

Plasma Hemoglobin Determination: Two Manual Methods Compared

An Honors Thesis (HONRS 499)

by

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ABSTRACT

Plasma hemoglobin levels are clinically significant in the determination of the amount of in vivo hemolysis. In this study, two manual methods for plasma hemoglobin were compared. The current method uses sodium carbonate to measure oxyhemoglobin at three wavelengths: 380 nm, 415 nm, and 450 nm. The second method, from SIGMA Diagnostics, uses 3,3',5,5'-tetramethylbenzidine in a peroxidase reaction; the absorbance is then read at 600 nm. In the peroxidase method from SIGMA Diagnostics, timing plays a very crucial role in obtaining accurate results. The peroxidase procedure from SIGMA Diagnostics is less time consuming than the current method and is therefore more advantageous. The peroxidase method proves to be the better method due to better linearity and accuracy.

INTRODUCTION

Plasma, or free, hemoglobin levels are used to determine the degree of in vivo hemolysis. Plasma hemoglobin levels at St. Francis Hospital and Health Centers are being determined by a spectrophotometric method which uses sodium carbonate to measure oxyhemoglobin absorption. The purpose of this research is to evaluate the method in use at this institution and to determine the necessity of replacing this method. A peroxidase method from SIGMA Diagnostics was chosen to compare with the spectrophotometric method in use. The peroxidase method will be evaluated as a possible replacement of the current method.

MATERIALS AND METHODS

Equipment and Specimens

There were two methods used in this study for plasma hemoglobin determinations. The spectrophotometric measurements for both methods were performed on a Turner spectrophotometer, model 340.

Random heparinized blood samples with varying degrees of hemolysis were chosen for analysis. All identification was removed from the samples before being assayed. Blood specimens were centrifuged twice at 2000 rpm for seven minutes. If testing was delayed for more than six hours, the specimens were frozen at -20°C . These specimens were used for both methods.

Procedures Used

The current spectrophotometric method involves the use of a sodium carbonate solution to measure the absorbance of oxyhemoglobin at 415 nm. Readings were also taken at 380 nm

and 450 nm; these values were used in a calculation to correct for interferences. A stock solution of 1g/dl was made by dissolving 1 g of Na₂CO₃ in 100 ml of distilled water. A working solution was then made by diluting 1 ml of stock solution to 100 ml with distilled water. A mixture of 3.0 ml of 0.01 g/dl Na₂CO₃ and 0.3 ml of plasma was made. Using the working solution as a blank, absorbance values were read at 380 nm, 415 nm, and 450 nm for this mixture.¹ Plasma hemoglobin levels were determined by the following calculation:

$$\text{Plasma Hemoglobin mg/dl} = \frac{2 \times A_{415} - (A_{380} + A_{450})}{1.655} \times \frac{1000D}{E}$$

D = the dilution made (1:11)

E = absorptivity at 415 nm of a 1 g/dl solution of oxyhemoglobin (79.46)

$\frac{2}{1.655}$ and $\frac{A_{380} + A_{450}}{1.655}$ are corrections for nonspecific absorption.¹

The expected normal range for this method is 0.0 to 5.0 mg/dl.¹

The second method involves the use of a peroxidase reaction to measure plasma hemoglobin. Free hemoglobin will oxidize benzidine in the presence of hydrogen peroxide.² A kit from SIGMA Diagnostics which utilizes this reaction was used. SIGMA has replaced the carcinogen benzidine with 3,3',5,5'-tetramethylbenzidine (TMB), a noncarcinogenic derivative.² The procedure involves adding 2.0 ml of TMB reagent to 0.01 ml of heparinized plasma, mixing, and then adding 2.0 ml of H₂O₂. Exactly ten minutes after the addition of the H₂O₂, the absorbance is read at 600 nm.² The concentration of plasma hemoglobin is calculated with the following formula:

$$\text{Plasma Hemoglobin mg/dl} = \frac{A_{\text{test}} - A_{\text{blank}}}{A_{\text{standard}} - A_{\text{blank}}} \times 30 \text{ (standard)}$$

The normal range for this method is 5.0 mg/dl or less.²

Groups of ten specimens were analyzed per day for four days within a two week time span. A calibration standard and controls were run with each group of specimens. The two methods were performed within 30 minutes of each other.

RESULTS

Lab Data

The specimens analyzed by the peroxide and the sodium carbonate method contained varying degrees of hemolysis. The peroxidase method gave values ranging from 1.9 mg/dl to 83.3 mg/dl with a mean of 27.9 mg/dl. The sodium carbonate levels averaged 17.7 mg/dl with a spread of 0.0 mg/dl to 63.4 mg/dl. Control levels for both methods also showed a comparable difference.

Linearity

The peroxidase method from SIGMA Diagnostics proved to exhibit acceptable linearity (see Exhibit A). This method also demonstrated high precision and accuracy as compared to the sodium carbonate method which displayed high precision and low accuracy (see Exhibit B). Accuracy within the sodium carbonate method decreased as the concentration increased.

Controls

High and low control levels were run in duplicate for both methods. One set was analyzed at the beginning of the run and the other at the end. For the peroxidase method there were two occasions that the high control level fell outside the range set by SIGMA Diagnostics. The first reading outside of the expected range was due to an error in timing; the second was random.

After disregarding the first high control level outside the range, the control levels were acceptable for the peroxidase method according to Westgard rules⁶. The values obtained from the sodium carbonate method for both control levels were consistently low (see Exhibit C).

Statistics

The linear regression line is shown on Exhibit D. The calculated r value was 0.9744. This shows that there is a degree of correlation between the methods. The bias of the two methods was calculated at 10.2. Other statistics, such as the paired t test and the F test that are commonly used to compare two methods are not valid in this study. These statistics are based upon the assumption that the values obtained by the current method are accurate. In this study the peroxidase method has proven to be more accurate based on assayed standards and control levels.

DISCUSSION

Clinical Significance

Plasma hemoglobin levels are used clinically to determine the amount of in vivo hemolysis present. Plasma hemoglobin levels are also used to "monitor patients undergoing extra-corporeal membrane oxygenation, or as a quality control tool for the modified heart-lung bypass machine."⁵

Increased levels of plasma hemoglobin can be attributed to hemolytic transfusion reactions or a problem during extra-corporeal treatment of the patients blood. Researchers have observed that the human eye has difficulty detecting plasma hemoglobin levels of 30 mg/dl.⁴ Normal levels of plasma hemoglobin are 0 to 5.0 mg/dl. This makes it important to choose a testing method capable of detecting increased levels of plasma hemoglobin below 30 mg/dl.

Interferences

The problems associated with the many methods available for plasma hemoglobin determinations are the interferences caused by bilirubin, lipemia, and ceruloplasmin. Each method that is developed tries to avoid or correct for these interferences. The method of measuring oxyhemoglobin absorbance attempts to correct for interferences by reading the absorbance at three different wavelengths. Some researchers note that absorbance values at six wavelengths are necessary to eliminate the interference effects.³ The SIGMA peroxidase method is designed to avoid the interferences at low or moderate levels. The SIGMA kit is only effected by bilirubin results greater than or equal to 20 mg/dl.² Tietz states that when the serum bilirubin concentration is increased, the sodium carbonate method becomes unreliable.¹ Fairbanks et al. state that ceruloplasmin "does not cause a spurious increase in results by the peroxidase method and causes only slight deviations in assays by other methods."³ Many methods are also very time dependent. The way in which a method attempts to avoid interference is an important factor to consider when evaluating a method.

Selection of Method

Based on correlation studies of the current and new method, the peroxidase method is the method of choice. This method was proven to be more linear and to give reproducible results. Bilirubin, a potentially large source of error in most methods, must be greater than or equal to 20 mg/dl to interfere with the peroxidase method. There are drawbacks to the peroxidase method. Timing played a very crucial role in obtaining accurate results. Once the hydrogen peroxide solution was added to the test mixture, the timing became critical. The absorbance must be read exactly ten minutes after the addition of the hydrogen peroxide solution. Any delay in reading the

absorbance will cause a falsely increased plasma hemoglobin concentration. Another drawback to this method is the odor of the TMB reagent. Although not harmful, it may irritate the eyes; the odor is also unpleasant. The peroxidase method is a fairly quick method in determining plasma hemoglobin levels and has proven to be precise and accurate.

CONCLUSION

Method Evaluation

By evaluation of the control and standard levels obtained by both methods and the calculated statistics, the peroxidase method has proven to be the better method. The peroxidase method has demonstrated greater linearity and better accuracy than the sodium carbonate method. The peroxidase method has also been designed to be less affected by common interfering substances, such as bilirubin and ceruloplasmin. Plasma hemoglobin levels obtained by the peroxidase method for the standard and controls were accurate and reproducible. The sodium carbonate method demonstrated acceptable precision and poor accuracy, as shown in Exhibit B. The reason for this is unknown. The procedure required for measuring plasma hemoglobin by the sodium carbonate method is less technique and technologist dependent, but it is unreliable. The critical timing involved in the peroxidase method makes this method very technique and technologist dependent, yet the advantage of an accurate and precise method makes this method more advantageous.

Recommendations

Further studies are needed to establish a normal range for the patient population at this institution. The random error of plasma hemoglobin levels due to hemolysis during the drawing of blood should also be addressed. A standard technique developed to minimize trauma and decrease hemolysis will increase the accuracy of the test. A study which addresses how long a specimen can be allowed to sit before being analyzed will also be beneficial.

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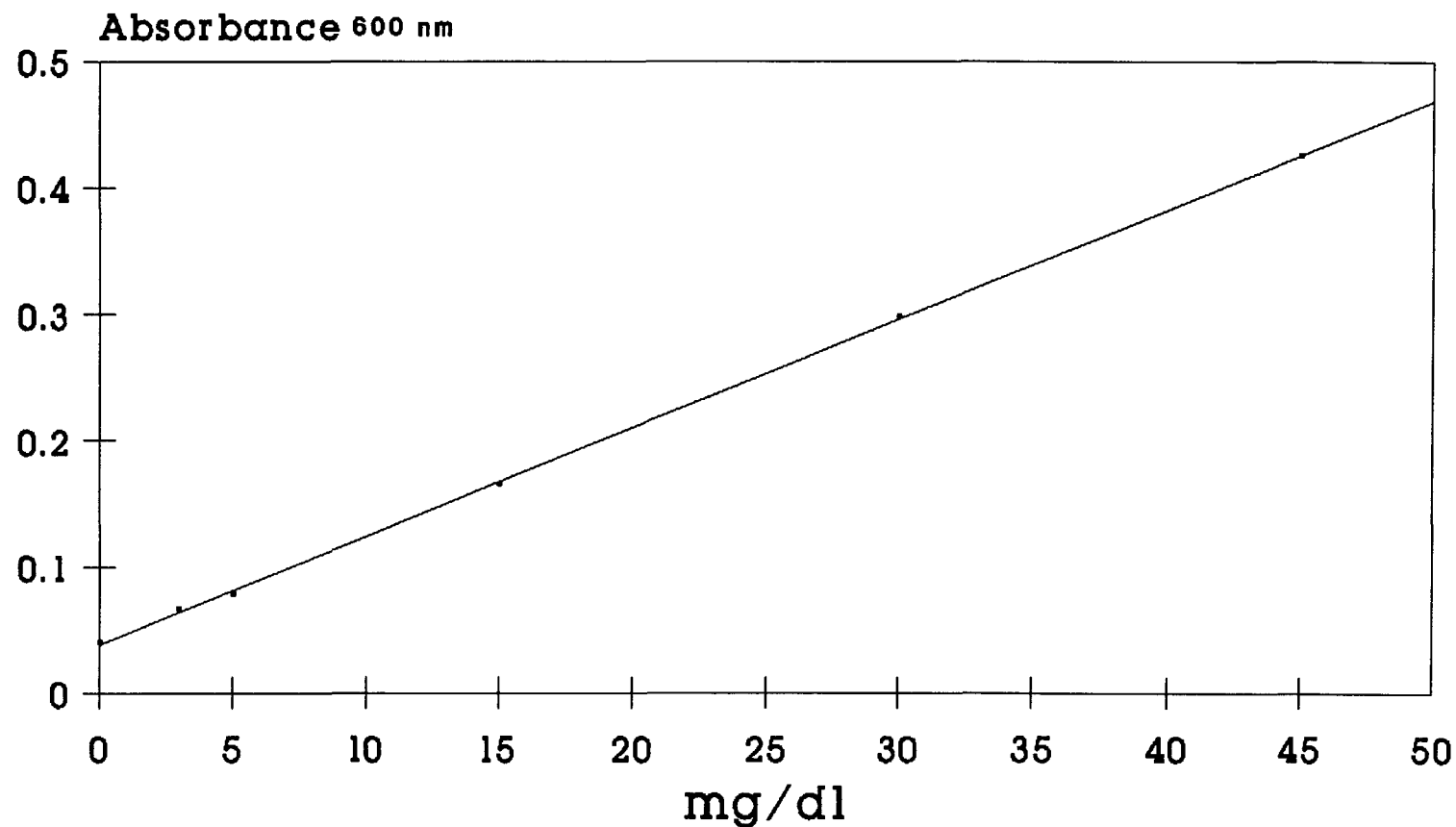
Robert W. Langhammer MT (ASCP)

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Plasma Hemoglobin

Standard Curve for Peroxidase Method



Shown here is the standard curve for the peroxidase method. Absorbance values for all four days have been averaged.

Exhibit A

Exhibit B

Standards for the Sodium Carbonate Method

Standards	Day 1	Day 2	Day 3	Day 4
3 mg/dl	2.4	2.0	0.5	0.7
5 mg/dl	2.7	2.7	3.0	1.2
15 mg/dl	10.1	9.7	9.5	8.8
30 mg/dl	17.9	18.7	17.6	15.6
45 mg/dl	27.2	24.8	24.3	23.5

Values obtained from standards analyzed by the sodium carbonate method exhibit acceptable precision. This method's accuracy decreases as the plasma hemoglobin concentration increases.

Exhibit C

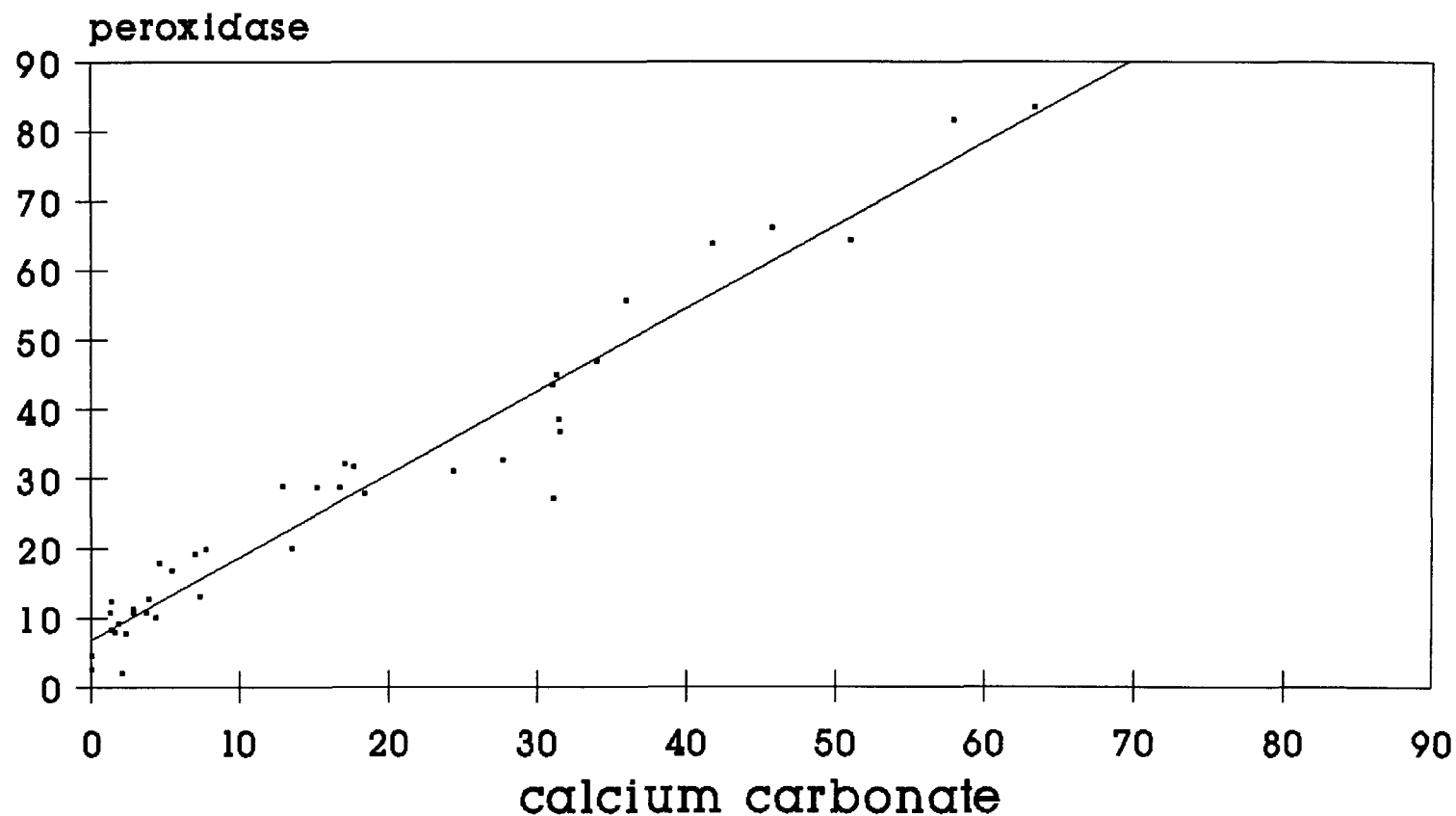
Control Values

	Sodium Carbonate		Peroxidase	
	Low	High	Low	High
Day 1	9.6	21.2	14.9	35.5
	8.6	25.0	14.1	35.6
Day 2	8.9	23.9	15.4	49.0
	10.1	24.4	15.9	44.4
Day 3	7.4	22.6	15.5	42.8
			16.6	48.3
Day 4		13		
	6.9	22.5	15.2	39.6
	6.9	21.7	12.7	32.5

A high and low control level were run each day in duplicate (except for the Na_2CO_3 method on day 3). The expected range for the low control is 12.0 to 20.0 mg/dl; it is assayed at 16 mg/dl. The high control is assayed at 38.0 mg/dl with a range of 31.0 to 45.0 mg/dl.²

Method Comparison

Linear Regression



This graph demonstrates the linear regression line for the two methods. The r value is .9744.

Exhibit D