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Note

Measurement of free amino acids in polymorphonuclear leukocytes by high-performance liquid chromatography

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The availability of a cellular model to investigate the intracellular metabolism of free amino acids (AAs) would be of value in several pathological states [1,2]. One of them is chronic renal failure, during which the measurement of free AAs in plasma is rather unreliable and provides clinical and pathogenetic information of limited value, since the concentrations of free AAs in extracellular fluid are very variable and differ considerably from those in the intracellular fluid [3]. To overcome these difficulties, intracellular AAs have been evaluated mostly in muscle tissue obtained by biopsy [4]. However, this procedure is quite traumatic and is not suitable, especially for very young children.

A possible alternative seems to be the measurement of intracellular AAs in circulating peripheral leukocytes, as proposed by Metcoff and co-workers [5,6]. Although precise and reproducible, the method is time-consuming and not readily applicable to clinical studies.

Furthermore, owing to the low sensitivity of traditional chromatographic methods for AA determination, some AAs of crucial importance in this metabolism cannot be determined unless large amounts of blood are analysed.

We describe here a new procedure for determining intracellular AAs based on the analysis of AAs by high-performance liquid chromatography (HPLC) after separation of cells by mono-poly resolving medium (M-PRM). The use of M-PRM reduces both the separation time and the exposure of cells to potentially damaging factors. The application of the HPLC technique, with au-

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tomatic precolumn derivatization and fluorescence detection of the o-phthaldialdehyde–2-mercaptoethanol (OPA–2ME) derivatives of AAs, improves both the sensitivity and precision and enables the quantitation of the very small amount of intracellular AAs contained in few millilitres of blood.

EXPERIMENTAL

Apparatus

A Beckman liquid chromatographic system, consisting of two high-pressure pumps (110 A), a system controller (420) and an autosampler (504) (Beckman Instruments, Fullerton, CA, U.S.A.) was used. The fluorescence of the OPA-AA derivatives was routinely monitored with a Model RF-530 fluorimeter with a 12-μl flow-cell and a xenon lamp (Shimadzu, Kyoto, Japan) at an excitation wavelength of 330 nm and an emission wavelength of 450 nm. Continuous on-line quantitation of the HPLC results was obtained with an C-R3A data processor (Shimadzu). Separation was carried out on 5-μm Ultrasphere ODS (250 mm$\times$4.6 mm I.D., Beckman) columns. The analytical column was coupled to a guard column (50 mm$\times$4.6 mm I.D.) filled with the same material.

Reagents

Sodium dihydrogenphosphate, potassium hydrogenphosphate, sodium hydrogenphosphate, sodium tetraborate, sulpho-5-salicylic acid (SSA), 2ME, methanol and tetrahydrofuran were obtained from Merck (Darmstadt, F.R.G.), Brij (30% solution) was from Pierce (Rockford, IL, U.S.A.), OPA from Sigma (St. Louis, MO, U.S.A.) and M-PRM from Flow Labs. (McLean, VI, U.S.A.). The AA standard containing 24 AAs was obtained by adding crystalline asparagine, glutamine, citrulline, 1-methylhistidine, 3-methylhistidine, taurine, ornithine, tryptophan and homoserine obtained from Sigma to the commercial Sigma standard. All chemicals and solvents were of analytical grade.

Preparation of samples

Plasma. A 1-ml volume of heparinized blood was centrifuged at 3000 *g* for 15 min. The plasma was deproteinized by adding SSA (30 mg of SSA per ml of plasma). The sample was kept on ice for 1 h and then centrifuged at 5000 *g* for 15 min. The supernatant was filtered through a 0.45-μm HA type filter (Millipore, Bedford, MA, U.S.A.) and stored at -80°C until analysis.

Leukocytes. Starting from 7 ml of fresh heparinized venous blood (lithium heparin), polymorphonuclear leukocytes (PMNLs) were isolated by layering 3.5 ml of blood onto 3 ml of M-PRM in a sterile 100 mm$\times$13 mm tube. Samples were processed within 30 min from the sampling [7]. Differential migration during centrifugation (300 *g* for 30 min at room temperature) resulted in the formation of two cell fractions, named 1 and 2, containing mononuclear cells and PMNLs, respectively, and a red blood cell pellet.

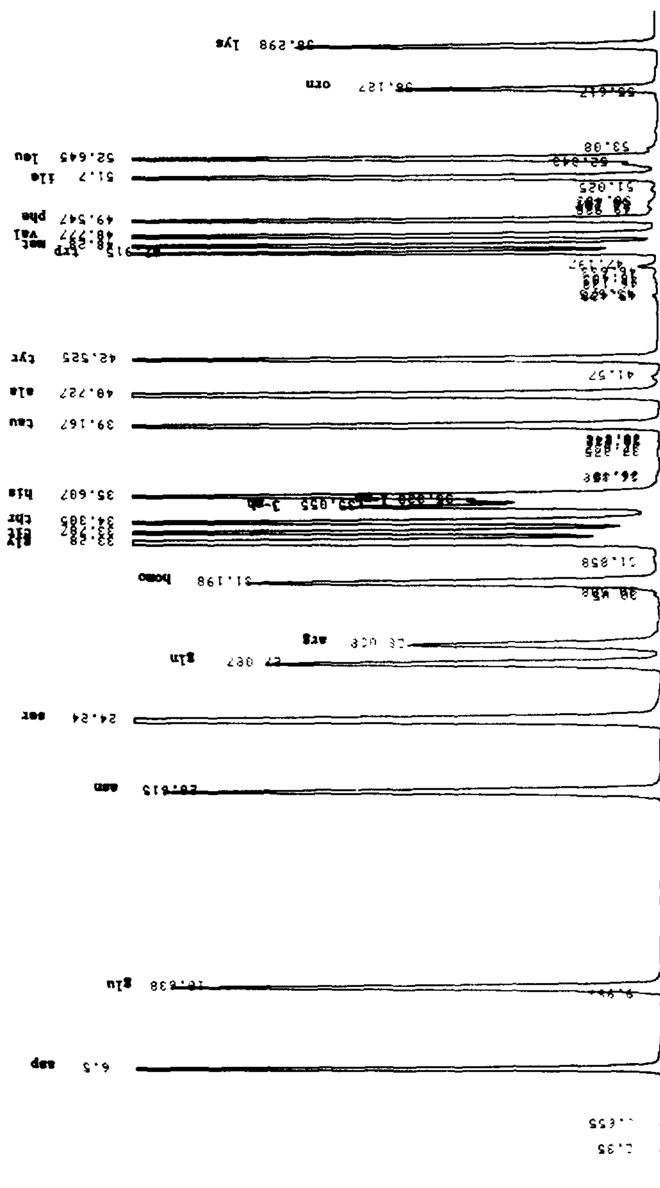

Fig. 1. Elution profile of OPA-2ME derivatives of a standard solution containing 24 AAs. Each AA had a concentration of 25 nmol/ml, and 20 μl of the derivatized solution were injected (1.2 pmol of each AA).

Plasma and fraction 1 were withdrawn and fraction 2 was transferred to a clean tube with a siliconized Pasteur pipette. Cells were washed in Hank's balanced salt solution (BSS), Ca- and Mg-free, then red cells lysed by adding 3 ml of distilled water to 1 ml of BSS and vortexed for 30 s. An aliquot of 3.5% sodium chloride was added to restore the isotonicity, then the solution was centrifuged for 5 min at 250 *g*. The pellet was resuspended with 3 ml of BSS.

The pellet obtained from a further slow centrifugation (250 *g* for 5 min) was suspended in 0.5 ml of 0.16 *M* potassium chloride. It contained more than 98% of PMNLs, as evaluated by the Wright method, and more than 95% of the cells were viable, as evaluated by the Trypan-Blue dye exclusion method. Cells were lysed by sonication and the suspension was deproteinized by adding SSA (7 mg of SSA per ml of suspension).

Derivatization

The preparation of the derivatization agents was performed by the method of Qureshi et al. [8]: the derivatizing reagent was prepared by dissolving 50 mg of anhydrous OPA in 1 ml of methanol; 9 ml of 0.4 *M* borate buffer (adjusted to pH 10.4 with 1 *M* potassium hydroxide containing 0.6% of 30% Brij) were added to this solution.

A 5-μl volume of the derivatizating agent was automatically drawn into a vial containing 5 μl of sample, 5 μl of internal standard (homoserine), 40 μl of sodium tetraborate (0.4 *M*, pH 10.4) and 45 μl of methanol. After a reaction time of 2 min at room temperature, 20 μl of the solution were injected into the column.

The use of an automatic injector system that involves mixing OPA and the AAs before injection avoids the time-dependent stability problem usually encountered with OPA adducts [9].

High-performance liquid chromatography

A multiple-step HPLC gradient system, with fluorescence detection of OPA–2ME derivatives of free AAs, as described by Qureshi et al. [8], was used for the analysis of free AAs.

Leukocyte alkali-soluble proteins, determined by the method of Lowry et al. [10], were used as a reference value for intracellular AA measurement, and the data were expressed as μM/mg of leukocyte proteins.

Patients

Two groups of children were studied. The first consisted of twenty children aged 10.3 ± 4.7 years with normal renal function and the second of ten patients aged 12.8 ± 3.7 years with chronic renal failure on maintenance hemodialysis (HD) for 16.0–18.8 months. All patients were in a stable condition with no concurrent illness. Blood was collected after an overnight fast, 36–48 h after

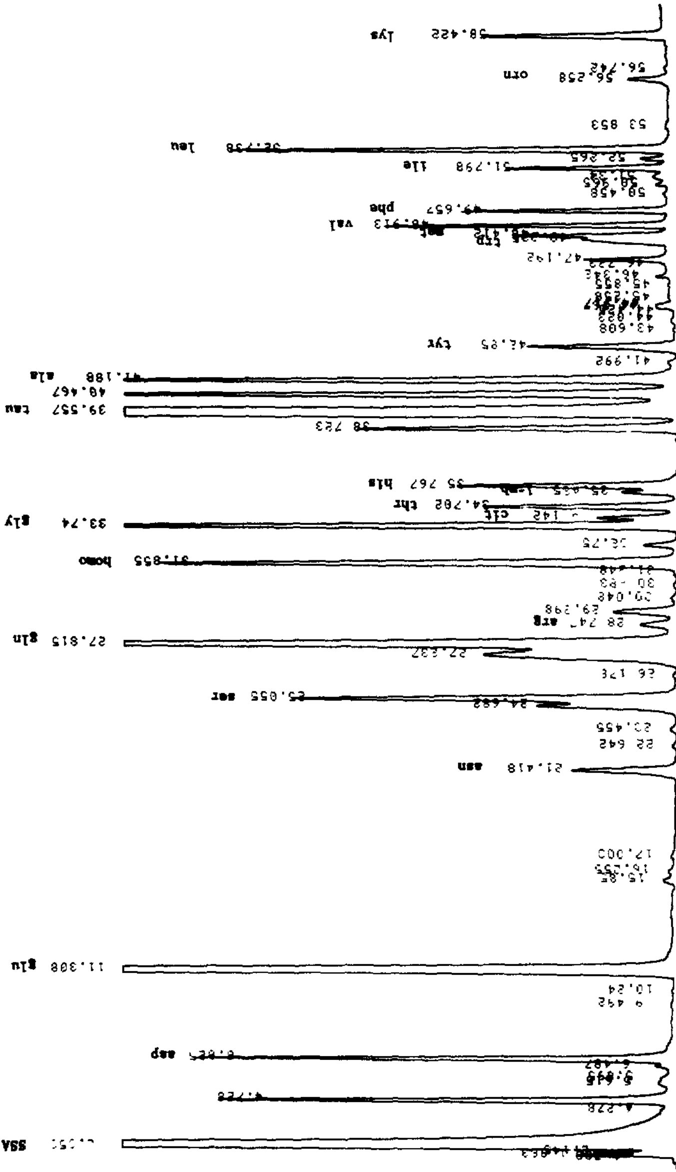

Fig. 2. Elution profile of leukocyte AAs from a uremic patient.

the last dialytic treatment and immediately before dialysis. Informed consent was obtained by the parents.

Statistics

Non-parametric statistical analysis based on the unpaired Mann Whitney U-test was used to compare the serum and the cellular AA concentrations in normal and uremic patients. Results are expressed as mean ±S.D.

RESULTS

A typical HPLC analysis of OPA–2ME derivatives of a standard solution is reported in Fig. 1, showing that all the 24 AAs analysed are separated in less than 60 min.

Several difficulties may arise in analysing intracellular AAs, owing to the potential damage to cells by the separation medium and procedure. The use of

TABLE I

PLASMA AA CONCENTRATIONS BEFORE AND AFTER THE CELL SEPARATION PROCEDURE

AA	Concentration (mean ± S.D., $n=5$) (μmol/l)		δ^a (%)
	Before	After	
Asp	2.44 ± 0.55	2.73 ± 0.64	5.24 ± 11.08
Glu	34.66 ± 16.47	35.32 ± 18.35	10.96 ± 6.49
Asn	37.40 ± 4.58	39.30 ± 6.31	6.06 ± 3.44
Ser	119.35 ± 23.61	124.16 ± 29.07	5.05 ± 2.82
Gln	520.96 ± 79.56	533.39 ± 100.84	4.62 ± 2.35
Arg	191.15 ± 22.05	188.84 ± 27.20	4.65 ± 2.06
Gly	213.41 ± 22.87	223.98 ± 28.90	5.91 ± 2.68
Cit	24.24 ± 5.60	24.92 ± 6.32	2.53 ± 7.11
Thr	112.55 ± 23.67	114.96 ± 29.57	5.30 ± 2.29
MH1	35.59 ± 9.46	37.85 ± 10.23	6.44 ± 3.80
MH3	9.66 ± 7.28	10.48 ± 8.0	11.56 ± 14.23
His	51.23 ± 11.49	51.92 ± 10.22	5.35 ± 3.60
Tau	40.94 ± 7.96	49.55 ± 14.05	9.72 ± 6.97
Ala	282.81 ± 58.43	299.24 ± 61.23	5.82 ± 3.01
Tyr	50.33 ± 11.26	51.52 ± 12.32	5.65 ± 2.12
Trp	40.88 ± 5.51	39.34 ± 8.15	8.13 ± 3.52
Met	21.63 ± 4.84	21.55 ± 5.8	6.40 ± 5.39
Val	194.13 ± 34.25	195.83 ± 47.89	8.16 ± 3.17
Phe	42.37 ± 5.60	42.39 ± 7.83	5.67 ± 3.25
Ile	52.57 ± 8.47	53.38 ± 13.73	9.35 ± 4.70
Leu	110.84 ± 22.71	112.02 ± 33.40	7.88 ± 5.83
Orn	64.09 ± 14.38	57.15 ± 12.27	5.41 ± 8.85
Lys	158.34 ± 34.17	160.75 ± 33.27	4.69 ± 5.30

[a]Mean of the percentage variation in the absolute value.

TABLE II

PLASMA AND INTRALEUKOCYTE AA CONCENTRATIONS IN CONTROLS AND UREMIC PATIENTS

AA	Plasma			Leukocytes		
	Concentration (mean ± S.D.) (μmol/l)		*p*	Concentration (mean ± S.D.) (μmol/mg)		*p*
	Control	Uremic		Control	Uremic	
Asp	5.84 ± 2.17	3.73 ± 1.78		5.23 ± 2.3	7.60 ± 3.71	
Glu	37.80 ± 9.22	38.43 ± 13.29		12.49 ± 4.18	27.34 ± 11.47	<0.001
Asn	75.28 ± 29.25	46.23 ± 12.70	<0.005	2.25 ± 1.03	2.37 ± 0.73	
Ser	90.83 ± 34.80	93.71 ± 18.74		17.15 ± 8.84	15.94 ± 9.21	
Gln	702.99 ± 115.63	539.16 ± 94.75		15.34 ± 5.37	18.69 ± 4.72	
Arg	153.99 ± 23.65	211.39 ± 42.97	<0.001	2.58 ± 2.63	8.63 ± 7.80	<0.01
Gly	222.55 ± 39.21	277.25 ± 77.17	<0.05	15.54 ± 9.04	17.93 ± 7.61	
Cit	20.89 ± 4.68	73.49 ± 17.88	<0.001	0.81 ± 0.60	0.45 ± 0.71	
Thr	144.53 ± 27.31	121.24 ± 45.57		8.57 ± 5.22	5.74 ± 2.86	
MH1	6.43 ± 2.03	52.95 ± 38.82	<0.005	1.41 ± 0.74	1.03 ± 1.15	
MH3	10.04 ± 3.21	110.25 ± 24.08	<0.001	1.27 ± 0.48	1.39 ± 1.91	
His	58.53 ± 10.88	74.39 ± 19.16	<0.05	7.22 ± 5.75	2.56 ± 1.74	<0.001
Tau	43.54 ± 14.52	49.29 ± 23.25		222.67 ± 99.23	338.61 ± 90.61	<0.001
Ala	311.72 ± 74.90	325.17 ± 167.10		16.86 ± 10.18	9.94 ± 3.15	<0.001
Tyr	61.51 ± 14.21	54.64 ± 19.55		5.45 ± 3.98	2.98 ± 1.49	<0.05
Trp	45.21 ± 8.79	23.72 ± 7.94	<0.001	2.56 ± 1.79	0.83 ± 0.46	<0.005
Met	33.29 ± 16.44	18.79 ± 6.43	<0.05	3.87 ± 1.58	2.0 ± 1.89	<0.001
Val	206.91 ± 31.26	156.07 ± 24.87	<0.05	8.06 ± 3.08	3.7 ± 1.85	<0.001
Phe	55.95 ± 11.79	40.67 ± 7.85	<0.01	6.40 ± 5.65	2.33 ± 1.80	<0.001
Ile	62.51 ± 14.01	49.89 ± 11.95		8.10 ± 5.89	2.45 ± 1.66	<0.001
Leu	115.40 ± 23.62	85.25 ± 16.82	<0.05	19.56 ± 9.61	6.18 ± 4.37	<0.001
Orn	80.44 ± 13.07	65.0 ± 16.88		10.14 ± 5.93	7.66 ± 5.53	
Lys	157.32 ± 27.49	156.04 ± 59.21		13.20 ± 7.57	7.80 ± 2.55	<0.05

the faster cell separation procedure could minimize the possible loss of AAs from cells. Fractionation of PMNLs by M-PRM was found to be rapid and specific for this purpose, since it allows the separation of PMNLs from monocytes and other blood constituents in less than 30 min; furthermore, no loss of AAs in the supernatant was observed even when cells were exposed to the separating medium for longer periods.

As reported in Table I, no important difference in the plasma concentration of any of the AAs was observed before and after the cell separation procedure; this demonstrates that there is no escape of AAs from cell to plasma and that the medium was not responsible for any variation of the chromatographic profile (Fig. 2).

The AA concentrations in both the plasma and intraleukocytes compart-

ment in normal and uremic patients are reported in Table II. Compared with the normal values, the intraleukocytes concentrations of several AAs in HD patients, including Val, Ile, Leu, Met, Trp, Phe, His, Ala, Lys and Tyr, were significantly reduced, whereas those of some other non-essential AAs (Glu, Arg, Tau) were significantly increased.

In some cases these variations (uremics versus normals) reflect the plasma imbalance but this is not a general feature. In fact, the levels of several AAs in uremic PMNLs are unmodified compared with the normal cells, whereas the plasma values are lower. This fact demonstrates that the AA plasma imbalance does not necessarily mirror the intracellular situation and prompts the use of intracellular AAs as a more reliable index of metabolic abnormalities in uremic patients, with potential applications in studies of other metabolic diseases.

DISCUSSION

The procedure described in this paper for determining intracellular AAs in PMNLs has several advantages over previous techniques. Various technical aspects should be emphasized.

(1) This method makes it possible to obtain a cellular suspension for the evaluation of intracellular AAs from a very small volume (6–8 ml) of blood. The plasma can be utilized for some other biochemical analysis.

(2) The gradient medium (M-PRM), which is widely used in haematology [9], has not yet been used for the detection of intracellular AAs and seems to work in a satisfactory way. M-PRM offers several advantages: the separation time is short, the sample is very pure (98% granulocytes) and the viability of cells is preserved, since more than 95% of cells are viable when evaluated by the Trypan-Blue dye exclusion method. Furthermore, it does not influence the chromatographic profile and does not determine leakage of individual AAs from cells to plasma.

(3) The application of the HPLC technique with fluorescence detection of OPA derivatives is very sensitive and appears to be adequate for quantifying the very small amounts of different AAs present inside these cells.

(4) By this technique it is possible to detect, in uremic children, alterations in the concentrations of free AAs in plasma and in leukocytes, characterized by a decrease of some essential AAs and, in particular, of all branched-chain AAs and by the increase of some non-essential AAs.

These changes are similar to those observed in studies performed on muscle biopsies, both in children [11,12] and in adults [3], and in good agreement with data on PMNL AAs in uremia previously reported by other authors [13,14], who employed traditional separation and AA analysis techniques.

The reasons for these changes and their relationship to dietetic and replacement treatment are so far unclear, and further studies are necessary to clarify these aspects.

In view of the striking difference between plasma and intracellular AA concentrations, the determination must be performed both in plasma and cell compartments.

(5) The use of PMNLs for the evaluation of intracellular AA concentrations offers a cell model that is easily accessible by a non-traumatic procedure.

These advantages over traditional methods allow the determinations to be repeated serially and performed even in very young children.

REFERENCES

1 M. Zachman, W.W. Cleveland, D.A. Sandberg and W.L. Nyhan, Am. J. Dis. Child., 112 (1966) 283.
2 J. Bergstrom, P. Furst, L.O. Noree and E. Vinners, J. Appl. Physiol., 36 (1974) 693.
3 A. Alvestrand, P. Furst and J. Bergstrom, Kidney Int , Suppl., 16 (1983) S9.
4 J. Bergstrom, P. Furst, L.O. Noree and E. Vinners, Clin. Sci. Mol. Med., 54 (1978) 51.
5 T. Yoshida, J. Metcoff, S. Frenk and C. De la Palma, Nature, 214 (1967) 525.
6 J. Metcoff, R. Lindeman, D. Baxter and J. Pederson, Am. J. Clin. Nutr., 31 (1978) 1627.
7 A. Ferrante, and Y.H. Thong, J. Immunol. Methods, 36 (1980) 109.
8 G.A. Qureshi, L. Fohlin and J. Bergstrom, J. Chromatogr., 297 (1984) 91.
9 R.F. Adams, F.L. Vandermark and G.J. Schmidt, J. Chromatogr. Sci., 15 (1977) 63.
10 N.I. Rosebrough, A.L. Ferr, R.J. Randall and O.H. Lowry, J. Biol. Chem., 193 (1951) 265.
11 M. Broyer, G. Jean, A.M. Dartois and C. Klein, Am. J. Clin. Nutr., 33 (1980) 1396.
12 C. Delaporte, G. Jean and M. Broyer, Am. J. Clin. Nutr., 31 (1978) 1647.
13 J. Metcoff, J. Pederson, B. Matter and S. Dutta, in R.N. Fine, K. Schärer and O. Mehls (Editors), CAPD in Children, Springer Verlag, Heidelberg, New York, Berlin, 1985, p. 1
14 K.E. Bonzel, G. Distler, K. Bonatz, T.A. Groser, D.E. Muller-Wiefel, J. Metcoff and K. Schärer, Perit. Dial. Bull., 7 (Suppl.) (1987) S8.