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AN ENZYME SENSOR FOR UREA BASED ON CONDUCTIVITY MEASUREMENT

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ABSTRACT

An enzyme-based sensor for the determination of urea is described. The system uses urease immobilized to porous glass and conductivity electrodes to measure the increase in conductivity of the sample solution resulting from hydrolysis of urea into charged products. A linear relationship between the changes in conductivity and urea concentrations was obtained with concentration up to 8 mM (correlation coefficient, r = 0.996 ± 0.002). The response of the enzyme column was stable when used intermittently for up to 48 days, or > 60 h operation time. Good agreement was obtained when the concentrations determined by this system were compared with the diacetylmonoxime colorimetric method (r = 0.994).

INTRODUCTION

Determination of urea is one of the most frequent analyses in routine clinical laboratory work, and currently the most popular screening test for evaluating renal function. The most common method for urea determination employs the reaction with diacetylmonoxime. However, this method requires heating and some of the reagents involved are quite noxious[1]. Many clinical laboratories now employ an indirect method where the free enzyme urease is used to hydrolyse urea and the product(s) is determined[1]. Recently the technologies used to immobilized protein have become readily available[2] and several analytical procedures using immobilized urease as sensors for urea have been reported[3-10].

The enzyme urease, which is known to be extremely specific to urea, catalyses the hydrolyis of urea to charged products according to the formulae

$$H_2NCONH_2 + H_2O + 2H^+ \longrightarrow 2NH_4^+ + CO_2$$

$$CO_2 + H_2O \longrightarrow HCO_3^- + H^+$$

Most urease-based sensors have been based on the detection of the ammonium ions or ammonia gas[3-10]. The ammonium ions can be monitored by ammonium ion selective

electrodes. However, these electrodes are also sensitive to other ions. Ammonia gas systems most often involve the addition of base to increase the sensitivity.

Here we propose an alternative urease-based system for the determination of urea. Since the catalysis reactions of urea by urease produce charged products, the conductivity of the solution should increase and the effect should be possible to detect using conductivity electrodes. The responses of enzyme to urea concentrations can then be quantified as the changes in conductivity and the relationship between urea concentrations and changes in conductivity can be determined.

MATERIALS AND METHODS

Materials

Urease (urea amidohydrolase EC 3.5.1.5 from Jack Beans Type IV 69 units/mg) and the reagents for the colorimetric determination of urea (UREA NITROGEN No.535 Colorimetric) were obtained from Sigma (St. Louis, Missouri, USA). Glass beads (mean diameter 41 mm, mean pore diameter 20 nm) were supplied by EKA Nobel AB (Surte, Sweden). All other chemicals used were of analytical grade.

Immobilization of urease

The preparation of alkylamine glass with 3-aminopropyltriethoxysilane and later aldehyde glass with glutaraldehyde, were carried out following procedures described by Weetall[11]. In a typical preparation, 25 mg of urease was dissolved in 5 ml of 0.05 M sodium phosphate buffer pH 7.0 and added to 2.5 ml (sedimented volume) of activated glass. The mixture was tumbled end over end at room temperature. After 4-5 h 100 mg of sodium cyanoborohydride was added to reduce the Schiffs bond between aldehyde and enzyme, thus stabilizing the coupling. The mixture was tumbled again for another 18 h and was then washed on a glass filter with the coupling buffer. To this enzyme preparation 50 ml of 1.0 M ethanolamine (adjusted to pH 8.0 with 6.0 M HCl) was added and two hours of reaction allowed. This step was to occupy all the aldehyde groups which did not couple to the enzyme. The preparation was then washed with the coupling buffer and was packed into a small column (inner diameter 4 mm, length 30 mm) to be used in the analysis. When not used, the column was stored in the coupling buffer + 0.02% sodium azide at 4°C.

Instrumentation

Fig.1 shows the basic principle of the system. The sample is pumped through the enzyme column which has conductivity electodes connected to its outlet. The column is filled with urease immobilized to porous glass. When the solution containing urea passes through the enzyme column, urea is converted to charged products, thus increasing the conductivity of the solution. The response is measured as the change in the conductivity by comparing the conductivity of the solutions with and without urea.

The conductivity circuit is shown in Fig.2. The electrodes are made from stainless steel tubes (outer diameter 0.9 mm), approximately 9-10 mm in length, glued to the ends of a 17 mm long glass tube (inner diameter 1.0 mm) (Fig.1). The ends of the electodes

inside the glass tube are approximately 8 mm apart. The oscillator frequency is about 1.6 kHz, chosen to avoid disturbances from other signals. The alternating current will also reduce the polarization effects at the electrodes.

Calibration of enzyme response

Solutions of urea (5, 10, 15, 20, 25, 30, 40 and 50 mM) were prepared in 0.05 M glycine-NaOH buffer pH 8.8, chosen because of its low conducutvity. The sample solutions were introduced as pulses in the continuous flow of buffer. Durations of the pulses used were eight minutes. When the solution containing urea passed through the enzyme column urea was degraded by the immobilized urease into charged products, thus increasing the conductivity of the solution. The effects were measured along the length of the glass tube using conductivity electrodes (Fig.1), and the signals registered on a chart recorder. The full response of the enzyme column for each urea concentration was measured from a chart recording, and the relationship between the changes in conductivity and urea concentrations was determined.

Similar procedures were carried out for urea solutions of lower concentration (2, 4, 6, 8, 10 mM). In this case the amplification of the output signal from the conductivity circuit was increased.

Stability of response

To investigate the response stability of the enzyme column the activity was tested intermittently over a period of 48 days.

Comparison between clinical analyses and enzymatic analyses

Urea solutions (2, 4, 6 and 8 mM) were prepared in 0.05 M glycine-NaOH buffere pH 8.8. They were analysed using a Urea Nitrogen test kit (Sigma). The absorbance of each sample was measured spectrophotometrically at 530 nm and a calibration curve was constructed.

Samples of various concentrations were analysed and the urea concentrations were determined from the calibration curve.

The same calibration solutions were used to calibrate the response of immobilized urease. The sample solutions were subsequently passed through the analytical system. The change in conductivity of each sample was used to calculate the urea concentration from the calibration done prior to the test.

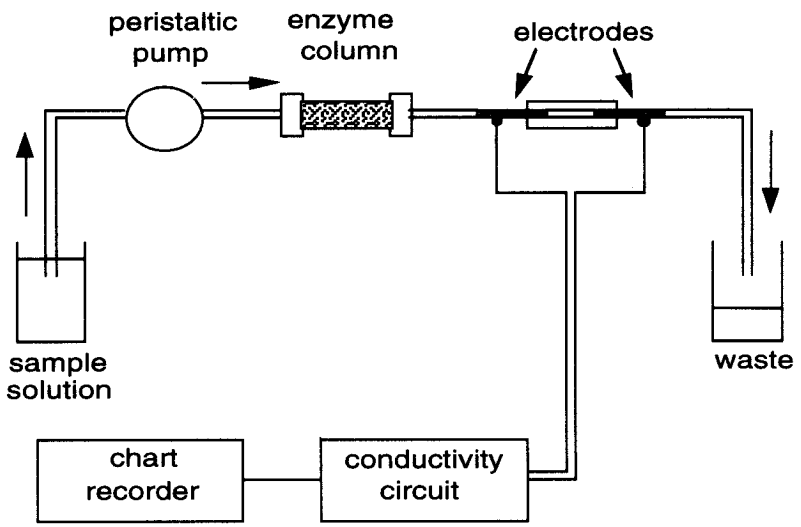

Fig.1 Schematic diagram showing the basic principle of the analytical system. The sample is pumped through the enzyme column where immobilized urease catalyses the hydrolysis of urea into charged products. The change in the conductivity of the solution is measured by the conductivity electrodes.

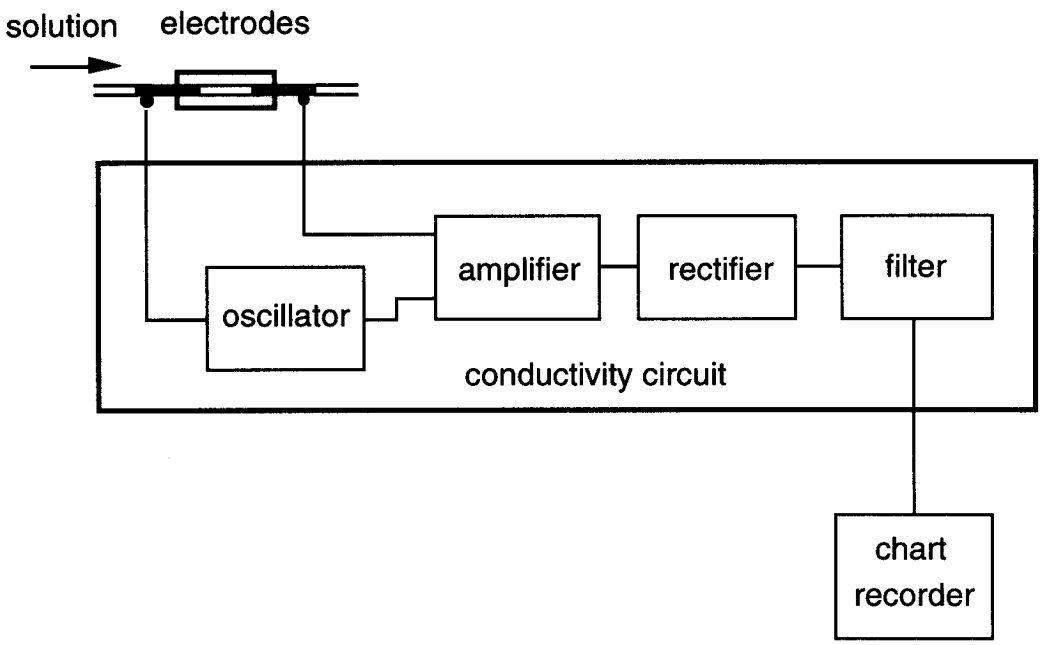

Fig.2 Block diagram of the conductivity circuit.

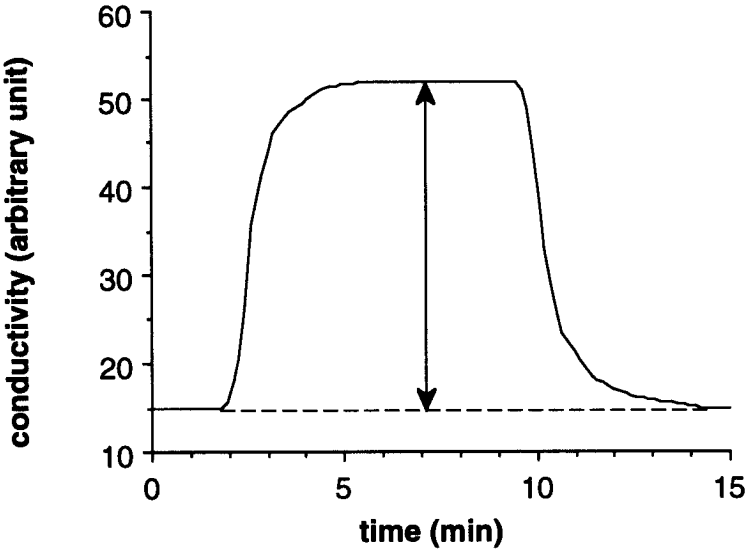

Fig.3 Response of immobilized urease to urea measured as the change in conductivity of the solution as recorded by the analytical system.

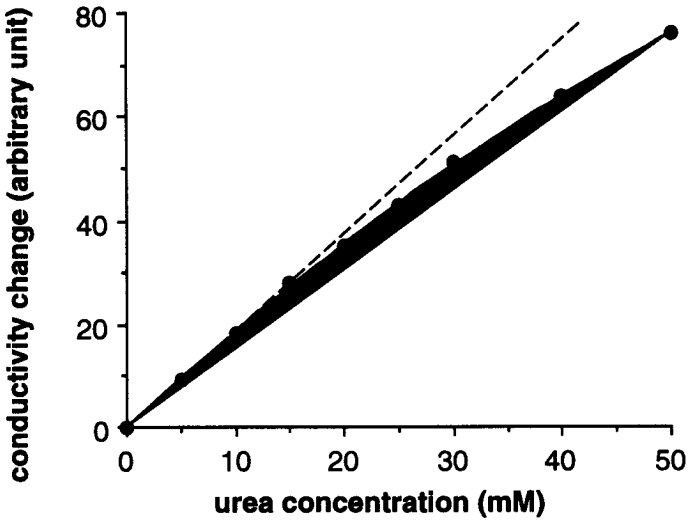

Fig.4 Calibration curve of urea. Plot of amplitude of the change in conductivity, as shown in Fig.2, as a function of urea concentrations (5-50 mM).

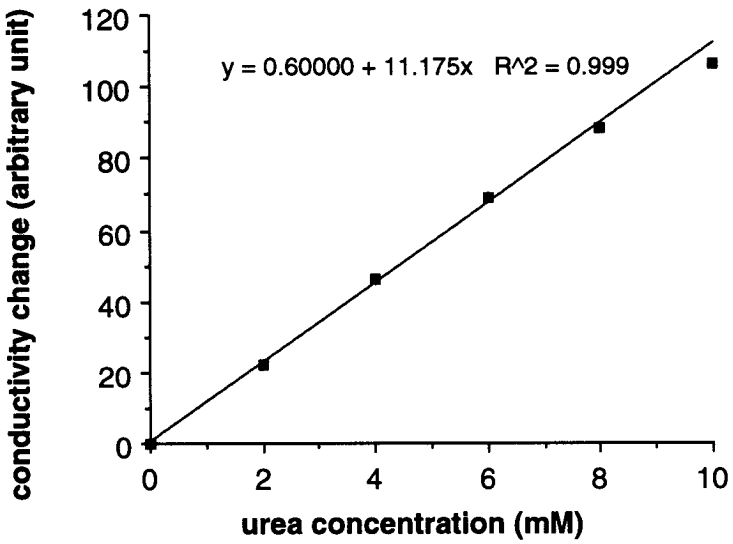

Fig.5 Calibration curve of urea. Plot of amplitude of the change in conductivity, as shown in Fig.2, as a function of urea concentrations (2-10 mM).

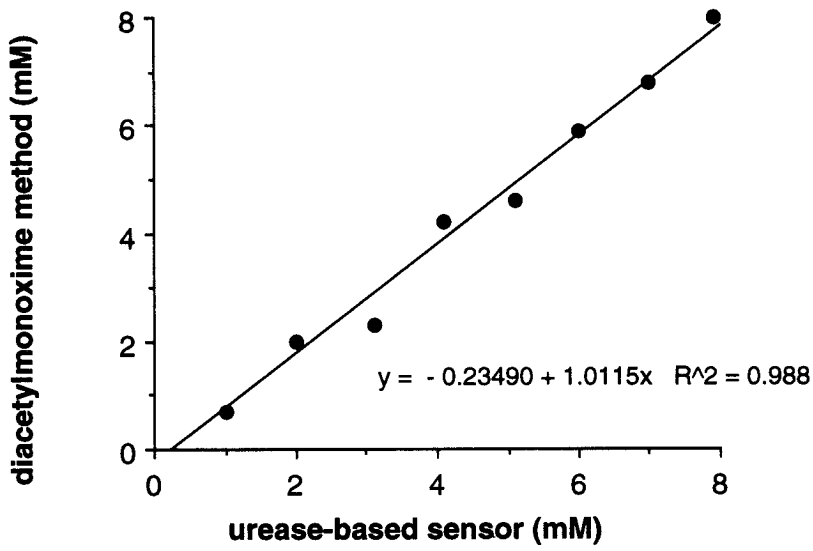

Fig.6 Comparison of urea concentrations determined by diacetylmonoxime colorimetric method and with the urease-based sensor.

RESULTS AND DISCUSSION

A typical response is shown in Fig.3. The amplitude of the signal was measured from the chart as indicated. At a flow rate of 0.5 ml/min, it took approximately 4-5 minutes for a full enzyme response. This response time is not critical for the analysis in clinical applications since a change in body fluid composition is also a slow process. However, if a shorter response time is needed, the system can be modified to a great extent, e.g. by altering the pump speed and/or choosing appropriate dimensions for the tubing.

Linearity

In the urea concentration range studied, 5-50 mM, the response of the enzyme column is linear up to about 10 mM as shown in Fig.4. The concentration range 5-50 mM was studied since it represents the concentration range of urea in human blood. For the analysis system to be useful, the response should be linear. From the results it seems that the linear range of this system may not be suitable to measure urea in blood. This is, however, not a problem since dilution of the sample before passing it through the enzyme column is possible.

Fig.5 shows the response of enzyme column to urea in the lower concentration range. The linearity of the response is in fact up to 8 mM. Repeated experiments on this concentration range (2-8 mM) indicated very good linear correlation. Five different series of experiments were carried out on the same column and the correlation coefficient was 0.996 ± 0.002.

Accuracy

In order to determine the accuracy of the analytical system both chemical analysis and analysis using this sytem were done on the same samples. The results are shown in Fig.6 where the chemical analysis data is plotted against the results obtained from this system. It can be seen that the concentrations determined by the two methods are in good agreement.

Stability

Generally after prolonged use of the enzyme, denaturation or inhibition of the enzyme may effect the response. The enzyme column was tested and found that in the linear range (2-8 mM) good responses were still obtained after more than one and a half months, or more than 60 h operation time. The average slope of the response was 11.6 ± 0.6 unit/mM, after with a correlation coefficient of 0.996 ± 0.002. If longer operation time is needed the column may still be used by using a larger amount of immobilized urease. Replacement of columns in this system can also be done easily.

CONCLUSIONS

It is clear from the results that with further development, this system could be used for the determination of urea concentration with accuracy. The relatively easy preparation of the immobilized enzymes, together with the good response stability of the enzyme column makes this a very attractive system for the analysis of urea.

The experiments reported here show that it is possible to use conductivity measurements in conjunction with enzyme based analysis. For this particular system it is best to operate in the concentration range 8 mM. Higher concentrations can also be analysed by diluting the samples prior to the analysis.

However, to be able to analyse a real sample, for example whole blood, additional sample handling systems need to be considered to seperate the particles in the sample being analysed before passing them through the enzyme column. Therefore, some form of filtration is required, perhaps a dialysis membrane system[12].

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บทคัดย่อ

เอนไซม์เซนเซอร์สำหรับยูเรียใช้เอนไซม์ urease ตรึงบน porous glass ร่วมกับ conductivity อิเล็กโทรดในการวัดสภาพนำไฟฟ้าที่เพิ่มขึ้นในสารละลาย เนื่องจากในปฏิกิริยาไฮโดรไลซิสของยูเรียจะทำให้เกิดผลผลิตที่มีประจุซึ่งจะทำให้สภาพนำไฟฟ้าของสารละลายเพิ่มขึ้น ความสัมพันธ์ระหว่างการเพิ่มของสภาพนำไฟฟ้ากับความเข้มข้นของยูเรียเป็นเชิงเส้นในช่วงความเข้มข้นไม่เกิน 8 mM (สัมประสิทธิ์สหสัมพันธ์ r = 0.996 ± 0.002) การตอบสนองของคอลัมน์ของเอนไซม์จะสม่ำเสมอในช่วง 48 วันที่ทำการทดสอบ หรือ > 60 ชั่วโมง operation time เมื่อเปรียบเทียบความเข้มข้นที่ได้จากการวิเคราะห์โดยใช้ระบบนี้กับวิธี diacetylmonoxime พบว่าได้ผลตรงกัน (r = 0.994)