An Improved Radioisotopic Assay for the Determination of Free and Esterified Carnitine in Serum and Urine

SERUM VE İDRARDA SERBEST VE ESTERİFİYE KARNİTİNİN BELİRLENMESİNDE YENİ BİR RADYOİZOTOPİK METOD

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SUMMARY

An improved radioisotopic assay adapted to the determination of L-carnitine and acylcarnitines in biological samples is described. The applicability of the assay to both normal and pathological conditions due to carnitine deficiency is discussed.

The use of N-ethylmaleimide as trapping agent has been shown to have no inhibition on carnitine acetyltransferase and the kinetic constant has been calculated (K_m=28). The superiority of this compound over other trapping agents has been discussed. Several modifications have been assayed and optimum recovery rates (99.7-100.1%) were obtained with: a) N-ethylmaleimide used in a much lower concentration than other methods, b) a mixture of ^{14}C acetly CoA and non-labeled acetyl CoA in a ratio of 21 to 5.6 μg, c) Carnitine acetyltransferase used in lower activitiy, d) Herpes buffer at a lower pH, e) The introduction of a correction factor for the calculation of total carnitine, f) a standard solution of L-acetyl carnitine run through the extraction procedure, g) 3 times extraction on ice following Dowex addition. The assay was applied to serum and urine samples of 15 healthy children and of 7 children with organic acidemias and hypotonia. The within assay coefficient of variation (CV%) was 1.3±0.15 and the within day CV% was 1.9+0.2. The possibility of accurately quantifying a carnitine content as low as 50 picomoles with high recovery and precision without any interference from acetylcarnitines, makes this assay eminently suitable for the study of patients with abnormal carnitine metabolism and excretion.

Key Words: Carnitine acetyltransferase, Acylcarnitines, Organic acidemias, N-Ethylmaleimide

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ÖZET

Biyolojik örneklerde L-karnitin ve açilkarnitinlerin tesbiti amacıyla geliştirilmiş yeni bir metod bu yazıda tarif edildi ve bu metodun karnitin eksikliğine bağlı normal ve patolojik durumlarda uygulanılması tartışıldı.

Trapping madde olarak N-etilmaleomid kullanımının karnitin asetiltransferazı inhibe etmediği gösterildi ve kinetik sabitesi (Km=28) hesaplandı. Bu bileşiğin diğerlerine olan üstünlüğü tartışıldı. Bu çalışmada çeşitli modifikasyonlar değerlendirildi ve optimum recovery rate (99.7-10.1%). Şu durumlarda elde edildi. a) N-etilmaleimid diğer metodlardan daha düşük konsantrasyonda kullanıldı, b) İşaretli ve işaretsiz asetil-CoA oranı 21:5.6 olan karşım hazırlandı, c) karnitin asetil transferaz aktivitesi daha düşüktü, d) Mepes tampon pH değeri düşüktü, e) total karnitin hesaplamasında düzeltme faktörü katıldı, f) standart L-asetil karnitin solusyonu ekstraksiyon işleminden geçirildi, g) Dowex ilavesinden sonra buzda 3 kez ekstraksiyon yapıldı. Bu metod 15 sağlıklı ve organik asidemi veya hipotonisi olan 7 çocuğun serum ve idrar örneklerine uygulandı. Bu metodla 50 pikomol kadar düşük karnitinin güvenle asetilkarnitinle interferans gösermeden tesbit edilebilmektedir. Bu nedenle tarif edilen metod anormal karnitin metabolizması ve itrahı gösteren hastalarda kullanılabilecek uygun bir metoddur.

Anahtar Kelimeler: Karnitin asetiltransferaz, Açil karnitinler, Organik asidemiler, N-etilmaleimid

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Carnitine (β-hydroxy-y-trimethylaminobutyric acid) is a carrier with an essential role in the transport of fatty acids into mitochondria, and hence in β-oxidation and energy balance (1). Intracellular free CoA and acetyl-CoA concentrations are regulated by carnitine acetyltransferase (CAT) (EC 2.3.1.7) via reversible formation of acetylcarnitine (1). Carnitine has also a detoxifying role in trapping excessive acyl-CoA metabolites and converting them into acylcarnitines which are excreted in the urine (2).

The discovery of carnitine deficiency states due to primary defects in carnitine transport (3,4) or secondary to inherited metabolic disorders like organic acidemias and β-oxidation defects (5,6) or to valproic acid therapy (7), has led the scientists to develop various techniques for physiologic carnitine measurement (8-13). Spectrophotometric methods using acid deproteinization (14,15), dialysis (16) or ultrafiltration (17), present same disadvantages, furthermore they are tedious and time

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consuming and are relatively insensitive to low concentrations. Radioisotopic assays (9,12,18-20) are selectively used because they are suitable to the accurate detection of picomole quantities of carnitine in physiological fluids.

Here we describe an alternative modification of the previously described radioisopotic methods (9,12). We report serum and urine carnitine concentrations measured by this technique in healthy children and in pediatric patients with organic acidemias and hypotonia.

MATERIALS AND METHODS

The principle of the assay is based on the following reaction conducted by CAT:

L-carnitine+Acetyl-CoA ⇄ Acetyl-L-carnitine+(CoASH)

The equilibrium of the reactions is deplaced to the right by the capture of Coenzyme A (CoASH= by N-ethylmaleimide (NEM). We use ^{14}C-acetyl CoA, and the acetyl-^{14}C-carnitine formed is seperated from acetyl-CoA by the use of an anion-exchange resin. For the determination of total carnitine, the acylated esters of carnitine are hydrolysed; free carnitine is measured together with carnitine liberated from its esters.

Sources of chemicals were as follows: Acetyl-DL-carnitine and L-carnitine HCl from Sigma; (Acetyl-L-C)-acetyl CoA (specific activity 51.9 mCiper mmole), Carnitine acetyltransferase (CAT) and Acetyl-CoA (trilithium salt) from Boehringer; N-ethylmaleimide (NEM), Hepes and Dowex 2x8 anion-exchange resin in its chloride from (200-400 mesh) from Merck; Aquasol-2 from Dupont New England Nuclear. Standart solutions of Acetyl-DL-carnitine (20 μmol/L) and L-carnitine (0.5, 1.25, 2.5, 5, 7.5, 10, and 12.5 μmol/L) were prepared. The anion exchange resin (Dowex) was prepared as fallows: 200 g of the resin was stirred (without use of magnetic bar) with 400 ml H_2O and 600 ml HCl (2 mol/4) and let to stand an overnight. The mixture was filtered and rinced with demineralized water until the pH of the rincage solution became neutral. The resin was then dried and kept at 4°C. The Dowex was suspensed in 3 volumes of bidistilled H_2O before the day of analysis, mixed and conserved at 4°C.

The experimental procedure involved mainly 2 steps;

a) Hydrolysis of carnitine esters (Total carnitine): After the addition of 100 μl of KOH (0.6 N) to 500 μl of the sample, the standard of acetyl-DL-carnitine (20 μmol/L), and the standard blank, the mixtures were vortexed and incubated 60 min at 56°C. 70 μl of HCl (0.5 N) and Hepes tampon solution (2.5 mol/L, pH=6.7) were then added, vortexed and put on ice. The pH was controlled in order to be 6.7. A factor of correction was introduced for the calculation of the concentration of free carnitine from the standard curve. 100 μl of the hydrolysate was pipetted into Eppendorf tubes and proceeded as follows with free carnitine.

b) Determination of free and total carnitine: To 100 μl of the sample, carnitine standarts, standart blank and the hydrolysates of total carnitine was added 100 μl of an ingredient mix (MIX) containing: (^{14}C) acetyl-CoA (26 nmoles, 1.4 μCi), acetyl-CoA (6.8 nmoles) in cold H_2O; NEM (16 nmoles); and Hepes buffer (23 μmoles, pH=6.7). The MIX was made immediately before use and stored on ice reactions were initiated by addition of 20 μl of a carnitine acetyltransferase (CAT= solution (0.8 U), diluted 10 fold with bidistilled H_2O just prior to use. The mixture was vortexed and allowed to stand for 30 min at room temperature. At this time, 300 ml of Dowex 2x8 anion exchange resin was added, vortexed 15 to 20 sec. and placed on ice for 5 min. This step was repeated three times, after which the tubes were centrifuged at 4°C for 10 min at 10.000 rpm. 300 μl of the supernatant fluid was mixed with Aquasol-2 scintillation fluid and the vials were counted in the β-counter TRI CARB 2500 TR. Quench correction was set up automatically (Quench indicator: tSIE/AEC, Quench Set: ^{14}C). The quantity of (^{14}C) acetyl-carnitine was stoichiometrically related to the amount of carnitine present.

All determinations were made in duplicate. The method was applied to the determination of carnitine in plasma and urine.

RESULTS AND DISCUSSION

Various trapping agents have been used both in spectrophotometric and radioisotopic assays. 5.5'-dithiobis-2-nitrobenzoic acid (DTNB) (8,14,16,17), tetrathionate (12,18), oxidized glutathione (GSSG) and 4.4'-dithiopyridine have been reported to result in non-linear standard curves (13). In the conditions of the technique described in this report, optimum linearity of different concentrations of carnitine versus the counts of desintegration per minute (DPM) and a perfect stoichiometry for the conversion of carnitine to acetyl carnitine was observed (r>0.997) with NEM used in a much lower concentration than that of other studies (13,19,20) (Figure 1). Analytical recovery of L-carnitine and acylcarnitine added

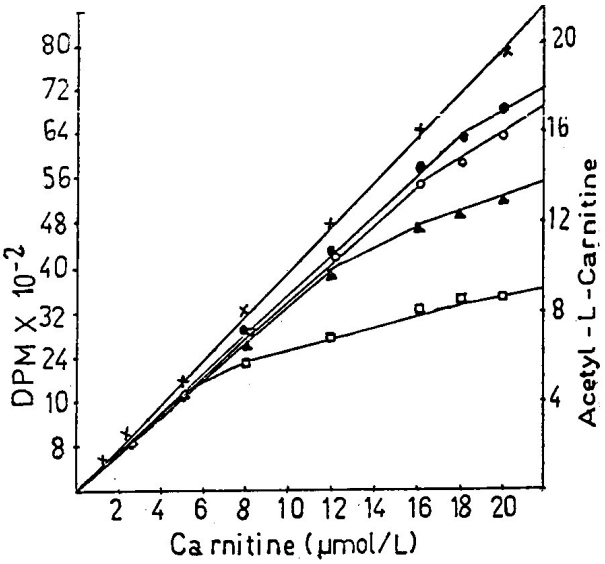

Figure 1. Effect of different trapping agents on the linearity of the standard curve for L-carnitine. Without any addition (-□-□-), with 1.6 μM (-x-x), 2 mM sodium tetrathionate (-O-O-), 5 mM GSSG (-O-O-) and 0.09 mM DTNB (-s-s-). With NEM, a linear response was obtained up to 260 mmol/L L-carnitine.

Table 1. Effect of Modifications on the Recovery of Serum Free Carnitine in the Presence of added Acetyl-L-Carnitine and L-Carnitine

Addition		L-Carnitine (0.25 nmol) (DPM)	Acetyl-L-Carnitine (20 nmol) (DPM)	Found (DPM)	Expected (DPM)	Recovery (%)
	2 mM (13)	1268	650	2119	1918	110.5
NEM	4 mm (19)	1289	625	2067	1914	108.0
	10 mM (20)	1275	638	1963	1913	102.6
	72 μM (*)	1310	695	2003	2005	99.9
RL/NL	0.9 (18)	1280	682	2115	1962	107.3
Acetyl	1.8	1293	715	2168	2008	108.0
CoA	3.75 (*)	1320	700	2022	2020	100.1
	0.4	1256	636	1828	1892	96.6
CAT	0.8 (*)	1273	718	1987	1991	99.8
	0.9 1.6 (13)	1315	694	2021	2009	100.6
	7.3 (12,18,20)	1289	715	1904	2004	95.0
pH	7.5 (16)	1278	721	1871	1999	93.6
	7.6 6.7 (*)	1318	697	2009	2015	99.7

Assays were carried out as described under Materials and Methods.
Average of duplicates are given.
(*) Conditions used in this assay.
RL/NL: Radiolabelled/Non-labeled
CAT: Carnitine Acetyltransferase
DPM: Desintegration Per minute
References are given in brackets.

to the assay medium with NEM used in higher concentrations is shown in Table 1.

Kinetic analysis of CAT activity was performed and the K_m value of the enzyme for acetyl CoA was found to be 28 μmol/L, which is in agreement with the findings of other studies (13). An inhibition of enzyme activity of 40% was observed with DTNB, of 29.7% with tetrathionate, and of 9.8% with GSSG (Figure 2). Figure 2 also shows that NEM (80 mmol/L) didn't inhibit CAT activity.

The useful lower limit of the method was 0.5 mmol/L (50 pmoles) of L-carnitine. The recovery of free carnitine in the presence of acetyl carnitine was found to be 99.7-100.1% for a molar ratio of acetyl to free carnitine as high as 1/80 (Table 1).

Since the calculation of total carnitine values on the basis of the free carnitine standard curve results in underestimation of the former by an average of 20% (20), a standard of acetyl-DL-carnitine for total carnitine and L-carnitine for free carnitine determination was run through the excretion. Moreover, to calculate the concentration of total carnitine after the carnitine curve, a correction factor was introduced which varied in relation to the volume of the HCl used. The highest recovery was obtained with CAT used in lower activity (0.8 U) than in previous studies (16,18-20) (Table 1).

To overcome the interference of endogenous acetyl CoA and acylcarnitines in biological samples, radiolabeled (RL) and non-labeled (NL) acetyl-CoA must be used in concentrations adapted to the conditions of the assay. Few reports have mentioned to the use of a mixture of these compounds in their assay medium (18,19). Thus, we assayed varying proportions of RL:NL acetyl-

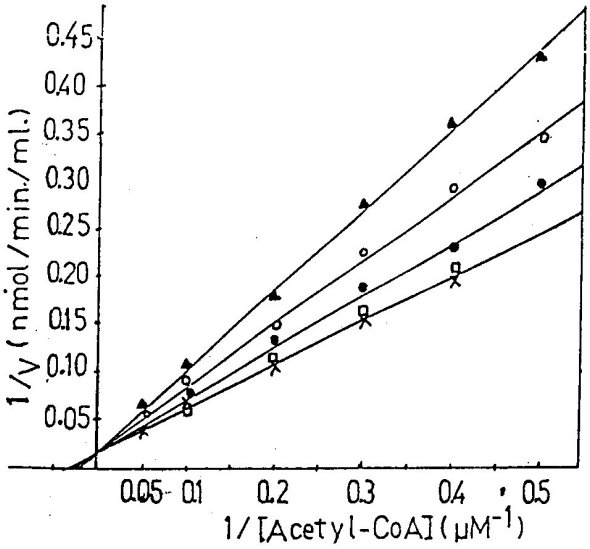

Figure 2. Lineaweaver-Buck Plots of CAT without any trapping agent (-n-n-), with 80 μM NEM (-x-x-), 6 mM sodium tetrathionate (-t-t-), 7 mM GSSG (-O-O-) and 0.4 mM DTNM (-s-s-). The reaction mixture contained, in a final volume of 0.2 m1, 1 M Hepes buffer (pH 6.7), 1.5 mM L-carnitine, 2.5 to 20 μM 14C Acetyl CoA (specific activity 51.9 nCi/nmol) and 10 ng CAT. Incubations were 2 min at 30°C. The reactions were stopped by the addition of 0.3 ml Dowex. Average of duplicates is shown.

CoA and found optimum recovery with a ratio of 21 μg: 5.6 μg (3.75) (Table 1).

We obtained higher recovery with Hepes buffer used in a lower pH than other studies (16,20) probably because the extraction with the Dowex of total acid so-

Table 2. Application of the Assay to the determination of Free Acyl and Total Carnitine in Serum and Urine of Healthy Children and of Pediatric Patients with Organic Acidemia and Hypotonia

		Serum Carnitine (μmol/L)			Urine Garnitine (mmol/g Creat.)		
Subjects	Age (months)	FC	TC	AC	FC	TC	AC
Propionic Acidemia	3	6.5	19.3	12.8	8.1	129.8	121.7
	6	5.0	16.1	11.1	12.0	165.0	153.0
Methylmalonic Acidemia	8	0.5	6.0	5.5	17.3	198.3	181.0
Methylmalonic Acidemia	4	8.0	17.8	9.8	53.5	268.5	215.0
Methylmalonic Acidemia	16	8.3	16.0	7.7	9.3	158.9	149.6
Hypotonia							
Hypotonia	22	15.1	21.0	5.9	17.6	118.6	101.0
Hypotonia	31	10.6	17.1	6.5	12.0	182.0	91.7
Hypotonia	31	10.6	17.1	6.5	12.0	182.0	91.7
Controls (n=15)	*	49.6 (1.31)	57.3 (1.97)	7.7 (0.6)	65.3 (33.1)	157.0 (51.6)	91.7 (12.3)

Values in brackets sepresent standard deviations.
*The age range of the controls was 6 to 34 months.
The "Microsta" software computer program was applied for statistical analyses (means and SD) of controls

luble carnitines (free+short chain acylcarnitines) is more efficace (Table 1).

Three times extraction on ice after the addition of the Dowex increased the sensitivity of the assay, preventing the desintegration of 14C-acetyl CoA (Table 1).

This assay was applied to serum and urine samples of 15 healthy children, 4 children with organic acidemia, and 3 children with hypotonia. Mean values were in agreement with the literature (20-22) (Table 2). Pediatric patients had significantly decreased serum free and total carnitine concentrations compared to controls. Acylcarnitine excretion was significantly increased, particularly in patients with methylmalonic acidemia.

The response of the assay was linear up to 200 μl of sample. The within assay coefficient of variation (CV%) for 10 analyses was 1.3%+0.15, and the within day CV% was 1.9±0.2. The presently described procedure offers the advantages of quantifying a L-carnitine content as low as 0.5 mmol/L (50 pmoles) in 100 ml of plasma or urine with optimum recovery and precision. No interference from acetylcarnitines is present. Thus the assay is suitable for the study of pathological states in concern with carnitine metabolism and excretion.

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