bar{x} \pm s$
Both ($n = 347$)	12.65–77.31	40.21 ± 8.75	18.14–89.25	50.30 ± 9.44	3.85–33.91	10.09 ± 3.22
Male ($n = 182$)	20.45–77.31	42.33 ± 8.27	33.67–89.25	52.20 ± 8.59	4.66–21.53	9.87 ± 2.93
Female ($n = 165$)	12.65–67.78	37.88 ± 8.69*	18.14–79.55	48.21 ± 9.91*	3.85–33.9	10.33 ± 3.50

* $P < 0.01$; compared with male group.

References

1. C.M. Ng, M.R. Blackman, C. Wang, and R.S. Swerdloff. The role of carnitine in the male reproductive system. *Ann. N.Y. Acad. Sci.* **1033:** 177–188 (2004).
2. N. Longo, C. Amat di San Filippo, and M. Pasquali. Disorders of carnitine transport and the carnitine cycle. *Am. J. Med. Genet. C Semin. Med. Genet.* **142(2):** 77–85 (2006).
3. E.D. Weinhandl, M. Rao, D.T. Gilbertson, A.J. Collins, and B.J. Pereira. Protective effect of intravenous levocarnitine on subsequent-month hospitalization among prevalent hemodialysis patients, 1998 to 2003. *Am. J. Kidney Dis.* **50(5):** 803–812 (2007).
4. R.A. Power, M.W. Hulver, J.Y. Zhang, J. Dubois, R.M. Marchand, and O. Ilkayeva et al. Carnitine revisited: potential use as adjunctive treatment in diabetes. *Diabetologia* **50(4):** 824–832 (2007).
5. G. Balercia, F. Regoli, T. Armeni, A. Koverech, and F. Mantero, M. Boscaro. Placebo-controlled double-blind randomized trial on the use of L-carnitine, L-acetylcarnitine, or combined L-carnitine and L-acetylcarnitine in men with idiopathic asthenozoospermia. *Fertil. Steril.* **84(3):** 662–671(2005).
6. M. Möder, A. Kießling, and H. Löster. Current Methods for Determination of L-Carnitine and Acylcarnitines. *Monatshefte für Chemie / Chemical Monthly* **136(8):** 1279–1291 (2005).
7. H.E. Indyk and D.C. Woollard. Enzymatic determination of free carnitine in milk and infant formulas. *J. AOAC Int.* **78(1):** 69–74 (1995).
8. A.K. Ghoshal and S.J. Soldin. Determination of total and free plasma carnitine concentrations on the Dade Behring Dimension RxL: integrated chemistry system. *Clin. Chim. Acta* **361(1–2):** 80–85 (2005).
9. L. Vernez, M. Wenk, and S. Krähenbühl. Determination of carnitine and acylcarnitines in plasma by high-performance liquid chromatography/electrospray ionization ion trap tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **18:** 1233–1238 (2004).
10. M.G. Murphy, J.F. Crocker, P. O'Regan, S.H. Lee, L. Geldenhuys, and K. Dooley et al. Carnitine, acylcarnitine and amino acid profiles analyzed by tandem mass spectrometry in a surfactant/virus mouse model of acute hepatic encephalopathy. *Chemosphere* **68(9):** 1692–1698 (2007).
11. P.E. Minkler, S.T. Ingalls, and C.L. Hoppel. Strategy for the isolation, derivatization, chromatographic separation, and detection of carnitine and acylcarnitines. *Anal. Chem.* **77(5):** 1448–1457 (2005).
12. Y. Maeda, T. Ito, A. Suzuki, Y. Kurono, A. Ueta, and K. Yokoi et al. Simultaneous quantification of acylcarnitine isomers containing dicarboxylic acylcarnitines in human serum and urine by high-performance liquid chromatography/ electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **21(5):** 799–806 (2007)
13. P.E. Minkler and C.L. Hoppel. Determination of free carnitine and 'total' carnitine in human urine: derivatization with 4'-bromophenacyl trifluoromethanesulfonate and high performance liquid chromatography. *Clin. Chim. Acta* **212(1-2):** 55–64 (1992).
14. B.J. Poorthuis, T. Jille-Vlckova, and W. Onkenhout. Determination of acylcarnitines in urine of patients with inborn errors of metabolism using high-performance liquid chromatography after derivatization with 4'-bromophenacylbromide. *Clin. Chim. Acta* **216(1-2):** 53–61 (1993).
15. C.J. McEntyre, M. Lever, and M.K. Storer. A high performance liquid chromatographic method for the measurement of total carnitine in human plasma and urine. *Clin. Chim. Acta* **344(1–2):** 123–130 (2004).
16. P.E. Minkler, J. Kerner, K.N. North, and C.L. Hoppel. Quantitation of long-chain acylcarnitines by HPLC/fluorescence detection: application to plasma and tissue specimens from patients with carnitine palmitoyltransferase-II deficiency. *Clin. Chim. Acta* **352(1-2):** 81–92 (2005).
17. Q.R. Cao, S. Ren, M.J. Park, Y.J. Choi, and B.J. Lee. Determination of highly soluble L-carnitine in biological samples by reverse phase high performance liquid chromatography with fluorescent derivatization. *Arch. Pharm. Res.* **30(8):** 1041–1046 (2007).
18. K. Li, W. Li, and Y. Huang. Determination of free L-carnitine in human seminal plasma by high performance liquid chromatography with pre-column ultraviolet derivatization and its clinical application in male infertility. *Clin. Chim. Acta* **378(1-2):** 159–163 (2007).
19. J.R. Opalka, F.N. Gellerich, and S. Zierz. Age and sex dependency of carnitine concentration in human serum and skeletal muscle. *Clin. Chem.* **47(12):** 2150–2153 (2001).
20. C.S. Ho, B.S. Cheng, and C.W. Lam. Rapid liquid chromatography-electrospray tandem mass spectrometry method for serum free and total carnitine. *Clin. Chem.* **49(7):** 1189–1191 (2003).
21. R. Alberty and D. Albertyova. Biological variation of free and total carnitine in serum of healthy subjects. *Clin. Chem.* **43(12):** 2441–2443 (1997).

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