MULTI-COMPONENT SPECTROPHOTOMETRIC DETERMINATION OF INACTIVE AND ACTIVE HEMOGLOBINS IN RAT AND MICE BLOOD

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Abstract. The fractions and concentrations of the inactive Hb pigments (such as sulfhemoglobin, SHb, methemoglobin, MetHb, carboxyhemoglobin, HbCO) and the active Hb (in the HbO_2 form) as well as the total blood Hb concentration were determined in mice and rat blood, using newly developed multi-component spectrophotometric methods. These methods have yielded values of MetHb% up to 3.514% and 2.914 in mice and rat blood, respectively. These methods have also yielded values of HbCO% up to 6.581% and 8.992% in mice and rat blood, respectively. The results revealed also values of SHb% up to 0.674% and 0.374% in mice and rat blood, respectively. The results revealed values of HbO_2% up to 93.842% and 92.103% in mice and rat blood, respectively. Furthermore, these methods have yielded values of the mice's total blood Hb concentration up to 15.994 g dL^{-1}, which are relatively higher than those values of rats (up to 12.410 g dL^{-1}). Moreover, these methods have yielded values of the mice's blood HbO_2 concentration up to 14.682 g dL^{-1}, which are relatively higher than the values of rats (up to 11.398 g dL^{-1}). In conclusion, the results obtained by the methods suggested here for Hb-derivatives determinations showed significantly higher values of HbCO% concomitant with significant lower values of the SHb% in rat blood, when compared to those values of mice blood. The results showed also insignificant differences in MetHb% and HbO_2% between mice and rat blood. The results revealed also insignificant differences in total Hb-concentration and HbO_2 concentration between mice and rats.

Key words: multi-component spectrophotometric analysis, hemoglobin, sulfhemoglobin, methemoglobin, carboxyhemoglobin, oxyhemoglobin, mice, rats.

INTRODUCTION

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 [5, 7, 8, 14, 22, 23]. 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,

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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 therefore interfere [14]. More recently, we have developed this technique theoretically and experimentally for determination of inactive and active hemoglobins in human [1, 2], bovine and canine blood [3]. However, significant differences in the millimolar absorpitivities between human, and bovine [20] and canine Hb-derivatives [19] and between human and rat Hb-derivatives [21] have been reported. These differences make the equations used for determination of Hb-derivatives in human, bovine and canine blood inapplicable for determination of these derivatives in rats and mice blood. Therefore, the aim of this paper was to develop this technique experimentally and theoretically to get more accurate results for the inactive Hb-derivatives (SHb, MetHb and HbCO) and the active component (in the HbO_2 form) in the blood of rats and mice, respectively.

MATERIALS AND METHODS

ANIMALS AND BLOOD COLLECTION

Blood from 7 healthy rats and 7 healthy mice of either sex was collected into heparinized tubes.

MULTI-COMPONENT SPECTROPHOTOMETRIC METHODS FOR THE SIMULTANEOUS DETERMINATION OF FOUR HEMOGLOBIN DERIVATIVES 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. In the case of mice, the packed erythrocytes were brought to 4 ml with ice-cold distilled water to obtain hemolysate. With respect to rats, the packed erythrocytes were brought to 3 times the original blood volume with ice-cold distilled water. After mixing well and waiting for erythrolysis to occur (3 min), one time of the original blood volume of ice-cold phosphate buffer (Na_2HPO_4 27.50 mmol/L and KH_2PO_4 13.16 mmol/L, pH 7.2) was added to obtain hemolysate. After mixing thoroughly, the hemolysate was centrifuged at 10,000 rpm for 20 minutes to remove erythrocytes ghosts. For absorbance measurements in the case of mice, the absorbances at λ =578 nm of

Hb-solutions were adjusted to values in the range (0.550–0.6), by using 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. For absorbance measurements in the case of rats, about 120 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 7.0) containing 0.4% Triton-X100. The concentration of Hb at this extreme dilution is in the range $3.5–4.4\times10^{-5}$ M.

Measurements and calculations

The absorbance measurements for the extremely dilute, air saturated Hb solutions were made at four wavelengths (λ = 500, 568, 576 and 620 nm) in the case of rats, and (λ = 500, 569, 577 and 620 nm) in the case of mice, 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. Then the absorbances of the extremely dilute Hb solution ($3.5–4.4\times10^{-5}$ M) were measured against air as a reference using the same blank cuvette without any further washing or cleaning. The absorbances A_{500}, A_{568}, A_{576} and A_{620} of the Hb solutions for rats, and A_{500}, A_{569}, A_{577} and A_{620} for mice, 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 ($3.1–4.4 \times 10^{-5}$ M).

The 16 millimolar absorptivities of HbO_2, HbCO, MetHb and SHb determined previously for mice [16] and rats [21], at four wavelengths (λ = 500, 568, 576 and 620 nm in the case of rats) and (λ = 500, 569, 577 and 620 nm in the case of mice) were substituted into four linear equations of the type described by the theory of multi-component spectrophotometric analysis [16], 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, 568 or 569, 576 or 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 for mice:

$$C_{\mathrm{SHb}} = \frac{A_{620} - 0.442293A_{500} + 0.1065519A_{569} + 0.0515769A_{577}}{18.896336} \quad (1)$$

$$C_{\mathrm{MetHb}} = \frac{9.0602343A_{500} - 2.6960235A_{569} - A_{577} - 35.295898C_{\mathrm{SHb}}}{66.750821} \quad (2)$$

$$C_{\mathrm{HbCO}} = \frac{A_{569} - 2.2316831A_{500} + 16.074415C_{\mathrm{MetHb}} + 7.9681188C_{\mathrm{SHb}}}{2.330495} \quad (3)$$

$$C_{\mathrm{HbO_2}} = \frac{A_{500} - 5.35C_{\mathrm{HbCO}} - 9.04C_{\mathrm{MetHb}} - 7.2C_{\mathrm{SHb}}}{5.05} \quad (4)$$

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 equations for rats were:

$$C_{\mathrm{SHb}} = \frac{A_{620} - 0.442360357A_{500} + 0.071415147A_{568} + 0.082292833A_{576}}{18.97374035} \quad (5)$$

$$C_{\mathrm{MetHb}} = \frac{5.441219158A_{500} - 1.238509918A_{568} - A_{576} - 21.47812966C_{\mathrm{SHb}}}{40.42875665} \quad (6)$$

$$C_{\mathrm{HbCO}} = \frac{A_{568} - 2.007518797A_{500} + 14.15759398C_{\mathrm{MetHb}} + 6.366887218C_{\mathrm{SHb}}}{3.419097744} \quad (7)$$

$$C_{\mathrm{HbO_2}} = \frac{A_{500} - 5.44C_{\mathrm{HbCO}} - 8.99C_{\mathrm{MetHb}} - 6.502C_{\mathrm{SHb}}}{5.32} \quad (8)$$

where A_{500}, A_{568}, A_{576} and A_{620} are the absorbances measured experimentally at wavelengths 500, 568, 576 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:

$$C^*_{\mathrm{Hb}} = \sum_{i=1}^{n=4} C\mathrm{i} = C_1 + C_2 \ldots + C_4 \quad (9)$$

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

$$C^*_{\mathrm{Hb}} = C_{\mathrm{SHb}} + C_{\mathrm{MetHb}} + C_{\mathrm{HbCO}} + C_{\mathrm{HbO_2}} \tag{10}$$

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

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

$$F_{\mathrm{MetHb}} = \frac{C_{\mathrm{MetHb}}}{C^*_{\mathrm{Hb}}} \tag{12}$$

$$F_{\mathrm{HbCO}} = \frac{C_{\mathrm{HbCO}}}{C^*_{\mathrm{Hb}}} \tag{13}$$

$$F_{\mathrm{HbO_2}} = \frac{C_{\mathrm{HbO_2}}}{C^*_{\mathrm{Hb}}} \tag{14}$$

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 total blood Hb concentration. The total blood Hb concentration for mice was measured according to the MetHb cyanide (MetHbCN) method [15], while those of rats can be determined by the multi-component spectrophotometric method, by using the following equation:

$$C_{\text{total Hb}} = 170.666 \times 1.6114 \times C^*_{\mathrm{Hb}}\ g\ \mathrm{d}L^{-1} \tag{15}$$

where 170.666 is the dilution factor and 1.6114 are the conversion factors for mmol L^{-1} to g dL^{-1}.

Data analysis

Data are presented as means ± S.D. Mann-Whitney and Student′s t-independent groups tests were used for determination of the level of significance of the difference between the two groups (SPSS for Window version 14.0 and Statistics Calculator) . The difference is considered significant at $P < 0.05$.

RESULTS

The percentage values of Hbs with different ligands (SHb, MetHb, HbCO and HbO_2) in normal mice and rat blood are shown in Tables 1, 2, respectively. Values of SHb% in the ranges (0.015–0.674%) and (0.007–0.374%) are observed in mice blood and rat blood, respectively. Values of MetHb% in the ranges (0.613–3.514%) and (1.118–2.914%) are observed in mice blood and rat blood,

respectively. Values of HbCO% in the ranges (3.857–6.581%) and (6.646–8.992%) are observed in mice blood and rat blood, respectively. Values of HbO_2% in the ranges (89.744–93.842%) and (88.026–92.103%) are observed in mice blood and rat blood, respectively.

Table 1

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

Sample number	SHb (%)	MetHb (%)	HbCO (%)	HbO_2 (%)
1	0.531	2.057	3.857	93.555
2	0.491	3.514	5.603	90.391
3	0.375	1.715	6.115	91.795
4	0.015	2.048	5.839	92.098
5	0.674	2.999	6.581	89.744
6	0.475	0.794	4.887	93.842
7	0.468	0.613	5.974	92.943

Table 2

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

Sample number	SHb (%)	MetHb (%)	HbCO (%)	HbO_2 (%)
1	0.018	1.154	6.725	92.103
2	0.374	2.358	7.788	89.479
3	0.133	2.168	7.258	90.440
4	0.007	2.726	7.814	89.454
5	0.068	2.914	8.992	88.026
6	0.100	1.118	6.872	91.910
7	0.101	1.411	6.646	91.842

The mean percentage values of Hbs with different ligands (SHb, MetHb, HbCO and HbO_2) in mice and rat blood are shown in Table 3. Significant decrease in the percentage of SHb concomitant with a highly significant increase in HbCO% in rat blood, when compared to mice blood, are observed. Whereas, insignificant differences in MetHb% and HbO_2 % between the two groups are observed.

Table 3

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

Animal	SHb (%)	MetHb (%)	HbCO (%)	HbO_2 (%)
Mice (n = 7)	0.433±0.205	1.963±1.060	5.551±0.909	92.053±1.550
Rats (n = 7)	0.114±0.123*	1.978±0.748	7.442±0.833**	90.465±1.560

* $P < 0.02$ by the Mann-Whitney test. ** $P < 0.002$ by the Student′s t-test.
Each value is the mean ± S.D. n is the number of animals for each group.

The values of total blood Hb concentration and the total concentration of active Hb (in the HbO_2 form), as determined by the MetHbCN and multi-component spectrophotometric methods, for mice and rats are shown in Tables 4,

5, respectively. Values of total blood Hb concentration in the ranges (10.075–15.994 g dL^{-1}) and (12.190–12.410 g dL^{-1}) are observed in mice and rats, respectively. Values of the HbO_2 concentration in the ranges (9.425–14.682 g dL^{-1}) and (10.836–11.398 g dL^{-1}) are observed in mice and rats, respectively.

The mean percentage values of total blood Hb concentration and the total concentration of active Hb (in the HbO_2 form), as determined by the MetHbCN and multi-component spectrophotometric methods, for mice and rats are shown in Table 6. Insignificant differences in both concentrations between the two groups are observed.

Table 4

The total Hb concentration and HbO_2 concentration in mice blood as determined by the MetHbCN and multi-component spectrophotometric methods

Sample number	**Total Hb concentration (g dL^{-1})**	**HbO_2 concentration (g dL^{-1})**
1	10.075	9.425
2	12.317	11.134
3	15.994	14.682
4	10.369	9.549
5	15.002	13.463
6	11.362	10.662
7	11.509	10.696

Table 5

The total Hb concentration and HbO_2 concentration in rat blood as determined by the multi-component spectrophotometric method

Sample number	**Total Hb concentration (g dL^{-1})**	**HbO_2 concentration (g dL^{-1})**
1	12.251	11.284
2	12.200	10.916
3	12.190	11.025
4	12.210	10.922
5	12.310	10.836
6	12.350	11.351
7	12.410	11.398

Table 6

The total Hb concentration and HbO_2 concentration in mice and rat blood as determined by the MetHbCN and multi-component spectrophotometric methods

Animal	**Total Hb concentration (g dL^{-1})**	**HbO_2 concentration (g dL^{-1})**
Mice (n = 7)	12.375±2.276	11.373±1.977
Rats (n = 7)	12.274±0.084 *	11.105±0.233 *

* Non significant differences between the two groups by the Student′s t-test.
Each value is the mean ± S.D. n is the number of animals for each group.

DISCUSSION AND CONCLUSIONS

The newly developed methods, suggested here for Hb derivatives 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 an 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 [6, 12, 17], 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 conditions, 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, 568, 576 and 620 nm in the case of rats, and at λ = 500, 569, 577 and 620 nm in the case of mice, respectively.

The newly developed multi-component spectrophotometric methods, suggested here in this work are 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) [14] 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) [14]. These errors were not easily overcome in previous methods [5, 7, 8, 14, 22, 23].

These methods have yielded values of MetHb% in mice blood up to 3.514%, and in rat blood up to 2.914%, which are higher than those values in human blood (< 1.0%) [1, 2, 4, 13, 14]. Similar high levels of MetHb% in rat blood, as compared to human blood, have been reported previously [10] and were attributed to the high rate of autoxidation of rat oxyhemoglobin [10]. These methods have also yielded values of HbCO% in mice blood up to 6.581%. Similar high levels of HbCO in normal mice blood [18] and human blood [11] have been reported. The results obtained by the rat's method revealed values of HbCO% up to 8.992%, which are relatively higher than those values in mice blood and human blood [1, 2, 4, 11, 13, 14]. The results obtained by the mice's method revealed values of SHb% up to 0.674%, which are relatively higher than those values in rat blood and human blood [1, 2, 14], respectively. The results obtained by these methods revealed values of HbO_2% up to 93.842% and 92.103% in mice and rat blood, respectively. Furthermore, these methods have yielded values of the mice's total blood Hb concentration up to 15.994 g dL^{-1}, which are relatively higher than those values of rats (up to 12.410 g dL^{-1}) and in complete agreement with the human values [1]. Moreover, these methods have yielded values of the mice's blood HbO_2 concentration up to 14.682 g dL^{-1}, which are relatively higher than the values of rats (up to 11.398 g dL^{-1}). HbO_2 concentration is the actual measure of the degree of anemia [2].

In conclusion, the results obtained by the methods suggested here for Hb-derivatives determinations showed significantly higher values of HbCO% concomitant with significant lower values of the SHb in rat blood, when compared to those values of mice blood. The results of these methods also showed insignificant differences in MetHb% and HbO_2% between mice and rat blood. The results of this study also revealed insignificant differences in total Hb-concentration and HbO_2 concentration between mice and rats.

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