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Fast, efficient capillary electrophoresis method for measuring nucleotide degradation and metabolism

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Abstract

An easy and fast method for the quantitative analysis of nucleotides by capillary zone electrophoresis was developed. The method employing a neutral-bonded capillary and reversed polarity mode provided a good resolution and a short analysis time of less than 5 min. The samples were injected electrokinetically using −6 kV voltage for 30 s and detected by their UV absorbance at 254 nm. Constant current (−45 μA) was applied, and a phosphate buffer, pH 7.4, was used. The detection limits for ATP, UDP, and UTP ranged between 0.14 and 0.28 μ*M*. This method was required for the investigation of the purity of the commercially available nucleotides used in pharmacological studies. In addition, the analytical method was applied to study the metabolism of nucleotides in a cell line, neuroblastoma×glioma hybrid cells (NG108-15), which is used in pharmacological studies with nucleotides, since it contains purine- and pyrimidine-sensitive nucleotide receptors. Furthermore, we used the new method for monitoring enzymatic studies using the enzyme hexokinase to convert nucleotide triphosphates to diphosphates.

Keywords: Electrokinetic injection; Nucleotide metabolism; Nucleotides; Hexokinase; Enzymes; Nucleotide phosphates; Phosphates

1. Introduction

Adenosine and uridine nucleotides, such as ATP, ADP, UTP and UDP, are physiological signaling molecules which bind to membrane receptors termed P2 (purine and pyrimidine nucleotide) receptors and produce a wide range of physiological responses [1]. Two distinct families of P2 receptors have been identified, the metabotropic P2Y and the ionotropic P2X receptors. At least seven different P2X ($P2X_{1-7}$) and seven different P2Y receptors subtypes ($P2Y_{1,2,4,6,11-13}$) are known to exist in mammals, but further subtypes have been postulated on the basis of pharmacological experiments [2]. While the physiological agonist at P2X receptors appears to be ATP, the various P2Y receptors show different preferences for adenosine and uridine di- and tri-phosphates. While $P2Y_{11}$ is activated by ATP, $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ are ADP receptors. $P2Y_2$ is activated by ATP and UTP, $P2Y_4$ by UTP, and $P2Y_6$ by UDP. P2 receptors activated by diadenosine polyphosphates, e.g., diadenosine tetraphosphate (Ap_4A) have also been described [1,2]. Nucleosides (e.g., adenosine, uridine) and nucleoside monophosphates, such as

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AMP and UMP, are virtually inactive at P2 receptors [3].

Major problems in pharmacological studies at P2 receptors using nucleotides are (i) a frequently low degree of purity of commercial nucleotides, e.g., contamination of triphosphates by di- and monophosphates (and vice versa), (ii) chemical instability of nucleotides, and (iii) fast enzymatic degradation or conversion of nucleotides by ecto-enzymes attached to the cell membranes [4].

We have been interested in a particular subtype of P2Y receptors, the $P2Y_2$ receptor, which is activated by ATP and UTP in similar concentrations (EC_{50} ca. 1 μ*M*), but insensitive to the nucleoside diphosphates ADP and UDP [5]. In our investigations we have been using a mouse neuroblastoma×rat glioma hybrid cell line (NG108-15), which is known to express $P2Y_2$ receptors coupled to phospholipase C resulting in an increase in the intracellular calcium concentration upon activation. We had previously determined a similarly high potency of UTP and UDP in the mobilization of intracellular calcium in NG108-15 cells [6]. However, UDP has been shown to be inactive at $P2Y_2$ receptors [5]. The measured activity of UDP might be due to (i) contamination by UTP, (ii) enzymatic conversion to UTP, or (iii) the existence of UDP receptors in addition to the ATP and UTP receptors on NG108-15 cells. This problem illustrates that it is extremely important for pharmacological experiments to determine the nucleotide concentrations in the assays.

In the present study we have developed an analytical method using capillary electrophoresis (CE) for the quantitative determination of the adenine and uracil nucleotides ATP, ADP, AMP, UTP, UDP and UMP. It was required as a fast method for purity control of the commercially available nucleotides. Furthermore it was applied to follow up the enzymatic conversion of nucleoside triphosphates to diphosphates by hexokinase, and to study the metabolism of nucleotides added to NG108-15 cells. The biological studies were performed in order to assess the stability of biologically active nucleotides under experimental conditions.

Shao et al. used a neutral capillary and electrokinetic injection mode to determine nucleotide pool levels in human Burkitt lymphoma cells [7]. The analysis time was 35 min. Pretreatment of samples included the precipitation of proteins and centrifugation of the extracts. Uhrova et al. performed the separation of 11 nucleotides on a fused-silica capillary and used 20 m*M* phosphate–borate buffer (pH 8.0–9.0). The detection limits (taken as three-times noise level) achieved for the nucleotides ranged between 0.62 and 3.8 μg/ml. The analysis time was about 14–16 min [8]. Huang et al. developed a method for the separation and quantitation of ribonucleotides using a neutral capillary and a mixture of Tris–HCl and phosphate buffer. Fourteen different ribonucleotides were separated within 50 min. The minimum detectable concentration was 5.4 μ*M* [9]. Blaschke et al. analyzed free intracellular nucleotides using borate buffer (140 m*M*, pH 9.4), and a fused silica capillary [10]. They performed the separation of nine nucleotides within 25 min.

In the present study we developed a simple and robust method for the separation of nucleoside mono-, di- and triphosphates, which provides short analysis times and does not require sample pretreatment to allow its application in bio-process monitoring. The method has been applied to investigate nucleotide degradation and metabolism by isolated enzymes and in the presence of cells.

2. Experimental

2.1. Apparatus

The experiments were performed on a P/ACE CE system MDQ glycoprotein (Beckman Coulter Instruments, Fullerton, CA, USA) equipped with a UV detection system. The electrophoretic separations were carried out using an eCAP coated neutral capillary [50 cm (40 cm effective length)×50 μm internal diameter (I.D.)×375 μm outside diameter (O.D.) obtained from Beckman Coulter]. An electrokinetic injection for 30 s was applied for introducing the sample. The separation was performed using an applied current of −45 μA and a data acquisition rate of 8 Hz. Analytes were detected using direct UV absorbance at 254 nm. The capillary was conditioned by rinsing with water for 1 min and subsequently with buffer (phosphate 20 m*M*, pH 7.4) for 1 min. Sample injections were made at the cathodic side of the capillary. The CE instrument was fully controlled

through a personal computer, which operated with the analysis software 32 KARAT obtained from Beckman Coulter. The evaluation of the electropherograms was done using the same software. The capillary temperature was kept constant at 25 °C. The temperature of the sample storing partition was adjusted to 25 °C.

2.2. Chemicals

Nucleotides (adenosine-5′-monophosphate (A17-52), adenosine-5′-diphosphate (A2754), adenosine-5′-triphosphate (A7699), uridine-5′-monophosphate (U6375), uridine-5′-diphosphate (U4125), uridine-5′-triphosphate (U6625)) were obtained from Sigma (Taufkirchen, Germany), hexokinase was from Roche (Mannheim, Germany). Stock solutions of nucleotides were prepared in Hepes (*N*-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) buffer with nucleotide concentrations ranging from 1 to 10 m*M*. The dipotassium hydrogenphosphate for the CE buffer was purchased from Fluka (Neu-Ulm, Germany). Sodium chloride, potassium chloride, potassium dihydrogenphosphate, sodium hydrogencarbonate, anhydrous D-glucose, Hepes (free acid), calcium chloride and magnesium sulfate (constituents of Krebs–Hepes buffer), Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin and fetal bovine serum for cell culture were obtained from Sigma. HAT (hypoxanthine, aminopterin, thymidine) supplement was from Gibco Life Technologies (Karlsruhe, Germany).

2.3. Cell culture and preparation of cells

Cells were cultured in DMEM containing 10% fetal bovine serum, HAT supplement and penicillin–streptomycin until cells were 80% confluent. After rinsing off the cells from the culture flasks they were washed three times with Krebs–Hepes buffer at 37 °C. Then the pellet was suspended in buffer at a cell concentration of 10^5 cells per ml (final concentration in the assay). The Krebs–Hepes buffer consisted of sodium chloride (118.6 m*M*), potassium chloride (4.7 m*M*), potassium dihydrogenphosphate (1.2 m*M*), sodium hydrogencarbonate (4.2 m*M*), anhydrous D-glucose (22 m*M*), Hepes (free acid, 10 m*M*), calcium chloride (1.3 m*M*) and magnesium sulfate (2.7 m*M*).

2.4. Sample preparation procedure

Nucleotides were first dissolved in Krebs–Hepes buffer to obtain 10.0 m*M* stock solutions. These were further diluted with the necessary amount of the same buffer to obtain a concentration of 100.0 μ*M* of nucleotide at the beginning of the assays. For experiments with hexokinase, enzyme solution was added to this solution (10 units/ml for the investigations with UDP or UTP, respectively, and 0.04 units/ml for ATP). For experiments with cells, the nucleotide solution was added to an NG108-15 cell suspension (10^5 cells/ml) to obtain a final concentration of 100.0 μ*M* of nucleotide. After starting the reaction, samples of 150.0 μl were withdrawn from the reaction flasks at appropriate time intervals, and diluted 1:100 with a solution of the internal standard in buffer to obtain a concentration of 1.0 μ*M* of internal standard (the appropriate nucleotide monophosphate). The samples were immediately heated to 99 °C for 3 min using an Eppendorf Thermomixer Comfort in order to inactivate enzymes. The experiments were repeated three times.

3. Results and discussion

3.1. Capillary electrophoresis method and conditions

3.1.1. Capillary preconditioning and rinsing

The capillary was conditioned every day by rinsing the capillary with water for 20 min before starting measurements. Between each run the capillary was rinsed with water for 1 min followed by rinsing with buffer for 1 min. The capillary was stored in vials containing water inside the CE instrument overnight after rinsing with water. Over the weekends the capillary was stored in water in a refrigerator (2–8 °C) after rinsing with water for 20 min. For rinsing procedures we applied 40 p.s.i. of pressure (1 p.s.i.=6894.76 Pa). Using the described rinsing procedures, the capillary could be used for several hundred separations.

3.1.2. Electrokinetic injection

The electrokinetic injection resulted in an increased sensitivity compared to the hydrodynamic injection. The limits of detection and quantitation are given in Table 1. Prior to electrokinetic injection samples had to be diluted. This step was necessary to decrease the ionic strength of the samples in order to increase the efficiency of the electrokinetic injection. The undiluted samples had a high ionic strength because they not only contained cells and enzymes but also a highly concentrated buffer; therefore, the samples were diluted with water 1:100. We found that the injection time and the applied voltage had a big impact on the amount of the injected analytes: long injection times and high voltages improved the sensitivity. Injection times longer than 30 s, however, led to peak broadening, and higher voltages than −6 kV led to overheating of the sample vial. The best results were obtained by injecting the sample with −6 kV for 30 s as illustrated in Fig. 1.

3.2. Validation of the method

3.2.1. Limits of detection and quantitation

For the determination of the limits of detection and quantitation different samples of ATP, UDP and UTP were measured. The concentrations of the nucleotides in this study were 0, 0.1, 0.25, 0.5, 0.75, 1.0 and 1.25 μ*M*. The concentration of the internal standard (AMP for ATP, UMP for UDP and UTP) added to the samples was kept constant at 1 μ*M*. Each sample was measured three to six times and the means were calculated as shown in Table 1.

3.2.2. Linearity and recovery

To determine the linearity and recovery rates, nine samples of different concentrations ranging up to 2.0 μ*M* were measured three to six times. The means of the peak areas for different concentrations were entered into a diagram as a function of concentration. Linearity was confirmed; as an example the equation for the measurement of ATP was determined as $y = 852.93x + 33.487$ ($R^2 = 0.999$). The found concentrations were calculated from the obtained linear regression curves and compared with the concentrations of the original samples to obtain the recovery equations; the recovery rates were generally high (see Table 1). For example, the equation for the recovery of ATP was $y = 0.9913x + 0.0114$ ($R^2 = 0.999$).

3.2.3. Precision

3.2.3.1. Precision of migration time

Each compound was measured at least 27 times. SD and RSD values were determined. There is only little difference between the migration times of each substance as shown in Table 1.

The variation coefficients are ranging between 0.19 and 0.40%. The reason for this good precision and reproducibility in migration times is first of all the use of a neutral-bonded capillary where the electroosmotic flow (EOF) is absent. Also the constant-current mode, which we used in our experiments proved to be the optimal solution as far as the precision in migration times is concerned [11,12].

Table 1
Limits of detection and quantitation, precision of migration time and peak area and recovery rate

Compound	ATP	UDP	UTP
Limit of detection (μ*M*)±SD	0.16±0.03	0.14±0.02	0.28±0.05
Limit of quantitation (μ*M*)±SD	0.23±0.03	0.21±0.02	0.42±0.05
Mean value of migration time (min) ($n = 30$)	3.2698	3.4458	3.0886
SD of migration time (min)	0.0129	0.0065	0.0082
RSD[a] (%) of migration time (min)	0.40	0.19	0.27
Recovery rate (RR) (%)±SD	99.13±0.8	97.22±2.1	98.85±1.8
R^2 of recovery rate	0.999	0.996	0.997
Mean value of peak area ($c = 1$ μ*M*)	866.23	486.92	492.62
SD of peak area	30.50	20.94	22.39
RSD[a] (% of peak area)	3.52	4.30	4.55

[a] RSD, relative standard deviation.

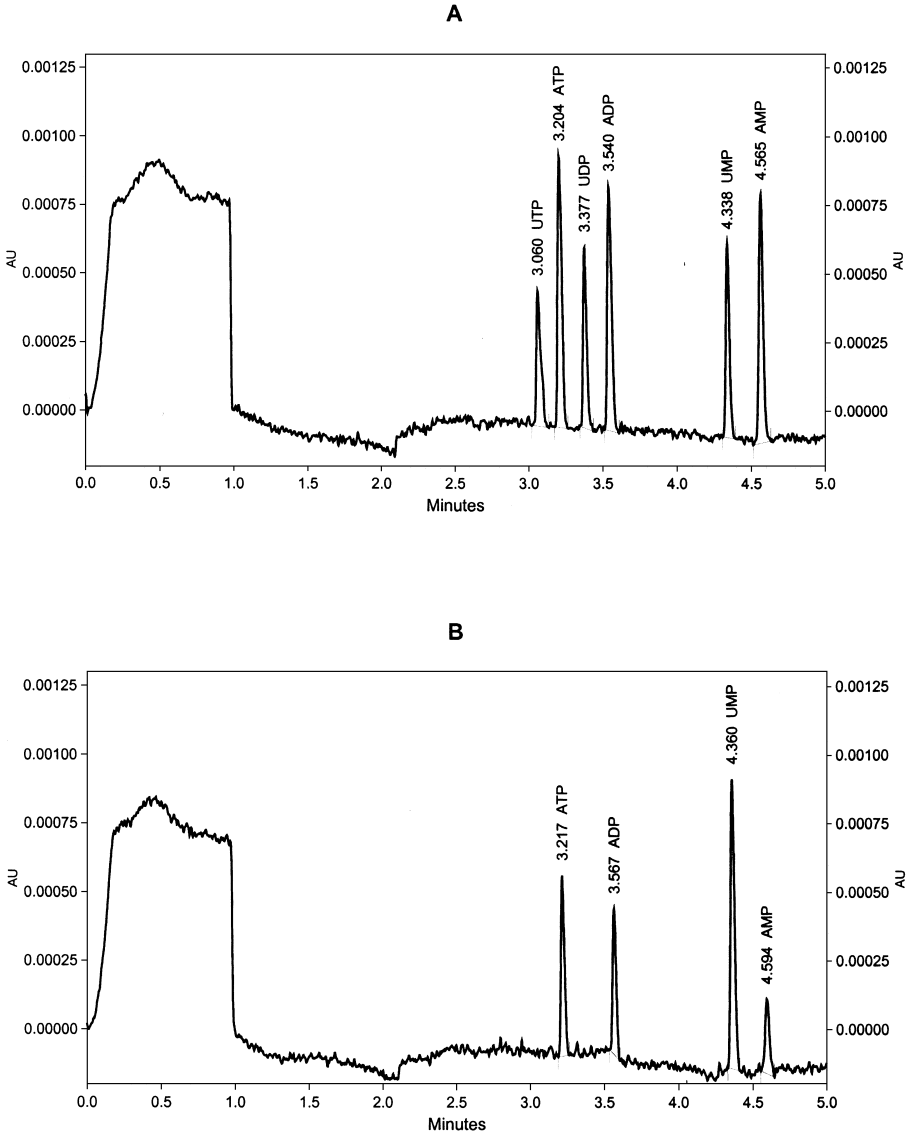

Fig. 1. (A) Electropherogram of separation of nucleotides (concentration of nucleotides: 1.0 μ*M* each); neutral capillary (50 cm×50 μm I.D.), phosphate buffer 20 m*M*, pH 7.4, separation at constant current of −45 μA, electrokinetic injection with −6 kV for 30 s, detection at 254 nm. (B) Electropherogram of biological sample: ATP was added to NG108-15 cells (for experimental details see Section 2.4). Sample was withdrawn 60 min after starting the reaction. UMP (1.0 μ*M*) was added as internal standard. Quantitative determination: the quantity of each nucleotide was calculated in relation to the internal standard (1.0 μ*M* UMP), taking into account the different absorption coefficients. Results (after 60 min): ATP, 32.4%; ADP, 35.48%; AMP, 20.99% (Σ=88.87%). The missing 11.13% is presumably adenosine formed by an ectophosphatase [14].

3.2.3.2. Precision of peak area

The precision of the peak area was evaluated by injecting a sample of ATP in a concentration of 0.75 μ*M* for six times. There were variations in the peak areas from run to run as shown in Table 1 but the ratio between analyte and the internal standard was always almost the same. Furthermore we tried to minimize the influence of electrokinetic injection on

the injected amount. Two major parameters that influence the amount of analyte migrating into the capillary during electrokinetic injection are the positions of the electrode and the capillary-end and the volume in the sample vial [13]. We tried to make sure that both of the capillary ends had the same length and the sample vials contained the same volume to keep the sample volume constant. In addition, we carefully ground off the capillary ends of each capillary with a Beckman capillary cutter in order to avoid any fluctuations during the injection.

3.2.4. Ruggedness of the method

In order to determine the ruggedness, the stability of the test solution and the robustness of the CE method were investigated. The test solution contained ATP (1.0 μM) and AMP as internal standard (1.0 μM) dissolved in Krebs–Hepes buffer. The solution was kept at room temperature for 3 days. The area under the curve (AUC) (889.32, SD = 27.96, RSD = 3.14%) did not change within 3 days ($y = 0.266x + 872.27$), indicating that the compound was stable under these conditions.

3.3. Determination of purity of nucleotides

Commercially available nucleotides were investigated for their purity (Table 2). The investigated nucleoside monophosphates AMP and UMP exhibited a high degree of purity (Table 2). The nucleoside tri- and particularly the diphosphates, however, were contaminated with other nucleotides. This result confirms previous reports that such impurities may complicate the interpretation of pharmacological investigations at P2 receptors since some receptors are selective for nucleoside diphosphates, and others for triphosphates [5].

3.4. Biological applications

3.4.1. Influence of hexokinase on nucleotide metabolism

Hexokinase phosphorylates glucose to glucose-6-phosphate using ATP, or UTP (in higher concentrations) as a cosubstrate. ATP and UTP are converted to ADP and UDP, respectively. The enzyme hexokinase has been used to convert UTP to UDP in experiments with UDP in which no UTP should be present [5]. UTP may be formed during the incubation of cells with UDP or may be present as an impurity in UDP. ATP and UTP were incubated with hexokinase in the presence of glucose and magnesium ions. These experiments were performed in order to investigate the efficiency and the time course of the enzymatic transformation. Under the applied conditions (100.0 μM ATP, 0.04 units/ml hexokinase, or 100.0 μM UTP, 10 units/ml hexokinase; at 37 °C, pH 7.4) the degradation was fast and complete (see Fig. 2). Thus, preincubation with hexokinase is an efficient method to convert even high concentrations of nucleoside triphoshates to the corresponding diphosphates. No further degradation of ADP and UDP could be observed. The enzymatic

Table 2
Purity of commercial nucleotides used in the study

Nucleotide	Lot number	Declared purity (%)	Determined purity (%)[a]	Determined impurity I (%)[a]	Determined impurity II (%)[a]
AMP	32H7806	99	100	–	–
ADP	028H7013	98	96.26	ATP (2.46)	AMP (1.29)
ADP	39H7006	98	93.59	ATP (1.53)	AMP (4.88)
ATP	069H7026	99	98.83	ADP (0.87)	AMP (0.30)
ATP	60K7063	99	99.19	ADP (0.82)	AMP (0.00)
UMP	38H5198	98	100	–	–
UDP	128H7031	96	92.85	UTP (3.41)	UMP (3.74)
UDP	79H7038	96	93.72	UTP (1.48)	UMP (4.80)
UTP	108H7011	98	97.85	UDP (1.98)	UMP (0.17)
UTP	40K7061	98	96.05	UDP (3.95)	UMP (0.00)

[a] Standard deviations were $<0.12\%$ in all cases determined.

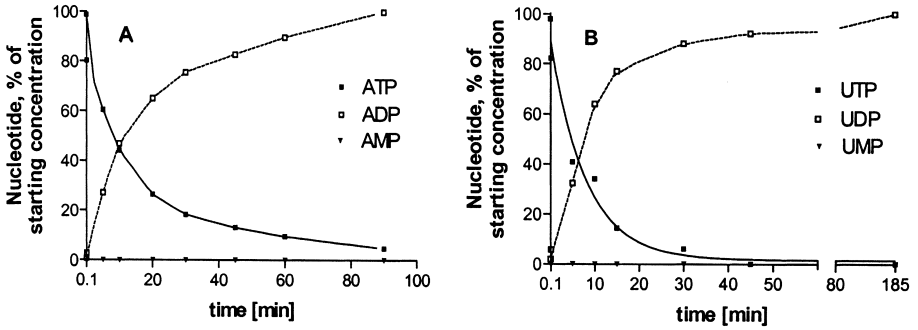

Fig. 2. (A) Time-dependent conversion of ATP to ADP after the addition of hexokinase (0.04 units/ml) to a 100.0 μ*M* solution of ATP; half-life for ATP: 8 min. (B) Time-dependent conversion of UTP to UDP after the addition of hexokinase (10 units/ml) to a 100.0 μ*M* solution of UTP; half-life for UTP: 4 min.

reaction can easily be monitored by the developed CE method.

3.4.2. Nucleotide metabolism by NG108-15 cells

The degradation of the nucleotides ATP, ADP, UTP, and UDP after addition to the mammalian hybrid cell line NG108-15 (mouse neuroblastoma× rat glioma) was investigated using the developed CE method. An electropherogram of ATP degradation by the cells is shown in Fig. 1B. The method was well applicable to measurements in biological samples such as live cell cultures. The complete results of this study will be reported elsewhere.

In conclusion, we developed an easy method for the quantitative analysis of nucleotides in a spiked, diluted cellular supernatant. The measuring time was less than 5 min.

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