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High-sensitivity capillary electrophoresis method for monitoring purine nucleoside phosphorylase and adenosine deaminase reactions by a reversed electrode polarity switching mode

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ABSTRACT

A simple, efficient, and highly sensitive in-line CE method was developed for the characterization and for inhibition studies of the nucleoside-metabolizing enzymes purine nucleoside phosphorylase (PNP) and adenosine deaminase (ADA) present in membrane preparations of human 1539 melanoma cells. After filling the running buffer (50 mM borate buffer, 100 mM SDS, pH 9.10) into a fused-silica capillary (50 cm effective length × 75 μm), a large sample volume was loaded by hydrodynamic injection (5 psi, 36 s), followed by the removal of the large plug of sample matrix from the capillary using polarity switching (−20 kV). The current was monitored and the polarity was reversed when 95% of the current had been recovered. The separation of the neutral analytes (nucleosides and nucleobases) was performed by applying a voltage of 15 kV. An about 10-fold improvement of sensitivity for the five investigated analytes (adenosine, inosine, adenine, hypoxanthine, xanthine) was achieved by large-volume stacking with polarity switching when compared with CE without stacking. For inosine and adenine detection limits as low as 60 nM were achieved. To the best of our knowledge, this represents the highest sensitivity for nucleoside and nucleobase analysis using CE with UV detection reported so far. The Michaelis–Menten constants (K_m) for PNP and ADA and the inhibition constants (K_i) for standard inhibitors determined with the new method were consistent with literature data.

1. Introduction

Purine nucleoside phosphorylase (PNP, EC 2.4.2.1) catalyzes the cleavage of the glycosidic bond of ribo- and deoxyribonucleosides in the presence of inorganic orthophosphate (P_i) as a second substrate, to generate the corresponding purine base and (deoxy)ribose-1-phosphate [1], (see Fig. 1).

PNP is mainly localized in the cytoplasm of both prokaryotic and eukaryotic organisms as a trimeric complex of ~90–100 kDa [2]. It has been suggested that PNP can also be expressed on the cell surface of lymphocytes [2] and on the surface of astrocytes as an *ecto*-enzyme (*ecto*-PNP) [3]. The enzymatic function of human PNP has an important medical implications. Its deficiency in humans leads to an impairment of T-cell function, usually with no apparent effects on B-cell function [4,5]. Therefore, T-cell leukemias and lymphomas can be impaired by designing efficient inhibitors that target PNP. Adenosine deaminase (ADA; EC 3.5.4.4) catalyzes the deamination of adenosine (Ado) and 2′-deoxyadenosine (dAdo) to inosine (Ino) and 2′-deoxyinosine, respectively [6]. ADA is widely expressed in the intestine, thymus, spleen and other lymphoid and non-lymphoid tissues [7]. Along with a cytosolic localization, ADA can be expressed as an *ecto*-enzyme on the surfaces of lymphocytes [8] and porcine brain synaptic membranes [9]. Two isoenzymes of ADA have been reported to exist, ADA_1 and ADA_2. However, the significance of the existence of two isoenzymes is not clear yet [10]. In order to evaluate the potential of selective PNP inhibitors as novel therapeutics, e.g. as antiviral and antitumour agents, selective inhibitors have to be developed. To investigate the inhibitory potential of a library of compounds, to identify lead structures, a fast and easy screening method is required.

CE is increasingly used to monitor enzymatic reactions [11–13]. An excellent review article has been published by Glatz [14] describing in detail the applications of CE for the determination of enzymatic activities. Previously, a normal MEKC–CE based method was developed for the measurement of adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP) and S-adenosyl-L-

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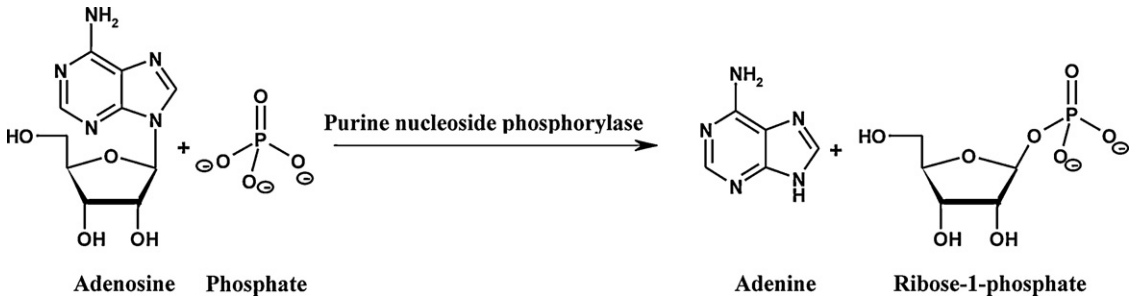


Fig. 1. Reaction catalyzed by purine nucleoside phosphorylase.

homocysteine-hydrolase (SAHH) activities for the diagnosis and monitoring of severe combined immunodeficiency disease (SCID) [15]. However, its sensitivity is a limiting factor, and some enzymatic reactions cannot be monitored by CE methods due to slow reaction and low amounts of product formation. In our previous work [16] we succeeded in developing a sensitive CE method for the determination of the enzymatic activity of *ecto*-nucleotide pyrophosphatases/phosphodiesterases (*ecto*-NPPs), an enzyme family that is not easy to monitor even by HPLC–UV [17]. However, the method developed for *ecto*-NPPs was only suitable for the determination of charged products, such as the nucleotide AMP. Therefore, there is a strong need for the development of a CE method applicable for the determination of neutral analytes such as nucleosides and nucleobases.

Several methods have been developed for the quantitative determination of nucleosides and nucleobases. Much research interest has been paid to the development and application of high performance liquid chromatography (HPLC) methods mostly coupled to UV detection [18]. MS-coupled techniques, including CE–MS [19,20] and LC–MS/MS [21] have also been described. However, these methods are time-consuming, expensive and LC-methods require the use of organic solvents. Compared to HPLC, CE has several advantages: high separation efficiency, minimum or no requirement of sample pretreatment, short analysis time, low operational costs, and low sample consumption [22–24]. Whereas CE is one of the most powerful separation tools but it suffers from the relatively poor sensitivity due to the small diameter of the capillary. To achieve better sensitivity in CE, several physical approaches have been reported, such as the use of a bubble cell [25] or derivatization with a fluorophore allowing CE with laser-induced fluorescence (LIF) detection [26]. However, such techniques require additional equipment and are not easily applicable. Therefore, a simple solution to improve the sensitivity of capillary electrophoresis is to integrate an in-line preconcentration process for sample analytes. In recent years, a number of novel sample stacking approaches have been developed that dealt with the preconcentration of analytes in-line within the capillary. Large-volume sample stacking with and without polarity switching (LVSSPS) is one of the in-line stacking techniques used in CE for improving the limit of detection [27–29]. A large amount of sample is injected into the capillary hydrodynamically. However, a higher volume leads to a reduction or loss of separation efficiency. Therefore, in order to maintain high resolution, a negative voltage is applied at the injection edge to electroosmotically push the sample matrix plug out of the column [30]. The sample species stack at the boundary between the sample zone and the BGE during the application of a negative voltage. The stacking and removal of solvent occurs concurrently. When the sample buffer is almost completely out of the column, which is monitored by the current, the polarity is switched to the normal configuration, and then separation of the analytes can proceed. Capillary electrophoresis with a reversed electrode polarity switching mode-micellar electrokinetic chromatography (REPSM-MEKC) had been used for improving the sensitivity of the analysis of 5-fluorouracil and its prodrug tegafur [31]. The LVSS method had been combined with dynamic pH junction for the monitoring of methotrexate and its metabolites [32]. Micellar electrokinetic chromatography has a broad area of application as it can be used to separate both, ionic and neutral analytes. In a recent article [33], the use of MEKC for enhancement of sensitivity by employing on-line enrichment schemes, like stacking and sweeping or their combination, has been reviewed. Another review paper published by Kim et al. [34] describes different sample stacking modes available in MEKC for the enhancement of sensitivity of analyte detection, including normal stacking mode (NSM), reversed electrode polarity stacking mode (REPSM), stacking with reverse migrating micelles (SRMM), field-enhanced sample injection (FESI), field-enhanced sample injection with reversely migrating micelles (FESI-RMM) and stacking using reversely migrating micelles and a water plug (SRW). The mechanism of sample stacking in all these approaches was similar involving stacking of the micelles on the sample/BGE boundary; however the methods differ in terms of the magnitude and direction of the EOF and the mode of injection, pressure or electrokinetic injection. Another newly developed MEKC based on-line sample preconcentration method was applied to get a large improvement in detection sensitivity of neutral analyte focusing using the micelle collapse technique [35]. A 60- to 80-fold increase in detection sensitivity of eight drugs was achieved using three sweeping strategies, i.e. analyte focusing by micelle collapse (AFMC), simultaneous field-amplified sample stacking (FASS), and normal sweeping [36]. In-line sample stacking has been used in MEKC for the improvement of the limit of detection of neutral analytes by reversely migrating micelles and injecting a small water plug before sample injection [37], by field-enhanced sample injection [38] and by reversed electrode polarity stacking [38]. Few attempts have been made to enhance the sensitivity of CE for the determination of nucleosides and nucleotides [11–13,16,39–41]. Britz-McKibbin et al. [42], developed a CE–UV method based on velocity-difference induced focusing (V-DIF) with a dynamic pH junction for the determination of nucleotides by using a dynamic pH junction. Using the sensitive CE method, a 40 nM detection limit for nucleotides was achieved and the method was suitable for the quantification of nucleotides in biological samples. On-line sample preconcentration techniques using dynamic pH junction was successfully used for weakly ionic species by CE [43].

Unfortunately, there is no sensitive CE-based method available for the determination of small concentrations of nucleosides in biological samples. Very recently Szymańska et al. have developed a CE-based method for the determination of urinary nucleosides, but again the limit of detection ranged from 0.41 to 2.26 μM [44] which is not high enough to be applicable in most biological samples. Jiang et al. developed a fast CE method for the separation and quantification of modified nucleosides in urinary samples using a borate-phosphate buffer containing 25 mM cetyltrimethylammonium bromide (CTAB) at pH 9.50. Ten different nucleosides

were separated within 10 min. The minimum detectable concentration was higher than 2.0 μM [45].

Recently, we have developed a sensitive CE–UV method for the determination of AMP by dynamically coating the capillary and using electrokinetic injection [16]. But this method is not applicable for the determination of nucleosides, which are uncharged and cannot be separated on a coated capillary. Thus, the goal of the present study was to develop a sensitive CE method suitable for the monitoring of PNP and ADA. The products of these nucleoside-metabolizing enzymes are nucleobases, or nucleosides, respectively. As an enzyme source we used membrane preparations obtained from a human melanoma cell line, which were found to express PNP and ADA activity. We decided to use the REPSM method, applying an in-line stacking technique induced by polarity switching in order to enhance the sensitivity of the analytical method [28]. Finally, the performance of this method was compared with that of a conventional CE method.

2. Materials and methods

2.1. Materials

Adenosine, adenine, inosine, hypoxanthine and xanthine were obtained from Sigma (Steinheim, Germany). Magnesium chloride, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), ethlylenediamine tetraacetic acid (EDTA) disodium salt, tris(hydroxymethyl)aminomethane (Trizma Base), Dulbecco's modified eagle medium (DMEM), penicillin–streptomycin and fetal calf serum for cell culture were from Sigma (Steinheim, Germany). Pentostatin was obtained from Tocris Bioscience Bristol, BS110QL, UK. Forodesine (Immucullin-H) was a generous gift from Professor V. Schramm, Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461, USA.

2.2. Cell culture and membrane preparation of human melanoma 1539 cells

Cells were grown in cell culture dishes in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 1% (w/v) penicillin/streptomycin, and 5% (w/v) glutamate at 37 °C in a 5% CO_2 atmosphere as described [39]. Membrane preparations were prepared as previously described [31] and stored at −80 °C until use.

2.3. Preparation of standard solutions and calibration curves

Nucleosides and bases were dissolved in deionized water to obtain 10.0 mM stock solutions. These were further diluted to obtain 1.0 mM solutions in assay buffer (1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.4). The 1 mM solutions were further diluted in the same buffer as required for standard calibration curves and enzyme assays. Injections of standards were performed in triplicate. Calibration curves were obtained by plotting the corrected peak areas of the nucleoside or nucleobase peaks against their concentrations. The concentrations of the nucleosides (adenosine and inosine) and nucleobases (adenine, hypoxanthine and xanthine) in conventional MEKC studies were 0.625, 1.25, 2.5, 5.0, 10.0 and 25.0 μM, and in REPSM-MEKC study concentrations of 0.025, 0.05, 0.1, 1.00 and 10.00 μM were used.

2.4. Capillary electrophoresis apparatus and conditions (conventional MEKC)

CE separations were carried out using a P/ACE MDQ system (Beckman Coulter Instruments, Fullerton, CA, USA) equipped with a DAD detection system. The electrophoretic separations were carried out using an eCAP fused-silica capillary [60 cm (50 cm effective length) × 75 μm internal diameter (I.D.) × 375 μm outside diameter (O.D.) obtained from Beckman Coulter]. The following conditions were applied: T = 25 °C, λ_{max} = 260 nm, voltage = 15 kV, running buffer 50 mM sodium borate buffer, 100 mM SDS, pH 9.10. The capillary was washed with 0.1 M NaOH for 2 min, deionized water for 1 min, and running buffer for 1 min before each injection. Injections were made by applying 0.5 psi of pressure to the sample solution for 5 s. The CE instrument was fully controlled through a personal computer, which operated with the analysis software 32 KARAT obtained from Beckman Coulter. Electropherograms were evaluated using the same software.

2.5. Capillary electrophoresis apparatus and conditions (reversed electrode polarity switching mode-micellar electrokinetic chromatography)

The CE instrument and capillary were the same as those used in the conventional MEKC (see Section 2.4). The separations were performed using an applied voltage of 15 kV and a data acquisition rate of 8 Hz. The capillary was conditioned by rinsing with 0.1 M aqueous NaOH solution for 2 min, water for 1 min, and subsequently with buffer (50 mM sodium borate buffer, 100 mM SDS, pH 9.1) for 1 min. The limit of detection (LOD) was calculated at a signal-to-noise ratio equal to 3, while the limit of quantitation (LOQ) was calculated at a signal-to-noise ratio equal to 10.

2.6. Reversed electrode polarity switching mode stacking process

Samples were diluted to obtain the required concentration in enzyme assay buffer (1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.4). Hydrodynamic pressure injections were performed at 5 psi for 36 s which corresponds to an injected volume representing 91.0% of the capillary volume. In a first step, a negative voltage (−20 kV) was applied and the large plug of sample was electroosmotically pumped out of the injection edge of the capillary. The electric current through the capillary was initially much lower than its value reached during a classical capillary electrophoresis separation applying the same conditions. When the current reached its normal value, the separation voltage was switched from a negative to a positive value. The experimental current was monitored very carefully and polarity switching was operated as the current reached 95% of its adjusted value.

2.7. Determination of Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) by REPSM-MEKC

PNP activity was measured in a mixture containing 50 mM sodium phosphate buffer, pH 7.4, whereas the *ecto*-ADA enzyme assay buffer consisted of 10 mM HEPES, 1 mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.4. Seven different concentrations of the substrate (inosine was used as a substrate of PNP and adenosine was used as a substrate of ADA) were used (5, 10, 20, 50, 100, 500 and 1000 μM). The reactions were started by adding 20 μl of melanoma membrane preparation (2.1 μg), and the reaction was incubated at 37 °C for 20 min in a final volume of 100 μl. The reaction was stopped by heating the mixture at 99 °C for 5 min. Precipitated membrane proteins were removed by centrifugation and 50 μL of reaction mixtures were withdrawn and 100-fold diluted with distilled water. Finally, 50 μl of diluted reaction mixtures were transferred into mini-CE vials for measurement. Each analysis was repeated twice (duplicates) in three separate experiments. The absorbance at 260 nm was monitored continuously and the nucle-

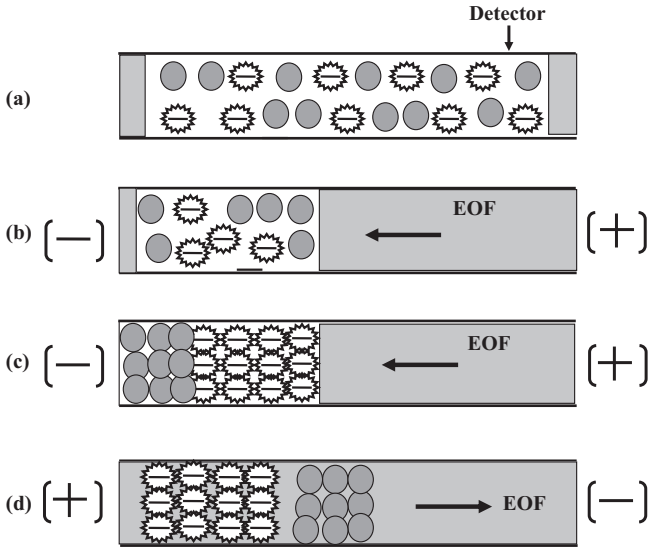


Fig. 2. The schematic steps of MEKC with REPSM conditions. (a) A large-volume sample (prepared in water) was injected hydrodynamically and a small buffer plug was subsequently injected; (b) voltage was applied with reversed polarity (reversed EOF direction), the sample-matrix was pushed back into the inlet vial by the EOF; (c) neutral analytes were focused on passing through the concentration boundary; (d) optimal stacking was achieved, the polarity was switched to normal mode and the separation voltage was reapplied for the analytes' separation and detection.

obase/nucleoside concentrations were determined from the area under each absorbance peak.

2.8. Enzyme inhibition assay of PNP and ADA by REPSM-MEKC

Inhibition of PNP and ADA was investigated over a range of 7–8 concentrations of antagonists spanning 3 orders of magnitude to determine K_i values of standard compounds. Each analysis was repeated twice (duplicates) in three separate experiments. The Cheng–Prusoff equation [46] and a K_D value of 15 μM for adenosine and 33 μM for inosine was used to calculate the K_i values from the IC_{50} values, determined by the non-linear curve fitting program PRISM® 4.0 (GraphPad, San Diego, CA, USA).

3. Results and discussion

In particular, we were interested in monitoring the enzymatic reactions of ADA and PNP, which we discovered to be expressed in human 1539 melanoma cell membranes as *ecto*-enzymes. In the current work, we therefore developed an in-line stacking REPSM-MEKC method to increase the applicability of CE in enzymatic reaction monitoring. In the REPSM-MEKC mode, analytes were dissolved in a low-conductive matrix and hydrodynamically injected into the capillary. Reverse polarity was applied for a short time, and the EOF pushed the sample matrix out of the capillary and stacked the analytes at the interface [47]. Schematic diagrams showing the REPSM processes under reversed electrode polarity are shown in Fig. 2. The REPSM-MEKC method was optimized with respect to sample loading time, separation buffer concentration, pH of separation buffer, and separation voltage. To validate the quantitative REPSM method, the results in terms of limit of detection and limit of quantitation were compared with a conventional MEKC method. It was observed that the new REPSM-MEKC method gave around ten-fold lower limits of detection and quantitation.

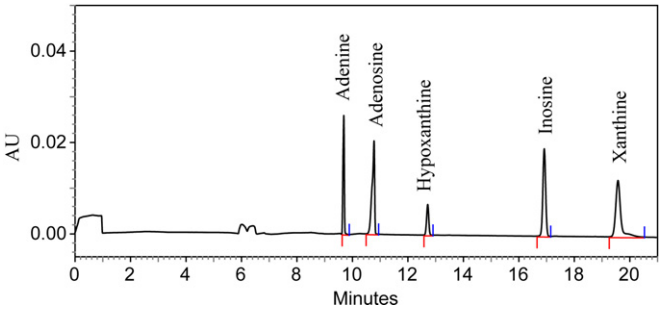


Fig. 3. Effect of sodium borate buffer concentration on the resolution and sensitivity of analytes. The concentration of all five analytes was 100 μM. Separation buffer was 100 mM sodium borate, 100 mM SDS and the pH was adjusted to 9.10. REPSM-MEKC conditions were as follows: sample loading, 5 psi for 36 s; stacking at −20 kV; uncoated fused-silica capillary, 50.0 cm (effective length) 75 μm I.D.; separation at 15 kV; wavelength, 260 nm.

3.1. Optimization of CE separation conditions

Capillary electrophoresis separation conditions were optimized for the nucleosides and nucleobases and the best conditions were selected. Initially, studies were focused on the optimization of the main parameters that could influence the separation and simultaneous quantification of the nucleosides and nucleobases by UV detection. The effect of running buffer concentration and pH value on the migration time and resolution between peaks was investigated. Different concentrations of sodium tetraborate buffer, 25, 50, 75 and 100 mM, were examined, while different pH values, 7.54, 8.00, 8.54, 9.10 and 9.54 were investigated. As the concentration of sodium tetraborate was increased, a longer migration time and better peak resolution was obtained. However, we did not observe a significant effect of running buffer concentration on the sensitivity of detection of the analytes. Different sample plugs were tested in order to determine the highest sample plug length to be injected without worsening the separation profile by REPSM. To improve the sensitivity of the method, the injection time was varied between 5 s and 50 s using a 5 psi hydrodynamic injection. The peak area of all five analytes was increased as the injection time increased, but poor efficiencies and bad peak shapes were obtained for times longer than 36 s. For that reason, 36 s was applied as injection time by applying a pressure of 5 psi.

The best resolution of analytes was obtained using a 50 mM sodium tetraborate buffer at pH 9.10 containing 100 mM SDS (Fig. 3). The addition of acetonitrile to the electrophoretic buffer had been reported to improve the peak resolution and the enhancement of detection sensitivity [48]. In the present study, a significant improvement in resolution was observed in the presence of 10% acetonitrile in the running buffer. The separation voltage was optimized between 10 and 25 kV so as to obtain a good compromise between satisfactory separation, analysis time and adequate current, avoiding the Joule heating effect. A voltage of 15 kV was applied as the optimum to achieve a base line separation of the analytes and a low electric current. The UV spectra of the analytes were registered at a wavelength of 260 nm which gave a high sensitivity.

3.2. Optimization of reversed electrode polarity switching mode-micellar electrokinetic chromatography (REPSM-MEKC) variables

Reversed electrode polarity switching mode-micellar electrokinetic chromatography (REPSM-MEKC) has been used for improving the sensitivity of ionic and neutral analytes [28,47,49–51]. However, only very few reports are available for REPSM and MEKC [51–54]. To the best of our knowledge, this is the first time a highly sensitive REPSM-MEKC method has been developed for the

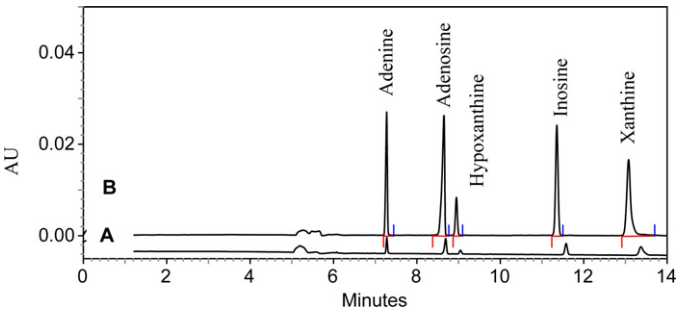


Fig. 4. Comparison of the electropherograms of a mixture of five analytes (100 μM 100 times diluted in deionized water) by (A) conventional MEKC, hydrodynamic injection of 5 s at 0.5 psi (ca. 25 nl) and (B) optimized REPSM-MEKC, hydrodynamic injection of 36 s at 5 psi (around 397 nl). Conditions: the running buffer consisted of 50 mM sodium borate, 100 mM SDS and had a pH of 9.10. The applied separation voltage was 15 kV.

determination of nucleosides and nucleobases, and in particular for monitoring enzymatic reactions yielding these products. Before starting the REPSM experiments the capillary was conditioned with micellar background solution and the analytes, dissolved in a low-conductivity matrix, were injected by the application of pressure for a much longer time than typically used in conventional MEKC methods. Separation voltage was then applied at negative polarity (positive at outlet). Micelles from the cathodic vial were carried and stacked the neutral analytes at the stacking boundary, which was the interface between the sample zone and the BGS, and the sample matrix was pumped out from the capillary by the EOF. When the analytes within the micelles were completely focused, and most of the sample matrix was removed, the negative voltage was stopped. The current decreased due to the low electrical resistance caused by the lower conductivity of the sample, but rose again as the sample was removed from the capillary [55]. The current shift was monitored carefully and initially the current was gradually increased with the removal of the sample matrix. When the measured electric current reached 95% of its value, the polarity was switched to positive (see Fig. 2). A negative polarity was required to focus the analytes, and a positive polarity was required to separate the analytes. Fig. 4 shows the electropherogram of the optimal MEKC conditions with REPSM technique for in-line concentration of five analytes (adenosine, adenine, inosine, hypoxanthine and xanthine). With the conditions given in Fig. 3, detector responses increased with an increasing sample injection time. An approximately 10-fold enrichment factor was achieved over the classical MEKC, comparing of the peak heights of the analytes. The major advantage of this stacking technique is that the analysis is performed on the sample itself, without the need for pre-treatment or prior manipulation.

3.3. Quantification of nucleosides and nucleobases and method validation

The linearity, regression, LOD and LOQ of five analytes were determined using the conventional MEKC and compared to the highly sensitive REPSM-MEKC method (Table 1). For the determination of the linearity and the limit of detection and quantitation, calibration curves of standard solutions were obtained from 5 to 6 different concentrations of nucleosides and nucleobases. The determined coefficients ($r > 0.9948$) indicated excellent correlations between the concentrations and relative peak areas for the investigated compounds within the tested ranges (Table 1). The limit of detection (LOD) calculated at a signal-to-noise ratio equal to 3 was found to be between 0.55 (inosine) and 1.61 μM (hypoxanthine) for conventional MEKC and ranged from 0.06 (inosine and adenine) to 0.17 μM (hypoxanthine) for REPSM-MEKC (see Table 1). Grune et al. previously performed the separation and quantitation of nucleosides and nucleobases on a fused silica capillary and used 20 mM borate buffer (pH 9.4). The detection limit (taken as three-fold noise level) achieved for the analytes was 0.5 μM [56]. This is more than ten times higher than the limit of detection in our REPSM-MEKC method.

For the determination of the reproducibility of the migration times and peak areas each compound was measured at least 6 times. The highest variation coefficient for the migration time was 1.2% (intra-assay), and 1.8% (inter-assay), respectively. The highest variation coefficient for the peak area was 2.1% (intra-assay), and 2.6% (inter-assay), respectively. These results showed that the REPSM-MEKC method had a very high reproducibility in migration times as well as with regard to peak areas. Good linearity was found in the range from the compounds' lowest measured concentration to the highest concentration. The limit of quantitation (LOQ) calculated at a signal-to-noise ratio equal to 10 was found in the range of 2.08 (inosine) to 5.99 μM (hypoxanthine) for conventional MEKC and 0.23 (adenine) and 0.64 μM (xanthine) for REPSM-MEKC (see Table 1). These results showed that the sensitivity was significantly enhanced by the newly developed method. The LOD and LOQ for investigated compounds were 10-fold lower with the new method than that obtained with a standard MEKC procedure.

3.4. Determination of Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) by REPSM-MEKC

Kinetic parameters were determined for both PNP and ADA in membrane preparations of human melanoma 1539 cells using the newly developed CE-based REPSM method. Michaelis–Menten constants (K_m) and maximal velocity (V_{max}) for PNP and ADA enzymes were determined by measuring the initial velocity as a function of substrate concentration to provide a saturation curve, which was fit by nonlinear regression analysis to the

Table 1
Linear regression data, correlation coefficients, LOD and LOQ of investigated compounds by conventional MEKC and MEKC with a reversed electrode polarity switching mode (REPSM-MEKC).

Compound	Conventional MEKC		Large volume sample stacking MEKC		Conventional MEKC[a]		Reversed electrode polarity switching mode MEKC[b]	
	Regression equation	Coefficient of correlation (r)	Regression equation	Coefficient of correlation (r)	LOD (μM) ± SD	LOQ (μM) ± SD	LOD (μM) ± SD	LOQ (μM) ± SD
Adenosine	$y = 306.34x - 39.46$	0.9999	$y = 12761x + 1466.21$	0.9999	0.91 ± 0.02	3.42 ± 0.04	0.09 ± 0.01	0.36 ± 0.01
Inosine	$y = 332.41x + 6.61$	0.9999	$y = 11964x + 487.87$	0.9999	0.55 ± 0.02	2.08 ± 0.06	0.06 ± 0.01	0.26 ± 0.02
Adenine	$y = 240.49x - 98.78$	0.9999	$y = 5038.8x + 287.28$	0.9889	0.59 ± 0.03	2.23 ± 0.07	0.06 ± 0.01	0.23 ± 0.01
Hypoxanthine	$y = 146.93x + 144.65$	0.9959	$y = 2590.5x + 620.49$	0.9948	1.61 ± 0.08	5.99 ± 0.05	0.17 ± 0.02	0.61 ± 0.02
Xanthine	$y = 710.89x + 471.66$	0.9979	$y = 35778x + 629.41$	0.9998	1.23 ± 0.07	4.61 ± 0.03	0.12 ± 0.02	0.64 ± 0.03

[a] The range of concentrations was 0.625–25.0 μM for nucleosides and nucleobases.
[b] The range of concentrations was 0.025–10.00 μM for nucleosides and nucleobases.

Table 2
Kinetic parameters for substrates of PNP and ADA. The results represent means ± SEM of three separate experiments each run in duplicate.

Substrate	REPSM-MEKC[a]		Literature values	
	K_m (μM)	V_{max} (nmol/min/mg)	K_m (μM)	V_{max} (nmol/min/mg)
Inosine (PNP)	15.2 ± 1.4	5.14 ± 0.1	13.1 ± 0.5 [57],b	31.1 ± 0.3 [57],b
Adenosine (ADA)	33.2 ± 6.8	24.4 ± 2.3	53 ± 8 [58],c 286.30 ± 40.38 [9],d	280.05 ± 7.06 [9],d

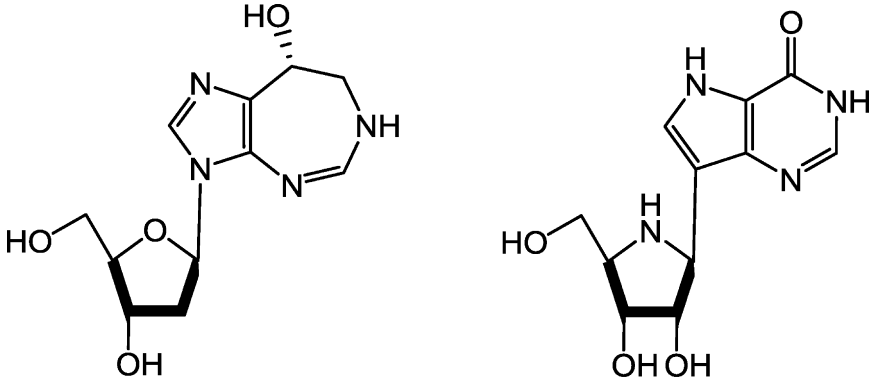
[a] Membrane preparations of 1539 melanoma cells were used for the assays.
[b] Calf spleen PNP and xanthine oxidase-coupled spectrophotometric assay was used for the measurement of *ecto*-PNP activity.
[c] Bovine spleen enzyme and CE was used.
[d] Synaptic membrane preparations from porcine cerebral cortex were used; the reaction was monitored using HPLC.

Michaelis–Menten equation. The Michaelis–Menten plots were constructed by using different concentrations of the substrates. Estimated K_m values of 15.2 and 33.2 μM were obtained for PNP and ADA, respectively. The initial reaction velocities were calculated from the amounts of product formed, hypoxanthine in the case of PNP, and inosine in the case of ADA. V_{max} values were 5.1 and 24.4 μmol/min/mg of protein (melanoma membrane preparation) for PNP and ADA, respectively (Table 2). The validity of the method was confirmed by a comparison with previously obtained K_m and V_{max} values by other analytical methods. Reported K_m values vary between species, sources and investigators. Since in most cases the enzymes are cytosolic and not *ecto*-enzymes, the values cannot be easily related to our data. However, the K_m and V_{max} values we have obtained for the *ecto*-PNP were in very good agreement with values obtained for the calf PNP (K_m 13.1 ± 0.5 and V_{max} 31.1 ± 0.3 nmol/min/mg) using same substrate, inosine [57]. Our obtained K_m value of *ecto*-ADA was somewhat lower than the K_m value of calf spleen (53 μM ± 8) using cytosolic ADA [58]. The K_m (286.30 μM) and V_{max} (280.05 nmol inosine/min/mg) values [9] obtained for *ecto*-ADA activity in synaptic membranes from cerebral cortex of porcine brain are varying from our K_m and V_{max} values obtained from melanoma cell membrane-bound *ecto*-ADA (Table 2). This difference could be due to species and location of enzyme.

3.5. Enzyme inhibition assay of PNP and ADA by REPSM-MEKC

A reversed electrode polarity switching mode sample stacking CE assay was also applied to study *ecto*-PNP and *ecto*-ADA inhibitors. Using the highly sensitive CE method inhibition constants (K_i values) for inhibitors of PNP and ADA were determined by a range of concentrations of inhibitors, in the presence of a fixed amount (100 μM) of substrate concentration, adenosine for ADA, and inosine for PNP (see Table 3). A typical electropherogram for the ecto-ADA control assay is shown in Fig. 5A, in which 100 μM of adenosine was present in the absence of an inhibitor. The percentage of conversion of substrate to product was less than 5%. The second electropherogram (Fig. 5B) shows a typical ecto-ADA inhibition experiment, in which the ecto-ADA inhibitor pentostatin (10 nM) had been added. In that electropherogram the peak size for inosine, the product of the enzymatic reaction, was significantly smaller as compared to the control assay. Similarly, in PNP inhibition assays inosine was used as a substrate and hypoxanthine was the product of enzyme reaction. In control assays, the peak of hypoxanthine was significantly larger as compared to inhibition assay, where forodesine [59,60] (3 nM) was added. The peak areas for hypoxanthine and inosine were used for the determination of IC_{50} values and for calculating K_i values for PNP and ADA, respectively. Using PNP and ADA and the newly devel-

Table 3
K_i values for standard inhibitors of PNP and ADA determined in 1539 melanoma cell membrane preparations comparing REPSM-MEKC with classical MEKC for quantitative determination of reaction products.



Pentostatin Forodesine

Inhibitor	K_i (pM) ± SEM (n = 3)	
	REPSM-MEKC[a]	Literature values (pM)
Forodesine (PNP)	75.6 ± 13.2	72 [61],b
Pentostatin (ADA)	14.3 ± 1.9	19 [10],c 2.5 [54],d

[a] Membrane preparations of 1539 melanoma cells were used for the assays.
[b] Purified human erythrocyte PNP and inosine as a substrate were used, xanthine oxidase-coupled spectrophotometric assay was used for the measurement of enzyme inhibition.
[c] ADA inhibition assay using partially purified human erythrocyte ADA and adenosine as a substrate.
[d] ADA inhibition assay using human erythrocyte ADA and adenosine as a substrate.

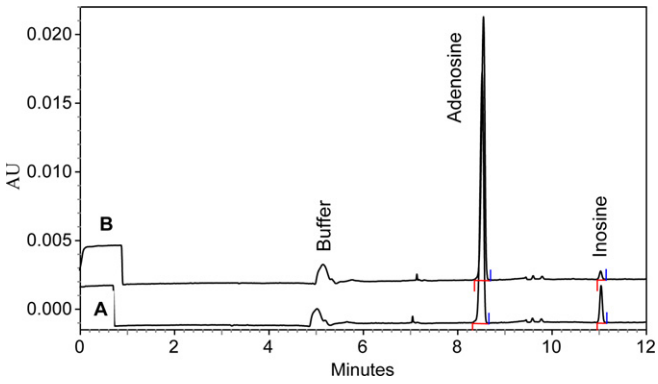


Fig. 5. Typical electropherograms of *ecto*-ADA inhibition assays by REPSM-MEKC. (A) Control without inhibitor. (B) Assay in the presence of the inhibitor pentostatin (10.0 nM). The concentration of adenosine was 100 μM. The separation conditions were 100 mM SDS, 50 mM sodium borate buffer pH 9.10, fused-silica capillary, 60 cm length (50 cm to the detector), 75 μM I.D.; 15 kV; 25 °C; detection at 260 nm. The quantitation of inosine was used to determine the inhibitory potency of the *ecto*-ADA inhibitor.

oped CE–REPSM method, concentration-dependent inhibition by the standard inhibitors was observed at both enzymes in the presence of their specific competitive inhibitors (Fig. 6). This allows a direct comparison of the effects of these inhibitors on PNP and ADA. The K_i values derived are summarized in Table 3. The K_i value (75 pM) obtained for the standard PNP inhibitor forodesine is in excellent agreement with the reported K_i value (72 pM) [59] obtained by spectrophotometric method using a pure human erythrocyte PNP enzyme preparation.

In that method, the reactions were initiated by the addition of various concentrations of inosine, and the product was monitored by UV detection of uric acid formed in the presence of xanthine oxidase from the PNP product hypoxanthine (Table 3).

Similarly, the K_i value (14 pM) obtained in our study for pentostatin at *ecto*-ADA is very close to the K_i value (19 pM) [60] reported for ADA from human plasma. The K_i value (2.5 pM) [60] at partially purified human erythrocyte ADA was about 6 times lower than the K_i value that we determined in melanoma cell membranes (Table 3).

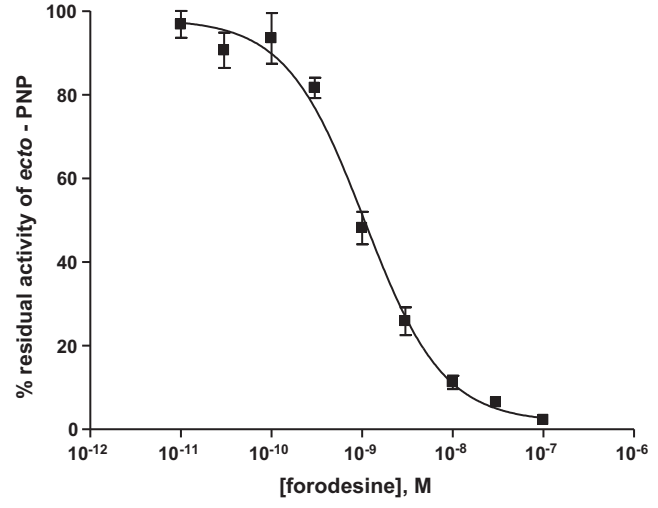


Fig. 6. Concentration-dependent inhibition of *ecto*-PNP by forodesine determined by capillary electrophoresis. A substrate concentration of 100 μM inosine, a reaction buffer consisting of 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Hepes, pH 7.4, and various concentrations of forodesine were used. The separation conditions were 50 mM sodium borate buffer pH 9.10, fused-silica capillary, 60 cm length (50 cm to the detector), 75 μM I.D.; 15 kV; capillary cartridge temperature 25 °C; detection at 260 nm. Data points represent means ± SD from three separate experiments, each run in duplicate. K_i value of 75.6 ± 13.2 pM was calculated.

4. Conclusions

The analytical procedure developed herein demonstrates that MEKC combined with the REPSM technique offers a reliable, simple, fast and highly sensitive and convenient analytical technique for the determination of nucleosides and nucleobases as products of PNP and ADA, and the investigation of potential inhibitors. The new REPSM-MEKC method has multiple advantages in comparison with standard PNP and ADA assays, e.g. no need for expensive radiolabeled or fluorescent substrates, the possibility to employ (various) natural substrates rather than artificial ones, no requirement for sample preparation prior to analysis, and a minimal use of reagents. The quantitative analysis of the samples can be carried out within a few minutes. It will therefore allow the screening of compound libraries in order to identify and develop selective inhibitors for these pharmacologically important classes of enzymes. In comparison with other CE methods described for the determination of nucleosides and nucleobases, it has clear advantages, such as an increased sensitivity allowing the monitoring of enzymes with low activity.

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