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Short communication

Determination of purine nucleoside phosphorylase activity in human erythrocytes by capillary electrophoresis

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Abstract

Purine nucleoside phosphorylase (EC 2.4.2.1) activity in human erythrocytes was measured using capillary electrophoresis. Enzyme samples were directly loaded into the capillary without preconcentration or purification. The electrophoretic separations were carried out in an uncoated fused-silica capillary (75 μm internal diameter, effective length of 45 cm, total 72 cm) at an electric field of 415 V/cm at ambient temperature (25°C). UV detection at 200 nm was used. Borate buffer (100 mmol/l, pH 9.5) was used as background electrolyte. The results obtained by CE compared favourably (r=0.989) with those of standard HPLC methods. The method presented is reliable, slightly faster and less expensive than the routinely performed HPLC method. © 1997 Elsevier Science B.V.

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1. Introduction

Purine nucleoside phosphorylase (PNP, EC 2.4.2.1) catalyses the reversible phosphorolysis of inosine (HR), guanosine (GR), and their 2′-deoxy derivatives to give either hypoxanthine (HX) or guanine (G) and the appropriate pentose-1-phosphate. Inheritable PNP deficiency is a rare autosomal recessive disease (33 cases described so far) [1,2]. The enzyme defect causes abnormalities in purine metabolism that are selectively toxic to lymphocytes and result in immune deficiency disease [2]. Immunodeficiency states are frequently classified according to whether they involve cellular immunity, humoral immunity, or both [3]. PNP-deficient children have a severe defect in cell-mediated immunity but may have normal, hyperactive, or reduced humoral immunity [1]. Clinical symptoms usually occurs between 4 months and 6 years of age. Clinical presentation includes recurrent otitis and sinus infections, pneumonia, diarrhoea, urinary tract infection, and pharyngitis [4]. The neurological abnormalities were described in more than half of PNP-deficient children [1]. Patients are usually hypouricemic and hypouricosuric but excrete abnormally large amounts of PNP-substrates HR, GR, deoxyinosine (dHR) and deoxyguanosine (dGR). Plasma HR and GR are abnormally high as well. The

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diagnosis is based on finding pathological metabolites in urine and plasma and on confirming lowered enzyme activity in erythrocytes. Earlier methods for determination of purine enzyme activities have relied on the use of radiolabelled substrates in conjunction with thin-layer chromatographic techniques [5]. More recently, HPLC measurements of enzyme activities in lysed erythrocytes were introduced. This eliminates the hazards due to isotopes, but also reduces costs and shortens the time taken to obtain a diagnosis [6]. The disease can be fatal, the only effective therapy reported is transplantation of HLA-identical allogenic bone marrow [2], but successful prenatal diagnosis has been reported [7]. Early diagnosis is crucial to enable prenatal diagnosis in families with one or more affected offspring.

Capillary electrophoresis (CE) is a current rapidly developing technique in bioanalytical sciences [8]. CE, with its ability to separate compounds of very similar structure (allows substrate loss and product formation to be followed simultaneously) in short analyses from small samples, offers excellent capabilities for studying enzyme activities [9–11].

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent grade. Inosine and hypoxanthine were obtained from Sigma (St. Louis, MO, USA), boric acid and potassium hydroxide were purchased from Merck (Darmstadt, Germany). Deionised water (Milli-Q, 18 MΩ cm) was used for preparation of all solutions.

2.2. Apparatus and conditions

All experiments were performed on a modular system of SpectraPHORESIS 100 (Thermo Separation Products, Fremont, CA, USA) equipped with a fast scanning SpectraFOCUS detector (Thermo Separation Products). The electrophoretic separations were carried out in an uncoated fused-silica capillary (75 μm I.D. × 375 μm O.D., Polymicro Technologies, Phoenix, AZ, USA). The capillary had an effective length of 45 cm (total length 72 cm) and was operated at an electric field of 415 V/cm at ambient temperature (25°C). UV detection was over the range 200–300 nm. Sample loading was by vacuum injection (0.5 s). All samples were filtered through a 0.45-μm membrane filter (Millipore, Milford, MA, USA) before loading. Borate buffer (100 mmol/l boric acid, pH adjusted with 50% NaOH to 9.5) was used for all experiments as background electrolyte (BGE). At the beginning of each working day, the capillary was washed with water, potassium hydroxide (0.1 mol/l), water and separation buffer for 5 min and after each sample injection the capillary was washed with potassium hydroxide for 2 min, water for 1 min and buffer for 3 min. These standard conditions were used for all experiments. The HPLC method was used as described elsewhere [12].

2.3. Erythrocytes and incubation

Erythrocytes were obtained from patients in which PNP activity was measured as a part of diagnostic screening for genetic purine disorders. Erythrocytes were separated from fresh heparinised blood, washed twice with isotonic saline and lysed by freezing–thawing in added water (1:6). Centrifuged lysate (stroma removed) was diluted (1:66) and added to preincubated 1.5 mmol/l HR in 50 mmol/l phosphate buffer, pH 7.4. The mixture was incubated for exactly 15 min at 37°C and the reaction was terminated by adding 25 μl of 40% trichloracetic acid while vortex mixing. Precipitated protein was removed by centrifugation and the supernatant was extracted with water-saturated diethylether to a pH above 5.0. The procedure used is described in detail elsewhere [12]. Samples were immediately quantified by HPLC and aliquots were freeze dried. The freeze-dried samples were reconstituted in water and used for CE determination.

3. Results and discussion

The migration characteristics of HR and HX (and other metabolically important purines) at different experimental conditions (pH, buffer concentration and composition) were studied earlier [13]. On the basis of these results 100 mmol/l borate buffer, pH

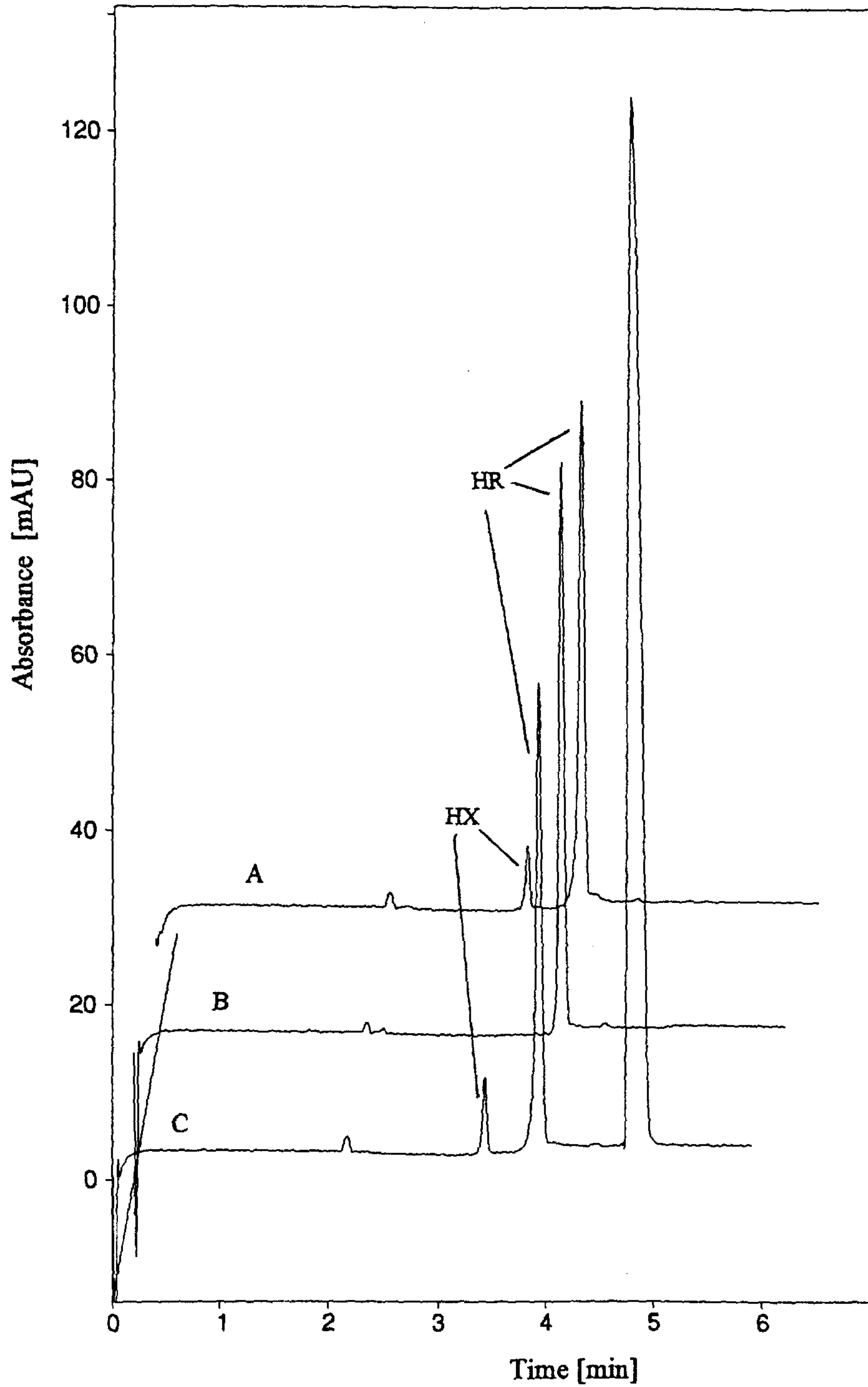

Fig. 1. Original electropherograms of standard mixture of HR and HX (1500 and 150 μmol/l, respectively, in incubation buffer) (A), pre- (B) and postincubation (C) sample from enzyme assay. Conditions: 100 mmol/l borate buffer, pH 9.5, as BGE; electric field of 415 V/cm at ambient temperature (25°C); detection, 200 nm.

Table 1
PNP activities (expressed as concentrations (μmol/l) of the product of enzymatic conversion, HX) in six samples of human erythrocytes measured by CE and HPLC

Sample	HX concentration (μmol/l)	
	HPLC	CE
PNP1	214.6	191.5
PNP2	136.3	131.3
PNP3	160.3	146.4
PNP4	180.4	161.4
PNP5	159.9	144.4
PNP6	199.3	185.2

9.5, was chosen for determination of PNP activity in lysed human erythrocytes.

Electropherograms of a standard mixture of HR and HX are shown in Fig. 1, together with enzyme assays. A very good separation of both substrate and product is seen in all samples. The pre-incubation assay blank contained no interfering compounds.

Linearity and the limit of detection were determined by measuring standards dissolved in incubation buffer (50 mmol/l phosphate buffer, pH 7.4) over a range up to 300 μmol/l of HX. Corrected peak areas at 200 nm were used for quantification. A linear correlation was found which could be expressed by the equation $y=83.47x-0.0014$, $R=0.9997$. The limit of detection of HX was 5 μmol/l (signal-to-noise ratio=3).

The run-to-run and day-to-day reproducibilities were tested at 100 μmol/l ($n=10$). The relative standard deviations (R.S.D.s) for the migration times were 0.4 and 1.8% for HX and 0.5 and 1.8% for HR. The R.S.D.s for the concentrations were 3.2 and 4.5% for HX and 3.3 and 4.4% for HR.

The usefulness of the capillary electrophoretic method described was proven by comparison with the results obtained by the HPLC method. Results obtained by CE and HPLC (see Table 1) correlate well ($r=0.989$). Correlation between the methods can be expressed by the equation $y=0.826x+14.973$. A slight underestimation (CE values are approx. 8% lower than HPLC) could be attributed to sample handling (freeze-drying and redissolving) prior to CE determination.

4. Conclusion

The data presented indicates that PNP activity in human erythrocytes can be measured by CE. The method is as reliable, slightly faster and less expensive than routinely performed HPLC methods.

The method reported here is potentially applicable to the measurement of other purine enzyme activities.

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