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# EFFECT OF ANTICOAGULANT TYPE DURING THE BLOOD SAMPLE COLLECTION PROCESS ON THE DETERMINATION OF THE ACYLCARNITINE PROFILE IN BLOOD AND PLASMA

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## EFFECTO DEL TIPO DE ANTICOAGULANTE UTILIZADO EN LA TOMA DE MUESTRAS, SOBRE LA DETERMINACIÓN DEL PERFIL DE ACILCARNITINAS EN SANGRE Y PLASMA

MS, inyección de electrospray espectrometría de masas en tándem; EIM, errores innatos del metabolismo.

**Palabras clave:** carnitina, acilcarnitinas, espectrometría de masas en tándem.

### RESUMEN

**Antecedentes:** la toma de muestras de sangre es un factor importante que puede influir los resultados finales de algunas pruebas de laboratorio. Las determinaciones de acilcarnitinas en sangre son utilizadas corrientemente para el diagnóstico y seguimiento de pacientes que sufren enfermedades metabólicas hereditarias. **Objetivo:** el presente estudio analizó la posible influencia del tipo de anticoagulante utilizado en la toma de la muestra de sangre, sobre el perfil de acilcarnitinas obtenido. **Metodología:** muestras de sangre y plasma fueron obtenidas utilizando heparina, potasio-EDTA, fluoruro de sodio-oxalato de potasio, y citrato de sodio y sin anticoagulante. **Resultados:** no fue encontrada diferencia estadísticamente significativa entre los anticoagulantes utilizados. **Conclusión:** el tipo de anticoagulante no tiene efecto sobre la determinación de acilcarnitinas en sangre o plasma. Para este tipo de análisis, puede ser utilizado cualquier anticoagulante de los antes mencionados. **Abreviaturas:** IEE-MS/

### ABSTRACT

**Background:** the collection process for blood sampling is a very important factor that can influence the final results of some laboratory tests. The acylcarnitine determinations in blood are usually used for diagnosis and follow up of patients suffering certain inherited metabolic illnesses. **Objective:** this study analyzed the possible influence of the type of anticoagulant used in blood sampling on the acylcarnitine profile. **Methodology:** blood and plasma samples were collected using anticoagulants such as: lithium heparin, potassium EDTA, sodium fluoride-potassium oxalate, and sodium citrate. Samples were also collected without the use of anticoagulants. Acylcarnitine levels were assessed for each sample via tandem mass spectrometry. **Results:** no significant differences were found among the anticoagulant used. **Conclusion:** the type of anticoagulant has no effect on the acylcarnitine determination on blood or plasma. For this type of analysis any of the above mentioned anticoagulants can be

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used. **Abbreviations:** ESI-MS/MS, electrospray injection tandem mass spectrometry; IEM, innate errors of metabolism.

**Key words:** carnitine, acylcarnitines, tandem mass spectrometry.

## INTRODUCTION

Electrospray injection tandem mass spectrometry (ESI-MS/MS) has become the mainstay for screening of IEM including amino acidopathies, defects of fatty acid oxidation, and organic acidopathies. Through the use of screening programs numerous metabolites can be detected that are relevant for the diagnosis of many IEM (1). The measurements of blood acylcarnitines by tandem mass spectrometry is currently used for the diagnosis and follow up of patients suffering from certain types of IEM. The blood sample collection is a very important factor, which can influence the final results for some laboratory tests. For the screening programs, dried blood spot cards are often prepared by applying the blood directly from the puncture site to the card. However, in many cases the blood is collected for step forward studies using different anticoagulants. The usual recommendation when sending samples for acylcarnitines analysis is to use tubes containing EDTA for samples collection, nevertheless in some situations when there are not the recommended tubes for sample collection, it can be at disposition tubes containing heparin, fluoride-oxalate, or citrate. The present study was performed in order to investigate whether the collecting of blood into a tube containing anticoagulant or the type of anticoagulant used had any effect on acylcarnitine analysis.

## MATERIALS AND METHODS

All the chemicals used were of analytical grade. Unlabelled acylcarnitine, and deuterated carnitine and acylcarnitines ( $[d_3]C_2cn$ ,  $[d_9]$

$C_2cn$ ,  $[d_3]C_3cn$ ,  $[d_3]C_8cn$ ,  $[d_9]C_8cn$ ,  $[d_3]C_{16}cn$ ,  $[d_3]C_{16}cn$ ) were obtained from Cambridge isotopes laboratories, (Andover, MA, USA). Butanolic HCL was prepared by passing HCL gas through anhydrous n-butanol (Sigma-aldrich Company, Ltd., Poole, UK) for 30 min. The concentration of the acid was determined by back tritiation and adjusted.

**Blood specimens and card preparation.** A fresh blood sample (120 ml) was taken from a volunteer and was immediately divided into 4 commercial tubes containing the following anticoagulants: lithium heparin, potassium EDTA, sodium fluoride-potassium oxalate, and sodium citrate. One sample was also placed into a commercial tube that contained no anticoagulant. Blood spot cards of plasma or serum from each tube were made. Acylcarnitine analysis of the above specimens were performed (each sample was analysed in replicates of 5). Aliquots of 20 ml were spotted on specimen collection filter paper cards (No. 903, 1.88 mm thick; Schleicher & Schuell, Dassel, Germany), dried overnight at room temperature, vacuum sealed and kept in the freezer (-80 °C) until analysis.

**Extraction of blood acylcarnitines using microtitre plates (2).** Blood spots were punched from the card in 6.35 mm diameter sections corresponding to the 12 ml of whole blood and placed into microtitre plates (96 samples each plate). 100 ml of the internal standard (containing the following labeled acylcarnitines in 100 ml methanol:  $[d_3]cn$ , 360pmol;  $[d_3]C_2cn$ , 120pmol;  $[d_3]C_3cn$ , 24pmol;  $[d_9]C_8cn$ , 12pmol;  $[d_9]C_{16}cn$ , 24pmol) were added, in addition to 500 ml of methanol to each sample. The plates were placed on an orbital shaker (setting 750

rpm) for 30 min and then sonicated for 15 min (sonic bath. 175SR). The plates were returned to the shaker for a further 2 hours and sonicated again for another 30 min. The filter discs from the card punch were removed and the resulting eluate was evaporated under air at 45°C until dry.

**Derivatization process.** 50 ml of 1 M Butanolic HCl was added to each sample and incubated at 60 °C for 15 min. Samples were immediately returned to the fume cupboard and evaporated under air at 45°C until dry and re-dissolved in 100 ml of 70% (v/v) acetonitrile in water prior to analysis by ESI-MS/MS.

**Tandem mass spectrometry analysis (3).**The MS/MS blood analysis for acylcarnitines was performed using the following scan function: parents of m/z 85, scan range 200-500 (m/z), collision energy 25 eV, cone voltage 30 V, scan time 2.0 sec., interscan time 0.1 sec, collision gas Argon, collision gas pressure  $1.6-2 \times 10^{-3}$  mBar. All analyses were performed using a Quattro II, triple quadrupole tandem mass spectrometer (Micromass, Manchester, UK) equipped with an ion spray source (ESI) and a micromass MassLynx data system. The samples were introduced into the mass spectrometer source using a Jasco AS980 autosampler and a Jasco PU980 HPLC pump.

The procedure was performed five times for each sample. Statistical comparisons were performed using one-way ANOVA (SigmaStat version 3.1 statistical software), followed by Dunnett's test.  $P < 0.05$  was considered significant. According to article 11 on its literal a. from resolution number 8430 promulgated by the health ministry for scientific, technical and administrative rules for research in health, the present study is considered without risk. The study was approved by the correspondent ethical committee.

## RESULTS

The results show that there are no significant differences ( $P > 0.05$  in all comparisons) in acylcarnitine levels when measured in different analysed specimens from tubes containing EDTA, heparin, fluoride-oxalate, or citrate as shown in Table 1. These results demonstrate that the type of anticoagulant has no significant effect on the measurement of blood or plasma acylcarnitines, and that any of the above mentioned anticoagulants can be use for collecting blood, for the analysis of acylcarnitine profile.

**Table 1.** Average values for concentration of acylcarnitines ( $\mu\text{mol/L}$ ) in whole blood, serum, and plasma, using different anticoagulants.

Anticoagulant	sample	Analyte (mM)			
		C2cn	C3cn	C16cn	C18cn
None	Whole blood	18.7	1.5	1.0	2.2
Heparin	Whole blood	19.0	1.4	0.9	2.2
EDTA	Whole blood	18.5	1.6	1.2	2.0
Fluoride-Oxalate	Whole blood	19.2	1.5	1.1	2.0
Citrate	Whole blood	19.0	1.4	1.0	2.1
None	Serum	9.4	0.7	0.2	0.8
Heparin	Plasma	9.0	0.7	0.2	0.7
EDTA	Plasma	9.3	0.6	0.2	0.8
Fluoride-Oxalate	Plasma	9.0	0.7	0.2	0.8
Citrate	Plasma	9.1	0.6	0.2	0.8

## DISCUSSION

Carnitine is an endogenous compound with well-established roles in intermediary metabolism. As an obligate carrier for optimal mitochondrial fatty acid oxidation, it is a critical source of energy and also protects the cell from acyl-CoA accretion through the generation of acylcarnitines (4). Inborn errors of metabolism in which acyl-CoA esters accumulate are associated with increased acylcarnitine concentrations in tissues and blood, and greatly increased urinary excretion of acylcarnitines. Therefore, the concentration of carnitine and these acylcarnitines is characteristic, and hence diagnostic of the metabolic disorder of interest (5).

Carnitine and acylcarnitines contain a quaternary ammonium functional group, making them positive ions (cations) that are polar and non-volatile. The butylester derivatives and underivatized carnitine and acylcarnitines share a common product ion, which is singly charged with a mass of 85 Da. This singly charged mass allows the use of ESI-MS/MS for the analysis of these compounds (6).

The methods used for measuring acylcarnitines can be influenced by several factors when samples are obtained or even during the laboratory procedure when samples are analyzed. Recent studies have shown that the temperature used during the procedure can affect the carnitine/acylcarnitine ratio (7). Other factors like hematocrit and localization of punch in dried blood spots can also modify the expected results. It has been reported that total acylcarnitines, free carnitines, and some long, medium and short chain acylcarnitines correlate positively with hematocrit levels and in samples with low hematocrit, levels of free carnitine were higher in the peripheral than in the central disk (8). Therefore both hematocrit and position of the disk within the dried blood spot also have a significant and sometimes additive effect on levels of acylcarnitines,

concluding that theoretically, diagnoses may be missed depending on hematocrit and position of the disk (8).

On the other hand, the conservation of samples also can influence the metabolite measurements. It is generally 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 1 h. Prior to analysis, the resulting plasma samples are kept at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  for short- and long-term storage, respectively (9). However, it has been reported that plasma samples from whole blood left for 3 h at  $37^{\circ}\text{C}$  and  $50^{\circ}\text{C}$  show moderate hemolysis compared with those in the  $0^{\circ}\text{C}$  and  $25^{\circ}\text{C}$  groups. Since the long-chain acylcarnitines are components of red cell membrane phospholipids (10), the erythrocyte membranes produce substantial amounts of long-chain acylcarnitines during 3 hr incubation at  $37^{\circ}\text{C}$  and  $50^{\circ}\text{C}$ , whereas no release of short-or medium-chain acylcarnitines occurs. Therefore, it is recommended that the test tubes for the analysis of plasma free carnitine and acylcarnitines be centrifuged immediately (or kept on ice for only a few hours prior to centrifugation), as this avoids miscalculation of acylcarnitines concentrations, particularly of the long-chain species (11). There is no report in the scientific literature related to the use of different anticoagulants, for the measurement of acylcarnitines in blood by tandem mass spectrometry; however the usual recommendation when sending samples for acylcarnitines analysis is to use tubes containing EDTA for samples collection. As it is shown in the present work, heparin, fluoride-oxalate, or citrate can be used as it is currently done with EDTA, and even blood without anticoagulant can be used, but in this particular case, the measurement has to be performed before the blood coagulation.

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