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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,