Determination of Uric Acid and Xanthine/Hypoxanthine in Blood Using Immobilized Enzymes in Flow Injection System

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Summary: A flow injection system containing on-line immobilized enzyme mini-columns of urate oxidase and xanthine oxidase/urate oxidase for the determination of uric acid and xanthine/hypoxanthine is described. For the determination of xanthine and hypoxanthine, a xanthine oxidase column was incorporated on-line prior to the urate oxidase column. The results of the uric acid analysis in serum were compared with the commercial kit method in use in hospitals with a correlation coefficient of 0.99 and r.s.d. of 1% (n = 6). The method is fast and the cost per analysis is greatly reduced.

Introduction

In the degradation of nucleotides, most of the purine liberated bases are reinforced in the metabolism but some are oxidized to uric acid via xanthine. Unfortunately, unlike most mammals, in humans, the necessary enzyme, uricase is lacking. Consequently, uric acid becomes the end-product of the purine metabolism.

Owing to its poor solubility in the body fluid, high concentration of uric acid is potentially dangerous because it may lead to sedimentation. Thus determination of uric acid is essential in clinical laboratories especially for the diagnosis and the treatment of gout and other haematologic disorders [1].

The amount of uric acid in serum depends on the genetic and environmental conditions of a person. The normal level in human beings is reported as 3.8 - 7.1 mg/dl and 2.6 - 5.6 mg/dl in serum of adult male and female respectively [2].

Conventional methods of determination of uric acid are based on reduction of phosphotungstate by uric acid in an alkaline solution [3], but they have been subjected to criticism mainly because of non-specificity. Many other compounds in the body fluid such as glutathione, cysteine and glucose in high concentration and drugs like caffeine and salicylate do interfere [4].

The development of enzymatic method based on the use of urate oxidase has been a real progress. Uric acid is oxidized by the enzyme urate oxidase and the decrease in uric acid concentration is monitored spectrophotometrically at 293 nm [5,8]. Alternatively the determination of hydrogen peroxide produced can be monitored amperometrically [7]. The present investigation is based on monitoring the H2O2 produced using an amperometric detector in a flow system as reported earlier [8]. The last step in uric acid biosynthesis is the oxidation of oxypurines (xanthine and hypoxanthine) by xanthine oxidase (XOD) which indirectly indicates the amount of uric acid in the body.

\[ \text{Hypoxanthine} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{XOD}} \text{Xanthine} + \text{H}_2\text{O}_2 \]

\[ \text{Xanthine} + \text{O}_2 + \text{H}_2\text{O}_2 \xrightarrow{\text{XOD}} \text{Uric acid} + \text{H}_2\text{O}_2 \]

\[ \text{Uric acid} + \text{O}_2 + 2\text{H}_2\text{O} \xrightarrow{\text{Urate oxidase}} \text{Allantoin} + 2\text{H}_2\text{O}_2 \]

The determination of these oxypurines has been increasingly required in clinical investigation on gout and other metabolic diseases related to purine metabolism. Therefore attempts were made to incorporate the xanthine oxidase immobilized enzyme column prior to the urate oxidase column in the system for the determination of xanthine and hypoxanthine.

Results and Discussion

Optimization of reaction

Effect of buffer and its pH

The flow injection system for the analysis of xanthine/hypoxanthine and uric acid is shown in

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Fig. 1. Enzymatic flow system for the determination of xanthine/hypoxanthine and uric acid in blood.

First the effect of pH on the activity of uricase was studied by omitting xanthine oxidase column. Soluble uricase exhibits optimal pH activity at 9.0, therefore we chose to study the effect of pH using Tris-HCl buffer having buffering capacity in the range 7.0 - 9.0. Buffer solution (0.1 M) at varying pH values was used as the carrier stream and the response of the uricase column for a standard of uric acid (6.0 mg/dl) was checked. An optimum response at pH 8.0 for the uricase column was observed. The experiment was repeated using another buffer system i.e. borate buffer which also gave the optimum pH 8.0, but the activity of uricase enzyme in this buffer system was low, therefore Tris-HCl buffer (0.1 M, pH 8.0) was used in all further experiments analysing uric acid. This shift to pH 8.0 when the enzyme is immobilized, may be attributed to the new environment for the enzyme and the changes in its kinetic properties after it is immobilized. In the same manner when xanthine oxidase column was incorporated, the effect of pH on the combined system was studied in the range pH 7.0 - 9.0 using Tris-HCl buffer (0.1M) of varying pH values as the carrier stream. A slight decrease in the response was noticed at higher pH values. But as pH 8.0 being the optimum for uricase enzyme as well, was therefore used in the rest of the experiments. Soluble xanthine oxidase exhibits optimum activity at pH 7.5.

Similarly the effect of temperature on the activity of immobilized enzyme system was also investigated by flowing water from a thermostated water bath through the outer water jacket of the enzyme column. The temperature was raised from ambient to about 50°C, and the response of enzyme tested. There was a continuous increase in response with increase in temperature up to 45°C and a slight decrease with further increase in temperature. But for the long life of the enzyme column all further experiments were carried out at 30°C.

The flow rate was also optimized and a flow rate of carrier stream, 2.5 ml/min. was selected for all further experiments as both the tasks, the sensitivity and reasonable throughput could be achieved with it.

Calibration for uric acid

Standard solutions of uric acid in the concentration range 0-10 mg/dl were injected into the flow system shown in Fig. 1, but containing only the urate oxidase column. Each standard was injected in triplicate Fig. 2 shows typical recorder output for the analysis of standard urate samples. The peaks show good within run precision and an excellent linearity in the range investigated as is shown by the calibration graph in Fig. 2. The limit of detection of the system was investigated and it was found that the system could detect easily down to 0.1 mg/dl.

This system offers a great deal of advantage over the conventional colorimetric methods used for urate determination or the use of soluble enzyme. Besides the reduction in cost per analysis, this simple FIA system can increase the speed, reliability and accuracy in routine testing for uric acid. Once the method was optimized, attempts were made to analyse uric acid in blood using the same procedure. Therefore blood samples were obtained from suspected patients. The uric acid concentration in these samples in the hospital is routinely determined using soluble urate oxidase followed by peroxidase treatment in the presence of
Determination of Uric Acid

Calibration for xanthine and hypoxanthine

Standard solutions of xanthine and hypoxanthine were prepared from stock solutions in Tris buffer covering the range 0.25 μmol/l. These standard solutions were simultaneously injected into the system containing the XOD followed by the urate oxidase column. The calibration data obtained is given in Table-2. From the calibration data it can be noticed that the system shows very low sensitivity towards the xanthine and hypoxanthine. This may partly be due to the very small amount of enzyme present in the column (approx. 8 units) which in such a short period of time may not be able to convert the maximum amount of xanthine/hypoxanthine injected. The system needs further investigation to make it sensitive. The two enzymes can be immobilized together and used as a single column for improving its conversion efficiency as has been achieved in several other cases [8,9].

Table-2: Calibration data for xanthine and hypoxanthine determination

<table>
<thead>
<tr>
<th>Concentration (μmol/l)</th>
<th>Xanthine peak height (mm)*</th>
<th>Hypoxanthine peak height (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td>10.0</td>
<td>31</td>
<td>17</td>
</tr>
<tr>
<td>15.0</td>
<td>42</td>
<td>21</td>
</tr>
<tr>
<td>20.0</td>
<td>51</td>
<td>24</td>
</tr>
<tr>
<td>25.0</td>
<td>63</td>
<td>29</td>
</tr>
</tbody>
</table>

*Mean of three injections.

Experimental

Materials and methods

Urate oxidase (EC 1.7.3.3., from hog liver) and xanthine oxidase (1.1.3.22., from butter milk) were obtained from Sigma Chemicals U.S.A. and immobilized on controlled porosity glass by cross linking with glutaraldehyde. The detailed immobilization procedure is described elsewhere [9]. 22.5 Units of uricase were immobilized per gram CPG.

Uric acid used was also obtained from Sigma. Stock solution (100 mg/dl) of uric acid was prepared by dissolving 100 mg of uric acid and lithium carbonate in 30 ml of distilled water at 50°C diluting to 100 ml with distilled water. This stock solution was further diluted to get standards...
in the range 0 - 10 mg/dl. Stock solution of xanthine/hypoxanthine (1.0 mmol/l) was prepared in Tris-HCl buffer (pH 8.0). All other reagents used were of AnalyR grade. The flow injection manifold for uric acid and xanthine/ hypoxanthine analysis is shown in Fig. 1. The components, including the amperometric detector, were as reported previously [10]. Substrate solutions (20 μl) were injected into the carrier stream (0.1 M Tris-HCl buffer, pH 8.0).

Conclusion

The method described for uric acid determination based on the use of immobilized enzyme mini-column in a flow system equipped with electrochemical detector is well suited for uric acid determination in clinical samples. The method can be extended to determine xanthine/hypoxanthine in biological samples.

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References