

# Analysis of 26 amino acids in human plasma by HPLC using AQC as derivatizing agent and its application in metabolic laboratory

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**Abstract** The present study reports the simultaneous analysis of 26 physiological amino acids in plasma along with total cysteine and homocysteine by high-performance liquid chromatography (HPLC) employing 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) as precolumn derivatizing reagent. Separations were carried out using Lichrospher 100 RP-18e (5 µm) 250 × 4.0 mm column connected to 100 CN 4.0 × 4.0 mm guard column on a quaternary HPLC system and run time was 53 min. Linearity of the peak areas for different concentrations ranging from 2.5 to 100 pmol/µL of individual amino acids was determined. A good linearity ( $R^2 > 0.998$ ) was achieved in the standard mixture for each amino acid. Recovery of amino acids incorporated at the time of derivatization ranged from 95 to 106 %. Using this method we have established the normative data of amino acids in plasma, the profile being comparable to the range reported in literature and identified cases of classical homocystinuria, cobalamin defect/deficiency, non-ketotic hyperglycinemia, hyperprolinemia, ketotic hyperglycinemia, urea cycle defect and maple syrup urine disease.

**Keywords** High-performance liquid chromatography · Physiological amino acids · 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate · Amino acid disorders

## Introduction

Amino acid analysis has become commonplace in metabolic laboratories. Over the last 30 years, amino acid analysis methods employing precolumn derivatization have become more popular than methods which employed post-column derivatization procedures. The major reasons for this include greater sensitivity with lower background levels, faster analysis, ability to minimize reagent consumption by specifying smaller reaction system allowing less expensive analysis, better resolution of hydrophobic derivatized analytes by reverse phase column and more flexible HPLC instrumentation rather than dedicated amino acid analysers (Cohen 2000).

The derivatizing reagents used for HPLC separation and analysis of amino acids after precolumn derivatization include 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) (Cohen 2000), *O*-phthaldehyde (OPA) (Soto et al. 1994), 9-fluoroenylmethyl-chloroformate (FMOC-Cl) (Einarsson et al. 1983), Edman's reagent, phenylisothiocyanate (PITC) (Hariharan et al. 1993), 1-fluoro-2,4-dinitrophenyl-5-*L*-alanine amide (Marfey's reagent) which allows separation and analysis of enantiomeric amino acids (Bhushan and Bruckner 2011), butylisothiocyanate (BITC) and benzylisothiocyanate (BZITC) (Woo 2003) and ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) (Cavalca et al. 2001).

Techniques have been described in which gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS/MS) are used to separate and analyse many more physiological amino acids (Dettmer et al. 2012); however, due to unaffordability of these instruments because of their higher costs, HPLC is still the cornerstone for the amino acid analysis in most of the laboratories in developing countries.

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*O*-Phthaldehyde and PITC are among the most widely used derivatizing agents, however, OPA has the biggest disadvantage that it does not react with secondary amino acids and some of the derivatives like those of glycine and lysine are unstable. PITC can react with secondary amino acids; however, its disadvantages include production of unstable derivatives and removal of excess PITC by evaporation prior to HPLC analysis to avoid column contamination and poor chromatographic separation (Cohen 2000).

Cohen and Michaud introduced 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) for precolumn derivatization in 1993 (Cohen and Michaud 1993). AQC has several advantages like reaction with both primary and secondary amino acids, production of stable urea derivatives within seconds that are highly fluorescent and there is no need to remove excess reagent as it is hydrolysed to 6-aminoquinoline (AMQ) that has different emission maxima and therefore does not interfere with the analysis of amino acids. Cohen and co-workers (Cohen 2000; Cohen and Antonis 1994; Cohen and Michaud 1993) applied this method to the determination of amino acids in different matrices. Wandelen and Cohen, in 1997 developed a quaternary eluent system to determine 24 amino acids in collagen hydrolysate and cell culture media (Wandelen and Cohen 1997).

The evaluation of amino acids in biological fluids is gaining interest in clinical laboratories that play a key role in the diagnosis of several amino acid disorders. The determination of homocysteine in plasma is useful both in the diagnosis and follow-up of folate and cobalamin deficiencies and the rare inborn errors causing homocystinuria (Allen et al. 1990; Skovby 1985). In addition, hyperhomocysteinemia is an independent risk factor for premature cardiovascular diseases (Kang et al. 1992). Thus, the accurate determination of plasma concentrations of homocysteine is essential for understanding the role of homocysteine in the pathogenesis of related diseases. Several methods have been described for analysis of homocysteine by HPLC, but most of them analyse homocysteine along with sulphur containing amino acids only and do not include other physiological important amino acids (Christine et al. 1999; Daskalakis et al. 1996; Dudman et al. 1996; Kuo et al. 1997). In order to optimize the method for the separation of clinically relevant amino acids, we modified the method described by Wandelen and Cohen (1997) to make it more relevant for metabolic laboratory by including total homocysteine, cysteine and citrulline so as to identify most of the amino acid disorders in a single run. The present work describes a RP-HPLC method, using AQC derivatization and fluorescence detection for simultaneous analysis of 26 amino acids including total homocysteine, cysteine and citrulline with

high sensitivity, accuracy and good repeatability in small amounts of plasma samples.

## Materials and methods

### Chemicals

AccQ-Fluor reagent kit (AQC, borate buffer, reagent diluent) was acquired from Waters (Milford, MA, USA). Acetonitrile, disodium ethylenediaminetetraacetic acid, phosphoric acid, sodium acetate trihydrate and triethylamine were from purchased from Sigma-Aldrich (Milwaukee, WI, USA). Amino acid standards were from Pierce (Rockford, IL, USA) and Sigma (St. Louis, MO, USA). A Perkin-Elmer HPLC system was used that included a quaternary gradient pump (Series 200) with a 50  $\mu$ L fixed volume injector coupled with an auto sampler and a fluorescent detector with excitation set at 250 nm and emission at 395 nm. The TotalChrom Navigator version 6.3.1.0504 was used for system control, data acquisition and processing.

### Methods

Aqueous 2.5 mM stock solutions of  $\alpha$ -amino butyric acid, asparagine, glutamine, hydroxyproline, ornithine, taurine, tryptophan and homocysteine were prepared in 0.1 M hydrochloric acid. Forty microliters of each stock solution and Pierce H standard mix (containing 17 amino acids) were mixed with 0.1 M hydrochloric acid to a final concentration of 0.1 mM per amino acid.

Plasma/CSF/urine sample (100  $\mu$ L) was pipetted into 2.0 mL micro-centrifuge tube and 10  $\mu$ L of DTT (for reduction of disulphide bonds and to release bound cysteine and homocysteine from proteins) was added to it. Then the solution was vortexed for 30 s. After that an equal volume (100  $\mu$ L) of 10 % sulfosalicylic acid was added to precipitate the proteins, followed by vortex mixing for 2 min. Then the samples were centrifuged at 25,000 rpm for 10 min at 4 °C yielding a clear supernatant ready to be derivatized.

For derivatization, 10  $\mu$ L standard solution or supernatant of deproteinized plasma sample was taken into the 1.5-mL Eppendorf tube. After that the standard or supernatant solution was buffered to pH 8.8 with 70  $\mu$ L of AccQ-Fluor borate buffer to yield a total volume of 80  $\mu$ L. Derivatization was done by the addition of 20  $\mu$ L of AccQ-Fluor reagent (3 mg/mL in acetonitrile) and was accelerated by heating for 10 min at 55 °C.

Separations of all the amino acids were carried out using Lichrospher 100 RP-18e (5  $\mu$ m), 250  $\times$  4.0 mm column connected to 100 CN 4.0  $\times$  4.0 mm guard column instead

of AccQ-Tag column on a quaternary HPLC system using modified gradient elution.

A series of experiments have been performed to obtain an optimum separation conditions by means of variation in the gradient blend of acetate buffer and its pH. Various gradient combinations and pH values of two acetate buffers were employed, which includes: (1) acetate buffer with pH range of 5.01–5.70; (2) acetate buffer with pH range of 6.40–6.90; (3) water and (4) acetonitrile.

Initially, the lower pH of 5.50 was found to be suitable for the separation of arginine, threonine, taurine, aspartate, proline, glutamate and higher pH of 7.50 was found to be appropriate for serine, glutamine, glycine and citrulline, however, after repetitive injections the peaks tended to broaden and merged. We could not observe good reproducibility using buffers with these pH values. Finally, satisfactory separation and reproducibility of all the amino acids were achieved using a blend of two buffers with pH values 5.25 and 6.80, water and acetonitrile.

Eluent concentrates were prepared by dissolving 148 g of sodium acetate trihydrate in 1 L of water and adding 7.06 g of triethylamine. The resulting solution was titrated to pH 5.25 and 6.80 with 50 % phosphoric acid solution or 1 M sodium hydroxide. Working eluents for separations were prepared by diluting 100 mL of eluent concentrates to 1,100 mL with water. Detection was accomplished by fluorescent detector with excitation at 250 nm and emission at 395 nm.

Amino acids were detected based on the retention time established for the individual amino acid under defined experimental conditions. Calculation was based on the area under peak established for a given amino acid of known concentration. Further, whenever a peak from test sample was required to be confirmed for a given amino acid, HPLC was repeated after spiking the amino acid(s) of interest to the sample.

Repeatability was assessed by repeatedly injecting working standard solutions on the same day and over a period of five consecutive days. The working standard solutions (5, 10, 20 and 40 pmol/ $\mu$ L) were derivatized and analysed according to the procedure. Three replicates of each concentration were injected on the same day. Inter-day and intra-day precision and accuracy were evaluated by spiking known amount of all the 26 amino acids. Precision was expressed as % CV (coefficient of variance) and % accuracy was expressed by using formula: measured concentration/nominal concentration  $\times$  100.

Three different concentrations (5, 10, 15 pmol/ $\mu$ L) were used and the samples were prepared as per the procedure described above. Inter-day precision and accuracy were assessed over a period of 3 days using replicates ( $n = 6$ ) determinations whereas intra-day precision and accuracy were assessed on three separate occasions on the same day

( $n = 6$ ) for each concentration, respectively. Limit of detection (LOD) was taken as the lowest concentration in the working standard solutions with accuracy between 80 and 120 % and limit of quantification (LOQ) was determined at signal to noise ratio of three. Linearity of the peak areas for different concentrations, ranging from 5 to 150 pmol/ $\mu$ L, of individual amino acids was determined (three points for each data).

#### High-risk screening for amino acid disorders

Overnight fasting blood samples or samples immediately before next feed in small babies were collected in heparinized and EDTA-fluoride vacutainers; centrifuged within 20 min at 3,500 $\times$ g for 10 min and processed for ammonia, amino acids and lactate, respectively. Preferably, first morning samples or random urine samples in very small babies were collected for amino acid analysis by HPLC. The CSF sample was collected when indicated.

## Results and discussion

The assay of the individual amino acids in biological fluids is of great importance in clinical laboratory. Accurate estimation of amino acids in biological fluids depends on efficiency of extraction, specificity and sensitivity of the method employed. The derivatization of amino acids with AQC is relatively very simple and fast and moreover the derivatized amino acids are stable for longer time. In the present studies, 26 amino acids including homocysteine, cysteine and citrulline were assayed with accuracy and good repeatability in plasma samples within 53 min. Cohen and Michaud (1993) introduced AQC for precolumn derivatization of amino acids and could separate 17 amino acids in approximately 35 min using ternary eluent system. We tried this method but we could not analyse some other amino acids especially citrulline, ornithine and homocysteine. In order to separate maximum amino acids in a single run, we tried method of Wandelen and Cohen (1997). We could analyse 24 amino acids with this method, however, to make it more relevant to metabolic laboratory, we modified the original method to include homocysteine, cysteine and citrulline so as to achieve separation of maximum number of physiologically important amino acids in a single run.

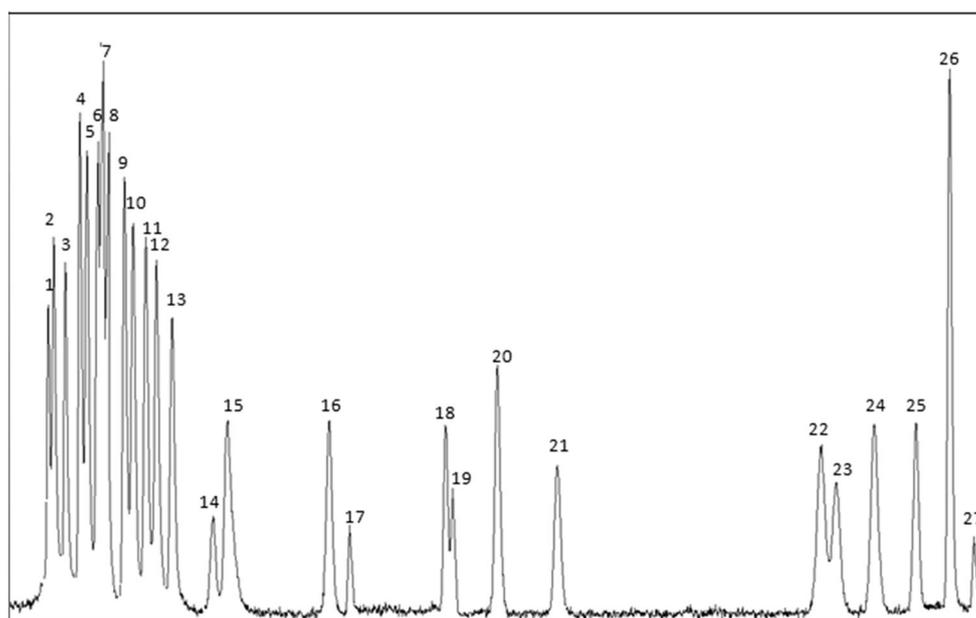
We modified the mobile phase using a blend of acetate buffer with two different pH values. The AQC linked to the amino acids does not make them completely hydrophobic, so we have adjusted the gradient system with the blend of buffers to improve the area under the curve. Here, we have used Lichrospher 100 RP-18e (5  $\mu$ m) 250  $\times$  4.0 mm column for separation which is much cheaper as compared to

**Table 1** Optimized gradient for the separation of amino acids at 37 °C

Time (min)	Flow rate (min/ml)	Acetate buffer (%) pH 5.25	Acetate buffer (%) pH 6.80	Acetonitrile (%)	Water (%)	Curve
Initial (0.5)	1	90	10	0	0	0
0.5	1	88	11	1	0	9.9 <sup>a</sup>
1	1	85	13	2	0	6 <sup>b</sup>
2.5	1	81	14	5	0	6
2.5	1	63	25	12	0	6
1.5	0.75	0	87.5	12.5	0	6
0.3	0.75	22	65.5	12.5	0	6
4.2	0.75	27	61	12	0	6
10.5	1	22	63	15	0	6
30	1	0	0	60	40	6

<sup>a</sup> Curve 9.9 is a step function

<sup>b</sup> Curve 6 is a linear segment



**Fig. 1** Elution profile of AQC-derivatized 26 standard amino acids (20 pmol/μL) Peaks: 1-aspartic acid; 2-glutamate; 3-OH-proline; 4-asparagine; 5-serine; 6-glutamine; 7-glycine; 8-histidine; 9-citrulline; 10-threonine; 11-arginine; 12-aurine; 13-alanine; 14-proline;

15-AMQ; 16- $\alpha$ -aminobutyric acid; 17-homocysteine; 18-tyrosine; 19-cysteine; 20-valine; 21-methionine; 22-ornithine; 23-isoleucine; 24-leucine; 25-lysine; 26-phenylalanine; 27-tryptophan

Waters AccQ-Tag column and can withstand 100 % aqueous media. To obtain hydrophobicity and a good retention, we have used triethylamine as a counter ion. A series of experiments have been performed to obtain an optimum separation conditions with variation in the gradient blend of acetate buffer and its pH. The optimized pH and gradient conditions are shown in Table 1. Initially, the lower pH of 5.50 was suitable for the separation of arginine, threonine, taurine, aspartate, proline, glutamate and higher pH of 7.50 was found to be more appropriate for serine, glutamine, glycine and citrulline, however, there was not good reproducibility using buffers with these pH values. Finally, satisfactory separation and reproducibility

of all the amino acids were achieved using a blend of two buffers with pH values 5.25 and 6.80, water and acetonitrile (Fig. 1).

The method was validated by evaluation of the following parameters: intra and inter accuracy, precision, limit of detection (LOD) and quantification (LOQ). LOQ values for all the amino acids were  $\geq 5$  pmol/μL and the LOD was found to be  $\geq 2$  pmol/μL. The intra-day accuracy was found to be 94.30–104.70 % with precision (RSD) less than 3.30 % (Table 2) and inter-day accuracy ranged from 93.70 to 103.10 % with RSD value from 2 to 9 % (Table 3). High resolution was achieved for a number of important amino acids including citrulline, threonine,

**Table 2** Intra-day accuracy and precision

	For 5 pmol			For 10 pmol			For 15 pmol		
	Mean	Precision	Accuracy	Mean	Precision	Accuracy	Mean	Precision	Accuracy
1	4.93 ± 0.15	3.10	102.00	9.93 ± 0.15	1.54	99.33	14.83 ± 0.30	2.06	98.89
2	4.93 ± 0.25	5.10	98.67	9.53 ± 0.05	0.61	95.33	14.83 ± 0.11	0.78	98.89
3	5.1 ± 0.20	3.92	102.00	9.80 ± 0.10	1.02	98.00	15.03 ± 0.20	1.38	100.22
4	4.90 ± 0.45	9.35	98.00	9.93 ± 0.25	2.53	99.33	15.06 ± 0.15	1.01	100.44
5	5.2 ± 0.20	3.95	105.33	10.03 ± 0.32	3.20	100.33	14.93 ± 0.37	2.54	99.56
6	4.90 ± 0.26	5.40	98.00	9.76 ± 0.15	1.56	97.67	14.96 ± 0.11	0.77	99.78
7	5.13 ± 0.20	4.06	102.67	9.93 ± 0.32	3.24	99.33	14.96 ± 0.28	1.93	99.78
8	4.83 ± 0.25	5.21	96.67	10.03 ± 0.32	3.20	100.33	14.9 ± 0.30	2.04	99.78
9	4.80 ± 0.34	7.22	96.00	9.93 ± 0.32	3.24	99.33	14.76 ± 0.23	1.56	98.44
10	4.73 ± 0.20	4.40	94.67	9.73 ± 0.05	0.59	97.33	14.93 ± 0.15	1.02	99.56
11	4.90 ± 0.26	5.40	98.00	9.93 ± 0.23	2.32	99.33	15.20 ± 0.30	1.97	101.33
12	4.76 ± 0.20	4.37	95.33	9.73 ± 0.15	1.57	97.33	15.03 ± 0.11	0.77	100.22
13	4.96 ± 0.30	6.15	99.33	9.90 ± 0.52	5.34	99.00	14.8 ± 0.26	1.79	98.67
14	4.83 ± 0.11	2.39	96.67	9.7 ± 0.20	2.14	97.33	15.33 ± 0.37	2.47	102.22
15	4.90 ± 0.20	4.08	98.00	9.97 ± 0.20	2.06	97.00	14.86 ± 0.05	0.39	99.11
16	4.83 ± 0.11	2.39	96.67	9.86 ± 0.30	3.10	98.67	15.03 ± 0.23	1.54	100.22
17	5.00 ± 0.45	9.17	100.00	9.96 ± 0.28	2.90	99.67	14.76 ± 0.15	1.03	98.44
18	5.1 ± 0.20	3.92	102.00	9.8 ± 0.58	5.96	98.33	14.80 ± 0.45	3.10	98.67
19	4.93 ± 0.15	3.10	98.67	9.6 ± 0.15	1.59	96.33	15.06 ± 0.37	2.51	100.44
20	4.83 ± 0.25	5.21	96.67	9.86 ± 0.05	0.59	98.67	15.00 ± 0.26	1.76	100.00
21	4.86 ± 0.25	5.17	97.33	10.06 ± 0.25	2.50	100.67	15.10 ± 0.40	2.65	100.67
22	4.96 ± 0.50	10.13	99.33	10.06 ± 0.15	1.52	100.67	15.20 ± 0.52	3.48	101.33
23	4.7 ± 0.15	3.23	94.67	9.7 ± 0.15	1.57	97.33	14.83 ± 0.30	2.06	98.89
24	4.90 ± 0.30	6.12	98.00	10.20 ± 0.10	0.98	102.00	14.76 ± 0.15	1.03	98.44
25	4.96 ± 0.30	6.15	99.33	9.73 ± 0.20	2.14	97.33	14.73 ± 0.15	1.04	98.22
26	4.93 ± 0.15	3.10	98.67	9.96 ± 0.20	2.07	99.67	15.00 ± 0.26	1.76	100.00

arginine, taurine, alanine, proline,  $\alpha$ -amino butyric acid, homocysteine, valine, methionine, leucine, lysine, phenylalanine and tryptophan.

Linearity ( $R^2$  value) ranged from 0.998 to 0.999 for different amino acids. Linearity of the peak areas for different concentrations, ranging from 2.5 to 200 pmol/ $\mu$ L of individual amino acids was determined (three points for each data). Linearity data were calculated by examining the area versus concentration of amino acid plots.

The optimized quaternary eluent blending conditions were used to analyse plasma samples. A typical chromatogram of control plasma sample is shown in Fig. 2. Even with the simple sample preparation procedure, separations of amino acids showed no significant interference from non-amino acid sample components. Amino acids containing sulfhydryl groups such as cysteine and particularly homocysteine are relevant in a clinical laboratory as it is increasingly implicated in cerebrovascular and cardiovascular disorders. This is the first method that has demonstrated analysis of homocysteine along with 25 other

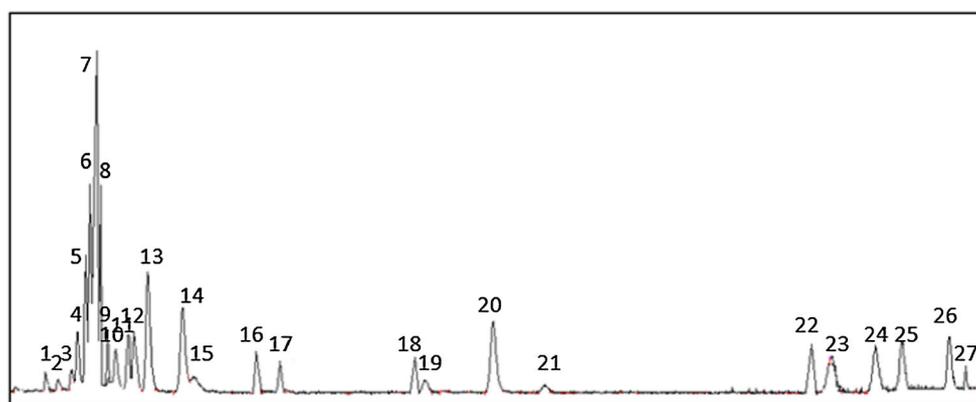
amino acids in a single run using AQC. Seo (2005) has reported analysis of homocysteine and cystathionine by AQC derivatization along with 17 other amino acids.

#### Screening of high-risk children

A total of 500 suspected cases of inborn error of metabolism had been enrolled from various wards of the Advanced Pediatric Centre like pediatric intensive care unit, neonatal intensive care unit, emergency and children attending neurodevelopmental clinic on the basis of clinical suspicion of inborn error of metabolism. Ten percent cases were neonatal presentations, 32 % were infants and 58 % cases were more than 1 year of age. On initial screening, hyperammonemia and hyperlacticacidemia were observed in 18 and 25 cases, respectively. The cyanide-nitroprusside test for cystine and homocystine was observed to be positive only in six patients and on further investigation all of these came out to be positive for homocystinuria with silver-nitroprusside test. However, on amino acid analysis

**Table 3** Inter-day accuracy and precision

	For 5 pmol			For 10 pmol			For 15 pmol		
	Mean $\pm$ SD	Precision	Accuracy	Mean $\pm$ SD	Precision	Accuracy	Mean	Precision	Accuracy
1	5.00 $\pm$ 0.17	3.46	100.00	10.03 $\pm$ 0.40	4.03	100.33	14.83 $\pm$ 0.12	0.78	98.89
2	5.03 $\pm$ 0.32	6.39	100.67	9.77 $\pm$ 0.15	1.56	97.67	15.27 $\pm$ 0.29	1.89	101.78
3	4.83 $\pm$ 0.32	6.65	96.67	9.87 $\pm$ 0.06	0.59	98.67	15.47 $\pm$ 0.15	0.99	103.11
4	4.90 $\pm$ 0.20	4.08	98.00	10.03 $\pm$ 0.21	2.07	100.33	15.33 $\pm$ 0.25	1.64	102.22
5	5.40 $\pm$ 0.20	3.70	108.00	10.23 $\pm$ 0.29	2.82	102.33	15.20 $\pm$ 0.10	0.66	101.33
6	5.03 $\pm$ 0.31	6.07	100.67	9.93 $\pm$ 0.23	2.32	99.33	15.13 $\pm$ 0.40	2.67	100.89
7	5.13 $\pm$ 0.25	4.90	102.67	9.93 $\pm$ 0.58	5.81	99.33	15.03 $\pm$ 0.51	3.41	100.22
8	5.00 $\pm$ 0.56	11.14	100.00	9.70 $\pm$ 0.26	2.73	97.00	15.20 $\pm$ 0.10	0.66	101.33
9	4.80 $\pm$ 0.46	9.55	96.00	9.64 $\pm$ 0.05	0.55	96.40	15.07 $\pm$ 0.25	1.67	100.44
10	4.97 $\pm$ 0.12	2.32	99.33	10.25 $\pm$ 0.26	2.53	102.50	48.40	120.42	322.67
11	5.10 $\pm$ 0.26	5.19	102.00	9.87 $\pm$ 0.15	1.55	98.67	15.23 $\pm$ 0.40	2.65	101.56
12	5.17 $\pm$ 0.06	1.12	103.33	9.80 $\pm$ 0.30	3.06	98.00	15.03 $\pm$ 0.40	2.69	100.22
13	5.13 $\pm$ 0.31	5.95	102.67	10.40 $\pm$ 0.20	1.92	104.00	15.07 $\pm$ 0.15	1.01	100.44
14	5.17 $\pm$ 0.40	7.82	103.33	10.27 $\pm$ 0.32	3.13	102.67	15.03 $\pm$ 0.21	1.38	100.22
15	4.80 $\pm$ 0.17	3.61	96.00	10.07 $\pm$ 0.46	4.59	100.67	15.23 $\pm$ 0.15	1.00	101.56
16	5.00 $\pm$ 0.26	5.29	100.00	9.93 $\pm$ 0.25	2.53	99.33	14.97 $\pm$ 0.21	1.39	99.78
17	4.93 $\pm$ 0.38	7.67	98.67	9.97 $\pm$ 0.21	2.09	99.67	15.37 $\pm$ 0.25	1.64	102.44
18	4.83 $\pm$ 0.65	13.46	96.67	10.17 $\pm$ 0.51	5.05	101.67	14.53 $\pm$ 0.40	2.78	96.89
19	5.03 $\pm$ 0.40	8.03	100.67	10.23 $\pm$ 0.23	2.26	102.33	15.17 $\pm$ 0.51	3.38	101.11
20	5.10 $\pm$ 0.26	5.19	102.00	9.93 $\pm$ 0.42	4.19	99.33	15.07 $\pm$ 0.38	2.51	100.44
21	5.23 $\pm$ 0.23	4.41	104.67	9.93 $\pm$ 0.15	1.54	99.33	15.20 $\pm$ 0.17	1.14	101.33
22	5.10 $\pm$ 0.20	3.92	102.00	9.80 $\pm$ 0.26	2.70	98.00	15.50 $\pm$ 0.53	3.41	103.33
23	5.33 $\pm$ 0.38	7.10	106.67	10.17 $\pm$ 0.40	3.98	101.67	14.97 $\pm$ 0.55	3.68	99.80
24	4.90 $\pm$ 0.17	3.53	98.00	9.80 $\pm$ 0.10	1.02	98.00	15.47 $\pm$ 0.51	3.32	103.11
25	5.03 $\pm$ 0.49	9.80	100.67	9.90 $\pm$ 0.17	1.75	99.00	15.33 $\pm$ 0.12	0.75	102.22
26	5.13 $\pm$ 0.40	7.87	102.67	9.93 $\pm$ 0.49	4.97	99.33	14.93 $\pm$ 0.15	1.02	99.56

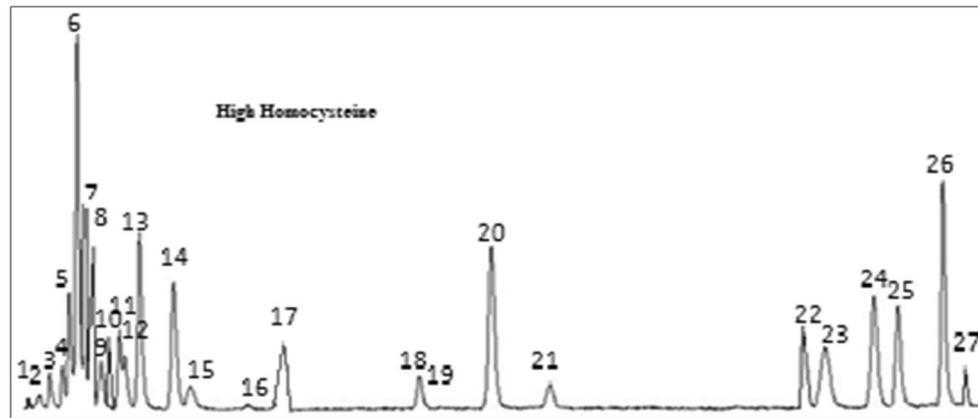


**Fig. 2** Elution profile of AQC-derivatized 26 amino acids in control plasma sample. Peaks: 1-aspartic acid; 2-glutamate; 3-OH-proline; 4-asparagine; 5-serine; 6-glutamine; 7-glycine; 8-histidine; 9-citrulline; 10-threonine; 11-arginine; 12-aurine; 13-alanine; 14-proline;

15-AMQ; 16- $\alpha$ -aminobutyric acid; 17-homocysteine; 18-tyrosine; 19-cysteine; 20-valine; 21-methionine; 22-ornithine; 23-isoleucine; 24-leucine; 25-lysine; 26-phenylalanine; 27-tryptophan

by HPLC, we were able to pick up 12 children who were found to have hyperhomocysteinemia and homocystinuria, out of which two children had classical homocystinuria,

four had cobalamin deficiency/defect and six had moderate hyperhomocysteinemia. Other cases which we could identify were non-ketotic hyperglycinemia (2),



**Fig. 3** Representative plasma HPLC profile showing hyperhomocysteinemia (peak 17). Peaks: 1-aspartic acid; 2-glutamate; 3-OH-proline; 4-asparagine; 5-serine; 6-glutamine; 7-glycine; 8-histidine; 9-citrulline; 10-threonine; 11-arginine; 12-taurine; 13-alanine;

14-proline; 15-AMQ; 16- $\alpha$ -aminobutyric acid; 17-homocysteine; 18-tyrosine; 19-cysteine; 20-valine; 21-methionine; 22-ornithine; 23-isoleucine; 24-leucine; 25-lysine; 26-phenylalanine; 27-tryptophan

hyperprolinemia (1), maple syrup urine disease (1), urea cycle defect (1) and ketotic hyperglycinemia (1).

#### *Classical homocystinuria: two cases*

Homocystinuria represents a group of hereditary metabolic disorders characterized by an accumulation of homocysteine in the serum and urine. Cystathionine- $\beta$ -synthase (CBS) deficiency is the most common cause associated with homocystinuria. The clinical manifestations include mental retardation, ectopia lentis, secondary glaucoma, retinal detachment, skeletal abnormalities, osteoporosis, neurological dysfunction and psychiatric symptoms. Thrombotic and cardiovascular diseases may also be encountered.

Eight-year-old female child, born by non-consanguineous marriage, normal vaginal delivery and without any adverse perinatal events was found to have developmental delay. On investigation, ammonia and lactate were normal with normal blood pH. Cyanide-nitroprusside and silver-nitroprusside tests for homocystine were positive. Plasma homocysteine levels by HPLC were 189  $\mu\text{mol/L}$  (normal 5–15  $\mu\text{mol/L}$ ) (Fig. 3), plasma methionine levels were 152  $\mu\text{mol/L}$  (normal 13–41  $\mu\text{mol/L}$ ) and urine homocystine levels were 208 mmol/mol creatinine (0.2–4.0 mmol/mol creatinine). On the basis of hyperhomocysteinemia and hypermethioninemia, the child was diagnosed to have CBS deficiency.

Seven-year-old male child was diagnosed to have classical homocystinuria. The child presented with swelling of left lower limb (deep vein thrombosis) and pain over left lower limb from 2 days. There was no history of trauma, constipation, abdominal distension and decreased urinary output. Developmental delay was present. His plasma homocysteine and methionine levels were 145 and

92  $\mu\text{mol/L}$ , respectively, and urine homocystine levels were found to be 367 mmol/mol creatinine. The child was started on treatment.

#### *Other homocystinuria cases: six cases*

The plasma and urine levels of homocysteine and clinical details of other homocystinuria patients have been provided in Table 4.

#### *Cobalamin deficiency/defect: four cases*

Vitamin B<sub>12</sub> (cobalamin, Cbl), in the form of the co-factors methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl), is required by the cytoplasmic enzyme methionine synthase and by methylmalonyl-CoA mutase, respectively. Inherited disorders of intracellular cobalamin are rare conditions and have been classified on the basis of eight different mutant groups (CblA to CblJ) (Coelho et al. 2012; Fowler et al. 2008; Kim et al. 2012). The defect of these two cofactors causes the accumulation of methylmalonic acid and homocysteine in body fluids and a decrease of methionine.

A 5-year-old female child presented with history of developmental delay, sub-acute progressive afebrile encephalopathy and on examination found to have bradycardia, microcephaly, severe encephalopathy with bilateral hypertonia and tremor-like movements of neck and dystonic posture of both hands. Her initial screening revealed positive silver-nitroprusside test for homocystine and MMA test for methylmalonic acid. HPLC analysis revealed hyperhomocysteinemia (185  $\mu\text{mol/L}$ ) and homocystinuria (213 mmol/mol creatinine). Her urine GCMS analysis showed very high levels of methylmalonic acid [5160 mmol/mol creatinine (normal <5 mmol/mol

**Table 4** Plasma and urine levels of homocysteine and clinical details of other homocystinuria patients

S. no.	Age, gender	Plasma Hcy ( $\mu\text{mol/L}$ )	Urine Hcy (mmol/mol creatinine)	Clinical findings
1	7 years, M	15	8.2	Referred from Eye OPD for workup of lens subluxation. H/o seizure onset at D <sub>20</sub> of age. CT scan showed left basal ganglia infarct. Long face, large ears, high arched palate, long fingers
2	11 years, M	9.2	6.02	Right side hemiparesis since birth. Focal seizures since past 4 years
3	12 years, F	43	9.4	Child presented with right-sided weakness and recurrent stroke
4	7 years, M	46	5.3	Global developmental delay with right subluxated lens
5	11 years, M	58	49	Progressive loss of vision for the past 4 years. Poor school performance. No history of trauma, seizures, pain in eyes. Bilateral lens dislocation
6	10 years, M	42	13	Tall stature from birth, defective vision from 3 years. Family h/o similar illness in mother. No history of altered sensorium, seizures, abnormal body movements, palpitation and breathlessness. Development was normal

creatinine)] which confirmed the diagnosis of cobalamin defect as her vitamin B<sub>12</sub> levels were also found to be normal. Other three cases with hyperhomocysteinemia were also found to have homocystinuria and methylmalonic aciduria and were labelled as cobalamin deficiency/defect and are on follow-up for further workup.

#### *Non-ketotic hyperglycinemia: two cases*

Non-ketotic hyperglycinemia (NKH) is an inborn error of glycine degradation in which large quantities of glycine accumulate in all body tissues, including the central nervous system. The diagnosis is established by calculating the cerebrospinal fluid/plasma glycine concentration ratio. A value of greater than 0.08 is diagnostic. Confirmation of the diagnosis requires measurement of the activity of the glycine cleavage system in liver tissue. The disorder is clinically divided into two groups: neonatal severe and variant late-onset (Hamosh and Johnston 2000).

A case of non-NKH was observed in a 23-day-old neonate having lethargy, hypotonia, intractable seizures. Plasma ammonia and lactate were normal. There was no acidosis and no urinary ketones. The plasma, CSF, urine glycine levels in this neonate were found to be 529  $\mu\text{mol/L}$  (normal 121–407) (Fig. 4), 54  $\mu\text{mol/L}$  (normal 3.7–7.6  $\mu\text{mol/L}$ ) and 1,207 mmol/mol creatinine (normal 283–1097). The ratio of CSF to plasma glycine was 0.10 and was diagnostic of NKH.

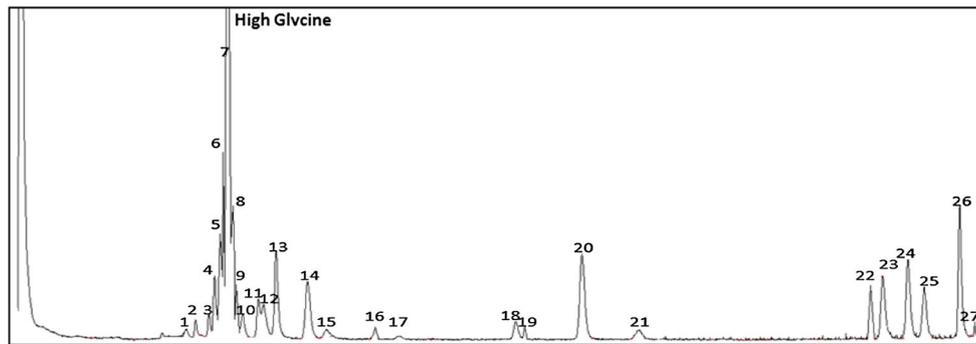
A 21-year-old girl presented with emotional lability for 9 months, impaired memory for 7 months, unsteadiness of gait and change in voice for 4 months. She had history of recurrent falls sideways and forwards, smearing of mouth while eating, slowness of all activities for 1½ months and medial deviation of left eye for 10 days. There was no metabolic acidosis and urinary ketones were also absent.

Her blood samples were sent to our laboratory to see if any metabolic cause was there. Her plasma glycine was found to be 498  $\mu\text{M/L}$  (normal 121–407) and urine analysis also showed higher peak of glycine with a value of 416 mmol/mol creatinine (43–173 mmol/mol creatinine). Then simultaneous samples of CSF and plasma were asked to look for NKH. The second plasma sample revealed a glycine level of 512  $\mu\text{mol/L}$  and CSF glycine level in this case was found to be 60  $\mu\text{mol/L}$  (normal 0.7–15  $\mu\text{mol/L}$ ) giving a CSF: plasma ratio of 0.12 which is more than diagnostic for late-onset NKH which was accepted by the neurologist.

#### *Hyperprolinemia: one case*

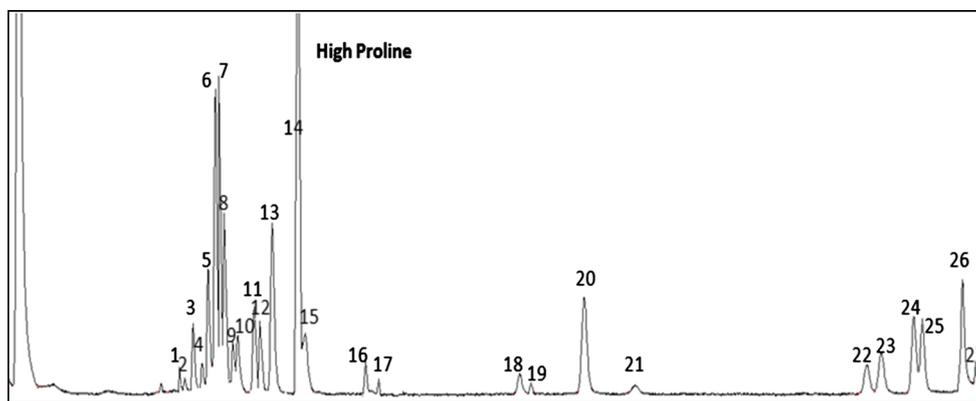
Hyperprolinemia generally occurs when proline is not broken down properly by the body. There are two inherited forms of hyperprolinemia, called type I and type II. Originally described in 1961, type I hyperprolinemia (HPI) is an autosomal recessive disorder caused by deficiency of proline oxidase. The phenotype of HPI has been considered benign or highly variable, but this view is under revision. Typically, plasma proline concentrations range from 500 to 2,200  $\mu\text{mol/L}$  (normal 51–271  $\mu\text{mol/L}$ ) and there is no P5C detectable in urine.

One female child at 11 months of age was seen in neurology clinic for global developmental delay with one episode of seizure, blue sclera with low-set bat-shaped ears with syndactyly. She was not able to hold her head, sit without support and roll over. She had previous history of neonatal jaundice sepsis/meningitis. Her mother had history of one previous spontaneous abortion. Her urine MR screen was negative, thyroid profile, routine biochemical tests like RFT, LFT, blood sugar were all normal. Only cholesterol levels were on higher side, i.e. 202 mg/dL. Ammonia and lactate levels were also within normal range.



**Fig. 4** Representative plasma chromatogram with non-ketotic hyperglycinemia. Peaks: 1-aspartic acid; 2-glutamate; 3-OH-proline; 4-asparagine; 5-serine; 6-glutamine; 7-glycine; 8-histidine; 9-citrulline; 10-threonine; 11-arginine; 12-taurine; 13-alanine; 14-proline;

15-AMQ; 16- $\alpha$ -aminobutyric acid; 17-homocysteine; 18-tyrosine; 19-cysteine; 20-valine; 21-methionine; 22-ornithine; 23-isoleucine; 24-leucine; 25-lysine; 26-phenylalanine; 27-tryptophan



**Fig. 5** Representative plasma HPLC profile of a baby with hyperprolinemia. Peaks: 1-aspartic acid; 2-glutamate; 3-OH-proline; 4-asparagine; 5-serine; 6-glutamine; 7-glycine; 8-histidine; 9-citrulline; 10-threonine; 11-arginine; 12-taurine; 13-alanine; 14-proline;

15-AMQ; 16- $\alpha$ -aminobutyric acid; 17-homocysteine; 18-tyrosine; 19-cysteine; 20-valine; 21-methionine; 22-ornithine; 23-isoleucine; 24-leucine; 25-lysine; 26-phenylalanine; 27-tryptophan

Her plasma amino acid levels revealed high proline levels (621  $\mu\text{mol/L}$ ) (Fig. 5), normal range being 52–343  $\mu\text{mol/L}$  for her age group. The plasma amino acid profile was repeated on three separate occasions to reconfirm the findings. The urine amino acid profile also showed massive amounts of proline (127 mmol/mol creatinine) (0–9 mmol/mol creatinine), glycine (357 mmol/mol creatinine) (91–246 mmol/mol creatinine) and hydroxyproline (45 mmol/mol creatinine) (0–13 mmol/mol creatinine). These findings clearly suggested hyperprolinemia. To further see whether it was type 1 or type 2, we got her urine pyrroline-5-carboxylate (P5C) levels checked from outside and her urine did not show any P5C levels. So the diagnosis of type 1 hyperprolinemia was made.

#### *Ketotic hyperglycinemia: one case*

Child was normal till day two of life, subsequently had poor feed intake and excessive sleepiness. Also child was

noted to have rapid breathing. So child was brought to Pediatric Emergency. At presentation, child was in decompensated state. He was having pulse of 180/min and respiratory rate of 80/min. Child was having hypothermia and hypoglycemia which were corrected at admission. Subsequently child was having repeated posturing. So this child was intubated and put on ventilator. He also developed shock so required inotropic support. On evaluation child was having anaemia, thrombocytopenia, transaminitis and hyperammonemia (1,800  $\mu\text{mol/L}$ ) with high anion metabolic acidosis. His lactate level was also elevated. His urine was positive for ketones. The qualitative methylmalonic acid test was negative. His plasma HPLC revealed increased glycine 890  $\mu\text{mol/L}$  (normal 121–407), along with moderately raised alanine 626  $\mu\text{mol/L}$  (135–590  $\mu\text{mol/L}$ ), leucine 283  $\mu\text{mol/L}$  (53–135  $\mu\text{mol/L}$ ), isoleucine 195  $\mu\text{mol/L}$  (53–135  $\mu\text{mol/L}$ ), valine 381  $\mu\text{mol/L}$  (112–242  $\mu\text{mol/L}$ ) and glutamine 1,184  $\mu\text{mol/L}$  (300–800  $\mu\text{mol/L}$ ). The child was suspected to have ketotic hyperglycinemia

or propionic acidemia which was later confirmed by GC/MS.

#### *Urea cycle defect: one case*

Eleven-year-old developmentally delayed male child presented to Pediatric Emergency with features of altered sensorium and ataxia for 5 days. This child was born to non-consanguineous marriage with no adverse perinatal events. Cardiovascular and respiratory examinations were unremarkable. His interactions with surroundings and parents were lost. He had increased tone in lower limb with brisk reflexes. Imaging studies were suggestive of periventricular hypo-intensity which was consistent with features of hypoxic ischemic encephalopathy. Ammonia was 227  $\mu\text{mol/L}$  and lactate was 0.4 mmol/L. His blood pH was normal. This child had high levels of arginine 632  $\mu\text{mol/L}$  (30–150  $\mu\text{mol/L}$ ), citrulline 158  $\mu\text{mol/L}$  (8–47  $\mu\text{mol/L}$ ) and glutamine 1,112  $\mu\text{mol/L}$  (<809  $\mu\text{mol/L}$ ). The TMS analysis on dried blood filter paper sample also showed high levels of arginine and citrulline. His urine sample showed elevated orotic acid levels. The child was probably having late-onset argininemia.

#### *Maple syrup urine disease (MSUD): one case*

One-year-old boy, developmentally normal, born of non-consanguineous marriage presented with a short history of multiple episodes of vomiting and altered sensorium for 2 days for which, child had received treatment (ceftriaxone and amikacin) by the referring physician. The child was also reported to have hypoglycemia. The past history of elder sibling death at the age of 2 years was present who also had short history of vomiting and altered sensorium, but the cause of death was not documented.

At admission child was in decompensated shock which was refractory to fluid therapy and required inotropic support. He also had respiratory failure for which he required ventilation. His anthropometry was satisfactory. On examination child had gross hypotonia with depressed reflexes, but no dysmorphic features or organomegaly.

On basis of acute symptoms and significant past history of sibling death due to similar complaint, child was screened for inborn errors of metabolism. On investigation, the child was having high anion gap metabolic acidosis, hypoglycemia with ketonuria. Ammonia was mildly elevated and lactate was normal. The dinitrophenylhydrazine test for MSUD was positive. HPLC analysis of his plasma sample revealed high level of branched chain amino acids: leucine 543  $\mu\text{mol/L}$  (50–140  $\mu\text{mol/L}$ ); isoleucine 448  $\mu\text{mol/L}$  (40–105  $\mu\text{mol/L}$ ) and valine 403  $\mu\text{mol/L}$  (112–242  $\mu\text{mol/L}$ ). So the diagnosis of MSUD was kept in this child.

## Conclusions

We describe an HPLC method that is suitable for simultaneous analysis of 26 amino acids including homocysteine in physiological fluids in a single run. This method demonstrates good precision, repeatability and reproducibility for all the amino acids analysed. Our study indicates that the hyperhomocysteinemia can be identified along with other aminoacidopathies in a single analysis. The prevalence of amino acid disorders in India is distinct from other countries. We have identified homocystinuria to be the most common (2.4 %) amino acid disorder among high-risk children in a tertiary care hospital from North India followed by NKH, hyperprolinemia, ketotic hyperglycinemia, urea cycle defect and MSUD.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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