Analysis of Plasma Amino Acids Using RP-HPLC and Pre-Column Derivatization with OPA/3-MPA

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ABSTRACT

Background and Objective: Measurement of amino acids is an important tool for metabolic studies and evaluation of patients' clinical condition. The aim of this study was to analyze the plasma amino acids using reverse phase high performance liquid chromatography techniques (RP-HPLC) with pre-column derivatization by o-phthalaldehyde (OPA) in combination with 3- mercaptopropionic acid (3-MPA).

Methods: Overall, 107 neonates and babies suspected of having metabolic disorder were enrolled in this study. The level of amino acids in plasma samples was analyzed within 65 minutes by HPLC with pre-column derivatization by OPA/3-MPA. This was a gradient RP-HPLC method that was performed using two solvents with a ratio of methanol and sodium acetate. L-norvaline internal standard was used as the reference peak for amino acids. Standard mixture of amino acids was used to determine the concentrations of amino acids.

Results: According to the values of coefficient of variation obtained for each amino acid, the results indicated a good chromatographic separation of amino acids by this method. The use of OPA/3-MPA derivative reagent increased the efficiency and resolution of amino acids chromatographic separation.

Conclusion: Due to simple preparation and accurate assessment, determination of plasma amino acids using OPA/3-MPA derivatives and RP-HPLC is a suitable method in many clinical samples.

Keywords: High Pressure Liquid Chromatography, OPA/3MPA, Amino Acids.

INTRODUCTION

Amino acids play a key role in various processes. These compounds are proteins' building blocks that can also act as precursors for biosynthesis of many important biological and physiological substances. They also have an essential role in energy metabolism, neurotransmission and fat transport (1).

Determining the concentration of amino acids is necessary to evaluate the pathophysiological processes of diseases (2). Analysis of these components is one of the most important practical programs in the field of biomedical and pharmaceutical sciences (3).

Measurement of amino acids in different matrices such as plasma, urine and cerebrospinal fluid has attracted a lot of attention (4). During the last 15 years, analysis of amino acids was performed using the ion exchange chromatography based on pre-column derivatization with ninhydrin or o-phthalaldehyde (OPA) (5).

However, this method is somewhat difficult, costly and time-consuming (6). Recently, the effective method of high performance liquid chromatography (HPLC) is used in combination with chemical derivatives in the form of pre- or post-column to overcome these problems. The Pre-column OPA-derivatization is one of the most widely used methods (7, 8).

This compound is an effective derivative factor for amino acids, due to its quick interaction with the fist amine group of amino acids that leads to formation of products with high fluorescence properties. Although the isoindole derivatives formed are not stable, accurate results can be obtained using these derivatives (9).

The stability of these derivatives depends on addition of sulfhydryl agents to the derivatives. Some studies indicate that 3-mercaptopropionic acid (3-MPA) produces a more stable product than the common 2-mercaptoethanol amine (2-ME). This study aimed to analyze main amino acids of plasma using pre-column derivatization with OPA combined with 3-MPA (10).

MATERIAL AND METHODS

The EDTA plasma samples were obtained from 107 neonates and babies suspected of having aminoacidopathy, after receiving informed consent from parents. The samples were stored at -70 °C until the time of analysis. Analysis of 19 amino acids was performed using KNAUER HPLC system (V7602 10/2003, Germany). This study was based on reverse-phase chromatography and performed using the gradient method. Amino acid analysis was done using column (UCLEOSHELL® RP 18, 2.7 μm, 75mm x 4.6mm ID.).The plasma proteins were precipitated by adding 10% trichloroacetic acid (TCA).

After centrifugation at 10000 rpm for 5 minutes, the supernatant was removed and filtration was done using syringe filter with a 0.45 μm pore size (Whatman SCA Syringe Filter, CHMLAB Inc.).The materials used were obtained at high purity grade (HPLC grade). All solutions used were filtered using Whatman filter papers with 0.45 μm pore size (CHMLAB Inc.).

In order to prepare borate buffer, 5.4 g of powdered tetraborate (Sigma, St. Louis, MO) were dissolved with 10 water molecules in 100 ml of water (HPLC grade) and then pH was brought to 9.5 using sodium hydroxide solution. The derivative solution was prepared by adding 2250 μl methanol (MERCK), 250 μl borate buffer and 25 μl 3-MPA (Sigma, St. Louis, MO) to 0.025 g OPA (Sigma, St. Louis, MO). Amino acid separation using this method required two mobile phases. The mobile phase A was prepared by mixing 790 ml of 0.05M sodium acetate buffer (Panreac) in 210 ml of methanol. The mobile phase B was prepared by mixing 250 ml of 0.05M sodium acetate buffer in 750 ml of methanol. The final pH of both phases was in the range of 7.02 - 7.06. The internal standard l-norvaline (Sigma, St. Louis, MO) was obtained at concentration of 1mM. In this study, the standard mixture of amino acids at concentration of 500mM/L was used (Amino acid standards, physiological analytical standard, acidics, neutrals, and basics, Sigma, St. Louis, MO).

The concentrations of 10, 25, 50 and 100 mM of the mobile phase A were prepared using the standard. The filtered plasma samples (200 μl) were prepared by adding l-norvaline (50 μl) and methanol (800 μl).

After thorough mixing, incubation was done at laboratory temperature for 5 minutes. After centrifugation at 5000 rpm for 5 minutes, 250 μl of the supernatant was taken and mixed with 100 μl of borate buffer. For derivatization of amino acids, 50 μl of OPA solution was added to the above solution and then incubation was performed for two minutes at room temperature. In this step, the mobile phase A was added as an organic compound to achieve baseline and better separation. After adding hydrochloric acid to the final mixture, 20 μl was injected into the column.

Table 1 shows the program given to the HPLC instrument for analysis and separation of amino acids at excitation wavelength of 330 nm and emission wavelength of 450 nm with flow rate of 1 ml/min.

Data analysisData

analysis was carried out using SPSS software (version 16). Data description was calculated as mean ± standard deviation (SD).

P-value of less than 0.05 was considered as statistical significance level. Kolmogorov-Smirnov test was used to assess the association of each amino acid with clinical symptoms by evaluating the normal distribution of data for levels of amino acids.

According to this test, P-value <0.05 was considered non-normal. In the case of normally distributed data, T-test was used to compare mean levels of the amino acid with each clinical symptom and Mann-Whitney test was used for non-normal data.

RESULTS

Table 2 shows the coefficient of variation (CV) along with the mean and SD for 19 amino acids of 107 neonates and babies tested with suspected aminoacidopathy. In order to achieve optimum baseline and high efficiency separation of each amino acid, the solvents and the gradient method were optimized. According to the two chromatograms, an appropriate separation of amino acids was achieved. The separation of some amino acids were overlapping, thus minor modification of methanol in the solvents composition led to better separation of these amino acids. The appropriate separation of tryptophan and phenylalanine was achieved by optimizing the methanol/water ratio in solvent B. Figure 1 shows the chromatographic separation of amino acids in the samples and the external standard with concentration of 10 μM/L. The resulted chromatogram in this method indicates an appropriate chromatographic separation of 19 amino acids along with the internal standard 1-norvaline.

Table 1- Program given to the HPLC instrument for amino acid analysis

Duration (minutes)	Solvent A (%)	Solvent B (%)
0	100	0
20	100	0
60	33	67
65	100	0

Table 2- Values of the coefficient of variation along with the mean and SD of amino acids

SD	Mean	CV of plasma samples (%)	Amino acid (Mol/μl)
15	165	9.2	Alanine
3.5	25	14	Arginine
3.27	34	10	Asparagine
1/09	8/2	13	Aspartic acid
0/9	11	8	Citrulline
13.5	211	6.4	Glutamine
17	206	8.3	Glycine
23.26	258	9	Glutamic acid
4.2	69	6	Histidine
2.1	51	4.1	Ornithine
17	61	2.8	Lysine
6.7	61	11	Leucine
1.6	14	11.4	Methionine
1.9	20	9.5	Isoleucine
1.9	39	9.5	Phenylalanine
10.4	109	9.5	Valine
6.4	68	9.4	Tyrosine
11.3	125	9.1	Threonine
14.01	137	10	Serine
2	23	8.7	Tryptophan

Figure 1- Chromatogram of the standard mixture of 19 amino acids at concentration of 10 µm. Peaks: 1 = aspartic acid; 2 = glutamic acid; 3 = asparagine; 4 = serine; 5 = histidine; 6 = glutamine; 7 = glycine; 8 = threonine; 9 = citrulline; 10 = arginine; 11 = alanine; 12 = tyrosine; 13 = valine, 14 = methionine; 15= norvaline; 16 = tryptophan; 17 = phenylalanine; 18 = isoleucine; 19 = leucine; 20 = ornithine; 21 = lysine
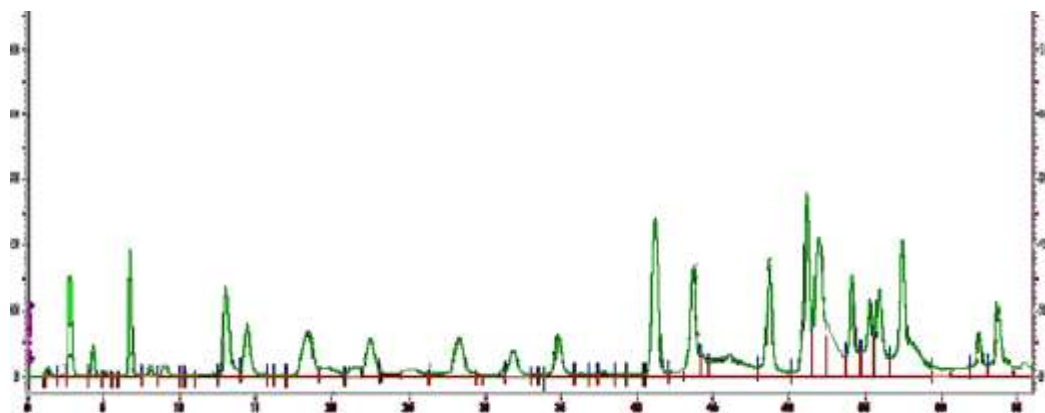
DISCUSSION

The present study aimed to assess the RP-HPLC method using the combination of OPA-3MPA derivatives for the separation of 19 amino acids as well as norvaline as the internal standard. According to the CV values obtained for each amino acid, the results indicate an appropriate chromatographic separation for amino acids using this method.

The column containing 2.7 µm particles was used to obtain high resolution. Moreover, the use of methanol and mobile phase A in the sample preparation process resulted in increased stability and peaks with good symmetry and quality.

Consistent with our findings, the Simons et al. study for quality assessment of derivatization using OPA by implementing polar solvents and thiol-containing compounds such as 3-MPA showed that the presence of these compounds have a big impact on the florescence property of isoindole derivatives. These compounds along with borate buffer had very good effects on the fluorescence intensity (11).

Similar to the present study, Turnell et al. demonstrated that the use of methanol alone as an organic compound in the process of sample preparation increases the quality of chromatogram and the resolution of amino acids separation (7). In this study, plasma deproteinization was performed using TCA. In line with the present study, Frank et al. evaluated a rapid and simple method for amino acid analysis using HPLC technique with pre-column derivatization using OPA/3-MPA and demonstrated that this simple and optimized method requires a small sample size and the use of OPA/3-MPA combination leads to high repeatability of the results. They also showed that TCA use for deproteinization has no interference with the chromatogram obtained and considered it a more suitable substance for plasma protein precipitation compared with 5- sulfosalicylic acid (SSA) (12).

However, the study of Qureshi et al. showed that the HPLC technique using OPA/2-ME combination was a suitable method with a high resolution and reproducibility for the analysis of amino acids in both patients and healthy individuals. This study showed no significant difference in the quality of amino acids chromatogram using SSA in comparison with TCA (13).

In line with the mentioned study, Chen et al. showed that SSA is a more suitable substance for deproteinization of plasma proteins compared to TCA (14).

Despite the results of these studies, a recent survey has shown that the SSA as a deproteinizing agent interferes with the chromatography of aspartic acid and glutamic acid (15). In the present study, no adverse effect was observed on the quantitative results of amino acids using the TCA. Moreover, 3- MPA was used as thiol-group-donor to increase the quality of derivatization. Many studies suggest that

this substance produces far more stable products compared to 2-ME (6, 16).

Kehr et al. study demonstrated that 3-MPA was more suitable than 2-ME for interactions with the OPA reagent, due to production of more stable products. Furthermore, this interaction is highly polarized due to the presence of additional carboxyl groups in the thiol group of this compound, which optimizes washing procedure in gradient chromatography (17).

Schwarz et al. study aimed to use RP-HPLC along with pre-column derivatization using OPA-3MPA for amino acid analysis and compared it with ion exchange chromatography (IEC). They demonstrated that HPLC had a good correlation with the IEC technique ($0.89 \leq r \leq 1.00$) with linearity higher than 2500 mol/μl, and recommended it as a suitable technique for separation of clinically important amino acids (18).

CONCLUSION

The reverse-phase chromatography with fluorescence intensity measurement using OPA-3MPA combination is a reliable and accurate method for measuring plasma amino acids.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest regarding this manuscript.

REFERENCES

1. Zhang X, Zhao T, Cheng T, Liu X, Zhang H. *Rapid resolution liquid chromatography (RRLC) analysis of amino acids using pre-column derivatization.* Journal of Chromatography B. 2012;906:91-5. doi:10.1016/j.jchromb.2012.08.030.

2. Gatti R, Gioia M, Andreatta P, Pentassuglia G. *HPLC-fluorescence determination of amino acids in pharmaceuticals after pre-column derivatization with phanquinone.* Journal of pharmaceutical and biomedical analysis. 2004;35(2):339-48. PMID:15063467.

3. Gheshlaghi R, Scharer J, Moo-Young M, Douglas P. *Application of statistical design for the optimization of amino acid separation by reverse-phase HPLC.* Analytical biochemistry. 2008;383(1):93-102. doi: 10.1016/j.ab.2008.07.032.

4. Piraud M, Ruet S, Boyer S, Acquaviva C, Clerc-Renaud P, Cheillan D, et al. *Amino acid profiling for the diagnosis of inborn errors of metabolism. Metabolic Profiling:* Springer; 2011. p. 25-53. doi: 10.1007/978-1-61737-985-7_2.

5. Herrndobler A. *Determination of amino acids in an infusion solution by reversed-phase HPLC and pre-column dansyl chloride derivatization.* Journal of High Resolution Chromatography. 1986; 9(10): 602-4. DOI: 10.1002/jhrc.1240091016.

6. Terrlink T, Van Leeuwen P, Houdijk A. *Plasma amino acids determined by liquid chromatography within 17 minutes.* Clinical chemistry. 1994; 40(2): 245-9.

7. Turnell D, Cooper J. *Rapid assay for amino acids in serum or urine by pre-column derivatization and reversed-phase liquid chromatography.* Clinical chemistry. 1982; 28(3): 527-31. PMID:7067099.

8. Kaspar H, Dettmer K, Gronwald W, Oefner PJ. *Advances in amino acid analysis. Analytical and bioanalytical chemistry.* 2009; 393(2): 445-52. doi: 10.1007/s00216-008-2421-1.

9. Jones BN, Pääbo S, Stein S. *Amino Acid Analysis and Enzymatic Sequence Determination of Peptides by an Improved o-Phthaldialdehyde Precolumn Labeling Procedure.* Journal of Liquid Chromatography. 2006; 4(4): 565-586.

10. Go K, Horikawa Y, Garcia R, Villarreal FJ. *Fluorescent method for detection of cleaved collagens using O-phthaldialdehyde (OPA).* Journal of biochemical and biophysical methods. 2008; 70(6): 878-82.

11. Simons SS, Johnson DF. *Reaction of o-phthalaldehyde and thiols with primary amines: Fluorescence properties of 1-alkyl (and aryl) thio-2-alkylisoindoles.* Anal Biochem. 1978; 90(2): 705-725. doi:10.1016/0003-2697(78)90163-X.

12. Frank MP, Powers RW. *Simple and rapid quantitative high-performance liquid chromatographic analysis of plasma amino acids.* Journal of Chromatography B. 2007; 852(1-2): 646-9.PMID:17254851.

13. Qureshi GA, Qureshi AR, Bergström J. *Quantitation of free amino acids in plasma and muscle samples in healthy subjects and uremic patients by high-performance liquid chromatography and fluorescence detection.* Journal of pharmaceutical and biomedical analysis. 1989;7(3):377-84.PMID:2488638

14. Chen B-M, Xia L-W, Zhao R-Q. *Determination of N(G),N(G)-dimethylarginine in human plasma by high-performance liquid chromatography.* Journal of Chromatography B: Biomedical Sciences and Applications. 1997; 692(2): 467-71.

15. Aristoy MC, Toldra F. *Deproteinization techniques for HPLC amino acid analysis in fresh pork muscle and dry-cured ham.* Journal of Agricultural and Food Chemistry. 1991; 39(10): 1792-5.

16. Molnár-Perl I, Bozor I. *Comparison of the stability and UV and fluorescence characteristics of the o-phthaldialdehyde/3-mercaptopropionic acid and o-phthaldialdehyde/N-acetyl-l-cysteine reagents and those of their amino acid derivatives.* Journal of Chromatography A. 1998; 798(1): 37-46. doi:10.1016/S0021-9673(97)01222-3.

17. Kehr J. Fluorescence detection of Amino Acids derivatized with o-phthalaldehyde (OPA) based reagents. CMA/Microdialysis AB, Stockholm, Sweden1993.

18. Schwarz EL, Roberts WL, Pasquali M. *Analysis of plasma amino acids by HPLC with photodiode array and fluorescence detection.* Clinica chimica acta. 2005; 354(1): 83-90. doi:10.1016/j.cccn.2004.11.016