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Research Article

Separation and Analysis of Mono-Di- And Tri- Phosphate Nucleotides from Cell Extract Using Reversed Phase HPLC

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ABSTRACT

The focus of the present study was to develop a simple, reliable, and highly sensitive ion pair reversed phase high performance liquid chromatography for the separation and quantification of purine, pyrimidine and pyridine nucleotides. First, we optimized the column conditions to achieve baseline separation and quantification of 15 nucleotides. The least amount required for detecting all the ribonucleotides with reproducibility and high accuracy was 10 pmol. Calibration curves for all nucleotide standards were linear within the range of 10-200 pmol. This method can be successfully used to quantitate all nucleotides simultaneously in studies with nucleotide inhibitors such as Tiazofurin and Benzamide Riboside in cancer cell lines. This is the first report showing the separation of all 15 nucleotides in a single injection.

Keywords: Nucleotide separation; Ion-Pair; Reversed phase HPLC; Nucleotide; Nucleoside; Nucleotide

INTRODUCTION

The Nucleotides, purines and pyrimidine's which are building blocks of nucleic acid play an important role in energy metabolism of the cell [1]. Nucleotides are composed of pentose sugar linked to a cyclic nitrogenous base with one to three phosphate groups thus making them co-factors for energy transfer in various transduction pathways thus maintaining the biochemical and physiological properties of the cells [1]. The energy status plays an important role in maintaining cellular integrity and redox state [2-4]. The nucleotides are involved in various signal transduction pathways; serve as allosteric regulators of enzymes; provide building blocks for both RNA and DNA synthesis and thereby play an important role in both anabolic and catabolic reactions [3,5,6]. The intracellular concentration of nucleotides reflects the energy status, metabolic stress and purine and pyrimidine nucleotide related metabolic disorders. Therefore, it is important to know the flux of each nucleotide within the cell and not just a single one at any given point of time. For measuring cellular levels of nucleotides, HPLC (UV-Vis) is currently the most widely used technique compared to other methods. This technique can separate wide range of nucleotides and quantify them simultaneously after a single injection [7].

Reversed phase separation is based on nonpolar, or hydrophobic, interaction between nonpolar sample molecules and the nonpolar stationary phase [8,9]. Based on the hydrophobic nature of the sample molecule, it can be retained, and its retention is altered by manipulating the aqueous to organic content of the mobile phase. Due to the highly charged and polar nature of nucleotides they cannot be retained under typical reversed phase high performance liquid chromatography conditions. The nonpolar end of the reagent is held strongly by the nonpolar stationary phase column (C8 or C18) leaving the charged functional group sticking out the mobile phase [10,11]. The simultaneous separation and retention of purine nucleotides was found to be difficult using a reversed phase anion exchange HPLC [2,4]. Therefore, a strong ion pair reversed phase chromatography was needed for nucleotide separation [12]. Ion pair reagent and organic solvent are the two major components of the aqueous mobile phase. Organic solvents, such as methanol or acetonitrile are used to reduce the running time to analyze samples. Recent studies on reversed phase separation of nucleotides are based on high strength ionic eluent with either potassium or ammonium phosphate buffer at appropriate pH for neutralizing polar nucleotides with a hydrophobic interaction at the stationary phase [5]. To further enhance the separation, positively charged reagents such as tetrabutylammonium hydrogen sulphate (TBA) are used to retain negatively charged nucleotides.

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Following drug treatment, particularly during chemotherapy with ribonucleoside inhibitors, cells die due to apoptosis by the reduction of nucleotides [13]. Therefore, the measurement of their cellular concentration is of importance, as it is a true indicator of in vivo effect of the drugs. Most of the publications to date concentrated to separate either only a few nucleotides or mostly nucleotides of interest and therefore, does not accurately give the ratio of interaction of their different phosphate groups in the microenvironment of the cell [8,14-16].

2-ß-D-ribofuranosylthiazole-4-carboxamide (Tiazofurin, TR) and 3-[1-D-ß-D-ribofuranosyl] benzamide (Benzamide Riboside, BR) are pro-drugs that needs to be activated to their respective NAD analog, thiazole-4-carboxamide adenine dinucleotide (TAD) and benzamide adenine dinucleotide (BAD), respectively by the action of nicotinamide 5'-mononucleotide adenylyltransferase (NMNAT). TAD and BAD inhibit IMP dehydrogenase causing cessation of guanylate synthesis (GTP, dGTP) in cancer cells resulting in cell death [17,18]. The level of GTP is critical for the signaling pathways required for cell function and the depletion of GTP pools can interfere with G-protein-mediated signal transduction thereby inducing phenotypic changes in gene expression. Therefore, using drugs to reduce intracellular GTP has proven to be very useful in clinical therapeutics since the reduction of GTP results in eventual cancer cell death. TR and BR drugs can down regulate GTP by targeting inosine 5'-monophosphate dehydrogenase (IMPDH) in the purine nucleotide synthetic pathway [19,20].

The goal of this study was to describe the method to separate and accurately measure all purine, pyrimidine and pyridine ribonucleotides at pmol concentrations using reversed phased ion pair HPLC method.

MATERIALS AND METHODS

Materials

All nucleotide standards were of highest analytical grade and were purchased from Sigma Aldrich (St. Louis, MO, USA). Potassium hydrogen phosphate, dipotassium hydrogen phosphate, tetrabutylammonium hydrogen sulphate and methanol were of HPLC grade from Fisher Scientific (Pittsburgh, PA, USA).

HPLC column and instrumentation

HPLC separation was conducted using Phenomenex Gemini 250 × 4.6 mm, 5 µm C18 110A° column (Torrance, CA). The column was washed and maintained as per manufacture's instruction. The Phenomenex security guard cartridge (Gemini C18 4 × 3.0 mm) was replaced after 200 injections. For the HPLC analysis Shimadzu High Performance Liquid Chromatography (Canby, OR) instrument was used utilizing diode array detector (SPD-M20A) and all the data for the work up were obtained by a UV diode array detector using chromatograms at 254 nm extracted from the UV spectra scanned from 230 to 310 nm for all runs. Chem station software version 4.0 was used for data collection and processing.

Methods

HPLC method: The HPLC method was carried out using a two solvent system. The mobile phase consisted of buffer A, 0.1 M potassium dihydrogen phosphate, pH 6.0; and buffer B, 0.1 M potassium dihydrogen phosphate containing 4 mM tetrabutylammonium hydrogen sulphate and 20% methanol at pH 6.0. The pH was maintained at 6.0 using 0.1 M dipotassium hydrogen phosphate in both buffers. The buffer stock solutions were filtered through 0.45 µm membrane filters, degassed and stored at 4°C; brought to room temperature before use. The column was equilibrated with buffer A, samples were injected using auto sampler (SIL-20AC) and the nucleotides were eluted off the column with gradient program as shown in Table 1.

Table 1: Gradient time program for nucleotide separation.

Time	Column temp. (°C)	Conc. of buffer solution (%)		Flow rate (ml/min)
		Buffer A	Buffer B	
0.01	25	100	0	0.5
6	29	100	0	0.5
7.5	29	99	1	0.5
15	25	95	5	0.75
17	25	85	15	0.75
50	25	20	80	0.75
55	25	0	100	0.75
57	25	100	0	0.5
66	25	100	0	0.5

Specificity, Linearity, precision, accuracy and recovery of the method: The specificity of each nucleotide was identified by comparing their retention times with those of known nucleotide standards. Linearity of the method was validated using five different standard solutions (10, 25, 50, 100 and 200 pmol) of 5 µl injections (n=3). A calibration curve was constructed by linear regression of the observed average peak versus concentration of the respective nucleotide using Chemstation software. Precision of the method was done for repeatability and reproducibility. Precision were determined by consecutive injections of 10, 25, 50, 100 and 200 pmol standard nucleotide concentrations with each injection repeated trice for calculating the relative standard deviation (RSD). Accuracy is expressed by calculating the percent recovery. For calculating, the percent recovery two different concentrations (50 and 100 pmol) of standard IMP, GTP, NAD and ATP were spiked with HeLa cell extract (sample) and 5 µl injected using auto sampler to evaluate spiked experiments for % recovery. % Recovery=[nucleotide (sample+standard) sample]/ standard × 100. All experiments were done in triplicates.

Cell culture: Human colorectal cancer cell lines (HT29 and Caco2) and cervical cancer cell line (HeLa) were obtained from the National Cancer Institute, Bethesda, MD, maintained in 5% CO2 at 37°C in minimum essential media (MEM, Life Technologies, Inc., Grand Island, NY) supplemented with 1% penicillin, streptomycin and amphotericin B (Invitrogen, Carlsbad, CA), and 10% fetal bovine serum for HT29 and HeLa while, Caco2 cell line required 20% fetal bovine serum for optimal growth.

Preparation of cell extracts: Logarithmically growing (1 × 106) cells were seeded in 10 cm Petri dishes and after 24 h, were exposed to sub lethal concentrations of Tiazofurin or Benzamide Riboside for 4 hrs. After 4 hrs, cells were trypsinized, centrifuged at 400 × g for 5 min at 4°C, washed once with 5 ml cold 1 × PBS

and re-suspended in appropriate volume of freshly prepared 10% trichloroacetic acid and kept on ice for 10 min. The suspension was centrifuged at 13,000 × g for 1 min and the supernatant was neutralized with trioctylamine (TOA): Freon (1:3, v/v), vortex for 20 sec, centrifuged at 13,000 × g for 1 min and the upper aqueous layer was collected and stored at -80°C.

Determination of nucleotide concentration: For nucleotide determination an aliquot of 5 µl of the nucleotide extract was injected on to HPLC column using auto sampler (SIL-20AC). Runs also included appropriate standards and controls. All nucleotide standard stock solutions were prepared in deionized water and stored at -20°C.

Statistical analysis: All experiments were conducted at least three times with each data point carried out in triplicate. The observed results in the two different groups were compared using Student's t-test. Significance in all tests was set at a 95% or greater confidence level ($P<0.05$).

RESULTS AND DISCUSSION

Chromatographic separation of nucleotides and its validation

Quantification of nucleotides requires proper baseline separation of their standards. The 15 nucleotides were separated in a single injection with ion pairing on a reversed phase HPLC column with reliable retention time (Figure 1). The baseline separation of all the nucleotides in the order of increasing polarity was achieved with a gradient buffer B. We optimized the TBA concentration at 4 mM and maintaining 11 minutes of equilibration time provided a reproducible separation with average retention times [7]. Of the different gradient time programs tested, we found that maintaining higher column oven temperature, low flow rate and 100% solvent A was important for the separating cytosine and uracil nucleotides followed by slow increase in solvent B concentration. We also observed that higher flow rate and oven temperature at 25°C affected their separation. The higher oven temperature at 29°C (Table 1) resulted in lowered viscosity of the mobile phase, thereby increasing the chromatographic efficiency and also increased temperature resulted in lesser retention time with improved resolution [21,22]. The gradient increase in temperature from initial 25°C to 29°C after 7 minute run and back to 25°C after 15 minutes for the separating CTP, UDP, GMP and UTP did not show desired results. In addition to the column temperature, initial high flow rate or higher solvent B concentration also led to the merger of CDP, UMP and CTP. We also observed that increased concentration of TBA from 4 mM led to precipitation with phosphate buffer and thereby changing the run profile and retention time (data not shown) of the nucleotides. The phosphate group of the nucleotides forms an iron pair with TBA in the mobile phase to become electrically neutral. The increased hydrophobic character of the ion pair resulted in a greater affinity for the reversed stationary phase and led to better resolution. Therefore, to ensure no shift in retention time and of peaks when all the 15 standard nucleotides are mixed, we spiked each of the nucleotides and analyzed as described in Table 1. As observed in Figure 1, the relative elution order of peaks corresponded to their polarity. The pyrimidine nucleotides were eluted before the purine; therefore, cytosine was eluted before uracil, and guanine was followed by adenine nucleotides due to the presence of pairing agent. Under this system, the separation of different molecules is related to their relative affinity between the stationary phase and the mobile phase and the elution time was delayed based on the number of phosphates. The nucleotides being strong acids are charged with a wide range of pH range and polarity. The addition of ionic group that could pair charged molecules, resulted in a greater retention time of the nucleotides on reversed phase columns. As predicted the monophosphates were less retained than diphosphates and triphosphates. The ion pairing with gradient elution led to successful separation of mono-di and triphosphates of cytosine, uracil, guanine, adenine, NAD and NADP. Related to the polarity of the base functional group, we also observed a gradual increase of elution time from cytosine to uridine, guanine, adenine, nicotinamide and their reduced forms when injected with 10 pmol of each standard (Figure 1). The retention time with clear peak were identified for each individual standard nucleotide, this was followed by pooling 15 nucleotides, were successfully separated without any shift in retention time. Under the present standardized method condition a minimum concentration of nucleotide with 10 pmol were detected and calibration for each ribonucleotide ranged from 10-200 pmol in a linear order. Linear response was observed for each compound at 5 different concentrations tested. The average peak area from three injections was plotted against 10, 25, 50, 100 and 200 pmol concentration; a linear relation with good correlation coefficient was obtained. From the calibration curve, correlation coefficient (r2) was between 0.996-0.999 for all the 15 nucleotides using Chem station software. The precision of the new method was tested at intra-day and inter-day repeatability of triplicate injection of 50 pmol standard solution. The studies were carried out at three different times within the same day and for 3 days. We found the percent RSD was less than two for both intra-day and inter-day analyses (data not shown). The next step was to determine the accuracy of the method for which, recovery studies were performed for four nucleotides that were relevant with ribonucleoside inhibitor Tiazofurin and Benzamide Riboside tested under in vitro conditions. A representative chromatogram of HeLa nucleotide extract is shown in Figure 2A, and a representative chromatogram of the sample spiked with 50 pmol of IMP, GTP, ATP and NAD are illustrated in Figure 2B. Three replicates of 50 and 100 pmol spiked IMP, GTP, ATP and NAD were injected and then the recovery values were calculated from the difference between the spiked sample versus the unspiked (normal) sample. We tested the recovery of four ribonucleotides, NAD and ATP had a recovery of approximately 125% at 100 pmol while IMP and GTP showed a recovery of 103% and 105% respectively at 100 pmol (Table 2). All the experimental results were in the range of the acceptability for precision and accuracy, which indicated that the newly developed method is sensitive and accurate for determination of ribonucleotides.

Table 2: Recovery studies were performed in cervical cancer cell line He La, spiked with known concentrations of IMP, GTP, ATP and NAD (n=3).

Nucleotide	Spiked with 50 pmol (% Recovery)	Spiked with 100 pmol (% Recovery)
IMP	103.5 ± 2.0	116.7 ± 3.5
GTP	102.9 ± 4.0	113.1 ± 5.6
NAD	121.9 ± 6.0	138.8 ± 5.2
ATP	102.9 ± 6.4	110.0 ± 5.7

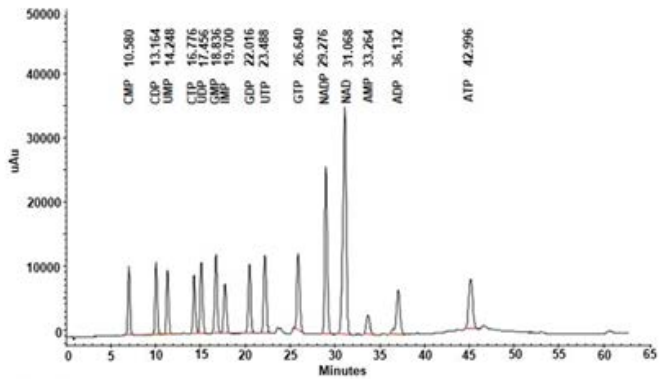

Figure 1: Representative chromatographic elution profile of 15 ribonucleotides on C18 using ion-pair reversed phase chromatography.

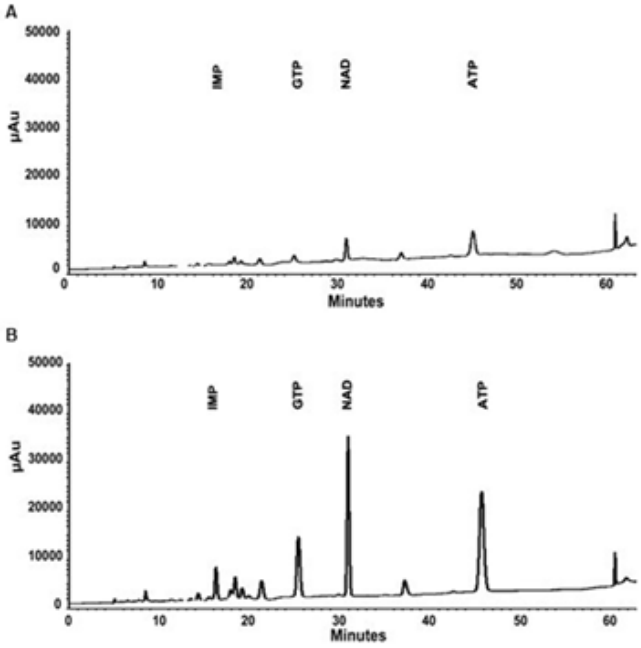

Figure 2: (A) Representative chromatogram of ribonucleotide separation in HeLa control sample. (B) Representative chromatogram of HeLa control sample spiked with standard ribonucleotides to show no shift in peak.

Estimation of nucleotides on Tiazofurin and Benzamide Riboside treatment *in vitro*

Next, we wanted to test this method for ribonucleotide separation and its quantification in in vitro or in vivo studies. We first isolated the nucleotides as described in method section from human cancer cell line HeLa without treatment (control). We then injected different volumes of the cell extract ranging from 5 µl to 50 µl and found that 5 µl injections were sufficient to estimate the nucleotides. Once these ribonucleotides were detected in control sample as shown in Figure 2A. The control samples were mixed with known amount of standard ribonucleotide, to make sure that the spikes correspond to their respective ribonucleotide (standard) (Figure 2B). The treatment of human cancer cell lines HT29, He La and Caco2 with TR and BR for 4 h with sub lethal dose [13] resulted in reduction of GTP, ATP and NAD (Table 2 and Figure 3). TR and BR acted on IMP dehydrogenase thereby depleting the cellular GTP pools, which was responsible for drug induced cytotoxic activity. The reduction of these nucleotides was mediated mainly by inhibiting IMP dehydrogenase resulting in the inhibition of DNA and RNA synthesis due to the depletion of guanine nucleotides. The reduction in NAD and GTP content of 20 to 89% compared to their respective control on treatment with Tiazofurin or Benzamide riboside was observed (Table 3) and these results were aligned with those reported in hepatic, colorectal and mice leukemic cell lines [19,20,23,24]. Logarithmically growing cells in culture were incubated with saline, Tiazofurin (TR) or Benzamide Riboside (BR) at the indicated concentrations for 4 hrs at 37°C. Cell pellets were extracted with 10% trichloroacetic acid, neutralized and analyzed on HPLC as described in the Methods section. Results are expressed as means ± SD of triplicate samples done with three different experiments. *denotes statistical significance ($P<0.05$). The reduction in GTP as a result of TR and BR incubation corresponds with increase of 111 to 733% in IMP levels in all the cell lines tested (Figure 3) compared to their respective controls as previously reported in leukemic K562 cell line [18]. Taken together, the newly established method can be used for detection, separation and estimation ofribonucleotides at pmol range from a single injection.

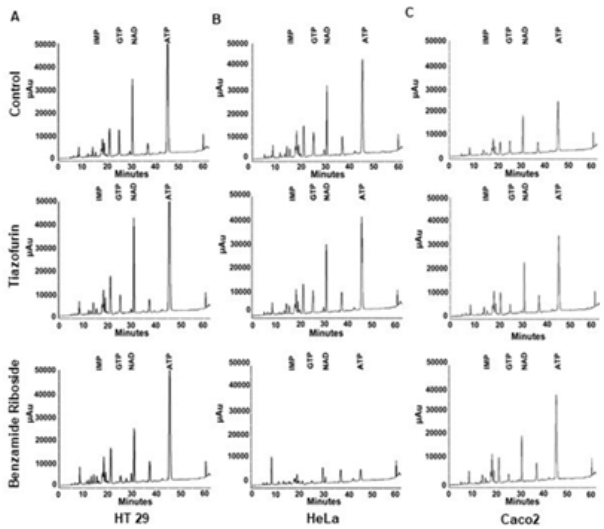

Figure 3: Chromatographic elution pattern of IMP, GTP, NAD, ATP and other nucleotides with and without Tiazofurin and Bezamide Riboside treatment in (A) HT29, (B) HeLa and (C) Caco2 cell line.

Table 3: Effect of Tiazofurin and Benzamide Riboside treatment on IMP, GTP, NAD and ATP concentration in cancer cell lines.

Cell line	Treatment	nmol of IMP/ 106 cells	% of control	nmol of GTP /106 cells	% of control	nmol of NAD /106 cells	% of control	nmol of ATP /106 cells	% of control
HT29	Control	0.09 ± 0.02		0.6 ± 0.13		0.8 ± 0.15		4.5 ± 0.85	
	TR(125 µM)	0.10 ± 0.01	111.1*	0.3 ± 0.07	50.0*	0.69 ± 0.09	86.2*	3.8 ± 0.61	84.4*
	BR (21 µM)	0.14 ± 0.02	155.6*	0.1 ± 0.02	16.7*	0.41 ± 0.08	51.3*	3.7 ± 0.44	82.2*
HeLa	Control	0.12 ± 0.02		0.9 ± 0.03		1.14 ± 0.02		3.5 ± 0.6	
	TR(100 µM)	0.15 ± 0.02	125	0.4 ± 0.01	44.4*	0.9 ± 0.01	78.9*	3.0 ± 0.1	85.7*
	BR(120 µM)	0.15 ± 0.01	125	0.1 ± 0.01	11.1*	0.3 ± 0.01	26.3*	0.5 ± 0.2	14.3*
Caco2	Control	0.3 ± 0.03		0.5 ± 0.04		0.40 ± 0.03		3.5 ± 0.2	
	TR(400 µM)	1.1 ± 0.02	366.7*	0.3 ± 0.04	60.0*	0.42 ± 0.01	105	4.2 ± 0.1	120.0*
	BR (18 µM)	2.2 ± 0.03	733.3*	0.2 ± 0.02	40.0*	0.35 ± 0.01	87.5*	4.5 ± 0.3	128.6*

CONCLUSION

An Ion pair reversed phase HPLC method was developed for the simultaneous separation of 15 ribonucleotides with required sensitivity, accuracy, and precision from a single injection. This method can be used to closely monitor the intracellular level of nucleotide in in vitro and in vivo studies.

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CONFLICT OF INTEREST

None of the authors have any conflicts of interest with this work

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