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Research Article

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Theoretical and Experimental Contributions about the Usual Application of the Pseudo-First-Order Model in Kinetic Analysis of Oxyhemoglobin Deoxygenation

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Abstract The reaction of hemoglobin with oxygen allows oxygenation of the body, ensuring homeostasis and cell survival. Although it occurs in four reversible, successive, and cooperative steps, the reaction is usually treated as a one-step reaction. This article aims to point out experimental and theoretical aspects relevant to the kinetic study of this reaction, but which are generally not taken into account. Just the oxyhemoglobin deoxygenation is discussed, since this step is easier to perform experimentally when compared to oxygenation. Oxyhemoglobin was obtained from the reduction of meta-hemoglobin by size exclusion liquid chromatography using sodium dithionite. Deoxygenation of oxyhemoglobin was performed by rapid mixing with interrupted flow, also using sodium dithionite, but in the absence of oxygen. From the experimental results, the dissociation constant was determined considering the pseudo-first order model. Subsequently, experimental questions were discussed, such as the contribution of at least two hemoglobin conformations in the measured absorbance and theoretical questions, such as limitations of the first order pseudo model. The results indicate that a more complete model is needed to describe oxyhemoglobin deoxygenation as well as a more sophisticated treatment in the experimental data, considering that more than one hemoglobin conformation has a significant absorbance value at a given wavelength.

Keywords oxyhemoglobin; deoxygenation;rapid mixing method; pseudo-first order kinetics

1. Introduction

The reaction of hemoglobin with oxygen is essential for maintenance of cellular metabolism [1]. Therefore, the understanding of its mechanism plays a primordial role in the prevention of various human pathologies, besides contributing to the development of several studies, such as the elaboration of blood viable substitutes [2] and the formulation of new therapeutic approaches [3,4].

The mechanism of this reaction, presented in Figure 1, was proposed in 1960 by Antonini e Gibson. The authors assumed that the reaction occurs in four reversible, successive and cooperative steps [5]. Gibson [6] was the first and the only one researcher to estimate the eight kinetic constants of the mathematical model corresponding to this mechanism. Although the reaction occurs in four steps, it is commonly treated in the literature as a reversible single-step reaction containing only two reaction rate constants, k_{on} andk_{off} [7-9].

The kinetic study of the reaction of hemoglobin with oxygen requires the experimental realization of hemoglobin oxygenation and deoxygenation. Conducting these two reactions simultaneously is not a trivial procedure, so it is common to analyze them individually. A widely used methodology for this individual analysis is the rapid mixing method with interrupted flow. Several studies have used this method to evaluate the hemoglobin deoxygenation and

to estimate the dissociation kinetic constant [9,10,11]. The study of oxygenation is unusual due to several experimental limitations.

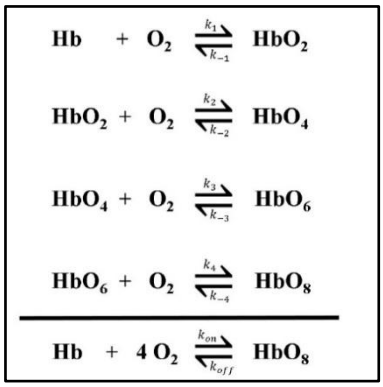

Figure 1: Scheme of the reaction mechanism of hemoglobin with oxygen. The first proposal considers that the reaction occurs in four reversible, successive and cooperative steps. The second proposal considers a single-step reaction and shows its association (k_{on}) and dissociation (k_{off}) constants.

Although several articles present the kinetic study of oxyhemoglobin deoxygenation reaction, some experimental and theoretical aspects are not discussed extensively. Regarding to the experiment, it is not usual consider that, on a same wavelength, there are two or more hemoglobin conformations contributing to the measured absorbance. As for the theory, the pseudo-first order model, widely used to represent the reaction kinetics, does not fit the experimental data at the first milliseconds of the deoxygenation. Thus, the present work aims to obtaining experimental data of oxyhemoglobin deoxygenation in order to analyze the problem in a new perspective, discussing experimental characteristics and limitations that must be taking into account for the reaction kinetic study. In addition, it is intending to discuss the validity and limitations of the pseudo-first order model, that is widely used to represents the reaction dynamic of hemoglobin with oxygen.

Materials and Methods

In order to achieve the objectives of this paper, it is necessary, first of all, to obtain the oxyhemoglobin. So, the meta-hemoglobin was converted to oxyhemoglobin by the size-exclusion liquid chromatography method. The reducing agent used was sodium dithionite and the process was carried out with HEPES buffer in order to maintain constant the pH. After this, the oxyhemoglobin obtained was deoxygenated by the rapid mixing method with interrupted flow. To this procedure, the sodium dithionite was also used, but now in the absence of oxygen. Finally, the dissociation constant of the deoxygenation reaction of oxyhemoglobin was estimated considering the pseudo-first order model. The reagents, methods, and procedures performed are described in this section.

2.1. Reagents

The main reagents used to carry out the experimental procedure were: lyophilized human methemoglobin purchased from Sigma-Aldrich (catalog code H7379); HEPES sodium salt ($C_8H_{18}N_2O_4S$, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt) purchased from Vetec (catalog code V900477); sodium dithionite or sodium hydrosulfite ($Na_2S_2O_4$) purchased from Vetec (catalog code V000593); Sephadex G-25 purchased from Sigma-Aldrich (catalog code G25150); reagent for the determination of hemoglobin purchased from Diagnostic Labtest AS (reference number 43); and distilled water.

2.2. Preparation of oxyhemoglobin from methemoglobin

First of all, the chromatography column was prepared using the Sephadex G-25 resin, according to the manufacturer's instruction. Prior to its use, the column was equilibrated at room temperature (25 ºC) and pH 7.4 with 50 mM HEPES buffer. Then, 2 mL of the 574 mM sodium dithionite solution was added. The sodium dithionite acts

as a reducing agent of meta-hemoglobin in order to convert it to oxyhemoglobin [12,13]. Lastly, a solution prepared from the dissolution of 200 mg of lyophilized meta-hemoglobin in 10 mL of 50 mM HEPES buffer, pH 7.4, was placed in column [13]. The HEPES buffer was used like eluent throughout the procedure.

In order to purify the obtained oxyhemoglobin and remove the excess of sodium dithionite, the sample obtained by chromatography was dialyzed during 24 h under constant stirring and temperature of 8 °C [14, 15]. The dialysis buffer was 50 mM HEPES, pH 7.4. During dialysis, the sample was maintained in a semipermeable membrane, containing pores with molecular weight cut-off of 30 kDa. After dialysis, the oxyhemoglobin was quantified according to the method of Drabkin and Austin [16].

2.3. Oxyhemoglobin deoxygenation

One of the commonly used methods for the analysis of oxyhemoglobin deoxygenation reaction is the rapid mixing method with interrupted flow [9,10,11]. The sodium dithionite is the reagent normally used for deoxygenation because it is a potential reducer of heme pigments [17,18]. Based on these studies, the deoxygenation of oxyhemoglobin with sodium dithionite was performed in the *Stopped Flow Mixer 4000/S* (*SFM 4000/S*), a equipment based on the rapid mixing with interrupted flow method.

The *SFM 4000/S* has four independent syringes (S1, S2, S3 and S4), where the reactants are stored before being mixed. The three mixers of the equipment (M1, M2 and M3) can be viewed in detail in the Figure 2. The M1 is responsible for mixing the solutions of the syringes S1 and S2, whose resulting solution is used as reagent blank. The M2 is directly connected to syringe S3 and M3 to syringe S4. The M3, which is located below the microcuvette, is responsible for finalizing the homogenization of the reagents [19]. The equipment's microcuvette, called μFC-08, is one of the most efficient on the market for measuring micro volumes of reagents (μL) and can be used for both absorbance or fluorescence spectroscopy. It presents an optical path of 0.08 cm in all directions. When the solution coming from the mixer M3 reaches the microcuvette, the flow is interrupted by the closing of a solenoid valve located inside the system of abrupt stop, called hard stop, activating the trigger. The trigger sends the data via a logic board connected to the computer, which is coupled to the *SFM 4000/S* [19].

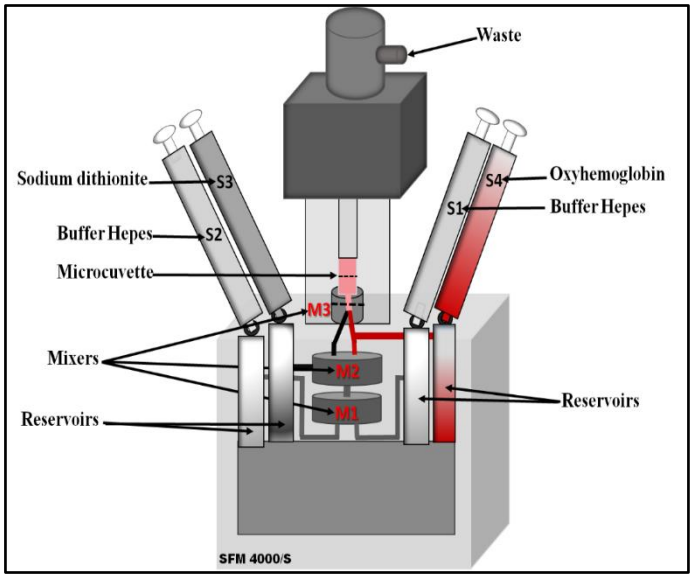

Figure 2: Representation of the configuration of the SFM 4000/S equipment showing the three mixers, named M1, M2 and M3. The reagents are placed in the S3 and S4 syringes. The sodium dithionite solution passes by the mixer M2 and reaches the last mixer, M3, which is located under the microcuvette, to be mixed with the oxyhemoglobin solution of the syringe S4.

For the kinetic study of oxyhemoglobin deoxygenation, HEPES buffer was placed in syringes S1 and S2, sodium dithionite in syringe S3 and oxyhemoglobin in syringe S4, as can be seen in Figure 2. In all cases, the sodium dithionite was used at a concentration of 1.5 mg/mL, as recommended by Jia et al. [9]. Different concentrations of oxyhemoglobin (20, 40, 60, 80 and 100 μM) were placed in syringe 4. As the reactants were mixed in the ratio of 1: 1, the initial concentrations of oxyhemoglobin for each evaluated kinetic run were 10, 20, 30, 40 and 50 μM, which corresponds to half the concentrations of the stock solutions. It is important to note that, prior to being placed in the syringe, oxyhemoglobin solutions were deaerated with nitrogen to remove the free oxygen.

All the experimental procedures were performed using a volumetric flow of 9.0 mL/s and a total volume in the microcuvette of 202 μL (101 μL of oxyhemoglobin and 101 μL of sodium dithionite). This setting resulted in a dead time of 0.4 ms. The total time of reaction was set at four seconds, and every 500 μs the absorbance of the solution in the microcuvette was recorded by the software.

2.4 Method validation

Before and after every experimental procedure be realized, each conformation of hemoglobin was identified by wavelength scanning between 390 nm and 650 nm. The spectra were measured on the SpectraMaxPlus 384 spectrophotometer and compared to the spectra presented by Lister et al. [20].

Another step for validation of the experimental methodology is the verification of the independence of the obtained results in relation to the initial oxyhemoglobin concentration [17]. For this analysis, the concentration values obtained for every kinetic run were divided by the correspondent initial concentration value in order to obtain the only curve that represents the reaction dynamics. This curve is here called characteristic curve.

2.5. Obtaining the concentration values over time as a function of the absorbance data

The oxyhemoglobin concentration values over time could be obtained from the absorbance measured experimentally using a previously constructed calibration curve. However, it is known that the deoxyhemoglobin has a significant absorbance value at the wavelength of 415 nm, which was the wavelength set to all the experiments performed on SFM 4000/S. Thus, the total measured absorbance value corresponds to the sum of the oxyhemoglobin and deoxyhemoglobin absorbances of in this wavelength. This consideration is expressed in the Equation (1).

$$\text{Absorbance} = A_{oxyHb} + A_{deoxyHb} \qquad (1)$$

Knowing that each term of Equation (1) can be replaced by a calibration curve, here considered as the linear relation of the Beer-Lambert Law, the Equation (2) can be obtained. The Beer-Lambert relation consists in multiplying the molar extinction coefficient (ε_i), the optical path of the microcuvette ($L = 0.08\,cm$) and the concentration of oxyhemoglobin or deoxyhemoglobin (C_{oxyHb} or $C_{deoxyHb}$).

$$\text{Absorbance} = \varepsilon_{oxyHb} \,.\, L.\, C_{oxyHb} + \varepsilon_{deoxyHb} \,.\, L.\, C_{deox\,yHb} \qquad (2)$$

Since the total hemoglobin concentration in the medium is constant and equal to the initial concentration, C^o_{oxyHb} . Also assuming that, in this case, hemoglobin can only be in the oxygenated or deoxygenated form, the total concentration is the sum of the concentration of these two forms. Thus, the concentration of deoxyhemoglobin can be written according to Equation (3).

$$C_{deoxyHb} = C^o_{oxyHb} - C_{oxyHb} \qquad (3)$$

Substituting $C_{deoxyHb}$ from Equation (3) in the Equation (2), the expression for calculate oxyhemoglobin concentration as a function of the measured absorbance is obtained, Equation (4).

$$C_{oxy\,Hb} = \frac{\text{Absorbance} \;\; -\varepsilon_{deoxyHb} \;\; .\text{L} \;.C^o_{oxyHb}}{\varepsilon_{oxyHb} \;.\text{L} - \varepsilon_{deoxyHb} \;\; .L} \qquad (4)$$

2.6. Calibration curves

The data used for the oxyhemoglobin calibration curve were obtained in the SFM 4000/S. After the plot of the absorbance as a function of oxyhemoglobin concentration, the linear behavior for the concentration range used in the experiments (10 to 50 μM) was verified. In this way, a linear regression was performed using Origin® software.

Considering that the reaction of oxyhemoglobin with sodium dithionite is complete, the data used for the deoxyhemoglobin calibration curve correspond to the final absorbance values in each experiment realized. After the construction of the absorbance graph as a function of the deoxyhemoglobin concentration, the linear behavior was also observed, being possible to perform a linear regression using the software Origin®.

2.7. Dissociation constant calculation (k_{off})

The deoxygenation reaction of oxyhemoglobin is irreversible when carried out by means of sodium dithionite [21], so that the conversion of oxyhemoglobin to deoxyhemoglobin can be approximated by means of a pseudo-first order reaction, disregarding the possibility of intermediate steps. Thus, the dynamics of oxyhemoglobin concentration over time can be given by Equation (5).

$$C_{oxyHb} = C^{o}_{oxyHb} \cdot e^{-k_{off} \cdot t} \qquad (5)$$

The k_{off} values were obtained by adjusting exponential functions to the oxyhemoglobin concentration curves over time through non-linear regression using Origin® software.

3. Result and Discussion

The results show the validation of experimental methodology and, they are used to discuss aspects related to experimental characteristics and to the use of the pseudo-first order model to represent the dynamics of oxyhemoglobin deoxygenation.

3.1. Method validation

3.1.1. Verification of hemoglobin conformations. Before passing through the chromatographic column, it was identified that the absorption peaks of the solution containing methemoglobin were in 405, 500 and 630 nm, as shown in Figure 3 (dotted line). These values correspond to the methemoglobin spectrum [20]. After performing the size-exclusion liquid chromatography, the spectra presented peaks in 415, 540 and 577 nm (Figure 3 - dashed line).

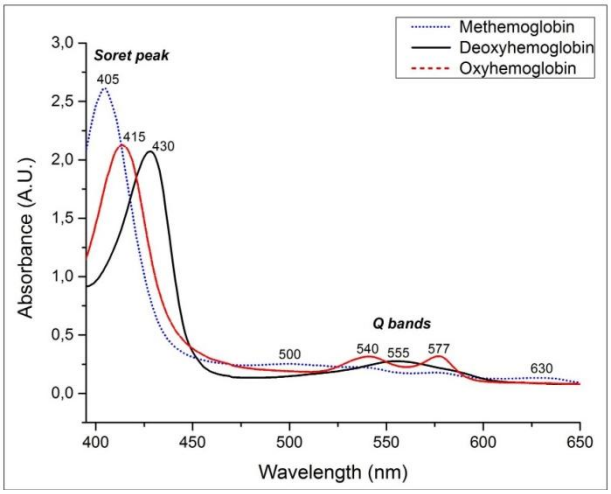

Figure 3: Illustration of the hemoglobin conformations. In this graph, it can be observed that the reduction of methemoglobin (dotted line) to oxyhemoglobin (dashed line) occurred after the execution of the liquid chromatography. In addition, oxyhemoglobin was converted to deoxyhemoglobin (solid line) by the rapid-blending method with interrupted flow.

These values correspond to the oxyhemoglobin peaks [20], indicating that the methemoglobin was converted to oxyhemoglobin by the reducing agent sodium dithionite. Oxyhemoglobin was reduced by sodium dithionite in the absence of oxygen using the rapid mixing method with interrupted flow. The spectrum of the resulting solution showed peaks in 430 and 555 nm (Figure 3 - solid line), which correspond to the deoxyhemoglobin peaks [20]. The spectrum obtained after deoxygenation of oxyhemoglobin agrees with the arguments of Brittain and Simpson [22], Coin and Olson [23], Dalziel and O'Brien [17], Elmer et al. [24], Jia et al. [9] and Jorge et al. [25], whose proposition is that excess sodium dithionite totally removes the oxygen that is bound to oxyhemoglobin. Thus, this method can be used for the study of oxyhemoglobindeoxygenation.

3.1.2. Characteristic curve. The curves obtained experimentally after the deoxygenation of oxyhemoglobin by the rapid mixing method with interrupted flow are presented in Figure 4a. Figure 4b corresponds to the visualization of the graph only up to 0.2 s in order to enlarge the visualization of the dynamic part of the reaction. The value of 0.2 s was chosen after plotting the absorbance derivative (Figure 5) and verifies that at 0.15 s, approximately, it assumes values very close to zero. It was decided to use a safety margin, which led to the choice of 0.2 s.

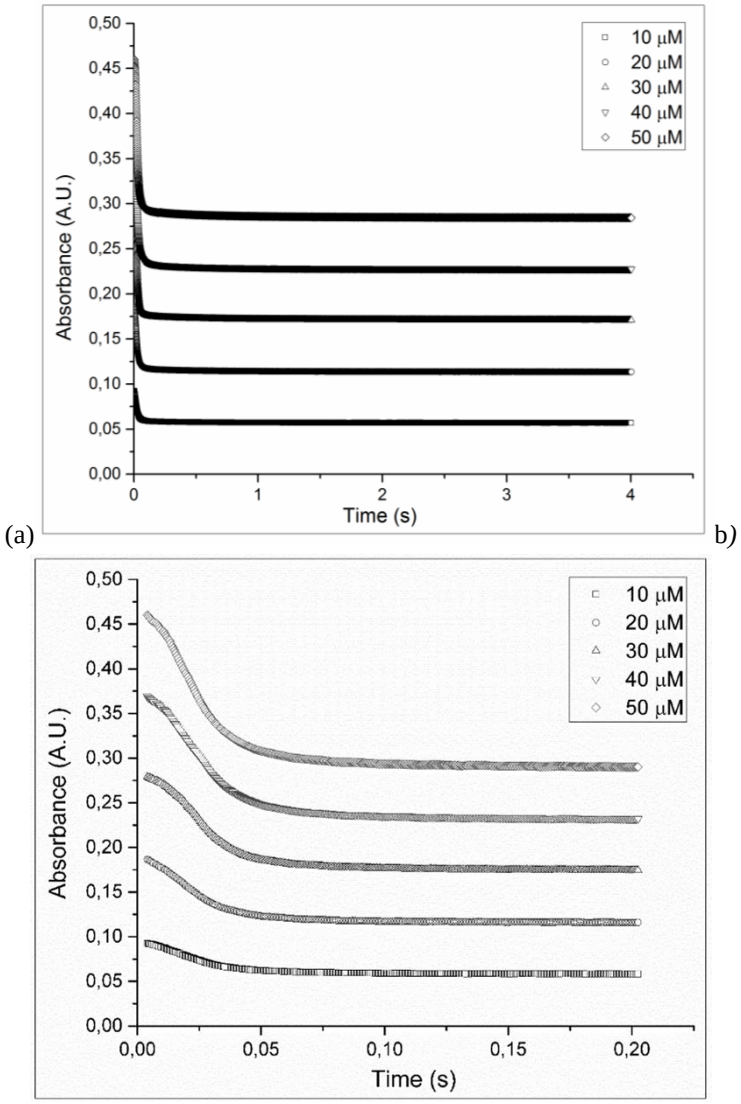

Figure 4: Graph of deoxygenation of oxyhemoglobin in different initial concentrations (10, 20, 30, 40 and 50 μM). a) total analysis time, 4s. b) time up to 0.2 s for better visualization of the dynamic part.

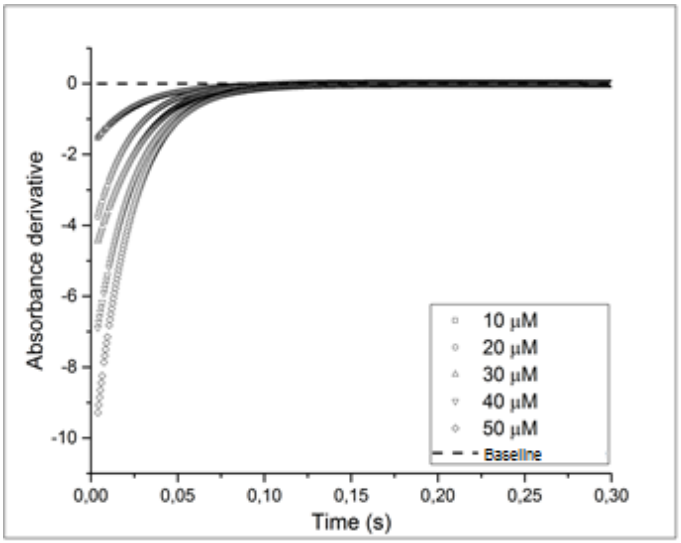

Figure 5: Derivative of the absorbance as a function of time. The graph shows the values only up to 0.3 s, since after that time all values are equal to zero. From 0.15 s it is already possible to state that the derivative is very close to zero.

One way of validating the experimental results is to normalize the absorbance data as a function of the absorbance value corresponding to the initial oxyhemoglobin concentration for each run. This is done because the reaction dynamics are independent of the initial concentration [17]. The result of the normalization is presented in Figure 6 and, as can be observed, validates the experimental data obtained. Due to the similar dynamics, it is possible to establish the characteristic curve, also shown in Figure 6, which represents the reaction dynamics for any initial concentration used.

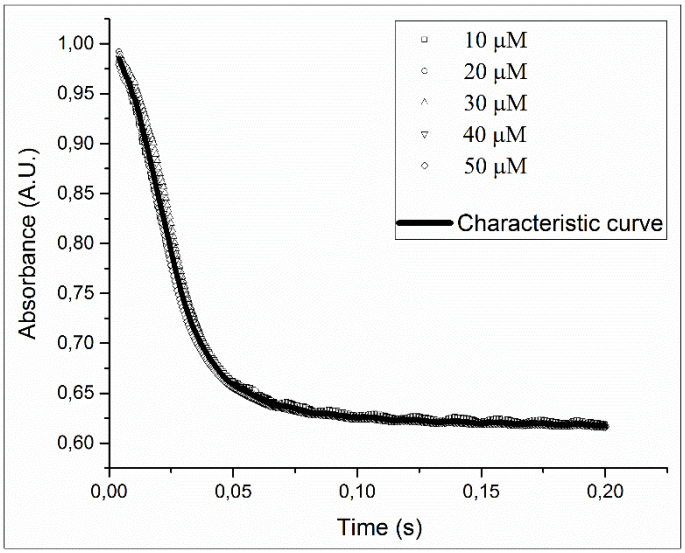

Figure 6: Normalized curve of oxyhemoglobindeoxygenation to different initial concentrations (10, 20, 30, 40 and 50 µM).

3.2. Experimental issues

As can be seen in Figure 3, at the oxyhemoglobin maximum absorption wavelength (415 nm), deoxyhemoglobin exhibits a significant absorbance value. At the deoxyhemoglobin maximum absorption wavelength (430 nm), the absorption value of oxyhemoglobin, although not as significant, is different from zero. Thus, when analyzing the

reaction at any of these wavelengths, it is possible to conclude that there is the contribution of both conformations of hemoglobin.

In the results obtained by the rapid mixing method with interrupted flow using the wavelength of 415 nm, the absorbance at the end of the reaction does not reach zero (Figure 6). Considering that the reaction is complete [9,17,22,23,24,25] and knowing that the deoxyhemoglobin presents a significant absorbance value of at this wavelength, it is possible to conclude that the final value corresponds to the absorbance of deoxyhemoglobin in this wavelength. This conclusion requires that the contribution of deoxyhemoglobin in the absorbance measured during all the reaction be taking into account.

Several papers evaluate the deoxygenation reaction at the wavelength of 430 nm. Although the contribution of oxyhemoglobin at this wavelength is lower when compared to the contribution of deoxyhemoglobin at the wavelength of 415 nm, it does exist. This is evident when evaluating the work of Jia et al. [26], for example, whose absorption curve starts above zero. In order to overcome this problem, several authors have working in terms of relative absorbance, discounting the absorbance value at the beginning of the measurement [9,25]. This artifice discounts the contribution of oxyhemoglobin considering that it is constant throughout the whole process, not taking into account that its influence decreases over time because it is the reagent of the reaction.

Another experimental evidence that it is necessary to consider the contribution of both hemoglobin conformations during deoxygenation is the difference between the graphs of hemoglobin saturation percentage presented by Dalziel and O'Brien [17] at different wavelengths (415 nm and 430 nm). The curves are not coincident, because the contributions of each of the conformations are different for each wavelength. Thus, it is concluded that the experimental analysis is compromised without considering that the measured absorbance corresponds to the contribution of each of the hemoglobin conformations.

3.3. Theoretical issues

One aspect to be highlighted before discussing the pseudo-first-order model, widely used to represent the deoxygenation dynamics of oxyhemoglobin, is that the papers usually discuss absorbance charts as a function of time [21,26,27]. However, absorbance values cannot serve as a general model since each equipment will register a value that varies depending on the manufacturer and the optical path of the microcuvette. In the present work, it was chosen to use concentration values instead of absorbance, what seemed a more viable alternative for validation of the experimental methodology. The graph presented in Figure 7 shows the calibration curves obtained experimentally, and the graph of Figure 8a shows the concentration values as a function of time, obtained from Equation (4), for each initial oxyhemoglobin concentration analyzed.

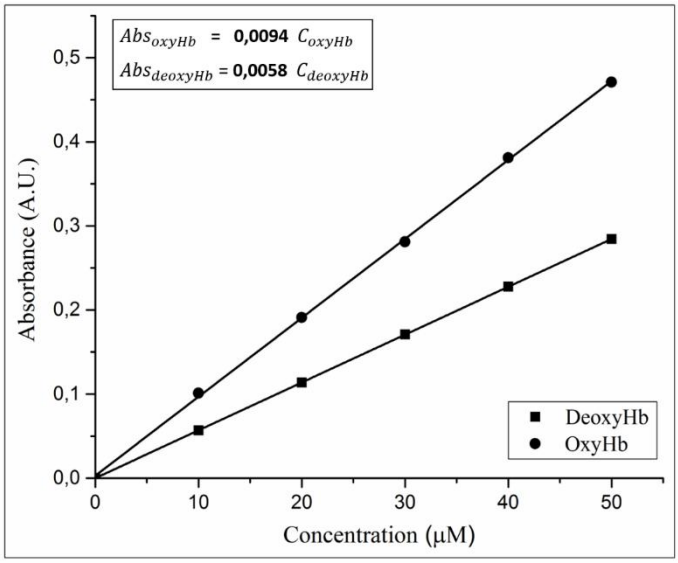

Figure 7: Calibration curve of oxyhemoglobin and deoxyhemoglobin at 415 nm.

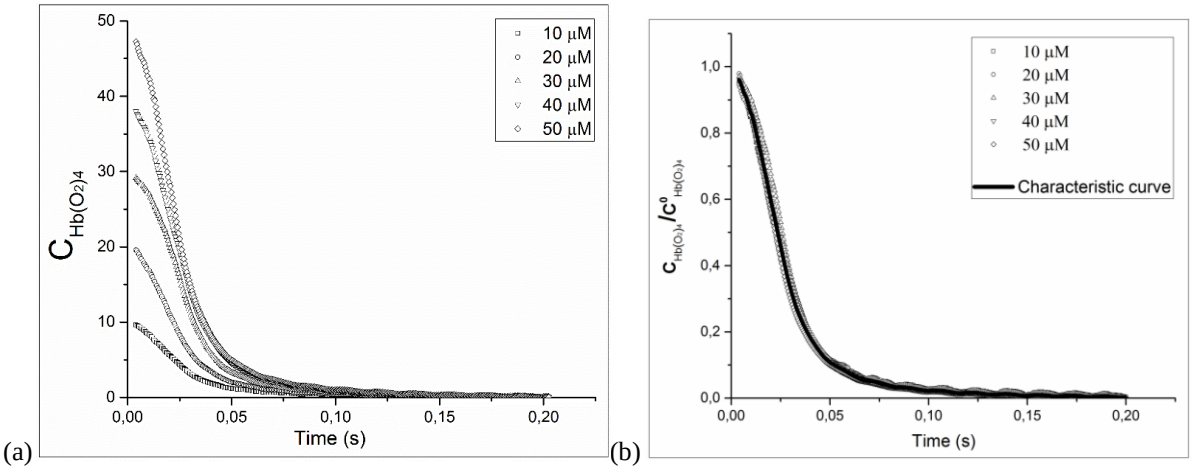

Figure 8: a) graph of oxyhemoglobin concentration as a function of time for all initial oxyhemoglobin concentrations assessed. b) presentation of the characteristic curve for the oxyhemoglobindeoxygenation.

It is possible to divide the concentration values over time by the initial concentration, in the same way as was done for the absorbance, in order to identify a characteristic curve. This curve is shown in Figure 8b. Using the data of Figure 8b, a nonlinear regression was performed on the Origin® software to obtain the dissociation constant of the pseudo first order model. Table 1 shows the k_{off} value for each of the initial concentrations of oxyhemoglobin used. From these values, it is possible to estimate an average value, which is equal to 35.8 ± 2.5 s^{-1}. It is also possible to estimate the dissociation constant of the characteristic curve, whose k_{off} corresponds to 35.2 s^{-1}. This value is compatible with the values already estimated in the literature: 34 s^{-1} [9]; 34.6 s^{-1} [10] and 38 to 43 s^{-1} [11].

Table 1: Estimated dissociation constant values for each of the kinetic runs.

$C^o_{oxi-Hb}(\mu M)$	$k_{off}(s^{-1})$
10	35.4
20	36.8
30	32.8
40	34.4
50	39.5

Although the pseudo-first order model is widely accepted for the mathematical description of oxyhemoglobin deoxygenation with sodium dithionite, an analysis of the comparison between experimental and model results, showed in Figure 9, reveals that the fit fails in the description of the reaction dynamics. The results obtained by Jia et al. [9] show discrepancies in the fit for the initial instants of the reaction. Thus, the pseudo-first order model must be used cautiously for the study of this reaction.

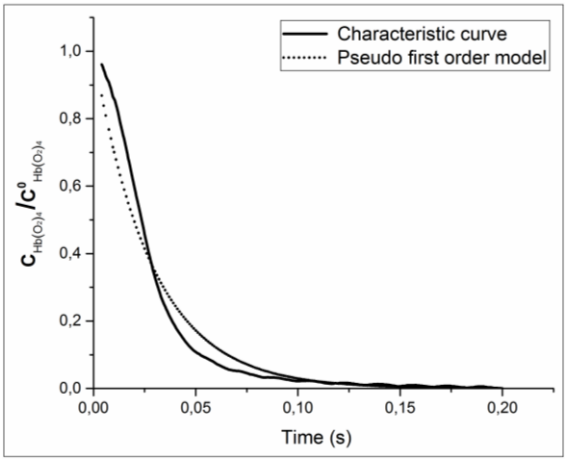

Figure 9: Comparison between the result of the characteristic curve and the pseudo first order model.

The non-adequacy of this model in the initial instants is compatible with the prediction of Legge and Roughton [28], whose assertion is that the pseudo-first order model only fits the experimental data for values below 70% of hemoglobin saturation. In order to find the saturation value below which the pseudo-first order model represents the reaction dynamics for the experimental data of the present work, different saturation values were choose and the model was evaluated from the moment that the saturation reach these values. This analysis requires an adaptation in the equation of the model, as presented in Equation (6). This adaptation takes into account that the exponential equation will be evaluated from time t_0. When $t = t_0$, the concentration assumes the value C'^{o}_{oxyHb}, which corresponds to the concentration value from which the model could fit most properly. Choosing one, the other gets predetermined based on the experimental results. In order to work with dimensionless quantities, C'^{o}_{oxyHb} will be normalized in terms of the initial concentration of oxyhemoglobin.

$$\frac{C_{oxyHb}}{C^{o}_{oxyHb}} = \frac{C'^{o}_{oxyHb}}{C^{o}_{oxyHb}} . e^{-k_{off} .(t-t_0)} \qquad (6)$$

Table 2 presents the saturation values chosen and the time associated with these saturations, as well as the optimized dissociation constant for the case evaluated and the error with respect to the experimental results.

In order to visualize the behavior of the error as a function of the saturation value, the graph of Figure 10 was constructed. From this graph it can be seen that, for values below 71.1%, the fit considering a pseudo-first order model is appropriate.

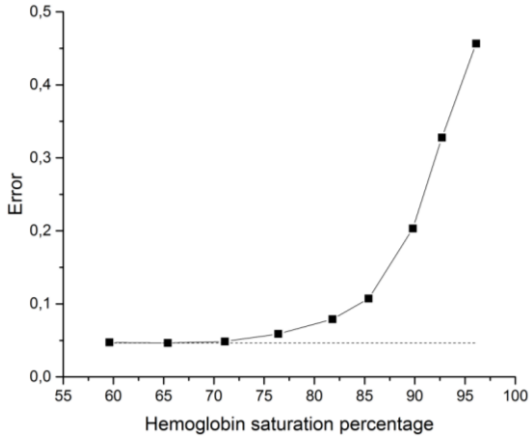

Figure 10: Graph of the error as a function of the saturation value below which the pseudo-first order model was considered.

Table 2: Evaluation of the errors and dissociation constants to different oxyhemoglobin saturation values from which the pseudo-first order model was used to represent the dynamics of the reaction

t_0(s)	$\frac{C'^{o}_{oxyHb}}{C^{o}_{oxyHb}}$	Hemoglobin saturation percentage	k_{off} (s^{-1})	Error
0,004	0,961	96,1%	39,5	0,456
0,006	0,927	92,7%	41,6	0,328
0,008	0,898	89,8%	44,2	0,203
0,0105	0,854	85,4%	47,5	0,107
0,012	0,818	81,8%	49,1	0,079
0,014	0,764	76,4%	51,0	0,059
0,016	0,711	71,1%	52,9	0,049
0,018	0,654	65,4%	54,3	0,046
0,020	0,596	59,6%	55,1	0,047

Figure 11 shows the comparison between the experimental and the model results for saturation below 71.1%. It is possible to observe that the adjustment improves significantly. Thus, it is possible to conclude that the pseudo-first order model may represent a part of the reaction, but it is not able to describe the beginning of its dynamics.

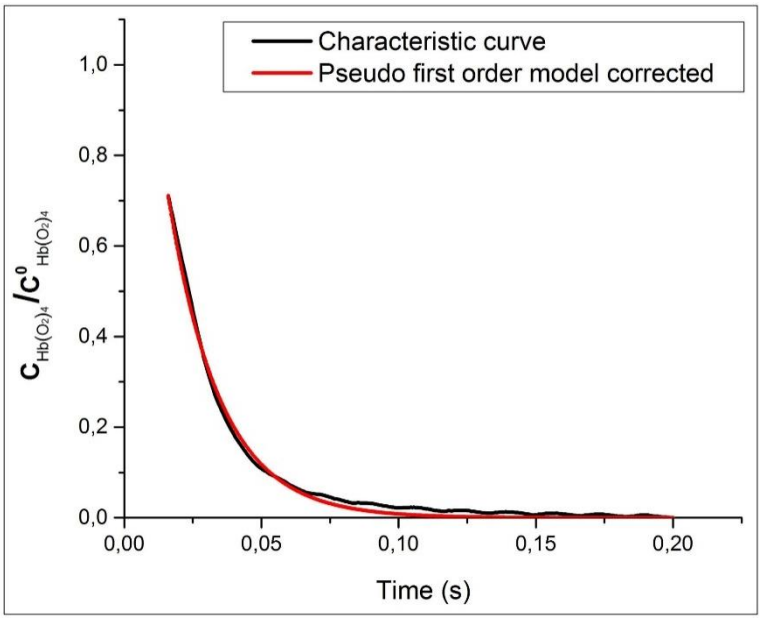

Figure 11: Comparison between the results of characteristic curve and of the pseudo-first order model disregarding oxyhemoglobin saturation values above 71.1%.

It is important to note that this saturation value was reached in the present work at a time around 16.4 ms whereas, for Legge and Roughton [28], the time was 40 ms. This difference is associated to the different temperatures used in the experiments, 25ºC and 11ºC, respectively. As the temperature influences the speed of the reaction, the reaction of the present work is faster than that of Legge and Roughton [28], reaching the same saturation in a smaller time interval. In this way, it is worth noting that, in order to use the model, it is necessary to know this time, t_0, which is dependent on temperature. Thus, it is possible to affirm that the model loses its predictive capacity, being always necessary the experimental realization in order to be able to determine this parameter.

Another questionable aspect is the utility of a model for describing the reaction between hemoglobin and oxygen that is only representative for values below 70% saturation, since the saturation of the inlet and outlet in the capillaries is approximately 98% and 78%, respectively [29]. Thus, the model is not able to predict the dynamics of the reaction in the interval in which it actually occurs. This fact shows the need to study this reaction in a more complete and complex way, considering, for example, that it occurs in four stages, as proposed by Antonini and Gibson [5].

4. Conclusion

The present work allowed, through validated methodologies to obtain oxyhemoglobin from the methemoglobin by the method of liquid chromatography by molecular exclusion and for the deoxygenation of oxyhemoglobin by the rapid mixing method with interrupted flow, the discussion of some experimental and theoretical aspects regarding deoxygenation of oxyhemoglobin. Firstly, we questioned the non-consideration in several studies of the contribution of more than one hemoglobin conformation to the absorbance measured experimentally, which leads to an error in the analysis. It was also possible to point out that the pseudo first order model, widely used in the literature, is not adequate to describe the reaction process as a whole because it does not fit the initial instants of the reaction. Considering that saturation of hemoglobin varies from 98 to 78% in capillaries, a model that adjusts below 70% is not satisfactory. Thus, the analysis presented contributes to a better understanding not only of the kinetic study of oxyhemoglobin deoxygenation reaction, but also indicates important issues that should be evaluated in future studies

on the subject. It is important to note that, in order to estimate the constants of a more sophisticated model such as Antonini and Gibson [5], it is necessary to take into account the absorption of all hemoglobin conformations present in the reaction medium. The author

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be built as a potential conflict of interest.

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