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Abstract: Carnitine is a major molecule which plays a great role in the transfer of long chain fatty acids into the mitochondrion. Measurement of its total and free forms is essential in the clinical evaluation of carnitine deficiency that can arise from disorders either primarily or secondarily.
In this study, different deproteinization methods (precipitation with perchloric acid, heat denaturation of freeze thawing and filtration) were evaluated for the optimization of fluorometric measurement of total and free carnitine in serum. The most appropriate method for the carnitine measurement was established by additional studies of deacylation at different temperatures, pH optimization of the reaction, tests with reagents of different concentrations and intensity alteration at reaction ends. Recovery of free carnitine and octanoylcarnitine added to serum was 104-92% and 87-91%, respectively. The method was found to be very sensitive for measuring concentrations from 5 to 200 µM. In conclusion, it was observed that there was a significant correlation (r=0.977, slope:1.055, intercept:-2.59) when the optimized method was compared with the UV-kinetic method.

Key Words: Carnitine, fluorometric measurement

Introduction

Carnitine (β-hydroxy-ϒ-trimethylammonium butyrate) is an original molecule functioning for the transport of long-chain fatty acids through the inner mitochondrial membrane. Therefore, it is evident that this system involving carnitine has a major role in the metabolism of fatty acids. Impaired oxidation of fatty acids can occur owing to enzyme deficiencies in this route, or due to primary and secondary carnitine deficiency. The etiology of the deficiency should be determined for proper diagnosis and treatment of impaired fatty acid oxidation. That is, measuring carnitine levels is of great importance for the determination of both its deficiency and the etiopathology of the disorders (1-6). Hypoketotic hypoglycemic attacks, dilated cardiomyopathy, myoglobinuria, valproic acid treatment in children, and post-dialysis application in patients with renal insufficiency could be shown as indications for measurement (1,2,6-10).

Carnitine is found in its acyl form or as free carnitine in the body. The acylcarnitine form, 10-30% of total carnitine, binds to free fatty acids with ester bonds via its carboxylic moiety. Although the measurement of free carnitine in serum is assumed to be efficient for the diagnosis of the aforementioned diseases, total and free carnitine analyses in both serum and urine samples should also be performed in order to eliminate some other pathological conditions (1,2,6,9).

Currently, measurement of carnitine is carried out by colorimetry with or without an autoanalyzer (11,12,13), or kinetic (14), fluorometric (15), radioimmunological (16) and radioenzymatic (17) methods. It can also be measured using high performance liquid chromatography (HPLC) and gas chromatography (GC) (18,19,20), especially in research studies and screening for blood spots by electrospray tandem mass spectrometry (21). The radioenzymatic method was preferred in the past, and it is still the method of choice for carnitine measurement (17).

In this study, our aim was to optimize the fluorometric measurement of carnitine, thereby choosing the appropriate method for the assessment of free carnitine in serum, and to compare it with the method using a UV-kit.

Materials and Methods

Acetyl CoA:carnitine O-acetyltransferase (EC 2.3.1.7), acetyl CoA, L-carnitine, octanoylcarnitine, and N-(9-acridinyl)-maleimide (NAM) were purchased from Sigma, St. Louis, MO (USA). The carnitine ultraviolet (UV) kit and Ultrafree–MC 10.000 NMWL filter units were obtained from Boehringer Mannheim No: 1242008 (FRG) and from Millipore Corporation, Bedford, MA, respectively. Other chemicals were of analytical grade and were obtained from Merck, Darmstadt (FRG).

There are three important steps involved in the fluorometric measurement of free and total carnitine in serum.

1. Deproteinization

2. Deacylation (valid only for total carnitine measurement)

3. Assays used for the measurement of carnitine

1. Deproteinization: For the measurement of carnitine, deproteinization should be performed for removing molecules containing –SH groups. In order to determine the most appropriate deproteinization method for the fluorometric measurement, various methods such as filtration, denaturation by heat precipitation and perchloric acid were examined. After serum or plasma was obtained, they were kept at –20°C until use.

We applied 3 types of methods for the deproteinization of serum:

a) Perchloric acid ($HClO_4$) method (12,22): 0.5 ml concentrated cold perchloric acid was added to 2 ml of serum. It was centrifuged for 10 min at 10,000 g after being kept at +4°C for 30 min. To 1 ml of the supernatant was added 300 µl 2 M potassium hydroxide (KOH) consisting of 0.5 M 3-[N-morpholino] butanesulfonic acid (MOPS), until the final pH of the mixture was between 7 and 8. Then, it was centrifuged at 10,000 g for 10 min after being left at +4°C for 30 min. Finally, clear supernatant was used for the study. If the percloric acid method is used, the neutralization step should be applied.

b) Heat denaturation of freeze-thawing (23): Initially, 2 ml of serum was kept in a thermal block at 100°C for 5 min and then at –80°C for 5 min. A frozen sample was dissolved the aid of an injector tip, and the supernatant obtained after centrifugation at 4,200 g for 5 min was used for the assay.

c) Filtration (24): ~300 µl serum was centrifuged for 30 min at 10,000 g in filter units permeable to proteins with a molecular weight of less than 10,000. The resultant clear filtrate was used for the measurement.

2. Deacylation: The measurement of total carnitine can be carried out by deacylation of carnitine in its acyl form via heat alkaline medium. For the determination of acylcarnitine, total carnitine should be measured after deacylation with KOH incubation. Therefore 10 µl of 1M KOH was added to 100 µl of deproteinized sample followed by incubation at 60°C and 80°C for an hour. Then 5 µl 1M HCl was added to the sample for neutralization. Thus, the effects of incubation of samples at 60°C and 80°C on the measurement of total carnitine at different pH values were also investigated.

3. Assays used for the measurement of carnitine

a) Fluorometric method

Free CoA, formed by the reaction of acetyl carnitine with carnitine acetyltransferase, was nonenzymatically reacted with NAM and CoA-NAM, and the complex compound formed had the fluorescein activity (15,25).

All solutions were prepared in various concentrations (Table 3). Standards of carnitine, acetyl CoA, EDTA Na_2 (etylenediaminetetraacetic acid-disodium salt) and CAT were all dissolved in distilled water, but NAM was dissolved in acetone.

The reaction mixture contained 250 µl phosphate buffer, 25 µl acetyl CoA and 25 µl EDTA Na_2 (24 mM).

The order of procedures in a run is listed below with its constituents:

Procedure	Sample (µl)	Blank (µl)
Deproteinized sample and standards	50	50
Reagent mixture	50	60
Carnitine acyltransferase (CAT)	10	-
Incubation at room temperature for 15 min		
NAM	50	50
Incubation of the final mixture on ice for 15 min		
Distilled water	1000	1000

Then the measurement of carnitine was performed by fluorometer (Perkin Elmer LS 50B,UK) with excitation at 365 nm and emission at 430 nm in 30-50 min. Slits of excitation and emission were 5 nm and each sample with its blank without enzyme was run simultaneously. Finally, the concentration of carnitine was determined using graphics obtained from the measurements of carnitine standards obtained via the same route.

Different concentrations of phosphate buffer, acetyl-CoA, NAM and CAT were tested and each of their optimal concentrations was determined (Table 3).

b) Carnitine kinetic (UV kit) method

Acetylcarnitine is formed by the reaction of L-carnitine with acetyl CoA catalyzed by CAT. AMP is obtained after free CoA is converted into acetyl CoA by the reaction between ATP and acetate with the catalysis of acetyl CoA synthetase. After the formation of AMP, the synthesis of myolinase and adenosine diphosphate precedes the generation of ATP and pyruvate by the enzyme of pyruvate kinase. In the final step, via catalysis of lactate dehydrogenase, pyruvate and NAD^+ are obtained and carnitine is measured by the absorbance at reading 340 nm.

Measurement of carnitine was consequently performed using the optimized fluorometric method on individuals in different age groups who had no known pathologies.

Results

a. Deproteinization

In this study, to determine the most appropriate method of deproteinization for the fluorometric measurement of carnitine, we compared three different deproteinization methods.

To determine the efficiency of deproteinization, the amount of protein remaining was measured after deproteinization by perchloric acid, heat denaturation and filtration, and the effects of each method on carnitine measurement were evaluated. According to the data in Table 1, the carnitine level was found to be increased because the precipitated protein concentration was high using the method of heat denaturation. The best method with a low coefficient of variation (CV) was filtration, since the perchloric acid method had a relatively high CV, despite its better efficiency (Table 1).

b. Deacylation and neutralization

According to recovery studies, no significant difference was found between the samples incubated at 60°C and 80°C and the most appropriate pH was about 7.0 (Table 2).

c. The effect of reaction pH on carnitine measurements

In fluorometric carnitine measurements, pH should be kept between 6 and 8 for an optimal CAT activity (14).

Table 1. Comparison of the efficiency of deproteinization methods for fluorometric carnitine measurements

Deproteinization methods	Protein* (mg/dl)	Carnitine µM (n=10)	%CV
Perchloric acid method	<20	42.1	7.7
Heat denaturation of freeze-thawing	150-250	51.3	3.6
Filtration	<20	46.2	2.9

CV: Coefficient of Variation
* Protein concentration in supernatant

Table 2. The effect of pH of the samples (5.5, 6.5, 7.0 and 7.5) on the mmeasurement of free and total carnitine after the procedure of deacylation and neutralization at different temperatures (60°C and 80°C).

	60°C				80°C			
Sample pH	5.5	6.5	7.0	7.5	5.5	6.5	70	7.5
Serum carnitine (µM)			56.2				56.2	
Serum carnitine +25 µM octanoylcarnitine (µM)	75.7	77.9	79.9	77.7	76.2	78.7	79.4	78.2
Recovery (%)	78	87	95	86	80	90	93	88

The most appropriate pH of the study was determined by measurements using different pH values (Figure 1).

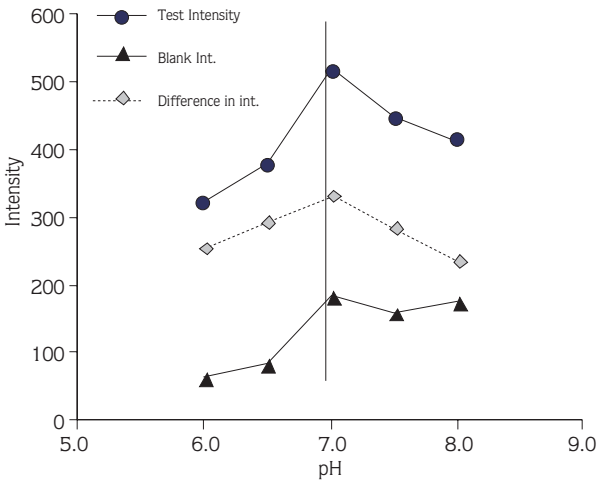

Figure 1. Effect of different pH values (5.0, 6.0, 7.0, 8.0 and 9.0) on intensity for test and blank samples.

d. Determination of optimal concentrations of reagents

50 mM carnitine standard was measured using various concentrations of phosphate buffer, acetyl-CoA, NAM and CAT in order to define the optimal concentrations of reagents. The optimal concentrations of phosphate buffer, acetyl-CoA, NAM and CAT were found to be 90 mM, 5 mM, 0.1 mM, and 0.05 U/ml, respectively (Table 3).

e. Stabilization of reaction intensity

Because the intensity at the end of the reaction could change with time, the intensity evaluation was performed in 5 different samples with blanks in order to find out the exact measurement time (Figure 2).

f. Calibration curve

By using carnitine standards with various concentrations between 5 and 200 µM, regression analysis of the methods was performed and a significant correlation between these methods was found (Figure 3).

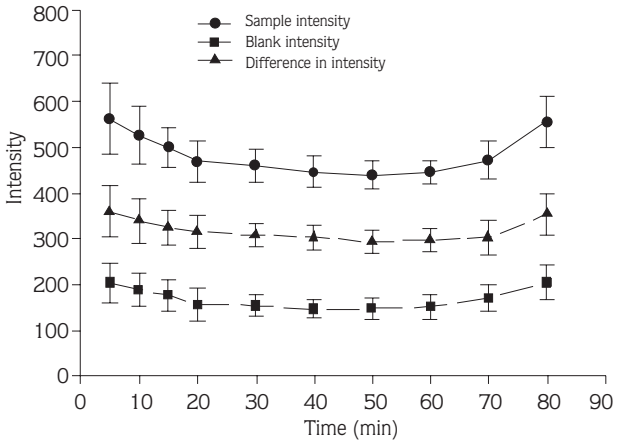

Figure 2. Time dependent variation of fluorescent intensity of the reaction to determine the optimal measurement time.

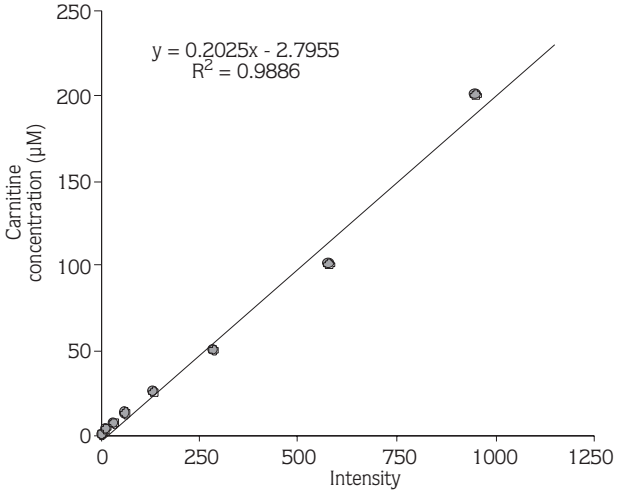

Figure 3. The calibration curve of fluorometric carnitine measurement.

g. Evaluation of the method

A previously prepared serum pool was run consecutively 10 times in one assay to determine within-

Table 3. Optimal concentrations determined for phosphate buffer, acetyl CoA, NAM and CAT used in the measurement of free and total carnitine.

Phosphate buffer		Acetyl CoA		NAM		CAT	
C (mM)	Int	C (mM)	Int	C (mM)	Int	C (U/ml)	Int
40	209	2,5	274	0,05	230	**0,05**	**288**
90	**288**	**5**	**291**	**0,1**	**294**	0,1	286
120	242	7,5	290	0,2	292	0,2	290

C: Concentration, Int: Intensity

run precision. The same sample was used in runs on 10 different days for the evaluation of day-to-day precision. Precision values were calculated using the formula (SD / mean value) x 100. In modified fluorometric measurements, within-run and day-to-day precisions were found to be less than 10%.

Recoveries were 104% and 92% when 25 µM and 50 µM free carnitine were added respectively, but lower (87% and 91%, respectively) when 25 µM and 50 µM of octanoylcarnitine were added (Table 4).

f. Interference study

Several compounds were tested for the possible assay interference of lipemia (prepared with Ultralipid), hemolysis. Bilirubin did not interfere with carnitine determination.

g. Method comparison

Measurements of the modified fluorometric method (filtration method for deproteinization, deacylation at 60°C, sample pH of 7.0, 90 mM phosphate buffer (pH=7.0), 5 mM CoA, 0.1 mM NAM, 0.05 U/ml CAT and measurement within 30-60 minutes) and UV kit were compared using 23 samples. As a result, as seen in Figure 4, significant correlation, slope and intercept values were in accordance with each of the methods (r= 0.976, p<0.001, slope= 1.055, intercept =-2.55).

Table 5 represents normal values of free and total carnitine levels in 104 healthy people determined by the modified fluorometric method.

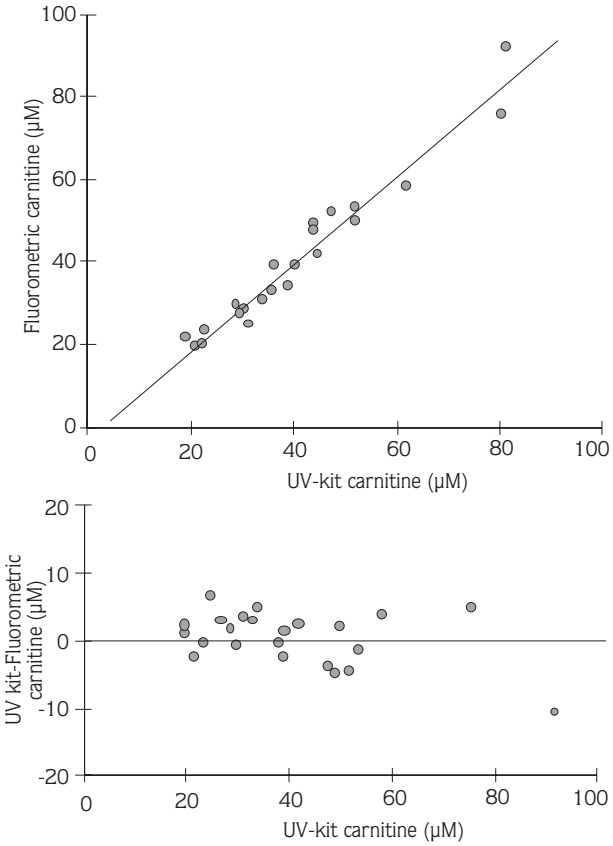

Figure 4. Comparison of modified fluorometric method and UV kit method.
(a) Regression analysis of flourometric carnitine measurement versus carnitine UV kit .
(b) The difference between the two methods is plotted against the results obtained by using UV-kit.

Table 4. Within-run and day-to-day precision and recovery results of the modified and optimized free and total fluorometric carnitine assay.

	Carnitine	Precision (n=10)		
	Levels µM	Within-run %	Day-to-day %	Recovery %
Serum pool (free)	45.2	1.9	4.5	
Serum pool +25 µM carnitine	78.1	2.8	6.2	104
Serum pool +50 µM carnitine	91.2	5.4	6.5	92
Serum pool (total)	56.2	5.4	8.1	
Serum pool + 25 µM octanoylcarnitine	77.9	6.3	8.4	87
Serum pool + 50 µM octanoylcarnitine	99.7	5.1	7.2	91

Table 5. Free and total carnitine levels of healthy people using modified fluorometric method.

	n	Free carnitine µM	Total carnitine µM
Cord blood	7	20.8 ± 4.5	25.2 ± 5.4
0-1 year	11	28.4 ± 5.9	36.1 ± 7.3
2-5 years	15	33.2 ± 6.1	39.4 ± 8.6
6-15 years	22	40.3 ± 8.7	51.7 ± 9.5
15-65 years	30	47.2 ± 9.3	55.2 ± 12.6
> 65 years	19	59.0 ± 9.7	76.3 ± 13.5

Discussion

Deficiency of carnitine, which has a major role in the oxidation of long chain fatty acids, is rarely seen because of adequate dietary intake and its synthesis in vivo. Carnitine deficiency could also develop due to some neonatal defects and secondary to various pathological conditions in adults. However, the diagnosis of symptomatic carnitine deficiency seems to be difficult since carnitine levels which appear clinically have not yet been determined or ranged completely (1,2,6,9).

Carnitine measurement by colorimetric, fluorometric and radioenzymatic methods is principally based on the measurement of free -SH groups found within the structure of CoA produced. Because proteins possess a large amount of -SH groups in their structures, protein interference has to be eliminated by the deproteinization of samples (12,22,23). Methods of precipitation with perchloric acid, heat denaturation of freeze-thawing and filtration were used to find out the most appropriate technique for this study (14,24,26).

Efficacies of deproteinization and pH alterations observed during the assay are major factors which affect the accuracy of carnitine measurement. Deproteinization, the most important step of carnitine measurement, should be easily applicable and quick, and have good precision. Our results showed that the most appropriate deproteinization method was filtration on account of its precision, easy applicability and stability at pH degree (Table 2).

Amongst the acid deproteinization methods, precipitation with perchloric acid is preferable to the others. However, in our study, this method was not appropriate due to the large range of pH variations and precision (Table 1). It was found that heat denaturation caused false-positive results. It was also observed that 150-250 mg/dl of protein in supernatant increased the carnitine level by 10-15% in the fluorometric measurement (Table 1). In methods using acid denaturation, the pH should be kept at 7.0. Although pH is easily adjusted manually by a pH meter when a large volume of reagent is used, strict attention should be paid to this neutralization procedure in fluorometric measurement in which very small volumes can be used. This problem can be relatively overcome by using an indicator (usually phenol red) instead of a pH meter in radioenzymatic measurement of carnitine (16).

After the optimization of deproteinization procedures, the reagents used in the fluorometric measurement of carnitine were standardized by using different concentrations. Maehera et al. used similar concentrations in their study (15), and reported that serum carnitine values obtained by fluorometric measurement were found to be higher than those obtained by colorimetric measurement (15). This might be due to running the procedures within 30 minutes, since the findings of the measurement by the "read" mode of the fluorometer in 30-60 minutes were found to be compatible with those of the colorimetric method (Figure 2). In addition, Mahera et al. used N-(p-(2-benzimidazoyl)-phenyl)-maleimide (BIPM), whereas we used NAM. It was also found that it was absolutely necessary to use a sample blank in spite of the fact that results with high precision could be obtained by the fluorometric method.

As seen in the calibration curve, the sensitivity of the method was found to be 5-200 µM, which is an adequate range for the diagnosis of carnitine deficiency.

In recovery studies, 104% and 92% recoveries were found for the free carnitine measurement and 87% and 91% for total carnitine measurement. As reported by Roe et al., the reason for low recovery in the measurement of total carnitine is suggested to be the binding of octanoylcarnitine to proteins and its being reserved within the supernatant during filtration (27). A second neutralization procedure can also affect the recovery in total carnitine measurement as well.

In addition, 23 samples with different concentrations of carnitine were studied using a commercial enzymatic-kinetic carnitine kit. Our results obtained by a modified fluorometric method showed that both methods were in high correlation with each other (slope=1.055, intercept=2.54, r=0.977) (Figure 2).

Our results concerning free and total carnitine levels in healthy children and adults, determined using a modified fluorometric method, are in good accordance with those found in other laboratories (15,23,24,28-31).

However, the radioenzymatic and automated method is the method of choice for carnitine measurement, and the fluorometric method can also be used because of its being applicable, easy and high precision.

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