



INFLUENCE OF USING HYDROCHLORIC ACID FOR MEASURING THE CONCENTRATION OF TOTAL CARNITINE IN HUMAN PLASMA BY TANDEM MASS SPECTROMETRY

INFLUENCIA DEL USO DE ÁCIDO HIDROCLÓRICO PARA MEDIR LA CONCENTRACIÓN DE LA CARNÍTINA TOTAL EN EL PLASMA HUMANO POR ESPECTROMETRÍA DE MASAS EN TANDEM

SHORT TITLE: INFLUENCE OF USING HYDROCHLORIC ACID FOR MEASURING THE CONCENTRATION OF TOTAL CARNITINE

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ABSTRACT

The present study establishes whether the pH of samples is crucial for measuring total carnitine in human plasma by tandem mass spectrometry and if it is necessary to neutralize the samples after alkaline hydrolysis of acylcarnitines. Free and total carnitine of ten plasma samples were measured by a radioenzymatic assay as a reference method, and forward there were analyzed by tandem mass spectrometry divided into two groups: treated with hydrochloric acid and without hydrochloric acid measuring each sample five times for both variables. Free and total carnitine concentrations were similar for the radioenzymatic assay and tandem mass spectrometry. There was no significant difference between the two analyzed variables (treated with hydrochloric acid and without hydrochloric acid). It could be concluded that the pH of samples is no crucial for measuring total carnitine in plasma by tandem mass spectrometry, and it is not necessary to neutralize the samples after alkaline hydrolysis of acylcarnitines, then the shorter method, without adding hydrochloric acid can be used for total carnitine measurement in plasma by tandem mass spectrometry.

Keywords: carnitine; tandem mass spectrometry; radioenzymatic assay.

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RESUMEN

El presente estudio establece, si el pH al cual se procesan las muestras, condiciona la determinación de la carnitina total en plasma, mediante el uso de espectrometría de masas en tándem; así mismo analiza, si es necesario neutralizar las muestras luego de la hidrólisis alcalina de las acilcarnitinas. Carnitina libre y total de 10 muestras de plasma fueron medidas mediante el ensayo radioenzimático, como método de referencia. Posteriormente fueron medidas cinco veces cada muestra, mediante espectrometría de masas en tándem utilizando ácido hidrocloclorhídrico y sin usar ácido hidrocloclorhídrico. Las concentraciones de carnitina libre y total, fueron similares utilizando ensayo radioenzimático y espectrometría de masas en tándem. No se encontró diferencias significativas entre las dos variables analizadas (con ácido hidrocloclorhídrico, o sin ácido hidrocloclorhídrico). Puede concluirse que el pH de las muestras no es crucial para medir carnitina total en plasma por espectrometría de masas en tandem y no es necesario neutralizar las muestras luego de la hidrólisis alcalina de las acilcarnitinas. El método más corto puede ser utilizado para tal fin.

Palabras clave: carnitine; espectrometría de masas en tandem; ensayo radioenzimático.

INTRODUCTION

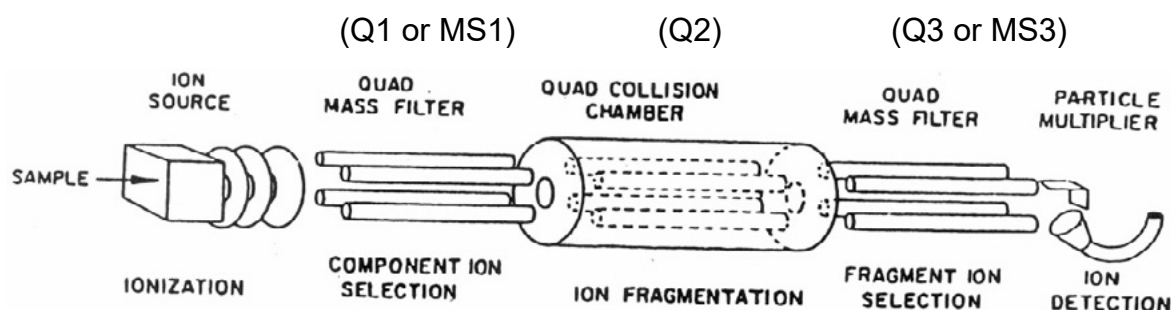
The study of inherited inborn errors is a big field that needs a basic technology for its diagnosis¹. The alterations of fatty acid mitochondrial β -oxidation an important part of the inherited inborn errors have as the main guide, the measurements of concentrations of free carnitine, total carnitine, and acylcarnitines in different tissues and fluids². All of the currently identified mitochondrial fatty acid β -oxidation defects are autosomal recessive. The clinical manifestations result from the inability of FA-oxidizing tissues to keep up with increased energy demands; therefore, target organs of FA oxidation defects include skeletal and cardiac muscles in addition to the liver³. Hypoketotic hypoglycemia is present in nearly all the defects, and usually occurs following an intercurrent illness but may occasionally be seen after a short fast. These diseases have been associated with sudden infant death syndrome, cyclic vomiting syndrome, Reye syndrome, and maternal complications of pregnancy³. Tandem mass spectrometry provides an easy way to measure acylcarnitines⁴, and new developments have been designed for the study of free and total carnitine in blood⁵ and plasma⁶ by tandem mass spectrometry. A quaternary ammonium functional

group is distinctive for carnitine and acylcarnitines, which enable them to form positive ions (cations) that are polar and non-volatile⁷. Ions produced in the source are selected by MS1 for transmission to the collision cell (figure 1). The fragments generated after collision-induced dissociation (CID) are transmitted to MS2 where they are again selected for transmission to the detector. Ions transmitted by MS1 to the collision cell are called precursor ions (commonly referred as "parent" ions), and the fragments produced from CID are product ions (known as "daughter" ions). During the derivatization process, butyl esters of acylcarnitines are formed. These butyl esters are well suited for analysis by MS/MS since they already carry a positive charge and accordingly no additives are needed in the mobile phase. Both butyl esters derivatives and underivatized carnitine and acylcarnitines share a common product ion upon CID, which is singly charged with a mass of 85 Da, corresponding to $+CH_2-CH=CH-COOH$. This fragment results from the loss of elements of both $(CH_3)_3N$ and C_4H_8 and the side chain acyl group as $RCOOH$. Selective analysis of only those molecules that produce an 85 Da product ion is accomplished using Pre ion scan mode. For acylcarnitine profiles, precursor ion scans of the peak at m/z 85 are monitored in the

range m/z 250-500. As such, they are detected as their respective molecular ions⁸. The precursor ion spectra thereby obtained shows the molecular ion species corresponding to the butylated carnitine and acylcarnitines in the sample⁹ and therefore provides information about the characteristic profile for the majority of mitochondrial fatty acid oxidation disorders,

and some organic acidurias. For the analysis of L-carnitine by MS/MS it is possible to obtain a characteristic fragment of 103 Da derived from the loss of both the trimethylamine group and the elements of C_4H_8 , and both the unlabelled and labelled forms of carnitine as butyl esters exhibit prominent molecular cations at m/z 218 and 221 respectively¹⁰.

Figure 1. Tandem mass spectrometer¹¹



The machine is divided in five parts: the ion source where the ionization takes place; the first mass analyzer (Q1 or MS1) for component ion selection; the quad (quadrupole) collision chamber (Q2) where ion fragmentation by CID is performed; the second mass analyzer (Q3 or MS2) for fragment ion selection; and the particle multiplier for ion detection.

In spite of for many laboratories around the world radioenzymatic assay is still a useful method for free and total carnitine measurement^{12,13}, tandem mass spectrometry constitutes the more precise technique for this kind of sizes; however, when total carnitine is measured by tandem mass spectrometry, total carnitine is measured after alkaline hydrolysis of acylcarnitines¹⁴. Plasma samples are neutralized by the addition of hydrochloric acid and then buffered before the analysis by tandem mass spectrometry, but these steps spend more chemicals and are time-consuming¹⁵. The present work ana-

lyzes this situation searching to save time and chemicals used during this process.

MATERIALS AND METHODS

The present study is experimental. Random blood samples from 10 adult volunteers were collected by venipuncture and centrifuged (2300g x 5min) to separate the plasma. All the samples were stored at $-80^{\circ}C$ until they were assayed. Before the analysis, all the samples were centrifuged (13000g x 2min). All the chemicals used were of analytical grade. Deuterated carnitine ($[d_3]Cn$) was obtained from Cambridge isotopes laboratories (Andover, MA, USA).

Radioenzymatic assay (REA) for the quantitative determination of plasma free and total L-carnitine¹⁶

The assay is based on the reaction catalyzed by carnitine acetyltransferase (CAT) (EC number 2.3.1.7) as follows:

L-Carnitine + [1-¹⁴C] acetyl-CoA -----
-> [1-¹⁴C] acylcarnitine + CoASH

This method measures the production of radio-labelled carnitine. For the total carnitine measurement, the plasma needed to be hydrolyzed: 100µl of plasma was mixed with 100µl of 1M KOH, centrifuged (3500g x 1min) and incubated at 45°C in shaking water-bath for 60min. 100µl of 1M HCl was added and vortexed. 100µl of 0.8M HEPES hemi-Na salt (pH 7,80) was added, the solution was vortexed and centrifuged (10000g x 1min), being ready for the analysis.

Assay procedure

All samples and control plasma (pool of stored plasma samples), as well as standard (L-carnitine 0.1 mM) were analyzed in triplicate. 50µl of plasma or 100µl of hydrolyzed plasma or 50µl of carnitine standards were placed in polystyrene (75 X 11.5mm) tube and made up to 200µl with nanopure water. Pre-mixed reaction mix was added (750µl) (Reaction mix: Buffer solution 400µl (240mM HEPES, 4mM EDTA, pH 7.3); H₂O 288µl; Tetrathionate 50µl (sodium tetrathionate 10mg/ml in nanopure water prepared on the day of the assay); Acetyl-CoA 10 µ (Acetyl-CoA (Li salt) 25mM (final concentration 25µM)); [1-¹⁴C] Acetyl-CoA 2µl ([1-¹⁴C] Acetyl-CoA about 14500 dpm/ml assay), and the tubes were kept on ice for at least 15min as the temperature of the contents of the tubes should be below 10°C before adding the CAT. The reaction was started by adding 50 µl of the CAT solution (30U/ml) to all samples, while on ice (total reaction volume 1.0 ml). The tubes were capped and centrifuged at 4°C (2579g x 1min). All of the tubes were transferred into the shaking water bath at 30°C and incubated for 30min. Then the tubes were placed on ice for 5min, and exactly 0.5 ml of a continuously stirred slurry of AG1-X8 resin (chloride form) was added. The samples were vortexed and placed on ice for

30min with occasional mixing. After centrifuging (3500g x 1min, 4°C), 800µl of the supernatant was put into a scintillation vial, and 5ml of scintillation fluid was added. The counting program for ¹⁴C was used.

Calculations

A calibration curve from the standard (L-carnitine 0.1 mM) (dpm - blank) of 20, 40, 60, 80 and 100 µmol/l carnitine was drawn. The dpm minus blank of each sample was plotted on the calibration curve, and the corresponding carnitine concentration was determined. For total carnitine, 2 multiplied the value. Acylcarnitine = Total - Free. Samples were analyzed using a Packard Liquid Scintillation Counter (model: Tri-carb 2100 TR).

Plasma sample analysis by tandem mass spectrometry

Card preparation for free and total carnitine measurement by tandem mass spectrometry. Osorio and Pourfarzam developed the method used, in 2002¹⁷. A standard internal volume of 20µl (300µM [d₃] Cn in 50% (v/v) methanol: water) was added to 180µl of plasma, and the solution was mixed before being left to stand for 15min. Aliquots of 20µl from this plasma were spotted on filter paper card, and the cards were put in the fume-cupboard for 60min to dry. These cards were used for the measurement of free carnitine concentrations. For total carnitine determination 16µl of 1m KOH was added to 100µl of the above internal standard-plasma mixture. After mixing and centrifugation (3000g x 1min) samples were incubated at 45°C in a shaking water-bath for 60min to hydrolyze the acylcarnitines. 16µl 4n hydrochloric acid was then added to the first group of samples before mixing and centrifuging again. The procedure was repeated for the second group of samples but without the addition of hydrochloric acid. Samples were

spotted on filter paper cards and dried at room temperature. Spots were punched from the card (6.35mm diameter, corresponding to 12µl of plasma) into microtiter plates and 500µl of pure methanol was added to each sample. The plates were placed on an orbital shaker for 30min and sonicated for 15min for extraction of acylcarnitines. The plates were returned to the shaker for a further 2 hours and sonicated again for another 30min. The discs were removed, and the resulting eluate was evaporated under air at 45 °C until dry and re-dissolved in 100µl of 70% (v/v) acetonitrile in water before to analysis by ESI -MS/MS. The study was performed with a scan range of 150-170 (m/z). The ratio value for carnitine/internal standard intensities was obtained and multiplied by 30µM after smoothing the peaks twice (peak width 0.80 Da- Savitzky Golay method), centering (min peak width at the half height two channels, centroid top 90%) without background subtraction. All the analyses were performed using a Quattro II, triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an ion source (ESI) and a micro mass MassLynx data system. The samples were injected into the mass spectrometer using a Jasco AS980 autosampler and a Jasco PU980 HPLC pump (Jasco International Co., Tokyo, Japan).

Statistical analysis

To determine the relationship between the two used methods a regression analysis was performed by using the Least squares method ($y = a + bx$), to find regression lines of best fit, and a correlation coefficient was calculated. The student's *t*-test was used to look for a statistical difference between the two analyzed variables.

Declaration on ethical aspects

Written consent was obtained from 10 adult volunteers from who the samples were collected.

RESULTS

Free and total carnitine concentrations were similar for radioenzymatic assay and tandem mass spectrometry ($Y=0,96X+0,45$ and $Y=0,95X-0,69$ respectively). There are no significant differences ($P>0.05$ in all comparisons) between the two analyzed variables (treated with hydrochloric acid and without hydrochloric acid). Table 1 shows the values for free and total carnitine measured by radioenzymatic assay compared to values for free carnitine using tandem mass spectrometry, as well as the values obtained for the two groups of samples treated with and without hydrochloric acid for total carnitine measurement by tandem mass spectrometry.

DISCUSSION

Radioenzymatic assay^{18,19} is regarded as the standard gold method for carnitine measurement. This procedure determines free carnitine by enzymatically converting it to radioactively labeled acetylcarnitine. Total carnitine is measured after alkaline hydrolysis of acylcarnitines. The acylcarnitine concentration is thus calculated as the difference between total and free carnitine values. The use of tandem mass spectrometry to measure carnitine in dried plasma spots is advantageous by permitting to confirm systemic carnitine deficiency after obtaining the acylcarnitine profile, and also later on to monitor the response to treatment. Carnitine contains a quaternary ammonium functional group, making them positive ions (cations) that are polar and non-volatile. The underivatized carnitine shows a common product ion, which is singly charged with a mass of 103 Da. This singly charged mass allows the use of ESI-MS/MS for the analysis of these compound²⁰. However, the methods used for measuring carnitine can be influenced by several factors when samples are obtained or even during the laboratory procedure when samples are analyzed.

When this study was commenced, an improved method for the analysis of acylcarnitine esters by ESI-MS/MS had been established in the laboratory. Different factors including the type of solvent for acylcarnitine extraction, extraction conditions, the effect of derivatization, type of derivatization, derivatization conditions (concentration of acid, temperature, time), mobile phase composition and MS/MS condition had been investigated, and optimum conditions had been established. Recent studies have shown that the temperature used during the procedure can affect the carnitine/acylcarnitine ratio^{21,22}. The conservation of samples also can influence the metabolite measurements. It is accepted that the correct quantification of free carnitine and acylcarnitines in plasma relies on rapid centrifugation of the blood after collection or storage on ice and centrifugation within one h. Before to analysis, the resulting plasma samples are kept at -20°C or -80°C for short- and long-term storage, respec-

tively. Scientific literature includes the analysis of some factors which are determinants in carnitine measurement as it has been reported for different acetonitrile concentrations during tandem mass spectrometry analysis, peaked significantly increased at an acetonitrile concentration of 90% and above^{23,24} or for the presence or not of an internal standard^{125,26} or the anticoagulant used during sampling²⁷. However, there is no any report in the scientific literature related to using or not HCl for plasma sample preparation. According to the results of the present work, it can be concluded that the pH of samples is no crucial for measuring total carnitine in plasma by tandem mass spectrometry, and it is not necessary to neutralize the samples after alkaline hydrolysis of acylcarnitines, then the shorter method, without adding hydrochloric acid can be used for total carnitine measurement in plasma by tandem mass spectrometry, saving time and materials which enables this method as more economic and easy to perform.

Table 1. Concentration of free and total carnitine in plasma samples, using two methods

Sample No.	Free carnitine	Free carnitine	Total carnitine	Total carnitine MS/MS	Total carnitine MS/MS
	REA $\mu\text{mol/L}$ (SD \pm 0,5)	MS/MS $\mu\text{mol/L}$ (SD \pm 0,3)	REA $\mu\text{mol/l}$ (SD \pm 0,4)	With HCl $\mu\text{mol/l}$ (SD \pm 0,4)	Without HCl $\mu\text{mol/l}$ (SD \pm 0,3)
1	44.8	45.2	55.1	55.3	54.9
2	33.0	32.3	41.0	40.1	40.5
3	38.5	39.0	52.1	52.0	52.5
4	48.0	47.8	52.4	53.1	53.3
5	36.0	35.8	47.9	48.5	48.9
6	35.5	35.9	43.0	42.8	42.1
7	29.9	29.3	39.9	40.0	40.8
8	37.5	38.0	48.5	49.1	49.0
9	32.0	31.2	46.0	45.6	44.2
10	47.1	48.1	68.1	67.3	65.8

DECLARATION ON CONFLICTS OF INTEREST

There are no conflicts of interest.

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INFLUENCE OF USING HYDROCHLORIC ACID FOR MEASURING THE CONCENTRATION OF
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