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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR IDENTIFICATION AND QUANTITATION OF NUCLEOTIDES IN LYMPHOCYTES AND MALIGNANT LYMPHOBLASTS

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SUMMARY

A method for the identification and quantitation of nucleotide pools in lymphocytes and leukemic blasts is described. Separation of these metabolites was performed by anion-exchange high-performance liquid chromatography using a pH and concentration gradient consisting of several linear steps.

The mono-, di- and triphosphates of adenosine, cytidine, guanosine, inosine, uridine and xanthosine could conveniently be separated together with NAD^+, cyclic AMP, $NADP^+$ and uridinediphosphoglucose (UDPG).

In addition, data on the accuracy and precision of the method are given and its potentials for use in the analysis of nucleotide pools in leukemic lymphoblasts are illustrated.

INTRODUCTION

Purine and pyrimidine metabolites are essential for human cells: they play an important role in the transfer of chemical energy for metabolic processes, in DNA and RNA synthesis, in cell proliferation and in cell regulation. Although more and more information is accumulating about purine and py-

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rimidine metabolism in relation to leukemia, our present insights are far from complete. Recent studies have demonstrated marked differences in adenosine deaminase, purine nucleoside phosphorylase and 5'-nucleotidase activities in leukemic blasts of various immunologically characterized subsets of acute and chronic lymphatic leukemia [1—4]. Selective toxicity of deoxyribonucleosides towards malignant human T-cell lines has been reported [5—7]. Determination of the levels of purine and pyrimidine metabolites in leukemic cells may contribute to a better understanding of the purine and pyrimidine metabolism in these cells.

In the present investigation an anion-exchange high-performance liquid chromatographic (HPLC) method was developed in order to determine nucleotide pools in these cells. The described method might also be used to measure nucleotide pools in lymphoid cells from patients with immune dysfunctions where deficient activities of enzymes of the purine interconversion pathway were observed [8—10].

Previously published methods [11—13] did not seem satisfactory for our purposes. To optimize the separation of the various nucleotides a multi-step gradient was chosen.

EXPERIMENTAL

Chemicals

The nucleotides cyclic AMP, NAD^+ and UDPG, used for standardization of the method, were obtained from Sigma, St. Louis, MO, U.S.A., and from Boehringer, Mannheim, G.F.R. The other chemicals were obtained from E. Merck, Darmstadt, G.F.R. Water used for all buffers was purified in a Milli-Q System (Millipore, Bedford, MA, U.S.A.).

Apparatus

A Spectra-Physics Model SP 8000B (Spectra-Physics, Santa Clara, CA, U.S.A.) with automated data system and a two-channel printer-plotter was used. A variable- and a fixed-wavelength UV detector (Spectra-Physics) were used for monitoring the peaks. Chromatographic columns were operated at 40°C. The columns were pre-packed Partisil-10 SAX columns (250 × 4.6 mm I.D., particle size 10 μm; Whatman, Maidstone, Great Britain). Injections were made using a 50-μl high-pressure Valco Valve loop injector.

Chromatographic procedure

The separation is based in principle on that described by Brown [11]. However, we used a modified system, changing the pH of the buffers and using a multi-step gradient.

Chromatography was carried out with two potassium dihydrogen phosphate buffers and water in a ternary system. The buffers and water contained 2% acetonitrile. The gradient is given in Table I. The low-concentration buffer consisting of 0.05 *M* potassium dihydrogen phosphate and 2% acetonitrile was adjusted to pH 3.35 with phosphoric acid. The high concentration buffer of 0.25 *M* potassium dihydrogen phosphate, 0.50 *M* potassium chloride and 2% acetonitrile was adjusted to pH 5.25 with potassium hydroxide. During

TABLE I

MOBILE PHASE SEQUENCE USED FOR THE SEPARATION OF NUCLEOTIDES IN THE CHROMATOGRAPHIC PROCEDURE

A = 2% acetonitrile in water; B = 0.05 *M* potassium dihydrogen phosphate buffer with 2% acetonitrile, pH 3.35; C = 0.25 *M* potassium dihydrogen phosphate buffer with 0.50 *M* potassium chloride and 2% acetonitrile, pH 5.25.

Time (min)	Volume per cent		
	A	B	C
0	80	20	0
10	60	40	0
20	44	44	12
25	40	40	20
50	25	25	50
75	10	10	80
85	7.5	7.5	85
90	7.5	7.5	85

elution the solutions were deaerated with helium. The flow-rate was kept constant at 1.3 ml/min.

Lymphoid cell fractionation

Lymphoid cells from children with non-B-non-T-cell acute lymphoblastic leukemia (non-B-non-T ALL) before treatment, were fractionated from bone marrow aspirates or from peripheral blood. Lymphocytes from normal persons were isolated from the peripheral blood. Bone marrow aspirates (1.0—2.0 ml) were collected in heparin and purified on a one-step Ficoll—Isopaque gradient according to the description of Bφyum [14]. Peripheral blood lymphocytes were fractionated in the same way. Cells floating at the interface were collected in 1 ml of Tris-buffered minimum essential medium supplemented with 5% foetal calf serum.

Nucleotide extraction

Nucleotides were extracted in the cold by addition of perchloric acid (PCA). A cell suspension ($10—20 \cdot 10^6$ cells) was centrifuged at 900 *g* for 3 min at 4°C. One hundred microlitres of 0.4 *M* ice-cold PCA were added to the cell pellet; the suspension was vortexed and kept on ice for 15 min. Proteins were precipitated by centrifugation at 2200 *g* for 5 min at 4°C. The supernatant was collected and adjusted to a pH between 6.0 and 6.5 with an ice-cold mixture of 4 *M* potassium hydroxide and 1 *M* dipotassium hydrogen phosphate (approximately 30 µl). The neutralized supernatant was kept on ice for another 15 min and the precipitated potassium perchlorate was removed by centrifugation. The extract was kept at —20°C until injection.

RESULTS AND DISCUSSION

Chromatographic conditions

The retention behaviour of a standard mixture of 5'-mono-, 5'-di- and

5′-triphosphates, cyclic AMP, NAD^+, $NADP^+$ and UDPG is shown in Fig. 1. A multi-step gradient was essential for optimal separation of the 5′-monophosphates, cyclic AMP and NAD^+, and of $NADP^+$ and the 5′-diphosphates. In one-step linear gradients poor resolutions were obtained either between the monophosphates or between $NADP^+$, CDP and UDP, or between ADP and IDP.

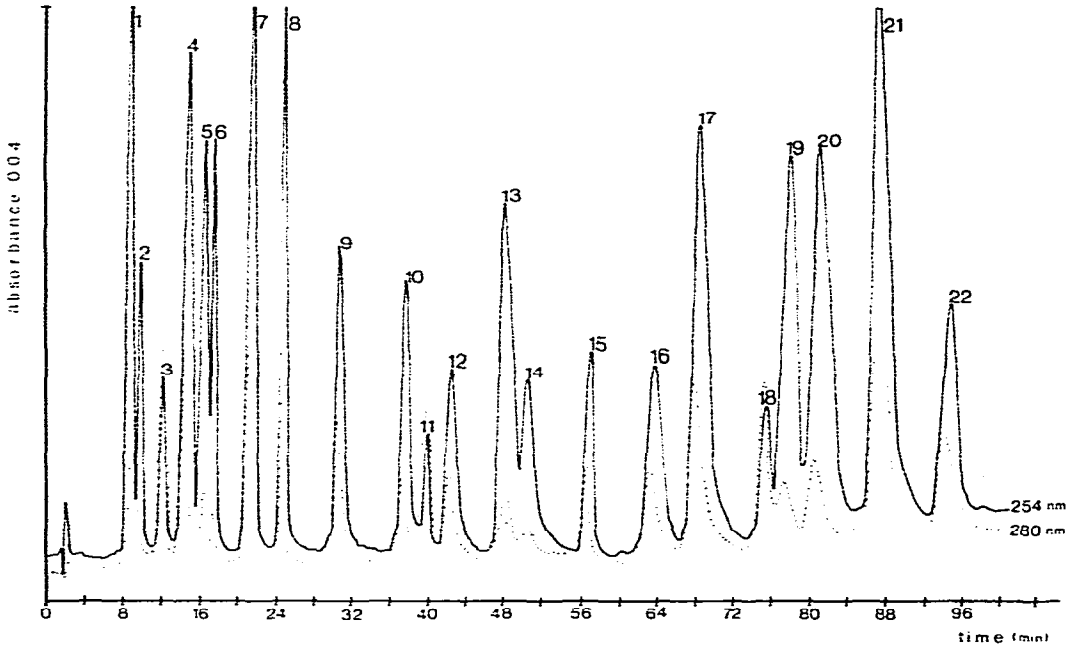

Fig. 1. Elution profile of a test mixture of cyclic AMP, NAD^+, $NADP^+$, UDPG and 5′-mono-, 5′-di- and 5′-triphosphates, as detected at 254 nm and 280 nm (separation conditions are described in Experimental). Retention times in minutes are as follows: (1) cyclic AMP, 8.9; (2) NAD^+, 10.0; (3) CMP, 12.0; (4) UMP, 15.1; (5) AMP, 16.6; (6) IMP, 17.6; (7) GMP, 21.4; (8) UDPG, 24.8; (9) XMP, 30.8; (10) $NADP^+$, 37.4; (11) CDP, 39.2; (12) UDP, 42.0; (13) ADP, 47.7; (14) IDP, 50.2; (15) GDP, 56.8; (16) XDP, 63.4; (17) UTP, 68.0; (18) CTP, 75.1; (19) ITP, 77.7; (20) ATP, 80.8; (21) GTP, 86.9; (22) XTP, 94.4.

Between successive runs the column was equilibrated for 30 min under initial conditions (Table I). In this way the retention times were reproducible. Before and after each series of runs, a standard mixture was analysed. Calibration curves were made by means of standard solutions of different concentrations. Integrated peak areas were calculated from UV scan patterns (254 nm). In addition, peak identification was facilitated using the E_{280}/E_{254} ratios. In Figs. 1—3 the scan patterns at both wavelengths (254 nm and 280 nm) are given. NAD^+, $NADP^+$, UDPG, cyclic AMP and the monophosphates could be quantitated with a lower detection limit of 10 pmol per injection. The diphosphates and triphosphates could be quantitated with a lower detection limit of 20 pmol and 40 pmol, respectively. The linearity of the calibration curves was considered to be sufficient, correlation coefficients being at least 0.997 in a concentration range between detection limit and 5 nmol per in-

jection. The relative standard deviation of peak areas for twenty successive injections of 1 nmol was 0.5%.

Nucleotide recovery after extraction

In order to check the stability of the nucleotides under experimental conditions, standard solutions of AMP, ADP, ATP, CTP, GTP and UTP were extracted with PCA (see Experimental). Thus, 10 μl of a 0.3 m*M* nucleotide standard solution was mixed at 0°C with 100 μl of 0.4 *M* PCA, neutralized with cold KOH—K_2HPO_4 solution and centrifuged.

In the supernatant, CTP and GTP appeared to be the most labile nucleotides with maximal losses of 8% and 5%, respectively. AMP, ADP, ATP and UTP had recoveries greater than 97%.

Analysis of nucleotide pools in human cells

Typical scans of PCA extracts from normal peripheral blood lymphocytes and lymphoblasts of children with non-B-non-T-cell ALL are shown in Figs. 2 and 3, respectively.

Results of studies with 25 patients and 21 controls are given in Table II. The leukemic blasts from patients with ALL before treatment have significantly higher ATP and UTP concentrations than normal lymphocytes. The ATP/ADP, CTP/CDP, GTP/GDP and UTP/UDP ratios (Table III) were higher than those reported by others [13, 15]. Furthermore, the energy charges, (TP + ½DP)/(TP + DP + MP), of the adenosine, guanosine, cytidine and uridine nucleotides were all higher than 0.90 (Table III). The high ratios and the

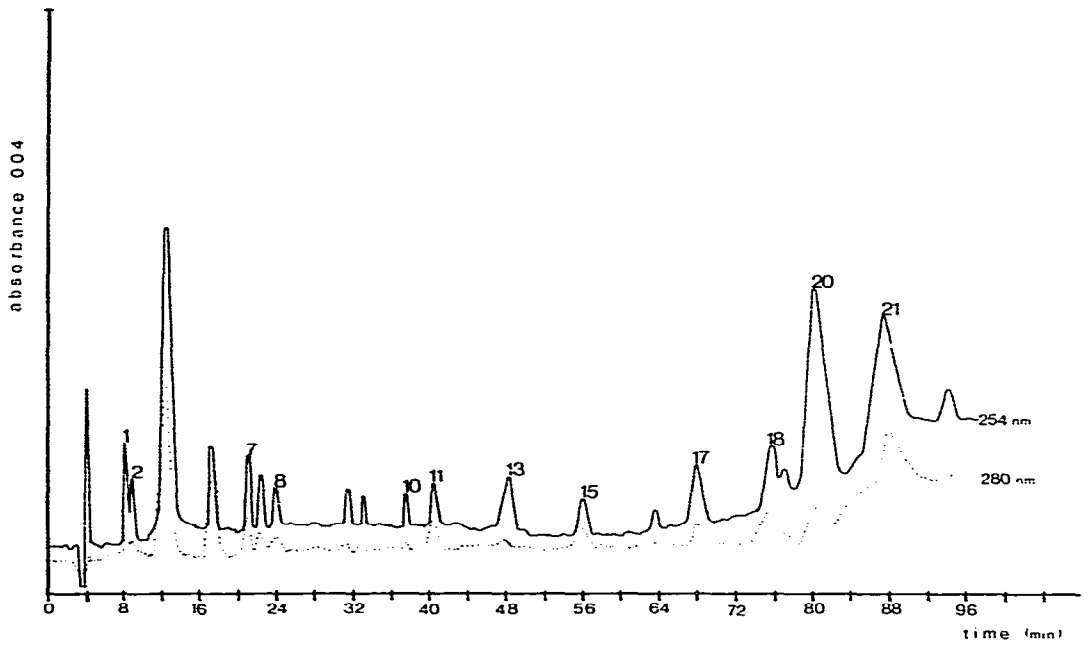

Fig. 2. HPLC profile of nucleotides in a cell extract from normal peripheral blood lymphocytes (for extraction procedure, see Experimental). The peaks can be identified by referring to the legend for Fig. 1.

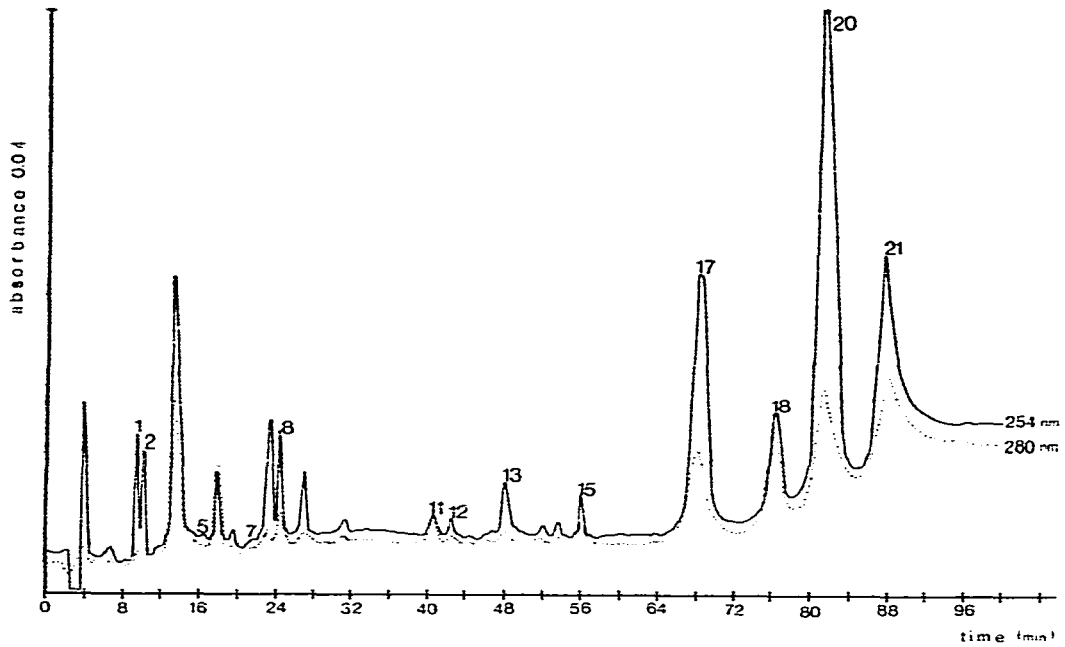

Fig. 3. HPLC profile of nucleotides in a cell extract from lymphoblasts from a patient with non-B-non-T ALL (for extraction procedure, see Experimental). The peaks can be identified by referring to the legend for Fig. 1.

TABLE II

NUCLEOTIDE CONCENTRATIONS IN BONE MARROW (BM) AND PERIPHERAL BLOOD (PB) LYMPHOBLASTS FROM PATIENTS WITH NON-B-NON-T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA AND IN NORMAL PERIPHERAL BLOOD LYMPHOCYTES

n = number of individuals.

Nucleotide	Concentration (pmol per 10^6 cells; mean ± S.D.)		
	Normal lymphocytes	Leukemic lymphoblasts	
	PB (n = 21)	BM (n = 25)	PB (n = 12)
AMP	25 ± 11	34 ± 11	28 ± 15
ADP	53 ± 40	66 ± 22	62 ± 38
ATP	500 ± 140	1005 ± 205	912 ± 210
GMP	7 ± 5	15 ± 10	12 ± 6
GDP	22 ± 10	33 ± 14	21 ± 10
GTP	128 ± 47	211 ± 43	213 ± 55
CDP	12 ± 12	21 ± 18	21 ± 21
CTP	73 ± 44	138 ± 64	108 ± 40
UDP	14 ± 12	23 ± 11	18 ± 7
UTP	102 ± 31	295 ± 107	347 ± 119

TABLE III

NUCLEOTIDE TRIPHOSPHATE/NUCLEOTIDE DIPHOSPHATE RATIOS (TP/DP) AND ENERGY CHARGES, (TP + ½DP)/(TP + DP + MP), CALCULATED FROM MEASURED ADENOSINE, GUANOSINE, CYTIDINE AND URIDINE NUCLEOTIDE CONCENTRATIONS IN NORMAL LYMPHOCYTES AND IN LYMPHOBLASTS FROM PATIENTS WITH NON-B-NON-T ACUTE LYMPHOBLASTIC LEUKEMIA

n = number of individuals.

Nucleotides	Lymphocytes (n = 21)		Lymphoblasts (n = 25)	
	TP/DP (mean ± S.D.)	Energy charge (mean ± S.D.)	TP/DP (mean ± S.D.)	Energy charge (mean ± S.D.)
Adenosine nucleotides	13 ± 7	0.90 ± 0.04	18 ± 8	0.93 ± 0.01
Guanosine nucleotides	6 ± 3	0.91 ± 0.03	9 ± 5	0.92 ± 0.05
Cytidine nucleotides	9 ± 7	0.91 ± 0.03	11 ± 8	0.92 ± 0.03
Uridine nucleotides	11 ± 6	0.93 ± 0.04	17 ± 11	0.95 ± 0.03

high energy charges are supplementary indications that the extraction procedure and the chromatographic method presented here are reliable enough to measure the nucleotide concentrations in lymphoid cells. In conclusion, a more refined separation of a great number of purine and pyrimidine nucleotides can be achieved using our multi-gradient system.

Further studies are now being performed in our laboratory to make an inventory of the nucleotide levels in different immunologically characterized lymphoid cells and to study the effect of cytostatics on these levels.

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