A simplified method for determining erythrocyte pyrimidine 5'-nucleotidase (P5N) activity by HPLC and its value in monitoring lead exposure

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ABSTRACT The method for determining erythrocyte pyrimidine 5'-nucleotidase (P5N EC 3.1.3.5) activity has been simplified using an automated high performance liquid chromatograph (HPLC). The activity determined by the simplified method agreed closely with that obtained by conventional methods. In 161 lead workers P5N activity declined linearly with increasing blood lead concentrations (Pb-B) between 20 and 80 $\mu\text{g}/100\text{ g}$, and correlated well with Pb-B ($r = -0.87$). For the same group of workers, correlation coefficients between Pb-B v ALA-D activity, zinc protoporphyrin, ALA-U, and coproporphyrin were -0.87 , 0.73 , 0.70 , and 0.32 , respectively. At Pb-B $\geq 40\text{ }\mu\text{g}/100\text{ g}$, the validity of P5N (1.86 at a cut off of $10 \leq \text{units}$) was higher than that of other indicators examined. P5N activity was fairly stable during the storage of samples for two weeks at 4°C . Determination of P5N activity by this method may be a useful indicator in screening for moderate exposure to lead.

Pyrimidine 5'-nucleotidase (P5N, EC 3.1.3.5) was first reported by Valentine *et al* in 1974 in their description of haemolytic anaemia.¹ The enzyme in the red cell cytosol catalyses the hydrolytic dephosphorylation of pyrimidine 5'-monophosphates but is ineffective on purine nucleotides. The reaction is thought to be a necessary step in the degradation of ribosomal RNA in maturing red cells. Through this mechanism, the erythrocyte is able to diffuse pyrimidine nucleotides as soluble nucleosides and retain purine nucleotides as a source of ATP.

The activity is extremely reduced in certain hereditary non-spherocytic anaemias and in lead poisoning.¹⁻³ Many investigators have confirmed that the measurement of P5N activity has a diagnostic value for these disorders.⁴⁻⁶ Several workers have also reported negative linear correlations between blood lead concentrations and P5N activity in lead workers.^{3,7-9} Thus the measurement of P5N activity appears to be a useful index of lead exposure or lead poisoning. Despite this, there have been few reports in which P5N activity has been determined as an index of lead exposure.

The conventional method for measuring P5N activ-

ity is based on the liberation of inorganic phosphorus from pyrimidine 5'-nucleotides and requires time-consuming and complicated procedures, limiting its application in a clinical laboratory.¹

We have previously developed a rapid and sensitive method of determining P5N activity, using reversed phase high performance liquid chromatography (HPLC).¹⁰ In the present paper we report on a further simplification of the method and its use in evaluating lead exposure. We have also studied the relation between P5N activity and other biological parameters of lead exposure.

Materials and methods

Heparinised venous blood was obtained from 161 lead workers who were employed in secondary smelters, glass factories, storage battery plants, and printing offices. To determine P5N activity, the erythrocytes were washed twice with a tenfold volume of saline (0.9% NaCl) and then suspended in the saline (about 50% suspension; RBC/saline). P5N activity was determined by the method previously reported¹⁰ with some modifications. We modified the preparation of the reaction mixture as follows: the enzyme solution was prepared by diluting $50\text{ }\mu\text{l}$ of RBC/saline to $600\text{ }\mu\text{l}$ with distilled water. Commercially available UMP-

Na (uridine 5'-monophosphate disodium salt) was used as the substrate without any purification instead of buffered substrate in the original method.⁸ The standard assay mixture contained 600 μ l of enzyme solution, 100 μ l of 0.2M Tris-HCl buffer (pH 7.7) containing 75 mM MgCl₂, and 50 μ l of 50 mM UMP-Na (Sigma Chemical Co, Saint Louis, USA). The reaction was started by adding the substrate at 37°C. After 60 minutes the reaction was terminated by placing the tubes in boiling water for three minutes. Then 2.25 ml of distilled water was added to the mixture and agitated. After centrifugation, the resulting supernatant was used for the HPLC analysis. A Shimadzu liquid chromatograph (Shimadzu, Kyoto, Japan) consisting of a pump (LC-3A), an automatic sample injector (SIL-2A), a column oven (CTO-2A), a variable wavelength spectrophotometer (SPD-1), and an integrator (C-R1A) was used. Flow rate, oven temperature, and detector wavelength were set at 1.0 ml/min, 40°C, and 254 nm, respectively. The column (150 $\times$ 4 mm) used was packed with reversed phase (ODS) silica. The mobile phase was 5% methanol containing 5 mM KH₂PO₄ and 0.2 mM 1-decanesulphonic acid. Samples were cooled at 4°C during a series of analyses, and 10 μ l were automatically injected at seven minute intervals. The activity in dialysed erythrocytes was also determined by the original method,¹⁰ using buffered substrate purified by ion exchanger. The unit of activity was expressed as micromoles of uridine formed per g Hb per hour.

Haemoglobin (Hb) concentrations in RBC/saline were determined using a haemoglobin counter (TOA Medical Electronic Co, Tokyo, Japan), which directly measured Hb concentrations spectrophotometrically as cyanmethemoglobin. Blood lead concentrations (Pb-B) were determined by the method previously reported¹¹; ALA-D by the standardised European method¹²; zinc protoporphyrin (ZP) by means of a haematofluorometer (Buchler Instruments, Fort Lee, NJ, USA); delta-aminolaevulinic acid in urine (ALA-U) by the method of Tomokuni and Ogata¹³; and coproporphyrin in urine (CP-U) by the method of Soulsby and Smith.¹⁴

Results

In our original method commercially available UMP was purified by ion exchange chromatography to provide the low blank values for the assay. UMP (free acid) is a labile compound and spontaneously decomposes into uridine, which is found in small quantities as a contaminant in commercial UMP. UMP-Na is rather more stable than the free acid and in the present study UMP-Na was used as the substrate without any purification. Figure 1 shows the chromatographic separation of the product (uridine) from the substrate

Fig 1 Separations of uridine from UMP and blood components. A; 10 μ M uridine, B; blank without enzyme solution, C; blank without substrate (UMP), and D; UMP incubated with enzyme solution.

and from erythrocyte constituents. Peak height or area of uridine shows P5N activity. Excellent resolution is achieved in less than four minutes. In the blank without addition of substrate there is no peak corresponding to uridine. Hence the blank for each sample may be omitted in routine analyses. In the blank without addition of enzyme solution a minute peak corresponding to uridine is detected, indicating that the substrate used contains a small amount of uridine as an impurity or a decomposition product. Thus the blank value must be subtracted from the uridine concentrations obtained. The data obtained by the present method (y) compared well with those using the original method (x): $y = 1.003 \times + 0.119$, $n = 28$, $r = 0.99$ (data not shown).

In table 1 the data showing the relation between Pb-B and P5N activity and other indicators of lead effect are given. A good negative correlation was found between Pb-B and P5N activity (fig 2) and between Pb-B and ALA-D activity. The correlation coefficient for Pb-B v P5N is greater than those for Pb-B v ZP, ALA-U, or CP-U. The dose-effect relation for the various biological parameters are

Table 1 Correlations between Pb-B and its biological effect

Regression equation	Correlation coefficient
$\log \text{P5N} = -0.00793 \text{ Pb-B} + 1.312$	$r = -0.80$
$\log \text{ALA-D} = -0.0128 \text{ Pb-B} + 1.819$	$r = -0.87$
$\log \text{ZP} = 0.0158 \text{ Pb-B} + 1.622$	$r = 0.73$
$\log \text{ALA-U} = 0.00876 \text{ Pb-B} + 0.154$	$r = 0.70$
$\log \text{CP-U} = 0.00677 \text{ Pb-B} + 1.443$	$r = 0.32$

Table 2 Mean values (SDs) of Pb-B ($\mu\text{g}/100\text{ g}$), P5N (u), ALA-D (u), ZP ($\mu\text{g}/100\text{ g}$), ALA-U (mg/g Crn), and CP-U ($\mu\text{g}/\text{g Crn}$) in the different groups; less than 10, 20, 30, 40, 50, and over 50 $\mu\text{g Pb-B}/100\text{ g}$

Groups	No	Pb-B	P5N	ALA-D	ZP	ALA-U	CP-U
< 10	35	6.6 (1.7)	17.7 (3.0)	62.3 (11.3)	63.7 (31.7)	2.3 (0.8)	50.9 (19.3)
< 20	33	15.7 (3.4)	17.4 (1.2)	61.7 (1.2)	57.5 (1.5)	1.5 (1.3)	34.7 (1.7)
< 30	44	25.2 (3.3)	15.3 (2.6)	39.9 (10.4)	78.0 (42.7)	2.7 (1.0)	49.6 (25.7)
< 40	32	33.9 (3.0)	15.1 (1.2)	38.9 (1.3)	69.2 (1.6)	2.2 (1.3)	37.2 (1.8)
< 50	8	44.1 (2.3)	13.7 (2.7)	30.7 (7.3)	102 (49.4)	3.0 (1.0)	53.6 (29.8)
≥ 50	7	62.8 (11.7)	13.5 (1.2)	30.2 (1.3)	93.3 (1.6)	2.4 (1.3)	38.9 (1.7)
			12.1 (2.4)	24.0 (5.8)	179 (94.9)	3.3 (1.0)	57.0 (35.9)
			12.0 (1.2)	23.4 (1.3)	158 (1.6)	2.6 (1.3)	38.0 (1.8)
			8.5 (2.0)	18.8 (3.4)	287 (139)	5.1 (0.9)	90.0 (64.0)
			8.2 (1.3)	18.6 (1.2)	257 (1.6)	4.0 (1.4)	61.7 (2.1)
			5.8 (1.9)	13.1 (3.7)	493 (353)	6.4 (3.7)	200 (162)
			5.6 (1.4)	12.6 (1.3)	398 (2.1)	5.6 (1.8)	155 (2.2)

1st row: arithmetic mean (arithmetic SD). 2nd row: geometric mean (geometric SD).

shown in fig 3 and table 2. The group was subdivided into groups with Pb-B levels at less than 10, 20, 30, 40, 50, and over 50 $\mu\text{g}/100\text{ g}$, and the geometric means and SD of P5N, ALA-D, ZP, ALA-U, and CP-U were plotted against the Pb-B values (fig 3). ALA-D

Table 3 Correlations between P5N and other biological effects of lead

Regression equation	Correlation coefficient
$\log \text{P5N} = 0.497 \log \text{ALA-D} + 0.371$	$r = 0.73$
$\log \text{P5N} = -0.316 \log \text{ZP} + 1.756$	$r = -0.68$
$\log \text{P5N} = -0.504 \log \text{ALA-U} + 1.311$	$r = -0.62$
$\log \text{P5N} = -0.618 \log \text{CP-U} + 1.397$	$r = -0.34$

Fig 2 Relation between Pb-B and P5N activity.

activity shows 37% and 70% reductions with Pb-B in the range 10–20 and 40–50 $\mu\text{g}/100\text{ g}$, respectively, compared with a Pb-B of less than 10 $\mu\text{g}/100\text{ g}$. The rate of inhibition of ALA-D decreased considerably at Pb-B values over 40 $\mu\text{g}/100\text{ g}$, levels that are important for monitoring lead exposure. By contrast, P5N shows a more linear reduction in activity with a

Table 4 Validity of P5N, ALA-D, ALA-U, and ZP at Pb-B $\geq 40\text{ }\mu\text{g}/100\text{ g}$

	SE	SP	VD
P5N (u):			
12	1.00	0.74	1.74
10	0.93	0.93	1.86
8	0.60	1.00	1.60
ALA-D (u):			
25	1.00	0.74	1.74
23	0.93	0.88	1.81
20	0.80	0.92	1.72
ALA-U (mg/g Crn):			
4	0.67	0.88	1.55
4.5	0.67	0.97	1.64
5	0.53	0.98	1.51
ZP ($\mu\text{g}/\text{dl RBC}$):			
100	1.00	0.64	1.64
150	0.93	0.83	1.76
200	0.73	0.92	1.65

SE, sensitivity; SP, specificity; VD, validity.

Table 5 Effect of sample storage on the activity

Sample No	P5N activity ($\mu\text{mol}/\text{h/g Hb}$)			
	Days after sampling			
	0	7	14	28
1	16.2	16.8	15.7	15.1
2	18.4	18.3	17.8	14.7
3	16.4	16.1	16.2	13.8
4	16.9	16.9	16.4	14.0
5	14.3	14.0	14.0	13.1
6	15.3	15.3	15.0	15.1
$\bar{x}$	16.25	16.23	15.85	14.30
(%)	(100)	(99.9)	(97.5)	(88.0)
SD	1.40	1.48	1.30	0.80

Fig 3 Relation between Pb-B and some haematological parameters. Geometrical means (○) and range of SD (vertical line) are shown in different Pb-B groups; less than 10, 20, 30, 40, 50, and over 50 µg/100 g.

rise in Pb-B values. P5N activity at Pb-B of 40–50 µg/100 g is only about 50% of that of levels of less than 10 µg/100 g. Since a 13.2% inhibition in P5N activity is found even with Pb-B segment of 10–20 µg/100 g, the threshold (no effect) level of Pb-B for P5N is less than 10 µg/100 g. The threshold concentration of Pb-B for ZP is less than 20 µg/100 g and ZP concentrations increase exponentially as Pb-B exceeds 30 µg/100 g. ALA-U and CP-U increase significantly only when Pb-B concentrations exceed 40 µg/100 g.

The data in table 3 shows the relation between P5N and the other biological indicators of effect. Highly significant ($p < 0.01$) correlations were found between all the indicators examined. With respect to P5N the correlations were ALA-D > ZP > ALA-U

> CP-U. The validity (sensitivity + specificity) of P5N, ALA-D, ZP, and ALA-U was compared for Pb-B values over 40 µg/100 g (table 4). The validity of P5N (1.86 at a cut off of ≤ 10 units) is higher than that of all other indicators examined. Table 5 shows the changes in P5N activity during the storage of blood at 4°C. There is no significant change a week after sampling, whereas at two weeks, the reduction in P5N activity is only 2.5%. Thus P5N activity is fairly stable if the sample is stored at 4°C.

Discussion

P5N catalyses the hydrolysis of pyrimidine 5'-monophosphate to yield pyrimidine nucleosides and inor-

ganic phosphate. Techniques for assay of P5N activity fall into two categories; one based on the determination of inorganic phosphate and the other on the determination of pyrimidine nucleosides. In the conventional method for determining P5N activity the amount of inorganic phosphate released is determined colorimetrically.¹ Inorganic phosphate (and other sources of inorganic phosphate) are normally present in erythrocytes, so to reduce the blank values the phosphate must be removed by dialysing the lysate before the enzyme assay. It is also necessary to incubate the samples for relatively long periods to release sufficient phosphate. Because of these disadvantages the determination of P5N activity has not been used frequently in the routine monitoring of lead exposure. The other type of assay of P5N activity was reported by Torrance *et al.*¹⁵ In this method radioactive CMP (¹⁴C-CMP, cytidine 5'-monophosphate) was used as the substrate, which was adsorbed on to barium sulphate after the reaction had finished and the ¹⁴C-cytidine formed was counted with a liquid scintillation counter. This method eliminates the need for dialysis of the enzyme solution and is also extremely sensitive. The method cannot easily be carried out in every clinical laboratory, however.

In our assay P5N activity is also determined by measuring liberated pyrimidine nucleosides. The product is separated from the substrate and from red cell constituents by means of HPLC. In this method dialysis of the samples is not necessary and the incubation time is shortened because the amount of endogenous uridine is non-detectable and the intensity of the absorption of uridine at 254 nm is high. In our original method¹⁰ commercial UMP was purified by ion exchange chromatography to obtain UMP free from uridine. In the present method we used UMP-Na which is more stable than UMP (free acid) and eliminated the purification step. By these modifications the procedure was further simplified. Both methods gave identical results.

The mean control values ($\pm$ SDs) for P5N activity have been reported as 7.1,¹ 6.6 $\pm$ 2.0,² 7.38 $\pm$ 2.56,⁴ 9.9 $\pm$ 2.5,³ 7.51 $\pm$ 0.86,⁵ and 12.9 $\pm$ 2.9.¹⁶ These results were determined by the method of Valentine *et al.*,¹ using UMP as the substrate. Mean values obtained with CMP as the substrate are 70.5–83.3% of those given above. Sato *et al.* established the normal value for P5N in Japanese subjects ($n = 209$)⁷ by the method of Valentine *et al.*,¹ using CMP as the substrate. Their mean values $\pm$ SDs for 109 men, 100 women, and all subjects are 13.8 $\pm$ 3.0, 14.0 $\pm$ 3.4, and 13.9 $\pm$ 3.2, respectively. These values are almost twice those found in American and European subjects. Sato *et al.* suggested that the higher activity found in Japanese subject is due to lower Pb-B values.⁷ They obtained a mean Pb-B value ($\pm$ SD) of

7.34 ($\pm$ 3.7) μ g/100 g in their subjects. No concurrent Pb-B values, however, were reported in the studies of the American or European subjects. In the present study the geometric and arithmetic means ($\pm$ SD) of P5N activity are 17.4 ($\pm$ 1.2) and 17.7 ($\pm$ 2.95), respectively, in subjects whose Pb-B values are less than 10 μ g/100 g (mean $\pm$ SD 6.58 $\pm$ 1.6 μ g/100 g, table 2). The mean activity of the group is similar to that reported by Sato *et al.*,⁷ taking into account that the substrate used in our methods is UMP.

In a previous study we found a significant correlation between P5N and Pb-B (log P5N = -0.0732 Pb-B + 1.27, $r = 0.79$, $n = 68$) at Pb-B values less than 65 μ g/100 g.⁸ In the present investigation we obtained almost the same equation (log P5N = -0.00793 Pb-B + 1.312, $r = -0.80$, $n = 161$) in lead workers whose Pb-B were between 3 and 80 μ g/100 g. These correlation coefficients are compatible with the data of Mohammed-Brahim *et al.*⁹ who determined P5N activity in a large population that included workers exposed to lead and other heavy metals; they obtained a correlation coefficient of 0.79 between log P5N and Pb-B (4.9–73.5 μ g/100 ml). According to Paglia *et al.*, correlation coefficients between P5N and erythrocyte lead concentrations are -0.5 and -0.7 (obtained with UMP and CMP as substrate, respectively) in 23 lead intoxicated subjects with Pb-B values over 100 μ g/dl RBC.³ They noted that P5N activity was progressively inhibited until erythrocyte lead concentrations reached 200 μ g/dl RBC, at which point the activity was maximally depressed. Our data indicate that P5N activity was sensitive to lead in blood as low as 10–20 μ g/100 g (fig 3 and table 2). The activity at Pb-B of 10–20 μ g/100 g was reduced by 13.2% compared with that at Pb-B levels of less than 10 μ g/100 g. Thus the threshold (no effect) values of Pb-B for P5N activity may be less than 10 μ g/100 g. Based on these findings and the description by Paglia *et al.*,³ it appears that P5N activity is progressively inhibited over a wide range of Pb-B values between 10 and 100 μ g/100 g, and has a wide field of application in the monitoring of lead effects. By contrast, other parameters seem to have a somewhat restricted application. Although ALA-D is highly sensitive to, and closely correlated with, Pb-B, the activity is almost completely inhibited with Pb-B increases beyond 40 μ g/100 g (fig 3), as we have described previously.¹⁷ ZP increases exponentially when Pb-B exceeds 30 μ g/100 g (fig 3) and ALA-U and CP-U also increase exponentially with Pb-B over 40 μ g/100 g (fig 3). These results are comparable with those reported previously.¹⁸

Strong correlations were found between P5N activity and other biological indicators of lead effect (table 3). Correlation coefficients between log P5N and log ALA-D, log ZP, log ALA-U, and log CP-U were

0.73, -0.68, -0.62, and -0.36, respectively. These data compare with the correlations reported by Mohammed-Brahim *et al.*,⁹ whose correlation coefficients of log P5N v log ALA-D, log ZP, and log ALA-U were 0.76, -0.79, and -0.60, respectively. The strong correlations between P5N and other biological parameters suggest that the decrease in P5N activity is able to predict the disturbance which lead induces in haem biosynthesis. In male workers exposed to lead and other heavy metals Mohammed-Brahim *et al.* reported the validity of P5N and ZP as 1.71 and 1.73, respectively, when Pb-B values were greater than 40 $\mu\text{g}/100\text{ ml}$.⁹ In the present study we obtained a high validity for P5N (1.86 at a cut off of ≤ 10 units) at Pb-B $\geq 40\text{ }\mu\text{g}/100\text{ g}$ and this was higher than those of the other indicators examined (table 4). This indicates that P5N is a good discriminator at moderate Pb-B values.

Although ALA-D activity is the most sensitive indicator of lead exposure and widely used for this purpose, one disadvantage of the test is that the assay must be carried out immediately after sampling. The enzyme is vulnerable and inactivated during storage. To solve this problem we routinely determine the activity ratio by heat treatment or zinc activation or both, of ALA-D in stored samples.¹¹⁻¹⁹ P5N, by contrast, is relatively stable at 4°C (table 5). Torrance *et al.* reported that the reduction of P5N activity was only 1.4% after storage of samples for seven days at 4°C.¹⁵ As the assay of P5N can be carried out with stored samples, it seems to be more suitable than ALA-D for screening lead exposure.

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