Ion-Pairing Liquid Chromatography Coupled with Mass Spectrometry for the Simultaneous Determination of Nucleosides and Nucleotides

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Adenosine and its corresponding nucleotides adenosine 5’-monophosphate (AMP), adenosine 5’-diphosphate (ADP) and adenosine 5’-triphosphate (ATP) are important biomolecules that provide energy and substrates for various cellular biochemical processes. There have been strong demands for sensitive and reliable analysis of these nucleotides because the determination of their levels in cells may provide valuable information for understanding cellular energy metabolism. Analysis of adenosine nucleotides is a big challenge because these chemicals exist together with high cellular background in samples. Moreover, nucleotides are extremely polar due to the presence of multiple phosphate groups that may interfere with chemical determination. In particular, the nucleotide phosphates are not retained under conventional reversed-phase chromatographic conditions because of their extremely high polarity. The most common approach for quantitative analysis of nucleotide phosphates in cellular extracts is to determine indirectly the corresponding parent nucleosides resulted from enzymatic dephosphorylation after the nucleotides were separated by using an anion-exchange solid phase extraction. The generated nucleosides were analyzed by using liquid chromatography with ultra violet absorption detection LC/UV radioimmunoassay and liquid chromatography-mass spectrometry-mass spectrometry LC-MS-MS. Because nucleotides are not retained under conventional reversed-phase conditions ion-suppression high performance liquid chromatography HPLC capillary electrophoresis and ion-pairing HPLC have been employed to circumvent the poor retention problem. The high selectivity of MS and MS/MS techniques provides great separation and detection power making them attractive alternatives for the trace analysis of nucleotides. The application of negative ion electrospray ionization ESI-MS to nucleotide analysis has been reviewed. The use of negative ion ESI-MS seemed to be a logical starting point for nucleotide phosphate analysis due to the existing phosphate groups. However, the application of positive ion ESI-MS coupled with ion-pairing HPLC for the quantitative analysis of intracellular nucleotides has recently been explored. Cai and co-workers reported the selection of most abundant MS-MS quantitative ions of 6 ribo- and ribodeoxy-nucleotides by using ion-pairing HPLC coupled with positive ESI-MS. Fung et al. discussed the advantage of positive ion mode in comparing with negative ion mode for the simultaneous determination of nucleotides and nucleosides when dimethylhexylamine DMHA was used as the ion-pairing agent. Recently, Cai and co-workers also reported an assay of ion-pairing HPLC coupled with positive ion electrospray ionization-time of flight mass spectrometry ESI-TOFMS for determining cellular levels of ATP. This presentation describes the method development and applications of the ion-pairing HPLC and positive ion ESI-MS for the simultaneous determination of nucleosides and nucleotides.
1 Experimental

DMHA was purchased from Aldrich Chemicals Co.[1] Milwaukee, WI, USA and used as ion-pairing agent. Liquid chromatography experiment was carried out on an HPLC system equipped with an auto-sampler and a micro mode pump[2] Capillary HP1100[3] Agilent Technologies[4] Palo Alto, CA, USA. A reversed-phase microbore column Waters[5] Xterra MS-C8, 1.0 mm i. d. ×100 mm[6] 3.5 µm[7] was used throughout the entire study. The temperature of the auto-sampler was set at 10 °C. The mobile phase consisted of two eluents. Eluent A was 8 mmol/L DMHA adjusted to pH 7 with formic acid and eluent B was acetonitrile-water[8] 70:30[v/v]. The gradient program was from 5% B to 65% B within 8 min and hold for 1 min and then returned back to 5% B at a flow rate of 55 µL/min. The sample injection volume was 5 µL. The effluent from the LC column was diverted to waste for the first 5 min after injection[9] then diverted to the MS ion source in order to avoid the non-volatile salts and interference background in the sample from contaminating the MS spectrometer. Mass spectrometric experiments were performed on a quadrupole-time of flight Q-TOF[10] tandem mass spectrometer[11] API Q-STAR Pulsar I[12] Applied Biosystems[13] Foster City, CA, USA. Positive ion mode ESI was used for the analysis of the nucleotides.

2 Results and discussion

Because of one or more negatively charged phosphate groups existing in the molecules[14] AMP[15] ADP and ATP are too polar to be retained on the reversed-phase HPLC column under conventional chromatographic conditions. However[16] the presence of the ion-pairing agent allowed the retention and separation of nucleotides on the column. The use of DMHA in the mobile phase also enabled the detection of the adenosine nucleotides with positive ion mode of ESI-MS. The retention of the nucleotides can be explained by the formation of “ion-pairs” between the positively charged ion-pairing agent and negatively charged nucleotides. It has been reported that DMHA has advantages over other ion-pairing agents such as tetraalkyl ammonium salts for LC-MS analysis of nucleosides and nucleotides[17]. To retain ATP[18] guanosine triphosphate[19] GTP[20] dATP and dGTP on a 2 mm diameter reversed-phase HPLC column[21] an addition of 20 mmol/L DMHA into the mobile phase was needed. Cai et al.[22] reported that DMHA concentration could be reduced to 10 mmol/L when using a microbore column[23] 1.0 mm i. d. [24] whereas only 5 mmol/L DMHA was needed when using a capillary[25] 0.5 mm i. d. [26] C18 column. Furthermore the use of lower concentration of DMHA in the capillary HPLC method resulted in a sensitivity increase of 5–10 times for the positive ion ESI-MS analysis of the nucleotides. In this study[27] 8 mmol/L DMHA in the aqueous mobile phase was found to be adequate to retain the adenosine nucleotides and provide good peak shapes for the separation[28] Fig. 1[29]. The data showed that 8 mmol/L DMHA in the aqueous mobile phase at pH 7 provided adequate retention and good peak shape for the separation of the adenosine nucleotides. The detection of the adduct ions of the nucleotides and DMHA by using LC-MS provided good sensitivity for the quantitative analysis of the cellular sample extracts.

![Fig 1](attachment:image.png)

Fig. 1 Ion-pairing LC-MS chromatograms of DMHA adduct ions at m/z 477 for AMP m/z 557 for ADP and m/z 637 for ATP

The developed LC-MS assay has been successfully applied for analyzing AMP[30] ADP and ATP in biological cellular samples[31]. Rat brain cells[32] C6 glioma[33] were treated with various concentrations of Zn. The cells were cultured and prepared by using a procedure containing acetonitrile precipitation and solid-phase extraction. AMP[34] ADP and ATP were identified in the cellular samples by using high-resolution mass spectrometry and confirmed with MS-MS analysis. The levels of the adenosine phosphorylated metabolites
were quantitated and compared with those from cellular blank matrices. The data indicated that an addition of Zn to the rat cells at a range of 40–120 mg/L did not affect the cellular energy status of the treated rat brain cells with the heavy metal.

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