Evaluation of chemiluminescence method for the analysis of plasma homocysteine and comparison with HPLC method in children samples

Avaliação do método de quimioluminescência na análise de homocisteína plasmática e sua comparação com o método de HPLC em amostras de crianças

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ABSTRACT

Objective: To compare the results for homocysteine concentration using chemiluminescence and HPLC methods in samples from school-age children. In addition, to determine the reference values for patients of this age group and assess the real prognostic value of homocysteine in healthy children. **Methods:** A prospective observational study was undertaken to determine plasma levels of homocysteine using two different assays, HPLC and chemiluminescence, in 185 samples from school-age children living in Santo Andre, with no chronic or inflammatory diseases, and absence of pubertal development. **Results**: The results were presented in percentiles and reference values were determined within this age group (7-9 years old). Homocysteine concentration ranged from 2.0 to 9.9 μmol/l (r = 0.821 and p < 0.001). **Conclusions**: It was verified that chemiluminescence is comparable to HPLC when both techniques are used to detect homocysteine in school-age children. There is an important correlation between both methods, which allows investigation of this amino acid as a risk factor for heart diseases.

RESUMO

Objetivo: Comparar os resultados da concentração de homocisteína usando os métodos de quimioluminescência e HPLC em amostras de crianças escolares. Determinar os valores de referência desse grupo etário e avaliar o valor prognóstico real da homocisteína em crianças saudáveis. **Métodos:** Um estudo prospectivo observacional foi executado para determinar os níveis de homocisteína usando dois ensaios diversos, o HPLC e a quimioluminescência, em 185 amostras de crianças em idade escolar moradoras da cidade de Santo André, que não apresentassem doenças crônicas ou inflamatórias, na ausência de desenvolvimento puberal. **Resultados:** Os resultados foram apresentados em percentis e os valores de referência foram determinados para esse grupo etário (7-9 anos). As concentrações de homocisteína variaram de 2,0 a 9,9 μmol/l (r = 0,821 e p < 0,001). **Conclusões:** Verificamos que o método da quimioluminescência é

comparável ao método HPLC quando as técnicas são usadas para detectar a homocisteína em crianças em idade escolar. Houve importante correlação entre os dois métodos, o que permite a investigação desse aminoácido como um fator de risco para doenças cardíacas.

Descritores: Homocisteína; Cromatografia líquida de alta pressão; Medições quimioluminescentes

INTRODUCTION

Homocysteine is a sulfur-containing amino acid found at the crossroads of two metabolic pathways: remethylation to form methionine, and transsulfuration, resulting in irreversible cystathionine[1].

Homocysteine is derived from dietary methionine and is an intermediate metabolite in the biosynthetic pathway which converts methionine into cysteine. Therefore, cysteine is not an essential amino acid in humans, as it can be synthesized from methionine, via transsulfuration process. Dietary methionine is converted into S-adenosylmethionine (SAM) by the enzyme methionine-adenosyltransferase (MAT), which forms high energy disulfide bonds between carbon-5' of ATP ribose and methionine sulfur. A small amount of SAM is used for polyamine synthesis, and most of it donates its methyl group to other compounds, such as creatine, DNA and RNA. Thus, SAM is metabolized to S-adenosylhomocysteine, which, in turn, is hydrolyzed in homocysteine and adenosine[2].

The intracellular and plasma levels of homocysteine are normalized with the activity of enzymes that participate in its metabolic pathway, as well as appropriate levels of vitamin B12 and, mainly, folate[2].

Several diseases and situations have been associated with higher levels of homocysteine, for example chronic heart failure[3], effects of vascular diseases[4] besides oxidative stress[5], type 1 diabetes[6], obesity[7] and homocystinuria[8]. However, it is not very safe to say if these changes are the cause or the consequences of primary diseases. In some cases, however, this situation seems clearer, as in neural tube defects and heart diseases[9].

Among a number of methods to analyze and determine homocysteine, HPLC (high performance liquid chromatography) is considered to be the method of reference (gold standard). Nevertheless, when comparing the cost/benefit ratio, its use may be impaired, for being expensive and depending on the analyst skills to be performed. However, other laboratory techniques have already been described and can be used with no implications in quality and results of analysis. One of these techniques is chemiluminescence

and, when compared to the reference method, it does not constrict the clinical analysis of results and may be more advantageous regarding its cost/benefit ratio and easy-to-use features[10].

OBJECTIVES

The aim of this study was to compare the results for homocysteine concentration using chemiluminescence and HPLC methods in samples comprised of school-aged children. In addition, the reference values for patients in this age group were determined and the real prognostic value of homocysteine in healthy children was assessed.

METHODS

After being approved by the Institutional Review Board, this prospective observational study was carried out between 2006 and 2008. The study group consisted of 185 prepuberal children, aged 7-9 years, enrolled at a public school in the city of Santo André, State of São Paulo, Brazil.

Children with any chronic, genetic, congenital or acquired immunodeficiency and infectious or inflammatory disease were excluded. We also excluded those who were on corticosteroids and who had been admitted to hospital in the past three months.

Blood samples were collected to determine the plasma levels of homocysteine, using both HPLC e chemiluminescence assays. We collected 185 samples by venipuncture, after fasting for at least four hours.

The 10-ml samples were separated into two test tubes with EDTA (ethylenediaminetetraacetic acid), which were kept under refrigeration until centrifugation for 6-8 minutes, at 3000 rpm. The obtained plasma samples were stored until analysis.

The samples were analyzed using a Shimadzu HPLC (SIL -10 Dvp) coupled with fluorimetric detection (RF-10 AXL) and isocratic elution at the Inborn Errors of Metabolism Laboratory, Center for Medical Genetics, Universidade Federal de São Paulo (UNIFESP). The chromatographic separation used Phenomenex Prodigy ODS2 with a column (3.2 x 150 mm, 5-μ microparticles). Fluorescence-separated compounds were captured by the detector adjusted for excitation of 385 nm and an emission of 515 nm. Total homocysteine was estimated using a calibration curve with known amounts of homocysteine[11].

The immunoenzymatic assay was performed using automated chemiluminescence apparatus model Immulite 2000 at the Laboratory of Clinical Analysis of Faculdade de Medicina do ABC (FMABC).

At the beginning of the chemiluminescense routine analysis in this equipment, the adjustments were performed in four replicates, following the manufacturer's recommendations.

In order to compare the results in both techniques, we measured 20 samples with hyperhomocysteinuria (well-known concentrations determined by HPLC), which were measured again and determined by chemiluminescence.

We used the SPSS 18.0 software to calculate Pearson's correlation coefficient, thus estimating sensitivity and specificity, and determining the Receiver Operating Characteristic (ROC) curve. However, when analyzing hyperhomocysteinaemia, we used Spearman's correlation coefficient.

RESULTS

The 50th percentiles of reference values were presented for both techniques: HPLC and chemiluminescence.

The percentile values for HPLC analysis demonstrate that 50% of 7-year-old patients presented values below 5.3 μmol/l whereas the 8 and 9-year-old children presented values below 5.4 μmol/l and 5.9 μmol/l, respectively. For the chemiluminescence assay, the following values were found: 3.2 μmol/l; 3.3 μmol/l; and 3.6 μmol/l. In children aged between 7 and 9 years, the minimum value was 2.0 μmol/l, the maximum was 9.9 μmol/l, and the mean value was 3.6 μmol/l. Consequently, the verified reference values were between 2.0 μmol/l to 9.9 μmol/l (Table 1).

Table 1. Percentile values, for age, HPLC and chemiluminescence techniques

Percentile	HPLC			Chemiluminescence		
	7 years	8 years	9 years	7 years	8 years	9 years
99.0	12.317	11.320	13.033	9.9	8.4	9.3
95.0	8.916	9.010	8.646	5.6	5.5	6.1
90.0	7.613	7.407	8.098	5.3	5.0	5.4
75.0	6.689	6.270	6.610	4.2	4.1	4.2
50.0	5.312	5.410	5.860	3.2	3.3	3.6
25.0	4.189	4.716	4.953	2.3	2.6	3.0
10.0	3.634	3.917	4.443	2.0	2.0	2.6
5.0	3.119	2.857	4.091	2.0	2.0	2.4
1.0	2.497	0.000	3.598	2.0	2.0	2.0

Using linear regression analysis in both techniques, HPLC and chemiluminescence, a significant correlation between them was obtained (r = 0.821, p < 0.001), as shown in Figure 1.

The values obtained from HPLC and chemiluminescence analyses show a positive and
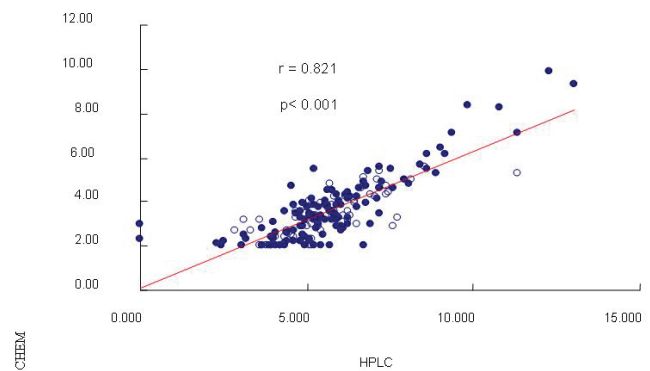

Figure 1. Correlation between chemiluminescence and HPLC

significant correlation. Thus, when the concentration obtained via HPLC increases, the level via chemiluminescence also rises.

Therefore, we found that the sensitivity for this method is 87.5%, the specificity is 97.0%, with positive and negative predictive values of 98.8% and 96.2%, respectively (Figure 2).
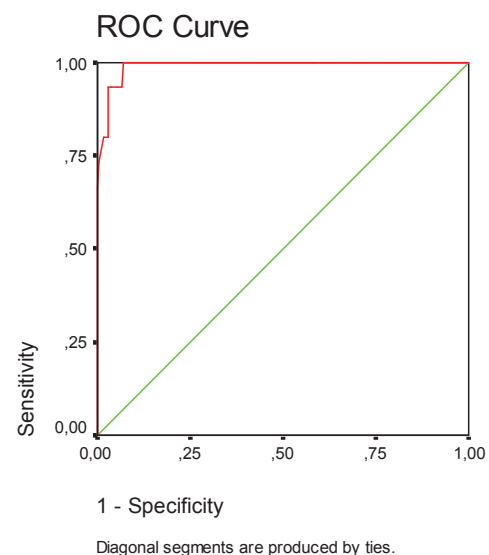

Figure 2. ROC curve (sensitivity and specificity)

The results for hyperhomocysteinemia measured by chemiluminescence assay showed, through Spearman's coefficient, that there was a positive and significant correlation between both techniques (r = 0.889 and p < 0.001).

Using chemiluminescence, urine homocysteine samples presented a mean value of 167.9, a median value of 187.5, and minimum and maximum values of 4.7 and 505.0. With the HPLC technique, the mean value was 148.3, the median value was 134.8, and the minimum and maximum values were 333.8 (Figure 3).
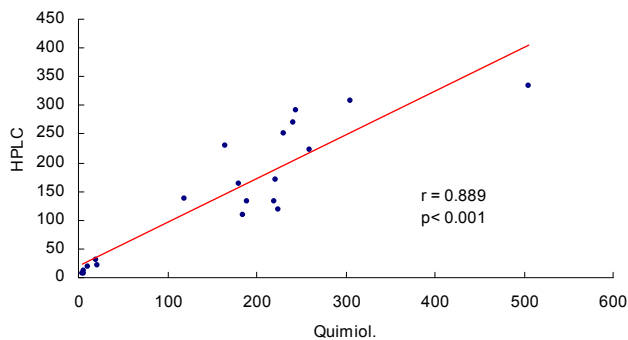
Figure 3. Correlation between both techniques to measure hyperhomocysteinemia

DISCUSSION

In the present study, we demonstrated that the use of chemiluminescence technique to measure plasma homocysteine presents a positive and significant correlation, assessing the real prognostic value of homocysteine levels in healthy children. The results suggest that it is possible to use chemiluminescence technique as a diagnostic method for the metabolic disorder involving homocysteine.

In most researches, the concern is to appraise the total concentration of homocysteine in adults, in order to establish a reference value to help prevent or even to define a clinical prognosis for cardiovascular risk.

For the time being, this standard quantification for children at preschool age has not been completely elucidated, as demonstrated by a study carried out with Belgian children, which determined reference values in three age groups, measured in both sexes, using HPLC technique, as follows: 5 to 9 years (6.21 μmol/l), 10 to 14 years (7.09 μmol/l) and 15 to 19 years (8.84 μmol/l). An increase in homocysteine levels when approaching adulthood is clear distinguishable, probably caused by effects of hormones during puberty[12].

Our results, when compared to the study mentioned above, both of them concerning young populations, indicate that plasma levels of homocysteine found are consistent, even though the methodology used is chemiluminescence assay instead of HPLC.

In this article, the authors cite other studies that provide guidelines to assess homocysteine concentration averages among young subjects. Vilaseca et al. analyzed homocysteine concentration in children aged 2 to 10 years (5.8 μmol/l), 11 to 15 years (6.6 μmol/l), and 16 to 18 years (8.1 μmol/l)[12].

The National Institutes of Health performed a study on homocysteine levels in a representative sample of American adolescents and adults. Three samples were collected in different moments related to folate fortification. The analytical method used to determine homocysteine was modified during the course of

the study. They started with HPLC (1991-1999) and completed the analysis using the immunoassay analyzer Abbott IIMX® (1999-2001) and Abbott AxSYM® (2002-2004). SAS and SUDAAN statistical software was used, proving that the use of different analytical techniques did not involve detrimental changes in the final analysis of the results, as our results demonstrated[13].

The laboratory techniques to quantify homocysteine (HPLC) are costly, hindering routine analysis, mainly when concerning the elucidation of results from Public Health Services users.

Thus, plasma homocysteine analysis would be less time-consuming, reduce expenditure with reagents and provide assistance to the majority of population in doing these tests.

There are few studies published on homocysteine determination in healthy or unhealthy children, but other analytical techniques had already been described and can be used without interfering in quality and results.

According to Stauffenberg, the chemiluminescence technique, when compared to the reference method (HPLC), does not impair the clinical evaluation of homocysteine concentration and can be even more favorable concerning its cost/benefit ratio and easiness to operate[14].

Notwithstanding, homocysteine is a risk factor, its determination is unusual, maybe because of the high costs of HPLC technique. Thus, our results suggest that it is possible to effectively measure it using chemiluminescence technique, as the samples presenting homocysteinuria were statistically significant when we compared both methods.

CONCLUSIONS

Our results proved that the determination of plasma homocysteine levels using chemiluminescence could be used as a routine assay in samples within the reference values, as well as in samples presenting high levels of homocysteine.

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