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ASSAY OF HUMAN ERYTHROCYTE PYRIMIDINE AND DEOXYPYRIMIDINE 5′-NUCLEOTIDASE BY ISOCRATIC REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We report a rapid and reproducible assay for activity of human erythrocyte pyrimidine 5′-nucleotidase and deoxypyrimidine 5′-nucleotidase. The nucleotides CMP, UMP, dUMP, dCMP or dTMP are individually incubated 30 min at 37°C with erythrocyte hemolysate and 4 m*M* magnesium chloride in Tris, pH 7.5. Data are provided for standardization of the reaction with each substrate. Individual nucleoside products are assayed in less than 10 min by reversed-phase high-performance liquid chromatography at 280 nm with 0—14% methanol in 0.01 *M* potassium dihydrogen phosphate. This is the first report of a high-performance liquid chromatographic assay system which allows quantitation of the activity of pyrimidine 5′-nucleotidase isozymes using five individual pyrimidine and deoxypyrimidine nucleotides as the substrates.

INTRODUCTION

This report describes the use of reversed-phase high-performance liquid chromatography (HPLC) to quantitate the activity of isozymes of erythrocyte pyrimidine 5′-nucleotidase (P5N). There are at least two isozymes of erythrocyte P5N, which dephosphorylate the 5′-pyrimidine and 5′-deoxypyrimidine mononucleotides [1—4]. P5N deficiency is one of the more common causes of congenital hemolytic anemia but the occurrence of abnormalities of deoxypyrimidine 5′-nucleotidase (dP5N) has not been reported. Assay for activity of both P5N and dP5N can confirm deficiencies of P5N and offers the possibility of exploring abnormalities of the dP5N. Erythrocyte P5N activity is increased in reticulocytosis and anemias characterized by a young population of red cells but decreased in β-thalassemia

trait [5], certain leukemias [6] and in lead poisoning [7]. Activity of dP5N in similar disorders is under investigation.

The method reported has several advantages over current assays. Assays based on the liberation of inorganic phosphorus from a specific mononucleotide such as cytidine monophosphate (CMP) require overnight dialysis of hemolysate to remove endogenous phosphate followed by a 2-h incubation [8]. The radiometric procedure of Ellims [9] requires overnight dialysis; that of Torrance et al. [10] requires approximately 2 h for sample preparation and incubation and with it, we have been unable to achieve acceptable reproducibility. Harley and Berman [11] utilized HPLC separation of labelled products of uridine. Rocco et al. [12], Ericson et al. [13], and Sakai et al. [14] have used HPLC to measure P5N by release of uridine from uridine monophosphate (UMP) but not with other nucleosides including the deoxyribonucleosides which are necessary for quantitation of dP5N activity. Reversed-phase HPLC for analysis of deoxypyrimidine nucleotides and their nucleosides has not been applied to the enzymatic assay of biological material [15, 16]. The method we report requires less than 10 min of HPLC running time for identification of cytidine, deoxycytidine, uridine, deoxyuridine and deoxythymidine allowing rapid quantitation of these products from a methanol—potassium dihydrogen phosphate buffer system.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Model 110A reciprocating pump (Beckman-Altex, St. Louis, MO, U.S.A.), a fixed-wavelength UV Monitor III (Laboratory Data Control, River Beach, FL, U.S.A.) and a syringe-loading injector, Model 7120 (Rheodyne, Berkeley, CA, U.S.A.) with a 10-μl fixed-volume sample loop. Samples were injected by Hamilton syringe with a blunt 22-gauge needle. An Ultrasphere ODS (25 cm $\times$ 4.5 mm I.D., 5 μm particle diameter) column was used in conjunction with a Brownlee guard cartridge RP-18 Spheri 5, particle size 5 μm (Rainin Instrument, Woburn, MA, U.S.A.). Peak heights were recorded with a Series 5000 Recordall (Fisher Scientific, Pittsburgh, PA, U.S.A.). The mobile phase was filtered using a Millipore vacuum system and cellulose membrane filters, 0.5 μm pore size, and degassed by vacuum before mixing.

Materials

HPLC-grade potassium dihydrogen phosphate and methanol for the mobile phase were purchased from Fisher Scientific. The hemoglobin determination kit, Tris buffer, UMP, deoxythymidine monophosphate (dTMP), deoxyuridine monophosphate (dUMP), deoxycytidine monophosphate (dCMP) and their corresponding nucleosides were purchased from Sigma (St. Louis, MO, U.S.A.). CMP was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Anhydrous magnesium chloride was obtained from Aldrich (Milwaukee, WI, U.S.A.).

HPLC conditions

The isocratic system utilized an aqueous 0.01 *M* potassium dihydrogen

phosphate solution, pH 4.0. Varying amounts (0—14%) of methanol were added, depending on the retention time of the product (see Table I). The mobile phase was pumped at 1.0 ml/min. The chart speed was set at 0.5 cm/min. The UV detector had a fixed wavelength of 280 nm. The a.u.f.s. (range) was set at 0.016, 0.032, or 0.064 depending on the product peak height. The column was maintained at room temperature (approximately 23°C).

Hemolysate enzyme preparation

Heparinized venous blood was washed with 3 vols. of 0.85% saline followed by centrifugation at 4500 *g* and removal of the supernatant after each wash to yield leucocyte-free red blood cells. The erythrocytes were diluted with cold distilled water to four times the original volume of whole blood to initiate hemolysis, then centrifuged at 14 750 *g* for 3 min. Samples were chilled throughout to minimize enyzme degradation. Hemolysate could be stored frozen up to two weeks, but subsequently lost activity. Data reported here were obtained using hemolysate frozen for one to seven days.

Hemolysate can be prepared using as little as 100 μl of whole blood. Data reported here are based on hemolysates prepared from approximately 10 ml of whole blood. The amount of blood used to prepare hemolysate should be consistent, since this variable may affect the amount of product. A 100-μl aliquot of whole blood yields 250—350 μl hemolysate, sufficient for four to five assays, and permits replicate assays of two isozymes.

Hemoglobin determination

A 20-μl aliquot of hemolysate was analysed for hemoglobin by the cyanmethemoglobin procedure, utilizing Drabkin's reagent [17].

Enzyme assay

The assay for enzyme activity is based on the modification of the procedures reported for UMP [12, 14] as substrate. A micromethod was employed which uses minimum amounts of substrate and hemolysate and optimizes magnesium chloride concentration and incubation time. Substrate was prepared in 0.05 *M* Tris, pH 7.5, to deliver the final substrate concentration in 50 μl. Magnesium chloride in 50 μl of 0.05 *M* Tris, pH 7.5, to yield 4 m*M* final concentration, and 50 μl hemolysate were added to substrate in microcentrifuge tubes. The mixture was incubated for 30 min in a 37°C water bath with gentle agitation. The reaction was halted by placing the microtubes in a boiling water bath for 1.5 min. Blanks were prepared by boiling samples of each hemolysate 1.5 min, then adding substrate and magnesium chloride prior to incubation.

Samples were centrifuged (Fisher Model 235 microcentrifuge) at 22 500 *g* for 4 min. Supernatant fractions were removed and recentrifuged before injection onto the HPLC column. We found, unlike Rocco et al. [12], that reaction mixture frozen at −20°C could be rechromatographed without loss of product for up to six weeks.

Standard preparation

Standard solutions of uridine, cytidine, deoxythymidine, deoxyuridine and

deoxycytidine were prepared in 0.05 *M* Tris buffer, pH 7.5, at concentrations of 2.5, 5.0, 10.0, 20.0 and 40.0 μg/ml. Linear regression equations based on standard injections were used to convert peak heights from μg/ml to μmol nucleoside released after 30 min incubation. International units were calculated as μmol nucleoside per h per g hemoglobin.

RESULTS AND DISCUSSION

Retention times of the pyrimidine monophosphates and their nucleosides eluted at varying concentrations of methanol in 0.01 *M* potassium dihydrogen phosphate, pH 4.0 are presented in Table I. Substrate—product separation after the reaction of P5N with CMP and UMP assays were satisfactory at 2—6% methanol; dCMP and dUMP and their deoxynucleosides were separated at 6—9% methanol and the formation of thymidine from dTMP was best measured at 14% methanol. Rocco et al. [12] employed 10% methanol in 0.01 *M* potassium dihydrogen phosphate, pH 5.1, for the assay of rat erythrocyte UMPase but the chromatographic conditions involved other differences with longer retention times for uridine.

Typical chromatograms of the substrate—product pairs obtained at 2% methanol are shown in Fig. 1A and B. Each nucleotide—nucleoside pair was cleanly separated using the specified conditions of Table I. Supernatants from a series of deoxythymidine-producing reactions were combined. Repeated injections from this combination contained 2.41 ± 0.05 mg/dl deoxythymidine ($\bar{x}$ ± S.E.M., n = 10).

The linearity of standards in Tris buffer is illustrated in Fig. 2. Standards were run daily. The relative variability of deoxythymidine was 4.4%, a typical value. Similar linearity was obtained when standards were added to aliquots of inactivated hemolysate instead of Tris buffer assuring that quantitation of product was complete. The recovery of nucleoside standards added to appropriate reactions after incubation averaged 96% (n = 16).

TABLE I

RETENTION TIMES (min) OF PYRIMIDINE MONOPHOSPHATES AND THEIR NUCLEOSIDES

	Methanol concentration (%) in 0.01 *M* potassium dihydrogen phosphate, pH 4.0					
	0	3	6	9	13	14
CMP	4.5	3.3	2.9	2.6		
UMP	4.6	3.4	3.1	2.7		
dCMP	7.2	4.5	3.4	3.1		
dUMP	7.8	4.7	3.5	3.0		
dTMP	16.0	8.1	5.3	4.0	3.7	3.5
Cytidine	12.0	6.4	4.7	3.3		
Uridine	17.0	9.0	6.1	4.5		
Deoxycytidine	23.0	9.5	6.6	4.7		
Deoxyuridine	28.4	13.2	8.4	5.6		
Deoxythymidine	35	34.1	19.5	11.0	8.2	7.0

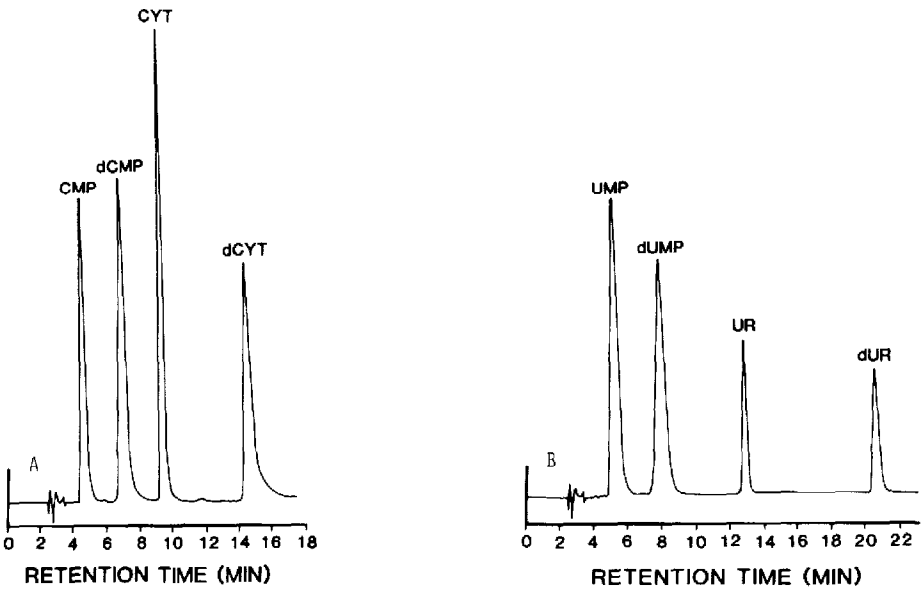

Fig. 1. Separation of the standards for the substrate nucleotides and their nucleoside products by 2% methanol in 0.01 *M* potassium dihydrogen phosphate, pH 4.0. Flow-rate 1.0 ml/min; UV detector wavelength 280 nm; 0.032 a.u.f.s. (A) 0.033 m*M* CMP, 0.033 m*M* dCMP, 0.055 m*M* cytidine (CYT), 0.059 m*M* deoxycytidine (dCYT). (B) 0.06 m*M* UMP, 0.06 m*M* dUMP, 0.04 m*M* uridine (UR), 0.04 m*M* deoxyuridine (dUR).

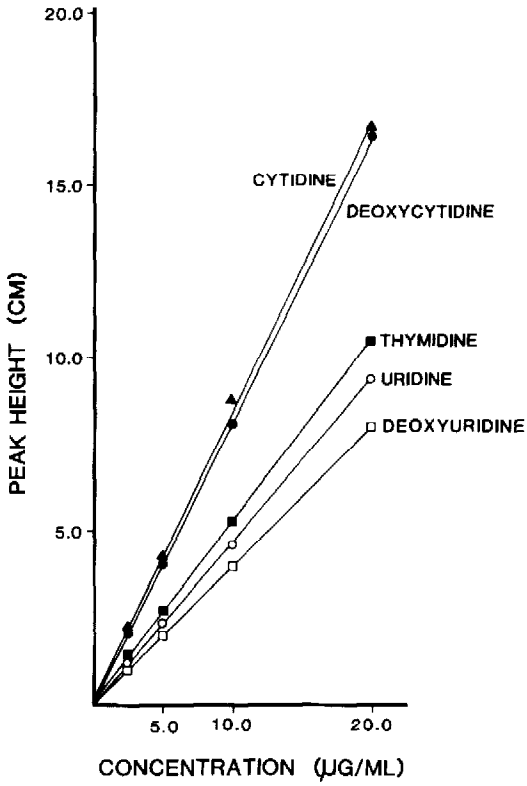

Fig. 2. Peak height (cm) versus concentration (µg/ml) for the nucleosides and deoxynucleosides at 0.032 a.u.f.s.

To investigate the possible interference of extraneous peaks with the enzymatic assay, chromatograms were run of inactivated hemolysate and buffer (Fig. 3A), inactivated hemolysate and substrate (Fig. 3B) and fresh hemolysate plus substrate which permitted product identification (Fig. 3C). These figures are representative of the quantitative product formation after reaction of hemolysate with each of the pyrimidine nucleotides.

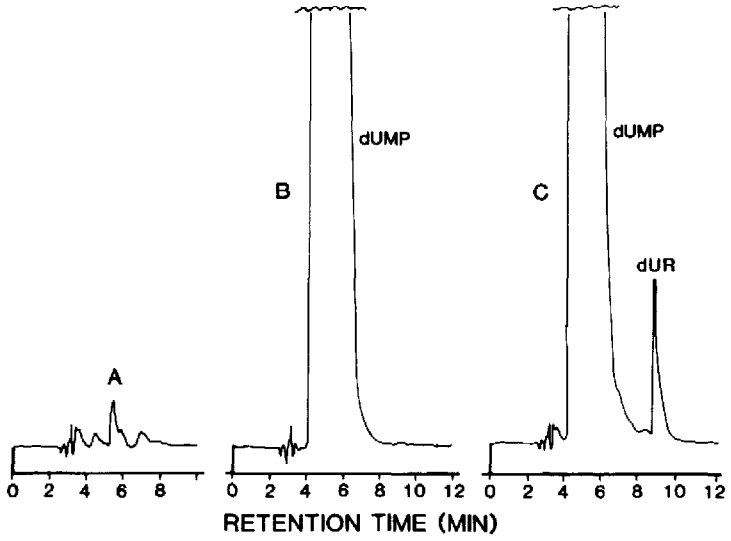

Fig. 3. Chromatographic assay of dP5N. Methanol 6% in 0.01 *M* potassium dihydrogen phosphate, pH 4.0. (A) Erythrocyte hemolysate, boiled, showing baseline peaks. (B) Hemolysate, boiled, and incubated with 2 m*M* dUMP. (C) Hemolysate incubated 30 min with 2 m*M* dUMP showing formation of deoxyuridine (dUR).

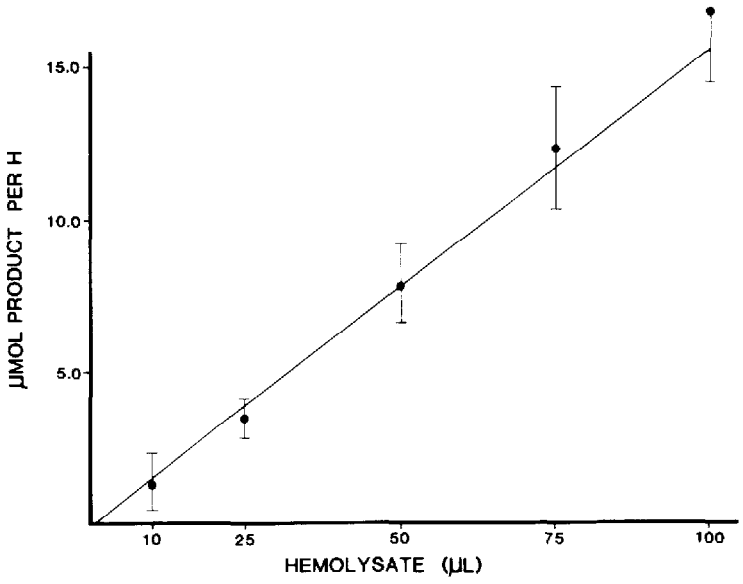

Fig. 4. Enzymatic release of deoxythymidine in μmol/h from 2 m*M* dTMP, pH 4.0, after 30 min incubation, at varying concentrations of hemolysate.

When nucleotide pairs such as CMP and dCMP or UMP and dUMP were incubated with hemolysate, chromatographic separation of the resulting mixture of substrates and products was possible. However, nucleoside yields from simultaneous assays were not equal to yields from the assay of the corresponding individual nucleotides possibly reflecting cross-reactivity of P5N and dP5N with the nucleotides tested. The presence of purines, pyrimidines and nucleosides, especially adenosine, adenine, guanosine, guanine, inosine and cytidine, also inhibits nucleotidase activity [8].

The linearity of enzymatic release of the nucleoside from 2 m*M* dTMP with increasing amounts of hemolysate or hemoglobin is shown in Fig. 4. A 50-μl aliquot, (containing approximately 0.001 g hemoglobin) is on the linear portion of the hemolysate–product relationship.

Fig. 5 illustrates the linearity of deoxythymidine formation per g hemoglobin versus incubation time, employing 50 μl hemolysate with 2 m*M* dTMP. A 30-min incubation produced suitable peak heights for quantitation of all nucleosides.

The release of nucleosides in international units (μmol/h/g hemoglobin) at

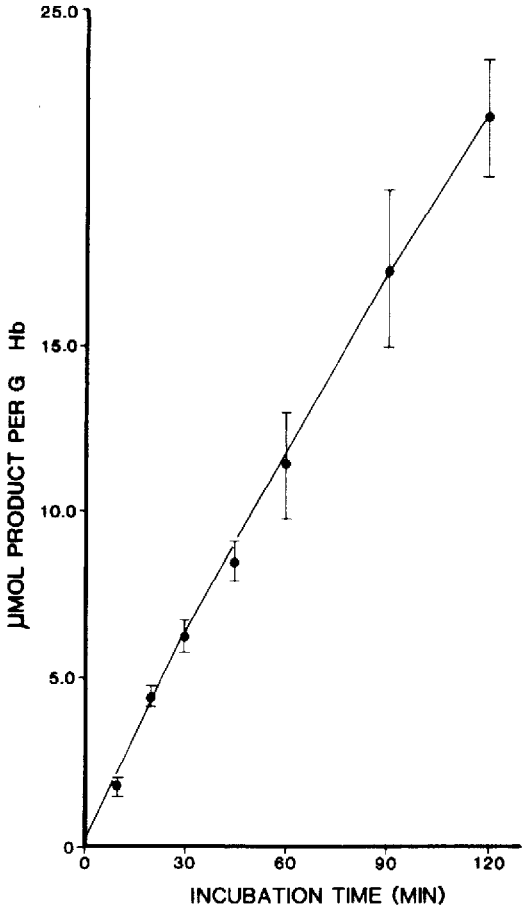

Fig. 5. Enzymatic release of deoxythymidine in μmol/g hemoglobin versus incubation time of 2 m*M* dTMP with 50 μl hemolysate at pH 4.0.

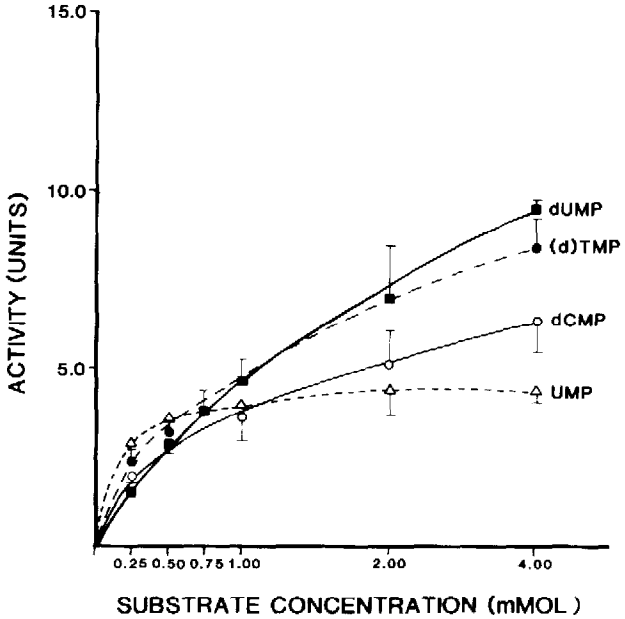

Fig. 6. Nucleoside formation in international units (μmol/h/g hemoglobin) versus concentration of the nucleotide substrates.

varying concentrations of nucleotides is shown in Fig. 6. The reaction appears linear at approximately 0.1—0.5 m*M* concentrations, depending on the nucleotide, with maximum product above 1.0 m*M*. Paglia and Valentine [8], Rocco et al. [12] and Sakai et al. [14] all employed a nucleotide concentration of 2.0—2.3 m*M* while Ericson et al. [13] utilized 0.3 m*M* UMP. Normal erythrocyte enzyme activity in μmol/h/g hemoglobin for 2 m*M* UMP is similar to that of Paglia and Valentine [8] but somewhat lower than that of Sakai et al. [14]. Paglia et al. [4] reported maximal nucleotidase activity for dTMP and dUMP at pH 6.2; activity for dCMP peaked at pH 6.8 but declined more rapidly at pH 7.0 to 8.0 than did activity for UMP and CMP. At pH 7.5, normal erythrocyte nucleotidase activity for all five substrates is the range of 5—10 I.U. An average 9.2 ± 0.8 I.U. ($\bar{x}$ ± S.E.M.) deoxythymidine was produced when ten controls were assayed using 4 m*M* dTMP substrate concentration.

TABLE II

MAGNESIUM CHLORIDE CONCENTRATION AND dP5N ACTIVITY WITH dTMP 1.0 m*M* AS SUBSTRATE

Each value is the mean ± S.D. of six subjects.

$MgCl_2$ (m*M*)	Activity (I.U.)*
0	8.8 ± 0.8
4	12.1 ± 1.0**
8	10.8 ± 1.2
12	9.2 ± 0.8

*International units (I.U.) are μmol/h/g hemoglobin.
**$p < 0.05$ for 4 m*M* magnesium chloride versus zero.

Table II summarizes the effect of varying concentrations of magnesium chloride on the activity of dP5N with 1 m*M* dTMP. The reported assays for P5N and dP5N utilize 0—12 m*M* magnesium chloride [4, 8, 13]. Our data show that magnesium chloride increases the activity of both P5N and dP5N with maximal activity at 4 m*M* magnesium chloride for all five nucleotides tested.

This report of the range of conditions for the HPLC analysis of the nucleoside products from the reaction of human erythrocytes with two pyrimidine nucleotides (CMP, UMP) and three deoxypyrimidine nucleotides (dCMP, dUMP, dTMP) simplifies the assay of both P5N and dP5N. The relative ease of this assay should facilitate biomedical screening for congenital deficiencies of erythrocyte P5N or dP5N, or acquired deficiencies such as seen with increased blood lead concentrations [7]. Standardization of methodology is relevant to investigations of the biological significance of the two or more isozymes of human erythrocyte P5N [4] and to mechanisms of abnormal erythrocyte accumulation of pyrimidine nucleotides and their nucleoside phosphodiesters with enzyme deficiency [18, 19].

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