A Flow-Through Enzyme Reactor System for Urea Determination in Blood Serum Using Conductimetric Measurement

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ABSTRACT A flow-through enzyme reactor system for the determination of urea using conductimetric measurement is described. Samples are introduced as pulses in a continuous flow of buffer which is passed through a dialyser. The dialysis solution on the other side of the dialysis membrane collects urea molecules from the samples which diffuse through the membrane and is pumped through an enzyme reactor containing urease immobilized to porous glass. Conductivity electrodes are used to measure the increase in conductivity of the dialysis solution resulting from the hydrolysis of urea into charged products. The effects of the flow rate and the possible interferences of cells or NaCl in the sample solutions to the response of the enzyme sensor system have been investigated. The system is used for the determination of urea in standard solutions as well as in human blood serum samples. The enzyme sensor is operated in a linear mode in the concentration range 5-70 mmol/L (correlation coefficient, r = 0.998). Good agreement is obtained when the urea concentrations of human blood serum samples are determined using the enzyme sensor system compared to the diacetyl monoxime colorimetric method (r = 0.997) and to the results obtained by a commercial automated analysis system (r = 0.998).

KEYWORDS: urea, serum, conductivity, enzyme sensor, immobilized urease.

INTRODUCTION

Urea levels in body fluids are one of the most frequently analyzed items in clinical laboratories since they serve as a rough predictive index of symptomatic renal failure and as a diagnostic aid in distinguishing among the various causes of renal insufficiency.[1] A widely accepted reference interval for serum urea is 2.3-8.3 mmol/L, derived from young men on a normal diet,[2] and the value between 24.9-41.5 mmol/L is taken as a conclusive evidence of severe renal impairment.[1]

The common direct method for the determination of urea employs the Fearon reaction where urea reacts with diacetyl to form a coloured chromagen, which is then quantified photometrically.[1] Disadvantages are the heat requirement and the noxious nature of some of the reagents[2]. Indirect methods employ the enzyme urease to catalyse the hydrolysis of urea and the product is determined. Urease has been used as free or immobilized enzyme. A major advantage of the latter is the reduction in cost through the reusability of expensive enzyme reagent[2] and several enzyme sensors using immobilized urease have been reported.

The enzyme urease, which has great specificity for urea, catalyses urea to ammonium and bicarbonate ions:

$$NH_2 - CO - NH_2 + 2H_2O + H^+ \xrightarrow{urease} 2NH_4^+ + HCO_3^-$$

Most urea biosensors are based on potentiometric mode of detection. The detectors are electrodes sensitive to ammonium ions,[3-4] ammonia gas[5-9] or pH.[10] Recently amperometric detection[11-12] and optical sensing methods[13-17] have also been reported. Conductimetry is another transducer principle that can be applied to determine urea since the conductivity in the solution would increase due to ammonium and bicarbonate ions produced by the hydrolysis reaction of urea. Conductimetric urea sensors often use planar interdigitated electrode arrays as transducers[18-21] where the enzyme was immobilized directly onto the transducer. This approach may seem attractive but special manufacturing technique is needed. An alternative approach is to separate the immobilized enzyme and the transducer. Several biosensors for urea using enzyme reactors and various types of detector have been proposed.[5,22-23] Preliminary study in our

laboratory has demonstrated the feasibility of an enzyme reactor system for urea based on conductivity measurement.[24] Here we report the development of a conductimetric flow-through system for the determination of urea using an immobilized urease enzyme reactor and its application for the determination of urea in serum.

Materials and Methods

Materials

Urease (urea amidohydrolase EC 3.5.1.5 from Jack Beans Type IV 35 units/mg) and the reagents for the colorimetric determination of urea were obtained from Sigma (St. Louis, Missouri, USA). Glass beads (mean diameter 41 µm, 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 by aqueous silanization and the activation by glutaraldehyde to yield an active carbonyl derivative were carried out following procedures described by Weetall.[25] 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 5 h 50 mg of sodium cyanoborohydride was added to reduce the Schiff's bond[26-27] between aldehyde and enzyme, thus stabilizing the coupling.[28] The mixture was tumbled again for another 12 h and was then washed on a glass filter with the coupling buffer. To this enzyme preparation 25 ml of 1.0 M ethanolamine (adjusted to pH 8.0 with 6.0 M HCl) was added and two hours of reaction were allowed. This step was to occupy all the aldehyde groups which did not couple to the enzyme. The enzyme glass preparation was then washed with the coupling buffer and was packed into a small reactor (inner diameter 4 mm, length 30 mm) to be used in the analysis. When not used, the reactor was stored in the coupling buffer + 0.02% sodium azide at 4°C.

Instrumentation

Fig 1 shows the diagram of the enzyme sensor system. The sample is pumped through a dialyser before being sent to waste. The dialyser has a cellulose ester dialysis membrane with a MW cut-off 6,000-8,000 (Spectra/Por 1, Spectrum Medical Industries, Inc, Los Angeles, USA) with the area of 4.9 x 0.19 cm^2. This allows small molecules to pass through the membrane and to be collected in the dialysis solution which is pumped through on the other side of the membrane. The dialysis solution containing urea molecules is then passed through the enzyme reactor which is filled with urease immobilized to porous glass. When the dialysis solution containing urea passes through the enzyme reactor, urea is converted into charged products, thus increased the conductivity of the solution. The outlet of the enzyme reactor is connected to a conductivity cell. The electrodes of the cell 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 glass tube (inner diameter 1.0 mm) (Fig 1). The ends of the electrodes inside the glass tube are approximately 8 mm apart. The enzyme response to urea is measured as the change in the conductivity within the conductivity cell by comparing the conductivity of the solutions with and without urea. The temperature during the experiments was 26 ± 2°C.

Flow-through system optimization

The background solution used throughout the experiments was 0.05 M glycine-NaOH buffer pH 8.8, chosen for its low conductivity. Urea calibration solutions were diluted from 0.1 mol/L urea solution prepared in glycine-NaOH buffer. The calibration solutions were introduced as pulses in the continuous flow of buffer with a flow rate of 0.5 ml/min, by switching the tube between buffer and sample containers. Durations of the pulses used were 2 min, *ie*, a sample pulse of 1.0 ml . The dialysis solution on the other side of the dialysis membrane was the same buffer flowing at the same flow rate of 0.5 ml/min. When the dialysis solution containing urea passed through the enzyme reactor the immobilized urease catalysed the hydrolysis reaction of urea and the conductivity of the solution increased. The change in conductivity were measured using the electrodes (Fig 1), and the signals were registered on a chart recorder (Linear Instrumental Corp, USA). The response of the enzyme reactor for each urea concentration was read from a chart recording, and the relationship between the changes in the conductivity electrodes signal and urea concentrations determined.

Calibration curves of the enzyme responses for 3 different sample flow rates (0.25, 0.50 and 0.80 ml/min) were also determined. The durations of the pulses used were 4, 2 and 1.25 min respectively which corresponded to the sample pulse of 1.0 ml.

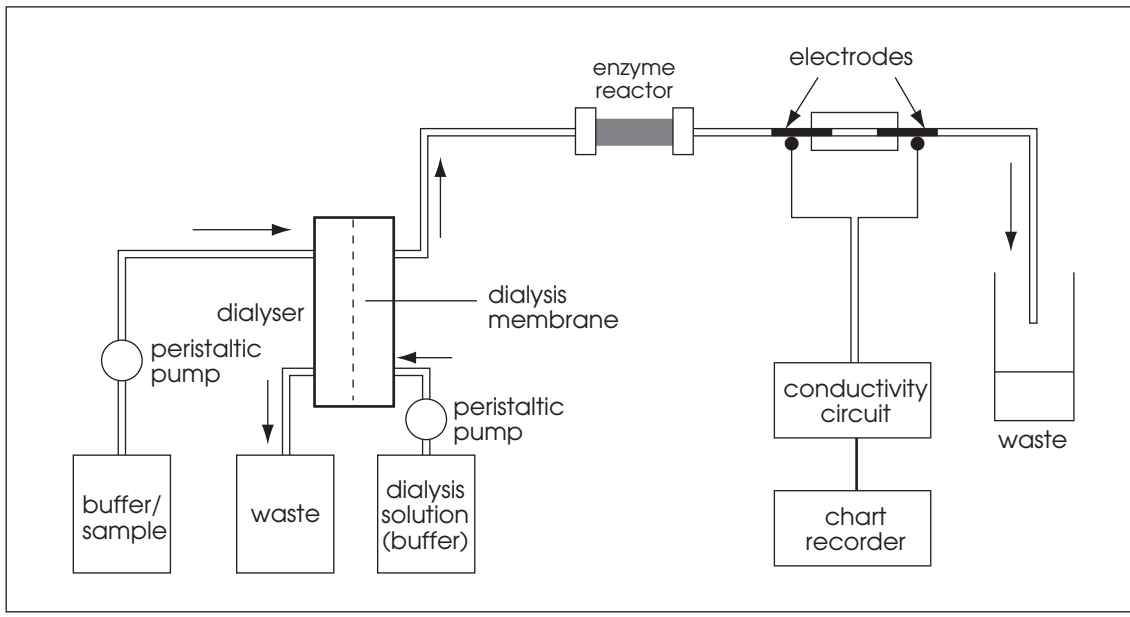

Fig 1. Schematic diagram showing the basic principle of the enzyme sensor system. The sample is introduced as pulse in the continuous flow of buffer by switching the tube between buffer and sample containers. The sample is pumped through a dialyser to waste. On the opposite side of the membrane in the dialyser, the dialysis solution (the same buffer) is being pumped through, urea from the sample passes through the membrane into the dialysis solution and to the enzyme column where urea is degraded by the immobilized urease. The change in the conductivity of the solution passing through the conductivity cell is measured by the conductivity electrodes.

The flow rates on the other side of the dialysis membrane were set to be the same as those of the samples.

Interferences

Urea may be determined in whole blood, plasma or serum and there is normally little difference between them.[2] In whole blood about 45% of its volume is occupied by the formed elements, i.e. red and white blood cells and blood platelets.[29] These may interfere with the diffusion of urea across the dialysis membrane. To test this effect, yeast cells of different concentrations (0.5% to 8.0% (w/v) at 0.5% step) were added to the urea calibration solutions and the responses compared to those without cells.

The liquid portion of blood is the blood plasma where the usual concentration of salts is isotonic to a 0.9% (w/v) NaCl solution.[29] These salts in the sample when diffuse through the membrane go through the enzyme reactor and then through the conductivity cell may effect the response in two folds. That is (i) the salts will increase the baseline conductivity of the solution and (ii) they may effect the enzyme activity since it has been reported that some ions such as Na^+ and K^+ are inhibitors of urease.[30] To examine this effect urea calibration solutions were prepared in 0.05 M glycine-NaOH buffer pH 8.8 with and without 0.9% (w/v) NaCl and the responses of the system were determined.

Determination of urea in blood serum

Urea calibration solutions were prepared in 0.05 M glycine-NaOH buffer pH 8.8 with 0.9% (w/v) NaCl. They were analysed using a Blood Urea Nitrogen test kit (535-A, Sigma, USA). The absorbance of each sample was measured spectrophotometrically at 530 nm (Spectrum 351, Trans Orchid Consultant inc., USA) and a calibration curve was constructed. Human blood serum samples were obtained from Songklanagarind Hospital, Prince of Songkla University, Hat Yai. They were diluted using the buffer with 0.9% (w/v) NaCl at a serum:buffer ratio of 1:9. These samples were analysed using the test kit and the urea concentrations were determined from the calibration curve. The same calibration solutions were used to calibrate the response of the enzyme sensor. One ml of each diluted serum samples were subsequently passed through the enzyme sensor system. The change in conductivity of each sample was used to calculate the urea concentration from the calibration done prior to the test. The results were also compared to those obtained by the hospital (Automatic Analyzer, Hitachi Model 704, Japan).

Results and Discussion

Typical responses are shown in Fig 2. The amplitude of the signal was determined from the chart as indicated.

Flow-through system optimization

Linearity

For urea concentration range studied, 5-90 mmol/L, the response of the enzyme reactor is linear up to 70 mmol/L, as shown in Fig 3. This is well over the range of urea concentration in human blood which is between 5 to 50 mmol/L.

The results from the experiments with different flow rates are shown in Fig 4. The response at the lower flow rate is more than the higher flow rate since the urea solution would spend more time in the enzyme reactor resulted in more hydrolysis of urea. The sensitivity of the system at 0.25, 0.50 and 0.8 ml/min are 0.837, 0.487 and 0.309 unit/mmol/L respectively. Taking 0.50 ml/min as a reference the sensitivity at 0.25 and 0.80 ml/min are 170% and 63% respectively. The 0.80 ml/min flow rate gave very low sensitivity so no further study was done using this flow rate. Although the sensitivity of the the lower flow rate was higher the analysis time was much longer. At the flow rate of 0.25 ml/min the analysis time per sample pulse was between 7 to 17 min, depending on the concentration, compared to 5-13 min at 0.50 ml/min. The choice of the flow rate was then a balance between sensitivity and the analysis time per pulse. To shorten the analysis time per pulse for the 0.25 ml/min flow rate the amount of a urea sample pulse was decreased to 0.65 ml which provided a slightly higher sensitivity (110%) than the 0.50 ml/min, however, the analysis time per pulse was still longer by about 2 min. Therefore, the 0.50 ml/min flow rate was chosen for the remaining experiments.

Interferences

Effect of yeast cells in the sample

The sensitivity of the system decreases as the amount of yeast cells increases. Fig 5 shows the calibration curves of urea with 2.5% and 8.0% yeast cells compared to the curve without yeast cells. The sensitivity of the 8.0% curve, 0.307 unit/mmol/L, is only 42% of the one without yeast cells. This is likely caused by the blocking effect of yeast cells to the diffusion of urea molecules through the dialysis membrane. Therefore, if whole blood which has

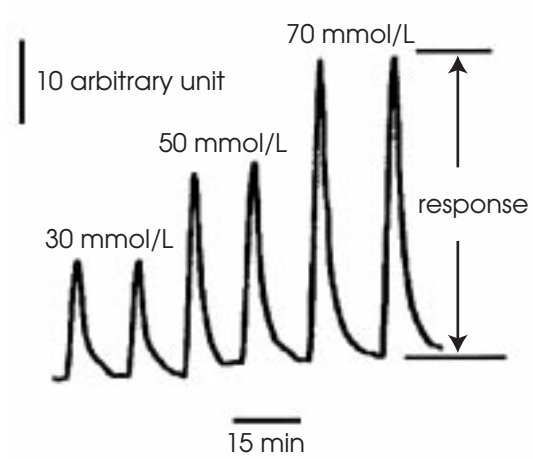

Fig 2. Responses of immobilized urease to urea measured as the change in conductivity of the solution recorded by the enzyme sensor system. This figure shows the responses when two one-minute pulses of urea solutions of 30, 50 and 70 mmol/L were passed through the system at 0.5 ml/min.

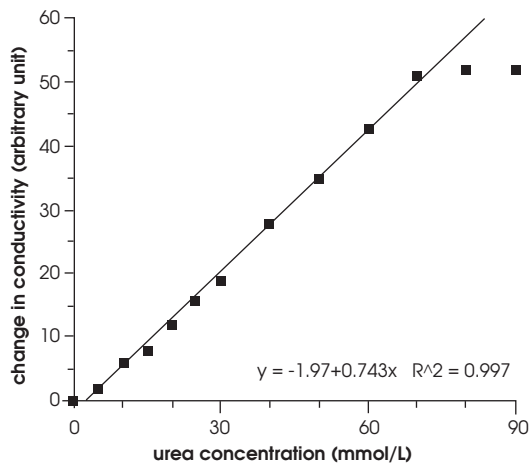

Fig 3. Calibration curve of urea showing the amplitude of the change in conductivity, as shown in Fig 2, as a function of the concentration of urea.

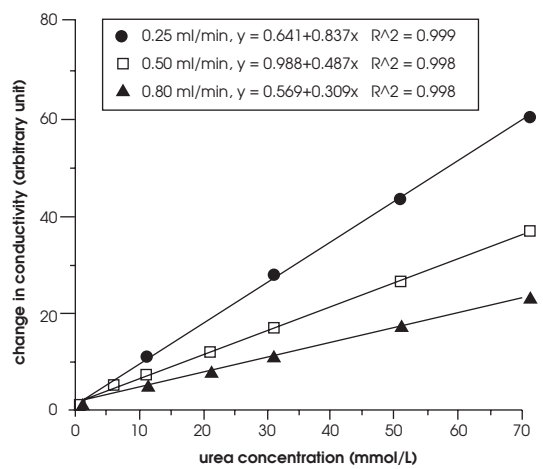

Fig 4. Calibration curves of the enzyme sensor at different flow rates.

about 45% of formed elements was used in the analysis the urea concentration given by this system would be much lower than the real value. In view of this blood plasma or serum should be used for the detection of urea. Blood plasma is about 92% water, only about 7% protein and the remaining 1% is made up of salts and some other compounds and serum would have even a lower percentage of proteins than blood plasma since it does not contain fibrinogen.[20] Dilution of the blood plasma or serum sample may also be needed in order to reduced the percentage of the proteins as much as possible so they would have the least interference to the analysis. In practice when serum was used the serum samples were diluted at a serum:buffer ratio of 1:9 and the system was sensitive enough for the analysis of these diluted samples.

Effect of NaCl

When the buffer solution on the sample side of the membrane contained 0.9% (w/v) NaCl the baseline of the signal increased. This increase in conductivity was caused by the Na^+ and Cl^- ions that diffused through the membrane into the buffer solution that passed through the enzyme reactor and the conductivity electrodes. This should not be a matter of much concern as long as the increase of conductivity due to these ions are much less than the increase in conductivity due to the enzymatic reaction.

The calibration curves of urea with and without NaCl are shown in Fig 6 and it seems that NaCl does have a slight effect on the responses of the enzyme sensor system. The sensitivity of the curve with NaCl, 0.400 unit/mmol/L, is about 13% lower than the one without NaCl, 0.457 unit/mmol/L. This may due to the interference of NaCl to the diffusion of urea across the dialysis membrane or it is possible that Na^+ may have some inhibitory effect on urease as reported.[30] This effect could easily be solved, by using 0.05 M glycine-NaOH buffer pH 8.8 with 0.9% (w/v) NaCl as the working solution on the sample side both during calibration and during the analysis of real samples so the effect of NaCl would be presented in the background at all time.

Determination of urea in blood serum

The analyses using the enzyme sensor system, colorimetric analysis, and the automated analysis system at the hospital were done on the same serum samples. The results obtained from the enzyme sensor are compared to the ones using diacetyl monoxime colorimetric analysis as shown in Fig 7. The linear regression equation was y = -2.706 +

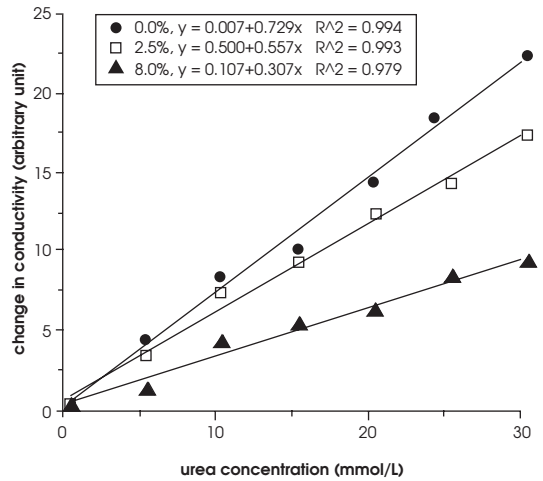

Fig 5. Calibration curves of the enzyme sensor showing the effect of yeast cells in the sample.

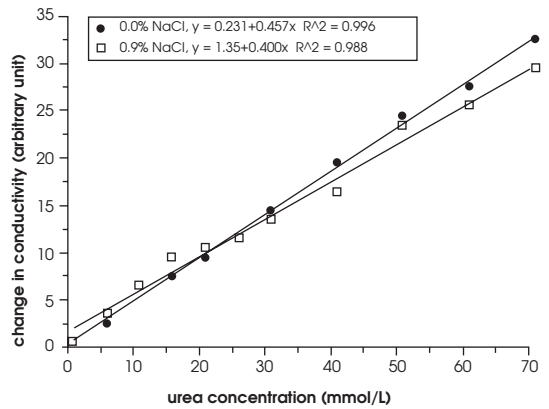

Fig 6. Calibration curves of the enzyme sensor showing the effect of 0.9% NaCl (w/v) in the sample.

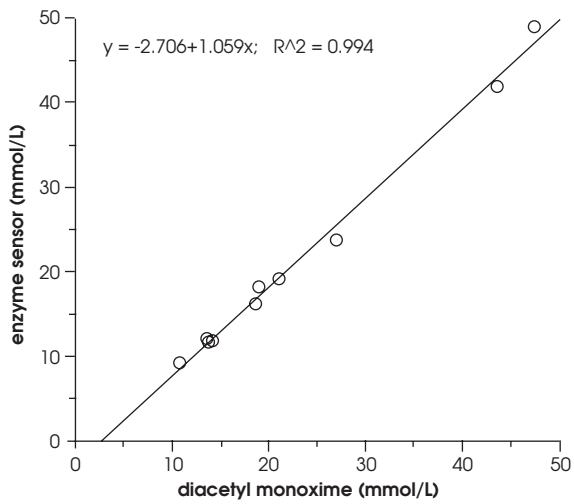

Fig 7. Comparison of urea concentration of human blood serum samples determined with the enzyme sensor and with the diacetyl monoxime colorimetric method.

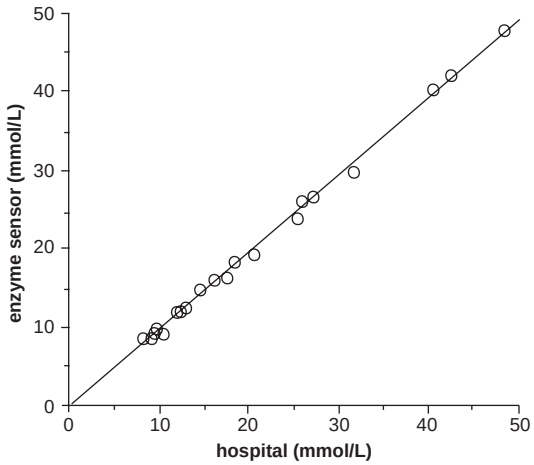

Fig 8. Comparison of urea concentration of human blood serum samples determined by the enzyme sensor and with the hospital automated analysis system.

1.059x with a correlation coefficient of 0.997. Fig 8 shows the comparison between the results from the enzyme sensor and those obtained from the hospital. In this case the linear regression equation was even better with y = -0.238 + 0.988x with a correlation coefficient of 0.998. It can be seen that concentrations determined by the enzyme sensor are in good agreement with the other two methods.

The enzyme sensor system when compares to the method using diacetyl monoxime is much more simpler since it requires less procedures, fewer reagents and using less time, 5-13 min and 25-30 min for the enzyme sensor and diacetyl monoxime respectively. On the other hand the automated coupled-enzyme method used by Songklanagarind Hospital, Prince of Songkla University, Hat Yai, requires about the same length of time for each analysis as the enzyme sensor. However, the former requires more reagents that includes two enzymes, urease and glutamate dehydrogenase, as well as 2α-oxoglurate and NADH. The reusability of the enzyme reactor in our system also helps to reduce the cost of the expensive enzyme reagent. In our earlier report[24] the enzyme reactor was tested and found that good linear responses were still obtained after one and a half months, or more than 60 h operation time. Since the average analysis time per pulse is 9 min this means that about 400 samples can be analysed using just one reactor. If longer operation time is needed the column may still be sufficient, otherwise larger amounts of immobilized urease can extend the operational life. Replacement of columns in this system can also be done easily.

Conclusion

The experiments reported here show that it is possible to use conductivity electrodes in conjunction with an enzyme based analysis. It is clear from the results that this system could be used for the determination of urea concentration with accuracy. The simple buffer reagent and the use of immobilized enzyme makes it more economical than the colorimetric or the automated systems.

Acknowledgements

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