An Enzymic Method for the Determination of Bilirubin Using an Oxygen Electrode

Johanna Klemm, Mamas I. Prodromidis, and *Miltiades I. Karayannis**

Department of Chemistry, Laboratory of Analytical Chemistry, University of Ioannina, Dourouti 45110, Ioannina, Greece

Received: June 18, 1999
Final version: September 10, 1999

Abstract
An enzymic method for the determination of bilirubin is described, based on the indirect electrochemical monitoring of bilirubin via its reaction with oxygen to biliverdin in the presence of bilirubin oxidase. Amperometric signals due to oxygen depletion were taken by means of an oxygen electrode incorporating an immobilized bilirubin oxidase membrane. The calibration plot shows linearity up to 2.0×10^{-4} M. This concentration range corresponds to elevated bilirubin concentrations in adults and in newborn infants. The RSD of the method is 3.1 % (1.0×10^{-4} M bilirubin, $n = 6$). The method is interference free, fast (< 3min) and easy to perform. The membrane retains almost 80 % of its initial activity after 30 successive measurements.

Keywords: Bilirubin, Oxygen electrode, Bilirubin oxidase, Enzymic method

1. Introduction

Bilirubin exists in human serum as two major forms: unconjugated bilirubin (Bu) and conjugated bilirubin (Bc), where Bc can be bilirubin monoglucuronide or bilirubin diglucuronide. The importance of bilirubin analysis in clinical chemistry is related to diagnosis and prediction of haemolytic disorders in adults as well as in newborn infants [1]. Bu is noncovalently bound to serum albumin, and the binding is readily cleaved by treatment with anionic detergents or organic solvents. It is a nonpolar, water insoluble molecule. In contrast, bilirubin conjugated with glucuronate is a polar, water insoluble compound that exists in plasma not bound to any protein.

There is a growing number of methods using the enzyme bilirubin oxidase (BOD) since Murao and Tanaka reported its purification and isolation from *Myrothecium verrucaria* MT-1. They reported that BOD (MW 52 000 Daltons) catalyzed the oxidation of bilirubin (both conjugated and uncunjugated) to biliverdin followed by further oxidation of biliverdin to an unknown purple compound or compounds [2].

Most of the bilirubin oxidase methods employ free enzyme in combination with spectrophotometry [2, 3–5]. A graphite epoxy electrode incorporating BOD has been described by Wang and Ozsoz [6]. Recently an enzymic method based on the amperometric detection of hydrogen peroxide using immobilized bilirubin oxidase is described [7], however, according to the literature hydrogen peroxide is not produced in the course of the enzymic reaction [1, 2, 8]. A fiber optic fluorescence biosensor [9] and a multilayer enzyme electrode [10] have also been reported. Here we describe an enzymic method for the determination of bilirubin by using immobilized bilirubin oxidase in combination with a Clark-type oxygen electrode. The reaction sequence in the assay is:

$$\text{Bilirubin} + \tfrac{1}{2}\,O_2 \xrightarrow{\text{BOD}} \text{Biliverdin} + H_2O \qquad (1)$$

$$\text{Biliverdin} + O_2 \longrightarrow \text{Purple compound(s)} + H_2O \qquad (2)$$

Oxygen depletion is monitored amperometrically at a platinum cathode, poised at 0.8 V with respect to an Ag/AgCl reference electrode. A three membrane sequence consists of a polyethylene membrane, an enzymic membrane (Nylon 66) where bilirubin oxidase has been immobilized through covalent bonding and an outer Nylon fabric, has been employed. This configuration provides a fast and easy to perform method. Additionally the nature of the oxygen sensor ensures an interference free sensor in respect to the electroactive species present in serum samples.

2. Experimental

2.1. Apparatus

An oxygen electrode assembly of Rank Brothers Ltd. (Bottisham, UK) was utilized to measure the amperometric current of the bilirubin oxidase electrode. The cell comprised a central 2 mm diameter platinum disk with a surrounding 12 mm outer diameter, 1 mm wide silver ring (Ag/AgCl), acting as an internal reference electrode. Both platinum and silver electrodes were sealed into the base of a thermostated incubation chamber.

The current output was recorded with an Autolab potentiostat PGSTAT 10 from Eco Chemie (Utrecht, The Netherlands). An adapter for a two-electrode cell is kindly donated by Dr. G. Brug from Eco chemie.

Preactivated Immunodyne ABC membranes (0.45 pore size, 120 μm thickness) were purchased from Pall Italia S.r.l. (Milan, Italy), oxygen membranes (YSI 5793 Standard membranes) were obtained from YSI Incorporated (Ohio, USA), and nylon fabrics (38 % free surface, 80 μm thickness, 100–120 threads/cm), were from Locertex Nylon textiles (Warrington Cheshire, UK). Polycarbonate membranes (0.5 μm pore size) were supplied by Nucleopore (Cambridge, MA) and Cuprofan membranes were kindly donated from the University hospital of Ioannina (Greece).

2.2. Chemicals

Bilirubin oxidase (bilirubin:oxygen oxidoreductase, EC 1.3.3.5, lyophilized powder) from *Myrothecium verrucaria*, bilirubin, bovine serum albumin (BSA), glutaraldehyde (grade II) and serum samples (Accutrol Abnormal Chemistry Controls, AACC) were purchased by Sigma (St. Louis, USA). All other (analytical-reagent) chemicals were supplied from Aldrich (Gollingham, Germany)

  1040–0397/00/0403–0292 $17.50+.50/0

Bilirubin oxidase solution: The lyophilized bilirubin oxidase, containing approximately 25 U of enzyme activity, was reconstituted with 0.2 mL of 0.1 M phosphate buffer, pH 7.5.

Bilirubin solution: A 0.005 M solution was prepared by dissolving 60 mg of bilirubin in 3 mL of 0.05 M phosphate buffer pH 7.5 and 20 μL of 4 M NaOH, then diluting to 20 mL with 0.05 M phosphate buffer pH 7.5. Solutions were filled in portions of ca. 0.5 mL in eppendorff tips, stored at −20 °C and prepared weekly, owing to their instability [1]. The laboratory lighting was kept as low as practicable while preparing and using the standards.

2.3. Electrode Assembling

The bilirubin oxidase membrane was prepared by crosslinking the enzyme with BSA and glutaraldehyde and then covalently bonding on preactivated membrane. 10 mg of BSA were dissolved in 300 μL of 0.1 M phosphate buffer, pH 7.5. 10 μL of this solution were gently mixed with 15 μL of bilirubin oxidase, then 5 μL of 9 % aqueous glutaraldehyde solution were added carefully. This mixture was placed in the center of a 1 cm^2 piece of Immunodyne ABC membrane. After air-drying for 30 min the membrane was washed in phosphate buffer (2×10 min) to remove the unbound protein.

The electrolyte chamber was filled with half-saturated potassium chloride as internal solution. The bilirubin oxidase membrane was then placed over the oxygen membrane previously stretched over the electrodes and the electrolyte chamber. The platinum working electrode is poised automatically at a constant potential of −800 mV (versus the Ag/AgCl internal reference) by the Autolab potentiostat at the beginning of each measurement.

2.4. Procedure

$(1.0 - x)$ mL of 0.1 M glycylglycine pH 9.0 was introduced in the reaction cell of the oxygen electrode assembly and allows the pressure of the oxygen in the cell to reach equilibrium with atmospheric oxygen while adjusting the solution temperature to 34 °C. The solution is stirred at a moderate speed with a magnetic stirrer. When a stable current value was monitored appropriate values (x) mL of bilirubin solution were added and current changes due to the oxygen depletion were recorded. Current response at 180 s was taken as a measure of the analyte concentration.

3. Results and Discussion

3.1. Optimum Working Buffer

Before investigating the behavior of immobilized enzyme, the optimum working buffer solution was determined using soluble enzyme. The following buffer solutions, all pH 8.5, have been investigated: phosphate buffer, morpholinopropansulfonsaeure (MOPS) and glycylglycine. Responses obtained for a concentration of 5.0×10^{-5} M bilirubin are shown in Table 1. The glycylglycine buffer proved to be the most suitable solution to perform the bilirubin measurement.

Table 1. Influence of working buffer solution on the response, using soluble BOD. Parameters: 5.0×10^{-5} M bilirubin; buffer, 0.1 M, pH 8.5; temperature, 25 °C.

Buffer solution	*R.E. (%) [a]*
Phosphate buffer	59
MOPS	87
Glycylglycine	100

[a] With respect to the highest response.

3.2. Enzyme Membranes

Two types of enzyme membranes have been investigated: untreated nylon fabric where the bilirubin oxidase was immobilized by O-alkylation and preactivated Immunodyne ABC membrane. In both cases the enzyme is attached to the membrane by chemical bonding.

The Immunodyne ABC membrane seemed to be slightly more effective. Also the immobilization procedure is much simpler compared to the procedure with untreated nylon, where hazardous chemicals like dimethyl sulfate have to be used for successful activation. The latter procedure includes several immobilization steps and therefore is time consuming. Another advantage of the Immunodyne ABC membrane is its sharp baseline, because stirrer effects on oxygen diffusion were limited by using membranes of smaller porosity.

3.3. Covering Membranes

Three different types of membranes were compared as outer membrane for the proposed configuration: cuprofan, polycarbonate and nylon fabric. Measurements were taken at a bilirubin concentration of 1.0×10^{-4} M. Responses for the different types of covering membranes are shown in Table 2.

The pore sizes of cuprofan and polycarbonate should theoretically allow bilirubin to pass, nevertheless only unsatisfactory results were obtained with these membranes. The reason for the low responses is mainly due to the strong tension of bilirubin to be adsorbed on the cuprofan and polycarbonate membranes. Therefore the membranes were blocked rapidly and no further bilirubin was able to pass the covering membrane. The nylon fabric, however, did not provide any protection of the immobilized enzyme (i.e., microbial attack). Its only purpose was to hold the enzyme membrane in place and to protect it from contact with the magnetic bar. A circular piece punched out of the 1×1 cm^2 piece of membrane was used throughout the experiments.

3.4. Preparation of Bilirubin Standard Solution

Four different solvents have been investigated for the preparation of bilirubin standards: 0.1 M glycylglycine, pH 8.7; 0.1 M NaOH; 0.05 M phosphate, pH 8.7 or pH 7.5.

Table 2. Efficiency of the sensor for different covering membranes. Parameters: 1.0×10^{-4} M bilirubin; buffer, 0.1 M glycylglycine, pH 8.5; temperature, 25 °C.

Membrane	*R.E. [%] [a]*
Cuprofan	4.8
Polycarbonate	1.1
Untreated nylon	100

[a] With respect to the highest response.

The obtained results show that the glycylglycine buffer is not suitable for the preparation of the bilirubin standards, although it is the most suitable as working buffer. Bilirubin in sodium hydroxide turned out to decay rather fast. This observation is in accordance to previous studies where the decomposition rate of bilirubin in 0.1 NaOH was found to be 0.18–0.21 % per minute [11]. Even by storage at −20 °C the color of the solution turned from orange to brown within a few days.

Standard solutions in 0.05 M phosphate buffer pH 7.5 gave a higher signal than those in 0.1 M phosphate buffer pH 8.7, although the buffer of the measurement was glycylglycine at pH 8.7. Phosphate buffer at pH 7.5 was therefore used for further experiments. The relative signals obtained with the different buffer solutions for a bilirubin concentration of 1.0×10^{-4} M are presented in Table 3.

3.5. Working Conditions

The effect of pH on the maximum rate of bilirubin oxidation in the present method has been studied. Figure 1 shows the pH profile of the system, attained at a concentration of bilirubin 2.0×10^{-4} M. It indicates that the maximum response was obtained at pH 8.5–9.0, therefore in the present procedure pH 9.0 was chosen for the measurements. This result is in good correlation to the maximum activity of bilirubin oxidase at pH 8.4 given in the literature [2]. The slight shift in the pH of maximum activity is probably due to the immobilized form of the enzyme.

The sensitivity of the biosensor increased with the temperature, leveling at a maximum value of 37 °C. After this point thermal inactivation dominates over the increase of the collision frequency resulting in the decrease of the signal. In addition the signals were getting unstable. Temperature of 34 °C was chosen as the working temperature for our experiments.

Copper acts here as a cofactor of the enzyme bilirubin oxidase, which is known to belong to the group of copper-based enzymes [5]. Copper effect on the performance of the bilirubin biosensor was tested in the concentration range 1.0×10^{-5}–2.5×10^{-4} M copper acetate (data not shown). A concentration of 2.5×10^{-5} M $Cu(CH_3COO)_2$ was found to be optimum for the performance of the sensor.

3.6. Application to Standards and Recovery Studies

Under optimum conditions, a calibration plot was constructed, applying the least squares method. Using Immunodyne ABC membrane a linear relationship was obtained in the range 1–2.0×10^{-4} M bilirubin with a correlation coefficient, $r = 0.998$. The detection limit was 8×10^{-6} M bilirubin for a signal to noise ratio of 3 ($S/N = 3$).

Table 3. Relative signals received with differently prepared bilirubin standards. Parameters: 1.0×10^{-4} M bilirubin; buffer: 0.1 M glycylglycine, pH 8.5; temperature, 25 °C.

Buffer solution	*R.E. [%] [a]*
0.1 M glycylglycine pH 8.7	53
0.1 M NaOH	86
0.1 M Phosphate buffer pH 8.7	85
0.05 M Phosphate buffer pH 7.5	100

[a] With respect to the highest response.

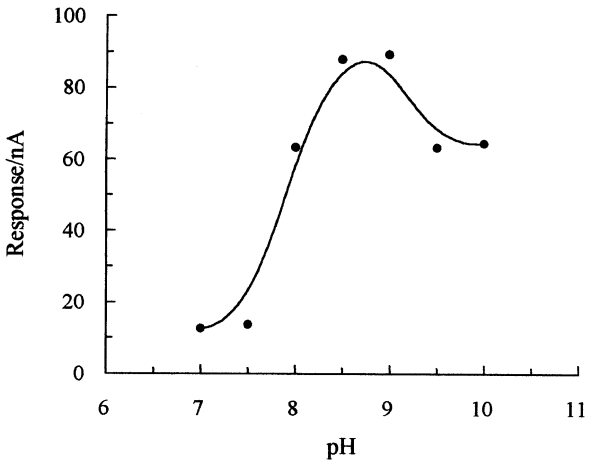

Fig. 1. pH profile of the bilirubin sensor. Parameters: 2.0×10^{-4} M bilirubin; buffer 0.1 M glycylglycine; temperature, 25 °C.

Recovery studies were carried out by adding standard solutions of bilirubin to AACC serum samples. The calibration plot, however, shows restricted linearity and lower response, probably because of the high level of HSA present in the working solutions. It is reported that bilirubin binds strongly to HSA in order to prevent its oxidation by BOD [8]. Both plots are shown in Figure 2.

In previous articles [4, 5] detergents like sodium dodecyl sulfate (SDS) or sodium cholate were used to disrupt this protective binding, increasing thus the rate of the reaction. Sodium cholate at a concentration of 2.8 mM and sodium dodecylsulfate at a concentration of 7.5 mM, however, turned out to be unsuitable for cleaving the HSA-bilirubin binding, as it caused a decrease of the signal of 90 % and 25 %, respectively. However in other studies [1, 5] concentrations of 15 mM and 46 Mm were used without any significant interference. Denaturation of bilirubin oxidase in the presence of SDS at concentration higher than 5 mM has been reported [8].

The interference effect of other compounds present in real samples was investigated by applying the method of mixed solutions in the presence of 1×10^{-4} M bilirubin. No interference effect was observed in the presence of 5 mM triolein, 5 mM triacetin, 5 mM glucose, 1 mM ampicillin, and 20 mM uric acid.

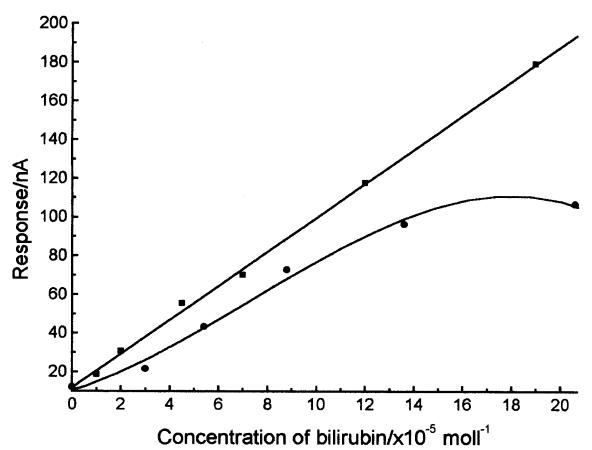

Fig. 2. Calibration plots of bilirubin in buffer (■) and AACC serum samples (●). Parameters: buffer, 0.1 M glycylglycine, pH 9.0; temperature, 34 °C; $Cu(CH_3COO)_2$, 2.5×10^{-5} M.

Recoveries of 95 %, 95 %, 96 %, 94 %, and 103 % were obtained in the presence of the above compounds, respectively.

The operational stability of the sensor was verified by performing successive measurements of 0.1 mM bilirubin solutions and was found to retain almost 80 % of its initial activity after 30 runs.

4. Conclusion

Bilirubin oxidase does not produce hydrogen peroxide and therefore the proposed approach by means of an oxygen based amperometric biosensor is promising. These results show the feasibility of the development of a bilirubin biosensor based on an oxygen electrode. The method is interference free, fast and easy to perform, but at the present state of the art there are still some questions to look upon. Additional studies have to be done in order to cleave the binding of bilirubin with HAS in a way that does not affect the efficiency of the bilirubin biosensor.

5. References

[1] J.E. Sherwin, R. Obernolte, in *Clinical Chemistry. Theory, Analysis and Correlation* (Eds: L.A. Kaplan, A.J. Pesce), The C.V Mosby Company, St. Louis **1989**, p. 1009.
[2] S. Murao, N. Tanaka, *Agric. Biol. Chem*. **1981**, *45*, 2383.
[3] B. Perry, B.T. Doumas, G. Buffone, M. Glick, C. Ou, K. Ryder, *Clin. Chem*. **1986**, *32*, 329.
[4] S. Otsuji, K. Mizuno, S. Ito, S. Kaawahara, M. Kai, *Clin. Biochem.* **1988**, *21*, 33.
[5] A. Kosaka, C. Yamamoto, Y. Morishita, K. Nakane, *Clin. Biochem.* **1987**, *20*, 451.
[6] J. Wang, M. Ozsoz, *Electroanalysis* **1990**, *2*, 647.
[7] A. Fortuney, G. Guilbault, *Electroanalysis* **1996**, *8*, 229.
[8] A Kosaka, in *Methods of Enzymatic Analysis*, Vol. 8, 3rd ed. Ed: H.V. Bergmeyer, VCH, Weinheim, **1985**, pp. 591–598.
[9] X. Li, A. Fortuney, G. Guilbault, A. Suleiman, *Anal. Lett.* **1996**, *29*, 171.
[10] B. Shoham, Y. Migron, A. Riklin, I. Willner, B. Tartakovsky, *Biosens. Bioelectron*. **1995**, *10*, 341.
[11] K.L.J Vink, J Dreumel, W. Schuurman, H. Wikkeling, R. Van Gansewinicel, C.J Phielix, H.C Koedam, *Clin.Chem*. **1987**, *33*, 1817.