Oxidation and estimation of bilirubin by using carbon microelectrode

M Boopathi & M Subbaiyan*
Department of Analytical Chemistry, University of Madras, Guindy Campus, Madras 600 025, India
Received 15 December 1998; revised 8 October 1999

Bilirubin is one of the major bile pigment, clinically and biologically important among the bile pigments present in the mammals. When the concentration of bilirubin exceeds the allowable limit, it leads to a number of disorders and ailments in the human beings including jaundice. Bilirubin is present in the reduced form which is highly toxic whereas its oxidised form biliverdin is nontoxic. Hence the oxidation and estimation of bilirubin is essential in the clinical arena. A differential pulse voltammetric method has been recommended for the estimation of bilirubin with a bare carbon microelectrode in the aqueous medium and indirect oxidation of bilirubin to biliverdin. The oxidation has been carried out using in situ electrogenerated hydrogen peroxide produced from the continuous reduction of oxygen at its reduction potential. Estimation of bilirubin has been done using its reduction peak observed at around -1.1 V. The kinetic studies of indirect oxidation of bilirubin has been followed spectrophotometrically in the visible region.

Bilirubin is a naturally occurring, clinically and biologically important tetrapyrole cytotoxic yellow bile pigment composed of two dipyrrine chromophores linked at their alpha carbons by a methylene group. It is the major pigment produced by the mammals due to the disintegration of blood cell13. A survey of literature shows that various methods available for the oxidation and estimation of bilirubin49 have several shortcomings. In the case of direct electroanalytical methods a much higher potential (>1.1 V) is required for oxidation and the estimation requires preconcentration and electrode cleaning for every measurement5. The enzymatic methods require rigorous conditions for proper functioning and also the electrode preparation and preservation involve elaborate procedures59. Photometric method produces a number of reversible isomers during oxidation. Also, it is an indirect method and a concentration loss is observed due to improper coupling of the dye with bilirubin89. To overcome these drawbacks in the above methods the present study was carried out.

A number of advances have been achieved in the electroanalysis of biological systems with the help of microelectrodes10. Metal microelectrodes which are generally used as good catalysts decompose easily the \( \text{H}_2\text{O}_2 \) generated during the electrochemical reduction of oxygen in biological systems14. Carbon electrodes are stable towards \( \text{H}_2\text{O}_2 \) decomposition, hence carbon microelectrodes find wide use in the study of biological compounds. The present study was carried out to illustrate the application of bare carbon microelectrode for the oxidation and estimation of bilirubin in synthetic samples of various concentrations by using differential pulse voltammetry. Hydrogen peroxide was continuously electrogenerated in situ by reducing the oxygen at its reduction potential and used for the indirect chemical oxidation of bilirubin to biliverdin. The reduction peak of bilirubin was used for its estimation at very low concentrations. The kinetics of bilirubin oxidation was also studied by following its absorbance by spectrophotometry.

Materials and Methods
Analytical grade reagents and Millipore water were used throughout. The carbon micro needle (0.5 mm) electrode used was obtained from Japan. Dulbecos physiological buffer solution (DBPS) (pH 7.4 to 11.4) was used for all the studies. Bilirubin (CDH-India) with molar extinction coefficient of 58,000 in chloroform was used.

Differential pulse voltammetric studies were carried out using Elico (model CL-90) instrument provided with X-Y recorder (model LR-180). A three-electrode cell assembly was used for the experimental studies consisting of bare carbon microelectrode as working electrode, a saturated calomel electrode as reference and platinum wire as counter electrode. The kinetic studies of oxidation were carried out by with a Varian double beam UV-visible spectrophotometer (model DMS 80). A Systronics digital pH meter (model 335) with ± 0.01 accuracy was used to adjust pH of the buffer solution.
**Fabrication of carbon microelectrode**

The carbon needle electrode (0.5 mm) was washed with millipore water and dried in an air-oven for one hour at 60°C. It was inserted into a glass microcapillary and fixed with epoxy resin by exposing 1 cm of the needle out of the microcapillary and dried at room temperature for six hours as reported earlier[13]. The electrode was in situ cleaned by allowing for hydrogen evolution[14] for 2 min.

**Estimation of bilirubin**

Since bilirubin is insoluble in water, desired quantity of bilirubin was dissolved in 2 ml of 0.1M sodium hydroxide and made up to the volume with DBPS (pH 7.4-11.4) to obtain the stock solution of pHs 7.4-11.4. The higher pHs were achieved by adding appropriate volumes of 0.1M sodium hydroxide to the DBPS and monitoring them with pH meter. The stock solutions were prepared freshly just before experimentation and were covered with an aluminium foil to avoid light sensitive decomposition[5].

The estimation of bilirubin was carried out in the pH 7.4 due to its instability in the acidic medium[5] as well as in highly basic solutions and physiological requirements at optimised instrumental conditions of pulse height 50 mV, pulse duration 80 ms, sensitivity 1 µA, drop time 0.5 s, temp. 22°C and scan rate 5 mV/s. The cell assembly was covered with an aluminum foil throughout the experiment to avoid light sensitive decomposition[5]. The differential pulse voltammogram was scanned from -0.5V to -1.8V for the reduction of bilirubin and oxygen (Fig.1). The calibration plot was constructed (Fig. 2) by plotting the peak currents of bilirubin reduction versus various concentrations of bilirubin.
**Oxidation of bilirubin**

Bilirubin (6.8x10^{-7} M) dissolved in different buffer solutions (pH 7.4-11.4) were taken in the cell assembly in order to study the effect of pH on the oxidation of bilirubin. The potential was fixed constant at the oxygen reduction potential (-0.7 to -0.84 V) with respect to the pH of the medium (Table 1). The electrolysis was carried out from 0 to 210 minutes and the rate of oxidation was followed by measuring the absorbance spectrophotometrically at λ_{max} 450 nm of the bilirubin to avoid the photochemical isomerisation reactions. Sample solution (1ml) taken from the electrolysis cell at required time (0-210 minutes) was used for absorbance measurement and then the above sample solution was transferred back to the electrolysis cell. This process was carried out at optimised instrumental conditions as in the case of bilirubin estimation.

**Results and Discussion**

The differential pulse voltammetry of DBPS buffer gave rise to a peak for the reduction of oxygen to
H₂O₂ (Fig.1) at -0.710 V at pH 7.4. With increasing pH, it was marginally shifted to more negative potential -0.730 to -0.780 V (Table 1). This oxygen reduction peak potential was used for the in situ electrogeneration of H₂O₂ which chemically oxidised bilirubin. The differential pulse voltammetry of bilirubin in DBPS gave rise to a peak at more negative (~ -1.1 V) potential corresponding to the bilirubin reduction (Fig.1). This reduction peak was used for estimation purposes. The bilirubin peak half width was calculated by earlier reported method¹⁵ and was found to vary between 46.8 mV and 55.35 mV with respect to pH. This suggests the involvement of two electrons in the reduction of bilirubin.

Estimation of bilirubin

The bilirubin reduction was carried out in the basic region at pHs 7.4-11.4. The peak potential was found to increase with increase in the pH up to 9.4 and beyond that a decrease in peak potential was observed suggesting the instability of bilirubin in highly basic medium as reported earlier.¹⁷

In the case of lower pulse heights, peak current increased with increase in pulse height and concentration (3.4 to 10×10⁻⁷ M) without any distortion in the shape of the peak. At higher pulse height up to a certain concentration (3.4 to 6.8×10⁻⁷ M) peak current increased; beyond this concentration a distortion in the peak was observed.¹⁵

The effect of pulse duration was studied in order to understand the kinetics of the bilirubin reduction. At lower pulse durations (10-40 ms) the reduction was not effective but in the case of higher pulse duration (80 ms) an increase in the bilirubin reduction current was observed. This may be due to the slow rate of the bilirubin reduction process.

The peak current linearly increased with concentrations in the lower pulse heights (5-50 mV) where the increase was marked (3.4-10×10⁻⁷ M with a relative standard deviation of 0.3-1.1%, n=5), whereas in the case of higher pulse height (100 mV) the current increase was marked up to (3.4 to 6.8×10⁻⁷ M) after which a distortion in the peak was observed.¹⁵

Hence lower pulse height was used for the construction of calibration plot for the estimation of bilirubin (Fig.2) which gives a slope of 1.1 μA/10⁻⁷ M. Due to the physiological requirement of bilirubin, estimation was carried out at pH 7.40.

Oxidation and kinetic studies

The bilirubin oxidation studies were carried out in the pH range 7.4-11.4 due to its instability in the acidic medium and also at higher pH values. For oxidation studies the optimum instrumental conditions were used as in the case of bilirubin estimation. The effect of pH on oxygen reduction potential was carried out in the pH region of 7.4-11.4 (Table 1). From the experimental results it is found that the peak potential increases with increase in pH (Table 1) in conformity with previous reports.¹⁶

The bile pigment bilirubin which is pale yellow in colour turns to green on oxidation to biliverdin. The oxidation of bilirubin to biliverdin by H₂O₂ is well established and extensively used in evaluation studies. Bilirubin was chemically oxidised in situ generated H₂O₂ produced from the O₂ reduction. The absorbance at 450 nm (Fig.3) showed a decrease at potentials corresponding to different pHs with time on continuous electrolysis (Table 1). It is observed from the Fig. 3 that at pH 7.4, the oxidation is more effective and at lower time intervals the indirect chemical oxidation is marked but at higher time intervals, the oxidation was rather slow. When the pH of the medium was increased from 7.4-11.4 it was observed that at lower time intervals the indirect oxidation is effective. With increase in pH and time intervals, constancy in the absorbance was observed in the pH ranges under investigation, indicating the slow rate of oxidation after a certain time of electrolysis. It is concluded from these studies that in all pH ranges the rate of oxidation is faster at lower time intervals while higher time intervals are effective for oxidation but rate of oxidation was slow. The O₂ reduction, bilirubin oxidation mechanism and its chemical reactions involved are as proposed earlier.¹⁷⁻¹⁸

\[ \text{O}_2 + \text{H}_2 \text{O} \xrightarrow{\text{Electrochemical}} \text{H}_2\text{O}_2 \]  

(i)

\[ \text{Bilirubin} + \text{H}_2\text{O}_2 \xrightarrow{\text{Chemical}} \text{Biliverdin} + \text{H}_2\text{O} \]  

(ii)

The kinetics of bilirubin indirect oxidation process was studied. Since its reduction process was very slow by differential pulse voltammetric experiments. It was followed spectrophotometrically at fixed wavelength to avoid isomer formation that occurs in the entire visible region. Rate constant was calculated using various kinetic equations. Constancy in rate constant with respect to time was observed for this system while using a pseudo first order reaction equation indicating the operation of pseudo first order kinetics during the oxidation of bilirubin to biliverdin (Table 2). The kinetics of the reaction was also compared with absorbance versus rate relationship (Table 3). It showed a linear variation with
absorbance in the entire studies (pH 7.4-11.4) which again indicates the operation of pseudo first order kinetics in the conversion of bilirubin to biliverdin oxidation.

References