Research Article

Aqueous normal phase retention of nucleotides on silica hydride-based columns: Method development strategies for analytes revelant in clinical analysis

An aqueous normal phase HPLC method coupled with UV or ESI/MS detection was used for the determination of a wide variety of nucleotides, essential in metabolomics studies. Fifteen nucleotides were tested in clinically relevant mixtures at levels of 100 μg/mL for UV detection and 1 μg/mL for ESI-MS detection. Analysis times for all protocols developed were less than 20 min. The chromatographic conditions were changed to achieve optimized retention and separation of the nucleotides studied. The aqueous normal phase-HPLC methods were developed utilizing two columns, one having a minimally modified hydride surface another having an undecanoic acid moiety on a hydride surface. Volatile, low ionic strength mobile phases were used. Negative ion mode ESI-MS at near neutral pH mobile phase, combined with a TOF detector provided a highly sensitive and specific method, which is equally suitable for quadrupole and ion trap instruments.

**Keywords:** Aqueous normal phase retention / Nucleotides / LC-MS / Uridine 5'-diphosphate-galactose / Uridine 5'-diphosphate-glucose

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1 Introduction

In recent years there has been a strong interest in robust and sensitive methods for the analysis of nucleotides, which are found in cell extracts and can be used as metabolomic markers of various disease states. It is important to note that determination of the concentration of nucleotides in living organisms can provide information on mitochondrial status and cellular metabolic stress as well as inherited diseases of purine and pyrimidine nucleotide metabolism [1]. The field is already substantial and growing rapidly. Examples of metabolic nucleotide analyses include: the ratio of the adenine nucleotides, adenosine 5'-triphosphate (ATP)/adenosine 5'-diphosphate (ADP)/adenosine 5'-monophosphate (AMP), is measured to indicate cell energy status or cell apoptosis/death, or ischemia in a tissue [2, 3], while the occurrence in red cells of the purine nucleotides deoxy-ATP and deoxy-guanosine 5'-triphosphate (GTP) are diagnostic for adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency, respectively [4].

Due to their highly charged and polar nature (the presence of one or more phosphate groups), nucleotides are not retained under typical reversed-phase conditions. Historically, ion-pair reversed phase chromatography and strong anion-exchange HPLC were methods of choice for determination of nucleotides in biological samples [2, 5]. However, these methods are not readily compatible with MS detection, due to high concentration of salts or ion pair agents in the mobile phases needed to separate nucleotides. Nucleotides have thus remained challenging compounds to analyze. As HPLC linked to MS is a powerful, sensitive technique, which specificity allows detection and identification of metabolites in the presence of a complex matrix, we have investigated a method of analysis for nucleotides based on an aqueous normal phase (ANP) retention mechanism for highly polar compounds. The mobile phase solvents used with the developed methods are compatible with LC-MS detection. In addition, the modifier or the salts selected for the analysis can also be used with LC-MS (volatile and at low concentration). The main purpose of this study was to test the ANP method using various combinations of nucleotides that are present in clinical samples and...
would have relevance to energy metabolism, disease diagnosis or pharmaceutical evaluations. The results reported below expand upon an earlier investigation using hydride-based stationary phases for the analysis of nucleotides [6]. All of the developed methods for the analysis of nucleotides can be readily used for challenging matrices such as erythrocyte and tissue samples. These methods using actual clinical samples will be the subject of subsequent reports.

2 Materials and methods

2.1 Materials

Stock solutions of standards analyzed in this study: GTP, 1 mM, inosine 5’-triphosphate (ITP), 40 mM, AMP, 1 mM, ADP, 1 mM, ATP, 1 mM, β-nicotinamide adenine dinucleotide (NAD), 1 mg/mL, NAD-phosphate (NADP), 1 mg/mL, uridine 5’-diphosphate-galactose (UDP-galactose), 1 mg/mL, uridine 5’-diphosphate-glucose (UDP-glucose), 1 mg/mL, S-adenosyl-L-methionine (SAM-e), 1 mg/mL, cytidine-5’-monophosphate (CMP), 1 mg/mL, cytidine-5’-diphosphate (CDP), 1 mg/mL, uridine 5’-monophosphate disodium (UMP), 1 mg/mL, uridine 5’-diphosphate (UDP), 1 mg/mL, uridine 5’-triphosphate (UTP), 1 mg/mL, xanthosine 5’-monophosphate (XMP), 1 mg/mL. All standards were purchased from Sigma-Aldrich, St. Louis, MO, USA. HPLC grade acetonitrile was obtained from Fisher Scientific (Pittsburgh, PA, USA), ammonium formate and ammonium acetate were purchased from Sigma-Aldrich. Deionized water was used for all HPLC buffers and standards (Milli-Q system, Millipore, Billerica, MA, USA).

2.2 HPLC columns

Cogent diamond hydride (DH): 2.1 x 150 cm, with a particle diameter of 4.2 µm, pore size of 100 Å, surface area of 350 m²/g and 2.5% carbon as reported by the manufacturer, MicroSolv Technology (Eatontown, NJ, USA).

Undecanoic acid (UDA): 2.1 x 50 cm, column material was synthesized at San Jose State University (SJSU). The bonding of the carboxylic acid to a silica hydride intermediate was confirmed by DRIFT (Diffuse Reflectance Fourier Transform) spectrometry and 13C CP-MAS NMR (Cross Polarization Magic Angle Spinning Nuclear Magnetic Resonance) spectroscopy [7].

A Cogent Bidentate-C18 silica hydride column, 2.1 x 150 cm, MicroSolv Technology, was also tested.

2.3 HPLC instrument

The HPLC system (Agilent 1100 binary LC system, Agilent Technologies, Wilmington, DE, USA) consisted of vacuum degasser, thermostatted automatic sampler, diode-array UV detector with standard flowcell. ChemStation software (version B.03.01) was used for data collection and processing. All data for the work-up were obtained by a UV diode array detector, using chromatograms at 254 and 280 nm, extracted from the UV spectra scanned from 230 to 310 nm for all runs.

2.4 LC-MS instrument

The HPLC was an Agilent (Little Falls, DE, USA) 1200SL Series LC system, including degasser, binary pump, temperature-controlled autosampler and temperature-controlled column compartment. The mass spectrometer was an Agilent (Santa Clara, CA, USA) Model 6220 MSD TOF instrument with a dual electrospray source.

2.5 HPLC method

Two solvent systems were adopted, both being compatible with LC-MS. The mobile phase for UV experiments consisted of solvent A (15.9 mM ammonium formate or 13.0 mM ammonium acetate) and solvent B (90% acetonitrile/10% v/v water containing 15.9 mM ammonium formate or 13.0 mM ammonium acetate). Both solutions A and B were prepared using a stock solution of the salt (159 mM ammonium formate or 130 mM ammonium acetate – kept refrigerated) by 1:10 dilution and were filtered through a 0.45 µm nylon filter (MicroSolv Technology) before use. The flow rate was 0.4 mL/min, injection volume was 1 µL. Several gradient profiles were used (see Table 1). A 5 min re-equilibration between runs was sufficient to restore initial conditions.

The mobile phase for LC-MS analysis consisted of the following: solvent A (5 mM ammonium acetate adjusted to pH 7.2 with ammonia) and solvent B (90% acetonitrile/10% v/v water containing 10 mM ammonium acetate adjusted to pH 6.5 with ammonia). Solution A was prepared using stock solution of the salt (100 mM ammonium acetate – kept refrigerated) by dilution. Solution B was prepared as follows: the pH of 100 mM solution of ammonium acetate

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Post time: 5 min.
was adjusted to pH 6.5 with ammonia and diluted 1:10 in acetonitrile. Solutions A and B were filtered through 0.45 μm nylon filters.

Stock standard solutions for analysis were prepared in deionized water (see Section 2.1 for concentration of each sample) and were stored at −20°C. For UV analysis, samples were diluted 1:10 into 50% acetonitrile/50% water mixture. For LC-MS analysis, samples were diluted 1:1000 in 50% acetonitrile/50% water. Due to the small injection volume (1 μL) little band broadening was observed. Larger injection volumes (>5 μL) cause some band broadening, which can be minimized or eliminated by using 70:30 acetonitrile/water for dilution of the sample.

2.6 Preparation of blood cell extracts

Whole blood samples (with EDTA anticoagulant) were collected from healthy people and from galactosemia patients. Bloods were centrifuged and the plasma and buffy coat (containing the white cells) were discarded. The packed erythrocyte pellet was then washed twice in saline, and used to prepare physiological nucleotide extracts. For deproteinization the following procedure was used: 100 μL packed erythrocytes and 200 μL of 10% TCA were mixed rapidly in a 1.5 mL capped Eppendorf tube. Samples were microfuged for 10 min at 12 000 rpm, and the supernatant was back extracted with water-saturated diethyl ether to remove the TCA. Immediately before analysis, each erythrocyte extract was filtered through a disposable syringe filter (0.45 μm) and was diluted 1:1 with acetonitrile and then injected into the HPLC system. Peak assignment was done by comparison with the retention time of standards as well as by comparison of UV spectra of the individual standards.

3 Results and discussion

Two of the columns investigated in this study were found to be useful for the studies of retention of nucleotides: a Cogent DH HPLC column, already used in the analysis of many metabolites and polar compounds [8, 9], and a UDA weak cation exchange, carboxylic acid HPLC column. A hydride-based C-18 was also tested for the sake of completeness. Although it might be predicted that a hydrophobic modifier such as C-18 would not bind hydrophilic nucleotides, the often counter-intuitive behavior of ANP interactions in practice justified checking that C-18 may bind, for example, the base or ribose moieties. It was found that the hydride C-18 indeed did retain mononucleotides, but the poor performance with di- and tri-nucleotides meant it was not suited for the purposes of this report.

Figure 1 illustrates the clear separation of several standards, featuring adenine, guanine and nicotinamide nucleotides with varying numbers of phosphate residues, using the UDA column with an LC-MS compatible mobile phase and a simple ANP gradient (decreasing amount of acetonitrile) [8]. UV detection was used, by diode array. The somewhat broader peak for AMP may be due to the high water content of the sample solvent and the fact that this compound is eluted early in the gradient when the acetonitrile content of the mobile phase is high. For comparison when using the DH column under Gradient 1 conditions (Table 1) the selectivity (α) for GTP/AMP is 1.06 (compared with 1.95 on UDA). The selectivity for NADP/NAD is 1.06 on the DH column compared with 1.39 on UDA. The NADP/NAD and GTP/AMP selectivity data presented indicate different separation mechanisms of the two columns studied. In the case of the UDA column a weak cation-exchange interaction is also possible in addition to the ANP contribution of the hydride surface and/or the solvent layer on the surface. The exact nature of the ANP mechanism on silica hydride materials is not understood at this time but various spectroscopic studies are currently under way to help elucidate how solutes are retained by these types of stationary phases. The relative contributions of each mechanism cannot be determined from the data in this comparison.

The ratio of NAD and NADP has biological relevance when studying redox profiling [10], and redox potential being exploited by new generation NAD depleting cytotoxic drugs [11]. For metabolic screening, the erythrocytes of Lesch-Nyhan Disease patients have grossly raised levels of NAD relative to NADP, while GTP is very low [12–14]. Sensitive assay of GTP levels is also relevant to binding studies of G-proteins [15]. Grossly raised levels of pyrimidine nucleotides (UTP, CTP and CDP-choline) in erythrocytes can be used as a screening method for pyrimidine nucleotidase deficiency, the second most-common erythrocyte metabolic disorder [16].

Figure 2 shows the separation of the three energy nucleotides AMP, ADP and ATP. The analysis using the UDA column and Gradient 1 (see Table 1) is shown in Fig.
All three nucleotides are baseline separated, in order of increasing polarity (i.e. mono-, di-, tri-nucleotides) as expected for ANP/hydrophilic interaction liquid chromatography (HILIC) elution and is the same as that obtained with anion exchange [4]. When the same mobile phase and Gradient 1 were used with the DH column, the separation was partial and the retention order was different: ATP eluted before ADP (Fig. 2B). To confirm this change in selectivity between the UDA and DH columns a sample with eight times higher concentration of ADP was used and the gradient conditions were modified (Gradient 2 – Table 1) to achieve the separation (Fig. 2C). The change in selectivity observed was not consistent with an ERLIC (electrostatic repulsion liquid interaction chromatography) mechanism: using an ammonium acetate buffer, the retention of ADP and ATP would be predicted to be drastically reduced with respect to AMP whereas on the hydride phases retention is increased as the buffer concentration is increased. We propose that the phosphate groups may interact more strongly with the surface structure on the hydride phase than the bonded moiety and thus this phenomenon may be responsible for the difference observed. Estimates of intracellular ATP/ADP/AMP ratios are widely used, to study energy metabolism, particularly in muscle metabolism studies for sports training [17], purine nucleotide metabolism [1] and in other areas of metabolomics.

Figure 3 shows the separation of XMP in relation to GTP, using both columns. Separation of the two compounds was greater when the UDA column was used (Fig. 3A); however better peak shape for these nucleotides was obtained on the DH column (Fig. 3B). This result suggests a difference in the mechanism of retention on the two phases with perhaps the carboxylic acid on the UDA column playing a significant role. Slower mass transfer at the weak cation exchange site could be responsible for the lower efficiency on this column. Although XMP does not normally appear in free nucleotide cell extracts, it is the product of the important cell differentiation enzyme IMP dehydrogenase, the target of mycophenylate and up-regulated by ribavirin therapies [18], and a putative regulator of adipose cell growth [19].

Deficiency of the enzyme ITP pyrophosphohydrolase is a common genetic defect in human populations and has aroused recent interest for its putative pharmacogenetic relevance to thiopurine therapy [20]. The enzyme is part of a nucleotide “futile cycle”, which converts IMP to IDP and ITP then back to IMP [21]. Figure 4A shows the optimized
separation of ITP, IDP and IMP, when the UDA column was used with Gradient 3. The three targeted analytes were separated in the order of increasing phosphate content similar to anion exchange [4] but the presence of at least one impurity near ITP and possibly a second near IMP precluded accurate determination of peak symmetry. When Gradient 1 was used on the UDA column the nucleotides were also well separated but the peaks exhibited some tailing (Fig. 4B). If the DH column was used with Gradient 1 (Fig. 4C), IDP and ITP were not separated, although the peaks were symmetric and efficiency was improved over the UDA column under identical conditions. Interestingly, the DH column did not reverse the order of elution of the ITP nucleotide group, in contrast with the ATP nucleotide group, illustrating the specific response of the silica hydride medium to the purine base composition.

SAM-e is used as a primary donor of methyl groups by many methylation processes including gene silencing and folate/methionine metabolism. As a drug, it is providing an effective alternative therapy for a number of neuropsychiatric disorders including bipolar disease, dementia and more recently schizophrenia [22]. SAM-e may also have an important role in treating alcoholic liver disease [23]. Figure 5 shows the separation of several nucleotides along with SAM-e. The retention time of this compound was much longer than any of the nucleotides studied most likely due to its fixed positive charge on the sulfur atom. This method could be used for the determination of SAM-e in serum or cell extracts in order to monitor the level of the drug after administration.

Separation of pyrimidine nucleotides was also achievable by ANP HPLC. The uracil nucleotides are essential not only for RNA, but their sugar esters are central to synthesis of polysaccharides – glycogen in animals and starch/cellulose in plants – as well as plant metabolomics [24]. Figure 6 shows the separation of the three primary uracil nucleotides using an LC-MS compatible mobile phase for the UDA column (as shown in Fig. 1) with Gradient 1. Again the separation was based on phosphate number. When the DH column was used with Gradient 1 the retention times were far closer (\( \alpha_{UDP/UMP} = 1.04 \) and \( \alpha_{UTP/UDP} = 1.02 \)) showing the greater selectivity offered by UDA column for the uracil nucleotides.

On the other hand, for a mixture of the cytosine nucleotides CMP and CDP, the two compounds were separated on the UDA column but peak tailing (either as a result of slow mass transfer or equilibration between ionization states at the mobile phase composition at the time of elution) occurred for CDP (Fig. 7A). For the DH column with Gradient 1, the peak shape for CDP was improved (Fig. 7B), although the separation was less. CTP was not tested. Repeatability of results was tested using the UDA column with Gradient 1 and injecting CMP 20 times. The mean retention time was 6.82 min, ranging from just 6.79 to 6.84 min, with an RSD of 0.3%. Cytosine nucleotides are utilized for DNA and RNA synthesis while the lipid esters, such as CDP-choline and CDP-ethanolamine, are important for cell membrane synthesis. Grossly raised erythrocyte levels of pyrimidine nucleotides are markers for pyrimidine
5’-nucleotidase deficiency [16], which is presently under-diagnosed but may – like many other nucleotide diseases – become more accessible to routine diagnosis using ANP HPLC with MS.

As part of our interest in nucleotide analyses we also wished to achieve a simplified MS-compatible separation for UDP-glucose and UDP-galactose, to measure levels in galactosemic patients. From Fig. 5 it can be seen that a more challenging analysis was involved in the separation of these two nucleotide-sugar esters. The UDA column was tested using several different gradients with a mobile phase containing ammonium formate or acetate as additives, but no separation was achieved for all conditions tested. However, the DH column produced the desired separation between these two molecules, and – using the DH column with Gradient 4 and ammonium acetate as the additive – all other nucleotides eluted after these two compounds and did not interfere with the analysis, as illustrated in Fig. 8. A similar separation was observed with ammonium formate but when ammonium acetate was used the peaks were narrower and the \( \alpha \) value increased. The first injection of the mixture of UDP-glucose, UDP-galactose and ATP (Fig. 8A) was done after the column had already been used for the analysis of many nucleotides and erythrocyte extracts (around 450 injections total). Figures 8B and C show the second and third injection of the mixture after another ten erythrocyte extracts were injected. Retention times were very reproducible with the %RSD around 0.4. Figure 9A shows an erythrocyte extract spiked with UDP-galactose from a patient analyzed using the gradient conditions in Fig. 8, demonstrating that even with UV detection the separation facilitates detection of individual nucleotides in the presence of a biological matrix. The retention time can be compared with the standard (Fig. 9B) showing that the values of the real sample and the standard were very close. Also detected in the erythrocyte extract was ATP, for which the retention time was also very close to that of the standard.

The above investigations utilizing UV detection demonstrated the ability of the two columns to provide adequate separation of a range of nucleotides. However, MS provides...
greater sensitivity as well as specificity, and an increasing number of metabolomic studies are utilizing mass spectrometers for screening. The typical detection limits for the UV studies described above are in the range of 0.1 to 0.2 nmol. These are generally sufficient for a wide range of metabolite analyses and are comparable to those reported for anion exchange methods [1, 4]. The advantage of the ANP HPLC methods described here are that they utilize volatile salts at low concentrations and high percentages of volatile solvents, making them ideal for MS. This is demonstrated in Fig. 10, which shows the separation and identification by TOF-MS using the (M–H)- ion for NAD (m/z 662) and NADP (m/z 742). These nucleotides were easily measurable at concentrations at least 100 times lower than used for UV detection and thus the methods reported here are readily applicable to physiologically relevant samples [1, 4]. While it was not essential to separate species with different m/z values completely, in general some degree of separation provides better quantitation. Of the nucleotides presented in this investigation, it was essential to separate only UDP-glucose and UDP-galactose since they are isomeric. For most physiologically relevant nucleotides, use of MS is the most practical approach for the analysis of biological samples.

A single report has described using ion pair HPLC to separate several mononucleotides for assay using an ion trap detector, but lacked the broad application of the ANP methods presented here [25]. In a small trial study (data not shown), we found that nucleotides were readily measurable using triple-quad (tandem) mass spectrometer, while noting that the separation remains important because the phosphate moieties are stripped off under some conditions in the

Figure 7. CMP and CDP separation using Gradient 1: (A) UDA column; (B) DH column. Conditions same as in Fig. 1.

Figure 8. Repeatability on the DH column. (A) First injection, (B) injection after analysis of eight red blood cell extracts, (C) injection after analysis of ten red blood cell extracts. Solvents: A: water/13.0 mM ammonium acetate, B: 90% acetonitrile/10% v/v water containing 13.0 mM ammonium acetate. Gradient 4 (see Table 1). Flow rate: 0.4 mL/min.
second stage of the spectrometer, converting mono-, di- and tri-nucleotides to their nucleoside equivalents. There have been a small number of proprietary applications published on the internet for HILIC separation of nucleotides [26]. But surprisingly, there has been little focus on screening constitutive cellular nucleotides, despite their possible relevance to “metabolomics”. The extensive review of HILIC applications by Ikegami et al. [27] shows one figure only with a nucleotide, UDP-glucose. In the several hundred references there is only one reference to nucleotide screening [28], which is limited in its scope. There is also a problem throughout the literature that “nucleotides” usually refers to nucleic acids or nucleotide bases/nucleosides or oligonucleotides, not the constitutive cell nucleotides – ATP/ADP/AMP and GTP/GDP and NAD/NADP, etc. Some specialized applications are for plant nucleotides, which are often quite different to animal cell nucleotides because of the emphasis in plants towards complex biosyntheses. The previous publication by our group [6] focuses on plant nucleotides while this paper focuses on animal cell nucleotides specifically to open up analyses of (i) energy metabolism, such as studied in sports medicine etc. and (ii) metabolic diseases of purines and pyrimidines, where nucleotides are used as markers [4].

4 Concluding remarks

It has been demonstrated that hydride-based stationary phases provide an effective tool for the separation of nucleotides in the aqueous normal phase mode. For gradient
separations there is a short equilibration time between runs on both columns. LC-MS compatible mobile phases (ammonium formate and ammonium acetate as additives) were used in all methods developed. AMP, ADP and ATP have a different retention order on UDA than on DH suggesting that both ion-exchange and ANP mechanisms may be present on the UDA column. Substitution of formic acid for ammonium formate in the water component of the mobile phase shortened all of the retention times. At lower pH more of the carboxylic acid groups on the nucleotides are protonated. Less overall charge produces lower retention. Interestingly, UDP-glucose and UDP-galactose were separated on the DH column as well as separated from ATP and other nucleotides present in erythrocyte extracts. This is in contrast to anion exchange HPLC, where the UDP-sugars usually elute close to other dinucleotides, and between the mononucleotides and trinucleotides. The behavior of the UDP-sugar isomers on the DH column was thus suggestive of a geometric element to the separation. This has furthered our understanding of ANP using DH columns, and more LC-MS studies involving the DH column and nucleotides in erythrocyte extracts are under way.

The methods reported here were simple, potentially specific (with MS detection), sensitive and fast. In addition, the ANP analysis of polar compounds is achieved at high concentration of the organic solvent in the mobile phase (usually acetonitrile, but acetone may be used as well), which provides increased sensitivity. When MS detection is used, the analysis can be performed without derivatization of any substrates, including highly polar amino acids, sugars and presumably sugar-phosphates, which are also present in biological samples [8, 9].

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5 References