Determination of Urea in Serum by Using Naturally Immobilized Urease in a Flow Injection Conductimetric System

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A flow injection method was developed, aimed at the determination of urea in human serum. The system makes use of the naturally immobilized urease present in *Canavalia ensiformis* DC (jack bean). A column is filled with small pieces of this bean, and the sample (50 μl) containing urea passes through it carried by a 1% NaCl solution. On leaving the column the stream is merged with an alkaline reagent (0.5 mol dm^{-3} NaOH; 0.5% disodium dihydrogen ethylenediaminetetraacetate). The ammonium ions, arising from the enzymatic reaction that occurs inside the column, are changed into the molecular form, which permeates a polytetrafluoroethylene membrane and is received in a de-ionized water acceptor stream. The ammonia ionizes causing an increase in the conductance, which is proportional to the urea content of the sample. About 40 samples can be processed in 1 h with negligible carry-over and with a relative standard deviation of 1% or less. The results are in agreement with those obtained by a standard spectrophotometric method.

Keywords: *Serum urea determination; naturally immobilized urease; flow injection*

Several papers describing artificial urease-immobilization processes and the construction of urea sensors have been published.[1–4] Most of them were aimed at the development of potentiometric sensors to be used in batch procedures for the determination of urea. Artificial enzyme immobilization has also been pointed out to be one of the most suitable approaches to automating biochemical reactions in flow injection (FI) systems.[5,6] However, it has been demonstrated that, sometimes, it is possible to make use of the raw material containing the enzyme, naturally immobilized inside vegetable cells, in the construction of a biosensor. Wang and Lin[7] described a biosensor that was developed, from the natural occurrence of polyphenol oxidase in banana, to provide the enzyme source in the determination of dopamine. Plant tissues have also been used for constructing electrochemical sensors for glutamate,[8] phosphate and fluoride[9] and tyrosine.[10] Meal, obtained from the jack bean (*Canavalis ensiformis* DC), was used in the construction of a potentiometric biosensor for urea,[11] and good results were obtained in terms of stability and detection limits.

The use of naturally immobilized enzymes is very attractive as no immobilization process is required. However, the enzyme should exhibit sufficient activity, and the conditions under which the determination is made should allow a rapid transport of both the substratum to the inside of the cell and of the products to be detected outside of the cell membrane. The diffusion through the cell membrane is the rate-limiting factor for an FI system that is based on the use of natural immobilization.

This paper describes an FI system developed for the determination of urea in human serum by using a methodology that employs the naturally immobilized urease present in the jack bean. The ammonium ion originating in the enzymic hydrolysis of urea is detected by using the conductimetric methodology previously described.[12]

Experimental

Apparatus

The same instruments, conductimetric flow cell and polytetrafluoroethylene membrane previously described were used.[12] The diffusion cell was modified and had its dimensions enlarged to contain a shallow groove 0.5 mm deep, 4 mm wide and 10 cm long.

The FI conductimetric manifold used for the determination of urea is outlined in Fig. 1. Column C_1 was filled with a mixed-bed resin, to effect a final purification of the de-ionized water, and was constructed as previously described.[12] Polyethylene tubing (0.8 mm i.d.) was used throughout. All the experiments were performed at ambient laboratory temperature (near 25 °C).

Column C_2, in Fig. 1, was made from a Tygon tube (2.5 mm i.d.) and filled with small pieces of bean. These pieces were directly cut from a grain of the bean, from which the skin had been removed, and had the form of small cubes with edges nearly 2 mm long. Two small plugs of glass wool were used to retain the bean pieces inside the column. A 5 cm long column contained between 15 and 20 pieces, with an average total mass of 0.13 g. The column was conditioned by passing a 1% NaCl solution (flow-rate: 1.0 ml min^{-1}) for 15 min. When not in use the column should be kept empty and stored in a refrigerator.

For comparison purposes an FI manifold was assembled to work with the free urease reagent solution. This system is the same as that shown in Fig. 1, but has the bean column replaced by a 50 cm reaction coil made from a polyethylene tube (0.8 mm i.d).

Reagents

Standard solutions of urea were prepared daily by suitable dilution of a 1000 μg ml^{-1} stock standard solution. Carbonate-free sodium hydroxide solutions were made by dilution of approximately 12 mol dm^{-3} sodium hydroxide prepared with

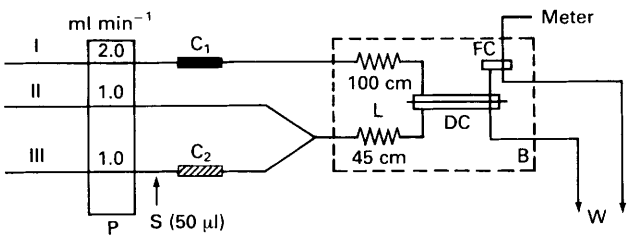

Fig. 1 Flow injection manifold for the determination of urea in serum using naturally immobilized urease and conductimetric determination. P, Peristaltic pump; S, sample; C_1, mixed bed ion-exchange column; C_2, bean tissue column; L, thermal equilibration coil; B, water-isolated bath; DC, diffusion cell; FC, conductimetric flow cell; and W, fluid discharge. I, De-ionized water; II, alkaline reagent: 0.5 mol dm^{-3} NaOH, 0.5% Na_2EDTA; and III, sample carrier fluid

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freshly boiled de-ionized water. Disodium dihydrogen ethylenediaminetetraacetate (Na_2EDTA) (0.05%) was used in the reagent solution.

Free urease solutions were prepared by dissolving 0.30 g of the Merck product (83.35 nkat mg^{-1}) in 5 ml of water and adjusting the volume to 500 ml with 1% NaCl solution. Portions (20 ml) of this solution were placed in 100 ml calibrated flasks and the volume of each was adjusted with a suitable 0.02 mol dm^{-3} tris(hydroxymethyl)amino methane (Tris)–HCl buffer containing 1% of NaCl. The pH of these solutions was measured by a standard pH-measurement procedure using a calibrated glass electrode. All other Tris–HCl buffer solutions were 0.02 mol dm^{-3} and contained 1% of NaCl.

Standard solutions of urea, containing various amounts of the urease inhibitors F^- and HSO_3^-, were prepared from stock 0.100 mol dm^{-3} NaF and $NaHSO_3$ solutions. Analytical-reagent grade solutions and freshly prepared de-ionized water were used throughout.

Serum Samples

Human serum samples were obtained from the Clinical Hospital of the State University of Campinas by a single centrifugation of the whole blood. All samples were analysed for urea in the hospital laboratory and by the proposed FI method on the same day. For the proposed method, 100 µl of serum were diluted to 100 ml with water in a calibrated flask.

Results and Discussion

Preliminary experimental data were obtained using a 5 cm column constructed as described under Experimental. The reagent solution was 0.5 mol dm^{-3} NaOH containing 0.5% m/v of EDTA. If no salt is present in the carrier stream the rate of urea hydrolysis is very low and barely detectable. Adding a salt such as NaCl or KCl to the carrier causes a marked increase in ammonium ion production and a decrease in the sample washing-out time. Fig. 2 shows the dependence of the FI peak height on the concentration of NaCl in the carrier stream. The same behaviour and quantitative increase in ammonia production was observed for KCl.

Additional experiments were carried out on the evaluation of the effect of the column length and on the reproducibility among columns. Fig. 3 shows how the length of the column affects the hydrolysis of the standard urea solutions. The results for urea are compared with those obtained for standard ammonia solutions prepared to contain an equivalent amount of nitrogen. The comparison shows that the hydrolysis yields are about 70 and 95% for a 3 and a 5 cm long column, respectively. Use of longer columns also increases the wash time interval, thereby reducing the sample-processing capability of the system.

Five columns (each 5 cm long) were prepared as described under Experimental, and the conductimetric signals obtained for each, using standard urea solutions in the concentration range 1–5 µg ml^{-1}, were compared. A maximum relative difference of 5% was observed among signal peak heights.

The effect of the concentration of sodium hydroxide in the reagent stream was investigated. The results showed that the signals for a standard solution containing 1–5 µg ml^{-1} of urea were slightly affected by changing the hydroxide concentration from 0.1 to 1.0 mol dm^{-3}.

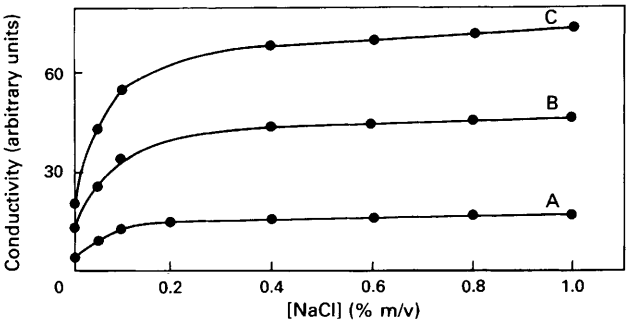

Fig. 2 Effect of the concentration of salt in the carrier stream on the analytical signal. Urea concentration: A, 1.0; B, 3.0; and C, 5.0 µg ml^{-1}

Effect of pH and Inhibitors

By using a 3 cm long column of beans, the effect of changing the pH of the carrier stream and of two urease inhibitors (F^- and HSO_3^-) was investigated. The results were compared with those obtained by using a solution of free urease as carrier in the manifold described under Experimental. The system employing free urease reagent showed a sensitivity that was about one-tenth of that employing the bean column. Therefore, the comparison was made with solutions that contained 3 µg ml^{-1} of urea for the column system and 30 µg ml^{-1} for that employing the free urease. No effort was made to optimize the free urease system as only relative results were required.

Fig. 4 shows how the pH of the carrier stream affects the peak height for both the systems. It can be observed that the free enzyme is more affected by the activity of the hydrogen ion in solution. The pH was previously reported as a critical parameter for a potentiometric biosensor constructed with use of a meal of jack bean[11] and for an artificially immobilized-urease potentiometric sensor.[13]

Fig. 5 shows the effect of the presence of F^- and HSO_3^- in the sample solution on both free and naturally immobilized-enzyme FI systems. In the absence of pH control the effect on the free enzyme is critical while the naturally immobilized enzyme is only slightly affected. The total effect on the inhibition of the enzyme activity comes from the presence of

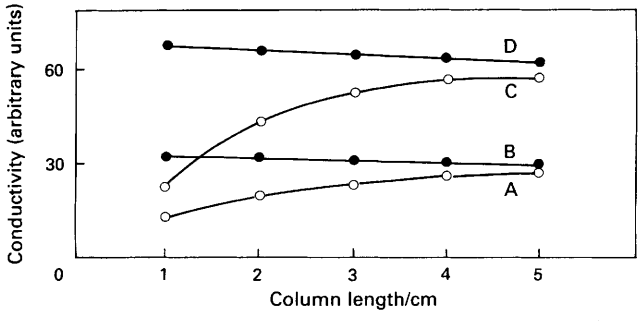

Fig. 3 Effect of the column length on the production of ammonium. ●, Ammonium standard solutions; and ○, urea standard solutions. Nitrogen concentration of the NH_4Cl and urea solutions: A and B, 1.0; and C and D, 2.0 µg ml^{-1}. Carrier solution, 1% NaCl

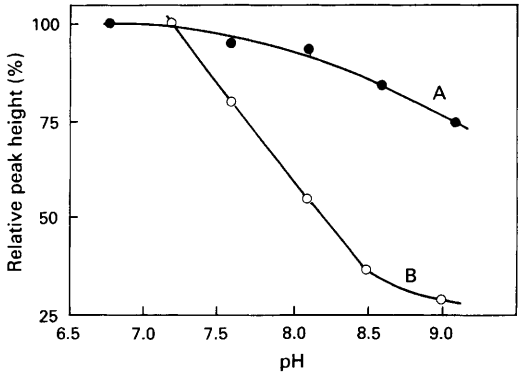

Fig. 4 Effect of the pH of the carrier solution on the analytical signal: A, using naturally immobilized urease (urea concentration of the test solution, 3 µg ml^{-1}); and B, using a solution of free urease (urea concentration, 30 µg ml^{-1})

the inhibitor itself and from the change it causes in the pH of the sample solution. It is worthwhile pointing out that the ratio of inhibitor to substratum is ten times greater when the bean column is employed as the urea concentration is ten times lower than that used in the free urease experiment. If a Tris–HCl buffer of pH 7.5 is used the inhibitor effect is cancelled for the naturally immobilized-enzyme system while it is still present in the free-enzyme system.

Although the response mechanism for the plant or animal tissue biosensors has not yet been well established[14] the results obtained in this work suggest that the cell integrity is maintained for the pieces of bean. The presence of a salt such as NaCl or KCl, frequently neglected when biosensor methodologies are developed, is essential to promote the rapid transport of the substratum to the inside of the cell where the enzyme-catalysed reaction occurs. The cell membrane acts as a selective filter, which is not permeable to some substances such as the inhibitors reported here, hence making the method less prone to interference. Also, the pH of the sample does not represent a critical parameter when the bean column is used as the urease source. To an extent, this is also a consequence of the fact that the reaction will occur inside the cell where the pH should be appropriate for the enzyme action. Furthermore, the conductimetric FI methodology described here does not require the enzymic reaction to occur at the same pH as that at which the detection is made, as is required for potentiometric sensors used in batch procedures.

Conditions for Determination of Urea in Serum

Based on the above results, the conditions for the determination of urea in serum were selected. A 3 cm long column was chosen along with a carrier solution containing 1% m/v of NaCl and a reagent solution, 0.5 mol dm^{-3} in NaOH, containing 0.5% m/v of Na_2EDTA. No buffer solution need be used owing to the high sample dilution employed. A sample volume of 50 μl was injected. A calibration run followed by signals obtained for some samples is shown in Fig. 6. Calibration runs obtained under these conditions showed a linear dependence of the peak height of the conductimetric signal in relation to the urea concentration in the range 1–10 μg ml^{-1}, with a typical correlation coefficient of 0.9997. The relative standard deviation for the analysis of ten replicates of a standard solution containing 3.0 μg ml^{-1} of urea was found to be 0.8%. About 40 samples could be processed in 1 h.

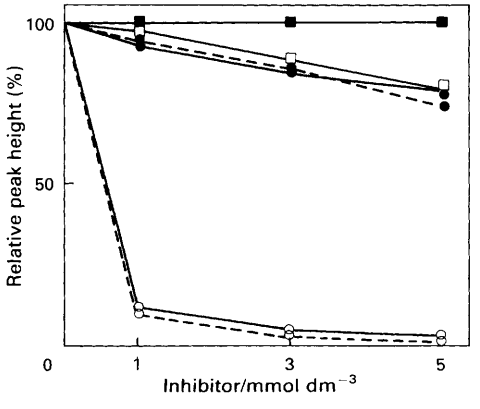

Fig. 5 Effect of the presence of F^- and HSO_3^- in the urea sample solution on the analytical signal. ● and ■, Naturally immobilized urease; and ○ and □, free enzyme reagent solution. Solid line, F^- added to the sample; and broken line, HSO_3^- added to the sample. Square symbols used when the pH was kept at 7.5 using Tris–HCl buffer. Urea concentrations were 3 μg ml^{-1} for the naturally immobilized urease and 30 μg ml^{-1} for the free enzyme solution system, respectively. All carrier solutions contained 1% NaCl

Column Lifetime

A 3 cm long column, prepared as described under Experimental, was fitted in the FI manifold and its long-term performance was evaluated for 10 h, injecting about 350 standard solutions containing 2 or 5 μg ml^{-1} of urea. The results show that, after this period, the activity of the column is reduced by about 10% in relation to its initial value. The rate of change in the column activity is slow. Therefore, periodical re-calibration can ensure good accuracy. In routine determinations a calibration involving use of three standard urea solutions was repeated every 30 min. The same column could be used to perform up to 1000 determinations, although it was found preferable to replace it every day in view of the low cost and ease of construction.

Accuracy of the Proposed Method

Sixty-five samples of human serum were analysed by the proposed method and by a spectrophotometric–enzymic method based on the reaction of the ammonium ion, produced in the hydrolysis of urea, with 2-oxoglutarate and reduced nicotinamide adenine dinucleotide (NADH) in the presence of glutamate dehydrogenase.[15] The decrease in the NADH concentration was monitored in the ultraviolet region. The method was performed in a Cobas Mira automatic analyser. Fig. 7 shows a comparison between the results obtained by the two methods. Least-squares statistical results show that the correlation between the two methods can be expressed as:

$$c_p = (0.47 \pm 0.31) + (0.9942 \pm 0.0237)\, c_s$$

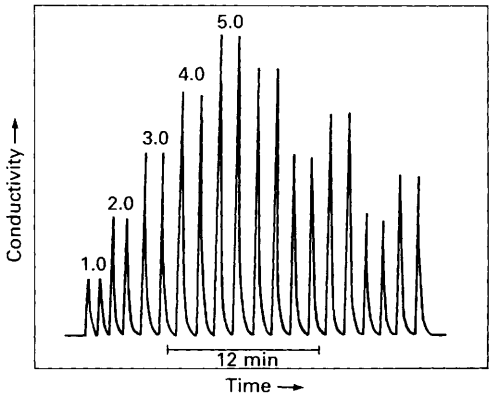

Fig. 6 Typical calibration run for serum urea determination using the naturally immobilized enzyme FI system. The signals for five standard solutions and for the five samples, introduced in duplicate, are shown. The numbers above the peaks are the urea standard solution concentrations in μg ml^{-1}

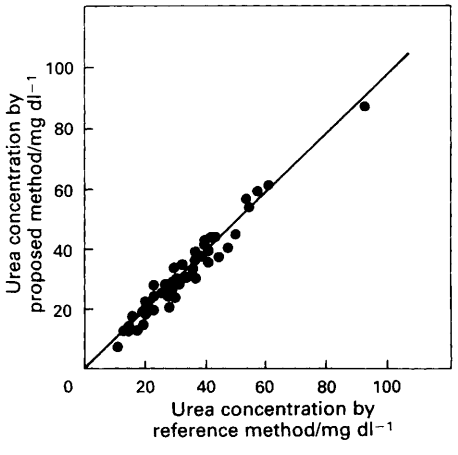

Fig. 7 Correlation of the results for 65 serum urea concentrations found by the proposed method and by a standard spectrophotometric method

where c_p is the urea concentration (mg dl^{-1}) found by the proposed method and c_s is the urea concentration (mg dl^{-1}) found by the reference spectrophotometric method. The correlation coefficient is 0.987 and the error of the estimate is ± 2.4 mg dl^{-1}. These results lead to the conclusion that the proposed method compares well with the conventional spectrophotometric method. The sensitivity of the conductimetric method permits a high sample dilution that helps in overcoming matrix effects. The method also presents advantages in relation to the cost and demonstrates that the naturally immobilized enzyme can be used to replace artificial immobilization in FI reactors when the raw material presents sufficient activity.

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