Artificial Organs
21(12):1255–1267, Blackwell Science, Inc.
© 1997 International Society for Artificial Organs

Plasma Hemoglobin Measurement Techniques for the In Vitro Evaluation of Blood Damage Caused by Medical Devices

Richard A. Malinauskas

Office of Science and Technology, Center for Devices and Radiological Health, U.S. Food and Drug Administration, Rockville, Maryland, U.S.A.

Abstract: A sensitive measure of the in vitro blood damage potential of a medical device is the rate at which hemoglobin is released into the plasma from red blood cells flowing through the device. Presently there is no one widely accepted method for measuring the plasma hemoglobin concentration. Nine currently used assays, classified as either direct optical or added chemical techniques, were evaluated for accuracy, reproducibility, sensitivity, interference effects, and ease of use by adding hemoglobin (1–200 mg/dl) to saline, lipid, and bilirubin solutions and to normal cow plasma. Most of the assays displayed good linearity, accuracy, and reproducibility down to 1 mg/dl when interferents were absent. However, representative of the effects caused by interferents, the endogenous hemoglobin concentration of a typical cow plasma sample measured by the 9 techniques ranged from −2 to 39 mg/dl. Although used by fewer organizations, some of the direct optical spectrophotometric methods (e.g., the Cripps and Harboe baseline correction methods) are safer, easier, and more precise and accurate than the chemical addition methods used to measure plasma hemoglobin concentration from in vitro blood damage testing of medical devices. **Key Words:** Plasma hemoglobin concentration—Medical device testing—Hemolysis—Assays—Blood damage—Bovine plasma.

To evaluate the extent to which a cardiovascular medical device (e.g., an oxygenator, extracorporeal blood pump, left ventricular assist device, or hemodialysis catheter) may damage blood elements when used with human patients, an in vitro mock circulatory flow loop for testing the device is often used with bovine blood because it is readily available. As a sensitive measure of blood damage, the amount of hemoglobin liberated into the plasma from injured or destroyed red blood cells is monitored as a function of time (1,2). An index of hemolysis, which is based on the rate of plasma hemoglobin generation normalized by the blood hematocrit, total hemoglobin concentration, and volumetric flow through the test loop, can be calculated for comparison with measurements from other medical devices (1–3).

A review of techniques for evaluating blood trauma used by 50 academic, clinical, manufacturing, and standards organizations revealed that there is no standard protocol or one widely accepted method for measuring the critical test parameter (i.e., the plasma hemoglobin concentration). There are over 20 different assays in use today which were developed for clinical use with human blood in which the plasma hemoglobin concentration is normally less than 10 mg/dl. These assays are routinely used with cow blood for in vitro device assessment in which plasma hemoglobin concentrations can be higher than 1,000 mg/dl ([4], and confidential information submitted to the U.S. Food and Drug Administration). The goal of this study was to evaluate the most commonly used plasma hemoglobin measurement techniques for their accuracy, reproducibility, sensitivity, interference effects, ease of use, instrumentation requirements, and appropriateness for use with bovine plasma in the in vitro blood damage assessment of medical devices.

Received June 1997; revised August 1997.
Address correspondence and reprint requests to Dr. Richard A. Malinauskas, U.S. Food and Drug Administration, Center for Devices and Radiological Health, Office of Science and Technology, 12721A Twinbrook Pkwy (HFZ-132), Rockville, MD 20852, U.S.A.
The statements made within this article regarding specific products or technologies are the private views of the author and do not represent an official endorsement or criticism by the U.S. Food and Drug Administration.

MATERIALS AND METHODS

Nine currently used plasma hemoglobin assay techniques were compared by adding aliquots of isolated bovine hemoglobin to phosphate buffered saline (PBS) and to distilled water (for calibration purposes), and to atraumatically collected cow plasma (as a test for analytical recovery). The assays were also evaluated for interference from excessive bilirubin and lipids and for degradation of oxyhemoglobin to methemoglobin.

All animals and procedures used in this study were approved by the Center for Devices and Radiological Health Institutional Animal Care and Use Committee. Cow blood from a jugular vein was collected using a 14 gauge needle into a flexible blood bag (2 L, Metrix Co., Dubuque, IA, U.S.A.) containing 150 ml of phosphate buffered saline (PBS, #1000-3, Sigma Chemical Co., St. Louis, MO, U.S.A.) and heparin (3 IU/ml blood). To aspirate the blood quickly but atraumatically, the bag was sealed in a custom-designed Plexiglas box, and a partial vacuum of less than 100 mm Hg was created within the box using a vacuum hand pump.

The whole blood was filtered (80 μm blood filter #4C7744, Baxter Healthcare Corp., Deerfield, IL, U.S.A.) and centrifuged at 1,000 *g* for 10 min. The plasma was drawn off and recentrifuged before being frozen in 1,500 μl aliquots at −60°C. To isolate hemoglobin, the remaining red blood cells were gently washed by mixing them with cold PBS and centrifuging them at 1,000 *g* for 10 min a total of 3 times (5). After each centrifugation step, the PBS/plasma supernatant and the buffy coat were withdrawn for disposal. To lyse the red blood cells, 4 parts water were added to 1 part packed red blood cells in each centrifuge vial, mixed, and incubated for 10 min. The hemoglobin rich supernatant from these vials was retained after they were centrifuged for 30 min at 1,200 *g* to remove the cell stroma. The purity and concentration of the isolated hemoglobin was confirmed by creating a cyanmethemoglobin (HiCN) solution (approximately 70 mg/dl), which conformed to the International HiCN Standard characteristics: $1.59 \leq A_{540}/A_{504} \leq 1.63$ and $A_{750} \leq 0.003$ (6,7), where A_λ denotes the absorbance at wavelength λ (nm). Aliquots of 1,500 μl of approximately 5,500 mg/dl of hemoglobin were maintained frozen at −60°C.

For calibration and comparison of the analytical sensitivity, accuracy, reproducibility, and range of the 9 assay methods, frozen or freshly isolated hemoglobin stock was used to create nine 1,000 μl dilutions in PBS or water with varying hemoglobin concentrations (1, 4, 10, 20, 40, 75, 115, 160, and 200 mg/dl) over a 9 month period. On each separate day (n = 17 total), the hemoglobin dilutions were assayed by the 9 different methods, except for the tetramethylbenzidine method, which was only performed on 14 of the 17 days. A realistic assessment of reproducibility was achieved by evaluating the between-day precision of each method as performed by 2 separate investigators. Reproducibility (precision) was determined by calculating the percent coefficient of variation (%CV) as follows: $\%CV = (SD/X) \times 100\%$ where X is the mean and SD is the standard deviation of the hemoglobin sample. Also, for these hemoglobin solutions, the percent total analytical error (%TAE), which includes both the accuracy and precision of the measurement, was calculated as follows: $\%TAE = (|X - \mu| + 2 \times SD)/\mu \times 100\%$ where μ is the actual hemoglobin concentration.

Because the plasma hemoglobin assays are used with bovine blood, analytical recovery and interference were evaluated by creating similar dilutions of hemoglobin in cow plasma with low levels of native hemoglobin (n = 4 multiple day experiments) and in PBS with added lipids (n = 3) for turbidity (Intralipid 20% used as 1.2 μl Intralipid/ml solution, Clintec Nutrition Co., Deerfield, IL, U.S.A.). For hemoglobin dilutions in cow plasma, the percent total analytical recovery error (%TARE) was calculated as follows: $\%TARE = (|X' - \mu| + 2 \times SD')/\mu \times 100\%$ where X' is the amount of hemoglobin recovered (i.e., measured concentration minus background concentration) and μ is the actual amount of hemoglobin added to the plasma.

The effect of bilirubin interference was evaluated (n = 4) by creating hemoglobin aliquots of 10 and 75 mg/dl with varying amounts of bilirubin in them (0, 0.2, 1, 4, 10 mg/dl, all with 3.5 g/dl albumin). The range of plasma bilirubin concentration in normal cows is 0.01–0.47 mg/dl, but it can be over 10 times greater in animals being used to test medical devices in vivo (8,9). Briefly, 10 mg of bilirubin (#B4126, Sigma) was dissolved in 1 ml of dimethyl sulfoxide, diluted in 2 ml Na_2CO_3 (0.1 mol/L), and then diluted to 25 ml with PBS (10). Because plasma bilirubin is complexed with albumin in vivo, bovine albumin (#A7906, Sigma) was added to a final concentration of 3.5 g/dl. The final bilirubin concentration was determined by the method of Jendrassik-Grof using Sigma Kit #605. Aliquots of the bilirubin (35 mg/dl)/albumin (3.5 g/dl) solution were maintained frozen at −60°C and used within 10 weeks.

To test the effect of oxyhemoglobin degradation to methemoglobin, hemoglobin calibration

curves were made in PBS using freshly isolated hemoglobin which had a methemoglobin content of less than 2.5% of the total hemoglobin concentration on 3 separate days (n = 3) (methemoglobin assay modified from Reference 11). These results were compared to calibration curves made using aged (filtered) hemoglobin which had a 9.5% methemoglobin content (n = 1) and assayed by each of the 9 hemoglobin measurement techniques. In vivo, methemoglobin normally accounts for less than 1.5% of the total hemoglobin concentration (11,12).

Spectrophotometry

A UV/VIS spectrophotometer (DU 640, Beckman Instruments, Fullerton, CA, U.S.A.) with traditional optics (resolution of 0.1 nm) and a spectral bandwidth of less than 1.8 nm was used. The wavelength calibration was checked 30 times over the course of 9 months using deuterium emission peaks at 486.0 and 656.1 nm and was found to be within 0.1 nm of these values. The absorbance calibration of the instrument was confirmed using a neutral density filter. Disposable UV grade methacrylate plastic microcuvettes (#14-385-938, Fisher Scientific, Pittsburgh, PA) were used throughout the experiments.

Assay methods

Plasma hemoglobin assays can be classified as either direct optical techniques (i.e., quantification based on oxyhemoglobin's absorbance peaks at 415, 541, or 577 nm, Fig. 1) or added chemical techniques in which hemoglobin forms a colored reaction product when mixed with chemicals such as cyanmethemoglobin reagent (Fig. 1A) or tetramethylbenzidine. The various added chemical techniques accounted for about 60% of the methods used by the 50 reviewed organizations.

Upon repeated inversion of fresh plasma samples, most of the hemoglobin is in the oxygenated form (oxyhemoglobin) (13–15). As with human oxyhemoglobin, freshly isolated bovine oxyhemoglobin in PBS has 3 distinct absorbance peaks between 400 and 700 nm, at 414.3, 541.5, and 576.7 nm (Fig. 1A). The exact location of the absorbance peaks varies based on the instrument which is used (i.e., the wavelength calibration, spectral bandwidth, optics), the purity of the sample, and the dilution media. The advantage for measuring at the 414–415 nm wavelength peak is that it offers about a 9 times larger absorbance signal than at the 541 nm or 577 nm peaks (millimolar absorptivity of 131 versus 14.37 and 15.37 mmol/L × cm, respectively [12]). The disadvantages are that the solution usually has to be diluted, and bilirubin complexed with albumin (with an absorbance peak around 460–464 nm), plasma, and lipids can have significant absorbance in this region (Fig. 2). The advantages for measuring oxyhemoglobin using the 577 nm peak are that it is furthest from the bilirubin and plasma peaks, the influence of albumin and lipids is less there, and the samples may not have to be diluted. Possible disadvantages are that the absorbance signals are smaller, the decay of oxyhemoglobin over time may reduce the signal (13), and a relatively expensive spectrophotometer with a spectral bandwidth of around 2 nm is preferred to make the most accurate measurements (14).

Direct optical techniques 1 (undiluted samples to measure the 577 nm oxyhemoglobin peak relative to a PBS or water blank): Cripps, Kahn, Porter, Shinowara, and first derivative methods

The absorbance equations used to calculate plasma hemoglobin concentration for the Cripps, Kahn, Porter, Shinowara, and first derivative methods are presented in Table 1.

Cripps method. The Cripps method (16) quantifies oxyhemoglobin in an undiluted plasma sample by using a 3 wavelength Allen baseline correction method (17). As used in this study (Fig. 1B), the partial absorbance of oxyhemoglobin at 576.5 nm was calculated relative to a linear baseline between 560 and 593 nm. The strength of the technique is that substances that may increase the absorbance at the 576.5 nm oxyhemoglobin peak (i.e., cause positive spectral interference: $A_{576.5}^{Sample} = A_{576.5}^{Hemoglobin} + A_{576.5}^{Background}$) are corrected for using absorbance values at wavelengths on both sides of the peak. Error arises when the assumption that the interference is changing linearly with the wavelength fails to hold.

Kahn method. The Kahn method (14) is essentially the same as that of Cripps, but it uses the partial absorbance at 578 nm relative to a linearly varying baseline between 562 and 598 nm. Based upon the theoretical absorbance of oxyhemoglobin at 578 nm, Kahn et al. (14) derived the absorbance constants and incorporated them directly into the final equation for this method so that a reference standard would not be needed.

Porter method. Except for substances that cause turbidity (e.g., lipids), most interfering substances for measuring hemoglobin absorb at wavelengths well below 700 nm (Fig. 2). To account for background interference from human plasma, Porter (13) used the absorbance difference between A_{578} and A_{700} to quantify oxyhemoglobin (Fig. 1B).

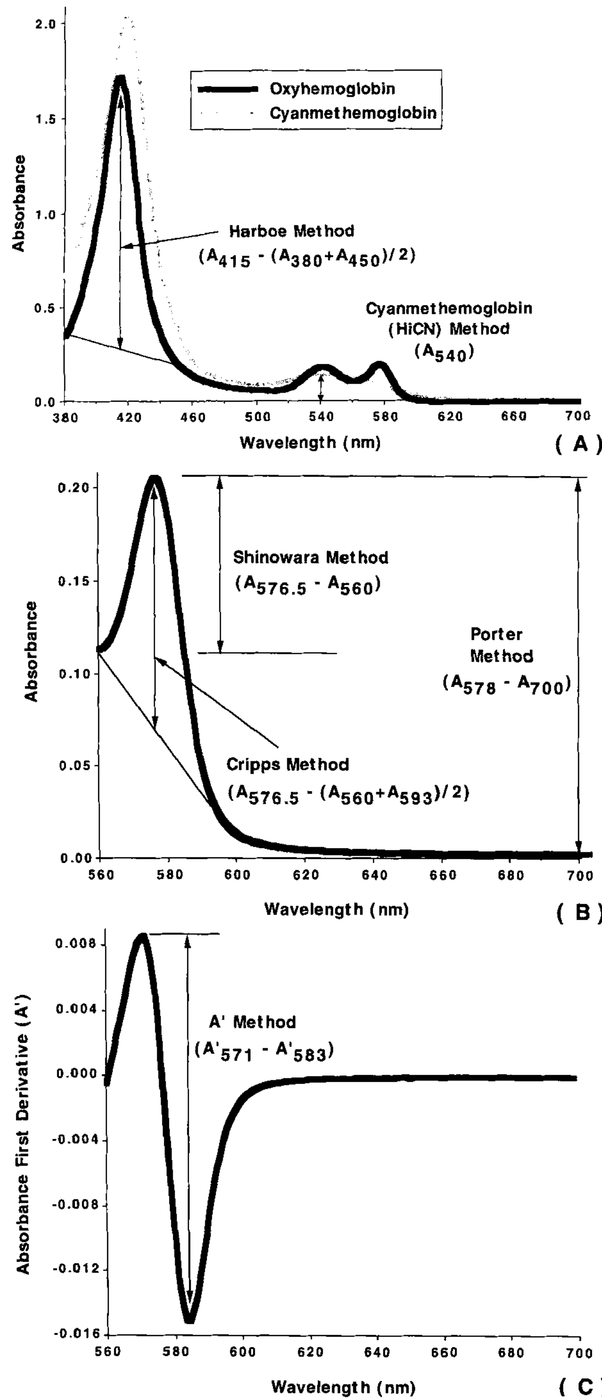

FIG. 1. The graphs show how hemoglobin isolated from cow red blood cells is quantified based on the absorbance spectra of oxyhemoglobin (direct optical read techniques about the 415 and 577 nm oxyhemoglobin peaks) and cyanmethemoglobin (added chemical technique). The fractional oxyhemoglobin absorbance at 415 nm is used by the Harboe method to compensate for background interference whereas the HiCN technique uses the absorbance at 540 nm and requires a separate background correction step **(A)**. Direct optical read techniques about the 577 nm oxyhemoglobin peak are shown **(B)** with the absorbance quantities used to measure oxyhemoglobin indicated for the Cripps, Porter, and Shinowara methods. The A′ method utilizes the difference between the local peak and trough values of the first derivative absorbance spectrum about the 577 nm hemoglobin absorbance peak **(C)**.

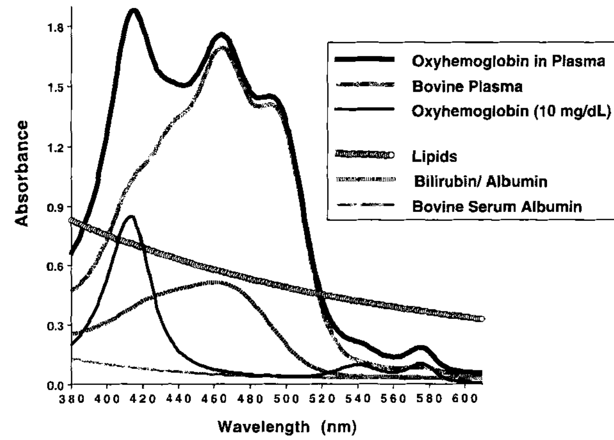

FIG. 2. The graph shows spectra of oxyhemoglobin (10 mg/dl) and the following common interferents: normal bovine plasma, lipids (Intralipid 0.6 μl/ml), bilirubin (0.5 mg/dl) with bovine serum albumin (3.5 g/dl), and bovine serum albumin (3.5 g/dl). As seen in the top curve, the absorbance spectrum of a solution of oxyhemoglobin (10 mg/dl) in bovine plasma is the superposition of the separate oxyhemoglobin (10 mg/dl) and bovine plasma (background) curves. For nonreactive substances, $A_\lambda^{Sample} = A_\lambda^{Hemoglobin} + A_\lambda^{Background}$ where A is the absorbance value and λ is the wavelength (nm).

Shinowara method. Shinowara (15) suggested using the absorbance difference between $A_{576.5}$ and A_{560} to quantify hemoglobin in an undiluted plasma sample (Fig. 1B). Since these 2 sampling wavelengths are near each other, interferents within the plasma should affect the absorbances similarly and cancel each other out upon taking the absorbance difference.

First derivative (A′) method. The first derivative method, adapted from Soloni et al. (18), was performed on an undiluted sample by obtaining the first derivative of the absorbance spectrum scanned from 560 nm to 590 nm in 0.5 nm increments. Since the absorbance spectra of most plasma interferents are slowly changing in the region around the 577 nm hemoglobin peak (Fig. 2), the difference between the first derivative absorbance peak (A'_{571}) and trough (A'_{583}) may be used to quantify oxyhemoglobin (Fig. 1C).

Direct optical techniques 2 (samples diluted to measure about the 415 nm hemoglobin peak): Harboe and Fairbanks AII methods

For the Harboe and Fairbanks AII techniques (for equations see Table 1), 100 μl of the samples were diluted in 1,000 μl of 10 mg/dl Na_2CO_3 (5). The alkaline pH of this medium minimizes turbidity in the mixtures (17). Absorbance values were measured relative to a 10 mg/dl Na_2CO_3 blank.

Harboe method. This method (19) utilizes a 3 point (380, 415, and 450 nm) Allen correction

TABLE 1. *Hemoglobin assay methods: absorbance equations and calibration coefficients*

Method	Absorbance equation[a]	Calibration coefficient–k values Original author (others)	Calibration coefficients (k values) this study	% error (compared to original author)
Cripps	$k1 \times \{A_{576.5} - [(A_{560} + A_{593})/2]\}$	181.7[16], (180.5[23], 176[b], 126[b])	177.6 ± 3.8	2.3
Kahn	$k2 \times [155 \times A_{578} - 86.1 \times A_{562} - 68.9 \times A_{598}]$	1[14]	1.160 ± 0.024	16.0
Porter	$k3 \times (A_{578} - A_{700})$	100.3[13], (105[b], 120[b])	115.4 ± 1.9	15.1
Shinowara	$k4 \times (A_{576.5} - A_{560})$	256[15], (323[17], 460[b])	262.5 ± 5.6	2.5
First Derivative	$k5 \times (A'_{571} - A'_{583})$		1792 ± 39	
Harboe	$k6 \times [167.2 \times A_{415} - 83.6 \times A_{380} - 83.6 \times A_{450}]$	1[19]	1.017 ± 0.015	1.7
Fairbanks AII	$k7 \times [154.7 \times A_{415} - 130.7 \times A_{450} - 123.9 \times A_{700}]$	1[5]	1.017 ± 0.014	1.7
HiCN	$k8 \times A_{540}$	329.6[6], (324[20])	335.5 ± 5.7	1.8
TMB (1–200 mg/dl)	$k9 \times [(A_{sample} - A_{blank})/(A_{stnd} - A_{blank}) \times C_{stnd}]$	1[22]	1.253 ± 0.073	25.3
(4–40 mg/dl)			1.065 ± 0.066	6.5

[a] The k values in the absorbance equations are the calibration coefficients used to convert an absorbance quantity to hemoglobin concentration (mg/dl). Those methods which use predetermined coefficients are assigned a calibration coefficient of 1 in column 3.
[b] Confidential information submitted to the U.S. Food and Drug Administration.
Numerical superscripts refer to reference citations in this paper.
A_λ, absorbance value at wavelength λ (nm); A'_λ, first derivative value of absorbance scan at wavelength λ (nm); HiCN, cyanmethemoglobin; TMB, tetramethylbenzidine; C_{stnd}, concentration of TMB standard.
Sample preparation: Methods 1–5, undiluted samples; Methods 6 and 7, diluted 1:10 in 0.1 mg/ml Na_2CO_3; Methods 8 and 9, added chemical techniques.

method to quantify the hemoglobin concentration using the 415 nm relative hemoglobin absorbance peak (Fig. 1A). A correction for "irrelevant absorption" due to impurities in hemoglobin solutions was incorporated by Harboe into the original equation to convert absorbance measurements to concentration directly (Table 1).

Fairbanks AII method. Fairbanks et al. (5) derived this method based on studies with human plasma that had normal or elevated levels of hemoglobin, bilirubin, and lipids. The coefficients in the equation (Table 1) were derived to compensate for excessive bilirubin (at 450 nm) and for turbidity (at 700 nm).

Added chemical techniques: Cyanmethemoglobin and tetramethylbenzidine methods

Cyanmethemoglobin (HiCN) method. The conversion of most forms of hemoglobin to the stable HiCN form for the quantification of total blood hemoglobin concentration (typically 11,000–18,000 mg/dl) is a common hematological technique which is supported by an international standard (6,7). Moore (20) suggested that this added chemical technique could be modified and used to assess plasma hemoglobin concentrations (over the range 5–2,000 mg/dl) when an appropriate plasma blank was used to correct for background interference. As used here, 400 μl of sample was added to 500 μl of HiCN reagent (200 mg $K_3Fe(CN)_6$, 50 mg KCN, 140 mg KH_2PO_4, and 1 ml Triton X-100 diluted to 1 L with water, pH 7.0–7.4, stored in a brown borosilicate glass bottle at room temperature [6,7]) and mixed by inversion. The reaction was complete within 5 min, at which time the 3 oxyhemoglobin peaks at 415, 541, and 576.5 nm were replaced by 2 peaks at 420 and 540 nm upon conversion to HiCN (Fig. 1A). To quantify the amount of hemoglobin present, the absorbance at the wide 540 nm peak was measured (relative to an HiCN reagent blank) and compared to the International HiCN Standard (Batch 70600, Centers for Disease Control, Atlanta, GA, U.S.A.).

Tetramethylbenzidine (TMB) method. This method is popular because of its availability in kit form with standards composed of hemoglobin diluted in human plasma (Kit 527A, Sigma). It is based on hemoglobin catalyzing the oxidation of a chromogen (TMB) by hydrogen peroxide to yield a reaction product which is proportional to color formation (21,22). TMB is representative of several chromogens (e.g., o-tolidine, chlorpromazine, diansidine, and leucomalachite green) which have been used as replacements for the carcinogen benzidine in the original method. For convenience and to conserve the working solutions, the TMB kit method was modified by reducing the suggested volumes by a factor of 2.5. To test tubes containing 800 μl of TMB in acetic acid (5 mg/ml), 4 μl of hemoglobin

sample or standard was mixed. At timed intervals, 800 μl of 0.3% H_2O_2 was added, and the tubes were mixed by inversion after being covered with parafilm. The absorbance at 600 nm was measured exactly 10 min after adding the H_2O_2 against a distilled water blank. Measurement at this wavelength insures minimal spectral interference. Due to the interrun variability in this technique, a TMB reagent blank and 2 of the 15, 30, or 45 mg/dl hemoglobin standards (#527-11, Sigma) were used in each run, along with up to 7 hemoglobin dilution samples. The reported dynamic range of this technique is 5–50 mg/dl (22).

RESULTS

Each of the 9 assays displayed good linearity (correlation coefficients 0.993 to 1.000) when calibrating the stock serial dilutions in PBS or water up to a hemoglobin concentration of 200 mg/dl (Fig. 3). The calibration coefficients (slopes) were obtained by linear least squares fitting the measured absorbance values to the known hemoglobin concentrations (with y-intercept at the origin) and were all within 4% of what the original authors or other researchers have used (Table 1), except for the Kahn and TMB methods. The %CV of the calibration coefficients for each of the methods was less than 2.2%, except for the TMB method (5.8%). Because the Kahn, TMB, Harboe, and Fairbanks methods use preestablished coefficients to convert absorbance readings to hemoglobin concentration directly (Table 1), the newly derived calibration coefficients were only used for the other methods to calculate the absolute hemoglobin concentrations.

When interferents were not present in the hemoglobin solutions, most of the methods displayed comparable accuracy. However, the Kahn and the TMB methods displayed a systematic error which was proportional to the hemoglobin concentration, causing them to underestimate the 200 mg/dl hemoglobin sample by 16–22% (Fig. 3). For hemoglobin dilutions within its reported dynamic range of 5–50 mg/dl, the percent error of the TMB method was less than 8.3%. Assay imprecision, as determined by the between-day coefficient of variation, and the percent total analytical error were greatest for the TMB method at nearly all of the hemoglobin concentrations (Table 2).

Analytical sensitivity is defined as the change in the signal absorbance relative to the change in the concentration of hemoglobin (11). For the dilution volumes used in this study, the TMB and Porter methods were the most sensitive to changes in hemoglobin concentration while the HiCN method was the least sensitive (Fig. 4).

When the percent of the total hemoglobin concentration that was methemoglobin was increased from 2.5% to 9.5% in hemoglobin dilutions in PBS, the slopes of the calibration curves for the assay methods based on the 577 nm oxyhemoglobin peak (Cripps, Kahn, Porter, Shinowara, and A′ methods) were affected by 3.8–7.5%. The other hemoglobin assay techniques were affected by less than 2.9%, particularly the Harboe and Fairbanks methods (about 1%).

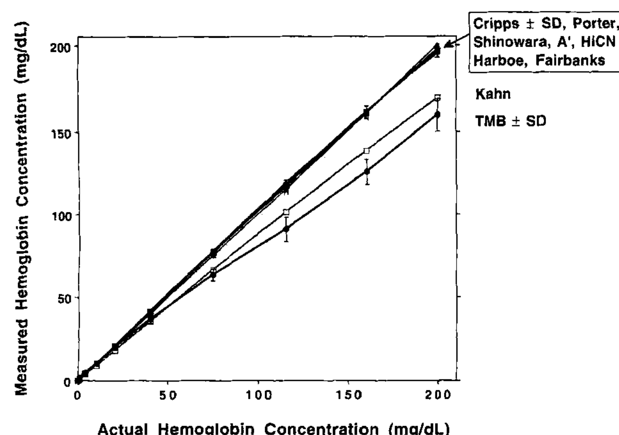

FIG. 3. Plotted is isolated hemoglobin (diluted in phosphate buffered saline or water to make calibration curves) as measured by the 9 hemoglobin assays.

Dilutions in bovine plasma

Absorbance scans performed on cow plasma samples obtained from 2 separate sites in Maryland, the Cleveland Clinic Foundation (Cleveland, Ohio, U.S.A.), and the Baylor College of Medicine (Houston, Texas, U.S.A.) revealed similar spectra (Fig. 5) with prominent absorption peaks at 464 nm (probably due to complexed bilirubin/albumin and to carotenoids [5,17]) and at 490 nm (due to carotenoids [5,17]), and the lack of a substantial oxyhemoglobin peak at 415 nm. The absorbance of the 464 nm peak varied by a factor of 2 to 3 times between all of the spectra.

Cow plasma from Maryland Site 2 (Fig. 5) had an apparent measured endogenous plasma hemoglobin concentration between −2.2 mg/dl (Shinowara method) and 38.8 mg/dl (HiCN method) when assayed by the 9 different methods (Table 3). Because the Cripps, A′, and Harboe methods were least influenced by low concentrations of interferents in the other tests, the true endogenous hemoglobin concentration of this plasma sample was assumed to be 1.2 mg/dl, based on averaging the values obtained from these 3 methods.

TABLE 2. *% Coefficient of variation and % total analytical error of the measurement assays for hemoglobin (1 to 200 mg/dl) added to PBS or water (n = 17)*

	% Coefficient of variation			% Total analytical error					
	Hemoglobin concentration (mg/dl)								
Method	1	4	(10–200)[a]	1	4	10	20	40	(75–200)[a]
Cripps	8.5	6.1	(2.7)	26	12	9	9	10	(6.5)
Kahn	8.9	5.8	(2.7)	36	24	18	16	17	(17)
Porter	7.7	5.6	(2.2)	20	12	9	8	8	(5)
Shinowara	9.4	6.4	(2.8)	27	13	10	11	12	(7.5)
A′	16.3	7.0	(2.8)	49	16	8	9	10	(7)
Harboe	10.9	5.5	(2.4)	37	18	12	8	7	(5)
Fairbanks	29.1[b]	5.4	(2.4)	62[b]	17	12	8	7	(5)
HiCN	13.5	5.3	(2.1)	29	13	7	5	5	(4)
TMB[c]	43.2	10.7	(6.6)	192	34	17	15	21	(30)

[a] Over the hemoglobin concentration range in parentheses, the table values were averaged due to low variability.
[b] Due to one erroneous absorbance measurement at 700 nm, the % CV of the Fairbanks method increased from 9.0 to 29.1%, and the % TAE increased from 33 to 62% at a hemoglobin concentration of 1 mg/dl.
[c] n = 14.

For the analytical recovery tests with hemoglobin spiked cow plasma, the HiCN and Porter methods displayed a systematic constant offset type of error while the Kahn method displayed the systematic proportional type of error that was evident in the hemoglobin dilutions in PBS (Fig. 6). By subtracting the intrinsic hemoglobin concentration of the plasma measured by each respective method (analytical recovery), most of the methods could accurately measure when 40 mg/dl of hemoglobin had been added to the plasma (Fig. 6A). A similar result occurred when 200 mg/dl had been added to the plasma (Fig. 6B), except for the TMB and Kahn methods which underestimated the added hemoglobin concentration. Reflecting these results, the percent total analytical recovery errors were also greatest for the TMB and Kahn methods above an added hemoglobin concentration of 20 mg/dl (Table 4) while being lowest for the Harboe method. As explained in Table 4, for a 1 mg/dl increase in hemoglobin concentration in plasma, the Cripps and TMB methods would measure a hemoglobin concentration increase within 0.2 and 1.01 mg/dl, respectively, of the true 1 mg/dl value 95% of the time.

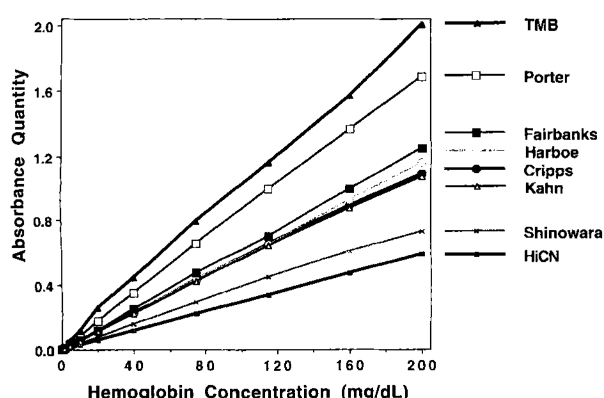

FIG. 4. This is a typical sensitivity plot (change in primary absorbance quantity versus the change in the hemoglobin concentration) for the hemoglobin assays.

Hemoglobin dilutions with elevated lipids

Similar to the background interference caused by normal plasma, elevated lipids in the absence of any added hemoglobin caused the Shinowara method to calculate a negative value (−11 mg/dl) for the hemoglobin concentration while the Porter (27 mg/dl) and HiCN (120 mg/dl) methods registered high values (Fig. 7). The other 6 methods were within 1.5 mg/dl of the true zero hemoglobin concentration value. As for the plasma dilutions, when lipids were added to the hemoglobin dilutions and the individually measured intrinsic background was subtracted from each respective method, all of the methods were within

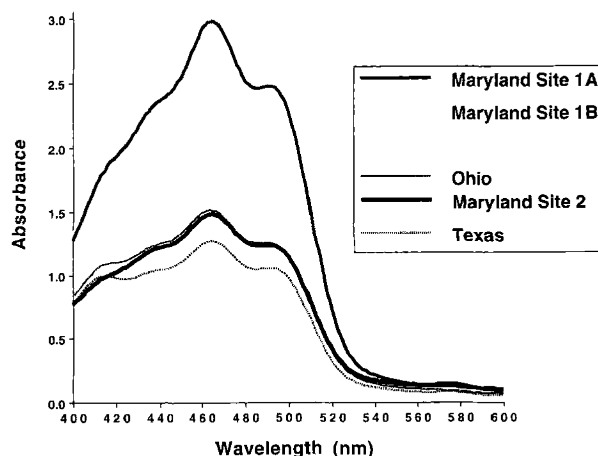

FIG. 5. Shown are spectra of bovine plasma obtained from 4 different sources. Note the common absorbance peaks around 464 and 490 nm in each sample.

TABLE 3. *Measured hemoglobin concentration in normal cow plasma and in a 45 mg/dl hemoglobin (in human plasma) standard solution (for TMB assay kit 527A, Sigma Chemical Co.)*

Method	Normal cow plasma (mg/dl) (n = 4)	45 mg/dl TMB hemoglobin standard (mg/dl) (n = 3)
Cripps	1.6 ± 0.1	6.1 ± 0.1
Kahn	1.4 ± 0.1	5.8 ± 0.1
Porter	10.9 ± 0.6	29.4 ± 0.6
Shinowara	−2.2 ± 0.2	−6.4 ± 0.2
A′	1.8 ± 0.2	4.4 ± 0.2
Harboe	0.2 ± 0.1	33.2 ± 0.8[b]
Fairbanks	−1.8 ± 0.1	38.4 ± 0.8[b]
HiCN	38.8 ± 0.8	64.0 ± 4.4
TMB	2.1 ± 0.4[a]	

[a] n = 3.
[b] n = 6.

TABLE 4. *% Total analytical recovery error of the measurement assays for hemoglobin (1 to 200 mg/dl) added to cow plasma (n = 4)*

	% Total analytical recovery error[a]								
	Hemoglobin concentration (mg/dl)								
Method	1	4	10	20	40	75	115	160	200
Cripps	20	20	11	14	13	15	11	9	4
Kahn	17	23	13	16	17	18	15	16	18
Porter	61	71	8	11	11	13	10	9	6
Shinowara	37	26	14	17	16	17	13	9	6
A′	44	18	10	14	13	15	15	9	5
Harboe	9	15	6	5	11	10	7	8	6
Fairbanks	60	19	6	5	11	10	6	8	7
HiCN	169	57	18	19	14	16	11	13	10
TMB[b]	101	38	12	23	16	22	22	16	23

[a] By multiplying the table values by the hemoglobin concentration in each column, the maximum absolute error (at a 95% level) can be determined for each method. For example, for a 1 mg/dl increase in hemoglobin concentration, the Cripps and TMB methods would measure a hemoglobin concentration increase within 0.2 and 1.01 mg/dl, respectively, of this value 95% of the time. For a 200 mg/dl increase in hemoglobin concentration, the Cripps value would be within 8 mg/dl of the true value (i.e., 200 mg/dl) while the TMB value would be within 46 mg/dl.
[b] n = 3.

5.5% of the 200 mg/dl hemoglobin value, except for the Shinowara (within 7%), Kahn (18%), and TMB methods (25%).

Hemoglobin dilutions in the presence of elevated bilirubin

The 577 nm oxyhemoglobin based assay methods and the TMB method were not significantly affected as the concentration of bilirubin was increased in solutions with constant hemoglobin concentrations. However, bilirubin had a linearly positive interferent effect on the HiCN method while it had a negative effect on the Harboe and, most noticeably, on the Fairbanks method (Fig. 8).

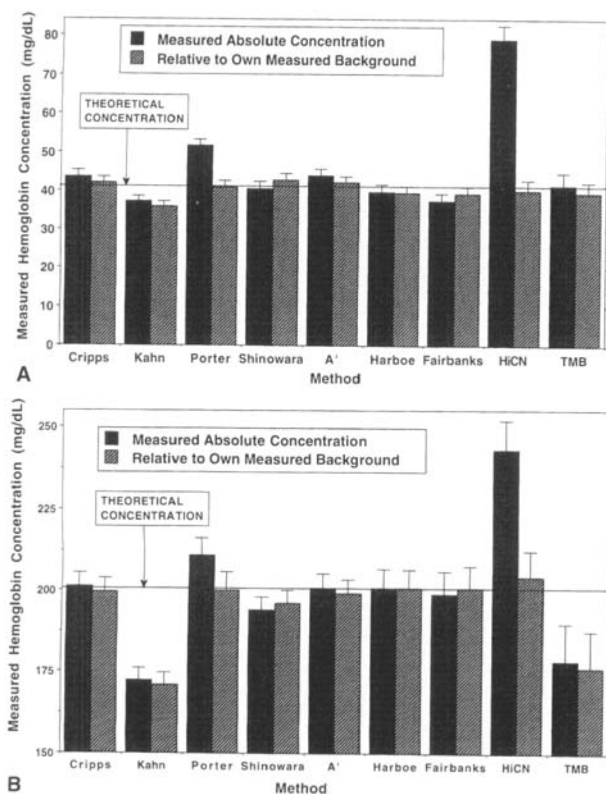

FIG. 6. The graphs show the absolute and relative measurements (analytical recovery) of hemoglobin added to normal bovine plasma (n = 4 experiments) for 40 mg/dl of hemoglobin added **(A)** and 200 mg/dl of hemoglobin added **(B)**.

DISCUSSION

Because the in vivo or ex vivo evaluation of a medical device for hematological damage can be very complex (e.g., due to the surgery and injectates involved, time-varying levels of blood chemistry components which can affect the blood damage assays, haptoglobin binding and removal of plasma hemoglobin, and general physiological differences between various test animals and humans), in vitro testing using blood in recirculating loops is a much simpler yet valuable measurement tool. Because of its ease of measurement, sensitivity, and typical linear increase over time, the plasma hemoglobin concentration is widely used as the most reliable indicator of overall blood damage in in vitro device testing (1). Although in vitro (and in vivo) evaluations of platelet and white blood cell counts and activation assays are necessary, they can be more difficult to perform, reproduce, interpret, and compare between testing institutions.

In vitro testing involves the collection of multiple samples from the same blood source over several hours. Although absolute measurement of the plasma hemoglobin concentration may be affected

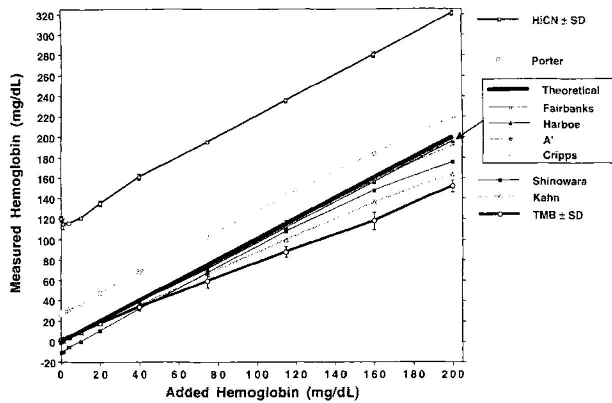

FIG. 7. The plot shows measurements of 1–200 mg/dl of hemoglobin added to a lipid solution (n = 3). The theoretical hemoglobin concentration is highlighted in the plot.

by background interference from plasma constituents, the relative hemoglobin concentration (synonymous with the analytical recovery experiments), which can be determined by subtracting the measured concentration at time zero from the concentration at the other time points, corrects for background interference directly (assuming that the background does not change with time and that the hemoglobin does not degrade or react with other substances). This relative increase in the plasma hemoglobin concentration over time is the fundamental parameter of in vitro hematological testing. However, the absolute plasma hemoglobin concentration is important as well because a blood pool to be used in device testing may be required to be below a certain absolute level as a quality control measure of the condition of the blood. Moreover, accuracy, sensitivity, specificity, and reproducibility of the plasma hemoglobin assays must be similar between testing institutions using different measuring photometers and assays so that comparisons of the index of hemolysis can be appropriately used in evaluating medical devices.

Although plasma absorbance spectra from only 5 different cows (from 4 different herds) were presented in this study (Fig. 5), they demonstrate the general similarities between the spectra of human (5) and cow plasma (and hemoglobin). This implies that the use and limitations of the plasma hemoglobin test methods are similar whether human or cow blood is being used for in vitro device testing. Similar to clinical limits reported for these methods (5), most of the assays could reliably measure down to 1 ± 1 mg/dl of added hemoglobin in the cow plasma used in this study. However, researchers should be aware that the magnitude of the background correction may be markedly different between human and cow plasma, especially because most cow blood is obtained from slaughterhouses, which provide an uncontrolled and highly variable blood source. As will be addressed in a subsequent study, the magnitudes of the plasma hemoglobin values are expected to be greater when human blood is used for in vitro device testing because of the apparent lower mechanical fragility of cow red blood cells due to their smaller size (1,2).

Based on information from several resources, an ideal plasma hemoglobin assay for medical device testing should meet the following criteria: measure plasma hemoglobin values from 0 to 1,000 mg/dl in human and animal plasma accurately and precisely; be unaffected by elevated bilirubin or lipid concentrations; be easy to use, time independent, and able to assay large batches of plasma samples quickly; be performed using small sample volumes and without diluting the samples; use no dangerous chemicals; be quantified against a readily available stable hemoglobin standard solution; and use an inexpensive spectrophotometric instrument. As tested here, the upper hemoglobin concentration limit that the assays could measure in bovine plasma without undergoing a dilution was around 200–250 mg/dl, depending on the level of background plasma components. Above this limit, the absorbance values exceed the linear range of many spectrophotometers (assuming a maximum linear absorbance limit around 2, which corresponds to a percent transmittance of 1%).

Although none of the assays meet all of the criteria completely, there are distinct advantages for using some of the methods over the others, as summarized in Table 5. Overall, the Cripps, A′, and Harboe methods were found to be the most robust in terms of accuracy, reproducibility, ease of use, sensitivity, and ability to compensate for lipid interference (Table 5). However, they do have disadvantages which must be considered. Although the Harboe method gave consistent results without the use of

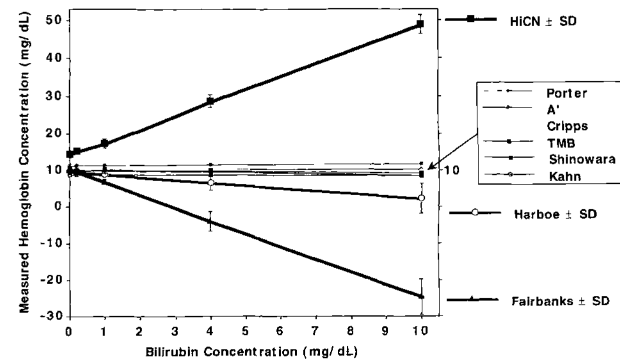

FIG. 8. The effects of increasing bilirubin concentration on the hemoglobin assays when measuring a constant concentration of hemoglobin (10 mg/dl, n = 4) are shown.

TABLE 5. *Comparison of the plasma hemoglobin assays on a scale of 1 (worst) to 9 (best)*

Method (media)	Absolute accuracy (plasma)	Relative accuracy (plasma)	Bilirubin interference (PBS)	Lipid interference (PBS)	Absolute accuracy (PBS/water)	Precision (PBS/water)	Sensitivity (PBS/water)	Ease of use	Time for 20 samples	Instrumentation (bandwidth)	Overall use	Convert abs to mg/dl
Cripps	9	9	9	8	8	9	6	9	9 (10 min)	7 (2 nm)	9	Self calibration
Kahn	2[a]	2[a]	8	8	3[a]	9	6	9	9 (10 min)	7 (2 nm)	4	Equation given
Porter	4[b]	8	8	4[b]	9	9	8	9	9 (10 min)	7 (2 nm)	6	Self calibration
Shinowara	5[a]	6[a]	8	5[a]	8	9	4	9	9 (10 min)	7 (2 nm)	5	Self calibration
A′	9	9	9	9	8	8		8	8 (15 min)	6 (2 nm)	8	Self calibration
Harboe	9	9	5[a]	9	8	8	6	8	8 (17 min)	8 (4 nm)	8	Equation given
Fairbanks	8	9	1[c]	9	8	7	6	8	8 (17 min)	8 (4 nm)	7	Equation given
HiCN	1[b]	8	1[d]	1[d]	9	8	3	7	7 (19 min)	9 (6 nm)	5	Self calibration
TMB	3[a]	3[a]	8	8	3[c]	4	9	2	1 (80 min)	9	3	Kit standards

[a] Negatively interferes with the assay.
[b] Positively interferes with the assay.
[c] Greatly negatively interferes with the assay.
[d] Greatly positively interferes with the assay.
The scores (from 1 to 9) presented in the table are based on relative comparisons between the accuracy, precision, or utility of the methods under each respective column heading as evaluated over the hemoglobin concentration range of 1–200 mg/dl.
Relative accuracy refers to analytical recovery evaluated by subtracting the baseline concentration obtained by each method from the concentration measured by that method when a known amount of hemoglobin was added.
In last column "abs" refers to the measured absorbance quantity and its conversion to a hemoglobin concentration (mg/dl) using a calibration coefficient.

hemoglobin standards, its absolute accuracy was affected as the bilirubin concentration was elevated above normal levels. This can be important because the bilirubin level in blood used for in vitro device testing is rarely measured, and bilirubin can be significantly increased during in vivo testing. Although the Cripps, A′, and other methods which quantitate oxyhemoglobin about the 577 nm peak can be performed in undiluted plasma, the criticisms for using them are that they cannot measure other forms of hemoglobin (5); the width of this peak is small (natural bandwidth of 23 nm) and requires a spectrophotometer with a spectral bandwidth of about 2 nm (or accuracy and sensitivity will be decreased) (14); oxyhemoglobin degradation can affect this peak within a few hours (13); and there are no commercially available stable standards (12).

The problems with the Cripps and A′ techniques can be addressed by using the simple methods presented in this paper. Hemoglobin can be isolated from PBS washed red blood cells easily and maintained frozen at −70°C for several months (personal communication: Dr. Virgil Fairbanks, Mayo Clinic, Rochester, MN, U.S.A.) and even as long as 2 years (23,24). Because the hemoglobin assays have high reproducibility, the slope of a hemoglobin calibration curve should not change much when monitored as part of a quality control plan (23,24). Individual laboratory calibration such as this would also compensate for the variability caused by different spectrophotometers (variations due to spectral bandwidth, type of cuvettes, wavelength calibration, and traditional measurement optics versus fixed-wavelength diode array sensors). The range of calibration coefficients reported for the Cripps (including the equation coefficient of 155 in the essentially identical Kahn method), Porter, and Shinowara methods in Table 1 suggests that each lab should perform its own calibration.

Although the methods that quantitate the plasma oxyhemoglobin concentration about the 577 nm peak do not measure the total amount of hemoglobin present, most of the hemoglobin should be in the oxygenated form if the fresh plasma samples are repeatedly inverted prior to measuring them in a spectrophotometer (14,15) and there is no chemical conversion of the hemoglobin. Typical proportions of hemoglobin derivatives determined by a cooximeter from hemoglobin isolated by freeze-thawing freshly obtained human erythrocytes include: 91.3–92.7% oxyhemoglobin, 1.9–7.7% methemoglobin (normally less than 1.5%), and 0.4–5.7% carboxyhemoglobin (normally 0.5–1.5% in nonsmokers) (11,12,25,26). Although oxyhemoglobin may degrade to methemoglobin/methemalbumin quicker in bovine plasma than it does in human plasma (27), it should not affect the hemoglobin measurements significantly. Similar to conditions which may be imposed in an in vitro blood test experiment, Porter (Fig. 3) (13) reported that oxyhemoglobin (15–150 mg/dl) incubated at 37°C for 6 h in human plasma decreased its 578 nm absorbance measurement by only about 4–5.2% as it was converted to methemalbumin. However, after 10 h of exposure at room temperature or up to 14 days when refrigerated at 4°C, no significant decrease in oxyhemoglobin concentration was observed (13). Hence, the error associated with using the oxyhemoglobin measurement techniques for a 6 h in vitro loop test and refrigerating the centrifuged plasma samples until they can be assayed is probably

less than 5%. Although further tests with bovine hemoglobin are needed to confirm this value, researchers may monitor whether methemalbumin is being formed by monitoring the growth of the 625–635 nm methemalbumin peak over time in their blood test loop samples (5,13).

Although methods that use TMB or similar chromogens are popular due to the small needed sample size and their availability in kit form with standards, it can take up to 8 times as long to assay 20 plasma samples using the manual TMB method compared to the direct optical read methods, and the results are not as precise or accurate. Although it is not difficult to perform, multiple pipetting and precise reaction timing are necessary, the number of plasma samples that can be run at one time is limited, and possible reaction inhibition (by as much as 40% [21]) may occur from calcium chelating anticoagulants (e.g., citrates, oxalates, and EDTA) (22), albumin (5), or other nonspecific plasma components (21). Another major disadvantage is that the test samples, which usually number over 20, should be diluted until the hemoglobin concentration is within the low dynamic range for this assay (5–50 mg/dl) (22). As for other added chemical techniques, the chemical stability, handling, and disposal are of greater importance than they are for the direct optical read methods.

For the HiCN chemical method, the disadvantages are that it uses a dangerous chemical to handle (which must be carefully disposed of and kept away from acids), it has the lowest sensitivity of all of the tested methods (as used here), and its absorbance is substantially increased by plasma constituents. Its main advantages are that a stable internationally accepted HiCN standard is available, HiCN has a wide peak at 540 nm so that a less expensive spectrophotometer with a wide spectral bandwidth may be used, it quickly converts most common forms of hemoglobin to cyanmethemoglobin, it is the accepted method for quantifying the total blood hemoglobin concentration, and it can be effectively used to make relative measurements of plasma hemoglobin concentration (>1 mg/dl) when corrected using an appropriate background blank.

Interestingly, the Fairbanks AII and Harboe methods, which were derived for use clinically with human plasma and do not require a calibration curve, had good results with normal cow plasma and when lipid and methemoglobin levels were elevated. This again emphasizes the basic similarities in the absorbance spectra of normal human plasma and the cow plasma used in this study. However, the Fairbanks equation significantly underestimated the hemoglobin concentration when the bilirubin concentration exceeded 1 mg/dl. This is partly due to the use of the absorbance at 450 nm to correct for bilirubin in human plasma (5) while the bilirubin-bovine albumin mixture used in these experiments had a peak around 460–464 nm, which was similar to the peak observed in the native cow plasma.

Finally, in the course of reviewing the measurement practices at various institutions, a few other important concerns arose about performing plasma hemoglobin assays. First, chemical assays which require the use of serum due to significant nonspecific plasma inhibition (e.g., leucomalachite green [28]) should be avoided because the clotting process used to obtain the serum can markedly increase the liberation of hemoglobin from erythrocytes (5,17). Second, cooximeters and hemoglobinometers used for the determination of total blood hemoglobin concentration do not have the resolution to measure low plasma hemoglobin concentrations. Along with the standard HiCN technique (6,7), these instruments should be used to measure the concentration of freshly isolated hemoglobin before it is diluted to make a calibration curve. Third, the hemoglobin (in human plasma) standards available from Sigma Chemical Co. for use with their TMB assay kit should not be used as hemoglobin standards for any other nonpseudoperoxidase type of method. As demonstrated in Table 3, substantial errors in estimating the hemoglobin concentration may result. Fourth, the direct optical measurement of plasma oxyhemoglobin at its 541 nm peak, adapted from the common red blood cell osmotic fragility test, has also been used as an assay to evaluate chemically and mechanically induced blood damage caused by medical materials and devices. Although the 541 nm hemoglobin peak is wider (but about 7% smaller) than the 577 nm peak (Fig. 1), it should only be used for relative measurements of plasma hemoglobin concentration because it is subject to greater spectral interference, which makes direct background correction more difficult (Fig. 2). Fifth, methods that utilize the absorbance at only 2 wavelengths (e.g., the Porter and Shinowara methods) and especially at only 1 wavelength (e.g., the cyanmethemoglobin method, 541 nm direct optical read method) cannot directly correct for spectral interference completely and are most effective for reporting the relative plasma hemoglobin concentration. Although not ideal, some clinical laboratories attempt to compensate for these interference problems by using a background blank composed of pooled human plasma. By using hemoglobin standards diluted in human plasma, this background correction method is also used with the TMB assay kit (Sigma). However, due to the variability in

the magnitude of the normal plasma absorbance values between cows (Fig. 5), background correction by subtracting the time zero measured concentration should be the preferred method for in vitro device testing.

CONCLUSIONS

Although direct optical read spectrophotometric methods are used by fewer testing institutions to measure plasma hemoglobin, some of them (e.g., the Cripps, A′, and Harboe methods) are safer, easier, and more precise and accurate than the typical chemical addition methods used to assay the large number of samples obtained from in vitro medical device testing. The method described by Cripps (16) for use in undiluted plasma, which is based on a 3 wavelength baseline correction about the 577 nm oxyhemoglobin peak, was ranked the highest overall among the 9 assays tested over the hemoglobin concentration range of 1 to 200 mg/dl. To compensate for the lack of a commercially available standard and for variations in spectrophotometers, a standard calibration curve using freshly isolated bovine hemoglobin, the hemoglobin concentration of which has been determined against an established HiCN standard solution or similarly validated hemoglobinometer, should be generated by each laboratory.

Acknowledgments: I would like to thank the many researchers at the various institutions who shared their plasma hemoglobin measurement techniques and experiences with me. In particular, I must thank Dr. Virgil Fairbanks (Mayo Clinic, Rochester, MN, U.S.A.) and Dr. Onno van Assendelft (Centers for Disease Control, Atlanta, GA, U.S.A.) for their many suggestions and review of the manuscript. Thanks also go to Dr. William Smith (Cleveland Clinic Foundation, Cleveland, OH, U.S.A.) and Mr. George Damm (Baylor College of Medicine, Houston, TX, U.S.A.) for supplying cow plasma samples, and to Dr. Warren Karp (Medical College of Georgia, Augusta, GA, U.S.A.) and Ms. Linda Wessels (University of Minnesota Hospital, Minneapolis, MN, U.S.A.) for data on the stability and use of hemoglobin in the Cripps method. At the U.S. Food and Drug Administration, Dr. Richard Cullison, Mr. Larry Brindza, and Mr. Ronald Robinson also contributed to this project. Finally, my thanks go to Mr. Maurice Reid who performed some of the experiments as part of the Public Health Service's co-step summer internship program.

REFERENCES

1. Mueller MR, Schima H, Engelhardt H, Salat A, Olsen DB, Losert U, Wolner E. In vitro hematological testing of rotary blood pumps: Remarks on standardization and data interpretation. *Artif Organs* 1993;1:103–10.
2. Naito K, Mizuguchi K, Nosé Y. The need for standardizing the index of hemolysis. *Artif Organs* 1994;18:7–10.
3. Mizuguchi K, Damm GA, Aber GS, Bozeman RJ, Bacak JW, Svejkovsky PA, Orime Y, Ohara Y, Naito K, Tasai K, Makinouchi K, Takatani S, Nosé Y, Noon GP, DeBakey ME. Does hematocrit affect in vitro hemolysis test results? Preliminary study with Baylor/NASA prototype axial flow pump. *Artif Organs* 1994;18:650–6.
4. Jakob H, Kutschera Y, Palzer B, Prellwitz W, Oelert H. In-vitro assessment of centrifugal pumps for ventricular assist. *Artif Organs* 1990;14:278–83.
5. Fairbanks VF, Ziesmer SC, O'Brien PC. Methods for measuring plasma hemoglobin in micromolar concentration compared. *Clin Chem* 1992;38:132–40.
6. National Committee for Clinical Laboratory Standards. Reference and selected procedures for the quantitative determination of hemoglobin in blood (2nd edition, approved standard). Document H15-A2, 1994.
7. Recommendations for reference method for haemoglobinometry in human blood (ICSH standard 1995) and specifications for international haemiglobincyanide standard (4th edition). *J Clin Pathol* 1996;49:271–4.
8. Smith BP. *Large animal internal medicine.* St. Louis, MO: C.V. Mosby, 1990.
9. Qian KX, Wang SS, Chu SH. In vivo studies of pulsatile implantable impeller assist and total hearts. *Artif Organs* 1995: 19:328–33.
10. Doumas BT, Kwok-Cheung PP, Perry BW, Jendrzejczak B, McComb RB, Schaffer R, Hause LL. Candidate reference method for determination of total bilirubin in serum: Development and validation. *Clin Chem* 1985;31:1779–89.
11. Burtis CA, Ashwood ER, eds. *Tietz textbook of clinical chemistry* (2nd edition). Philadelphia: W.B. Saunders, 1994.
12. Henry JB, ed. *Clinical diagnosis and management by laboratory methods* (18th edition). Philadelphia: W.B. Saunders, 1991.
13. Porter GA. Spectrophotometric method for quantitative plasma hemoglobin resulting from acute hemolysis. *J Clin Lab Med* 1962;60:339–48.
14. Kahn SE, Watkins BF, Bermes EW. An evaluation of a spectrophotometric scanning technique for measurement of plasma hemoglobin. *Ann Clin Lab Sci* 1981;11:126–31.
15. Shinowara GY. Spectrophotometric studies on blood serum and plasma. *Am J Clin Path* 1954;24:696–710.
16. Cripps CM. Rapid method for the estimation of plasma haemoglobin levels. *J Clin Path* 1968;21:110–2.
17. Henry RJ, Cannon DC, Winkelman JW. *Clinical chemistry—Principles and technics* (2nd edition). Hagerstown, Maryland: Harper & Row, 1974.
18. Soloni FG, Cunningham MR, Amazon K. Plasma hemoglobin determination by recording derivative spectrophotometry. *Am J Clin Pathol* 1986;85:342–7.
19. Harboe M. A method for determination of hemoglobin in plasma by near-ultraviolet spectrophotometry. *Scand J Clin Lab Invest* 1959;11:66–70.
20. Moore GL, Ledford ME, Merydith A. A micromodification of the Drabkin hemoglobin assay for measuring plasma hemoglobin in the range of 5 to 2000 mg/dl. *Biochem Med* 1981; 26:167–73.
21. Standefer JC, Vanderjagt D. Use of tetramethylbenzidine in plasma hemoglobin assay. *Clin Chem* 1977;23:749–51.
22. Sigma Diagnostics. Plasma hemoglobin: Quantitative, colorimetric determination in plasma at 600 nm (Procedure No. 527). St. Louis, MO: Sigma Diagnostics, April 1991.
23. Karp WB. Hemoglobin in the plasma of infants on ECMO by multiple wavelength spectrophotometry. *Clinical perinatal laboratory procedure manual* (Augusta, GA: Medical College of Georgia, June 1995).

24. Hemoglobin in plasma by multiple wavelength spectrophotometry, *University of Minnesota Hospital procedure manual* Minneapolis, MN: University of Minnesota, 1992.
25. Wians FH, Miller CL, Heald JI, Clark HC. Evaluation of a direct spectrophotometric procedure for quantitating plasma hemoglobin. *Lab Med* 1988;19:151–5.
26. Noe DA, Weedn V, Bell WR. Direct spectrophotometry of serum hemoglobin: An Allen correction compared with a three-wavelength polychromatic analysis. *Clin Chem* 1984;30:627–30.
27. Smith JE, Beutler E. Methemoglobin formation and reduction in man and various animal species. *Am J Physiol* 1966;210:347–50.
28. Slaunwhite D, Clements J, Tuggey RL, Reynoso G. Leucomalachite green assay for free hemoglobin in serum. *Am J Clin Pathol* 1979;72:852–5.