HPLC–UV Method for Simultaneous Determination of Adenosine Triphosphate and Its Metabolites in *Mycobacterium smegmatis*

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Method for the analysis of intracellular adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) in *Mycobacterium smegmatis* that involves rapid extraction procedure based on sonication of cells in perchloric acid, as well as separation of nucleotides by ion-pair reversed-phase high-performance liquid chromatography and ultraviolet (UV) detection at 254 nm, is developed. The analytes were separated with mobile phase consisted of acetonitrile and 50 mM monobasic potassium phosphate (pH 4.6) with 25 mM tetrabutylammonium hydrogensulfate in a ratio of 0.5:99.5% within 30 min. The calibration curves were linear in the range of 20–1000 pmol of ATP and 10–1000 pmol of ADP and AMP with correlation coefficient (r^2) of ≥0.9998. The proposed method is applicable for mycobacterium cultures taken over a wide range of optical density and physiological states. Concentrations of ATP, ADP, and AMP in mycobacterial extracts varied from 2.61 ± 0.27 to 9.60 ± 0.19 nmol/mg dry weight, from 1.75 ± 0.12 to 5.86 ± 0.09 nmol/mg dry weight, and from 0.55 ± 0.08 to 4.40 ± 0.07 nmol/mg dry weight, respectively, depending on the physiological state.

Keywords: Adenine nucleotides, ATP, energy charge, HPLC, mycobacterium

1. Introduction

Adenine nucleotides are the ubiquitous components of biological systems that are not only used as the units for DNA and RNA synthesis but are also required for the regulation of other biosynthetic processes and energy metabolism. Adenosine triphosphate (ATP) is known to be a universal energy carrier. Furthermore, the relative ratio of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) designated as adenylate energy charge is an important indicator of cell energy status and metabolic potential [1]. ATP and the adenylate energy charge are widely used to estimate the total microbial biomass [2, 3], survival of bacteria exposed to various unfavorable conditions [4], and antibiotic susceptibility of microorganisms by minimal inhibitory concentration determination [5, 6]. Furthermore, analysis of nucleotide pools is useful for screening of new antimicrobial compounds targeting oxidative phosphorylation that are considered to be promising antituberculous drugs [7].

Analysis of adenine nucleotides in biological samples includes two steps: extraction and quantification. Efficient extraction procedure requires rapid cell death and lysis, complete and irreversible inactivation of enzymes, and guaranteed stability of the extracted substances. The majority of procedures developed for the nucleotide extraction can be divided into several categories depending on the nature of extractant: extraction with boiling buffers [5, 8], extraction with cold acids [9–11], or extraction with organic solvents [2, 8]. Various techniques are available to quantify adenine nucleotides including capillary electrophoresis [12], ion-exchange chromatography [13, 14], thin-layer chromatography [15, 16], different sensors [17, 18], high-performance liquid chromatography [9, 10, 11, 19, 20], and bioluminescence methods [2, 5, 8, 21, 22]. The last two methods are the most commonly used. The bioluminescence tests are usually applicable for ATP measurement, although some modifications have been developed to determine ADP and AMP [5, 22]. Nevertheless, it is becoming increasingly apparent that high-performance liquid chromatography is the most appropriate for simultaneous analysis of different nucleotides.

Non-pathogenic *Mycobacterium smegmatis* is often used as a model organism for pathogenic mycobacteria. Determination of adenine nucleotides in mycobacteria might encounter some difficulties, such as providing of entire and stable nucleotide extraction that is complicated by massive waxy mycobacterial cell envelope. Therefore, the procedure providing rapid cell wall destruction but stability of nucleotides is required. Furthermore, bacterial extracts, unlike the eukaryotic ones, contain fewer amounts of adenine nucleotides but greater variety of related compounds that complicate separation and analysis of ATP, ADP, and AMP. Thereby, most of the available high-performance liquid chromatography (HPLC) methods that were developed for the analysis of nucleotides extracted from eukaryotic organisms cannot be applied for bacteria. Few methods that are reported to be used for the analysis of bacterial extracts usually refer to ion-pair version of HPLC [2, 10, 11].

Here, we proposed the ion-pair reversed-phase high-performance liquid chromatography method in combination with a rapid extraction procedure that allows a simultaneous determination of ATP, ADP, and AMP in *M. smegmatis*.

2. Experimental

2.1. Chemicals and reagents. Adenosine 5′-triphosphate disodium salt hydrate (99%), adenosine 5′-diphosphate sodium salt (98%), adenosine 5′-monophosphate sodium salt (99%), perchloric acid (99%), and tetrabutylammonium hydrogensulfate (97%) (TBAS) were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Acetonitrile (Cryochrom, Russia), potassium dihydrogen phosphate anhydrous (HiMedia Laboratories Pvt. Limited, India), and potassium carbonate (Applichem, Germany) were of analytical grade.

2.2. Instrumentation. Chromatographic analyses were performed with an LC-20A system (Shimadzu, Japan) equipped with SPD-M20A UV/VIS photodiode array detector, CTO-20AC

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column oven, DGU-20A3 degasser, and CBM-20A communications bus module. Analytes were separated on a Luna C18 column (5 μm, 250 mm × 4.6 mm i.d.) (Phenomenex, CA, USA) provided with C18 Security Guard cartridge (5 μm, 4 mm × 3 mm i.d.) (Phenomenex, CA, USA). pH measurements were carried out with HI 2210 pH meter (Hanna Instruments, RI, USA). Shaking water bath GFL1092 (GFL, Germany), 5415R centrifuge (Eppendorf, Germany), UV-1650PC spectrophotometer (Shimadzu, Japan), microscope (LOMO, Russia), and CPX 130 ultrasonic processor (Cole-Parmer, IL, USA) were used for the bacterial cultivation and biological sample preparation.

2.3. Chromatographic conditions. The mobile phase consisted of (A) acetonitrile and (B) 50 mM monobasic potassium phosphate (pH 4.6) with 25 mM TBAS in a ratio of 0.5 (A):99.5 (B). The chromatographic separation was carried out at 25 °C and flow rate of 1 mL/min. The injection volume was 20 μL. The identification of peaks was based on the comparison of retention times and diode-array spectra of biological samples and chemical standards. ATP, ADP, and AMP were detected at 254 nm.

2.4. Standard solution preparation. Stock solutions of 10 mM ATP, ADP, and AMP were prepared by dissolving the appropriate amounts of these compounds in 10 mL of deionized water. Aliquots of the standard solutions were kept at −20 °C. The working solutions were prepared daily by appropriate dilutions with deionized water.

2.5. Bacterial strains and growth conditions. Experiments were performed with *M. smegmatis* strain mc^2155 (ATCC Number: 700084).

A loop of *M. smegmatis* culture maintained on Middlebrook 7H10 agar (Difco, France) plate was inoculated into 5 mL Middlebrook 7H9 broth (Difco, France) supplemented with Tween 80 (0.05%) and ampicillin (50 μg/mL) and cultivated at 37 °C and 200 rpm for about 36 h. The resulting culture of approximate concentration of 10^8 CFU/mL was diluted (1:200) in 50 mL of fresh Middlebrook 7H9 broth supplemented with Tween 80 (0.05%) and cultivated at 37 °C and 200 rpm. Aliquots of the culture for the analysis were sampled at time specified in the text below.

The culture growth was monitored by the absorbance at 600 nm.

Cell viability was tested by cell plating on Luria-Bertani agar (Sigma, USA) and counting of colony forming units (CFU) after 3 days of incubation at 37 °C.

Cell biomass was estimated by optical density (at 600 nm), preliminary calibrated in values of absolutely dry weight. The calibration curve was plotted by measuring the mass of equal volumes of cell suspensions of different optical densities deposited on filters and heated for 18 h at 105 °C.

2.6. Sample preparation. Cells were placed into 550 μL of 0.4 N $HClO_4$ and sonicated for 30 s (230 V, 20 kHz, amplitude 50%). The resulting suspension was centrifuged (1 min, 16,000 *g*, 0 °C), and the supernatant was neutralized with 2 M K_2CO_3 (pH 7.0). All manipulations were performed on ice. Neutralized samples were stored at −20 °C and then thawed and centrifuged (1 min, 16,000 *g*, 25 °C) before HPLC analysis.

2.7. Microscopy and smear preparation. Bacterial smears stained with gentian violet (15 min) or carbolic fuchsin (Ziehl-Neelsen method) were viewed using oil immersion microscopy (1000× multiplication; not less than 20 fields were examined).

3. Results and discussion

3.1. Extraction of nucleotides. A thick cell wall of *M. smegmatis* makes difficulties for the intracellular component extraction. Treatment of such kind of bacteria with acid is less effective as compared to gram-negative bacteria, and the boiling extraction procedure usually takes more than 5 min [5, 23]. Here, we proposed a rapid method of extraction based on sonication of cells in perchloric acid. The completeness of cell destruction was estimated microscopically and by the viability test. The treatment of bacterial cells during 30 s resulted in a complete destruction of cell walls (Figure 1) and allowed an entire release of cell components into media. In agreement with, no colony forming units were detected in the cell suspension after sonication. The similar results were obtained after sonication of 24 hour old culture (5×10^6–10^7 CFU/mL) (depicted in Figure 1), 48 hour old culture (5×10^8–10^9 CFU/mL), and 72 hour old culture (5×10^8–10^9 CFU/mL) (data not shown). Less time of sonication in perchloric acid or an application of the influencing factors separately did not lead to the desired effect. It should be noted that the usage of perchloric acid was of importance since it causes inactivation and precipitation of proteins.

The influence of the extraction procedure on adenine nucleotide stability was tested in 100 μM standard solution. Variation of the nucleotide amount after the treatment was within the uncertainty of the method, specifically 98.73 ± 0.79% for ATP, 100.85 ± 0.94% for ADP, and 100.83 ± 0.79% for AMP against 100% of the untreated nucleotide solution (data presented as mean ± SD of 4 replicates).

3.2. Chromatographic separation. The proposed method allowed the separation of ATP, ADP, and AMP simultaneously within 30 min (Figure 2). The adequate selectivity of adenine nucleotides was achieved by using of tetrabutylammonium hydrogensulfate as ion-pair reagent and 0.5% acetonitrile. The application of the elution solvent containing more than 0.5% acetonitrile reduced the analysis time but impaired the selectivity in early of the analysis making the determination of AMP in biological samples impossible. An increase in acetonitrile concentration up to 1–5% of the elution solvent from 10 min after the start time resulted in a decrease of the ATP retention time and an improving of its resolution. However, such a modification of the chromatographic program required additional time for equilibration of the column cancelling the time saving and reduced the selectivity of AMP and ADP in the next run.

An increase in retention time of the target peaks, especially AMP, occurred in biological samples as compared to standard solution. This shift was observed in all extracts and seemed to be due to the presence of other compounds. The correctness of peak identification in biological samples was confirmed by the standard addition method and ultraviolet (UV) spectrum data.

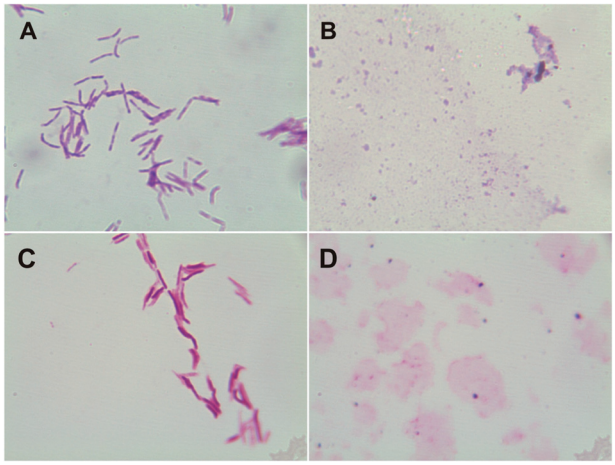

Figure 1. Effect of sonication in 0.4 N $HClO_4$ on *Mycobacterium smegmatis* cells. A, C — control cultures without treatment; B, D — cells sonicated for 30 s. A, B — smears stained with gentian violet; C, D — smears stained with carbolic fuchsin

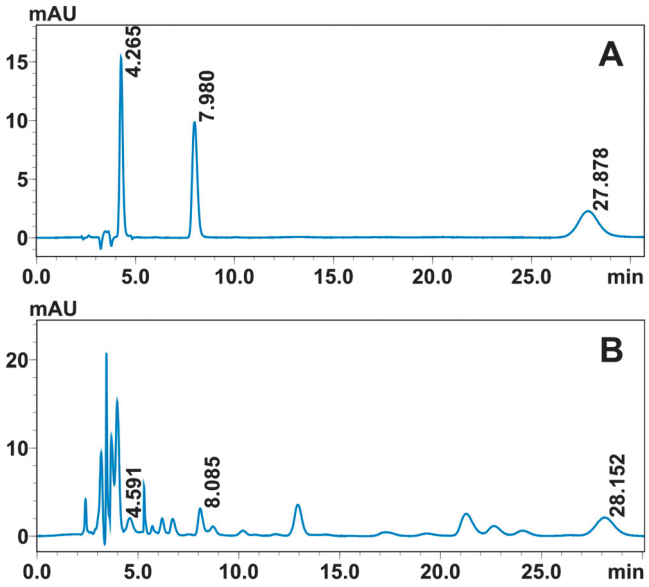

Figure 2. Separation of AMP (1), ADP (2), and ATP (3) in standard solution (A) and *Mycobacterium smegmatis* cell extract (B)

3.3. Linearity and limits of quantification of the method. The linearity of the method was assayed using nine-point calibration curves representing the relation between the peak area values and concentration of the compounds. Calibration data are shown in Table 1. The limit of detection was defined as the amount of a substance that produced a detector signal that could be clearly distinguished from the baseline (6 fold higher than the baseline noise). Equations for calculating the amount of nucleotides in injection volume are presented in Table 1.

3.4. Determination of adenine nucleotide intracellular concentration in *M. smegmatis*. Adenine nucleotide pools were analyzed in *M. smegmatis* cells of different growth phases. Extraction and HPLC analysis were performed as described in Experimental section. External standard method and, in some cases, standard addition method were used for the quantification of adenine nucleotide concentration in biological samples. The amount of nucleotides in injection volume of mycobacterial extracts ranged, at an average, from 18 to 200 pmol for ATP, 10–90 pmol for ADP, and 20–100 pmol for AMP depending on the culture density (number of cells) and physiological state.

To calculate the intracellular nucleotide concentration, the obtained values of nucleotide concentration in extracts (nmol/L) were divided by the values of dry weight of the biomass which nucleotides were extracted from (mg/L). The data presented in Table 2 demonstrate an applicability of the proposed method for mycobacterium cultures of different optical density and physiological states.

Energy charge (EC) which is considered as a measure of energy stored in the adenine nucleotides and, in turn, as a parameter of the metabolic potential of cells can be calculated according to the formula: EC = ATP + 0.5ADP/(AMP + ADP + ATP) [1]. The data in Table 2 indicate that energy charge of actively growing mycobacteria was 0.7–0.8 and declined when cells enter stationary phase. The energy charge values of *M. smegmatis* we obtained were in agreement with those reported by Lee and Colston, who used the boiling in buffer for the extraction of nucleotides and the fluorescence method for their analysis [5].

3.5. Precision and accuracy. Precision is expressed in terms of relative standard deviation (RSD) calculated based on the values of at least 30 replications within 3 months (Table 3). Coefficient of variation for retention time involves both values in standard solutions and biological samples and demonstrates a sufficient repeatability of the method. The variation of the peak area values in standard nucleotide solution was no higher than 1.8%, but the intracellular concentration determination was less precise (4–7.5% RSD). This discrepancy could be due to the additional random errors originated from sample collection and processing.

Table 1. Detection limits and linear ranges for quantification of adenine nucleotides

Nucleotide	Retention time (min)	Detection limit (pmol)	Linear range (pmol)	Regression equation	R^2
ATP	28.37	10	20–1000	$y = 11{,}897x$	0.9988
ADP	8.09	2	10–1000	$y = 12{,}052x$	0.9989
AMP	4.49	2	10–1000	$y = 12{,}029x$	0.9988

Table 3. Inter-day mean precision

Nucleotide	Relative standard deviation (%)		
	Retention time	Peak area (standard solution)	Intracellular concentration (biological sample)
ATP	3.28	1.77	7.23
ADP	2.11	1.45	4.21
AMP	3.39	1.64	7.36

Based on at least 30 replications during 3 months.

4. Conclusion

The proposed method could be applied for measurements of ATP, ADP, and AMP in cells of *M. smegmatis* and other non-pathogenic gram-positive bacteria. The extraction procedure allows the release of the nucleotides from bacteria with thick cell wall and is efficient for the high-density cell suspensions even (up to 10^9 CFU/mL, at least). The sensitivity of the HPLC method enables analysis of the intracellular ATP, ADP, and AMP content in *M. smegmatis* cultures of different growth phases and densities as from about 0.5 g/L dry weight of log phase cells.

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Table 2. Intracellular concentration of adenine nucleotides in *M. smegmatis*

Time of cultivation	A_{600}	Concentration, nmol × mg dry weight^{-1}			EC
		ATP	ADP	AMP	
18 h (log phase)	1.626	4.69 ± 0.26	1.75 ± 0.12	0.55 ± 0.08	0.80 ± 0.03
24 h (late log phase)	3.246	2.61 ± 0.27	3.57 ± 0.01	2.80 ± 0.13	0.49 ± 0.03
72 h (stationary phase)	2.820	9.60 ± 0.19	5.86 ± 0.09	4.40 ± 0.07	0.63 ± 0.01

Data presented as mean ± SE of one biological replicate but 3 replicates of adenine nucleotides' determination method including the extraction procedure.

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