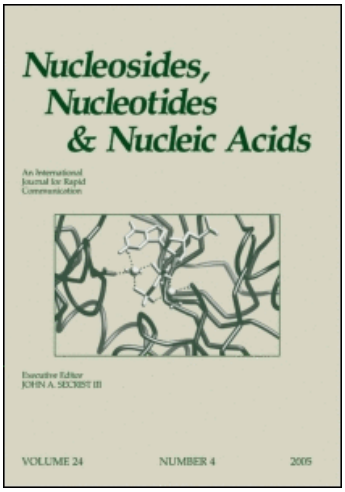

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ANALYSIS OF INTRACELLULAR NUCLEOTIDES BY CAPILLARY ELECTROPHORESIS–MASS SPECTROMETRY

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□ *A pilot study using capillary electrophoresis with mass spectrometry for the analysis of nucleotides in human erythrocytes is presented. Erythrocytes were incubated with 5-amino-4-imidazolecarboxamide riboside in order to mimic situation in defect of purine metabolism—AICA-ribosiduria. Characteristic AICA-ribotides together with normal nucleotides were separated by capillary electrophoresis in acetate buffer (20 mmol/L, pH 4.4) and identified on line by mass spectrometry.*

Keywords Capillary electrophoresis; Mass spectrometry; Metabolism; Erythrocyte; Purine disorders

INTRODUCTION

Ion exchange high performance liquid chromatography is commonly used technique for analysis of nucleotides. Capillary electrophoresis (CE) also can be utilized for separation of nucleotides. Compared to chromatographic methods, CE allows the significantly better separation efficiencies (>100,000 theoretical plates per meter) with shorter analysis time. Coupling

Authors contributed equally to the work.

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of capillary electrophoresis with mass spectrometry (CE/MS) allows a fast analysis of ionic analytes in complex matrixes with simultaneous structural identification as clearly reviewed by Schmitt-Kopplin and Frommberger.[1] In this study CE/MS was used for analysis and identification of nucleotides in human erythrocytes.

MATERIALS AND METHODS

Instrumentation

A PrinCE-C660 capillary electrophoretic apparatus (Prince Technologies, Emmen, the Netherlands) was used for coupling with an ESI-MS instrument (ion trap, Finnigan MAT LCQ, Finnigan, San Jose, CA, USA). Experimental set-up of ion optics, temperature of the heated capillary, spray voltage and sheath gas flow-rate were optimized. Tuning was done using a direct infusion of 1 mmol/L methanol solution of 5-amino-4-imidazolecarboxamide ribotide standard (ZMP, Sigma-Aldrich, St. Louis, MO, USA) into the MS ion source (flow-rate of 3 μl/min). The optimized conditions for MS used in all further experiments are as follows: negative ESI mode, sheath gas flow-rate of 20 arbitrary units, spray voltage of 4.5 kV, temperature of the heated capillary of 200°C. Pure methanol offered the highest response of analyte when used as sheath liquid. The optimal flow rate of sheath liquid was 3 μl/min. Analytes were quantified by the use of molecular ion peak areas. Samples were measured in triplicates.

Separation Conditions

Separations were performed with electrolyte consisting of 20 mmol/l acetic acid—ammonium (pH 4.4) by using voltage of –30 kV (inlet). After each sample injection, the capillary was washed with the electrolyte for 1.5 minutes. A fused-silica capillary, length of 65 cm, 50 μm I.D. without internal coating was used. The outlet capillary end was positioned into the electrospray needle. Sample injection: 0.3 min/50 mbar. The supporting overpressure of 70 mbar was applied on inlet vial during analysis in order to enable the electromigration of analytes into the ion source (elimination of the influence of electroosmotic flow).

Erythrocyte Incorporation and Sample Preparation

Washed erythrocyte cells (0.9% NaCl, 3 times) were incubated with 5-amino-4-imidazolecarboxamide riboside (ZR, Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 10 mmol/L in Minimum Essential Medium Eagle (Sigma-Aldrich, St. Louis, MO, USA) at 20% hematocrit. Mixtures were incubated at 37°C for 4 hours. The incubated cells were washed (0.9%

NaCl, 3 times) and centrifuged. One volume of washed erythrocytes was pipetted to 2 volumes of trichloracetic acid (10%) while vortex mixing. Samples were sonicated (10 seconds) and centrifuged (10,000 g, 1 minute). Supernatant containing TCA was immediately back-extracted into ether –5× with 6 volumes of ether for 20 seconds while vortex mixing (pH ∼ 7). Water phase was left open for 10 minutes to evaporate residual ether. Sample was injected into capillary or stored at −50°C.

RESULTS

It is well known that the composition of sheath liquid strongly influences the detection sensitivity of electrospray equipped MS instruments. In our case methanol with addition of water (0–30%, v/v) and/or ammonium (0–0.25%, v/v) or ammonium acetate (0–0.15%, v/v) were tested in order to obtain maximum sensitivity of mass spectrometer. Surprisingly, the best results (the best S/N ratio) were obtained using pure methanol as sheath liquid. Note however, that the results are strongly dependent on the geometry of ion source as well as the positioning of the separation capillary in the spray needle. The used ion source has "off axis" geometry. The separation capillary protruded from the spray needle around 0.3 mm toward the heated capillary in our case. Flow-rate was tested within the range of 1–20 μl/min and optimal response was found at a flow-rate of 3 μl/min.

Full MS spectrum of ZMP obtained by direct infusion contained molecular ion of ZMP $[M\text{-}H]^-$ ($m/z = 337.3$), the peaks of dimer $[2^*M\text{-}H]^-$ ($m/z = 675.3$) and adduct of 2 molecular ions with sodium ($m/z = 697.3$). The fragment ion $m/z = 294.3$ obtained after collision induced dissociation of isolated parent ion in ion trap corresponds to the cleavage of amido group. 5-Amino-4-imidazolecarboxamide di- and triphosphate (ZDP, ZTP) and adenine nucleotides also were detectable under the above described conditions as $[M\text{-}H]^-$ with $m/z = 417$, 497, 346, 426, and 506 for ZDP, ZTP, AMP, ADP, and ATP, respectively. CE/MS analyses of samples of control erythrocytes and erythrocytes incubated with ZR are shown in Figure 1. Traces in figure were extracted from full MS spectra records.

DISCUSSION

Incubation of erythrocytes with ZR resulted in its transport through membrane and conversion of ZR into mono-, di-, and triphosphates by intracellular kinases. This situation corresponds to the recently discovered inherited metabolic disorders of purine metabolism—AICA-ribosiduria[2] and to Lesch-Nyhan syndrome.[3] Figure 1b shows these characteristic nucleotides at concentrations of 31, 17, and 12 μmol/L for ZMP, ZDP, and ZTP, respectively. Adenosine nucleotides were found at concentrations of

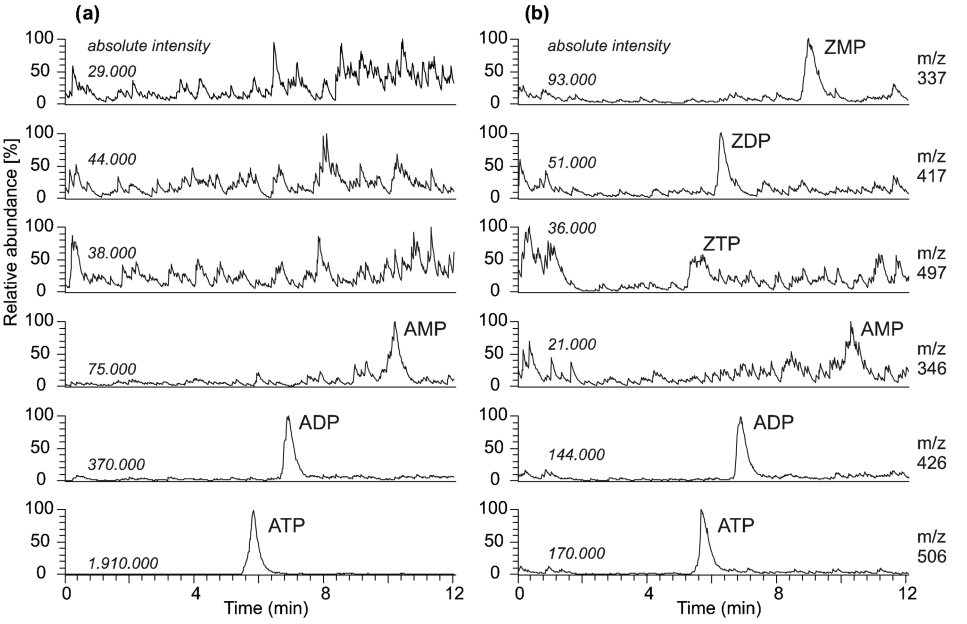

FIGURE 1 (a) Analysis of control erythrocytes and (b) erythrocytes incubated with ZR. Traces at m/z specific for ZMP, ZDP, ZTP, AMP, ADP, and ATP were extracted from full MS spectra records. For conditions see Materials and Methods.

25, 123, 633 μmol/L and 7, 47, 57 μmol/L for AMP, ADP, ATP in control and incubated samples, respectively. Decrease of adenine nucleotides is associated with formation of AICA-ribotides as also observed in patients with AICA-ribosiduria and probably with the inhibition effect of ZR during incubation.

The presented data indicate that CE technique may be a useful tool for the separation and identification of nucleotides in erythrocytes. In this work quantification of analytes is based on peak areas of molecular ions which does not preclude eventual ion suppressions in MS. The use of internal standards (e.g., deuterated) will improve the assay. The procedure for simultaneous fragmentation spectra collection (CE/MS/MS) increases the certainty of identification of normal as well as abnormal nucleotides.

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