A SIMPLE ACCURATE METHOD FOR SIMULTANEOUS DETERMINATION OF TOTAL HEMOGLOBIN AND ITS DERIVATIVES IN HUMAN AND MICE BLOOD

A.M.M. ATTIA[#], FATMA ABD EL-HAMID ABD EL-SAMEE IBRAHIM*, NOHA AHMED ABD EL-LATIF*, S.W. AZIZ*, AZHAR MOHAMED ELWAN*, W.M. ABOULTHANA*, S.A.A. MOUSSA***, M.S. ELALFY****

[*]Biochemistry Department, National Research Centre, 33 Bohouth Street, Dokki, Giza, Egypt,
[#]e-mail: yousatef@yahoo.com
[**] Department of Physics, College of Science, Al-Imam Muhammad Ibn Saud Islamic University, Riyadh, Saudi Arabia
[***] Pediatric Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt

Abstract. A new multi-component spectrophotometric method was developed experimentally and theoretically to determine the accurate concentrations of the total hemoglobin (Hb) and its inactive derivatives including methemoglobin (MetHb), carboxyhemoglobin (HbCO) and sulfhemoglobin (SHb)) as well as the active derivative in the form of oxyhemoglobin (HbO_2) in the human and mice blood. With respect to the experimental technique, the method of preparation of Hb solutions has been developed, like the separation of erythrocytes ghosts, plasma leukocytes and plasma lipid aggregates and preparation of diluted Hb solutions, in order to overcome the drawbacks of the previous methods. It was found that results of this experimental method revealed values of normal human SHb% in the range (0.263–0.472) and MetHb% (0.174–1.003), HbCO% (0.683–1.365) and HbO_2% (97.469–98.810). Furthermore, the results of this method revealed values of normal mice SHb% in the range (0.033–0.091) and MetHb% (0.033–0.357), HbCO% (1.689–3.369) and HbO_2% (96.430–98.086). The method is non-expensive, highly sensitive, accurate and reproducible and have the advantages of small sample volume, simplicity and speed and can be computerized.

Key words: Carboxyhemoglobin (HbCO), hemoglobin (Hb), humans, methemoglobin (MetHb), mice, oxyhemoglobin (HbO_2), sulfhemoglobin (SHb).

INTRODUCTION

The protein hemoglobin (Hb) exhibits a vital function in red blood cells (RBCs) through its role in carrying oxygen present in inspired air from lung to tissue cells. Although Hb is naturally present in trace amounts, it is well known that there are three Hb species which have not the ability to transport oxygen. These

Received: January 2017;
in final form January 2017.

ROMANIAN J. BIOPHYS., Vol. 26, Nos. 3–4, P. 163–174, BUCHAREST, 2016

species, collectively called the dyshemoglobins, are methemoglobin, carboxyhemoglobin and sulfhemoglobin.

Methemoglobin (MetHb) is a form of oxygen carrying metalloprotein hemoglobin. It is characterized by presence of iron in the ferric (Fe^{3+}) state in the heme portion instead of ferrous (Fe^{2+}) as in the normal Hb. The condition which is characterized by elevation of MetHb level to be higher than normal level (1%) in the blood cells is called methemoglobinemia. As a result of presence of iron in the ferric state, the MetHb has not the ability to bind oxygen. However, iron in the ferrous state has a high affinity to bind oxygen. Due to oxygen binding to MetHb, affinity of oxygen to bind the three other heme sites that are still ferrous increases within the same tetrameric Hb molecule. This leads to an overall reduction in ability of the RBCs to release oxygen to tissues. Subsequently, the oxygen-hemoglobin dissociation curve shifted to the left [9, 20]. Elevation of MetHb concentration in RBCs results in the tissue hypoxia which is characterized by appearance of some severe symptoms such as shortness of breath, changing of the mental status, headache, fatigue, exercise intolerance and loss of hairlines [1, 3, 33].

Carboxyhemoglobin (HbCO) is a stable complex produced as a result of binding of carbon monoxide (CO) with Hb in RBCs upon the exposure to CO. It is well known that there are four heme groups in the Hb molecule. Each heme portion is able to bind reversibly to one oxygen molecule. The conformational changes which occur in the protein portion after oxygen binding to any of these sites facilitate oxygen binding to the other sites [15]. In the normal conditions, oxygen would bind to Hb in the lungs and would be transported carried to peripheral areas with low oxygen partial pressure. When CO binds to Hb, it has higher affinity to bind Hb at the same sites specific to oxygen, but approximately 200 times more tightly [15]. So, it is not released as easily as oxygen. Due to the slow release rate of CO, the CO-bound Hb molecules accumulate as CO exposure continues. This leads to lowering of the number of Hb molecules which are available to bind and deliver oxygen. This subsequently results in gradual suffocation associated with case of toxicity induced by CO exposure. This condition is known medically as carboxyhemoglobinemia. Small quantities of HbCO are sufficient to cause body oxygen deprivation and hence tiredness, dizziness and unconsciousness [16, 18].

Sulfhemoglobin (SHb) is a stable molecule formed by insertion of a thiol group within the tetra-pyrrol ring of Hb molecule. In this condition, the blood is incapable to carry oxygen. The condition in which the sulfhemoglobin is elevated in the blood is medically known as sulfhemoglobinemia. In this case, the Hb molecule is converted into a greenish pigment of the Hb derivative which cannot be converted back to a functionally normal molecule. It causes cyanosis even at low SHb levels. The sulfhemoglobinemia is usually induced by various drugs such

as sulphonamides, sulfasalazine and sumatriptan [10]. Also, it may occur due to the occupational exposure to sulfur compounds, such as phenazopyridine [14].

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 [8, 11, 12, 22, 35, 36]. These spectrophotometric methods suffer from some errors that arise from turbidity of the solutions caused by erythrocyte ghosts, plasma-leukocytes and plasma lipid aggregates, as in lipaemia [22]. Also, these methods are also affected of errors arising from light scattering by Hb-molecules aggregates, because these methods are based on absorbance measurements of concentrated Hb-solutions. For this reason, we have developed this technique based on the theoretical and experimental principles for determination of inactive and active Hb derivatives in human blood [6]. However, this method is laborious, time-consuming, expensive, and requires a large sample volume. Moreover, it was reported that there are significant differences between the millimolar absorptivities among human, bovine [32] and canine Hb-derivatives [31] and between human and rat Hb-derivatives [29]. These differences make the equations which are suitable for determination of Hb-derivatives in rat, bovine and canine blood, inapplicable for determination of these derivatives in human and mice blood. 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 mice, respectively.

MATERIALS AND METHODS

ANIMALS AND SUBJECTS

Eight male mice aged 3 months were obtained from the Animal House, National Research Centre, Dokki, Giza, Egypt. All animals were treated in accordance to the principles and guidelines of Laboratory Animal Facilities of the World Health Organization (WHO), Geneva, Switzerland (2003) [37]. With respect to humans, eight healthy male adult volunteers, who had to fulfill the following criteria: Hb >13.8 g/dL and normochromic-normocytic erythrocytes, not to smoke. The written consent of the volunteers was obtained. The study was approved by the Research Ethics Committee of the National Research Centre.

BLOOD COLLECTION

Blood samples were collected from humans by venipuncture and from animals by puncturing the retro-orbital sinus, into heparinized tubes. The blood samples were preserved in a refrigerator (at 4 °C) until analysis.

DETERMINATION OF HEMOGLOBIN DERIVATIVES

The levels of the inactive Hb (methemoglobin, carboxyhemoglobin and sulfhemoglobin) and active Hb (oxyhemoglobin) as well as the total Hb concentration in the human and mice blood were determined by the following multi-component spectrophotometric method.

Materials and sample preparation

The measurements were made within 24 h 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. After mixing vigorously, these erythrocytes hemolysates were centrifuged at 10,000 rpm for 10 minutes to remove erythrocytes ghosts, plasma leukocytes and plasma lipid aggregates. Then the purified Hb solutions were separated for absorbance measurements. The concentration of Hb at this extreme dilution is in the range $3.5–5.8\times10^{-5}$ M.

Measurements and calculations

The absorbance measurements for the extremely dilute, air saturated, purified Hb solutions were made at four wavelengths (λ = 500, 569, 577 and 620 nm absorption maxima of MetHb, HbCO, HbO_2 and SHb), using a Cary UV/VIS double-beam spectrophotometer (model 100 UV-VIS), manufactured by Agilent Technologies, Australia. The spectrophotometer was adjusted at a spectral band width of 2.0 nm and a quartz cuvette of 1.0 cm lightpath 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 first using a cleaned quartz cuvette against air as a reference. Then the absorbances of the extremely dilute Hb solution ($3.5–5.8 \times 10^{-5}$ M) were measured against air as a reference using the same blank cuvette without any further washing or cleaning. The absorbances A_{500}, A_{569}, A_{577} and A_{620} of the Hb for mice and humans were calculated by subtracting the absorbances of the blank from the absorbances of the Hb solutions measured at the same wavelengths.

The absorbance at 700 nm, where the Hb pigments have low absorption coefficients, was also recorded in order to confirm the absence of any turbidity or light-scattering in the Hb sample. The absorbance should not exceed 0.009, corresponding to the very low absorbance expected for Hb pigments at this wavelength for Hb samples of low concentration ($3.5–5.8 \times 10^{-5}$ M).

The mathematical formalism is based on the theory of multi-component spectrophotometric analysis [26] and the details, including formulas, are published in our previous reports [4, 7].

The concentrations of Hb pigments (SHb, MetHb, HbCO and the functional or active Hb in the HbO_2 form) 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:

$$C_{\text{total Hb}} = 167.666 \times 1.6114 \times C^{*}_{\text{Hb}} \quad \text{g}\cdot\text{dL}^{-1} \tag{1}$$

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

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 the difference between the two groups, using statistical programs (Statistical Package for the Social Sciences, version 14 [SPSS Inc., Chicago, IL]). The difference is considered significant at $p < 0.05$. The t distribution was used to calculate the 95% confidence interval of the parameter assessed. Computer programs written in the Clipper language were constructed and called Atef's programs. These programs are suggested and available by the author. The percents and concentrations of the Hb-derivatives (SHb, MetHb, HbCO and HbO_2) as well as the total Hb in human and mice blood were estimated easily by means of these programs.

RESULTS

The results of reproducibility and accuracy of the multicomponent spectrophotometric method, suggested in this article, for determination of Hb derivatives, are shown in Tables 1, 2. The reproducibility and accuracy of the method were evaluated by measuring the percentages of inactive and active Hbs for 6 samples from a single healthy subject or three samples from single healthy mice and then calculating the values of standard deviation (SD) for each Hb derivative. The results of small SDs and the small ranges of 95% confidence intervals indicate the high reproducibility and accuracy of the method.

Percents 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 mice blood are illustrated in Table 3. The data revealed that values of SHb% are noticed in the ranges (0.263–0.472%) and (0.033–0.091%) in the blood of normal human and

mice, respectively. In addition, values of MetHb% are observed in the ranges (0.174–1.003%) and (0.033–0.357%) in human and mice blood, respectively.

Furthermore, values of HbCO% are observed in the ranges (0.683–1.365%) and (1.689–3.369%) in human and mice blood, respectively. Values of HbO_2% are reported in the ranges (97.469–98.810%) and (96.430–98.086%) in human and mice blood, respectively. The data revealed also that the concentrations of the total Hb are noticed in the ranges (13.912–17.935 $g \cdot dL^{-1}$) and (8.262–14.029 $g \cdot dL^{-1}$) in humans and mice, respectively. Moreover, concentrations of the HbO_2 were recorded in the ranges (13.598–17.582 $g \cdot dL^{-1}$) and (7.969–13.689 $g \cdot dL^{-1}$) in humans and mice, respectively.

Table 1

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

Sample number	SHb (%)	MetHb (%)	HbCO (%)	HbO_2 (%)
1	0.2288	0.1361	0.9444	98.6907
2	0.2510	0.2396	0.9668	98.5426
3	0.2836	0.2158	0.9547	98.5459
4	0.3163	0.1432	1.0744	98.4661
5	0.3366	0.2416	1.1007	98.3210
6	0.3496	0.2147	1.0688	98.3759
Mean ± SD	0.2943±0.0482	0.1985±0.0470	1.0183±0.0702	98.4904± 0.1329
95% Confidence interval for mean	0.2437–0.3449	0.1491–0.2478	0.9446–1.0919	98.3509– 98.6298
Range of the 95% confidence interval	0.1012	0.0987	0.1473	0.2789

Table 2

Reproducibility and accuracy of the multi-component spectrophotometric method for mice Hb-derivatives

Sample number	SHb (%)	MetHb (%)	HbCO (%)	HbO_2 (%)
1	0.05854	0.1808	3.4173	96.3432
2	0.03896	0.1616	3.3689	96.4304
3	0.04915	0.1786	3.3967	96.3754
Mean ± SD	0.0489±0.00978	0.1737±0.0105	3.3943±0.0242	96.3830±0.0441
95% Confidence interval for mean	0.0246–0.0732	0.1476–0.1998	3.3341–3.4546	96.2735–96.4925
Range of the 95% confidence interval	0.0486	0.0522	0.1205	0.219

The results of Table 3 showed also significantly higher values of SHb% ($p < 0.0005$) and MetHb% ($p < 0.05$) and significantly lower values of HbCO% ($p < 0.0005$) in human blood as compared to mice blood. Also, the results showed significantly higher values of HbO_2% ($p < 0.01$) in human blood as compared to mice blood.

Table 3

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

Parameters	Humans (n = 8)	Mice (n = 8)	p-value
SHb (%)	0.3583±0.0828 (0.263–0.472)	0.0586±0.0256 (0.033–0.091)	<0.0005
MetHb (%)	0.4898±0.3202 (0.174–1.003)	0.1719±0.0968 (0.033–0.357)	<0.05
HbCO (%)	1.0737±0.2302 (0.683–1.365)	2.5502±0.5704 (1.689–3.369)	<0.0005
HbO_2 (%)	98.0782±0.4545 (97.469–98.810)	97.2193±0.6257 (96.430–98.086)	<0.01
Total Hb (g/dL)	16.0263±1.5223 (13.912–17.935)	11.4909±1.9173 (8.262–14.029)	<0.0005
HbO_2 (g/dL)	15.7212± 1.5290 (13.598–17.582)	11.1783±1.9071 (7.969–13.689)	<0.0005

The values expressed as mean ± S.D. n is the number of the individuals in each group; the values between parentheses represent the ranges of various parameters.

DISCUSSION

During the current study, a new method, based on principles of the multi-component spectrophotometric analysis, was developed to be suitable for estimation of Hb derivatives. This developed method requires taking into account all the absorption contributions of all Hb derivatives. The four absorbance values were estimated for extremely dilute Hb solution, this being the main improvement comparing with our previous works on the same topic [8, 11, 12, 22, 35, 36]. Under air-saturated conditions and at this extreme dilution, the deoxyHb should be converted completely into HbO_2 (*i.e.* full oxygenation) [19, 27]. This provided with the possibility to measure the concentration of the active Hb which is represented

by HbO_2. Since the fifth component (deoxyHb) 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, 569, 577, and 620 nm.

After the dilution, the centrifugation step resulted in the removal of scattering particles (cells, ghosts, lipid aggregates) improving the conditions for absorption measurements. Therefore, the sources of errors arising from turbidity induced by these components and aggregates were eliminated. As reported by various experimental studies, these sources of errors were not eliminated during the previous methods [8, 11, 12, 22, 35, 36], since no centrifugation of Hb solutions was performed.

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.

This method is characterized by the high sensitivity because it was able to detect SHb, MetHb and HbCO% as low as 0.033, 0.033 and 0.683, respectively, at this extreme dilution. Furthermore, this method yielded percentage values of Hb derivatives with a high accuracy and reproducibility.

Also, the method is non-expensive, rapid and simple. Furthermore, the method requires small sample volume (30 μL), in contrast to our previous method (2 mL) [6]. The results of human Hb-derivatives obtained in our new study are in agreement with previous studies, which reported values of MetHb up to 1.0% [6], SHb up to 1.0% [17, 24] and HbCO up to 1.5% [2, 6, 21, 23] in normal human blood, using other methods.

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 the concentration of total Hb by using distilled water as a solvent instead of the MetHb-cyanide method, which is based on the use of expensive chemicals [25]. Moreover, our method yields correct values of the total Hb concentration, since it takes into account the sum of concentrations of all Hb-derivatives, including SHb, which is not taken during the measurement by the MetHb-cyanide method. This is because the SHb cannot be converted into cyano-MetHb by the reagent used in that method [25]. In addition, our method can be used to simultaneously determine concentrations of the total Hb and its Hb-derivatives using distilled water instead of the expensive chemicals used in our previous method [6]. Also, it was suitable to determine the concentration of active Hb-derivative (HbO_2), which is considered as the actual measure of the degree of anemia [5], rather than the concentration of total Hb.

According to the results (Table 3) obtained with our new method, significant differences have been revealed between the Hbs distributions characterizing human and mice blood samples as follows: MetHb and SHb relative concentrations are higher in human than in mouse, while HbCO is higher in mouse. Similar findings represented by high levels of HbCO in normal mice blood (up to 3.8%) have been reported previously [28]. On the other hand, HbO_2 and total Hb concentrations are higher in human than in mouse.

CONCLUSIONS

In conclusion, the method which was suggested for the determination of Hb-derivatives during this experimental study showed that the SHb% and MetHb% present with significant higher values concomitantly with significant lower values of HbCO% in the human blood, when compared to those values of mice blood. Also, this method showed that HbO_2% was present in the human blood with significantly higher values than those values in mice blood. In addition, results of this study showed concentrations of the total Hb and HbO_2 present in the human blood with values significantly higher than those values in mice blood. This method is characterized by high reproducibility, accuracy, simplicity and speed as well as small sample volume. Moreover, the method can be used simultaneously to determine the concentrations of total Hb and its derivatives in the blood of human and mice, respectively.

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

REFERENCES

1. ADAMS, V., J. MARLEY, C. McCARROLL, Prilocaine induced methaemoglobinaemia in a medically compromised patient. Was this an inevitable consequence of the dose administered?, *Br. Dent. J.*, 2007, **203**, 585–587.
2. ALLAN, L.G, Carboxyhemoglobin levels in primary and secondary cigar and pipe smokers, *Chest,* 1977, **1**, 33–35.
3. ASH-BERNAL, R., R. WISE, S.M. WRIGHT, Acquired methemoglobinemia: a retrospective series of 138 cases at 2 teaching hospitals, *Medicine* (Baltimore), 2004, **83**, 265–273.
4. ATEF, M.M.A., A.M. EL-HEFNAWY, Conformational stability against auto-oxidation for mice and human oxyhemoglobins, *Romanian J. Biophys.*, 2009, **19**, 187–198.
5. ATTIA, M.M.A., F.A.A. IBRAHIM, N.A. ABD EL-LATIF, S.W. AZIZ, S.A. ABDELMOTTALEB MOUSSA, Biophysical study on conformational stability against autoxidation and erythrocytes oxidative status in humans and rats, *Wulfenia*, 2015, **22**, 264–281.

6. ATTIA, A.M., F.A. IBRAHIM, N.A. ABD EL-LATIF, S.W. AZIZ, S.A. ABDELMOTTALEB MOUSSA, M.S. ELALFY, Determination of human hemoglobin derivatives, *Hemoglobin*, 2015, **39**, 371–374.
7. ATTIA, A.M.M., M.N. GHADA, A.I. FATMA, S.H. NAHED, Multi-component spectrophotometric determination of inactive and active hemoglobins in rat and mice blood, *Romanian J. Biophys.*, 2011, **21**, 267–276.
8. BRUNELLE, J.A., A.M. DEGTIAROV, F. MORANR, L.A. RACE. Simultaneous measurement of total hemoglobin and its derivatives in blood using CO-oximeters: Analytical principles; their application in selecting analytical wavelengths and reference methods. A comparison of the results of the choices made, *J. Clin. Lab. Invest.*, 1996, **224** (Suppl), 47–69.
9. CORDONE, L., A. CUPANE, M. LEONE, V. MILITELLO, E. VITRANO, Oxygen binding to partially oxidized hemoglobin, Analysis in terms of an allosteric model, *Biophysical Chem.*, 1990, **37**, 171–181.
10. FLEXMAN, A.M., G. DEL VICARIO, S.K. SCHWARZ, Dark green blood in the operating theatre, *Lancet,* 2007, **369**, 1972.
11. FOGH-ANDERSEN, N., O. SIGGAARD-ANDERSEN, F.C. LUNDSGAARD, P.D. WIMBERLEY, Diode-array spectrophotometry for simultaneous measurement of hemoglobin pigments, *Clin. Chim. Acta,* 1987, **166**, 283–289.
12. FOGH-ANDERSEN, N., O. SIGGAARD-ANDERSEN, F.C. LUNDSGAARD, P.D. WIMBERLEY, Spectrophotometric determination of hemoglobin pigments in neonatal blood. *Clin. Chim. Acta*. 1987, **166**, 291–296.
13. GERALD, C.F., Solving sets of equations. In: C.F. Gerald (ed.), *Applied Numerical Analysis*, 2nd ed. London, UK: Addison-Wesley Publishing Company, 1978, pp. 78–80.
14. GOPALACHAR, A.S., V.L. BOWIE, P. BHARADWAJ, Phenazopyridine-induced sulfhemoglobinemia, *Ann. Pharmacother*., 2005, **39**, 1128–1130.
15. GREGORY, B.V., J. XINHUA, F. CLARA, L.G. GARY, Human Carboxyhemoglobin at 2.2 Å Resolution: Structure and Solvent Comparisons of R-State, R2-State and T-State Hemoglobins, *Acta Crystallogr.*, 1998, **D 54**, 355–366.
16. LOPEZ-HERCE, J., R. BORREGO, A. BUSTINZA, A. CARRILLO, Elevated carboxyhemoglobin associated with sodium nitroprusside treatment, *Intensive Care Med.*, 2005, **31,** 1235–1238.
17. NICHOL, A.W., D.B. MORELL, Spectrophotometric determination of mixtures of sulphaemoglobin and methaemoglobin in blood, *Clin. Chim. Acta*, 1968, **22**, 157–160.
18. ROTH, D., N. HUBMANN, C. HAVEL, H. HERKNER, W. SCHREIBER, A. LAGGNER, Victim of carbon monoxide poisoning identified by carbon monoxide oximetry, *J. Emerg. Med.*, 2009, **40**, 640–642.
19. SAVICKI, J.P., G. LANG, M. AKEDA-SAITO, Magnetic susceptibility of oxy- and carbonmonoxyhemoglobin, *Proc. Natl. Acad. Sci. USA*, 1984, **81**, 5417–5419.
20. SHANNON, H., C. ROHIT, S.E. CHARLES, G.S. MITCHELL, Laboratory assessment of oxygenation in methemoglobinemia, *Clin. Chem.*, 2005, **51**, 434–444.
21. SHIMIZU, S., Y. ENOKI, H. KOHZUKI, Y. OHGA, S. SAKATA, Determination of Hüfner's factor and inactive hemoglobins in human, canine, and murine blood, *Japanese J. Physiol.*, 1986, **36**, 1047–1051.
22. SIGGAARD-ANDERSEN, O., B.N. PEDERSEN, J. REM, Hemoglobin pigments spectrophotometric determination of oxy-, carboxy-, met- and sulfhemoglobin in capillary blood, *Clin. Chim. Acta*, 1972, **42**, 85–100.
23. TURNER, J.A., M.W. McNICOL, R.W. SILLETT, Distribution of carboxyhemoglobin concentrations in smokers and non-smokers, *Thorax*, 1986, **41**, 25–27.

24. UTHAI, T., N. PRASIT, K. CHINNAPHAT, C. SURACHATE, Methemoglobin and sulfhemoglobin levels in students of Walailak University, *Walailak J. Sci. & Tech.*, 2008, **5**, 173–180.
25. VAN KAMPEN, E.J., W.G. ZIJLSTRA, Determination of hemoglobin and its derivatives, *Adv. Clin. Chem.*, 1965, **8**, 140–187.
26. VAN KAMPEN, E.J., W.G. ZIJLSTRA, Spectrophotometry of hemoglobin and hemoglobin derivatives, *Adv. Clin. Chem.*, 1983, **23**, 199–257.
27. WALLACE, W.J., R.A. HOUTCHEN, W.S. MAXWELL, W.S. CAUGHEY, Mechanism of autoxidation for hemoglobins promotion of superoxide production by protons and anions, *J. Biol. Chem.*, 1982, **257**, 4966–4977.
28. WATSON, E.S., A.B. JONES, M.K. ASHFAQ, J. TODD BARRETT, Spectrophotometric evaluation of carboxyhemoglobin in blood of mice after exposure to marijuana or tobacco smoke in a modified Walton horizontal smoke exposure machine, *J. Anal. Toxicol.*, 1987, **11**, 19–23.
29. ZIJLSTRA, W.G., A. BUURSMA, H.E. FALKE, J.F. CATSBURG, Spectrophotometry of hemoglobin: absorption spectra of rat oxyhemoglobin, deoxyhemoglobin, carboxyhemoglobin, and methemoglobin, *Comp. Biochem. Physiol.*, 1994, **107B,** 161–166.
30. ZIJLSTRA, W.G., A. BUURSMA, W.P. MEEUWSEN-VAN DER ROEST, Absorption spectra of human fetal and adult oxyhemoglobin, de-oxyhemoglobin, carboxyhemoglobin, and methemoglobin, *Clin. Chem.*, 1991, **37**, 1633–1638.
31. ZIJLSTRA, W.G., A. BUURSMA, Spectrophotometry of hemoglobin: a comparison of dog and man, *Comp. Biochem. Physiol.*, 1987, **88B**, 251–255.
32. ZIJLSTRA, W.G., A. BUURSMA. Spectrophotometry of hemoglobin: absorption spectra of bovine oxyhemoglobin, deoxyhemoglobin, carboxyhemoglobin, and methemoglobin, *Comp. Biochem. Physiol.*, 1997, **116B**, 743–749.
33. ZOSEL, A., K. RYCHTER, J.B. LEIKIN, Dapsone induced methemoglobinemia: case report and literature review, *Am. J. Ther.*, 2007, **14,** 585–587.
34. ZWART, A., A. BUURSMA, E.J. VAN KAMPEN, W.G. ZIJLSTRA, Multicomponent analysis of hemoglobin derivatives with a reversed-optics spectrophotometer, *Clin. Chem.*, 1984, **30**, 373–379.
35. ZWART, A., A. BUURSMA, E.J. VAN KAMPEN, B. OESEBURG, P.H.W. VAN DER PLOEG, W.G. ZIJLSTRA, A multi-wavelength spectrophotometric method for the simultaneous determination of five hemoglobin derivatives, *J. Clin. Chem. Clin. Biochem.*, 1981, **19**, 457–463.
36. ZWART, A., E.J. VAN KAMPEN, W.G. ZIJLSTRA, Results of routine determination of clinically significant hemoglobin derivatives by multicomponent analysis, *Clin. Chem.*, 1986, **32**, 972–978.
37. * * *, Laboratory Animal Facilities, in *Laboratory Biosafety Manual*, 2nd ed (revised), Geneva, Zwitzerland: World Health Organization, 2003, pp. 22–24.