MULTI-COMPONENT SPECTROPHOTOMETRIC DETERMINTION OF INACTIVE AND ACTIVE HEMOGLOBINS IN CANINE AND BOVINE 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 oxyhemoglobin form) as well as the total blood Hb concentration were determined in canine and bovine blood, using newly developed multi-component spectrophotometric methods. The results obtained by the cow's method revealed values of MetHb% up to 4.607% and of HbCO% up to 4.814%, which are relatively higher than those values in canine blood (up to 1.385% and 3.0%, respectively). These high values of MetHb% and HbCO% in bovine blood may account for the observed decrease in the oxygen-carrying capacity of bovine blood Hb (HbO_2%), when compared to canine blood. Furthermore, these methods have yielded values of the cow's total blood Hb concentration up to 16.161 g dL^{-1} which are relatively lower than those values of dogs (up to 19.992 g dL^{-1}). Moreover, these methods have yielded values of the cow's blood HbO_2 concentration up to 15.233 g dL^{-1} which are relatively lower than those values of dogs (up to 19.599 g dL^{-1}). In conclusion, the results obtained by the methods suggested here for Hb-derivatives determinations showed significantly higher values of inactive Hbs % (MetHb % and HbCO %) concomitant with significant lower values of the active Hb (in the HbO_2 form) in bovine blood, when compared to those values of canine blood. The results of these methods showed also significantly higher values of the total blood Hb-concentration and HbO_2 concentration in dogs than in cows.

Key words: multi-component spectrophotometric analysis, hemoglobin, sulfhemoglobin, methemoglobin, carboxyhemoglobin, oxyhemoglobin, cows, dogs.

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, have been described [4, 6, 7, 12, 17, 18]. 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:

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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 therefore interfere [12]. More recently, we have developed this technique theoretically and experimentally for determination of inactive and active hemoglobins in human blood [1, 2]. However, significant differences in the millimolar absorptivities between human and bovine Hb-derivatives [16] and between human and canine Hb-derivatives [15] have been reported. These differences make the equations used for determination of Hb-derivatives in human blood inapplicable for determination of these derivatives in bovine and canine 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 cows and dogs, respectively.

MATERIALS AND METHODS

ANIMALS AND BLOOD COLLECTION

Blood from 7 healthy cows was collected by veni-puncture and withdrawn into EDTA containing tubes. Blood from 7 healthy dogs of either sex was collected by veni-puncture and withdrawn 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 EDTA in the case of cows and on heparin in the case of dogs. 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 7.15 in the case of dogs and 7.25 in the case of cows) containing 0.4% Triton×100. The concentration of Hb at this extreme dilution is in the range $3.1–4.9\times10^{-5}$ 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), 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 ($3.1–4.9\times10^{-5}$ 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 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 ($3.1–4.9 \times 10^{-5}$ M).

The 16 millimolar absorptivities of HbO_2, HbCO, MetHb and SHb determined previously for cows [16] and dogs [15], at four wavelengths (λ = 500, 568, 576 and 620 nm) were substituted into four linear equations of the type described by the theory of multi-component spectrophotometric analysis [13], 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, 576 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 [8], to yield the following equations for cows:

$$C_{\mathrm{SHb}} = \frac{A_{620} - 0.453094995A_{500} + 0.06954985A_{568} + 0.090090228A_{576}}{18.93529402} \quad (1)$$

$$C_{\mathrm{MetHb}} = \frac{5.050968372A_{500} - 1.065932198A_{568} - A_{576} - 20.09457368C_{\mathrm{SHb}}}{40.06395646} \quad (2)$$

$$C_{\mathrm{HbCO}} = \frac{A_{568} - 1.998076923A_{500} + 15.16176923C_{\mathrm{MetHb}} + 6.305496154C_{\mathrm{SHb}}}{3.620269231} \quad (3)$$

$$C_{\mathrm{HbO_2}} = \frac{A_{500} - 5.34C_{\mathrm{HbCO}} - 9.48C_{\mathrm{MetHb}} - 6.502C_{\mathrm{SHb}}}{5.20} \quad (4)$$

The equations for dogs were:

$$C_{\mathrm{SHb}} = \frac{A_{620} - 0.431445425A_{500} + 0.064983115A_{568} + 0.091806782A_{576}}{19.05517307} \quad (5)$$

$$C_{\mathrm{MetHb}} = \frac{4.90821089A_{500} - 1.008517325A_{568} - A_{576} - 19.55024037C_{\mathrm{SHb}}}{38.97138399} \quad (6)$$

$$C_{\mathrm{HbCO}} = \frac{A_{568} - 2.019120459A_{500} + 15.4389675C_{\mathrm{MetHb}} + 6.442321224C_{\mathrm{SHb}}}{3.650191205} \quad (7)$$

$$C_{\mathrm{HbO_2}} = \frac{A_{500} - 5.22C_{\mathrm{HbCO}} - 9.36C_{\mathrm{MetHb}} - 6.502C_{\mathrm{SHb}}}{5.23} \quad (8)$$

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.

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

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

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

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

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

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

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 cows and dogs can be determined by using the following two equations, respectively:

$$C_{\text{total Hb}} = 251.5 \times 1.6133 \times C^{*}_{\text{Hb}} \text{ g dL}^{-1} \quad (15)$$

$$C_{\text{total Hb}} = 251.5 \times 1.6114 \times C^{*}_{\text{Hb}} \text{ g dL}^{-1} \quad (16)$$

where 251.5 is the dilution factor and 1.6133 and 1.6114 are the conversion factors for mmol L^{-1} to g dL^{-1} for cows and dogs, respectively.

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 canine and bovine blood are shown in Tables 1 and 2, respectively. Values of SHb% in the ranges (0.043–0.309%) and (0.124–0.714%) are observed in canine blood and bovine blood, respectively. Values of MetHb% in the ranges (0.023–1.385%) and (1.509–4.607%) are observed in canine blood and bovine blood, respectively. Values of HbCO% in the ranges (1.453–3.076%) and (3.107–4.814%) are observed in canine blood and bovine blood, respectively. Values of HbO_2% in the ranges (95.230–98.057%) and (90.132–94.992%) are observed in canine blood and bovine blood, respectively. The mean percentage values of Hbs with different ligands (SHb, MetHb, HbCO and HbO_2) in canine and bovine blood are shown in Table 3. Highly significant increases in the percentages of MetHb and HbCO concomitant with a highly significant decrease in HbO_2% are observed in bovine blood, when compared to canine blood. Whereas, an insignificant difference in SHb% between the two groups was observed.

Table 1

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

Sample number	SHb (%)	MetHb (%)	HbCO (%)	HbO_2 (%)
1	0.241	0.235	1.534	97.989
2	0.215	0.265	1.825	97.694
3	0.043	0.023	1.876	98.057
4	0.083	0.034	1.851	98.032
5	0.221	0.183	1.771	97.825
6	0.196	0.381	1.453	97.969
7	0.309	1.385	3.076	95.230

Table 2

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

Sample number	SHb (%)	MetHb (%)	HbCO (%)	HbO_2 (%)
1	0.293	1.608	3.107	94.992
2	0.124	3.313	4.814	91.748
3	0.714	3.290	4.127	91.869
4	0.514	4.607	4.746	90.132
5	0.148	1.509	4.086	94.256
6	0.501	2.770	4.145	92.584
7	0.419	3.301	4.470	91.808

Table 3

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

Animal	SHb (%)	MetHb (%)	HbCO (%)	HbO_2 (%)
Dogs (n = 7)	0.187±0.093	0.358±0.470	1.912±0.539	97.542±1.028
Cows (n = 7)	0.388±0.213	2.914±1.081*	4.214±0.572**	92.484±1.651**

* $P < 0.0001$ by the Mann-Whitney test. ** $P < 0.00001$ 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 multi-component spectrophotometric methods, for dogs and cows are shown in Tables 4, 5, respectively. Values of total blood Hb concentration in the ranges (16.550–19.992 g dL^{-1}) and (11.394–16.161 g dL^{-1}) are observed in dogs and cows, respectively. Values of the HbO_2 concentration in the ranges (16.190–19.599 g dL^{-1}) and (10.274–15.233 g dL^{-1}) are observed in dogs and cows, 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 multi-component spectrophotometric methods, for dogs and cows are shown in Table 6. Highly significant increases in both concentrations are observed in dogs, when compared to cows.

Table 4

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

Sample number	Total Hb concentration (g dL^{-1})	HbO_2 concentration (g dL^{-1})
1	19.403	19.013
2	18.085	17.668
3	19.987	19.599
4	17.353	17.012
5	16.550	16.190
6	19.769	19.367
7	19.992	19.038

Table 5

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

Sample number	Total Hb concentration (g dL^{-1})	HbO_2 concentration (g dL^{-1})
1	12.686	12.051
2	12.892	11.828
3	13.010	11.970
4	11.399	10.274
5	16.161	15.233
6	13.015	12.050
7	11.394	10.461

Table 6

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

Animal	Total Hb concentration (g dL^{-1})	HbO_2 concentration (g dL^{-1})
Cows (*n*=7)	12.937±1.593	11.981±1.624
Dogs (*n*=7)	18.734±1.401*	18.270±1.315*

* $P < 0.00001$ 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 are based on the principle of multi-component spectrophotometric analysis, taking into account all the absorption contributions of all Hb derivatives. The three 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) should result [5, 10, 14], 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, 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) [12] 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 been easily overcome in previous methods [4, 6, 7, 12, 17, 18].

This method has yielded values of MetHb% in dog blood up to 1.385%, which is slightly higher than those values in human blood (< 1.0%) [1, 2, 3, 11, 12]. These methods have yielded values of HbCO% in dog blood up to 3%, which are in agreement with normal values in human blood [9]. The results obtained by the cow's method revealed values of MetHb% up to 4.607% and of HbCO% up to 4.814%, which are relatively higher than those values in canine and human blood [1, 2, 3, 11, 12]. These high values of MetHb% and HbCO% in bovine blood can account for the observed decrease in the oxygen-carrying capacity of bovine blood Hb (HbO_2%), when compared to canine blood. Furthermore, these methods has yielded values of the cow's total blood Hb concentration up to 16.161 g dL^{-1}, which are relatively lower than those values of dogs (up to 19.992 g dL^{-1}) and in complete agreement with those human values [1]. Moreover, these methods have yielded values of the cow's blood HbO_2 concentration up to 15.233 g $dL^{-}1$, which are relatively lower than those values of dogs (up to 19.599 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 inactive Hbs % (MetHb % and HbCO %) concomitant with significant lower values of the active Hb (in the HbO_2) in bovine blood, when compared to those values of canine blood. The results of these methods showed also significantly higher values of the total blood Hb-concentration and HbO_2 concentration in dogs than in cows.

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