APPLICATION NOTE

Measuring CO-Oximetry Parameters in Bovine Hemoglobin Utilizing Slope Spectroscopy®

Carolina Cabral, Prasanna Reddy, Shaunak Pandya, Nigel Herbert, and Joe Ferraiolo

Variable. Pathlength. Technology.

Abstract

In this work we demonstrate the application of the CTech™ SoloVPE® System combined with multicomponent analysis to measuring CO-oximetry parameters in bovine hemoglobin samples and its applicability to the measurement of different binding states: carboxyhemoglobin (HbCO), deoxyhemoglobin (RHb), oxyhemoglobin (HbO_2), and methemoglobin (MetHb).

The flexibility of the SoloVPE platform, in conjunction with multicomponent analysis, allows for quick measurements of molar extinction coefficients, the total concentration, and individual hemoglobin derivative concentrations. The range of concentrations presents a challenge for UV-fixed pathlength spectroscopy due to the spectral limitation of low concentration derivatives (Wagh et al., 2018). Slope Spectroscopy resolves this by varying the pathlength to attain the ideal range of absorbance. Quantifying hemoglobin derivatives in clinical and research environments allows for the development of hemoglobin-based oxygen carriers (HBOCs), which can be used to treat a variety of diseases.

DOC0235 Rev. 00 05/27/2021

APPLICATION NOTE

Introduction

Hemoglobin is an iron-rich protein in red blood cells that is responsible for transporting oxygen throughout the body. The molecule comprises four subunits, each containing an iron atom bound to a heme group. The heme is a prosthetic group, which has different states of oxidation depending on the binding compound. The four main binding states that were analyzed in this paper include HbO_2, HbCO, RHb, and MetHb. HbO_2 is formed when the heme binds to oxygen. HbCO is formed when the heme binds to carbon monoxide. RHb is formed when the heme binds to nothing. MetHb is formed when the heme breaks down. There are a variety of factors that affect hemoglobin binding and release, such as temperature, pH, and O_2/CO_2 pressure.

One of the most important components of critical care assessment is oxygenation. The ability to assess the oxygenation status in critical care with a high degree of accuracy allows for the development of artificial blood substitutes that can be used to treat a variety of diseases related to anemia and ischemia: HBOCs. The many advantages that HBOCs have over human blood include availability, compatibility, and long-term storage.

CO-oximetry involves measuring the absorption of light passing through blood at several wavelengths. CO-oximeters use multicomponent analysis of UV absorbances to determine concentrations of oxygen-binding states of whole blood and hemoglobin products. Although CO-oximeters may be a valuable tool, they do have their limitations. To start, they have fixed wavelengths of interest for each hemoglobin derivative. If the wavelength shifts, the CO-oximeter will not be able to measure the sample at the appropriate lambda max. Additionally, CO-oximeters utilize single, fixed pathlength absorbance readings. As a result, dilutions are required for the samples to be within the linear range of the instrument. Dilutions can be a major source of error, affecting the optical density and calculated concentration of the sample.

The SoloVPE System is an alternative UV-Vis spectroscopic technology that can overcome the limitations of the CO-oximeter. The Slope Spectroscopy method utilizes variable pathlength technology and the Slope Spectroscopy equation to determine the slope value of a sample at the wavelength of interest. The Slope Spectroscopy equation is a manipulation or derivation of the Beer-Lambert law. Beer's law is expressed as $A = \varepsilon lc$ where A is the measured absorbance, ε is the molar extinction coefficient, l is the pathlength, and c is the concentration of the sample. The Slope Spectroscopy equation moves the pathlength to the left side of the equation, resulting in the change in absorbance per change in pathlength ($A/l = \varepsilon * c$). This results in a new expression called the slope value which allows for the concentration or extinction coefficient to be calculated. The Slope Spectroscopy equation can now be expressed as $m = \varepsilon * c$.

The SoloVPE System operates by measuring absorbance values at varying pathlengths. The pathlength is defined as the distance between the light-delivering Fibrette® and the bottom of the sample vessel. The instrument's hardware and software control the pathlength by precisely moving the Fibrette up and down within the sample. The SoloVPE System can take measurements between 5 µm and 15 mm, with a pathlength resolution of 5 µm. The software calculates the linear regression coefficient (R^2) of each sample, which verifies the validity of the measurement. Values close to one confirm a strong correlation with Beer's law by demonstrating that the

absorbance values change proportionally with the pathlength values. The System's R^2 criteria is ≥0.999, demonstrating near-perfect correlation with Beer's law. This allows the System to accurately measure samples with a wide range of concentrations (for further details, see https://ctech.repligen.com/solovpe/).

Materials and Methods

CO-oximetry standards for hemoglobin derivatives HbO_2, HbCO, RHb, and MetHb were generated following standard preparation methods described in literature (Meng & Alayash, 2017). The OSM3 radiometer was first used to determine the total hemoglobin concentration (tHb) of all the derivatives. It uses the known maximum absorption wavelengths for each derivative; 535 nm (HbCO), 560 nm (RHb), 577 nm (HbO_2) and 636 nm (MetHb). The SoloVPE System was used to determine the extinction coefficients of the derivatives from the tHb concentration result from the OSM3. First, the tHb concentration result from the OSM3 was used to calculate the extinction coefficient from the known protein at 280 nm with the SoloVPE System. The calculated extinction coefficient was then used to measure and verify the tHb concentration on the SoloVPE System. Once the calculated tHb from the System was determined, this value was used to determine the extinction coefficients of the remaining derivatives.

The MultiQ-M feature within the SoloVPE System software allows for up to five wavelength measurements simultaneously. This feature, in conjunction with multicomponent analysis, makes it possible to determine the percentage of the derivative concentration relative to the total tHb. In order to calculate these values, the inverse matrix of extinction coefficients (K) must be multiplied by the slope vector (m).

The Beer's Law Multicomponent Analysis Equation is as follows:

$$\mathrm{A}_{\mathrm{Total}} = \varepsilon_{535}\mathrm{C}_{CO}l + \varepsilon_{560}\mathrm{C}_{\mathrm{RHb}}l + \varepsilon_{577}\mathrm{C}_{\mathrm{O2}}l + \varepsilon_{636}\mathrm{C}_{Met}l \ldots..\varepsilon_n\mathrm{C}_n l$$

Beer's law can be applied to a multicomponent system where the total absorbance is equivalent to the extinction coefficient, concentration, and pathlength of each component.

Slope Spectroscopy Conversion is as follows:

$$\mathrm{A}_1 = \varepsilon_1 \mathrm{c}_1 l \qquad \varepsilon_1 c_1 = \frac{\mathrm{A}_1}{l} \qquad \frac{\mathrm{A}_1}{l} = m \qquad C = \mathrm{m}[\varepsilon]^{-1}$$

Slope Spectroscopy can be applied by varying the pathlength to attain a slope value. The slope can be multiplied by the inverse matrix of extinction coefficients to determine the concentration of the intended component.

The Multicomponent Analysis Slope Equation is as follows:

$$\begin{bmatrix} m_{CO} \\ m_{RHb} \\ m_{O2} \\ m_{Met} \end{bmatrix} = \begin{bmatrix} \varepsilon_{535} + \varepsilon_{560} + \varepsilon_{577} + \varepsilon_{636} \\ \varepsilon_{535} + \varepsilon_{560} + \varepsilon_{577} + \varepsilon_{636} \\ \varepsilon_{535} + \varepsilon_{560} + \varepsilon_{577} + \varepsilon_{636} \\ \varepsilon_{535} + \varepsilon_{560} + \varepsilon_{577} + \varepsilon_{636} \end{bmatrix} \begin{bmatrix} C_{CO} \\ C_{RHb} \\ C_{O2} \\ C_{Met} \end{bmatrix}$$

The equation can be rewritten in terms of a matrix using a model known as Classical Least Squares as below:

$$c(HbCO) = (K_{CO} * m_{535}) + (K_{CO} * m_{560}) + (K_{CO} * m_{577}) + (K_{CO} * m_{636})$$

$$c(RHb) = (K_{Red} * m_{535}) + (K_{Red} * m_{560}) + (K_{Red} * m_{577}) + (K_{Red} * m_{636})$$

$$c(HbO_2) = (K_{O2} * m_{535}) + (K_{O2} * m_{560}) + (K_{CO} * m_{577}) + (K_{CO} * m_{636})$$

$$c(MetHb) = (K_{Met} * m_{535}) + (K_{Met} * m_{560}) + (K_{Met} * m_{577}) + (K_{Met} * m_{636})$$

The equation can then be rewritten to determine the concentration of each component. The slope of each component can be multiplied by the inverse matrix of the intended component, and then added together to determine the component's concentration.

A set of standards was prepared to measure MetHb impurity in HbO_2. MetHb derivatives (98%) were spiked into HbO_2 (100%) to demonstrate specificity, linearity, accuracy, range, and repeatability. Samples were prepared in 1 mL tubes with 10 µL of sample being used in the plastic vessels for analysis. The values measured on the SoloVPE System were compared to those from a standard clinical CO-oximeter. The extinction coefficient matrix was based on multilinear regression values obtained by the CO-oximeter and the SoloVPE System.

Results

Deriving Extinction Coefficients

The UV spectra was generated for all four hemoglobin derivatives (see Figure 1).

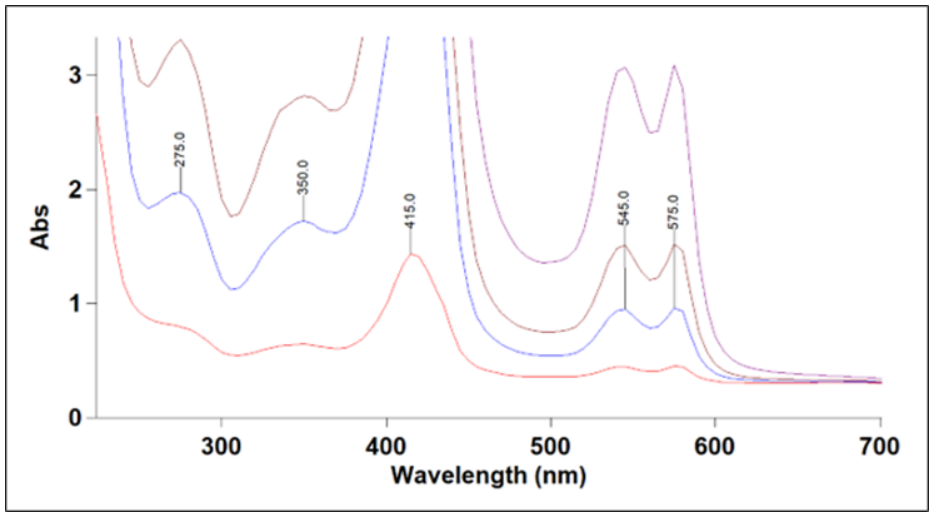

Figure 1. Example Quick Survey of deoxyhemoglobin standard.

APPLICATION NOTE

These samples were closest to 100% of their major derivative, exhibiting strong absorbance profiles at their intended wavelength of interest. Utilizing the SoloVPE software and its MultiQ-M feature, the extinction coefficients ((mg/mL)-1cm-1) for each derivative were calculated and demonstrated in a matrix (see Table 1).

Table 1. Extinction coefficients for hemoglobin derivatives

$\varepsilon =$

HbCO	RHb	HbO_2	MetHb
0.75386	0.60553	0.72269	0.42873
0.65654	**0.78702**	0.47165	0.25938
0.57873	0.60341	**0.85233**	0.25175
0.00399	0.11589	0.00122	**0.25945**

The inverse matrix of extinction coefficients was then generated from Table 1 and demonstrated in Table 2.

Table 2. Inverse matrix of extinction coefficients

$[\varepsilon^{-1}] =$

HbCO	RHb	HbO_2	MetHb
3.81782	0.01598	-3.24142	-3.17954
-3.19730	**2.48782**	1.33216	1.50362
-0.73426	-1.44582	**2.59570**	0.14010
1.37290	-1.10470	-0.55740	**3.23091**

Method Specificity

Buffer solutions of phosphate-buffered saline (PBS) and water were analyzed to determine the specificity of the sample. The slopes of the buffers were less than 0.01 (see Table 3), which is the baseline correction threshold for the SoloVPE System. In other words, there is no pathlength-dependent absorbance contribution by buffer components at the method wavelength.

Table 3. Average slope of PBS and water solutions

Sample	PBS	Water
Wavelength (nm)	Average Slope (Abs/mm)	Average Slope (Abs/mm)
535.00	-0.03075	0.00075
560.00	-0.00113	0.00074
577.00	-0.00156	0.00072
636.00	-0.00134	0.00086

Method Linearity

MetHb was spiked in HbO_2 to produce seven standard solutions (see Table 4). The linearity was analyzed to demonstrate the relationship between the nominal spiked %MetHb and the experimental values. The linear regression is illustrated in Figure 2 from the data in Table 4, where the coefficient of determination was ~0.999.

Table 4. Relationship between nominal spiked %MetHb and experimental values.

Sample	Spiked %MetHb	Measured %MetHb
No spike	0	1.5
CAL-1	0.5	2.0
CAL-2	1	2.4
CAL-3	2	3.3
CAL-5	3	4.2
CAL-7	4	5.1
CAL-9	5	5.8
CAL-10	6	6.9

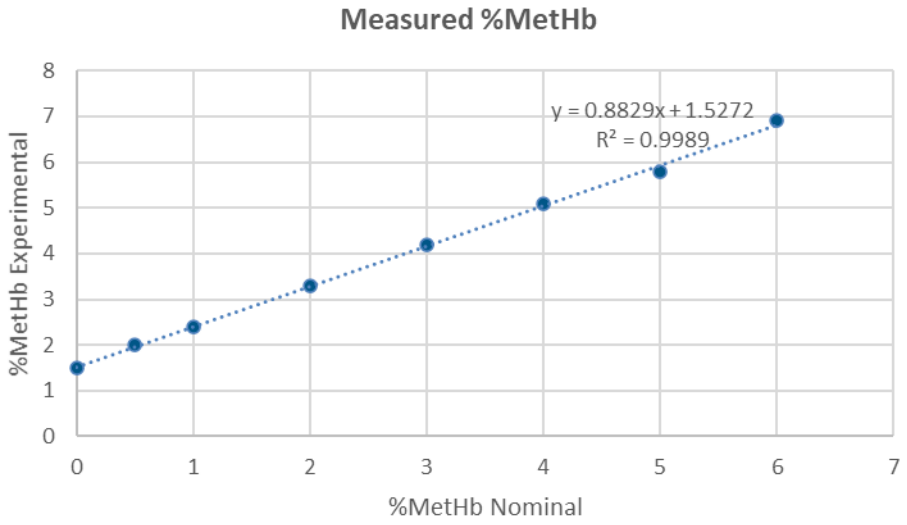

Figure 2. Linear regression of absorbance in measured %MetHb.

Method Accuracy

The accuracy was evaluated by measuring three standard solutions: HbO_2 spiked with 2.5%, 3.5%, and 4.5% MetHb. The measured %MetHb was divided by the nominal spiked %MetHb to determine the accuracy. The accuracy varies from ~97% to 102% (see Table 5).

Table 5. Method accuracy results

Sample	%Met Spiked	Measured %Met	Calculated	%Recovery
CAL-4	2.5	3.7	2.5	100.00
CAL-6	3.5	4.5	3.4	97.14
CAL-8	4.5	5.6	4.6	102.22

Method Range

The range for this assay was established based on the precision, linearity, and accuracy results. Since all samples are analyzed neat and device response for each sample is optimized based on observed absorbance, any sample that falls within the precision, linearity, and accuracy of the method is capable of being analyzed. Therefore, no additional studies are required for determination of method range.

Method Repeatability

The repeatability was demonstrated by analyzing six replicates of a sample containing HbO_2 with 2.5% excess spiked MetHb (see Table 6). The $\%HbO_2$ and %MetHb were calculated for each sample. From those six replicates, the average, standard deviation, and percent relative standard deviation (%RSD) was calculated.

Table 6. Repeatability of method results

Sample	$\%HbO_2$	%MetHb
Replicate 1	99.9	3.7
Replicate 2	100.2	3.9
Replicate 3	100.0	3.8
Replicate 4	99.9	3.7
Replicate 5	99.5	3.8
Replicate 6	99.5	4.0
Average	99.83	3.82
Standard Deviation	0.28	0.12
%RSD	0.28	3.06

Discussion

MetHb is a derivative of hemoglobin that is incapable of carrying oxygen. It has high clinical significance even at low concentrations. In this work, MetHb impurity in HbO_2 was determined using the SoloVPE System and multicomponent analysis. Multicomponent analysis is an effective analytical method in measuring the concentration of hemoglobin CO-oximetry derivatives. It is important to understand that the concentration of MetHb is not the only contributing factor to the final calculation. The percentage depends heavily on the main derivative and stability of the sample. This is evident within the results of the slope vector and the inverse matrix.

The %MetHb results obtained from the SoloVPE System were compared to the OSM3 radiometer. While radiometers and CO-oximeters are useful tools, they are, however, limited to fixed wavelengths and are tailored to clinically relevant ranges of CO-oximetry. Traditional UV-Vis methods utilizing multicomponent analysis are also useful; however, dilution errors can directly impact the optical density readings and calculated sample calculations.

Slope Spectroscopy with multicomponent analysis was successfully employed to measure the concentration of hemoglobin derivatives. The SoloVPE System was able to effectively demonstrate specificity, linearity, accuracy, range, and repeatability of the CO-oximetry derivatives. The System's ability to rapidly generate section data at a given wavelength is one of its most powerful features. The variable pathlength technology eliminates the time-consuming and error-prone use of serial dilutions. One measurement can determine the concentration of hemoglobin derivatives at multiple wavelengths. The SoloVPE System's accuracy, simplicity, and wide range of concentrations make it one of the best methods for this application.

References

Meng, F., & Alayash, A. I. (2017). Determination of extinction coefficients of human hemoglobin in various redox states. Analytical Biochemistry, 521, 11–19. https://doi.org/10.1016/j.ab.2017.01.002

Wagh, A., Song, H., Zeng, M., Tao, L., & Das T. K. (2018). Challenges and new frontiers in analytical characterization of antibody-drug conjugates. MAbs, 10(2), 222–243. https://doi.org/10.1080/19420862.2017.1412025