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Analytical Determination of Hemoglobin by Spectrophotometric Method

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***Abstract*—Devolvement of rapid, simple, Sensitive, and stable spectrophotometric method for the assay of Hemoglobin using 2,4-dimethylaniline (DMA) and Hydrogen peroxide (H_2O_2) is presented. This study is based on the intermolecular coupling of oxidized DMA in the presence of H_2O_2 and Hemoglobin yields intense pink chromogenic coupled product having absorption maximum at 540 nm. The linearity of Hemoglobin by both rate and fixed time methods was found in the rage of 15.3 - 138 mM and 1.53 – 61.2 nM indicating the sensitivity of this method. H_2O_2 sensitivity range is found to be 20.2 - 331 µM, Michaelis-Menten constant for DMA and H_2O_2 were found to be 0.75 mM and 0.45 µM respectively. Catalytic efficiency and catalytic power were found to be 75×10^4 and 0.33 min^{-1} respectively. The reaction was applied for the assay of Hemoglobin and the results of proposed method correlated with Standard reference method.**

***Index Terms*—2,4-dimethylaniline, Hydrogen peroxide, Hemoglobin.**

I. INTRODUCTION

Majority of the proteins need metal ion for their structure and function [1] . This gaining a popularity and creates interest in the designing of artificial metal targeted site into protein structure [2]. Hemoglobin (Hb) is a Iron (Fe) contain oxygen carrying Heme metalloprotein having a molecular weight nearly 6,4500 D [3] which is found in RBC of all vertebrates and some invertebrates [4]. Chemically these Hemoglobin is a Ball-shaped tetrameric globular protein [5]. each Hemoglobin is having a capacity to transport 4 oxygen molecule from the lungs and delivered to the tissues, This oxygen is used in the energy production which is needed for cell metabolism and bring back carbon dioxide to lungs from the tissues [5]. The function of Hemoglobin is a balance between two structures, one with the low affinity for the oxygen and other with high [6]. It also controls the blood flow and blood pressure. A very high Hemoglobin concentration in the blood results in increasing in its viscosity which also affects cerebrovascular problems in it [7]. Evolution and minute mutations in the structure of Hemoglobin protein causes anemia (i.e., lower concentration of oxygen the blood) and other pathological conditions like nutritional deficiency [8], Kidney failure [9], bone marrow disorder [10] etc. Many surveys on this have proven that it will also affect the poor pregnancy risk [11]. Total Hemoglobin count in a regular blood test will assist to diagnose many anomalous condition in the human body [12] (i.e., 10.3-12.4 mg/dL for children, 10.9-15.7 mg/dL for male and 10.7-13.5 mg/dL for female). On that note, it is very important to quantify the Hemoglobin

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in blood by using standard method which is approved by Clinical and laboratory standard institute (CLSI) named Drabkin's method which is also well known as Cyan-Methemoglobin method [13]. In this method, blood which must be tested is mixed with the solution of potassium ferricyanide and potassium cyanide, iron in Hemoglobin is oxidized to Methemoglobin by potassium ferricyanide. Methemoglobin then reacts with potassium cyanide to form cyan-Methemoglobin. Which is having absorption maxima 540nm. Except Sulhemoglobin, all forms of Hemoglobin can be measured by using this standard method [14]. Other accepted methods used for the measurement of Hemoglobin concentration approved by CLSI are Hemoglobinometry [15], Multi-wavelength oximeters [16], Pulseoximetry [17]. These methods are based on idea that Oxy and deoxyhemoglobin having different absorption spectra [18] i.e., 660nm for red (visible light) and 900 to 940nm (infrared light).

II. PROCEDURE

A. Instrumentation

A JASCO UV-Visible spectrophotometer model UVIDEC-610 with 1.0 cm compatible cuvette cells was used to measure the absorbance of the solution throughout the experiment. A water bath shaker model NSW 133 was used to maintain a fixed temperature for color devolvement. An Equip-tonics digital pH meter model EQ-614 was used for the pH measurements of the solution and Micropipettes was used in this assay.

B. Chemicals

All the reagents used in this assay of Hemoglobin were analytical grade. DMA Hemoglobin was purchased from Merck, Germany. And H2O2 (30% v/v) were purchased from E Merck, Mumbai, India Respectively. DMA (175.4 mM) was prepared by dissolving 0.268g in 1ml of alcohol and 9 ml of water to a total volume of 10ml. Hemoglobin (4.61 µM) was prepared by dissolving 3 mg in 0.8ml of alcohol and 0.2 ml of 0.1N Sodium hydroxide and 9ml of water to a total volume of 10ml. H2O2 (100mM) stock was prepared daily and its concentration was standardized by using a standard potassium permanganate solution. Preparation of working standard solution can be done by further dilution from the stock solution. Potassium chloride-Sodium hydroxide buffer (0.5M) of pH 12.5 was prepared by mixing 0.5M sodium hydroxide of 1:4 v/v ratio and 0.5M Potassium chloride. Throughout the experiment double distilled water was used and the prepared solution was stored under the mild condition of 1-10 ^{0}C.

III. PROPOSED EXPERIMENTAL PROTOCOL

A. Assay procedure for the quantification of H_2O_2

Kinetics studies of 3 ml aliquot sample of a solution containing 5.84 mM DMA, 138 nM Hemoglobin, and potassium chloride – sodium hydroxide buffer 100 mM to maintain pH 12.5. The absorbance change in the solution was measured against the control solution containing all the reagents except H_2O_2 at 530 nm for 5 min at a regular 1 min interval. by using a plot of rate versus concentration, Linearity was determined. And it was observed that a good increment in the absorbance is observed after every increase in time and concentration of H_2O_2. The linearity was studied between 20 to 331 µM. observed linearity rate was found to be $0.0003CH_2O_2$ + 0.0035 with R2 = 0.998 as regression coefficient as shown in Fig. 1. Linearity by the rate method was determined using various concentrations of H_2O_2 by absorbance versus time response shown in Fig. 2.

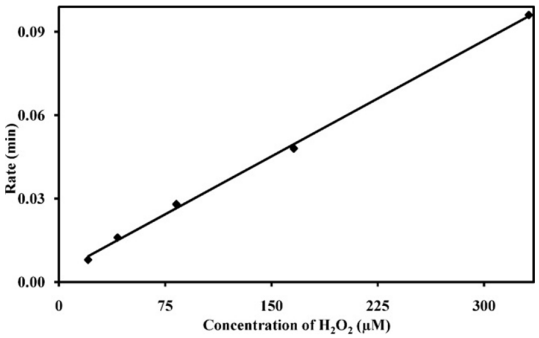


Figure.1. Linearity Plot for the quantification of H_2O_2 by rate method

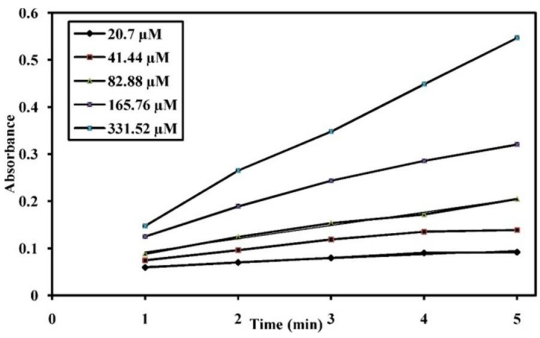


Figure.2. Absorbance-time response for varying concentrations of H_2O_2, inset shows the concentration of H_2O_2 used

B. Assay procedure for the quantification of Hemoglobin

An aliquot sample of 3 ml reaction mixture containing DMA (5.384 mM) and H_2O_2 (331 µM) and potassium chloride-Sodium hydroxide buffer (100 mM) of pH 12.5 was examined for 5 min at 1 minute time interval for the quantification of Hemoglobin by rate method. The linearity observed is 0.0005CHb + 0.0021 with linear regression coefficient of 0.998 Fig. 3. The kinetic change of the absorbance of the solution containing 5.384 mM DMA and 331 µM H_2O_2 in a final volume of 3 ml in 100 mM potassium chloride/sodium hydroxide buffer of pH 12.5 was measured for 5 min for the quantification of Hemoglobin. The linearity by the rate method was found between 15.3 and 138 nM. The linearity by the rate method is 0.0005CHb + 0.0021 with a linear regression coefficient of 0.998 shown in Fig. 3. The linearity was observed by plotting the response of absorbance versus time by rate method for various Hemoglobin concentrations as shown in Fig. 4.

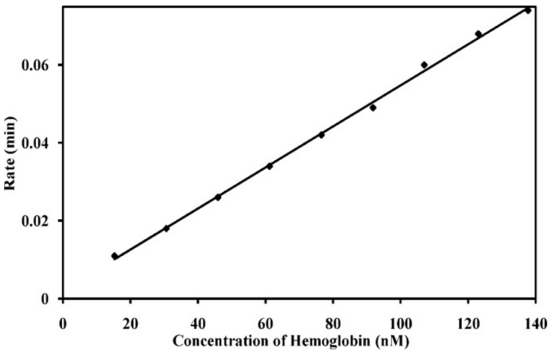


Figure.3. Calibration graph for the quantification of haemoglobin by rate method by the rate method. The calibration graph was constructed from the solution containing 5.84 mM DMA, 331 µM Hemoglobin, and Hemoglobin (15.3 – 138 nM) in a potassium chloride/sodium hydroxide buffer of pH 12.5 in a total volume of 3 ml of the solution

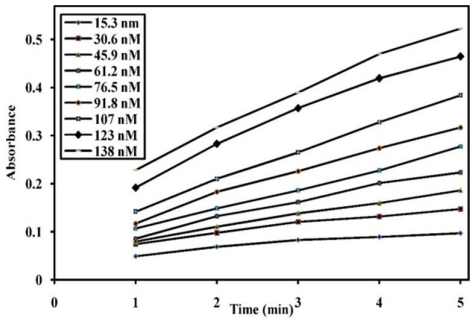


Figure.4. Absorbance-time response for varying concentrations of Hemoglobin, inset shows the concentration of Hemoglobin used.

One time assay method was carried out with the same concentration of the reagents. The reaction mixture was incubated for 20 min and the reaction was seized using 0.5 ml of 0.1M H_2SO_4, which yielded a pink-colored product with absorbance maxima peak at 530 nm as shown in Fig. 5. The graph of linearity lies in the range between 1.53 and 61.2 nM Hemoglobin. The Calibration curve for Hemoglobin quantification is shown in the Fig. 6. The linearity by the rate observed is 0.015 CHb + 0.005 with the linear regression coefficient is 0.999.

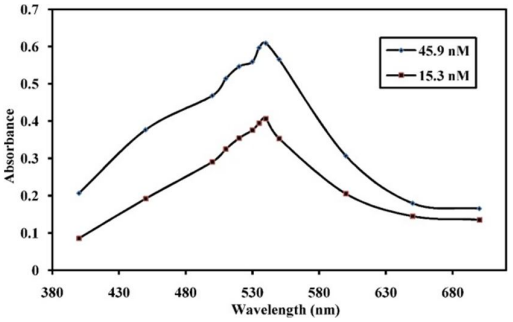


Figure.5. Absorption spectra of coloured product

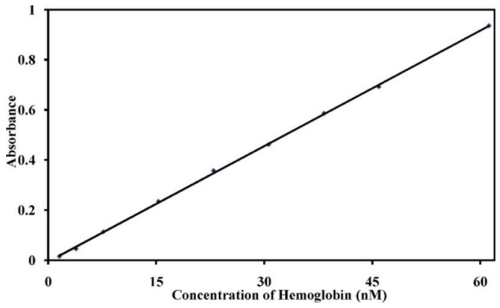


Figure.6. Calibration graph of Hemoglobin by fixed time assay method. Above Calibration graph was obtained by the total 3 ml solution containing DMA (5.84 mM), Hemoglobin (331 µM) and Hemoglobin (1.53 – 61.2 nm) in a buffer solution of Potassium Chloride-Sodium hydroxide of pH 12.5 with 0.5 ml 0.1 M H_2SO_4

IV. Optimization of Conditions for the Assay Protocol

A. Effect of H_2O_2

The impact of varying concentrations of H_2O_2 was studied. The results show that the increase in the concentration of H_2O_2 leads to an increase in the rate up to 331µM. We observed that the rate is dependent on

the concentration of H_2O_2. Beyond which further increase in the concentration of H_2O_2 we observed a very small change in the rate. Thus 331 µM was the concentration of H_2O_2 selected for the assay.

B. Effect of pH on the reaction rate

The rate of a reaction is specifically pH dependent. Hence the rate was studied based on catalytic constant and different pH values ranging from 5.8 to 13.8 as shown in Fig. 7. Maximum catalytic activity was observed when 100 mM potassium chloride-Sodium hydroxide of pH 12.5. The rate of the reaction started from 5.8 and reached 12.5. beyond which further increase in the pH leads to a decrease in the rate. Hence the pH of 12.5 was selected for the assay.

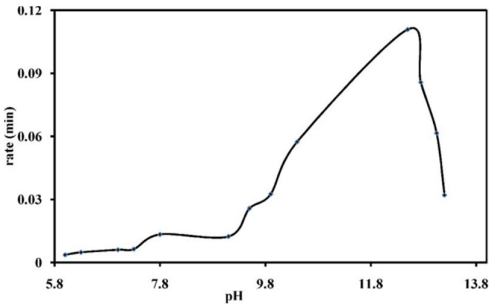


Figure.7. pH effect on the rate of the reaction

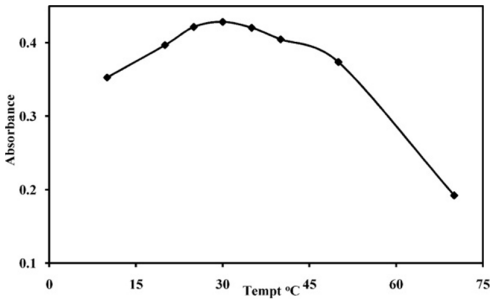


Figure.8. Effect of incubation temperature on the the rate of the reaction

C. Effect of temperature

The rate of the reaction is temperature sensitive. Hence it was measured with different temperatures ranging from 5-70 ^{0}C. It has been observed that initially, the rate of the reaction was increasing with every increment in the temperature to a certain extent. The rate of the reaction was maximum at the temperature of 30 ^{0}C. beyond which the activity started decreasing. Fig. 8**.** shows the change in the rate of the reaction at different temperatures.

D. Spectral characteristics of the colored product

The yellow-colored product was obtained by mixing the solution of DMA(5.84mM), H2O2(331 µM), Buffer (pH=12.5), and different concentrations of Hemoglobin were scanned against control which consist of similar reagents except for Hemoglobin. The wavelength range of 380 to 620 nm was selected. The above-colored solution shows maximum absorbance in the absorption spectra at 450 nm as shown in Fig. 9.

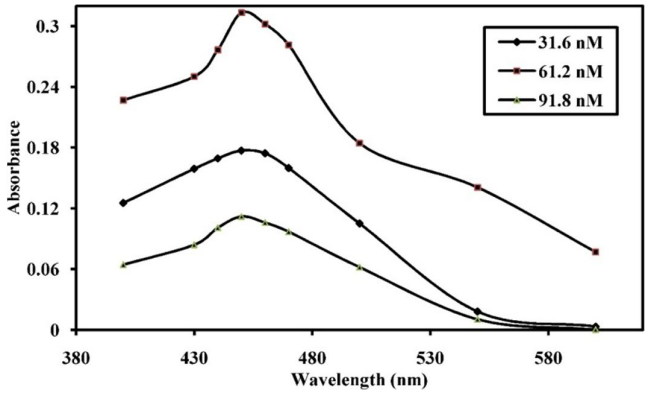


Figure.9. Absorption spectra (rate method) were recorded for 3 ml of aliquot sample include DMA (5.84 mM), H2O2 (331 µM) in a potassium chloride/sodium hydroxide buffer of pH 12.5 at 25 ^{0}C with three different concentrations of Hemoglobin of 31.6, 61.2, 91.8 nM

V. Effect of Catalytic and Substrate Parameter

In the present proposed method, the rate of different concentrations of Hemoglobin (Hbo) was measured concerning the different concentrations of DMA (Do) and H_2O_2 (Ho). Determination of Michaelis-Menten constant for DMA with the concentration ranging from 0.59 – 5.84 mM and H_2O_2 from 33.1 – 331 mM

respectively. Hemoglobin concentrations of 30.6, 61.2, and 91.8 µM was used in the 3 ml of the reaction mixture for the study of its kinetics by keeping constant temperature and pH. By assuming Vo is the initial rate, with respect to the function of all reagent concentrations, the general equation for the mechanism was given by

$$\frac{1}{V_o} = \frac{1}{V_{max}} + \frac{K_D}{D_o V_{max}} + \frac{K_H}{H_o V_{max}} \quad (1)$$

The value of initial rates was estimated regarding the function of reagent concentration. Calculation of the Michaelis-Menten constant can be done by changing one constant at a time by keeping the other fixed. The initial velocities were determined as a function of reagent concentration. The Michaelis-Menten constant was evaluated by varying one constant at a time and keeping the other fixed. Keeping Ho constant, equation (1) is given by

$$\text{Intercept} = \frac{1}{V_{max}} + \frac{K_D}{D_0 V_{max}} + \frac{K_H}{h_0 V_{max}} \quad (2)$$

$$\text{Slope} = \frac{K_D}{V_{max}} \quad (3)$$

Keeping P_o as constants, equation (1) is given by

$$\text{Intercept} = \frac{1}{V_{max}} + \frac{K_D}{D_0 V_{max}} \quad (4)$$

$$\text{Slope} = \frac{K_H}{V_{max}} \quad (5)$$

The constants K_D and K_H can be determined from equations (3) and (5), respectively. Here, K_H, and K_D are Michaelis-Menten constants of H_2O_2 and DMA which acts corresponds to substrate and co-substrate, Ho and Do are its initial concentration and Initial rate were determine by the function of substrate concentration.
Michaelis-Menten constant values for the reagents can be calculated by the equation (1). By using Do and Ho initial velocities (Vo) can be determined. In one experiment, Hbo and Do keep constant and Ho was changed. in another experiment by keeping Ho constant Do was changed. The constant slope obtained by the line-weaver Burk plot of Vo versus Do and at Vo versus Ho is shown in **Fig. 10** and **Fig. 11**. The K_D and K_H were found to be 0.75 mM and 0.45 µM, respectively and V_{max} was 0.015 µM min^{-1}.
Different catalytic parameters like K_{pow} (Catalytic power), K_{sp} (Specific constant), K_{cat} (catalytic constant), and K_{eff} (catalytic efficiency) were evaluated using a line weaver Burk plot of 1/rate vs $1/[H_2O_2]$ which gave the following results K_{cat} is 0.108 min^{-1}, K_{eff} is 4.75 × 104, K_{pow} is 0.33 min-1 and K_{sp} is 0.24min-1 µM-1.

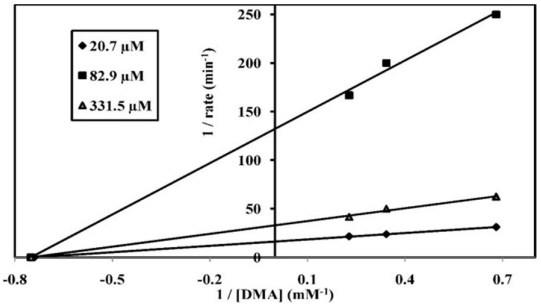


Figure.10 . Evaluation of Michaelis-Menten constant of DMA. Inset is the various concentration of H_2O_2 used

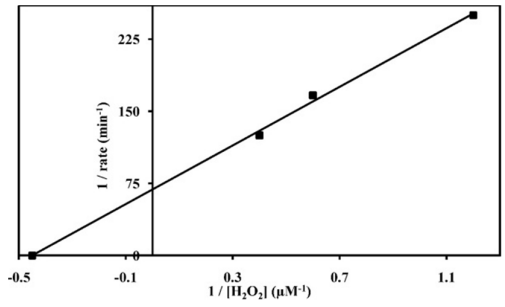
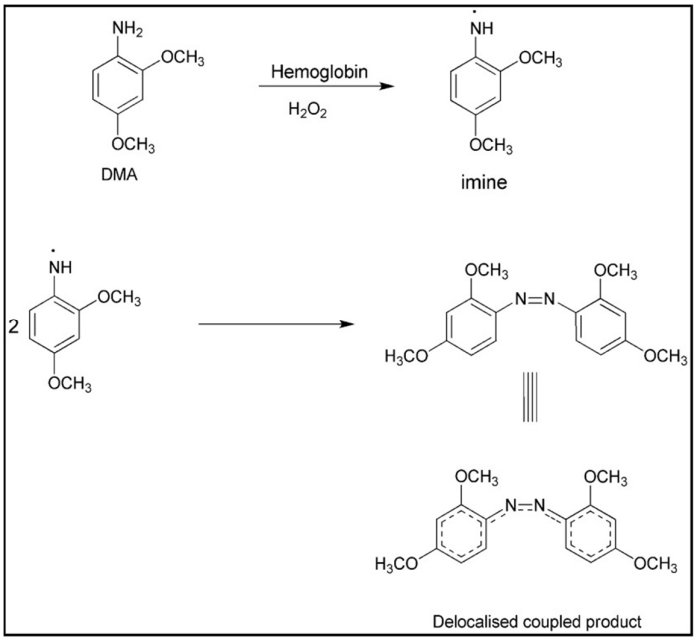


Figure.11 . Evaluation of Michaelis-Menten constant of H_2O_2

VI. Proposed Reaction Mechanism

The reaction mechanism of Hemoglobin canalized DMA reaction is shown in Scheme (1). Oxidation of H_2O_2 and Hemoglobin releases free radicle. DMA in the solution undergoes oxidation by losing one electron and one proton in the presence of H_2O_2 releases a cation radical. This radical of cation undergoes coupling reaction with another radical forming a pink-colored coupled product exhibiting absorption maxima at 540nm.



Scheme 1: Proposed reaction mechanism for the quantification of Hemoglobin

VII. Interferance Studies

The interference from an external source was examined in a solution of 3 ml reaction mixture containing 5.84 mM DMA, 331 μM H_2O_2, 1 mM Potassium chloride/sodium hydroxide buffer at pH 12.5, and a fixed hemoglobin concentration of 61.2 nM. Deviation from the primary value was found to be ± 3%. The ratio of tolerance is listed in Table.7.1. From the table, it is very clear that under the given condition, compounds like hemoglobin, ascorbic acid, Fe (II), Fe (III), and Uric acid, Nitrite, and some drugs showed low tolerance limit. some compounds like creatinine, glucose, ammonia, nitrate, urea, and common inorganic ions showed a reliable tolerance.

TABLE I. Interference Study

Interferants	Tolerance ratio[a]
Heparin* Iron (II) and (III)**	8
Ascorbic acid	18
Uric acid, Ammonium, Acetone	28
Glycine, Potassium, Sodium	38
Glucose, Glycine, Iron (III)**	90
Nickel, Phosphate, , citrate*	120
Urea	145
Lactose, Sucrose	200
Carbonate Bicarbonate, Calcium	220
Creatine, Creatinine	200
Iron II, Iron III, Nitrite, Bilirubin	400
Chloride, Sodium fluoride***	500
EDTA*	700
Magnesium, Aluminum, Nitrate,	1000

[a] Tolerance ratio corresponds to the ratio of limit of inhibiting species concentration to that of 45.9 nM hemoglobin used.
*anti coagulants
** Masked
*** Masking agent

VIII. Applications in Human Blood Sample

Blood samples were obtained from volunteer donors with their prior consent. The samples were diluted with double distilled water to the linearity range and measurement was done using the proposed method by spiking a standard solution of Hemoglobin. The assay procedure exhibited minimum interference with the good reproducibility recovery of 95.3% - 103.2% in the blood sample. The result obtained is summarized in Table.II.

TABLE II. ANALYTICAL RECOVERY OF HEMOGLOBIN IN HUMAN BLOOD SAMPLE

Sample number	Hemoglobin nM Proposed[a]	Added nM	Found nM	Recovered nM	Recovery * %
1	2.6	5.3	7.7	5.1	96.2
		15.3	17.6	14.9	97.4
2	5.2	15.3	53.8	20.7	103.8
		30.6	36.1	30.9	100.9
3	10.4	15.3	25.9	15.5	101.3
		30.6	39.7	29.3	95.8
4	20.8	5.3	25.9	5.1	96.2
		30.6	51.9	31.1	101.6

[a] mean of duplicate measurement
* [(final concentration – initial concentration)/added concentration]

Blood samples (n=24) were collected from the Pathology laboratories and Hospitals were analyzed by the standard Drabkins method and these data were collected for further investigation by the proposed method. **Fig .12.** Shows the measurement of the sample by the proposed method. It has been clear that the proposed method and standard method show a good correlation with the Correlation coefficient = 0.982, slope = 0.920, and Intercept = 0.811.

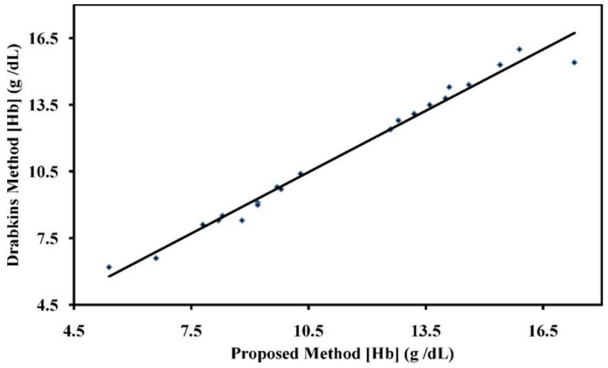


Figure.12. Comparison of results of 24 blood samples as obtained with Drabkin's method and by proposed method

IX. Conclusion

The proposed method is rapid, simple, sensitive, selective, and stable and was developed by using Chromogenic reagent 2,4-dimethoxyaniline for the assay of Hemoglobin in the human blood sample. This method involves the intermolecular coupling of oxidized DMA with the aid of H_2O_2 and Hemoglobin. This coupled product is having a maximum absorption peak at 540 nm which disables background interference by the biological samples. Therefore, this method is suitable for the quantification of Hemoglobin. The linearity of Hemoglobin by both rate and fixed time methods was observed in the range of 15.3 - 138 mM and 1.53 – 61.2 nM indicating the sensitivity of this method. The sensitivity toward H_2O_2 is in a range of 20.2 – 331 µM respectively. The Michaelis-Menton constant for DMA is 0.75 mM and H_2O_2 is 0.45 µM respectively. Vmax was 0.015 µM min^{-1}. The kinetic parameters such as K_{cat}, K_{eff}, K_{pow}, and K_{sp} were 0.108 min^{-1}, 4.75×10^4, 0.33 min^{-1} and 0.24min^{-1} μM^{-1} respectively. A recovery study was carried out with a human blood sample by spiking standard Hemoglobin showed 95.3% - 103.2%. The proposed method was compared with the well-established Drabkin's method, the correlation graph showed a good agreement with the proposed method.

Acknowledgment

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