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Capillary liquid chromatographic–high-resolution mass spectrometric analysis of ribonucleotides

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Abstract

A method of capillary HPLC–high-resolution MS was developed for the trace analysis of ATP, GTP, dATP and dGTP. Dimethylhexylamine (DMHA) was used as ion-pairing agent for the HPLC retention and separation of the nucleotides and positive ion electrospray time-of-flight MS was used for the detection. The application of capillary HPLC allowed minimal usage of DMHA while providing excellent peak retention and resolution, which significantly reduced the ion suppression in electrospray ionization-MS analysis and thus increased the sensitivity. Adduct ions of nucleotides and DMHA were used as quantitative ions in order to achieve the best sensitivity. DMHA concentration at 5 m*M* in the aqueous mobile phase at pH 7 was found to be the optimal conditions for the C_{18} capillary column. The method was applied to determine ATP level in cultured *C6 glioma* cells that were treated with toxic concentrations of Zn. The results showed that the cellular ATP level decreased from 2.7 pmol/cell (<10% cell death) in average control cell samples to 0.36 pmol/cell as the concentration of Zn increased to 120 mg/l (>35% cell death) in culture medium.

Keywords: Ion-pairing reagents; Ribonucleotides; Nucleotides; Phosphates; Dimethylhexylamine

1. Introduction

Deoxyribo- and ribonucleotides are important biomolecules [1]. In addition to forming the basic units of genetic materials, they also act as energy carriers (ATP, GTP), and form the basic structure of cofactors (coenzyme A, NAD^+, FAD^+), and signal mediators (cAMP, ppGpp). Nucleoside analogues have been developed and were found to possess antiviral activity against human immunodeficiency virus (HIV). The determination of the corresponding generated nucleotides in cells and tissues may provide valuable information on understanding the mechanism of action of these analogs [2,3], and facilitate future drug development. There have been strong demands for suitable procedures for both accurate determination and high-throughput screening of nucleoside and nucleotides. Accurate quantification of ribonucleotides such as adenosine 5′-triphosphate (ATP), guanosine 5′-triphosphate (GTP) as well as the related deoxygenated nucleotides (dATP and dGTP) (Fig. 1) is a unique challenge to conventional high-performance liquid chromatography (HPLC). First, nucleotides retain weakly on reversed-phase HPLC columns with conventional mobile phases, so it is necessary to establish the chromatographic conditions to improve the retention

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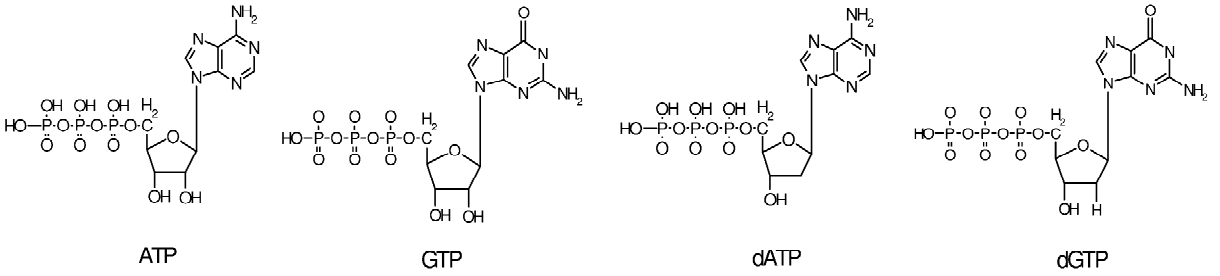

Fig. 1. Structures of ATP, GTP, dATP and dGTP.

time. Secondly, because the chemical structure and properties of many nucleotides as well as the triphosphate metabolites of nucleoside drug analogues may be similar, their complete chromatographic separation is often difficult. This can be overcome by a second-dimensional separation, for example, mass spectrometric separation. Finally, nucleotides may be present in trace levels in cellular or intracellular compartments. Therefore, techniques with high sensitivity are needed in order to monitor their concentrations at the cellular level.

Chromatographic procedures with UV or radiometric detection have been applied to isolate and determine nucleotides [2–8]. Methods using ion-exchange liquid chromatography have been successfully applied to quantify nucleosides and nucleotides to support in vitro studies [2,3]. Ion-suppression HPLC [4], capillary electrophoresis [5] and ion-pairing HPLC [6–8] were developed for the separation and determination of these biomolecules and their analogues.

Mass spectrometry has evolved as an important technique for the characterization of nucleotides, mainly because of its high sensitivity compared to other detection methods [9]. The high selectivity of MS and MS–MS techniques provides great separation and detection power, making LC–MS and LC–MS–MS attractive alternatives for the trace analysis of nucleosides and nucleotides. LC–MS with electrospray ionization (ESI) has become widely used for both qualitative and quantitative analyses of nucleosides and nucleotides [10–12]. This paper reports an ion-pairing capillary HPLC–high-resolution MS method for fast, specific and sensitive analysis of nucleotides. In addition, the method was applied to quantify cellular ATP content, and was used to monitor the toxicity of Zn.

2. Experimental

2.1. Chemicals

ATP, GTP and the corresponding deoxynucleotides (dATP and dGTP) were purchased from Sigma (St Louis, MO, USA). *N*,*N*-Dimethylhexylamine was obtained from Aldrich (Milwaukee, WI, USA). HPLC-grade acetonitrile and water were purchased from Fisher (Pittsburgh, PA, USA), ammonium acetate was from Panreac Quimica (Barcelona, Spain) and glacial acetic acid was from Merck (Darmstadt, Germany). Other chemicals (analytical grade) were purchased from Sigma.

2.2. Apparatus

2.2.1. HPLC system

On-line HPLC experiments were carried out on a capillary liquid chromatography system equipped with an auto-sampler and a capillary pump (Capillary HP1100, Agilent Technologies, Palo Alto, CA, USA). A reversed-phase capillary column (Zorbax XDB-C_{18}, 150×0.5 mm, 5 μm) from Agilent Technologies was used throughout the entire study, except in special cases noted. The temperature of the auto-sampler was set at 10 °C. The mobile phases consisted of two eluents. Eluent A was 5 m*M* dimethylhexylamine (DMHA) at pH 7 and eluent B was acetonitrile–water (70:30). The gradient program was from 10% B to 90% B within 2 min at a

flow-rate of 30 μl/min. The sample injection volume was 0.2 μl.

2.2.2. MS instrument

All mass spectrometry experiments were carried out on a quadrupole/time of flight (Q-TOF) tandem mass spectrometer (API Q-STAR Pulsar *i*, Applied Biosystems, Foster City, USA). Positive ion mode ESI was used for the analysis of the nucleotides. The following parameters of the turbo-ionspray source were used: ionspray voltage 4600 V; declustering potential (DP1) 45 V; focusing potential (FP) 165 V; declustering potential 2 (DP2) 15 V; collision energy (CE) 12 eV. The ion source gas 1 (GS1), gas 2 (GS2), curtain gas (CUR) and collision gas (CAD) were 25, 10, 12 and 3, respectively. The temperature of GS2 was 400 °C.

2.3. Sample preparation

2.3.1. Standard solutions

Stock solutions of the nucleotides were each prepared in 10 m*M* ammonium acetate at a concentration level of 5 m*M* and stored at −20 °C. Calibration standards were prepared by diluting the stock solutions with the HPLC aqueous mobile phase.

2.3.2. Cell extract samples

The *C6 glioma* cells were cultured in the Ham's F-12K medium supplemented with 10% fetal bovine serum. The culture condition was similar to that reported in Yang et al. [13]. Cells were incubated in a culture medium containing 0 or 120 μg/ml $ZnCl_2$ (Sigma) for 3 h. At the end of the incubation, cells were harvested and washed with phosphate-buffered saline (PBS). The numbers of both alive and dead cells were counted. The PBS was then removed by centrifugation (12 000 rpm for 10 min), and 1 ml of solution containing 0.3 *M* perchloric acid and 1 m*M* EDTA (Sigma) was added to digest the cells and precipitate proteins. The supernatant, containing the non-enzymatic metabolites, were neutralized with 150 μl of a solution containing KOH–imidizole–KCl (2 *M*:0.4 *M*:2 *M*) (Sigma). After centrifugation at 12 000 rpm for 10 min, the supernatant was used for the ion-pairing LC–MS analysis.

3. Results and discussion

3.1. Method development for ion-pairing capillary liquid chromatography

3.1.1. Capillary HPLC

The advantages of using capillary HPLC column for bioanalysis have been discussed [14]. Nano-scale capillary HPLC has been rapidly developed recently not only because it requires much less solvent consumption but also due to its strong chromatographic resolving power. The technique has the potential of offering a chromatographic peak concentration enhancement compared to a larger diameter HPLC column. Moreover, capillary LC is an ideal technique to couple with ESI-MS. The sensitivity of ESI-MS is high because the signal is concentration-dependent, providing an ideal feature for quantitative analysis. The challenge, however, is that the nucleotides have poor retention on the reversed-phase column due to their high polarity. When the aqueous mobile phase containing ammonium acetate at pH 7 or lower and any percentages of organic phase were used, the nucleotides were not retained but eluted with the solvent front. However, the retention of the nucleotides on the C_{18} capillary column increased with the addition of DMHA as an ion-pairing agent. Addition of 5–20 m*M* of the DMHA improved the retention of the nucleotides on the column.

Unlike some ion-pairing agents, which have been reported to cause a significant negative effect on ESI-MS detection, DMHA was found to be by far the most-friendly ion-pairing agent for ESI-MS analysis. Previous studies indicated that DMHA has advantages over other ion-pairing agents such as tetraalkyl ammonium salts used for the ion-pairing LC–MS analysis of nucleosides and nucleotides [8]. Still, it was observed that the use of DMHA decreased the MS detection sensitivity, especially when negative ion mode was applied [15,16]. This problem actually gives an additional advantage of using the capillary column for ion-pairing LC–MS analysis of the nucleotides because the use of the small column allowed for the lowest delivery of mobile phase containing the ion-pairing agent into the ESI-MS ion source. The concentration of the ion-paring agent was reduced to a minimal level (5 m*M* in aqueous

phase) when using a capillary LC column, while still meeting the chromatographic and mass spectrometric requirements for the nucleotides analysis (see below).

3.1.2. Concentration of the ion-pairing agent in mobile phases

The concentration of DMHA in mobile phases is critical to the LC retention of the nucleotides and ESI-MS sensitivity. It was reported that 20 m*M* DMHA was needed in the aqueous mobile phase in order to achieve better retention of nucleotides for adenine nucleotide-containing metabolites of bisphosphonates [15]. Also, to retain ATP, GTP, dATP and dGTP on a 2-mm diameter reversed-phase HPLC column, an addition of 20 m*M* DMHA into mobile phases was needed [8]. For a microbore column (1.0 mm I.D.), 10 m*M* DMHA was needed (Fig. 2). However, the utilization of the capillary column allowed the reduction of DMHA concentration, while still providing excellent retention of the nucleotides on column. It was found that 5 m*M* of DMHA in aqueous mobile phase was adequate when using the 0.5 mm capillary C_{18} column (Fig. 3). Compared to the microbore LC analysis, the use of a lower concentration of DMHA (5 m*M*) in the capillary LC method resulted in a sensitivity increase of 5- to 10-fold for the positive ion ESI-MS analysis of the nucleotides.

3.1.3. pH value of mobile phases

It was observed that the pH value of mobile phases was critical to the retention and peak shape of the nucleotides (Fig. 2). Fig. 2 demonstrated a significant effect of pH in the aqueous mobile phase on peak shapes and separation of ATP and dGTP. In addition, ATP was found unstable at pH 3 or lower (data not shown).

3.2. Positive ion ESI-MS analysis of the nucleotides

It was reported that the use of ion-pairing agents enabled the detection of nucleotides using positive ion mode ESI [7,8]. The formation of ion pair or adduct ion greatly enhanced the positive ionization of nucleotides. The protonated parent ion and its product ions are often monitored for the quantitative analysis. However, we found that full-scan positive ion ESI-MS analysis of the nucleotides produced a signal from the nucleotide–DMHA adduct ion as the most intensive peak. Fig. 4 demonstrated that the intensity of the adduct ion of ATP–DMHA ($[ATP+DMHA+H]^+$) was approximately three times that of the ATP molecule ion $[ATP+H]^+$ under the optimized ESI-MS conditions, indicating that the current method selecting the adduct ion as the quantitative ion could provide better sensitivity than those methods selecting the $[ATP+H]^+$ ion as quantitative ion. It should be noted that the intensity of the adduct ion was consistently higher than that of the $[ATP+H]^+$ ion under various ESI-MS conditions.

The approximate threefold intensity difference between the adduct ion $[ATP+DMHA+H]^+$ and the

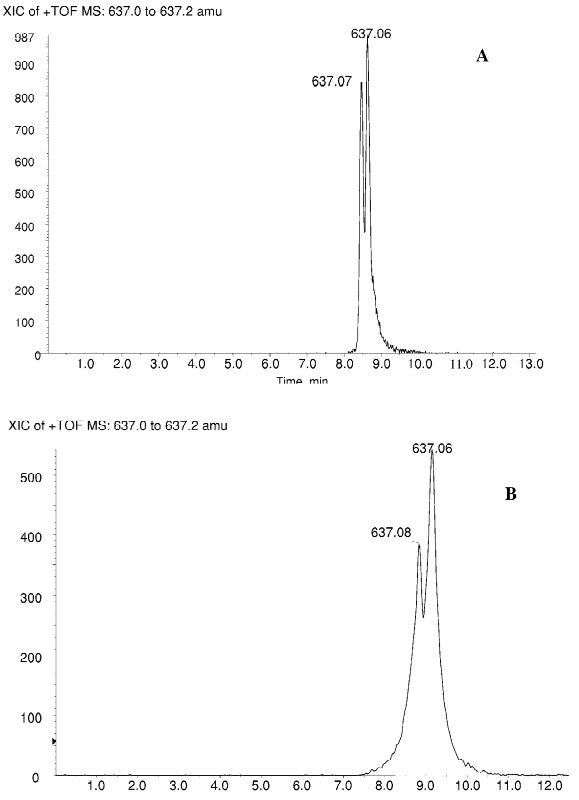

Fig. 2. LC–MS chromatograms of dGTP and ATP. The nucleotide retention was achieved on a microbore column (Waters Xterra C_8, 100×1.0 mm, 3.5 μm) with the aqueous mobile phase containing 10 m*M* DMHA and pH at 7 (A) and 6.2 (B). The organic phase was acetonitrile–10 m*M* NH_4Oac (70:30). A gradient program (18% B–50% B within 6 min) was applied. The ATP injection amount was 500 pmol on-column.

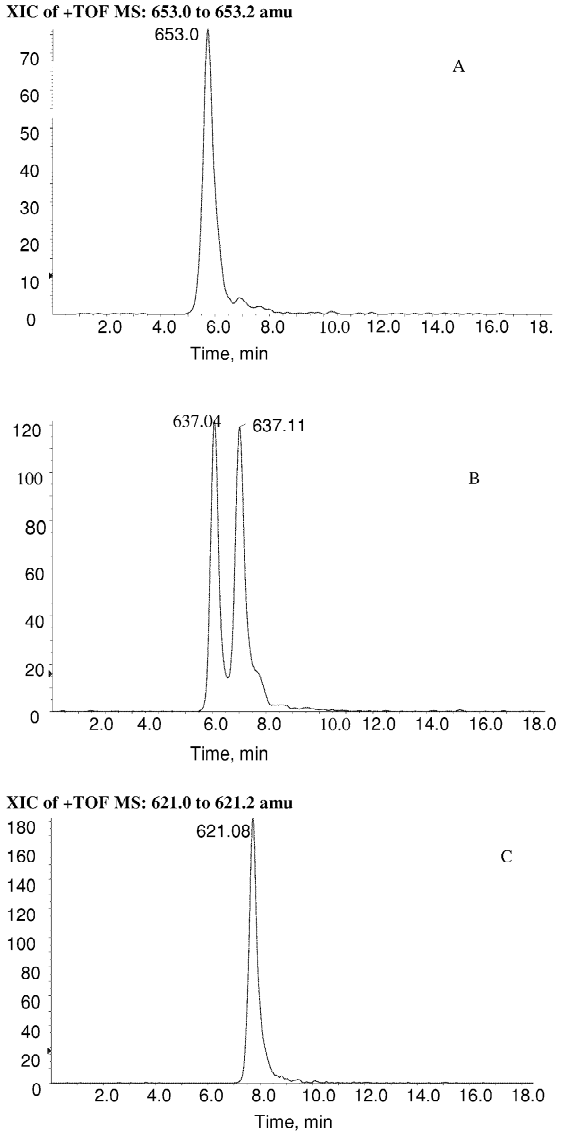

Fig. 3. LC–MS chromatograms of GTP at m/z 653 (A), dGTP and ATP at m/z 637 (B) and dATP at m/z 621 (C) obtained from a injection of 20 pmol of each.

$[ATP+H]^+$ ion was not only observed under the current mobile conditions containing 5 m*M* DMHA, but was also seen when 10 and 20 m*M* were used, respectively. The use of a lower DMHA concentration (5 m*M*) in the capillary LC method did not result in decreasing the peak intensity ratio of the adduct ion to $[ATP+H]^+$ ion. This indicated that the reduction of DMHA concentration from 20 to 5 m*M* in the mobile phase did not have an effect on the formation of the adduct ion. The addition of 5 m*M* DMHA in the aqueous mobile phase seemed adequate for forming the adduct ion.

To confirm the detection of the ATP–DMHA adduct ion, an MS–MS experiment was conducted on the selecting ion mass at m/z 637 as the parent ion of $[ATP+DMHA+H]^+$ (Fig. 5). The base peak of fragmentation detected in the collision-induced dissociation (CID) mass spectrum of the nucleotide was very informative because it resulted from the loss of the DMHA, i.e. the observed base peak represented $[ATP+H]$ ion. This fragment ion is clearly indicative of the formation of the ATP–DMHA adduct ion. Moreover, two other major fragment ions resulting from logical cleavages on the ATP–DMHA molecule were also observed (Fig. 5).

In addition to the MS–MS results, the ATP–DMHA adduct ion was also confirmed by measuring the accurate masses of both the parent and the major fragment ion. Table 1 lists high-resolution mass data for parent ions (the DMHA adduct ions) as well as the corresponding major fragment ions of ATP, GTP, dATP and dGTP. The exact mass measurement from the high-resolution MS analysis provided the elemental composition information of all ions (Table 1). The comparison of the experimental and theoretical masses of the assigned elemental compositions is also presented in Table 1. The mass difference between the measured and theoretical exact masses of both the parent and fragment ions was less than 10 ppm. Thus, the high-resolution MS–MS analysis provided conclusive confirmation for the formation of the nucleotide–DMHA adduct ions.

3.3. Determination of ATP in cultured C6 glioma cells treated with zinc chloride

The developed ion-pairing LC–MS method was used to determine ATP levels in cultured *C6 glioma* cells after treatment with a high concentration of zinc chloride for toxicity assessment. The sample extract was directly injected onto the capillary column. The high sensitivity of the ESI-MS and good peak resolution from the capillary HPLC separation allowed a direct injection of 0.2 μl of the sample extract while still maintaining required low detection limits. The low injection volume also dramatically

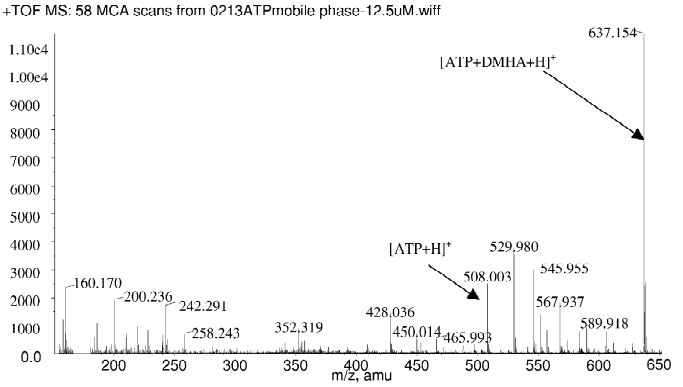

Fig. 4. Positive ion ESI-HRMS spectrum of ATP obtained from the direct infusion of 10 μ*M* ATP in 5 m*M* DMHA with a flow-rate of 10 μl/min. The intensity of the DMHA–ATP adduct ion at *m*/*z* 637 was approximately three times of that of [ATP+H] ion at *m*/*z* 508.

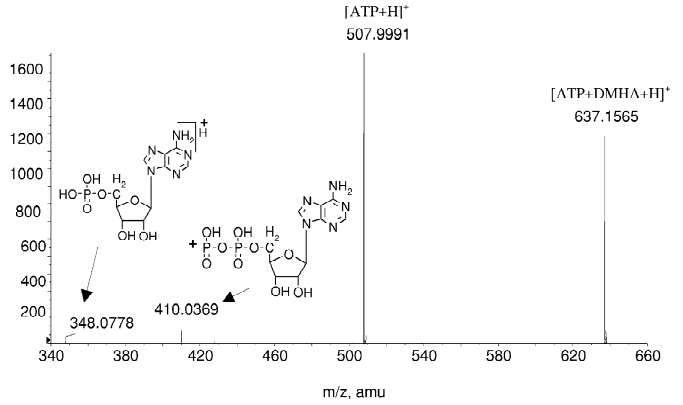

Fig. 5. High-resolution MS–MS spectrum of ATP–DMHA adduct ion. The spectrum was obtained from a direct infusion of ATP at 10 μ*M* with a flow-rate of 10 μl/min.

Table 1
High-resolution ESI-MS–MS data of the parent DMHA–nucleotide adduct ions and major fragment ions

Ions	Theoretical value (*m*/*z*)	Measured value (*m*/*z*)	Elemental comp.	Mass accuracy (ppm)
[ATP+DMHA+H]	637.1553	637.1565	$C_{18}H_{36}N_6O_{13}P_3$	1.8425
[ATP+H]	508.0035	507.9991	$C_{10}H_{17}N_5O_{13}P_3$	−8.5306
[dATP+DMHA+H]	621.1604	621.1599	$C_{18}H_{36}N_6O_{12}P_3$	−0.8232
[dATP+H]	492.0086	492.0093	$C_{10}H_{17}N_5O_{12}P_3$	1.2975
[GTP+DMHA+H]	653.1502	653.1500	$C_{18}H_{36}N_6O_{14}P_3$	−0.3684
[GTP+H]	523.9984	523.9970	$C_{10}H_{17}N_5O_{14}P_3$	−2.8452
[dGTP+DMHA+H]	637.1553	637.1550	$C_{18}H_{36}N_6O_{13}P_3$	−0.5116
[dGTP+H]	508.0035	508.0036	$C_{10}H_{17}N_5O_{13}P_3$	0.0467

reduced the negative impact of different chemical compositions between the sample and the initial mobile phase condition on chromatographic peak shape. It should be noted that the injection volume could be increased (up to 1 μl) in order to achieve lower detection limits. When a large volume of sample was injected onto the capillary column, the high amount of salts and other impurities in cell extracts may result in peak shape deterioration. By dissolving or diluting the sample extracts into a solution of the initial conditions of mobile phases, however, it could maintain the retention of the nucleotides on the reversed-phase HPLC column, reduce matrix interference and provide a good peak shape for subsequent analysis.

3.3.1. Identification of ATP in the cell extracts

ATP was identified in the cell extracts of both control and Zn-treated samples. The identification was achieved by detecting the ATP–DMHA adduct ion and by measuring its accurate mass (Fig. 6A). The measured exact masses of the $[DMHA+ATP+H]^+$ and $[ATP+H]^+$ ions were at m/z 637.1541 and m/z 507.9989, which had mass differences of −1.9 and 9.0 ppm from the theoretical values, respectively. Moreover, tandem mass spectrometry was used to induce the formation of fragment ions for the purpose of confirmation of ATP identified in the cell extract samples. The obtained high-resolution MS–MS spectrum was the same as that presented in Fig. 5, clearly indicating the presence of ATP in the cell extracts.

High-resolution MS (HRMS) and MS–MS analyses could also be extremely useful for identifying other unknowns, e.g. the mono- and diphosphated compounds. In addition to the capillary chromatographic separation, this technique also provided another dimension of separation with high specificity due to its high mass resolving power. The mass separation power is particularly important for the identification of nucleotides as well as their trace quantitative analysis in complex biological matrices.

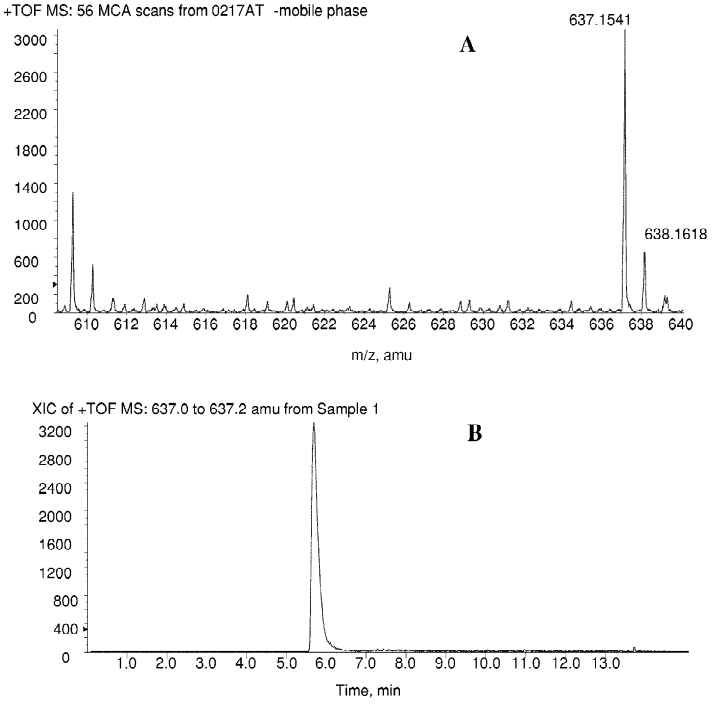

Fig. 6. TOF-HRMS spectrum for identifying ATP in the rat brain cell extracts (A) and mass chromatogram of ATP at m/z 637 used for quantifying ATP levels. See Table 2 for quantitative results.

Table 2
ATP levels determined in the rat brain cells (*C6 glioma*) treated with Zn of different concentrations

Code	C21	C22	S85	S90	S91
Conc. of Zn (μl/ml)	0	0	60	100	120
No. of live cells	6.60×10^6	1.06×10^7	7.12×10^6	4.44×10^6	5.72×10^6
No. of dead cells	5.20×10^5	4.80×10^5	2.04×10^6	3.72×10^6	3.44×10^6
Total no. of cells	7.12×10^6	1.11×10^7	9.16×10^6	8.16×10^6	9.16×10^6
Conc.±SD (μM; $n=3$)	17.9±0.9	19.5±0.4	7.7±0.3	4.8±0.4	2.6±0.1
Cell conc. (pmol/cell)[a]	3.2	2.2	1.1	0.75	0.36

[a] Note: A dilution factor of 0.78 from the sample preparation was considered.

3.3.2. Quantitation of ATP levels

The levels of ATP in the cell samples were determined by monitoring the adduct ion of ATP–DMHA in the TOF-MS experiments. A high-resolution mass chromatogram of the extracted adduct ion was used for the quantitative analysis (Fig. 6B). Due to the use of HRMS, little background noise was observed. For better specificity, high resolution MS–MS of the mass transition from the adduct ion to the $[ATP+H]^+$ ion could be used. For example, m/z 508 produced from the parent ion at m/z 637 could be monitored for the quantification of ATP during the MS–MS analysis. However, it was found that the sensitivity dropped approximately fivefold when using HRMS–MS compared to HRMS scan. For the low-resolution quadrupole MS, this loss of sensitivity might be compensated from the reduction of background noise so that MS–MS analysis could eventually have similar or better signal-to-noise. However, the background noise was less important for the TOF-MS quantitative analysis because the noise level was already low under the high-resolution MS conditions. The absolute detection sensitivity played a more significant role for the quantitative analysis using the TOF-MS.

The limit of detection, defined with a signal-to-noise ratio better than 3, was 0.2 μM. With the injection volume of 0.2 μl, the lowest on-column detectable amount of ATP was 0.04 pmol per injection. A linear calibration curve was obtained for a concentration range of 0.4–20 μM with a correlation coefficient of 0.999, a slope of 276±19.2 and an intercept of −181±11.7. The possibility of obtaining a wider dynamic range was not examined because the current calibration curve already covered the ATP concentration levels in the rat brain cell samples.

The results of ATP quantitative analysis are listed in Table 2. Each concentration value represented the average from three analyses±S.D. The calculated average ATP level in control cells (C21 and C22) was 2.7 pmol/cell. This is in the same order of magnitude as that detected by the fluorimetric method (M.S. Yang, unpublished results). A high concentration of $ZnCl_2$ added to the incubation medium caused a significant amount of cell death. ATP content also decreased significantly (see results for S80, S90 and S91 samples). The decrease may be attributable to direct effects (e.g. inhibition) of the metal on some enzyme systems because Zn is an important metal that participates in a wide variety of enzymatic reactions [17]. It was reported that over 80% of Zn in brain is bound to proteins [18]. But the precise mechanism is currently under further investigation.

Although the scale of the investigation was small, the data obtained clearly indicated that ATP concentration was sensitive to Zn effects. Thus, the determination of ATP levels in cells by using the developed ion-pairing LC–MS assay might provide an important biochemical parameter in toxicological studies. Further study would include the simultaneous determination of ATP, adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate (AMP) and the investigation of their concentration ratio in relation to cell viability.

4. Conclusion

The developed capillary LC–high-resolution MS method using DMHA as the ion-pairing agent provides good sensitivity, selectivity, and a dynamic range suitable for measuring cellular ATP concen-

tration in a biological application. The ion-pairing HPLC coupled with ESI-MS with TOF detection proved to be an excellent analytical approach for determining cell concentrations of nucleotides. The use of capillary HPLC minimized the negative effect of the ion-pairing agent on ESI-MS due to the possible reduction of the DMHA concentration in mobile phases. Additional specificity obtained from the high-resolution and tandem mass spectrometric analysis is very suitable for the trace analysis of complex biological samples. The method has been successfully applied to determine ATP concentration levels in rat brain cells treated with different Zn concentrations and to support investigations of zinc's toxic effects on the cells.

Acknowledgements

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