Bi-enzyme reactor for electrochemical detection of low concentrations of uric acid and glucose

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Abstract

An enzyme-based flow-injection amperometric analysis system (FIA) for monitoring of uric acid and glucose is described. The oxidase and peroxidase enzymes are physically co-immobilised in a sandwich-type reactor and ferrocene serves as a mediator. The assays are based on the measurement of a reduction current resulting from the enzymatic reactions, at a glassy carbon electrode held at 0.00 mV (vs. Ag/AgCl). The high selectivity (ascorbic acid did not interfere) is coupled to high sensitivity (a detection limit of 30 and 60 nmol/l for uric acid and glucose, respectively; signal/noise = 3) and good stability (the enzymes remained active for more than 6 weeks at 30°C). The usefulness of the assay in clinical chemistry is illustrated by the measurement of human serum uric acid and glucose concentration. The results obtained were in fairly good agreement with those obtained using conventional hospital laboratory methods.

Keywords: Uric acid; Glucose; Enzyme reactor; Amperometry; Mediator, ascorbic acid

1. Introduction

Assays using enzyme reactors have been shown to be useful in HPLC and flow injection analysis (FIA) of a variety of metabolites, including choline, glucose and lactate [1,2]. Such assays are fast and highly sensitive. The enzyme immobilisation
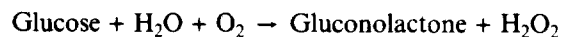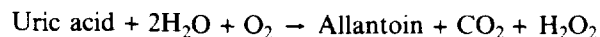
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is accomplished by various means, including electrochemical immobilisation, covalent binding, cross linking and entrapment in a gel or polymer, sometimes implicating elaborate and time consuming preparation procedures (e.g. chemical and physical pretreatments) which may, however, influence the enzyme activity [3]. Consequently, innovative routes for preparing versatile enzyme reactors are highly desirable. The coupling of the specificity of biocatalytic recognition processes with the high sensitivity of amperometric transducers makes enzyme based electrochemical methods practical for routine clinical and industrial applications. In the present communication we describe two applications of a simple and versatile enzyme reactor, allowing sensitive electrochemical detection of uric acid and glucose.

Levels of uric acid in the serum and urine increase in disorders of purine metabolism [4]. Enhanced adenine nucleotide degradation occurs in clinical conditions associated with tissue hypoxia and uric acid has the capability to scavenge oxygen free radicals [5,6]. Wayner et al. estimated that uric acid contributes to 35–65% of the total plasma antioxidant capacity [7]. Already a large number of colorimetric [8–10] and electrochemical [11–14] methods have been described. Most of these methods have inherent problems: lack of sensitivity, susceptibility to interferences, time requirement, operational complexity. Because of the relatively low concentration of urate in plasma (< 0.5 mmol/l) there is the risk of interference of other plasma constituents. Although there is substantial progress in sensitivity and linearity of the determination of uric acid [8,10,13], the selectivity is still a problem [12–14].

Glucose is a routine clinical assay to monitor plasma levels in several diseases or during hospitalisation. Recently, we showed the possibility of monitoring glucose transcutaneously, both in premature babies and in adults [15,16]. Such transcutaneous devices are particularly attractive in intensive care units, neonatology and sports medicine, where blood collection is often difficult. Therefore, there is a need for a simple and sensitive assay of glucose which is not yet available.

The enzyme reactor described here contains physically co-immobilised uricase and horseradish peroxidase, or glucose oxidase and horseradish peroxidase (HRP), integrated in a flow injection system.

The oxidase enzymes catalyse the oxidation of substrates according to the reactions:

$$\text{Uric acid} + 2H_2O + O_2 \rightarrow \text{Allantoin} + CO_2 + H_2O_2$$

$$\text{Glucose} + H_2O + O_2 \rightarrow \text{Gluconolactone} + H_2O_2$$

In the usual electrochemical assay methods, either the consumption of oxygen or the formation of hydrogen peroxide is monitored; they are often affected by the ambient concentration of oxygen and require a large overpotential for the oxidation of hydrogen peroxide that may also detect electro-oxidable contaminants. Efforts to exclude such faradaic interferences include the use of a preoxidation cell or ion-exchange column to permselective membranes, leading to a larger response time and a lower sensitivity of the assays. In order to avoid these problems we used a combination of HRP and a ferrocene derivative (Fc) as a solution mediator, according to suggestions of Frew et al. [17].

The hydrogen peroxide is reduced by HRPred (reduced form) forming the oxidised form (HRPox) which oxidizes the ferrocene (FcII) to the ferricinium (FcIII) ion. The reactions are:

$$HRPred + H_2O_2 \rightarrow HRPox + H_2O + H^+$$

$$HRPox + Fc(II) \rightarrow Fc\ (III) + HRPred + e^-$$

FcIII is subsequently reduced back to FcII at the glassy carbon electrode. The reduction current related to the concentration of hydrogen peroxide is monitored amperometrically.

By using the second enzymatic reaction and the mediator two distinct advantages are obtained. First, it allows low potential detection (0 mV vs. Ag/AgCl) of substrates thus avoiding interferences from electroactive species and ensuring the specificity of the assay, and second, the assay sensitivity is enhanced as a result of increasing the efficiency of the electrochemical detection, as decay of the formed H_2O_2 remains very low.

2. Materials and methods

2.1. Materials

Glucose oxidase (EC 1.1.3.4, grade I), uricase (EC 1.7.3.3), horseradish peroxidase (EC 1.11.1.7, grade I) were obtained from Boehringer Mannheim (Germany). Glucose and uric acid for standard solutions (0.3, 0.6 and 0.9 nmol/l), ferrocenemono-
carboxylic acid (FcA) were obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals were of pro-analysis quality and purchased from Merck (Darmstadt, Germany). The cellulose nitrate filters (thickness, ~100 μm; pore size, 0.01 μm; cutoff, 50 kDa) were from Sartorius (Göttingen, Germany). Double quartz distilled water was used for all aqueous solutions containing 0.1% (by volume) Kathon CG (Rhom and Haas, Croydon, UK) to inhibit bacterial growth. The composition of Dulbecco's phosphate-buffered saline (PBS) was (mmol/l): NaCl (136.9), KCl (2.7), KH_2PO_4 (1.5), CaCl_2 (0.9), MgCl_2 (0.5), Na_2HPO_4 (8.1). The carrier solution contains FcA (0.5) and EDTA (2) dissolved in PBS and adjusted to pH 7.4 with NaOH. EDTA was added to suppress catalysed degradation of hydrogen peroxide and inhibition of enzymes by heavy metal ions [17]. Helium was bubbled into the carrier medium to remove air.

The stock glucose solution (10 mmol/l) was prepared by dissolving glucose in PBS and allowed to reach mutarotational equilibrium before use (24 h). The standard solutions were prepared from the stock solutions by diluting with the carrier medium.

2.2. Flow injection analysis system

The schematic set-up of the analysis is shown in Fig. 1A. An HPLC pump (LKB 2150, Pharmacia, Bromma, Sweden) equipped with a splitter (made in the laboratory; split ratio, 1:4) in order to maintain a constant and pulse free flow
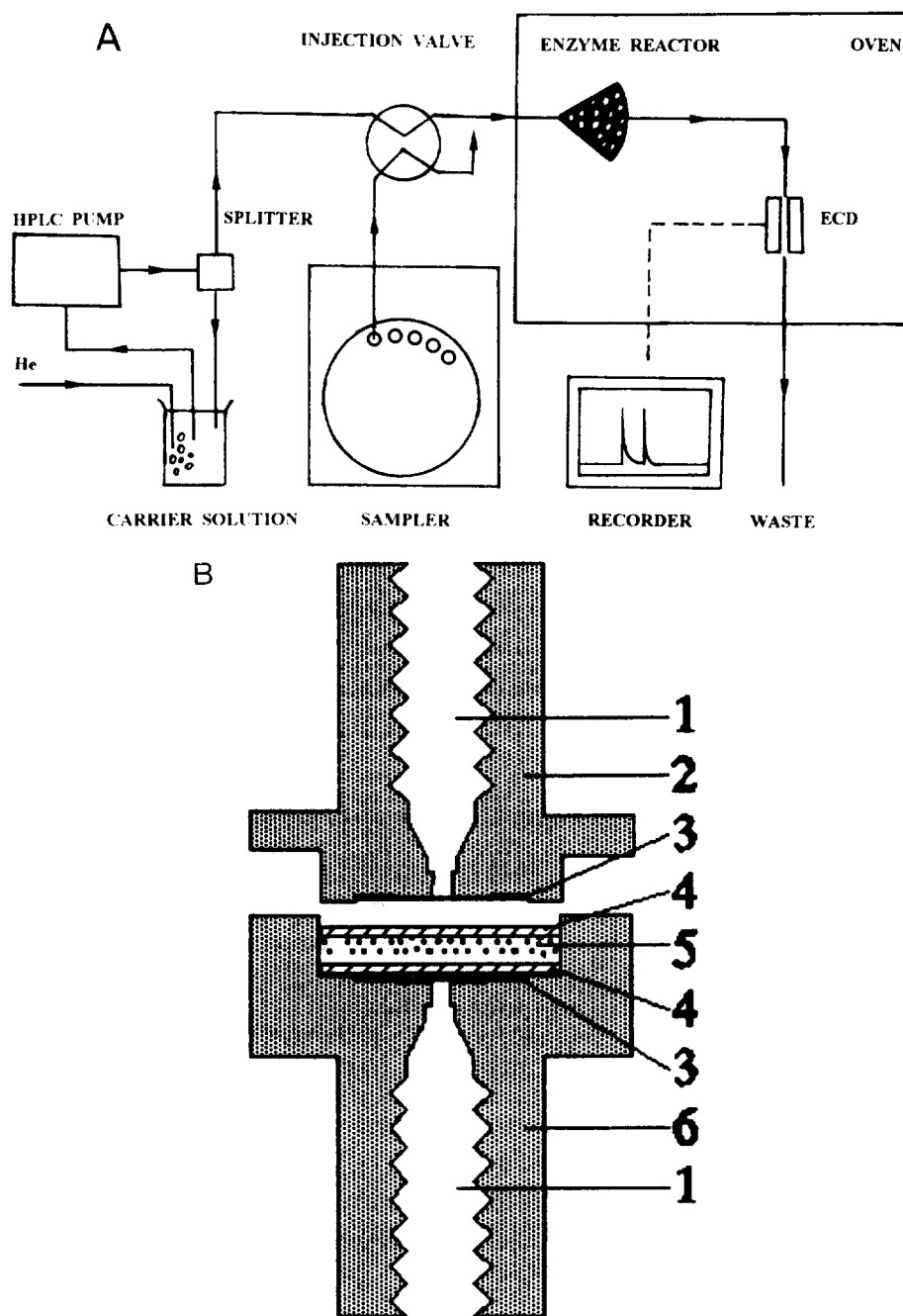
Fig. 1. Schematic drawing of the electrochemical FIA system and the enzyme reactor. (A) Schematic drawing of the electrochemical FIA set-up for uric acid and glucose. (B) The bi-enzyme reactor. The fluid passes through the in- and outlet connections (1) to the reactor that is enclosed by the polyacetal adaptors (2,6). The enzymes (5) are immobilized between the cellulose nitrate filters (4), which are located between two stainless steel screens (3).

delivered the samples at a pre-set flow rate (150 μl/min). A Marathon HPLC Autosampler (Spark Holland, Emmen, The Netherlands) was used to inject the samples into the analysis system. The sampler was equipped with a Rheodyne 7010 Valve (Cotati, CA) with a loop (home-made 2.5 μl). After injection, the sample reached the enzyme-reactor (Fig. 1B) and the electrical current was monitored. The electrochemical cell was a thin layer-type cell (AMOR, purchased from Spark Holland, Emmen, The Netherlands), with a glassy-carbon working electrode held at 0.00 mV relative to an Ag/AgCl reference electrode and a Teflon/carbon counter electrode connected to the potentiostate (Decade, Antec Leyden B.V., Leiden, The Netherlands). The signal output was recorded with a flatbed recorder (model BD112, Kipp & Zonen Delft BV, Delft, The Netherlands). The enzyme reactor and the electrochemical cell were placed in the oven of the potentiostat to keep the temperature constant at 30°C (minor fluctuations in temperature result in instabilities of the electrochemical detector, ECD).

Under the assay conditions for glucose the electrode drifts were less than 5% per 5 days when continuous in use and tested when 0.01 mmol/l glucose was at full scale.

2.3. Enzyme reactor preparation

The enzymes (either 5 U uricase and 250 U HRP or 250 U glucose oxidase and 250 U HRP) were dissolved in 2–4 μl PBS and gently pipetted on the cellulose nitrate filters kept in a special holder of polyacetal with minimal dead space [19] (Fig. 1B shows the present version). The uricase (solution in glycerol) was directly pipetted on the filter. The reactor was stored at 30°C and continuously flushed with carrier solution when not in use.
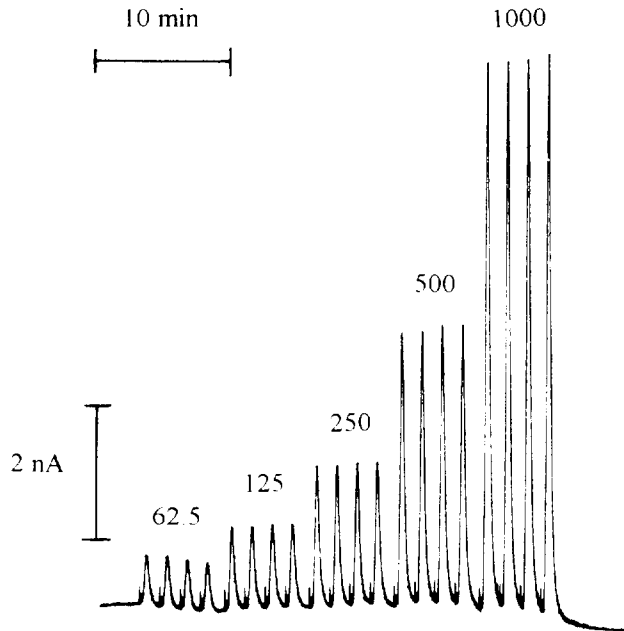

Fig. 2. Original chart record of uric acid calibration curve. Numbers on the peaks represent the uric acid concentration in nmol/l.

2.4. Sample preparation

Blood serum samples of male and female patients with a wide variety of pathological conditions were from the local hospital (University Hospital of Groningen) collected in tubes containing NaF, to prevent glycolysis by erythrocytes. Before being placed in the autosampler, 4-μl specimens were diluted 50- to 100-fold with carrier solution and left to stand at room temperature for at least 6 min.

2.5. Presentation of the results

The measured serum metabolite concentrations were correlated to those obtained by standard methods with linear regression analysis.

In order to establish the suitability of this system to measure uric acid and glucose in biological fluids, human serum with low, normal and high levels of the metabolites were analyzed with the current method and with routine assays.

Thus, the present serum uric acid values were compared with those obtained by colorimetric method using the automated SMAC system based on continuous flow analysis [20]. The results of our glucose analysis were compared with those obtained with the APEC glucose analyser using an oxygen electrode [21]. The latter method has been correlated to a hexokinase reference method (AAll Technicon).

3. Results and discussion

3.1. Calibration curves

An example of a recording of uric acid is shown in Fig. 2. The calibration curves of uric acid and glucose respectively, are shown in Fig. 3A,B. The uric acid assay was linear up to 200 μmol/l (correlation coefficient $r = 0.999$). Examples of calibration curves in the nanomolar range are presented in the same figures.

For glucose the response is linear up to 1 mmol/l with a correlation coefficient $r = 0.998$. The signal/noise (S/N = 3) characteristics indicate a detection limit of 30 nmol/l and 60 nmol/l for uric acid and glucose, respectively. These ranges allow the analysis of 50- or 100-fold diluted blood samples.

3.2. Effect of interferences

The selectivity of the method was tested with potentially interfering compounds, such as ascorbic acid (0.2 mmol/l), lactic acid (2 mmol/l), acetaminophen (1 mmol/l), cysteine (0.1 mmol/l) and glutathione (0.1 mmol/l). Uric acid (0.3 mmol/l) was tested in glucose measurement. Dissolved oxygen remaining after the degassing with He did not interfere at the conditions chosen. A possible reduction due to cathodic PO_2 reduction was too low to be observed. The tested substances were dissolved individually or in combination with the substrates in the carrier medium (FcA 0.5 mmol/l) or in PBS. Of the above mentioned compounds only ascorbic acid may interfere in the assay of either metabolite.

3.3. Studies on ascorbic acid response

In spite of the low potential used for the detection, the ascorbic acid gave a response that was highly time dependent. The time course of the decay of the oxidation current appeared to be concentration dependent. For example, the current pro-
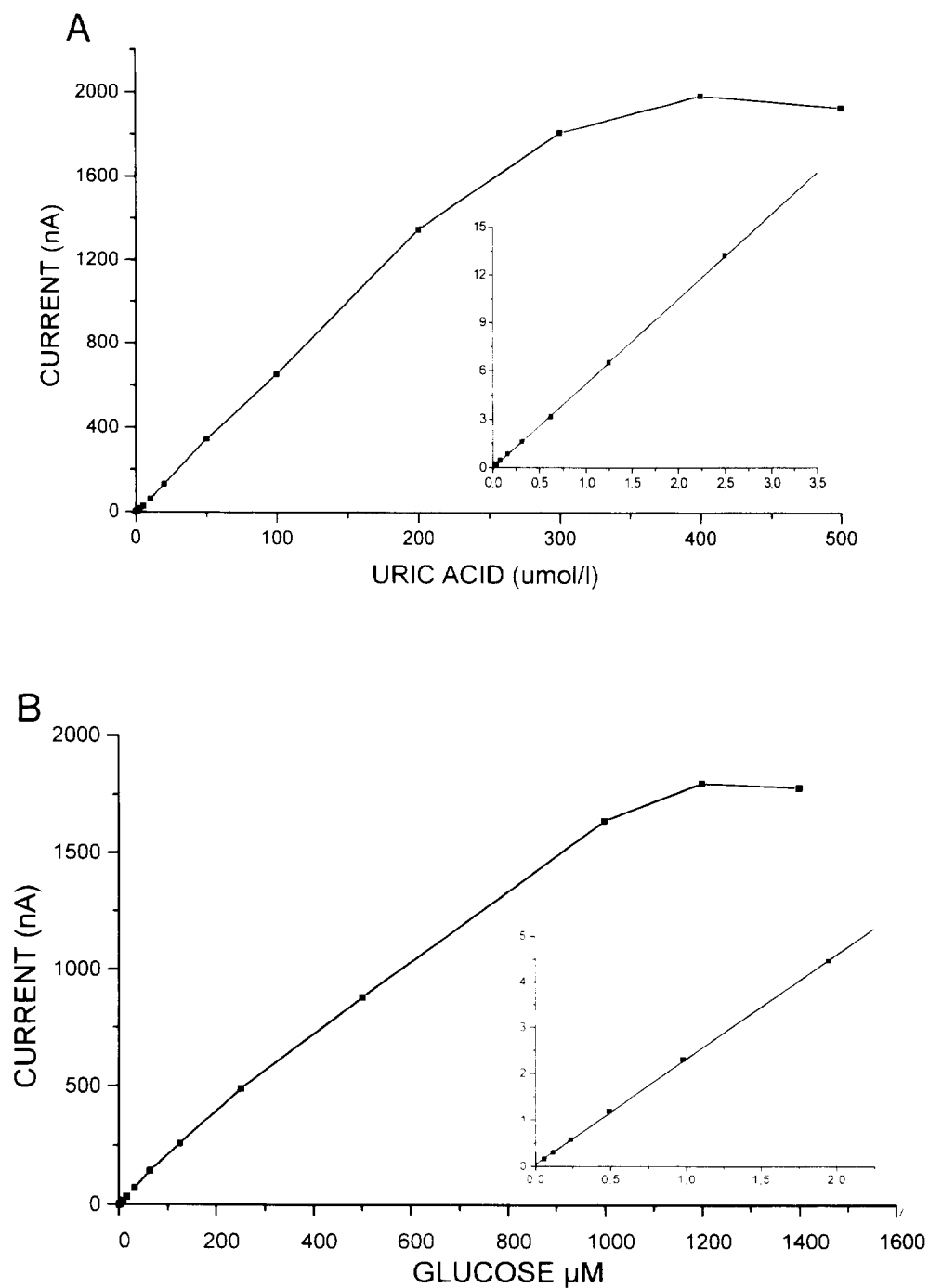
Fig. 3. (A) Calibration curve for uric acid. (B) Calibration curve for glucose. (pH = 7.4; T = 30°C; flow rate, 150 μl/min; carrier medium, ferrocene monocarboxylic acid (0.5 mmol/l).)

duced by the 200 μmol/l ascorbic acid solution gradually decreased over the first 30 min and then reached an almost steady level (approximately 5% of the initial current). For the lowest concentrations of ascorbic acid the decay is much quicker. Omitting the FcA from the carrier medium, the ascorbic acid being dissolved in PBS, a stable oxidation current was observed (Fig. 4). In the 50- to 100-fold diluted samples the possible ascorbic acid concentration is around 4 μmol/l (corresponding to a 200–400 μmol/l serum concentration) and apparently it requires approximately 6 min for complete elimination. When the ascorbic acid was added to the substrate solutions, it had a transient negative effect on the size of the substrate peaks. Thus, a combination of 20 μmol/l ascorbic acid with 10 μmol/l uric acid leads, at first, to a lower uric acid response, but after 10 min the uric acid current attained the standard level (Fig. 5)

In order to determine the nature of the response, the enzyme reactor was excluded from the analysis system. With or without reactor the ascorbic acid gave the same response in the ECD. We conclude that the ascorbic acid is a direct electrochemical interference and this interference problem could be resolved by protocol design, ensuring sufficient reaction time for FcA to eliminate the ascorbic acid. All analyses described in the following section took into account 10 min to allow a virtually complete elimination of ascorbic acid.
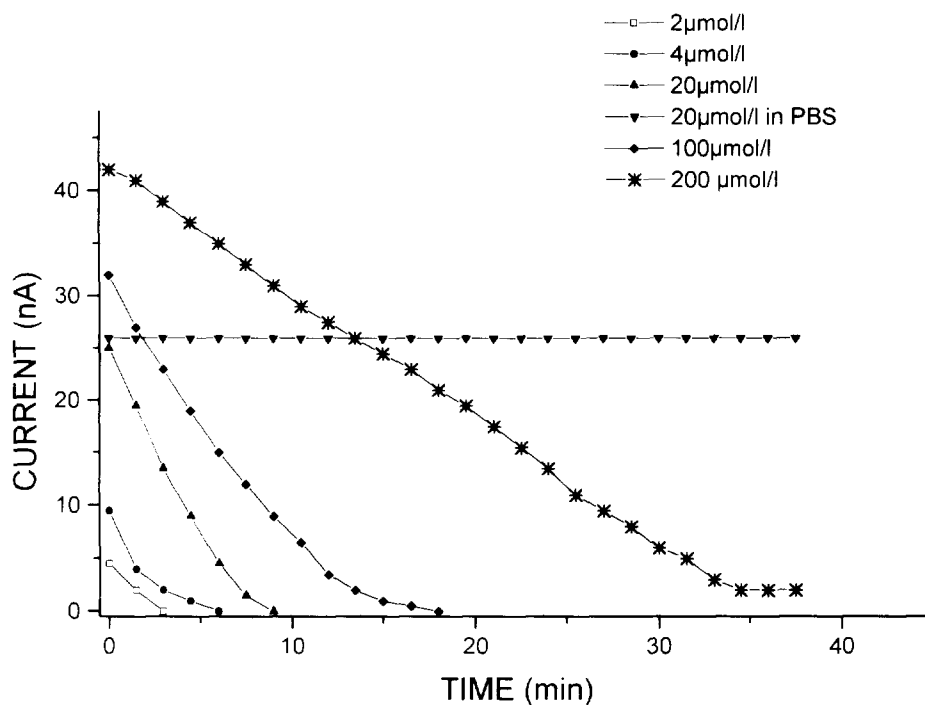

Fig. 4. The time course of various ascorbic acid concentrations in presence and absence (i.e. in PBS) of ferrocenemonocarboxylic acid (0.5 mmol/l).
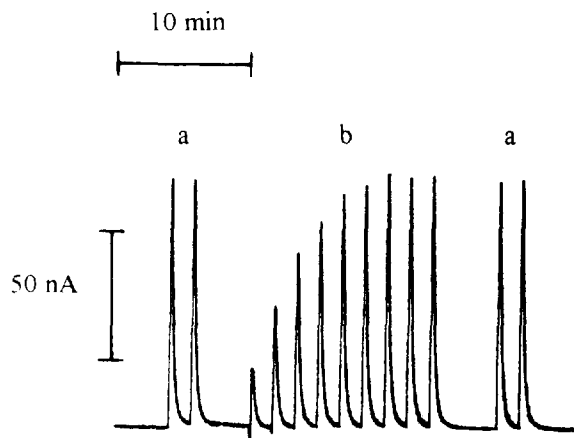
10 min

a   b   a

50 nA

Fig. 5. Effect of 20 μmol/l ascorbic acid on the 10 μmol/l uric acid response. (a) 10 μmol/l uric acid standard (without ascorbic acid). (b) 10 μmol/l uric acid and 20 μmol/l ascorbic acid added just before repetitive analysis of the same sample solution.

3.4. Recovery and correlation studies

The accuracy of the method was evaluated by analytical recovery studies on pooled sera of 15 patients and increasing concentrations of uric acids or glucose, respectively were added. The results show a 99.8% mean analytical recovery for uric acid (Table 1) and 102.8% for glucose (Table 2). As shown in Fig. 6A,B, there was a linear correlation with the reference methods, with correlation coefficients over 0.99, thus validating the reliability of the method for the determination of uric acid and glucose.

The variation of the methods was estimated within runs, between days and during 4 weeks of operation. With 10 samples of 100 μmol/l glucose, these values were 3%, 3% and 7%, respectively. Similar data were found for 100 μmol/l uric acid and were 2%, 4% and 5%, respectively.

4. Discussion

The assays described here are based on the application of an electrochemical detection technique after passage of the sample through an enzyme reactor.

The enzyme immobilisation method used is simple, thus avoiding the possible enzyme denaturation that occurs often in more complicated immobilisation procedures, while the amount of enzyme to be immobilised is not a function of accessibility of the binding sites as is often limiting when chemically immobilised. Therefore, it is cost effective since a small amount of enzymes can be used for a large number of determinations possessing an excellent operating life. The reactors were generally in use for 6 h/day (200-300 samples). The immobilised uricase retained its enzyme activity for at least 6 weeks and glucose oxidase remained active for more
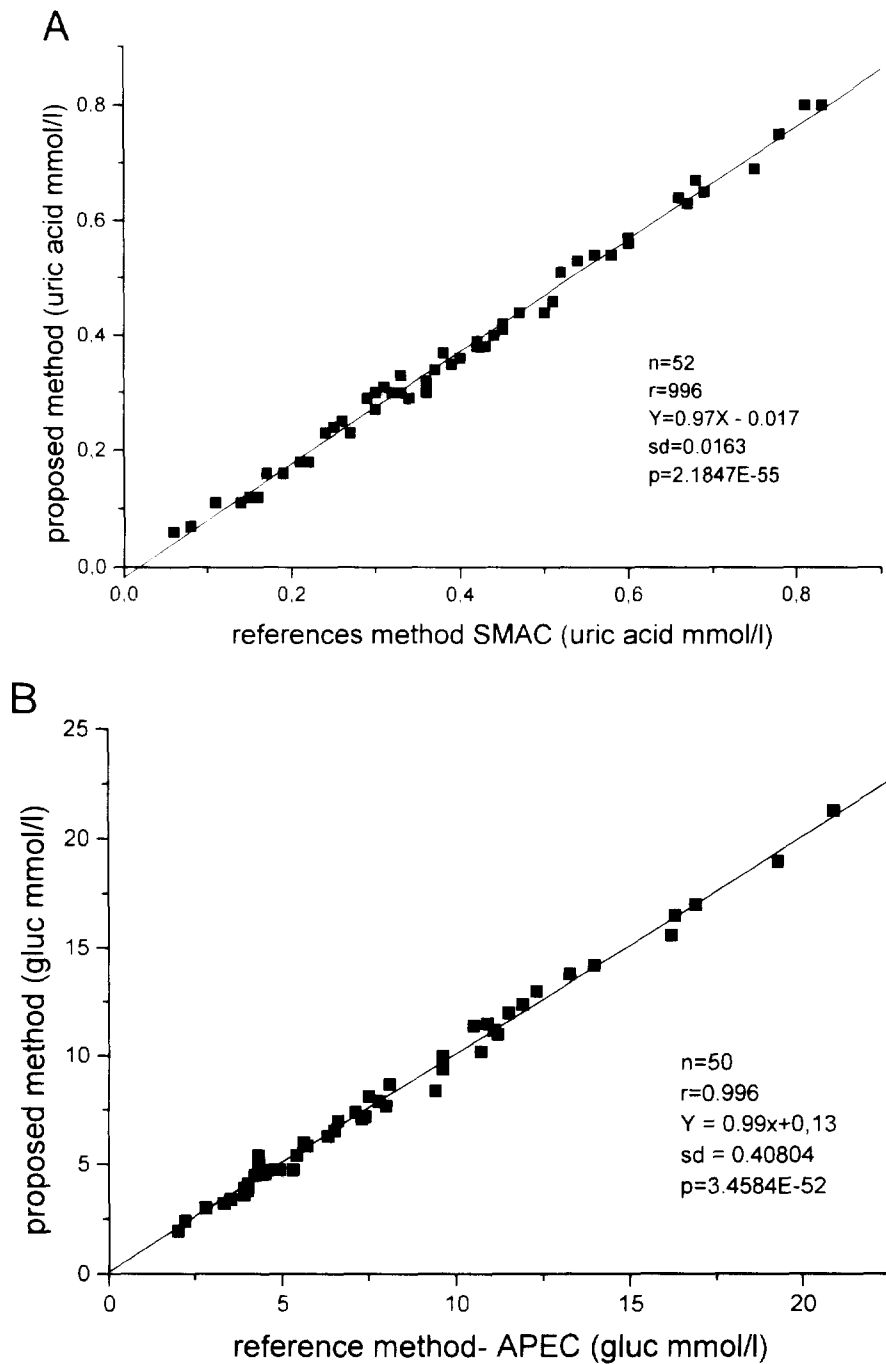
Fig. 6. (A) Serum uric acid assay by present method versus a standard routine colorimetric method. (B) Plasma glucose assay by the present method versus a standard routine electrochemical method.

Table 1
Recovery study for the determination of uric acid added to pooled sera of 15 human subjects

Added (mmol/l)	Expected (mmol/l)	Found (mmol/l)	Recovery (%)
0	—	0.13	—
0.05	0.18	0.18	100
0.1	0.23	0.23	100
0.2	0.33	0.32	99
0.3	0.43	0.42	98
0.4	0.53	0.54	102

Table 2
Recovery study for the determination of glucose added to pooled sera of 15 human subjects

Added (mmol/l)	Expected (mmol/l)	Found (mmol/l)	Recovery (%)
0	—	6.3	—
3	9.3	10.1	109
6	12.3	12.3	100
9	15.3	15.0	98
12	18.3	19.0	104
15	21.3	22.0	103

than 8 weeks without needing special storage. The operational stability of the reactor is sufficient to allow the analysis of at least 5000 samples.

By integrating the reactor into an electrochemical flow injection analysis system we succeed in realising a reliable assay dedicated to routine analysis of a large series of samples permitting the handling of about 60 samples per h. Only 4 μl of specimen is required and the precision and accuracy are very good. A similar concept has been used by Boutelle et al. for measuring glucose, lactate, and glutamate in rat brain dialysate [22]. However, their immobilisation procedure was more complicated, requiring more enzyme and the glucose assay was approximately 100 times less sensitive compared with our procedure (60 nmol/l).

Our uric acid assay showing a detection limit of 30 nmol/l, is approximately 400 times more sensitive than that of a recently published amperometric method [13] and 1900 times that of a recently published spectrophotometric method [10].

A major advantage of the use of ferrocene is that ascorbic acid no longer interferes, so that the assays become specific for uric acid and glucose. The data obtained by the present method agree well with those of routine hospital methods validating the clinical applicability of the system.

The application of the enzyme reactor is not limited to monitoring uric acid and glucose. Previously, we have applied the reactor to assay lactate and choline [1,2]

and it can be anticipated that other oxidising enzymes (e.g. glutathione oxidase, cholesterol oxidase, xanthine oxidase, ethanol oxidase) can be immobilized between the filters of the reactor leading to greater analytical possibilities. Nanomolar quantities of target analyte are quantified in microliter amounts of tissue fluid, ensuring the usefulness of the system in biomedical research, including transcutaneous monitoring [15,16]. Moreover, due to constructional and operational simplicity, the present approach may successfully compete with current microelectronic technology, and can also be miniaturized.

Acknowledgements

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