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Simultaneous Determination of Homocysteine, Cysteine, Glycine, Glutamic Acid, Methionine, Serine and Taurine in Human Plasma by Using Reverse Phase-High Performance Liquid Chromatography

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Abstract

Alterations in the plasma aminothiol levels can be considered as biomarkers for the diagnosis and screening of human disorders namely cardiovascular diseases. Among the common method, ELISA is available for homocysteine estimation, the analytical method like HPLC is considering as a gold standard. In the proposed study, we have developed a RP-HPLC method for the simultaneous determination of homocysteine, cysteine, glycine, glutamic acid, methionine, serine and taurine in human plasma using fluorescence detector. Separation was achieved on C18 column with gradient elution using 80% methanol, 0.05 M acetate buffer (pH-7.0) and the column was maintained at 26°C and the mobile phase was pumped at flow rate of 1.0 mL min-1. The detection of eluent was carried out at fluorimetrically (excitation 340 nm, emission 450 nm) with a total run time 30 min. The calibration curve ranged from 25 to 100 ng/mL for the analytes. Inter and intraday precision and accuracy, stability studies and sample reanalysis were investigated for all the analytes, and the results met the acceptance criteria. This method will be a useful tool in the investigation of the roles of these important thiol-containing compounds and their corresponding disulfides in physiological and pathological processes.

Keywords: Amino acids, Homocysteine, Fluorescence detector, Eales' disease, Age related macular degeneration

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Introduction

Determination of thiol-containing amino acids involved in methionine pathway namely methionine, homocysteine, cysteine, glycine, serine, taurine and glutamic acid is important for clinical diagnosis and screening of various human disorders [1,2]. Hyperhomocysteinemia is associated with the risk of retinal vascular diseases, such as diabetes, cataract, Eales' disease, age related macular degeneration, central retinal vein occlusion, cardiovascular diseases, ischemic heart disease and stroke [2-4]. The aetiology of hyperhomocysteinemia in Eales' disease and age related macular degeneration is unclear till date. These amino acids are having numerous functions in redox homeostasis, cellular protection against reactive oxygen species and reactive nitrogen species, heavy metal detoxification, and control of cell signalling besides normal metabolism [5,6]. Amino acids methionine is involved in the synthesis of cysteine *via*, homocysteine, thus extracellular cystine reduced by disulfides through N-acetylcysteine to produce cysteine which is transported into the cells and involved in the synthesis of antioxidants glutathione during oxidative stress [7].

Till date, to our knowledge, there is no method available to simultaneously determine these amino acids involved in methionine pathway. Numerous methods for the determination of thiol compounds have been reported, including HPLC using variable wavelength detector, fluorescence detector and electrochemical detector [8-12], gas chromatography-mass spectrometry [13], ion-exchange chromatography [14] and capillary electrophoresis [15]. In the year 2013, Sochor et al. [16] estimated the Cysteine (Cys) and Homocysteine (Hcy) using electrochemical techniques which was a time-consuming analysis of Hcy and Cys in plasma. In the year 2013, Pattengale et al. [17] estimated the Hcy, Methionine (Met), methylmalonic acid and

methyl citric acid without the need of additional derivitization by LC/MS/MS. In the year 2003, Guan et al. [18] estimated simultaneously glutathione (GSH), Hcy and Cys in biological samples using LC/MS, it is a selective and sensitive assay for the estimation of endogenous thiols. But in this method, Cys was not able to be estimated in biological samples due to the strong interferences of derivitizing agents. In the year 2001, Tcherkas et al. [19] estimated Hcy, Cys and Glutamic Acid (Glut) in plasma using isocratic reverse phase liquid chromatography with fluorescence detector. Each of these methods have their own limitations in terms of complexity, sample processing, run times, number of thiols simultaneously quantitated, and validation parameters assessed which create challenges and render them impractical for high-throughput routine clinical or research purposes. Thus, the aim of the present study was to establish, validate, and implement a simple, reproducible, and robust method for simultaneous determination of total concentrations of Hcy, Cys, Gly, Glut, Met, Serine (Ser) and Taurine (Tau) in human plasma.

Materials and Methods

Materials

β-mercaptoethanol (β-ME), iodoacetic acid, Ortho-pthalaldehyde (OPA), Hcy, Cys, Glut, Gly, Met, Ser, Tau and Norvaline (internal standard) were obtained from Sigma (St Louis, MO, USA). Methanol and acetic acid used were of HPLC grade. Sodium acetate trihydrate, Triethyl amine, Disodium tetra borate, Sodium hydroxide, perchloric acid were obtained from E-Merck chemicals (Mumbai, India). Water used in this study was purified through Millipore Water Purification System (Millipore Co., India).

Solutions

Stock solutions of Hcy, Cys, Met, Tau, Glut, Gly, and Ser (1 mg/mL compounds) were prepared in 0.05 M Perchloric acid and stored at -20˚C. Working solutions of standards 25 ng were prepared freshly before use. Weighing of each compounds dissolved in 0.05 M perchloric acid at 25 ng, 50 ng, 75 ng and 100 ng concentrations respectively for calibration standards 20 ng, 80 ng 40 ng, and 60 ng concentrations, respectively for QC samples. OPA–β-ME reagent was prepared according to the previously published method [17]. The norvaline internal standard (IS) was dissolved in HPLC grade water to prepared 1 mg /mL stock solution. Standards and QC samples containing all the compounds were prepared at the beginning of the validation experiment by appropriate dilution with HPLC grade water and subsequent mixing of the individual stock solutions. All solutions, standards and QC samples were stored at -20°C to stimulate the storage conditions of the samples.

Equipment

The HPLC system consisted of a Agilent LC 1100 series, G1312A Binary pump, a Rheodyne 7125 injection valve (Agilent, Santa Clara, CA, USA) fitted with a 50 µL sample-loading loop and Agilent 1200 series fluorescence detector (Agilent, Santa Clara, CA, USA) with a 150W Xenon lamp. The detector was set at excitation and emission wavelengths of 340 nm and 450 nm, respectively. Signal output was captured with the Agilent Chemstation software (Agilent, Santa Clara, CA, USA). Separation of the analytes was achieved with a Agilent Symmetry C18 guard column (3.0 mm × 20 mm; 5 µm particles) connected to a Thermo Hypersil octadodecyl silicane (ODS) C18 analytical column (4.6 × 150 mm; 5 µm particles) at 26 °C. The two mobile phases consisted of A: 0.05 M acetate buffer (pH 7.0), 45 µl triethylamine in water and B: 0.05 M acetate buffer (pH-7.0), 45 µl triethylamine in methanol. Hcy and its related compounds were separated over 5 min with 100 % A at a flow rate of 1.0 mL /min, followed by 40 %: 60 % A:B (changed linearly over 5 min) at a flow rate of 1.0 mL /min for 20 min, and 5 min of column re-equilibration for a total run time of 30 min.

Preparation and derivatization of biological samples

Following approval from the Institutional Ethics committee, Informed consent was obtained from all the participants in the study. Among the seventy two (72) subjects, patients with the age group 16 – 80 years along with age matched healthy controls were enrolled in the study.

The patients and controls included in the study were non-smokers, non-alcoholics and not using any vitamin and are not any antibiotic supplements. They were not on any corticosteroid treatment or antioxidant supplement. Venous blood was collected from the participants after overnight fasting into EDTA containing tubes (Vacutainer, Becton Dickinson, Franklin Lakes, and New Jersey). Plasma was separated immediately from blood cells by centrifugation at 3,000 rpm at 25 ºC for 10 min and stored at – 80 ºC until use.

Sample preparation

The reduction of disulfide bonds using 20 µL of β-ME were added to 100 µL of plasma sample. The sample was mixed and after incubation for 30 s at room temperature. Plasma proteins were removed by precipitation with 380 µL of ice cold 100 % methanol followed by centrifugation at 10,000 rpm for 5 min in cooling centrifuge. Then 20 µL of amino acid standard mixture and the internal standard (50 µL) was then added to samples (50 µL) and briefly vortex-mixed. Following addition of 40 µL IAA solution (0.8 M iodoacetic acid in 0.1 M sodium borate buffer, pH 10.5) and 120 µL of 0.1 M sodium borate buffer (pH 11.5). After incubation for 1 min at room temperature, 20 µL of OPA–β-ME reagent was added and incubate for 3 min at room temperature. After incubation, 50 µL of the mixture was injected into the Agilent HPLC system.

Results

HPLC analysis

Retention times for Glut, Cys, Hcy, Gly, Tau, Met, Ser and IS were approximately 10.8, 11.6, 12.2, 13.0, 13.3, 16.2, 17.3 and 18.2 min, respectively. The peaks of interest were well separated and there was no interference from endogenous compounds.

Calibration, linearity and recovery

Calibration curves were constructed using six standard concentrations of all the Hcy related amino acids (analytes) Hcy, Cys, Glut, Gly, Met, Ser and Tau in water and were run in duplicate. Curves were obtained daily for three days. Individual standard concentrations in water were shown in Table 1.

Duplicate standard curves for all the analytes were analyzed for three runs (lowest standard in triplicate). Calibration curves for Hcy, Cys, Glut, Gly, Met, Ser, and Tau were generated by linear regression analysis. Linear calibration curves were obtained for Glut, Cys, Hcy, Gly, Tau, Met, Ser and IS over the concentration ranges of 25-100 ng / mL, with the correlation co-efficient for all analytes were shown in Table 1 respectively.

Samples at each concentration for the determination of inter-day precision and Intra-day precision were determined. CV % of means, standard deviations were calculated from the QC values and used to estimate the inter- and intra-day precision were shown in Table 1.

Recovery study

Recovery of all the analytes was assessed by analyzing human plasma obtained from normal healthy subjects spiked with the known standard concentration levels shown in Table 1. Unspiked plasma samples and spiked samples of standard were analyzed using calibration curves generated from water based standards. Recovery was determined by comparing the nominal analytes concentration, calculated as the sum of the concentration in unspiked plasma and the spiked QC concentration, to their corresponding measured concentration. Measured concentrations were defined as 100%. Means, standard deviations and relative standard deviations were calculated.

Detection limit

The Lower Limit of Quantitation (LOQ) for each analytes and signal-to-noise ratios of at least 5:1, and the intra- and inter-day and accuracy were within ± 15% (Table 1). There was no evidence of carryover in any of the blank reagent samples. The mean recoveries of all analytes from plasma at all concentrations ranged from 85% to 100% (Table 1).

This method was developed for the simultaneous determination of Hcy and its related amino acids in ocular diseases. This method has been used to quantitative AAs in plasma samples from healthy subject and patients. Seventy-two subjects were received among that thirty-six subjects having retinal diseases (20 ED,16 AMD and 36 controls) and plasma concentrations of Glut, Cys, Hcy, Gly, Tau, Met, and Ser were estimated and the mean ± SD given in Table 2 and Table 3 with respective controls.

Most of the amino acids in Hcy pathway can be measured with baseline chromatographic separation. Only 50 µL of plasma

Table 1: Inter-assay, intra-assay coefficient, linear regression and reproducibility of samples.

	Conc in ng	Glut	Cys	Hcy	Gly	Tau	Met	Ser
Inter-assay CV %	25	5.1	9	4	6.1	5.0	2.5	7.3
	50	4.1	4	9	9.1	1.5	11.6	8.0
	75	12.0	3	9	5.7	10.1	12.8	6.8
	100	5.0	7	4	2.6	5.3	9.2	4.3
Intra- assay CV %	25	2.6	7	9	2.2	8.8	9.7	3.5
	50	9.8	4	8	3.0	9.4	2.6	3.9
	75	8.7	5	3	4.1	12.9	1.9	1.8
	100	1.5	6	9	7.6	6.9	8.2	6.7
R^2		0.995	0.986	0.945	0.985	0.924	0.986	0.990
% Recovery		91.7	89.7	93.8	89.4	95	90	95

Table 2: Amino acid concentrations in plasma of healthy subjects and patients with age group 16 – 40 years (ED).

S.no	Amino acids	Controls (n=20) µM	ED (n=20) µM	P valve
1	Glutamic acid	29 ± 4	19 ± 1	0.018
2	Cysteine	228 ± 20	197 ± 12	0.193
3	Homocysteine	19 ± 2	49 ± 1	0.001
4	Glycine	108 ± 15	78 ± 6	0.08
5	Taurine	117 ± 9	149 ± 9	0.014
6	Methionine	18 ± 4	15 ± 1	0.79
7	Serine	137 ± 19	149 ± 9	0.60
All values are expressed as mean ± standard deviation				

Table 3: Amino acid concentrations in plasma of healthy subjects and patients with age group 50 – 80 years (AMD).

S.no	Amino acids	Controls (n=16) µM	AMD (n=16) µM	P valve
1	Glutamic acid	47 ± 5	30 ± 2	0.006
2	Cysteine	386 ± 68	227 ± 53	0.070
3	Homocysteine	11 ± 1	27 ± 2	0.01
4	Glycine	114 ± 14	122 ± 14	0.70
5	Taurine	53 ± 8	49 ± 8	0.70
6	Methionine	60 ± 7	54 ± 6	0.49
7	Serine	349 ± 33	275 ± 15	0.050
All values are expressed as mean ± standard deviation.				

volume is required without compromising sensitivity, making the assay suitable in patient populations in which only small blood volumes are available for testing.

Discussion

In the laboratory practice of clinical biochemistry, a simple optimized method for the simultaneous determination of sulfur containing amino acids namely, homocysteine, methionine, and cysteine is not available. It was necessary for this research on homocysteine metabolism, to have a protocol which has the power to determine these amino acids, and the other amino acids involved in homocysteine metabolism namely glutamic acid, glycine, serine and taurine. A simple and convenient, HPLC method for the estimation of these related amino acids was developed with fluorescence detector in healthy donors and retinal vascular disease patients. This attempt indeed, generated a fully validated, simple precise and sensitive analytical method for the determination of all amino acids involved in homocysteine pathway and it's useful for clinical and routine diagnostic purposes.

Many methods have been described for quantification of Hcy in the laboratory. HPLC is one of the most used technologies, coupled with UV/Visible detection [18], fluorescence [19] electrochemical [20] and mass spectrometry (MS) [21]. Also, the gas chromatography coupled to MS and tandem mass spectrometry have been utilized [22]. The selection of a particular method depends on the required sensitivity and equipment available in the laboratory. Each of these methods has limitations in terms of equipment cost, complexity, processing time per sample and number of metabolites simultaneously analyzed. Ultraviolet detection presents low sensitivity and specificity, while the loss of selectivity is one of the disadvantages of electrochemical detection [23]. Furthermore MS detection is expensive and it has a good precision but a superior technical complexity. MS/MS requires deuterated patterns, while immunoenzymatic methods do not allow multiple simultaneous determinations of thiols and generally there are commercial kits with spectrophotometric detection [24,25]. In the other hand, HPLC with fluorescence detection is very used in the quantification of thiols by its high sensitivity, relative simplicity, easy automation and high performance.

This method is useful for the simultaneous determination of amino acids involved in homocysteine pathway, still now there is no method available. All the above methods mentioned were able to estimate plasma Hcy only, some methods able to estimate Met, whereas some are able to estimate Glutamic acid, they were not able to estimate all the above mentioned amino acids involved in Homocysteine pathway.

Hcy is unstable in biological matrixes due to oxidation and it exists in multiple forms: the most abundant is linked by disulfide bonds to albumin, while the rest circulates freely. Therefore Hcy and other analytes determinations require the application of reducing agents to obtain the free amino acid. The developed method in this work uses this reducing agent, which allows the release of sulfhydryl groups, enabling their quantification. Furthermore, there is not interference because it elutes with a different retention time to Hcy. The sulfur containing amino acids react with OPA as a thiol-containing component of OPA-β-mercaptoethanol reagent. The amino acids cysteine weakly reacts with OPA-β-mercaptoethanol derivative depend on presence of sulfhydryl groups on cysteine molecule. It was necessary to protect sulfhydryl groups of Homocysteine, cysteine and methionine before their reaction with OPA. Iodoacetic acid was added to standard mixture of amino acids after deproteinization of samples before addition of OPA reducing agent β -mercaptoethanol was used to sample to release Hcy and Cys.

The amino acids glutamic acid was significantly decreased whereas Hcy was significantly increased in ED and AMD. Hcy estimation was done by this in-house developed HPLC method and compared with gold standard ELISA are highly comparable, thus validating the HPLC method is most reliable one [26,27]. It shows the power of the assay, ELISA measure only Hcy, whereas HPLC being a separation method capable of measuring all the amino acids associated with Hcy in methionine metabolism. In the year 2009, Coral et al. [27] developed a method to estimate Hcy using OPA derivation with UV detector at 190 nm, in both the methods estimation of Hcy are correlated well. The increased levels of Hcy in both ED and AMD confirm the role of Hcy as a risk factor in vascular eye diseases.

Glycine was significantly decreased in ED, while it was slightly increased in AMD. Glycine has protective role against vascular damage. It decreases the expression of VEGF *via* reducing the advanced glycation end products expression results in lowering nuclear factor kappa B in bovine retinal endothelial cells and thus glycine acts as anti-angiogenic molecule in endothelial cells by Barathi et al. [28]. Glycine is an abundant amino acid in extracellular matrix, as collagen is a major protein accounting for bulk of ECM, which contains glycine as 1/3 of its composition. Recently it is reported that MMP 9 activity is high in people with AMD, justifying the increased levels of glycine in the plasma of people with AMD [29,30]. Similar to Glutamic acid, the amino acids cysteine decreased in both ED and AMD, due to the decreased level of cysteine, glycine and glutamic acid, the plasma level of GSH was decreased in ED and AMD. Surprisingly the level of methionine was not changed in both the diseases.

Taurine is another sulfur amino acid, which is not a constituent of proteins, but produced endogenously in Met metabolism. There was a mild elevation in the level of Taurine in ED, while it was slightly lowered in AMD. The levels were not statistically significant in both cases. Taurine is getting importance as a nutritional supplement as this amino acid is reported to antioxidant and anti-aetherogenic functions in human [31]. These interesting results warrant further research in order to get the insights into their roles in endothelium.

Conclusion

In summary, it is a simple RP-HPLC method was developed for the rapid and simultaneous estimation of the ocular vascular disease markers, namely homocysteine, cysteine, glutamic acid methionine as well as serine, glycine and taurine. The sample preparation and method is simple and reliable. Importantly, as this method achieves accurate and precise results from small volumes of plasma, it is particularly useful for research into critical

illness and comparable to those published earlier. This rapid method can be useful for the routine analysis of blood samples.

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Conflict of Interest

The authors declared no conflict of interest.

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