A SIMPLE ACCURATE MULTI-COMPONENT SPECTROPHOTOMETRIC METHOD FOR SIMULTANEOUS DETERMINATION OF TOTAL HEMOGLOBIN AND FOUR CLINICALLY SIGNIFICANT HEMOGLOBIN DERIVATIVES IN HUMAN AND RAT BLOOD

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Abstract. Our recent multi-component spectrophotometric method for determination of hemoglobin derivatives has been developed theoretically and experimentally to get more accurate and precise concentrations of sulfhemoglobin, methemoglobin, carboxyhemoglobin and oxyhemoglobin in the blood of humans and rats. Theoretically, the equations used for determining the hemoglobin derivatives were derived by using more recent millimolar absorptivity matrices and are based on the theory of multi-component spectrophotometric analysis and the mathematical Gaussian elimination method for matrix calculation. New millimolar absorptivities at λ = 576 and 630 nm have been used for the first time to derive these equations. Experimentally, the method has been standardized with respect to the pH and temperature of hemoglobin solution. The results on methemoglobin percentage showed high correlations (r = 0.997 and r = 0.998) with our old multi-component method and the chemical method, respectively. Also, the results of oxyhemoglobin determined by our new method showed a high correlation (r = 0.999) with those determined by our old multi-component method. Moreover, the results of total hemoglobin concentration determined by our new method showed a high correlation (r = 0.998) with the chemical method. These results indicate the high accuracy of our new method. Furthermore, the percent values of sulfhemoglobin, carboxyhemoglobin and oxyhemoglobin showed a coefficient of variation of 1.52%, 5.85% and 0.137%, indicating the high reproducibility and precision of our new method. The method is non-expensive, highly sensitive, accurate, precise and reproducible and have the advantages of small sample volume, simplicity and speed and can be computerized.

Key words: carboxyhemoglobin, hemoglobin, methemoglobin, sulfhemoglobin, oxyhemoglobin, human, rats.

INTRODUCTION

Normal red cells contain a mixture of hemoglobin (Hb) derivatives; the physiologically important ones are deoxyhemoglobin and oxyhemoglobin (HbO_2), but there are another inactive Hb-derivatives, such as carboxyhemoglobin (HbCO),

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methemoglobin (MetHb), and sulfhemoglobin (SHb), the concentrations of which increase in many pathological conditions.

HbCO is an inactive Hb-derivative formed by binding of carbon monoxide (CO) with Hb. A direct measurement of HbCO in blood can document exposure to and poisoning from CO. The percentage of HbCO correlates with clinical symptoms. Carbon monoxide intoxication gives widespread disorders of the nervous system; impaired driving skills and decreased exercise tolerance have been observed in individuals with HbCO levels of 6%; more overt symptoms, such as headache and fatigue develop at 10–20%; a saturation level of about 20–30% is sufficient to cause severe headache, nausea, vomiting, dizziness, blurred vision and fainting; 30–40% causes nausea, vomiting, fainting, increased heart and respiratory rate and impaired neurological function; 40–50% causes coma, convulsion, impaired cardiovascular and neurological function, at 50–60% levels coma, convulsion, depressed respiration and depressed cardiovascular status occur; 60–70% levels cause coma, convulsions, cardio-respiratory depression, bradycardia and severe hypotension; at >70% levels respiratory failure and death occur [52]. In normal non-smoker subjects values within the range 0.2–2% HbCO are found [40, 48, 52], they are related to the ambient level of CO. Chronic intoxication with HbCO values up to 10−15% can arise from smoking and air pollution [2, 13, 26, 28, 33, 44].

MetHb is another inactive Hb in which the heme iron of hemoglobin is oxidized from ferrous (Fe^{2+}) to ferric (Fe^{3+}) and therefore is unable to bind and carry oxygen, resulting in a tissue hypoxia and related diseases [1, 3, 57]. In the normal physiological state, MetHb is formed by autoxidation of heme iron of oxyhemoglobin (HbO_2), by the reactive oxygen species induced by normal metabolic processes. The rate of autoxidation is about 3% per day [27]. Oxidized Hemoglobin *in vivo* undergoes reduction by NADH-methemoglobin reductase system and auxiliary mechanisms such as ascorbate and reduced glutathione (GSH) [38] in order to achieve a steady-state MetHb level; this amounts in normal blood to 0.5−1% [14, 34, 40].

If MetHb formation exceeds reduction to Hb, there is an increased MetHb level in blood and cyanosis may appear. There are two mechanisms for methemoglobinemia: acquired methemoglobinemia [12, 49] and congenital methemoglobinemia. The latter is a hereditary disease caused either by a deficiency of an antioxidant enzyme (glucose-6-phosphate dehydrogenase, G6PD), which leads to intravascular hemolysis and increased rate of Hb-oxidation, as in G6PD deficiency anemia [18, 42], or by a deficiency of NADH-dependent methemoglobin reductase [30, 43] or to the inheritance of an abnormality in the hemoglobin structure as in hemoglobins M [11, 23]. In congenital methemoglobinemia, values up to 30% occur, and exceptionally 40–50% levels have been reported [13]. Acquired methemoglobinemia is that caused by toxic substances such as nitrate or oxidant drugs, such as sulfone and aniline [29]. In newborns, since NADH-methemoglobin reductase activity is normally low [35], ingestion of toxic substances, such as nitrate-contaminated water, can give rise to a severe methemoglobinemia [15].

Another inactive Hb derivative is sulfhemoglobin (SHb,) in which a thiol group is inserted in the tetra-pyrrol ring of the Hb molecule, which makes it unable

to bind and carry oxygen. Hydrogen sulfide (H_2S) is produced and absorbed in the intestine. It is normally excreted from the lungs or destroyed. If H_2S is in excess or if substances are present that promote its reaction with hemoglobin, SHb is obtained. The sulfhemoglobinemia is usually induced by various drugs such as sulphonamides, sulfasalazine and sumatriptan [19]. Also, it may occur due to the occupational exposure to sulfur compounds, such as phenazopyridine [24]. In normal blood SHb is very low and pathological values range between 1 and 10% [17].

Due to the various diseases which occur by significant elevation of these derivatives, it is necessary for the recent studies to suggest new innovated diagnostic methods to facilitate diagnosis of these diseases. The previous studies reported that there are several multi-component spectrophotometric methods for determination of these Hb-derivatives [10, 20, 21, 41, 58–60]. These methods suffer from some errors arising from light scattering through erythrocyte ghosts, plasma lipid aggregates and Hb-aggregates. For this reason, recently, we have developed this method experimentally and theoretically [4–8]. However, this method is time-consuming, expensive, and requires a large sample volume. Recently, we have developed this method to overcome these drawbacks [9]. However, this developed method needs standardization with respect to the pH and temperature of the Hb solution and has not applied on the Hb-derivatives in the rat blood. Moreover, the differences among the millimolar absorptivities of canine, bovine, human and rats Hb-derivatives [53, 55, 56] make the equations used for determination of Hb-derivatives concentrations in human, bovine and canine blood, inapplicable for determination of these derivatives in rat blood. Also, the equations used for determination of Hb-derivatives in human and rat blood, are based on old millimolar absorptivities at λ = 577 and 620 nm [47]. Therefore, this practical study aimed to develop this method to be easier experimentally and theoretically in order to overcome the drawbacks of previous methods and to get more accurate results of the Hb-derivatives (SHb, MetHb, HbCO and HbO_2) in the blood of humans and rats, respectively.

MATERIALS AND METHODS

ANIMALS AND SUBJECTS

Seven female rats aged 3 months were purchased from the Animal House, National Research Centre, Dokki, Giza, Egypt. All animals were handled in accordance to the principles and guidelines of Laboratory Animal Facilities of the World Health Organization (WHO), Geneva, Switzerland (2003) [61]. With respect to humans, nine healthy female adult volunteers, who have Hb > 12.1 g/dL and normochromic-normocytic erythrocytes and not smokers. The written consent of the volunteers was obtained. The study was approved by the Research Ethics Committee of National Research Centre.

BLOOD COLLECTION

Blood samples were withdrawn from humans by venipuncture and from animals by puncturing the retro-orbital sinus, into heparinized tubes.

DETERMINATION OF HEMOGLOBIN DERIVATIVES

The levels of the inactive MetHb, HbCO and SHb and the active HbO_2 as well as the total Hb concentration in the human and rat blood were determined by the multi-component spectrophotometric methods as described below.

SAMPLE PREPARATION

The measurements were made directly after collecting the blood on heparin. For absorbance measurements, 30 μL of the whole blood is added to 5 mL of ice-cold distilled water, pH = 7.4 for humans and pH = 6.75 for rats. After mixing vigorously, to remove erythrocytes ghosts and plasma lipid aggregates, we centrifuged these erythrocytes hemolysates at 10,000 rpm for 10 minutes. Then, we separated the purified Hb solutions for absorbance measurements. The concentration of Hb at this extreme dilution is in the range $3.5–5.3\times10^{-5}$ M.

MEASUREMENTS AND CALCULATIONS

We measured the absorbances of extremely dilute, air saturated, equilibrated Hb solutions, at temperature 35°C, at four wavelengths (λ = 500, 569, 578 and 630 nm and λ = 500, 568, 576 and 630 nm for humans and rats, respectively), using a Cary UV/VIS double-beam spectrophotometer (model 100 UV-VIS), manufactured by Agilent Technologies, Australia. The spectral band width of the spectrophotometer was 2.0 nm and a quartz cuvette of 1 cm light path was used for absorbance measurement. A cuvette filled with distilled water was used as a blank. The absorbances of the blank (distilled water) were measured against air as a reference. The absorbances A_{500}, A_{568}/A_{569}, A_{576}/A_{578} and A_{630} of the Hb were calculated by subtracting the absorbances of the blank from the absorbances of the Hb solutions, measured at the same wavelengths, by using the same blank cuvette.

The millimolar concentration of human Hb-derivatives (SHb, MetHb, HbCO and HbO_2) in diluted Hb-solution were calculated from the absorbances at 500, 569, 578 and 630 nm, and the millimolar absorptivities determined previously [47, 54]. These millimolar absorptivities of HbO_2, HbCO, MetHb and SHb, were substituted into four linear equations of the type described by the theory of multi-component spectrophotometric analysis [47], with the four unknown concentrations of Hb pigments (C_{HbO2}, C_{HbCO}, C_{MetHb} and C_{SHb}).

$$A_{500} = 5.154 \cdot C_{\mathrm{HbO_2}} + 5.279 \cdot C_{\mathrm{HbCO}} + 9.067 \cdot C_{\mathrm{MetHb}} + 7.2 \cdot C_{\mathrm{SHb}} \quad (1)$$

$$A_{569} = 11.27 \cdot C_{\mathrm{HbO_2}} + 14.27 \cdot C_{\mathrm{HbCO}} + 4.10 \cdot C_{\mathrm{MetHb}} + 8.1 \cdot C_{\mathrm{SHb}} \quad (2)$$

$$A_{578} = 15.36 \cdot C_{\mathrm{HbO_2}} + 10.24 \cdot C_{\mathrm{HbCO}} + 4.22 \cdot C_{\mathrm{MetHb}} + 5.68 \cdot C_{\mathrm{SHb}} \quad (3)$$

$$A_{630} = 0.115 \cdot C_{\mathrm{HbO_2}} + 0.170 \cdot C_{\mathrm{HbCO}} + 3.80 \cdot C_{\mathrm{MetHb}} + 15.28 \cdot C_{\mathrm{SHb}} \quad (4)$$

where, A_{500}, A_{569}, A_{578} and A_{630} are the absorbances at 500, 569, 578 and 630 nm, respectively. The absorption bands at wavelengths 500, 569, 578 and 630 nm represent the absorption maxima of MetHb, HbCO, HbO_2 and SHb, respectively. The above linear system of equations can be represented in the matrix form as:

$$\begin{bmatrix} 5.154 & 5.279 & 9.067 & 7.2 \\ 11.27 & 14.27 & 4.1 & 8.1 \\ 15.36 & 10.24 & 4.22 & 5.68 \\ 0.115 & 0.170 & 3.80 & 15.28 \end{bmatrix} \times \begin{bmatrix} C_{\mathrm{HbO_2}} \\ C_{\mathrm{HbCO}} \\ C_{\mathrm{MetHb}} \\ C_{\mathrm{SHb}} \end{bmatrix} = \begin{bmatrix} A_{500} \\ A_{569} \\ A_{578} \\ A_{630} \end{bmatrix} \quad (5)$$

This linear system of equations was solved by mathematical manipulation using the Gaussian elimination method for matrix calculation [22], to yield the following equations of human's Hb pigments:

$$C_{\mathrm{SHb}} = \frac{A_{630} - 0.50894752 \cdot A_{500} + 0.125060228 \cdot A_{569} + 0.071535595 \cdot A_{578}}{13.0348879} \quad (6)$$

$$C_{\mathrm{MetHb}} = \frac{7.384939433 \cdot A_{500} - 2.014372479 \cdot A_{569} - A_{578} - 31.17514683 \cdot C_{\mathrm{SHb}}}{54.48031867} \quad (7)$$

$$C_{\mathrm{HbCO}} = \frac{A_{569} - 2.186651145 \cdot A_{500} + 15.72636593 \cdot C_{\mathrm{MetHb}} + 7.643888242 \cdot C_{\mathrm{SHb}}}{2.726668607} \quad (8)$$

$$\mathrm{C_{HbO_2}} = \frac{\mathrm{A_{500}} - 5.279 \cdot \mathrm{C_{HbCO}} - 9.067 \cdot \mathrm{C_{MetHb}} - 7.2 \cdot \mathrm{C_{SHb}}}{5.154} \quad (9)$$

where, A_{500}, A_{569}, A_{578} and A_{630} are the absorbances measured experimentally at wavelengths 500, 569, 578 and 630 nm, respectively, for purified, extremely dilute aqueous Hb solution.

For rats, the millimolar absorptivities of HbO_2, HbCO, MetHb and SHb, determined previously [53] were substituted into four linear equations of the type described by the theory of multi-component spectrophotometric analysis [47], with the four unknown concentrations of Hb pigments (C_{HbO2}, C_{HbCO}, C_{MetHb} and C_{SHb}).

$$A_{500} = 5.32 \cdot C_{\mathrm{HbO_2}} + 5.44 \cdot C_{\mathrm{HbCO}} + 8.99 \cdot C_{\mathrm{MetHb}} + 6.502 \cdot C_{\mathrm{SHb}} \quad (10)$$

$$A_{568} = 10.68 \cdot C_{\mathrm{HbO_2}} + 14.34 \cdot C_{\mathrm{HbCO}} + 3.89 \cdot C_{\mathrm{MetHb}} + 6.686 \cdot C_{\mathrm{SHb}} \quad (11)$$

$$A_{576} = 15.72 \cdot C_{\mathrm{HbO_2}} + 11.84 \cdot C_{\mathrm{HbCO}} + 3.67 \cdot C_{\mathrm{MetHb}} + 5.62 \cdot C_{\mathrm{SHb}} \quad (12)$$

$$A_{630} = 0.22 \cdot C_{\mathrm{HbO_2}} + 0.27 \cdot C_{\mathrm{HbCO}} + 3.92 \cdot C_{\mathrm{MetHb}} + 15.28 \cdot C_{\mathrm{SHb}} \quad (13)$$

where, A_{500}, A_{568}, A_{576} and A_{630} are the absorbances at 500, 568, 576 and 630 nm, respectively. The absorption bands at wavelengths 500, 568, 576 and 630 nm represent the absorption maxima of MetHb, HbCO, HbO_2 and SHb, respectively. The above linear system of equations can be represented in the matrix form as:

$$\begin{bmatrix} 5.32 & 5.44 & 8.99 & 6.502 \\ 10.68 & 14.34 & 3.89 & 6.686 \\ 15.72 & 11.84 & 3.67 & 5.62 \\ 0.22 & 0.27 & 3.92 & 15.28 \end{bmatrix} \times \begin{bmatrix} C_{\mathrm{HbO_2}} \\ C_{\mathrm{HbCO}} \\ C_{\mathrm{MetHb}} \\ C_{\mathrm{SHb}} \end{bmatrix} = \begin{bmatrix} A_{500} \\ A_{568} \\ A_{576} \\ A_{630} \end{bmatrix} \quad (14)$$

This linear system of equations was solved by mathematical manipulation using the Gaussian elimination method for matrix calculation [22], to yield the following equations of rat's Hb pigments:

$$C_{\mathrm{SHb}} = \frac{A_{630} - 0.5175576 \cdot A_{500} + 0.1012385 \cdot A_{568} + 0.0923778 \cdot A_{576}}{13.110884} \quad (15)$$

$$C_{\mathrm{MetHb}} = \frac{5.4412192 \cdot A_{500} - 1.2385099 \cdot A_{568} - A_{576} - 21.47813 \cdot C_{\mathrm{SHb}}}{40.428757} \quad (16)$$

$$C_{\mathrm{HbCO}} = \frac{A_{568} - 2.0075188 \cdot A_{500} + 14.157594 \cdot C_{\mathrm{MetHb}} + 6.3668872 \cdot C_{\mathrm{SHb}}}{3.4190977} \quad (17)$$

$$\mathrm{C_{HbO_2}} = \frac{\mathrm{A_{500}} - 5.44 \cdot \mathrm{C_{HbCO}} - 8.99 \cdot \mathrm{C_{MetHb}} - 6.502 \cdot \mathrm{C_{SHb}}}{5.32} \quad (18)$$

where A_{500}, A_{568}, A_{576} and A_{630} are the absorbances measured experimentally at wavelengths 500, 568, 576 and 630 nm, respectively, for plasma-free, purified, extremely dilute Hb solution.

The total Hb concentration (C^*_{Hb}) in mmol·L^{-1} for this diluted Hb solution was calculated by summing the concentrations of the individual Hb derivatives as given by the formulas (9, 10) from a previous paper [7]. Whereas, the fraction of Hb derivatives can be determined from the formulas (11–14) from a previous paper [7].

The concentrations of Hb pigments (SHb, MetHb, HbCO and the functional, active HbO_2) in the collected blood can be determined by multiplying the fraction of each Hb derivative by the whole blood total Hb concentration. The whole blood total Hb concentration can be determined by the multi-component spectrophotometric method, by using the following equation:

$$C_{\text{totalHb}} = 167.666 \times 1.6114 \times C^{*}_{\text{Hb}}(\text{g} \cdot \text{dL}^{-1}) \tag{19}$$

where 167.666 is the dilution factor and 1.6114 is the conversion factor from $\text{mmol}\cdot\text{L}^{-1}$ to $\text{g}\cdot\text{dL}^{-1}$ andC^{*}_{Hb} is the concentration of diluted Hb solution in $\text{mmol}\cdot\text{L}^{-1}$.

PREPARATION OF BLOOD SAMPLES OF VARIOUS METHB LEVELS

Blood samples of various MetHb levels were prepared by adding phosphate buffer (Na_2HPO_4 27.5 $\text{mmol}\cdot\text{L}^{-1}$ and KH_2PO_4 13.16 $\text{mmol}\cdot\text{L}^{-1}$, pH = 7.4) containing 3.04 mmol of $K_3Fe(CN)_6$ of volumes shown in Table 1 to 400 mL blood. After mixing vigorously, the samples were stored at room temperature for 1h until oxidation reaction of Hb is completed. Take samples from the mixture of columns (1) and (2) of volumes shown in Table 1 into 4 mL of distilled water (pH = 7.4) or $K_3Fe(CN)_6$ solution shown previously. Measure the levels of MetHb in the taken samples by the old multi-component spectrophotometric method [8] and the new method shown in this paper and the chemical method to determine the accuracy of the new method.

Table 1

Method of preparation of blood samples of various MetHb levels

Blood (μL)	**$K_3Fe(CN)_6$-solution (μL)**	**Samples taken* (μL)**
(1)	**(2)**	**(3)**
400	100	30
400	200	35
400	300	40
400	400	45
400	500	50
400	600	60

Mixtures of columns (1) and (2) must be stored, after mixing vigorously, at room temperature for 1h until oxidation reaction of Hb is completed. *Samples taken from a mixture shown in columns (1) and (2).

DETERMINATION OF *MetHb*% BY THE CHEMICAL METHOD

Take samples from the mixture of columns (1) and (2) of volumes shown in Table 1 into 4mL of distilled water (pH = 7.4) or 4 mL of phosphate buffer (Na_2HPO_4 27.5 $\text{mmol}\cdot\text{L}^{-1}$ and KH_2PO_4 13.16 $\text{mmol}\cdot\text{L}^{-1}$, pH = 7.4) containing 3.04 mmol of $K_3Fe(CN)_6$. After mixing vigorously wait for 30 min at room temperature until

complete oxidation of Hb occurs. Centrifuge the samples at 10,000 rpm for 10 min to remove erythrocytes ghosts. Measure the absorbance of Hb-solutions at 630 nm. Then calculate *MetHb*% by the following equation:

$$MetHb\% = \frac{A_S^{630}}{A_{630}^*} \times 100 \tag{20}$$

where A_S^{630} is the absorbance at 630 nm of aqueous Hb-solution and A_{630}^* is the absorbance at 630 nm of Hb-solution after complete oxidation in $K_3Fe(CN)_6$ solution of composition shown previously.

PREPARATION OF HEMOGLOBIN SOLUTIONS OF VARIOUS CONCENTRATIONS

Hemoglobin solutions of various concentrations were prepared by serial dilution of whole blood with distilled water (pH = 7.4) as shown in Table 2. Samples of volume 30 µL were taken into 5 mL distilled water (pH = 7.4) or Drabkin's reagent to determine the Hb-concentration in each sample, by the new multi-component method and chemical method, respectively.

Table 2

Method of preparation of Hb-solutions of various total Hb-concentrations

Blood (mL)	**Distilled water (mL)**	**Samples taken* (mL)**
(1)	**(2)**	**(3)**
300	0	30
300	75	30
300	150	30
300	225	30
300	300	30
300	375	30

Mixtures of columns (1) and (2) must be stored prepared. *Samples taken from a mixture shown in columns (1) and (2).

DETERMINATION OF TOTAL HB-CONCENTRATION BY THE CHEMICAL METHOD

Total Hb-concentration was determined by using the method of Van Kampen and Zijlstra [46]. Blood samples (30 µL) were taken into 5 mL of the reagent (200 mg $K_3Fe(CN)_6$, 50 mg KCN, 140 mg KH_2PO_4, pH = 7.2). After mixing vigorously, store the samples at room temperature for 10 min until the reaction occurs. Centrifuge the samples at 10,000 rpm for 10 min to remove erythrocyte ghosts. Measure the absorbance at 540 nm against reagent as a blank. Calculate the total Hb-concentration from the following equation:

$$C_{\text{totHb}}(\text{g / dL}) = \frac{A_{540} \times 16114 \times 167.666}{11 \times 10000} \tag{21}$$

where A_{540} is the absorbance at 540 nm, 16114 is the molecular weight of one Hb subunit, 167.666 is the dilution factor and 11 is the millimolar absorptivity of MetHb-cyanid.

DATA ANALYSIS

Data were presented as the mean ± standard deviation (SD) values. The Student's *t*-test was used for determination of the level of significance of difference between the two groups, using statistical programs (Statistical Package for the Social Sciences, version 14 [SPSS Inc., Chicago, IL] and the Statistics Calculator). The difference is considered significant at $p < 0.05$. The *t*-distribution was used to calculate the 95% confidence interval of the means. The 95% confidence interval is a parameter for precision of the mean values of data. On the other hand, the coefficient of variation (*CV*) was used to assess the precision of the individual values.

Linear regression analysis was used to determine the best fit curves, with the corresponding calibration functions, for the relationships between the *MetHb*% and HbO_2% determined by our old [9] and new multi-component methods and for the relationship between *MetHb*% determined by our new multi-component method and those determined by the chemical method by using a statistical Origin 8 software (USA). While the linear-quadratic analysis was used to determine the best fit curve for the relationship between total Hb-concentration determined by our new method and the chemical method. The Pearson's correlation coefficient (*r*) was used to determine these relationships, assessing the strength of association between the two used variables.

By using the advanced database Clipper-language, computer programs called Atef's programs, were constructed. By means of these programs we can estimate easily the percentages and concentrations of various hemoglobin derivatives as well as the total hemoglobin concentration in human and rat blood, based on the equations (6–9 and 15–19) mathematically derived for human and rat Hb-derivatives. The software is characterized by the simplicity, speed, and high accuracy. This software is suggested and available by the author for request.

RESULTS

The results of reproducibility and precision of the multicomponent spectrophotometric method, suggested in this article, for determination of Hb derivatives and total Hb-concentration, are shown in Tables 3 and 4. The reproducibility and precision of the method were evaluated by measuring the percentages of inactive and active Hbs as well as the total Hb-concentration for

3 samples from a single healthy subject and then calculating the values of standard deviation (SD) for each Hb derivative. The results of small *CV* and the small ranges of 95% confidence intervals indicate the high reproducibility and precision of the method.

Table 3

Reproducibility and precision of the multi-component spectrophotometric method for human Hb-derivatives

Sample number	***SHb*%**	***MetHb*%**	***HbCO*%**	***HbO₂*%**
1	0.6760	0.5351	0.6973	98.0916
2	0.6763	0.7851	0.6491	97.8895
3	0.6585	0.5762	0.6214	98.1438
Mean ± SD	0.6703±0.0102	0.6321±0.134	0.6559±0.0384	98.0416±0.1343
CV (%)	1.52	21.2	5.85	0.137
95% confidence interval for mean	0.645–0.695	0.303–0.962	0.561–0.750	97.711–98.372
Range of 95% confidence interval	0.050	0.659	0.189	0.661

Table 4

Reproducibility and precision of the multi-component spectrophotometric method for human HbO_2 and total Hb concentrations

Sample number	***HbO₂* (g/dL)**	**C_{totHb} (g/dL)**
1	14.8401	15.1289
2	14.8989	15.2201
3	15.0337	15.3181
Mean ± SD	14.9242±0.0992	15.2224±0.0946
CV (%)	0.665	0.621
95% Confidence interval for mean	14.680–15.168	14.990–15.455
Range of 95% confidence interval	0.488	0.465

A relationship between the *MetHb*% values determined by our new multi-component spectrophotometric method and the old method [9] is illustrated in Fig. 1. A high correlation (r = 0.997) between the two parameters is observed, indicating the high accuracy of our new method.

A relationship between the *MetHb*% values determined by our new multi-component spectrophotometric method and the chemical method is shown in Fig. 2. A high correlation (r = 0.998) between the results of the two methods is obtained, indicating the high accuracy of our new method.

In Fig. 2, the values of y-axis coordinate represent the mean±SD values of 4–6 replicates of blood samples of various *MetHb*% values determined by our new multi-component spectrophotometric method. The values of the x-axis coordinate represent the mean value of two replicates of blood samples of various *MetHb*% values determined by the chemical method.

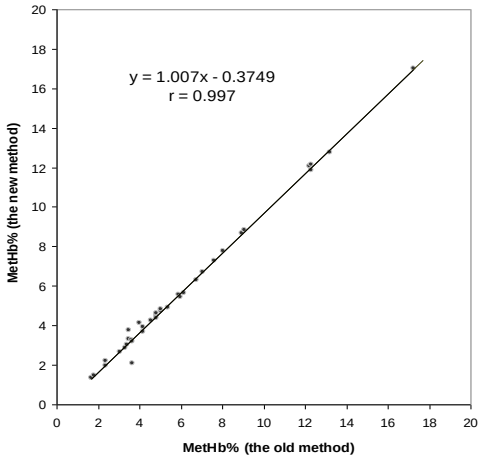

Fig. 1. Relationship between the *MetHb*% values determined by our new multi-component spectrophotometric method and the old method. The solid line represents the best fit line determined by linear regression analysis represented by the equation: $y = 1.007x - 0.3749$ and a correlation coefficient $r = 0.997$.

A relationship between the HbO_2% values determined by our new multi-component spectrophotometric method and the old method [9] is shown in Fig. 3. A high correlation ($r = 0.999$) between the results of the two methods is obtained, indicating the high accuracy of our new method.

A relationship between the total Hb concentration values determined by our new multi-component spectrophotometric method and the chemical method [46] is shown in Fig. 4. A high correlation ($r = 0.998$) between the results of the two methods is obtained, indicating the high accuracy of our new method.

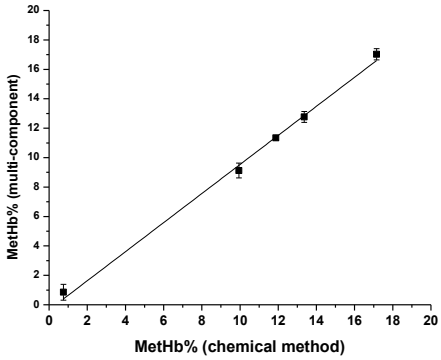

Fig. 2. Relationship between the *MetHb*% values determined by our new multi-component spectrophotometric method and the chemical method. The solid line represents the best fit line determined by linear regression analysis represented by the equation: $y = 0.9762x - 0.1436$ and a correlation coefficient $r = 0.998$.

In Fig. 4, the values of y-axis coordinate represent the mean±SD values of 3 replicates of blood samples of various total Hb values determined by our new multi-component spectrophotometric method. The values of the x-axis coordinate represent the mean value of 3 replicates of blood samples of various total Hb values determined by the chemical method.

Percentages of the Hbs with different ligands (SHb, MetHb, HbCO and HbO_2), and the concentrations of total Hb and HbO_2 in normal human and rat's blood are illustrated in Table 5. The data revealed that values of *SHb*% are noticed in the ranges (0.553–1.012%) and (0.371–1.213%) in the blood of normal human and rats, respectively. In addition, values of *MetHb*% are observed in the ranges (0.371–0.997%) and (0.0714–2.647%) in human and rat blood, respectively.

Furthermore, values of *HbCO*% are observed in the ranges (0.649–1.761%) and (4.029–4.911%) in human and rat blood, respectively. Values of HbO_2% are reported in the ranges (96.458–97.889%) and (91.229–95.209%) in human and rat blood, respectively. The data revealed also that the concentration of the total Hb are noticed in the ranges (12.10–15.220 $g \cdot dL^{-1}$) and (10.369–14.292 $g \cdot dL^{-1}$) in humans and rats, respectively. Moreover, concentrations of the HbO_2 were recorded in the ranges (11.873–14.899 $g \cdot dL^{-1}$) and (9.625–13.238 $g \cdot dL^{-1}$) in humans and rats, respectively.

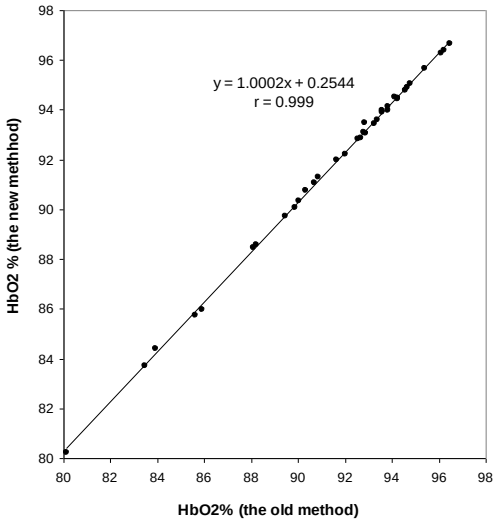

Fig. 3. Relationship between the HbO_2% values determined by our new multi-component spectrophotometric method and the old method. The solid line represents the best fit line determined by linear regression analysis represented by the equation: $y = 1.0002x - 0.2544$ and a correlation coefficient $r = 0.999$.

The results of Table 5 showed also insignificant difference between *SHb*% and *MetHb*% in the human and rat blood. The results showed also significantly higher values of *HbCO*% ($p < 0.00005$) in rat blood as compared to human blood. Also,

the results showed significantly higher values of HbO_2% ($p < 0.00005$) in human blood as compared to rat blood. Furthermore, the results showed significantly higher values of the total-Hb ($p < 0.01$) and the HbO_2 ($p < 0.005$) concentrations in human blood as compared to rat blood.

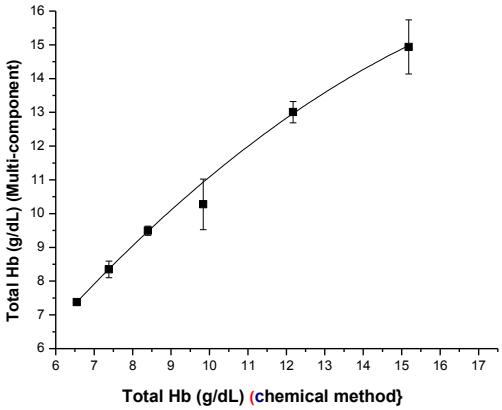

Fig. 4. Relationship between the total Hb concentration values determined by our new multi-component spectrophotometric method and the chemical method. The solid line represents the best fit line determined by linear quadratic analysis represented by the equation: $y = -2.118 + 1.6953x - 0.0375x^2$ and a correlation coefficient $r = 0.998$.

Table 5

Percents of active (HbO_2 form) and inactive Hb-derivatives and concentrations of total Hb and HbO_2 in normal human and rat's blood

Parameters	**Humans (n = 9)**	**Rats (n = 7)**	***p*-value**
SHb (%)	0.8192±0.1548 (0.553–1.012)	0.6638±0.3229 (0.371–1.213)	INS
MetHb (%)	0.7438±0.2209 (0.371–0.997)	0.9151±0.9957 (0.0714–2.647)	INS
HbCO (%)	1.4363±0.3457 (0.649–1.761)	4.5737±0.3457 (4.029–4.911)	<0.00005
HbO_2 (%)	97.1107±0.7166 (96.458–97.889)	93.8471±1.5985 (91.229–95.209)	<0.00005
C_{totHb}(g / dL)	13.5227±1.1582 (12.10–15.220)	11.6773±1.4084 (10.369–14.292)	<0.01
HbO_2(g/dL)	13.1313±1.1227 (11.873–14.899)	10.9471±1.2046 (9.625–13.238)	<0.005

The values are expressed as mean±SD; n is the number of the individuals in each group; the values between parentheses represent the ranges of parameters; INS indicate insignificant difference.

DISCUSSION

During the current study, a new method based on principles of the multi-component spectrophotometric analysis, were developed to be suitable for estimation of Hb derivatives. These developed methods require taking into account all the absorption contributions of all Hb derivatives. The four absorbance values were estimated for extremely dilute Hb solution. Under air-saturated conditions and at this extreme dilution, the deoxyhemoglobin should be converted completely into HbO_2 (i.e. full oxygenation) [16, 50]. This provided with the possibility to measure concentration of the active Hb which is represented by HbO_2. Since the fifth component (deoxyhemoglobin) can be neglected, in this extremely diluted Hb solution, under air saturated conditions, concentrations and fractions of other Hb derivatives (SHb, MetHb, HbCO, and HbO_2) can be determined, based on 4-absorbance measurements at the wavelengths λ = 500, 568/569, 576/578, and 630 nm.

Theoretically, our new method for determination of Hb-derivatives in human blood is based on absorbance measurement and millimolar absorptivities at more recent wavelengths λ = 500, 569, 578 and 630 nm [54] instead of old wavelengths 500, 569, 577 and 620 nm used in old methods [4–6, 47]. The new method for determination of Hb-derivatives in rat blood is based on absorbance measurement at more recent wavelengths and millimolar absorptivities at λ = 500, 568, 576 and 630 nm [53] instead of old wavelengths 500, 568, 576 and 620 nm used in old methods [8, 47, 53]. The absorption at λ = 630 represents the absorption maximum of SHb of millimolar absorptivity 15.28 as compared 3.8 of MetHb.

Technically, our new method is simple and rapid as compared to the old rat's method, since it involves dilution of 30 µL blood with 5 mL distilled water and centrifugation at 10,000 rpm for 10 min, instead of removal of plasma by centrifugation at 3,000 rpm for 5 min and then washing of packed erythrocytes 3 times with saline by centrifugation at 3000 rpm for 5 min each time and finally centrifugation at 10,000 rpm for 20 min of washed packed erythrocytes after dilution 1.5 times with 1% Triton-X100, in the previous methods [4–6]. At the extreme Hb dilution used in our study, the source of errors arising from scattering of light by hemoglobin aggregates was eliminated. This source of errors was not eliminated in previous methods, since they based on absorbance measurements of concentrated Hb-solutions. Centrifugation at 10,000 rpm for 10 min removes erythrocytes ghosts and plasma lipid aggregates which may interfere during absorbance measurement. These sources of errors were not eliminated during the previous methods [10, 20, 21, 41, 58–60], since no centrifugation of Hb solutions was performed.

This method is characterized by the high sensitivity because it was able to detect *SHb*%, *MetHb*% and *HbCO*% as low as 0.371, 0.0714 and 0.649, respectively, at this extreme dilution. Furthermore, these methods yielded percentage values of Hb derivatives with a high accuracy and reproducibility.

Also, the method is non-expensive, since it depends on using of non-expensive distilled water as a solvent instead of our old methods [4–6], which depends on the

use of expensive chemicals. Also, it requires small sample volume (30 μL), in contrast to our previous method (2 mL) [4–6]. As compared to the other previous methods, it was found that our new method is economically more suitable because it can be used to measure concentration of the total Hb by using distilled water as a solvent instead of the MetHb-cyanide method, which is based on the use of expensive chemicals [46]. Also, it was suitable to determine concentration of the active Hb-derivative (HbO_2), which is considered as the actual measure of the degree of anemia [5], rather than concentration of the total Hb.

The results obtained for human Hb-derivatives in our new study are in agreement with previous studies, which reported values of MetHb up to 1.0% [4–6, 40], SHb up to 1.0% [32, 45] and HbCO up to 2.0% [40, 48, 52] in normal human blood, using other methods.

According to the results (Table 5) obtained with our new method, significant differences have been revealed between the hemoglobins distributions characterizing human and rat blood samples as follows: HbCO is higher in rat and HbO_2% is higher in human blood. Similar findings represented by high levels of HbCO in normal rat blood (up to 6.322%) have been reported previously [5]. On the other hand, HbO_2 and total Hb concentrations are higher in human than in rats.

CONCLUSIONS

In conclusion, the method which was suggested for determination of Hb-derivatives during this experimental study showed that the *HbCO*% is higher in rat than in human blood. Also, this method showed that the HbO_2% present in human blood with significantly higher values than those values in rat blood. The total Hb and HbO_2 concentrations determined by this method were significantly higher in human than in rat blood. The method is highly reproducible, accurate, simple, rapid, and requires a small sample volume. Moreover, the method can be used simultaneously to determine concentrations of the total Hb and its derivatives in the blood of human and rats, respectively.

Declaration of interest. There are no declared conflicts of interest by the authors who are responsible for content and writing of this manuscript.

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