

Research Article Insights into Sulfhemoglobin Detection: UV-Vis and Fluorescence Spectroscopy Correlations

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The mechanisms by which drugs and several sulfur chemicals induce sulfhemoglobin formation have not yet been elucidated. However, enzymes producing hydrogen sulfide in mammalian tissues and organs suggest sulfhemoglobin and sulfmyoglobin formation mechanisms are more complex than previously hypothesized. The process involves the interaction of H₂S with hemoglobin or myoglobin in the presence of O₂ or H₂O₂ to generate sulfhemoglobin or sulfmyoglobin, respectively. Structurally, the sulfheme product chromophore is a covalent heme modification. This modification involves the incorporation of one sulfur atom within carbon atoms to form a sulfur-carbons ring moiety across the β - β double bond of heme pyrrole B, which shows a characteristic optical band around 623 nm and 618 nm for sulfhemoglobin and sulfmyoglobin, respectively. The results show a linear correlation between the sulfHb electronic charge transfer transition at 623 nm and the emission wavelength of 460 nm upon Soret excitation at 420 nm. The data show no such linear relationship for oxy-Hb or met-aquo Hb. This new approach allows us to measure from 0.02% to 13.5% sulfhemoglobin in mixtures of met-aquo hemoglobin and oxy-hemoglobin. Although additional work is needed, the results suggest that simultaneously monitoring sulfHb electronic transition at 623 nm and emission wavelength at 460 nm upon Soret excitation at 420 nm is a powerful technique to determine the percentage of sulfhemoglobin in blood. The data and techniques presented indicate that fluorescence spectroscopy coupled with UV-vis spectroscopy provides a fast and accurate method for detecting sulfhemoglobin in the blood, facilitating the diagnosis of sulfhemoglobinemia in patients.

1. Introduction

Hydrogen sulfide (H₂S), a highly lipophilic gas endogenously produced in tissues and organs, has been associated with myriad conditions and diseases [1–11]. H₂S plasma values in the 100 μ M range are associated with the fibrosis phenomena [12]. SARS-CoV-2 survivors show serum H₂S levels higher (\geq 150 μ M) than those of nonsurvivors [13, 14], suggesting that the production of H₂S acts as a defense mechanism against COVID-19 [15, 16]. Reduced H₂S levels are also observed in Parkinson's, Alzheimer's, and atherosclerosis [17, 18]. In addition, H₂S has been correlated with chronic diseases [19–21], inflammation [22–24], immune system regulation [25, 26], cancer [27, 28], oxidative stress [29–34], oral diseases [35], glaucoma [36], subarachnoid hemorrhage [37], infertility in men [38], arterial oxygen saturation [39], and skin diseases [40]. Furthermore, clinical studies have emphasized the potential of modulating H_2S synthesis for therapeutic use [41–43]. However, the investigation results are limited by the lack of reliable H_2S measurements in body fluids and tissues and the absence of specific biomarkers [1].

Hydrogen sulfide could interact with metals in the body like iron, copper, nickel, and zinc to carry out specific functions. The interactions of H_2S with metal-binding proteins have been shown to aid signal transduction and cellular metabolism [44]. Furthermore, H_2S is capable of reducing heme iron from Fe(III) to Fe (II) and providing a cytoprotective role against the gas. This reduction process depends strongly on heme pocket distal mutations showing faster reduction for those mutants exhibiting the most robust H-bonding interactions [45]. Also, hydrogen sulfide can exert a biological role via widespread interactions with metalloproteins in maintaining redox homeostasis by eliminating reactive oxygen species [46]. Moreover, hydrogen sulfide interacts with oxy-myoglobin and oxyhemoglobin to generate sulfmyoglobin (sulfMb) and sulfhemoglobin (sulfHb) complexes. This process leads to sulfhemoglobinemia, a bluish skin color associated with the lack of oxygen, and cyanosis. Furthermore, common causes of sulfhemoglobinemia-induced cyanosis include a wide range of drug overdoses like acetanilide metoclopramide, phenacetin, dapsone, sulfanilamide, cimetidine, paracetamol, ibuprofen, naproxen, and exposure to sulfur compounds [47]. This phenomenon has been observed in a newborn [48], during gamma-ray irradiation [49, 50], in cancer and neurodegenerative disease [51], in dimethyl sulfide dermal applications [52] and applications of sodium nitrate formulations [53], sulfur dioxide [54, 55], hydroxylamine sulfate [56], and hydrogen sulfide [57], in chronic constipation [58] and urinary tract infection [59], and in applications of thiocolchicoside (Miorel) [60], metoclopramide, and N-acetylcysteine [61].

The mechanisms by which drugs and chemicals containing sulfur induce sulfhemoglobin formation have not yet been elucidated but are postulated to be similar to how H₂S is produced by intestinal bacteria [62]. However, the enzymatic process by which hydrogen sulfide is produced in mammalian tissues and organs suggests that the chemical-induced sulfHb and sulfMb mechanisms are more complex than previously hypothesized. This problem can be understood through red blood cell (RBC) analysis. For example, a hemoglobin (Hb) level of 13.5 g/dL is equivalent to a 2.0 mM Hb concentration. Normal levels of physiological sulfHb are estimated to be below 0.037 g/dL (5.5 μ M, ~0.28%), whereas a concentration of 0.5 g/dL (74.4 μ M, ~3.8%) in the blood is enough to present clinically detectable cyanosis symptoms [1, 57-61]. Severe sulfHb cyanosis seldom exceeds 10% ($\sim 200 \,\mu$ M). However, 16% and 23% SulfHb measurements have also been reported [62]. Analogously, methemoglobinemia is a blood disorder characterized by a higher-than-average level of met-aquo Hb (Fe(III))-H₂O. Toxic agents like oxidizing chemicals or drugs such as nitrites, nitrates, aniline dyes, aniline derivatives, sulfonamides, and lidocaine can also convert oxy-Hb into met-aquo Hb [60-63]. A 1.5 g/dL (223.0 µM) of met-aquo hemoglobin in the blood also causes detectable cyanosis. This represents approximately 10% met-aquo Hb in total blood hemoglobin [63]. At the same time, it is estimated that 3.0% of oxy-heme (Fe^{II}-O₂) groups autoxidize to met-aquo heme (Fe^{III}-H₂O) and superoxide (O_2^-), leading to reactive oxygen species (ROS) reactions and free radical chemistry [64]. In summary, only 0.5 g/dL (74.4 μ M) sulfhemoglobin is needed to cause clinically detectable cyanosis, compared with 1.5 g/dL $(223.0 \,\mu\text{M})$ met-aquo hemoglobin and $5.0 \,\text{g/dL}$ (744.0 μM) of deoxygenated hemoglobin in a matrix of ~2.0 mM oxy-Hb [63]. Thus, it is a challenge to determine precise concentrations of sulfheme in oxy-hemoglobin red blood cell environments.

Structurally, the sulfheme product chromophore is a covalent heme modified by incorporating one sulfur with carbon atoms to generate a sulfur-carbons ring moiety across the β - β double bond of heme pyrrole B. This sulfMb structure is supported by X-ray [65], NMR [66-69], and resonance Raman [70, 71] spectroscopy. Figure 1 shows a visualization model of the sulfHb system containing partial sulfheme structures in different subunits. The sulfHb and sulfMb Soret chromophore transitions in the Soret region occur from 402 nm to 423 nm, coupled with a unique charge transfer band in the 565 nm to 623 nm region. These transition energies depend on the nature of the hemeprotein, iron oxidation, ligation, spin states, and pH [72-80]. The most prominent transitions for oxysulfhemoglobin are the Soret band at 412 nm, characteristic of a π to π^* transition, and the 623 nm band characterized by π to $d\pi$ (dyz, dxz) charge transfer transition associated with the sulfur ring attached to pyrrole B and the heme iron [73, 81, 82].

Roman-Morales et al. demonstrated that distal histidine in the E7 position (HisE7) is essential in sulfheme formation [71]. The hydrogen bonds between heme Fe(III)-H₂O₂ or heme Fe(II)-O₂, heme distal His64, and H₂S regulate this process. Mechanistically, the sulfheme formations for metaquo Mb (Fe(III))-H₂O adduct and oxy-Mb (Fe(II)) in the presence of H₂S show different heme intermediates and energy barriers towards the reaction intermediate heme Cpd 0 (Heme Fe(III)-OOH) and the generation of a thiyl radical (HS•) [82-85]. This reactive sulfur species, suggested previously [78, 86], explicitly attacks the heme pyrrole B at the β - β double bond to generate the heme-sulfur five-member ring formation. Favorable energy pathways of -135.3 kcal/ mol and -69.1 kcal/mol to the five-member ring met-aquo sulfMb structure have been established [82-85]. These mechanisms support the antioxidant role of hydrogen sulfide by avoiding the strained formation of the highly reactive heme compound I (Fe (IV) = $O + \bullet$, Cpd I). Upon forming sulfHb and sulfMb, hemoglobin and myoglobin have an oxygen reduction affinity of 135 and 2,500 times, respectively [72, 81]. Experimental results suggest that the difference in charge transfer intensity between sulfMb and sulfHb in the 620 nm region could be attributed to the lack of sulfheme formation in oxy-Hb heme centers upon reaction with H₂S [87-89]. As time progresses, the autoxidation process leads to the formation of met-aquo-, hydroxyl, deoxy-, and oxy-sulfheme species, resulting in energy shifts in the Soret transitions, intensity decrease, and a broad 623 nm band [72-80]. A possible sulfHb change in the transition state from R to T, along with the ligand gateway, plays a role in such an outcome [89]. This idea was beneficial in explaining the 10% to 15% sulfHb yield in a patient-derived sample [87, 88], but the mechanistic pathway controlling such behavior remains unknown. Therefore, there is a practical need, as has been done for other human hemoglobin derivatives [90-94], to quantify, with high accuracy and precision, the sulfHb percentage in the blood as a function of met-aquo Hb and oxy-Hb concentrations [62-64, 95-100].



FIGURE 1: Visualization of sulfhemoglobin system containing partial sulfheme structures.

Gas oximeters are the most widely accepted and costeffective technique in the biomedical and toxicology community for addressing this challenge of sulfHb and met-aquo Hb quantification in the presence of oxy-Hb. However, the concern for this heme quantification method is the overlap between the characteristic electronic transitions of sulfHb (623 nm) and met-aquo Hb (635 nm) [62, 101–104]. Alternative techniques have also been explored [55, 90, 101, 105-107]. For example, highpressure liquid chromatography normal phase (NP-HPLC) and reverse phase (RP-HPCL), coupled with fluorescence detection, may be used for rapid heme quantification [90]. The heme Soret is excitable from 390 nm to 410 nm, while its emission is monitored in the 600 nm to 630 nm range. Emission at 662 nm has also been observed [108]. Similarly, fluorescence spectroscopy with excitation in the 460 nm region and emission in the 475 nm to 580 nm has been use ranged to investigate the reaction between the heme system and hydrogen reactive peroxide-producing oxygen species [64, 109-114]. However, due to fluorescence signal quenching by the heme group, UV-vis absorption spectroscopy is necessary to monitor these reactions [62, 102-104]. Therefore, a unique combination of UV-vis and fluorescence spectroscopy is presented here to measure sulfhemoglobin formation in the presence of met-aquo Hb and oxy-Hb. The results demonstrate a linear correction between the sulfHb electronic charge transfer transition at 623 nm and the emission wavelength of 460 nm upon Soret excitation at 420 nm. The data indicate no oxy-Hb or met-aquo Hb interference in this linear relationship. Furthermore, this new approach enables the measurement of sulfhemoglobin from 0.02% to 13.5% in mixtures of met-aquo hemoglobin with excess oxy-hemoglobin. Although additional work is needed, the results strongly suggest that simultaneous monitoring of sulfHb electronic transition at 623 nm and emission wavelength 460 nm upon Soret excitation at 420 nm is a new and revolutionary approach for determining the percentage of sulfhemoglobin in blood.

2. Methods, Materials, and Sample Preparation

2.1. HEPES Buffer 0.05 M, pH 7.4 Preparation. The buffer was prepared by adding 2.38 g of 99% HEPES salt ($C_8H_{18}N_2O_4S$) to a volumetric flask containing 150 mL of ultra-pure deionized water. The solution was stirred for about a minute until it completely dissolved. Next, the pH of the solution was monitored, and NaOH was added until the solution reached a pH of 7.4. Then, deionized water was added to a volume of 200 mL of this solution. The resulting solution was stored in a crystal bottle in the refrigerator for up to 4 months.

2.2. Oxy-Myoglobin, Oxy-Hemoglobin, Met-Aquo Hemoglobin, and Hydrogen Sulfide Stock Solutions. The oxy-Mb stock solution was prepared by dissolving lyophilized powder of equine muscle myoglobin (Sigma Aldrich) in 0.05 mM HEPES buffer (pH 7.4) in a sealed vial. The solution was degassed by purging it with nitrogen. A minimum excess (~2 fold) of sodium dithionite (Na2O4S2) solution was added to the vial to obtain the deoxy Mb (MbFe(II)) form. Once the deoxy hemeprotein was obtained, the solution was purged for 15 min with 99% oxygen. A purple-to-red color change was observed, corresponding to oxy-Mb formation. The excess sodium dithionite was removed by passing the solution through Amicon® ultrafiltration devices from Millipore. The solution spectra were recorded on a UV-vis Shimadzu 2700 Spectrophotometer using a 1 cm pathlength quartz cuvette (Starna Scientific) to confirm the presence of the oxy-myoglobin. Protein concentration was calculated utilizing the Beer-Lambert law, = $A/\varepsilon b$, where *c*, A, b, and ε represent concentration (mM), absorbance, path length (1 cm), and extinction coefficient, respectively. The extinction coefficient used for oxy-myoglobin is $136 \text{ cm}^{-1} \cdot \text{mM}^{-1}$ at 418 nm [115]. The oxy-hemoglobin stock solution was prepared using the same methodology as the oxy-myoglobin stock solution [71]. Lyophilized human hemoglobin was obtained from Sigma Aldrich, and the extinction coefficient used to calculate oxy-hemoglobin concentrations was $125 \text{ cm}^{-1} \cdot \text{mM}^{-1}$ at 415 nm [116]. The met-aquo hemoglobin stock solution was prepared by dissolving the protein powder in HEPES buffer 0.05 mM (pH 7.4). A 10% molar excess of (K₃(Fe(CN)₆), ACS reagent, \geq 99.0%, was used to fully oxidize the hemeprotein to metaquo Hb. A UV-vis spectrophotometer was used to confirm the presence of met-aquo Hb. Potassium ferricyanide excess in the solution was removed using Amicon® ultrafiltration devices. The met-aquo Hb concentration was determined using the molar extinction coefficient $179 \text{ cm}^{-1} \cdot \text{mM}^{-1}$ at 405 nm [117]. The hydrogen sulfide stock solution (83 mM) was prepared anaerobically in an amber vial by dissolving 0.020 g of sodium sulfide nonahydrate salt (Na₂S·9H₂O, ≥99.99%, Alfa Aesar) in 1.0 mL of HEPES 0.05 M buffer at pH 7.4. The solution was purged with nitrogen.

2.3. SulfMb and SulfHb Formation Reactions and UV-Vis Spectrophotometric Measurements. The samples were prepared using 0.05 M HEPES buffer at pH 7.4 and 25°C. Oxy-

myoglobin and oxy-hemoglobin stock solutions were used to prepare eight individual solutions, each with a concentration of $55 \,\mu$ M, in a 1 cm quartz cuvette with a cap from Starna Scientific. To generate sulfMb and sulfHb, aliquots of the hydrogen sulfide stock solution (83 mM) were added to the oxy-myoglobin or oxy-hemoglobin solutions in a quartz cuvette, resulting in individual hydrogen sulfide concentrations ranging from $55 \,\mu\text{M}$ to $1,155 \,\mu\text{M}$. The formation of sulfMb and sulfHb was monitored by spectral scanning using a Shimadzu UV 2700 spectrophotometer every minute over four hours. The presence of sulfMb was evaluated by observing characteristic Q bands around 618 nm, while sulfHb was identified by Q-bands around 623 nm as described in previous studies [72, 77, 118]. The formation reactions of sulfHb and sulfMb using 3-21 fold H₂S molar excess follow a pseudo-first-order reaction [45]. Using the OriginLab[®] Origin 9, the pseudo-first-order constants (k_{obs}) values were determined by plotting $Ln (A-A\infty)$ versus time (seconds) for each H₂S concentration. The second-order rate constant was obtained from the slope of the kobs versus H₂S molar concentration plot. This process was performed in triplicate for each sample. The corresponding extinction coefficient used for analysis was 20.8 [97].

2.4. Sulfheme Formation Reaction: Spectrofluorometric Measurements. Fluorescence spectroscopy measurements were conducted using a Jasco FP-8500 spectrofluorometer with the following settings. Excitation and emission slit: 10 nm/10 nm, excitation wavelength: 420 nm, and an emission wavelength of 460 nm. Measurements were performed at a temperature of 25°C. Initially, an oxyhemoglobin solution with a concentration of $55 \mu M$ was mixed with the H_2S solution in a concentration ratio of 1:21. The reaction spectra for sulfhemoglobin formation reaction were recorded using both UV-vis and fluorescence spectroscopy over 65 minutes. Next, the spectra of the hemoglobin species were obtained. Three 1 cm quartz cuvettes with caps filled with met-aquo Hb (55.0 μ M), oxyHb (55.0 μ M), and sulfHb (5.6 μ M), respectively, were analyzed using a spectrophotometer and spectrofluorometer at 25°C. Following this, the sulfhemoglobin formation reaction was initiated by mixing oxy-hemoglobin (55.0 μ M), met-aquo hemoglobin (5.5 μ M), and H₂S solution (1,155 μ M) in a 1 cm quartz cuvette with a cap. The cuvette was filled with HEPES 0.05 M buffer at pH 7.4 and maintained at 25°C. The progress of the reaction was monitored using UV-vis and fluorescence spectroscopy for the first 65 minutes.

3. Results and Discussion

3.1. Optical Absorption of SulfMb and SulfHb Formation at Prolonged Times. UV-vis spectroscopy was utilized to understand the conversion from oxy-Mb and oxy-Hb to sulfMb and sulfHb in the presence of H_2S and the progression of heme byproducts over longer reaction times. The aim was to evaluate the formation and stability of sulfMb and sulfHb over a four-hour reaction period at various concentrations. In Figure 2(a), the visible transition of

sulfmyoglobin is depicted, with peaks observed at 545 nm and 582 nm, which are assigned to π to π * excitations. A distinctive charge transfer band is also seen at 618 nm, representing π to $d\pi$ (dyz, dxz) charge transfer transitions [73, 81, 82]. The changes in the intensity of these three electronic transitions are associated with the formation of met-aquo sulfMb and a mixture of its heme ligand states, such as sulfMbSH₂, deoxy-sulfMb, oxy-sulfMb, and MbSH₂ derivatives [72-80]. Theoretical calculations support these observations [82]. Figure 2(b) displays the 618 nm transition intensity variation as a function of H₂S concentration and time. This analysis enabled the determination of the average reaction constant, which was found to be $0.61 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the process. This value provides insight into the rate at which sulfmyoglobin is formed in the presence of different H₂S concentrations.

Similarly, sulfHb formation is observed through the appearance of the 540 nm and 576 nm electronic transition, along with the characteristic π to $d\pi$ charge transfer band at 623 nm (Figure 2(c)) [73, 81, 82]. As the reaction progresses, autoxidation occurs, resulting in the formation of different sulfheme derivatives, which have decreased intensities and broadening of their 623 nm bands. Furthermore, it is noted that not all monomeric subunits of Hb generate sulfheme, suggesting a mixture of heme and sulfheme within the tetrameric structure of Hb, which limits the intensity at 623 nm. Figure 2(d) illustrates the changes in the strength of the 623 nm transition as a function of H₂S concentration and time. The data obtained allow for calculating a reaction constant of $1.22 \text{ M}^{-1} \cdot \text{s}^{-1}$. It should be noted that these constants for sulfMb (0.61 M^{-1} ·s⁻¹) and sulfHb (1.22 M^{-1} ·s⁻¹) do not fully characterize the kinetics of sulfheme formation. Instead, they represent the long-term presence of unknown derivatives. A separate kinetic constant $\sim 1.0 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$, determined for the reaction between met-aquo Mb and hydrogen peroxide (H₂O₂) in the presence of H₂S, supports the previous assignments [77].

The energetic barrier to the heme intermediate compound 0 [Fe (III)-OOH, Cpd-0] is 1.9 kcal/mol for the peroxide reaction between met-aquo hemoglobin and H_2O_2 , whereas for the oxy-Mb in the presence of H₂S, the heme Cpd-0 has a higher energy of 23.3 kcal/mol [82-85]. However, the relatively small 21.4 kcal/mol difference in energy barrier cannot account for the kinetic factor difference of 10³. This observation further supports the idea that the low values in the reaction constant represent a mixture of sulfheme species in sulfMb and sulfHb. Nonetheless, it remains an open question to determine which specific derivatives could be present that explain the kinetic behavior of sulfheme in patients displaying symptoms of sulfhemoglobinemia. After a reaction time of seven hours between oxy-Mb, oxy-Hb, and H₂S, the spectral changes of sulfMb (Figure 3(a)) and sulfHb (Figure 3(b)) were analyzed by converting them into absorbance ratios, namely (A_{618}/A_{582}) and (A_{623}/A_{576}) , as a function of hydrogen sulfide concentration. These ratios are commonly used to estimate the yields and purity criteria for sulfheme in samples [72, 73, 86-88, 118].



FIGURE 2: Sulfheme complex spectra as a function of hydrogen sulfide concentration $(165 \,\mu\text{M}-1155 \,\mu\text{M})$ and time. (a) Sulfmyoglobin product spectra. (b) Sulfmyoglobin 618 nm absorbance as a function of initial hydrogen sulfide concentration over a period of four hours. (c) Sulfhemoglobin product spectra and (d) sulfhemoglobin 623 nm absorbance as a function of initial hydrogen sulfide concentration over a period of four hours.



FIGURE 3: Sulfmyoglobin A_{618}/A_{582} and sulfhemoglobin A_{623}/A_{576} ratios as a function of hydrogen sulfide concentration after seven hours of reaction time.

Figure 3 shows that sulfHb exhibits smaller (A_{623}/A_{576}) ratios than sulfMb (A_{618}/A_{582}) . Also, a nonlinear behavior, specifically a quasi-sigmoidal curve, is observed for the sulfhemoglobin formation reaction (Figure 3(b)), whereas

a linear behavior was observed for sulfMb (Figure 3(a)). These observations support the existing literature, which suggests that these ratios do not follow a linear relationship with sulfheme concentration. Higher values (1.0 to 3.28)

than those presented in Figure 3 have been reported for sulfheme formation [73]. These values are associated with different percentages of sulfMb purity (i.e., 90%) and sulfHb purity (40%, 60%, and 75%) [73, 86, 88]. It has been suggested that as the values of the ratios decrease, indicating a decrease in the absorbance at higher wavelengths, the mixture of the sulfheme derivatives increases [86]. Our findings suggest that the absorbance ratios (A_{618}/A_{582}) and (A_{623}/A_{576}) indicate sulfheme formation but do not directly correlate with sulfheme concentration. The purity and mixture of sulfheme derivatives in the samples influence the ratios.

The approach of analyzing the (A_{623}/A_{576}) and (A_{618}/A_{576}) A_{582}) absorbance ratio has also been applied to examine the yield of sulfHb in patient-derived samples, ranging from 10% to 15% [73, 87, 88]. Therefore, the smaller ratios observed for sulfHb (A_{623}/A_{576}) and sulfMb (A_{618}/A_{582}) in Figure 3, after seven hours of reaction times, suggest that this sulfheme preparation method could provide further insight into patient-derived sulfHb samples. The data supports sulfHb in the presence of sulfHb with inhomogeneity heme groups, some in their native form and others with the sulfur atom incorporated. The factors determining the extent to which heme groups are sulfurated or remain in their native state remain challenging to predict [87, 88]. The presence of hybrid groups, consisting of sulfurated and native heme groups, could explain the observed behavior during the formation of sulfhemoglobin at different H₂S concentrations. Various factors contribute to this phenomenon, including (i) the techniques used for sulfheme synthesis, (ii) the nature of the oxidizing agent (oxygen or hydrogen peroxide), (iii) reaction time, (iv) sulfheme ligand species, (v) autoxidation processes, and (vi) pH. The (A_{618}/A_{582}) and (A_{623}/A_{576}) absorbance ratios provide valuable insights into the concentration of these sulfheme derivates in red blood cells, but caution should be exercised in interpreting the results. In summary, analyzing the absorbance ratios can contribute to understanding the landscape of sulfheme derivates in red blood cells and provide helpful information concerning the complexities and factors influencing their formation and presence.

3.2. Sulfhemoglobin UV-Vis Absorption and Fluorescence Response Spectra. In the reaction between oxy-hemoglobin solution (55μ M) and H₂S at a concentration ratio (1:21), sulfhemoglobin formation and additional sulfheme products were observed using UV-vis and fluorescence spectroscopy (Figure 4). The Soret spectrum, which corresponds to the absorption maximum at 416 nm, suggests the presence of a mixture of sulfheme derivatives. Pure SHbO₂, SHb, and met-aquoSHb have maximum absorptions at 412 nm, 423 nm, and 403 nm, respectively [73, 81]. A mixture of sulfHb species is expected in our experiments due to the long reaction times. In addition, excess hydrogen sulfide in the reaction plays a significant role in product formation as it can lead to heme reduction under our experimental conditions [45]. For sulfhemoglobin fluorescence detection, the



FIGURE 4: A sulfhemoglobin absorption spectra (open circles) and emission spectra (squares).

parameters used were excitation and emission slits 10/10, an excitation wavelength of 420 nm, and an emission wavelength of 460 nm. Figure 4 shows a fluorescence profile signal observed upon sample excitation at 420 nm, with a maximum emission of 460 nm. The normalized spectra show a characteristic Stokes shift of 40 nm (UV-vis absorption maximum minus fluorescence maximum), which indicates these processes. It is worth noting that the heme group typically quenches the fluorescence signal of hemo-globin. However, in the case of sulfhemoglobin, the sulfur ring attached to pyrrole B may allow for the observed fluorescence signal, potentially overcoming, possibly by another mechanism, the quenching effect.

To investigate the potential of fluorescence spectroscopy to detect sulfHb in solution, individual UV-vis and fluorescence spectra were obtained for oxy-hemoglobin (550 μ M), met-aquo hemoglobin (55 μ M), and sulfhemoglobin (5.63 μ M), as shown in Figure 5. In the 600 nm region, the absorption spectra of met-aquo hemoglobin and sulfhemoglobin show similar electronic transitions between the two species. However, the two species have a concentration difference of approximately tenfold (55 μ M and 5.53 μ M). This contrast is not observed in the case of oxy-Hb (Figure 5(a)), where the absorption spectrum differs significantly from those of met-aquo hemoglobin and sulfHb.

As discussed earlier, the smaller intensity of the (A_{623}/A_{576}) absorbance ratio suggests the presence of a mixture of sulfHb species. This ratio indicates the relative concentrations of the absorbance at 623 nm and 576 nm, respectively, corresponding to characteristic transitions for sulfHb. The different ratios observed for met-aquo hemoglobin further support the notion of a mixture of sulfHb species in the solution. These findings suggest that fluorescence spectroscopy could be a valuable tool for detecting sulfHb in solution, as the fluorescence properties of sulfHb may differ from those of the other hemoglobin species. Upon excitation at 420 nm, which corresponds to the Soret π to π^* transitions (415 nm oxy-Hb, 405 nm met-aquo Hb, and 421 nm sulfHb),



FIGURE 5: (a) UV-vis absorption and fluorescence (b) spectra of oxy-hemoglobin (55.0 μ M), met-aquo hemoglobin (55.0 μ M), and sulfhemoglobin (5.63 μ M).



FIGURE 6: UV-vis (a) and fluorescence (b) spectra of sulfhemoglobin formation upon the reaction of oxy-hemoglobin (55μ M) in the presence of met-aquo hemoglobin (5.5μ M) and H₂S solution (1,155 μ M) as a function of time. (c) Same conditions but in the absence of met-aquo hemoglobin.



FIGURE 7: Sulfhemoglobin fluorescence (460 nm) and visible absorbance (623 nm) intensity relationship.



FIGURE 8: Fluorescence intensity at 460 nm vs. percentage of sulfhemoglobin in the sample. (a) Sulfhemoglobin from oxy-hemoglobin ($55 \,\mu$ M) in the presence of met-aquo hemoglobin ($5.5 \,\mu$ M) and H₂S solution ($1,155 \,\mu$ M). (b) Sulfhemoglobin upon oxy-hemoglobin reaction ($55 \,\mu$ M) H₂S solution ($1,155 \,\mu$ M).

a fluorescence response is observed at 460 nm (Figure 5(b)). Interestingly, the fluorescence response of sulfhemoglobin is greater in intensity than that of met-aquo hemoglobin or oxy-hemoglobin, despite the sulfheme species having a concentration ten times lower than the other hemoglobin species. This finding further supports the hypothesis that fluorescence spectroscopy can detect sulfHb. To evaluate sulfHb formation in a mixture of oxy-hemoglobin (55.0 μ M) and met-aquo hemoglobin $(5.5 \,\mu\text{M})$, hydrogen sulfide $(1,155 \,\mu\text{M})$ was added, and the reaction was monitored for 65 minutes. The presence of H₂S excess ensures the formation of a mixture of sulfHb derivatives. Under these conditions, Figures 6(a) and 6(b) show an increase in intensity in the 623 nm and the 460 nm fluorescence spectra, respectively. The same experiment was performed without met-aquo hemoglobin, yielding similar results. These results strongly support the notion that excitation at the sulfheme Soret transition at 420 nm leads to an increase in fluorescence intensity at 460 nm, indicative of sulfHb formation.

3.3. Sulfhemoglobin Correlations of UV-Vis and Fluorescence Data. Figure 7 illustrates the relationship between the charge transfer intensity of the transition at 623 nm (Figure 6(a)) and the fluorescence intensity at 460 nm (Figure 6(b)) for sulfHb. The data demonstrate a linear behavior between these spectroscopic properties. It is worth noting that a similar trend is observed for the relationship between the UV-vis spectra of sulfHb formation over time, as oxy-hemoglobin (55μ M) reacts with H₂S solution (1,155 μ M), and the fluorescence intensity at 460 nm. Although this figure is not shown, the linear relation between these measurements further supports the significance of sulfHb fluorescence and its correlation with the classical 623 nm sulfheme transition characterized by π to $d\pi$ (dyz, dxz) charge transfer.

Figure 8 demonstrates the relationship between the fluorescence intensity at 460 nm and the percentage of sulfHb in the samples, as calculated from Figures 6(a) and 6(b). Physiological levels of sulfHb have been estimated to be

below 0.037 g/dL (5.5 µM, ~0.28%), while clinically detectable cyanosis symptoms can occur at levels around 0.5 g/dL (74.4 µM, ~3.8%) in the blood [1, 57-61]. Severe sulfHb cyanosis rarely exceeds 10% ($200 \,\mu$ M) [62]. Interestingly, smaller (A_{623}/A_{576}) absorbance ratios have been associated with a 10% to 15% sulfHb yield in patient-derived sample [73, 87, 88]. Figure 8 also indicates a direct relationship between the fluorescence data at 460 nm and the percentage of sulfheme in the sample. These values are similar to the reported sulfHb range observed in patients. Therefore, this approach suggests that the relationship between sulfHb Soret excitation at 420 nm, the induced fluorescence at 460 nm, and its correlation with the charge transfer absorption at 623 nm is a valuable development that can enhance the detection of sulfHb in patients. Nevertheless, further in vitro and in vivo studies are necessary to better understand the mixture of sulfheme derivatives present under the 623 nm transition over extended periods.

4. Conclusion

The data presented in the study support the idea that the combination of fluorescence spectroscopy and UV-vis spectroscopy can be a powerful and effective method for detecting sulfhemoglobin in the blood. The fluorescence response at 460 nm, coupled with the absorbance ratios at specific wavelengths, provide valuable information about the presence and concentration of sulfhemoglobin. This approach offers a fast and accurate detection method for sulfhemoglobinemia in patients. This technique may allow healthcare professionals to better understand and determine sulfhemoglobin levels, allowing for appropriate medical interventions and treatments as needed.

Data Availability

The data used to support the findings of this study are included within the article. Additional information is available upon request to Lysmarie Santos Velázquez.

Disclosure

A provisional patent application number 63/601, 191/2023, incorporating part of this report has been filed.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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