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Analysis of adenosine phosphates in HepG-2 cell by a HPLC–ESI-MS system with porous graphitic carbon as stationary phase

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ABSTRACT

A high performance liquid chromatography coupled with electrospray ionization/mass spectrometry method was developed for the determination of adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), and adenosine 5′-triphosphate (ATP) in the extract of HepG-2 cells. The chromatographic conditions were optimized by using porous graphitic carbon as the stationary phase for the retention and separation of the AMP, ADP and ATP. Negative-ion mode ESI-MS in basic mobile phase was applied to improve the method sensitivity. An external calibration method with linear ranges from 0.22 to 57.80 μM for AMP, from 0.59 to 117.37 μM for ADP, and from 0.49 to 98.81 μM for ATP was used for quantitative analysis. The levels of ATP, ADP, and AMP in HepG-2 cells treated with benzo[a]pyrene with different time periods were determined. Total adenine nucleotides and the energy charge potential were calculated for the investigation of the effect of benzo[a]pyrene on cell energy metabolism.

1. Introduction

Adenosine and its corresponding phosphates are crucial biomolecules that represent energy currency of living cells through the cleavage of its phosphate groups [1]. There have been strong demands for sensitive and reliable analysis of the nucleotides because their cell concentrations may provide valuable information of energy state in cells for understanding the mechanism of cell death.

Adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), and adenosine 5′-triphosphate (ATP) are chemical compounds that consist of heterocyclic base, sugar and one or more phosphate groups. Due to the presence of multiple phosphate groups that are extremely polar, the nucleotide phosphates are not retained well on columns under conventional reversed-phase chromatographic conditions [2,3]. Therefore, indirect determination of the corresponding parent nucleosides resulted from enzymatic dephosphorylation after the nucleotides were separated with solid-phase extraction has been used for the quantitative analysis of nucleotide phosphate in cellular extracts. Direct HPLC methods have also been investigated to isolate and analyze the nucleotides, including the use of strong anion-exchange HPLC [4] and ion-pairing HPLC [5–6]. Tomiya et al. developed a method with anion-exchange chromatography for the simultaneous detection of nucleotides and sugar nucleotides in Chinese hamster ovary cells [7]. The method gave better separation than the HPLC method using an ion-pairing reagent. Cichna et al. reported an ion-paring method for the separation of 18 nucleotides, nucleosides, and nucleobases with a 55 min isocratic elution [8]. Ganzera et al. investigated an extraction method for the analysis of nucleotides by using ion-pairing HPLC [9].

Considering the strong background interference of cellular matrix and the submicromolar levels of adenine nucleotides in cells [10], HPLC–MS has been considered a powerful technique for directly monitoring the energy state in cells and organisms. The high sensitivity and specificity of the MS-related techniques provide specific detection and potential identification of chemicals in the presence of complex matrix [11]. Electrospray ionization (ESI) has become popular in bioanalysis, especially for the compounds with high polarity. Cai and co-workers developed a sensitive LC–MS method for the determination of adenosine nucleotides in cultured cells [3]. Dimethylhexylamine (DMHA) was used as ion-pairing agent to retain and separate the analytes on a reversed-phase microbore column with a gradient program. Luo et al. developed a highly selective and sensitive method using tributylamine as a volatile ion-pairing reagent for the identification and quantification of intracellular metabolites involved in central carbon metabolism (including glycolysis, pentose phosphate pathway and tricarboxylic acid cycle) [12]. The method was successfully applied to detect the intracellular metabolites in *Escherichia coli*.

However, ion-exchange and ion-pairing chromatography are often not compatible with mass spectrometric detection because

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of the high salt concentrations in mobile phases used to elute the nucleotides [13–15]. Salt precipitation in ion source may not only affect the detection sensitivity but also require frequent cleanings of ESI source. Therefore, it is necessary to develop a suitable LC–MS method with better chromatographic retention for sensitive detection of nucleotides in cellular extracts without using ion-exchange or ion-pairing agent. The modifier or the buffer salt selected for LC separation should be kept at low concentration and volatile during the ESI-MS analysis.

It has been reported that porous graphitic carbon (PGC) column can provide significantly better retention and selectivity for polar compounds because of the special chemical and physical properties of the packing material [16–18]. Hsieh et al. reported a HPLC system using a PGC stationary phase for the analysis of cytarabine in mouse plasma samples [19]. They found that cytarabine and endogenous peaks from mouse plasma samples could be separated by using a PGC column under the reversed-phase conditions. The solvents used for PGC column were similar to those used in mobile phases of traditional reversed-phase HPLC. HPLC with PGC column would be more compatible for MS detection because the source contamination with salts from mobile phases could be avoided. Another advantage of using PGC column is the capacity of tolerating a wide pH range (pH 0–14) while most reversed-phase columns can only tolerate the mobile phase with pH <9. The sensitivity of ESI-MS in negative ion mode which is suitable for detection of adenosine nucleotides may be enhanced when moderately strong basic mobile phase is chosen.

In this paper, a simple and reliable LC–ESI-MS method for the determination of adenosine nucleotides by using PGC column was described. The method was applied to measure the levels of ATP, ADP, and AMP, which are useful for understanding the energy state of the human hepatoma cells (HepG-2) during the course of cell death following treatment with benzo[a]pyrene. The retention and separation capabilities of different reversed-phase columns for the polar compounds were investigated and compared.

2. Materials and methods

2.1. Chemicals and reagents

Standards of AMP, ADP and ATP were purchased from Sigma (St. Louis, MO, USA). Benzo[a]pyrene was from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade acetonitrile (ACN) and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Other chemicals used were of analytical grade. Distilled water filtered with a Milli-Q Academic ultrapure water system (Millipore, Bedford, MA, USA) was used to prepare standard solutions and HPLC mobile phases. Stock solutions of AMP, ADP and ATP were prepared at the concentration of 1 mM in 10 mM ammonium acetate and stored at −20 °C until use. Further dilutions were made by using the Milli-Q water.

2.2. Chromatographic conditions

Chromatographic separations were performed by using the Agilent 1100 HPLC system equipped with an autosampler. The Hypercarb column (150 × 2.0 mm, 5 μm) was purchased from Thermo Electronics (Thermo-Hypersil-Keystone, Bellefonte, PA); the Synergi Hydro-RP (2.00 × 150 mm, 4 μm) and the Synergi Max-RP 80A (2.00 × 150 mm, 4 μm) columns were obtained from Phenomenex (Phenomenex, Torrance, CA); the Zorbax SB-C8 column (2.10 × 150 mm, 4 μm) was purchased from Agilent (Agilent Technologies, Madrid, Spain); the Symmetry Shield TM RP8 column (150 × 2.1 mm, 3.5 μm) was purchased from Waters (Waters, Milan, Italy) and the Alltima HP HILIC column (2.00 × 150 mm, 3 μm) was purchased from Grace (Grace, Deerfield, IL). The columns were kept at ambient temperature. A solvent gradient program was used unless otherwise specified. The mobile phase consisted of 10 mM ammonium acetate that was adjusted with ammonia to pH 10 (eluent A) and 100% acetonitrile (eluent B). The initial solvent condition of 10% B was kept for 2 min after the injection and the gradient program began from 10% B to 50% B within 8 min, which was held for 5 min before returned to 10% B. The flow rate of HPLC elution was at 300 μL/min. Under the LC conditions, the re-equilibration time of column was about 4 min.

2.3. Mass spectrometer conditions

Mass spectrometric experiments were performed in negative ion mode on a Bruker Esquire-4000 ion trap mass spectrometer (Bruker-Fransen, Bremen, Germany). A narrow mass range from m/z 250–550 as set for the analysis of the adenine nucleotides. Nebulizer gas flow rate was set at 40 psi. Dry gas flow rate was set at 8.0 L/min and the dry temperature was set at 350 °C. The ionization voltage was −3500 V.

2.4. Cellular extract samples

The HepG-2 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in minimum essential medium (MEM) supplemented with fetal bovine serum, antibiotics (amphotericin B and penicillin–streptomycin), and fungizone as described previously [20]. Approximately 10^6 cells were used in each sample. After the treatment with benzo[a]pyrene with different time, the acid soluble metabolites were extracted according to the method described by Yang and Gupta [20]. Briefly, cells were washed twice with prewarmed (37 °C) phosphate buffer saline to remove the incubation medium. Then 950 μL of perchloric acid (0.3 M) containing Na-EDTA (1 mM) were directly added to the culture plate. The cells were scraped off the bottom and moved to an Eppendorf tube. The extract was neutralized carefully with 170 μL KOH (2 M). After removing the precipitate ($KClO_4$) by centrifugation (9000 g), the neutralized supernatants were stored at −20 °C prior to the LC–MS analysis.

2.5. Sample analysis

Extract of cells treated with benzo[a]pyrene for different periods of time were analyzed. Peak responses of the three adenine nucleotides were recorded and the concentration of each nucleotide was calculated with the area of corresponding peak. The total adenine nucleotides (TAN = ATP + ADP + AMP) was used as a base for calculating the relative amount of ATP, ADP and AMP in the sample.

2.6. Statistics

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 10.0 (SPSS, Chicago, IL, USA). Correlations were considered statistically significant if calculated P values were 0.05 or less. F-test and ANOVA were applied to calculate linearity, precision and accuracy of the method.

3. Results and discussion

3.1. Selection of HPLC columns for the analysis of adenine nucleotides

Several modified reversed-phase columns, namely Zorbax SB-C8, Symmetry Shield TM RP8, Synergi Max-RP 80, Synergi Hydro-RP,

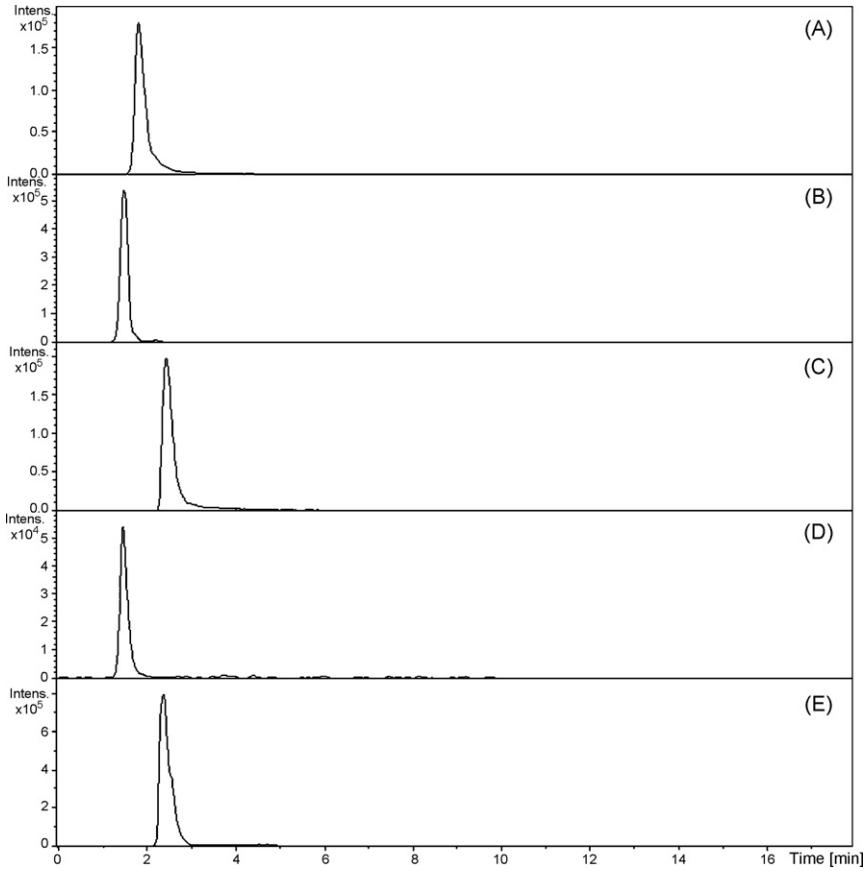

Fig. 1. LC–ESI-MS chromatograms of ATP retained on various reversed-phase HPLC columns: (A) Zorbax SB-C8 (150 × 2.1 mm, 4 μm); (B) Symmetry Shield TM RP8, (150 × 2.1 mm, 3.5 μm); (C) Synergi Max-RP 80A (150 × 2.0 mm, 4 μm); (D) Synergi Hydro-RP (150 × 2.0 mm, 4 μm); and (E) Alltima HP HILIC (150 × 2.0 mm, 3 μm).

Synergi Polar-RP and Alltima HP HILIC were used in order to select the optimal chromatographic conditions for the better retention of adenosine phosphates. Fig. 1 shows the HPLC chromatograms of ATP from the LC–MS analyses by using various modified C18 columns. Retention time of the highly polar ATP was less than 3 min even when using nearly complete aqueous mobile phase (Fig. 1A–E). The ESI-MS sensitivity was also poor because of the high water content of the mobile phase. Furthermore, when the method was applied for the analysis of complex biological samples, the specificity and sensitivity suffered from the co-eluted interference. The use of porous graphitic carbon (PGC) column, however, provided much better retention of ATP (8.8 min), ADP (9.5 min) and AMP (9.9 min) under the traditional reverse-phase conditions due to the special properties of the packing material (Fig. 2). In this case, the high proportion of organic phase could be used, which was more suitable for the subsequent mass spectrometry with electrospray ionization. The use of higher percentage of the organic solvent and higher flow rate compared to conventional reversed-phase LC methods also provided good peak shape for the nucleotides. Peak tailing of AMP, ADP, and ATP during the LC–MS analysis due to the interaction between stainless steel hardware and phosphate groups was reported [21–23]. Applications of various buffers including phosphoric acid or phosphate buffer [21], 25–50% ammonium hydroxide (∼pH 12) [22] or hydrogen

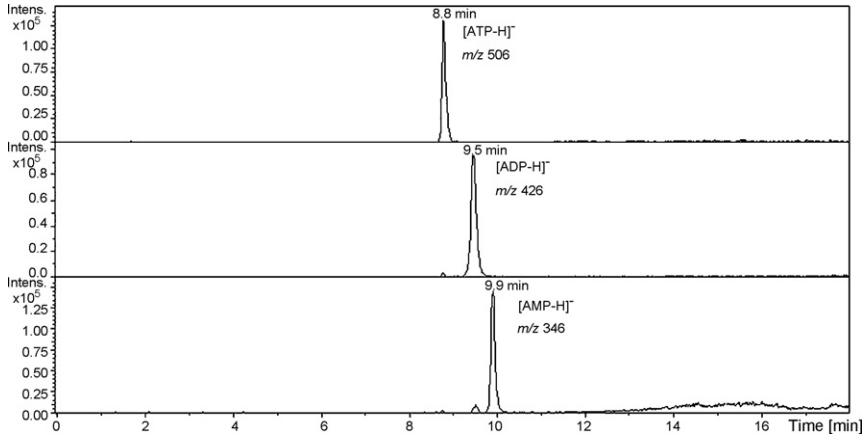

Fig. 2. LC–MS chromatograms of the mixture of adenosine phosphate standards at *m*/*z* 346 for AMP, *m*/*z* 426 for ADP and *m*/*z* 506 for ATP when using the PGC column.

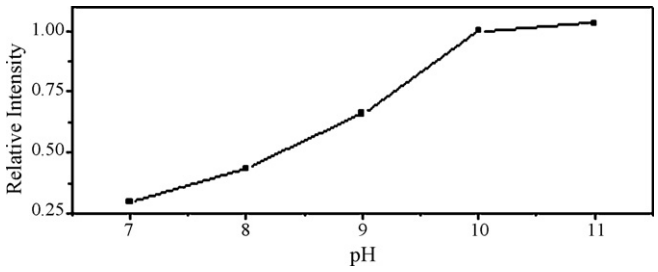

Fig. 3. Effect of pH in the LC mobile phase on ESI-MS detection sensitivity of nucleotides.

carbonate to mobile phase [23] in the mobile phase might solve the peak tailing problem.

3.2. Influence of pH in mobile phase

The influence of pH in the aqueous mobile phase on the detection sensitivity of the nucleotides was investigated over the range of pH 7–11 which was adjusted with the addition of ammonia when using the PGC column. With the increase in pH value over the range of 7–10, the ESI-MS intensity of nucleotides was clearly enhanced. However, there was no obvious improvement when the pH value was further increased (Fig. 3). Therefore, the mobile phase at pH 10 was used for the determination of nucleotides. It should be noted that the use of PGC column provided advantage of using basic condition in mobile phase while other reversed-phase HPLC columns could not tolerate the mobile phase at high pH. It should be noted, however, that the higher pH condition may have a deleterious effect on the seals, fittings or other instrument parts. An aqueous normal phase method used neutral to slightly acidic pH was reported recently for the sensitive analysis of the nucleotides, which was more instrument friendly [24].

3.3. Validation of the method

3.3.1. Specificity and selectivity

Specificity of the method was examined with the presence of endogenous interference from the cellular samples. The samples were extracted according to the sample preparation procedure and separated by the PGC column. Adenosine phosphates showed good retention time (8.8–9.9 min) on the PGC column and were separated well from the inorganic salts and other interferences in the complex biological matrix. Ion trap mass spectrometry analysis provided not only high sensitivity, but also high selectivity for the further separation of the analytes from interfering compounds. The determination of $[M\text{-}H]^-$ ion was applied for identifying the adenosine phosphates. Fig. 2 shows the detected peaks with good response of ATP at m/z 506, ADP at m/z 426 and AMP at m/z 346 in the mass chromatograms. The ionization voltage was carefully optimized from 1500 V to 5000 V. 3500 V was chosen for the best method sensitivity in our experiments, which showed that higher voltage could also cause fragmentation of parent ions.

Table 2
Stability data from the replicate analysis of ATP stock solutions.

Storage conditions	Concentration (μM)	Average recovery (%)[a]	RSD (%)
4 °C for 24 h	0.49	95	7.0
	1.98	93	3.5
	19.76	97	2.2
−20 °C for 14 days	0.49	105	4.1
	1.98	101	2.7
	19.76	99	1.3

[a] $N = 6$.

3.3.2. Limit of detection and limit of quantitation

The limit of detection (LOD) was defined as the concentration with a signal-to-noise ratio of 3. The LODs were 0.066 μM for AMP, 0.18 μM for ADP and 0.15 μM for ATP. The limit of quantitation (LOQ) was defined as the signal-to-noise ratio of 10 with the precision and accuracy being less than 20%. The LOQs were found to be 0.22 μM for AMP, 0.59 μM for ADP and 0.49 μM for ATP.

3.3.3. Linearity, precision and accuracy

Calibration curves were obtained by analyzing seven standard solutions with the concentrations in the range of 0.22–57.80 μM for AMP, 0.59–117.37 μM for ADP, and 0.49–98.81 μM. The linearity of the curves were observed when using F-test for lack-of-fit ($\alpha = 0.05$) as an indicator of linearity of the regression model. Correlation coefficients (R^2) of calibration curves determined by least-squares linear regression analysis were more than 0.998.

Accuracy as well as inter-day and intra-day precisions of the method were determined from the analyses of three standard samples in three independent runs. The accuracy was calculated as the percent deviation from the nominal concentration. The intra-day and inter-day precision were obtained from the analysis of variance (ANOVA) for each test concentration by using the analytical run as the grouping variable.

Table 1 shows accuracy and precisions data from the analysis of ATP in the spiked samples at levels of 0.49, 1.98 and 19.76 μM. The averaged recovery was 98.2–103.7% of the nominal concentrations and the intra-day precision and inter-day precision were from 1.2 to 5.0% and from 1.7 to 8.5%, respectively. Good accuracy and precision were also obtained from the analysis of ADP and AMP.

3.3.4. Stability

The stock solutions were stored at 4 °C for 24 h and at −20 °C for 14 days. Six replicates of the samples were analyzed immediately at the end of the storage period. The stability of nucleotides was listed in Table 2. The stability of the samples in the HPLC autosampler was tested by preparing a duplicate series of calibration samples. The second sample at each level was analyzed 24 h after the first sample was injected on the column. Nucleotides appeared to be stable when kept in autosampler at 4 °C for 24 h and the loss was less than 5%.

3.3.5. Extraction recovery

In order to assess the extraction recovery, the standard solutions of adenosine nucleotides were added into the blank HepG-2 cells

Table 1
Accuracy and precision data obtained from the analysis of ATP in spiked standard samples.

Spiked level (μM)	Average measured value[a] (μM)	Intra-day precision RSD (%)[b]	Inter-day precision RSD (%)
0.49	0.51 ± 0.025	5.0	8.5
1.98	1.94 ± 0.045	2.3	5.3
19.76	20.06 ± 0.24	1.2	1.7

[a] $N = 6$.
[b] RSD = relative standard deviation.

which were cultured in MEM medium and extracted according to the procedure described in Section 2.4. The recovery comprising the loss during the sample preparation was calculated by comparing the difference between the experimental and theoretical values. The recovery was 78.5% for ATP, 80.5% for ADP and 87.1% for AMP.

3.4. Investigation on benzo[a]pyrene toxicity through the determination of energy metabolites

Benzo[a]pyrene is a toxic compound that has been found in coal tar, automobile exhaust fumes and tobacco smoke, etc. Recent studies have suggested the links between benzo[a]pyrene toxicity with cancer and cell death. Benzo[a]pyrene can cause cell death via either apoptosis [25] or necrosis [26], which are two different modes of cell death following exposure to the toxicant. The occurrence of the different modes of cell death depends on energy level. High level of ATP may cause cell death via apoptosis while inadequate ATP level is associated with necrotic cell death [27–29]. On the other hand, cellular ATP level is also used as a marker for determining cell viability. Thus, the only information of ATP level is inadequate for assessing whether the cell death is a progression through apoptosis or necrosis. A novel approach of examining the relation in energy state and the mode of cell death has been proposed [23]. It was reported that exposure of the HepG-2 cells to the toxic metal Cd resulted in apoptotic cell death [30]. Despite a decrease in total adenosine nucleotide (TAN) level, ATP/TAN was significantly increased together with a significant decrease in ADP/TAN prior to the loss of mitochondrial function [31]. Exposure to the same cell line to benzo[a]pyrene caused cell death via necrosis [26]. Therefore, determination of energy metabolites in the cells following benzo[a]pyrene exposure may lend support to the hypothesis that energy level is important in mediating the modes of cell death.

Four groups of HepG-2 cell were incubated in the medium with benzo[a]pyrene for 12, 24, 36, 48 h, respectively. The corresponding control groups were incubated in the medium without benzo[a]pyrene. Fig. 4 shows the comparison of adenine nucleotides concentrations in the four groups of treated samples with the corresponding control groups. ATP, ADP and AMP levels in the control groups were stable over 48 h, with the levels of ATP at 6.21–6.93 nmol/mL, ADP at 1.54–1.62 nmol/mL and AMP at 0.76–0.94 nmol/mL. The levels of ATP, ADP and AMP in the incubated samples were significantly lower than those of controls, even for the initial incubation point at 12 h. TAN was calculated to evaluate the relationship between the toxicity of benzo[a]pyrene and the exposure time. The changes in energy state of the HepG-2 cells following the exposure to benzo[a]pyrene are given in Table 3. The TAN values in benzo[a]pyrene-treated samples were apparently lower than those in control samples, indicating that the concentrations of the nucleotides in the samples treated with benzo[a]pyrene declined over the incubation period. The values of ATP/TAN in the treated samples were lower than those in control groups, but the ADP/TAN values in the treated samples were higher. The low

Table 3
Data for cellular energy state of HepG-2 cells treated with benzo[a]pyrene.

		12 h	24 h	36 h	48 h
TAN	Control	8.91	9.30	9.34	8.74
	Treated	8.08	7.13	6.49	5.70
ATP/TAN	Control	0.733	0.738	0.742	0.711
	Treated	0.615	0.619	0.631	0.599
ADP/TAN	Control	0.182	0.166	0.165	0.181
	Treated	0.345	0.369	0.369	0.401
AMP/TAN	Control	0.0857	0.0956	0.0929	0.108
	Treated	0.0401	0.0124	–	–

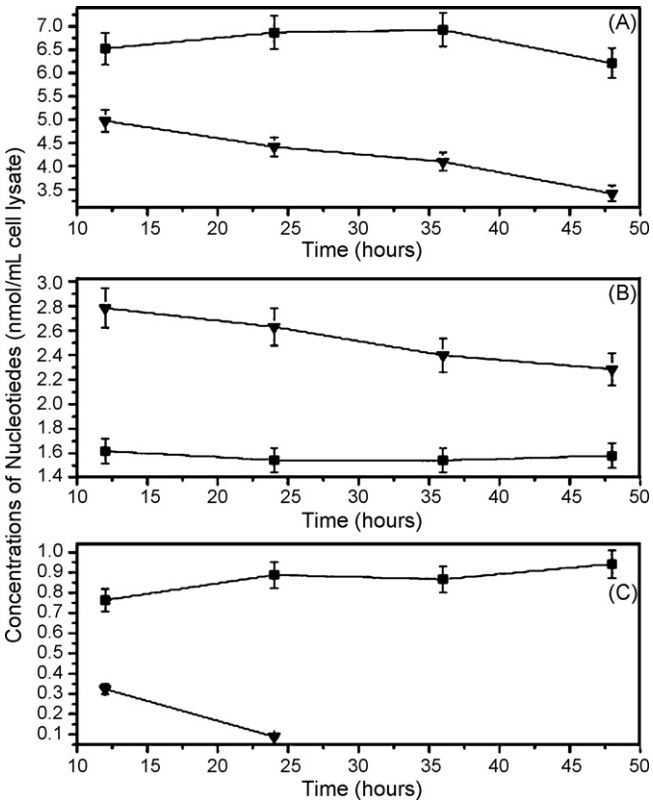

Fig. 4. Effect of benzo[a]pyrene on the adenosine phosphates levels in HepG-2 cells: (A) ATP; (B) ADP; and (C) AMP. The square points were for control groups, while the triangle points were for cell samples treated with benzo[a]pyrene ($n = 3$).

ATP/TAN together with an elevated ADP/TAN suggested that the cells might have difficulty in maintaining adequate ATP availability. The results are consistent with the hypothesis that lower ATP availability is associated with necrotic cell death. Besides a change in ATP/TAN and ADP/TAN, there was a significant decline in the AMP/TAN value in the treated samples. In the case of Cd, necrotic cell death was associated with an increase in AMP/TAN [30]. The difference in the pattern of change in energy metabolites may suggest that the change of energy state reflects the different mode of cell death. However, whether different toxicant may affect other features of energy metabolism should be further investigated.

4. Conclusion

LC–MS method with a porous graphitic carbon column provided sensitive and specific determination of adenine nucleotides in cells. The use of porous graphitic carbon column could enhance the retention and separation of the polar nucleotides under the conventional reversed-phase HPLC conditions. The HPLC method was well compatible with ESI-MS analysis because no ion-pairing or ion-exchange agent was needed. Negative-ion mode ESI-MS with basic mobile phase condition improved the sensitivity of the MS analysis. The method was successfully applied to determine ATP, ADP, and AMP in HepG-2 cells treated with benzo[a]pyrene. The obtained preliminary results helped understanding the effect of benzo[a]pyrene on energy metabolism.

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