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Quantitation of free amino acids in plasma and muscle samples in healthy subjects and uremic patients by high-performance liquid chromatography and fluorescence detection

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Abstract An automatic on-line high-performance liquid chromatographic (HPLC) method is developed to quantitate free amino acids in the biological samples The method is based on pre-column derivatization of amino acids with orthophthalaldehyde (OPA) in presence of 2-mercaptoethanol (2-ME) The derivatized amino acids were separated on a 5 μm C_{18} column using a multi-step gradient with two solvents and the detection was made at E_{ex} = 340 nm and E_{em} = 450 nm The results were highly reproducible with a relative standard deviation (RSD) between 0 5–2% for all amino acids Each chromatographic run was completed within 40 min to separate 24 amino acids The optimized method was applied to evaluate the levels of free amino acids in plasma and muscle samples of eight healthy subjects and 13 uremic patients under fasting conditions

Keywords *High-performance liquid chromatography, orthophthalaldehyde, uremic patients, amino acid assay, plasma amino acids, muscle amino acids*

Introduction

Amino acids are among the most important constituents of a human body in assessing the nutritional requirements under various pathological states Hence, the quantitation of amino acids in biological matrices is one of the most challenging tasks in the biomedical sciences Most of the amino acid analyses are performed by the classical ion-exchange chromatography based on a post-column derivatization with ninhydrin introduced nearly 30 years ago [1] This technique has improved, and analysis time has been shortened from 2 days to 3 h but, problems are yet to be solved in the broadening of peaks, inaccuracy in quantitation of basic amino acids, buffer contamination, baseline shift and the identification of amino acids in physiological samples [2] Some of the errors and interferences appear to be especially prominent when analysing samples from patients with renal failure, due to the presence of contaminating metabolites which accumulate in uremia [3–7]

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In search of new methodology, various post-column derivatization procedures have been developed until the introduction of orthophthalaldehyde (OPA) by Roth in 1973 [8] This reagent reacts with primary amines in presence of 2-mercaptoethanol (2-ME), producing a highly fluorescent product within a few minutes at room temperature The reaction product is very sensitive to ninhydrin and has been used successfully for the quantitation of amino acids in biological samples [9, 10] Since the product of OPA/2-ME amino acid has a hydrophobic character, this characteristic has been utilized in the pre-column derivatization mode for the separation of amino acids *in vivo* and *in vitro* [11–14]

In this study, we describe an automatic on-line procedure, based on a pre-column derivatization of amino acids with OPA/2-ME, to analyse free amino acids in plasma and muscle samples The system is automated to react OPA with amino acids for 2 min before the injection is made onto a 5-μm RP-C_{18} column, and the detection is made at E_{ex} = 340 nm and E_{em} = 450 nm The whole chromatographic run lasted for 40 min to separate 24 amino acids by the use of a multiple-step gradient with two solvents The reproducibility of the method is within the acceptable range giving relative standard deviations (RSD) between 0 5–2% for all amino acids in five duplicate runs

Abnormal patterns of plasma and muscle free amino acids are observed in patients with chronic renal failure which are to some extent similar to those found in protein malnutrition, but in other respects typical of the uremic state [15] The results obtained from eight healthy subjects and 13 uremic patients are compared by students *t*-test and the rôle of some of the essential and non-essential amino acids in chronic uremia is briefly discussed

Experimental

Chemicals

Individual crystalline salts of L-amino acids (AMAC Standard Kit No 20065), Brij (30%) and OPA were obtained from Pierce Eurochemie (The Netherlands) Phosphoserine, 3-methylhistidine, citrulline, carnosine, cysteine-sulphinic acid (Cys-S), α-, β-, γ-aminobutyric acid, taurine, β-alanine, and 2-ME were obtained from Sigma Chemical Co (St Louis, MO, USA) Methanol "HPLC grade" was obtained from Rathburn Chemicals (Walkerburn, UK) Anhydrous sodium dihydrogen phosphate, disodium hydrogen phosphate, 5-sulphosalicylic acid (SSA), boric acid, sodium hydroxide and hydrochloric acid were all analytical reagent grade and were obtained from Merck (Darmstadt, FRG)

Individual standard stock solutions of amino acids (1 mM) were prepared in distilled water by addition of few drops of 0 1 M HCl A standard mixture containing 24 amino acids was prepared with the similar concentration and stored at −70°C This standard mixture was diluted to 0 01, 0 02, 0 03, 0 05 and 0 1 mM to construct a linear relationship between the fluorescence intensity and the concentration of individual amino acids Water used for the preparation of buffers and standards was deionized and sterile (Milli-Q, Water Purification System, obtained from Millipore Corporation, MA, USA)

OPA reagent

Anhydrous OPA (100 mg) was dissolved in 2 ml of methanol to which 8 ml of 0 4 M borate buffer of pH 10 4 containing 0 6% Brij were added To this mixture, 200 μl of 2-

ME were added The reagent mixture was kept in dark at 4°C for 24 h before use The reagent remains stable for 1 week

Buffer and gradient used

Sodium phosphate buffer of pH 7 2 (0 05 M) was prepared by gradually adding 0 05 M NaH_2PO_4 to 0 05 M NaH_2HPO_4 This buffer was diluted to a concentration of 0 02 M with deionized water For pump A, a mobile phase consisting of phosphate buffer/methanol/tetrahydrofuran (98 1 1, solvent A) was used, and for pump B, a mobile phase consisting of phosphate buffer/methanol (32 68, solvent B) Both these solvents were filtered through 0 45 μm filter paper type HA and HV (Millipore, Bedford, MA, USA), respectively The gradient used is shown (%B) in Fig 1 The flow rate was maintained at 1 ml min^{-1} throughout the chromatographic run except for the first 2 min when it was increased linearly from 0 2 to 1 ml min^{-1} The column was equilibrated with solvent A for 6 min between the injections

Biological samples and their treatment

Venous plasma samples were obtained from eight healthy subjects (six males and two females, age 43 ± 11 years, mean ±SD) and from 13 chronic uremic patients (seven

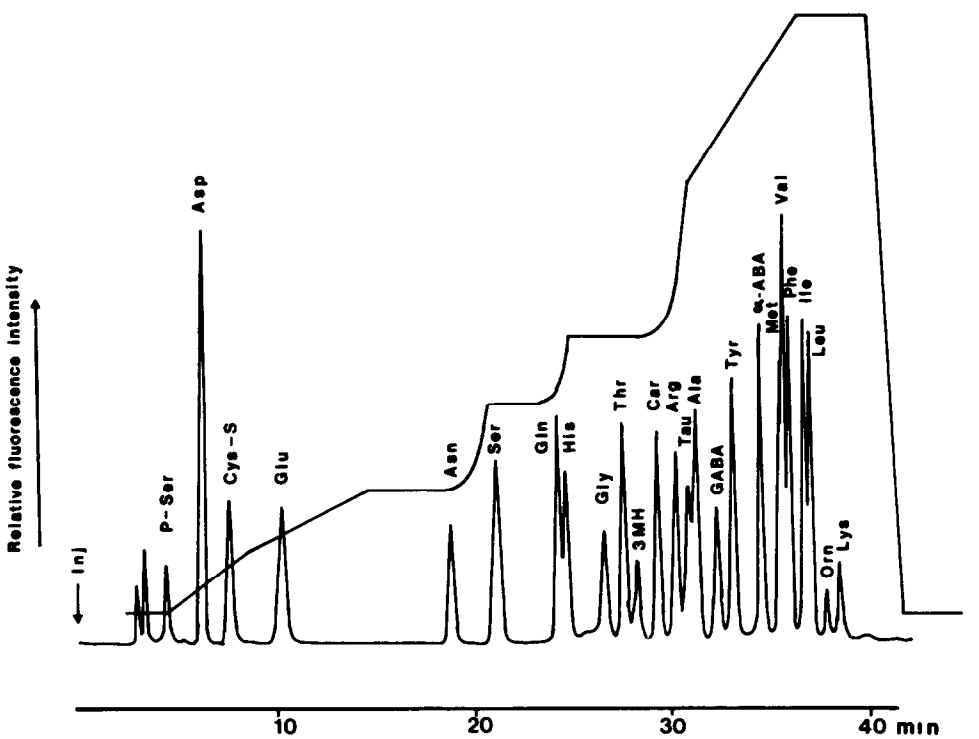

Figure 1

Typical chromatogram showing the separation of 24 standard (2 5 μmol) OPA/2-ME amino acid derivatives on 5 μm Lichrosorb-C_{18} (125 × 4 mm, i d) by the use of the mobile phases 1 1 98 (solvent A) tetrahydrofuran/methanol/0 02 M phosphate buffer (pH 7 1) and 32 68 0 02 M phosphate buffer (pH 7 1)/methanol (solvent B) The gradient used is shown in % B A flow rate of 1 ml min^{-1} was maintained throughout the chromatographic run except for the first 2 min when it was increased linearly from 0 2 to 1 ml min^{-1} The detection was made at E_{ex} = 340 nm and E_{em} = 450 nm The separation was performed at ambient temperature

males and six females, age 57 ± 12 years) Four of the uremic patients were non-dialysed, six were treated with continuous peritoneal dialysis (CAPD) and three with maintenance hemodialysis

Muscle samples were taken from eight healthy subjects (two males and six females, age 42 ± 11 years) and eight chronic uremic patients (six males and two females, age 57 ± 11 years) Six of the patients were non-dialysed and two were treated with CAPD Informed consent was obtained from each patient and control subject The study protocol was approved by the Ethics Committee of Karolinska Institute

All plasma and muscle samples were collected in the morning after an overnight fast Muscle tissue was obtained by percutaneous needle biopsy from the lateral portion of the quadriceps femoris muscle as previously described [16] Aliquots of the muscle samples (15–25 mg) were carefully dissected to remove all visible connective tissue and fat Water was determined by weighing before and after drying at 90°C to constant weight The amount of neutral fat was calculated by weighing the dried samples after extraction with petroleum ether The amino acid content of muscle was expressed as $\mu M\ g^{-1}$ fat-free solids

Plasma samples (100 μl) were diluted to 800 μl with distilled water and 100 μl of 100 μM internal standard (β-amino butyric acid) was added before deproteinization with 100 μl of 30% SSA The sample was centrifuged at 3000*g* for 15 min and the supernatant collected Muscle samples were deproteinized with 0 5 ml of 5% SSA, centrifuged at 1500*g* for 15 min and the supernatant collected All samples were stored at −70°C if not analysed immediately

The derivatization procedure

The derivatization of OPA with sample or standard was done according to Qureshi *et al* [12]

Apparatus

The chromatographic system consisted of two high-pressure delivery pumps M45 and 6000A, a multiple sampler WISP 710B, a data module, a system controller 730B and fluorescence detector model 420-AC with the monochromator set at 340 nm with 450 nm cut-off filter, all supplied by Water Associates (Milford, MA, USA)

The separation of amino acids was carried out by 5 μm Lichosorb C_{18} (100 × 4 mm, i d) obtained from Merck (Darmstadt, FRG) A pre-column (4 × 4 mm, i d) containing similar material was connected between the injector and analytical column The column was conditioned first with water/methanol (50 50) and then with solvent A for 1 h before use

Results and Discussion

Chromatographic methodology

Figure 1 shows the elution profile of OPA-derivatized amino acids chromatographed under our experimental conditions Each peak represents 2 5 μM of amino acid Running the standard 7 times, the quantitation of individual amino acids showed high reproducibility in terms of area and retention time giving RSD between 0 73–3 86 and 0 05–1 86%, respectively The detection limit for each amino acid is <1 pmol A linear relationship was found between the fluorescence intensity and the amount of amino acids injected in the concentration range of 5–100 μM per injection of 20 μl with r^2 equal to

unity The linearity of fluorescence response was maintained over a wide range of amino acid concentrations However, it is important to use 10-fold excess of derivatization reagent (OPA) to the total amount of amino acids in order to maintain a linear response

The whole separation of 24 amino acids is accomplished within 40 min which constitutes a definite improvement over the commonly used post-column ion-exchange chromatographic system Pre-column systems on the similar derivatization reaction have already been used requiring shorter or longer analysis time depending on the particle size and length of the column in operation [11–13, 17, 18] But in a complex biological sample, the precision for quantitation and complete separation of amino acids without any overlap are the most important factors to be controlled, enabling a chromatographist to maintain a confidence on the method Among these methods, in one study [13], a shorter column was used to record the separation of amino acids within 20 min, but at the same time the authors were unable to detect glutamine (Gln) in a serum sample of a healthy person Gln has one of the highest concentrations among the amino acids On the other hand, use of smaller particles in a short column, decays the column performance from sample to sample, and this could be a disadvantage in securing the shortest possible analysis time at the cost of losing the separation Figures 2 and 3 show the chromatograms of plasma and muscle samples, respectively, from an uremic patient Most of the amino acids are well separated and it is noteworthy that there is no baseline shift, a problem which is often encountered when ion-exchange chromatography is used for amino acid analysis in uremic muscle samples

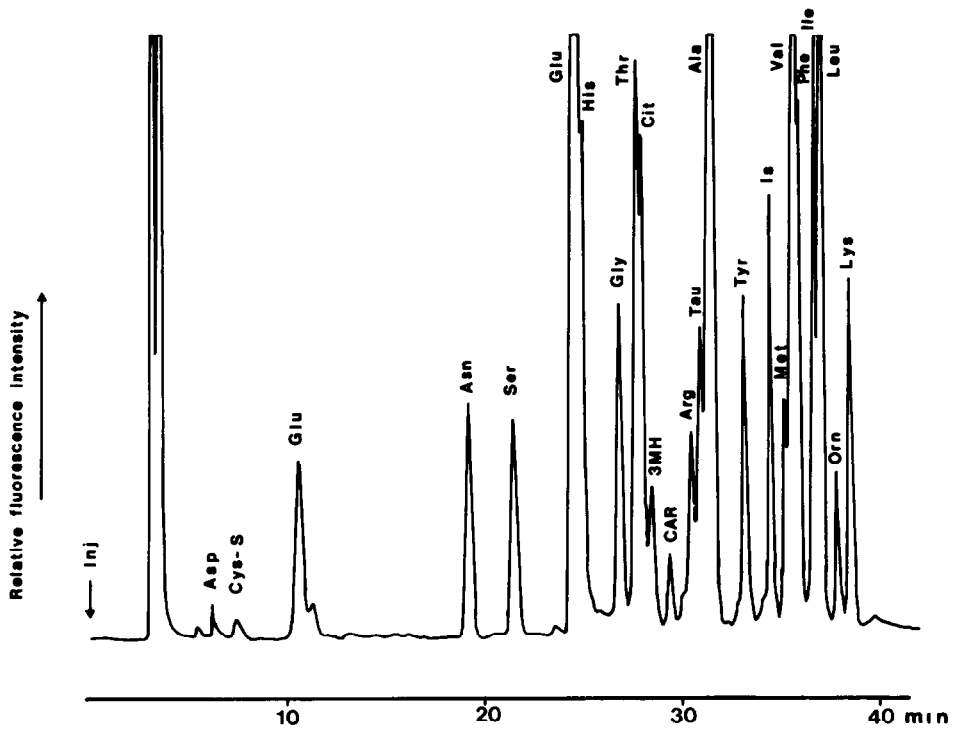

Figure 2
Chromatogram of plasma amino acids as OPA/2-ME derivatives from an uremic patient under identical conditions to those in Fig 1

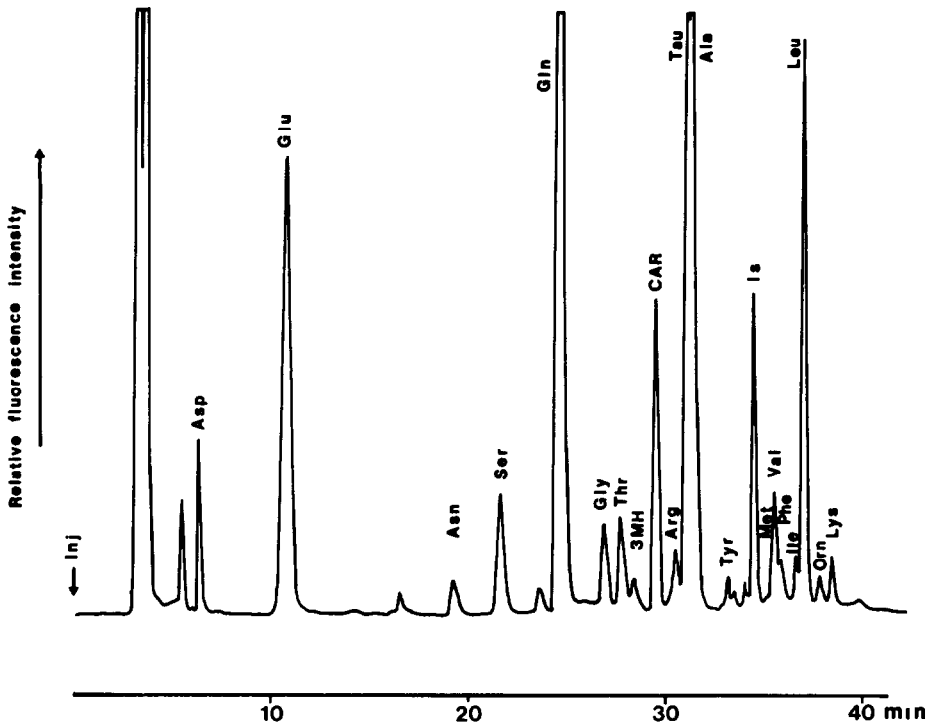

Figure 3
Chromatogram of muscle amino acids as OPA/2-ME derivatives from an uremic patient under identical experimental conditions to those in Fig 1

Clinical findings

Table 1 shows the concentrations of amino acids in plasma from eight healthy subjects and 13 uremic patients In the patients there were statistically decreased plasma concentrations of valine (Val), isoleucine (Ile), phenylalanine (Phe) [essential amino acids], serine (Ser), tyrosine (Tyr) and taurine (Tau) [non-essential amino acids], and increased concentrations of glutamate (Glu) and 3-methylhistidine (3-MH) Cys-S was not detected in normal plasma but was found to be present in plasma from the uremic patients

Table 2 shows the muscle concentration of amino acids in eight healthy subjects and eight uremic patients Val, threonine (Thr) [essential amino acids], arginine (Arg), Gln and Tau [non-essential amino acids], were significantly decreased in the uremic patients, while methionine (Met) and some of the non-essential amino acids were increased

Most of these results agree with earlier observations [15, 19–22] The decrease in plasma of essential amino acids in uremia has been considered as an indicator of protein deficiency, whereas the increase in 3-MH and decrease in Ser may be a consequence of reduced renal function and metabolism

The accumulation of Cys-S in uremic plasma in presence of low plasma and muscle Tau could be due to a block of the metabolic pathway leading to Tau biosynthesis in uremia [23, 24]

Skeletal muscle is the most abundant cellular tissue in the body and has an important rôle in the control of protein metabolism as it is known that the muscle protein synthesis

Table 1
Plasma free amino acids in normal subjects ($n = 8$) and uremic patients ($n = 13$) All values are expressed as mean ±SD in $\mu M\ l^{-1}$

Amino acid	Normal subjects	Uremic patients
Asp	11 5 ± 4 1	18 1 ± 7 0*
Cys-S	ND	13 7 ± 4 0
Glu	34 6 ± 11 3	57 4 ± 15 7**
Asn	87 4 ± 17 1	131 6 ± 28 4**
Ser	94 8 ± 8 2	61 2 ± 7 0***
Gln	460 0 ± 45 0	485 0 ± 97 3
His	81 8 ± 8 8	71 3 ± 12 8
Gly	225 5 ± 19 8	272 1 ± 27 0
Thr	112 1 ± 18 9	136 0 ± 31 7
Cit	35 0 ± 6 0	53 0 ± 11 0
3-MH	7 8 ± 3 3	74 1 ± 12 3***
Arg	109 6 ± 23 6	99 5 ± 29 5
Tau	60 1 ± 8 5	46 0 ± 12 2
Ala	289 6 ± 46 7	252 3 ± 18 0
Tyr	64 7 ± 3 6	42 5 ± 9 6***
Met	27 6 ± 2 9	29 1 ± 5 1
Val	203 3 ± 25 2	115 6 ± 17 7***
Phe	62 5 ± 7 7	49 3 ± 9 9**
Ile	66 4 ± 10 4	50 7 ± 8 2**
Leu	151 8 ± 27 0	135 0 ± 14 8
Orn	49 4 ± 13 0	55 4 ± 7 3**
Lys	196 3 ± 16 0	98 7 ± 10 7***

* $P < 0\ 05$, ** $P < 0\ 01$, *** $P < 0\ 001$

Table 2
Muscle free amino acids in normal subjects ($n = 8$) and uremic patients ($n = 8$) All values are expressed as mean ±SD in $\mu M\ g^{-1}$ fat free muscle

Amino acid	Normal subjects	Uremic patients
Asp	2 07 ± 0 28	3 00 ± 0 35**
Glu	18 24 ± 1 90	16 84 ± 3 76
Asn	1 35 ± 0 22	1 74 ± 0 22*
Ser	4 80 ± 0 64	5 07 ± 0 68
Gln	77 30 ± 5 20	67 81 ± 6 61*
Gly	7 01 ± 0 71	8 30 ± 0 73*
Thr	4 78 ± 0 35	3 95 ± 0 39**
Arg	23 92 ± 1 75	20 40 ± 2 77*
Tau	82 04 ± 5 37	66 94 ± 9 04*
Ala	9 13 ± 1 09	16 91 ± 2 96**
Tyr	0 97 ± 0 08	0 99 ± 0 11
Met	0 65 ± 0 06	0 82 ± 0 05**
Val	2 18 ± 0 11	1 74 ± 0 26**
Phe	1 28 ± 0 09	1 34 ± 0 10
Ile	1 01 ± 0 07	0 98 ± 0 16
Orn	4 66 ± 0 55	4 11 ± 0 65**
Lys	4 66 ± 0 55	4 48 ± 0 92

* $P < 0\ 01$, ** $P < 0\ 001$

accounts for about 50% of the total body synthesis [25] Hence, studies of free amino acids in muscle may further elucidate the role of amino acids in uremia Low muscle concentrations of Val and Thr have been described earlier in non-dialysed patients with chronic renal failure, and intracellular Val depletion in presence of normal Ile and leucine (Leu) levels appears to be a typical feature of uremia [7, 26]

In the present study we also observed a slight but significant decrease in muscle Gln levels in the uremic patients This has not been observed earlier in uremia, but low intracellular Gln concentrations have been recorded in patients with post-traumatic catabolism [27–29]

By performing this study, it is shown that the HPLC system is an appropriate and suitable alternative to the classical ion-exchange chromatography for the quantitation of free amino acids with precision in biological samples for the following reasons

1 Small volumes of samples are required and the samples can be injected directly after simple deproteinization with SSA
2 The system is fully automated and 40 min is required to analyse 24 amino acids More than 25 samples can be run in a day
3 The reason kinetics of OPA/amino acid are strictly controlled to provide reproducible results
4 There is no baseline shift in chromatograms from uremic muscle, a problem usually encountered with ion-exchange chromatography
5 The analysis is conducted at room temperature

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