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Determination of Urinary Glucose by a Flow Injection Analysis Amperometric Biosensor and Ion-Exchange Chromatography

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ABSTRACT

A practical biosensor system has been developed for the determination of urinary glucose using a flow-injection analysis (FIA) amperometric detector and ion-exchange chromatography. Glucose oxidase was immobilized onto porous aminopropyl glass beads via glutaraldehyde activation to form an immobilized enzyme column. On the basis of its negative charge at pH 5.5, endogenous urate in urine samples was effectively retained by an upstream anion-exchange resin column. The biosensor system possessed a sensitivity of 160 ± 2.4 RU μM^{-1} (RU or relative unit is defined as 2.86 μV at the detection output) for glucose with a minimum detection level of 10 μM. When applied for the determination of urinary glucose, the result obtained compared very well with that of the widely accepted hexokinase assay. The immobilized glucose oxidase could be reused for more than 1000 repeated analyses without losing its original activity. The reuse of the acetate anion-exchange column before replacement would be about 25–30 analyses. Acetaminophen and ascorbic acid were also effectively adsorbed by the acetate anion exchanger. The introduction of this type of anion exchanger thus greatly improved the selectivity of the FIA biosensor system and fostered its applicability for the determination of glucose in urine samples.

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Index Entries: Urinary glucose; biosensor; immobilized glucose oxidase; ion-exchange chromatography; acetate anion resins.

INTRODUCTION

The determination of glucose levels in biological samples is an indispensable test for the diagnosis and therapy of diabetes mellitus, hypoglycemia, and suspected meningitis (*1*). The normal blood glucose level is about 90 mg/dL (5 m*M*), whereas the pathological value may increase up to 900 mg/dL. In the case of urinary glucose, the normal level found in healthy persons is about 18 mg/dL (*2*). Among several analytical procedures, electrochemical detection of enzymatically generated hydrogen peroxide is probably the most developed type of glucose biosensor. Amperometric glucose biosensors using immobilized glucose oxidase together with a sensitive hydrogen peroxide electrode have been used for in vitro and in vivo monitoring because of the high specificity of this enzyme for β-D-glucose (*3*). In such a biosensor, the enzyme glucose oxidase catalyzes the oxidation of glucose to D-glucono-δ-lactone and hydrogen peroxide. The latter then contacts with a platinum anode vs silver/silver chloride cathode poised at +0.7 V where electrochemical oxidation takes place, and the current generated is directly proportional to the glucose concentration in the measured sample. Unfortunately, hydrogen peroxide amperometric detection is also sensitive to several naturally occurring electron donors, such as ascorbate, urate, tyrosine, and so forth (*4*). As a consequence, amperometric biosensors based on hydrogen peroxide detection developed for measuring glucose are not directly applicable to blood and/or urine specimens, owing to the high urate and ascorbate concentrations that interfere with the electrochemical oxidation of hydrogen peroxide.

Several methods have been proposed to improve the selectivity of the glucose biosensor toward such electrochemically interfering substances. The simplest solution is to compensate the response by the addition of a second electrode, not associated with glucose oxidase, i.e., to form a differential system (*5*). In addition to its complexity, this approach, however, is only applicable for measurements in which the background signal is significantly lower than that of the enzyme electrode. In addition, it is somewhat difficult and/or expensive to produce electrodes that exhibit identical performance characteristics in view of response time and sensitivity. An elegant approach was patented by Clark (*6*) and then commercialized by Yellow Springs Instrument (OH) using a permselective membrane (cellulose acetate, for instance) to cover the platinum anode. This type of membrane only allows the diffusion of small molecules, such as oxygen, hydrogen peroxide, and so on, but excludes ascorbate and other

larger potential interfering substances. The main disadvantage of this approach is that it creates an additional diffusion layer that adversely affects the sensitivity and the response of the enzyme electrode. The linear range of this type of biosensor is limited at high glucose concentrations, because oxygen becomes a limiting factor, and of course, this type of composed membrane is more complicated to manufacture. An attempt to cover over the glucose oxidase membrane and the platinum electrode with a negatively charged dialysis membrane rejected only up to 0.0852 m*M* of ascorbic acid and 0.464 m*M* of uric acid (*7*). Higher concentrations of these substances, and also bilirubin and glutathione influence the glucose signal.

Less susceptibility to interference can be achieved by incorporating mediators, such as ferrocene and its derivatives, with glucose oxidase and by operating at applied potentials lower than 700 mV. Such systems have been designated as mediated biosensors, a second generation of amperometric biosensors (*8*). For disposable electrodes, such as the strips of the ExacTech™ pen-size glucose meter, the incorporation of mediator is very simple (*9*). However, for repeated analyses, complicated procedures must be used to anchor the mediator to the enzyme (*10*) or some polymer backbones (*11,12*). It should be noted that ascorbic acid at 0.13 m*M* still caused some significant interferences in ferrocene-based biosensors (*13*). Several organic conducting salts have been shown to provide good mediating properties without being anchored to high-molecular-weight entities (*14*). The best-known conducting salt is TTF-TCNQ, the salt of tetrathiafulvalene (TTF) and tetracyanoquinodimethane (TCNQ). Unfortunately, ascorbic and uric acids were still electroactive at the TTF-TCNQ structure, at a very low applied potential (*15*). In addition, the presence of oxygen significantly decreased the current response of the electrode. Undoubtedly, mediated biosensors can be useful for many applications, and a search for an ideal mediator is a current widespread subject. However, one must recall the original intention in the creation of mediated electrodes, i.e., to alleviate interference by operating at low potential.

In this study, a practical flow injection analysis (FIA) amperometric biosensor based on hydrogen peroxide detection has been used together with ion-exchange chromatography for the determination of urinary glucose. Glucose oxidase was covalently immobilized onto porous aminopropyl glass beads via glutaraldehyde activation to form an immobilized enzyme column. As a novel aspect of this study, an anion-exchange resin column was placed upstream for the removal of uric acid, ascorbic acid, and acetaminophen if present from urine specimens. The applicability of the newly developed biosensor in terms of detection limit, sensitivity, reliability, and reusability was investigated, and the result obtained was compared to that of the widely accepted reference hexokinase assay.

MATERIALS AND METHODS

β-D-glucose, glutaraldehyde (25% w v^{-1}), ATP, NADP, uricase (EC. 1.7.3.3), glucose oxidase type X-S from *Aspergillus niger* (EC. 1.1.3.4), hexokinase type VI, glucose 6-phosphate dehydrogenase type IX, catalase, and porous aminopropyl glass were purchased from Sigma (St. Louis, MO). Immunodyne™-activated membrane (3-μm pore size) was supplied by Pall Biosupport Corporation (Glencove, NY). Anion-exchange resins AG 1-X8 were obtained from Bio-Rad Laboratories (Grand Island, NY).

Immobilization of Glucose Oxidase on Aminopropyl Glass Beads

Two hundred and fifty milligrams of aminopropyl glass beads (80–120 mesh, 70 nm pore size) were washed extensively with phosphate-buffered saline, PBS (9 g L^{-1} sodium chloride, 20 m*M* phosphate, pH 7) and then activated by contacting with 3 mL of 2.5% (w v^{-1}) glutaraldehyde in PBS for 2–3 h at room temperature (20–24°C). The resulting orangish-pink beads were washed thoroughly with PBS followed by 20 m*M* phosphate, pH 7, to remove excess glutaraldehyde.

A 3-mL solution of glucose oxidase (547 U mL^{-1} and 205 U mg $solid^{-1}$) in 20 m*M* phosphate buffer, pH 7, was then covalently immobilized to the batch of activated beads and rotated end-over-end in a capped test tube overnight at 4°C. Experimental results confirmed that there was no evidence of any enzyme activity or protein content in the supernatant. After immobilization, the beads were packed into a piece of tygon tubing (2.54 mm id, 6 cm in length), furnished with glasswool at the ends to retain the beads (0.25 g of beads will pack into approximately two columns). The enzyme column was stored in 50 m*M* acetate 500 m*M* NaCl buffer, pH 5.5, at 4°C. Optimum operating conditions for glucose conversion were determined with respect to buffer type and strength, pH, NaCl concentration, enzyme column length, and flow rate.

Anion-Exchange Resin

Commercially available anion exchange resins AG 1-X8 have been known to exhibit very good solvent stability as well as thermal stability up to 150°C. Such resins were tested with respect to their binding capabilities toward both uric acid and glucose. The resins were packed into a 2.54-mm id piece of tygon tubing of 12 cm in length, furnished with glasswool at the ends to retain the resins. Three forms of resin were analyzed: hydroxyl, acetate, and chloride, as well as three different particle sizes: 20–50 mesh (1190–420 μm), 100–200 mesh (180–106 μm), and 200–400 mesh (106–45 μm). Optimum conditions for uric acid adsorption were determined with respect to flow rate, pH, and buffer concentration.

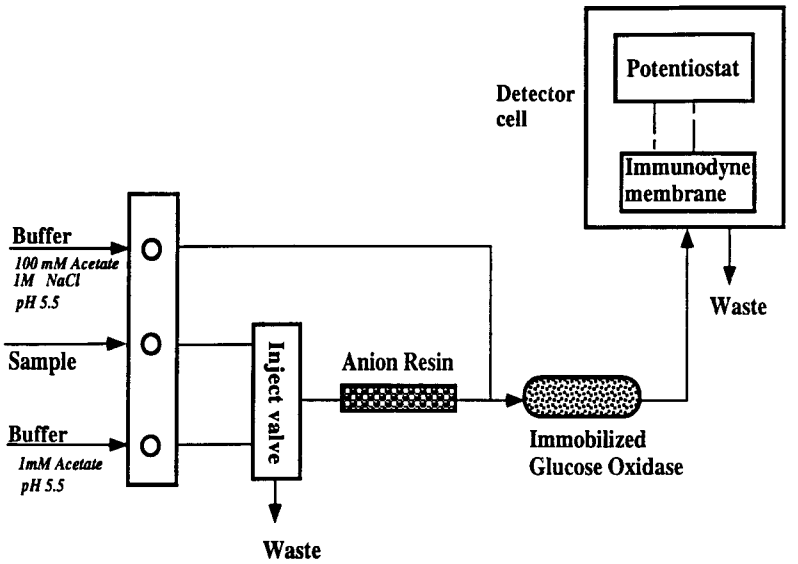

Fig. 1. A schematic diagram of the FIA biosensor system for glucose.

Apparatus

The FIA biosensor system consisted of a peristaltic pump (FIA Pump 1000, Eppendorf North America Inc., Madison, WI) that delivered the sample and different buffers at a preset flow rate. A 75-μL sample was injected into the acetate buffer (1 m*M*, pH 5.5) stream by a motorized injection valve (EVA injector, Eppendorf North America Inc., Madison, WI). The sample flowed through the anion-exchange column for the removal of endogenous uric acid. The resulting sample stream then merged with the buffer stream containing 1*M* NaCl and 100 m*M* sodium acetate, pH 5.5, before entering the immobilized enzyme column reactor (Fig. 1). The response was determined by the FIA detector system (EVA-zyme 500, Eppendorf North America Inc., Madison, WI) consisting of a temperature-controlled flow cell and an amperometric hydrogen peroxide electrode (platinum vs silver/silver chloride at +0.7 V). The Immunodyne™ membrane was tightly attached to the electrode and held in place by an O-ring to alleviate the interference caused by the liquid-flow pattern around the electrode. The sampling and injection into the FIA were controlled by a master module (Eppendorf North America Inc., Madison, WI) that also performed data acquisition in peak height or peak area mode. The output of the amperometric electrode after conversion of the current signal to voltage was recorded on a strip chart recorder. In peak height mode, the response was expressed as relative units (RU) in which 1 RU is equal to 2.86 μV at the detection output.

Measurement of Glucose Concentration in Urine Samples

The anion-exchange column was placed in the sample flow line before the glucose oxidase column in order to remove endogenous uric acid from the samples (Fig. 1). Urine samples were taken from healthy males (30–47 yr old) and diluted fivefold by 1 m*M* acetate buffer, pH 5.5. Calibration of the FIA biosensor for β-D-glucose was performed by spiking a diluted urine sample with known concentrations of β-D-glucose (0.1, 0.2, 0.3 m*M*). A 157-m*M* β-D-glucose solution equilibrated for 2–3 h will consist of 100 m*M* β-D-glucose and 57 m*M* α-D-glucose. The samples were analyzed for β-D-glucose by the FIA biosensor as well as the standard hexokinase assay. For the enzymatic assay, urine samples were further diluted twofold in 150 m*M* phosphate buffer, pH 7.8, and measured in the presence of excess glucose 6-phosphate dehydrogenase, NADP, ATP, and $MgCl_2$. The metabolite assay was initiated by the addition of hexokinase, and the change in absorbance was monitored at 340 nm. It should be noted that the hexokinase assay measures the total D-glucose pool, which contains 64% β-D-glucose and 36% α-D-glucose, whereas the FIA biosensor system only detects the β-D-glucose form. Consequently, this must be taken into account in comparison with the results obtained by these two methods.

The uric acid content of the urine samples could be determined by monitoring the blank amperometric response of the urine sample in the absence of both the anion-exchange and immobilized enzyme columns. The sample was then reanalyzed after reaction with excess uricase and catalase, which removed the uric acid component of the interfering blank.

RESULTS

Optimization of Immobilized Enzyme System

The glucose oxidase column was first used together with the FIA system without the anion-exchange column in place to establish optimal operating conditions. At the preset flow rate of 31 mL h^{-1}, the response to 1 m*M* glucose increased with an increase in the column length up to 2 cm (Fig. 2). Beyond this level, the response was no longer dependent on the column length. In view of the reusability of the immobilized glucose oxidase, a column length of 6 cm was chosen for all subsequent experiments. This series of experiments was performed in a peak area to account for the difference in peak heights caused by changing dispersions owing to varying column lengths.

The optimal pH for soluble glucose oxidase was reported to be 5.6, however, after immobilization, glucose oxidase responded maximal to

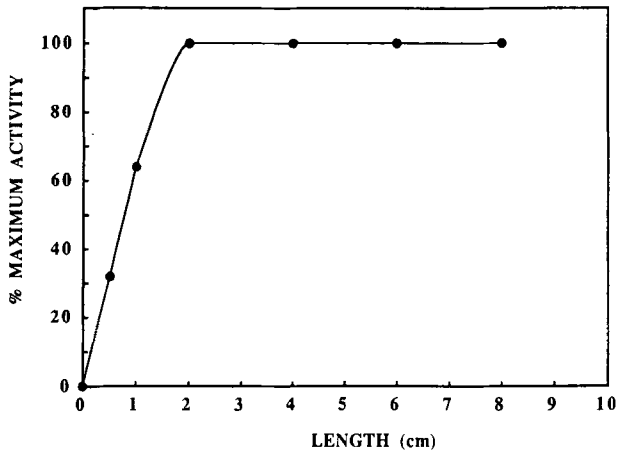

Fig. 2. Relationship between the enzyme column length and the FIA response to 1 m*M* glucose (in 1 m*M* acetate, pH 5.5) at a flow rate of 31 mL h^{-1} with 100 m*M* acetate and 1*M* NaCl, pH 5.5 buffer.

glucose over the pH range 4.5–7. Such behavior should be anticipated since a very large excess of glucose oxidase immobilized onto aminopropyl glass beads could easily overcome the pH dependency in the pH range tested. It should be important to note that at pH 5.5, uric acid exists mainly as negatively charged urate, which could be retained by the anion-exchange resins as discussed later. Therefore, a pH of 5.5 was chosen for all subsequent experiments. Among four different buffers tested at pH 5.5 —acetate, citrate, imidazole, and phosphate—the response was somewhat similar, and acetate was selected in view of its buffering capacity over the desired pH range. Acetate buffer strength in the range 20–500 m*M* had no effect on the response to glucose, and 100 m*M* acetate was considered sufficient for maintaining the pH of the buffer in the column after mixing with the sample stream. Addition of sodium chloride to the buffer was necessary to prevent fouling of the immobilized enzyme column as well as the electrode surface. Concentrations of NaCl between 50 m*M* and 1.5*M* exhibited very little effect on the glucose response, and 1*M* NaCl was used for further experiments.

The response to 1 m*M* glucose remained constant as the sample flow rate increased over the range of 15–75 mL h^{-1}. Theoretical predictions for FIA systems expect a decrease in response as flow rates increase (*16*), however, in this case, the large excess of glucose oxidase immobilized on the beads likely overcomes this phenomenon in the range tested, and this characteristic may only be observed at even higher flow rates. A flow rate of 31 mL h^{-1} was selected for this study, and the total flow rate through the immobilized enzyme column will be 62 mL h^{-1} after the two streams merge.

Selection of the Type of Anion Resin for Removal of Uric Acid

As a novel aspect of this study, effort was focused on the removal of endogenous uric acid from urine samples by anion-exchange resins. Anion-exchange resin AG 1-X8 containing quaternary ammonium functional groups is capable of exchanging anions and possesses the following order of selectivity: Cl > acetate > OH. Columns containing the three above resin forms were monitored using the configuration shown in Fig. 1. The injection of uric acid to the FIA biosensor should provide minimal or no response since at pH 5.5; uric acid exists mainly as negatively charged urate ($pK = 5.4$) and will be retained effectively by the anion exchanger. On the other hand, the response to glucose of the FIA biosensor should be similar with or without the ion-exchange resin, since glucose is not ionized at this pH and passes through the ion-exchange column.

To select the most suitable type of resin, a series of experiments was performed using a very large particle size resin, 20–50 mesh, in order that the effects would be more pronounced. Among the three different types tested (hydroxyl, chloride, acetate), the response to uric acid (1 m*M*) was 2.0, 0.40, and 0.25%, respectively, when compared with the signal obtained without the ion-exchange in place. As a result, the acetate form of the resin was chosen for subsequent experiments to establish optimal operating conditions for the removal of uric acid.

The acetate buffer concentration used in the sample stream affects the binding capacity of the acetate resin (20–50 mesh) for uric acid. At low concentration of acetate (< 5 m*M*) very little uric acid passed through (< 1%), whereas at higher concentrations (100 m*M*) a larger amount of uric acid was detected (5%). Also as expected, the sample stream flow rate affected the uric acid binding capacity of the acetate resin column. At higher flow rates (> 90 mL h^{-1}), 4% of the uric acid was observed to pass through the column. Obviously, there was a minimum residence time required for a complete interaction between uric acid and the anion exchanger.

Maximal Uric Acid Binding of the Acetate Resin Column

To be practical in the FIA biosensor system, the acetate resin must be effective for an extended period, so that adsorbed uric acid does not dissociate during the course of repeated measurements. The binding capacity of the two smaller particle sizes (higher binding surface areas) of acetate resins, 100–200 and 200–400 mesh, were evaluated by repeated injections of 5 m*M* uric acid (in 1 m*M* acetate). Uric acid began to pass through the column after 370 injections as detected by the biosensor with the 200–400 mesh and after only 200 injections with the 100–200 mesh. Based on this result, the maximum binding capacity of 200–400 and 100–200 mesh acetate resins was estimated to be 23.3 and 12.5 mg of uric acid,

respectively. As expected, increasing the surface area for binding resulted in an increased uric acid binding efficiency, and as a consequence, the 200–400 acetate mesh was used for real samples.

Optimal Conditions of the FIA System Equipped with the Acetate Resin Column

The binding capacity of the acetate resin (200–400 mesh) column as a function of the acetate buffer concentration used in the sample stream was reconfirmed. Under continuous injections of 1 m*M* uric acid at high acetate conditions (100 m*M*), the column was only good for 16 injections. However, as the acetate concentration was decreased, the efficiency of the column was improved. At 20 m*M* acetate, the column could be reused for 80 repeated injections before uric acid was detected. As a result, 1 m*M* acetate was chosen as the optimal running condition, since the column was observed to last for about 900 repeated injections of 1 m*M* uric acid.

Unlike the behavior of the 20–50 mesh resin, samples containing uric acid passed through the 200–400 mesh acetate resin at flow rates up to 90 mL h^{-1} without significant detection (<1%). By decreasing the particle size, the residence time is no longer critical, and the selected speed of 31 mL^{-1} was considered compatible with the immobilized glucose oxidase column. The pH of the sample in the range of 5.5–7.5 did not have any noticeable effect on the binding efficiency of uric acid.

Response of the Biosensor System to Glucose and Interference Studies

In peak height mode, there was an excellent linear response of the FIA biosensor to glucose up to 1 m*M* (correlation coefficient of 1). The sensitivity of the biosensor was determined to be 160 ± 2.4 RU μM^{-1} (95% confidence interval, $n = 10$) with a minimum detection level of 10 μM. A good reproducibility ($\pm 0.23\%$) was obtained as reflected by the average response for 20 repeated analyses of 1 m*M* glucose ($158{,}700 \pm 358$ RU at 95% confidence interval). Each assay could be performed in 4 min, including washing giving a throughput of 15 h^{-1}. Similarly, the response was also linear using peak area mode, however, the minimum detection level was considerably higher (50 μM). The immobilized enzyme column could be reused for at least 1000 repeated analyses without loss of activity and was stable for several months if stored at 4°C in 50 m*M* acetate 500 m*M* NaCl, pH 5.5.

Without the anion exchanger, the FIA biosensor using immobilized glucose oxidase detects both uric acid and glucose in a similar manner with respect to sensitivity. The response to a mixture containing an equimolar ratio of glucose and uric acid was further found to be additive. Such a result thus leads to a conclusion that uric acid must be removed from the sample, otherwise, it will interfere with amperometric detection of

glucose and produce a falsely elevated result. The introduction of the acetate column to the FIA biosensor system (Fig. 1) completely blocks the uric-acid-interfering signal leaving just the detection of glucose. The peak height obtained for glucose (0.5 m*M*) was identical to that for a mixture of glucose and uric acid (0.5 m*M* each). The peak height for glucose was slightly lower (85%) because of dispersion effects when compared to the peak heights for glucose without the acetate resin.

Other electroactive substances known to interfere in amperometric detection were injected into the sample stream to determine whether the addition of the resin would alleviate the interference. Without the ion exchanger in place the injection of ascorbic acid (1 m*M*) and acetaminophen (1 m*M*) resulted in responses of 100 and 20%, respectively, in comparison to glucose. However, with the resin in place, the acetaminophen signal was completely suppressed, and the ascorbic acid was reduced by 90%. Both of these interferents may be present in urine, and therefore, their removal will further improve the selectivity of the biosensor for detecting glucose.

Measurement of Background Signal Owing to Uric Acid in Urine Samples

The interference of electroactive uric acid in urine was first investigated, since the platinum electrode (poised at +0.7 V vs silver/silver chloride) should respond to urine owing to its high uric acid content (4–10 m*M*). In this experiment, the acetate anion-exchange column was removed from the FIA biosensor system and a blank porous glass beads column instead of the immobilized glucose oxidase column was used. As expected, the urine sample was a very significant interfering signal. The response to uric acid was then confirmed by treating the urine sample with uricase and catalase to convert uric acid to noninterfering allantoin and water. In this case, the background signal was reduced by approx 90%.

The measurement of urinary glucose was first attempted by replacing the blank column with the glucose oxidase enzyme column. The resulting signal, however, was only ca. 10% higher than the background signal. Such a result was somewhat anticipated, since the urine sample normally contains about 1 m*M* glucose or less. Therefore, the differential measurement was not considered satisfactory for the determination of urinary glucose in view of the signal-to-background ratio. Consequently, the removal of uric acid from urine is a prerequisite for reliable determinations of glucose by amperometry.

With the anion-exchange resin in place together with the blank column of glass beads, the background signal was reduced by more than 99%, an indication of strong binding between urate and the anion exchanger. When the blank column was replaced by the immobilized enzyme column for the measurement of urinary glucose, the resulting signal-to-back-

ground ratio was always higher than 3, depending on the level of glucose in urine. Such data thus provided confidence for using the FIA biosensor system consisting of both the acetate anion-exchange and immobilized glucose oxidase columns for the determination of urinary glucose.

Measurement of Urinary Glucose

The biosensor system was first run continuously with a urine sample in the absence of the immobilized glucose oxidase column to determine the binding capacity of the anion-exchange column. Urine samples were diluted fivefold with a corresponding concentration of 0.6 m*M* for uric acid. Uric acid was first detected after about 65 injections, i.e., the column only retained up to 0.56 mg uric acid. The lower binding capacity of the column when compared to the pure uric acid samples is likely the result of other anions in the sample that will bind to the resin lowering the effective binding of uric acid. As well, the ionic strength of the sample will certainly be higher than the pure uric acid sample, which would cause the bound uric acid to release more rapidly. Since the level of uric acid in real samples varies from 4 to 10 m*M*, a conservative estimate for the reuse of the acetate column before replacement would be about 25–30 injections.

The FIA biosensor equipped with the acetate resin column was then applied to determine glucose in the urine. The standard calibration for glucose was performed by spiking a urine sample with known concentrations of glucose and determining the peak heights by their differences compared to the urine sample alone. These peak height were about 10–15% lower than those obtained using glucose alone. This phenomenon occurs because of a widening of the peak base when using real samples. Alternatively, peak area could be used, but owing to the lack of sensitivity and the low level of glucose in many urine samples, the above spiking protocol was preferred. The background signal was subtracted from the total signal by measuring the urine sample with a blank glass bead column rather than the glucose oxidase enzyme column.

The data obtained by the biosensor compared well with those of the standard hexokinase enzyme assay. The biosensor values plotted against those of the enzyme assay resulted in a straight line with a slope of 0.99 and a correlation coefficient of 0.97 ($n=15$) (Fig. 3). Such good agreement thus validated the applicability of the glucose FIA biosensor for measuring glucose levels in urine.

In brief, a FIA biosensor system using immobilized glucose oxidase was used together with a hydrogen peroxide electrode for the direct determination of glucose in urine. The effective removal of endogenous uric acid by a anion-exchnge resin was successfully demonstrated. In addition to uric acid, acetaminophen and ascorbic acid were also effectively adsored by the acetate ion exchanger. The introduction of this type of ion exchanger thus greatly improved the selectivity of the FIA biosensor system.

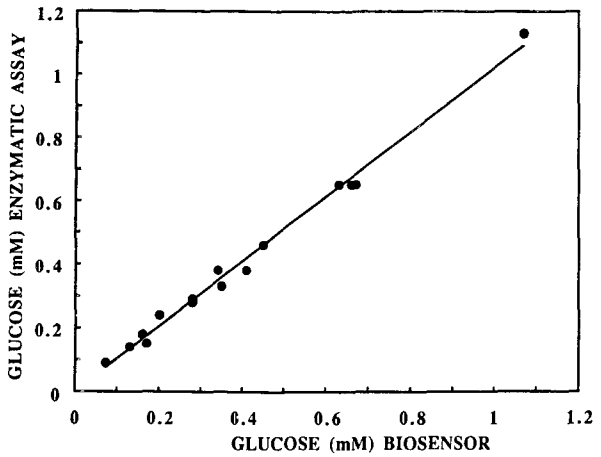

Fig. 3. Glucose concentration determined by hexokinase enzyme assay and the FIA biosensor for human urine samples ($C_{HEXO} = 0.99\ C_{BIO}$, $R^2 = 0.97$, $n = 15$).

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