Journal of Chromatography, 277 (1983) 137—144
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1764

RAPID, ISOCRATIC SEPARATION OF PURINE NUCLEOTIDES USING STRONG ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

B. BURNETTE**, C.R. McFARLAND and P. BATRA

The Departments of Biological Chemistry and Microbiology & Immunology, Wright State University, School of Medicine and the College of Science & Engineering, Dayton, OH 45435 (U.S.A.)

(First received November 30th, 1982; revised manuscript received April 18th, 1983)

SUMMARY

A method is presented for the rapid, isocratic separation of purine nucleotides using strong anion-exchange high-performance liquid chromatography at ambient temperature. The last peak of interest guanosine 5′-triphosphate (GTP) is eluted within 30 min and immediate reinjection is possible. All adenine and guanine nucleotides can be assayed with a single injection without the use of a gradient for elution. The procedure is particularly useful for the assay of NTP:AMP phosphotransferase reactions and/or the determination of changes in size of cellular purine nucleotide pools and computation of energy charges. An Altex Ultrasil AX prepacked column was used, and virtually identical results were obtained under similar conditions with the Whatman Partisil-10 SAX column. The eluting solution was 200 m*M* potassium phosphate, pH 6.85.

INTRODUCTION

The degree of energy charge loss from cells, and in particular the size of the purine nucleotide pool, is of importance in determining the recoverability of cells from periods of ischemia. Since AMP may be lost from cells irreversibly as adenosine through the action of 5′-nucleotidase, two enzymes that favor the formation of AMP during ischemia are of interest. These are adenylate kinase and guanosine 5′-triphosphate (GTP):AMP phosphotransferase, which catalyze the following freely reversible reactions:

*Some of the studies reported here are in partial fulfillment of the Ph.D. dissertation requirement.

0378-4347/83/$03.00

Adenylate kinase: 2 ADP ⇌ ATP + AMP
GTP:AMP phosphotransferase: ADP + GDP ⇌ GTP + AMP

Because of interest in the activities of these enzymes as well as determination of whole-cell energy charge under various conditions, a quick, accurate, and reproducible method for separating and quantitatively measuring purine nucleotides is required. Thin-layer chromatography was slow, with poor reproducibility, because of low concentration of reaction products. High-performance liquid chromatography (HPLC) seemed the method of choice, but the more universal reversed-phase ion-pairing technique failed to produce acceptable resolution of compounds of interest, probably due to interactions with the Tris buffer and other reaction components of the enzyme—substrate system. However, strong anion-exchange columns seemed to have none of the problems associated with multiple interactions between solvent, ion pairs, and reversed-phase support.

Other investigators have used the HPLC technique to determine the adenylate energy charge and/or specific nucleotide content of cells [1—10], or to assay various kinase reactions [10—12]. These preparations often require considerable sample cleanup for prolonged column life and all have used gradients to separate the mono-, di-, and triphosphate nucleotides, which produces a rising baseline with increasing ionic strength, and requires column re-equilibration before reinjection. By optimizing the eluent with respect to ionic strength, we have developed a method for the rapid, isocratic separations of adenine and guanine nucleotides with minimal sample cleanup. Elution of the slowest nucleotide, GTP, is complete within 30 min and immediate reinjection is possible. Only AMP and GMP have a resolution of less than 1 (0.93), which was within discrimination limits of the desk-top computing integrator.

METHODS

HPLC analysis of nucleotides

The HPLC assembly was a Tracor 900 series consisting of a Model 995 isochromatographic pump with a Model 970 variable-wavelength detector (Tracor Instruments), and a Rheodyne Model 7125 continuous-flow loop injector. The ultraviolet—visible detector was interfaced with a strip chart recorder and a Spectra-Physics System I computing integrator. The column was a prepacked, strong anion-exchange column (25 cm × 4.6 mm I.D.), the Altex Ultrasil AX with 10-μm diameter silica (Beckman Instruments). It was found that the Partisil-10 SAX (Whatman), of same dimensions and particle size, could be substituted with virtually identical results. A pre-column (25 cm × 4.6 mm I.D.) and a guard column (6.5 cm × 2.0 mm I.D.), both packed with 10—18 μm silica, were also used.

The eluent was 200 m*M* potassium phosphate buffer, pH 6.85, which was passed through a Millipore 0.45-μm filter and degassed prior to use. Flow conditions were typically 2.1 ml/min at ambient temperature with 186-bar back pressure of which about 48 bar was attributed to the precolumn. The column was stored under 100% ethanol when not in use, and equilibrated with about 40 ml buffer before use. Nucleotides were identified on the basis of

retention times (t_R) and detected by absorbance at 260 nm.

Concentrations of compounds of interest were computed by the external standard method utilizing standard predetermined time functions. The integrator was simultaneously calibrated for all six purine nucleotides by injection of an aqueous mixture containing the six standards. All standards were prepared from nucleotides of the highest purity available from Sigma, and as aqueous solutions of the sodium salt forms.

Enzyme assays

All enzyme assays were conducted in snap-cap polypropylene vials at 37°C. Reaction mixtures contained 4 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, 50 m*M* Tris—HCl (pH 7.9), 1 m*M* nucleotide substrates, and an aliquot of enzyme preparation in a total volume of 100 μl. The substrates were either ATP plus AMP or ADP only for the adenylate kinase reaction, and either AMP plus GTP or ADP plus GDP for the GTP:AMP phosphotransferase reaction. Reactions were stopped by addition of 100 μl of 95% ethanol and after mixing were chilled to 0°C until analyzed.

Determination of purine nucleotide content and energy charge of isolated heart cells

Adult-rat cells were isolated by collagenase perfusion as described by Altschuld et al. [13]. The isolated cells were suspended in a calcium-free Krebs—Ringer phosphate buffer containing Na^+ (150 m*M*), K^+ (6 m*M*), Cl^- (126 m*M*), $MgSO_4$ (1.2 m*M*), phosphate (17.2 m*M*), taurine (7.5 mg/ml), creatine (3.35 mg/ml), 10 μl/ml vitamin mix (Gibco 100X), 10 μl/ml essential amino acid mix (Gibco 100X), 10 μl/ml non-essential amino acid mix (Gibco 100X), 0.68 m*M* glutamine, and bovine serum albumin (20 mg/ml), at pH 7.2. Cell suspensions (5 mg cell protein per ml) were incubated at 37°C, with and without glucose (11 m*M*), in a metabolic water bath under an atmosphere of oxygen or nitrogen. After 45 min, the cells from a 1-ml sample were centrifuged through 350 μl of bromododecane into 100 μl of 2 *N* perchloric acid, according to McCune and Harris [14]. The resulting acid extracts were neutralized with 50 μl of 0.2 *M* triethanolamine containing 2 *M* K_2CO_3 and centrifuged. A 50-μl sample aliquot was used for nucleotide analysis by HPLC.

RESULTS AND DISCUSSION

Fig. 1 illustrates the total resolution of all purine nucleotides at 2.5 nmoles each, except for AMP and GMP, which were near totally resolved. Table I lists these resolution (R) values, defined as $R = 2\,(t_2 - t_1)/(w_2 + w_1)$. AMP and GMP were eluted as slightly fused peaks and the resolution value of 0.93 rises or falls when the load was decreased or increased, respectively. A representative quantity of 2.5 nmoles was chosen as a midrange value that would be expected in nucleotide analyses. For other nucleotides, the resolution remained complete to 50 nmoles.

Table I also shows t_R values, theoretical plates per meter (N), and capacity

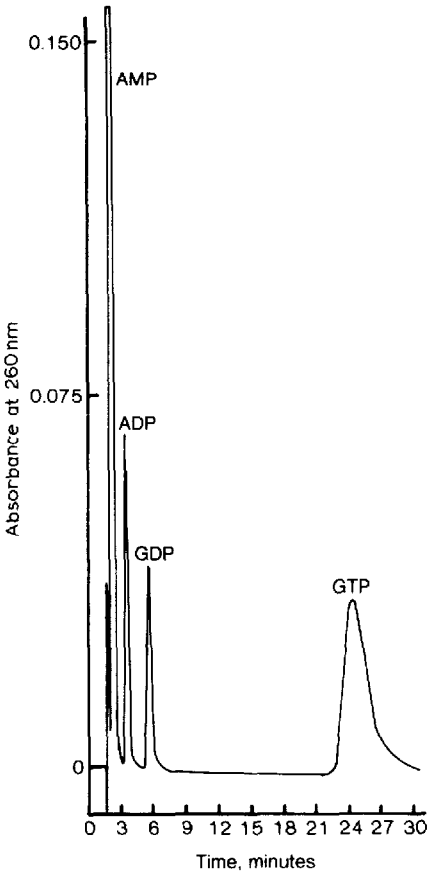

Fig. 1. Separation of purine nucleotide standards, 2.5 nmoles of each. For chromatographic conditions see Methods.

factor (k') values for all nucleotides. The number of plates per meter were calculated by:

$$N = 5.5\left[\frac{t_R}{w_{1/2}}\right]^2 \cdot \frac{1000 \text{ mm/m}}{250 \text{ mm column}}$$

where $w_{1/2}$ is the peak width at one-half of total peak height. The capacity factor was defined as $k' = (t_m - t_R)/t_m$, where t_m = 60 sec = one-half column volume/flow-rate. AMP and GMP had k' values at or above the recommended minimum of 1.00 and by fine-tuning the run parameters, the integrator had little difficulty computing individual peak areas. GTP has a rather high k' value but no other nucleotide elutes close enough to interfere with its resolution.

In order to determine linearity and reproducibility of the method, injections were made in triplicate. The average standard deviation was found to be 6.52% for adenine and guanine nucleotides in the range of 0.25 to 50 nmoles. The linear-regression equations indicated the correlation coefficient value for each nucleotide to be 0.99. These high values confirm the linearity of the detector response despite the wide range involved.

TABLE I

PURINE NUCLEOTIDE CHROMATOGRAPHIC SEPARATION DATA BASED UPON 2.5 NMOLES INJECTED FOR EACH NUCLEOTIDE

For chromatographic conditions see *HPLC analysis of nucleotides*. Equations used for plates per meter (N), capacity factor (k'), and resolution (R) values are described in Results and Discussion.

Nucleotide	t_R (sec)	N	k'	R
AMP	120	3912	1.00	
				0.93
GMP	142	3804	1.37	
				2.16
ADP	208	3372	2.47	
				3.08
GDP	342	3808	4.70	
				4.93
ATP	740	2832	11.5	
				4.42
GTP	1505	3140	24.1	

Figs. 2 and 3 illustrate the application of this HPLC method to the separation of nucleotides following GTP:AMP phosphotransferase and adenylate kinase reactions, respectively. Fig. 4 depicts the usefulness of the method for determining the size of purine nucleotide pools in isolated myocardial cells following incubation under aerobic conditions in the presence and absence of glucose. As expected, cells incubated in the presence of glucose (Fig. 4B) had a higher ATP content compared to cells that were incubated in its absence (Fig. 4A). The amount of GMP and GTP of cells under these conditions was too low to be detected. It appeared that the first peak (perhaps nucleosides) of both parts A and B to Fig. 4 might interfere with the quantitation of AMP. Though resolution was incomplete, refinement of integrator run parameters provided a trapezoidal baseline correction which maintained linearity of the peak area—concentration response.

The results in Table II show the effects on adenine nucleotide levels in heart cells subjected to anaerobic conditions. For comparison purposes, the effect of glucose is also shown. Although the amounts of adenine nucleotides generally declined when heart cells were incubated in an atmosphere of nitrogen, the decrease was much larger when glucose was unavailable. This was true regardless of whether the incubation was carried out under nitrogen or oxygen. The total nucleotide concentrations reported in Table II at time zero (before starting incubation), and after incubation without glucose (both aerobic and anaerobic), compare very well to previously reported values [13].

In summary, the HPLC procedure described here has many attractive features.

(a) Interference from enzyme reaction components does not occur in nucleotide determinations as shown by the chromatographic constants and analytical reproducibility.

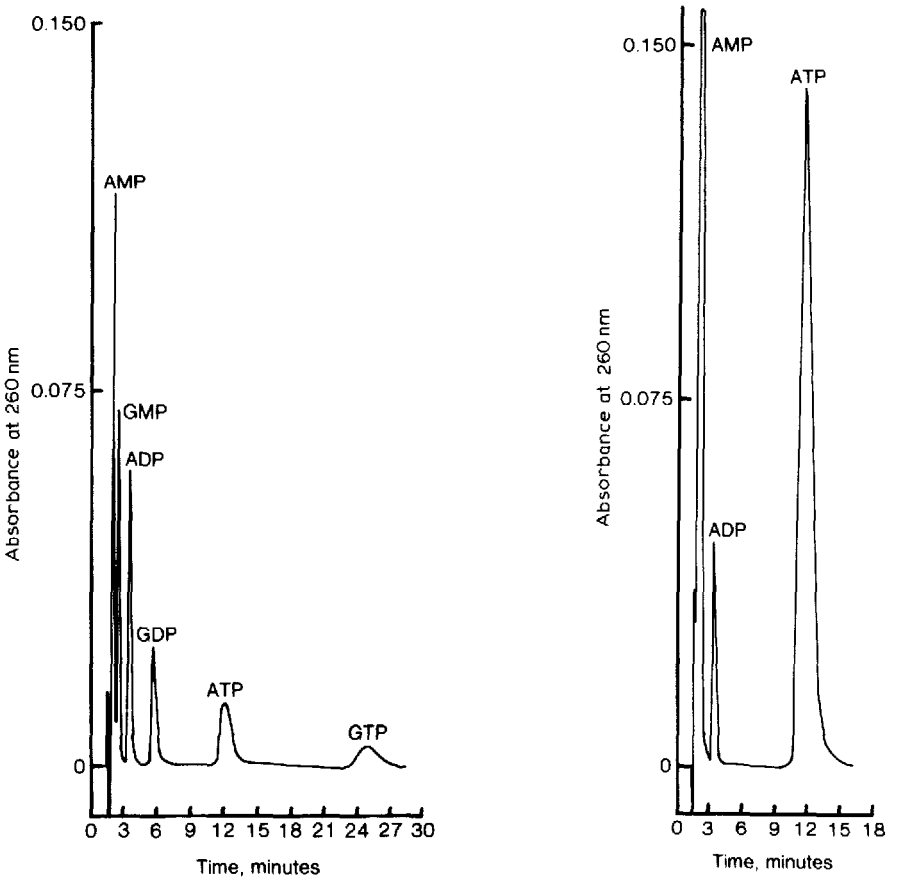

Fig. 2. Separation of reactants (AMP and GTP) from products (ADP and GDP) for a GTP:AMP phosphotransferase reaction, from a 50-μl injection of the sample directly without cleanup, as described in Methods.

Fig. 3. Separation of reactants (AMP and ATP) from product (ADP) for an adenylate kinase reaction, from a 50-μl injection of the sample without cleanup, as described in Methods.

(b) A simple eluting buffer at a pH value below 7.0 and nominal pressure assures reasonable column life and does not require any partial purification of the sample to be analyzed.

(c) The use of ambient room temperature does not require elevated temperatures that would contribute to solvent outgassing over extended periods of operation.

(d) The nucleotides of interest in these experiments are readily separated by isocratic elution employing a buffer of optimal ionic strength and pH. Analyses are rapid and time functions more constant than might be expected with a gradient.

(e) The utility of anion-exchange HPLC nucleotide analyses for heart cell extracts, standards, and enzyme-catalyzed reaction mixtures are shown by identical chromatographic constants.

TABLE II

EFFECT OF GLUCOSE UPON ADENINE NUCLEOTIDE POOLS OF ISOLATED MYOCARDIAL CELLS

Nucleotides were extracted into perchloric acid, which was then neutralized and a 50-μl aliquot used for HPLC analysis. Details of procedures provided in Methods. Time zero is a control value for nucleotides extracted from cells prior to incubation.

Nucleotide	Nucleotide concentration as nmoles per mg cell protein				
	Time zero	Incubated 45 min with glucose		Incubated 45 min without glucose	
		Aerobic	Anaerobic	Aerobic	Anaerobic
AMP	0.86	0.77	0.82	0.19	0.10
ADP	1.52	1.95	1.68	1.44	1.28
ATP	3.35	3.89	3.19	1.66	1.17
Total	5.73	6.60	5.68	3.29	2.55

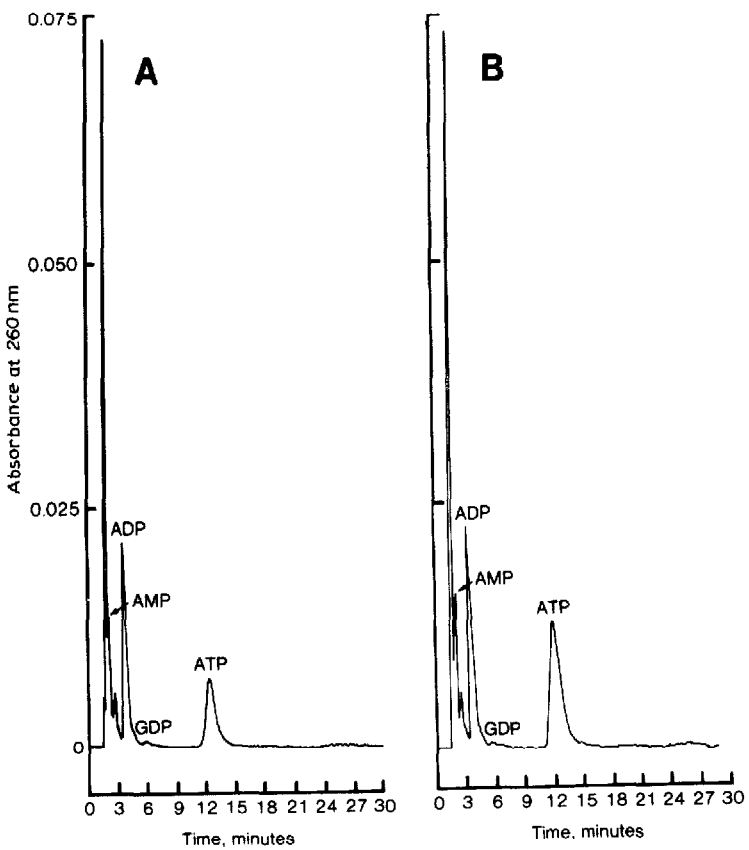

Fig. 4. Separation of purine nucleotides from 50-μl injections of neutralized perchloric acid extract of isolated myocardial cells incubated aerobically for 45 min. Part A represents incubation without glucose, and part B with glucose (11 m*M*) as described in Methods.

ACKNOWLEDGEMENTS

We are grateful to Dr. Gerald Brierley and his research group at Ohio State University for their assistance with studies on isolated heart cells.

These studies were supported in part by grants from the Miami Valley Chapter of the American Heart Association and the Biomedical Sciences Ph.D. Program at Wright State University.

REFERENCES

1 R.A. De Abreu, J.M. van Baal, J.A.J.M. Bakkeren, C.H.M.M. de Bruyn and E.D.A.M. Schretlen, J. Chromatogr., 227 (1982) 45.
2 H.W. Chmukler, J. Chromatogr. Sci., 10 (1972) 38.
3 R.A. Henry, J.A. Schmit and R.C. Williams, J. Chromatogr. Sci., 11 (1973) 358.
4 A. Floridi, C.A. Palmerini and C. Fini, J. Chromatogr., 138 (1977) 203.
5 K.A. Reimer, M.L. Hill and R.B. Jennings, J. Mol. Cell. Cardiol., 13 (1981) 229.
6 E.M. Scholar, P.R. Brown, R.E. Parks and P. Calabresi, Blood, 41 (1973) 927.
7 D. Pruneau, E. Wulfert, M. Pascal and C. Baron, Anal. Biochem., 119 (1982) 274.
8 M.P. Reinhart and M.J. Koroly, Anal. Biochem., 119 (1982) 392.
9 E. Juengling and H. Kammermeier, Anal. Biochem., 102 (1980) 358.
10 P.R. Brown, J. Chromatogr., 52 (1970) 257.
11 P.R. Brown and A.M. Krstulovic, Anal. Biochem., 99 (1979) 1.
12 S. Ronca-Testoni and A. Lucacchini, Ital. J. Biochem., 30 (1981) 190.
13 R.A. Altschuld, J.R. Hostetler and G.P. Brierley, Circ. Res., 49 (1981) 307.
14 S.A. McCune and R.A. Harris, J. Biol. Chem., 254 (1979) 10095.