Flow Injection Analysis of Blood Urea in Combination with a Trinitrate Cellulose Based Mini Enzyme Reactor and a Modified Potentiometric Ammonium Ion Transducer

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Summary: A flow injection analysis method for blood urea has been described in combination with a modified poly (vinyl chloride)-nonaction potentiometric ammonium ion transducer. The urease enzyme has been effectively immobilized on a 5 mm trinitrate cellulose membrane by covalent cross linking. Reusability, stability and durability regarding the activity of the immobilized enzyme has been successfully demonstrated. It has been proposed that such membrane filters can be an effective base material in comparison to a column type enzyme reactor for immobilization of enzyme due to their large available hollow space.

Introduction

Analysis of body fluids for various non-ionic bioproducts is important regarding diagnosis of various diseases [1]. One of these bioproducts is urea, whose concentration in blood alongwith creatinine is related to renal function [2-5]. On the other hand, urea concentration in water reservoirs is also monitored from the environmental point of view [6]. In general the ascending tendency of nitrogenous material is considered as a depletion of oxygen concerning marine life [7]. Urea can be determined either using optical methods or electrochemical techniques [8].

A fiber optic urea biosensor based on the ammonium ion measurements has been presented. However, it lacks the required long term stability [9]. Fluorescence detection based urea biosensors have also been reported [10,11]. An enzyme urea biosensor based on an ammonium ions selective bulk optode membrane has been described. However, it suffers from lengthy response time and control of sample pH is also required [12]. Among the electrochemical methods, particularly potentiometry has been used extensively because of its simple instrumentation, low cost and considering its merits and demerits regarding matrix effects [13]. The potentiometric assay of urea involves either the monitoring of pH [14] or ammonium ions [15] after the bio-reaction of substrate and biocatalyst (urease) under the optimum conditions. The monitoring of pH is mostly carried out with glass membrane based pH electrodes. The certain disadvantages associated with glass pH transducer are buffer capacity of the measuring buffer, shorter linear analytical range and slow diffusion of hydrogen ions through proteins [16]. An alternative to pH type transducers is, polymer membrane based potentiometric ammonium ions transducer. It offers advantages of easy and cost effective self manufacturing, and comparatively larger linear analytical range. In our previous communication a price worthy self prepared modified poly vinyl chloride nonaction (PVC-nonactin) ammonium sensitive electrode has been reported for the fabrication of a combination type biosensor for the assay of urea in blood samples [17].

The application of this modified ammonium ions transducer is now being extended to the flow injection analysis (FIA) of urea by incorporating a mini urease enzyme reactor. The urease has been immobilized on trinitrate cellulose membrane filter. The efficiency of the FIA is comparable with the results of earlier reported enzyme electrode and photometric diacetyl methods.

Results and Discussion

Flow Injection Analysis of Urea

The system was conditioned for half an hour with ground electrolyte. A urea concentration of $1x10^{-5}$M was opted for the optimization of experimental conditions. The tested flow rate of ground electrolyte were 0.38, 0.47 and 0.67 ml/ minute. Among these 0.38 ml/ min. was found most suitable. Here the system exhibited minimum drift, improved sensitivity and response time, better

reproducibility and minimum noise in the background potential. This optimum performance may be due to the provided sufficient contact time between the substrate and enzyme. For higher flow rates, although the response time was shorter, however, sensitivity was reasonably reduced and splited peaks signals were encountered as well. Reduction in response signal at high flow rates may be because of inefficient substrate enzyme interaction and splitting of signals could be related to the uneven flow of the substrate in the flowing stream. The reproducible potentiometric responses of the mini enzyme reactor to varied standard urea concentration are shown in Fig. 3. The Nernstian slope of the FIA system is 38 mV/ decade urea concentration. The linear analytical range of the calibration curve is $4x10^{-5}$ to $1x10^{-3}$M. Because of the small cell volume, the combined efficiency of the transducer and mini enzyme reactor is good for the 50 µl injected urea standard. The linearity of the calibration curve for higher urea concentration can be extended by incorporating additional enzyme activity. The results of analyses performed on the known urea concentration solutions using calibration curve method are summarized in Table-1. In general, the system is capable of analyzing 40 samples per hour when urea contents are in the linear analytical range.

Table-1: Flow injection analysis of standard urea solutions with mini enzyme reactor, at a flow rate 0.38 ml/min.

	Urea concentration [mole/L]		Relative error [%]
	Standard	Found	
1.	$1x10^{-6}$	$1.3x10^{-6}$	+30
2.	$5x10^{-5}$	$5.5x10^{-5}$	+10.6
3.	$6x10^{-5}$	$6.2x10^{-5}$	+4.2
4.	$8x10^{-5}$	$7.9x10^{-5}$	-1.4
5.	$1x10^{-4}$	$1.02x10^{-4}$	+2

The relative percentage error tends to decrease from 30 % to 2 % as the urea concentration was increased from 10^{-6} to 10^{-4}M. It indicates the relative error tends to improve as the urea concentration reaches the levels as it is normally in blood samples $5x10^{-3}$M [5]. The high levels of uncertainties at low urea concentration are due to the nonlinear response of the reactor. In addition to that, lack of reproducibility was also observed at low urea concentration. The enzyme reactor functioned almost for four weeks without any appreciable change in its efficiency.

Analysis of Urea in Human Blood

The reliability of the developed FIA system was evaluated by analyzing blood urea in blood samples collected from various volunteers. The blood plasma samples were obtained from whole blood samples by centrifugal process at 4 °C. The samples were analysed within 12 hours after their collection and were kept under refrigeration at 0 °C when not in use. Pre-determination of ammonium ions in these samples was carried out with a self developed combination type PVC-nonactin based potentiometric ammonia gas sensor [30]. A sample volume of 0.5ml was employed for the analysis. The average concentration of NH_4^+ in these blood samples was 40µ mole/L. This concentration is comparable to the literature value of 20 to 100μ mole/L [31].

Blood plasma samples were diluted ten times with ground electrolyte because of their viscous nature. This helped in injecting reproducible volumes, lowered the amount of the accompanied proteins and brought the substrate concentration in the linear analytical range as well. These conditions provided optimum interaction between urea and enzyme. In addition to these, dilution brought the endogenous NH_4^+ concentration to approximate 4µ mole/L and reduced its interference at reasonable level. The results of analyzed human blood plasma samples are summarized in Table-2. The samples were also analyzed with self developed potentiometric urea biosensor (static method) [17] and diacetyl photometric techniques [32]. It can be seen that the reported results are fairly in agreement. The reproducibility of FIA is relatively better than urea biosensor because of a stable baseline potential achieved for the earlier. Whereas in case of urea biosensor the ammonia diffused through the gas membrane, sometimes reaches beyond the inner thin buffer film between gas and PVC-nonactin membranes which affect the baseline potential. Moreover, a slight positive trend in FIA values regarding urea concentration in blood samples with respect to urea biosensor may be because of K^+ ions interference. It may be mentioned here that a detail ion exchange chromatography based investigation for the removal of ammonium and potassium ions from urea containing samples has been optimized, which will be presented in a subsequent communication.

Table-2:Comparison of blood plasma urea analysis (mg/100 ml) using different techniques

Sample	FIA[α]	Urea biosensor[α]	Photometric[α]
1.	19.2±0.5	18.7±0.8	21.3±1
2.	22.1 ±0 4	21.3±0.7	22.3±0.8
3	28 .0 ±0.5	27.1±1.2	29.1±0.5
4.	34.8 ±0.8	34.1±1.8	34.7±1
5.	26.2 ±0.4	25.1±0.1	26.4±0.7

α:Average of three measurements.

Experimental

Instrument

A Knick pH-meter type 510 and a strip recorder SE 110, Gorz, Metrawatt, Germany was used for potential measurements. Injector and flow through cell of plexi glass were self machined. A Gilson Puls 2 peristaltic pump was employed for delivering grond electrolyte 0.05 M Tris-HCl buffer of pH 7.5. Sample and standard were injected with 250 μl injecting syring. Alternating current Impedance spectroscopy was carried out using Princeton Applied Research Potentiostat/Galvanostat 273 EG&G, USA and a Solartron, Schlumberger 1255 HF frequency response analyzer, Germany in combination with an IBM comptible computer and impedance software M 388 from EG&G. The pH-electrode was an Orion combination type USA. A variable volume micro pipette of 1-10 μl capacity was of Eppendorf, Germany. All the measurements were carried out at room temperature (23±2) °C.

Reagents

Nonactin, dibutylsebacate ester, urea (ACS), Tri[hydroxymethyl] amino methane and urease type V11 (E.C. 3.5.1.5.) were products of Sigma, USA. Tetrahydrofuran was of Riedel de Haen, Germany. High molecular weight PVC was a product of EGA-Chemie. A 0.5 mm diameter silver wire was purchased from Aldrich, USA. Tetraheptyl-ammonium tetraphenylborate was prepared as decribed in the literature [18]. Buffers and required urea solutions were prepared biweekly and were kept under refrigeration at 4 °C, when not in use. The trinitrate cellulose membrane filter was a product of Sartorious, Germany.

The Modified Potentiometric Ammonium Ions Transducer

The details regarding the casting/fabrication of modified ammonium ions transducer are described [17] elsewhere. However, a brief introduction of its potentiometric behavior will be outlined here. The preliminary potentiometric response of the sensor made of PVC pipe (4Φ x 70) mm was measured in a 20ml of 0.05 M Tris-HCl buffer of pH 7.5 (measuring buffer). The reference electrode was Ag/AgCl with double jacket arrangement. The Nernst slope and the linear analytical range was 59 mV/decade NH_4^+ ions concentration and $4x10^{-6}$ to $6x10^{-3}$M NH_4^+ ions, respectively. The early potentiometric measurements were carried out under proper Faraday shielding to overcome the problem of encountered high background noise. However, the modification of the sensing membrane by the incorporation of lipophilic salt has almost eliminated the background noise and shielding was no more required. The presence of lipophilic salt did not significantly alter the earlier potentiometric characteristics of sensing membrane. The impedance of membranes without and with lipophilic salt was 500 and 30 KΩ, respectively.

Enzyme Immobilization

Various types of polymers are being utilized as base material for the immobilization of urease [19]. Among these are poly(vinyl alcohol) [20], poly (propalene) [21], poly (urethane) [22] and poly (N-vinyl-2-pyrolidone) [23]. Chitosan membrane has also been utilized for the covalent bonding of urease [24]. Incorporation of urease in conductive polymere polypyrrole by galvanostatic film formation has been carried out for FIA [25]. The in-situ enzyme entrapment from gel solutions of triacetate cellulose (TAC), trinitrate cellulose (TNC), ethyl cellulose and poly (vinylchloride) has also been proposed [26]. Here the enzyme containing gel was physically coated on the sensitive surface of the glass electrode and the response time and sensitivity parameters were optimized. The effective use of TAC and TNC as supporting material has been demonstrated.

The Enzyme Immobilization on TNC Membrane

TNC is also available as a membrane filter with varied porosity. Such a membrane filter is made of 80 percent hollow space and its structure is sponge like [27]. However, unlike sponge, membrane filter has pressure resistance, a rigid structure and it behaves like a multi layered sieved with tight and uniform mesh hole. On these grounds the membrane filters are being used successfully for residual analysis of microorganism. Having these physical characters, such membranes can be a good supporting media for the cross liking immobilization of enzyme. Due to the large available internal space, the cross-linking could be multidirectional. By this it means, the additional active sites are also available for substrate-enzyme interaction as compared to the planer film structure of immobilization. Moreover, such an immobilization is compact and involves less enzyme activity as compared to column type enzyme reactor [6]. Considering these parameters, a 5mm

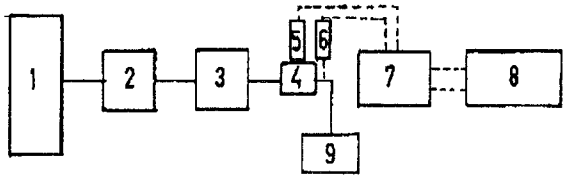


Fig. 1 Block diagram of flow injection analysis system

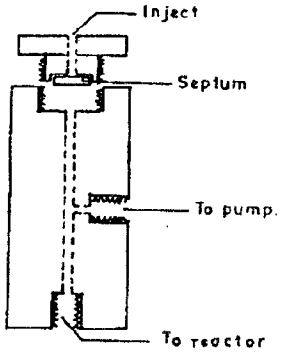
1. Ground electrolyte, 2. Peristaltic pump,

3. Injector, 4. Flow through cell,

5. Ammonium sensitive electrode,

6. Ag/AgCl electrode, 7. pH meter,

8. Recorder, 9. Adopter.



Injector

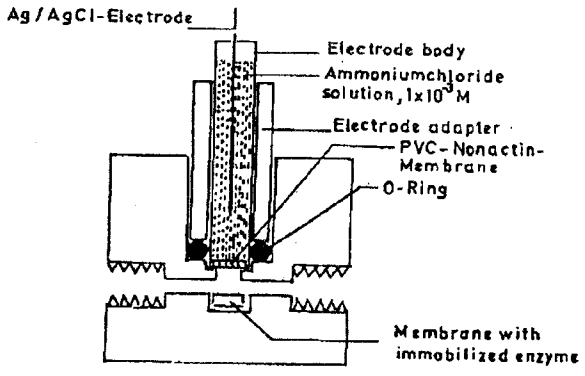


Fig. 2: The injector and cell for flow injection analysis of Urea.

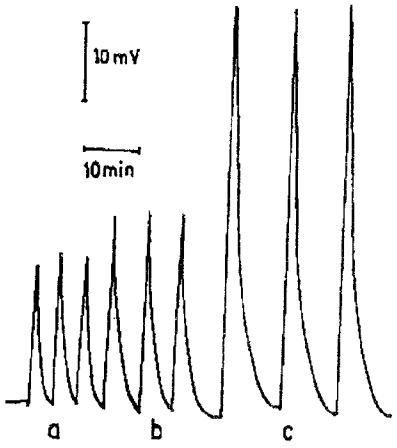


Fig. 3. F.I.A. of urea in combination with a mini enzyme reactor Enzyme immobilized on a trinitrate cellulose membrane by chemical cross-linking Urea standard solution. a. ($1x10^{-6}$M) b. ($1x10^{-5}$M) c.($1x10^{-4}$M) Buffer: 0.05M Tris-HCl, pH 7.5, Flow rate: 0.38ml/min.

diameter TNC based membrane filter has been utilized as a solid support for the immobilization of urease by chemical cross-linking procedure. The method involves the reaction of bovine serum albumin (BSA) with glutardialdehyde in the presence of enzyme. 150mg of BSA was dissolved in 1ml of 0.2 M Tris-HCl buffer of pH 7.5. Seventy µl of this BSA-enzyme solution containing 210 units of urease was transferred on the 5 mm TNC membrane. Three microliter of 25 % glutardialdehyde was mixed with a BSA-enzyme solution using a close end of a capillary tube within one minute. Thereafter, the material becomes hard to be mixed. The mixture was allowed to react for 30 minutes. The end product was a tough and light brown polymer film and it strongly adhered to support. The enzyme containing membrane was thoroughly washed with distilled water and was kept in 0.1 M glycerin solution for half an hour to remove any left over unreacted glutardialdehyde.

Experimental Procedure for FIASystem

The FIA system used is described in Fig. 1. The injector port and the flow though cell of plexi glass were self fabricated. The enzyme containing membrane was placed in the cell right in the stream of flowing ground electrolyte, as shown in Fig. 2. Innner part of the cell was properly engraved for the

membrane fixing. Ammonium ions sensor was positioned near by the enzyme containing membrane. The approximate created cell volume between these membranes was 0.01 ml. The connection between various parts of the system was made with Tygon tubing.

Urea in the proposed system is assayed indirectly by measuring ammonium ions, produced after the reaction of urea with urease. Considering this aspect, system performance was evaluated first with standard ammonium solutions. Moreover, such a preliminary FIA investigation was also a necessity considering ammonium ions interference in case if it is present in the actual samples to be analyzed. The ground electrolyte 0.05 M Tris-HCl pH 7.5 was purged with pure nitrogen for 15 minutes to avoid formation of gas bubbles and to attain stable baseline potential. The FIA system for NH_4^+ analysis exhibited a Nernstian slope of 57mV/decade ammonium ions concentration and a drift of 3mV/hour. In an earlier static (batch) study, Li^+ and Na^+ ions, each having a concentration of 10^{-3} M did not interfere. The log of selectivity coefficients for Li^+ and Na^+ ions were –3.8 and –3.12 respectively [28] using mixed solution method [29]. However, log of K^+ selectivity coefficient and its Nernstian slop was –1.6 and 52 mV/ decade K^+ concentration respectively.

Conclusions

The proposed enzyme immobilization method is simple and efficient as regards its efficiency and life time workability. The immobilized enzyme activity is much lower than the column type enzyme reactor. Moreover, the sensitivity for urea in blood samples is attractive. The minimum drift and background noise of the FIA system are additional advantages for improved accuracy and precision

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