Clinica Chimica Acta, 149 (1985) 263–268
Elsevier

CCA 03204

Brief technical note

An improved method for the determination of free and esterified carnitine

Claudia Rössle [a], K.P. Kohse [b], H.-E. Franz [b] and P. Fürst [a]

[a] *Institute of Biological Chemistry and Nutrition, University of Hohenheim, Garbenstr. 30, D-7000 Stuttgart 70 and* [b] *Nephrology Section, Department of Internal Medicine I, University of Ulm, Steinhövelstr. 1, D-7900 Ulm (FRG)*

(Received November 13th, 1984; revision March 6th, 1985)

Key words: Carnitine; Acylcarnitines; Radiochemical–enzymatic assay; Normal plasma values

Introduction

With the growing interest in the role of L-carnitine (3-hydroxy-4-*N*-trimethylaminobutyric acid) in a variety of metabolic processes (for review, cf. [1,2]), there is an increased need for a reliable, specific and routinely manageable assay for the determination of this compound and its esterified derivatives in biological fluids.

The majority of available methods for determination of free carnitine is based upon a combined radiochemical–enzymatic test as described by Cederblad and Lindstedt [3] and modified subsequently by others [4–6]. Carnitine esters are determined after alkaline hydrolysis [7].

There are only few publications in which normal values of all plasma carnitine fractions are documented [8–11]. In these reports, remarkable variations, especially for long-chain acylcarnitine, are to be found. Thus, a carefully controlled methodology and adequate normal data base might be of utmost importance in evaluating the results obtained in patient material.

In the present communication, a modified, sufficiently sensitive and reproducible radiochemical–enzymatic assay for free and esterified carnitine fractions is described, and the results obtained in healthy males and females are statistically evaluated.

Materials and methods

Chemicals

The following chemicals were used: [^{14}C]acetyl–coenzyme A (spec act > 50 Ci/mol), Amersham, Buckinghamshire, UK; acetyl–coenzyme A (sodium salt), Sigma, St. Louis, MO, USA; carnitine acetyltransferase (CAT; ca. 80 U/mg), Boehringer, Mannheim, FRG. RIALUMA scintillation cocktail, Baker, Deventer, The Netherlands; ion exchange resin AG 2X8 (200–400 mesh, Cl-form), Bio-Rad,

Richmond, VA, USA; *N*-(2-hydroxyethyl)-piperazine-*N'*-ethane sulphonic acid (HEPES), SERVA, Heidelberg, FRG and sodium tetrathionate dihydrate, Fluka, Buchs, Switzerland. Crystalline L-carnitine was kindly provided by Nefro-Pharma, Bad Aibling, FRG. All other chemicals used were reagent grade and supplied by Merck, Darmstadt, FRG.

Plasma samples (heparinized) were obtained after an overnight fast from 10 females (mean age 27.5 yr, range 20–51) and 10 males (mean age 30.8 yr, range 24–47) in good health and on normal diet prior to the study. Plasma was kept at −80°C until analyzed.

Plasma sample preparation

0.5 ml plasma were deproteinized with 0.1 ml $HClO_4$ (300 g/l; PCA), or alternatively, 0.05 ml 5-sulphosalicylic acid (300 g/l; SSA). After 10 min, the samples were centrifuged at maximally $600 \times g$ in order to obtain a pellet which could be resuspended easily.

Fraction 1 (free carnitine; FC): 0.12 ml of the supernatant were mixed with 0.24 ml 0.48 mol/l HEPES/KOH (pH 7.3) and 0.12 ml 0.9 mol/l KOH (for PCA-precipitation), or with 0.36 ml 0.48 mol/l HEPES/KOH (pH 7.3) for SSA-precipitation of plasma.

Fraction 2 (total acid-soluble carnitine; TASC): 0.1 ml of the supernatant were mixed with 0.2 ml 0.9 mol/l KOH and incubated for 2 h at 37°C. Thereafter, 0.2 ml 0.48 mol/l HEPES/KOH (pH 7.3) were added and the samples are neutralized with 0.06 ml 0.1 mol/l HCl.

Fraction 3 (long-chain acylcarnitine; LCC): the pellet was washed twice by resuspension in 1 ml PCA (50 g/l) [12] and centrifuged at $600 \times g$. After careful removal of the supernatant, the pellet was suspended in 0.4 ml 1.8 mol/l KOH. Conditions for the hydrolysis were identical to those for fraction 2. 0.15 ml 0.48 mol/l HEPES/KOH (pH 7.3) were added and each sample is neutralized with an individual amount of 3 mol/l HCl.

Following centrifugation at $2{,}000 \times g$ for 10 min, 0.2-ml portions of each of the 3 fractions were used for the carnitine assay or as blank, respectively.

Preparation of the ion exchange resin

Fifty grams of AG 2X8 were stirred in 300 ml 1 mol/l HCl for 15 min, stored overnight at 4°C and washed with ca. 2 l H_2O until neutral pH of the effluent was reached. The washed resin was resuspended in water to obtain a concentration of 0.75 g resin/ml.

Carnitine assay

The following reaction mixture was used: 0.15 mol/l HEPES/KOH (pH 7.3), 2.5 mmol/l $Na_2S_4O_6$, 31 μmol/l acetyl-coenzyme A, 31 nCi/ml [^{14}C]acetyl-coenzyme A, 31 μg/ml carnitine acetyltransferase (CAT). For blanks 18 mmol/l $(NH_4)_2SO_4$, 31 μg/ml bovine serum albumin was used instead of CAT.

0.8 ml of the reaction mixture and 0.2 ml sample were incubated at room

temperature for 30 min. 0.3 ml of continuously stirred resin suspension were added. The reaction tube was vortexed immediately and repeatedly. All tubes were centrifuged at 2,000 × *g*, exactly 60 min after the reaction in the first tube was started. 0.8 ml of the supernatant were used for scintillation counting.

Aqueous standard solutions (0–0.24 mmol/l carnitine) were prepared from crystalline L-carnitine (stored over P_2O_5 in vacuo at 4°C). Portions of them were treated like the plasma samples for the determination of FC (fraction 1). The stock solutions were stored at −20°C not exceeding 6 wk.

Results and discussion

Influence of blank preparation on the reliability of the carnitine assay

In order to obtain reliable carnitine values, the preparation of appropriate blanks (i.e. assays without CAT) is essential. Differences in protein content and ionic strength of the reaction mixtures for blanks and samples, respectively, have to be considered. As shown in Table I, simple replacement of the enzyme by H_2O in the reaction mixture resulted in a significant reduction of the dpm values (by 25–35%) measured in a carnitine-free blank, i.e. a falsely low blank. This discrepancy in the recovered radioactivity could be resolved by successively mimicing the salt and protein composition of the CAT suspension. Complete recovery of the dpm values was only achieved when both ammonium sulphate and protein were added to the reaction mixture.

Furthermore, since an increased pH was found to be associated with a considerable elevation of blank values, individual neutralization of each sample was needed.

Thus, protein concentration, ionic strength and pH are factors seriously influencing the reliability of the method.

The dpm values of the blanks for LCC were consistently found to be higher than those for TASC which, in turn, exceeded those for FC. Consequently, we found an overestimation of LCC and TASC of up to 150 and 12%, respectively, when not using individual blanks for each fraction. These observations might be an explana-

TABLE I

Influence of ionic strength and protein content of the reaction mixture on the blank for the carnitine assay [a]

Reaction mixture	Radioactivity recovered in blank (%)
CAT containing	100
−CAT, + H_2O	64–74
−CAT, + $(NH_4)_2SO_4$	81–83
−CAT, + $(NH_4)_2SO_4$ + BSA [b]	93–103

[a] Carnitine-free samples were used. The radioactivity recovered in the blanks with the CAT-containing reaction mixture was set equal to 100%. For further conditions, see text.

[b] BSA, bovine serum albumin.

TABLE II

Precision and reproducibility of the carnitine assay

Carnitine fraction	Mean (μmol/l)	SD (μmol/l)	CV (%)
Intraassay ($n = 19$)			
Free (FC)	29.6	1.16	3.9
Total acid-soluble (TASC)	39.9	1.25	3.1
Long-chain acyl (LCC)	2.64	0.35	13.3
Inter-assay ($n = 10$)			
Free (FC)	29.0	1.16	4.0
Total acid-soluble (TASC)	37.0	2.06	5.6
Long-chain acyl (LCC)	1.82	0.59	32.2

tion for the markedly high LCC levels reported by Genuth and Hoppel [11].

Determination of carnitine in different plasma samples based on a common blank, prepared from a single reference plasma, might be an attractive and inexpensive approach. This procedure, however, resulted in inconsistent, highly variable values for LCC, deviating ±40% from those when using individual blanks. On the other hand, the variations in FC and TASC were negligible (< 3%). Therefore, a common blank for determinations of FC and TASC in the same set of assays can be used without loss of reliability while each plasma sample needs its individual blank for the determination of LCC.

Statistical evaluation

The detection limit of the assay corresponds to plasma concentrations of 1.5 and 0.4 μmol/l for FC and LCC, respectively. The sensitivity, assessed as mean slope of the standard curves ($n = 9$), was 63 dpm/μmol per l, 45 dpm/μmol per l, and 240 dpm/μmol per l (plasma concentration) for FC, TASC, and LCC, respectively. Standard curves were linear up to 0.24 mmol/l L-carnitine (mean r^2, 0.9994; $n = 12$). Analytical recovery in the assay was found to be 94–105%, depending on the batch of [^{14}C]acetyl–coenzyme A used.

Precision and reproducibility of the method are illustrated by Table II. With regard to precision and reproducibility of FC and TASC, our results compare favourably with previously published data [4] and may emphasize the reliability of the assay procedure as recommended in the present communication. The rather poor precision for the LCC is not an unexpected finding, keeping in mind that physiological plasma concentrations of this fraction are extremely low, being close to the detection limit. A higher sensitivity of the assay can be achieved by using a [^{14}C]acetyl–coenzyme A mixture of higher specific activity, yet the outlined conditions imply a compromise between analytical and economic needs allowing reliable determination of all carnitine fractions in normal and pathological plasma samples.

Normal values

Plasma concentrations for free carnitine and carnitine esters determined in

TABLE III

Plasma carnitine concentrations in healthy subjects (after an overnight fast). Values are given in μmol/l

Carnitine fraction	All subjects		Males		Females	
	($n = 20$)		($n = 10$)		($n = 10$)	
	Mean	SD	Mean	SD	Mean	SD
Free (FC)	42.1	9.7	46.2 [a]	11.1	38.1 [a]	6.2
Total acid-soluble (TASC)	47.8	9.9	52.1 [a]	11.6	43.5 [a]	5.7
Short-chain acyl (SCC) [b]	5.68	2.82	6.00	2.85	5.41	2.91
Long-chain acyl (LCC)	1.64	0.57	1.87 [a]	0.59	1.40 [a]	0.47
Total (TC) [c]	49.4	10.3	54.0 [a]	11.9	44.9 [a]	6.00
ratio of acyl-/ free carnitine	0.182	0.085	0.176	0.074	0.188	0.099

[a] Significant difference ($p < 0.05$) between males and females.
[b] Calculated as difference between total acid-soluble and free carnitine.
[c] Calculated as sum of free, short- and long-chain acylcarnitine.

healthy males and females are shown in Table III. The results for FC and SCC are in good agreement with previous results [8,10,13]. However, in the present study, plasma LCC levels were found to be considerably lower than those reported by Genuth and Hoppel [11] or Fuller and Hoppel [10]. The possible reason for this discrepancy has been discussed above. Among the different fractions, the highest inter-individual variation was observed for SCC followed by that of LCC. Since SCC is estimated as the difference of TASC and FC, the high variation may arise as a consequence of cumulative errors in the determination of the two fractions.

The mean values for FC, TASC, LCC and TC differ significantly between males and females when analyzed by the non-parametric U test of Mann–Whitney. These findings confirm earlier results from Cederblad [14] concerning TASC, and from Leschke et al [15] regarding FC values.

In conclusion, the optimized carnitine assay as described in the present communication represents a reliable, precise and well reproducible method, suitable for accurate determination of all carnitine fractions in plasma.

Acknowledgement

The support of Fresenius AG (Bad Homburg, FRG) is gratefully acknowledged.

References

1 Borum PR. Carnitine. Ann Rev Nutr 1983; 3: 233–259.
2 Bremer J. Carnitine – metabolism and functions. Physiol Rev 1983; 63: 1420–1480.
3 Cederblad G, Lindstedt S. A method for the determination of carnitine in the picomole range. Clin Chim Acta 1972; 37: 235–243.

4 McGarry J, Foster DW. An improved and simplified radioisotopic assay for the determination of free and esterified carnitine. J Lipid Res 1976; 17: 277–281.
5 Secombe DW, Hahn P, Novak M. The effect of diet and development on blood levels of free and esterified carnitine in the rat. Biochim Biophys Acta 1978; 528: 483–489.
6 Böhmer T, Rydning A, Solberg HE. Carnitine levels in human serum in health and disease. Clin Chim Acta 1974; 57: 55–61.
7 Brass EP, Hoppel CL. Carnitine metabolism in the fasting rat. J Biol Chem 1978; 253: 2688–2693.
8 Penn D, Schmidt-Sommerfeld E. Carnitine and carnitine esters in plasma and adipose tissue of chronic uremic patients undergoing hemodialysis. Metabolism 1984; 32: 806–809.
9 Roe CR, Millington DS, Maltby DA, Bohan TP, Hoppel CL. L-Carnitine enhances excretion of propionyl-coenzyme A as propionyl-carnitine in propionic acidemia. J Clin Invest 1984; 73: 1785–1788.
10 Fuller RK, Hoppel CL. Elevated plasma carnitine in hepatic cirrhosis. Hepatology 1983; 3: 554–558.
11 Genuth SM, Hoppel CL. Acute hormonal effects on carnitine metabolism in thin and obese subjects: responses to somatostatin, glucagon and insulin. Metabolism 1981; 30: 393–401.
12 Fishlock RC, Bieber LL, Snowsnell, AW. Sources of error in determination of carnitine and acyl-carnitine in plasma. Clin Chem 1984; 30: 316–318.
13 Savica V, Bellinghieri G, DiStefano G, et al. Plasma and muscle carnitine levels in hemodialysis patients with morphological-ultrastructural examination of muscle samples. Nephron 1983; 35: 232–236.
14 Cederblad G. Plasma carnitine and body composition. Clin Chim Acta 1976; 67: 207–212.
15 Leschke M, Rumpf KW, Eisenhauer Th, Becker K, Bock U, Scheler F. Serumspiegel und Urinausscheidung von L-Carnitin bei Patienten mit nomaler und eingeschränkter Nierenfunktion. Klin Wochenschr 1984; 62: 274–277.