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DETERMINATION OF 5′-NUCLEOTIDASE ACTIVITY IN HUMAN ERYTHROCYTES AND PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the determination of 5′-nucleotidase activity in human erythrocytes and plasma. Using reversed-phase high-performance liquid chromatography; the product (uridine) was separated from the substrate (uridine-5′-monophosphate) in less than 4 min. The activity determined closely agreed with that determined by the conventional method, in which the inorganic phosphate released is measured. The present method eliminates the need for dialysis of enzyme solution prior to the assay, and offers several advantages over other assay methods, including high sensitivity.

INTRODUCTION

5′-Nucleotidase is widespread in various animal tissues and determinations of the activity in human erythrocytes and serum have diagnostic value for some disorders. The enzyme activity in erythrocytes (pyrimidine 5′-nucleotidase) is deficient in hereditary haemolytic anaemia and is inhibited in lead poisoning[1–5]. Serum 5′-nucleotidase activity is increased in diseases of the liver and bilary tract[6,7].

High-performance liquid chromatography (HPLC) has been utilized for the assay of several enzymes[8–12]. This paper describes an HPLC method for the determination of the activity in human erythrocytes and in plasma. Uridine 5′-monophosphate (UMP) is used as the substrate and the product (uridine) formed is separated by automated reversed-phase chromatography.

EXPERIMENTAL

Materials

UMP, uridine and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma (St. Louis, MO, U.S.A.). Methanol, potassium dihydrogen phosphate, hydrochloric acid, and magnesium chloride were purchased from Wako (Osaka, Japan) and 1-decanesulphonic acid from Tokyo Kasei Kogyo (Tokyo, Japan).

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Preparation of buffered substrate

For low blank values to be obtained in the assay, commercially available UMP was purified by ion-exchange chromatography[13]. All procedures for purification were carried out at 4°C. A 25-ml volume of 50 m*M* UMP was applied to a 3 × 4 cm column of 400 mesh Dowex 1-X8 (Cl^-). The column was washed with 1 l of water to remove uridine. Subsequent elution with 20 m*M* HCl gave UMP free from uridine. Peak fractions (about 160 ml) were pooled and the concentration of UMP was determined from the absorbance at 262 nm in 10 m*M* HCl, with an extinction coefficient of $10.0 \cdot 10^3$ (ref. 14). A 160-ml volume of purified UMP (the pooled fractions) was added to 10 ml of 1 *M* Tris, and carefully adjusted the pH to 7.7 with 1.5 *M* HCl. After adjusting the volume to 200 ml by adding water, the concentration of UMP was also adjusted to 3.12 m*M* with 50 m*M* Tris–HCl buffer pH 7.7. Thus prepared Tris-buffered UMP was stored at −20°C.

Enzyme solution and incubation procedure

Heparinized venous blood from normal subjects was the enzyme source. Erythrocytes were washed twice with a 10-fold volume of saline (0.9%), and then suspended in the saline (about 50% suspension). The enzyme solution was prepared by diluting 50 µl of erythrocyte suspension or 100 µl of plasma to 300 µl with distilled water. The standard assay mixture contained 300 µl of the enzyme solution, 400 µl of Tris-buffered UMP, and 50 µl of 150 m*M* $MgCl_2$. The incubation was carried out at 37°C for 60 min, and the reaction was terminated by placing tubes in boiling water for 3 min. The mixture was diluted 2-fold with distilled water and then centrifuged. The resulting supernatant was used for the HPLC analysis.

Automated HPLC

The chromatographic system employed was a Shimazu LC-3A (Shimazu, Kyoto, Japan), consisting of a Model LC-3A pump, an automatic sample injector (SIL-2AS), a column oven (CTO-2AS), a variable-wavelength spectrophotometer (SPD-1) and an integrator (C-R1A). A disposable MPLC guard column (RP-18 cartridge, 30 × 4.6 mm; Brownlee Labs., CA, U.S.A.) and a 5-µm reversed-phase column (Shodex ODSpak, 150 × 4.6 mm; Showa Denko, Tokyo, Japan) were used for the separations. The mobile phase was 5% methanol containing 5 m*M* potassium dihydrogen phosphate and 0.25 m*M* 1-decanesulphonic acid. The flow-rate, column temperature, and wavelength were set at 1.0 ml/min, 40°C and 254 nm, respectively. Samples were cooled to 4°C during a series of analyses, and 10 or 20 µl were injected at 7-min intervals.

Other analyses

The pyrimidine 5′-nucleotidase activity of erythrocyte lysate dialysed was also determined by the method of Valentine *et al.*[1]. Hb concentrations in erythrocyte suspension and lysate were determined using a haemoglobin counter (TOA Medical Electronic Co., Tokyo, Japan), which directly measured Hb concentrations spectrophotometrically as cyanmethaemoglobin.

RESULTS AND DISCUSSION

In the present system, the desired separation of the product (uridine) from the substrate (UMP) and blood components was achieved in less than 4 min (Fig. 1). The substrate used here was almost free from uridine. In the blank with no added UMP no uridine peak was found in either erythrocytes or plasma, from normal and abnormal subjects. There was also no detectable peak of uridine even if the samples stored at 4°C for 2 weeks were incubated without UMP, although two or three other peaks that were completely separated from the uridine peak appeared in the chromatogram. Hence the blank for each sample can be omitted in routine analysis.

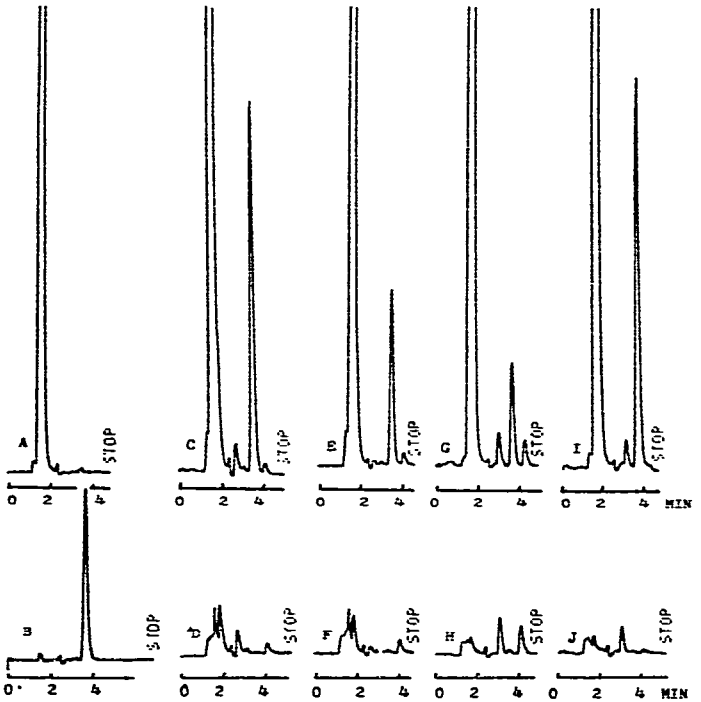

Fig. 1. Separations of uridine from UMP and blood components. A and B were the chromatogram of UMP in the reaction mixture without enzyme, and uridine standard (50 μM), respectively. Samples were incubated with (C, E, G and I) or without UMP (D, F, H and J). C and D: erythrocytes from a normal subject; E and F: erythrocytes from a lead-poisoned subject; G and H: plasma from a normal subject; I and J: plasma from a person suffering from hepatobiliary disease.

There was no effect of boiling on the uridine level in the reaction mixture. The reaction of both enzymes was linear over a broad range with respect to the amount of enzyme added (less than 100 μl).

Fig. 2 indicates the relationship between reaction time and the activity. A reaction time of 60 min was adopted because it seemed to be sufficiently long for the decreased activity to be detected. In the present method measuring uridine with high sensitivity, the reaction time was shortened for erythrocyte enzyme. In the conven-

TABLE I

PRECISION OF THE ASSAY

	Mean ± S.D. (n = 10)	*C.V. (%)*
Erythrocytes		
normal	16.0 ± 0.342 μmol/h/g Hb	2.14
abnormal*	7.28 ± 0.376 μmol/h/g Hb	5.16
Plasma		
normal	13.3 ± 0.299 μmol/min/l	2.25
abnormal**	43.2 ± 0.879 μmol/min/l	2.03

* Lead poisoning.
** Hepatobiliary disease.

tional method it is incubated for 2 h for enough amount of phosphate to be released[1]. Michaelis constants for UMP in normal erythrocyte enzyme were 0.154 m*M*.

To evaluate the precision of the method, we calculated within-run coefficients of variation for normal and abnormal samples (Table I). We assayed 10 replicates for each sample in a single analytical run. The results indicate that the method is sufficiently accurate to detect the abnormal activity.

The enzyme activity in erythrocyte lysate that had been dialysed overnight was measured both by the method of Valentine *et al.*[1] and by the present method (Table II). The data for the uridine formed measured by the present method agreed well with those obtained by the conventional method, measuring inorganic phosphate released. This suggests that all of the uridine formed from UMP can be measured by the present method. The S.D. shown in Table II included both the biological variation

TABLE II

COMPARISON OF THE HPLC METHOD WITH THE METHOD OF VALENTINE *ET AL.*[1]

Sample No.	*Pyrimidine 5'-nucleotidase activity (μmole/h · g Hb)*	
	HPLC method	*Method of Valentine et al.*[1]
1	14.6	15.2
2	11.7	12.1
3	14.4	14.7
4	12.9	13.2
5	13.6	14.8
6	15.3	15.9
7	12.6	12.2
8	10.8	11.1
9	11.4	12.0
10	13.3	13.0
Mean ± S.D.	13.1 ± 1.40	13.5 ± 1.54

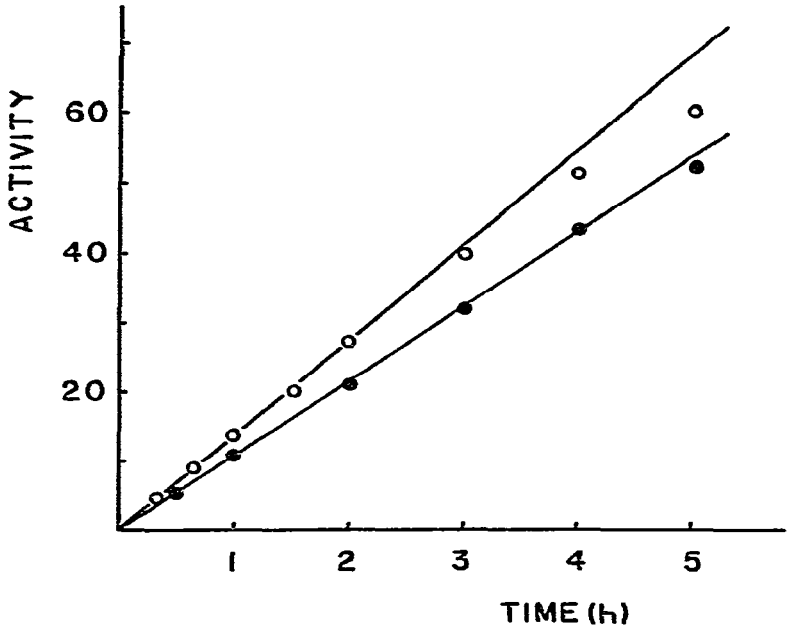

Fig. 2. Relationship between time of incubation and the activity. O, Erythrocyte enzyme (μmol/h/g Hb); ●, plasma enzyme (μmol/min/l).

and the S.D. of the method mentioned above. The biological one or the normal limit of the activity remains to be determined.

The method of Valentine *et al.*[1] requires dialysis of erythrocyte lysate prior to enzyme assay, to remove inorganic phosphate that is already present in erythrocytes. It is also necessary to incubate the samples for relatively long periods of time for enough phosphate to be released. In our method, in contrast, dialysis of the samples is not necessary and the incubation time is shortened, because of the negligible amount of endogenous uridine and the high intensity of the absorption of uridine at 254 nm. Hence the present method should be more useful for routine analysis in clinical laboratories and for biochemical research on the enzyme.

REFERENCES

1 W. N. Valentine, K. Fink, D. E. Paglia, S. R. Harris and W. S. Adams, *J. Clin. Invest.*, 54 (1974) 866.
2 D. E. Paglia, W. N. Valentine and J. G. Dahlgren, *J. Clin. Invest.*, 56 (1975) 1164.
3 I. Ben-Bassat, F. Brok-Simoni, G. Kende, F. Holtzmann and B. Ramot, *Blood*, 47 (1976) 919.
4 W. N. Valentine, D. E. Paglia, K. Fink and G. Madokoro, *J. Clin. Invest.*, 58 (1976) 926.
5 D. E. Paglia, W. N. Valentine and K. Fink, *J. Clin. Invest.*, 60 (1977) 1362.
6 T. F. Dixon and M. Purdom, *J. Clin. Pathol.*, 7 (1954) 341.
7 A. Belfield and D. M. Goldberg, *J. Clin. Pathol.*, 22 (1969) 144.
8 S. N. Pennington, *Anal. Chem.*, 43 (1971) 1701.
9 J. Uberti, J. J. Lightbody and R. M. Johnson, *Anal. Chem.*, 80 (1977) 1.
10 R. Hartwick, A. Jeffries, A. Krstulovic and P. R. Brown, *J. Chromatogr. Sci.*, 16 (1978) 427.
11 N. D. Danielson and J. A. Huth, *J. Chromatogr.*, 221 (1980) 39.
12 S. S. Chen and D. Hsu, *J. Chromatogr.*, 210 (1981) 186.
13 M. Smith and H. G. Khorana, *Methods Enzymol.*, 6 (1963) 645.
14 R. M. Bock and N.-S. Ling, *Arch. Biochem. Biophys.*, 62 (1956) 253.