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Nucleotides in Erythrocytes – Incorporation Study*

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ABSTRACT
Objective: Analysis of nucleotides for diagnosing inborn errors of metabolism. Transport studies of purine ribosides into erythrocytes.
Material and Methods: Erythrocytes were incubated with purine ribosides. Ribosides were transported through erythrocyte membrane and converted into ribotides. Nucleotides were separated and identified by capillary electrophoretic method using background electrolyte 40 mmol/L citric acid – 0.8 mmol/L cetyltrimethylammonium bromide-γ-aminobutyric acid, pH 4.4.
Results: We found accumulated deoxynucleotides and nucleotides in erythrocyte extracts, which mimics metabolic situation of enzyme defects of purine metabolism. Under optimal electrophoretic conditions all compounds of interest were separated.
Conclusion: The method can be successfully used to analyze intracellular nucleotides and diagnose inherited disorders of nucleotide metabolism.
Key words: capillary electrophoresis, membrane transport, metabolism, erythrocyte, purine disorders.

Introduction

Number of inherited metabolic disorders is typically accompanied by accumulation of specific nucleotides in erythrocytes. Most of purine/pyrimidine analogs/drugs are metabolized via nucleotides. Membrane transport and biostransformation of purine and pyrimidine analogues acting as anticancer and antiviral drugs (e. g. 5-fluorouracil, cytarabine, methotrexate, dideoxyadenosine) is addresses by number of studies (1–3).

Separation of nucleotides is usually performed by chromatographic techniques based on ion exchange, ion pairing and reversed phase, respectively. Generally, LC methods offer good sensitivities and separation efficiencies of up to 10.000 theoretical plates per meter at analytical times of about half an hour. Nucleotides are ideally suited for electrophoretic separations and have been the subject of enormous attention of electroseparation scientists (e.g. 4, 5).

Capillary electrophoretic methods allow good separation of basic nucleotides with efficiencies of up to 150.000 theoretical plates per meter in times of 3 to 20 minutes. We have optimized separation of 21 nucleotides and applied it for analysis of erythrocytes (separate paper concerning method development will be published elsewhere).

In this study, incorporations of purine ribosides of metabolic relevance into erythrocytes were performed in order to model situations similar to enzyme defects of purine metabolism and to prove diagnostic usefulness of the method.

Material and Methods

Washed erythrocytes were incubated with ribosides (deoxyadenosine, deoxyguanosine, aminoimidazolecarboxamide riboside (AICAr)) at concentrations 10 mmol/L in Minimum Essential Medium Eagle (20% hematocrit). Mixtures were incubated at 37 °C in an incubator for 4 hr. The incubated cells (3 times washed with 0.9% NaCl) were disrupted and deproteinated with trichloracetic acid (TCA). One volume of erythrocytes was pipetted to two volumes of TCA (10%) followed by vortex mixing, sonicated (10 s) and centrifuged (10.000 g, 1 min). TCA from supernatant was immediately extracted into ether – 5 times with 1 ml of ether for 20 s while vortex mixing (pH ~ 7). Water phase was injected into capillary or stored at -50 °C.

All experiments were performed on home made instrument consisting of CE-975 UV detector (Jasco Corp., Tokyo, Japan), CZE 3000 HV power (Spellman, New York, USA) and CSW 1.7 station (DataApex, Prague, Czech Republic). Uncoated silica capillary with effective length of 89 cm (total length 100 cm) and inner diameter of 75 μm was used. UV detection was performed at 250 nm. Background electrolyte consisted of 40 mmol.l^{-1} citric acid and 0.8 mmol.l^{-1} cetyltrimethyl-ammonium bromide adjusted using γ-aminobutyric acid to pH 4.4. The pH 4.0 was used in the case of comigration of accumulated nucleotides. Voltage of 25 kV was applied. Nucleotides were identified by their UV spectra and by electromigration behavior.

Results and Discussion

Method was optimized and statistical parameters were obtained. The limit of detection for compounds of interest was in the range 0.3–0.9 μmol/L. The method

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was linear over the range 0.01–10 mmol/L, (r > 0.995). The recoveries of the method were measured by assaying erythrocyte extracts (N = 6) with added standard mixture of ATP, ADP, AMP, NAD and NADP. Values were in range 94–104%, CV = 3.8–5.6% (addition of 20 μmol/L) and 97–101 %, CV = 2.9–4.5 % (addition of 200 μmol/L). Imprecision of the method was tested on pooled erythrocyte extracts of six healthy volunteers containing physiological levels of ATP, ADP, AMP, NAD and NADP (28–1493 μmol/L) over 10 consecutive days. The imprecisions were 1.4–2.8% and 1.5–5.2% for within-day and between-day, respectively.

Incubation of erythrocytes with 2'-deoxyadenosine, 2'-deoxyguanosine and AICAr, respectively, results in transport of ribosides through erythrocyte membrane and enzymatic conversion into their mono-, di- and triphosphates by kinases (Fig. 1). No accumulation of unmodified ribosides was found within the cells (data not shown) and nucleotides corresponding to incorporated riboside were quantitatively formed within the cells. They were easily analyzed by capillary electrophoresis (Fig. 2).

Fig. 1. Conversions of incorporated ribosides in erythrocytes

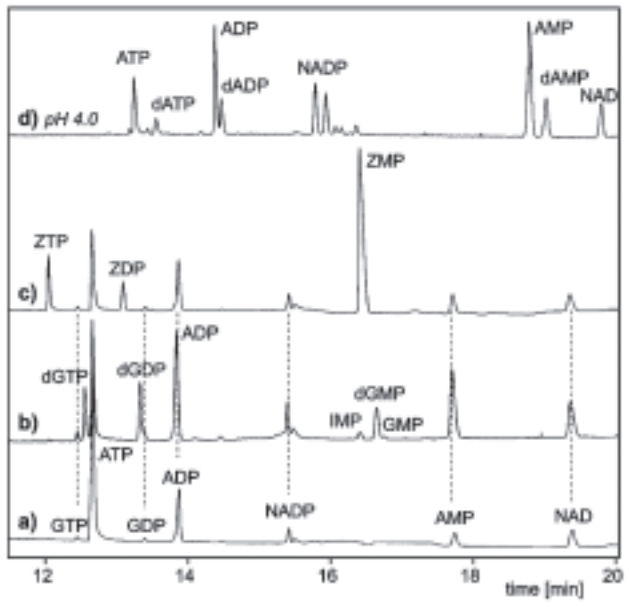

Fig. 2. Analysis of intracellular nucleotides by capillary electrophoresis – erythrocyte incorporation studies. Incubation: a) without riboside addition; b) with 2'-deoxyguanosine; c) with AICAr; d) with 2'-deoxyadenosine; conditions – see experimental; a,b,c – pH 4.4; d – pH 4.0.

These experiments model situation in defects of purine metabolism – adenosine deaminase [EC 3.5.4.4] (incubation with 2'-deoxyadenosine) and purine nucleoside phosphorylase [EC 2.4.2.1] (incubation with 2'-deoxyguanosine). In the case of incubation with AICAr, erythrocytes accumulate nucleotides corresponds to a new defect purine *de novo* synthesis – AICAribosiduria – defect of the bifunctional enzyme AICAr transformylase/IMP cyclohydrolase [EC 2.1.2.3, EC 3.5.4.10] (6).

Conclusion

The method can be successfully used to analyze nucleotides in erythrocytes. It can be applied for diagnosing inherited metabolic disorders and potentially finding new defects. There are also many other applications, e.g. studying effects of drugs on nucleotide energetic metabolism of the cells and measurement of enzyme activities.

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Abbreviations:
AICAr – aminoimidazole-carboxamide riboside
ZMP/ZDP/ZTP – AICAr mono-, di-, triphosphate
dAMP/dADP/dATP – 2'-deoxyadenosine mono-, di-, triphosphate
dGMP/dGDP/dGTP – 2'-deoxyguanosine mono-, di-, triphosphate

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