Levels of carnitine and acylcarnitines in reconstituted red blood cell samples washed with different concentrations of saline solutions

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SUMMARY

Objective: To evaluate the percentage of carnitine and acylcarnitines remaining in red blood cells after washing them with different concentrations of saline solution.

Materials and methods: Human blood samples were centrifuged and the blood cells were washed with different saline solutions. The final pellet was resuspended in PBS for card preparation and tandem mass spectrometry analysis.

Results: It was found that carnitine, as well as short-chain, medium-chain, and long-chain acylcarnitines remain in red blood cells at average percentages of 19.3; 34; 34; and 32%, respectively. Significant differences were found for carnitine and acylcarnitine levels in blood washed with an isotonic solution compared to their levels using several hypotonic solutions ($p < 0.05$).

Conclusion: Because carnitine and acylcarnitines remained associated with the blood cells, we recommend using whole blood to measure these metabolites.

Niveles de carnitina y acilcarnitinas en muestras de glóbulos rojos reconstituidos y lavados con solución salina a diferentes concentraciones

RESUMEN

Objetivo: Evaluar el porcentaje de carnitina y acilcarnitinas que permanece en los glóbulos rojos, luego de lavarlos con solución salina a diferentes concentraciones.

Materiales y métodos: Muestras de sangre humana fueron centrifugadas y los glóbulos rojos fueron lavados con solución salina a diferentes concentraciones. El pellet obtenido, fue resuspendido en PBS para preparación de tarjetas y análisis por espectrometría de masa en *tandem*.

Resultados: La carnitina, así como las acilcarnitinas de cadena corta, cadena media y cadena larga, permanecen en los glóbulos rojos a porcentajes promedio de 19,3; 34; 34; and 32%, respectivamente. Se encontró diferencia significativa al comparar los niveles de carnitina y acilcarnitinas de sangre lavada con una solución salina isotónica *vs.* varias hipotónicas ($p < 0.05$).

Conclusión: debido a que la carnitina y las acilcarnitinas permanecen en los glóbulos rojos, se recomienda el uso de sangre entera para medir los niveles de estos metabolitos.

Carnitine and acylcarnitine identification in body fluids by using tandem mass spectrometry was developed in the late 1980s[1,2] and represents a valuable tool for the diagnosis of some inherited metabolic defects, which are difficult to diagnose by classical chromatographic method. The method has the potential of effectively screening for at least a dozen other disorders[3-7]. Some authors suggest that a plasma carnitine and acylcarnitine profile should be performed in all patients presenting acute episode of hypoketotic hypoglycemia, Reye

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syndrome, hypertrophic cardiomyopathy, pericardial effusion, cardiac failure, or rapid unexpected death during the neonatal period or during infancy; also, heart beat disorders during neonatal period, hypotonia with unexplained failure to thrive, retinitis pigmentosa, or even muscle pain triggered by exercise[8]. Measurement of acylcarnitines by using tandem mass spectrometry has been reported in whole blood[9], plasma[10], urine[11], amniotic fluid[12], and bile[13]. There has been a permanent question about the ideal fluid for carnitine and acylcarnitine measurement. This study evaluates the percentage of carnitine and acylcarnitines remaining in red blood cells, after washing with different concentrations of saline solution to establish the recommended fluid for carnitine and acylcrnitine analysis by tandem mass spectrometry.

MATERIALS AND METHODS

The current study is experimental. 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. Blood samples used in this study were from ten adult healthy volunteers who signed written consent. Blood was collected into a tube containing EDTA (23.5 μmol/tube). Aliquots of 20 ml were spotted on specimen collection filter paper cards (N° 903, 1.88-mm thick; Schleicher & Schuell, Dassel, Germany), dried overnight at room temperature, vacuum sealed, and kept in the freezer (-80ºC) until analysis.

Extraction of blood acylcarnitines using microtiter plates. Blood spots were punched from the card, (6.35-mm diameter corresponding to 12 ml of whole blood, as described (Hannon *et al.*, 1997) and placed into microtiter plates (96 samples for each plate). A total of 100 ml of the internal standard (containing the following labeled acylcarnitines in 100 ml methanol: $[d_3]cn$, 360 pmol; $[d_3]C_2cn$, 120 μmol; $[d_3]C_3cn$, 24 μmol; $[d_9]C_8cn$, 12 μmol; $[d_9]C_{16}cn$, 24 μmol) were added, plus 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 an additional 2 h 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. An amount of 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 (MS/MS) analysis. The blood analysis for acylcarnitines was performed by 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 x 10^{-3} mBar. All analyses were performed by 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 by using a Jasco AS980 autosampler and a Jasco PU980 HPLC pump. For these kinds of works, the use of selected reaction monitoring for each analyte could provide better quantification data; however, analysis for acylcarnitines using parents of m/z 85 is the routine method when analyzing samples from patients, then we adopted this scan function.

Analysis of carnitine and acylcarnitine leves in red blood cells. Blood samples (2 ml) collected with and without anticoagulant were centrifuged (2300 g x 5 min) and the serum or plasma was retained for acylcarnitine analysis. The pellet was resuspended to the volume of 2 ml in each of the following saline (NaCl) solutions: 0.9% (w/v) NaCl (*i.e.*, 0.9 g/dl); 0.75% (w/v) NaCl (*i.e.*, 0.75 g/dl); 0.65% (w/v) NaCl (*i.e.*, 0.65 g/dl); 0.55% (w/v) NaCl (*i.e.*, 0.55 g/dl); 0.5% (w/v) NaCl (*i.e.*, 0.5 g/dl); 0.45% (w/v) NaCl (*i.e.*, 0.45 g/dl); 0.4% (w/v) NaCl (*i.e.*, 0.4 g/dl); 0.3% (w/v) NaCl (*i.e.*, 0.35 g/dl); 0.3% (w/v) NaCl (*i.e.*, 0.33 g/dl); 0.3% (w/v) NaCl (*i.e.*, 0.3 g/dl); 0% (w/v) NaCl (*i.e.*, 0 g/dl). Samples were centrifuged (2,300 g x 5 min) and the procedure was repeated twice. The final pellet was resuspended in PBS for card preparation and tandem

Table 1
Concentration of carnitine and acylcarnitines in reconstituted red blood cell samples washed
with different concentrations of saline solution

	% of analyte relative to whole blood				
Saline NaCl (g/dl)	**Free carnitine**	**C_2cn**	**C_8Cn**	**C_{16}cn**	**C_{18}cn**
0.9	53.3	49.4	49	86.0	64
0.75	38.8	42.4	43.2	85.0	62
0.65	39.2	45.0	45.4	87.5	60
0.55	39.8	47.3	46.5	75.0	60
0.5	41.1	48.0	47.0	76.4	61
0.45	37.9	48.6	47.9	76.4	59.8
0.4	36.1	45.0	44.0	76.4	60
0.35	27.1	34.1	35.3	56.0	55.7
0.33	20.9	33.5	32.5	31.9	50.1
0.3	19.0	33.5	34.0	32.0	38
0	19.3	34.0	34.0	30.0	34.5

Abbreviations: C_2cn , acetylcarnitine; C_{16}cn , hexadecanoylcarnitine; C_{18}cn, octadecanoylcarnitine; ud, undetectable.

mass spectrometry analysis. All the samples were extracted and free carnitine and acylcarnitines were analysed. The procedure was performed five times for each sample. Statistical comparisons were performed by 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 Ministry of Health for scientific, technical and administrative guidelines for health research, the present study is considered without risk. The study was approved by the corresponding ethical committee.

RESULTS

The results obtained for the different saline solutions are shown in Table 1. It was found that carnitine, as well as short-chain, medium-chain and long-chain acylcarnitines remain in red blood cells at average percentages of 19.3, 34, 34, and 32%, respectively, in a complete hypotonic solution. It is evident that most acylcarnitines are associated with blood cells and this may have diagnostic implication as to whether whole blood or plasma/serum is the preferred sample for acylcarnitine analysis. It is clear that with decreasing saline concen-

tration the percentage of the remaining free carnitine and acylcarnitines in the washed cell preparation decreases, but this is probably due to the fact that at lower saline concentrations (<0.4) some degree of haemolysis occurs with the resulting leakage of carnitine species in the matrix. Significant differences were found for carnitine and acylcarnitine levels in blood washed with an isotonic solution compared to its levels using several hypotonic solutions ($p<0.05$).

DISCUSSION

It is unexpected to find carnitine and acylcarnitines remaining in RBC because of the absence of mitochondria and because they are energetically independent of fatty-acid oxidation, with no demand for classical carnitine-mediated fatty-acid transport. However, there have been reports of the activity of carnitine palmitoyltransferase as the essential enzyme for the physiological expression of deacylation-reacylation process, within the phospholipid fatty acid membrane of human erythrocytes[14,15]. The results, however, seem to have a far more important diagnostic implication in that although free carnitine and short-chain acylcarnitine are distributed equally between

plasma and blood cells, long-chain acylcarnitines are more associated with the latter. Serum carnitine concentrations reflect less than 0.5% of the total body carnitine pool of which 98% is represented by the muscle mass, the remaining 1.5% being distributed between the different organ systems and blood cells[16]. The contribution of red blood cell to whole blood level of carnitine increased significantly at delivery[17]. However according to Mares-Perlman *et al.*[18], carnitine content found in red blood cells represents 73.6±4% of whole-blood carnitine by human preterm neonates at birth but declined to 42.2±14.1% by day 14. This finding agrees with the percentage found in the present study, and then it is possible to postulate that after this day this percentage remains constant even to adulthood.

There is a close correlation between the plasma and muscle carnitine levels, but carnitine in red blood cells seems to represent a carnitine compartment of its own. Carnitine level in red blood cells is probably less related with fatty-acid metabolism of the mitochondrial than with cell-membrane stabilization or buffer function for Na-K-ATPase[19]. The role of the carnitine system is to maintain homeostasis in the acyl-CoA pools of the cell, keeping the acyl-CoA/CoA pool constant even under conditions of very high turnover of the acyl-CoA[20]. The enzyme carnitine palmitoyltransferase (CPT) properties and locations are consistent with this[21]. Above all, the carnitine derivatives can be moved across intracellular barriers, so the carnitine system provides a shuttle mechanism between microsomes, peroxisomes, and mitochondria for complex lipid-synthetic and breakdown pathways[22]. However, it was also demonstrated that the acyl-carnitine pool could act as a source of acyl groups, via the CoA pool, for the incorporation into lipids when energy, required to activate free fatty acids, is limited[23].

CONCLUSION

Carnitine, along with short-chain, medium-chain and long-chain acylcarnitines remain in red blood cells at average percentages of 19.3, 34, 34, and 32%, respectively, after washing with a total hypotonic solution. Although some researchers recommend using serum or plasma to measure these metabolites, we recommend using whole blood.

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