------ Raf. Jour. Sci., Vol.16, No.3 Chemistry, Special Issue, pp. 201-211, 2005 ------

Flow Injection Analysis (FIA) Technique as a Method for Determination of Haemoglobin (Total) Quantity in Human Blood Serum

Amer Th. Al-Taee
Department of Chemistry
College of Science
Mosul University

(Received 24/5/2005 , Accepted 15/8/2005)

ABSTRACT

A new method for the determination of Haemoglobin quantity in human blood serum based on Flow Injection Analysis (FIA) was described. This method is relay on the decrease in the reduction peak of Potassium Ferricyanide $K_3[Fe(CN)_6]$ which appeared at (-0.5 V) vs. (Ag/AgC1,Sat. KCl) using Phosphate buffer (pH = 7.0) as a supporting and carrier solution. The method is sensitive and rapid. The procedure was successfully applied to the determination of Haemoglobin in various blood samples represent normal and different diseased cases such as: anemia, polysythemia.

A comparison between the proposed method (FIA) and colorimetric method was carried out using (36 samples), the relation was linear with a correlation coefficient (r = 0.9998).

تقدير كمية الهيموكلوبين في مصل الدم البشري باستخدام تقنية التحليل بالحقن الجرياني

الملخص

يتضمن البحث وصف طريقة جديدة لتقدير كمية الهيموكلوبين (Haemoglobin) في مصل دم الانسان باستخدام تقنية التحليل الحقني الجرياني (FIA). تعتمد الطريقة على قياس الانخفاض في قمة اختزال مادة سيانيد البوتاسيوم الحديديكي (Potassium Ferricyanide $K_3[Fe(CN)_6]$) عند جهد 0.5- فولت المستهلك خلال التفاعل الانزيمي للهيموكلوبين. تم تطبيق الطريقة المقترحة لقياس كمية الهيموكلوبين في (36) نموذج من عينات مصل الدم لاشخاص اصحاء وكذلك المصابين في حالات مرضية مختلفة مثل: فقر الدم وسرطان نخاع العظم (لدى الكبار). تم مقارنة النتائج المستحصلة من هذه الطريقة مع الطريقة الطيفية وكانت العلاقة خطية وبمعامل ارتباط (r = 0.9998).

INTRODUCTION

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 injection valve. 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. It is easy to see that compared to manual analyses, the tubing lines serve as solution containers and transfer vessels, the injection valve serves as a micropipette, and the pump replaces the lab technician using all this labware (Ruzicka and Ivaska, 1997, Ivaska and Kubika, 1997).

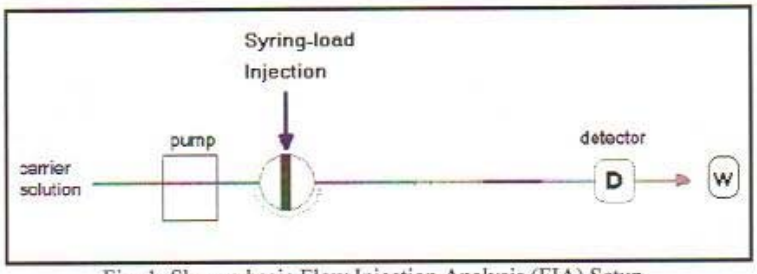

Fig. 1: Show a basic Flow Injection Analysis (FIA) Setup.

Many methods have been applied for determination of Haemoglobin among these methods, Haemoglobin determination with an automated high performance liquid chromatography (HPLC) method in patients with clinically silent Haemoglobin variants was carried out with high resolution mode aids the identifications of interferences (Lahousen et al., 2002).

A screen printed carbon has been modified in order to developed biosensor for Haemoglobin determination and the biomedical application areas are discussed (Hart et al., 2003).

The bright colour of Haemoglobin has played a significant role in both the investigation of this compound as well as in the study of blood oxygen transport, visible and near infrared absorption spectra of human and animal Haemoglobin determination and application studied by Zijlstra et. al. (2000).

In this work a FIA method is describe for indirect determination of the quantity of Haemoglobin in different samples represent different diseased cases. The method depends on measuring the decrease in the reduction peak of Potassium Ferricyanide $K_3[Fe(CN)_6]$ which appeared at -0.5 V vs. Ag/AgC1,Sat. KCl using Phosphate buffer pH = 7.0 as a supporting and carrier solution.

EXPERIMENTAL

Apparatus:

The experimental set-up as mentioned (Al-Taee, 2002) (components of the Flow

Injection Analysis System) are shown in (Fig. 2} was constructed as simple flow injection system. The carrier stream (C) is pumped by pump (P) through the electrode electrochemical flow through 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 using an x-t recorder.

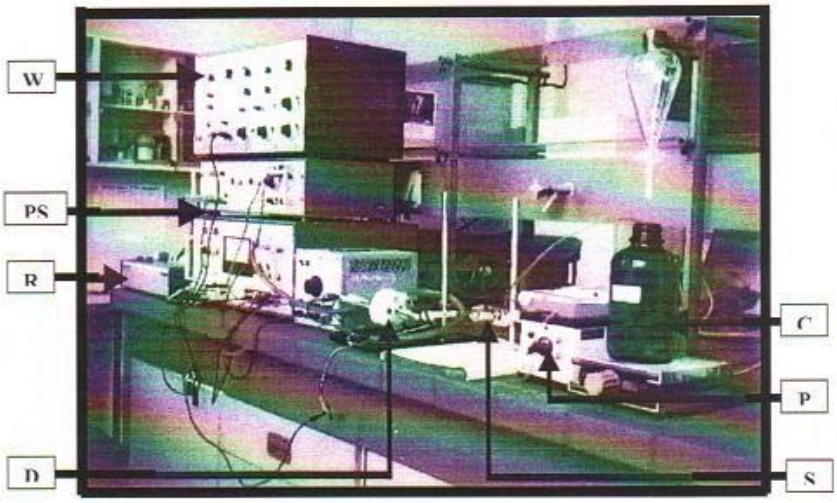

Fig. 2: The Experimental Set-up: (C) carrier stream, (P) pump, (S) sample injection, (D) detector, (R) recorder, (PS) Potentiostat unit and (W) Wave form generator.

The detection system was a house-built single-electrode 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)] was 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).

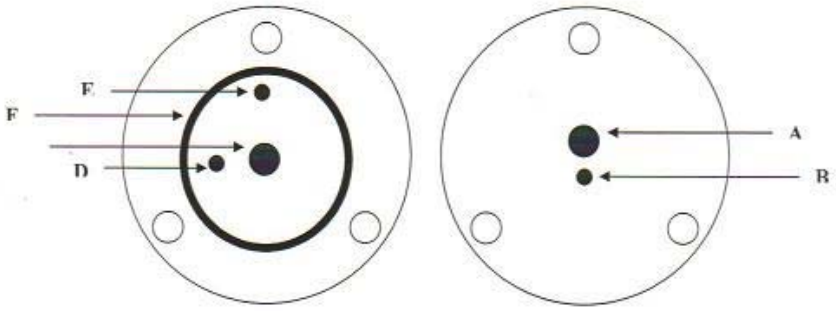

Fig. 3: Detector Configuration Internal-face view:

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

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.

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 is 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 scanning 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 and 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 and Reagents:

1. Drabkin's Reagent from HAEMOGLOBIN (TOTAL) (Cat. No. HG 980 (RANDOX): Sodium Bicarbonate 100 parts, Potassium Ferricyanide 20 parts & Potassium Cyanide 5 parts in a total volume 1000 ml.
2. Brij-35 Solution from HAEMOGLOBIN (TOTAL) (Cat. No. HG 980) (RANDOX): Brij-35 Solution 25% used for colorimetric method only.
3. Standard solution from HAEMOGLOBIN (TOTAL) (Cat. No. HG 980) (RANDOX): Methaemoglobin 18 mg/dl in a total volume 50 ml.
4. Drabkin's Reagent for FIA method:

Drabkin's solution that used for FIA-procedure was prepared by mixing one Bottle of Drabkin's Reagent with 980 ml deionized water. This solution was stable for about six months at +15 to +25°C.

5. Drabkin's Reagent for Colorimetric method:
Drabkin's solution used for colorimetric procedure was prepared by mixing one Bottle of Drabkin's Reagent with 980 ml deionized water followed by addition of (0.5 ml) of Brij-35 Solution to make colour with the sample. This solution was stable for about six months at +15 to +25°C.
6. Phosphate Buffer (0.2 M) at (pH 7.0):
Prepared freshly by dissolving 3.4836 gm of K_2HPO_4 and 2.7218 gm of KH_2PO_4 in a total volume of 100 ml of distill water.
7. (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.
8. (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.
9. (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 of Potassium Ferricyanide $K_3[Fe(CN)_6]$ before and after addition of 5µl of human blood serum sample to a solution containing 5 ml of Phosphate buffer (pH=7.0) and 500 µl of Drabkin's solution. Calculate the decrease in reduction peak of $K_3[Fe(CN)_6]$ which indicates the Haemoglobin quantity in the human blood serum sample using the following equation:

The concentration of Haemoglobin in serum $= \frac{H_{Blank} - H_{Sample}}{H_{Blank} - H_{Standard}} * C_H$, which in :-

H_{Blank}	represent the value of Peak height (cm) of Haemoglobin before serum addition.
H_{Sample}	represent the value of Peak height (cm) of Haemoglobin after serum addition.
$H_{Standard}$	represent the value of Peak height (cm) of the standard. the experimental value of $H_{Standard}$ equal to 2.5 cm.
C_H	Represent the value of the standard Haemoglobin concentration equal to 18.0 gm/dl.

Colorimetric-Procedure:

Colorimetric method was 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	Sample
Drabkin's solution	5.0 ml	5.0 ml
Sample	----------	20 µl

Mix well and allow to stand at least 15 minutes at 25°C. The colour is stable for several hours.

Calibration Curve for Colorimetric method:

For Calculation of the concentration of Total Haemoglobin in human serum (gm/dl). A calibration curve was constructed as shown in the following list by preparing the working standards by pipetting and mixing thoroughly the solution.

Tube No.	Standard Solution (ml)	Drabkin's Solution (ml)	Abs	Blood Haemoglobin (gm/dl)
1	0	6	0.000	0
2	2	4	0.141	6
3	4	2	0.290	12
4	6	0	0.442	18

The plot of Absorbance (Abs) versus the concentration of Blood Haemoglobin gives straight line with correlation coefficient (r = 0.9997) as shown in (Fig. 4), and the amount of unknown Haemoglobin calculated from the following equation: y = 0.0251x-0.01, (x = absorbance at 540 nm, y = peak height (cm).

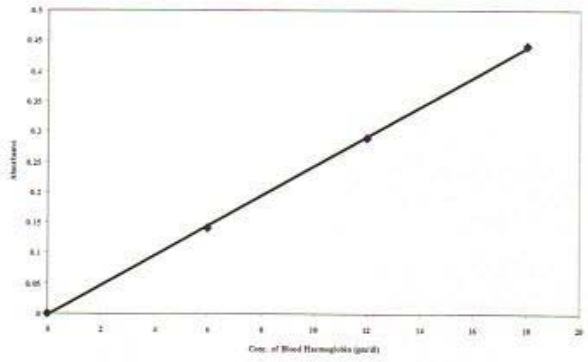

Fig. 4: The calibration curve of Haemoglobin using colorimetric method.

RESULTS AND DISCUSSION

In colorimetric method (Makarem, 1974), the presence of alkaline potassium ferricyanide, haemoglobin is oxidized to methaemoglobin, then reacts with potassium cyanide to form cyanmethaemoglobin which absorbs at 540 nm. The intensity of absorbance is directly related to total haemoglobin concentration.

In Flow Injection Analysis (FIA) (Sulaiman and Al-Taee, 2002), we found that potassium ferricyanide $K_3[Fe(CN)_6]$ gives a well defined peak at (-0.5 V) vs. (Ag/AgCl,

Sat. KCl) in phosphate buffer at (pH = 7.0) as shown in (Fig. 5B).

After addition of serum, we saw a decrease in the Peak height (H_{Sample}) of $K_3[Fe(CN)_6]$, with increasing addition of serum due to its consumption during the reaction as shown in (Fig. 5C).

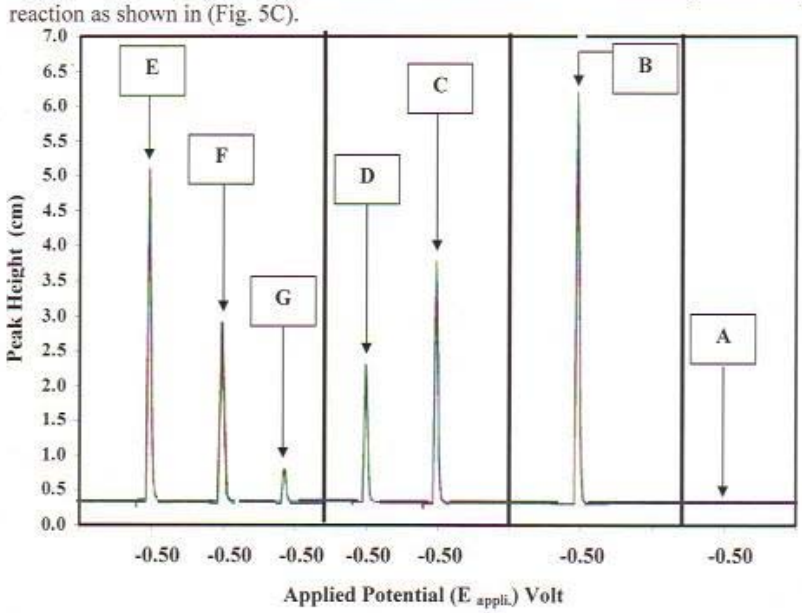

Fig.5: FIA -Voltammograms of $K_3[Fe(CN)_6]$ in Phosphate buffer (pH= 7.0) as supporting and carrier:

(A) For Phosphate buffer (pH= 7.0)
(B) For Drabkin's Solution Only
(C) After Serum Addition.
(D) After Standard Haemoglobin Addition
(E) After Low Human Serum Addition.
(F) After Normal Human Serum Addition.
(G) After Elevated Human Serum Addition.

Optimum Conditions for FIA Measurements:

Effect of Applied Potential:

The required potential for electrometric determination of Haemoglobin, DC-Voltammetric experiment was carried out using Drabkin's solution and Phosphate buffer (pH=7.0) as supporting and carrier. Different applied potentials were employed from (-0.3 V down to -0.7 V) with decreasing intervals of (0.05 V) and the peak heights were recorded. The result indicates that ($E_{appl.}$ = -0.5 V) represents the optimum potential for measurements obtained are shown in Table (1).

Table 1: Effect of applied potential on the reduction peak of $K_3[Fe(CN)_6]$ using (GC) electrode.

$E_{appl.}$ (V)	-0.30	-0.35	-0.40	-0.45	**-0.50**	-0.55	-0.60	-0.65	-0.70
Peak height (cm)	1.1	1.4	1.9	2.2	**3.0**	2.5	2.4	2.1	2.0

Effect of Flow Rate:

The effect of flow-rate on the peak current was studied using (500 µl) of Working solution added into phosphate buffer (pH = 7.0), ($E_{appl.}$ = -0.5 V). Different flow-rates were used, the plot of peak height versus flow-rates is shown in (Fig. 6), the peak height increase with the increasing of flow-rate up to (3.0 ml/min.) then become almost constant. During this work (3.0 ml/min.) was used.

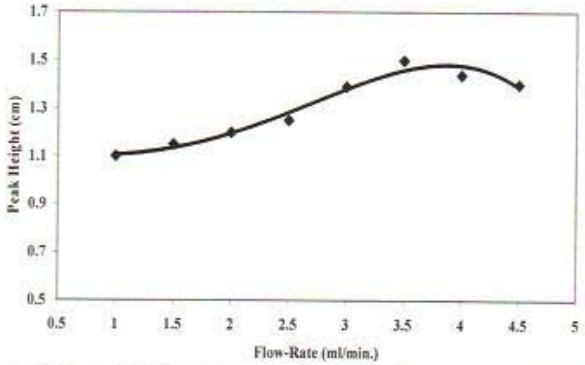

Fig. 6: Show the Effect of Flow-Rate on the reduction peak of $K_3[Fe(CN)_6]$ at E_{appli} = -0.5 V

Effect of Drabkin's Solution:

Different amounts of Drabkin's solution were added (100-500µl), and then the reduction peak was recorded for each solution. The results are shown in Table (2) which indicates that (500µl) is the optimum choice for Haemoglobin determination due to the highest reduction peak obtained.

Table 2: Effect of Drabkin's solution amount on the reduction peak of $K_3[Fe(CN)_6]$ at E_{appli} = -0.5 V

Addition of Drabkin's Solution (µl)	100	150	200	250	300	350	400	450	500
Peak height (cm)	3.30	3.70	4.00	4.60	5.00	5.40	5.90	6.30	6.80

Effect of Standard:

A serious of experiments were used for selecting the optimum amount of standard Haemoglobin that used for determination of Haemoglobin in serum. Different amount of Standard Haemoglobin (0.5-5) µl were used. The result indicates that 5.0 µl is suitable for Haemoglobin determination because it gives the highest reduction in the peak current (Table 3).

Table 3: Effect of Standard amount on the reduction peak of $K_3[Fe(CN)_6]$ at E_{appli} = -0.5 V.

Addition of Standard (µl)	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Peak height (cm)	6.20	5.70	5.30	5.10	5.00	4.80	4.20	3.40	2.70

Clinical Applications:

A comparison between the proposed FIA method and colorimetric was carried out

using (36 Samples) which consist of: Normal and some diseases cases such as: anemia, polysythemia. The results obtained are shown in Table (4).

Table 4: The results obtained for determination of Haemoglobin in Normal and Abnormal cases using two methods: FIA method and Colorimetric method.

	FIA method			Colorimetric Method	
Sample No.	H_{Blank} (cm)	H_{Sample} (cm)	Conc. (gm/dl)	A_{Sample}	Conc. (gm/dl)
1	6.5	5.9	2.700	0.074	3.122
2	6.5	5.8	3.150	0.085	3.581
3	6.5	5.7	3.600	0.096	4.039
4	6.5	5.6	4.050	0.108	4.498
5	6.5	5.5	4.500	0.119	4.956
6	6.5	5.4	4.950	0.130	5.415
7	6.5	5.3	4.918	0.127	5.284
8	6.5	5.2	5.505	0.139	5.775
9	6.5	5.1	5.701	0.145	5.998
10	6.5	4.9	6.875	0.175	7.238
11	6.5	4.6	7.900	0.197	8.120
12	6.5	4.4	8.745	0.225	9.281
13	6.5	3.8	11.610	0.289	11.885
14	6.5	3.7	11.880	0.294	12.074
15	6.5	3.5	12.741	0.325	13.352
16	6.5	3.4	13.140	0.333	13.658
17	6.5	3.2	14.389	0.354	14.530
18	6.5	3.1	14.702	0.370	15.149
19	6.5	3.0	15.007	0.374	15.333
20	6.5	2.9	15.523	0.393	16.089
21	6.5	2.8	15.758	0.396	16.225
22	6.5	2.7	16.267	0.399	16.344
23	6.5	2.6	16.999	0.421	17.228
24	6.5	2.5	17.441	0.438	17.940
25	6.5	2.2	18.685	0.467	19.108
26	6.5	1.8	20.180	0.512	20.931
27	6.5	1.7	20.846	0.529	21.608
28	6.5	1.6	21.080	0.534	21.847
29	6.5	1.5	21.902	0.555	22.685
30	6.5	1.1	23.315	0.588	24.024
31	6.5	1.0	23.781	0.602	24.598
32	6.5	0.9	25.200	0.638	26.044
33	6.5	0.8	25.650	0.649	26.503
34	6.5	0.7	25.072	0.630	25.714
35	6.5	0.6	26.550	0.672	27.420
36	6.5	0.5	27.000	0.683	27.878

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

The relation between the quantity of Haemoglobin in human blood serum measured by the two methods (Fig. 7) gives a straight line with correlation coefficient (r = 0.9998), (RSQ = 0.9996) 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 = -0.2153 + [0.9806 * 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 (5) and could be shown in (Fig. 5 E, F, G).

To ensure the accuracy of our purposed method (FIA method), we calculate the : Related Standard Deviation (RSD%), Relative Error (Error%) and (Recovery%), the result are shown in Table (6).

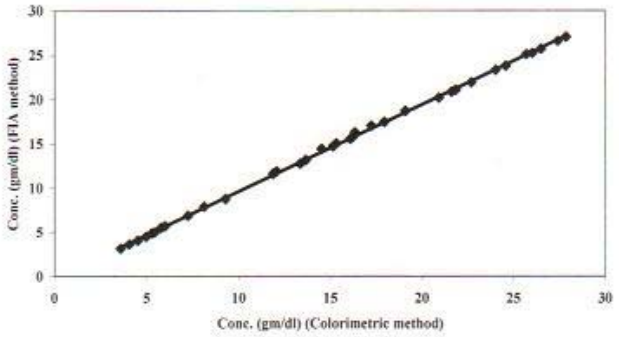

Fig. 7: Show the relation between FIA method and Colorimetric method for measurement the Haemoglobin quantity in normal and abnormal cases.

Table 5: Show the Quality Control of Haemoglobin quantity by the two methods: [(FIA-method) and (Colorimetric method)].

Standards	Colorimetric Method (gm/dl)	FIA Method (gm/dl)
Low Human Sera	5.977	5.502
Normal Human Sera	18.497	14.792
Elevated Human Sera	26.098	25.253

Table 6: The statistical results for accuracy of purposed method (FIA method).

	RSD %	Error %	Recovery %
Low Human Sera	± 0.05247	-0.07271	99.92730
Normal Human Sera	± 0.01789	-0.02028	99.97972
Elevated Human Sera	± 0.00990	-0.00792	99.99208

REFERENCES

Al-Taee, A. Th., 2002, Ph. D. Thesis, Mosul University, Mosul, Iraq, p.38.

Hart, J. P.; Abass, A. K.; Honeychurch, K. C.; Pemberton, R. M. and Wedge R., 2003, Sensors / Biosensors, Based on Screen-Printing Technology for Biomedical Applications, Indian Journal of Chemistry, 42A, 709-718.

Ivaska, A. and Kubiak, W., 1997, Application of Sequential Injection Analysis to Anodic Stripping Voltammetry, Talanta, 44, , p. 713-723.

Lahousen, T.; Roller R. E.; Lipp, R. W. and Schnedl, W. J., 2002, Silent Haemoglobin Variants and Determination of HbA1c With HPLC Bio-Rad Variant II, Journal of Clinical Pathology, 55, 699-703.

Makarem, A., 1974, Clinical Chemistry-Principles and Techniques, 2nd Ed., Henry, R. F.,Cannon D. C. and Winkelman, J. W., Editors, Harper and Row, Hagerstown [MD], 1128-1135.

Prissanaroon, W.; Brack, N.; Pigram, P. J.; Liesegang J. and Cardwell, T. J. 2002, Surface and Electrochemical Study of DBSA-doped Polypyrrole Films Grown on Stainless Steel, Surf. Interface Anal., 33, p. 653 - 662

Ruzicka, J. and Ivaska, A., 1997, Bioligand Interaction Assay by Flow Injection Absorptimetry, Analytical Chemistry, 69, , p. 5024-5030.

Sulaiman, S. T. and Al-Taee , 2002, Construction of Flow Injection- Electrochemical System Part I, Application to Fe^{2+} / Fe^{3+} System , Raf. Jour.Sci., Vol. 13, No. 4, 17-22.

Zijlstra, W. G.; Buursma, A. and van Assendel, O. W., 2000, Visible and Near Infrared Absorption Spectra of Human and Animal Haemoglobin (VSP: Utrecht).