Journal of Chromatography, 349 (1985) 275–282
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 649

SIMULTANEOUS HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF THE ACTIVITIES OF ERYTHROCYTIC HYPOXANTHINE–GUANINE PHOSPHORIBOSYL TRANSFERASE AND PURINE NUCLEOSIDE PHOSPHORYLASE

ANNE P. HALFPENNY and PHYLLIS R. BROWN*
Department of Chemistry, University of Rhode Island, Kingston, RI 02881 (U.S.A.)

SUMMARY

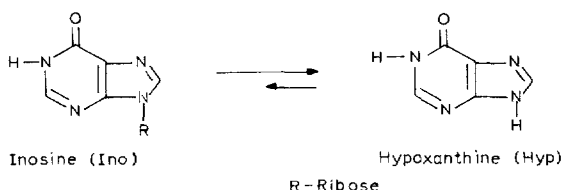
Hypoxanthine–guanine phosphoribosyl transferase (HGPRTase) and purine nucleoside phosporylase (PNPase) activities were simultaneously determined in erythrocyte lysates, using the reversed-phse mode of high-performance liuid chromatography. Reaction conditions were developed to provide zero-order kinetics for both enzymes. The activities of the individual enzymes were calculated after incubation of cell lysates with the PNPase substrate, inosine. After sufficient hypoxanthine had been formed to saturate the HGPRTase, the co-enzyme phosphoribosylpyrophosphate and co-factor magnesium (Mg^{2+}) were added to the incubation medium. The enzyme activities were calculated by measurement of the decrease in the PNPase substraste, inosine, and the increase in the HGPRTase product, inosine-5′-monophosphate.

INTRODUCTION

Purine nucleotides are formed through one of two pathways in physiological systems. The first pathway is *de novo* synthesis, which in a series of ten reaction steps, starting with the amino acid glutamine in the presence of 5′-phosphoribosyl-pyrophosphate (PRPP), utilizes six moles of adenosine-5′-triphosphate (ATP) and produces one mole of inosine-5′-monophosphate (IMP)[1,2]. The other route for nucleotide formation is through the salvage of purines from sources such as diet and/or the breakdown of nucleic acids. Although a balance between these pathways exists, its mechanism is unclear. Human erythrocytes lack the ability to perform *de novo* synthesis; however, they posses an extremely active salvage pathway. In recent years, the salvage pathway has been of clinical and biochemical interest as a result of the association of defects or deficiencies in the enzymes of this pathway in a variety of disease states[3,4].

Purine nucleoside phosphorylase (PNPase) (E.C. 2.4.2.1) and hypoxanthine–guanine phosphoribosyltransferase (HGPRTase) (E.C. 2.4.2.8) are enzymes in the purine salvage pathway (Fig. 1). PNPase converts the nucleosides inosine and guanosine to the products hypoxanthine and guanine, respectively. Human red blood

0021-9673/85/$03.30 © 1985 Elsevier Science Publishers B.V.



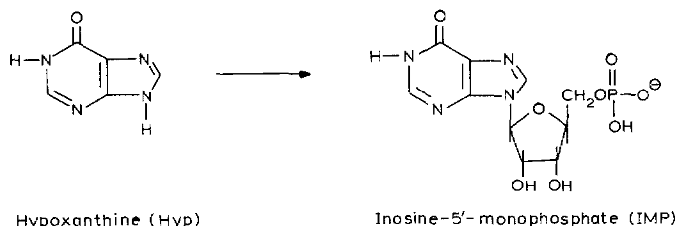


Fig. 1. The salvage pathway in erthrocytes. The coupled assay is for the enzymes PNPase and HGPRTase.

cell PNPase has little or no affinity for the nucleoside adenosine. Defects of this enzyme have been associated with disturbances in cellular immunity. PNPase deficiencies have been demonstrated in individuals with no T cell function and normal B cell function[5]. This information is intruiguing in view of the association of the absence of adenosine deaminase, a salvage enzyme, with the occurrence of severe combined immunodeficiency[6] and suggests multienzyme mediation in the immune response.

A deficiency or absence of HGPRTase has also been associated with a number of disease states. The best known, the Lesch-Nyhan syndrome, is characterized by severe neurological dysfunction and a metabolic disturbance which evidences the highest rate of *de novo* synthesis seen in man[7]. Additionally, there is an effective HGPRTase deficiency which is seen in individuals with defective PNPase[8]. In this case, the substrate hypoxanthine is not being produced, and the metabolism mimics HGPRTase deficiency. The cause and effect relation between enzyme function and clinical symptoms is not well understood nor are the relationships among enzymes within a pathway. In order for these areas to be fully studied, increasingly sensitive and specific assays must be developed.

High-performance liuid chromatography (HPLC) is a powerful tool for the multicomponent analysis of physiological fluids and has been used in many enzymes assays[9–15]. With HPLC, enzyme activity can be determined through the changes both in substrate and product, rather than only one of these. Furthermore, this technique is ideally suited for the assay of multiple enzymes in a pathway, since multiple substrates and products can be measured in one analysis. Thus, we utilized HPLC to determine in one analysis the activities of two enzymes, PNPase and HGPRTase, in the purine metabolic pathway of human erythrocytes. In our method, red blood cells were incubated with inosine. The inosine is subsequently converted to hypoxanthine by PNPase. This hypoxanthine is used by HGPRTase as its substrate (Fig. 1). The

decrease in inosine and the amount of IMP formed are used to determine the activities of PNPase and HGPRTase, respectively. Methods currently used for the assay of these enzymes, such as spectrophotometry, will not permit the simultaneous determination of two enzymes.

EXPERIMENTAL

Reagents

Inosine-5′-monophosphoric acid (IMP), hypoxanthine, inosine, guanine, tris-(hydroxymethyl)amine methane (Trizma base), magnesium sulfate, and PRPP were obtained from Sigma (St. Louis, MO, U.S.A.). All eluents for HPLC and water for standards were doubly distilled, deionized and filtered through membrane filters, pore size 0.45 μm (Millipore, Redford, MA, U.S.A.). Distilled-in-glass methanol was obtained from Burdick & Jackson (Muskegon, MI, U.S.A.). Potassium dihydrogen and disodium hydrogen phosphate were purchased from Fischer Scientific (Waltham, MA, U.S.A.) and were HPLC grade.

Instrumentation

All analyses were performed on a Waters (Milford, MA, U.S.A.) liquid chromatograph. Integration was performed with a 3380A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). Samples were incubated in a thermostated water bath (Lab-Line Instruments, Melrose Park, IL, U.S.A.). All separations were performed on a 25 × 4 cm Partisil-5-ODS column, particle size 5 μm (Whatman, Clifton, NJ, U.S.A.). A guard column, packed with Co-Pell CDS (Whatman), was inserted ahead of the analytical column for all analyses. Hematocrit determinations were made with a microhematocrit centrifuge (Clay Adams, Parsippany, NH, U.S.A.).

Chromatographic conditions

All separations were performed isocratically at ambient temperature. The eluent consisted of 0.02 *M* potassium dihydrogen phosphate–methanol (95:5). The volumetric flow-rate was 1.5 ml/min.

Sample preparation

Whole blood was collected into vacutainers containing acid–citrate–dextrose anticoagulant. Erythrocytes were separated from the plasma and other formed elements of blood by centrifugation for 15 min at 750 *g*. The cells were washed with 10 volumes of cold isotonic (0.9%) saline. The cells were recentrifuged and suspended in saline at a hematocrit value of 5. The use of cells as a suspension rather than as packed red cells provides greater precision in the pipetting and, thus, in the assay.

Incubation procedures

The reaction conditions leading to maximum activity of the individual enzymes have previously been examined[14]. The concentration of inosine and the pH for the present assay were determined by simplex modeling of the response surface made up of these factors[15]. The protocol adopted for all assays was as follows: 50 μl of a 5% red cell suspension in saline was pipetted into glass test tubes. The cells were lysed

by the addition of 100 μl of cold water, followed by two rapid freeze-thaw cycles, using either liquid nitrogen or a dry ice–acetone bath.

At time zero, 200 μl of a 2.08 mM inosine solution in 0.05 M phosphate buffer (pH 7.9) was added to the lysate. This concentration corresponds, after dilution, to the optimal concentration for the analysis, as determined by the simplex search[15]. The PNPase reaction mixture was incubated for 5 min at 37°C. The PNPase produced sufficient hypoxanthine to saturate the HGPRTase. The HGPRTase reaction was initiated by addition of 200 μl of a solution that contained 10 mM PRPP and 20 mM magnesium sulfate in 0.05 M phosphate buffer (pH 7.9). The reaction was incubated at 37°C for 10 min. After 10 min, the reaction was terminated by the addition of 25 μl of 3 M hydrochloric acid. The mixture was neutralized with potassium hydroxide, and a aliquot of the solution was analyzed by HPLC.

Activity calculation

In order to minimize the errors due to pipetting, the activities were calculated from the percent of the initial substrate inosine converted to hypoxanthine and IMP.

$$\% \text{ conversion} = \frac{(\text{area hypoxanthine} \times \text{RF}) + (\text{area IMP} \times \text{RF})}{(\text{area inosine} \times \text{RF}) + (\text{area IMP} \times \text{RF}) + (\text{area hypoxanthine} \times \text{RF})}$$

where RF = response factor (area/mole); units of PNPase = increase of IMP and Hypo or decrease of Ino; units of HGPRTase = increase in IMP.

One unit (U) is the amount of enzyme producing 1 μmol of product at 37°C in 1 min. The activity of PNPase can be calculated from the decrease in inosine or from the increase in hypoxanthine and IMP. The activity of HGPRTase is calculated from the increase in IMP or from the decrease in inosine minus the increase in hypoxanthine. The only source of hypoxanthine in this system is through reaction of inosine with PNPase. Similarly, the only source of IMP is through the action of HGPRTase on the previously formed hypoxanthine. Thus, the calculations of activity can all be obtained by the extent of the reaction.

RESULTS AND DISCUSSION

The isocratic HPLC separation of the participants in these reactions is shown in Fig. 2. This separation of IMP, hypoxanthine and inosine is achieved in less than 6 min.

The activity of an enzyme is determined by the change it causes in a substrate concentration. In order for an assay to be a reasonable measure of the quantity of enzyme in a given matrix, the concentration of substrate must be such that the enzyme is on the so-called Michaelis plateau. In other words, the concentration of substrate must insure that the velocity of the reaction is limited only by the enzyme concentration.

In the present assay, this requirement is met for both PNPase and HGPRTase. However, only one substrate, inosine, is added at saturating levels. The second sub-

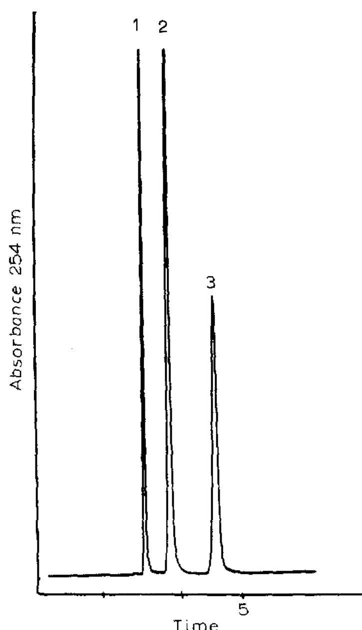


Fig. 2. The separation of the substrates and products in the reaction mixture. The separation is obtained isocratically in under 6 min. The chromatographic conditions are given in the Experimental section. Peaks: 1 = IMP; 2 = hypoxanthine; 3 = inosine.

strate, hypoxanthine, is the result of the reaction of PNPase with the added inosine. Therefore, the saturation of HGPRTase is a result of the activity of the PNPase. The formation of sufficient quantities of hypoxanthine is possible because the activity of the PNPase is 10 times greater than the activity of HGPRTase in red blood cells. Thus, it was possible to develop conditions which permit the assay of both enzymes. However, to determine the activity of HGPRTase, no conversion of hypoxanthine must take place, except that catalyzed by HGPRTase. This was confirmed by chromatographing an incubation mixture containing all components used in these reactions except the co-enzyme PRPP and the co-factor magnesium sulfate, which are required for the HGPRTase reaction (Fig. 3). From this chromatogram it can be seen that under these conditions, no hypoxanthine was converted to IMP. A similar incubation of IMP with HGPRTase showed no inosine or hypoxanthine formation. Thus, the reaction catalyzed by HGPRTase proceeded only in the forward direction (Hyp → IMP).

The total reaction, which includes the reactions of both enzymes, is linear over an incubation period of 30 min. The time chosen for the assay was 15 min: 5 min for the initial incubation of PNPase with inosine, and 10 min for HGPRTase. Although shorter incubation times for both reactions could be used, these times were chosen, since they gave the best precision. A chromatogram obtained from the HPLC analysis of an aliquot of the reaction mixture 5 min and 20 min after the HGPRtase co-factors had been added is shown in Fig. 4.

Using an incubation period of 15 min, the activities of PNPase and HGPRTase

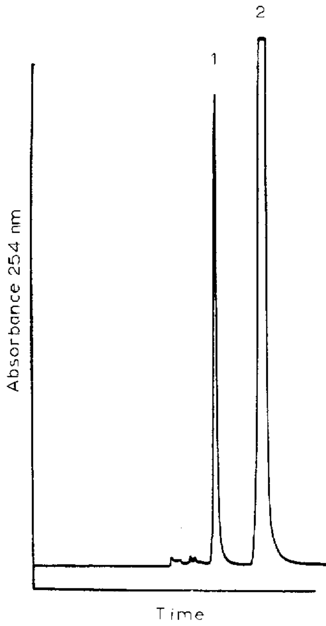


Fig. 3. Chromatogram of the incubation mixture containing all reaction components except the PRPP and Mg^{2+}. Since no IMP is formed, this chromatogram shows that only the PNPase is active in the incubation mixture. The chromatographic conditions are given in the Experimental section. Peaks: 1 = hypoxanthine; 2 = inosine.

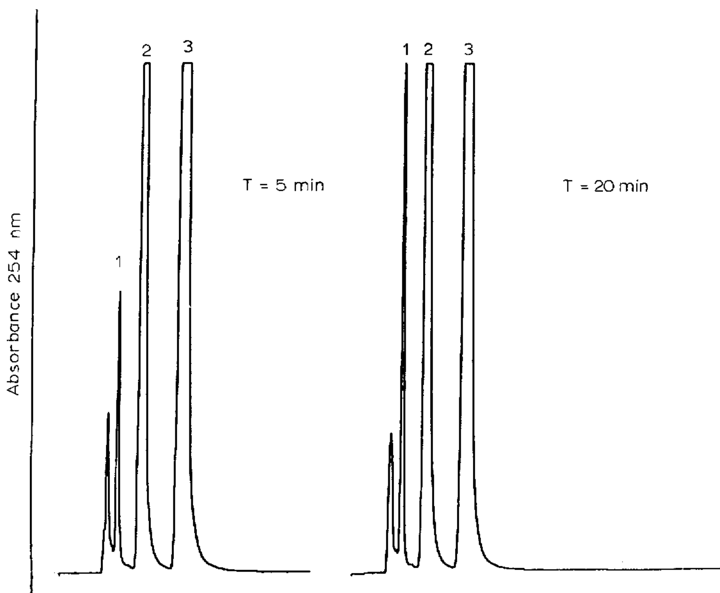


Fig. 4. Chromatogram of an aliquot of the incubation mixture, 5 min and 20 min after the HGPRTase co-factors had been added. The chromatographic conditions are given in the Experimental section. Peaks: 1 = IMP; 2 = hypoxanthine; 3 = inosine.

TABLE I

ACTIVITIES OF PNPase AND HGPRTase

The activities in U/ml of red blood cell lysates, obtained from seven individuals are listed. Conditions for combined assay: pH = 7.9, temperature = 37°C. Conditions for individual assays: for PNPase: pH = 7.4, temperature = 25°C; for HGPRTase: pH = 9.2, temperature = 37°C.

Sample	*PNPase*	*HGPRTase*
1	9.93	0.65
2	10.22	0.66
3	8.50	0.62
4	9.31	0.59
5	9.87	0.71
6	8.74	0.52
7	9.48	0.61
Average (in combined assay)	9.44 ± 0.64	0.63 ± 0.04
Average (in individual assays)	6.53 ± 0.57	1.26 ± 0.08

were measured in the red blood cells of seven subjects with no known disease (Table I). The average activity of the PNPase was 9.44 ± 0.67 U/ml of packed cells and 0.63 ± 0.04 for HGPRTase. The precision of the assay was calculated by twenty replicate assays from the same lot of blood. The precision was found to be 2.4% (relative standard deviation) for PNPase, and 2.6% for HGPRTase. The small difference in the precision for the two enzymes is a function of the integrations. Direct comparison of the activity values obtained with this assay with literature values is somewhat difficult, since the activity of an enzyme is highly dependent upon assay conditions. For example, in optimizing experiments for the assay for PNPase activity alone in erythrocytes, the activity was found to be 6.53 U/ml of packed cells at 25°C. Since the present assay is conducted at 37°C, a higher activity would be expected. The value obtained for HGPRTase is lower than the value obtained under optimized conditions for the separate assay of this enzyme. The separate HGPRTase assay was performed at a pH of 9.2, where HGPRTase has maximum activity, but the optimal pH for the combined enzyme assay was 7.9. Thus, the value obtained in the simultaneous assay of two enzymes may be extrapolated and essentially agrees with the values obtained when the enzyme activities are determined individually.

In conclusion, our results illustrate the great potential of HPLC as a tool for the study of enzyme activities and enzyme-catalyzed reactions. The assay presented has many advantages. It is sufficiently sensitive to detect all the substrate and products at the picomol level, and the results are available less than 6 min after the reaction is terminated. Most importantly, the activities of two enzymes can be determined in a single incubation period and in a single analysis; thus, the activities of two enzymes in a metabolic pathway are determined simultaneously. Finally, the sensitivity of the assay permits extremely small amounts of blood to be used; *e.g.*, 2.5 μl in this assay. Therefore, this technique is eminently suitable for examining enzymes in pediatric or geriatric medicine.

REFERENCES

1 J. F. Henderson, *Regulation of Purine Biosynthesis*, American Chemical Society, Washington, DC, 1972.
2 A. L. Lehninger, *Biochemistry*, Worth Publisher, 1976.
3 M. E. Balis, J. S. Salser, P. P. Trotta and E. Wainfran, in R. W. Ruddon (Editor), *Purine and Pyrimidine Metabolism as Tumor Markers, Biological Markers of Nesoplasia: Basic and Applied Aspects*, Elsevier, Amsterdam, 1978.
4 A. W. Murray, *Ann. Rev. Biochem.*, 40 (1971) 811.
5 E. Giblet, J. A. Amman, R. Sandman, D. Ware and L. K. Diamond, *Lancet*, i (1975) 1010.
6 H. Meuwissen, R. J. Pickering, B. Pollara and J. H. Porter (Editors), *Combined Immunodeficiency Disease and Adenosine Deaminase Deficiency*, Academic Press, New York, 1975.
7 J. E. Seegmiller, R. M. Rosenbloom and W. N. Kelley, *Science*, 155 (1967) 1692.
8 J. E. Seegmiller and H. Harris (Editors), *Adv. Hum. Genetics, 6*, Plenum Press, New York, 1976.
9 S. N. Pennington, *Anal. Chem.*, 43 (1971) 1202.
10 R. H. Hartwick, A. Jeffries, A. M. Krstulovic and P. R. Brown, *J. Chromatogr. Sci.*, 16 (1978) 427.
11 A. M. Krstulovic, R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 163 (1979) 19.
12 A. P. Halfpenny and P. R. Brown, *J. Chromatogr.*, 199 (1980) 275.
13 I.-W. Yu and R. S. Fager, *Anal. Chem.*, 54 (1982) 1904.
14 A. P. Halfpenny, *Master of Science*, University of Rhode Island, 1980.
15 A. P. Halfpenny, *Ph.D. Dissertation*, University of Rhode Island, 1983.