Journal of Chromatography B, 889–890 (2012) 110–115

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A sensitive HPLC-based method to quantify adenine nucleotides in primary astrocyte cell cultures

Dhaval P. Bhatt, Xuesong Chen, Jonathan D. Geiger, Thad A. Rosenberger *

Department of Pharmacology, Physiology and Therapeutics, University of North Dakota, School of Medicine and Health Sciences, Grand Forks, ND 58203, USA

ARTICLE INFO

Article history:
Received 2 November 2011
Accepted 7 February 2012
Available online 13 February 2012

Keywords:
Adenylate energy charge
AMP
Chloroacetaldehyde
Etheno-adenine nucleotides
Fluorescence
Primary astrocyte cultures

ABSTRACT

In mono-layered primary cell cultures baseline AMP and ADP levels are found nominally in the mid to low picomolar range and are thus difficult to measure with conventional HPLC methods that often require the pooling of samples or require indirect detection methods using radiotracers or enzyme coupled assays. To address this issue, we developed a highly sensitive and selective ion-pairing HPLC method with fluorescence detection to quantify adenine nucleotides and the adenylate energy charge in primary astrocyte cell cultures. To accomplish this, we optimized the fluorescence derivatization conditions and the HPLC parameters to achieve baseline separation and quantification of all adenine nucleotides. Nucleotides were converted to their respective 1, N^6-etheno derivatives by incubating with chloroacetaldehyde at pH 4.5 and 60 °C for 60 min. Under these conditions, the loss of the adenine nucleotides due to hydrolysis was minimized with a derivatization yield of 94.1% for 1, N^6-ethenoadenosine. The optimal concentration of tetrabutylammonium phosphate, the ion-pairing reagent, required to achieve a reproducible separation of the adenine nucleotides was found to be 0.8 mM. Calibration curves of nucleotide standards were linear within the range of 0.16–10.4 pmol for adenosine, 0.16–20.6 pmol for AMP, 0.15–19.2 pmol for ADP, and 0.15–19.5 pmol for ATP. The limits of detection and quantification for all adenine nucleotides were approximately 0.08 and 0.16 pmol, respectively. The intra- and inter-day variability for this method was less than 5.1 and 3.4%, respectively. This method was successfully used to measure all adenine nucleotides and an adenylate energy charge of 0.92 ± 0.02 in primary astrocyte cell cultures.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Adenine nucleotides and adenosine form an important class of molecules that are central to both intra and extracellular metabolic processes. Inside the cell, adenine nucleotides are essential for energy transfer, function as co-factors in various signal transduction pathways, are allosteric regulators of enzymes, and are building blocks for both DNA and RNA [1]. Outside the cell, ATP and adenosine are known to modulate synaptic activity *via* specific cell surface purinergic and adenosine receptors located throughout the body including the central nervous system [2,3]. Based on the important role that adenine nucleotides have in various physiological and pathological conditions, the adenylate energy charge (AEC) has been adopted to describe the energetic status of a cell or tissue preparation. The AEC is the ratio of the compete adenylate pool defined as [[ATP + (0.5ADP)]/(AMP + ADP + ATP)] [4]. In this regard, the measurement of nucleotides from mono-layered cell cultures is a challenging task often requiring pooling of samples in order to obtain detectable concentrations or the use of indirect modes of measurement that employ radiotracers or enzyme-linked assays. Thus, a highly sensitive and selective method is required to quantify small changes in adenine nucleotides and to accurately calculate the AEC in primary cell cultures.

Several analytical procedures utilizing either isocratic or gradient, reversed phase or ion-exchange or ion-pairing HPLC in combination with UV or fluorescence detection are available for nucleotide analysis [5–10]. The method of choice is ion-pairing reversed phase HPLC due to its ability to provide stable and reproducible results [11]. Depending upon the amount of sample available for analysis the detection mode varies between UV detection for μmol–nmol range and fluorescence detection for nmol–pmol range. However, when using *in vitro* cell culture models current methodology does not provide adequate reproducibility to quantify ADP, AMP and adenosine levels directly. To bolster the detectability Kochectkov et al. developed a method using chloroacetaldehyde to form fluorescent 1, N^6-ethenoderivatives of the

Abbreviations: AEC, adenylate energy charge; HPLC, high performance liquid chromatography; ADO, adenosine; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; AMPCP, α, β-methylene adenosine diphosphate; GTP, guanosine triphosphate; NAD, nicotinamide adenine dinucleotide; TBAP, tetrabutylammonium phosphate.

* Corresponding author at: University of North Dakota, School of Medicine and Health Sciences, 501 North Columbia Road, Room 3742, Grand Forks, ND 58203, USA. Tel.: +1 701 777 0591; fax: +1 701 777 4490.

E-mail address: thad.rosenberger@med.und.edu (T.A. Rosenberger).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2012.02.005

Table 1
Percentage purity of individual and mixed nucleotide standards.

	Percentage nucleotide content of standards				
	Adenosine	AMP	ADP	ATP	Mixture
Adenosine	100.0 ± 0.0	3.5 ± 0.4	–	–	103.5 ± 0.4
AMP	–	96.5 ± 0.4	6.6 ± 0.8	–	103.1 ± 0.6
ADP	–	–	93.4 ± 0.8	2.5 ± 0.3	95.9 ± 1.1
ATP	–	–	–	97.5 ± 0.3	97.5 ± 0.3

adenylate nucleotides in aqueous solution at pH 4.5. The reaction scheme involves formation of an etheno-bridge between the 1st nitrogen of the purine ring and amino group nitrogen on the 6th carbon of the purine ring as described [12]. Variations of this method are used to quantify adenosine, adenine nucleotides and adenine analogues in different tissues, fluids, and cell culture systems [5–10,13]. However, the derivatization conditions described in these reports vary and depend on the tissue being analyzed making quantification of the nucleotides difficult to reproduce. Also, the derivatization conditions if not properly controlled leads to the hydrolysis of the adenine nucleotides adding yet more variation [6]. More importantly, a wide range of ion-pairing reagent concentrations (0.2–7.5 mM) are used to separate and quantify the 1, N^6-etheno adenylate derivatives using reversed phase HPLC which is highly dependent on the amount of sample analyzed, and the specific HPLC conditions used to perform the separation [5,6,8].

Therefore, to measure energy metabolism in primary astrocyte cell cultures we developed an ion-pairing HPLC method coupled with fluorescence detection to accurately measure all adenine nucleotides and the AEC. The method was optimized to limit the loss of native adenylate nucleotides to hydrolysis, provided baseline separation to quantify picomolar levels of nucleotides, did not require the pooling of cell cultures, and was suitable to detect small variations in both adenine nucleotides and adenosine levels. Given the importance of adenine nucleotide metabolism and the current interest in understanding the role of brain energy metabolism in both normal and pathologic physiology the method described herein provides a clear advantage to quantify these processes *in vitro*.

2. Experimental

2.1. Materials

Nucleotide standards (adenosine, ATP, ADP, AMP, α, β-methylene adenosine diphosphate (AMPCP), NAD, GTP), sodium acetate (99%), trichloroflurομethane (Freon), chloroacetaldehyde (50%), trioctylamine, glucose, hexokinase, and, myokinase were purchased from Sigma–Aldrich (St. Louis, MO). The standard 1, N^6-ethenoadenosine was from Axxora, LLC (San Diego, CA), tetrabutylammonium phosphate (TBAP) was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ), and HPLC grade acetonitrile, mono-basic potassium phosphate (KH_2PO_4), perchloric acid, acetone, sodium hydroxide, and bovine serum albumin were obtained from EMD Chemicals Inc. (Gibbstown, NJ). Bradford reagent was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Poly-L-lysine was obtained from MP Biomedicals, LLC (Solon, OH). Acetic acid and all other cell culture supplies were from VWR International, LLC (Batavia, IL).

2.1.1. Preparation of standards

All standard stock solutions were prepared by dissolving 10–25 mg of a pure standard nucleotide in 1–1.5 mL of deionized water. All standards were stored at −20 °C until use. Standard solutions were diluted 1000-fold and the absorbance was recorded at 259 nm for adenine nucleotides, 253 nm for GTP, and 275 nm for 1, N^6-ethenoadenosine. The molar concentration of the stock standards was calculated using the molar extinction coefficients. Stock standards were further diluted in deionized water to prepare working individual and mixed nucleotide standards. All calibration procedures were performed using freshly made stock standards while the working standards used for method development, analyte stability, and other aspects described herein were stored at 4 °C for no more than one month prior to use. The purity of the adenine nucleotide working standards was determined using HPLC with UV detection [14] and are listed in Table 1.

2.2. Methods

2.2.1. Cell culture

Primary cortical astrocyte cultures were prepared from 3- to 4-day old Sprague–Dawley rat pups as described [15] in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23) as approved by the University of North Dakota animal care and use committee. The cell culture media (DMEM/F12 containing 10% FBS supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B, and 10 mM HEPES) was replaced every alternate day and on the 8th day the cells were transferred to a shaking incubator at 37 °C (260 RPM) for 24 h under air-restricted conditions to remove oligodendrocytes and microglia. The astrocyte-enriched cell cultures were allowed to grow for another 4 days and then on day 12 the astrocyte cell cultures were divided onto 100 mm × 20 mm poly-L-lysine coated culture dishes at a density of 1.0×10^6 cells/dish. The enrichment of astrocytes was determined to be greater than 95% by GFAP immunofluorescence staining as described [16]. When the cells became confluent, media was removed and washed three times with Ca^{2+}/Mg^{2+} free Hanks balanced salt solution then flash frozen by rapid immersion of dishes in liquid nitrogen. The cells were stored at −80 °C prior to nucleotide analysis.

2.2.2. Nucleotide extraction

Frozen cells were scrapped off culture dishes kept on dry ice and were transferred to a micro-centrifuge tube chilled in liquid nitrogen. The frozen cells were homogenized using a Polytron® PT1200E homogenizer (Kinematica Inc, Bohemia, NY) in 500 μL ice-cold 0.6 N perchloric acid to extract the nucleotides. The homogenate was centrifuged at 13,000 × *g* for 2 min at 4 °C. The supernatant was collected and neutralized with 1 mL ice-cold freon/trioctylamine (4:1, by Vol.). The pellet was saved for protein determination. The neutralized nucleotide extract was vortexed for 30 s then centrifuged at 13,000 × *g* for 2 min to induce phase separation. The upper aqueous layer was collected and stored at 4 °C until derivatization. The protein pellet was washed once with acetone, dried using a nitrogen evaporator and then re-suspended in 1 N sodium hydroxide for 24 h. The dissolved pellets were boiled for 5 min, sonicated, then the protein content was measured using the Bradford method as described [17].

2.2.3. Nucleotide derivatization

For conversion of nucleotides into fluorescent 1, N^6-etheno derivatives, a 50 μL aliquot of nucleotide extract was mixed with

150 μL freshly prepared mix of chloroacetaldehyde (7.8 M) to 1 M acetate buffer (pH 4.5) [11.2:138.8, by Vol.] in a 13 mm capped glass tube. Tubes were vortex mixed, centrifuged at 450 × *g* for 2 min at 22 °C, and then heated to 60 °C for 60 min in a Techne DRI-Block DB-3A (Techne, Cambridge, UK). After the reaction, the tubes were immediately placed in a 4 °C refrigerator to stop the reaction, centrifuged at 450 × *g* for 2 min at 22 °C and then diluted with water to 1:6 for AMP and 1:80 for ATP analysis. A 100 μL aliquot of this solution was placed in a microvial and 50 μL of the derivatized sample was used for analysis.

2.2.4. Chromatographic separation and analysis

The HPLC analysis of etheno-adenine nucleotides was performed on System Gold® 125 Solvent Module (Beckman Coulter, Inc., Fullerton, CA) equipped with a System Gold® 508 auto-sampler and an in-line Jasco FP-2020 fluorescence detector (Jasco Corporation, Tokyo, Japan). Separation was performed on a Waters Sunfire™ ODS column (5 μm, 250 mm × 4.6 mm, Milford, MA) equipped with a C18 SecurityGuard cartridge (Phenomenex, Torrance, CA) at 22 °C. Pump control and peak integration was achieved using the 32 Karat™ software (Ver. 7.0, build 1048, Beckman Coulter, Inc., Fullerton, CA). The mobile phase consisted of buffer A, 30 mM KH_2PO_4 + 0.8 mM TBAP, pH 5.45; and buffer B, acetonitrile/30 mM KH_2PO_4 (1:1, by Vol.) + 0.8 mM TBAP, pH 7.0. The pH of buffer B was adjusted to 7.0 prior to the addition of acetonitrile. All buffers were filtered using a 0.45-μm Supor-450 membrane filter (Pall Corporation, Ann Arbor, MI) prior to addition of the ion-pairing reagent (TBAP).

The etheno-adenine nucleotides were eluted off the column with the following gradient. The initial buffer B concentration was maintained at 10% for 0.5 min, increased to 20% over a period of 2.5 min, and then held constant for 4 min. At 7.0 min the concentration of solvent B was increased to 50% over a 4 min period and then held constant for 10 min. At 21 min the proportion of buffer B was decreased back to the starting concentration of 10% over a 4 min period and then held constant for 5 min until the end of the run. The re-equilibration time between samples and the flow rate was held constant at 20 min and 1 mL/min, respectively. The quantification of the etheno-adenine nucleotides was performed using an excitation wavelength of 280 nm with an output emission wavelength set at 410 nm. The identity of etheno-adenine nucleotides were determined by comparing retention times to known nucleotide standards and were further confirmed by enzymatic peak shift analysis.

2.2.5. Statistical analysis

The nucleotide levels obtained from primary astrocyte cell cultures were expressed as mean ± SD in units of nmoles of nucleotide per mg of protein. Each fluorescence derivatization reaction was performed in triplicate and the coefficient of variance was calculated to determine the precision of each reaction and linear regression analysis was performed to determine the linearity of calibration curve (SigmaPlot, Ver. 10, Systat Software Inc., Chicago, IL). Percentage error was calculated using known standards at three different concentrations that were extracted and derivatized as samples.

3. Results and discussion

3.1. Optimum ion-pairing reagent concentration and gradient elution

Figs. 1 and 2 show the separation of the etheno-adenine nucleotides from a standard nucleotide mixture and astrocyte cell extract, respectively. The optimum TBAP concentration

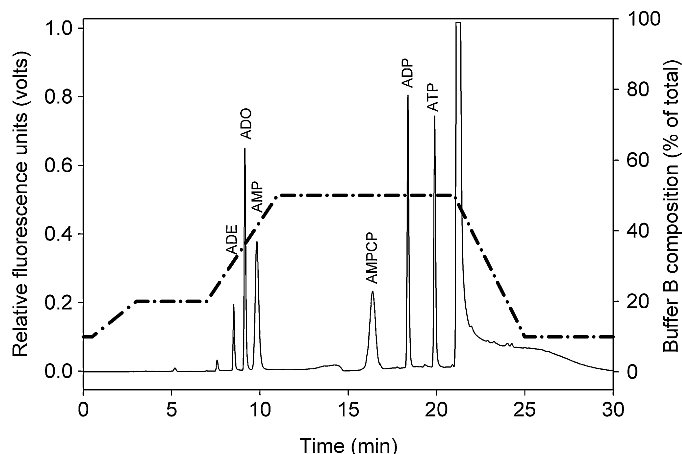

Fig. 1. Representative chromatogram of a standard mixture of adenine nucleotides and α, β-methylene adenosine diphosphate. 10 pmol mixed etheno-derivatized standard of adenine nucleotides (adenine, adenosine, AMP, AMPCP, ADP, ATP) with fluorescence detection at Em: 280 nm and Ex: 410 nm. ADE, adenine; ADO, adenosine; AMP, adenosine monophosphate; AMPCP, α, β-methylene adenosine diphosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

was 0.80 mM and maintaining a 20 min equilibration time provided a reproducible separation with average retention times of 8.32 ± 0.06, 9.04 ± 0.03, 10.36 ± 0.04, 16.67 ± 0.03, 18.46 ± 0.01, and 20.25 ± 0.03 min for adenine, adenosine, AMP, AMPCP, ADP and ATP, respectively. The excitation maxima for etheno-adenine nucleotides were between 276 and 282 nm and the emission maxima were between 408 and 415 nm. Thus, the fluorescence detection was performed at 280 nm excitation and 410 nm emission wavelengths.

Current ion-pairing HPLC methods to quantify etheno-adenine nucleotides use a wide range of tetrabutylammonium ion concentrations (0.20–7.50 mM) to achieve separation [5,6,8]. However, we found that 7.50 mM TBAP did not allow for baseline separation of the etheno-derivatives and their retention on the column was independent of buffer B composition. Moreover, at this TBAP concentration (7.50 mM) after elution of the ATP peak the fluorescence background saturated the detector and remained saturated for at least five minutes. Thus, to reduce the retention time of etheno-adenine nucleotides, we tried 5.00, 2.50, and 1.25 mM TBAP concentrations, and investigated the effect of varying buffer B gradient. Reducing TBAP concentration did not adequately alter the retention times of etheno-derivatives, however it did reduce the

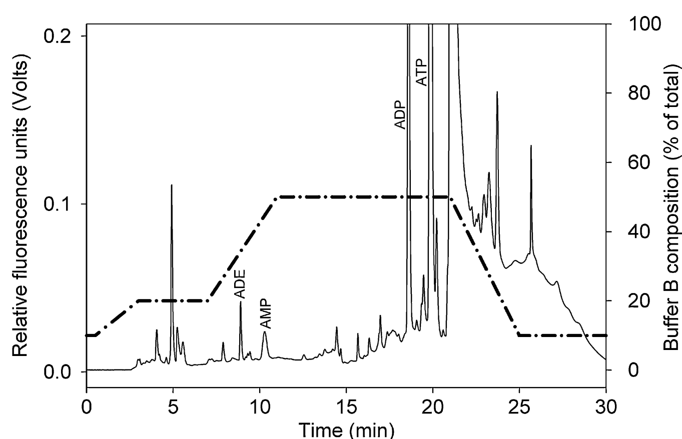

Fig. 2. Representative chromatogram of adenine nucleotides found in a primary astrocyte cell culture. ADE, adenine; ADO, adenosine; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

fluorescence background and allowed better control of elution by altering buffer B composition.

Based on the proportionate decrease in the fluorescence background observed, we predicted that a 4-fold reduction in TBAP concentration was required to control background fluorescence. Considering this and the reported method by Ramos-Salazar and Baines [5] we tried 0.20 mM TBAP which dramatically reduced the retention time and altered the elusion order of etheno-derivatives. Under these conditions, AMP eluted before adenosine which co-eluted with ADP followed by ATP and the fluorescence background eluted as a non-saturating ghost peak. However, variation in buffer B composition was not adequate resulting in either insufficient separation or broad peaks. At this point, we increased the concentration of TBAP to 0.40 mM, which improved the resolution and sharpness of the ADP peak but AMP was not separated from the adenosine peak and the reproducibility was inconsistent. In ion-pair chromatography the effective concentration of the pairing ion covering the stationary phase of the column dictates the separation rather than the absolute mobile phase concentration [18]. Hence, when working at sub-saturating concentrations of TBAP it is critical to determine the optimum equilibration time required to load the stationary phase with sufficient TBAP and maintain it between sample runs. Finally, 0.80 mM TBAP with a 20 min equilibration time provided adequate separation of etheno-adenine nucleotides and allowed us to control elution with solvent B.

Earlier studies have used excitation wavelengths ranging from 225 to 305 nm and emission wavelengths from 380 to 420 nm for etheno-adenine nucleotide detection [5–8,13,19]. We determined the extinction and emission maxima of etheno-adenine nucleotides in buffer A, a 50:50 mixture of buffer A and B, and in buffer B. The excitation maxima for etheno-derivative of adenosine, AMP, ADP and ATP were determined using a DU®-530 spectrophotometer (Beckman Coulter Inc., Fullerton, CA) and the emission maxima were determined using a Varian Cary Eclipse spectrofluorometer (Mulgrave, Victoria, Australia). We found that the excitation maxima for different etheno-derivatives ranged from 276 to 282 nm while the emission maxima were between 408 and 415 nm. Hence, in the current study we used 280 and 410 nm as the excitation and emission wavelengths for the analysis, which are in agreement with the spectral information of etheno-adenine nucleotides originally reported by Barrio et al. [13].

3.2. Optimum incubation temperature and time for derivatization

The fluorescent derivatization of adenine nucleotides and adenosine is most frequently performed at 80 °C for 40 min [6,7,19]. However shorter times of 20 min have also been reported [8]. Under these conditions, 1.2–10% hydrolysis of nucleotides has been observed [6]. In contrast to this, we observed a 46% hydrolysis in ATP and 25% in ADP at 80 °C using a 40 min incubation, which dramatically reduces the accuracy of the method. Ramos-Salazar and Baines reported a similar hydrolysis pattern and instead performed the reaction at 37 °C for 24 h to reduce the loss of the adenine nucleotides to hydrolysis, however the reaction yields using this method are very low [5]. Thus, we performed the derivatization reaction for each individual nucleotide at 60, 70 and 80 °C for 20, 40, 60 and 80 min in an attempt to optimize yield while limiting loss due to hydrolysis. We found that the hydrolysis of nucleotides increased with an increase in incubation time and temperature. The least amount of hydrolysis occurred at 60 °C however the reaction required 60 min to attain maximum yield with a simultaneous increase in hydrolytic products (Fig. 3). When compared to pure 1, N^6-ethenoadenosine standard, the derivatization yield of adenosine at 60 °C for 60 min was 94.1% which is much higher than 74% at 80 °C for 1 h [19]. At 70 and 80 °C the maximum yields were obtained within 20 min, however the hydrolytic products

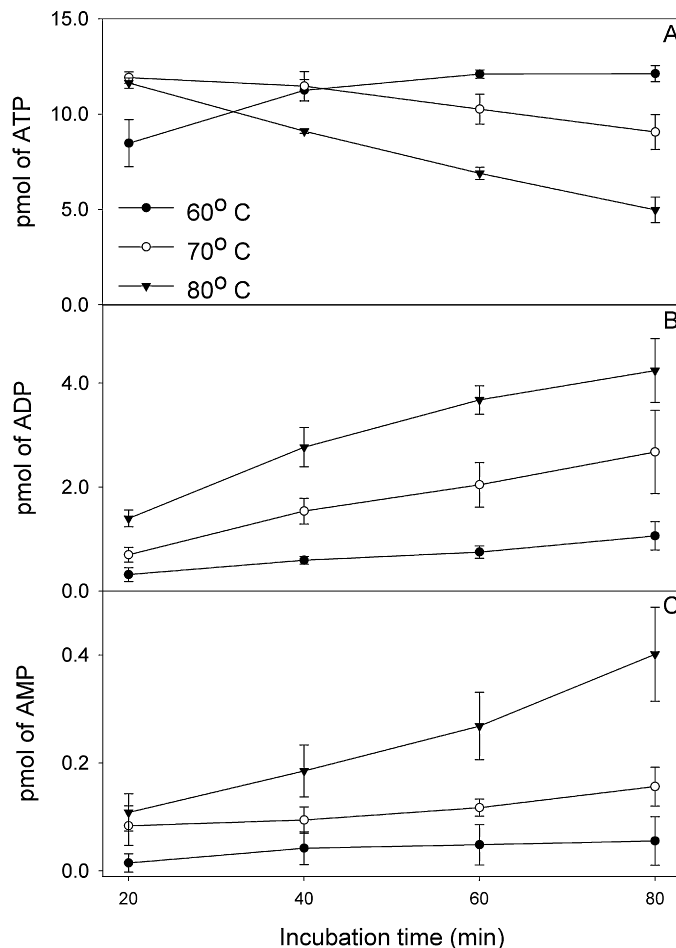

Fig. 3. Optimum incubation temperature and time for derivatization. Fluorescent derivatization of ATP at 60, 70, and 80 °C for 20, 40, 60 and 80 min. Increase in temperature at different incubation time results in hydrolysis of ATP and formation of ATP (A), ADP (B), and AMP (C).

for different nucleotides were either comparable or much higher than the reaction at 60 °C for 60 min. Fig. 3 shows different etheno-adenine nucleotides formed as a result of ATP derivatization and similar curves for ADP, AMP and adenosine were generated that showed similar patterns (data not shown). Therefore, we selected 60 °C and 60 min for the derivatization reaction and determined the percentage hydrolysis for all adenine nucleotides under these conditions (Table 2) which is similar to that previously reported using a reaction condition performed at 80 °C for 40 min [6]. Since all standards and samples underwent the same derivatization procedure the hydrolytic percentage of nucleotides present were assumed to be similar thus requiring no background correction.

3.3. Selectivity, linear range, and limits

The selectivity of the 1, N^6-etheno derivatization reaction between adenine nucleotides and chloroacetaldehyde is achieved by maintaining the reaction pH at 4.5 [13] with 1–2 M acetate buffer (final concentration not below 500 mM). Further selectivity is obtained by using the excitation wavelength of 280 nm and recording the emission at 410 nm which is specific for etheno-adenine compounds in contrast to 300 nm and 347 nm for etheno-cytidine compounds [13]. We performed the derivatization of adenine nucleotides in addition to GTP, NAD, and AMPCP. We found that GTP did not produce any fluorescent derivatives at pH 4.5 while NAD degraded to AMPCP and formed etheno-AMPCP which was

Table 2
Percentage conversion of adenine nucleotide standards and identification of products following the derivatization reaction at 60 °C for 60 min.

	Percentage of nucleotide following conversion to etheno-derivative				
	adenine	adenosine	AMP	ADP	ATP
Adenosine	–	100.0 ± 0.0	–	–	–
AMP	–	13.8 ± 0.0	86.2 ± 0.0	–	–
ADP	0.5 ± 0.2	1.4 ± 0.0	12.7 ± 0.9	85.4 ± 1.1	–
ATP	0.6 ± 0.2	–	0.9 ± 0.2	8.7 ± 0.9	89.8 ± 0.9
Mixture	1.1 ± 0.3	115.2 ± 0.1	99.7 ± 0.9	94.2 ± 1.8	89.8 ± 0.9

confirmed by co-elution with a known AMPCP standard. Reports have suggested the use of AMPCP as an internal standard [5,6], however based on our results endogenous NAD levels artificially elevate AMPCP levels and hence we suggest that it should not be used as an internal standard when measuring adenine nucleotide from biological samples containing NAD.

For peak purity analysis, enzyme digested nucleotide extracts and standards were derivatized with chloroacetaldehyde. For digesting ATP peak, 70 U of hexokinase and 10 μmol of glucose were incubated with the nucleotide extract at 22 °C for 30 min. Following incubation and derivatization we found a 97.5% decrease in ATP and a consequent increase in ADP and AMP by 95.4 and 1.5%, respectively. Similarly, when nucleotide extracts were successively incubated with 70 U hexokinase and 10 μmol glucose followed by 80 U myokinase for 30 min at 37 °C, 94.2% of ADP peak disappeared with a subsequent 93.4% increase in AMP. This demonstrates that there were no co-eluting peaks that interfered with the quantification of ATP, ADP and AMP using this method.

All commercially available nucleotide standards decompose over time which is unavoidable due to the highly labile nature of the high energy phosphate bond. In fact the product literature states a spontaneous rate of breakdown of 0.5% per year in the dry desiccated form which will increase once in solution. Considering this limitation and to ensure that the standard curves were calculated accurately, the purity of each individual nucleotide standard was measured after it was placed in solution and immediately prior to use using HPLC with UV detection [14] (Table 1). The percent nucleotide content of each individual standard was used to calculate the actual amount (pmol) of nucleotide present in the mixed standards and to develop standard curves. Based on these calibration curves we found a linear relationship between 0.16 and 10.4 pmol for adenosine, 0.16 and 20.6 pmol for AMP, 0.15 and 19.2 pmol for ADP, and 0.15 and 19.5 pmol for ATP. Liner regression analysis of the calibration curves provided the following equations: pmol ADO = (Peak area ADO − 32,173)/411,348, r^2 = 0.9997; pmol AMP = (Peak area AMP + 5559)/373,835, r^2 = 0.9999; pmol ADP = (Peak area ADP − 41,356)/414,606, r^2 = 0.9996 and pmol ATP = (Peak area ATP + 46,849)/388,094, r^2 = 0.9997. The limit of detection was found to be 0.08 pmol and the limit of quantification was measured at 0.16 pmol for AMP with signal to noise ratios of 3 and 10, respectively. The limits of detection of the other nucleotides were approximately 0.08 pmol and the limit of quantification ranged between 0.15 and 0.16 pmol. The signal to noise ratio of ADP, ATP, and ADO were similar to that found with AMP.

3.4. Accuracy and precision

To determine the accuracy of the method, mixed nucleotide standards (36, 18, and 5.4 μM) were extracted using the nucleotide extraction protocol and derivatized using the method described above. Table 3 shows the average experimental values obtained from 9 replicate observations performed in triplicate on three different days. The percentage error demonstrates that the method is accurate (87.8–107.9%) within the linear range of measurement. The intra- and inter-day variability was less than 5.1 and 3.4%, respectively; this demonstrates the repeatability and precision of the method. The fluorescence adenine nucleotide derivatives were stable for up to 4 weeks when stored at 4 °C and were stable for up to 24 h when stored at 22 °C.

3.5. Nucleotide levels in primary astrocyte cultures

Analysis of nucleotides is often required when the direct endpoint of interest is a change in nucleotide levels or to determine the integrity of the preparation based on the AEC. However, studies measuring alterations in energy metabolism in cell culture models are only able to quantify ATP due to low levels of ADP and AMP. Thus the measurement of ADP and AMP *in vitro* have required the pooling of samples to increase analyte quantities or have employed kinetic studies using radiolabeled tracer or enzyme coupled reactions to measure the rate of release of nucleotides. However, the application of this method using fluorescence detection with a stable HPLC separation allowed us to measure adenine nucleotides and calculate the AEC.

Table 3
Accuracy and precision of the etheno-adenine nucleotide analysis.

Adenine nucleotide	Theoretical value (pmol)	Experimental value[a] (pmol)	Error (%)	Precision (RSD, %)	
				Intra-day[b]	Inter-day[a]
Adenosine	10.3	10.6 ± 0.3	2.5	1.5	3.3
	5.2	5.6 ± 0.2	7.9	1.6	2.8
	1.6	1.6 ± 0.0	1.7	1.6	2.9
AMP	10.3	10.0 ± 0.2	−3.3	0.6	2.4
	5.2	5.2 ± 0.1	1.8	1.5	2.4
	1.6	1.6 ± 0.1	6.4	3.7	3.1
ADP	9.6	9.3 ± 0.2	−3.5	5.1	2.6
	4.8	4.7 ± 0.1	−3.0	2.3	2.0
	1.4	1.4 ± 0.0	−1.1	4.3	1.9
ATP	9.8	8.7 ± 0.2	−11.0	1.8	2.9
	4.9	4.2 ± 0.1	−13.4	2.9	3.4
	1.5	1.3 ± 0.0	−12.2	3.2	0.6

[a] Nine replicates.
[b] Three replicates.

Table 4
Nucleotide levels and adenylate energy charge in primary astrocyte cultures compared to reported values.

	nmol nucleotide per mg protein		Reference
	Current study	Reported range	
AMP	0.22 ± 0.07	0.2–2.3	[22,25]
ADP	4.59 ± 0.77	2.5–5.0	[23,25,27]
ATP	25.4 ± 2.93	17.5–47.3	[21,23–27]
AEC	0.92 ± 0.02	0.82–0.83	[20,25]

Average protein per 100 mm × 20 mm petridish was 0.344 ± 0.058 mg from six samples.
AEC, [[ATP + (0.5ADP)]/(AMP + ADP + ATP)].

The physiological levels of nucleotides from primary astrocyte cells measured using this method are listed in Table 4. We found that ATP, ADP, AMP levels, and the AEC in control astrocyte cultures were 25.4 ± 2.93, 4.59 ± 0.77, 0.22 ± 0.07, and 0.92 ± 0.02, respectively. These values are in agreement with other published values found in astrocyte cultures or astocytoma cell lines using alternative methods or pooling of samples [20–27]. In this study, the cells were flash frozen in liquid nitrogen and extracted on dry ice to prevent the loss of ATP due to enzymatic conversion to ADP and AMP. However, most studies do not attempt to limit ATP hydrolysis but rather immediately deproteinize samples using either perchloric acid or trichloroacetic acid at room temperature. This is reflected in the AEC of 0.92 obtained using this method compared to reported value of 0.82–0.89 [20,25].

4. Conclusion

To study adenine nucleotide metabolism *in vitro*, it is essential to have a method that can simultaneously measure adenine nucleotides and reliably report AEC. Thus, here we report a highly sensitive, accurate, and selective method to measure AMP, ADP and ATP levels in primary astrocyte cultures. This method is reproducible and can be applied to other cell culture models where the detection of AMP and ADP is a limiting factor.

Acknowledgements

This publication was made possible by a Grant from the National Center for Research Resources (NCRR), a component of the National Institute of Health (NIH) (2P20RR017699).

References

[1] M. Erecinska, I.A. Silver, J. Cereb. Blood Flow Metab. 9 (1989) 2.
[2] S. Latini, F. Pedata, J. Neurochem. 79 (2001) 463.
[3] H. Zimmermann, Trends Neurosci. 17 (1994) 420.
[4] D.E. Atkinson, Biochemistry 7 (1968) 4030.
[5] A. Ramos-Salazar, A.D. Baines, Anal. Biochem. 145 (1985) 9.
[6] Y. Kawamoto, K. Shinozuka, M. Kunitomo, J. Haginaka, Anal. Biochem. 262 (1998) 33.
[7] K. Mohri, K. Takeuchi, K. Shinozuka, R.A. Bjur, D.P. Westfall, Anal. Biochem. 210 (1993) 262.
[8] M. Katayama, Y. Matsuda, K. Shimokawa, S. Tanabe, S. Kaneko, I. Hara, H. Sato, J. Chromatogr. B: Biomed. Sci. Appl. 760 (2001) 159.
[9] B. Levitt, R.J. Head, D.P. Westfall, Anal. Biochem. 137 (1984) 93.
[10] M. Yoshioka, K. Nishidate, H. Iizuka, A. Nakamura, M.M. El-Merzabani, Z. Tamura, T. Miyazaki, J. Chromatogr. 309 (1984) 63.
[11] M. Zakaria, P.R. Brown, J. Chromatogr. 226 (1981) 267.
[12] N.K. Kochetkov, V.N. Shibaev, A.A. Kost, Tetrahedron Lett. 22 (1971) 1993.
[13] J.R. Barrio, J.A. Secrist 3rd, N.J. Leonard, Biochem. Biophys. Res. Commun. 46 (1972) 597.
[14] D.F. Hammer, D.V. Unverferth, R.E. Kelley, P.A. Harvan, R.A. Altschuld, Anal. Biochem. 169 (1988) 300.
[15] K.D. McCarthy, J. de Vellis, J. Cell Biol. 85 (1980) 890.
[16] D.E. Weinstein, Current protocols in neuroscience/editorial board, Jacqueline N. Crawley... [et al.] Chapter 3 (2001) Unit 3 5.
[17] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[18] E. Tomlinson, T.M.R.C.M. Jefferies, J. Chromatogr. 159 (1978) 315.
[19] Y. Zhang, J.D. Geiger, W.W. Lautt, Am. J. Physiol. 260 (1991) G658.
[20] F. Balestri, M. Giannecchini, F. Sgarrella, M.C. Carta, M.G. Tozzi, M. Camici, Neurochem. Int. 50 (2007) 517.
[21] B. Gabryel, A. Pudelko, H.I. Trzeciak, P. Cieslik, Acta Pol. Pharm. 57 (2000) 289.
[22] O. Hori, M. Matsumoto, Y. Maeda, H. Ueda, T. Ohtsuki, D.M. Stern, T. Kinoshita, S. Ogawa, T. Kamada, J. Neurochem. 62 (1994) 1489.
[23] R. Reinhardt, A. Manaenko, M. Pissarek, A. Wagner, P. Illes, Neurochem. Int. 40 (2002) 427.
[24] R.A. Swanson, S.H. Graham, Brain Res. 664 (1994) 94.
[25] K.S. Tang, S.W. Suh, C.C. Alano, Z. Shao, W.T. Hunt, R.A. Swanson, C.M. Anderson, Glia 58 (2010) 446.
[26] J.Y. Yager, G. Kala, L. Hertz, B.H. Juurlink, Brain Res. Dev. Brain Res. 82 (1994) 62.
[27] E. Zoref-Shani, Y. Bromberg, G. Lilling, I. Gozes, S. Brosh, Y. Sidi, O. Sperling, Int. J. Dev. Neurosci. 13 (1995) 887.