A QUICK REPRODUCIBLE RADIOMETRIC ASSAY METHOD FOR FREE AND TOTAL CARNITINE IN PLASMA AND URINE

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The determination of carnitine in body matrices has proved a difficult task over the years because of the errors inherent in the various radiometric methods that have been used. In this research the potential sources of error in the routinely used radio-isotopic assays for carnitine have been recognised and eliminated by the careful assessment of each step in the process. The assay described in this paper allows for quick, reproducible and reliable determinations of free and esterified carnitine in two body matrices, viz. serum and urine.

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Carnitine (3-hydroxy-4-N-trimethylamino butanoic acid) has long been known to play an important role in fatty acid oxidation. More recently, many other functions of carnitine in intermediary metabolism have become apparent. These discoveries have led to the use of carnitine as a therapeutic supplement in a number of pathological conditions.

The total body carnitine store in a 70 kg adult is approximately 100 mmol,[1] of which 98% is in muscle, 0.6% in extracellular fluid and only 1.6% in the liver and kidneys.[2] In tissues and physiological fluids, carnitine is present in free form and in esterified form bound to short-chain and long-chain fatty acids. Acylcarnitine esters account for 9 - 42% (mean 22%) of total carnitine in serum or plasma, and about 4 - 33% (mean 13%) of total carnitine in muscle and liver of normal humans.[2] As much as 50 - 60% of total carnitine in urine may be in the form of acylcarnitines. However, these proportions may vary considerably with nutritional status, exercise and disease states.[3]

The first carnitine-deficient patient was described in 1973 by Engel and Angelini[4] and subsequent clinical investigations have demonstrated primary carnitine deficiencies, which may be life-threatening, and some deficiencies secondary to a variety of genetic defects of intermediary metabolism or other disorders.

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Decreased tissue or blood levels can result from the consumption of a diet deficient in carnitine or the various nutrients involved in its biogenesis or function. Various groups of individuals, in addition to those with the classic carnitine deficiency syndrome, may require exogenous carnitine. Carnitine may be an essential nutrient for the newborn, since a switch to lipid metabolism for energy production takes place at parturition.[5] Others include the normal pregnant woman, the normal lactating woman, and the normal adult with a diet low in lysine, methionine and the necessary micronutrients, viz. vitamins B_3, B_6, C and ferrous iron.[5] Following these discoveries it became necessary to develop an adequate means of determining the levels of carnitine and its esters in body matrices. In the past, carnitine was measured by a number of relatively insensitive biological and chemical assays. With the discovery of the carnitine acyltransferase enzymes and their use with labelled acetyl co-enzyme A, more direct and specific measurements of the concentrations of carnitine and its derivatives became possible. The methods developed invariably utilised carnitine acetyltransferase to form a radioactive acetyl derivative from labelled acetyl CoA and free carnitine, or carnitine liberated by the hydrolysis of its esters, as shown in the following reaction:

$$1\text{-}^{14}\text{C-acetyl CoA} + \text{carnitine} \overset{\text{CAT}}{\rightleftharpoons} 1\text{-}^{14}\text{C-acetylcarnitine} + \text{CoASH}$$

The resulting amount of radioactive acetylcarnitine (a β-emitter) was then determined radiometrically with the use of a scintillation counter. There was, however, considerable disparity in both the methodology and the results obtained from the various methods.

The present report describes an improved method for the determination of free and total carnitine in body matrices, which eliminates to a large extent the deficiencies of earlier methods, but retains speed and simplicity of operation.

METHODS

Materials

L-carnitine hydrochloride, palmitoyl-L-carnitine chloride, carnitine acetyltransferase from pigeon breast muscle (CAT; ca 94 U/mg), acetyl co-enzyme A (sodium salt), Dowex AG1-X8 anion exchange resin in its chloride form (200 - 400 mesh), and N-(2-hydroxyethyl)-piperizine-N'-ethane sulphonic acid (HEPES) were supplied by Sigma Chemical Company, St Louis, USA. 1-^{14}C-acetyl CoA (specific activity 50 μCi/mol) was supplied by NEN Research Products, Boston, USA and Amersham, Buckinghamshire, UK. Pico-Fluor 40 was purchased from the Weil Organisation. N-ethylmaleimide was bought from Aldrich-Chemie, Steinheim, Germany. All other chemicals used were of reagent grade and supplied by Sigma Chemical Co., St Louis, USA.

The following reagents were used: 250 mM HEPES buffer, pH 7.6; 2 mM N-ethylmaleimide (NEM), freshly made daily; 0.2

µCi/100 nmol ^{14}C acetyl CoA, stored in aliquots at –70°C; 50 mM L-carnitine standard, stored in aliquots at –70°C; 11.45 mM palmitoyl-L-carnitine standard, stored in aliquots at –70°C; carnitine acetyltransferase, diluted daily to 1:20 with distilled water; Dowex AGl-X8 resin (200 - 400 mesh), stored covered at 4°C; 1.0 M KCl in 500 mM HEPES buffer; 1.0 M KOH in 500 mM HEPES buffer; 1.0 M HCl in 500 mM HEPES buffer; Pico-Fluor 40 scintillation fluid. Serum and urine samples were either assayed immediately or kept at –20°C until analysed.

Experimental procedure

Assays were carried out in triplicate in disposable plastic tubes (13 x 100 mm). Four L-carnitine standards (1.25, 2.5, 3.75 and 5.0 nmol) and four palmitoyl-L-carnitine standards (1.25, 2.5, 3.75 and 5.0 nmol) were routinely included with each series of samples, as was a blank (50 µl H_2O), which corrects for nonspecific supernatant radioactivity.

Assay for free carnitine. The basic reaction mixture contained 600 µl 250 mM HEPES buffer, 200 µl 2 mM NEM, 100 µl labelled acetyl CoA, 100 µl 1.0 M KCl, and 50 µl of the sample to be analysed. For the blank and the standards the same reaction mixture was used with either 50 µl water or standard, respectively.

After the addition of 50 µl of freshly diluted enzyme to the tubes in order to initiate the reaction, the mixture was well mixed and allowed to stand at 37°C for 30 minutes. Following this, 1.0 ml of a continuously stirred slurry of Dowex AGl-X8 resin was added. The mixture was vortexed again and placed on ice for 20 minutes, with regular mixing at 10-minute intervals. The tubes were then centrifuged and 0.5 ml of the supernatant fluid (containing the formed 1-^{14}C acetylcarnitine) was mixed with 10 ml of scintillation fluid and assayed for radioactivity in a liquid scintillation counter. Since, in this system, the quantity of labelled acetylcarnitine formed was stoichiometrically related to the amount of carnitine present in the sample, the carnitine concentration of the sample could be read off from the standard curve obtained by plotting the radioactivity of the four standards against their respective concentrations.

Assay for total carnitine. The procedure was identical to that described above except that 50 µl of the sample was added to 50 µl 1.0 M KOH in HEPES buffer, vortexed, and allowed to stand at 56°C for 1 hour in order to hydrolyse any acylcarnitines present. This was repeated for the blank and the four palmitoyl-L-carnitine standard solutions. Following this hydrolysis procedure, the pH was adjusted to 7.3 by the addition of 50 µl 1 M HCl. Thereafter, the previously described method for free carnitine was followed, omitting the addition of KCl.

RESULTS AND DISCUSSION

The recommended definition[6] for the detection limit used in this study was: $y\text{-}y_B = 3S_B$, where y_B represents the disintegrations per minute given by a blank and S_B the standard deviation of the blank. The latter two quantities can be obtained from the regression line. y_B is then the intercept and S_B is equivalent to the standard deviation of the regression line, calculated in the usual way. The calculated detection limit is 0.2 nmol for L-carnitine (N = 20), corresponding to a concentration of 3.3 µM and 0.3 nmol for palmitoyl-L-carnitine (N = 20), corresponding to a concentration of 6.0 µM.

Typical standard curves obtained for free L-carnitine and palmitoyl-L-carnitine standards with the method described are given in Table I. Analyses carried out on human serum in this laboratory in 1993 gave values of 40.5 ± 1.3 µM and 53.2 ± 2.5 µM for free and total carnitine, respectively, in women (mean ± SD; N = 10), and 50.4 ± 2.1 µM and 67.0 ± 3.0 µM for free and total carnitine, respectively, in men (mean ± SD; N = 10).[7] Since then, numerous analyses using this technique have been performed: these now accepted normal values remain essentially unchanged.

Table I. Calibration graphs for free and total carnitine

Free carnitine standard (nmol)	1.25	2.5	3.75	5.0
Disintegrations per minute	2 187	3 785	5 351	6 935
r = 0.9999				
y = 63.64x + 582				
Total carnitine standard (nmol)	1.25	2.5	3.75	5.0
Disintegrations per minute	2 011	3 496	5 101	6 682
r = 0.9997				
y = 61.864x + 464				

Some authors extract urine samples with a 3:2 chloroform/methanol mixture while others analyse urine samples without prior extraction. Given that chloroform/methanol did not extract the carnitine (Table II), the urine samples are analysed using the method for plasma samples, without prior extraction. Twenty-four-hour urine samples are required to assess the carnitine concentration in urine, since the excretion of carnitine and its esters varies with the ingestion of carnitine during the day. Most workers calculate the carnitiine content of urine in µmol/24 hours. As 24-hour samples are difficult to collect, the carnitine concentrations in random urine samples are given as nmol carnitine/mg creatinine. Normal values for children were determined in this laboratory using the method for serum reported here (Table III).

NEM was demonstrated to be superior to sodium tetrathionate in preventing the reversal of the carnitine

Table II. The extraction of labelled acetylcarnitine into chloroform/methanol*

Water layer measured after extraction†	5 204 dpm
Organic layer measured after extraction	118 dpm
300 µl labelled acetylcarnitine without extraction	5 942 dpm

* 300 µl labelled acetylcarnitine was extracted twice with an equal amount of 3:2 chloroform/methanol mixture. The organic phases were pooled and evaporated, and the residue was dissolved in 300 µl distilled water which was then counted.
† 200 µl of the water phase was counted.
dpm = disintegrations per minute.

Table III. Urine carnitine levels in children (nmol carnitine/mg creatinine)

Age (yrs)*	Free carnitine	Acylcarnitine	Total carnitine
0 - 1	113 - 241	322 - 482	437 - 721
1 - 2	162 - 244	327 - 401	466 - 596
2 - 3	122 - 338	218 - 404	350 - 730
3 - 4	112 - 410	283 - 425	403 - 827
4 - 5	83 - 139	213 - 259	299 - 395
5 - 6	59 - 211	141 - 203	239 - 425

* $N = 10$ per age group.

acetyltransferase (CAT) catalysed acetylation of carnitine, since it removes the CoASH product of the reaction rapidly.[8] As the ratio of acetylcarnitine to free carnitine in the sample increased, Parvin and Pande[8] found an increasing overestimation of free carnitine, when using sodium tetrathionate, illustrating increased action of CAT in the reverse direction. Further, as the concentration of CAT enzyme was increased, the overestimation was exacerbated. The use of NEM allowed the accurate determination of free carnitine even when the molar ratio of acetylcarnitine to free carnitine was as high as 90:1. Their findings were confirmed by Cederblad *et al.*[9] It is also worth noting that unlike sodium tetrathionate, NEM does not inhibit the activity of the enzyme.[8]

Christiansen and Bremer[10] observed very high tissue carnitine values, especially when (trishydroxymethyl) methylglycine (Tricine) was used in the cell suspension medium. They also found that TRIS, tris(hydroxymethyl) aminoethansulphonic acid (TES), and phosphate interfered in the carnitine analysis, since these buffers can be acetylated by CAT. Evidence of this was the formation of radioactive products after incubation of these buffers on their own with and without CAT, in the presence of labelled acetyl CoA. These radioactive products were not retained on a Dowex column. Therefore, the substrate properties of these commonly used buffers can also appear as false carnitine depending on the kind of blank used. Further, a high concentration of TRIS prevented the binding of labelled acetyl CoA to the Dowex column, resulting in high blank values. The use of HEPES buffer eliminates these deficiencies since it is not acetylated under these conditions.[10]

Although McGarry and Foster[11] allowed the enzymatic reaction to proceed for 30 minutes at room temperature, a temperature of 37°C was chosen in this study as it is the temperature at which this reaction occurs in the body. Furthermore, potential errors that might occur due to variations in the ambient temperature are eliminated, ensuring more constant conditions, and therefore better comparison between the different runs. Methods other than the radio-isotopic method, such as the automated method for the determination of carnitine described by Seccombe *et al.*,[12] use a temperature of 37°C for the enzymatic reaction. The alkaline hydrolysis of carnitine esters is known to be a slow reaction. To increase the rate of reaction, the mixture was heated at 56°C for 1 hour.

The method used by McGarry and Foster[11] routinely employs 0.1 µCi/100 nmol of labelled acetyl CoA. However, these authors and others[13] state that the sensitivity of the assay can be enhanced by increasing the specific activity of the labelled acetyl CoA. This would minimise the possible interference of the assay for free carnitine by endogenous acetyl CoA which would otherwise dilute the specific activity of the labelled acetyl CoA. However, plasma and urine do not normally contain either acetyl CoA or carnitine acetyltransferase, in contrast to perchloric acid extracts of other tissues such as muscle. Should the sample contain significant amounts of either acetyl CoA or carnitine acetyltransferase, this potential source of interference would be eliminated in the assay for total carnitine, since any acetyl CoA present in the sample would be hydrolysed during the preliminary treatment with alkali.[11] Pace *et al.*[14] suggested a fourfold increase in the concentration of labelled acetyl CoA over that used for the plasma assay to eliminate interference of endogenous acetyl CoA in the sample. An alternative is to purify the sample first by passing it through an anion-exchange resin as suggested by Parvin and Pande[8] and Kerner and Bieber.[15] This, however, results in a more complicated and lengthy procedure. As radiaoctive acetyl CoA is very expensive, a compromise between analytical expediency and economic considerations can be achieved by using labelled acetyl CoA of specific activity 0.2 µCi/100 nmol. This twofold increase significantly improves the sensitivity of the method and allows the reliable determination of carnitine in serum and urine samples.

CONCLUSION

A careful analysis of each step in the analytical procedure has led to the development of an optimal assay method for carnitine, which retains advantages of precision, speed and relative simplicity.

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