924393 Application of Slope Spectroscopy® to the Measurement of Co-oximetry Parameters in Bovine Hemoglobin: The Case of Measuring Low Concentration Met Hemoglobin in Bovine Hemoglobin Samples

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PURPOSE

To describe the application of the Slope Spectroscopy® method (measurement of absorbance at variable pathlength (1)) in the measurement of hemoglobin derivatives by multicomponent analysis. The example of bovine hemoglobin and the quantitation of hemoglobin derivative concentrations is shown. Multicomponent analysis has been routinely used to measure concentration of antibody drug conjugates (ADCs). The range of protein concentrations compared to the conjugated drug presents a challenge for UV fixed pathlength spectroscopy due to the spectral limitation of low concentration derivatives (2). The Slope spectroscopy resolves the issues with fixed pathlength by manipulating the Beer Lambert Law and varying the pathlength to attain the ideal range of absorbance. With this approach, strong linear correlation can be achieved, and a wide range of concentrations can be measured.

METHOD

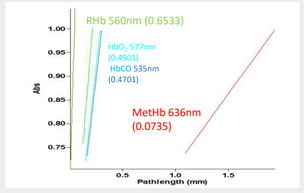
Co-oximetry standards for hemoglobin derivatives (Carboxy-hemoglobin (HbCO), Deoxy hemoglobin (RHb), Oxy- hemoglobin (HbO_2) and Met hemoglobin (MetHb) were generated following standard preparation methods described in the literature (3) with slight modifications.

Coefficient of extinction (CE) were measured utilizing SoloVPE system (Repligen Inc.) at operating in quick slope mode with multiple wavelength acquisition at 535, 560, 577 and 636nm. These values correspond to the maximum absorption wavelengths of major bovine hemoglobin derivatives Carboxy-hemoglobin (535 nm), DeoxyHb (560 nm), OxyHb (577nm) and MetHb (636nm). The user concentration set by an alternative measurement in OSM3 (Radiometer) for total hemoglobin, tHb, which is the sum of the four components described above.

Since absorbance is an additive property of the matter, multicomponent analysis is applicable, the slope equation can then be written as: $[m] = [\mathcal{E}] \bullet [C]$, therefore, : $[m] \bullet [\mathcal{E}]^{-1} = ([\mathcal{E}] \bullet [\mathcal{E}]^{-1}) \bullet [C]$, and therefore,

$$[\mathbf{C}] = [\mathbf{m}] \bullet [\boldsymbol{\mathcal{E}}]^{-1}$$

where **[m]** is $m = [S_{535}\ S_{560}\ S_{577}\ S_{636}]$, the vector of Slopes obtained for a given sample (S_{WL} = SoloVPE Slope measured at the given WL), **$[\mathcal{E}^{-1}]$** = Inverted matrix of coeff. of extinction. The concentration of hemoglobin derivatives was calculated by, multiplying the Slope vector [m] by the Inverse matrix of CEs **$[\mathcal{E}^{-1}]$.**



Typical slope values for Hemoglobin component wavelengths in a pure DeoxyHb sample.

To measure MetHb impurity in oxyhemoglobin preparations, MetHb derivative (98% MetHb) were spiked into OxyHb (100%) to generate a set of standards to demonstrate the method capability of measuring low concentrations of MetHb in OxyHb samples and to demonstrate linearity, range, precision and accuracy. Standards were in MetHb% (Neat, 0.5, 1, 2, 3, 4, 5 and 6%), QC's were 2.5, 3.5 and 4.5% MetHb. Samples were prepared in 1.0 mL volume (minimum pipetted volume was 10 µL) and measured in Multi-Quick Slope method.

The values obtained were compared to values that were measured in a standard clinical co-oximeter. Adjustments and optimization of the extinction coefficient matrix were based on multilinear regression values obtained by the co-oximeter and the SoloVPE.

RESULTS

Deriving Coefficients of Extinction

All four bovine hemoglobin derivatives were generated and confirmed by for their distinct UV profiles (Figure 1).

Figure 1. UV-vis spectrum of derivatives generated. Note the contribution of the sample in multiple WL. These samples were closest to 100% its major derivative, the lines point to the wavelengths were coefficient of extinction were measured.

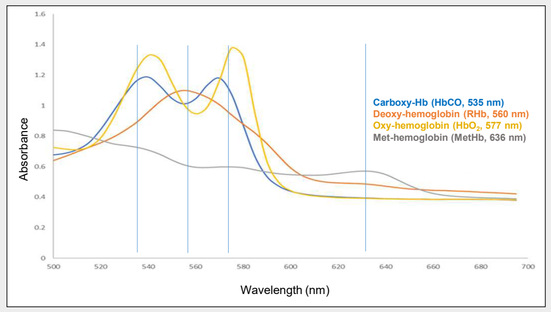


Table 1. Matrix of coefficient of extinction (mL/(mg•cm)) obtained from generated samples utilizing SoloVPE. is shown in :

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

Coefficients of extinction of hemoglobin derivatives, given in mL/(mg•cm)

Table 2. Calculated Inverse matrix of CEs

$[\mathcal{E}^{-1}]$ =

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

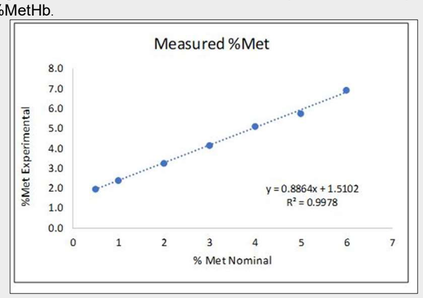
Method specificity was demonstrated by analyzing blank buffer solution and sample solution. The slopes obtained for the blank buffer were less than 0.01 (Table 3), which is the baseline correction threshold for the SoloVPE System. These results confirm that MetHb is not present in buffer. The method was found to be specific.

Table 3. Specificity and typical slopes for standards utilized in the experiments.

Sample	Blank PBS Buffer	WATER	HbO_2 Std	MetHb Std
WL (nm)	Avg-M Abs/mm	Avg-M Abs/mm	Avg-M Abs/mm	Avg-M Abs/mm
535.00	-0.03075	0.00075	2.24629	7.51011
560.00	-0.00113	0.00074	1.46181	4.96518
577.00	-0.00156	0.00072	2.69589	7.43075
636.00	-0.00134	0.00086	0.02319	1.46775

Method Linearity was evaluated by analyzing 7 standard solutions of calibrators by spiking Met hemoglobin in Oxy hemoglobin (Figure 2). The linear regression presented in Figure 1 illustrates the linear relationship of nominal spiked % Met with experimental values, the regression has a coefficient of determination = 0.9978.

Figure 2. Linear correlation of % MetHb experimental with spiked %MetHb.



Method accuracy was evaluated by assaying 3 calibrators generated in the 2.5, 3.5 and 4.5% MetHb. The calculated accuracy was calculated by dividing the percentage MetHb obtained by the method divide by the nominal MetHb spike. Accuracy varies from 97.5% to 101.9% (Table 4).

Table 4. Linearity Values of calibrators and accuracy samples

	%Met spiked	Measured %Met		
No spike	0	1.5		
CAL-1	0.5	2.0		
CAL-2	1	2.4	**Intercept**	1.5102
CAL-3	2	3.3	**Slope**	0.8864
CAL-5	3	4.2	**R2**	0.9978
CAL-7	4	5.1		
CAL-9	5	5.8		
CAL-10	6	6.9		
			Calculated	% Accuracy/Recovery
CAL-4	2.5	3.7	2.5	100.3
CAL-6	3.5	4.5	3.4	97.5
CAL-8	4.5	5.6	4.6	101.9

Method range for Met hemoglobin for this assay was established based in the precision, linearity and accuracy (Figure 2).

Method precision was demonstrated by analyzing 6 replicates (Table 5) of a sample containing a 2.5% excess MetHb spike (Cal-4, from accuracy study).

Table 5. Precision of the measurements

Sample	% Oxy Hemoglobin	% Met Hemoglobin
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	**99.8**	**3.8**
SD	**0.3**	**0.1**
%RSD	**0.3**	**2.5**

Lower Limit of Quantitation LLOQ was established by the precision of the slope at the lowest concentration observed. The criterion for slope precision as per manufacture validation is %RSD≤ 2%. The experiment for the LLOQ was performed in diluted hemoglobin solution generated from whole blood lysates, where slopes reached average value of $S_{AVG(n=6)}$ = 0.01443 Abs/mm (STD =0.00021, **%RSD 1.4**%, in 6 independent determinations). The amount of MetHb being measured was at **0.37 mg/mL** ($C_{AVG(n=6)}$= 0.37mg/mL , STD= 0.01 and %RSD=2.6%) in samples containing total hemoglobin of 11.0 mg/mL and 3% MetHb ($\%Met_{AVG(n=6)}$= 3.2%, STD =0.1, %RSD=2.6%).

RESULTS (CONTD)

Detection Limit was measured to be the lowest slope where a sample contribution to slope could be measured. The criterion of regression having a minimum of 5 points in the linear regression and the coefficient of determination be $R^2 \geq 0.999$.

It is important to note that in the case of multicomponent analysis, MetHb slope is not the only contributor to the final calculation. The multiplication of the slope vector with the inverse matrix, carries weight of other components' contributions to each wavelength. The percentage depends heavily on the type (main component being Oxy, Deoxy, Carboxy or Met) and stability of the sample.

The results reported in this work were confirmed side-be-side with a commercial co-oximeter (Radiometer OSM3) to verify %MetHb with an alternative platform. In research space, co-oximeters have the great limitation of being tailored for clinically relevant set ranges of co-oximetry. UV-Vis platform utilizing multicomponent analysis can be utilized to measure co-oximetry parameters, however the limitation on conventional UV platforms rises from the large discrepancy of extinction coefficients in the respective wavelengths for each hemoglobin derivative, as a direct consequence, linearity would not be attained without dilution and/or modification of the pathlength.

The ability of the SoloVPE System to rapidly generate section data at a given wavelength (absorbance vs. pathlength) is one of its most powerful features. More importantly, the variable pathlength technology eliminates the time-consuming and error-prone use of dilution factors commonly seen with fixed pathlength technology to attain linearity range of Beer-Lambert Law. Dilution errors can significantly impact the optical density readings and calculated sample concentrations.

CONCLUSIONS

Slope spectroscopy with multicomponent analysis was successfully employed to measure the concentration of hemoglobin derivatives. MetHb is a derivative of hemoglobin that is unable to carry oxygen and has high clinical significance even at low concentrations.

In this work we present the feasibility to determine MetHb impurity in oxy hemoglobin preparations. The Met hemoglobin concentration in the example shown was approximately 0.3 – 2.0 mg/mL (i.e. 1.0 - 6.0%) in solutions at ~30mg/mL of total bovine hemoglobin.

With this application it is possible to calculate content of carboxy hemoglobin, oxy hemoglobin, deoxy hemoglobin along with the met hemoglobin with accuracy, reproducibility and precision levels matching that of commercial co-oximeters.

This method is a lot simpler and cheaper than using a clinical co-oximetry device. An external calibration is not necessary and clean up does not require running cycles of proprietary buffers and standards.

REFERENCES

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3 - Determination of extinction coefficients of human hemoglobin in various redox states. Anal Biochem. 2017 March 15; 521: 11–19. doi:10.1016/j.ab.2017.01.002.

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