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ADDIS ABABA UNIVERSITY
Addis Ababa, ETHIOPIA

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Date June 19/1998

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Ref.No. CD/92/3-12a/98

To: **Prof Theodros Solomon**
Dean, SGS

From: **Wendimagegn Mammo**
Chairman, Department of Chemistry

Subject: **MSc Theses**

The following MSc Candidates in Chemistry have successfully defended their MSc theses between June 8 and 12, 1998.

1. Tesfaye Refera
2. Abi Tadesse
3. Derib Shewangizaw
4. Yohannes Ali
5. Kahsay G/Medhn
6. Kasim Ahmed

The research supervisors of all have testified that they have incorporated all comments made by the examining panel in their final versions of their theses.

Thank You

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AMPEROMETRIC SENSOR BASED ON MnO_2 FILM MODIFIED CARBON PASTE ELECTRODE COUPLED WITH GLUCOSE OXIDASE IMMOBILIZED REACTOR FOR THE DETERMINATION OF GLUCOSE

BY
KAHSAY G/MEDHIN

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SCHOOL OF GRADUATE STUDIES

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by

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DEDICATION

To my Family

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Amperometric Sensor Based on MnO_2 Film Modified Carbon Paste Electrode Coupled with Glucose Oxidase Immobilized Reactor for the Determination of Glucose

By Kahsay G/Medhin

Research Advisors: Dr. Hailemichael Alemu and Prof. Theodros Solomon.

ABSTRACT

An amperometric system based on a carbon paste electrode modified with MnO_2 was investigated as a sensor for glucose in flow-injection analysis (FIA). Enzymatic oxidation of glucose and catalytic oxidation of the intermediate (H_2O_2) by MnO_2 is exploited for the amperometric monitoring of glucose, at an operating potential of + 0.59 V vs. Ag/AgCl.

Experimental parameters, such as flow rate, injection volume and concentration dependence have also been optimized. The amperometric signals are linearly proportional to the concentration of glucose in the range of 10^{-1} to 10^{3} μM. The method was applied for the determination of glucose in human serum. Data showing the effect of interfering substances on the response of the sensor are also optimized.

1. INTRODUCTION

Hydrogen peroxide is a very important intermediate in environmental and biological reactions. It also plays an important role in various industrial applications. It has also been used as a bleaching agent for textiles, as a hypoeliminator in photography, as a depolymerization agent in the modification of resins and adhesives and as an active chemical reagent for the preparation of many chemicals (e.g. hydrazine, iodic acid, etc.) [1,2].

In environmental control, it is used for detoxicating organic pollutants (e.g. formaldehyde, phenol, surfactants, etc.) [1]. Moreover, the determination of hydrogen peroxide produced as a result of various oxidase reactions has been widely used in clinical assays of biological fluids. Clearly, a reliable, rapid and economical method for monitoring H_2O_2 is very essential and desirable.

Several analytical techniques have been employed for its determination but most of them suffer from interferences, long analysis time, and use of expensive reagents [1,3,4].

Electrochemical methods have offered improved analytical characteristics such as high sensitivity, large dynamic range and rapid response time [5]. Several different electrode materials have been reported. These include carbon electrodes operating at high over-potentials, e.g., + 900 mV vs SCE, carbon paste electrodes doped with platinium, palladium, or ruthenium at + 800 mV vs Ag/AgCl and platinium electrodes at + 700 mV *vs* Ag/AgCl. The main drawback of these techniques is the high voltage applied to the working electrode, which makes such a sensor susceptible to interfering substances [6]. However, electrodes modified by immobilizing some substances with catalytic activity onto the electrode surfaces can dramatically decrease the over-potential for the reduction of hydrogen peroxide and improve its electrochemical response. An important approach in this field is to immobilize transition metals, their oxides or complexes onto the electrode surfaces by various techniques.

Immobilization of $[Ru(NH_3)_6^{2+}]$ into a montmorillonite clay coating on graphite resulted in an electrode that mediated the reduction of hydrogen peroxide at - 0.2 V (vs SCE) [7].

Modifying the electrode surface of semiconductors, glassy carbon, graphite and polymers via film formation was investigated [8, 9]. Noble metals, metal oxides, metal complexes and enzymes were used as modifiers for the determination of hydrogen peroxide.

An indium-tin oxide film deposited on a glassy substrate exhibits response towards hydrogen peroxide [10]. Glassy carbon as a frequently used electrode material was modified with a film containing palladium and iridium, irdium oxide, manganese dioxide, prussian blue, ferrocene, pyrrole iron(III)-octaethylporphyrin or copper-heptacyanonitrosyl ferrate [11]. Johnston *et al.* investigated an H_2O_2 sensitive electrode by deposition of a gold /palladium film on a plastic substrate [12].

Hydrogen peroxide, involved in numerous enzymatic reactions allows the tailoring of enzyme modified sensors for monitoring biologically important molecules. Investigations on film deposited mediators for lowering detection limits and working electrode potentials were performed frequently [13-17]. A glassy carbon electrode modified with an electrodeposited MnO_2 film as described by Taha *et al.* responds to H_2O_2 in strongly alkaline media. However, this excludes its modification with enzymes [5].

The modification of carbon paste electrodes (CPE) with catalytic metals, metal oxide micro-particles or their complexes for the determination of H_2O_2 has received considerable attention [18]. A screen printed carbon, a carbon paste and film modified electrodes modified with MnO_2 were investigated as simple and fast procedures in the amperometric determination of hydrogen peroxide in flow- injection analysis (FIA) [11, 18, 19]. Manganese dioxide oxidises H_2O_2 to O_2 whereas it is reduced to oxides containing manganese in lower oxidation states. This reaction was investigated for its applicability to the electroanalytical determination of hydrogen peroxide. The low detection limit as well as the applicability to neutral media suggests that these electrodes can probably act as useful electrode materials for the development of a sensor for glucose.

Wang *et al.* [20] reported on the electrocatalytic oxidation of H_2O_2 at a poly (*m*-phenylene diamine)-modified carbon paste electrode and its use for biosensing of glucose.

Incorporation of enzymes and mediators into carbon paste matrices represents an attractive approach to the preparation of reagentless biosensors [21]. Carbon paste is a mixture of graphite powder and an organic liquid. The pasting liquid is immiscible with the contacting aqueous solutions and includes paraffin oil, vaseline oil, nujol, and silicon oil, which functions to fill up the gaps between the graphite particles and insulate the graphite from the contacting aqueous solution.

Liu *et al.* have investigated a reagentless amperometric sensor that is highly sensitive to H_2O_2 by incorporating fumed silica, horseradish peroxidase (HRP) and meldola blue into carbon paste [21]. The enzyme horseradish peroxidase (HRP) catalyzes the reduction of hydrogen peroxide in the presence of an electron transfer mediator according to the following scheme:

$$H_2O_2 + Med_{red} \xrightarrow{HRP} H_2O + Med_{ox} \qquad (1)$$

The electrochemical reduction of the oxidized mediator generated by the enzymatic reaction provides the amperometric signal for the measurement of hydrogen peroxide [22].

Enzymes can be used in homogeneous analysis and bring about many benefits since analysis based on enzymatic methods is rapid, selective, and environmentally friendly. The incorporation of these biomolecules into the sensing device gave rise to the development of a new type of sensors called biosensors [23].

Biosensors combining the selectivity of enzymes with the sensitivity of amperometric detection, are in rapid progress since the development of the first electrochemical glucose sensor by Clark and Lyons in 1962 [24]. The abundance of glucose in nature and its importance in life processes has made necessary the construction of glucose sensors in light of the fast-expanding techniques of enzyme immobilization and chemical modification of electrodes [25-27]. Because of the complexity and serious specificity problems associated with direct electro-oxidation of glucose, the oxidoreductase enzyme glucose oxidase (GOD, E.C. 1.1.3.4) is most often used in the development of glucose sensors.

Several flow injection analysis (FIA) systems combined with immobilized glucose oxidase for the analysis of glucose based on electrochemical detection have been described [28-30]. The electrochemical mode of detection in these reactors is based on the measurement of electron exchange from the anodic oxidation of hydrogen peroxide produced by the enzymatic reaction of glucose oxidase. However, the use of a mediator permits electrochemical detection at a lower-overvoltage than the the oxidation of hydrogen peroxide, thereby decreasing the effects of potential interfering species.

Thus, the objectives of this thesis work are :

i) to devise a sensor for glucose using glucose oxidase based on the hydrogen peroxide detecting MnO_2 film electrode using flow injection analysis;

ii) optimization of the sensor with respect to flow rate, injection volume and working potential; and

iii) to couple the sensor in flow injection analysis to assay glucose in serum samples.

2. LITERATURE REVIEW AND THEORETICAL BACKGROUND

2.1. Biosensors

Biosensors are analytical devices based on the combination of a biological component (in an immobilized form) with a suitable transducer, which can convert a biochemical response into a quantifiable electrical signal [31]. The biocomponent typically limits biosensor life time, stability, reproducibility and calibration requirements, while the physical transducer does not tend to limit those characteristics.

An ideal biosensor should be sensitive enough to allow detection of the analyte at reasonably low concentrations, selective to discriminate response from undesired interfering species, fast to respond to changes in the concentration of the analyte, stable in terms of operation and storage, and reproducible in terms of the recorded signal. In addition a broad linear range and a high signal to-noise-ratio are required [32].

2.1.1. Amperometric sensors

Amperometry is based on the oxidation or reduction of an electroactive species at the surface of a working electrode while a constant potential is applied to the electrode with respect to a reference electrode. The resulting current is measured by using either these two electrodes (working and reference) or a three-electrode arrangement (working, counter and reference electrodes) to compensate for the potential drop caused by passage of the current through the solution.

Amperometric techniques give a current response which is linearly dependent on the analyte concentration. The recorded current, I, is a direct measure of the electrochemical reaction rate as described by Faraday's law:

$$I = nF(dN/dt) \qquad (2)$$

where dN/dt is the oxidation/reduction rate, n and F have their usual significance.

By increasing the applied voltage the rate of the heterogeneous charge transfer reaction can be enhanced so as to cause the rate of the whole process to become controlled by mass transfer. Under these conditions the diffusion current, I_d, is directly proportional to the concentration C, of the substance to be determined.

$$I_d = nFAD_s C/\delta \quad (3)$$

where A is the electrode surface area; D_s is the diffusion coefficient and δ equals the thickness of the diffusion layer (which is constant at a given convection) [31].

2.2. Flow Analysis

Flow injection analysis (FIA), introduced by Ruzicka and Hansen [33] and Stewart and coworkers [34] in the middle of 1970's, is now a common tool in the modern analytical world. The principle of flow injection analysis is based on the combination of three elements: sample injection, controlled dispersion and reproducible time of its movement.

In FIA a liquid sample is injected into a constant moving, nonsegmented continuous carrier stream of a suitable liquid that may contain reagents. The injected sample forms a zone, which is then transported towards a detector. Each sample is dispersed reproducibly and the recorded flow injection peak is proportional to concentration. FIA has the advantage of being inexpensive, simple, easy to construct and to adopt for different analytical purposes.

Electrochemical detection in flowing systems is usually performed by controlling the potential of the working electrode at a fixed value (corresponding to the limiting current plateau region of the compounds of interest) and monitoring the current as a function of time. This technique is called flow amperometry. The current response thus generated reflects the concentration profiles of these compounds as they pass through the detector [35].

The applied potential affects not only the sensitivity and signal-to-noise characteristics, but also the selectivity of amperometric measurements. In general, a lower potential is more

selective, and a higher one more universal. Thus, compounds undergoing redox reactions at lower potentials can be detected with greater selectivity.

2.3. Cell Design

A wide range of cell designs have been used for electrochemical monitoring of flow streams. The cell design must fulfil the requirements of high signal-to-noise ratio, low dead volume, well-defined hydrodynamics, small ohmic drop, and a stable reference electrode [35]. The most widely used amperometric detectors are based on the thin layer and wall-jet configuration [35, 36]. The limiting current equation for the wall-jet detector is given by [35, 36].

$$i = 0.898\, nFCD^{2/3}\, \vartheta^{-5/12}\, a^{-1/2}\, A^{3/8}\, V^{3/4} \qquad (4)$$

where a is the inlet diameter, A is electrode area, C is concentration, D is diffusion coefficient, ϑ is Kinematic viscosity, and V is the average volume flow rate.

2.4. Chemically Modified Electrodes

Chemically modified electrodes (CMEs) represent a modern approach to electrode systems. Such deliberate alteration of electrode surfaces, through the incorporation of an appropriate surface modifier, can meet the needs of many electroanalytical applications and different sensing devices [37].

There are different directions by which CMEs can benefit analytical applications. These include acceleration of electron-transfer reactions, preferential accumulation, or selective membrane permeation. Such steps can impart higher selectivity, sensitivity, or stability to electrochemical devices [35].

One of the most common approaches for incorporating a modifier onto the surface has been coverage with an appropriate polymer film. Polymer-modified electrodes are often prepared by casting a solution containing the dissolved polymer onto the surface and allowing the solvent

to evaporate, or via electropolymerization in the presence of the dissolved monomer. The latter offers precise control of the film thickness (and often the morphology) and is particularly attractive in connection with miniaturized sensor surfaces. Other useful modification schemes include bulk modification of composite carbon materials, covalent (chemical) attachement, physical adsorption, and spontaneous chemisorption [35, 37].

2.5. Enzymes

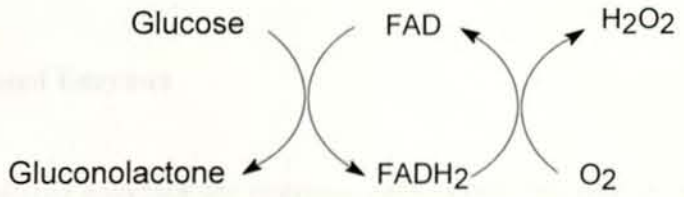
Enzymes are biological catalysts (i.e. they speed up the rates of reactions without themselves undergoing any permanent change) [38]. They accelerate reactions through a lowering of the activation energy. As for other catalysts, enzymes catalyze both the forward and reverse reactions, and promote the attainment of equilibrium without altering it. Besides their catalytic function, the most striking features of enzymes is their specificity. Enzymes catalyze a certain kind of reactions and they react with a single reactant, or a number of structurally similar reactants. These reactants are called substrates.

Enzymes are proteins, sometimes containing a non-protein component, which can be a cofactor or prosthetic group. The region in the enzyme where the substrate binds and where the reaction takes place is called the active site. It is small compared to the enzyme, and a large part of the aminoacid residues in the enzyme is required for maintaining the three-dimensional integrity of the net work.

On the basis of the kind of chemical reactions they catalyze enzymes are classified into six main classes. These are oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases [39]. Those which catalyze redox reactions are known as oxidoreductases. They are further divided into dehydrogenases, oxidases and peroxidases, depending on the substrates to which they promote electron transfer. Oxidoreductases, both in the free and immobilized forms, are the most important catalytic reagents in bioanalysis because they involve substrates or products that can be conveniently monitored by a wide choice of detectors [24, 40-42].

2.5.1. Oxidases

Oxidases catalyze the transfer of hydrogen atoms to oxygen forming either H_2O_2 (2 H^+, 2e^- transfer) or H_2O ($4H^+$, 4e^- transfer). The substrate is oxidized to a product with the concomitant reduction of the enzyme bound cofactor. The oxidation of glucose with glucose oxidase (GOD, EC 1.1.3.4) is one of the most common reactions used for enzymatic analysis. The active site of this enzyme is a flavine adenine nucleotide (FAD), which exists in one of two forms-oxidised (FAD) or reduced ($FADH_2$). FAD oxidizes glucose to gluconic acid, and the $FADH_2$ generated by this reaction can be oxidized to FAD by oxygen (hydogen peroxide is a by-product of this reaction). The mechanism can be shown as follows [43].

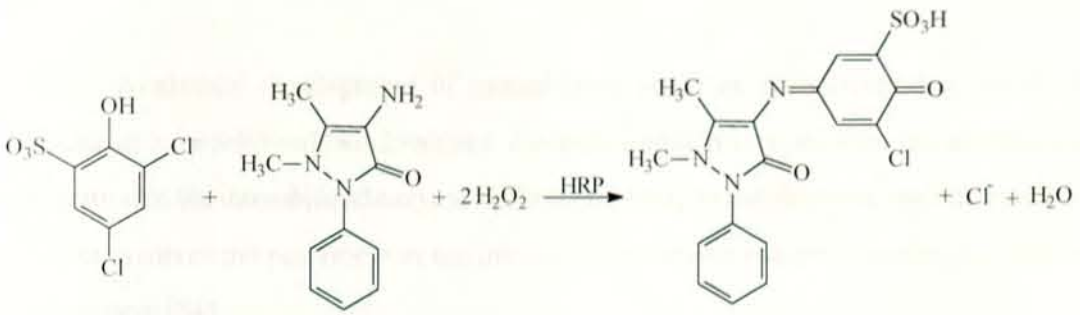


2.5.2. Peroxidases

Peroxidases catalyze the transfer of hydrogen from a reducing co-substrate to hydrogen peroxide or other organic peroxides. The prosthetic group of most peroxidases, including horseradish peroxidase, HRP (EC 1.11.1.7), is ferriprotoporphyrin IX, a redox-active porphyrin with central iron(III) bound to this class of enzymes [44]. HRP catalyzes the reduction of hydrogen peroxide by substrates.

Chromogenic substrates whose oxidative products strongly absorb in the visible region are commonly used in spectrophotometric detections of hydrogen peroxide [45, 46]. Oxidative coupling of aromatic amino compounds with other aryl compounds in presence of HRP is another class of reaction that produce colored products [47-51]. This includes the chromogenic couple 4-aminoantipyrene (4-AP) and 2,4-dichlorophenol-6-sulphonate (DCPS) reported by Barham and Trinder. The reagent has been used for the determination of glucose [48] and uric acid in

biological fluids [49], using the respective oxidase to generate hydrogen peroxide. The reaction (Trinder reaction), represented in the equation below, gives a red quinoneimine complex which absorbs at 512-514 nm.



(5)

2.6. Immobilized Enzymes

Immobilized enzymes are enzymes constrained one way or another within a limited confines of a solid support. The solid support can be polysaccharides (e.g. agarose, cellulose), inorganic supports (such as porous glass, porous ceramics, alumina), synthetic polymers (e.g. nylon) [52]. Enzymes can be constrained or held to these supports by adsorption, cross linking, microencapsulation and covalent attachment [52, 53].

Immobilization by covalent attachment is the most frequently used method. There are several methods of preparing covalent conjugates, but the most popular techniques involve the reaction of an aqueous solution of enzyme with an activated, derivatized water insoluble support. Triethoxysilane derivatization and glutaraldehyde activation on controlled pore glass was used in this work.

The major aim of research in immobilized enzymes for biotechnological and analytical applications is to increase the life-time i.e. thermal, operational as well as mechanical stability of the biocatalyst [54]. Applications of immobilized enzymes in analysis and biochemical synthesis offers significant advantages over their free counterparts. Most enzymes are expensive and the cost of the analysis, as a result, becomes high. The same batch of immobilized

biocatalyst can be used repeatedly because of the convenience in separating the reaction mixture from the catalytic phase, making the unit very practical for continuous flow analysis. Integration of immobilized enzyme reactors or biosensors in flow injections (FIA) of biochemical substances has been a major advance in all fields where bioanalysis is applied [55].

Analytical development of immobilized enzymes with electrodes, as the physical transducer, have followed two directions: the most common version of the biosensor involves the integration of the immobilized enzyme in close proximity to the electrode, and the enzyme reactor version involves the placement of the immobilized enzyme reactor preceding an electrode in a flow system [54].

2.7. ENZYME KINETICS

For one substrate enzyme catalyst reaction, the mechanism of the reaction for the conversion of the substrate, S to product P is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} P + E \qquad (6)$$

where E is the enzyme and ES is the enzyme-substrate complex [39, 56]. The second reaction is rate-limiting at very high substrate concentrations and almost all the enzyme is present as the enzyme-substrate complex, making the rate of formation of the product negligible, i.e. $k_2 << k_{-1}$. Hence, at the initiation of the reaction, [p] = 0, and the rate of formation of ES from the product can be ignored. Equation 6 is, therefore, reduced to

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} P + E \qquad (7)$$

At steady state the rate of formation of ES equals the rate of its decomposition (i.e. $k_1[E][S_0] = (k_{-1} + k_2)[ES]$). With this assumption, the well known Michaelis-Menten relationship, shown in eq. 8, is valid [41]. The equation predicts the following relationship between the rate of the enzymatic reaction and the substrate concentration.

$$V_o = V_{max}[S_o] / (K_m + [S_o]) \quad (8)$$

where V_o is the initial rate, V_{max} is the maximum rate at a saturating concentration of S (i.e. [S] >> k_m, $V_{max} = k_2[E_T]$) and K_m is the Michaelis constant defined by $K_m = (k_{-1} + k_2) / k_1$ or $K_m = [S]$ at $1/2V_{max}$.

2.7.1. Electrochemical Michaelis-Menten Formulations

In amperometric biosensors the current registered at an electrode is a measure of the reaction rate as a result of the enzymatic reaction. An electrochemical expression of eq. 8, can thus be given as:

$$i = i_{max} C / (K_m^{app} + C) \quad (9)$$

where i and C denote the current measured at steady state conditions and the bulk concentration of the substrate, respectively. The calculated K_m for an immobilized enzyme usually differs from the K_m value of the enzyme in solution. This is caused by changes in the enzyme microenvironment, enzyme conformation, or diffusion effects [57]. Therefore, for immobilized enzymes an "apparent" K_m (K_m^{app}) is defined.

The calculation of i_{max} and K_m^{app} from the Michaelis-Menten relationship eq.(9) is not straight forward. Rearrangement of eq. 9 gives relationships for the determination of i_{max} and K_m^{app}. The reciprocal of equation 9 gives the most common linearised form of Michaelis-Menten equation, called Line weaver-Burk equation. Since the Line weaver-Burk equation is a double reciprocal transformation, it gives a higher weight to points at lower substrate concentrations.

$$1/i = K_m^{app} / i_{max} C + 1/i_{max} \quad (10)$$

A plot of 1/i vs1/C gives a straight line with a slope of K_m^{app} / i_{max} and an intercept of $1/i_{max}$ which can further be applied to evaluate i_{max} and K_m^{app}. If the enzymatic reaction is controlled

by mass transfer rather than by the enzyme kinetics, the Line weaver-Burk plot may deviate from linearity.

Cross multiplication of eq. (9) leads to the Eadie-Hofstee equation;

$$i = - K_m^{app} i / C + i_{max} \qquad (11)$$

plotting i vs i / C gives a straight line with a slope $- K_m^{app}$ and an intercept of i_{max}. Response limitation due to mass transfer or depletion of cosubstrate is often more obvious in the Eadie-Hofstee plot.

Another statistically superior linearized form of C / i vs. C with a slope of $1/i_{max}$ and an intercept of K_m^{app} / i_{max}, Hanes plot, is obtained by multiplying eq. 10 by C.

$$C / i = C / i_{max} + K_m^{app} / i_{max} \qquad (12)$$

Both eq. 11 and 12 decrease the overemphasis on lower substrate concentrations.

3. EXPERIMENTAL

3.1. Flow injection system

A diagram of the flow injection system is shown in Fig. 1. The flow injection system consisted of a two channel peristaltic pump (Gilson model M 321), a Rheodyne 7125 injection valve and a wall-jet electrochemical cell. The cell consisted of film modified carbon paste working electrode, a Ag/AgCl reference electrode and a built-in platinium counter electrode. The response of the reaction was monitored with an amperometric detector (Zata LC 4, Hoor, Sweden) and the currents obtained were recorded with a strip-chart recorder (Kipp and Zonen, Sweden).

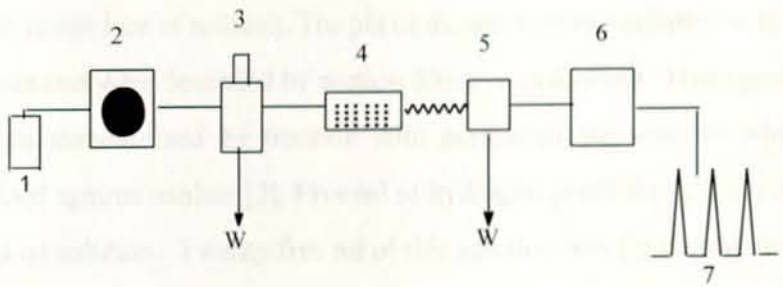


Fig. 1. A schematic diagram of the FIA biosensor.

1. Buffer, 2. Pump, 3. Injection port, 4. Immobilized enzyme reactor
5. Cell, 6. Detector, 7. Recorder.

3.2. Working electrode

A Teflon rod with a cavity for carbon paste filling served as the electrode body. Electrical contact was made with graphite through one end of the rod. Unmodified carbon paste was prepared by adding 0.35 ml of paraffin oil (Uvasol, Merk) to 1 g of spectral carbon powder (RWB, Ringsdorff-Werke, Bad Godesberg, Germany). The carbon paste was packed into the hole of the electrode and the surface was smoothed with a spatula. Film formation was made by the electrolysis of Mn (II) solutions (containing 20 mg/l) in phosphate buffer (0.1 M, pH 7) and /or in (0.2 M NH_3/NH_4Cl) on to the surface of the paste with a plating potential of 0.65 V vs Ag/AgCl for 120 seconds [19].

3.3. Reagents and solutions

The reagents 4-Amino-antipyrene, AP(BDH), D-(+)-glucose(Sigma), 2,4-dichlorophenol-6-sulphonate (DCPS) which was already synthesized in our laboratory from DCP and concentrated sulphuric acid according to the method of Barham and Trinder [48], were used as such.

Ammonia-ammonium chloride buffer (0.2 M, pH 9.5) was prepared by dissolving 5.35 g of ammonium chloride in 7.5 ml of 25 % ammonia and diluting to 1000 ml with water.

Phosphate buffer (0.1 M, pH 7) was prepared by dissolving 11.98 g of sodium hydrogen phosphate in one litre of solution. The pH of the solution was adjusted with NaOH solutions. The carrier solutions were deaerated by suction filtration before use. Hydrogen peroxide (30% (w/v), BDH) was standardized by titration with permanganate solution which in turn had been standardized against oxalate [3]. Five ml of hydrogen peroxide solution was diluted with water to 500 ml of solution. Twenty five ml of this solution was further diluted to 200 ml and 20 ml of dilute sulphuric acid (1:5) was added to it. The resulting solution was titrated with standard 0.1 N potassium permanganate solution. An aqueous stock solution containing 10 g/l H_2O_2 was prepared freshly each day. Solutions of lower concentrations were prepared with doubly distilled water immediately before use. Manganese chloride was obtained from BDH chemicals Ltd. Ten mM of D-(+)-glucose was used as a stock solution for glucose. The reagent for the spectrophotometric determination of glucose was prepared in a 0.1 M phosphate buffer (pH 7) that consisted of 2.5 mM DCPS, 0.5 mM 4-AP and 1 mg/100 ml of horseradish peroxidase (HRP, E.C. 1.11.1.7. 268 purpurogallin units/mg of solid, Sigma). The carrier in the flow system was phosphate buffer containing 0.5 mM EDTA.

3.4. Optimization of FIA parameters

The parameters such as flow rate, injection volume, working potential and linear range were optimized in both ammonia-ammonium chloride, and phosphate buffers.

3.5. Immobilization of enzymes

Two hundred milligrams of controlled pore glass (CPG-10 with 0.08-0.12 mm particle size and 530 angestrom pore size, Serva) was treated with concentrated nitric acid for 2 hours. It was then thoroughly washed with doubly distilled water and dried over night at 190 °C in an oven. The glass was then silanized by refluxing in a boiling water bath with 10% 3-aminopropyltriethoxy silane in dry toluene for 40 min. It was thoroughly washed with toluene and acetone over a G-3 filter and then dried over night in an oven at 115 °C. Test on the silanization of the CPG was made by adding 3-4 drops of 1.5 % 2,4,6-trinitrobenzene sulphonate solution in ethanol in a test tube containing small amount of the derivatized CPG mixed with saturated sodium borate solution. Positive test for the product would give a bright orange coloured surface within about 5 min [58]. The silanized support was activated at reduced pressure with 2.5 % glutaraldehyde in 0.1 M phosphate buffer at pH 7 for 30 min. The activated support was thoroughly washed with doubly distilled water over a G-3 filter. Prior to activation the stock 25 % glutaraldehyde solution (Sigma, G-6257) was mixed with activated charcoal and centrifuged to remove any possible polymeric product.

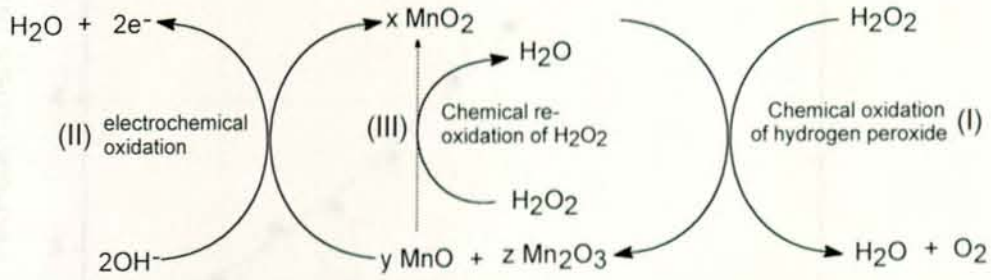
Fifteen milligrams of glucose oxidase (GOD, E.C.1.1.3.4, 128,000 units/gm of solid from Aspergillus niger, Sigma), was dissolved in 3.5 ml of 0.1 M phosphate buffer (pH 7). The enzyme solution was added to 150 mg of silanized and activated controlled pore glass according to a previously described procedure [59]. The immobilized enzyme-support was packed in a 50 µl plexiglass tube and stored at 4 °C in 0.1 M phosphate buffer at pH 7 when not in use.

3.6. Analysis of sample (human serum)

Different serum samples were collected from the Police Hospital, Addis Ababa and each of these samples was diluted 1:20 (v/v) with distilled water. Evaluation of the glucose concentrations for each sample was made from the slope of linear regression of the data collected within one hour. Reference measurements were performed by spectrophotometric determinations of each samples (using Trinder reagent).

4. RESULTS AND DISCUSSION

Hydrogen peroxide reacts chemically with the mediator (MnO_2) by forming H_2O, O_2, and manganese oxides (I); the latter are electrochemically reoxidized which produce the catalytic current (II). As can be expected, chemical reoxidation of oxides of manganese in lower oxidation states should also occur (III), which corresponds overall to a mere decomposition of H_2O_2 catalyzed by MnO_2. Reaction (III) is obviously kinetically slower than (II) because the catalytic activity of the modifier can be electrochemically monitored [11].



4.1. FIA of hydrogen peroxide

For flow injection analysis the film modified electrode was used as an amperometric detector in a wall-jet cell using both ammonia/ammonium chloride and phosphate buffers.

4.1.1. Ammonia buffer (0.2 M, pH 9.5)

Hydrogen peroxide has been studied very recently using flow injection analysis and MnO_2 bulk modified carbon paste electrode [11], MnO_2 film modified carbon paste electrode [18], and MnO_2 modified screen printed electrode [19] in ammonia buffer. The purpose of the present study using ammonia buffer was intended to check the reproducibility of the previous studies using our experimental set-up.

The dependence of the amperometric signal on the applied working potential is shown in Fig. 2. A maximum current is observed at a potential of around 0.58 V and this was used for further mesurements. At lower potential values the current decreases significantly. This could be due to the less efficient electrochemical oxidation that allows the chemical decomposition of H_2O_2 to become dominant.

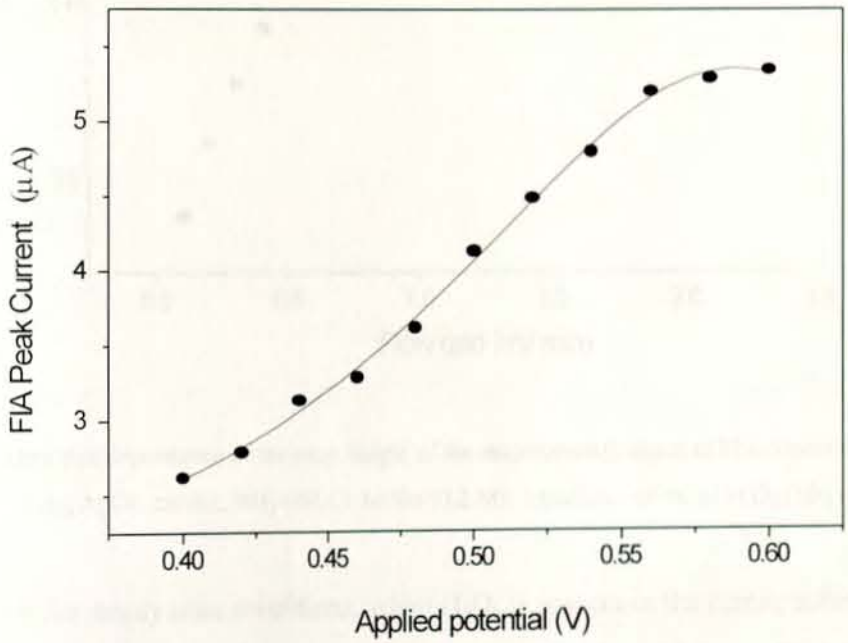


Fig. 2. Dependence of the current response in FIA on the applied working potential. Flow rate, 0.5 ml/min; carrier, NH_3-NH_4Cl buffer (0.2 M); injections of 50 μl H_2O_2 (100 mg/l).

The dependence of the amperometric response on the flow rate could be observed under injection as well as steady state conditions. Under injection conditions the peak height increases up to a flow rate of about 1 ml/min, then it decreases at higher flow rates (Fig 3). This might be probably due to dispersion of the analyte in the carrier. The decrease is significant and suggests that apart from increased dispersion of H_2O_2 in the carrier, a chemical reaction is involved which precedes the electrochemical reaction and which is kinetically slow.

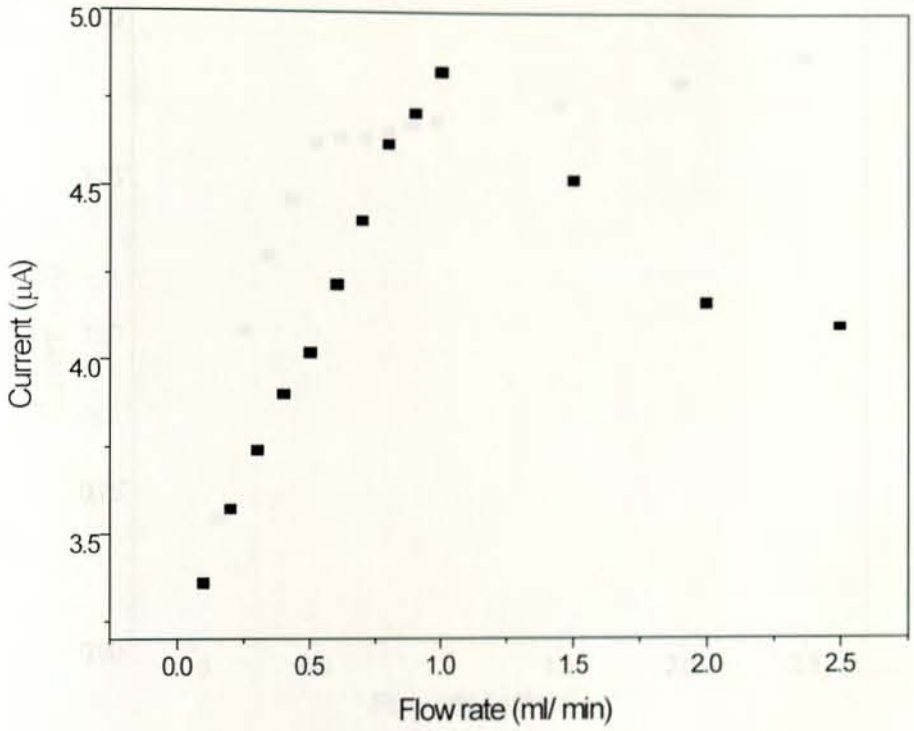


Fig. 3. Flow rate dependence of the peak height of the amperometric signal in FIA. Operating potential 0.58 V vs. Ag/AgCl; carrier, NH_3-NH_4Cl buffer (0.2 M); injections of 50 µl H_2O_2 (100 mg/l).

Under steady state conditions, when H_2O_2 is present in the carrier solution, the response increases sharply with slow flow rates, and only gradually with faster flow rates (Fig 4). For Faradaic processes under steady state conditions, and for a wall-jet arrangement, the relation between the current response and the flow rate raised to a power of 3/4 is expected to be linear [35]. However, the result obtained is different from this indicating that besides the Faradaic process a chemical process is involved, that is most probably the catalytic decomposition of H_2O_2 at MnO_2.

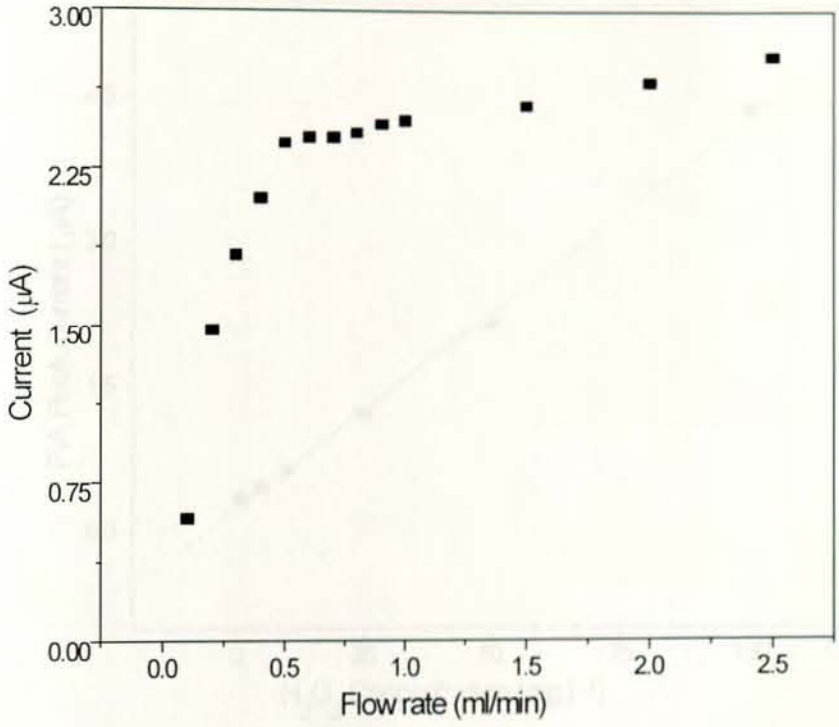


Fig. 4. Flow rate dependence of the peak height of the amperometric signal under steady state conditions. Operating potential, 0.58 V vs. Ag/AgCl; carrier, NH_3-NH_4Cl buffer (0.2 M) containing H_2O_2 (50 mg/l).

The relationship between the FIA peak current and the concentration of hydrogen peroxide was investigated for the range 1-500 mg/l. Linear relationship exists up to 100 mg/l and the linear regression y (μA) = 0.041X (mg/l H_2O_2) + 0.232 gives a correlation coefficient of 0.999 (Fig. 5). With higher concentrations a deviation occurs probably due to an unfavourably high ratio of H_2O_2 to MnO_2. It may also be assumed that in the presence of large amounts of H_2O chemical oxidation of the reduced modifier becomes more and more dominant.

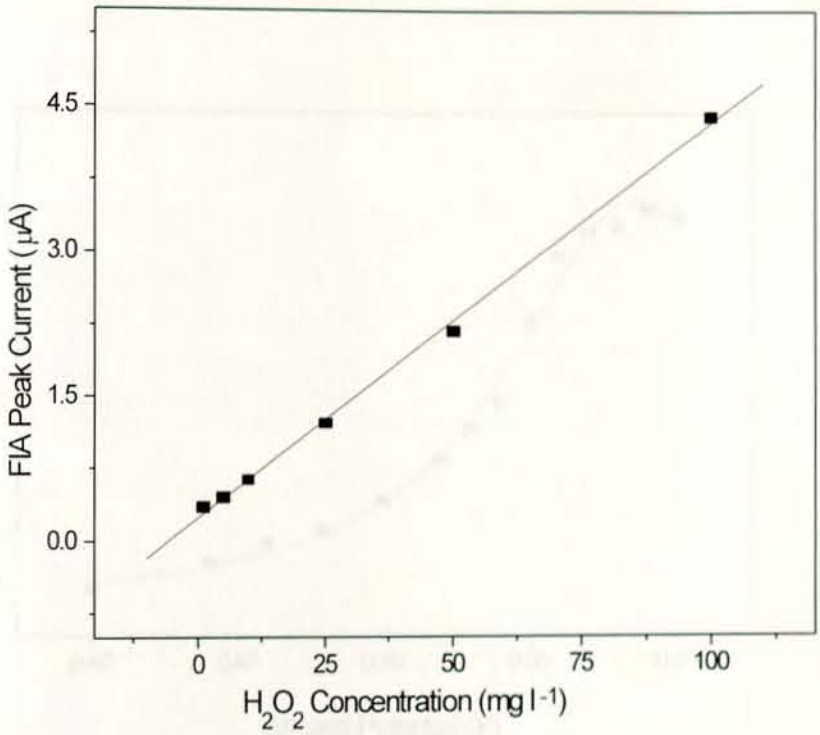


Fig. 5. Linear range. Operating potential, 0.58 V; carrier NH_3-NH_4Cl (0.2 M); injections of 50 µl H_2O_2; flow rate, 1 ml/min.

4.1.2 Phosphate buffer

Fig. 6. illustrates the influence of the working potential on the amperometric signal of hydrogen peroxide (100 mg/l). As illustrated in the figure the response increases with increasing applied potential showing a levelling off at + 0.57 V vs. Ag/AgCl. Obviously, this value is sufficient enough to reoxidise electrochemically all the manganese which has been reduced by chemical reaction with the analyte. Therefore, an operating potential of 0.57 V was selected for further investigations. At lower potential values the current decreases significantly; probably, due to less efficient electrochemical oxidation allowing the chemical decomposition of H_2O_2 to become dominant.

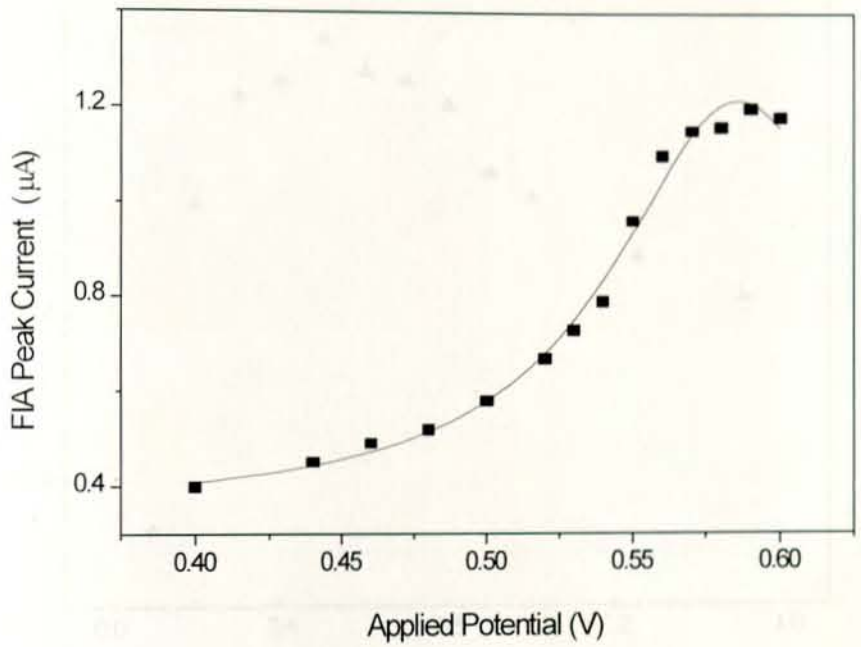


Fig. 6. Dependence of the current response in FIA on the applied working potential. Flow rate, 0.5 ml/min; carrier phosphate buffer (0.1 M); injections of 50 μl H_2O_2 (100 mg/l).

The influence of the flow rate both under flow injection conditions and steady state conditions (analyte in the carrier) on the amperometric signal is shown in Figures 7 and 8, respectively. Under injection conditions the current response increases up to about 0.5 ml/min, whereas it decreases at higher flow rates. The overall shape of the figure is similar to the study made using ammonia buffer as carrier and suggests that there exists here again a slow chemical reaction that precedes the electrochemical reaction.

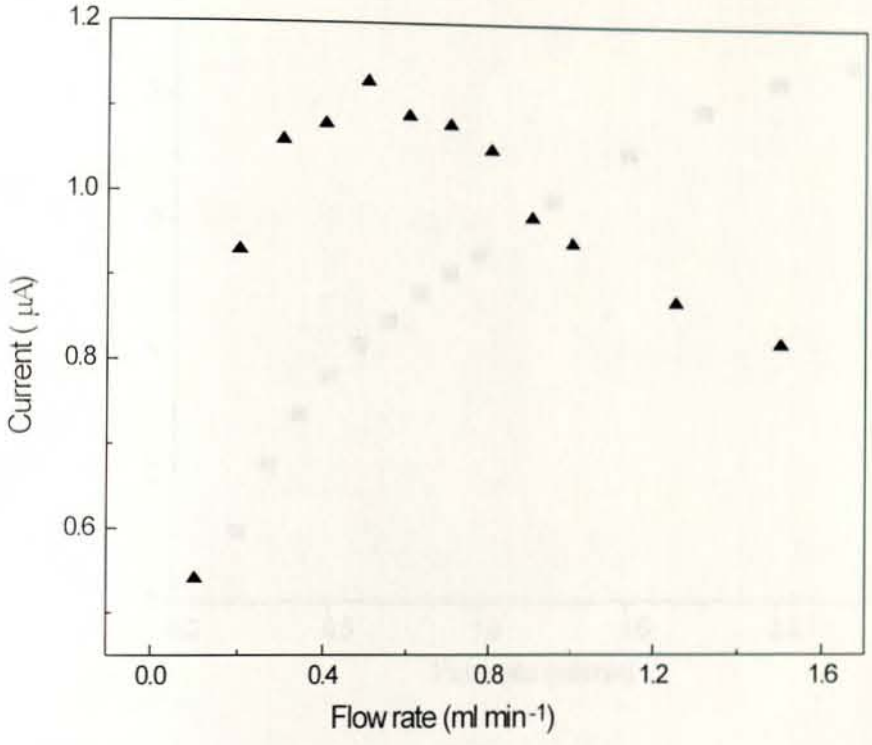


Fig. 7. Flow rate dependence of the peak height of the amperometric in FIA. Operating potential, 0.57 V; carrier, phosphate buffer (0.1 M); injections of 50 μl H_2O_2 (100 mg/l).

Under steady state conditions, the response increases for low flow rates and gradually with higher flow rates . For faradaic processes one would expect a dependence of the current on the three-fourth root of the flow rate in a wall-jet cell [35, 36]. However, a plot of the three-fourth root of the flow rate vs. the amperometric response doesn't give a linear relationship. Increasing flow rates causes a diminishing of the diffusion layer at the electrode surface which will lead to an increase of the diffusion current.

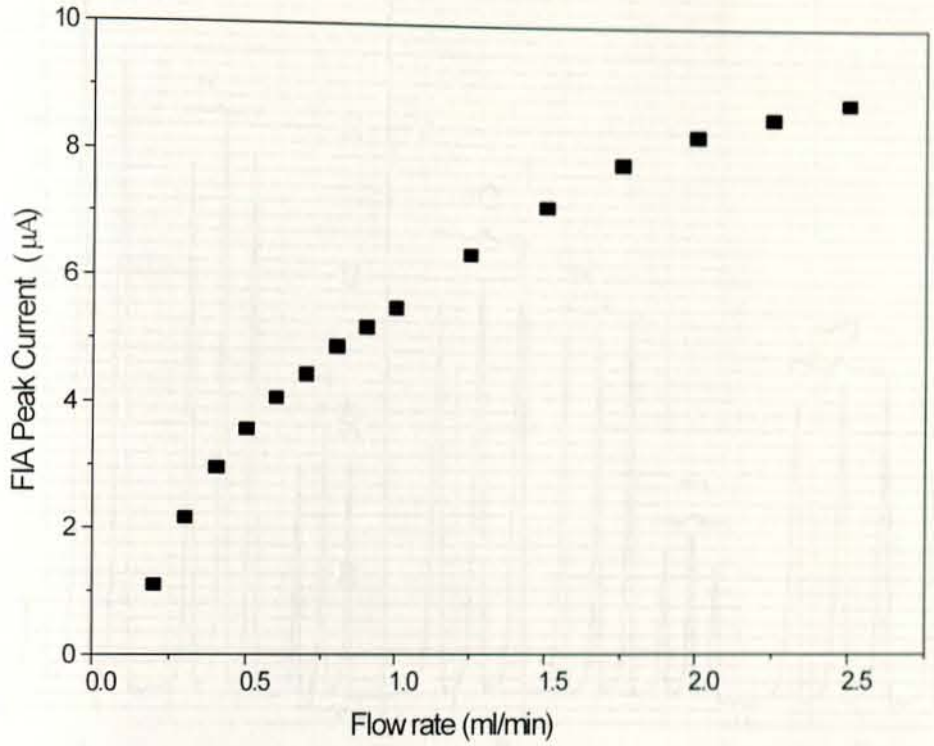


Fig. 8. Flow rate dependence of the peak height of the amperometric signal under steady state conditions. Operating potential, 0.57 V; carrier, phosphate buffer (0.1 M) containing H_2O_2 (50 mg/l).

The injection volume also influences the amperometric signal. The current increases with increasing the injection volume. Better sensitivity and reproducibility was obtained with 50 μl of injection sample loop (Fig. 9). Considering the low injection volume and sensitivity of the system a 50 μL sample injection volume was chosen for further analysis.

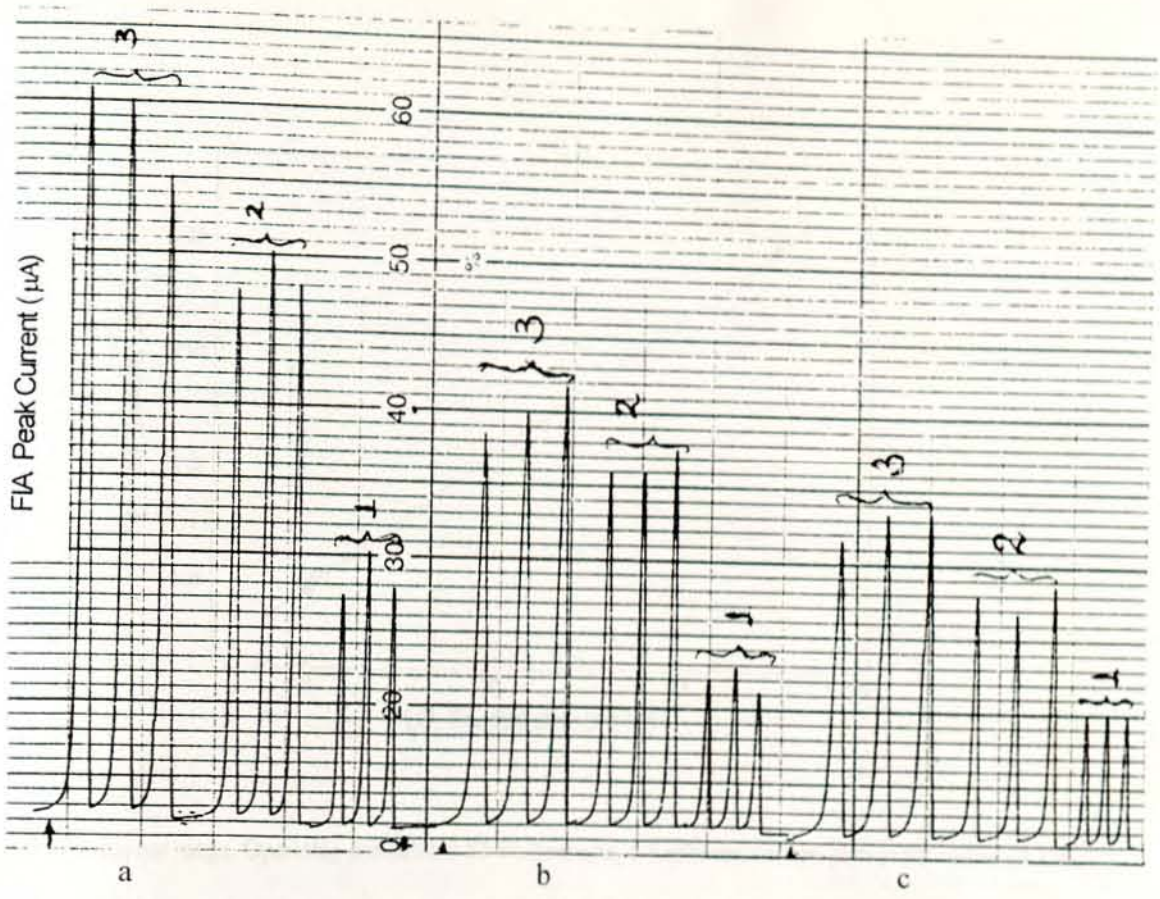


Fig. 9. FIA responses of H_2O_2. Operating potential 0.57 V; carrier, phosphate buffer (0.1 M), flow rate 0.5 ml/min; H_2O_2 concentration, (a) 30, (b) 20, and (c) 10 mg/l; injection volume (1) 20, (2) 50, and (3) 100 μl.

Linear Range, Detection Limit and Reproducibility

The relationship between the amperometric peak current and the concentration of H_2O_2 was investigated for the range 0.2-500 mg/l (Fig. 10a and 10b). Two linear ranges (0.2-50 mg/l and 50-500 mg/l) with linear regression y (μA) = 0.0024X (mg/l H_2O_2) + 0.077 and y (μA) = 0.0113X (mg/l H_2O_2) - 0.327, respectively, have been obtained. In both cases the correlation coefficient was found to be 0.999. With concentrations greater than 500 mg/l a deviation occurs, probably owing to an unfavourably high ratio of H_2O_2 to MnO_2. The detection limit (3 σ values, standard deviation for 10 injections of 50 μl H_2O_2 in a concentration of 0.2 mg/l) was determined to be 31 μg/l. For a concentration of 0.2 mg/l (10 injections of 50 μl H_2O_2 solutions) the relative standard deviation was determined as 0.4 %. A reproducibility study made on a series of 20 repetitive injections of 100 mg/l of H_2O_2 showed a relative standard deviation (RSD) of 0.97 % and a sampling frequency of around 72 samples/h could thus be realized.

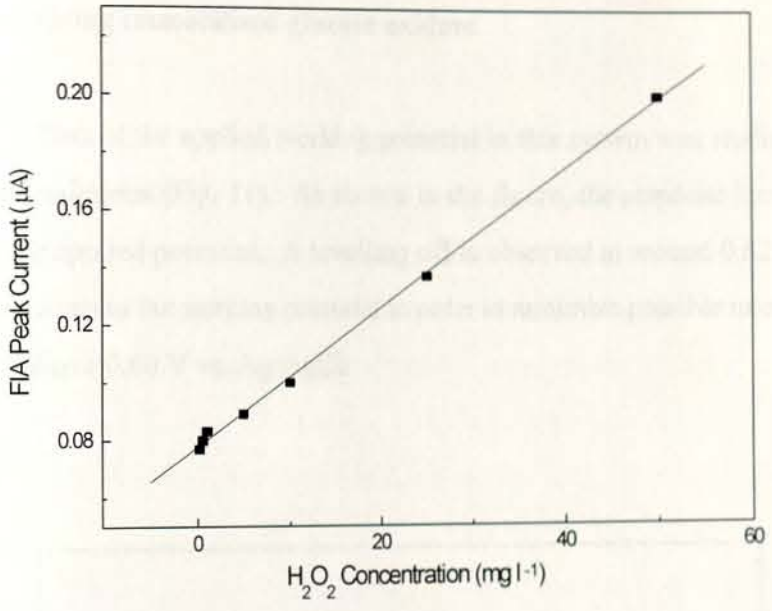


Fig. 10a. Linear range. Operating potential, 0.57 V; flow rate, 0.5 ml/min; carrier, phosphate buffer (0.1 M).

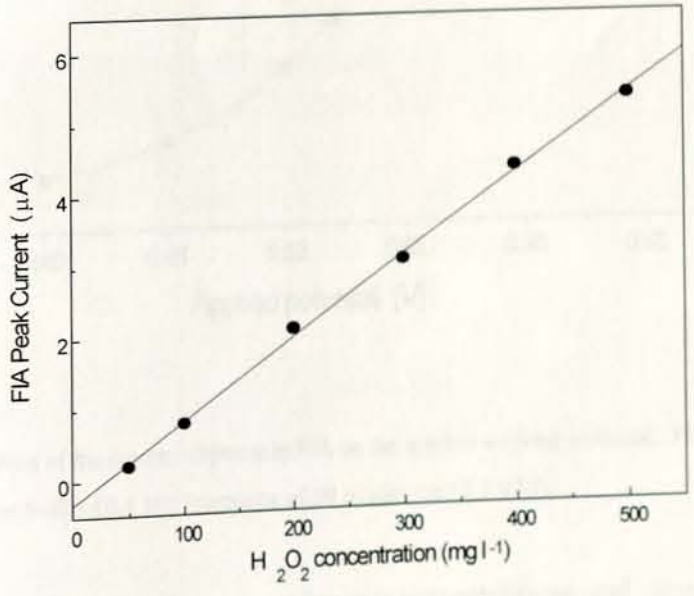


Fig. 10b. Linear range. Operating potential, 0.57 V; flow rate, 0.5 ml/min; carrier, phosphate buffer (0.1 M).

4.2. Optimization of FIA parameters for glucose determination in the sensor containing immobilized glucose oxidase

The effect of the applied working potential in this system was studied by injecting 0.5 mM glucose solutions (Fig. 11). As shown in the figure, the response increases steadly with increasing the applied potential. A levelling off is observed at around 0.62 V. A potential of 0.59 V was chosen as the working potential in order to minimize possible interferences that could be oxidized above 0.60 V vs. Ag/AgCl.

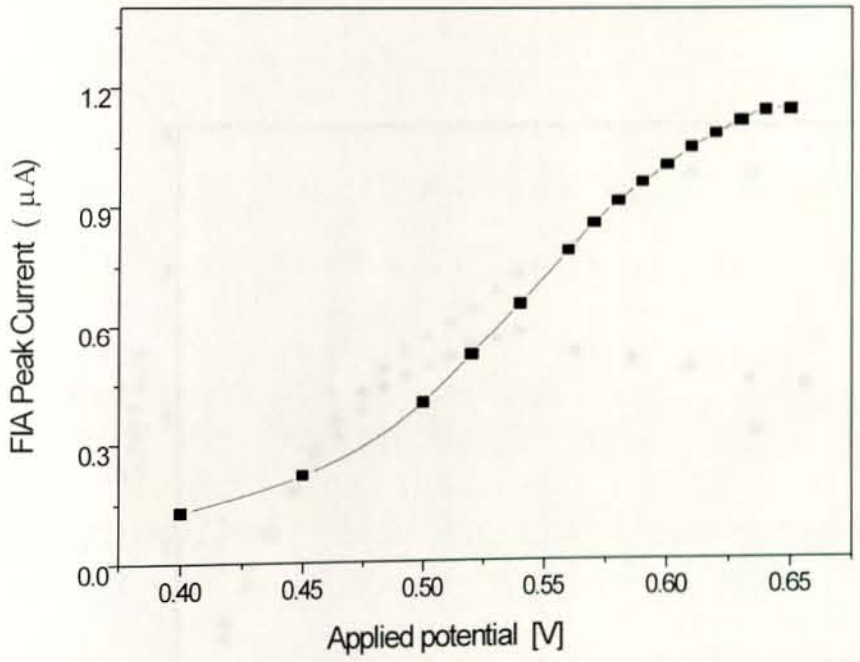


Fig. 11. Dependence of the current response in FIA on the applied working potential. Flow rate, 1 ml/min; carrier, phosphate buffer (0.1 M); injections of 50 μl glucose (0.5 mM).

The influence of the flow rate under injection conditions and steady state conditions on the amperometric signal is shown in Fig. 12. Since flow rate is inversely related to the residence time of the substrates and products within the enzyme reactor [60], decreasing the flow rate will increase the conversion efficiency of the reactor and higher flow rates produce a decrease of the

signal. Hence, there is a minimum flow rate required for equilibrium to set-in, depending on the activity of the enzyme in a reactor.

Both under steady state and injection conditions, the current responses increase significantly and similarly with low flow rates up to about (0.9 ml/min). The response keeps on increasing steadly with higher flow rates for the steady state condition (curve b). Whereas the current signal levels off with increasing flow rates under injection condition. A plot of the three-fourth root of the flow rate vs the current response shows a linear relationship (R= 0.998) for the range 0.7 - 2.25 ml/min (Fig. not shown), with steady state condition as expected theoretically [35]. Hence for the subsequent determination of glucose with the sensor, a flow rate of 1 ml/min was used.

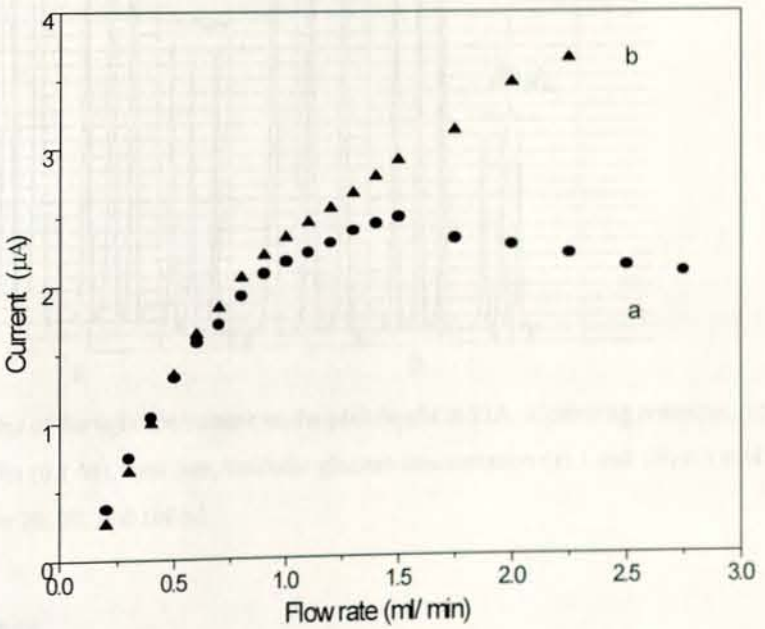


Fig. 12. Flow rate dependence of the peak height of the amperometric signal.
a. Carrier phosphate buffer (0.1 M); injections of 50 μl glucose (0.5 mM);
b. Carrier phosphate buffer containing glucose (0.5 mM).

The effect of injection volume on the response of the FIA sensor is shown in Fig. 13. There is an increase in the response with increasing injection volume. With respect to analysis time, sensitivity and reproducibility, a 50 µl sample injection volume was chosen for further measurements.

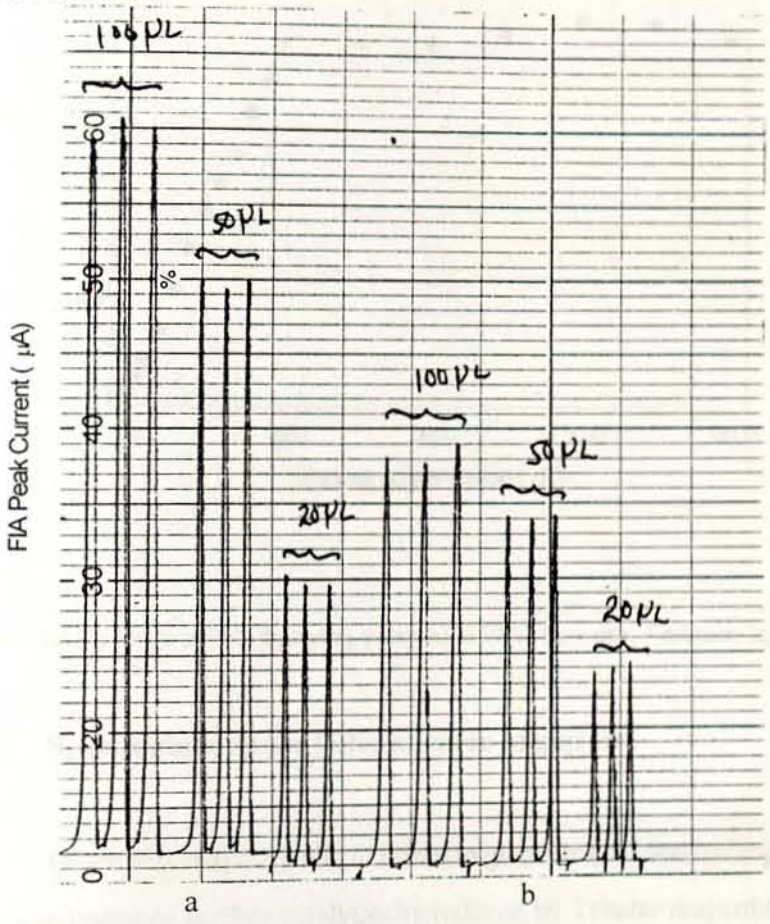


Fig. 13. Effect of the injection volume on the peak height in FIA. Operating potential, 0.59 V; carrier, phosphate buffer (0.1 M), flow rate, 1ml/min; glucose concentration (a) 1 and (b) 0.5 mM; injection volumes used were 20, 50, and 100 µl.

Linear range

The dependence of peak current on the glucose concentration is shown in Fig.14. There is an excellent linear response to glucose in the range 0.1 - 1000 µM and the linear regression y (µA) = 0.000876X (µM glucose) + 0.03451 shows a correlation coefficient of 0.9996. At higher concentrations the current signal of the sensor becomes constant indicating that the enzyme attains saturation. The detection limit (3 σ values, standard deviation for 10 injections of 50 µl glucose in a concentration of 0.1 µM) was determined to be 5.5 nM.

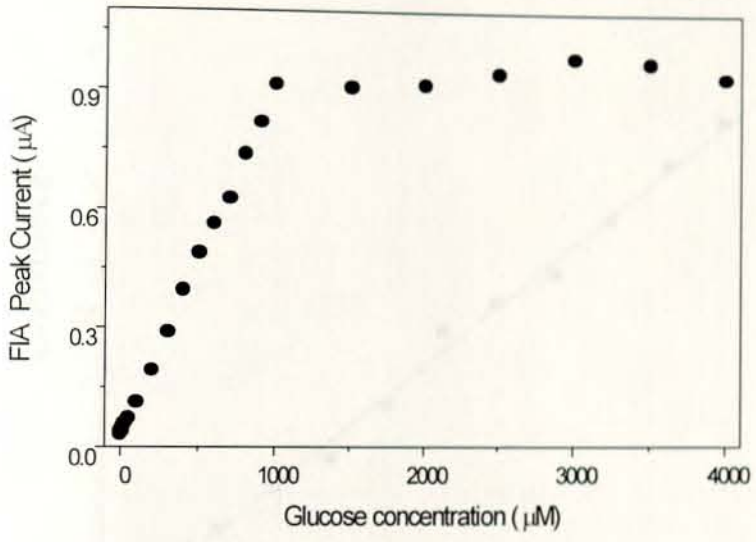


Fig. 14. Linear range in FIA. Operating potential, 0.59 V; flow rate, 1 ml/min; injections of 50 μl glucose.

4.2.1. Spectrophotometric Determination of glucose

Glucose is enzymatically oxidized to gluconic acid and hydrogen peroxide. The resulting hydogen peroxide is, then catalytically reduced by Trinder reagent to give a red quinoneimine complex which absorbs at 512 nm as indicated by equation 5. Spectrophotometric measurements of glucose were carried out in order to use the resuts obtained as reference data.

The relationship between the absorbance and the concentration of glucose was investigated in the range 1 - 1300 μM (Fig. 15). A linear relationship exists up to 1000 μM and the linear regression y (AU) = 0.00346X - 0.0429 gives a correlation coefficient of 0.998.

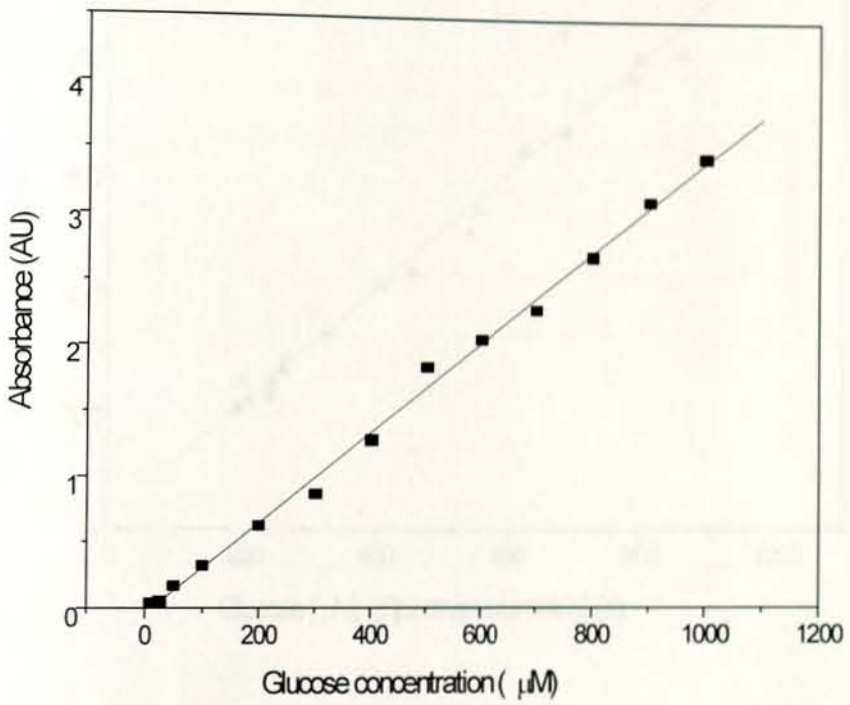


Fig. 15 Spectrophotometric linear range; flow rate, 0.4 ml/min.

4.2.2. Assay of glucose in human serum sample.

The FIA sensor has been applied for the analysis of glucose in human serum samples. Each sample was analyzed at a flow rate of 1 ml/min. For comparison, the results obtained by this method (Y) and those by the spectrophotometric method (X) are plotted in Fig.16. As can be seen from the plot, there is a very good linearity between these two sets of data showing the agreement of the two methods. Least squares analysis of the relationship between the two methods is $Y = 0.98x + 17.455$ μM (correlation coefficient = 0.982, n = 19).

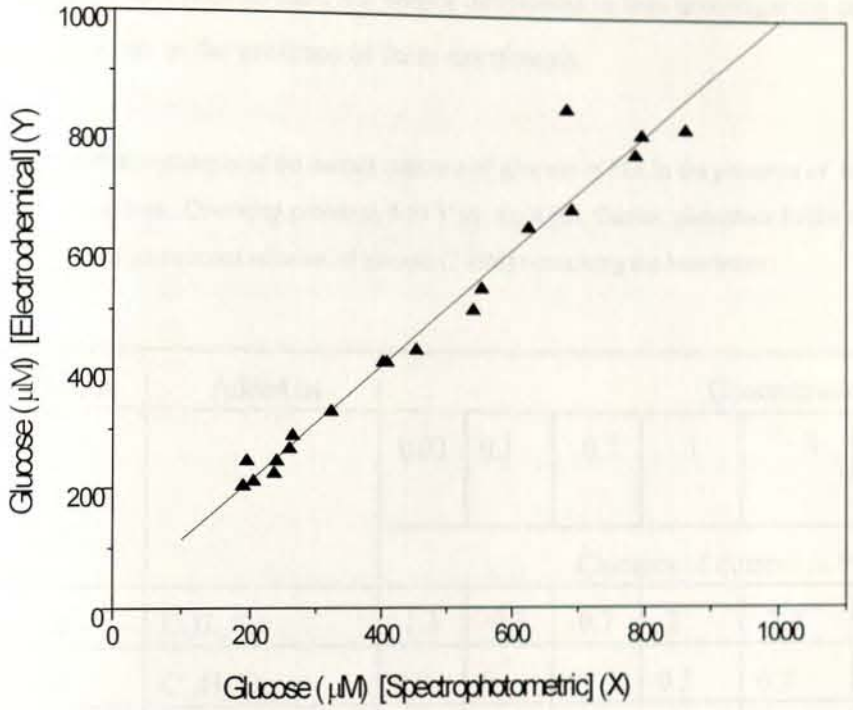


Fig. 16. Results of glucose analysis in human serum. The data were obtained with the FIA sensor (y) and spectrophotometric method (x) using Trinder reagent.

4.2.3. Interferences

The effect of possible organic interferents on the response of the FIA sensor was investigated and the results are shown in Table1. The carbohydrates: (fructose, sucrose and DL-xylose), oxalic acid, pyruvic acid, glutamic acid, aspartic acid, and L- glutamine have very little or no effect in a 50-fold concentration excess with respect to glucose. DL-Tartaric acid, hypoxanthine and xanthine interfere at higher concentrations (10-fold concentration excess) probably due to their reaction with the generated H_2O_2. Ascorbic acid, uric acid, dopamine, and L-cysteine interfere very strongly even if they are present at concentration that is comparable to glucose. The observed increase in the current response when any of these interferents is present is presumably due to the chemical reduction of MnO_2 by the interferents. The approximate concentration of these species in human serum is : 0.1 mM ascorbate, 0.08 mM cysteine, 0.5 mM urate [61]. The sensor described here was very sensitive only above their maximum interfering levels. The concentration of glucose in human serum is in the range 3.6 - 6.1 mM in normal

humans [55], this implies that, the sensor developed in this investigation can be employed to quantify glucose in the presence of these interferents.

Table. 1. Relative changes of the current response of glucose in FIA in the presence of interferents. Flow rate, 1 ml/min.; Operating potential, 0.59 V vs. Ag/AgCl; Carrier, phosphate buffer (pH 7); injection of 50 µl of an aqueous solution of glucose (1 mM) containing the interferent:

Interferent	Added as	Concentration (mM)						
		0.02	0.1	0.2	1	5	10	50
		Changes of current in %						
Fructose	$C_6H_{12}O_6$	-1.3	-0.3	-0.7	-2	-2.4	-2.6	-7.3
Sucrose	$C_{12}H_{22}O_{11}$	0.00	0	0	0.3	6.8	8	8.3
(DL-) Xylose	$C_5H_{10}O_5$	-1	-0.3	-1.6	-4.3	-1.3	-1.6	-1.4
Ascorbic acid	$C_6H_8O_6$	3.3	51	133	721	-	-	-
Uric acid	$C_5H_4N_4O_3$	5.6	27.5	65	143	-	-	-
Oxalic acid	$C_2H_4(NH_4)_2.H_2O$	-3.3	-3	-2	-1.6	-1.6	-2.6	0.6
Dopamine	$C_8H_{11}O_2N.HCl$	5.66	41.6	94.6	732	-	-	-
Pyruvic acid	$C_3H_3O_3Na$	-2.6	-2.9	-2.9	-2.90	-1.2	-2.9	-7.2
Glutamic acid	$C_5H_9NO_4$	-0.6	-2.6	-2.3	-0.3	0	-2	-6
DL-Tartaric acid	$C_4H_6O_6$	1	-0.7	-0.7	-4	-15	-23.7	-
Hypoxantine	$C_5H_4N_4O$	0	0	0	0	- 5.33	-18	-
Xanthine	$C_5H_4N_4O_2$	0	0	0	-2	-20	-28	-
Aspartic acid	$C_4H_7NO_4$	1.4	2.4	1.4	-0.3	-1	-3.6	-9.6
L-Cysteine	CH_7NO_2S	-1	67.7	160	910	-	-	-
L-Glutamine	$C_5H_{10}N_2O_3$	-2.3	-1.6	-0.6	-2.3	-3.6	-2.3	-3.6

5. CONCLUSION

A carbon paste electrode modified with MnO_2 film can be used as a useful electrode material in the construction of a hydrogen peroxide based amperometric glucose sensor in flow injection analysis (FIA). The developed method was optimized with respect to flow rate, working potential, concentration and injection volume. As compared to platinium electrodes, (+ 700 mV vs Ag/AgCl [6]) these electrodes are operated at a lower oxidation potentials.

Investigation of the interferents on the sensors performance showed a notable current change by ascorbic and uric acid above their maximum biologically relevant concentrations. The method is applied for the determination of glucose in serum and the results obtained are in good agreement with that of the spectrophotometric method.

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DECLARATION

I, the undersigned, declare that this thesis is my original work, has not been presented for a degree in any other university and that all sources of material used for the thesis have been duly acknolwedged.

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