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OPTIMIZED ASSAY FOR PURINE NUCLEOSIDE PHOSPHORYLASE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The reversed-phase mode of high-performance liquid chromatography (HPLC) was used to assay purine nucleoside phosphorylase (PNPase E.C. 2.4.2.1) in human erythrocytes. The reaction conditions were optimized with respect to pH, concentration of enzyme, concentration of substrate and time. In this method, a sample of erythrocytes was incubated with substrate and necessary cofactors. After termination of the reaction, both the decrease in substrate and the increase in product were measured. HPLC is highly suitable for PNPase as both the forward and reverse reactions can be monitored. The complete separation of products from reactants allows the determination of any competing or side reactions.

INTRODUCTION

Purine bases and their nucleoside and nucleotide derivatives are of primary importance in biological systems. These compounds are components of the nucleic acids and are participants in intermediary metabolism[1]. Purine nucleotides may be produced in the body either directly through *de novo* synthesis, or through the salvage pathway. The purine salvage pathway re-utilizes purines formed from the breakdown of the nucleic acids[2]. Purine nucleoside phosphorylase (PNPase) is a key mediator in the salvage pathway. This enzyme catalyzes the reversible conversion of a purine riboside to its purine base analog (Fig. 1). Human erythrocytes, while lacking the ability to synthesize purines *de novo*, possess a highly active salvage pathway. Erythrocytic PNPase acts primarily upon inosine and guanosine with reduced affinity for xanthosine, and essentially no affinity for adenosine[3]. Deficiency of PNPase has been demonstrated in defects of T-cell immunity and is associated with the ultimately fatal severe combined immuno-deficiency[4–6].

Current methods for the assay of PNPase are radioisotopic and spectrophotometric. Spectrophotometric assays, first developed by Kalckar[7], then by others[8,9], are based upon the differential absorption of uric acid at 293 nm. The uric acid is produced by reaction of hypoxanthine, formed from inosine, with xanthine oxidase. This method may lack specificity, as many components of blood might absorb at 293 nm. Additionally, the reverse reaction of PNPase, in the direction of synthesis of the nucleoside, is known to exist in erythrocytes lysates and fibroblasts in higher

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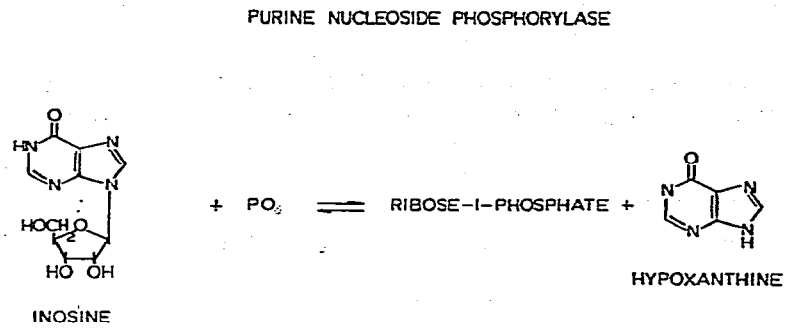

Fig. 1. Reversible reaction of erythrocytic PNPAse with inosine as the substrate and hypoxanthine and ribose-1-phosphate as the products.

activity than the forward reaction[6,10]. With the spectrophotometric methods, one is unable to monitor both the forward and reverse reactions.

A number of radiochemical assays exist[11,12]. While these assays may be sensitive and specific, they suffer the disadvantages of being time consuming and expensive.

High-performance liquid chromatography (HPLC) has been shown to be a powerful tool when used in the analysis of constituents of physiological fluids, since a large number of compounds in a complex matrix may be assayed simultaneously[13–15]. Thus, HPLC is highly suitable for enzyme analysis where the substrate can be rapidly separated from reaction product(s). A number of enzyme assays utilizing HPLC exist[16–18]. These assays have demonstrated that the technique provides a sensitive, reproducible and accurate means of activity measurement. Therefore, HPLC was chosen for development of an assay for PNPase. The reaction optimized was the conversion of the nucleoside inosine to the base hypoxanthine. Additionally, a preliminary investigation of the reverse reaction was undertaken.

EXPERIMENTAL

Instrumentation

A Waters Model ALC 204 liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model U6K injector and a Model 440 dual-wavelength absorbance detector was used for the analysis. Detection was at 254 and 280 nm. Integration was performed electronically with a Hewlett-Packard 3380A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). Samples were incubated at 25°C in a thermostated shaker bath (Lab-Line Instruments, Melrose Park, IL, U.S.A.). All separations were performed on a pre-packed 25 × 4 cm I.D. C-18 column (Whatman Partisil 5-ODS, 5 μm particle size). In order to protect the analytical column, a 5-cm column packed with pellicular C-18 (particle size 30–37 μm) was installed before the analytical column. Centrifugations were performed in a Dynac centrifuge (Clay Adams, Parsippany, NJ, U.S.A.).

Reagents

Inosine, hypoxanthine, xanthine, uric acid, xanthine oxidase, ribose-1-phosphate, guanine, guanosine, inosine-5′-monophosphoric acid and tris(hydroxymethyl)-

aminomethane (Trisma Base) were purchased from Sigma (St. Louis, MO, U.S.A.). Distilled, deionized water used for preparation of eluents and standard solutions filtered through membrane filters, pore size 0.45 μm (Millipore, Medford, MA, U.S.A.). Distilled-in-glass methanol was obtained from Burdick and Jackson (Muskegon, MI, U.S.A.) and potassium dihydrogen phosphate from Mallinckrodt (St. Louis, MO, U.S.A.).

Chromatographic conditions

All analyses were performed in the isocratic mode, at ambient temperature, and at a flow-rate of 2 ml/min. The eluent used was 0.02 *F* KH_2PO_4 (pH 4.2)–3% methanol.

Sample preparation

Whole blood was collected by subcubital venipuncture into Vacutainer tubes, purple top, EDTA(K_3) anticoagulant. Blood not used immediately was refrigerated at 4°C.

Erythrocytes were isolated by centrifugation for 10 min at 1145 relative centrifugal force (RCF). After centrifugation, the plasma and the buffy coat were removed and the remaining cells washed in ten volumes of cold isotonic saline (0.9%, w/v, NaCl). The cells were then packed by centrifugation for 20 min at 1145 RCF. Two hundred microliters of packed cells were suspended in 10 ml of isotonic saline and served as the stock of blood used in the individual assays.

Enzymatic assays

After optimization of reaction conditions, standard protocol was adopted. Two hundred microliters of stock blood (2% suspension) were pipetted into 100 × 13 mm glass tubes. Cells were lysed by the addition of 200 μl of water, followed by freezing and thawing. At time zero, 200 μl of xanthine oxidase (activity *ca.* 0.2 U/ml in incubation mixture) and 1 ml of 2 m*M* inosine, in 0.1 *M* phosphate buffer, were added to initiate the reaction. After incubation for 10 min at 25°C, with continuous shaking, the reactions were terminated by immersion of the test-tubes in boiling water for 1 min. Immersion for less than 1 min did not completely terminate the reaction. The samples were then centrifuged for 3 min and the supernatant was transferred to a clean tube. Samples were then analyzed by HPLC.

The reverse reaction was performed in a similar manner. The substrate added was 1 ml of 1.5 m*M* hypoxanthine and in addition 200 μl of 7 m*M* ribose-1-phosphate, both dissolved in Tris–HCl buffer (pH 7.4), were added. The activity of the enzyme was determined by measurement of the decrease in substrate or the increase in product. One unit is defined as the amount of enzyme converting 1 μmole of substrate to product in 1 min at 25°C.

RESULTS AND DISCUSSION

Fig. 2 shows rapid separation of the substrate inosine from the reaction products hypoxanthine and uric acid. The analysis time is less than 10 min. Chromatograms of samples (Fig. 3) taken from the reaction mixture at 5, 25 and 45 min show the decrease of inosine and the concomitant increase of uric acid with time. Excellent

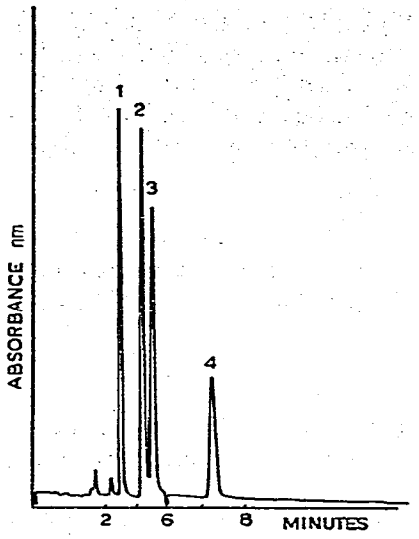

Fig. 2. Separation of the components of the reaction studied by HPLC. Chromatographic conditions: isocratic elution, 2 ml/min, 0.02 *F* KH_2PO_4 (pH 4.2)–3% methanol. Peaks: 1 = uric acid; 2 = hypoxanthine; 3 = xanthine; 4 = inosine.

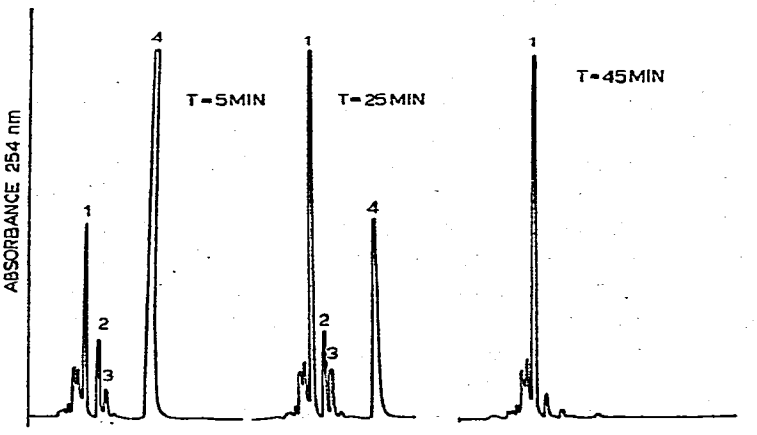

Fig. 3. Reaction of PNPase as a function of time. Chromatograms at various time intervals show the decrease of the substrate inosine (4) and the increase of the products uric acid (1), hypoxanthine (2) and xanthine (3).

instrumental linearity was observed over the concentration range used in the assay. Fig. 4 shows the calibration graphs for uric acid and inosine.

pH

The behavior of PNPase with different pH values is shown in Fig. 5. As reported by other workers[17,18], the enzyme exhibits a rather broad pH optimum between 6.8 and 8, with a maximum of pH 7.48.

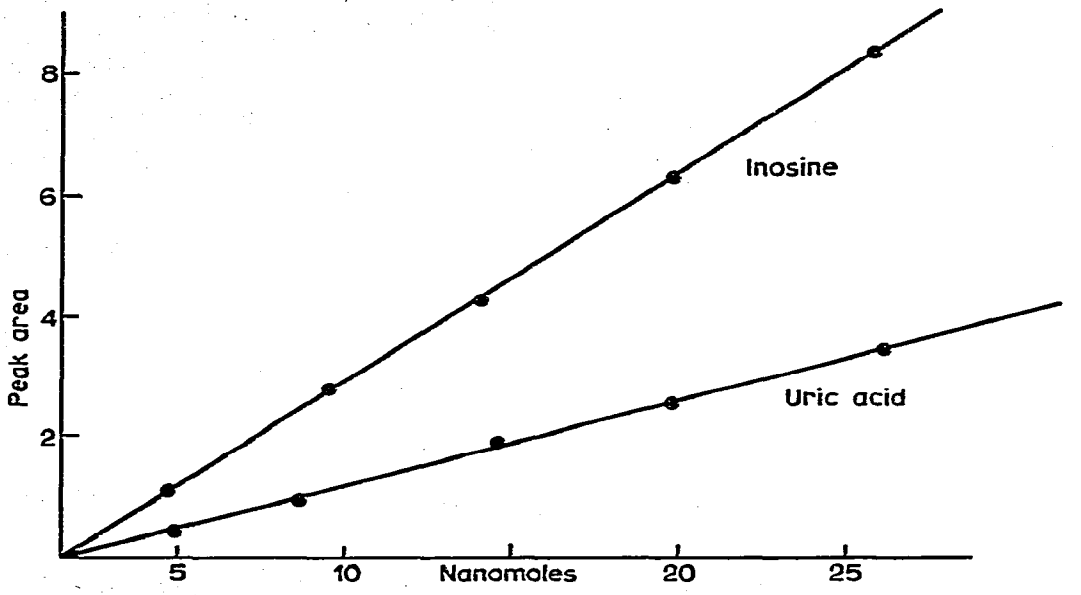

Fig. 4. Instrument response curve for inosine and uric acid over the range of 5–25 nmole, under the conditions given in Fig. 2.

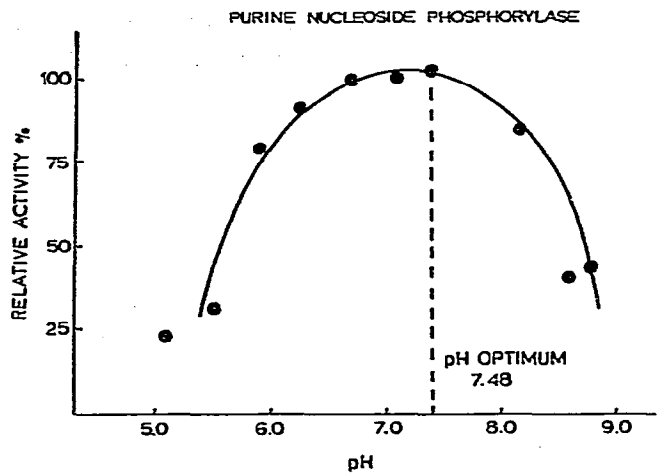

Fig. 5. Effect of pH on activity of PNPase. The pH chosen for subsequent assays was 7.4.

Enzyme concentration

The fractional volume of erythrocytes was varied under constant total incubation volume (Fig. 6). The activity was found to be linear in the range used in the assay; larger volumes of blood can be used provided that sufficient xanthine oxidase is added. PNPase is fairly sensitive to end-product inhibition by the base. The presence of xanthine oxidase removes the hypoxanthine by converting it to uric acid. Four

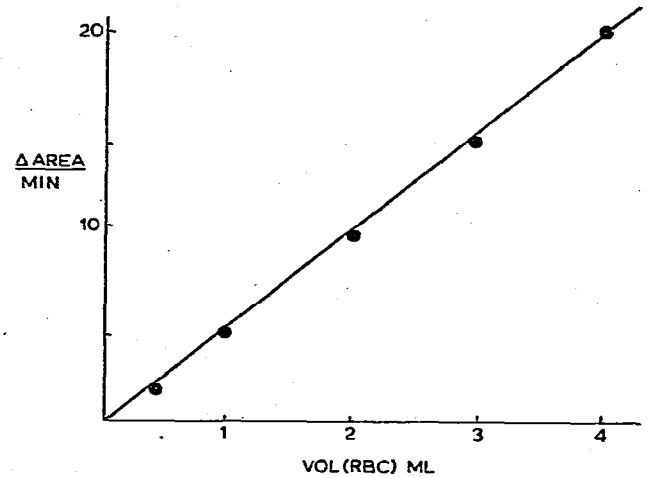

Fig. 6. PNPase activity as a function of volume of erythrocytes. A volume of 4 μl was chosen for the final protocol conditions.

microliters was chosen as the volume for the assay in order to obviate the need for excessive amounts of xanthine oxidase and to minimize the incubation time.

Time

The reaction of PNPase was found to be linear up to 26 min. Fig. 7 shows the linear increase of uric acid and linear decrease of inosine with time. Ten minutes was chosen as the reaction time.

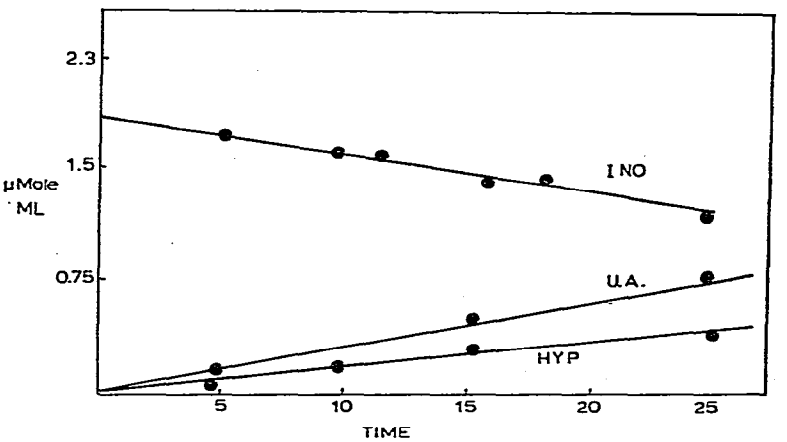

Fig. 7. Linearity of the reaction with time. A final incubation time of 10 min was chosen for the assay.

Substrate

The Michaelis–Menten constant (K_m) was determined for the enzyme under the previously established assay conditions. This constant is specific for an enzyme under

a given set of reaction conditions. The K_m was generated from a modified Edie–Hofstee transformation of the Michaelis–Menten equation:

$$\frac{[S]}{V} = \frac{[S]}{V_m} + \frac{K_m}{V_m} \quad (1)$$

where [S] is the initial substrate concentration in the incubation medium and V is the reaction velocity. K_m was found to be 0.13 mM with a regression coefficient of 0.998. The substrate concentration in the incubation medium was, therefore, set at 1.3 mM, a value equal to 10 times K_m. This substrate concentration insured saturation of the enzyme and hence zero-order kinetics for the assay. As reported by other workers[8,10], the enzyme is erratically activated by high substrate concentrations. This activation is thought to be the result of multivalency of the enzyme causing cooperative binding by the active sites[8]. In the present assay, activation occurred at substrate concentrations of greater than 3 mM in the incubation mixture.

Reverse reaction

In vivo, PNPase is believed to function primarily in the direction of breakdown of the nucleoside. However, a system in which organic phosphate has been excluded, the enzyme catalyzes the formation of the nucleoside. In measurement of formation of inosine from hypoxanthine and ribose-1-phosphate care was taken to avoid contamination from phosphate.

Activity

Using the described procedure, the average activity of eight normal erythrocyte samples was 6.53 ± 0.57 U/ml of packed cells for the forward reaction and 12.17 ± 0.63 U/ml of packed red cells for the reverse reaction. The precision of the assay (relative standard deviation) was calculated from ten identical assays and found to be 3.6%. If needed, the overall precision of the assay can be improved through increasing the incubation time and/or increasing the amount of enzyme (volume of erythrocytes) in the system. Table I shows the range of values obtained. Direct comparison with literature values is not meaningful, as different reaction conditions and units for reporting enzyme activity were used. A value of 13 units/ml of packed cells has been

TABLE I

PURINE NUCLEOSIDE PHOSPHORYLASE ACTIVITY FROM HUMAN ERYTHROCYTES

Sample No.	*Inosine → hypoxanthine (units/ml)*	*Hypoxanthine → inosine (units/ml)*
1	7.34	13.16
2	5.79	11.71
3	5.97	11.66
4	7.12	12.79
5	6.16	11.34
6	6.98	12.63
7	6.29	12.05
8	6.60	11.98
Average	6.53 ± 57	12.17 ± 63

reported[19]. The assay used to obtain this value was performed at 37°C. As one would expect an approximate doubling of the activity with a 10°C rise in temperature, this activity appears to agree with the value obtained by our assay.

CONCLUSIONS

The use of reversed-phase HPLC for the determination of enzyme activity offers a number of advantages over other methods. HPLC allows the rapid determination of both substrate and products. The analysis time of the present assay is less than 10 min after reaction termination. Detection limits for all nucleosides and bases are in the picomole range, offering good sensitivity. As both the substrate and the product are determined simultaneously, activity can be calculated from the decrease in substrate and from the increase in product. Any interference would then be seen from a discrepancy in these values. For example, in the present assay, IMP could be formed through hypoxanthine–guanine–phosphoribosyl transferase. This reaction could be detected through an increase in IMP or by noting the unequal changes in products and substrates.

The present assay, although performed in erythrocytes, is applicable to any biological matrix, with minor modification.

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