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Serum and Tissue Carnitine Assay Based on Dialysis

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Carnitine (L-β-hydroxy-γ-trimethylaminobutyric acid) aids mitochondrial energy production by transferring fatty acids across the membranes for β-oxidation. We describe here a modified enzymatic assay for free serum and tissue carnitine based on dialysis to remove interfering substances in the serum, with subsequent conversion of carnitine to the acyl derivative by carnitine acetyltransferase (EC 2.3.1.7) in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid). The method compared well with a radioenzymatic assay. The reference interval for serum is 28–70 μmol/L. Patients with advanced diabetes and those undergoing valproic acid treatment displayed lower mean values; a statistically significant number of them showed serum carnitine values below the reference interval. The method was also applied to carnitine measurement in cerebrospinal fluid and human tissues.

Additional Keyphrases: *valproic acid · diabetes · enzymatic assays*

Carnitine is an essential carrier compound abundant in muscle and liver that transports long-chain fatty acids across the mitochondrial membrane to aid in β-oxidation, i.e., energy production (*1*) and detoxification of excess organic acids. In addition to primary carnitine deficiency, several conditions such as organic acidurias, chronic hemodialysis, and administration of high doses of valproic acid (*2*) may be associated with low serum concentrations of this compound. Low tissue carnitine concentrations lead to intramuscular lipid accumulation and muscular weakness (*2, 3*). These symptoms can be corrected by dietary L-carnitine supplementation (*2*).

Several spectrophotometric methods that use the enzyme carnitine acetyltransferase (CAT) (EC 2.3.1.7) have been described for the assay of serum carnitine (*4*). These assays differ mainly in respect to removal of the sulfhydryl-containing compounds, principally proteins, which interfere in the reaction. Some methods remove proteins by acid deproteinization (*4–6*), filtration (*7, 8*), alcohol precipitation (*9*), or heat denaturation (*10*). All of these methods suffer from certain disadvantages. For example, filtration is expensive, requires long centrifugation time, yields very small volumes of filtrate, and does not remove peptides. Acid deproteinization requires neutralization of the supernatant fluid and results in a high concentration of salts, which slows the reaction. Heat denaturation produces a minute volume of sample and does not remove small peptides. In general, these methods do not allow the use of large sample volumes in the assay to obtain good precision, especially for the low concentrations. Though time consuming and expensive, radioisotopic assays (*11, 12*) can precisely determine low concentrations, and for this reason are often used.

Here we describe an enzymatic method that has adequate sensitivity to measure low concentrations of carnitine in serum and tissues. The method removes proteins by dialysis. The small pores of the dialysis membrane also exclude many other interfering substances such as peptides, coenzyme A, lipids, and bilirubin. Carnitine in the dialysate is conjugated enzymatically into an acyl derivative and the free coenzyme A is reacted with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). Because this method does not require pH adjustment, it is easier to perform, and it lacks the undesirable aspects of other methods. We used it to measure serum carnitine in patients having advanced diabetes and those being treated with valproic acid.

Materials and Methods

Reagents

HEPES buffer, hydroxyethylpiperazine ethanesulfonic acid (50 mmol/L), was adjusted to pH 7.5. It is stable for 2 mo refrigerated. We dissolved 1.2 g/L acetyl coenzyme A (Sigma Chemical Co., St. Louis, MO) in water, aliquoted it, and kept it frozen at −18 °C. The DTNB reagent (Sigma Chemical Co.) was placed in the HEPES buffer (2.0 g/L). It is stable for 3 mo refrigerated. We dissolved stock L-carnitine standard (Sigma Chemical Co.) at 500 μmol/L in distilled water. It is stable for 3 mo refrigerated. The working carnitine standard (50 μmol/L) was diluted 10-fold in water. It is stable for 2 wk refrigerated. CAT from pigeon muscle without the preservative mercaptoethanolamine (Sigma Chemical Co. catalog number C 8757) gave the highest activity in this reaction and was added directly (2 μL), without dilution, to the reaction mixture.

Dialysis Cell

We fitted a dialysis block (1-mL chamber size) that holds five patients' samples at one time (Scienceware, Bel-Art, Pequannock, NJ) with a type C dialysis membrane, pore size 4–6 nm (Acculab, Precision Technology, Norwood, NJ).

Care of Dialysis Cell

To get reproducible results, it is important to clean and remove the proteins bound to the dialysis mem-

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[3] Nonstandard abbreviations: CAT, carnitine acetyltransferase; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); and HEPES, hydroxyethylpiperazine ethanesulfonic acid.
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branes by soaking the cell for 5 min with 1 mol/L NaOH followed by three water washes. On the basis of control assays, the membranes did not exhibit any loss in performance even after 20 trials. When a new membrane is installed, the cell is washed by the same procedure. The membranes are kept wet at all times by storing the cells with water. However, immediately before use the water must be aspirated thoroughly to prevent sample dilution.

Procedure

Serum, control, working standard, tissue homogenates, or cerebrospinal fluid (500 μL) was placed in one side of the dialysis chamber and dialyzed at 25 °C for 1 h with constant shaking against an equal volume of 50 mmol/L HEPES buffer. An aliquot sample (400 μL) of the dialysate (buffer) was removed and placed in a semimicro spectrophotometer cuvette (1 mL volume). Patient samples were assayed in duplicate. Pediatric serum samples can be diluted with an equal volume of water if necessary.

DTNB (100 μL) and acetyl coenzyme A (25 μL) were added to the cuvette and the reaction was monitored on the recorder (blank absorbance) at 410 nm, at room temperature, until there was no major change in absorbance (1 min). CAT 1 U (2 μL) was added to trigger the reaction, and the cuvette was monitored again until the reaction reached a plateau in ~4 min.

Calculation

The difference between the initial and final absorbance readings was used to calculate the carnitine concentration based on a molar absorbance of DTNB of 13.6:

$$\text{Carnitine } (\mu\text{mol/L}) = \text{absorbance difference} \times (2/13.6) \times (527/400) \times (1000/0.95)$$
$$= \text{absorbance difference} \times 203.9$$

The numbers in the above formula represent the following: 2 = distribution of carnitine between the two sides of the membrane; 527 = total volume of assay; and 400 = the aliquot volume used for assay; 0.95 = recovery. We added 10 μL of the carnitine stock standard (500 μmol/L) to some cuvettes after the reaction reached a plateau, to test for the presence of inhibitors.

Patients

Ambulatory individuals visiting an outpatient clinic but with normal chemistry profiles were used to establish the reference interval for this method.

Tissue

Human tissues from autopsy samples (20–100 mg wet weight) were homogenized in 1 mL of HEPES buffer, and centrifuged at 14 000 × *g* for 1 min. The supernatant was dialyzed and used for the assay.

Results and Discussion

Dialysis, which has been used extensively on the continuous flow analyzer, is a simple and efficient method for removing proteins. In addition, the small pores of the dialysis membrane, as tested, also exclude peptides and retard other molecules larger than carnitine, producing a clear filtrate free from hemolysis, lipemia, and bilirubin. Because the dialysate is clear, a large volume of the dialysate can be used in this assay without a high initial absorbance or side reactions, which permit a short preincubation period (Figure 1). After the addition of the CAT enzyme, the reaction is complete in <4 min. If serum is used directly without dialysis, the secondary reactions proceed for a considerable time (Figure 1).

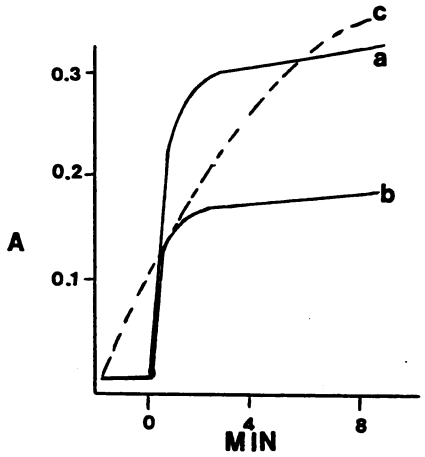

Fig. 1. Kinetics of the reaction for (*a*) standard 60 μmol/L, (*b*) dialyzed serum sample (full reaction mixture), and (*c*) undialyzed serum sample
The enzyme CAT was added at 0 time for a and b but not for c

The sensitivity of this method results from the use of a large sample volume relative to the total assay volume. Other methods' reliance on smaller sample sizes to reduce side reactions or interference results in a decrease in the assay sensitivity and precision. On the other hand, sample deproteinization with acids requires careful sample neutralization, with the formation of salts, which slows the reaction rate of the enzymatic CAT step (*5, 6*).

To detect the presence of inhibitors, at the end of the reaction we added 10 μL of the stock standard to all cuvettes containing patient samples having low carnitine values. The reaction was monitored for another 4 min. The average recovery of the added standard (n = 22 samples) was 96% (range 88–105%), indicating the absence of any major inhibitors.

Figure 2 displays the effect of dialysis time on the absorbance change for a 60 μmol/L standard and a serum pool. After 1 h the dialysis reaches ~97% of its concentration at equilibrium. The dialysis time will depend on the cell configuration and the degree of shaking. Thus, each laboratory should determine the optimum time for the cell used. The average recovery of carnitine standards (30 μmol/L) added to five pools of serum of low carnitine concentration is 95% (range 90–97%). Using aqueous standards, the reaction is linear from 2 to 80 μmol/L with the linearity line passing through the zero point. The between-run CV was 6.3% (n = 23, mean = 42 μmol/L) and the within-run CV was

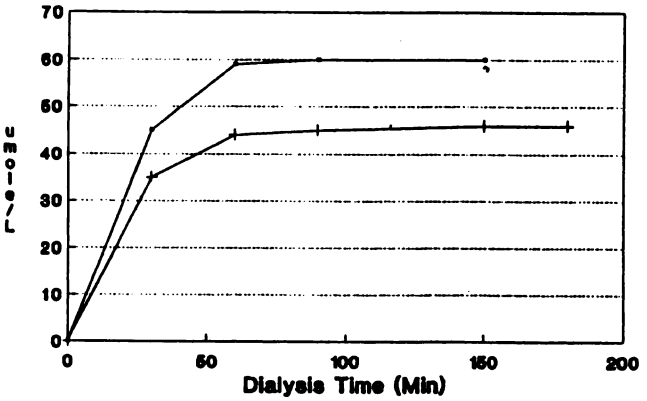

Fig. 2. Effect of dialysis time (min) on the absorbance for a patient's serum sample (+) and a standard (■; 60 μmol/L)

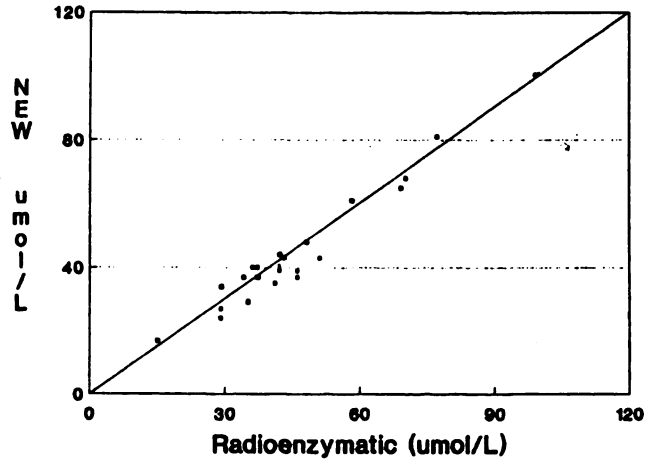

Fig. 3. Comparison of the dialysis method (new) with a radioenzymatic assay (*12*)
New method: 0.98 x − 0.77; r = 0.96

4.0% (n = 16, mean = 38). The use of duplicate assays improves the precision further by a factor of 1.4. This is very important in the case of low values, which can be verified also by adding 10 μL of the stock standard to the cuvette as described earlier. Comparison of this method with a radioenzymatic assay (*12*) of 22 samples is illustrated in Figure 3 (new method = 0.98 x − 0.77; r = 0.96, P = 0.0001, $S_{y|x}$ = 4.41).

The mean serum carnitine concentration for the ambulatory individuals is 43 μmol/L (Table 1). Based on a nonparametric statistical method (*13*), we determined the reference interval to be 28–70 μmol/L, close to the value described by others (*7, 8, 10, 11*).

We investigated two groups of patients who may have low carnitine concentrations: valproate-treated individuals and patients with advanced diabetes (Table 1). Both groups contain a statistically significant number of individuals with carnitine values below the reference interval (21.6% and 24.5%, respectively). The first group has a low mean for serum carnitine of 36 μmol/L (Table 1), as reported previously (*2, 14, 15*). The low concentrations encountered by this group result from conjugation of carnitine with valproic acid to form acyl derivatives of carnitine. Patients with serum carnitine values <28 μmol/L had a higher mean value for valproic acid (86 vs 62 mg/L). A moderate negative correlation exists between carnitine and valproic acid serum concentrations in serum (r = −0.41, P = 0.02, n = 33).

Serum carnitine concentrations in diabetes have not been well investigated. Because uncontrolled diabetic patients metabolize more fatty acids and produce more organic acids than do controlled diabetic patients, we expected that they would have lower carnitine concentrations than healthy individuals because of the excretion of these acids as carnitine acyl derivatives, as in patients on valproic acid. Diabetic patients with glycated hemoglobin (by affinity chromatography) >15% had a mean serum carnitine of 34 μmol/L; many individuals had concentrations below the reference interval (Table 1). Administration of carnitine to diabetic rats reduced hyperglycemia and decreased serum triglycerides (*16*). We do not know if carnitine supplementation might also help decrease elevated triglycerides in human diabetic patients.

Some tissues, such as those of the liver, contain high concentrations of coenzyme A (*17*) and glutathione, which may interfere with the colorimetric assays. Because of the high molecular weight of these two compounds compared with carnitine, they dialyze at a slower rate (0.15% and 25% after 1 h, respectively). The reaction of these two compounds with DTNB is very rapid, so the small amounts that dialyze do not interfere with the test. This method was applied to carnitine measurement in some human tissues (Table 2). Skeletal muscle contained the highest amounts of carnitine

Table 1. Carnitine Concentrations (μmol/L) in Serum and Cerebrospinal Fluid

Group	n	Mean	Range	Values <28 μmol/L			Reported range	Ref.
				%	χ^2	P^a		
Serum								
Valproate	74	36	8–67	21.6	13.6	0.0002		
Diabetic	52	34	6–57	24.5	14.8	0.0001		
Ambulatory[b]	102	43	26–79	2.5			25–74	8
							27–64	7
							30–72	11
							29–66	10
Cerebrospinal fluid	12	4	2–9					

[a] Compared with the ambulatory group.
[b] With a routine chemistry profile within the reference interval.

Table 2. Carnitine Concentrations (μmol/g Wet Weight) in Some Human Tissues

Tissue	n	Mean	SD	Reported range/SD	Ref.
Liver	7	0.85	0.35	0.66 ± 1.9	3
Brain, cortex	7	0.09	0.02		
Heart	7	0.74	0.21		
Kidney, cortex	7	0.71	0.29		
Skeletal muscle	9	2.72	1.03	1.99 ± 0.68	3
				1.2–1.7	18

among the tissues we analyzed, values close to those reported in the literature. It is interesting to note that despite the small size of the carnitine molecule, its concentration in cerebrospinal fluid is much lower than that in serum (Table 1). The brain cortex also contains a small amount of carnitine, probably reflecting the use of glucose for energy rather than fatty acids. The clinical significance and physiological role of carnitine in spinal fluid have not been explored.

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