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SEPARATION OF NUCLEOTIDES BY ION-PAIR, REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

USE OF Mg(II) AND TRIETHYLAMINE AS COMPETING HETAERONS IN THE SEPARATION OF ADENINE AND GUANINE NUCLEOTIDES

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SUMMARY

A sensitive, rapid, and reproducible method for the separation and quantitation of adenine and guanine nucleotides has been developed. Nucleotides are loaded onto an octadecyl silica column (BioRad ODS-5S) in the presence of triethylamine, the primary hetaeron, and then eluted with a gradient of Mg(II), the competing hetaeron. The Mg(II) elution regime used here eliminates the background absorbance observed with commonly used acetonitrile or methanol gradient elution methods. It gives satisfactory line shapes and allows for quantitation of samples containing less than one nanomole of each nucleotide phosphate. Moreover, the replacement of the commonly used quaternary ammonium "solvophobic ions" by triethylamine in this method leads to extended column lifetimes.

INTRODUCTION

The determination of energy charge in intracellular compartments (such as mitochondria) requires an analytical technique for measuring adenine and guanine nucleotide levels that is both rapid and highly sensitive (able to easily detect less than 1 nmole). Over the past 15 years, a plethora of methods for the quantitation of these nucleotides have appeared in the literature, many as a recent outgrowth of high-performance liquid chromatography (HPLC). Modern chromatographic separations of nucleotides fall into three categories: ion-pair, ion-exchange[2,3] and metal chelate (ligand exchange) chromatography[2].

Ion-pair separations typically have employed amphiphilic, quaternary ammonium compounds, such as tetra-*n*-butylammonium hydrogen sulfate[3], as hetaerons (companion ions[1,4,5]) in order to increase the relative retention of charged nucleotides on the reversed-phase support and maximize the selectivity of the separation. Unfortunately, the use of quaternary ammonium hetaerons over long periods of time alters irreversibly stationary phase characteristics[6] and, in some cases, drastically shortens column life, possibly by enhancing the "washing off" of stationary

phase alkyl chains[1,6]. As an alternative, Mg(II) has been used as a hetaeron in reversed-phase HPLC of nucleotides[1,7,8], but the low retention times observed for the Mg(II) complexes do not allow for satisfactory analytical separations. This method has been used successfully, however, for the measurement of association constants of Mg(II)–nucleotide complexes[8].

By far, the largest number of published nucleotide separations use a microparticulate, anion-exchange, silica or resin bonded phase as the ion-exchange stationary phase[9-18]. With the advent of porous silica supports, these methods became "state of the art" for nucleotide separation. But the long analysis and reequilibration times plus the need for high salt eluants limit the usefulness of this approach, especially when compared to reversed-phase HPLC. The last problem, high salt, is especially detrimental, since it leads to rapid deterioration of pump seals.

As an alternative to the ion-pair reversed-phase and ion-exchange modes of analysis, several researchers have developed metal chelate chromatography for nucleotide separation. These methods typically use immobilized bidentate ligands such as dithiocarbamate in conjunction with Mg(II), Cd(II), or Co(III) to effect analytical separations[19,20]. An interesting mixed mode separation, introduced by Karger et al.[20] employs a C_{18} phase and a non-covalently bound, bidentate ligand, 4-dodecyldiethylenetriamine, in the presence of Cd(II). Acetonitrile (25%) is used as the organic eluent. Unfortunately, these metal chelate methods also have drawbacks: (1) sensitivity is decreased by gradient-induced baseline shifts, and (2) the covalently coupled, bidentate ligand supports such as dithiocarbamate silica[19] are not commercially available.

We have developed an alternative to these established methods that incorporates several of the advantages of both "metal chelate" and "ion-pair" chromatography. Our C_{18} reversed-phase nucleotide separation method uses triethylamine (TEA) as the primary hetaeron and Mg(II) as the competing hetaeron to yield sensitive (0.25–3.0 nmoles), high resolution, rapid elution of nucleotides without the application of organic solvent, pH, or salt gradients.

EXPERIMENTAL

Chemicals

Aqueous buffers were prepared from phosphoric acid (H_3PO_4; Baker, Phillipsburg, NJ, U.S.A.) in three-times-filtered, deionized water prepared by the Milli-RO4 system (Millipore, Bedford, MA, U.S.A.). Magnesium sulfate was also from Baker, and was added from a 0.5 M stock solution. The buffers were made up to the phosphoric acid concentrations as noted in the figure legends, then titrated to the proper pH by addition of Sequenol Grade triethylamine (Pierce, Rockford, IL, U.S.A.). Acetonitrile of highest purity available was purchased from Burdick and Jackson (Muskegon, MI, U.S.A.). All buffers were filtered through an 0.4 μm membrane filter prior to use (HA, aqueous; FH, organic; Millipore).

The 5'-mono-, -di-, and -triphosphate nucleotides of adenine, guanine, cytosine, and uracil were purchased from Sigma (St. Louis, MO, U.S.A.). Standard solutions of 0.25 mM, 1.0 mM, and 5.0 mM were prepared and frozen in aliquots prior to use. Standard solutions were kept on ice during use.

Apparatus

The high-performance liquid chromatograph apparatus from Waters Assoc. (Milford, MA, U.S.A.) consisted of a Model 720 system controller, a Model 730 data module, two Model 6000A solvent delivery systems, a Model 450 absorbance detector, and a Model U6K sample injector fitted with a 2-ml loop.

Separations were carried out on a BioRad (Richmond, CA, U.S.A.) ODS-5S (150 × 4 mm I.D., octadecyl stationary phase bound to fully capped 5-μm spherical porous silica) column.

Quantitation of peaks

Peak areas were quantified by the direct direct cutting and weighing of the peaks on each chromatogram. Corresponding peaks were cut from multiple chromatograms, and their weights averaged.

RESULTS

Use of TEA as a hetaeron

Our primary interest in the separation of nucleotides has been to determine the energy charge of yeast mitochondrial preparations. Since the nucleotide levels which contribute to energy charge (*i.e.*, ATP, ADP, GTP and GDP) are known to be low in isolated yeast mitochondria [0.5–5 nmoles/mg protein[21]], our goal was to develop a separation system of optimal sensitivity. Our main concerns in the development of such a system were: (1) sensitivity down to 0.5 nmoles per sample; (2) resolution of all nucleotides which contribute to energy charge; and (3) the use of solvents that would be easy to obtain and use and that would not substantially shorten column or equipment lifetime.

Since we have observed that TEA, unlike N-alkyl quaternary amines, does not lead to the rapid deterioration of reversed-phase columns, our initial separations have made use of it as the hetaeron. Fig. 1 shows the separation of a mixture of nucleotides in the presence of TEA-phosphate under optimal conditions of pH and hetaeron (TEA) concentration. In this system nucleotide–TEA complexes are eluted from the stationary phase by an acetonitrile gradient. At the detector sensitivity required for easy quantitation of samples containing nucleotides at levels of 8 nmoles or above, the resolution, line shapes and baseline are good. However, at detector sensitivities required for lower nucleotide levels (1–5 nmoles per injected sample), a rise in background absorbance obscures the good resolution and line shapes of nucleotides eluted late in the gradient (*i.e.* GTP, ADP, ATP; Fig. 2). This absorbance starts at a point corresponding to the onset of the acetonitrile gradient and worsens as the gradient progresses. Since the magnitude of the baseline absorbance is proportional to the initial concentration of TEA in the mobile phase, we believe that the rise in baseline absorbance upon the onset of the acetonitrile gradient is due to the elution of TEA from the stationary phase. While this elution and increased absorbance are easily tolerated for separations containing 8 nmoles of each nucleotide per sample, where detector sensitivities are set low, they are not tolerable for separations of low levels of nucleotides. Reproducible quantitation of samples containing less than 5 nmoles of each nucleotide can not be obtained with this solvent system.
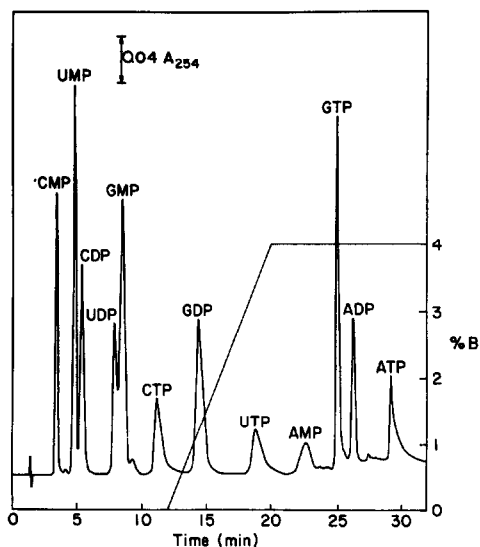
Fig. 1. Separation of nucleotide mono-, di- and triphosphate by ion-pair, reversed-phase chromatography. Acetonitrile elution. Conditions: column, BioRad ODS-5S. Mobile phase, Solvent A: 100 mM TEA-Pi, pH 5.8; solvent B: acetonitrile. Gradient, 12 min isocratic, then 0 to 4% B at 0.5%/min. Flow-rate, 1 ml/min. Detector, 254 nm fixed wavelength, 0.4 a.u.f.s. Chart speed, 0.5 cm/min. Sample size, 8 nmoles of each component.
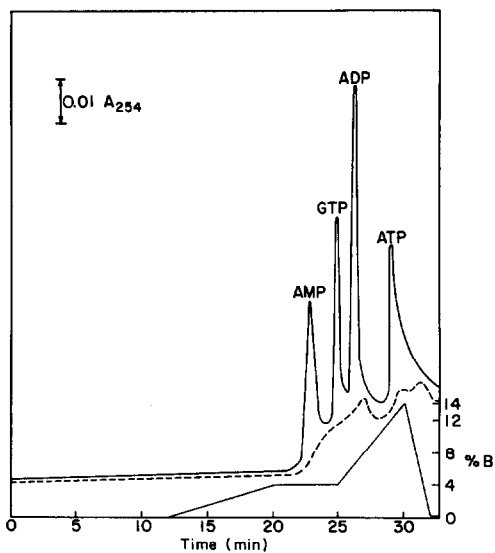

Fig. 2. Composite trace. Blank and standards. Acetonitrile elution. Conditions as Fig. 1, but with continuation of 4% B isocratically for 5 min, then 4 to 14% B at 2%/min. Detector, 254 nm fixed wavelength, 0.1 a.u.f.s. ---------, Blank run; ———, composite standard runs. Sample size, UTP, AMP, ADP: 4 nmoles; ATP, GTP: 3 nmoles.

Use of Mg(II) and TEA as competing hetaerons

In the above separation scheme, TEA was added to the phosphate mobile phase to increase the relative retention of each nucleotide on the reversed-phase column. Ideally, in ion-pair, reversed-phase separations, all of the hetaeron-eluite complexes elute ahead of, and well-separated from, the bulk elution peak of the hetaeron. Unfortunately, whereas TEA is an ideal ion from the point of view of complex retention and column stability, it elutes too early in our gradient separation; increasing background absorbance and hence limiting quantitation of low amounts of ATP, GTP and ADP.

To obviate the difficulties brought about by the elution of TEA, we explored an alternative separation scheme which, during elution, would not alter the concentration of TEA in the mobile phase. Our final separation borrowed from work on metal chelate chromatography, specifically the use of Mg(II) in nucleotide separations[7,14]. Horvath *et al.*[8] have demonstrated that Mg(II) could be employed as a hetaeron in nucleotide–Mg(II) binding studies; the weak retention behavior exhibited by these complexes suggested to us that Mg(II) could be added to our system as a competing hetaeron, facilitating the elution of nucleotides without altering the concentration of the primary hetaeron, TEA, in the mobile and stationary phases. This separation takes advantage of the fact that free Mg(II), unlike TEA, is restricted entirely to the mobile phase. As seen in Fig. 3, this separation scheme gives excellent resolution of nucleotides with little, if any, increase in background absorbance. It should be noted that the separation shown is for 0.75 nmoles of each nucleotide. The conditions for optimal separation of adenine and guanine nucleotides are given in Table I. A Mg(II) concentration of less than 10 mM was used to avoid precipitation.
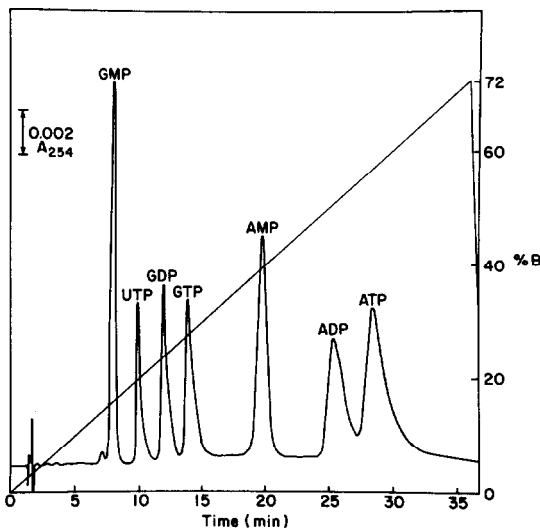

Fig. 3. Separation of nucleotide mono-, di- and triphosphates by ion-pair, reversed-phase chromatography. Mg(II) elution. Conditions: column, BioRad ODS-5S. Mobile phase, Solvent A: 30 mM TEA-Pi, pH 6.5; solvent B: 30 mM TEA-Pi, 5 mM magnesium sulfate, pH 6.5. Gradient, 0 to 72% B at 2%/min. Flow-rate, 1 ml/min. Detector, 254 nm fixed wavelength, 0.02 a.u.f.s. Chart speed, 0.5 cm/min. Sample size, 0.75 nmoles of each component.

TABLE I

OPTIMIZED PROTOCOL FOR REVERSED-PHASE COMPETITIVE ION ELUTION OF GUAN-
INE AND ADENINE NUCLEOTIDES

Column	BioRad ODS-5S (150 × 4 mm I.D.), fully capped
Solvents*	A: 30 mM TEA phosphate, pH 6.5
	B: 30 mM TEA phosphate, 5 mM magnesium sulfate, pH 6.5
Gradient	Initial conditions: 100% A, 0% B
	Gradient: 2%/min, 36 min.
	Flow-rate: 1 ml/min
Sensitivity	Detector: 0.02 a.u.f.s. (254 nm)
	Lower range limit: 0.25–3.0 nmoles/nucleotide

* See Experimental section for solvent preparation.

As predicted from ion pair theory[1], nucleotides are eluted in the order: mono-, di-
and triphosphate.

In order to determine the concentration range over which the separation
scheme could be used to quantitate adenine and guanine nucleotide levels, calibration
curves relating absorbance to nmoles were determined. As can be seen from Fig. 4,
the quantitation from absorbance is linear down to at least 0.5 nmoles of each nu-
cleotide. Thus, this separation scheme allows for both the resolution and quantitation
of very low levels of adenine and guanine nucleotides.

DISCUSSION

In this paper, we describe a method for separating adenine and guanine nu-
cleotides by ion-pair, reversed-phase chromatography. This method is capable of
separating and quantifying samples containing less than 1 nmole of each nucleotide.
It makes use of TEA as the primary hetaeron and Mg(II) as the secondary, competing
hetaeron. Since TEA has both a hydrophobic domain, which can interact with the
C_{18} stationary phase, and an ionized domain, which can interact with the negatively
charged nucleotides, it is able to moderately increase the retention times of the nu-
cleotides on the reversed-phase column. In addition, TEA has a low absorbance in
the UV, and, unlike quaternary ammonium ions[6], does not have a deleterious effect
on the stationary phase. The secondary, competing hetaeron Mg(II) is known to
form complexes with nucleotides[4,7]. Since it is small and polar, it is confined to the
mobile phase in our system. An increase in the Mg(II) concentration in the mobile
phase favors formation of Mg-nucleotide complexes, which are retained much less
than the corresponding TEA–nucleotide species. Thus, the Mg(II) gradient decreases
retention times of the nucleotides. The use of Mg(II) to directly elute nucleotides
from the reversed-phase column virtually eliminates the problems of background
absorbance encountered during more conventional organic solvent (e.g., acetonitrile)
elution of TEA–eluite complexes [see refs. 3, 22–26, and this paper].

Our competitive hetaeron method is derived from both standard ion-pair and
metal chelate chromatography. By combining the best features of both, we have
realized several advantages over either method. These include:

(1) Column/equipment stability. This method avoids the use of corrosive high
salt solutions and replaces quaternary ammonium salts with the relatively innocuous
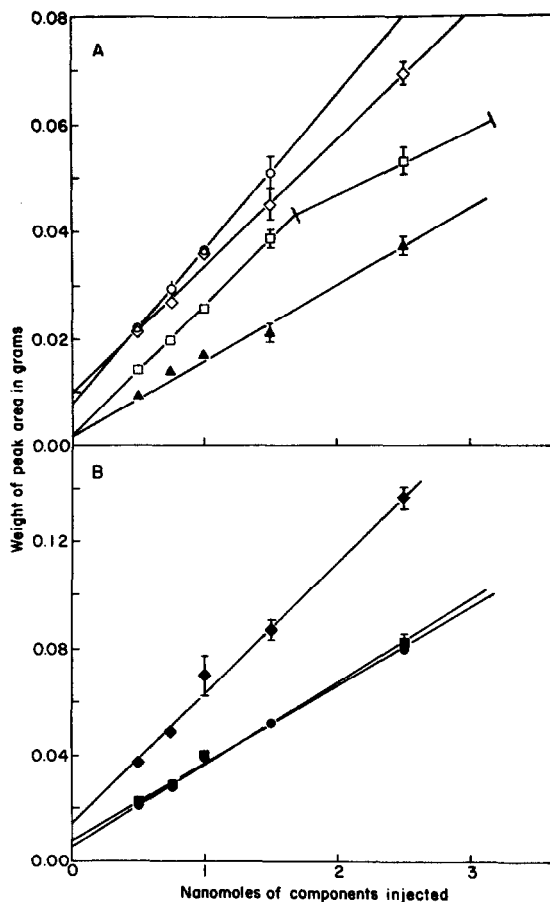TEA ion.

Fig. 4. Calibration curve. Mg(II) elution. Chromatographic conditions as in Fig. 3. Each point represents the average weight (in grams) of peak areas obtained from at least 4 copies of each chromatogram. I-bars represent ± 1 standard deviation unit. Lines were drawn by linear regression analysis to give correlation coefficients with absolute values in the range 0.994 to 0.999. Panel A: O, GMP; □, GDP; ◇, GTP; ▲, UTP. Panel B: ●, AMP; ■, ADP; ◆, ATP.

(2) *Specificity*. Although other hydrophobic molecules may bind to the C_{18} stationary phase, only those which can complex Mg(II) (*i.e.*, nucleotides) will be specifically eluted by our gradient method. Non-specific elution with acetonitrile cannot offer this advantage.

(3) *Sensitivity with speed*. This competitive hetaeron system can reproducibly quantify samples containing less than 1 nmole of each adenine and guanine nucleotide phosphate. This sensitivity has allowed us to measure easily levels of adenine nucleotides in cell extracts[27] of as few as $3 \cdot 10^5$ cultured PtKI cells[28]. Furthermore, since diffusion rates are faster in siliceous stationary phase particles than in ion-exchange resins, separations can be more rapid[1]. Analysis time, including re-equilibration, for this system is less than 40 min.

(4) *Versatility and convenience*. The investigator can select the hetaeron and competing ion with respect to both their chemical constitutions and concentrations.

Gradient steepness can also be adjusted to provide conditions best suited for a particular separation requirement. A single reversed-phase column, without chemical modification, can serve as a basis for many different types of separation.

Our competitive ion elution system has been successfully utilized in the determination of energy charge in isolated mitochondria of *Saccharomyces cerevisiae* and in cultured PtKI cells treated with and without metabolic inhibitors (*e.g.*, 2,4-dinitrophenol; data not shown). Of course, it is suitable for any application where nucleotide concentrations are low and rapid quantitation of extracts is desired.

The broader applications possible using a reversed-phase column, and primary and competing hetaerons seem limited only by the types of ions available. The necessary characteristic for a hetaeron is simply that it contain both a hydrophobic domain that can interact with, but not damage, the stationary phase and a specific complexing domain that can interact reversibly with the molecular species of interest. The competing hetaeron should be confined to the mobile phase and should form a relatively tighter, earlier eluting complex with the eluite. Both hetaerons should have a low absorbance at the monitoring wavelength. Interactions of the primary and competing hetaerons with the compound of interest must be mutually exclusive (*i.e.*, competitive). Using hetaerons with these basic characteristics, a sensitive, reversed-phase, competitive hetaeron elution system can be designed for the separation and quantitation of many types of compounds.

ACKNOWLEDGEMENTS

REFERENCES

1 W. R. Melander and Cs. Horvath, in Cs. Horvath (Editor), *High Performance Liquid Chromatography*, Vol. 2, Academic Press, NY, 1980, p. 114.
2 H. A. Scoble and P. R. Brown, in Cs. Horvath (Editor), *High Performance Liquid Chromatography*, Vol. 3, Academic Press, NY, 1982, p. 1.
3 N. E. Hoffman and J. C. Liao, *Anal. Chem.*, 49(14) (1977) 2231.
4 Cs. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129.
5 E. Tomlinson, T. M. Jefferies and C. M. Riley, *J. Chromatogr.*, 159 (1978) 315.
6 R. M. Cassidy and S. Elchuk, *Anal. Chem.*, 54 (1982) 1558.
7 F. K. Chow and E. Grushka, *J. Chromatogr.*, 185 (1979) 361.
8 Cs. Horváth, W. Melander and A. Nahum, *J. Chromatogr.*, 186 (1979) 371.
9 B. Bakay, E. Nissinen and L. Sweetman, *Anal. Biochem.*, 86 (1978) 65.
10 M. B. Cohen, J. Maybaum and W. Sadée, *J. Chromatogr.*, 198 (1980) 435.
11 P. E. Hare and E. Gil-Av, *Science*, 204 (1979) 1226.
12 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 112 (1975) 651.
13 C. D. Lothrop, Jr. and M. Uziel, *Anal. Chem.*, 26 (1980) 1430.
14 C. Lush, Z. H. A. Rahim, D. Perrett and J. R. Griffiths, *Anal. Biochem.*, 93 (1979) 227.
15 M. McKeag and P. R. Brown, *J. Chromatogr.*, 152 (1978) 253.
16 J. D. Karkas, J. Germershausen and R. Liou, *J. Chromatogr.*, 214 (1981) 267.
17 R. T. Pon and K. K. Ogilvie, *J. Chromatogr.*, 205 (1981) 202.
18 E. J. Ritter and L. M. Bruce, *Biochem. Med.*, 21 (1979) 16.
19 E. Grushka and F. K. Chow, *J. Chromatogr.*, 199 (1980) 283.

20 B. L. Karger, W. S. Wong, R. L. Viavattene, J. N. Lepage and G. Davies, *J. Chromatogr.*, 167 (1978) 253.
21 E. A. Munn, *The Structure of Mitochondria*, Academic Press, NY, 1974.
22 J. H. Knox and J. Jurand, *J. Chromatogr.*, 203 (1981) 85.
23 A. A. Darwish and R. K. Pritchard, *J. Liquid Chromatogr.*, 4 (1981) 1511.
24 S. M. Payne and B. N. Ames, *Anal. Biochem.*, 123 (1982) 151.
25 G. H. R. Rao, J. D. Peller, K. L. Richard, J. McCullough and J. G. White, *J. Chromatogr.*, 229 (1982) 205.
26 E. G. Brown, R. P. Newton and N. M. Shaw, *Anal. Biochem.*, 123 (1982) 378.
27 C. D. Lothrop and M. Uziel, *Clin. Chem.*, 26 (1980) 1430.
28 T. Spurck, L. S. Folley and S. Power, unpublished observations.