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ANALYSIS OF PURINE RIBONUCLEOTIDES AND DEOXYRIBONUCLEOTIDES IN CELL EXTRACTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic (HPLC) assay for the fourteen major cellular purine ribonucleotides and 2′-deoxyribonucleotides is presented. Following an initial separation by anion-exchange HPLC, the nucleotides are hydrolyzed to their respective nucleosides by alkaline phosphatase and quantified by reversed-phase HPLC and UV absorbance detection. The assay is reproducible, specific, and has a detection limit of 10 pmol/sample. The recovery of nucleosides derived from nucleotides is 85%. Purine nucleotide pool sizes have been measured in cultured mouse T lymphoma (S-49) cells before and after treatment with 2.0 μM mycophenolic acid, an inhibitor of the enzyme IMP dehydrogenase, for 3 h. Control nucleotide levels obtained by this method are consistent with those reported for S-49 cells using other methods, and the observed decrease in guanine nucleotides and increase in IMP after treatment with mycophenolic acid agree with previous reports.

INTRODUCTION

The measurement of the acid-soluble purine and pyrimidine mononucleotide pools is important in the study of the regulation of cellular function and its alteration by enzyme abnormalities or antimetabolite chemotherapeutic treatment. Current assay procedures for the measurement of the purine and pyrimidine mononucleotides are not capable of detecting all of the major ribonucleotides and 2′-deoxyribonucleotides at physiological concentrations[1–4]. High-performance liquid chromatographic (HPLC) assays are usually limited to the analysis of ribonucleotides by the incomplete separation of 2′-deoxyribonucleotides from the more abundant ribonucleotides[1,2]. HPLC analysis of ribonucleotide monophosphates is often hindered by their low intracellular concentrations and interfering endogenous compounds such as dinucleotide cofactors. The selective chemical degradation of ribonucleotides by periodate oxidation has been used to measure 2′-deoxyribonucleotide triphosphates, but not the corresponding mono- or di-phosphates[3]. The *in vitro* DNA template assay is also limited to quantitation of 2′-deoxyribonucleotide triphosphates[4].

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We now report an HPLC–UV assay procedure for the determination of all the major purine mononucleotides. The assay consists of an initial separation of the nucleotides by anion-exchange HPLC followed by hydrolysis of the collected nucleotides to nucleosides with alkaline phosphatase and quantitation by reversed-phase HPLC with UV detection. Our laboratory has previously reported an assay procedure for the measurement of the pyrimidine mononucleotides[5], based on the same analytical principles, which can be combined with the purine assay presented here to allow a comprehensive analysis of cellular mononucleotide pools.

MATERIALS AND METHODS

Reagents

Nucleosides and nucleotides of analytical grade and alkaline phosphatase (orthophosphoric monoester phosphohydrolase; E.C. 3.1.3.2) type III, from *Escherichia coli*, at an activity of 22 I.U./mg protein, were obtained from Sigma, St. Louis, MO, U.S.A. [2-^{3}H]Adenosine 5'-monophosphate (^{3}H-AMP), specific activity 15.8 Ci/mmol, 1 mCi/ml ethanol–water (1 : 1) was purchased as the ammonium salt from Amersham, Arlington, IL, U.S.A.

Apparatus

HPLC analysis was performed on a Model ALC/GPC 204 liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) with an additional Model 6000A solvent delivery system and a Model 660 solvent programmer.

Cell culture

The growth and lymphocytic properties of wild type mouse T lymphoma (S-49) cells have been described in detail previously[5]. Cells were grown to a density of 10^6 cells/ml in Dulbecco's modified Eagle's medium with 10% horse serum at 37°C. A 100-ml volume of the cell suspensions was then incubated in the presence or absence of 2.0 μM mycophenolic acid for 3 h. An acid-soluble extract was obtained from the cells by centrifugation, PBS wash of the cell pellet, perchloric acid extraction of the cell pellet and neutralization of the extract by ion pair extraction as previously reported[5,7].

HPLC separation of nucleotides

Nucleotides were separated by anion-exchange HPLC on an Aminex A-29 column (30 cm × 4.0 mm I.D.; resin supplied by Bio-Rad Labs., Richmond, CA, U.S.A.) using the chromatographic conditions previously reported by this laboratory for the analysis of pyrimidine nucleotides[5]. Briefly, a 30 min isocratic elution using 0.025 M sodium citrate, pH 8.2, was followed by a 2-h linear gradient to a final eluent concentration of 0.500 M sodium citrate, pH 8.2. The flow-rate was maintained at 0.3 ml/min throughout the procedure and the column temperature was 50°C. Nine effluent fractions were collected (Fig. 1) and concentrated by lyophilization.

Enzymatic conversion of nucleotides into nucleosides

Lyophilized nucleotide fractions 1–7 were reconstituted with 1 ml of water. Fractions 8 and 9 were reconstituted with 1.5 ml of warm water (*ca.* 60°C) to dissolve

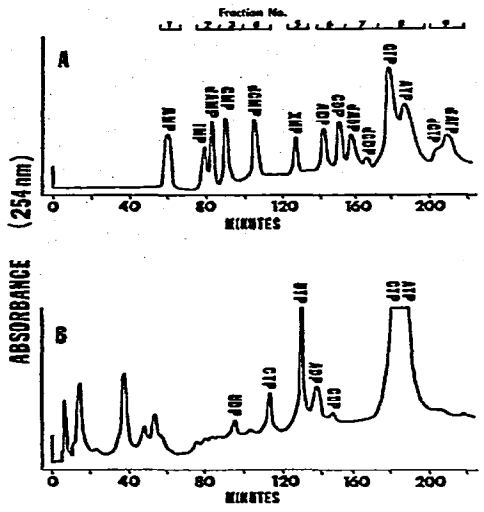

Fig. 1. Separation of purine nucleotides by Aminex A-29 anion-exchange HPLC. (A) Chromatogram of purine nucleotide standards and fraction collection intervals. (B) Chromatogram of an acid-soluble extract from mouse T lymphoma (S-49) cells. The detector sensitivity is 0.5 absorbance units full scale.

the larger amount of citrate present. One unit of *E. coli* alkaline phosphatase was added and the fractions were incubated at 37°C for 30 min. This enzyme preparation replaces the potato acid phosphatase previously used for this procedure[5]. *E. coli* alkaline phosphatase has the following advantages over potato acid phosphatase: (1) the commercially available enzyme has a sufficiently high activity that protein denaturation and centrifugation are no longer required before HPLC analysis; rather the incubation mixture can be directly subjected to HPLC analysis, with a resultant increase in analytical recovery; (2) no pH adjustment is necessary since the pH of the anion-exchange HPLC eluent is close to the pH optimum of the enzyme; (3) a component of the potato acid phosphatase preparation degrades adenosine and 2′-deoxyadenosine to the base adenine, which precludes the separate analysis of adenine ribonucleotides and 2′-deoxyribonucleotides with this enzyme.

Analysis of resultant nucleotides

A 10-μl volume of 99.7% glacial acetic acid was added to samples containing inosine, guanosine, or 2′-deoxyguanosine after phosphate hydrolysis in order to obtain reproducible retention times during reversed-phase chromatography. Fraction 4, containing xanthosine, required the addition of 100 μl of 99.7% glacial acetic acid to obtain reproducible retention times. An aliquot of each sample up to the entire volume was injected onto a LiChrosorb RP-18 reversed-phase column (25 cm × 10 mm I.D.; Altex Scientific, Berkeley, CA, U.S.A.). Samples were eluted with acetonitrile–water (3.5:96.5) at a flow-rate of 7.0 ml/min. Absorbances at 254 nm and 280 nm were monitored. Peaks were quantitated by peak height measurement and comparison with an external standard solution.

High sensitivity analysis of nucleotides

Five- to ten-fold higher sensitivity was attained, when necessary, by collecting the appropriate effluent fraction from the 10 mm I.D. reversed-phase column, concentrating it to *ca.* 250 μl under a stream of nitrogen at 70°C, and reinjecting it onto a μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm I.D.; Waters Assoc.) using an optimized eluent at a flow-rate of 2.5 ml/min. Guanosine, 2'-deoxyguanosine, inosine, and xanthosine were eluted with acetonitrile–water (2.5:97.5) buffered at pH 4.7 with 0.01 *M* sodium acetate. Adenosine and 2'-deoxyadenosine were eluted with acetonitrile–water (2.0:98.0) buffered at pH 3.0 with 0.01 *M* potassium phosphate.

RESULTS AND DISCUSSION

HPLC analysis of nucleotides

Separation. Fig. 1 shows (A) the separation of a solution of pure purine nucleotides and (B) the separation of nucleotides from an extract of S-49 cells on Aminex A-29. Weak anion-exchange chromatography with citrate buffer at pH 8.2 was chosen over strong anion-exchange methods with phosphate buffer at acidic pH values[2] because phosphate buffer inhibits alkaline phosphatase, an integral part of this assay. The fully porous anion-exchange resin Aminex A-29 was chosen over pellicular resins because of its higher sample capacity. Moreover, Aminex A-29 has a high separation efficiency for mononucleotides, including the partial or complete separation of ribo- from 2'-deoxyribonucleotides. This porous resin does, however, require lower flow-rates for proper performance and therefore a longer total analysis time of 4 h. Fig. 2 shows the nucleosides derived from nucleotides in an extract of S-49 cells on the 10 mm I.D. reversed-phase column. This high-capacity column was

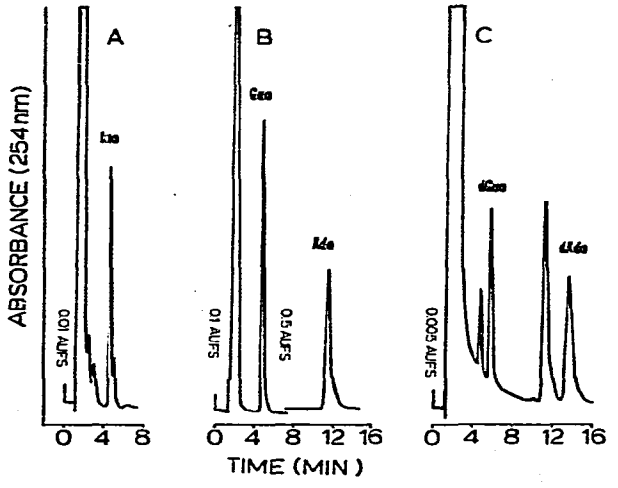

Fig. 2. Chromatography of nucleosides derived from the nucleotides in a biological sample consisting of the extract of S-49 cells on LiChrosorb RP-18 reversed-phase column (10 mm I.D.). (A) 1.48 nmol of inosine derived from IMP). (B) 13.3 nmol of guanosine derived from GTP and 74.3 nmol of adenosine derived from ATP. (C) 0.59 nmol of deoxyguanosine derived from dGTP and 1.08 nmol of deoxyadenosine derived from dATP. The peaks immediately preceding each deoxyribonucleoside are small amounts of the respective ribonucleoside derived from GTP and ATP.

used because of the large amount of citrate in samples after anion exchange, which can overload a smaller column. Fig. 3 shows the increased sensitivity attainable when the nucleosides eluted from the 10 mm I.D. column are collected, concentrated, and reinjected onto an analytical reversed-phase column.

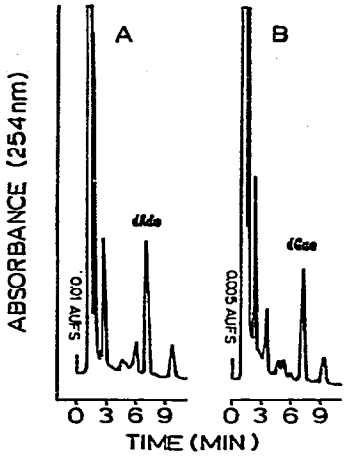

Fig. 3. Chromatography of nucleosides derived from the nucleotides in a biological sample consisting of the extract of S-49 cells which were collected from the 10 mm I.D. column and reanalyzed on a μBondapak C_{18} reversed-phase column (3.9 mm I.D.). (A) 0.103 nmol of deoxyguanosine derived from dGDP. (B) 0.218 nmol of deoxyadenosine derived from dADP.

Minimum detection limit. The detection limit for nucleosides isolated from cell extracts on the 10 mm I.D. reversed-phase column ranges from 40 pmol/sample for guanosine to 100 pmol/sample for 2′-deoxyadenosine. An ultimate sensitivity of at least 10 pmol per sample is attainable by reanalysis on the analytical reversed-phase column. In a sample of 10^8 cells only dGMP and dAMP are present at levels that require the higher sensitivity of the final analytical step. XMP was not present in detectable quantities (< 10 pmol/*ca.* 10^8 cells) in the S-49 cell line.

Analytical recovery. The analytical recovery of the method was determined by the addition of trace amounts of ^{3}H-AMP to samples at the beginning of the procedure. ^{3}H-AMP recovery after quantitation on the 10 mm I.D. reversed-phase column was 85 ± 3%. The effect of the different citrate concentrations of mono-, di-, and triphosphate fractions on quantitation by reversed-phase HPLC was studied by analyzing nucleoside standards in solutions of citrate ranging from 0.1 to 2.5 *M*. No difference was observed. There was also no difference (< 5%) observed between quantitation on the 10 mm I.D. reversed-phase column and upon collection, concentration, and reanalysis of nucleosides by analytical reversed-phase column, suggesting complete analytical recovery of nucleosides during this step. Alkaline phosphatase quantitatively hydrolyzes nucleotides present in amounts far in excess of those found in biological samples in 30 min. Leaving samples in solution with the active enzyme longer than this does not result in any degradation of the nucleosides.

Precision. The precision of the assay was studied by the measurement of the

nucleotide pools of three separate aliquots of the same S-49 cell extract. The standard deviations of these measurements for each nucleotide, given in Table I, are below 10% except for the dNMPs, which are present in levels approaching the sensitivity limit of the assay.

TABLE I

PRECISION OF NUCLEOTIDE ANALYSIS

The nucleotide contents of three aliquots from the same extract of S-49 cells were separately determined.

	nmol/10⁸ cells	*S.D. (%)*		*nmol/10⁸ cells*	*S.D. (%)*
AMP	3.66	6	dAMP	0.023	12
ADP	12.4	6	dADP	0.12	3
ATP	141	6	dATP	1.93	10
GMP	0.18	*	dGMP	0.009	19
GDP	2.27	1	dGDP	0.062	5
GTP	24.8	5	dGTP	0.80	9
IMP	0.42	*			
XMP	**				

* S.D. not calculated because of sample mishandling.
** Nucleotide not present in detectable amounts.

Specificity. The specificity of the method for the desired nucleosides has been confirmed by retention times on both anion-exchange and reversed-phase HPLC, peak symmetry, and the ratio of their absorbances at 254 nm to those at 280 nm. dADP cannot be quantitated under the conditions used for the high capacity reversed-phase HPLC step because of an interfering compound with a UV absorbance ratio different from that of authentic 2′-deoxyadenosine; however, the interference is separated from 2′-deoxyadenosine under the conditions used for high sensitively analysis, pH 3.0. Dinucleotide cofactors are resistant to treatment by the phosphatase enzyme, *i.e.*, NAD is not converted to adenosine + P_i + NMN by alkaline phosphatase and therefore do not interfere with the assay[8].

Comparison with other methods. Table II shows the nucleotide levels in S-49 cells determined by this method and those determined by other methods. There is consistency between the different methods.

TABLE II

NUCLEOTIDE CONTENT OF S-49 CELLS AS DETERMINED BY THIS AND OTHER METHODS

	Nucleotide content (nmol/10⁸ cells)				
	IMP	*ATP*	*GTP*	*dATP*	*dGTP*
This method*	0.59	130	22.7	2.02	0.78
Other methods	—	185**	24.2**	1.44***	0.98***

* Values are the mean obtained from six separate S-49 cell extracts.
** Determined by anion exchange with UV absorbance[12].
*** Determined by DNA template[13].

Nucleotide pool size changes in S-49 cells following treatment with mycophenolic acid.

Mycophenolic acid is a potent inhibitor of the enzyme inosinate dehydrogenase, an essential enzyme in the biosynthesis of guanine ribo- and deoxyribonucleotides from inosinate[9]. We are studying the toxic effects resulting from depletion of either guanine ribonucleotides or guanine deoxyribonucleotides in S-49 cells. As a part of this study, we treated wild type S-49 cells with 2.0 μM mycophenolic acid for 3 h and observed the resulting changes in purine nucleotide pool sizes. Mycophenolic acid reduced the concentrations of GTP and dGTP to 30 and 55% of control values, respectively, while the IMP level increased to *ca* 170% of control values. These changes can be attributed to inosinate dehydrogenase inhibition. In addition, the level of ATP was relatively unaffected while the level of dATP increased slightly to 130% of control values. Similar purine nucleotide pool size changes caused by mycophenolic acid have been observed in the murine lymphoma L5178Y cell line[10,11].

CONCLUSION

The presented assay for purine mononucleotides yields reproducible results that are consistent with previous methods for the analysis of selected nucleotides. Moreover, the analytical approach combining high-resolution anion-exchange and reversed-phase HPLC with one enzymatic step allows the flexibility to determine any or all of the endogenous purine or pyrimidine mononucleotides as well as nucleotide derivatives of administered antimetabolite drugs. The analytical features of the method make it a more generally applicable tool than previous procedures in the study of the mononucleotide metabolism of cells.

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