High sensitivity analysis of human red blood cells nucleotides by Micellar Electrokinetic Capillary Chromatography

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ABSTRACT

We evaluated a method for the extraction and analysis, by Micellar Electrokinetic Capillary Chromatography (MECC), of all adenine nucleosides from human erythrocytes. The simultaneous detection of adenine nucleosides at different state of phosphorilation allows the calculation of the energy parameters ATP/ADP ratio and energy charge (EC). We have studied these parameters in the erythrocytes of cancer patients. The results obtained show an higher ATP/ADP ratio and an high energy charge in cancer patients with respect to normal controls. Although further experiments will be required to clarify these issues, the results reported here demonstrate the interesting potentiality of this analytic method, for a systematic study of energetic parameters in large populations such as neoplastic cachetic subjects and patients with AIDS.

RIASSUNTO

Un metodo altamente sensibile per l'analisi di nucleotidi nei globuli rossi umani mediante Elettroforesi Capillare Micellare

Abbiamo valutato un nuovo metodo di estrazione ed analisi dei nucleosidi adenilici dai globuli rossi mediante Elettroforesi Capillare Micellare (MECC). La valutazione simultanea dei nucleosidi adenilici a differenti stadi di fosforilazione permette di calcolare alcuni importanti parametri energetici quali il rapporto ATP/ADP e la carica energetica delle cellule (CE). Abbiamo studiato questi parametri negli eritrociti di pazienti affetti da cancro. I risultati ottenuti mostrano che i pazienti con cancro hanno un rapporto ATP/ADP più elevato ed una CE più elevata dei controlli normali. Anche se ulteriori esperimenti saranno necessari per chiarire questi rilievi, i risultati che riportiamo dimostrano una interessante potenzialità di questo metodo d'analisi, che permette uno studio sistematico dei parametri energetici in ampie popolazioni come pazienti oncologici, cachettici e pazienti con AIDS.

INTRODUCTION

Quantitative methods for cell nucleotides are of interest in clinical studies dealing with therapeutic drug monitoring or cell metabolic studies (1).

Separation and analysis of intracellular nucleotides have been performed traditionally by high performance liquid chromatography (HPLC) and enzymatic analysis (2, 3, 4, 5). These procedures are time and labor consuming and are unfit to a clinical laboratory performing analyses on a large number of samples.

Micellar electrokinetic capillary chromatography (MECC) offers a number of advantages in comparison with the traditional techniques (6). The size of the sample is small (in the order of nanoliters), run times are typically much shorter than those using HPLC (10-15 vs 30 min), excellent peak efficiency and resolution are achievable with resulting high numbers of theoretical plates.

We have reported previously a method for nucleoside triphosphate analysis using MECC (7).

We report here the extension of our MECC method for the simultaneous extraction and analysis of all adenine nucleosides (mono-di and triphosphates) in a single run, from human red blood cells (RBCs).

The method described here allows the acquisition of data witch can provide important clues about the metabolic state and the energy content of the cellular system under study.

MATERIALS AND METHODS

A capillary electropherograph (ISCO model 3140, Lincoln, NE, USA) was equipped with an on-column UV absorbance detector at 254 nm and a vacuum system for hydrostatic injection of samples. Untreated fused-silica capillary tubes (Beckman Instruments, Fullerton, CA USA) of 50 μm i.d. x 70 cm total length were used.

All nucleotide standards, sodium phosphates, EDTA, dodecyltrimethylammonium bromide (DTAB), trichloroacetic acid (TCA), tri-n-octylamine and Phosphate Buffered Saline (PBS) were obtained from Sigma (St. Louis, MO, USA). Freon was purchased from Aldrich (Milwaukee, WI, USA). All solutions employed in MECC were filtered before use with 0,45 μm membrane filters (Corning Costar, Acton MA, USA).

Blood sampling and nucleotide extraction

One ml of venous whole blood, collected in heparinized vials was diluted 1:5 and washed with cold PBS and

centrifuged at 4°C and 200 g in a refrigerated centrifuge (Microfuge® R Centrifuge, Beckman Instruments, Fullerton, CA USA).

Fifty µl of packaged red cells were resuspended in 1 ml of cold PBS and washed twice. After these washes, a platelet count was performed on electronic counter. Platelet counts were found to be lower than 1000/µl (Fig.I).

RBCs were resuspended in 100 µl of a lysis solution of 10% TCA in PBS, for 30 min in ice. The extracts were centrifuged at 4°C for 5 min at 13000 g in order to spin down cellular debris. The supernatant, containing the ribonucleotides, was collected and neutralized twice with 1.1 vol of Freon (1,1,2-trichlorotrifluoroethane) containing 0.5 M tri-n-octylamine: the neutralizing solution was added and, after vigorous vortexing, the sample was centrifuged for 5 min at 15000 g and the clear supernatant phase was collected. The superior phase was then collected again and stored at -80°C until the analysis.

MECC analysis

A buffer 50 mM sodium phosphate, 100 mM DTAB and 1mM EDTA at pH 7 was employed to obtain the separation of all adenine nucleotide (ATP, ADP and AMP) from the red cell extract. The running conditions were: T = 25°C, constant voltage = 14 kV and maximum current limit of 100 µA, polarity reversed (positive ground), sample's injection under pressure of 8 kPa/sec, for 3 sec. Before the runs the capillary was conditioned with 0.1 M NaOH, then sequentially washed for 5 min with H_2O, 0.1 M HCl, H_2O again and with sodium phosphate buffer without DTAB; finally the capillary was equilibrated with the analysis buffer.

In order to provide a better reproducibility of analysis, between the runs the column was sequentially rinsed with 0.1 M NaOH, buffer without DTAB and running buffer.

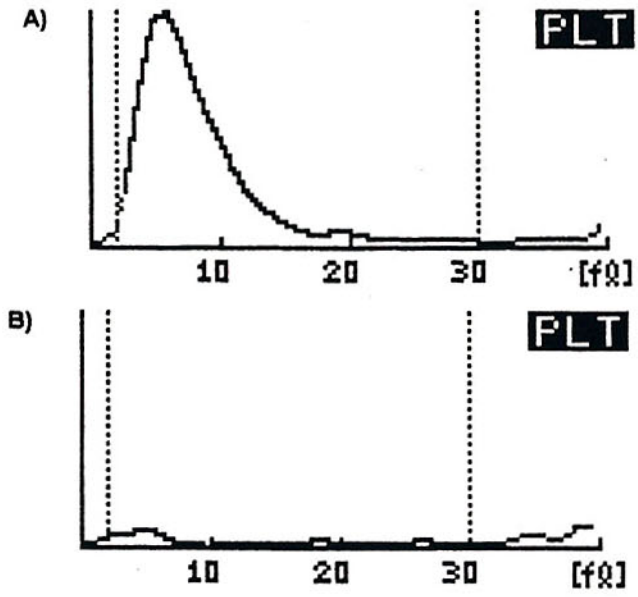

Figure I
Platelet count in red blood cells suspension before (A) and after (B) washings: the typical peak referring to platelets is clearly visible in A), and it is almost disappeared in B), showing absence of platelet contamination in red cell pellet

Identification and quantitation of nucleotides

Nucleotide standard solutions were analyzed at concentrations ranging from 0.09 to 1.5 pmol to obtain for each nucleotide the calibration curves of known concentrations vs peak areas. The linearity between nucleotide concentration and peak areas is evident in Fig. II, were each point

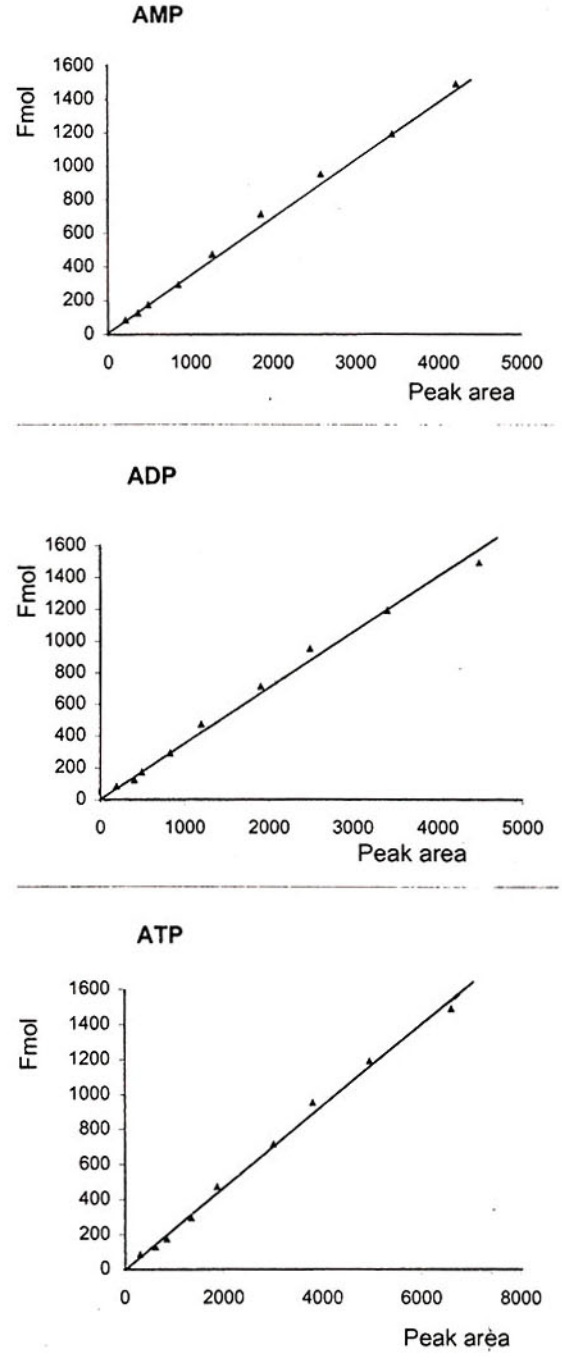

Figure II
Plots of concentrations vs peak areas of standard nucleotides. Four runs were carried out for each single calibration point

represents a mean of four runs. Nucleotides in cell extracts were identified by comparing the migration times of the unknown peaks with those of standard nucleotides eluted under the same conditions. In addition, the identification was checked by spiking with pure single nucleotide standard added to the sample. The nucleotides quantitation was carried out by reading the concentration from its respective calibration curves.

Statistical evaluation was performed by conventional one-way analysis of variance, taking p values <0.05 as significant.

RESULTS

Run to run repeatability and day to day variation

Run to run repeatability was determined by injecting the samples four times and calculating the mean, the standard variation (SD) and the percentage relative standard variation (% RSD) for retention times and peak areas. In addition, the day to day variation of retention times was calculated. Table 1 shows the reproducibility of migration times and peak areas of standard nucleotides. Table 2 shows an example of data obtained for a single blood sample.

Energy parameters

The simultaneous detection of adenine nucleotides at different state of phosphorilation allows the calculation of ATP/ADP ratio and energy charge (EC).

These energy parameters were calculated according to literature, in fact the ATP/ADP ratio represent the amount of cell energy (8), in addition the energy charge, an important index of the energy status of a system, was defined as follows: EC= ATP +1/2 ADP/(ATP +ADP +AMP) (9).

Red blood cells represent an ideal system because their are easily obtained and their adenine triphosphate pools are mainly derived from the glycolitic pathway.

Evaluation of adenine nucleotide (ATP) content in whole blood, has recently been obtained with Capillary Chromatography for the monitoring of the energy charge of an organism in different metabolic situation (hypoxic stress) (10).

We have evaluated these energy parameters in the red blood cells of three different groups of subjects:

A) 21 healthy blood donors taken as controls, aged 45 to 64 years.

B) 9 cancer patients undergoing an adjuvant chemotherapy after a radical resection of the tumor. Out of these, one patient aged 29 and the others 40 to 70 years.

C) 31 patients with more advanced metastatic disease. Out of these, two patients aged 24 and 29 years and others 42 to 73 years.

Both controls and patients gave informed consent to the study.

Figure III shows a typical nucleotide electropherogram of RBCs from healthy subjects (group A), and cancer patients belonging to the B and C group.

The ATP peak was increased in red cell lysate of cancer patients, especially in advanced cases (Group C).

The results are reported in Table 3, in witch the ribonucleotides are expressed as mean and standard error of

Table 1
Run to run repeatability and day to day variation of standard nucleotides

Parameter	Compound	Retention time (seconds)			Peak area (arbitrary units)		
		Mean	SD	RDS (%)	Mean	SD	RSD (%)
Run to run repeatability (same day, n 0 4)	AMP	459,6	3,17	0,69	479,7	40,53	8,45
	ADP	599,6	6,89	1,15	490,3	37,02	7,55
	ATP	843,9	10,94	1,30	888,3	71,68	8,07
Day to day variation (6 days)	AMP	462,4	3,22	0,7			
	ADP	608,1	4,64	0,76			
	ATP	848,8	10,89	1,28			

Table 2
Run to run repeatability and day to day variation of nucleotides in blood sample

Parameter	Compound	Retention time (seconds)			Peak area (arbitrary units)		
		Mean	SD	RDS (%)	Mean	SD	RSD (%)
Run to run repeatability (same day, n 0 4)	AMP	489,2	2,10	0,43	59,50	4,95	8,32
	ADP	633,7	3,51	0,55	794,0	50,91	6,41
	ATP	872,1	9,19	1,05	6296	316,1	5,02
Day to day variation (6 days)	AMP	491,0	2,82	0,57			
	ADP	644,6	9,22	1,43			
	ATP	892,7	14,58	1,63			

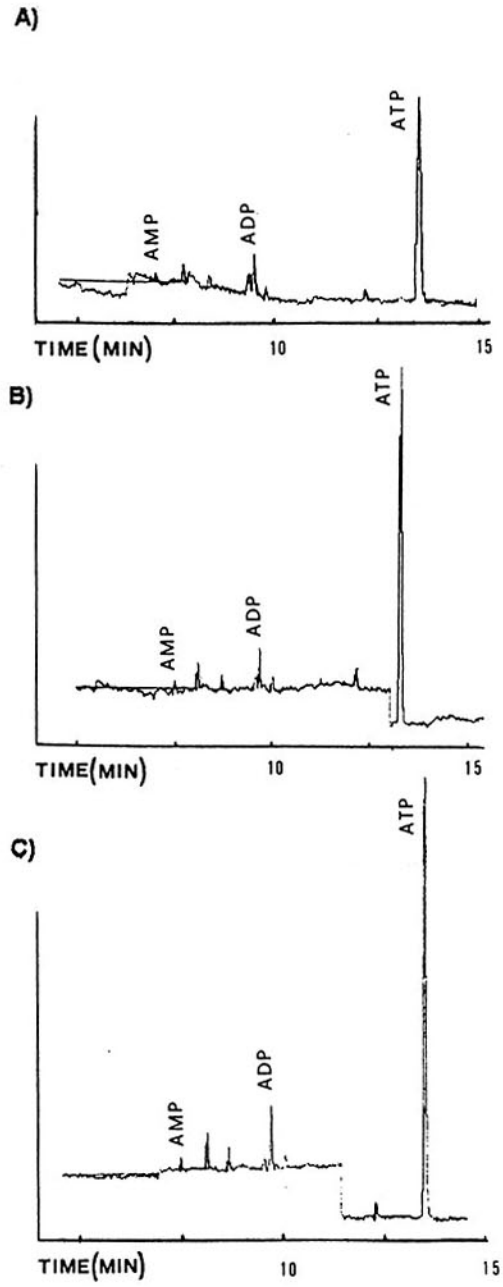

Figure III
Individual electropherograms of ATP, ADP and AMP in: A) normal controls; B) patients undergoing adjuvant chemotherapy and C) patients with advanced disease

the mean: the ATP content in erythrocytes of advanced cancer patients was significantly higher than normal controls.

Furthermore the ATP/ADP ratio and the EC were also increased in group B and C of patients, as compared to the healthy controls.

These differences were statistically significant at $p< 0.05$.

Table 3
Ribonucleotides in red blood cells. Data are expressed as mean and standard error of the mean, from N cases, of ribonucleotides expressed in pmol / ml of red blood cells ***

	Group A N = 21	Group B N = 9	Group C N = 32
AMP	24,01±0,65	23,59±0,88	24,45±0,80
ADP	88,45±3,72	76,91±4,56	74,54±2,73*
ATP	435,49±15,08	473,82±15,34	545,79±20,64*
ATP/ADP	5,01±0,17	6,37±0,49*	7,47±0,29*
EC	0,88±0,003	0,89±0,005*	0,90±0,002*

***The red cell parameters including MCH, MCV and MCHC and the platelet counts were determined using a ADVIA 120 Hematology System instrument (Bayer, Swords, Dublin, Eire).
* $p< 0,05$ with respect to controls

DISCUSSION

Our results demonstrate an higher ATP/ADP ratio and an high energy charge (EC) in RBCs of cancer patients with respect to normal controls. This clinical finding is in agreement with biochemical data coming from some experimental systems (11, 12). In these experimental models, ATP levels in rat erytrhocytes increased during the logarithmic phase of tumor growth and returned toward normal levels during the plateau phase of tumor development. The expansion of intraerytrhocyte nucleoside triphosphate pools mirrored the neoplastic growth (11). The same feature has been observed in other experimental tumours as well, such the C3HA ICR mice during Hepatoma 22 tumor growth (12).

The clinical situation is more intricate, however.

ATP content in circulating red cells of patients with solid tumors has been studied previously in a smaller series of less advanced case, by other investigators who measured red cells nucleotides using reverse phase high-pressure liquid chromatography (RP-HPLC) (13). In contrast with our results, cancer patients showed a lower level of ATP with respect to normal controls (13).

These differences may be due to many factors such as disease stage, age and nutritional status.

Although further experiments will be required to clarify these issues, the results reported here demonstrate the interesting potentiality of this analytic method. The use of MECC, that allows a simple and simultaneous separation of all adenine nucleotide in a single run, looks promising for the study of fundamental metabolic parameters in easily obtainable cells like RBCs.

This open the possibility to study systematically energetic parameters in large populations, such as neoplastic cachetic subjects and patients with AIDS.

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