Brazilian Journal of Medical and Biological Research (2002) 35: 1265-1271
ISSN 0100-879X

Plasma free and total carnitine measured in children by tandem mass spectrometry

J.H. Osorio[1,2] and M. Pourfarzam[2]

[1]Universidad de Caldas, Manizales, Colombia
[2]Child Health Department, University of Newcastle upon Tyne, Newcastle upon Tyne, England

Abstract

Free and total carnitine quantification is important as a complementary test for the diagnosis of unusual metabolic diseases, including fatty acid degradation disorders. The present study reports a new method for the quantification of free and total carnitine in dried plasma specimens by isotope dilution electrospray tandem mass spectrometry with sample derivatization. Carnitine is determined by looking for the precursor of ions of m/z = 103 of N-butylester derivative, and the method is validated by comparison with radioenzymatic assay. We obtained an inter- and intra-day assay coefficient of variation of 4.3 and 2.3, respectively. Free and total carnitine was analyzed in 309 dried plasma spot samples from children ranging in age from newborn to 14 years using the new method, which was found to be suitable for calculating reference age-related values for free and total carnitine (less than one month: 19.3 ± 2.4 and 23.5 ± 2.9; one to twelve months: 28.8 ± 10.2 and 35.9 ± 11.4; one to seven years: 30.7 ± 10.3 and 38.1 ± 11.9; seven to 14 years: 33.7 ± 11.6, and 43.1 ± 13.8 µM, respectively). No difference was found between males and females. A significant difference was observed between neonates and the other age groups. We compare our data with reference values in the literature, most of them obtained by radioenzymatic assay. However, this method is laborious and time consuming. The electrospray tandem mass spectrometry method presented here is a reliable, rapid and automated procedure for carnitine quantitation.

Correspondence
J.H. Osorio
Instituto de Bioquímica Clínica
Mejía Lequerica, s/n
Edificio Helios III
Planta Baixa
08028 Barcelona
Spain

Received October 18, 2001
Accepted August 6, 2002

Key words
• Carnitine
• Tandem mass spectrometry

Introduction

The number of inherited metabolic diseases is increasing as a result of many reports of cases related to alterations in several metabolic pathways, thus contributing to the advancement of research and technology developed in this field. The inborn errors of metabolism are usually caused by defective enzymes or cofactors and most are of autosomal recessive inheritance. This results in the accumulation of abnormal metabolites that might be detectable in biological fluids of affected patients.

The differential diagnosis of metabolic disorders can be difficult even when more detailed biochemical analysis is performed. The main objective of such analysis is to identify the cause of the metabolic disturbance, which might be temporary or perma-

nent, and eventually arrive at a possible diagnosis by further detailed testing, which includes specific enzyme assays or DNA testing.

The determination of acylcarnitine levels by tandem mass spectrometry using dried blood spots on filter paper for the detection of defects in fatty acid oxidation, organic acidemias, and the selective screening of these disorders has been described (1-5). The altered ß-oxidation is followed by the accumulation of representative acylcarnitines depending on the type of enzymatic deficiency in the pathway. However, the production of these acylcarnitines depends on the availability of free carnitine in the mitochondria, with the possibility of undetectable alterations in acylcarnitine accumulation if the cell becomes carnitine depleted. It is necessary to know the occurrence and distribution of free carnitine and acylcarnitines in blood as an indication of unusual metabolic conditions, including fatty acid degradation disorders. Therefore, the diagnosis and management of these metabolic diseases require knowledge of both free carnitine and acylcarnitine concentrations.

Radioenzymatic assay (6) is regarded as the standard 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, but the use of a derivatization process in dried plasma spots remains somehow controversial. The present study reports a new method for the accurate and reliable measurement of free and total carnitine in plasma specimens by stable isotope dilution electrospray tandem mass spectrometry (ESI/MS/MS) using derivatized samples. Reference values for children are provided.

Material and Methods

Materials

All chemicals used were of analytical grade. L-carnitine was obtained from Sigma-Aldrich (Poole, UK). Stable isotope [$^2H_3$]-carnitine was purchased from Cambridge Isotopes Laboratories (Andover, MA, USA). During the present study, 309 blood samples from children were sent to the Genetics Biochemistry Unit Laboratory (Dr. Pourfarzam) for the investigation of metabolic disorders but subsequently showed that the children did not have such disorders.

Card preparation for free and total carnitine measurement

Random blood samples from a normal adult volunteer were collected by venipuncture and centrifuged (2300 *g* for 5 min) to separate the plasma.

An internal standard volume of 20 µl (300 µmol/l [$^2H_3$]-carnitine in 50% (v/v) methanol:water) was added to 180 µl of plasma. The solution was mixed and then left to stand for 15 min. Aliquots of 20 µl from this plasma were spread on filter paper cards (No. 903, 1.88 mm thick; Schleicher and Schuell, Dassel, Germany), which were dried in a hood for 60 min and then used for the measurement of free carnitine.

For total carnitine determination 16 µl of 2 M KOH was added to 100 µl of the above internal standard-plasma mixture. After mixing and centrifugation (3000 *g* for 1 min), samples were incubated at 45ºC in a shaking water bath for 60 min to hydrolyze the acylcarnitines. The samples were then mixed and centrifuged again and spots were prepared on filter paper.

Preparation of a plasma sample free of endogenous carnitine

Normal plasma samples contain a level of endogenous carnitine which does not allow the construction of standard curves starting with very low carnitine values (0 to 100 µmol/l). In order to obtain plasma without carnitine, normal plasma was dialyzed.

Dialysis membrane bags containing 1 ml of plasma were placed in 50 mM isotonic phosphate-buffered saline (PBS), pH 7.4, 250 ml PBS for each 1 ml sample, with gentle stirring for 24 h at 4ºC. The PBS solution was changed every 6 h.

Plasma sample analysis by tandem mass spectrometry on microtiter plates

Spots were punched from the card (6.35 mm in diameter, corresponding to 12 µl of plasma) into microtiter plates and 500 µl of pure methanol was added to each sample. The plates were placed on an orbital shaker (setting 750 rpm) for 30 min, sonicated for 15 min, returned to the shaker for a further 2 h and sonicated again for another 30 min. The filter paper discs were removed and the resulting eluate was evaporated dry in air at 45ºC, and 50 µl of 1.0 M butanolic HCl was added to each sample and incubated at 60ºC for 15 min. Samples were immediately returned to the chemical hood and evaporated dry in air at 45ºC and re-dissolved in 100 µl of 70% acetonitrile in water prior to analysis by ESI/MS/MS. The internal standard [$^2H_3$]-carnitine was used for quantification of L-carnitine using the ratio of the signals.

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 carnitine butylester passes analyzer 1, collides with nitrogen (fragmentation), and one of the typical fragments (*m/z* = 103) passes analyzer 2, which is fixed at *m/z* = 103. The detector registers it while the *m/z* that has passed analyzer 1 is recorded.

Plasma sample analysis by radioenzymatic assay

The radioenzymatic assay for analysis of plasma samples was performed as described (7).

Statistical analysis

The Student *t*-test was used to determine significant differences. A Bland-Altman plot was used to assess agreement between the two methods used for clinical measurement (8).

Linearity between concentration and scan function

To evaluate the linearity of the ion intensity ratio relative to carnitine concentration, samples of 24-h dialyzed plasma specimens were enriched with L-carnitine at concentrations of 5, 10, 20, 40, 60, 80, and 100 µmol/l, and cards for free carnitine analysis by ESI/MS/MS for the detection of parents of *m/z* 85 and *m/z* 103 patients with and without derivatization were prepared.

Results

The correlation coefficients for standard curves from 0 to 100 µmol/l for free carnitine using two scan functions with and without derivatization were all close to 1 and the best correlation was observed using parents of *m/z* 85 for derivatized samples. To expose any possible differences between them, the results of this scan function were compared to others using the *t*-test. No significant difference was found at any significance level.

Determination of a scan function to measure free and total carnitine

The accuracy of free carnitine measurement was further evaluated by analysis of 95 stored plasma specimens and by comparing the values with those obtained by radioenzymatic assay. Specimens were analyzed with and without derivatization using two scan functions (parents of *m/z* 85 and *m/z* 103 children). Some changes were detected for real samples without derivatization. It was difficult to obtain clear spectra due to high noise level and interferences from unknown peaks. These were reduced considerably after the derivatization procedure. When compared with radioenzymatic assay values a Pearson value of 0.55 was calculated for samples without derivatization and 0.89 for derivatized samples.

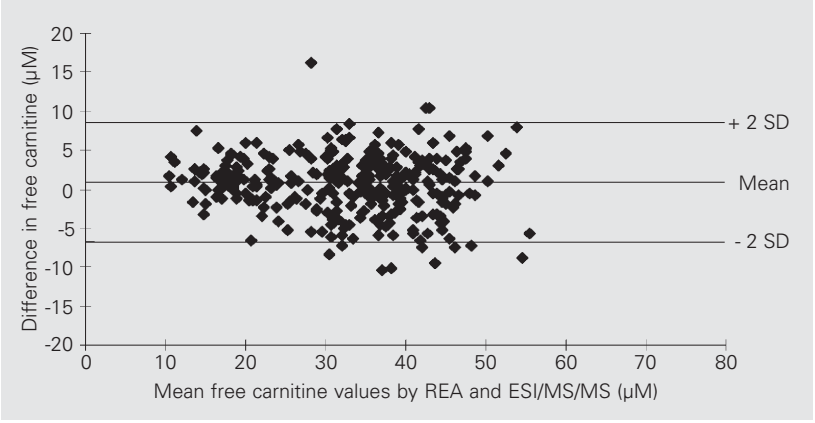

Figure 1. Bland-Altman plot of the difference, Vs, of the mean of paired values for free carnitine measurement comparing the radioenzymatic (REA) and ESI/MS/MS methods (N = 312, mean ± SD = 0.6 ± 3.7).

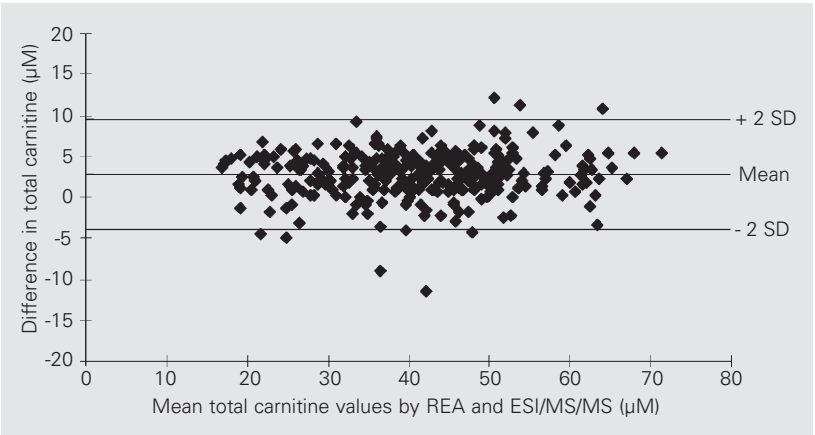

Figure 2. Bland-Altman plot of the difference, Vs, of the mean of paired values for total carnitine measurement comparing the radioenzymatic (REA) and ESI/MS/MS methods (N = 312, mean ± SD = 2.8 ± 2.8).

An analysis of 218/average of noise and 221/average of noise ratios was performed. The highest average values were found for parents of *m/z* 103 with derivatization and the lowest for parents of *m/z* 85 without derivatization. In order to further evaluate the accuracy of measuring free and total carnitine in plasma using this new method, 312 plasma samples received for routine analysis were analyzed in parallel for free and total carnitine using both the radioenzymatic method and ESI/MS/MS method with the scan function for the parent of *m/z* 103 and derivatization. The analysis of these data showed a correlation between the radioenzymatic method and ESI/MS/MS assay for free and total carnitine of $y = 0.96x + 0.45$ and $y = 0.95x - 0.69$, respectively.

The distribution of the mean versus the difference of paired values (Bland-Altman plot) is shown in Figures 1 and 2 for free and total carnitine, respectively. These data show that there is a good correlation between the two methods for both free and total carnitine over the concentration ranges studied.

An intra-day and inter-day assay for the new method was performed using a plasma sample with a free carnitine value of 41.8 µmol/l by radioenzymatic assay. The data are shown in Table 1.

Free and total carnitine in children

Table 2 shows average free and total carnitine values for four age groups. A significant difference ($P<0.01$) was found for the group of less than one month compared with the groups of one year to twelve years and one year to seven years. A significant difference ($P<0.05$) was found for the same first group compared with the group of more than seven-year-old children.

Discussion

The main function of carnitine is to shuttle activated long-chain fatty acids (fatty acyl-coenzyme A (CoA)) from the cytosol to the mitochondria matrix for ß-oxidation, and to remove from mitochondria short-chain, medium-chain and long-chain fatty acids that accumulate as a result of normal and abnormal metabolism, maintaining adequate cellular levels of free CoA. Carnitine can also interact with membranes to change their physiochemical properties (9). This means that carnitine modulates the acyl-CoA/free CoA ratio via the formation of acylcarnitines. If acyl-CoAs are produced faster than they are utilized, intramitochondrial free CoA is regenerated as carnitine binds acyl groups and the high intramitochondrial acyl-CoA/free CoA ratio is corrected (10).

Carnitine is present in tissues and body fluids in free and esterified form as short-chain and long-chain acylcarnitines. Total carnitine consists of the sum of free carnitine and all acylcarnitines. L-carnitine is synthesized in humans primarily in the liver; however, 50 to 75% of daily requirements are provided by dietary intake (10).

The importance of ESI/MS/MS for the diagnosis of inherited metabolic diseases and its utility for the confirmation of diagnostic errors have been demonstrated (11). The present study was undertaken in order to evaluate and further improve the reliability, reproducibility, sensitivity and specificity of carnitine ester analysis by electrospray tandem mass spectrometry.

Several methods for measuring L-carnitine have been described and the radioenzymatic assay is regarded as the method of choice. An MS/MS method for free and total carnitine using fast atom bombardment-tandem mass spectrometry (12) was found to be somewhat limited for application to plasma due to calibration problems with the internal standard and was recommended as ideal for urine analysis. A report using ESI/MS/MS for the determination of free carnitine and acylcarnitines in plasma and serum has been published (13). A method for plasma carnitine measurement using a stable isotope-labeled internal standard and MS/MS was published elsewhere (14). These investigators used a slightly different extraction procedure, i.e., a mixture of methanol, water and 6 M HCl (400:100:0.5, v/v/v) instead of the pure methanol used here. However, the most important difference was that their study was done without derivatization. A precursor ion scan of *m*/*z* 85 over the range *m*/*z* 160-210 was used and their new method was compared with a spectrophotometric assay based on an enzymatic reaction, showing good correlation between them (14).

As observed by our method for carnitine and the internal standard, the derivatization increased their relative molecular masses but not the impurities. Thus, key ions are removed from the region containing interfering compounds, with consequent improvement of the specificity of the analysis. The comparison between radioenzymatic assay and ESI/MS/MS by the Bland-Altman test

Table 1. Intra- and inter-day assay coefficients of plasma free carnitine determined by tandem mass spectrometry.

Assay	Carnitine (µM)				
	Min	Max	Mean	SD	CV
Intra-day	40	44.4	43.1	1.0	2.3
Inter-day	41	45	44	1.9	4.3

Min, minimum; Max, maximum; SD, standard deviation; CV, coefficient of variation. Four assays were carried out.

Table 2. Plasma reference values for free and total carnitine measured in children by tandem mass spectrometry.

Age	N	Free carnitine (µM)	Total carnitine (µM)	% Acylated
<1 month	35	19.3 ± 2.4	23.5 ± 2.9	17.9
1-12 months	43	28.8 ± 10.2	35.9 ± 11.4	19.8
1-7 years	118	30.7 ± 10.3	38.1 ± 11.9	19.4
7-14 years	113	33.7 ± 11.6	43.1 ± 13.8	21.8

Data are reported as means ± SD of the sample size shown.

looks for a significant difference rather than for a possible similarity between methods. For the newly developed method described here the difference from the radioenzymatic method was not significant and most of the values were within the acceptable limits for the test, showing good agreement for these two methods of clinical measurement.

The present results were obtained by a new reliable technique for measuring free and total carnitine in plasma as a good alternative replacing the traditional one, a fact that may be helpful for the diagnosis of carnitine alterations mainly occurring in inherited disorders of mitochondrial ß-oxidation of fatty acids.

Reference values for free carnitine and total carnitine, as well as acylcarnitines, in fluids and tissues of pediatric patients and during pregnancy have been reported previously (15-18). For free and total carnitine reference values in plasma, our ESI/MS/MS method showed a positive correlation between the plasma concentration and the age of the children. Patients younger than one month have significant lower levels of free and total carnitine. This agrees with a study (19) in which 353 samples were analyzed by the enzymatic radioisotopic method, showing a significant difference between the groups aged one month to one year compared with elderly individuals.

In several clinical conditions, the acyl/free carnitine ratio can be altered (acylcarnitine = total carnitine - free carnitine), and the present study shows values for this ratio to be within those found for healthy subjects (20). There are some differences from other reported data (Table 3) probably due to different methodologies used, different physiological characteristics of the subjects, or both. The distribution of free carnitine and carnitine esters varies according to fasting status, adiposity, renal function, and muscular exercise (21,22). The average values for free and total carnitine in the neonatal group (<1 month) do not agree with the postulate that free carnitine values <20 µmol/l and total carnitine ≤30 µmol/l determined by nonradioenzymatic methods are considered to indicate carnitine deficiency (23). However, low plasma carnitine concentrations are present in some inherited metabolic diseases, in infants fed carnitine-free soy formulas (24), in individuals receiving long-term total parenteral nutrition (25-27), valproate (28) or benzoate (29), in adults consuming carnitine-free enteral formulas, and possibly in persons consuming low-carnitine diets (30).

Table 3. Pediatric reference values for free and total carnitine (µM) in plasma and the acylcarnitine/free carnitine ratio.

Carnitine	Neonates	Infants	Children	Reference
Free	31.2 ± 2.5	36.6 ± 2.0	44.1 ± 1.7	31
Total	–	–	–	
Free	–	–	–	16
Total	22.4 ± 0.8	–	–	
Free	27.6 ± 9.7	35.5 ± 6.5	41.7 ± 7.9	19
Total	36.7 ± 10.5	47.6 ± 7.7	54.4 ± 9.9	
Acyl/Free	0.32	0.34	0.30	
Free	–	–	34.4 ± 6.3	32
Total	–	–	45.9 ± 5.6	
Acyl/Free			0.33	
Free	–	–	36.1 ± 4.5	25
Total	–	–	41.9 ± 5.2	
Acyl/Free			0.16	
Free	–	–	–	33
Total	–	15 to 39	18 to 37	
Free	–	33 ± 2.0	29.7 ± 1.0	34
Total	–	41 ± 2.0	34.8 ± 0.9	
Acyl/Free	–	0.24	0.17	
Free	20.2 ± 5.1	40.2 ± 6.1	35.7 ± 9.1	35
Total	29.4 ± 6.7	51.1 ± 9.2	45.4 ± 9.9	
Acyl/Free	0.45	0.27	0.27	
Free	19.3 ± 2.4	28.8 ± 10.2	30.7 ± 10.3	Present study
Total	23.5 ± 2.9	35.9 ± 11.4	38.1 ± 11.9	
Acyl/Free	0.21	0.24	0.24	

Data are reported as means ± SD. Neonates: <1 month; infants: 1-12 months; children: 1-7 years.

Acknowledgments

The authors would like to acknowledge Marie Appleton for valuable assistance with the laboratory work.

References

1. Millington DS, Kodo N, Norwood DL & Roe CR (1990). Tandem mass spectrometry: a new method for acylcarnitine profiling with potential for neonatal screening for inborn errors of metabolism. *Journal of Inherited Metabolic Disease*, 13: 321-324.
2. Van Hove JL, Zhang W, Kahler SG, Roe CR, Chen YT, Terada N, Chace DH, Iafolla AK, Ding JH & Millington DS (1993). Medium chain acyl-CoA dehydrogenase (MCAD) deficiency: diagnosis by acylcarnitine analysis in blood. *American Journal of Human Genetics*, 52: 958-966.
3. Rashed MS, Ozand PT, Bennet MJ, Barnard JJ, Govindaraza DR & Rinaldo P (1995). Inborn errors of metabolism diagnosed in sudden death cases by acylcarnitine analysis of postmortem bile. *Clinical Chemistry*, 41: 1109-1114.
4. Rashed MS, Bucknall MP & Little D (1997). Screening blood spots for inborn errors of metabolism by electrospray tandem mass spectrometry with a microplate batch process and a computer algorithm for automated flagging of abnormal profiles. *Clinical Chemistry*, 43: 1129-1141.
5. Chace H, Hillman SL, Vanhove JLK & Naylor EW (1997). Rapid diagnosis of MCAD deficiency: quantitative analysis of octanoylcarnitine and other acylcarnitine in newborn blood spots by tandem mass spectrometry. *Clinical Chemistry*, 43: 2106-2113.
6. Cederbland G & Lindstedt S (1972). A method for the determination of carnitine in the picomole range. *Clinica Chimica Acta*, 37: 235-243.
7. McGarry JD & Foster DW (1976). An improved and simplified radioisotopic assay for the determination of free and sterified carnitine. *Journal of Lipid Research*, 17: 277-281.
8. Bland JM & Altman DG (1986). Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet*, I: 307-310.
9. Heinoven OJ (1996). Carnitine and physical exercise. *Sports Medicine*, 22: 109-132.
10. Sewell AC & Bohles HJ (1995). Acylcarnitines in intermediary metabolism. *European Journal of Pediatrics*, 154: 871-877.
11. Osorio-Orozco JH & Pourfarzam M (2000). Diagnostic error of mental retardation of neurometabolic origin confirmed by mass sequential spectrometry. *Revista de Neurología*, 30: 728-730.
12. Kodo N, Millington DS, Norwood DL & Roe CR (1989). Quantitative assay of free and total carnitine using tandem mass spectrometry. *Clinica Chimica Acta*, 186: 383-390.
13. Vreken P, van Lint AEM, Bootsma AH, Overmars H, Wanders RJA & van Gennip AH (1999). Quantitative plasma acylcarnitine analysis using electrospray tandem mass spectrometry for the diagnosis of organic acidemias and fatty acid oxidation defects. *Journal of Inherited Metabolic Disease*, 22: 302-306.
14. Stevens RD, Hillman SL, Worthy S, Sanders D & Millington DS (2000). Assay for free and total carnitine in human plasma using tandem mass spectrometry. *Clinical Chemistry*, 46: 727-729.
15. Ingebresen OC, Strand H, Ingebrigtsen M & Hvidsen D (1998). Pediatric reference values for free and total carnitine and acylcarnitine in urine. *Clinical Chemistry*, 44 (Suppl): A14 (Abstract).
16. Shenai JP & Borum PR (1984). Tissue carnitine reserves of new-born infants. *Pediatric Research*, 18: 679-681.
17. Coude M, Chadefaux D, Rabier D & Kamoun P (1990). Early amniocentesis and fluid organic acid levels in the prenatal diagnosis of organic acidemias. *Clinica Chimica Acta*, 187: 329-332.
18. Novak M, Monkus EF, Chung D & Buch M (1981). Carnitine in perinatal metabolism of lipids. I. Relationship between maternal and fetal plasma levels of carnitine and acylcarnitines. *Pediatrics*, 67: 95-100.
19. Schmidt-Sommerfeld E, Werner D & Penn D (1989). Carnitine plasma concentrations in 353 metabolically healthy children. *European Journal of Pediatrics*, 147: 356-360.
20. Harper P, Wadström C & Cederbland G (1993). Carnitine measurements in liver, muscle tissue, and blood in normal subjects. *Clinical Chemistry*, 39: 592-599.
21. Rebouche CJ & Seim H (1998). Carnitine metabolism and its regulation in microorganisms and mammals. *Annual Review of Nutrition*, 18: 39-61.
22. Alberty R & Albertyová D (1997). Biological variation of free and total carnitine in serum of healthy subjects. *Clinical Chemistry*, 43: 2441-2443.
23. Roe DS, Terada N & Millington DS (1992). Automated analysis for free and short-chain acylcarnitine in plasma with a centrifugal analyzer. *Clinical Chemistry*, 38: 2215-2220.
24. Novak M, Wieser PB, Buch M & Mahn P (1979). Acetyl-carnitine and free carnitine in body fluids before and after birth. *Pediatric Research*, 13: 10-15.
25. Hahn P, Allardyce DB & Frohlich J (1982). Plasma carnitine levels during parenteral nutrition of adult surgical patients. *American Journal of Clinical Nutrition*, 36: 569-572.
26. Dalström KA, Ament ME, Moukarzel A, Vinton NE & Cederbland G (1990). Low blood and plasma carnitine levels in children receiving long-term parenteral nutrition. *Journal of Pediatric Gastroenterology and Nutrition*, 11: 375-379.
27. Schmidt-Sommerfeld E, Penn D, Bieber LL, Kerner J, Rossy TM & Lebenthal E (1990). Carnitine ester excretion in pediatric patients receiving parenteral nutrition. *Pediatric Research*, 28: 158-165.
28. Rebouche CJ (1998). Carnitine absorption: effects of sodium valproate and sodium octanoate in the Caco-2 cell culture model of human intestinal epithelium. *Journal of Nutrition and Biochemistry*, 9: 228-235.
29. van Hove JL, Kishani P, Muenzer J, Wenstrup RS, Summor ML, Brummond MR, Lachiewcz AM, Millington DS & Kahler SG (1995). Benzoate therapy and carnitine deficiency in non-ketotic hyperglycemia. *American Journal of Medical Genetics*, 59: 444-453.
30. Tanphaichitr V, Leerdvuthiospon N, Dhanamitta S & Brosquist HP (1980). Carnitine status of Thai adults. *American Journal of Clinical Nutrition*, 33: 876-880.
31. Battistella PA, Vergani L, Donzelli F, Rubaltelli FF & Angelini C (1980). Plasma and urine carnitine levels during development. *Pediatric Research*, 14: 1379-1381.
32. Lombard KA, Olson AL, Nelson SE & Rebouche CJ (1989). Carnitine status of lactoovovegetarians and strict vegetarian adults and children. *American Journal of Clinical Nutrition*, 50: 301-306.
33. Bonnefont JP, Specola NP & Vassault A (1990). The fasting test in pediatrics: application to the diagnosis of pathological hypo- and hyperketotic states. *European Journal of Pediatrics*, 150: 80-85.
34. De Sousa C, English NR, Stacey TE & Chalmers RA (1990). Measurement of L-carnitine and acylcarnitines in body fluids and tissues in children and adults. *Clinica Chimica Acta*, 187: 317-328.
35. Winter SC, Linn LS & Helton E (1995). Plasma carnitine concentrations in pregnancy, cord blood, and neonates and children. *Clinical Chemistry*, 243: 87-93.