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ARTÍCULO ORIGINAL

Measurement of blood acylcarnitines in adult canines using tandem mass spectrometry

José Henry Osorio[1], Luis Fernando Uribe-Velásquez[2]

[1]*Laboratorio de Enfermedades Metabólicas, Departamento de Ciencias Básicas de la Salud,* *Universidad de Caldas, Calle 65 No. 26-10. Manizales, Colombia.*
[2]*Departamento de Salud Animal, Universidad de Caldas.*

josheno@yahoo.com

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ABSTRACT: There is little data in the literature regarding the reference ranges of various acylcarnitines in filter paper dried blood spots for dogs and whether these reference ranges are sex dependent. This article describes establishing such reference ranges for adult canines. Blood samples from 60 normal adult canines (30 males and 30 females) that had fasted overnight and with no evidence of an acyl-CoA metabolism disorder were obtained. Dried blood spots were prepared for acylcarnitines analysis by means of tandem mass spectrometry. No significant differences between sexes were found, a normal profile for acylcarnitines in canines was obtained. It is important to highlight the absence of hydroxyacylcarnitines, and glutaryl carnitine processing normal samples.

Key words: β-oxidation, carnitine, canines, inherited inborn errors, lipids.

Determinación de acilcarnitinas en sangre de caninos adultos mediante espectrometría de masa tándem

RESUMEN: Existen pocos datos en la literatura relacionados con los rangos de referencia de acilcarnitinas en manchas de sangre seca en papel de filtro para perros y si estos rangos de referencia dependen del género. Este artículo describe el establecimiento de dichos rangos de referencia para caninos adultos. Se tomaron muestras de sangre de 60 perros adultos normales (30 machos y 30 hembras), ayunados y que no presentaban ninguna evidencia de desordenes del metabolismo del acil-CoA. Fueron preparadas manchas de sangre seca para el análisis de acilcarnitinas utilizando espectrometría de masa en tándem. No se encontraron diferencias significativas realcionadas con el sexo, se obtuvo un perfil normal de acilcarnitinas en los caninos. Es importante resaltar la ausencia de hidroxiacilcarnitinas y glutarilcarnitina cuando se procesan muestras normales.

Palabras clave: β-oxidación, carnitina, caninos, errores hereditarios, lípidos.

Introduction

Acylcarnitine identification in body fluids using tandem mass spectrometry (MS/MS) was developed in the late 1980s (Millington et al., 1990; 1991) and represents a valuable tool for the diagnosis of some inherited metabolic diseases which are difficult to diagnose by classical chromatographic methods. The method has the potential to screen effectively for at least a dozen of several disorders (Bartlett et al., 1997; Bartlett & Pourfarzam, 1997; 1999; Green & Pollit, 1999; Levy, 1998).

Since carnitine is a vehicle by which the acyl groups can leave the mitochondria and there is equilibrium between acylcarnitines and their respective CoA thioesters, the analysis of carnitine and acylcarnitines in blood is approximately equivalent to the analysis of acyl-CoAs in the mitochondria. The concept of an acylcarnitine profile rather than a urine organic acid profile was therefore indicated as a potentially more valuable diagnostic tool for the disorders of branched-chain amino acid and fatty acid catabolism (Millington *et al.,* 1991).

The present work presents an approach for the measurement of acylcarnitines in blood of canines using electrospray tandem mass spectrometry, and provides the acylcarnitines profile for this domestic specie.

Materials and Methods

All the chemicals used were of analytical grade. Deuterated acylcarnitines ($[d_3]C_2$cn, $[d_9]C_2$cn, $[d_3]C_3$cn, $[d_3]C_8$cn, $[d_9]C_8$cn, $[d_3]C_{16}$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 for 30 min. The concentration of the acid was determined by back tritiation and adjusted. The blood samples used in this study were from 60 normal adult dogs (30 males and 30 females older than 18 months) with no evidence of an acyl-CoA metabolism disorder and fasted overnight. Blood was collected into tube containing EDTA (23.5 μmol/tube). Aliquots of 20 μl were spotted on specimen collection filter paper cards (No. 903, 1.88 mm thick; Schleicher & Schuell), dried overnight at room temperature, vacuum sealed and kept in the freezer (-80°C) until analysis. For the extraction of blood acylcarnitines using microtitre plates, blood spots were punched from the card, (6.35 mm diameter corresponding to 12 μl of whole blood) and placed into microtitre plates (96 samples each plate). 100 μl of the internal standard (containing the following labeled acylcarnitines in 100μl methanol: $[d_3]$cn, 360 pmol; $[d_3]C_2$cn, 120 pmol; $[d_3]C_3$cn, 24 pmol; $[d_9]C_8$cn, 12 pmol; $[d_9]C_{16}$cn, 24 pmol) were added, plus 500μl 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. The derivatization process was performed as follow: 50 μl 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 100μl of 70% (v/v) acetonitrile in water prior to analysis by ESI -MS/MS. 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 30V, scan time 2.0 sec, interscan time 0.1 sec, collision gas Argon, collision gas pressure 1.6-2 x 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.

Statistical analysis was carried out using Student *t*-test to search significant differences related to sex.

Results and Discussion

Some authors suggest that a plasma acylcarnitine profile should be performed in all patients presenting an acute episode of hypoketotic hypoglycemia, Reye syndrome, hypertrophic cardiomyopathy, pericardial effusion, cardiac failure or rapid unexpected death in the neonatal period or during early stages of life, also heart beat disorders during neonatal period, hypotonia with unexplained failure to thrive, retinitis pigmentosa or even muscle pain triggered by exercise (Vianey-Saban et al., 1997). The measurement of acylcarnitines using tandem mass spectrometry has been reported in whole blood (Johnson et al., 1986), plasma (Millington & Chace, 1992), urine (Libert et al., 1999), amniotic fluid (Shigmatsu et al., 1996), and bile (Rashed et al., 1995).

To date most of the symptoms and signs for clinical suspicions of alterations in intermediate metabolism have been identified. As clinical diagnosis for these disorders is very difficult, laboratory findings are used to confirm the presence of an enzymatic deficiency responsible for the metabolic alterations. Levels of intermediary metabolites in urine (glucose, ketone bodies, lactate, pyruvate), and blood (non-sterified fatty acids) (Moser & Moser, 1991); urinary organic acid profile (Greter & Jacobson, 1987); body fluids and tissue acylcarnitine analysis (Millington et al., 1994); enzyme measurement and pathway intermediates in cultured cells and leukocytes (Pourfarzam et al., 1994; Schaefer et al., 1995), and DNA analysis (Ziadeh et al., 1995) are the recommended analysis when the clinician is thinking about a metabolic disease.

Table 1. Concentration of acylcarnitines in dried blood specimen from overnight fasted dogs with no evidence of any acyl-CoA metabolism disorder.

	m/z	Mean±SD
Short-chain acylcarnitines		
Acetylcarnitine (C2)	260	8.12±8.94
Propionylcarnitine (C3)	274	1.13±1.82
Butirylcarnitine(C4)	288	0.39±0.60
Isovalerylcarnitine (C5)	302	0.27±0.41
Medium-chain acylcarnitines		
Hexanoylcarnitine (C6)	316	nd±0.01
Octanoylcarnitine (C8)	344	nd±0.01
Decanoylcarnitine (C10)	372	0.01±0.08
Dodecanoylcarnitine (C12)	400	0.17±0.39
Long-chain acylcarnitines		
Tetradecenoylcarnitine (C14:1)	426	0.06 ±0.08
Tetradecanoylcarnitine (C14)	428	0.12±0.19
Hidroxytetradecanoylcarnitine (C14:OH)	444	0.02±0.13
Hexadecenoylcarnitine (C16:1)	454	0.10±0.16
Hexadecanoylcarnitine (C16)	456	0.78±1.02
Hidroxyhexadecanoylcarnitine (C16:OH)	472	0.01±0.05
Octadecdienoylcarnitine (C18:2)	480	0.10±0.12
Octadecenoylcarnitine (C18:1)	482	0.28±0.56
Octadecanoylcarnitine (C18)	484	0.52±0.79
Hidroxyoctadecenoylcarnitine (C18:1-OH)	498	nd
Hidroxyoctadecanoylcarnitine (C18:OH)	500	nd

Abbreviations: m/z, mass/charge. nd, undetectable (< 0.002). SD, standard deviation. n=60.

Blood acylcarnitine analysis is probably the most useful single test for the differential diagnosis of these disorders with most conditions having characteristic abnormal profile, e.g. increased levels of C_4cn and C_5cn in Short-chain acyl-CoA dehydrogenase deficiency; C_6cn, C_8cn and $C_{10:1}$cn in medium-chain acyl-CoA dehydrogenase deficiency; $C_{14:1}$cn and $C_{14:2}$cn in very-long chain acyl-CoA dehydrogenase deficiency; C_{14}cn and C_{18}cn in carnitine acyl-carnitine translocase deficiency; C_{16}cn, $C_{16:1}$cn, C_{18}cn, $C_{18:1}$cn and $C_{18:2}$cn in carnitine palmitoyl transferase II deficiency; and $C_{16:OH}$cn, $C_{18:1\,OH}$cn, and $C_{18:OH}$cn in long-chain 3-hydroyacyl-CoA dehydrogenase deficiency. C10:2-acylcarnitine species, identified as 2-trans, 4-cis-C10:2, an intermediate in the degradation of linoleic acid and a substrate for 2,4-dienoyl-CoA reductase, has been found in 2,4-dienoyl-CoA reductase deficiency, and other inherited disorders show an acylcarnitine-altered profile (e.g. organic

acidurias such as glutaryl-CoA dehydrogenase, 3-hydroxy-3-methylglutaryl-CoA lyase, β-ketothiolase, Propionyl-CoA carboxylase, Methylmalonyl-CoA mutase, and Isovaleryl-CoA dehydrogenase) (Vreken et al., 1999). The present study presents reference values for acylcarnitines in canines (Table 1) and no significant differences between sexes were found, it is important to remark the absence of hydroxyacylcarnitines and glutaryl carnitine processing normal samples. For diagnostic purposes, cards containing dried blood spot samples are normally received and therefore the method using blood spots on filter paper is suitable for ESI-MS/MS analysis.

The study of the diseases related to the intermediate metabolism are widely known in human medicine, however in veterinary medicine are rare probably due to the fact of lacking trained professionals in this field.

We did not find previous reports of blood acylcarnitine reference values in dogs in the literature consulted and only some isolated cases of reports of methylmalonic and hydroxyglutaric acidurias in canines associated to neurological signs have been described, but these authors did not measured acylcarnitines in body fluids, as they based the diagnosis on the urinary organic acids profile only (Podell et al., 1996; Faunt & O'Brien, 1998; O'Brien et al., 1999).

In order to establish the possible relation between acylcarnitine levels and carnitine levels, our group provided some time ago reference values for free and total carnitine in canines but using the radio enzymatic method (Osorio & Bassols, 2002).

Other studies related to acylcarnitines in dogs have been performed but analyzing the several changes that occurred specifically in some organs like heart during some periods of hypoxia and its relationship with the acyltransferasas system without focusing on reference values (Heathers et al., 1987; Yamada et al, 1991; McHowat et al., 1993; Vogel et al., 1994).

Conclusion

It is possible to use electrospray tandem mass spectrometry for measuring acylcarnitines in blood of dogs establishing reference ranges as a valuable diagnostic tool for some inherited metabolic diseases.

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