An automated method for the microbiological assay of serum pyridoxal

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SYNOPSIS A fully automated method for the measurement of serum pyridoxal has been developed. Acid phosphatase was used for dephosphorylation and precipitation of the serum proteins was not required. A chloramphenicol-resistant strain of *L. casei* was used as the test organism and this removed the need for sterilization. The method gives highly reproducible results, and is suitable for population and institutional studies.

The principal form of vitamin B6 found in serum is pyridoxal phosphate. This can be measured by enzymatic methods (Wada, Morisue, Nishimura, Morino, Sakamoto, and Ichihara, 1959; Walsh, 1966; Hines and Love, 1969) or after dephosphorylation by microbiological techniques such as those described by Baker, Frank, Ning, Gellene, Hutner, and Leevy, 1966; and Anderson, Peart, and Fulford-Jones, 1970. Few of these techniques have proved entirely satisfactory and they are not suitable for the measurement of large numbers of samples.

In this paper we describe a fully automated procedure using an acid phosphatase for dephosphorylation of the pyridoxal phosphate. Pyridoxal was measured microbiologically using a chloramphenicol-resistant strain of *Lactobacillus casei*. Chloramphenicol was added to the assay medium and this removed the need for sterilization and permitted the use of automated equipment.

Materials and Methods

**TEST ORGANISM**

The assay organism used was a chloramphenicol-resistant strain of *L. casei* (NCIB8010, ATCC7469). Chloramphenicol resistance was developed by the method described by Davis, Nicol, and Kelly, 1970. It was maintained by drying onto small ceramic beads (Annear, 1962).

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**ASSAY MEDIUM**

Double strength assay medium was prepared according to the protocol shown in table I. A magnetic stirrer was used to assist solution of the solids rather than heat, since it was found that the test organism grew better in a medium which had not been subjected to heat.

Some batches of casein hydrolysate were found to contain small quantities of pyridoxal; this may be destroyed by exposure of the casein to ultraviolet light before it is added to the medium.

The concentration of manganese sulphate is

| Casein hydrolysate (enzymatic NBC) | 200 ml |
| Glucose | 40 g |
| Sodium acetate (anhydrous) | 40 g |
| L-alanine | 5 g |
| L-asparagine | 0.6 g |
| L-cysteine HCl | 0.4 g |
| L-tryptophan | 0.1 g |
| KH₂PO₄ | 1.0 g |
| KCl | 1.0 g |
| Glutathione (reduced) | 5.0 mg |
| Adenine | 10 mg |
| Guanine HCl | 10 mg |
| Xanthine | 20 mg |
| Uracil | 10 mg |
| Calcium pantothenate | 0.8 mg |
| Nicotinic acid | 0.8 mg |
| Thiamine HCl | 0.4 mg |
| Pteroylglutamic acid | 0.02 mg |
| Riboflavin | 1 mg |
| Biotin (10 µg/ml) | 0.02 mg |
| Tween 80 (1:10 dilution) | 1 ml |
| NaCl | 20 mg |
| MgSO₄ | 0.4 g |
| MnSO₄.H₂O | 7.5 mg |
| Distilled water to | 1 litre |
| The pH was adjusted to 6.8 |

Table I Assay media double strength

1Dissolved by suspending in a small volume of water and then adding concentrated KOH drop by drop until solution was complete.
critical; levels above that recommended results in precipitation of the acid phosphatase.

MAINTENANCE MEDIUM
This comprised single strength assay medium supplemented with 0.8 μg of pyridoxal and 30 mg of chloramphenicol base per litre.

PREPARATION OF INOCULUM
On the day before the test, a tube containing 10 ml of maintenance medium was inoculated with one ceramic bead containing the dried organisms. After overnight incubation the optical density of the culture was adjusted to 0.68-0.70.

PREPARATION OF STANDARDS
Pyridoxal HCl was used for the preparation of standards as follows.

Stock solution A
Pyridoxal HCl (equivalent to 0.5 g pyridoxal), 0.609 g, was made up to 1 litre with 0.02 N HCl giving a concentration of 500 mg/l.

Stock solution B
Of solution A 1.0 ml was diluted to 200 ml with 0.02 N HCl giving a pyridoxal concentration of 2.5 mg/l. This solution was stable for one month when kept in the dark.

Working solution
On the day of the test 4 ml of solution B was diluted to 500 ml with distilled water to give a concentration of 20 μg/l. This working solution was used to prepare the final standard solutions shown in the protocol (table II) and these were sampled in the same manner as the serum to be assayed.

Table II Preparation of pyridoxal standards

<table>
<thead>
<tr>
<th>Stock Solution (20 μg/l)</th>
<th>Distilled Water</th>
<th>Final (μg/l)</th>
<th>Concentration (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>4.5</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>1.0</td>
<td>4.0</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>2.0</td>
<td>3.0</td>
<td>8</td>
<td>48</td>
</tr>
<tr>
<td>3.0</td>
<td>2.0</td>
<td>12</td>
<td>72</td>
</tr>
<tr>
<td>4.0</td>
<td>1.0</td>
<td>16</td>
<td>96</td>
</tr>
<tr>
<td>5.0</td>
<td>0.1</td>
<td>20</td>
<td>120</td>
</tr>
</tbody>
</table>

METHOD
A Joyce Loeb Mecolab M automated system was used for setting up and reading the results of the assays.

Serum samples contained in small cups were placed on the inner circular rack and disposable plastic tubes were loaded into the outer rack. Each rack holds 40 tubes.

A diluter on the universal sampler unit was adjusted to sample 0.025 ml of serum, and to dilute this with 2.5 ml of acid phosphatase solution (50 mg Sigma type II acid phosphatase was added to 1 litre of citrate buffer pH 4.6, 230 ml 0.1M citric acid plus 270 ml 0.1M trisodium citrate made up to 1 litre with distilled water). Standards were processed in the same manner except that citrate buffer was used without enzyme. To each standard tube was added 0.025 ml of serum which had been freed of pyridoxal by ultraviolet irradiation. Standards were set up in triplicate with two extra blanks and two extra top standards.

To each litre of double strength assay medium was added 40 mg of chloramphenicol base and 0.8 ml of the overnight culture of the test organism. The flask of medium was placed on a magnetic stirrer on top of the sampler unit. The instrument was set in operation, but this time 2.5 ml of the inoculated medium was delivered into each of the tubes making a final volume of 5.0 ml and giving a serum dilution of 1 in 200. The medium should be added as soon as the enzyme sampling cycle has been completed; this gives a constant enzyme contact time of 13.3 minutes. At least one pyridoxal-free serum is set up with each batch plus others with known pyridoxal levels.

When the medium had been added to all the tubes in a rack it was removed and placed in a 37°C incubator for approximately 24 hours or until the top standard reached an optical density of 0.33. The racks were then returned to the Mecolab, the contents of each tube were mixed and the growth was measured in the autocolorimeter using a 621 μm filter and a 1.0 OD grey screen. Results were recorded in digital form on paper tape.

Calculation of Results
Readings obtained from the standards can be used to plot a standard curve on linear graph paper and the concentration of the unknown determined by interpolation. Alternatively a programmable electronic calculator such as the Diehl Combition S may be used.

Results
RECOVERY OF PYRIDOXAL
Pyridoxal HCl was added to serum containing 240 nmol/l to bring the concentration to 54 and 84 nmol/l respectively. The samples were then assayed and results of 56.4 nmol/l and 82.8 nmol/l equal to recoveries of 108 and 98% were obtained. A similar experiment using Anderson's method (Anderson et al, 1970) gave recoveries of 104 and 92%.
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<table>
<thead>
<tr>
<th>Age</th>
<th>10-19</th>
<th>20-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
<th>60+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>64.5</td>
<td>59.6</td>
<td>55.1</td>
<td>43.9</td>
<td>41.9</td>
<td>35.6</td>
</tr>
<tr>
<td>Range</td>
<td>30.0-96.0</td>
<td>26.4-96.0</td>
<td>25.2-83.2</td>
<td>25.2-66.0</td>
<td>23.4-58.8</td>
<td>19.8-49.8</td>
</tr>
<tr>
<td>Female</td>
<td>54.3</td>
<td>51.2</td>
<td>40.3</td>
<td>39.0</td>
<td>35.1</td>
<td>34.3</td>
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<tr>
<td>Range</td>
<td>28.2-96.0</td>
<td>25.2-90.0</td>
<td>24.0-60.0</td>
<td>21.6-54.0</td>
<td>21.6-51.6</td>
<td>18.0-48.0</td>
</tr>
</tbody>
</table>

Table III  Range and significance of serum pyridoxal levels (nmol/l) related to age and sex

**REPRODUCIBILITY AND EFFECT OF STORAGE**
A sample of serum assayed 14 times over a period of five months gave results ranging from 66.0 to 78.6 nmol/l (mean 68.7, SD 4.9). A sample of serum was assayed 20 times in the same batch and results ranged from 39.0 to 49.2 nmol/l (mean 45.2, SD 3.2).

**COMPARISON WITH ANDERSON’S METHOD**
Twenty samples were assayed in triplicate by both methods. The results obtained with the present method were on an average 30% higher than those obtained with Anderson’s method.

**SERUM PYRIDOXAL CONCENTRATION IN CONTROL SUBJECTS**
Blood was obtained from 371 healthy volunteers, 194 males and 177 females, aged from 12 to 77 years. The results are shown in table III. There was a significant difference between males and females and there was a significant correlation between the serum pyridoxal level and age.

**Discussion**
A rapid, sensitive, fully automated method for the measurement of pyridoxal phosphate in serum has been described. The test appears to be specific for pyridoxal since the test organism is relatively insensitive to the other forms of the vitamin B6, pyridoxine and pyridoxamine.

The use of an acid phosphatase provides a simple method of dephosphorylation and is compatible with the requirements of an automated procedure.

Results obtained by the present method are higher than those obtained by Anderson et al (1970). This may be due to the destruction of serum pyridoxal during the hydrolysis procedure which requires prolonged heating; such treatment leaves the pyridoxal standards unaltered. This suggests that there may be some differences between naturally occurring and synthetic pyridoxal and this requires further investigation.

Using the method described it is possible to set up assays at a rate of 80 an hour and to read results at 160 an hour. The method is particularly well suited for use in population and institutional surveys.

We wish to thank the many volunteers who kindly gave samples of their blood for the establishment of the reference range. This work was supported in part by a medical research grant from the University of Western Australia.

**References**


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