CHAPTER - 5

DEVELOPMENT AND VALIDATION OF A SPECTROPHOTOMETRIC ENZYMATIC ASSAY METHOD FOR THE QUANTIFICATION OF URIC ACID BY USING 3 - HYDROXYTYRAMINE AS A CHROMOGENIC SUBSTRATE
DEVELOPMENT AND VALIDATION OF A SPECTROPHOTOMETRIC ENZYMATIC ASSAY METHOD FOR THE QUANTIFICATION OF URIC ACID BY USING 3-HYDROXYTYRAMINE AS A CHROMOGENIC SUBSTRATE

5.1 Introduction

3-Hydroxytyramine (3-HT) (4-(2-aminoethyl)benzene-1,2-diol), (Molecular formula = C₈H₁₁NO₂, Molecular mass = 153.18 g/mol) or commonly called as Dopamine, is a simple organic chemical in the catecholamine family, and is a monoamine neurotransmitter, which has a number of important physiological roles in the bodies of animals. In the laboratory, 3-HT can be synthesized by demethylation of 3,4-dimethoxyphenethylamine using hydrogen bromide.

Various analytical techniques have been developed for the determination of 3-HT or dopamine by utilizing the activities of enzymes and these include such as peroxidase (POD), 4-chlorophenol and 4-aminoantipyrine (4-AAP) based on inhibition effect of 3-HT on POD activity (spectrophotometric) [1], biosensor based on L-cysteine self assembled monolayer for 3-HT (square wave voltammeter) [2], bean sprout peroxidase, fluorescence quenching of dopamine (spectrofluorimeter) [3], crude extract of zucchini, on MWCNT for 3-HT determination [4], (Flow injection fluorescence) photo induced electron transfer boronic acid derivative [5], (flow injection analysis coupled with chemiluminescence) luminal-hexacyanoferrate(III) for 3-HT assay [6], and (flow injection spectrophotometric) p-toluidine and sodium periodate for catecholamine [7].

The structure of 3-Hydroxytyramine is shown below
A modest attempt has been made for the development of a method for the quantitative validation of uric acid by the use of 3-HT as a novel chromogenic reagent in the presence of POD, Uricase (UOx) and citric acid-tripotassium citrate buffer of pH 6.8 at room temperature. The reaction conditions such as temperature sensitivity, effect of pH, effects of reagent co-substrate and substrate and effect of incubation period have been determined and optimized for getting maximum color development of the colored solution and for securing maximum absorbance of the resulting product. The catalytic parameters of the enzymatic reaction have been evaluated. Lineweaver-Burk plot was used for the evaluation of Michaelis-Menten constant of uric acid concentration. The proposed enzymatic method has been tried for the quantification of uric acid in human serum samples.
5.2 Experimental

5.2.1 Instrumentation

A Jasco model UVIDEC-610 ultraviolet–visible (UV–Vis) spectrophotometer (Tokyo, Japan) with 1.0-cm matched cells was used in all the absorbance measurements. A water bath shaker (NSW 133, New Delhi, India) was used to maintain constant temperature during color development. All pH measurements and adjustments were done by using a digital pH meter (model EQ-614, Equip-tronics, Mumbai, India). Micropipettes used in the enzymatic assay were from Bohit Proline, Finland. Serum samples were centrifuged using Remi R-24 (Mumbai, India) Research centrifuge having 17,300 rpm and 27,440 ‘g’ RCF. Remi cyclomixer was used for mixing the reaction mixture.

5.2.2 Reagents and solutions

All chemicals used in the assay were of analytical grade and double distilled water was used throughout the assay procedure.

5.2.2.1 3-Hydroxytyramine (3-HT)

3-Hydroxytyramine (98 %) was purchased from SD fine-chem limited, Mumbai. 3-HT (78.33 mM) solution was prepared by dissolving the required quantity in water. The solution was kept under refrigeration for effective maintenance of molarity. Freshly prepared solution was always used.

5.2.2.2 Hydrogen peroxide (H_2O_2)

As presented in Section 3.2.2.2.

5.2.2.3 Citric acid – Tripotassium citrate buffer (pH 6.8)

Stock solution (500 mM) of the above was prepared by dissolving 5.253 g of citric acid monohydrate (Molecular formula = C_6H_8O_7, Molecular mass = 210.14 g/mol) in 50 mL and 8.560 g of tripotassium citrate ((Molecular formula = K_3C_6H_5O_7, Molecular mass = 324.42 g/mol)) in 50 mL of distilled water. The above stock solution of 0.5 M was diluted 8 times to get 62.5 mM. From this 0.6 mL of buffer solution was used for the assay.


5.2.2.4  Horseradish Peroxidase (HRP)

As presented in Section 3.2.2.5.

All solutions were kept under refrigeration at 0-10 °C temperature until use.

5.2.2.5  Uricase (UOx)

Lyophilized UOx (12 units/mg) (94310-5MG) from *Bacillus fastidious* was purchased from Sigma Aldrich Chemicals Pvt. Ltd, Bangalore, India. A stock solution of 12 units/mg was prepared by dissolving lyophilized enzyme in 5 mL of distilled water and stored in a refrigerator at 4 °C. Further dilution with double distilled water was made freshly as and when required.

5.2.2.6  Uric acid

Uric acid (98 %) was obtained from SD fine-chem limited, Mumbai and a stock solution (5.948 mM or 1000 ppm) was prepared using the procedure reported by Trivedi et al., [8]. Sixty mg of Li$_2$CO$_3$ were dissolved in 20 mL of distilled water at 60 °C. While still hot, this solution was filtered through Whatman No. 1 paper and exactly 100 mg of uric acid were dissolved in the hot filtrate. After cooling to room temperature, the solution was diluted with distilled water to 100 mL in a volumetric flask. Dilutions of this stock uric acid standard were used as and when required. The stock solution was stable for at least one month when stored at 4 °C.

5.2.3  Human serum sample collection and preparation for uric acid determination

Human blood samples collected from a local hospital and also from a clinical laboratory were preserved at - 20 °C for use. Blood samples were collected in heparinized tubes and centrifuged. The accuracy of the present method was assessed by comparing the results obtained with a commonly used uric acid assay method [9].

Necessary permission was obtained from Institutional Human Ethical Committee (IHEC-UOM No.22/Ph.D/2008-09) of University of Mysore for the use of human blood samples in the experiment. The patients were well informed and their consents were obtained before collecting the blood samples.
5.2.4 General assay procedure

5.2.4.1 Quantification of hydrogen peroxide

H$_2$O$_2$ concentration in the range of 12 - 1630 µM was used for its quantification by kinetic method containing 2.611 mM 3-HT, 4.73 nM POD, and 12.5 mM citric acid/tripotassium citrate buffer of pH 6.8 in 3 mL reaction mixture. The resulting slope from the regression equation, which is the initial rate of the reaction, was used to plot the graph against the concentration of H$_2$O$_2$ to obtain a standard curve.

5.2.4.2 Quantification of horseradish peroxidase

To a reaction mixture containing 2.611 mM 3-HT and 102 µM H$_2$O$_2$, and 12.5 mM citric acid/tripotassium citrate buffer of pH 6.8, peroxidase enzyme (100 µL) having different concentrations was added. The changes in the absorbance were continuously recorded in the experimental as also in the corresponding control containing all the reagents except peroxidase. The linearity for fixed-time method was also evaluated by incubating the reaction mixture for 5 min at 30 °C and measuring absorbance of the colored solution.

5.2.4.3 Quantification of Uricase

Similarly, UOx was determined in the range of 0 - 12 units/mg in a reaction mixture containing 2.611 mM 3-HT, 200 µL of 5.948 uric acid, 18.92 nM POD and 12.5 mM citric acid/tripotassium citrate buffer of pH 6.8 by the kinetic method. The linearity was observed between 0.022 and 0.18 units/mg in which 0.18 units/mg of UOx was fixed for uric acid assay.

5.2.4.4 Quantification of uric acid

Uric acid assay was carried out by adding to the reaction mixture of 3 mL containing 2.611 mM 3-HT, 0.18 units/mg UOx, 18.92 nM POD and 12.5 mM citric acid/tripotassium citrate buffer of pH 6.8 varying concentrations (3.09 – 991.3 µM) of uric acid solution. The reaction mixture was incubated for 5 min at room temperature (30 °C) and changes in the absorbance of the colored solution were recorded along with the control, which contained all reagents except uric acid. Reaction rate method was also carried out for the quantification of uric acid.
5.2.5 Units of enzymes

One unit of peroxidase activity is defined as that amount of enzyme, which utilizes 1 µmol of H$_2$O$_2$ quinone formation of 1 µmol of 3-HT to form orange colored product per min under standard assay conditions [10].

One unit of Uricase is defined as that amount of enzyme activity which is required to oxidize 1 µmol of uric acid to generate allointon and 1 µmol hydrogen peroxide per min under standard assay conditions [11].

5.3 Results and discussion

5.3.1 Absorption spectrum of H$_2$O$_2$ on the colored product

The absorption spectrum of the colored solution obtained at 76, 102, and 204 µM concentration of H$_2$O$_2$ was measured in the wavelength range of 400 - 600 nm. Then the spectrum was recorded on a spectrophotometer after incubating the reaction mixture for 5 min at 30 °C against the corresponding reagent blank. The results are shown in Figure 5.1. The optimum wavelength for maximum absorption of the colored product was 500 nm.

![Figure 5.1](image)

**Figure 5.1.** Absorption spectrum of H$_2$O$_2$ was recorded in a 3 mL solution containing 2.611 mM 3-HT + 9.46 nM peroxidase in 12.5 mM citric acid/tripotassium citrate at pH 6.8 at varying concentration of H$_2$O$_2$ (76, 102 and 204 µM) and the corresponding reagent blank. Spectrum was recorded after incubating the reaction mixture for 5 min at 30 °C.
5.3.2 Optimization of experimental conditions

Experimental protocol condition parameters such as substrates, co-substrates, different buffer concentrations with pH 3.0 to 8.0, temperature and incubation period, which affect enzyme assay, have been optimized.

5.3.2.1 Effect of pH on the rate of formation of colored product

The influence of pH on the uric acid assay was studied by using different buffers of varying concentrations (0.01 to 2.0 M) such as acetic acid/sodium acetate (pH 3.6–5.6), citric acid/tri potassium citrate (pH 3.6–7.0), KH$_2$PO$_4$/NaOH (pH 6.0–8.0), and KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 6.0–7.5) and tris buffer (pH 9.8). Maximum activity was observed in citric acid/tri potassium citrate buffer of pH 6.8 and it was selected for further assay. Also, any increase in the concentration of buffer showed no effect on the rate of reaction. Whereas decrease in the concentration of buffer from 500 mM to 62.5 mM, resulted in increase in the reaction rate and further decrease in the buffer concentration had no effect on rate of reaction and results in increase in the reagent blank color. Hence, buffer concentration of 12.5 mM (0.6 mL of 62.5 mM in a total 3 mL) was found optimum and selected for the assay procedure.

5.3.2.2 Effect of co-substrate 3-HT concentration on the rate of reaction

The effect of varying concentration of co-substrate 3-HT in the range of 0.652 mM to 10.44 mM was studied in the final 3 mL reaction mixture containing 102 µM H$_2$O$_2$, 4.73 nM POD and 12.5 mM citric acid/tri potassium citrate buffer of pH 6.8. It was observed that the reaction rate increased with increase in concentration of 3-HT up to 2.611 mM beyond which the rate remained constant. Hence for all further assays, 3-HT concentration was optimized at 2.611 mM and used. The results are shown in Figure 5.2.
Figure 5.2. Effect of 3-Hydroxytyramine concentrations on the reaction rate of UOx activity.

5.3.2.3 Effect of temperature on the sensitivity of uric acid assay

The temperature effect on the uric acid assay was studied by adding solutions containing 2.611 mM 3-HT, 4.73 nM POD, 102 µM H₂O₂ in 12.5 mM citric acid/tri potassium citrate buffer of pH 6.8 and incubating the 3 mL reaction mixture for 5 min in the temperature range of 10 – 45 °C. A proportional increase in the absorbance values of color formed was observed with increase in temperature up to 30 °C as evident from Figure 5.3 and the values decreased thereafter. Therefore, 30 °C was chosen as optimum temperature for the assay.
5.3.3 Analytical characteristics of the proposed method

The calibration curve for H$_2$O$_2$ assay was 12 – 102 µM and the relevant data are presented in Figure 5.4. The co-efficient of variation (CV) was 3.2 (n = 6) for 60 µM H$_2$O$_2$. The linear ranges for the quantification of HRP were 0.59 – 18.92 nM and 0.443 – 9.46 nM by the kinetic and fixed time methods, respectively, and the results are shown in Figure 5.5. The relative absorbance-time plot for ascertaining linearity by the reaction rate method for different concentrations of HRP (0.59 nM – 18.92 nM) is shown in Figure 5.6. The linearity for the UOx enzyme was observed between 0.022 and 0.18 units/mg and any further increase in the concentration of UOx, the rate remained constant and the relevant results are presented in Figure 5.7. The relative absorbance-time plot for ascertaining linearity by the rate method for different concentrations of UOx (0.022 units/mg – 0.18 units/mg) is shown in Figure 5.8.
Figure 5.4. Calibration graph for the quantification of H$_2$O$_2$.

Figure 5.5. Calibration graph for the quantification of HRP by the rate (+) and fixed time (■) methods.
Figure 5.6. Relative absorbance-time plot for varying concentrations of Horseradish peroxidase.

Figure 5.7. Effect of Uricase enzyme on the rate of reaction of uric acid assay.
Figure 5.8. Relative absorbance-time plot for varying concentrations of uricase.

Using the HRP assay and coupling with UOx-catalyzed reaction, the standard curve for uric acid was found linear in the range of 12.3 – 297.3 µM and 3.07 – 396.5 µM by rate method and fixed time method, respectively as shown in Figure 5.9. The relative absorbance-time plot for ascertaining linearity by the rate method for different concentrations of Uric acid (12.3 – 297.3 µM) is shown in Figure 5.10. The apparent molar absorptivity for uric acid was $0.1003 \times 10^4$ L/mol/cm and the determination of 250 µM uric acid has a CV of 2.87 ($n = 6$). The limits of detection (LOD) and quantification (LOQ) for uric acid were 1.5 µM and 2.9 µM, respectively.
Figure 5.9. Calibration graph for the quantification of uric acid by the rate (+) and fixed time (■) methods.

Figure 5.10. Relative absorbance-time plot for varying concentrations of uric acid.
5.3.4 Method validation and comparison plots

5.3.4.1. Precision and accuracy of the uric acid assay

Precision and accuracy of the method were determined by analyzing solutions containing known amounts of uric acid within linearity range [12]. The results showed that within-day precision was 1.50-3.07 % (n = 7) and day-to-day precision was 3.10-4.16 % (n = 7). The accuracy ranges for uric acid having concentrations of 24.7 μM, 198 μM and 297.3 μM were 87-101.50 %, 90-105 %, and 89-107 % respectively. The within day and day-to-day precisions of the method gave a very low standard deviation (SD) and CV indicating high precision and reproducibility of the method. The accuracy value was also high. Results are presented in Table 5.1.
Table 5.1. Within day and day-to-day precision and % of accuracy range.

<table>
<thead>
<tr>
<th>Uric acid (µM)</th>
<th>SD</th>
<th>CV</th>
<th>n</th>
<th>Accuracy range %</th>
<th>Day-to-day precision*</th>
<th>SD</th>
<th>CV</th>
<th>n</th>
<th>Accuracy range %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low conc. (24.7)</td>
<td>0.00248</td>
<td>2.22</td>
<td>7</td>
<td>89.10-94.14</td>
<td>Low conc. (24.7)</td>
<td>0.00346</td>
<td>3.10</td>
<td>7</td>
<td>87.36-101.50</td>
</tr>
<tr>
<td>Medium conc. (198)</td>
<td>0.00427</td>
<td>1.50</td>
<td>7</td>
<td>95.48-105.00</td>
<td>Medium conc. (198)</td>
<td>0.00913</td>
<td>3.24</td>
<td>7</td>
<td>90.12-102.00</td>
</tr>
<tr>
<td>High conc. (297.3)</td>
<td>0.0116</td>
<td>3.07</td>
<td>7</td>
<td>92.56-107.00</td>
<td>High conc. (297.3)</td>
<td>0.01591</td>
<td>4.16</td>
<td>7</td>
<td>89.16-103.96</td>
</tr>
</tbody>
</table>

Note. n = number of runs, SD = Standard deviation, CV = Co-efficient of variation, *duplicate measurement.
5.3.4.2. Analytical recovery

Recovery tests were performed with 6 different serum samples each spiked with known concentrations of uric acid based on standard curve of the assay. The uric acid levels recovered by proposed method were compared with the results obtained by reported assay method and the results are shown in Table 5.2. The uric acid recovery range by the proposed method was 87 – 107 % with a mean recovery of 96.87 %.

Table 5.2. Determination of Uric acid in human serum samples

<table>
<thead>
<tr>
<th>Serum samples</th>
<th>Uric acid (µM)</th>
<th>Added (µM)</th>
<th>Proposed methoda (µM)</th>
<th>Recovery (%)</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proposed method</td>
<td>Reference method [9]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>145.92</td>
<td>149.08</td>
<td>24.7</td>
<td>168.93</td>
<td>93.15</td>
</tr>
<tr>
<td>2</td>
<td>168.80</td>
<td>172.20</td>
<td>49.5</td>
<td>219.10</td>
<td>101.61</td>
</tr>
<tr>
<td>3</td>
<td>243.2</td>
<td>240.24</td>
<td>12.3</td>
<td>254.62</td>
<td>92.84</td>
</tr>
<tr>
<td>4</td>
<td>185.14</td>
<td>186.22</td>
<td>99.5</td>
<td>282.12</td>
<td>97.46</td>
</tr>
<tr>
<td>5</td>
<td>345.51</td>
<td>341.10</td>
<td>12.3</td>
<td>358.60</td>
<td>106.42</td>
</tr>
<tr>
<td>6</td>
<td>321.62</td>
<td>318.30</td>
<td>24.7</td>
<td>343.80</td>
<td>89.79</td>
</tr>
</tbody>
</table>

a mean of three replicate measurements; CV: co-efficient of variation

5.3.4.3. Method comparison plot

The uric acid assay was performed by the proposed method and compared the results with those obtained with reported uric acid assay method [9]. The correlation coefficient of 0.981 between the proposed method and reported method implies that the proposed method is on par with the reference method. Results are shown in Figure 5.11.
5.3.5 Interference studies

Interference by any of the common blood constituents in the quantification of uric acid was studied at uric acid concentration of 198 µM. The concentrations of interferants as well as their tolerance limits are summarized in Table 5.3. Most of the common ions present in serum samples showed minor effects on the determination of uric acid and it can be concluded that the proposed method is highly selective and specific for uric acid measurement.
Table 5.3. Tolerance ratios for the measurement of uric acid.

<table>
<thead>
<tr>
<th>Interferants</th>
<th>Tolerance ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin</td>
<td>0.0950</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.0980</td>
</tr>
<tr>
<td>Nitrite</td>
<td>0.1120</td>
</tr>
<tr>
<td>Iron (II)</td>
<td>0.1560</td>
</tr>
<tr>
<td>Iron (III), Mo (VI)</td>
<td>0.250</td>
</tr>
<tr>
<td>L-cystine</td>
<td>1.640</td>
</tr>
<tr>
<td>Copper (II)</td>
<td>3.58</td>
</tr>
<tr>
<td>F, L-cysteine, L-leucine, L-tryptophan, L-tyrosine, urea</td>
<td>5.48</td>
</tr>
<tr>
<td>Magnesium (II), chloride, calcium (II), lactose, maltose</td>
<td>10.00</td>
</tr>
<tr>
<td>EDTA, citric acid, mannose, L-histidine,</td>
<td>16.00</td>
</tr>
<tr>
<td>DL-methionine, glucose, DL-threonine</td>
<td>22.00</td>
</tr>
<tr>
<td>Creatinine, Isoleucine, fructose, Potassium (I)</td>
<td>28.00</td>
</tr>
<tr>
<td>Nitrate, L-serine, D-asparagine</td>
<td>42.00</td>
</tr>
<tr>
<td>D-galactose, sucrose</td>
<td>96.00</td>
</tr>
<tr>
<td>Carbonate, sodium, oxalic acid, sulphate,</td>
<td>150.00</td>
</tr>
<tr>
<td>Glycine, ammonium</td>
<td>300.00</td>
</tr>
<tr>
<td>Acetone</td>
<td>356.00</td>
</tr>
</tbody>
</table>

* Tolerance ratios for the measurement of 198 µM uric acid.

Note: Tolerance ratio is the ratio of limit of interferants concentration to that of concentration of uric acid used.

5.3.6 Evaluation of kinetic constants for the measurement of catalytic parameters

The Lineweaver–Burk plot [13], for the catalytic reaction of H$_2$O$_2$ in the presence of peroxidase is shown in Figure 5.12 and its linear regression equation having co-relation co-efficient of 0.996 was found to be $\frac{1}{V} = 3193 \left(\frac{1}{C_{H_2O_2}}\right) + 31.03$. The Lineweaver–Burk plot for uric acid in the presence of Uricase is shown in Figure
5.13 and its linear regression equation was found to be
\[
\frac{1}{V_0} = 5.960 \left( \frac{1}{C_{\text{uricacid}}} \right) + 15.52
\]
having co-relation co-efficient of 0.995. Kinetic constants data for the Lineweaver-Burk plot of HRP by the proposed method are as follows: Michealis-Menten constant \((K_m)\) is 102.90 \(\times 10^{-6}\) M, and maximum velocity \((V_{\text{max}})\) is 0.0322\(\times 10^6\) M\(^{-1}\) min\(^{-1}\), the catalytic constant \((K_{\text{cat}})\) is 6.80 min\(^{-1}\) and the specificity constant \(\frac{K_{\text{cat}}}{K_m}\) is 0.0660\(\times 10^6\) M\(^{-1}\) min\(^{-1}\). The catalytic efficiency and catalytic power at 4.73 nM peroxidase concentration are found to be 0.066 \(\times 10^6\) M\(^{-1}\) min\(^{-1}\) and 3.12 \(\times 10^{-4}\) min\(^{-1}\), respectively.

The Michaelis-Menten constant for the uric acid substrate using Uricase by the proposed method was found to be \(K_m = 0.384\) mM and \(V_{\text{max}} = 0.0644\) (mM) min\(^{-1}\). Lower \(K_m\) value of 3-HT system indicates that there is more interaction between active sites of substrate and co-substrate (3-HT) with the binding site of Uricase/peroxidase system. The catalytic efficiency and catalytic power of the proposed method are \(K_{\text{eff}} = 1.864\) (mM\(^{-1}\)) EU min\(^{-1}\) and \(K_{\text{pow}} = 0.1677\) min\(^{-1}\). The catalytic constant \((K_{\text{cat}})\) and specificity constant \(\frac{K_{\text{cat}}}{K_m}\) at saturated concentration of the co-substrates are 0.715 (mM) min\(^{-1}\) (EU\(^{-1}\)) and 1.8619 (EU\(^{-1}\)) min\(^{-1}\) by the proposed assay method.
Figure 5.12. Lineweaver-Burk plot for HRP by the proposed method. The kinetic study was carried out in 3 mL reaction mixture containing 2.611 mM 3-HT, 4.73 nM peroxidase in 12.5 mM citric acid/tripotassium citrate buffer of pH 6.8 with concentrations of H$_2$O$_2$ varying from 12 to 1630 µM.

Figure 5.13. Lineweaver–Burk plot for Uric acid by the proposed method. The kinetic study was carried out in 3 mL reaction mixture containing 2.611mM 3-HT, 4.73 nM peroxidase in 12.5 mM citric acid/tripotassium citrate buffer of pH 6.8 with concentrations of uric acid varying from 0.0123 mM to 0.396 mM.
5.3.7 Proposed reaction pathway for the Uricase catalyzed reaction of 3-HT in presence of uric acid and peroxidase

The probable reaction mechanism involved is based on peroxidase/H₂O₂ acting as a bio-mimetic oxidizing agent for the oxidation of 3-HT or dopamine resulting in the formation of quinone form of 3-HT which is similar to the report presented by A. Napolitano et al., [14], and Karon et al., [15]. UOx catalyzes the oxidation of uric acid in presence of dissolved oxygen and water to produce allotinton, CO₂, along with the liberation of H₂O₂ [16, 17]. Then POD consequently in oxidative coupling of peroxide formed in the UOx reaction in presence of 3-HT forms the colored product. The Uricase/peroxidase-catalyzed oxidation of 3-HT is shown in Scheme 5.1.

![Scheme 5.1](image)

**Scheme 5.1** Proposed reaction pathway for the formation of orange-colored product of quinone form of 3-HT involving POD, UOx, in the presence of uric acid.

The hydroxyl radical gets released by the oxidation of H₂O₂ through a ferryl intermediate (Fe⁷⁺=O-porphyrin π-cation radical) of the peroxidase [18]. Under the reaction condition examined, 3-HT loses one electron and one proton upon enzymatic oxidation in the presence of H₂O₂ to change to the quinone form of 3-HT producing an intense orange-colored product showing a strong absorption at 500 nm. The peroxidase catalyzed kinetic reaction mechanism may be ascertained for the formation of orange colored product by oxidation of 3-HT at the active sites of o-position to the hydroxyl group of 3-HT.
5.3.8 Application of uric acid assay to human serum samples

The levels of uric acid in human serum samples were determined by the proposed assay method. These samples were also analyzed by standard enzymatic reference method for comparison [9]. The results obtained by these two methods are summarized in Table 5.2. The recovery study done by the proposed method exhibited minimum interference from reducing substances as well as good reproducibility of the assay procedure.

Some of the disadvantages in the analytical methods that are reported for the uric acid determination include spectrophotometer based 3,5-Dichloro-2-hydroxy benzenesulfonic Acid/4-Aminophenazone (4-AAP) in which incubation period is of 15 min, interference from bilirubin is eliminated by use of ferrocyanide, and ascorbate oxidase is needed to be used to eliminate ascorbic acid interference and these are some of the demerits of the method [9]; and in p-hydroxybenzoate and 4-AAP based method, bilirubin at moderate conditions will interfere strongly and use of $K_2Cr_2O_7$ reagent is needed for the elimination of interference caused by catalase [8]. In 2,4,6-tribromophenol and 4-AAP, spectrophotometric method large volume of serum sample (1.0 mL) and deproteinization of serum sample using acetic acid are required and assay procedure takes around 45 min for extraction of the colored product by using n-butyl acetate which is time consuming process and suffers serious interference from ascorbic acid and bilirubin [19]; the main drawback of tetrazolium salt method, is that it uses three enzyme systems such as catalase, uricase and formaldehyde dehydrogenase and besides ascorbate oxidase is also used to eliminate ascorbic acid interference all resulting in high cost of the experimental procedure [20]. UV method using alkaline ferricyanide [21], is a simple and rapid spectrophotometric assay, but UV absorbance detection at 240 nm may be fraught with problems due to interferences by endogenous and exogenous UV-absorbing components present in or added to the biological sample [22]. Flow injection analysis using either dithiothreitol [23] or ferrocene-nafion [24] and spectrofluorimeter require high cost instrument and skilled operator to handle, which are the drawbacks of the system. Some of the spectrofluorimeter method reagents include $o$-phenylenediamine [25], pyronine Y [26], amplex red sol-gel matrix [27] and only uricase with uric acid [28]. HPLC methods using thymol and thimerosal [29], dithiothreitol [30] requires sample pretreatment before injection of the analyte for analysis. Liquid
Chapter – 5

3-HT-Horseradish peroxidase - Uricase-system

chromatography and tandem mass spectrometry using urate anion as internal standard [31] is a elevated expenditure procedure. Electrochemical methods using cyclic voltammetry or amperometry involve tedious procedure in preparing modified electrodes, take long time, use high cost instruments for the characterization of cross linkers and some of the methods that come under these include o-aminophenol [32], thiophene-3-boronic acid with Pt nano composites [33], polymer film of (N,N-dimethylaniline) [34], poly(thiophene-3-boronic acid) [33], gold/amino acid nanocomposite [35], thionine/single walled carbon nanotube [36] and 3-aminopropyltriethoxysilane [37]. Capillary electrophoresis along with chemiluminescence using luminol and potassium ferricyanide [38]; chemiluminescence detection using rhodanine derivative [39], pentacene in peroxalate nanoparticles [40] are having such defects wherein selectivity of the luminescence is poor. The linearity range of the proposed uric acid assay is found to be consistent from day to day, owing to excellent stability of the reagents. Determination of 0.198 mmol/L of uric acid has a CV of 1.50 (n = 7). The low values of CV signify high accuracy and reproducible measurement of uric acid with lesser error %. The lower values for limit of detection and limit of quantification for uric acid imply that the developed method is highly sensitive.

5.4 Conclusions

No work has been published so far on the oxidation of 3-HT by H$_2$O$_2$ using UOx-POD system in presence of uric acid for the determination of serum uric acid. This is the original contribution to the development of newer methods in the field of enzyme technology. The proposed method describes a low-cost, straightforward, selective, and highly sensitive method for the direct determination of uric acid with a detection limit of 1.5 µM using a UV–Vis spectrophotometer method. The procedure requires only small quantities of colorimetric reagents. The simplicity involved in the proposed method is determination of uric acid without extraction and preheating steps of serum samples. The kinetics of the assay using 3-HT system showed that the rate of color formation was “instantaneous”. Evidence from the kinetic study also explains that the lesser values ($K_m = 0.384$ mM and $V_{max} = 0.0644$ (mM) min$^{-1}$) for the enzyme substrate from the Lineweaver-Burk plot, indicate that there is a stronger affinity of active site of 3-HT to that of H$_2$O$_2$ molecules which signifies selectivity.
and specificity of the proposed assay. The within day and day-to-day precisions of the method gave a very low SD and CV indicating high precision and reproducibility of the method. The accuracy value was also high. Uric acid determined by this method has excellent correlation with that of reference method thereby justifying the authenticity of the method as well as its applicability. Even though some of the instrumental techniques have higher sensitivity compared to this proposed method, but they require costly instruments, skilled operators for handling the instruments and involve multiple steps for the preparation of biosensors which is a time consuming process.
Literature cited
