Quantitation of Intracellular Tryptophan and its Metabolite 5-Hydroxy-Indole-3-Acetic Acid in Uremic Patients

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Summary: High performance liquid chromatographic method with electrochemical detection was used to quantitate leveis of tryptophan (Trp) and its metabolite 5-hydroxy-indole-3-acetic acid (5-HIAA) levels in erythrocytes and leucocytes of 41 uremic patients. These patients were treated by maintenance hemodialysis (HD, n=18) and continuous ambulatory peritoneal dialysis (CAPD, n=25). The method of analysis is accurate, sensitive and reproducible. The results accumulated in uremic patients are statistically compared with age-matched healthy subjects (n=21). The results demonstrate the abnormal intracellular distribution of Trp and 5-HIAA in uremic patients.

Introduction

Abnormalities in electrolyte, muscular and plasma amino acid pools, impaired protein synthesizing enzymes and metabolites in the energy generation pathways have been identified in patients with chronic renal failure [1-5]. These changes are also observed in uremic patients undergoing various kinds of dialysis therapies [6-7]. Plasma amino acid levels at any time are the resultant of recent administration of the dietary intake, catabolic stress, protein degradation, cellular transport, interorganism transport and metabolism of specific amino acid. Various studies have shown that plasma amino acids do not necessarly reflect their intracellular concentration in skeletal muscle [8-9] or in leucocytes [10-12].

Tryptophan (Trp) is the only circulating amino acid which differs substantially from others by having a high degree of albumin binding in plasma [13]. In uremic patients, it is known that plasma Trp levels are reduced while the concentration of free Trp is normal or increased [14-15]. Abnormalities of plasma Trp and its distribution within the central nervous system are known to contribute to the neurological dysfunction [16-17]. In addition, uremic patients exhibit various other symptoms such as loss of appetite and fatigue which may be related to abnormal Trp metabolism.

We have recently reported, that in uremic patients which were treated with continuous ambulatory peritoneal dialysis (CAPD), Trp in muscle was high and was accompanied by low plasma total and free Trp level. There was also an accumulation of 5-hydroxy-indole-3-acetic acid (5-HIAA) in uremic plasma [9]. In an attempt to further understand the role of Trp and its distribution into the intracellular compartment, the present study was performed to investigate the transport of Trp from the extracellular into the intracellular compartments. Forty three Uremic patients of which 18 were on the maintenance hemodialysis and 25 on CAPD were studied by quantifying Trp and its metabolite 5-HIAA in erythrocytes and leucocytes. The results are compared with data obtained from 21 healthy subjects. Newly developed high performance liquid chromatographic method is used to monitor the levels of these substances. The method of analysis is highly sensitive with good reproducibility.

Results and Discussion

Figure 1 (A) shows a typical chromatogram of a standard mixture (2 μmol/L) demonstrating the base line separation of 5-HIAA, 5-HT, 5-HTrp, DOPAC, HMPG, HVA and Trp. All these substances are well separated to allow accurate quantitation. Among these substances the sensitivity of detection limit especially for Trp is dependent on the applied potential increasing with the increase of applied potential from +0.85 to +1.1 V but at the same time base-line

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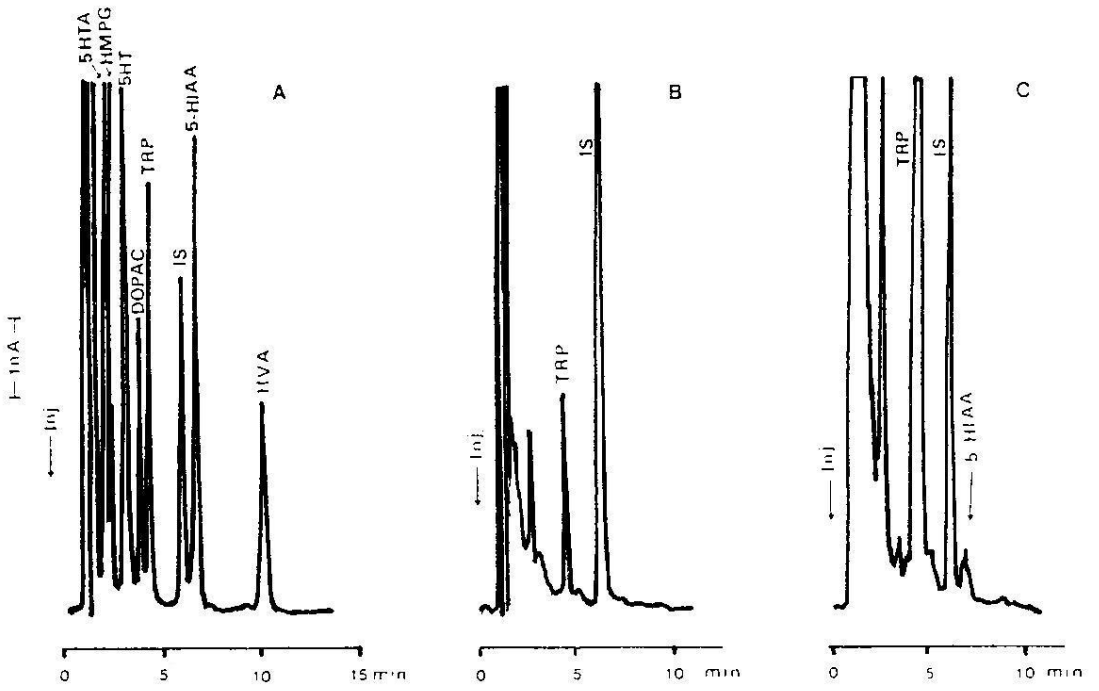

Fig.1: HPLC separation of (A) standard mixture containing 2 nmol/l of each substance (B) erthrocytes sample from healthy subject and (C) an uremic patient.

Table 1: Concentrations of tryptophan (Trp) and 5-hydroxy indole 3-acetic acid (5-HIAA) in leucocytes and erythrocytes of uremic patients undergoing HD and CAPD, and healthy subjects. All values are expressed as mean standard deviation (SD) in mol/mg protein.

		Erythrocytes		Leucocytes	
	n	Trp	5-HIAA	Trp	5-HIAA
Healthy subject	21	2.1 ±0.7	ND	0.4± 0.2	ND
HD patients	18	4.9 ±1.0**	1.1 ±0.6	0.7 ±0.3*	ND
CAPD patients	25	4.5 ± 0.9	0.8 ±0.3	0.8± 0.3*	ND

*p < 0.05, ** p < 0.001, ND = not detected

disturbances, risk of decreasing the performance of EC detector and the appearances of unknown endogenous substances, the applied potential of + 0.95 V was used which gave adequate sensitivity to quantitate Trp and its metabolite with accuracy. Figure 1 (A) also shows the chromatograms of erythrocytes, samples from a (B) healthy subject and an (C) uremic patient.

Figure 2 shows chromatograms of leucocytes samples from a (A) healthy subject and (B) an uremic patient. Identification of the peaks founds in these chromatograms was achieved by matching the retention times of the standards and internal addition method giving a linear relationship between the added concentrations of the substances and EC signal in the concentrations range of 0.5- 5.0 µmol/L. This linearity remained valied over a wide range of concentrations in both standards and spiked plasma samples, giving coefficient of correlation (r^2) from regression data equals to 1 for all substances. The limit of detection for most of the substances are <1 p mol under our experimental conditions.

Intracellular Trp uptake is a complex process which may be influenced by its plasma protein binding [15] and by competition among amino acids for the membrane transport sites [15,16,21,22]. Table 1 shows the levels of Trp and its metabolite 5-HIAA in erythrocytes and leucocytes. It has been suggested that small solutes especially indoles are retained in uremia and bind strongly by ionic, hydrophobic or other forces to albumin and may reduce Trp binding by competitive or non-competitive mechanisms [14]. The accumulation of indolic compounds in uremic

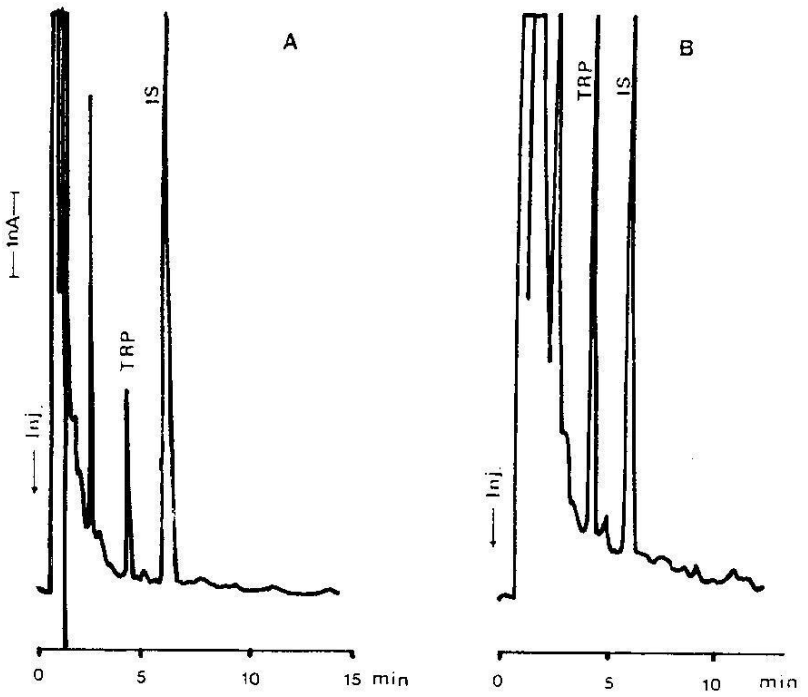

Fig.2: HPLC separation of leucocyte sample from (A) a healthy subject and (B) an uremic patient under the identical experimental conditions as figure 1.

Table 2: Leucocytes and erythrocytes contents of Tryptophan (Trp), large neutral amino acids (LNAA) and their ratios in uremic patients and healthy subjects. All values are expressed as mean standard deviation in μmol/mg proteins.

	n	Trp	LNAA	Ratio
Erythrocytes				
Healthy subjects	21	2.1± 0.7	15.3 ±2.9	0.14 ±0.02
HD patients	18	4.9 ±1.0**	9.1 ±1.8*	0.53 ±0.05***
CAPD patients	25	4.5± 0.9**	8.8 ±1.6*	0.5 1 ±0.07***
Leucocytes				
Healthy subjects		0.4± 0.2	3.4± 1.1	0.11±0.02
HD patients		0.7 ±0.2*	1.7 ±0.5**	0.41± 0.06***
CAPD patients		0.8± 0.3*	1.3 ±0.4***	90.57± 0.11***

*p<0.05, **p<0.01, ***p<0.001

plasma has been reported [21-24]. Apart from indole compounds, Trp binding to albumin is blocked by fatty acids, carbohydrates and various salts [25-26].

Table 2 shows the concentrations of Trp, total LNAA and their ratios in healthy and uremic patients. The sum of LNAA is significantly reduced in uremic patients, however, the ratio between Trp and LNAA, which is an important determinant of the rate at which tissues synthesize neurotransmitters such as catecholamines and serotonin, is increased in both groups. Similar to our previous study in uremic plasma [9], the accumulation of 5-HIAA is only observed in erythrocytes from the uremic patients whereas, the levels of Trp in both leucocytes and erythrocytes is significantly increased in uremic patients as compared to healthy subjects. There are few reported data on the cellular Trp levels in uremic patients apart from one study [12] on granulocytes where it was shown five folds increase in Trp in granulocytes in CAPD patients despite reduced total plasma Trp levels. Increase in muscle Trp levels is already reported by us [9]. Hence, it could be assumed that the reduced levels of plasma Trp in uremic patients may not necessarily reflect its deficiency but rather a shift of Trp from extracellular to intracellular space.

In various studies on uremic patients, abnormal amino acid pattern in plasma, muscle, erythrocytes and leucocytes are reported

Table 3: Clinical and biochemical data of uremic patients undergoing HD and CAPD and healthy subjects. All values are given as mean standard deviation (SD). Values of creatinine, urea and albumin are determined in plasma.

	n	Age years	Creatinine µmol/l	Urea µmol/l	Albumin g/l
Healthy subjects	21	42 ±13	83 ±15	5 ±1	42± 6
HD patients	18	53 ±15	978 ±63	25± 5	37 ±7
CAPD patients	25	47 ±12	903 ±56	24± 5	35± 6

[3,5,6,12,20] and it is well documented that extracellular concentrations of amino acids do not reflect their intracellular levels particularly in uremic patients who are in non-steady state conditions in which anabolism or catabolism is increased [27]. Hence, the change in Trp pattern in uremic patients could be the result of its competition with large neutral amino acids (LNAA) for the transport across the cell membrance. Secondly the uremic patients are known to have younger erythrocytes because of the shorter life span of their erythrocytes [28] and the possibility of Trp increase in the patients effected by the younger age of the erythrocytes or the presence of anaemia cannot be ruled out.

By conducting this study we have shown that HPLC method with EC detection is simple, sensitive and reproducible technique to quantitate cellular Trp and its metabolite in erythrocytes and leucocytes in the uremic patients. The results accumulated shows the abnormal transport of Trp from extracellular to intracellular space.

Experimental

Chemicals

All reference substances were obtained from Sigma Chemical Co., St. Louis, MO, USA. Methanol "HPLC grade" was obtained from Rathburn chemical Ltd., Walkerburn, Scotland. Anhydrous citric acid was obtained from Pierce Chemical Co., Rockford, Illinois, USA. Disodium hydrogen phosphate and sodium dihydrogen phosphate were obtained from Merck Damstradt, Germany. 1-Octane sulphonic acid (sodium salt) "HPLC grade" was obtained from Fisons Scientific Co., Loughborough England. All other chemicals used for analysis were (Anal-R) quality obtained from various commercial sources.

Chromatographic system

The chromatographic system consisted of a HPLC pump from LKB, Bromma, Sweden (Model No. 2150) and a syrine loading rotatory injection value from Rheodyne, Cotati, CA, USA (Model No. 7125) with 20 µl sample loop. 5 µM Microsorb C_{18} column (150 x 4 mm i.d.) was used for separation purpose and a pre-column (4 x 4 mm i.d.) of the similar material was inserted between the analytical column and injector. Both these columns were obtained from Rainin instruments, Woburn, MA, USA. Electrochemical detection was accomplished by using a LC-4A amperometric detector with a glassy carbon electrode TL-5A held at +900 mv versus Ag^+/Ag Cl as a reference electrode, obtained from Bioanalytical system, West Lafayette, IN, USA. To record the EC signals we used a data automation system chromatopac R-R6A obtained from Shimadzu corporation, Koyoto, Japan.

The mobile phase used consisted of 12% methanol (v/v) in 0.05 disodium hydrogen phosphate-citric acid 0.1 M (pH 3.8). To this calculated amount of octane sulphonic acid and EDTA were added for the final concentration of 1.2 mM and 0.2 mM respectively. The mobile phase was filtered through 0.45 m filter paper (Millipore, USA) and degased under vacuum by ultrasonic filtration. Helium gas was continuously passed through the mobile phase for an hour before use. All separation was performed at flow rate of 1.2 ml/min at ambient temperature.

Amino acid analysis was done with a pre-column derivatization procedure as described elsewhere [18].

Patients and control subjects

The control group consisted of 21 healthy subjects and 43 uremic patients of which 18 patients were undergoing maintenance hemodialysis (HD) and 25 uremic patients undergoing continuous ambulatory peritoneal dialysis (CAPD). Table-3 shows age, sex, plasma albumin, creatinine and urea levels of uremic patients and healthy subjects. 15 ml of venous

blood were collected from each individual who participated in this study in the morning after an overnight fast.

Isolation of blood cells

Granulocytes

On the basis of Boyum method of isolation [18], granulocytes and erythocytes were separated. 10 ml of blood was mixed with 4 ml of phosphate buffer (pH 7.4) and 2 ml of 6% Dextran 70. When the erythocytes had sedimented (after ca. 30-45 min), the supernatant was pipetted off and carefully layered onto 8 ml of Lymphoprep. The tube was then centrifuged at 400 x g for 30 min. The supernatant was sucked up, leaving the granulocytes in the last 0.5-1 ml. To this fraction were admixed with 5 ml of phosphate buffer containing 0.03 mol/1 EDTA. By centrifugation at 100 x g for 20 min, a granulocyte pallet was produced. Most of the contaminating lymphocytes and thermocytes were left in the supernatant which was removed. Leucocyte pellet supsension containing 75% to 97% granulocytes with greater than 97% viability by tryptan blue exclusion were isolated from the tube containing leucocytes and thrombocytes. Cells were hemolysed by sonication and the suspension was deproteinized rapidly with ε0.5 ml of 10% 5- sulfosalicylic acid. 20% of the total s uspension volume and the supernatant after centrifuged at 3000 g for 20 min was kept at -70°C if not immediately analysed.

Erythrocytes

The erythrocytes from the Dextran sedimentation step in the granulocyte separation as described above were centrifuged at 1000 x g for 10 min and the supernatant and buffy coat were removed. The erythrocytes were washed 3 times with 7 ml of 154 mmol/l NaCl in phosphate buffer by centrifugation at 2000 x g for 10 min. The erythrocyte fraction was almost free from other cells. Packed erythrocytes, 0.5-1 g were taken from bottom of the tube by pipetting rapidly and hemolysed by adding 1 ml of 1% Saponin, mixed and allowed to stand for 5 min on ice. Then 0.5 ml of 10% sulfosalicyclic acid (SSA) was added. The sample were mixed thoroughly and centrifuged at 3500 g for 15 min at 4°C. The supernatant was frozen at -70 °C until analyzed.

To each 50 µl of SSA extract, 50 ul of internal standard (5-hydroxy-indole-2-carboxylic acid, 1µM) were added, mixed thoroughly before injecting onto the HPLC system [9].

Alkali soluble protein in erythrocytes and leucocytes were determined by Lowery et al. method [20] and the contents of Trp and 5 HIAA are expressed as µM/mg of protein.

Serum albumin, creatinine and urea were determined by the Hitachi 737 Automatic Analyzer (Naka works, Hitachi Ltd., Tokyo, Japan).

Statistical methods

The test of significance for differences between groups was made by the parametric Student T-test. The level of significance was set at 5% for all analysed. All values are expressed as mean ± SD.

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