ABSTRACT

PLASMA HEMOGLOBIN DETERMINATION
BY FIVE WAVELENGTHS ANALYSIS

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In hospitals and other health-related laboratories, there is a need to know the hemoglobin concentration in the plasma of many patients. This helps the physicians in the diagnoses of the patients' malady. This is because hemoglobin can only be found in substantial amounts in the plasma when there is hemolysis of the red cells of the blood.

Two basic physico-chemical properties of the hemoglobin molecule help in the quantitation of plasma hemoglobin. One is it's ability to exhibit catalase activity. Here it catalyzes the split, the break-up, of hydrogen peroxide molecules, releasing nascent oxygen which reacts with a chromogenic antioxidant to form a "color solution", the intensity of whose color is proportional to hemoglobin concentration. This method is time consuming, expensive and some of the anti-oxidants are toxic.

The alternative method is by direct spectrophotometry. This method exploits the known ability of the hemo-

globin molecule to absorb light in some known wavelength of the visible light spectrum.

The objective of this research is to improve one of these spectrophotometric methods, by correcting for the absorption of bilirubin -- a byproduct of hemoglobin catabolism, and a major interference in the determination.

Instead of analyzing the hemoglobin absorption maxima at three wavelengths, to correct for background absorbance, the maxima at five wavelengths is analyzed to correct for both the background and the bilirubin absorption.

This improves plasma hemoglobin determinations, particularly when the bilirubin concentration is high, a condition often encountered in patients undergoing blood transfusion therapy. It is these very patients whose plasma hemoglobin levels need to be monitored the most.

ACKNOWLEDGEMENTS

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LIST OF SYMBOLS

SYMBOL	DEFINITION
Hb	Hemoglobin molecule
PO_2	Partial pressure of oxygen
Fe++	Iron in the ferrous state
H_2O_2	Hydrogen peroxide
A_x	Absorbance at wavelength, x
(O)	Nascent oxygen
mg/dl	Milligram per deciliter
$Hb \cdot H_2O_2$	Hemoglobin-peroxide complex
nm	Wavelength in nanometers
$[Hb]$	Hemoglobin concentration
$B.A_x$	Bilirubin absorption at a given wavelength, x
$\left[\begin{smallmatrix} Hb \\ corrected \end{smallmatrix}\right]$	Corrected Hemoglobin concentration
gm/dl	Grams per deciliter
g	Earth's gravitational constant

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CHAPTER I

THE HEMOGLOBIN MOLECULE

General Introduction and Historical Background

Hemoglobin, the oxygen-carrying molecule in the red blood cell, is made up of two parts. The first part is a protein with a polypeptide chain or globin. The second part is a prosthetic or heme component.[1]

The heme is an iron porphyrin. It is synthesized by the condensation of Glycine and Succinyl-CoA, which is then coupled with a ferrous ion (Fe^{++}). See Figure 1. The heme is then combined with a polypeptide chain to form one of the monomers that constitute the tetrameric hemoglobin molecule.

$$\text{Heme} + \text{Globin} \longrightarrow \text{Hemoglobin}$$

The polypeptide chains are of two kinds; the differences being in their primary structures. Two are alpha (α) chains; the other two are beta (β) chains.

Modifications in the amino-acid sequences of these chains lead to hemoglobin variants. Some of these are normal, like fetal hemoglobin (Hbf). Others are abnormal, like hemoglobin S (HbS).

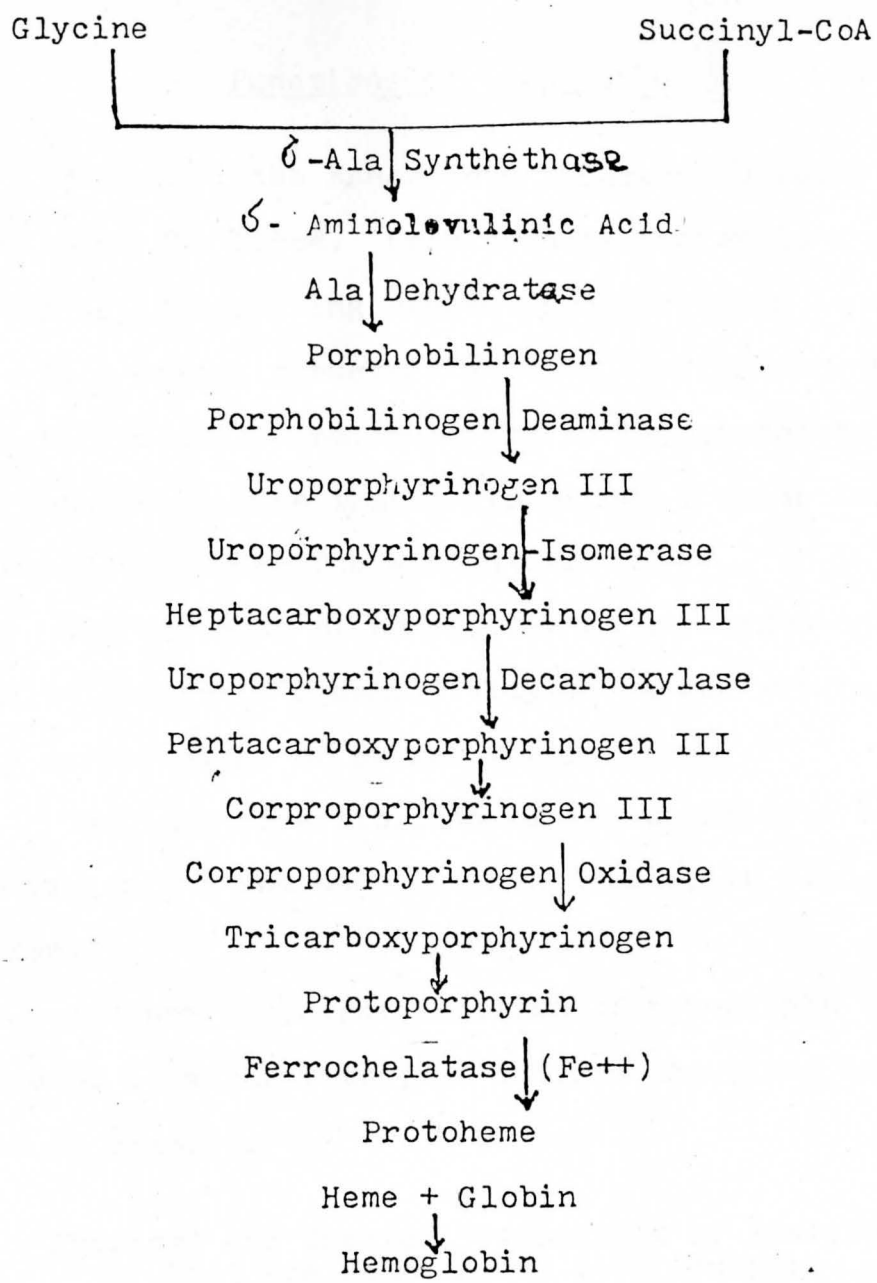


Fig. 1 The Stepwise Synthesis of Hemoglobin

Functions of Hemoglobin

Normally, the hemoglobin molecule is found in the red cells of the blood. From here it serves it's main function of transporting oxygen from the lungs to the tissues. It's unique structure makes co-ordinate bonding between the hemoglobin molecule and an oxygen molecule possible. Hemoglobin also transports carbon dioxide from the tissues to the lungs for exhalation.

The strength of hemoglobin as an oxygen-carrying molecule lies in it's sensitivity to oxygen within a relatively narrow range of oxygen tension (pO_2).[2]

The easy reversibility of the coordinate bond between oxygen and the hemoglobin molecule, is also an added advantage.

Another important function of hemoglobin is that because of it's chemistry, it buffers the blood against sudden pH changes.[3]

Physical and Chemical Properties of Hemoglobin That Are Relevant to It's Analysis

Physical

Hemoglobin has a characteristic absorption spectrum for each of it's forms (that is depending on the presence and nature of ligands, and the valence state of the iron).[4]

According to Linus Pauling, the characteristic absorption bands result from the electrical and magnetic interactions of the iron atom, and the porphyrin ring.[5]

The absorptions at these known wavelengths normally follows a Beer's Law relationship to the hemoglobin concentration. This makes it possible to determine the concentration of a given form of hemoglobin. Chemical reaction with a given reagent (for example, an acid or a cyanide) leads to a hemoglobin form with an analyzable absorption spectrum.

Chemical

Hemoglobin can catalyze the splitting of a hydrogen peroxide molecule into a water molecule and a nascent oxygen atom:

$$Hb + H_2O_2 \rightleftharpoons Hb.H_2O_2 \rightleftharpoons Hb + H_2O + (O)$$

This is called a pseudoperoxidase activity. In the presence of a chromogenic antioxidant, the oxygen is reacted with, producing a characteristic color, whose intensity bears a Beer's Law relationship to the concentration of hemoglobin. This is a second basis for plasma hemoglobin analysis.

Hemoglobin In Plasma And It's Clinical Significance

Virtually, all the hemoglobin in man exists as a saturated solution in the erythrocyte, where it gives blood it's characteristic red color.[6]

Under normal circumstances, there is a negligible amount of red cell destruction going on in the blood at any given moment. The hemoglobin released quickly binds the hemoglobin-binding-globin haptoglobin.[7] This is removed from the blood by liver cells.

The normal plasma hemoglobin level is 2-3 mg/dl. Plasma concentrations in excess of this amount are normally due to hemolysis. Levels of 25-30 mg/dl are normally due to hemolytic anaemia.

Higher levels of hemoglobin in plasma greater than 30 mg/dl are normally due to intravascular hemolysis.[8]

High plasma hemoglobin concentrations are also seen in adverse transfusion reactions, paroxysmal cold and nocturnal hemoglobinuria.[9]

Because of these facts, knowledge of the hemoglobin concentration in the plasma of patients could be a vital fact to know in order to effectively treat the problem.

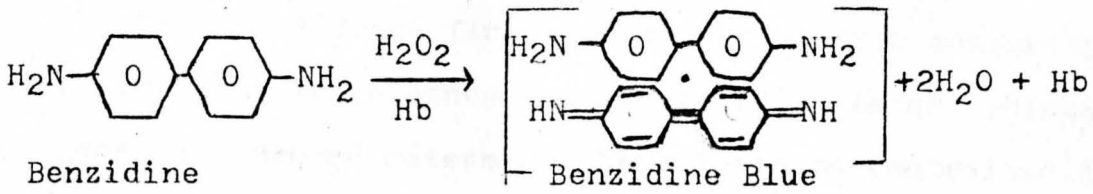
History of Methods for Plasma Hemoglobin Determination

Many methods already exist for the determination of the hemoglobin concentration of blood plasma. These procedures are based either on direct or indirect spectrophotometry.

One of the earliest works done on hemoglobin determination was done by Grovers.[10] In 1855, Angstrom determined the spectrum of hemoglobin.[11] And in 1887, Hufner carried out the first spectrophotometric quantitative determination of hemoglobin.[12]

Indirect Spectrophotometric Methods

Iron compounds of porphyrin exhibit pseudo-peroxidase activity,[13] that is, they oxidize antioxidants in the presence of hydrogen peroxide. To illustrate this point, the reaction of the antioxidant benzidine is shown below.



Benzidine — Benzidine Blue

Other substrates that can be oxidized to produce color are pyrogallol, ortho-toluidine, guaiac, phenolphthalein, hydriodic acid and ascorbic acid.[14]

Wu first introduced the benzidine-H_2O_2 method in 1923. The first major modification of Wu's method was done by Bing and Baker in 1931. Hanks and his co-workers modified the prodecure to increase it's sensitivity by about ten fold.[15]

Vanzetti and Valente further modified this procedure and improved its sensitivity by eliminating inhibiting substances in the medium.[16]

A micro method of the direct spectrophotometric method was developed by Crosby and Furth in 1956.[17]

These methods are the methods of choice in plasma hemoglobinometry - particularly due to their sensitivity. But, the drawback to these methods are the known carcinogenicity of some of the reagents and the suspected similar

toxicity of others. This has resulted in Food and Drug Administration restrictions on their use. Other pseudo-peroxidases in the medium interfere, too.

Direct Spectrophotometric Methods

Hufner did the first spectrophotometric measurement of plasma hemoglobin concentration (Ibid). Later, Shinowara, Childers and Berger determined hemoglobin concentration from the difference between the 575 nm (peak) and the 560nm (peak) of diluted plasma.[18] This method has very limited sensitivity, as the difference between these two absorption peaks is very small.

Porter later applied differences between absorbance at 578nm and absorbance at 700nm. In both the Shinowara and Porter methods, inactive hemoglobin is not measured. This is a drawback to their usefulness.[19]

Chiamori had used hemoglobin absorption at the Soret peak, 415nm and corrected for bilirubin absorbance at 460nm, but he did not correct for background absorption. So results obtained by this method are poor.[20]

Harboe made hemoglobin absorbance measurements at 380nm, 415nm and 450nm. See Figure 2. He corrected for background absorption, but did not correct for bilirubin interference.[21] So assay results were invalidated by the presence of bilirubin.

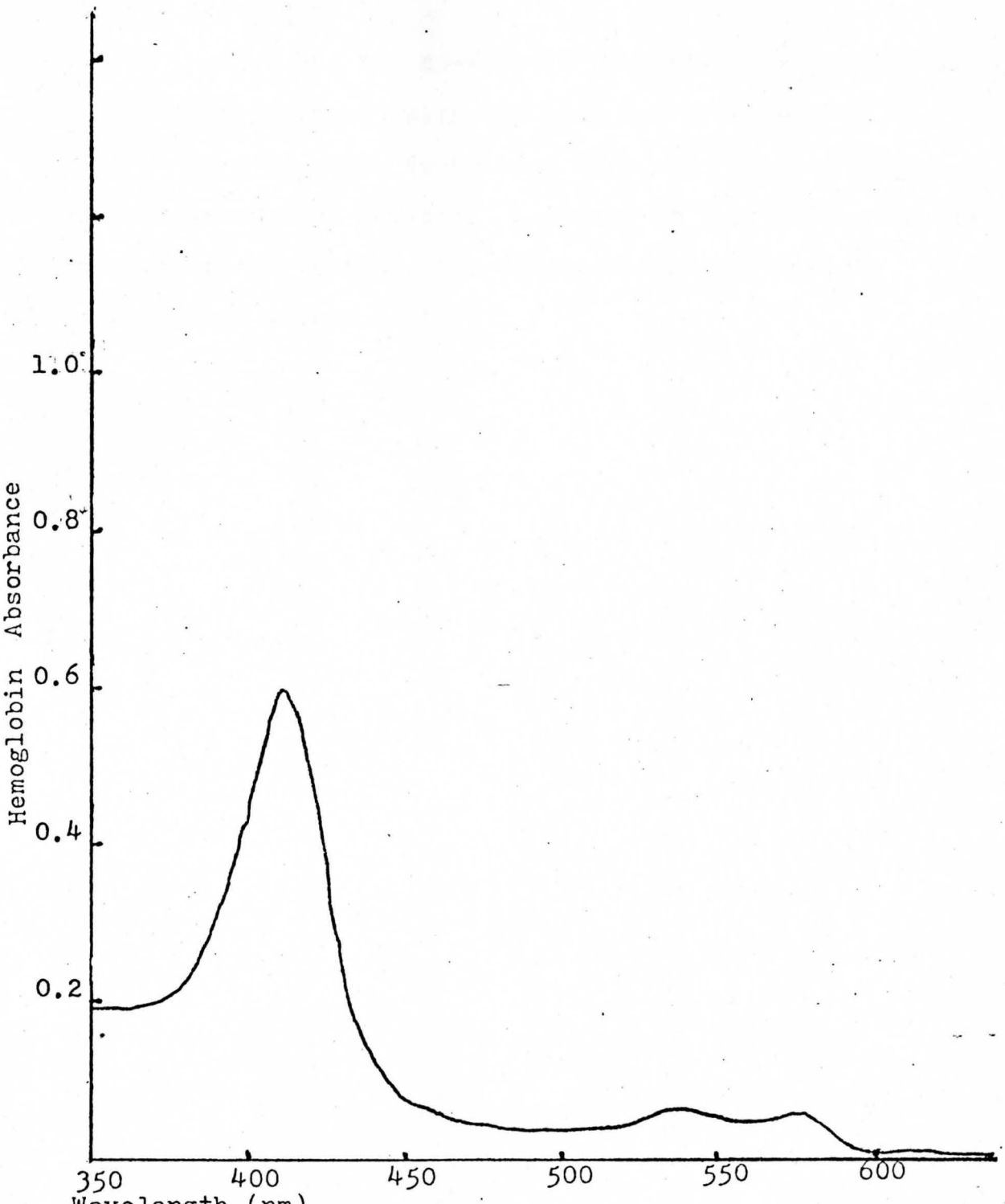


Fig. 2 Hemoglobin Absorption Bands.

Bilirubin is a tetrapyrrole, a byproduct of hemoglobin catabolism. Bilirubin has maximum absorbance at 460nm. See Figure 3.

Hunter and his co-workers converted hemoglobin to alkaline ferrohematin with 10% NaOH and hydrosulfite.[22]

Various other techniques have been applied with varying amounts of defects. Compared to the indirect spectrophotometric methods, the direct spectrophotometric methods come second best.

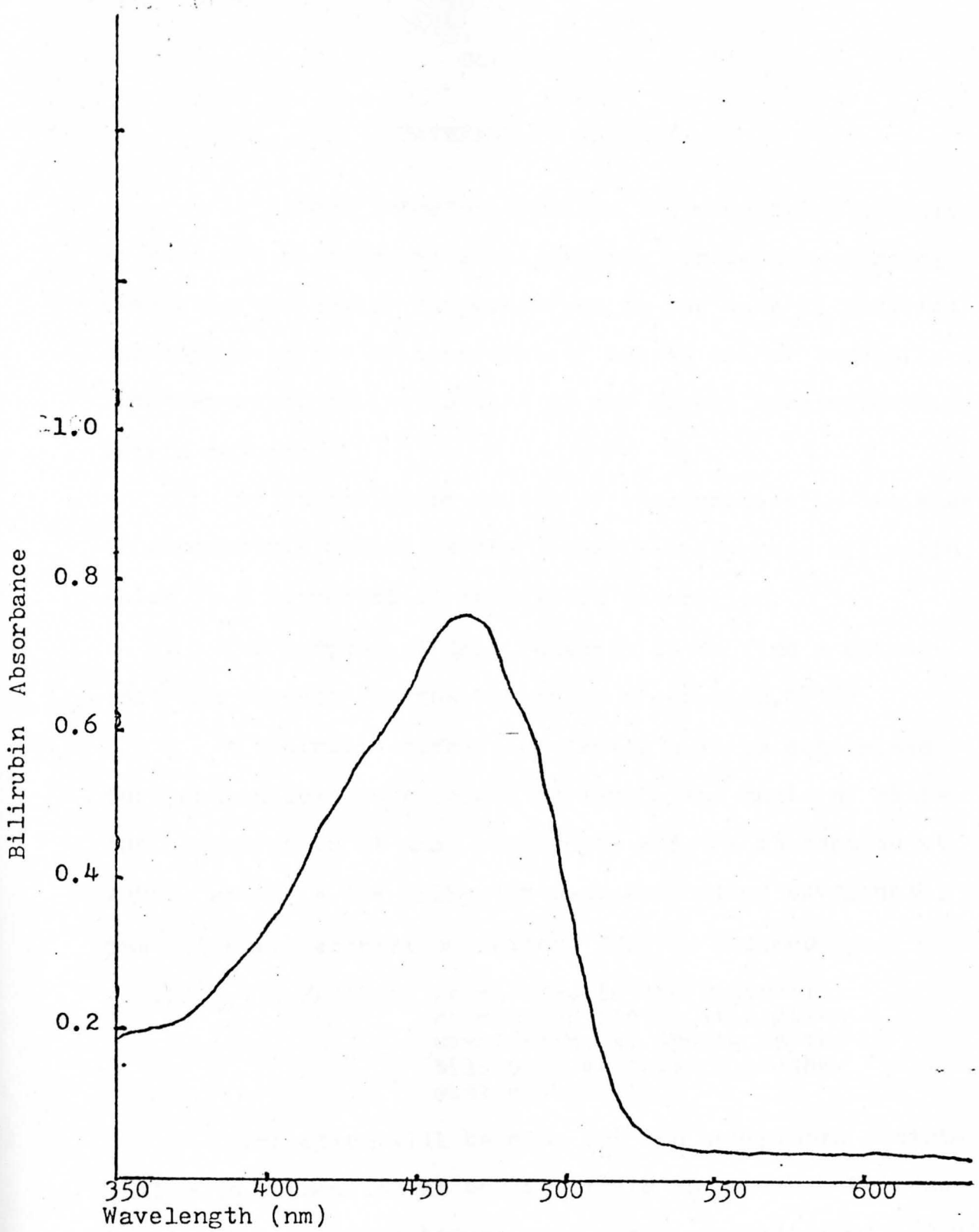


Fig. 3 Bilirubin Spectrum.

CHAPTER II

STATEMENT OF PROBLEM

In plasma hemoglobinometry, the need for a relatively accurate measurement still exists. Procedures already available are either dangerous, as is the case in indirect spectrophotometry or inaccurate[23] due to either turbidity or interferences, as in the case of the direct spectrophotometric methods.

One of the major causes of inaccuracies in the spectrophotometric method is the linear tetrapyrrole bilirubin, which is a byproduct of hemoglobin catabolism.

The purpose of this research is to find a method that can correct for the bilirubin absorption.[24,25]

A bilirubin correction factor will be determined for each relevant wavelength by taking the ratio of bilirubin absorption at that wavelength and the absorption at 460nm, which is the bilirubin peak absorption wavelength. The bilirubin correction factor, BCF_x is defined by:

$BCF_x = A_x/A460nm$, where BCF_x is the bilirubin correction factor at a given wavelength, x, and A_x is the bilirubin absorbance at the same wavelength.

Correction will be made for the hemoglobin contribution to absorption at 460nm. It is known that the hemoglobin absorption at 460nm equals it's absorption at 550nm.[26]

Therefore, readings will be taken at 550nm, and used to correct for this hemoglobin interference to bilirubin absorption.

The use of the correction factors is an application of the technique of Multicomponent Analysis as described in many analytical chemistry textbooks.[27] The overlap of the hemoglobin and bilirubin spectra is an example of two-way overlap, making the calculations somewhat more complicated.

CHAPTER III

EXPERIMENTAL

Reagents and Their Preparations

The essential reagents for the experiments are distilled water, standard solution of human or bovine serum albumin, standardized hemoglobin solution, standard solution of bilirubin and human blood plasma solutions.

Serum Albumin Solution

The expected concentration of the standard serum albumin is 4 g/dl.

4 g of dry serum albumin is dissolved in 100 ml of distilled water. By shaking and stirring, the solution is made as homogeneous as possible. Storage is at $4^{o}C$.

Standard Bilirubin Solution

The standard bilirubin solution is of a concentration of 20 mg/dl. The pH of the solution is 7.4. The solution is buffered with 0.1 M phosphate buffer. The buffer is prepared by adding 11.92 g of sodium biphosphate (Na_2HPO_4), and 2.8 g of potassium biphosphate to one liter of distilled water. 1 ml of 5 N sodium hydroxide and 20 mg of bilirubin are added to a 100 ml round flask, and with the phosphate buffer solution, this is made up to a 100 ml, to

obtain the standard bilirubin solution. This solution is employed in the experiments.

Hemoglobin Standard Solution

The method adopted for the preparation of plasma hemoglobin standard is as described by Tietz.[28] The procedure is as follows:

a. 1 ml of whole blood is centrifuged at 700 Xg for ten minutes.

b. The plasma is removed and discarded.

c. Erythrocytes are resuspended in 10 ml of 0.9% sodium chloride solution.

d. This suspension is centrifuged again at 700 X g and the supernatant removed and discarded.

e. The erythrocytes are lysed by alternate freezing and thawing twice.

f. The hemoglobin concentration is measured by the cyanmethemoglobin method and adjusted to required concentration by adding working buffer.

Plasma Specimens

By special arrangement plasma specimens were collected from the Clinical Laboratory of the Northside Unit of the Youngstown Hospital Association, Youngstown, Ohio.

The specimens were stored in refrigerators at $4^{o}C$ until they were used.

Apparatus

The central instrument for the experiments is the spectrophotometer. In this case, a Beckman Double-Beam 26

(D.B. 26) Spectrophotometer was used. The spectral region of interest used for the experiments is the visible light region.

Characteristics and Specifications
of Spectrophotometer

a. Optical Principle: Double Beam.

b. Wavelength Range: 190-900nm.

c. Wavelength Presentation: Linear Digital Counter.

d. Wavelength Accuracy: ± 0.5nm.

e. Wavelength Repeatability: 0.20nm.

f. Stray Light: Less than 0.1% at 220nm.

g. Resolution: 0.2nm.

h. Readout; Digital Display: 0-2A.

i. Data-Ready-Output: 0.5m sec.. - i.e. data is valid for 0.25 sec.

j. Photometric Accuracy: 0.5% of Reading or 0.001A, whichever is larger.

k. Baseline Stability: Greater than 0.0004A/hr at zero A.

l. Power Requirements: 120/240 Volts (± 10%), 50/60 H_z 2 amp. at 120V., 1 amp. at 240V.

Where; nm is nanometer
% is parts per hundred
A is absorbance unit
A/hr is absorbance per hour
H_z is cycles per second
V. is volts and
Amp is amperes of current

Other Equipment

a. A set of micropipettes in the 5-100 microliter range.

b. A set of pipettes, various sizes.

c. A pipette bulb.

d. A set of test tubes - 5 and 10 ml.

e. Test tube racks.

f. Round bottomed flasks.

g. Graduated cylinders.

h. Beakers.

i. A set of two matched 1 cm spectrophotometric cells.

j. Distilled water bottles.

Procedure for Absorbance Measurements

The steps for making a measurement are as follows:

Plasma:

a. 0.4 ml plasma or 0.4 ml human serum albumin-hemoglobin standard solution is made up to 4 ml by the addition of 3.6 ml distilled water.

b. Distilled water is used in both cells in order to zero the spectrophotometer.

c. The sample is measured against a distilled water blank and the absorbance measured at different wavelengths. Alternatively, a spectrum could be recorded.

d. The absorbances are put in the standard equation, and the corrected hemoglobin concentration is calculated.

In the case of bilirubin measurements, the amount of bilirubin solution taken is made up to 0.4 ml with human albumin. This is finally made up to 4 ml with distilled water. The readings are taken as above.

CHAPTER IV

RESULTS AND THEIR INTERPRETATIONS

Data on Assays of Pure Hemoglobin

A number of measurements were made to determine the structural characteristics of hemoglobin. The spectra of various concentrations of hemoglobin in bovine serum albumin solution were obtained. See Figure 4. Also see Table 1. From these spectra, it is seen that the maximum absorption of hemoglobin occurs at 414nm. Although it is also seen that at very low hemoglobin concentrations, the absorbance increases linearly as the wavelength increases within the relevant range of wavelengths.

But in general, the data fitted the literature description of the hemoglobin spectrum.[29] Maximum absorption is at 414nm.

Bilirubin Spectrum

A series of measurements were made with varying amounts of bilirubin in bovine serum albumin solution. The spectra of these samples were recorded. See Figure 5. The observed spectra agree with the expected literature values, with a maximum absorption at 460nm.[30]

A series of plasma samples were assayed using the Harboe method as described in Lynch's Medical Laboratory

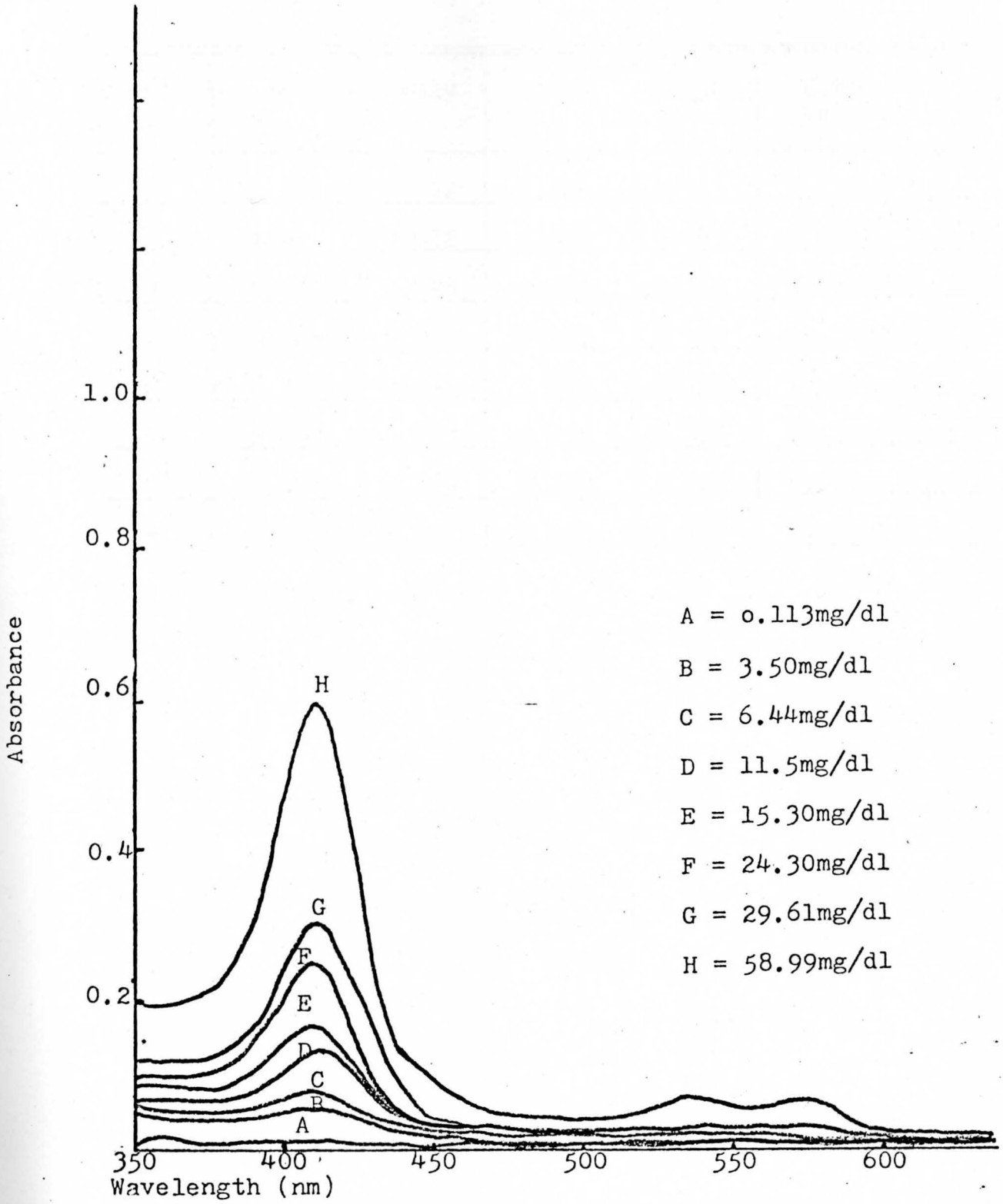


Fig. 4 Hemoglobin Spectra at Various Concentrations.

TABLE 1

HEMOGLOBIN ABSORPTION AT DIFFERENT WAVELENGTHS

Sample No.	A460	A429	A414	A398	Harboe Values
1	0.008	0.012	0.014	0.015	0.113mg/dl
2	0.06	0.032	0.054	0.045	3.50mg/dl
3	0.022	0.048	0.085	0.065	6.44mg/dl
4	0.024	0.071	0.142	0.102	12.54mg/dl
5	0.032	0.091	0.178	0.130	15.26mg/dl
6	0.034	0.123	0.258	0.178	24.30mg/dl
7	0.038	0.143	0.308	0.211	29.61mg/dl
8	0.067	0.281	0.605	0.407	58.99mg/dl

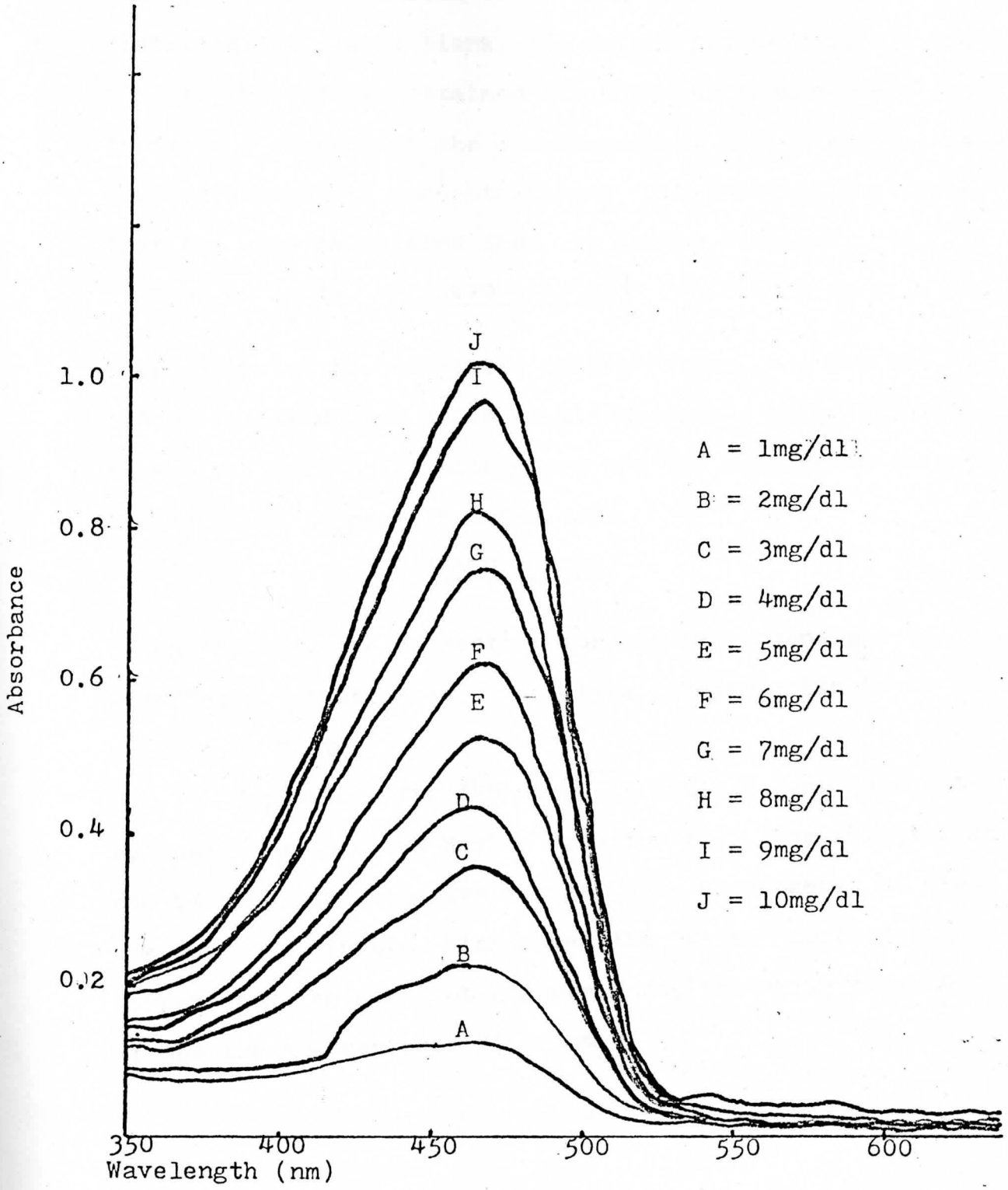


Fig. 5 Bilirubin Spectra at Various Concentrations.

Technology.[31] In this method, 0.4 ml of plasma is mixed with 3.6 ml of distilled water and the absorbances, with distilled water as a blank, are determined at 398, 414 and 429 nm. The values obtained for these absorbances are shown in Table 2 along with the absorbances at 460 nm and the calculated hemoglobin concentrations. The hemoglobin concentrations were calculated from the Harboe equation:

$$[Hb] = \left[A414 - \frac{(A429 + A398)}{2}\right] \times 226 \text{ in mg/dl} \qquad (1)$$

where [Hb] is the hemoglobin concentration, and the 226 factor is based upon a 0.4 ml plasma sample being diluted to 4.0 ml, 1 cm cells being used and the millimolar absorptivity of Hb being 11.5. The term

$$\frac{(A429 + A398)}{2}$$

is an "Allen" type correction for the background due to interfering substances. This type of correction is described by Tietz.[32]

The values for [Hb] shown in Table 2 were calculated by insertion into the Harboe equation (1). The results, as can be seen, are negative values. The absorbances at 460nm are due to bilirubin which makes the "Allen" correction larger than the Hemoglobin absorbance, primarily because of the large absorbance at 429nm.

TABLE 2

ASSAY TO OBSERVE VARIATION IN BILIRUBIN ABSORBANCE WITH CHANGES IN BILIRUBIN CONCENTRATION

Sample No.	A460	A429	A414	A398	Hb
1	0.123	0.104	0.086	0.073	-0.68mg/dl
2	0.228	0.174	0.140	0.112	-0.34mg/dl
3	0.338	0.241	0.194	0.149	-0.23mg/dl
4	0.425	0.294	0.228	0.171	-1.02mg/dl
5	0.508	0.354	0.279	0.207	-0.34mg/dl
6	0.600	0.416	0.321	0.237	-1.24mg/dl
7	0.663	0.468	0.367	0.276	-1.13mg/dl
8	0.725	0.519	0.412	0.315	-1.13mg/dl
9	0.800	0.561	0.435	0.316	-0.80mg/dl
10	0.928	0.656	0.504	0.361	-1.02mg/dl
11	0.986	0.698	0.544	0.391	-0.113mg/dl

Spectra and Data on Hemoglobin and Bilirubin In Various Concentration Mixtures

A series of assays on hemoglobin in combination with varying amounts of bilirubin were run. Observation was made on the effect of the presence of bilirubin on the hemoglobin spectra.

The result is illustrated in Figure 6. It is seen that the presence of bilirubin masks the hemoglobin absorption peaks, which are neccessary for the calculation of the hemoglobin concentration. Figures 7a, 7b, 7c show the effect of varying amounts of hemoglobin and bilirubin in solution.

Table 3 illustrates the practical implication of an increasing amount of bilirubin in a plasma sample, in which the hemoglobin concentration is to be determined. As the bilirubin increases, the apparent concentration of hemoglobin increases.

It is noteworthy that the hemoglobin is actually depressed if the absorption at 460nm is lower than 0.40 absorbance units.

The first two samples in the table are the values of plasma hemoglobin with no bilirubin added, averaging out to 5.70mg/dl.

If a way is found, therefore, to correct for the effect of bilirubin on the calculated value of hemoglobin, the value of each sample should turn out to be around 5.70mg/dl.

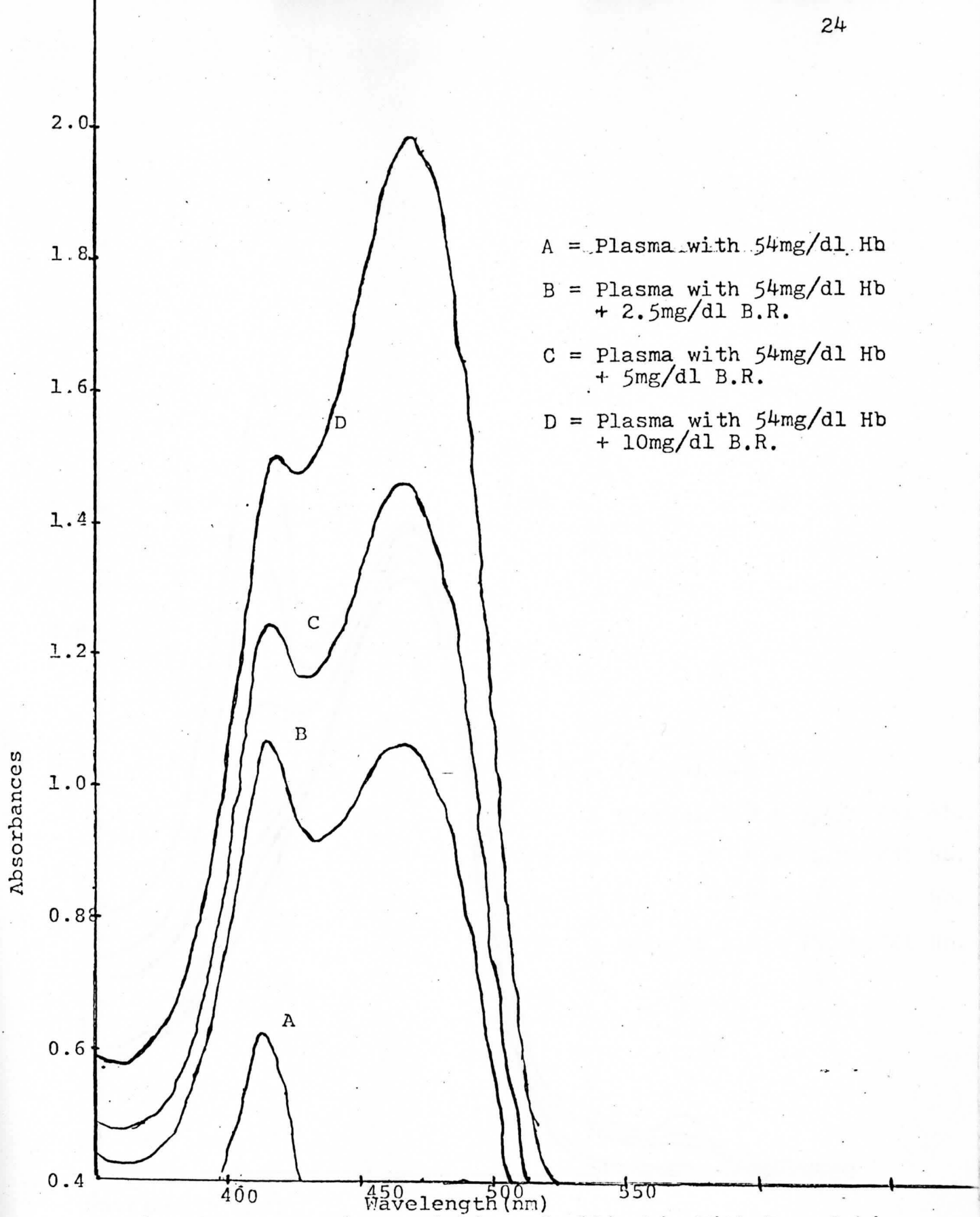


Fig. 6 Spectra of Hemoglobin and Bilirubin With Hemoglobin Concentration Held Constant as the Bilirubin Is Increased.

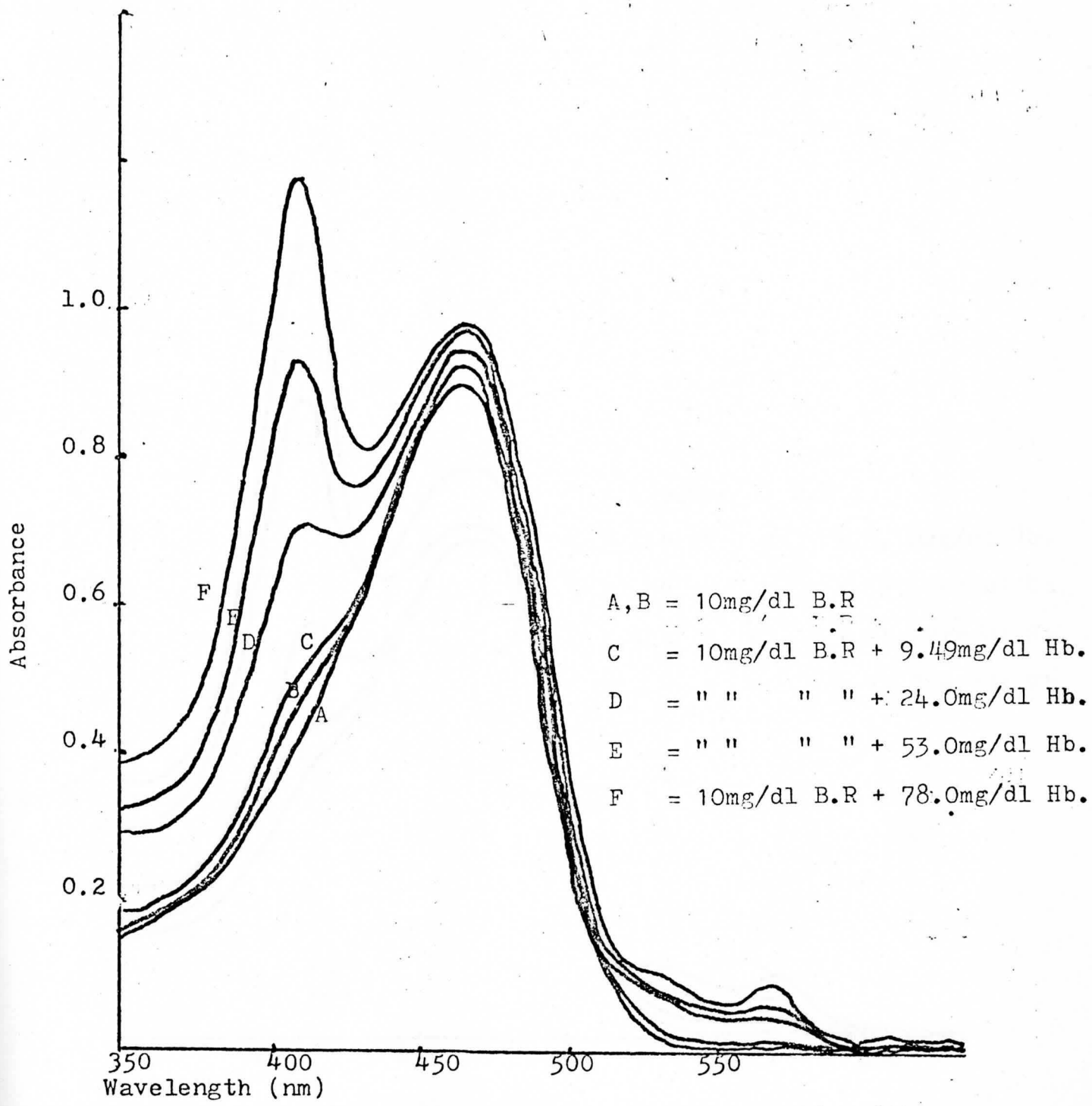


Fig. 7a Spectra of Hemoglobin and Bilirubin With Bilirubin Held Constant.

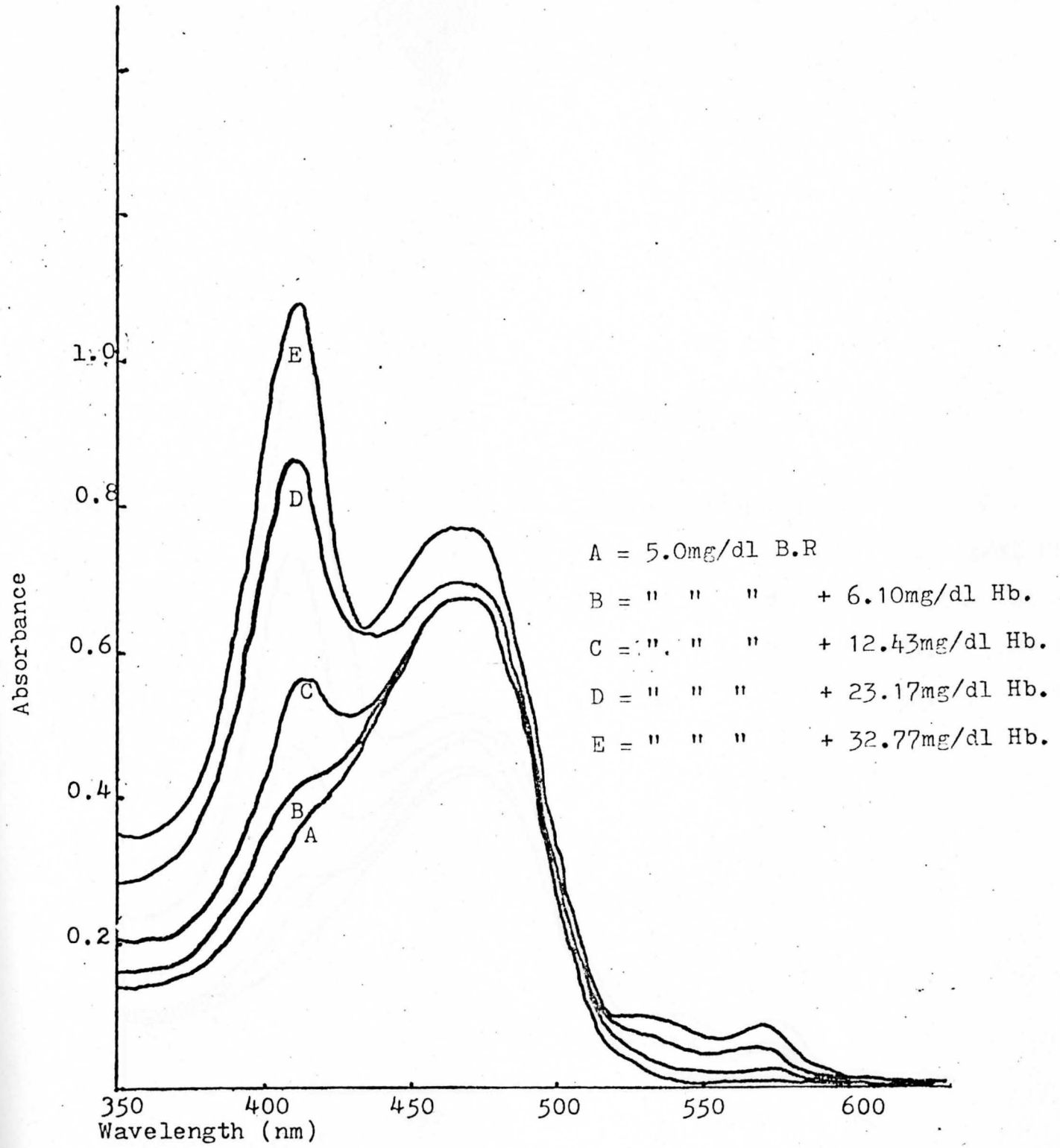


Fig. 7b. Spectra of Hemoglobin and Bilirubin With Bilirubin Held Constant.

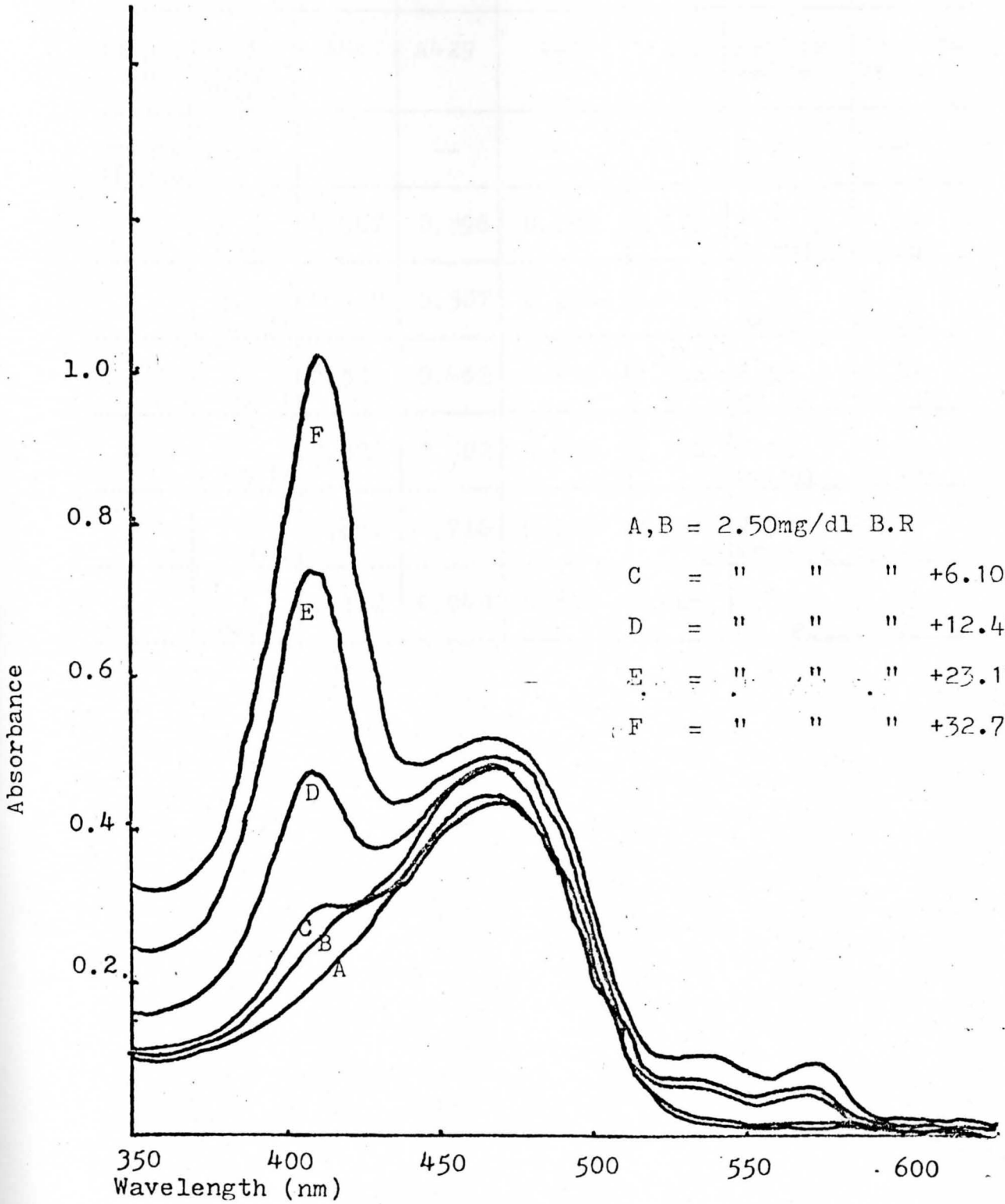


Fig. 7c. Spectra of Hemoglobin and Bilirubin With Bilirubin Held Constant.

TABLE 3

EFFECT OF INCREASING BILIRUBIN CONCENTRATION ON HEMOGLOBIN DETERMINATION BY HARBOE'S METHOD

Sample No.	Bilirubin Conc.	A460	A429	A414	A398	Harboe Value	Expected Value
Straight Plasma	--	--	--	--	--	5.70 mg/dl	--
1	1 mg/dl	0.307	0.296	0.280	0.227	4.30 mg/dl	5.70 mg/dl
2	2.5 mg/dl	0.428	0.387	0.358	0.269	6.78 mg/dl	5.70 mg/dl
3	3.75 mg/dl	0.524	0.462	0.416	0.311	6.55 mg/dl	5.70 mg/dl
4	5 mg/dl	0.571	0.502	0.450	0.336	7.01 mg/dl	5.70 mg/dl
5	7.5 mg/dl	0.860	0.716	0.622	0.458	7.91 mg/dl	5.70 mg/dl
6	10 mg/dl	1.128	0.940	0.810	0.605	8.36 mg/dl	5.70 mg/dl

Bilirubin Correction Factor

A series of measurements were made on bilirubin in 0.40ml human plasma albumin standard -- all made up to 4.0ml.

The results are shown in Table 4. Also shown is the ratio of the bilirubin absorption at 429nm, 414nm and 398nm.

Figure 8 shows a plot of bilirubin correction factors as a function of bilirubin absorbance. From the plot, the absorbing ratios of bilirubin at the different absorption bands can be taken and then used to correct for bilirubin before calculations are made.

It can be seen from the graph that from 0.425A and up, the ratio of absorbance is relatively constant as 0.70, 0.54, 0.40. Below 0.425A, this ratio changes progressively.

Correction for Hemoglobin Absorption at 460nm

A number of experiments were carried out at progressively higher concentrations to confirm that the hemoglobin absorption 460nm is approximately equal to it's absorption at 550nm.

The results from three samples are shown in Table 5. From the table, it can seen that, indeed, the absorption at 460nm is approximately equal to absorption at 550nm -- and therefore, subtraction of A550nm from A460nm will effectively correct for hemoglobin absorption at that wavelength.

TABLE 4

RELATIVE ABSORPTION OF BILIRUBIN
AT DIFFERENT WAVELENGTHS

Sample No.	A460	A429	A414	A398	A429/ A460	A414/ A460	A398/ A460
1	0.123	0.104	0.086	0.073	0.84	0.70	0.60
2	0.228	0.171	0.140	0.112	0.75	0.61	0.49
3	0.338	0.241	0.194	0.149	0.71	0.57	0.44
4	0.425	0.294	0.228	0.171	0.69	0.54	0.44
5	0.508	0.354	0.279	0.207	0.70	0.55	0.41
6	0.600	0.416	0.321	0.237	0.69	0.54	0.40
7	0.725	0.519	0.412	0.315	0.72	0.57	0.43
8	0.800	0.561	0.435	0.316	0.70	0.54	0.40
9	0.928	0.656	0.504	0.361	0.71	0.54	0.39
10	0.986	0.698	0.544	0.391	0.71	0.55	0.40

TABLE 5

COMPARISON OF HEMOGLOBIN
ABSORPTION AT 460nm and 550nm

Sample No.	A550	A460
1	0.006	0.006
2	0.030	0.031
3	0.056	0.062

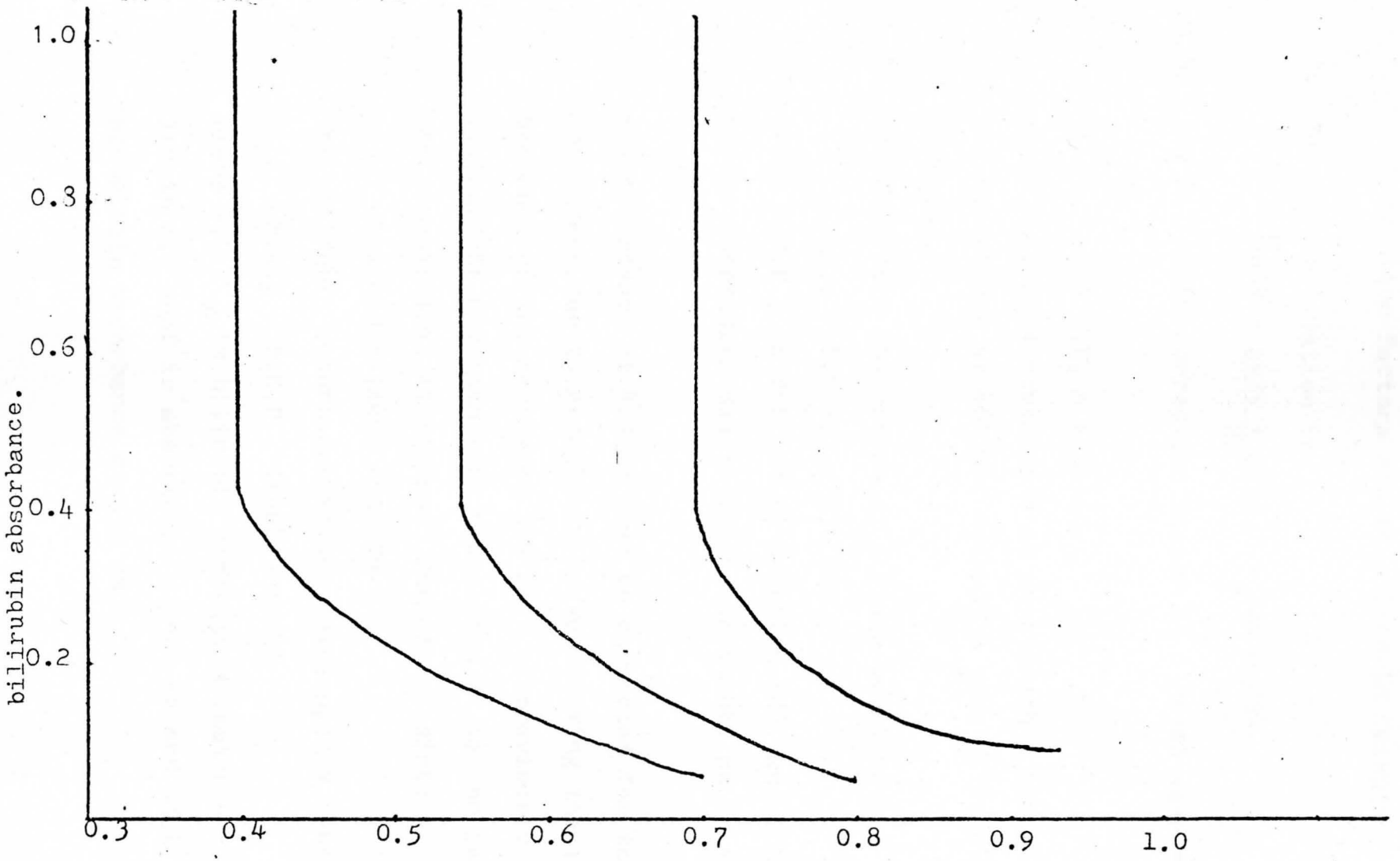


Fig. 8 Bilirubin Correction Factors.

Correction Factors and Equations in Calculations

A. The Harboe Equation is:

$$[Hb] = \left[A414 - \frac{A429 + A398}{2}\right] \times 226 \text{ mg/dl} \quad (1)$$

B. The Bilirubin correction factor at a given wavelength, x is:

$$B.C.F_x = A_x/A460 \quad (2)$$

where A_x is the absorbance at wavelength x and A460 is the absorbance at 460 nm, both of a pure bilirubin solution.

C. The bilirubin absorption at a given wavelength x is:

$$B.A._x = B.C.F._x \times B.A.460 \quad (3)$$

where $B.C.F._x$ is defined by Equation (2), and B.A.460 is the absorption due to bilirubin at 460 nm in the sample.

D. The absorbance at 460 nm must be corrected for the absorbance due to hemoglobin by subtracting the absorbance of the sample at 550 nm. As previously stated, the absorbance of hemoglobin at 460 nm is the same as that at 550 nm. Therefore, since

$$B.A.460 = (A460 - A550) \quad (4)$$

the bilirubin absorbance at any wavelength x becomes:

$$B.A._x = B.C.F._x (A460 - A550) \quad (5)$$

where $B.C.F._x$ is bilirubin correction factor at wavelength X. A460 is absorbance at 460 nm and A550 is hemoglobin absorbance at 550 nm.

E. Finally our equation corrected for bilirubin interference becomes:

$$[Hb] = \left[(A414 - B.A.414) - \frac{(A429 - B.A.429) + (A398 - B.A.398)}{2}\right] \times (226 \text{ mg/dl}) \quad (7)$$

Where [Hb] is the hemoglobin concentration, A414 is total absorption at 414nm, B.A.414 is bilirubin absorption at 414nm, A429 is total absorption at 429nm, B.A.429 is bilirubin absorption at 429nm, A398 is total absorption at 398nm, B.A.398 is bilirubin absorption at 398nm and 226 mg/dl is the background correction factor.

Results

The data in Table 3 on page 28 were analyzed using the correction equation and the results are shown in Table 6.

Because no readings were taken at 550nm, no corrections were made for hemoglobin absorption in this series of samples.

Table 7 compares the results from assays in which both bilirubin and the hemoglobin concentrations are varied. Bilirubin concentration is high. No corrections are made at 550 nm.

TABLE 6

COMPARISON OF HARBOE VALUES
WITH BILIRUBIN CORRECTED VALUES

Sample No.	Expected Values mg/dl	Harboe Values mg/dl	Corrected Values mg/dl
1	5.70	4.30	5.88
2	5.70	6.78	5.81
3	5.70	6.55	5.49
4	5.70	7.01	5.72
5	5.70	7.91	6.01
6	5.70	8.36	6.01

TABLE 7

COMPARISON OF RESULTS FROM ASSAYS IN WHICH BOTH BILIRUBIN AND HEMOGLOBIN CONCENTRATION ARE VARIED. BILIRUBIN CONCENTRATION IS HIGH. A550 CORRECTION NOT MADE.

Sample No.	A460	A429	A414	A398	Harboe Value mg/dl	Corrected Value mg/dl	Correction Factors
A1	0.465	0.300	0.256	0.179	3.62	4.29	0.70,0.55 0.40
A2	0.415	0.297	0.297	0.206	9.94	11.22	0.70,0.54 0.40
A3	0.465	0.397	0.482	0.324	27.35	28.50	0.70,0.54 0.40
A4	0.475	0.517	0.754	0.502	55.37	56.33	0.70,0.54 0.40
A5	0.511	0.653	1.036	0.680	83.39	84.67	0.70,0.54, 0.40
0.1 mlB.R.	0.421	0.264	0.196	0.45	-1.92	0.97	0.70,0.54, 0.40
B1	0.640	0.439	0.402	0.286	8.8	10.28	0.70,0.54, 0.40
B2	0.663	0.516	0.564	0.391	20.34	26.33	0.70,0.54, 0.40
B3	0.746	0.674	0.873	0.590	54.47	55.82	0.70,0.54, 0.40
B4	0.678	0.752	1.094	0.737	78.87	80.39	0.70,0.54, 0.40
0.15 mlB.R.	0.634	0.396	0.286	0.207	-3.62	2.10	0.70,0.54, 0.40

TABLE 7

COMPARISON OF RESULTS FROM ASSAYS (CONT.)

Sample No.	A460	A429	A414	A398	Harboe Value mg/dl	Corrected Value mg/dl	Correction Factors
C1	0.861	0.574	0.496	0.350	7.68	9.49	0.70,0.54, 0.40
C2	0.898	0.712	0.706	0.505	20.34	24.0	0.70,0.54, 0.40
C3	0.934	0.791	0.944	0.644	51.08	53.22	0.70,0.54, 0.40
C4	0.936	0.898	1.196	0.816	78.87	78.65	0.70,0.54, 0.40
0.2 mlB.R.	0.868	0.536	0.396	0.284	-15.82	1.20	0.70,0.54, 0.40

It is observed from the calculations that corrections for ordinary bilirubin absorption at different wavelengths lead to a positive apparent hemoglobin concentration. This means that absorption at 414nm is relatively increased in value with the corrections. An adjustment for this is made by increasing the relative value of the correction factor at 414nm by between 0.001 to 0.0015.

Tables 8 and 9 illustrate the effect of correction for hemoglobin absorption at 460nm on calculated hemoglobin concentration.

It can be seen from Tables 8 and 9 that in cases where the concentration of hemoglobin is high, correction for hemoglobin absorption at 460nm does not lead to any major difference in the calculated hemoglobin value. But if the concentration of hemoglobin is low, or the bilirubin concentration is low, the difference is significant.

Another advantage of correcting for absorption at 460nm is that background absorption might contribute to the apparent absorption at that wavelength. So subtracting absorption at 550nm will not only correct for hemoglobin absorbance, but it will also correct for background interference.

Derived Correction Factors

Attempts were made to derive a set of correction factors that would apply throughout all absorption ranges. Table 10 shows the results obtained. Both the bilirubin and hemoglobin concentrations were varied.

TABLE 8

EFFECT OF CORRECTION FOR HEMOGLOBIN ABSORPTION AT 550nm
ON CALCULATED HEMOGLOBIN CONCENTRATION

Sample No.	A460	A550	A460-A550	A429	A414	A398	Harboe Value mg/dl	Value Without Correction at 550nm mg/dl	Value With Correction at 550nm mg/dl
1	0.159	0.056	0.103	0.182	0.208	0.185	5.54	5.36	6.05
2	0.470	0.197	0.220	0.567	0.825	0.790	33.11	32.16	33.48
3	0.195	0.078	0.117	0.228	0.255	0.269	1.47	1.70	1.61
4	0.810	0.390	0.420	0.971	1.251	1.310	24.97	22.92	24.07
5	0.200	0.100	0.100	0.239	0.271	0.273	3.39	3.62	3.90
6	0.155	0.056	0.100	0.225	0.324	0.241	20.57	20.39	21.1
7	0.131	0.046	0.085	0.146	0.160	0.150	2.71	3.02	5.83
8	0.098	0.038	0.060	0.122	0.150	0.125	5.99	6.50	6.74
9	0.192	0.082	0.110	0.232	0.280	0.252	8.59	8.81	9.15
10	0.232	0.100	0.132	0.264	0.290	0.282	3.84	6.28	4.14

TABLE 9

COMPARISON OF HEMOGLOBIN VALUES WITH CORRECTION MADE FOR ABSORBANCE AT 550nm AND NO CORRECTION MADE

Sample No.	A550	A460	A429	A414	A398	Harboe Values mg/dl	Corrected at 550nm mg/dl	No Correction at 550nm mg/dl	Expected Value mg/dl
1	0.015	0.023	0.055	0.098	0.073	7.64	---	---	7.64
2	0.032	0.547	0.474	0.449	0.373	6.66	7.49	7.01	7.64
3	0.026	0.342	0.309	0.310	0.256	6.10	7.01	6.98	7.64
4	0.019	0.175	0.178	0.201	0.162	7.01	7.68	7.8	7.64
5	0.010	0.018	0.037	0.061	0.051	3.84	---	---	3.84
6	0.028	0.528	0.445	0.403	0.347	1.58	3.16	3.40	3.84
7	0.020	0.327	0.286	0.269	0.231	2.26	3.84	3.84	3.84
8	0.017	0.174	0.165	0.170	0.142	3.62	4.07	4.11	3.84

TABLE 10

ASSAYS WITH CORRECTION RATIOS APPLIED

Sample No.	A460	A429	A414	A398	Harboe Value	CF's .76, .61, .45	CF's .71, .56, .42	CF's .744, .592, .436	Standard Curve Corrected	Expected Value
1	0.634	0.598	0.562	0.444	9.27	8.59	10.62	8.93	10.62	9.49
2	0.337	0.342	0.350	0.276	9.27	9.04	9.94	9.04	9.49	9.49
3	0.607	0.563	0.510	0.413	4.97	4.29	5.88	4.52	4.52	5.34
4	0.335	0.328	0.318	0.258	5.65	5.20	6.33	5.42	5.92	5.34
5	0.676	0.631	0.604	0.494	9.34	8.59	9.49	8.81	9.04	8.65
6	0.361	0.359	0.370	0.304	8.70	8.4	9.27	8.59	9.94	8.65
7	0.700	0.648	0.591	0.496	4.29	3.39	5.88	3.84	4.75	4.31
8	0.365	0.351	0.337	0.290	3.73	3.39	4.29	4.29	4.29	4.31

In order to calculate the 'derived correction factors', two series of assays were performed and the average correction factors for each series found. One series was 0.81, 0.67, 0.49. A second series was 0.71, 0.55, 0.41. To derive one series of factors, the average of the factors at a given wavelength for the two series was taken. In the second, the square root of the multiple for the pair from the same wavelength was taken. The third derived ratio was obtained by adding three times the second series to two times the first series and dividing by five. No ratio showed a consistency over all absorption ranges.

Relative Absorbances

To facilitate the use of correction factors, the corrected values for bilirubin absorbances, CV, at the relevant wavelengths (i.e. 429nm, 414nm, 398nm) are calculated and set down in tabular form as a function of bilirubin absorbance at 460nm.

A user would only have to find the absorption at 460nm, correct it for hemoglobin absorption, and then pick the CV for the other wavelengths off the table. These values are then subtracted from the 'apparent' absorbances at 398, 414, 419 nm to get the corrected hemoglobin absorbances. These values are inserted into the Harboe equation and the corrected hemoglobin concentration is calculated.

Example Of Calculations:

Data;

A460	A429	A414	A398
0.365	0.351	0.337	0.290

a) Hemoglobin Concentration Determined By Harboe's Method:

$$Hb = A414 - \frac{(A429 + A398)}{2} \quad 226mg/dl$$

$$= 0.337 - \frac{(0.351 + 0.290)}{2} \quad 226mg/dl$$

$$=3.73mg/dl.$$

b) Hemoglobin Determination With Bilirubin Interference Corrected For:

$$Hb = (A414-B414) - \frac{(A429-B429)+(A398-B398)}{2} \quad 226mg/dl$$

The correction factors at429nm,414nm,and398nm are 0.71, 0.57,and 0.44. These factors are from the ratio of the absorbance closest to 0.365 on Table 4.

$$= (0.337-0.57x0.365) - \frac{(0.351-0.71x0.365)+(0.290-0.44x0.365)}{2} \quad x \quad 226mg/dl.$$

$$= (0.337-0.208) - \frac{(0.351-0.259)+(0.290-0.161)}{2} \quad 226mg/dl.$$

$$= (0.129) - \frac{(0.092+0.129)}{2} \quad 226mg/dl$$

$$= (0.129-\frac{0.221}{2}) \quad 226mg/dl \quad =0.0185x226mg/dl = 4.18mg/dl.$$

This shows a close agreement with an expected value of 4.30mg/dl.

TABLE 11

CORRECTED BILIRUBIN ABSORBANCES
RELATIVE TO ABSORBANCE AT 460nm

A460 - A550	CV429	CV414	CV398	
1.40	0.980	0.777	0.560	
1.39	0.973	0.772	0.556	
1.38	0.966	0.766	0.552	
1.37	0.959	0.760	0.548	
1.36	0.952	0.755	0.544	
1.35	0.945	0.749	0.540	
1.34	0.938	0.744	0.536	
1.33	0.931	0.738	0.532	
1.32	0.924	0.733	0.528	
1.31	0.917	0.727	0.524	
1.30	0.910	0.722	0.520	
1.29	0.904	0.716	0.516	
1.28	0.896	0.710	0.512	
1.27	0.889	0.705	0.508	
1.26	0.882	0.699	0.504	
1.25	0.875	0.694	0.500	
1.24	0.868	0.688	0.496	
1.23	0.861	0.683	0.492	
1.22	0.854	0.677	0.488	
1.21	0.847	0.672	0.484	
1.20	0.840	0.666	0.480	
1.19	0.833	0.661	0.476	

TABLE 11

CORRECTED BILIRUBIN ABSORBANCES (CONT.)

A460 - A550	CV429	CV414	CV398	
1.18	0.826	0.655	0.472	
1.17	0.819	0.649	0.468	
1.16	0.812	0.644	0.464	
1.15	0.805	0.638	0.460	
1.14	0.798	0.633	0.456	
1.13	0.791	0.627	0.452	
1.12	0.784	0.622	0.448	
1.11	0.777	0.616	0.444	
1.10	0.770	0.611	0.440	
1.09	0.763	0.605	0.436	
1.08	0.756	0.599	0.432	
1.07	0.749	0.594	0.428	
1.06	0.742	0.588	0.426	
1.05	0.735	0.583	0.420	
1.04	0.728	0.577	0.416	
1.03	0.721	0.572	0.412	
1.02	0.714	0.566	0.408	
1.01	0.707	0.561	0.404	
1.00	0.700	0.555	0.400	
0.99	0.693	0.550	0.396	
0.98	0.686	0.544	0.392	
0.97	0.679	0.538	0.388	
0.96	0.672	0.533	0.384	

TABLE 11

CORRECTED BILIRUBIN ABSORBANCES (CONT.)

A460 - A550	CV429	CV414	CV398	
0.95	0.665	0.527	0.380	
0.94	0.658	0.522	0.376	
0.93	0.651	0.516	0.372	
0.92	0.644	0.511	0.368	
0.91	0.637	0.505	0.364	
0.90	0.630	0.500	0.360	
0.89	0.623	0.494	0.356	
0.88	0.616	0.488	0.352	
0.87	0.609	0.483	0.348	
0.86	0.602	0.477	0.344	
0.85	0.595	0.472	0.340	
0.84	0.588	0.466	0.336	
0.83	0.581	0.461	0.332	
0.82	0.574	0.455	0.328	
0.81	0.567	0.450	0.324	
0.80	0.560	0.444	0.320	
0.79	0.553	0.435	0.316	
0.78	0.546	0.429	0.312	
0.77	0.539	0.424	0.308	
0.76	0.532	0.418	0.304	
0.75	0.525	0.413	0.300	
0.74	0.518	0.407	0.296	
0.73	0.511	0.402	0.292	

TABLE 11

CORRECTED BILIRUBIN ABSORBANCES (CONT.)

A460 -A550	CV429	CV414	CV398	
0.72	0.504	0.396	0.288	
0.71	0.497	0.391	0.284	
0.70	0.420	0.385	0.280	
0.69	0.483	0.380	0.276	
0.68	0.476	0.374	0.272	
0.67	0.469	0.369	0.268	
0.66	0.462	0.363	0.264	
0.65	0.455	0.358	0.260	
0.64	0.448	0.352	0.256	
0.63	0.441	0.347	0.252	
0.62	0.432	0.341	0.248	
0.61	0.427	0.336	0.244	
0.60	0.420	0.330	0.240	
0.59	0.413	0.325	0.236	
0.58	0.406	0.319	0.232	
0.57	0.399	0.314	0.228	
0.56	0.392	0.308	0.224	
0.55	0.385	0.303	0.220	
0.54	0.378	0.297	0.216	
0.53	0.371	0.292	0.212	
0.52	0.364	0.286	0.208	
0.51	0.357	0.281	0.204	
0.50	0.350	0.275	0.200	

TABLE 11

CORRECTED BILIRUBIN ABSORBANCES (CONT.)

A460 - A550	CV429	CV414	CV398	
0.49	0.343	0.272	0.196	
0.48	0.336	0.266	0.192	
0.47	0.329	0.261	0.188	
0.46	0.322	0.255	0.184	
0.45	0.315	0.250	0.180	
0.44	0.308	0.244	0.176	
0.43	0.301	0.239	0.172	
0.42	0.294	0.233	0.168	
0.41	0.287	0.228	0.166	
0.40	0.280	0.223	0.163	
0.39	0.273	0.218	0.160	
0.38	0.267	0.214	0.157	
0.37	0.260	0.209	0.154	
0.36	0.254	0.204	0.151	
0.35	0.248	0.200	0.149	
0.34	0.241	0.196	0.146	
0.33	0.235	0.191	0.143	
0.32	0.229	0.186	0.140	
0.31	0.223	0.181	0.137	
0.30	0.217	0.177	0.134	
0.29	0.210	0.174	0.132	
0.28	0.204	0.169	0.129	
0.27	0.198	0.165	0.126	

TABLE 11

CORRECTED BILIRUBIN ABSORBANCES (CONT.)

A460 - A550	CV429	CV414	CV398	
0.26	0.192	0.160	0.123	
0.25	0.185	0.155	0.120	
0.24	0.179	0.150	0.117	
0.23	0.173	0.145	0.113	
0.22	0.166	0.141	0.11	
0.21	0.160	0.135	0.107	
0.20	0.154	0.131	0.104	
0.19	0.147	0.125	0.101	
0.18	0.141	0.120	0.970	
0.17	0.134	0.115	0.933	
0.16	0.128	0.110	0.089	
0.15	0.121	0.104	0.080	
0.14	0.114	0.099	0.081	
0.13	0.108	0.930	0.077	
0.12	0.101	0.087	0.725	
0.11	0.095	0.081	0.068	
0.10	0.089	0.075	0.063	

CHAPTER V

SUMMARY AND CONCLUSIONS

As stated in the earlier part of this thesis, the aim of this research was to find a method for correcting for bilirubin interference in plasma hemoglobinometry.

The chosen method is a procedure that corrects hemoglobin absorbances for absorbances due to bilirubin. The corrections are calculated from the bilirubin absorbances at the absorption maximum, 460 nm.

The hemoglobin contibution to the bilirubin peak at 460 nm is corrected for by substracting the absorbance at 550 nm. This also corrects for possible background contribution to the apparent bilirubin absorbance at 460 nm.

It was found that no common ratio exists for the correction factors at different bilirubin concentrations. A standard curve was made to show correction factors at different absorbances. Also, a table of correction values were prepared so as to make the calculations in a routine clinical laboratory easier. From this table a technician can pick off the values and subtract them from the measured absorbances at the different wavelengths, thus saving time.

From the results obtained, it has been shown that use of the correction factors significantly improves the quality of the values obtained in direct spectrophotometric plasma hemoglobinometry. Computer reduction of data in this method is a logical extension.

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