CONFORMATIONAL STABILITY AGAINST AUTO-OXIDATION FOR MICE AND HUMAN OXYHEMOGLOBINS

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Abstract. The fractions and concentrations of the inactive Hb pigments (such as MetHb, carboxyhemoglobin, HbCO, and sulfohemoglobin, SHb) and the active Hb (in the oxyhemoglobin form) as well as the total blood Hb concentration were determined in human and mice blood, using a newly developed multi-component spectrophotometric method. The conformational stability of mice and human oxyhemoglobins was investigated by measuring the auto-oxidation kinetics of oxyhemoglobin, bioenergetics of this reaction and intrinsic viscosity. The results obtained by using the new method of multi-component spectrophotometric analysis revealed higher concentrations of all inactive Hb derivatives in mice blood, when compared to human blood. To determine whether the increase in methemoglobin (MetHb) is attributed to changes in conformational stability against auto-oxidation, the auto-oxidation kinetics, bioenergetics and intrinsic viscosity were determined. The results revealed difference in conformation between mice and human blood accompanied by changes in intermolecular interactions represented by the slope of the $\eta_{sp}/C = F(C)$ lines and Huggins' constant K', while no measurable change in intrinsic viscosity [η] of Hb was observed. These results indicate a lack of changes in the dimensions and shape of the Hb molecule. Study of the kinetics of oxyhemoglobin (HbO_2) auto-oxidation revealed an increase in the auto-oxidation reaction rate of mice HbO_2 by 58%, when compared to human HbO_2. This increase in auto-oxidation reaction rate was attributed to the decrease in the conformational stability as indicated by the smaller values of E_{act} (137.522 kJ/mole), ($\Delta H_{25}^{0\#}$) (134.047 kJ/mole) and hence the smaller value of $\Delta G_{25}^{0\#}$ (105.098 kJ/mole) for mice HbO_2, compared with those values for native human HbO_2 (E_{act} = 139.118 kJ/mole, $\Delta H_{25}^{0\#}$ = 136.640 kJ/mole and $\Delta G_{25}^{0\#}$ = 106.155 kJ/mole). Therefore, it can be concluded that human HbO_2 is more conformationally stable against auto-oxidation than mice HbO_2.

Key words: conformational stability, auto-oxidation, multi-component spectrophotometric analysis, intrinsic viscosity, hemoglobin.

INTRODUCTION

A higher level of MetHb in rats blood, up to 3%, compared to human, has been reported [16]. This increase in MetHb was attributed to the high rate of auto-

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oxidation reaction of rats HbO_2, which showed a two fold increase as that of human HbO_2. However, the MetHb% in mice blood and the reaction rate of auto-oxidation reaction of mice HbO_2 have not been studied. Therefore, one aim of this paper was to study the conformational stability of mice HbO_2 against auto-oxidation. This could be accomplished by measuring Hb-derivatives, including MetHb, kinetics of HbO_2 auto-oxidation [6, 27, 28], bioenergetics of this reaction [34] and intrinsic viscosity [12, 24, 25]. The Hb derivatives were measured by using a newly developed multi-component spectrophotometic method.

Several methods for the determination of 5-Hb derivatives (SHb, MetHb, HbCO, HbO_2 and deoxyHb), based on spectrophotometric and multi-component spectrophotometic analysis, had been described [2, 7, 8, 23, 36, 37]. Most of these methods based on using simple hemolysate without any efficient clearing procedure. Therefore, these methods suffered from some sources of errors that were not easily overcome. Such sources of errors can be summarized as follows: 1) Turbidity of the Hb hemolysate caused by erythrocytes ghosts, plasma-leukocytes, plasma paraproteins and plasma-lipid aggregates, as in lipemia; 2) Interference that arises from plasma pigments, like bilirubin, carotenes or diagnostic dyes, like Evans blue or cardio green. All these plasma pigments are not accounted for in the calculations and may interfere [23]. Therefore, a second aim of this paper was to develop this technique experimentally to get more accurate results for the inactive Hb-derivatives and the active component (in the HbO_2 form).

MATERIALS AND METHODS

BLOOD COLLECTION AND Hb EXTRACTION

Blood from 8 mice was obtained by decapitation. Eight normal individuals of average age 16 years were selected irrespective of sex. Blood was collected from these individuals by veni-puncture. The blood was collected on heparin. Purified aqueous stock Hb solutions were extracted from the collected blood according to the method described by Trivelli *et al.* [26].

ABSORPTION SPECTRUM OF OXYHAEMOGLOBIN

The absorption spectra and the corresponding spectral data were measured in the UV region for aqueous Hb solutions with a concentration of 3.4×10^{-5}M by using a double-beam UV/VIS spectrophotometer (model 240; Schimadzu Co, Japan). The Hb concentrations are given in terms of the concentration of heme subunits.

VISCOSITY MEASUREMENTS

Viscosity measurements were carried out using a capillary-type Ostwald viscometer with a distilled water flow time of 208 seconds. All the viscosity measurements were carried out at a constant temperature of 20°C. The intrinsic viscosity of Hb in an aqueous solution of pH 5.87 was calculated by means of the Huggins equation [11]. The average of 3 measurements of the flow times of the Hb solution was taken and the deviation from the mean value was found to be less than 0.12 seconds.

CONCENTRATION OF HAEMOGLOBIN SOLUTIONS

The Hb concentration was estimated spectrophotometrically in the cyanomet-hemoglobin, assuming a value of 11 for the millimolar absorption coefficient at $\lambda = 540$ nm for a molecular weight of 16114 Da [30].

AUTO-OXIDATION RATE MEASUREMENTS

Measurement of the auto-oxidation rate was carried out spectrophotometrically as described previously [10], in air-saturated phosphate buffer of pH 7.05 with 0.06 mmol L^{-1} HbO_2 in the temperature range of 25–40 ^{0}C. The reaction was followed by determining the absorbance at wavelength 630 nm (A_{630}) and using the equation:

$$(HbO_2)_0/(HbO_2)_t = (A_\infty - A_0)/(A_\infty - A_t) \quad (1)$$

where A_0, A_t and A_∞ are, respectively, the absorbances at time 0, t and after complete conversion to MetHb ($A_\infty = 3.8\ C$), where 3.8 is the millimolar absorptivity of MetHb, pH = 7.05, at $\lambda = 630$ nm; $C = C_{HbO2} + C_{MetHb}$, and C_{HbO_2} and C_{MetHb} are the HbO_2 and MetHb concentrations, determined by the method of multi-component spectrophotometric analysis, suggested in this paper, for Hb derivative estimation. In this method, the four absorbance values A_{500}, A_{569}, A_{577} and A_{620} were measured for Hb solutions. Then using equations (3) and (5), C_{HbO2} and C_{MetHb} can be determined. Spectral data were measured at the isobestic points (at $\lambda = 525.3$ and 590.7 nm) in order to confirm the absence of hemichrome formation during auto-oxidation rate measurements. Kinetic and thermodynamic analyses were carried out based on the initial rates of auto-oxidation. The activation energy and thermodynamic parameters for activating the auto-oxidation reaction were calculated by means of the Arrhenius and Eyring equations according to the theory of absolute reaction rates [4].

MULTI-COMPONENT SPECTROPHOTOMETRIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF FOUR HEMOGLOBIN DERIVATIVE CONCENTRATIONS

Materials and sample preparation

The measurements were made directly after collecting the blood on heparin. The blood was centrifuged at 3000 rpm for 5 minutes and the plasma was removed. The packed erythrocytes were washed three times with 4-fold phosphate-buffered saline (PBS) to remove the plasma remnant. After each procedure, erythrocyte PBS mixture was centrifuged at 3000 rpm for 5 min. The packed erythrocytes were brought to 1.5-times the original blood volume with ice-cold 1% Triton-X100 to obtain hemolysate. After mixing thoroughly, the hemolysate was centrifuged at 10,000 rpm for 20 minutes to remove erythrocytes ghosts. For absorbance measurements, about 30 μl of the purified hemolysate is added to 5 ml of temperature equilibrated (25 ºC) phosphate buffer (Na_2HPO_4 27.50 mmol/L and KH_2PO_4 13.16 mmol/L, pH 7.28) containing 0.4% Triton-X100. The concentration of Hb at this extreme dilution is about 3.8×10^{-5} M.

Measurements and calculations

The absorbance measurements for the extremely dilute, air saturated Hb solutions were made at four wavelengths (λ = 500, 569, 577 and 620 nm), using a Schimadzu UV/VIS double-beam spectrophotometer (model 240), with a spectral band width of 2.0 nm and a quartz cuvette of 1.0 cm lightpath. A similar cuvette filled with the phosphate buffer containing 0.4% Triton-X100 (diluting reagent) was used as a blank. The absorbances of the blank were measured first using a cleaned quartz cuvette. The absorbances of the extremely dilute Hb solution (3.8×10^{-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 solutions 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 0.005, corresponding to the very low absorbance expected for Hb pigments at this wavelength for Hb samples of low concentration (3.8×10^{-5} M).

The 16 millimolar absorptivities of HbO_2, HbCO, MetHb and SHb, determined previously [29, 35], at four wavelengths (λ = 500, 569, 577 and 620 nm) were substituted into four linear equations of the type described by the theory of multi-component spectrophotometric analysis [29], with the four unknown concentrations of Hb pigments (C_{HbO2}, C_{HbCO}, C_{MetHb} and C_{SHb}), where the visible absorption bands at wavelengths 500, 569, 577 and 620 nm represent the absorption maxima of MetHb, HbCO, HbO_2 and SHb, respectively. This linear system of equations was solved by mathematical manipulation using the Gaussian elimination method for matrix calculation [9], to yield the following equations:

$$C_{\mathrm{SHb}} = \frac{A_{620} - 0.46241784A_{500} + 0.10425144A_{569} + 0.066173573A_{577}}{19.28380181} \tag{2}$$

$$C_{\mathrm{MetHb}} = \frac{7.587561597A_{500} - 2.1061484A_{569} - A_{577} - 24.17452346C_{\mathrm{SHb}}}{56.06121255} \tag{3}$$

$$C_{\mathrm{HbCO}} = \frac{A_{569} - 2.186651145A_{500} + 15.72636593C_{\mathrm{MetHb}} + 6.117605743C_{\mathrm{SHb}}}{2.726668607} \tag{4}$$

$$C_{\mathrm{Hb)2}} = \frac{A_{500} - 5.279C_{\mathrm{HbCO}} - 9.067C_{\mathrm{MetHb}} - 6.502C_{\mathrm{SHb}}}{5.154} \tag{5}$$

where A_{500}, A_{569}, A_{577} and A_{620} are the absorbances measured experimentally at wavelengths 500, 569, 577 and 620 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:

$$C^*_{\mathrm{Hb}} = \sum_{i=1}^{n=4} C\mathrm{i} = C_1 + C_2 \ldots + C_4 \tag{6}$$

where i is the serial number of each Hb component, i.e.

$$C^*_{\mathrm{Hb}} = C_{\mathrm{SHb}} + C_{\mathrm{MetHb}} + C_{\mathrm{HbCO}} + C_{\mathrm{HbO_2}} \tag{7}$$

The fraction of Hb derivatives can be determined from the following equations:

$$F_{\mathrm{SHb}} = \frac{C_{\mathrm{SHb}}}{C^*_{\mathrm{Hb}}} \tag{8}$$

$$F_{\mathrm{MetHb}} = \frac{C_{\mathrm{MetHb}}}{C^*_{\mathrm{Hb}}} \tag{9}$$

$$F_{\mathrm{HbCO}} = \frac{C_{\mathrm{HbCO}}}{C^*_{\mathrm{Hb}}} \tag{10}$$

$$F_{\mathrm{HbO_2}} = \frac{C_{\mathrm{HbO_2}}}{C^*_{\mathrm{Hb}}} \tag{11}$$

The concentrations of Hb pigments (SHb, MetHb and HbCO and the functional or active Hb in the HbO_2 form) in the collected blood may be

determined by multiplying the fraction of each Hb derivative by the total blood Hb concentration. The total blood Hb concentration can be determined by using the following equation:

$$C_{\text{total Hb}} = 251.5 \times 1.6114 \times C^{*}_{\text{Hb}} \text{ g dL}^{-1} \quad (12)$$

where 251.5 is the dilution factor and 1.6114 is the conversion factor for mmol L^{-1} to g dL^{-1}.

The total blood Hb concentration as determined by the multi-component spectrophotometric method by using the last equation was compared with that determined by MetHb cyanide (MetHbCN) method [30].

Data analysis

Data are presented as means ± S.D. Student′s t-test was used for determination of the level of significance of the difference between different groups. The difference is considered significant at $P < 0.05$.

RESULTS

The results of Hb spectral analysis in the UV region are shown in Table 1. The spectral data show a significant increase in the absorbance of the globin band of mice HbO_2, when compared to that of human HbO_2.

Table 1

Absorption spectra of hemoglobins extracted from mice and human blood (at constant heme concentration: 3.4×10^{-5} M)

Animal	Globin band (λ = 275 nm)
Mice	1.238 ± 0.011 *
Human	1.209 ± 0.017

Each value is the mean ± S.D. for four pooled Hb samples.
* Significantly higher than that of human HbO_2 ($p < 0.05$).

The results of intrinsic viscosity [η] and the slope or Huggins' constant K' are shown in Table 2. The data show no significant difference between the intrinsic viscosity of mice and human HbO_2. However, a remarkable increase in the slope of the $\eta_{sp}/C = F(C)$ lines and Huggins' constant K' of mice HbO_2, when compared to those of human HbO_2, was observed.

The results of the reaction rate constant (k_r) of the auto-oxidation for mice and human HbO_2 are shown in Table 3. The results revealed a significant increase in the auto-oxidation rate of mice HbO_2, when compared to human HbO_2.

Table 2

Intrinsic viscosities of hemoglobins extracted from mice and human blood

Animal	Intrinsic viscosity η (dL g^{-1})	Slope (dL^2g^{-2})	Huggins' constant (K')
Mice (n=3)	0.033 ± 0.00029	0.00225 ± 0.00005*	2.039 ± 0.048 *
Human (n=4)	0.0326 ± 0.000115	0.001827 ± 0.0001	1.7206 ± 0.084

Viscosity measurements for aqueous Hb solution pH=5.87, concentration range 0–2.0 g $dL^{-1,}$ at temperature 20°C.
Each value is the mean ± S.D. n: is the number of animals for each group.
* significantly higher than that of human HbO_2 ($p < 0.002$).

The values of thermodynamic parameters for the activation of auto-oxidation for mice and human oxyhemoglobins are illustrated in Table 4. The obtained data revealed that HbO_2 is protected against auto-oxidation by a high energy barrier of approximately (138 KJ mol^{-1}) in terms of the activation energy (E_{act}). The data show also smaller values of E_{act}, $\Delta H_{25}^{0\#}$ and hence the smaller value of $\Delta G_{25}^{0\#}$ for mice HbO_2, compared with those values for native human HbO_2.

Table 3

Auto-oxidation rates for HbO_2 extracted from mice and human blood

Animal	HbO_2 – auto-oxidation rate k_r (h^{-1})
Mice (n=3)	0.0696 ± (0.00472) *
Human (n=3)	0.044 ± (0.00236)

Auto-oxidation kinetics: HbO_2 of concentration 0.06 mmol L^{-1}, in air-saturated 0.1 M phosphate buffer, pH = 7.05, at 37 ºC.
* significantly higher than that of human HbO_2 ($p < 0.002$).

Table 4

Thermodynamic parameters for the activation of auto-oxidation of HbO_2 extracted from mice and human blood

Animal	E_{act} (kJ/mole)	$\Delta H_{25}^{0\#}$ (kJ/mole)	$\Delta S_{25}^{0\#}$ (J/mole K)	$\Delta G_{25}^{0\#}$ (kJ/mole)
Mice	137.522	134.047	100.487	105.098
Human	139.118	136.640	102.298	106.155

Auto-oxidation kinetics: HbO_2 of concentration 0.06 mmol L^{-1}, in air-saturated 0.1 M phosphate buffer, pH = 7.05, at 25, 31, 34, 37, and 40 ºC.

The percentage values of Hbs with different ligands (SHb, MetHb, HbCO and HbO_2) in human blood are shown in Table 5. Values of SHb% in the range (0.0727–0.370%) and MetHb% (0.43–1.0%) and HbCO% (0.4–1.52%) and HbO_2% (97.06–98.62%) were observed. The values of total blood Hb

concentration as determined by the multi-component spectrophotometric method and the MetHbCN method are shown in Table 6. A complete agreement between the results of both methods is observed.

Table 5

Percentages of inactive hemoglobins and the active Hb (in the HbO_2 form) in human blood

Sample number	SHb (%)	MetHb (%)	HbCO (%)	HbO_2 (%)
1	0.0727	0.7431	0.8257	98.3585
2	0.2989	0.671	0.4033	98.6269
3	0.3615	1.0588	1.520	97.0587
4	0.2105	0.8988	1.1116	97.7791
5	0.2380	08560	0.4688	98.4371
6	0.2070	0.4349	1.4718	97.8863
7	0.2984	0.3869	1.3359	97.9787
8	03702	0.7824	1.3880	97.4593

The mean percentage values of Hbs with different ligands (SHb, MetHb, HbCO and HbO_2) in mice and human blood are shown in Table 7. Significant increases in the percentages of all inactive Hb-pigments (SHb, MetHb and HbCO) concomitant with a significant decrease in HbO_2% are observed in mice blood, when compared to human blood.

Table 6

The total Hb concentration in human blood as determined by the mult-component spectrophotometric method, in comparison to the MetHbCN method

Sample number	Total Hb concentration by the multi-component method (g dL^{-1})	Total Hb concentration by the MetHbCN method (g dL^{-1})
1	13.990	13.4942
2	12.6082	12.722
3	14.7234	14.6708
4	16.777	15.958
5	16.550	16.7299
6	16.2087	16.3254
7	16.6811	16.7300
8	16.4719	16.3260

Table 7

Percentages of inactive hemoglobins and the active Hb (in the HbO_2 form) in human and mice blood

Animal	SHb (%)	MetHb (%)	HbCO (%)	HbO_2 (%)
Mice (n=5)	0.686±0.087 *	2.175±0.581 *	3.93±0.284 *	93.2±0.492 *
Human (n=8)	0.257±0.0972	0.7289±0.228	1.0656±0.447	97.948±0.525

* $p < 0.00005$.

Each value is the mean ± S.D. n is the number of animals for each group.

DISCUSSION AND CONCLUSIONS

The significant reduction in the absorption of the mice Hb globin band (λ = 275 nm), when compared to human Hb, probably reflects a difference in the aggregation state and the local environment, which in turn indicates some conformational differences of the Hb protein moiety. These conformational differences are indicated by the viscosity measurements.

The results for the intrinsic viscosity of Hb indicate a lack of differences in the dimensions and shape of mice and human Hb molecules. The slope of the $\eta_{sp} / C = F(C)$ lines and Huggins' constant K' suggest some increases in intermolecular interaction of mice Hb, as compared to human Hb.

Similar changes in intrinsic viscosity and the slope of the $\eta_{sp}/C = F(C)$ lines were observed in the tertiary conformational transitions occurring in Hb oxygenation [12] and oxidation [24, 25]. The observed results for intrinsic viscosity and intermolecular interactions suggest some differences in the tertiary conformation between mice and human Hbs.

As demonstrated previously, the tertiary or quaternary conformation of the Hb molecule is related to its molecular stability against auto-oxidation [13, 15, 17–19, 27]. This means that the rate of auto-oxidation of HbO_2 to MetHb depends highly on the tertiary conformation of the Hb molecule. The conformational differences in the Hb protein moiety between mice and human Hbs may be reflected in its stability against auto-oxidation.

A normal physiological function of Hb is the reversible binding of O_2 which can occur with the haem iron in the reduced (ferrous) state. In human erythrocytes, HbA undergoes auto-oxidation to inactive Hb (MetHb) with the heme iron in the ferric state at a rate of about 3% per day [14]. The level of MetHb in the red cell depends on the rate of auto-oxidation of HbO_2 and the efficiency of the MetHb-reductase of the red cell [1].

In vitro, the Fe-O_2 bond of the HbO_2 molecule is inherently stable and so O_2 is unlikely to dissociate spontaneously. Oxygen is a rather poor single-electron acceptor, so a considerable thermodynamic barrier exists for such electron transfer. An additional thermodynamic barrier is provided by the high hydrophobicity of the amino acid residues constituting the heme pocket, which can protect the Fe-O_2 centre from easy access of a water molecule and the formation of an activated complex, reducing the extent of H_2O-promoted auto-oxidation of HbO_2. However, in vivo, it becomes evident that the Fe-O_2 bond is always subject to nucleophilic attack by an entering water molecule, with or without proton catalysis, and to attack by an entering hydroxide anion. These can cause irreversible oxidation of the Fe-O_2 group to the Met species with generation of the superoxide anion radical. This mechanism of HbO_2 auto-oxidation proposed previously [6, 16, 28, 31, 32, 34] is known as proton- and anion-promoted auto-oxidation of Hb.

Hemoglobin has thus evolved with a globin moiety that can protect the Fe-O_2 center from easy access by a water molecule or its conjugate ions OH^- and H^+. It also becomes evident that, even in these native proteins, the globin moiety has not yet attained a maximal ability to block entering water molecules from the Fe-O_2 centre. Nevertheless, the relative stability of the HbO_2, which is protected against auto-oxidation by a high activation energy barrier (E_{act}) of 138 kJ mol^{-1} provides the basis for Hb function in vivo. This functional stability of Hb is known to be easily lost on denaturation. Therefore, it must be linked to the integrity of the globin moiety so that it can act as a breakwater [21].

The smaller values of E_{act}, $\Delta H_{25}^{0\#}$ and hence the smaller value of $\Delta G_{25}^{0\#}$ (105 kJ mol^{-1}) for mice HbO_2, compared with those values for native human HbO_2, may account for the higher rate of auto-oxidation of mice HbO_2 compared with human HbO_2. This high rate of auto-oxidation may account for the relatively higher MetHb level in albino mice (mean value = 2.2%) compared with that in humans (0.73%). This is attributed also to the low activity of superoxide dismutase in albino mice [5].

The results for HbCO percentage reveal a high HbCO content in the blood of mice (2.0–4.0%), compared to the low values in human blood (0.4–1.5%). Similar high HbCO levels in mice blood (values up to 3.8%) have been reported previously [33]. These high values of MetHb% and HbCO% in mice blood may account for the observed decrease in the oxygen-carrying capacity of mice blood Hb (HbO_2), when compared to human blood.

The newly developed method suggested here for Hb derivative estimation is based on the principle of multi-component spectrophotometric analysis, taking into account all the absorption contributions of all Hb derivatives. The four absorbance values were measured for extremely dilute Hb solution. Thus, the sources of error arising from light scattering through aggregation of Hb molecules are highly eliminated. At this extreme dilution, under air-saturated conditions, complete conversion of deoxyHb to HbO_2 (i.e. full oxygenation) shoud result [3, 20, 31], providing the possibility for determining the concentration of active or functional Hb (in the HbO_2 form). Since we can neglect the fifth component (DeoxyHb), in this extremely diluted Hb solution,under air saturated condition, the concentrations and fractions of other four Hb derivatives (SHb, MetHb, HbCO, and HbO_2) can be determined, based on 4-absorbance measurements, at λ = 500, 569, 577, and 620 nm, respectively.

The newly developed multi-component spectrophotometric method, suggested here in this work, is provided with an efficient clearing procedure. By means of this clearing procedure, the sources of error arising from scattering particles (such as erythrocyte ghosts, plasma leukocytes, plasma paraproteins and plasma lipid aggregates, such as found in lipaemia) [23] were highly eliminated. It also prevents the interference that arises from plasma pigments (such as bilirubin, carotenes) or diagnostic dyes (such as Evans blue or cardio green) [1]. These errors have not easily overcome in previous methods [2, 7, 8, 23, 36, 37]. Moreover, this

method has yielded values of SHb%, MetHb%, HbCO% of 0.073–0.370, 0.43–1.0% and 0.4–1.5%, respectively, in human blood, which are in agreement with many previous results [22, 23]. Furthermore, this method has yielded values of the total blood Hb concentration which are in complete agreement with those determined by using MetHbCN method.

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