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Urea determination using pH–enzyme electrode

Robert Koncki *, Agnieszka Chudzik, Izabela Walcerz

Department of Chemistry, University of Warsaw, ul. Pasteura 1, 02-093 Warsaw, Poland

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Abstract

A pH-membrane electrode with n-tridodecylamine (TDDA) as the hydrogen-ion-selective ionophore was used for the construction of a potentiometric biosensor for urea determination. The electrode was enzymatically modified by covalent binding of urease molecules directly to the surface of the potentiometric membrane. Incorporation of the urea biosensor into simple double-channel flow injection analysis (FIA) system allows reproducible urea determination in a millimolar range of concentration. The utility and limitations of the presented biosensor–FIA system for analysis of various real samples has been investigated. The system can be useful for some biomedical and pharmaceutical applications such as analyses of urine, posthaemodialysis fluid and extracts from pharmaceutical ointments containing urea. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Biosensor; Flow injection analysis; Urea

1. Introduction

Traditional non-enzymatic methods of analysis of various substances containing urea are poorly selective and time-consuming. Among these the Caraway-Fanger [1] and Yatzidis [2] methods were the most often used. Enzymatic methods are now generally recommended for urea determination [3,4]. The methods are based on specific hydrolysis of urea catalyzed by urease. The products formed are detected mainly by spectrophotometry. Most frequently, Berthelot or Nessler reactions with ammonia are applied. However, the consumption of urease as a reagent in the course of urea determination significantly increases costs of the analysis. Therefore, immobilized enzymes are applied more frequently as they can be used several times. Immobilized enzyme can be connected intimately with a detector sensitive for the products of the biocatalytic process forming a urea biosensor. Most urea biosensors are enzymatically modified potentiometric sensors. As the products of the urea hydrolysis are alkaline, an increase of urea concentration in the analyzed solution causes an increase of pH inside the enzyme layer of the biosensor. Therefore, many urea biosensors are based on pH electrodes.

Recently, we have developed a urea biosensor produced by covalent binding of monomolecular enzyme layer directly to the surface of polymeric membrane of the pH electrode [5]. Good analyti-

* Corresponding author. Fax: +48-22-8225-996.
E-mail address: rkoncki@chem.uw.edu.pl (R. Koncki)

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cal parameters of such a biosensor, mainly high stability, sensitivity and short response time allow its application in FIA systems. FIA techniques are especially recommended for frequent use over a long period of time as the measurements in such mode are faster, more reproducible and less time-consuming [6]. In this paper results of the application of the biosensor–FIA system for analyses of real samples important from biomedical and pharmaceutical points of view are presented.

2. Experimental

2.1. Materials and reagents

n-Tridodecylamine (TDDA, ionophore) and bis-(2-ethylhexyl)sebacate (DOS, plasticizer) for ion-selective membrane preparations were from Fluka. Carboxylated poly(vinyl chloride) (PVC-COOH, membrane matrix and immobilization support) was purchased from Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) for enzyme immobilization and urease (EC 3.5.1.5, from jack bean, 50 U/mg) were obtained from Sigma. Analytical grade reagents and double-distilled water were used for all experiments.

2.2. Urea biosensor preparation

The detailed procedure of fabrication and the properties of the biosensor were described in a previous paper [5]. Ion-selective membranes were prepared according to the standard procedure with the composition: TDDA (1%), DOS (66%) and PVC-COOH (33%). For enzyme immobilization a freshly prepared solution containing urease (10 mg/ml) and EDAC (5 mg/ml) was deposited on the surface of an ion-selective membrane and left for 12 h. Then the electrode was washed in vigorously stirred 0.1 M phosphate buffer (pH 7.0) to remove excess unbound enzyme.

2.3. Apparatus and FIA set-up

The applied biosensor–FIA set-up was recently described [7]. Ion-selective electrode bodies (Philips Model IS 561, Moller Glasblaserei, Schwitzerland) were used for the construction of enzymatically modified membrane electrodes. The potentiometric biosensor and the reference calomel electrode (Philips Model R11) were coupled to a digital pH-meter (Radiometer Model PHM 85, Denmark) connected to a data-collecting computer by RS 232c interface. Both electrodes were mounted into a small flow cell (approximately 10 μl) through which carrier buffer was pumped (flow rate 1.7 ml/min) using a multichannel peristaltic pump (Minipuls 3, Gilson, France). The length of mixing coil was 20 cm. The sample dilution in the FIA system was approximately 6. For sample injection a home-made rotary injection valve was used. The sample injection volume was 150 μl.

2.4. Reference method of urea determination

For determination of urea in all tested real samples a spectrophotometric bienzyme method, so-called Wartburg test [8,9], was used. The method is based on two enzymatic reactions: (1) urea hydrolysis catalyzed by urease, and (2) α-ketoglutarate reaction with the formation of ammonia in the presence of glutamate dehydrogenase and NADH. Changes of absorbance resulting from enzymatic oxidation of NADH to NAD were measured at a wavelength of 340 nm. The decrease in NADH concentration is directly proportional to the urea concentration. The test reagent kit is commercially available from F. Hoffmann-La Roche.

3. Results and discussion

3.1. Performance of urea biosensor in FIA system

Calibration curves of the biosensor in the FIA system have a sigmoidal shape over a wide range of urea concentrations and are strongly dependent on the composition of the carrier buffer used (Fig. 1). Similar characteristics were obtained in the case of measurements performed in the steady-state mode [5].

The magnitude of the analytical signal (change of the potential of the biosensor) mainly depends

on the protolytic equilibria inside the enzyme layer sensitizing the pH electrode. Those connected with protolytic products of the enzyme reaction and with the carrier buffer components are the most significant. The increase of buffer concentration (and, therefore, buffer capacity) has a 'dumping' effect on the pH changes inside the enzyme layer and suppresses the sensitivity of the biosensor. Moreover, it causes an increase in the determination limit. As a result the calibration graphs are shifted towards higher urea concentrations. The buffer of pH 6.0 guarantees the maximal sensitivity of the analytical system. As the upper pH, caused by products of urea hydrolysis, is about 9.3, the increase of buffer pH limits the available pH changes inside the enzyme layer. In more acidic solutions ($pH < 6$) the urease activity significantly decreases causing a decrease in the biosensor sensitivity. Similar effects of buffer components on the analytical response are observed for all kinds of pH-based enzyme sensors and are well described by diffusional [10] and non-diffusional [11,12] models.

Besides protolytic equilibria the sigmoidal shape of calibration curves depends on the kinetic parameters of the enzyme reaction [10–12]. The upper determination limit observed for all calibrations (Fig. 1) results from the change of the order of the biocatalytic reaction. For high urea concentrations the enzyme process is a zero order reaction. This means that the increase of urea concentration does not raise the concentration of reaction products (causing changes of the pH at the electrode). However, for low concentrations of analyte it is possible to find experimental conditions for a linear response of the biosensor. This linear range of concentration (in linear scale) covers non-linear part of calibration curve between lower determination limit and quasi-linear part of calibration graph obtained in logarithmic scale. Fig. 2 shows typical behavior of the enzyme electrode in the FIA system in the linear response range. The measurements were performed in optimized conditions, using 5.0 mM phosphate buffer pH 6.00 as a carrier. Under the conditions, a linear analytical response in the range 1–13 mM ($\Delta E = 0.7 + 4.1 \times C_{urea}$) with satisfactory regres-

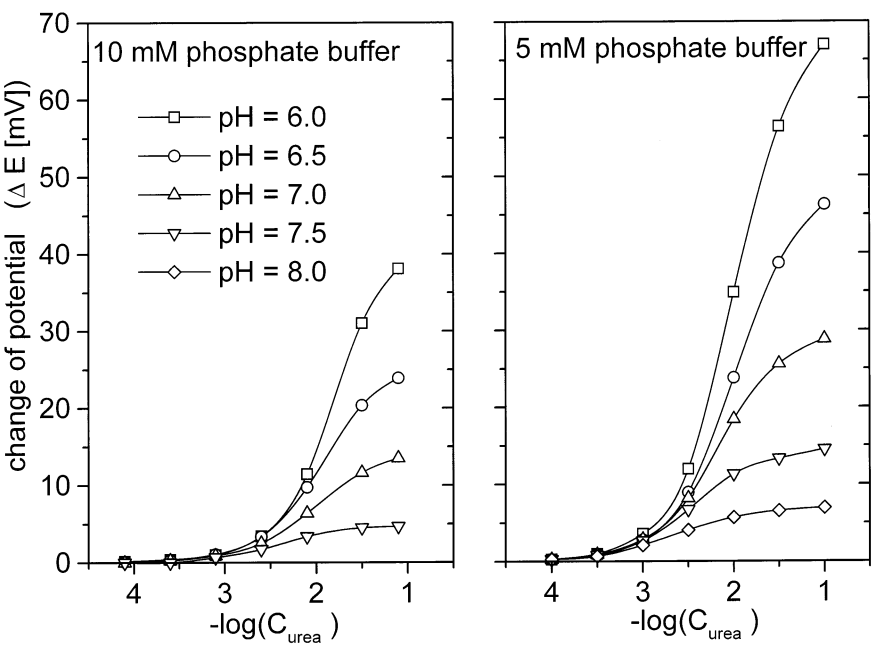

Fig. 1. Effect of pH on calibration curves of the urea biosensor in the FIA system. Measurements were performed in 5.0 mM (right) and 10 mM (left) phosphate buffer. pH values of buffers are given.

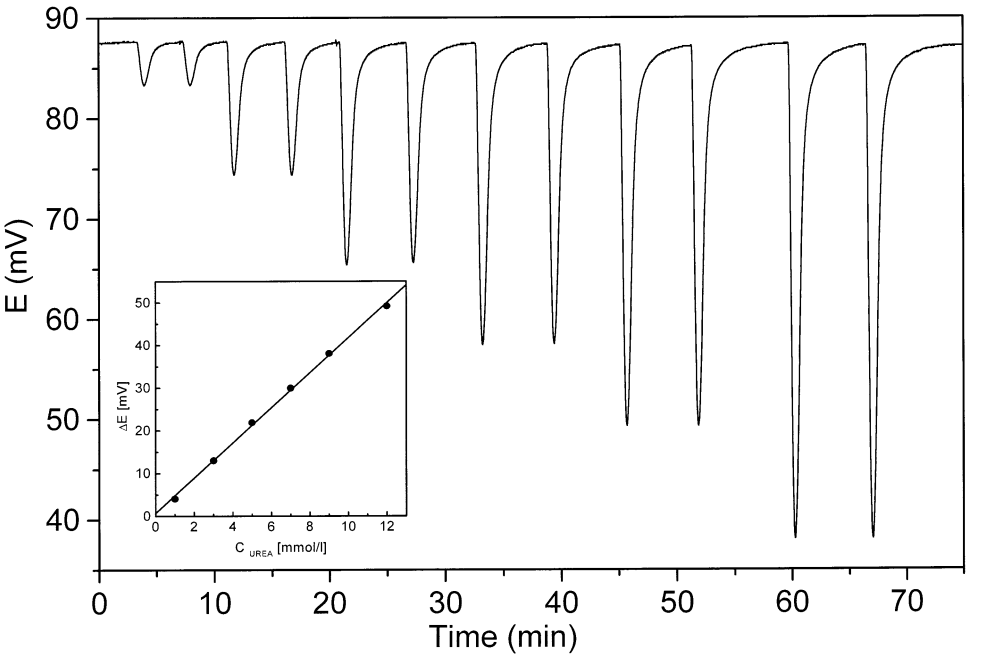

Fig. 2. Response of the biosensor in FIA system and corresponding calibration graph (insert). Measurements were performed in 5.0 mM phosphate buffer (pH 6.0).

sion coefficient ($r > 0.999$) has been obtained (Fig. 2, insert). The baseline of the system is stable and the peaks are reproducible. Under controlled conditions (pH and buffer capacity constant) cations and anions that commonly exist in real samples do not affect the analytical response. Taking into account the response and recovery times of the analytical system it is possible to do more than 15 injections per hour. As the system is stable, one calibration daily is sufficient to perform analysis. The analytical system exhibits a long operational stability retaining over 65% of initial sensitivity of the biosensor after 1 month of daily usage.

3.2. Biomedical analysis

Urea exists in all body fluids; however, from the clinical point of view the most important are determinations of this metabolite in blood and urine.

The analysis of blood (and serum) using the bioanalytical system was unsuccessful. The main problem of the analysis was connected with high buffering capacity of blood. The effect can be eliminated by high dilution of the samples with carrier buffer or by using more concentrated buffer. However, under such conditions the system is useful only for analysis of pathological samples with high levels of urea (over 20 mM). Moreover, in the case of such analysis the pH adjustment of the samples before injection is necessary making the analytical procedure much more complicated. It can be concluded that for blood/serum analysis the use of a similar FIA system with a urea biosensor based on an ammonium-ion-selective electrode [7] is more convenient. That biosensor is not sensitive for pH and buffer capacity of the samples and allows analysis of serum samples with physiological and pathological urea levels without the necessity of any sample pretreatment.

The bioanalytical system presented is useful for analysis of urine as the samples have low buffer capacity. Moreover, high content of urea in urine enables dilution of the samples before measurement and elimination of differences in pH of

analyzed samples. Fig. 3 presents the effect of dilution of the same sample of urine on the analytical response of the biosensor and on the response of the non-modified pH sub-electrode (Fig. 3, insert). It was found that 50 times dilution eliminates the effect of the pH of samples on the signal of the biosensor and that 100 times diluted urine samples can be analyzed using the analytical system. Samples diluted 20 times affected the response of the internal pH sensor due to significant differences in pH. Undiluted (more acidic) samples caused deformation of the peaks (Fig. 3) due to two opposite effects: i.e. increase of potential of the internal pH sensor caused by the pH of samples and decrease of pH inside electrode sensitizing layer caused by products of enzymatic hydrolysis of urea.

The results of urine analysis using the biosensor–FIA system are comparable with those obtained using a reference method (Fig. 4). It is worth to notice that contrary to the cited ammonium-ion-electrode-based biosensor, the presented pH-based enzyme electrode is not influenced by alkaline cations and, therefore, the results of analysis are independent of electrolyte level in the samples.

From a biomedical point of view analysis of posthaemodialysis fluid has become important. It was found that the presented biosensor–FIA system can be used for analysis of such type of samples in the appropriate of range of determination (1–13 mM of urea). Unfortunately the biosystem is not useful for continuous monitoring of the haemodialysis process because of the drift of the baseline potential. The effect is connected with slow changes of pH of the dialysate based on unstable carbonate buffer. Moreover, in the case of such application a shorter lifetime of the biosensor was observed. After several hours of permanent use the biosensor showed irreproducible parameters of calibration. It is supposed that the reasons for such unstability are changes inside of pH membrane of the biosensor contacted with dialysate. It is well known from the literature that TDDA used as an ionophore in the ion-selective membrane can slowly react with carbon dioxide forming insoluble salts and therefore loosing its ionophoric property [13].

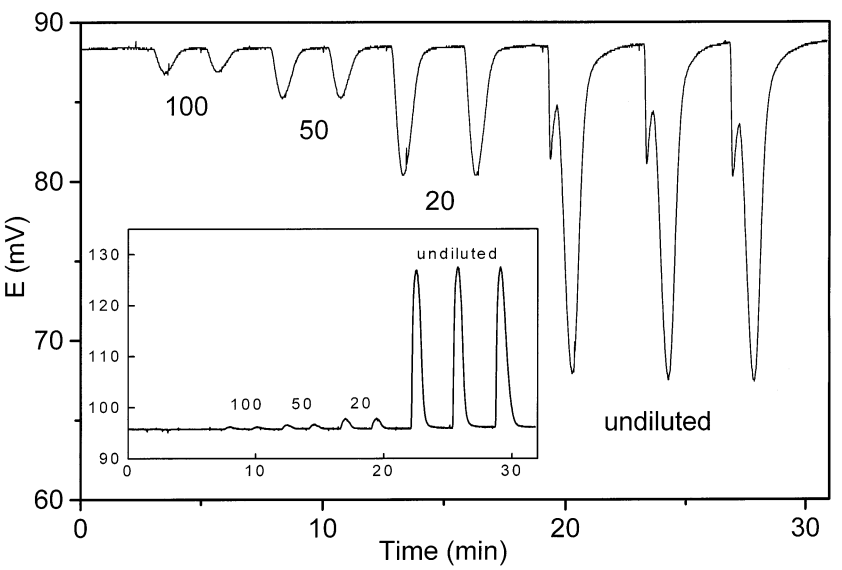

Fig. 3. Analytical signal of the biosensor for urine sample. Dilution of the sample with the carrier buffer before injection is given. The corresponding signal measured by a pH electrode without an enzyme layer is shown in the insert. Measurements were performed in 5.0 mM phosphate buffer (pH 6.0).

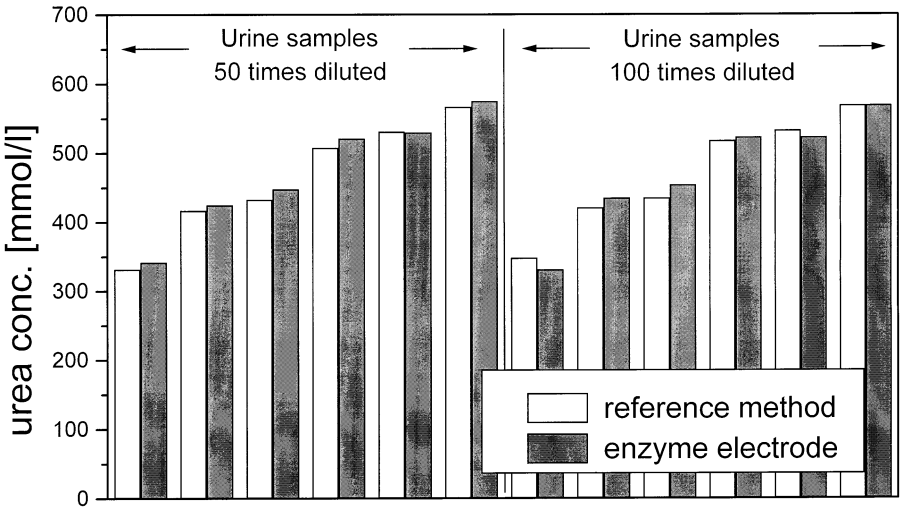

Fig. 4. Results of analysis of urine samples using the biosensor–FIA system and reference method. Sample dilution is given. Measurements were performed in 5.0 mM phosphate buffer (pH 6.0).

3.3. Pharmaceutical analysis

Urea is a component of several cosmetic creams and pharmaceutical ointments. At low contents in cosmetics (less than 1%) urea plays a role as a moistener. Due to its ceratolytic properties urea is applied as 3–10% ointments for peeling the epidermis or as 25–50% ointments for non-operational removal of nails.

The bioanalytical system was successfully applied for analysis of aqueous urea extracts from several pharmaceutical products: Elacutan (Leipziger Arzneimittelwerk), Xserial nos 3, 10 and 30 (Laboratories SVR Evry) and Mycospor Onychoset (Bayer). Urea was extracted from the ointment samples using 5 mM phosphate buffer pH 6.0. The same solution was used as a carrier in the FIA system. Addition of 5% NaCl was used to destroy the formed emulsions. Chloroform solutions of the ointments were applied in the case of non-water-wettable Xserial preparations for extraction. The results of analysis of all the obtained extracts using the proposed and reference method were fully comparable (Fig. 5).

The biosensor–FIA system is also useful for the analysis of cosmetic creams containing lower amounts of urea. For example, in the course of analysis of extracts from a moisture face mask (Etrebelle Cosmetic GmbH) $0.065 \pm 0.005\%$ of urea was found. Using the reference method $0.06 \pm 0.01\%$ of urea was determined.

The pH-based enzyme electrode presented is useful for the analysis of urea extracts from phar-

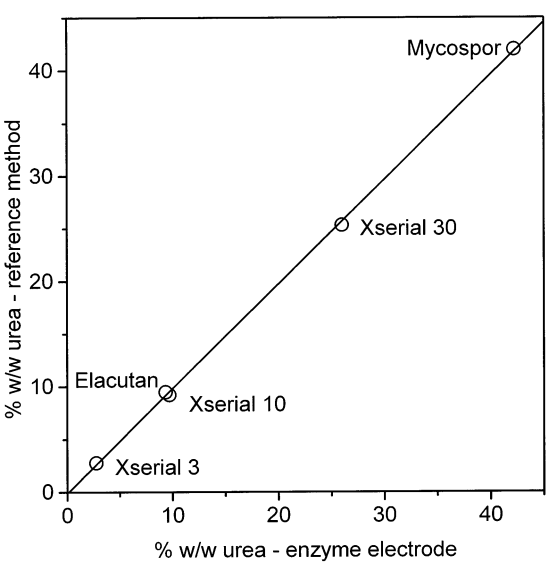

Fig. 5. Correlation of the results of ointment extract analysis obtained using the biosensor–FIA system and reference method. For extractions and measurements 5.0 mM phosphate buffer (pH 6.0) was used.

maceutical ointments, as the samples practically have no buffer capacity and do not influence the pH of extracts. Moreover, high contents of salts added in the course of extraction do not interfere in the course of the determination.

4. Conclusion

In this paper the utility and limitations of the FIA system based on pH–membrane electrode with covalently bound urease was discussed. This analytical system is an alternative for methods used currently in the biomedical and pharmaceutical analysis of real samples containing urea. The main advantages of the proposed method are the simplicity of the developed system, the short time of analysis and good selectivity.

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