Determination of Adenine Nucleotides in Biological Samples by Isocratic High-Performance Liquid Chromatography

Y. K. Chau[a]; P. T. S. Wong[b]; G. A. Bengert[a]

[a] National Water Research Institute, Burlington, Ontario, Canada [b] Great Lakes Laboratory for Fisheries and Aquatic Sciences, Canada Centre for Inland Waters, Burlington, Ontario, Canada

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DETERMINATION OF ADENINE NUCLEOTIDES IN BIOLOGICAL SAMPLES BY ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Y. K. CHAU,* P. T. S. WONG† and G. A. BENGERT*

National Water Research Institute and †Great Lakes Laboratory for Fisheries and Aquatic Sciences, Canada Centre for Inland Waters, Burlington, Ontario L7R 4A6, Canada

A simple, fast, and sensitive, isocratic high-performance liquid chromatography technique was developed for the determination of adenine nucleotides (ATP, ADP, AMP) in biological tissues. In conjunction with this technique, a new method of dissolving biological samples and extraction of nucelotides was introduced, using a tissue solubilizer, TMAH (tetramethylammonium hydroxide). The adenine nucleotides were separated on a C-18 column, eluted isocratically with a solvent system consisting of 0.15 M potassium dihydrogen phosphate and disodium hydrogen phosphate, and water at a 60:40 ratio, using an ultra-violet detector at 254 nm. The HPLC technique has sensitivity in the picomol level and is suitable for nucleotide pool and adenylate energy charge calculations. There was no interference from other nucleotides such as the cyclic AMP's, adenine and adenosine. Guanosine diphosphate and guanosine triphosphate gave peaks in the chromatograms but did not interfere.

KEY WORDS: HPLC, adenine nucleotides, ATP, ADP, AMP, mussels.

INTRODUCTION

The application of biochemical indicators in studying environmental stress, toxic effects and system health has been one of the major topics of interest to environmental scientists. The measurement of adenine nucleotides, adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) in living organisms, is essential in the calculation of Adenylate Energy Charge (AEC) and total nucleotide pool. The AEC is a ratio defined as (ATP + 1/2ADP)/(ATP + ADP + AMP), and has a value of 1 when all the adenylate is in the form of ATP, and a value of 0 when all the adenylate is in the form of AMP. Values of the AEC were observed to correlate with physiological conditions of the biota.[1] AEC values between 0.8 and 0.9 indicate that the organisms are growing actively and reproducing. Values in the range of 0.5 to 0.7 have been observed in organisms under stress where the growth and reproductive rates are reduced. Values under 0.5 have been associated with severe stress and loss of viability. The AEC has been applied to a wide range of organisms, such as bacteria,[2] marine worms,[3] bivalves,[4–8] microplankton[9] and fish[10] in the assessment of stress.

Several techniques are available for the determination of adenine nucleotides. ATP, ADP and AMP were determined spectrophotometrically after enzymatic assays with hexokinase,[11,12] pyruvate kinase and myokinase,[13] respectively, with sensitivities in the 10–100 nmole/ml level. Sensitivity is enhanced by fluorimetric measurement of the bioluminescence formed in the reaction of ATP with luciferin-luciferase, which is not only sensitive but also specific in the 0.1–80 pmole/assay range.[14,15] Radioenzymatic technique[16] following the phosphorylation of a labelled sugar can be applied to quantify ATP at the 3 pmole/assay level. Most of the methods for adenine nucleotide determination used in the past were based on enzymatic reactions which gave highly variable results and were difficult to control. More accurate and reproducible analysis has been obtained recently with high-performance liquid chromatography (HPLC) techniques, using ultra-violet or fluorescence detectors[17,18] following conversion of the analytes to fluorescent derivatives. However, many of the presently available HPLC techniques are lengthy in gradient operation modes and are designed for complete analysis of several homologous series of nucleotides for biochemical investigations.[7,19] The only HPLC method that is simple and fast is the one published by Walker *et al.*,[20] which requires derivatization of the extracted adenylates to form fluorescent derivatives for fluorimetric detection. The present paper describes an alternate method, which is direct, simple, and rapid, for the determination of adenine nucleotides in aquatic biota, using a phosphate buffer and water as the mobile phase. The method can analyze samples directly after dissolution without extraction and derivatization steps. The major adenine nucleotides are separated in about 15 min with picomolar sensitivity, indicating suitability for adenylate energy charge and nucleotide pool calculations in aquatic biota. The method has a wide dynamic range covering a concentration range of 4 orders of magnitude which minimizes the necessity of sample size adjustment for analysis. Both in our laboratory and the field, these procedures have been used extensively in conjunction with other biochemical indicators for environmental stress and pollutant effect studies.

MATERIALS AND METHODS

An HPLC model 600 and a WISP 712 Auto-sampler (Waters) were used with a Hewlett-Packard 1046A variable-wavelength UV detector. The column was a 30 cm × 3.9 mm I.D. μ-Bondapack C-18 column (particle size, 10 μm micron) from Waters. The mobile phase consisted of a buffer of 0.15 M potassium dihydrogen phosphate and disodium hydrogen phosphate (pH = 6.54), and water at 60:40 ratio. The flow rate was 1.0 ml/min and the system was run at room temperature (20 °C). The UV wavelength used was 254 nm, and the sensitivity was set at 0.05 AUFS. Peak areas were integrated by a Hewlett-Packard 3392A Integrator. Sample injections were made by the WISP Auto-sampler.

Chemicals

Adenine nucleotides (AMP, ADP, ATP) were obtained in the highest quality from

Sigma (St. Louis, MO). Disodium hydrogen phosphate and potassium dihydrogen phosphate were HPLC grade from Fisher Scientific. After dissolving the appropriate amounts (0.15 mol of each) in 1 liter of water, the pH of the buffer solution was adjusted to 6.54 ($\pm$0.10) with H_3PO_4 or NH_4OH. Other solvents were HPLC grade. Water was distilled and purified in a Milli-Q-water system. All solutions were filtered through a 0.45 μm membrane and degassed with nitrogen for 20 min in the solvent reservoirs before use.

Procedure

Before use, the HPLC column was conditioned first by flushing with water at 1 ml/min for 20 min to displace the methanol in which it was stored, followed by the HPLC eluent for 30 min at 1 ml/min. After use, the column was first washed with water for 20 min and then with methanol for 20 min and stored in the same medium until next use.

Extraction of Nucleotides from Mussels

Frozen tissue samples (mussels, fish, 1 g) were digested in 5 ml of 20% aqueous solution of TMAH at 60 °C for 20 min to a clear straw-yellow colour solution. After cooling, the solution was neutralized with 50% HCl to pH 7–7.5. The final volume of the solution was recorded for volume adjustment in the final calculation. The sample was then centrifuged and 0.5 ml of the supernatant was removed for dilution to 10 ml with the buffer solution. An aliquot (ca. 2 ml) of the diluted sample was filtered through a syringe filter (0.45 μm, hydrophilic membrane) into an Auto-sampler vial. A 20–50 μl aliquot was injected into the HPLC.

Stock nucleotide standard solutions (1 mM) were prepared in water. Working standards were prepared in replicates ($n=3$) by adding 0.2 ml of each of the nucleotide stock solutions to 5 ml of 20% TMAH and processed in parallel with the samples. After digestion, 0.2 ml of the mixed standards was diluted to 2 ml with the phosphate buffer, then filtered through a syringe filter (hydrophilic membrane, 0.45 μm) into the Auto-sampler vial. There was no need to neutralize the TMAH solution as the buffer itself has sufficient capacity to overcome the alkalinity and to maintain the required pH. The standards so processed have a concentration of 3.57 pmol/μl of each nucleotide.

In normal practice, 20 μl of the mixed standards (containing ca. 70–80 pmol of each nucleotide) were injected into the HPLC. The averaged peak areas from replicate analysis ($n=3$) were used for calculation of the sample concentrations.

Stock standard solutions maintained at -20 °C can be kept for a period of up to 3 months without noticeable deterioration. Diluted standards were kept refrigerated and were stable for at least two weeks.

RESULTS AND DISCUSSION

Extraction of Nucleotides from Biological Samples

Extraction of analytes from samples without altering their chemical form is one of

the major challenges in trace speciation techniques. The digestant used must be strong enough to release all the analytes from the sample matrix to achieve quantitative recovery, and yet mild enough not to cause destruction of the authentic form of the analytes.

Several extracting agents were investigated for their efficiency in releasing the adenine nucleotides from tissues. Phosphoric acid (cold and warm), boiling Tris buffer, and sulphuric acid (50%) did not dissolve the sample efficiently to yield quantitative recovery. Two other extracting agents, namely, perchloric acid which was reported to be efficient for the oyster,[8] and TMAH, a tissue solubilizer which was used for dissolving biological samples for speciation of alkyllead and butyltin compounds,[21] were investigated with fish and mussels for their suitability in releasing the nucleotides. TMAH is non-oxidizing and can dissolve complex biological tissues without the necessity of prolonged heating.

Both reagents were found to release the adenine nucleotides effectively. While perchloric acid occasionally gave higher but variable results for the three nucleotides, TMAH digestion gave more consistent and reproducible results. It had neither destructive effects on the authenticity of the nucleotides nor did it interfere with the UV detection. The recovery of the nucleotides after TMAH digestion ranged from 86 to 94%.

HPLC Techniques

Analysis of adenine nucleotides based on enzymatic reactions are difficult to reproduce because of the difficulties in obtaining high-purity enzymes and in controlling the reaction time. Reaction time is crucial in enzymatic reactions for reproducible results. From our experience, we have not been able to obtain commercial enzyme kits that have low blank values, and to obtain reproducible results even with standards.

HPLC techniques basically involve a physical separation and a detection system. There can be pre-column or post-column derivatization if fluorescence detection is to be used. Once the analyte compounds are isolated from the sample, the analytical part is highly reproducible. Thus the major difficulties in the analysis of adenine nucleotides are found in the quantitative extraction of these compounds from biological tissues. Adenine nucleotides are biologically active, but chemically stable compounds. As long as the organisms are still living, these compounds are active components in the anabolic (energy-utilizing) and catabolic (energy-forming) biochemical pathways. After isolation from the living organism, they are stable in the buffer solution at $-20\,°C$ for a period of at least two weeks.

All three adenine nucleotides have strong UV absorbance at 210 and 259 nm. The wavelength 254 nm was used for analysis for an overall more stable baseline with only ca. 6–8% sacrifice in sensitivity.

The Solvent System

Several buffer systems were investigated in an attempt to achieve better separation of the nucleotides on the C-18 column. Ammonium phosphate, potassium

phosphate and a combination of sodium and potassium phosphate were tried at different pH values. The best result occurred with a buffer system consisting of 0.15 M of potassium dihydrogen phosphate and disodium hydrogen phosphate. This method was further investigated with varying ratios of buffer to water, flow rates and pH, until the optimum sensitivity and resolution were achieved.

At a buffer-to-water ratio of 60:40, the three nucleotides were well separated in clear and well-formed peaks on a stable baseline. Retention times and peak areas were identical within experimental errors for the standards prepared in buffer solution and spiked to a biological sample digest. There was no interference of the biological matrices.

Reproducibility, Recovery and Detection Limit

The reproducibility of the method was evaluated by replicate analysis ($n = 6$) of a standard mixture, which had been taken through the TMAH digestion. The standard solution containing 80 pmol of each nucleotide was injected into the HPLC. At this level, the relative standard deviations of the method for ATP, ADP and AMP were 1.19%, 0.56% and 1.38%, respectively. Neither deterioration nor decomposition of the nucleotides by the action of TMAH was observed. The day-to-day reproducibility of both retention times and response are satisfactory with the same batch of reagent and column. There may be a slight shift of retention times on using new reagents and column. The absolute detection limit for the nucleotides was 2 pmols for a signal 5 times the noise level at a detector sensitivity of 0.005 AUFS. When a 2 g sample is used with the present procedure, the detection limit for the nucleotide is 1 pmol per gram of tissue. In ordinary practice, a detector sensitivity in the range of 0.10–0.02 AUFS is adequate.

The calibration curves for the three nucleotides are linear over a dynamic range of at least 4 orders of magnitude (8 to 20,000 pmols) as indicated by the coefficients of correlation (0.96–0.99 for all three nucleotides).

The same signal responses were observed for ATP and ADP. AMP, as the last nucleotide to elute, had a broader peak, and higher peak area count, resulting in a shifted but parallel calibration curve when compared to the others.

The calibration equations for the three nucleotides, as evaluated from replicate runs ($n = 3$) are:

$$\text{ATP: } Y = -1.75 + 4.62X \quad \text{slope} = 0.38 \quad \text{RSD} = 12.7\%$$

$$\text{ADP: } Y = -1.52 + 3.36X \quad \text{slope} = 0.33 \quad \text{RSD} = 14.8\%$$

$$\text{AMP: } Y = -0.94 + 5.53X \quad \text{slope} = 0.15 \quad \text{RSD} = 18.4\%.$$

Analytes which are biologically active and labile are difficult to recover after spiking to biological materials. The sample-spiking techniques commonly used for assessing recovery do not really assure complete extraction of the analytes from biological tissues. In spite of the discrepancy, standards (500 pmols of each nucleotide) were spiked to 1.0 g of mussel tissue (muscle) and digested in TMAH solution accordingly. The recoveries for the three adenine nucleotides from mussel tissue ranged from 82 to 86% at this level (Table 1).

Table 1 Recoveries of adenine nucleotides from mussel.[a]

	ATP	ADP	AMP
Mussel	0.924	1.202	0.642
Mussel + spikes	1.333	1.618	1.072
Recovery (%)	81.8	83.2	86.0
RSD (%)	14.2	17.4	21.5

[a]Mussel tissue, 1.0 g (muscle); adenine nucleotide spikes, 500 pmol each, adenine nucleotides expressed in μmol/g tissue (wet wt.) ($n = 5$). RSD, relative standard deviation.

There was no interference from other series of nucleotides. Guanosine diphosphate and guanosine triphosphate gave peaks at retention times of 4.95 and 4.65 min, respectively, under the present chromatographic conditions. There was no peak for 2,3-cyclic AMP, 3,5-cyclic AMP, adenine or adenosine. There were a few other peaks in the chromatograms of muscle sample, which were probably other components of the sample, but they did not interfere with the identification of the adenine nucleotides of interest. The first peak at 3.26 min was present in the blank, and was probably caused by the reagent. HPLC chromatograms of the adenine nucleotide standards and of samples are shown in Figures 1A and 1B.

Table 2 demonstrates the application of the present technique in the determination of adenine nucleotides in mussels. The obtained AEC values indicate that the biota have been under stress. Since the AEC ratio is not a specific biochemical indicator, we are using this value as a general indicator in parallel with other biochemical indicators, such as induced production of metallothioneins and enzymatic activities, for studying stress in particular due to metals. Results of these studies will be reported elsewhere.

Preservation of Sample

Biological samples were kept in liquid nitrogen immediately after sampling and remained there until analysis. After thawing, samples were immediately dissected and the desired tissue was digested in TMAH accordingly. If not immediately analyzed, the finished sample contained in buffer solution can be stored in the freezer for future analysis. No change in results was observed for a period of up to one month.

Table 3 summarizes some analyses of adenine nucleotides in aquatic organisms published in the literature. Most likely, the disparity in values may result from different techniques of sample preparation and analytical methodologies and also from sample varieties. There is indeed a need for a simple and reproducible procedure for the determination of adenine nucleotides in environmental studies.

(a)

3.26

ATP

8.80

9.73

ADP

12.42

AMP

ST

(b)
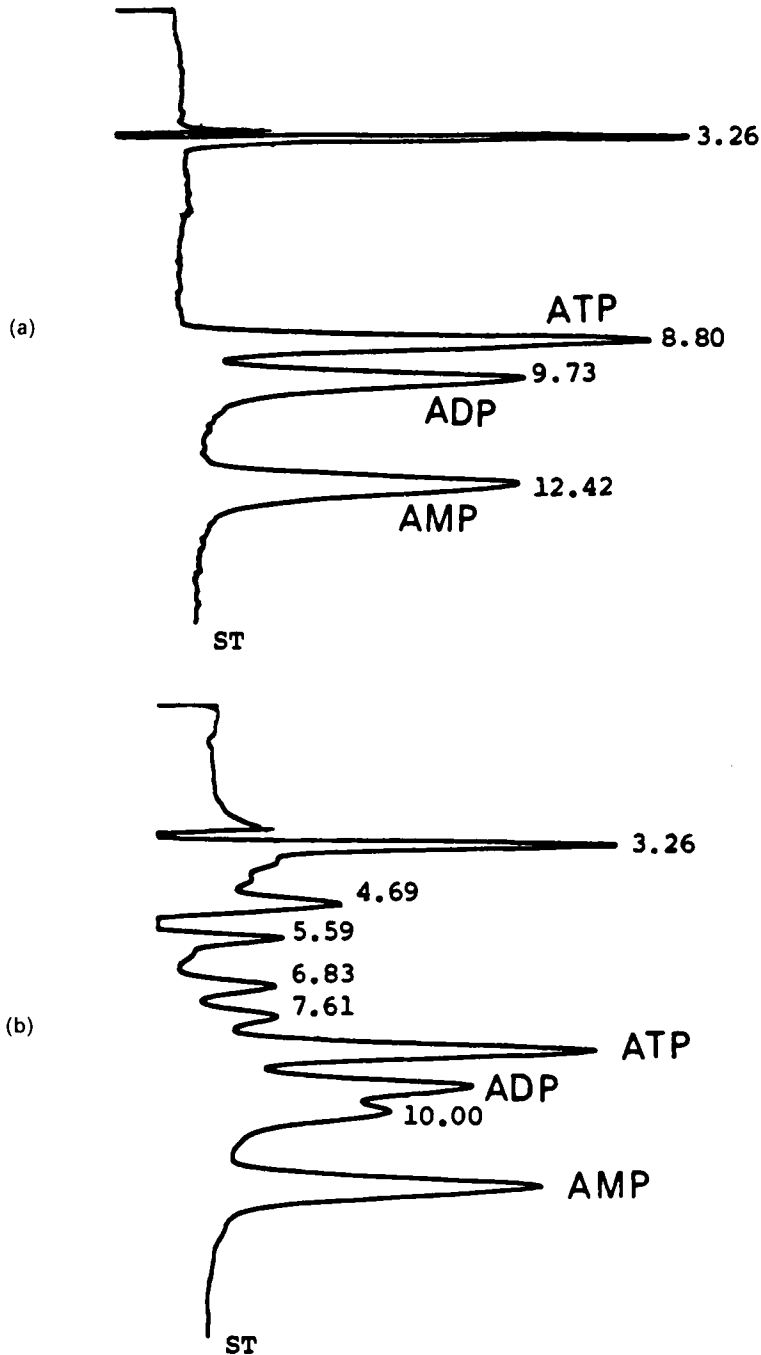
3.26

4.69

5.59

6.83

7.61

ATP

ADP

10.00

AMP

ST

Figure 1 (a) HPLC chromatograms of a standard mixture of adenine nucleotides. Detection at 254 nm, 0.01 AUFS; Each peak contained 60 pmol of nucleotide. (b) HPLC chromatograms of adenine nucleotides in the foot muscle of *Elliptic complanata*. Sample (0.280 g) dissolved in 5 ml TMAH, diluted 10 × with phosphate buffer; 30 μl injected into HPLC. Instrument parameters same as in Figure 1a.

Table 2 Adenine nucleotides in mussel (*Elliptio complanata*)[a]

Sample wt (g)	ATP	ADP	AMP	AEC
0.299	1.045	1.096	0.809	0.540
0.208	1.270	1.533	0.866	0.550
0.285	1.156	1.303	0.835	0.549
0.242	0.964	1.187	1.050	0.486

[a]Live mussel was dissected, and the foot muscle was immediately weighed and analyzed ($n=3$). Results in μmol nucleotide/g wet tissue. AEC=(ATP+0.5ADP)/ATP+ADP+AMP).

Table 3 Concentration of adenine nucleotides in aquatic biota[a]

Species	ATP	ADP	AMP	AEC	Method	Reference
Mytilus edulis	2.82	0.97	0.09	0.85	1	5
Mytilus edulis	3.63	0.93	0.14	0.87	1	22
Crassostrea virginica	0.94	1.49	1.59	0.42	1	22
Mytilus edulis	22.83[b]	6.00	0.28		2	7
Nereis diversicolor	1.30				3	3
Nephtys sp.	2.66					3
Pimephales promelas	3.8[c]			0.770	1	10
Homarus americanus						
Tail	4.67	0.75	0.12	0.911	1	23
Gills	0.74	0.09	0.08	0.866		

[a]Analytical methods: 1, enzymatic, using spectrophotometry; 2, HPLC; 3, enzymatic, firefly bioluminescence reactions.
All nucleotide concentrations in μmol/g tissue wet wt. except: [b]μmol/g protein and [c]μmol/g dry wt.

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