Flow Injection Analysis (FIA) Technique As a method for Indirect Determination of Urea Quantity in Human Blood Serum

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Abstract

A new method for the determination of Urea in human blood serum based on Flow Injection Analysis (FIA) is described. This method is relay on the decrease in the reduction peak height (Hp) of Sodium nitroprusside $Na_2[Fe(CN)_5NO].2H_2O$ which appeared at (-0.55 V) vs. (Ag/AgCl, Sat. KCl) using phosphate buffer (pH=6.8) as a supporting electrolyte and carrier through the enzymatic reaction in the presence of Urease. The method is sensitive and rapid and the procedure was successfully applied to determination of Urea quantity in various human blood serum samples represent different cases such as: Dehydration (for Children), Heart failure, Liver failure and Kidney failure (for Adults).

The results for FIA method have been compared with those obtained from the colorimetric method for (45) samples and show a good agreement between the two methods with correlation coefficient (r = 0.99997).

الخلاصة

يتضمن البحث طريقة جديدة لتقدير كمية اليوريا (Urea) في مصل الدم البشري باستخدام تقنية التحليل بالحقن الجرياني (FIA)، حيث تعتمد هذه الطريقة على قياس الانخفاض في ارتفاع قمة اختزال (Hp) مادة نايتروبرووسيد الصوديوم $Na_2[Fe(CN)_5NO].2H_2O$ المستهلك خلال التفاعل الانزيمي لليوريا. وقد تم تطبيق الطريقة المقترحة لقياس كمية اليوريا في (45) عينة من عينات مصل الدم لاشخاص اصحاء وكذلك المصابين في حالات مرضية مختلفة مثل:- الجفاف (عند الاطفال) ، اخفاق عضلة القلب ، التهاب الكبد والفشل الكلوي (عند الكبار). وكذلك تم مقارنة النتائج المستحصلة من هذه الطريقة المقترحة مع الطريقة اللونية وكانت العلاقة خطية بمعامل ارتباط (r = 0.99997).

Introduction

Urea is a common constituent of blood and other body fluids [(1)]. It is formed from ammonia in the kidney and liver. Ammonia is produced by the breakdown of protein during tissue metabolism. The conversion of ammonia to Urea, primarily in the liver, prevents ammonia toxicity. Urea is then excreted from the body in urine. Therefore, Urea is a compound containing nitrogen that occurs in the urine and other body fluids as a result of protein metabolism. [(2,3)]

The Blood Urea Nitrogen (BUN) test measures the level of Urea nitrogen in a sample of the patient's blood. The BUN level may be checked in order to assess or

monitor: The presence or progression of kidney or liver disease, Blockage of urine flow, Patients with kidney failure are sometimes disoriented and confused, Abnormal loss of water from the body (dehydration) and recovery from severe burns, the body uses larger than normal amounts of protein following serious burns. [4,5]

Normal BUN levels are (5-18) for children; (7-18) for adults; and (8-20) in the elderly [6,13]. BUN levels can be too low as well as too high. Low levels of BUN may indicate *overhydration*, *malnutrition , liver disease*. Low BUN may also occur in early *pregnancy*. High levels of BUN may indicate kidney disease or failure; blockage of the urinary tract by a kidney stone or tumor; a *heart attack* or congestive *heart failure*; *dehydration*; or bleeding in the digestive tract. High BUN levels can sometimes occur result from eating large amounts of protein-rich foods. A BUN level higher than (100 mg/dl) points to severe kidney damage. [4,7]

The flow injection analysis (FIA) technique was developed in the mid-1970's to automate wet chemistry assays. Automation is achieved by carrying out analyses in a flow system where a pump is used to continuously draw sample and reagent solutions into different lines or segments of plastic tubing, as well as push them forward through the system **[Fig. (1)]**. Precise aliquots of the sample solution are dispensed into the carrier stream by Syringe-load injection. Bringing together solutions from different lines in mixing tees, or including a reagent in the carrier stream enables seamless, automated reagent addition. By connecting a detector at the end of the sample's flow path, automated detection of the processed sample is ensured. [8-12]

The present paper describe a FIA method for the indirect determination (enzymatic) of the small quantity of Urea in different human blood serum samples repast different cases based on the decrease in the reduction peak height (Hp) of Sodium nitroprusside which appeared at (-0.55 V) vs. (Ag/AgCl, Sat. KCl) using phosphate buffer (pH=6.8) as a supporting electrolyte and carrier through the enzymatic reaction in the presence of Urease.

Experimental

Apparatus:

The experimental set-up (components of the Flow Injection Analysis System) as shown in **[Fig. (2)]** was constructed as simple flow injection system. The carrier stream **(C)** is pumped by pump **(P)** through the electrochemical cell then to detector **(D)** after which the stream is discharge to the waste. The sample is injected at position **(S)**, by means of a disposable plastic-syringe loaded injection, into the carrier stream where it is transported as a plug to the detector. The incoming solution impinges on the surface of the working electrode and the resulting signal was recorded by an (x-t) recorder. [13]

The detection system was a house-built flow-through cell has been constructed for FIA, **[Fig. (3)]**. The detection system constructed in two parts, the auxiliary electrode [1.5 mm diameter platinum (Pt wire)] and the working electrode [5 mm diameter Glassy Carbon (GC) electrode were placed in one part, whereas, the reference electrode [Sliver/Silver electrode, saturated potassium chloride (Ag/AgCl, Sat. KCl)] placed in the second part connected to the cell solution by a vycor ceramic frit. All electrodes were fitted into a Teflon body cell as it is a good insulator and easy to machine. (1mm) diameter inlet and outlet drilled into the body cell. The two parts of the cell were clamped together with three screws.

The pump is used to propel one or more streams through the detection system via narrow bore (0.3-0.8 mm internal

diameter) tubing. In this work the fluid is driven by using peristaltic pump (LKB type).

The injection unit used is made of Teflon. The unit involves three holes, one for carrier inlet, second for carrier outlet and third for sample injection. The sample was injected into the stream by means of plastic syringe-loaded injection.

Tubing material using to connect the units of the flow system was made of polytetrafluoroethane (PTFE) with a unique diameter of (0.4) mm of internal diameter. The flow system supplied by a buiret tap to prevent the disturbing of the sample zone during the sample injection.

All D.C. voltammetric measurements were performed using a potentiostat type (LB75) and potentiometer type (SMP72) supplied by Gerhard Bank Electronic, Germany, for supplying the required potential, peak current component was recorded using x-t Fisher recordall series 5000.

All colorimetric measurements were performed by using (Cecil Spectrophotometer) model (CE 10211 Ultra Violet & Visible Spectrohotometer) from Cecil Instruments Limited.

Preparing of Electrode Surface

To ensure reproducible results and low background current, (GC) electrode was polished using hand polishing with aluminum oxide coated paper (400 P mesh.), followed by fine polishing with aluminum oxide (0.3, 0.075 and 0.015 Mm) on a polishing cloth for about 10 min.

Chemicals & Reagents:

Reagent/R1: (buffer) *from* ***Fluitest® UREA, col. Urea Colorimetric, Endpoint Determination Urease - Berthelot Reaction (BD-511100-04) (BIOCON):***
phosphate buffer, pH 6.7 (60 mmol/l), EDTA (1.5 mmol/l), Sodium salicylate (60 mmol/l) & Sodium nitroprusside (5.2 mmol/l) in a total volume (100 ml).

Reagent/R2: (enzyme reagent) *from* ***Fluitest® UREA, col. Urea Colorimetric, Endpoint Determination Urease - Berthelot Reaction (BD-511100-04) (BIOCON):***
Urease (= 5000 U/l) in a total volume (100 ml).

Reagent/R3: (hypochloride solution) *from* ***Fluitest® UREA, col. Urea Colorimetric, Endpoint Determination Urease - Berthelot Reaction (BD-511100-04) (BIOCON):***
Sodium hypochloride (18 mmol/l) & (450 mmol/l) in a total volume (80 ml).

Reagent/R4: (standard) *from* ***Fluitest® UREA, col. Urea Colorimetric, Endpoint Determination Urease - Berthelot Reaction (BD-511100-04) (BIOCON):***
Urea 50mg/dl (8.235 mmol/l) in a total volume (5 ml).

Preparation of Working Solution
Working solution is prepared by addition 1 vial enzyme reagent/R2 to 1 bottle of buffer/R1. The working solution is stable for 4 weeks at (+2 to +8 ^{o}C) and 6 days at (+20 to +25^{o}C).

***Phosphate Buffer* (0.2 M) *at* (pH 6.8).**
Freshly prepared by dissolving 8.709 gm of K_2HPO_4 (0.2 M) and 6.804 gm of KH_2PO_4 (0.2 M) in a total volume (100 ml) of Distill water.

(Precision Multi-Sera Low Human)(Cat.No.UL2701)(RANDOX):
Reconstitute each vial of lypophilised serum with exactly (5 ml) of distilled water. then stand for (30 min.) out of bright light before use.

(Precision Multi-Sera Normal Human) (Cat. No. UN 1557) (RANDOX):
Reconstitute each vial of lypophilised serum with exactly (5 ml) of distilled water. then stand for (30 min.) out of bright light before use.

(Precision Multi-Sera Elevated Human) (Cat. No. UE 1558) (RANDOX):
Reconstitute each vial of lypophilised serum with exactly (5 ml) of distilled water. then stand for (30 min.) out of

bright light before use.

Specimen Collection and Preparation

Samples of human serum were obtained from routine clinical assays. Serum samples were prepared and assayed within (1 hr), otherwise the serum should be kept frozen.

FIA-Procedure:

The FIA-procedure depends upon measuring the reduction peak height of Sodium nitroprusside before and after addition of (5µl) of human blood serum sample to a solution containing (5 ml) of phosphate buffer (pH=6.8) and (500 µl) of working solution. Calculate the decrease in the reduction peak height of Sodium nitroprusside which indicates the Urea quantity in the human blood serum sample using the following equation:

The concentration of Urea in human blood serum $= \dfrac{\mathbf{Hp_{Blank} - Hp_{Sample}}}{\mathbf{Hp_{Blank} - Hp_{Standard}}} * \mathbf{n}$ **,** which in :-

Hp_{Blank}	Represent the value of peak height of Sodium nitroprusside before serum addition, the unit of **Hp_{Blank}** is (cm).
Hp_{Sample}	Represent the value of peak height of Sodium nitroprusside after serum addition, the unit of **Hp_{Sample}** is (cm).
$Hp_{Standard}$	Represent the value of peak height of Sodium nitroprusside after standard addition, the experimental value of **$Hp_{Standard}$** equal to **7.0 cm.**
n	Is the value of the standard concentration equal to **50 mg/dl.**

Colorimetric-Procedure:

Colorimetric method based on measuring the absorbance of sample against the blank at 540 nm wavelength. The instrument adjusted to zero by distilled water. The colorimetric procedure is shown in the following list :-

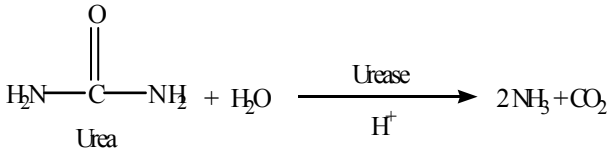
	Reagent blank	**Standard**	**Sample**
working solution	1000 µl	1000 µl	1000 µl
Standard / R4	----------	10 µl	----------
Sample	----------	----------	10 µl
Mix. and incubate at +25 ^{o}C for 10 minutes. Then add:			
Reagent / R3	200 µl	200 µl	200 µl
Mix. and incubate at +25 ^{o}C for 10 minutes. Then read the Absorbance.			

The concentration of Urea in human blood serum $= \dfrac{\mathbf{A_{Sample}}}{\mathbf{A_{Standard}}}$ **x n ,** which in :-

A_{Sample}	Is the value of the absorbance for each sample.
$A_{Standard}$	Is the value of the absorbance standard, the experimental value of **$A_{standard}$** equal to **0.704**
n	Is the value of the standard concentration equal to **50 mg/dl.**

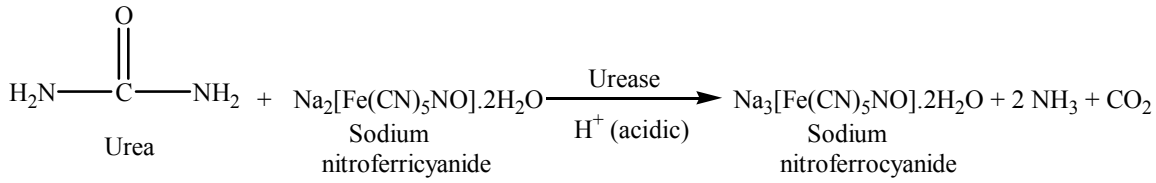
Results and Discussion

In enzymatic colorimetric method of Urea determination is based on the following reaction :



The ammonium ions formed react with salicylate and hypochloride to give a green dye (2,2 Dicarboxylindophenol). The normal values in serum that's dependent in the present work are (15-45 mg/dl). [14]

In Flow Injection Analysis (FIA) method, the principle of this method is based on the reduction process of Sodium nitroprusside (Sodium nitroferricyanide) on the surface of GC electrode when reacting with Urea that's found in serum in the presence of Urease to form Sodium nitroferrocyanide, Ammoina & Carbon dioxide in acidic medium (H^+), as shown in the following equation :-



From the above equation, Fe(III) is reduce to Fe(II) on the surface of GC electrode when reacting with Urea. Therefore we found that that Sodium nitroprusside gives a well defined reduction peak at (-0.55V) vs. (Ag/AgCl, Sat. KCl) in phosphate buffer at (pH = 6.8) as a carrier **[Fig. 4 (B)]** due to it's found in working solution.

The addition of human blood serum caused a decrease in the peak height of Sodium nitoprusside, as shown **[Fig. 4 (E)]**. The decrease is proportional to the quantity of Urea that's found in sample.

Optimum Conditions for FIA Measurements:

Effect of Applied Potential

In order to obtain the optimum required potential used for electrochemical reduction of sodium nitroprusside to determination the quantity of Urea. A set of DC-Voltammetric experiments were carried out using working solution and phosphate buffer [(pH = 6.8) as supporting and carrier]. Different applied potentials were employed from (-0.35 V down to -0.65 V) with decreasing intervals of (0.05 V) and the peak heights were recorded. The result indicates that ($E_{appl.}$= -0.55 V) represents the optimum potential for measurements obtained are shown in **Table (1)**.

Effect of Flow Rate

The effect of flow-rate on the peak current was studied by using (500 µl) of working solution into phosphate buffer (pH = 6.8), ($E_{appl.}$ = -0.55 V). Different flow-rates were used, The results obtained shown in **Table (2)**. The plot of peak height versus flow-rates is shown in **[Fig. (5)]**, the peak height increase with the increasing of flow-rate up to (3.5 ml/min.) then became approximately constant. During this work (3.5 ml/min.) was used.

Effect of Working Solution

Different amounts of working solution were added (100-800 µl), then the reduction peak was recorded for each solution. The results are shown in **Table (3)** which indicates that (500 µl) is the optimum choice for Urea determination due to the highest reduction peak obtained.

We can show the reduction peak of working solution in **[Fig. 4 (B)].**

Effect of (10^{-3}M) *Sodium Nitroprusside Solution:*

In order to determine the reduction peak that appeared at the reduction potential (-0.55 V) is related to the Sodium nitroprusside exactly. A series of experiments were carried out using (10^{-3}M) Sodium nitroprusside solution that preparing freshly which added in a range between (100-800µl) into phosphate buffer (pH = 6.8) as supporting and carrier. The results shows increasing in the Peak height of the Sodium nitroprusside at (-0.55 V). therefore the result for this experiment proved that the reduction peak is related to Sodium nitroprusside **Table (4)**. We can show the reduction peak of Sodium nitroprusside in **[Fig. 4 (C)].**

Effect of Standard(R4)

A serious of experiments were used for selecting the optimum amount of Standard Urea (R4) that used for determination of Urea in serum. Different amount of (R4) between (0.5-5.0 µl) were used. The results are shown in **Table (5)** which indicates that (5.0 µl) is suitable for Urea determination in serum. We can show the reduction peak of Standard Urea in **[Fig. 4 (D)].**

Clinical Applications

A comparison between the proposed FIA method and colorimetric was carried out using (45 Samples) which consist of : Normal and some diseases such as: Heart failure, Dehydration and Liver failure. The results obtained are shown in **Table (8)**.

The relation between the quantity of Urea in human blood serum measured by the two methods **[Fig. (6)]** gives a straight line with correlation coefficient (r = 0.99997), (RSQ= 0.99995) that's indicates the good agreement between the two methods. The relation between them can be represented by the following equation:

Conc. by FIA method = -1.2968 + [0.9997 * Conc. by Colorimetric method]

Quality Control

For accuracy and reproducibility control, we assayed Multi-Sera Low, Normal and Elevated by the two methods: FIA and Colorimetric methods. The results shown in **Table (6)** and could be shown in **[Fig. 4 (F) , (G) & (H)]**.

For ensure the accuracy of our purposed method (FIA method), we calculated each of : Related Standard Deviation (RSD %), Relative Error (Error %) and (Recovery %) to increase the accuracy of our purposed method, the results shown in **Table (7)**.

Table (1): Effect of applied potential on the reduction peak of Sodium nitroprusside using (GC) electrode.

$E_{appl.}$ (V)	-0.35	-0.40	-0.45	-0.50	**-0.55**	-0.60	-0.65
Peak height Hp (cm)	2.7	3.3	5.1	7.9	**10.6**	10.1	9.7

Table (2): Effect of Flow-Rate on the reduction peak of Sodium nitroprusside at E_{appli} = -0.55 V

Flow Rate (ml/min.)	Peak height Hp (cm)
0.5	8.6
1.0	8.9
1.5	9.1
2.0	9.3
2.5	9.5
3.0	9.7
3.5	**9.9**
4.0	9.8
4.5	9.6

Table (3): Effect of working solution amount on the reduction peak of Sodium nitroprusside at E_{appli} = -0.55 V

Addition of working solution (µl)	Peak height Hp (cm)
100	5.5
200	6.8
300	7.8
400	8.9
500	**9.6**
600	9.6
700	9.5
800	9.6

Table (4): Effect of Sodium nitroprusside amount on the reduction peak of Sodium nitroprusside at E_p = -0.55 V

Addition of (10^{-3}M) Sodium nitroprusside solution (µl)	Peak height Hp (cm)
100	5.0
200	6.4
300	7.2
400	8.5
500	9.0
600	9.4
700	9.7
800	10.0

Table (5): Effect of Standard amount on the reduction peak of Sodium nitroprusside at E_{appli} = -0.55 V

Addition of Standard R4 (µl)	Peak height Hp (cm)
1.0	8.0
2.0	7.4
3.0	6.8
4.0	5.7
5.0	**4.8**
6.0	4.7
7.0	4.8
8.0	4.7

Table (6): Show the Quality Control of Urea quantity by the two methods: [(FIA-method) and (Colorimetric method)].

Samples	Range (mg/dl)	Colorimetric Method (mg/dl)	FIA Method (mg/dl)
Low Human Sera	16.8 – 21.6	19.200	17.897
Normal Human Sera	33.0 – 42.0	37.800	36.491
Elevated Human Sera	99.0 – 134.0	116.000	114.668

Table (7): The statistical results for accuracy of purposed method (FIA method).

Samples	RSD %	Error %	Recovery %
Low Human Sera	± 0.01202	-0.01084	99.98916
Normal Human Sera	± 0.00806	-0.00810	99.99190
Elevated Human Sera	± 0.00371	-0.38568	99.61432

Table (8): The results obtained for determination of Urea in Normal and Abnormal cases using two methods: FIA method and Colorimetric method.

FIA method				*Colorimetric Method*	
Sample No.	**Hp $_{Blank}$ (cm)**	**Hp $_{Sample}$ (cm)**	**Conc. (mg/dl)**	**A $_{Sample}$**	**Conc. (mg/dl)**
1	9.0	0.3	**217.500**	3.072	**218.176**
2	9.0	1.0	**200.000**	2.840	**201.705**
3	9.0	1.1	**197.500**	2.798	**198.727**
4	9.0	1.2	**195.000**	2.764	**196.318**
5	9.0	1.7	**182.500**	2.576	**182.955**
6	9.0	2.0	**175.000**	2.492	**176.989**
7	9.0	2.1	**172.500**	2.442	**173.432**
8	9.0	2.2	**170.000**	2.418	**171.733**
9	9.0	2.4	**165.000**	2.348	**166.761**
10	9.0	2.5	**162.500**	2.306	**163.778**
11	9.0	2.6	**160.000**	2.270	**161.216**
12	9.0	2.7	**157.500**	2.232	**158.511**
13	9.0	2.9	**152.500**	2.164	**153.693**
14	9.0	3.0	**150.000**	2.136	**151.710**
15	9.0	3.1	**147.500**	2.094	**148.716**
16	9.0	3.3	**142.500**	2.026	**143.886**
17	9.0	3.5	**137.500**	1.956	**138.920**
18	9.0	3.6	**135.000**	1.928	**136.932**
19	9.0	3.7	**132.500**	1.888	**134.085**
20	9.0	3.8	**130.000**	1.850	**131.392**
21	9.0	4.0	**125.000**	1.780	**126.420**
22	9.0	4.2	**120.000**	1.710	**121.449**
23	9.0	4.4	**115.000**	1.638	**116.330**
24	9.0	4.7	**107.500**	1.534	**108.949**
25	9.0	4.9	**102.500**	1.464	**103.977**
26	9.0	5.0	**100.000**	1.430	**101.563**
27	9.0	5.1	**97.500**	1.380	**98.006**
28	9.0	5.3	**92.500**	1.310	**93.034**
29	9.0	5.4	**90.000**	1.286	**91.330**
30	9.0	5.7	**82.500**	1.200	**85.227**
31	9.0	6.0	**75.000**	1.074	**76.284**
32	9.0	6.1	**72.500**	1.034	**73.426**
33	9.0	6.2	**70.000**	1.010	**71.733**
34	9.0	6.4	**65.000**	0.940	**66.761**
35	9.0	6.7	**57.500**	0.822	**58.381**
36	9.0	7.2	**45.000**	0.648	**46.016**
37	9.0	7.4	**40.000**	0.586	**41.608**
38	9.0	7.5	**37.500**	0.547	**38.847**
39	9.0	7.6	**35.000**	0.517	**36.744**
40	9.0	7.8	**30.000**	0.441	**31.318**
41	9.0	7.9	**27.500**	0.403	**28.621**
42	9.0	8.0	**25.000**	0.370	**26.298**
43	9.0	8.1	**22.500**	0.335	**23.784**
44	9.0	8.3	**17.500**	0.262	**18.600**
45	9.0	8.5	**12.500**	0.185	**13.104**

Which in:- **Conc.** is the Concentration of Urea in human blood serum measured by two methods: FIA method and Colorimetric method.

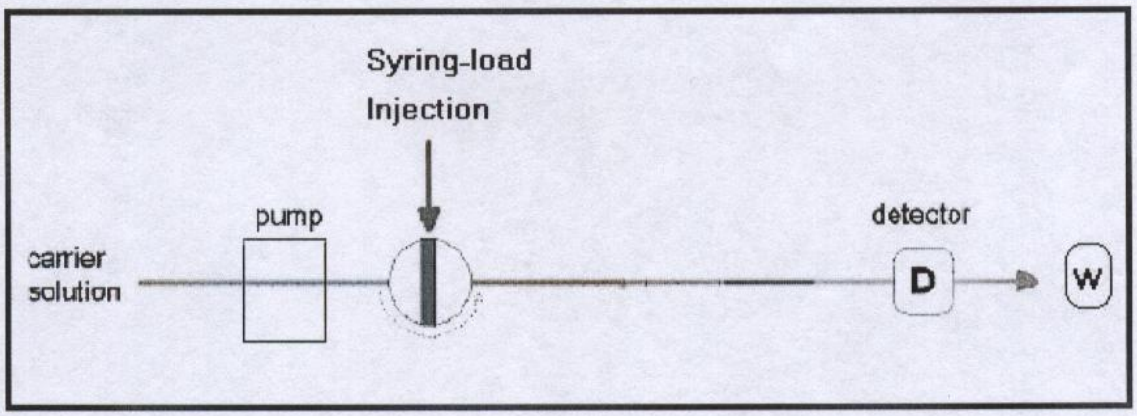


Fig. (1): One-line Flow Injection Analysis (FIA) Setup.

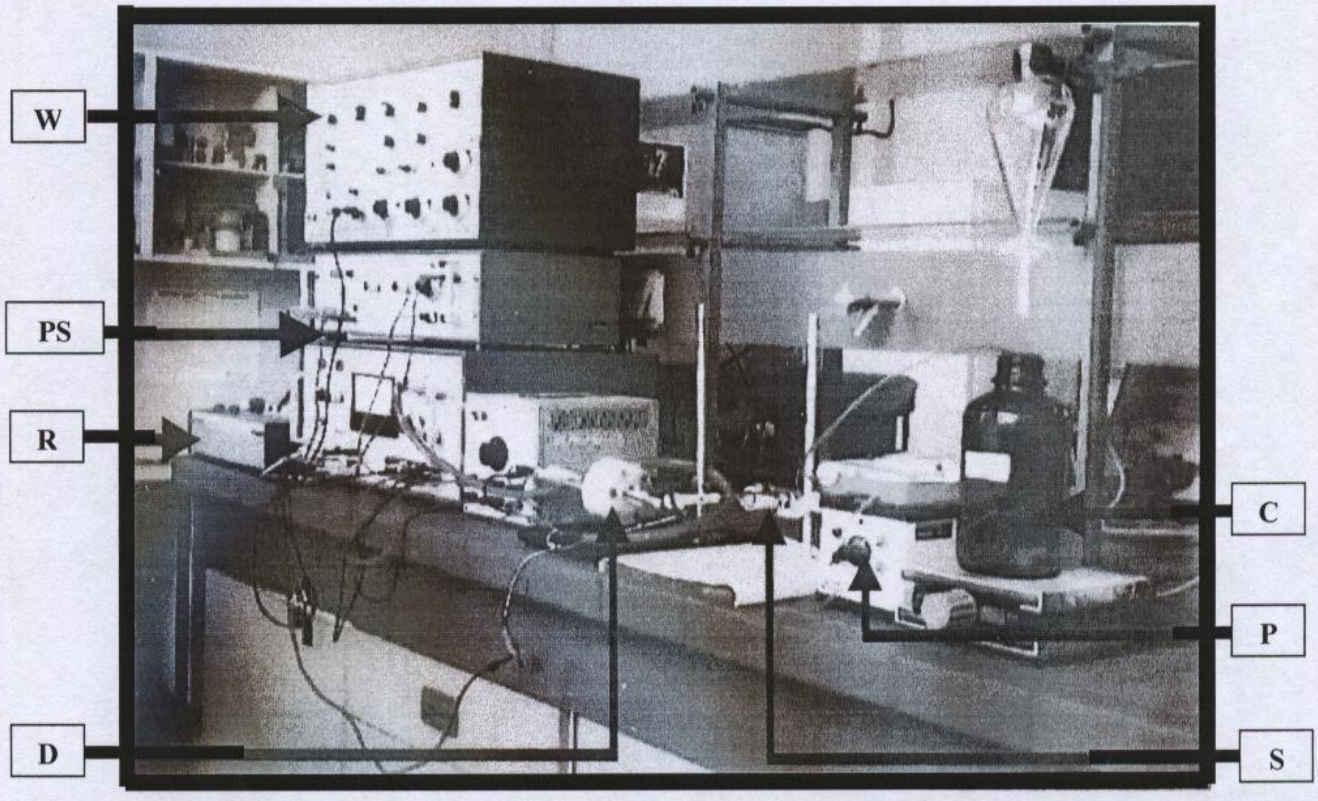


Fig. (2): The Experimental Set-up: (C) carrier stream, (P) pump, (S) sample injection, (D) detector, (R) recorder, (PS) Potentiostat unit & (W) Wave form generator.

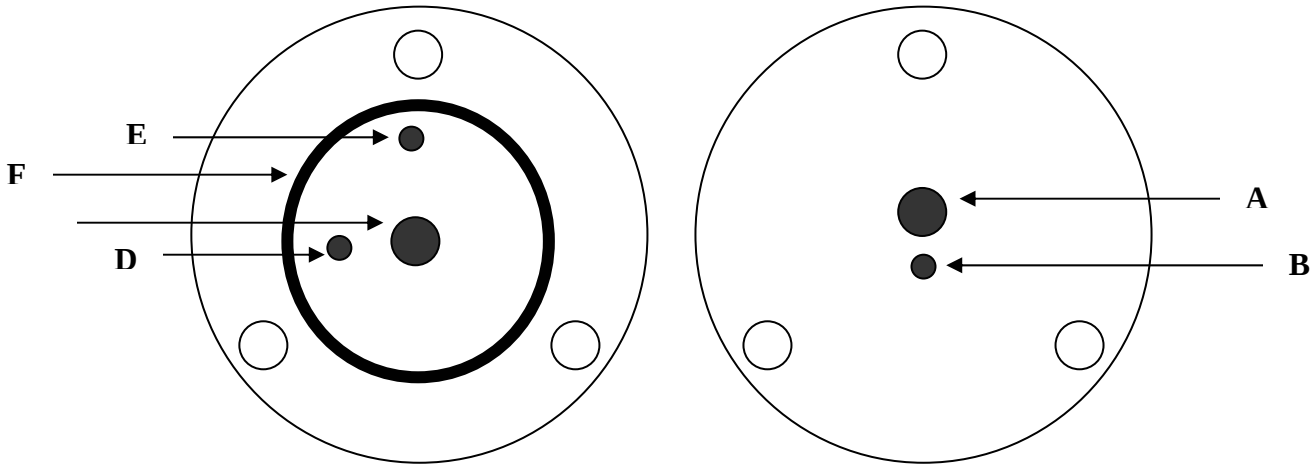


Fig. (3): Detector Configuration Internal-face view :

A: working electrode (5 mm diameter GC electrode)	**D: inlet**
B: Auxiliary electrode (1.5 mm diameter Pt wire)	**E: Outlet**
C: Reference electrode (Ag/AgCl, Sat. KCl).	**F: o-ring washer**

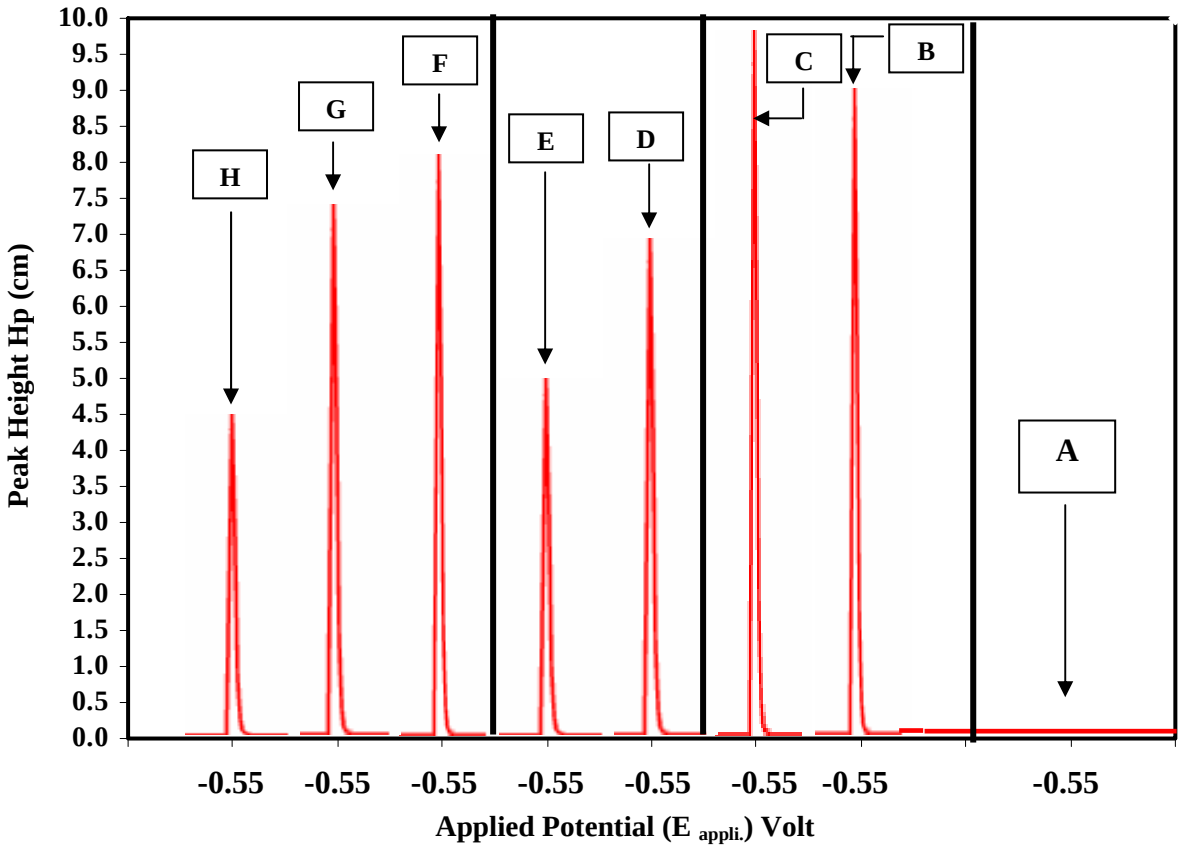


Fig.(4): FIA-Voltammograms of Sodium nitroprusside in phosphate buffer (pH= 6.8) as supporting and carrier:

(A) For phosphate buffer (pH = 6.8) only.	**(E) After human blood Serum Addition.**
(B) For working solution only.	**(F) After Low Human Serum Addition.**
(C) For (10^{-3}M) Sodium nitroprusside solution.	**(G) After Normal Human Serum Addition.**
(D) After Standard Urea (R4) Addition.	**(H) After Elevated Human Serum Addition.**

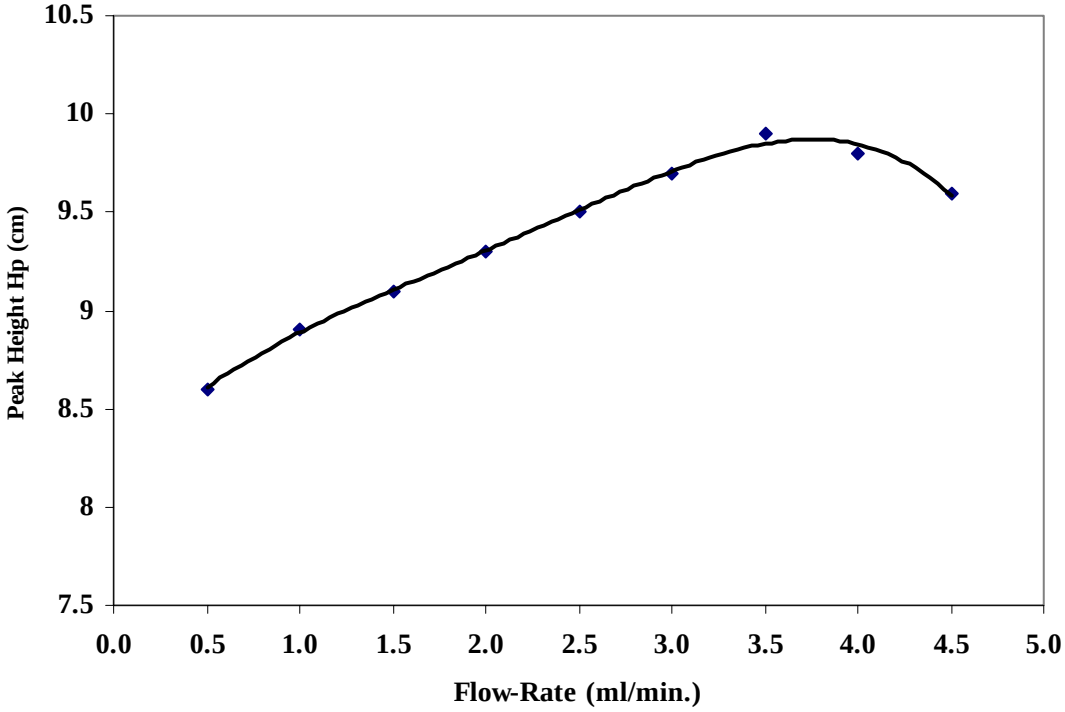


Fig.(5):- Show the effect of Flow-Rate on the reduction peak of Sodium nitroprusside at E_{appli} = -0.55 V.

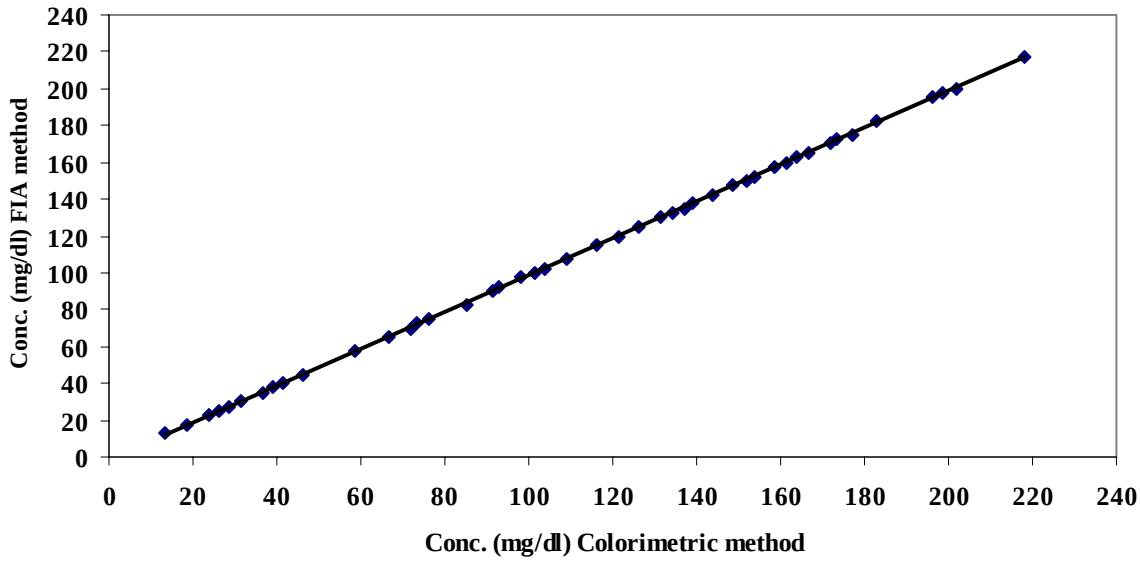


Fig.(6): Show the relation between Concentrations of Urea measured by FIA method and Colorimetric method for Normal & Abnormal Cases.

References

1. A. L. Lehninger, "Biochemistry", 2nd ed., Worth Publishers, Inc., New York (1975), p.831,833,840.
2. www.yahoo.com/Science/Chemistry/Biochemistrychemistry/Engormix_com-Dairy_Cattle-Milk_Urea_Nitrogen.htm, Yahoo! Inc.,(2004).p. 1-4.
3. K. Talaro, and A. Talaro, "Foundations In MICROBILOLOGY," 2nd ed., Wm. C. Brown Publishers, Chicago, (1996), p. 223,618,662.
4. J. F. Zilva, and P. R. Pannall, "Clinical Chemistry in Diagnosis and Treatment," 6th Ed., Lloyd-Luke (Medical Books) LTD, London, (1994), p. 17,18, 289,338.
5. www.yahoo.com/Science/Chemistry/Biochemistrychemistry/Blood Urea Nitrogen.htm, Yahoo! Inc.,(2004).p. 1-2.
6. www.yahoo.com/Science/Chemistry/Clinicalchemistry/Blood Urea Nitrogen Test AHealthyMe_com.htm, Yahoo! Inc.,(2005).p. 1-3.
7. www.yahoo.com/Science/Chemistry/Clinicalchemistry/Body1_com-Blood Urea Nitrogen (BUN).html, Yahoo! Inc.,(2004).p. 1-2.
8. J. Ruzicka and A. Ivaska, "Bioligand Interaction Assay by Flow Injection Absorptimetry" ***Analytical Chemistry***, 1997, **69**, 5024.
9. A. Ivaska and W. Kubiak, "Application of Sequential Injection Analysis to Anodic Stripping Voltammetry", ***Talanta***, 1997, **44**, 713.
10. J. Ruzicka and E. H. Hansen, "Flow Injection Analysis", 2nd edition, John Wiley & Sons, New York, (1981), p. 9,99.
11. L. Wang, T. J. Cardwell, R. W. Cattrall, M. D. Luque de Castro and S. D. Kolev, "Determination of Ammonia in Beers by Pervaporation Flow Injection Analysis and Spectrophotometric Detection", ***Talanta***, 2003, **60**, 1269.
12. W. Prissanaroon, N. Brack, P. J. Pigram, J. Liesegang and T. J. Cardwell, "Surface and Electrochemical Study of DBSA-doped Polypyrrole Films Grown on Stainless Steel", ***Surf. Interface Anal***., 2002, **33**, 653.
13. A. Th. Al-Taee, Ph. D. Thesis, Mosul University, Mosul, Iraq, (2002), p.38.
14. C. J. Patton and S. R. Crouch, ***Anal. Chem.***, 1977, **49**, 464.