Simple Determination of Erythrocyte Pyrimidine 5'-Nucleotidase Activity in Human Blood by High-Performance Liquid Chromatography

Katsumaro TOMOKUNI and Masayoshi ICHIBA

Department of Community Health Science, Saga Medical School, Nabeshima, Saga 840-01, Japan

(Received May 21, 1986 and in revised form July 3, 1986)

Abstract: We developed a method for the determination of erythrocyte pyrimidine 5'-nucleotidase (P5N) activity in human blood using a high-performance liquid chromatograph (HPLC). In this method, UMP (uridine 5'-monophosphate disodium salt) was used as a substrate and assay solutions were directly prepared from whole blood. Uridine formed by the enzyme reaction of P5N was determined spectrophotometrically at 260 nm after separation from the substrate and blood components by HPLC. The optimum pH of this reaction was 7.5.

This HPLC method was very rapid and simple compared to the conventional method, because of eliminating the need for dialysis of erythrocyte lysate prior to the assay.

The present method was applied to the examination of P5N activity in the blood of lead workers, indicating that the P5N activity is significantly inhibited in these workers.

Key words: Erythrocyte—Pyrimidine 5'-nucleotidase—Lead exposure—Inhibition—High-performance liquid chromatography—UMP substrate

INTRODUCTION

Erythrocyte pyrimidine 5'-nucleotidase (P5N) is an enzyme which catalyses the dephosphorylation of pyrimidine nucleotides, i.e., uridine and cytidine 5'-monophosphates (UMP and CMP). Its presence in erythrocytes was first reported by Valentine et al. in 1974.

Several investigators have indicated that erythrocyte P5N activity is strongly inhibited by lead in vivo and the degree of its inhibition has a close correlation with the concentration of lead in blood. These reports suggest that the erythrocyte P5N activity test is useful as an indicator of lead exposure or lead poisoning.

The erythrocyte P5N activity has been measured in most cases according to the conventional method of Paglia and Valentine, in which the amount of inorganic phosphate (Pi) formed in the enzyme assay is determined colorimetrically. However, this method is very time-consuming in large-scale routine
analysis, because it requires dialysis of erythrocyte lysate prior to the enzyme assay, to remove Pi which is already present in erythrocytes. On the other hand, Sakai et al. have developed a method for the determination of erythrocyte P5N activity using a high-performance liquid chromatograph (HPLC). In the latter method, the preparation of the enzyme was carried out using washed erythrocytes, and the product (uridine) formed by the enzyme reaction was separated from the UMP substrate and determined.

In the present study, we further simplified this HPLC method by using whole blood for the preparation of enzyme solution instead of washed erythrocytes. In addition, we investigated the reliability on UMP and CMP as the substrate, when the HPLC method was adopted for determining erythrocyte P5N activity.

**Materials and Methods**

**Blood specimen**

Heparinized blood samples were collected from 20 healthy male adults (age: 35-43) with no history of lead exposure (controls) and from 8 lead-exposed male workers (age: 30-52) being employed in a secondary lead refinery.

**Apparatus**

We used a Shimadzu LC-6A HPLC equipped with a UV-VIS spectrophotometric detector (Shimadzu Ltd., Kyoto, Japan) in this study. We also used an atomic absorption spectrophotometer equipped with deuterium background corrector and graphite furnace atomizer (GFA-3) (Shimadzu Ltd., Kyoto, Japan) for the analysis of lead in blood.

**Reagents**

All reagent used were of analytical grade. Uridine 5'-monophosphate disodium salt (UMP), cytidine 5'-monophosphate (CMP), uridine and cytidine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan). Tris-HCl buffer (50 mM, pH 7.5) and MgCl₂ solution (0.1 M) were used for P5N assay. UMP substrate (4 mM) was prepared by dissolving 74 mg of UMP-2Na in 50 ml of Tris-HCl buffer and stored at 4°C. CMP substrate (4 mM) was also prepared similarly. A stock solution of uridine (1 mM) was prepared by dissolving 12.2 mg of uridine in 50 ml of distilled water. The stock solution was stable for more than 6 months at -20°C. A uridine standard solution (100 μM) for HPLC was prepared by dilution of the stock solution 10-fold with distilled water. A cytidine standard solution (100 μM) was also made in the same way.

**Procedure**

The assay solution for P5N contained 0.6 ml of distilled water, 0.2 ml of whole blood, 0.2 ml of the MgCl₂ solution and 1.0 ml of the UMP substrate. The final volume of the reaction mixture was 2.0 ml. The reaction mixture was incubated for 1 h at 37°C. The enzyme reaction was terminated by placing
the tubes in a boiling water bath for 2 min. After cooling, they were centrifuged at 3,000 rpm for 5 min. An aliquot of the resulting supernatant was pipetted into another test tube and submitted to the HPLC analysis. The HPLC analysis was carried out according to the analytical conditions listed in Table 1. The P5N activity was expressed as μmol of uridine formed per 1 h per 1 g of hemoglobin (Hb) and it was calculated by the following formula:

\[
P5N \text{ activity} = \frac{\text{Peak area of sample}}{\text{Peak area of uridine standard} \times \frac{100}{\text{Hb (g/dl)}}}
\]

**RESULTS AND DISCUSSION**

In the present HPLC method for determining the erythrocyte P5N activity in human blood, a Shim-pack CLC-ODS column was used to separate the product from the substrate and blood components.

Figure 1 shows the separation patterns of uridine from the UMP substrate mixture and from the assay solution prepared with the blood samples from one of the healthy adults and of the lead workers. The relationship between peak area and quantity of uridine standard was linear. The chromatograms obtained from the assay solutions of blood of the healthy adult (Fig. 1B) and of the lead worker (Fig. 1C) showed good separation peaks of uridine. P5N activities of these blood samples, calculated from the peak areas using the above-described formula, were 12.5 and 5.1 μmol uridine h⁻¹(g Hb)⁻¹, respectively.

When blood samples were incubated without UMP or with UMP after deproteinization, no peak corresponding to uridine was found on the chromatogram. Hence the blank measurement for each sample can be omitted in the routine analysis. In the substrate mixture, no dephosphorylation of UMP to uridine by both incubation and heating was observed. In addition, there was no effect of heating on the amount of uridine in the reaction mixture.
Fig. 1. The chromatograms obtained from the UMP substrate (4 mM) containing uridine (100 μM) (A), the P5N assay for a normal blood (B), and the P5N assay for a lead worker’s blood (C).

Fig. 2. The effect of pH on erythrocyte P5N activity in normal blood. The pH values were measured at 37°C in the final incubation medium. P5N activity; μmol uridine h⁻¹ (g Hb)⁻¹.
Fig. 3. Relationship between time of incubation and erythrocyte P5N activity in normal blood.

P5N activity: \( \mu \text{mol uridine h}^{-1} (\text{g Hb})^{-1} \).

Fig. 4. The chromatograms obtained from the CMP substrate (4 mM) containing cytidine (100 \( \mu \text{M} \)) (A), the blank assay without CMP substrate (B), and the P5N assay for a normal blood (C).

a; cytidine, b; uridine
Figure 2 shows the data concerning a pH optimum of erythrocyte P5N activity. Tris-HCl buffer used was adjusted to the required pH with HCl. It was indicated that the P5N enzyme has a pH optimum at 7.5.

As shown in Fig. 3, the relationship between reaction time and the activity was linear within 4 h. On the other hand, it was found that both trichloroacetic acid and perchloric acid which are widely used for stopping the enzyme reaction had some disadvantages on the HPLC analysis. The former reagent gave a positive interference on UV absorption at 260 nm, and the latter induced in part the dephosphorylation of the UMP substrate. Therefore, we terminated the enzyme reaction by heating for 2 min.

As shown in Fig. 4, cytidine was separated from CMP substrate (Fig. 4A) and the peak corresponding to it was not found in the blank measurement (Fig. 4B). When the erythrocyte P5N assay was carried out with CMP instead of UMP as the substrate, two new peaks appeared on the chromatogram (Fig. 4C). One peak (a) was cytidine and the other (b) was identified to be uridine. Further, the peak of uridine also appeared when the normal blood sample was incubated with cytidine instead of UMP. These results suggest that an enzyme converting cytidine to uridine (i.e. cytidine deaminase) is present in human blood. Therefore, CMP is unsuitable as the substrate, when the HPLC method is used for the determination of the erythrocyte P5N activity in human blood.

The results concerning the erythrocyte P5N activity in human blood are shown in Table 2. The P5N activities for 20 normal blood samples measured using the present HPLC method averaged 16.3±5.1 (μmol uridine h⁻¹(g Hb)⁻¹), and ranged from 8.0 to 27.6. In contrast, the P5N activities for 8 lead-exposed workers whose blood levels of lead were 38±11 (μg/100 ml) averaged 7.9±2.3 (μmol uridine h⁻¹(g Hb)⁻¹) with a range of 5.1 to 12.5. In addition, these values had a good correlation with those measured using the conventional method of Paglia and Valentine⁹ (r=0.97).

Erythrocyte P5N activity is known to be inhibited by lead, cadmium and mercury in vitro.⁹,¹² On the other hand, it has been demonstrated by Mohammed-Brahim et al.⁹ that the inhibition of erythrocyte P5N activity in vivo is specific for lead but not for cadmium and mercury and that, therefore, the blood P5N test can be evaluated as a specific indicator of lead exposure.

<table>
<thead>
<tr>
<th>Normal</th>
<th>Mean±SD</th>
<th>16.3±5.1</th>
<th>8±3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lead</td>
<td>Mean±SD</td>
<td>7.9±2.3</td>
<td>38±11</td>
</tr>
<tr>
<td>workers</td>
<td>Range</td>
<td>5.1–12.5</td>
<td>20–57</td>
</tr>
</tbody>
</table>

**Table 2. The data concerning erythrocyte P5N activity and blood lead concentration obtained from normals and lead workers.**
In conclusion, the HPLC method for the determination of P5N activity becomes more rapid and simpler, when the whole blood instead of the washed erythrocytes is used for enzyme preparation and UMP instead of CMP is used as the substrate.

ACKNOWLEDGMENTS

We are grateful to Professor A. Kumon, Department of Biochemistry, Saga Medical School, for his helpful discussion. We also thank Miss K. Funatsu, Department of Community Health Science, for typing this manuscript.

REFERENCES