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Laboratory Management

# Lyophilized bovine hemoglobin as a possible reference material for the determination of hemoglobin derivatives in human blood

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**We investigated the suitability of a lyophilized bovine hemoglobin (LBH) preparation containing various fractions of oxyhemoglobin ($O_2$Hb), carboxyhemoglobin (COHb), and methemoglobin (MetHb) for quality assessment in multicomponent analysis (MCA) of hemoglobin derivatives. It was demonstrated that a stable preparation of these components after reconstitution yields a hemoglobin solution that is spectrophotometrically equivalent with a fresh bovine hemoglobin solution. The preparation was found to be stable for at least 1 year when it is kept at 2–8 °C and for 1 h after reconstitution. We determined the fractions of $O_2$Hb, COHb, and MetHb of several LBH preparations, using the complete spectra of 480–650 nm with 2-nm intervals and absorptivities as determined for pure LBH solutions. A field trial involving various types of multiwavelength hemoglobin photometers showed the suitability of LBH as a quality-control material. Computer models of the various common multiwavelength hemoglobin photometers may be useful for establishing more accurate target values of LBH preparations for each type of photometer and for studying the importance of the influence of specific factors such as wavelength selection, absorptivity values, and interfering dyes.**

Spectrophotometric multicomponent analysis (MCA)[5] of hemoglobin derivatives *(1, 2)* has become available in routine clinical chemistry through the development of multiwavelength hemoglobin photometers (CO-oximeters) of different design, using either the same number of wavelengths as components to be analyzed [exactly determined system *(3, 4)*] or more wavelengths than are strictly necessary (overdetermined system). In addition, various procedures are used for hemolysis and for eliminating the influence of turbidity. All instruments measure oxyhemoglobin ($O_2$Hb), deoxygenated hemoglobin (HHb), carboxyhemoglobin (COHb), and methemoglobin (MetHb); some also signal the presence of or measure sulfhemoglobin.

Comparative measurements have shown that most of these instruments are quite reliable when used within the specifications of the manufacturers *(5–8)*. Quality control is rather difficult. Of course, the instruments can be tested with stable dye solutions. However, these solutions show non-identical spectral behavior in comparison with blood. The ideal test material would be a solution of hemoglobin derivatives of known concentration. Such solutions are not stable for sufficient time periods. Therefore, we investigated whether a lyophilized bovine hemoglobin (LBH) preparation containing various fractions of $O_2$Hb, COHb, and MetHb may be suitable for quality control in MCA of hemoglobin derivatives.

## Materials and Methods

### PREPARATION OF LBH

A stroma-free hemoglobin (SFH) solution was prepared according to Sprokholt and co-workers *(9, 10)*. Briefly,

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[5] Nonstandard abbreviations: MCA, multicomponent analysis; $O_2$Hb, oxyhemoglobin; HHb, deoxygenated hemoglobin; COHb, carboxyhemoglobin; MetHb, methemoglobin; SFH, stroma-free hemoglobin; and LBH, lyophilized bovine hemoglobin.

fresh bovine blood was aseptically collected in a 3-L glass vessel containing sodium heparin solution (100 000 units/L; LEO Pharmaceutical Products B.V.). Plasma was separated from blood cells by centrifugation (2000*g* for 20 min at 4 °C; Jouan K110). Blood cells were suspended in NaCl solution (154 mmol/L). Leukocytes and platelets were removed by cotton wool filtration. Red cell concentrates were prepared by centrifugation, and erythrolysates were prepared from these concentrates ($V_c$) by adding water ($V_w$) in the volume ratio $V_c/V_w = 1/4$. The erythrocyte stromata were separated from the hemoglobin solution by centrifugation and tangential cross-flow filtration using the Pellicon system with an HVMP filter cassette (Millipore Corp.) in combination with a peristaltic pump (type 603, Watson-Marlow Ltd.). The diluted SFH solution was concentrated by ultrafiltration using the Pellicon system equipped with a PTGC filter cassette and a rotary vane pump, type 4 GPM (Millipore). Finally, erythrocyte remnants were removed by filtration through polycarbonate filters with a pore size of 0.8 $\mu$m, and the SFH solution was subsequently sterilized using a polycarbonate filter with a pore size of 0.22 $\mu$m (Millipore).

SFH solutions were equilibrated with $O_2$ and CO to obtain $O_2$Hb and COHb, respectively, and treated with an oxidizing agent (sodium nitrite) to induce formation of MetHb. The pH of these solutions was adjusted to 7.26 at 25 °C by the addition of diluted sodium hydroxide or hydrochloric acid solution. Solutions of pure $O_2$Hb, COHb, or MetHb and a series of four mixtures of these hemoglobin derivatives covering the pathophysiologically interesting ranges (*F*$O_2$Hb, 90–40%; *F*COHb, 5–20%; and *F*MetHb, 0–40%) were lyophilized in 3-mL vacuum-sealed vials, each vial containing 0.5 mL of material, and stored at 2–8 °C in the dark.

### RECONSTITUTION, MEASUREMENT, AND STABILITY OF LBH

For use, the contents of a vial were reconstituted by adding 0.5 mL of demineralized water with a $Po_2$ of ~20 kPa and proper mixing until a homogeneous solution was obtained, which takes ~2 min. The intervial precision was determined by measuring five vials of each mixture with an IL282 multiwavelength hemoglobin photometer. For practical reasons we decided not to use HHb because of its affinity to oxygen, which leads to unpredictable results after reconstitution. To ascertain that each mixture is free of HHb, five vials of each mixture were reconstituted with demineralized water with $Po_2$ >100 kPa and measured on an IL282 multiwavelength hemoglobin photometer.

The absorptivities of pure $O_2$Hb, COHb, and MetHb of LBH were established by measuring the absorbance of reconstituted vials of each derivative with an HP8450A reversed-optics spectrophotometer at room temperature (480–650 nm with intervals of 2 nm). Calibration of the spectrophotometer was verified with a Corning HT yellow filter calibrated by NIST. The absorptivities of LBH derivatives were compared with those of bovine and human hemoglobin, which had been published previously *(11, 12)*. Because of the dependence of the absorption spectrum of MetHb on the pH of the solution, we recorded the absorptivity as a function of wavelength for LBH-MetHb at various pH values. The pH of the solutions was measured at 25 °C with an ABL500 blood gas analyzer (Radiometer).

The stability of the LBH solutions after reconstitution of mixtures of LBH by adding 0.5 mL of water to the vials was established after storage of the solutions at 25 °C for various periods of time. The $O_2$Hb, COHb, and MetHb fractions of the solutions were measured at 10-min intervals with a Radiometer ABL520 multiwavelength hemoglobin photometer.

The shelf life was determined by storage of vials with mixtures of LBH at 2–8 °C. Measurements of reconstituted samples were performed at 15-min intervals with an IL282 multiwavelength hemoglobin photometer.

### COMPUTER PROGRAM SIMULATING MCA

A computer program has been developed to calculate the composition of mixtures of hemoglobin derivatives by simulating MCA, which is based on the Lambert-Beer law:

$$\mathbf{A} = \varepsilon^{\lambda} \cdot \boldsymbol{c} \cdot \boldsymbol{l} \qquad (1)$$

where **A**, the absorbance, is log $I_o/I$, $\varepsilon^{\lambda}$ is the millimolar absorptivity ($L \cdot mmol^{-1} \cdot cm^{-1}$) at wavelength $\lambda$, ***c*** is the concentration (mmol/L), and ***l*** is the lightpath length in centimeters. The equation can be solved for ***c*** when **A** has been measured at a fixed wavelength ($\lambda$) where $\varepsilon^{\lambda}$ is known, provided that the lightpath length is known exactly. For a solution containing several components, Eq. 1 becomes:

$$\mathbf{A} = \varepsilon_1^{\lambda} \cdot \boldsymbol{c}_1 \cdot \boldsymbol{l} + \varepsilon_2^{\lambda} \cdot \boldsymbol{c}_2 \cdot \boldsymbol{l} + \ldots\ldots \varepsilon_n^{\lambda} \cdot \boldsymbol{c}_n \cdot \boldsymbol{l} \qquad (2)$$

Writing $\mathbf{A}'$ for $\mathbf{A}/\boldsymbol{l}$, this becomes for a solution of $O_2$Hb, COHb, and MetHb:

$$\mathbf{A}' = \varepsilon^{\lambda}_{O_2Hb} \cdot \boldsymbol{c}_{O_2Hb} + \varepsilon^{\lambda}_{COHb} \cdot \boldsymbol{c}_{COHb} + \varepsilon^{\lambda}_{MetHb} \cdot \boldsymbol{c}_{MetHb} \qquad (3)$$

Measuring $\mathbf{A}'$ of the solution at three different wavelengths yields a set of three linear equations with three unknown concentrations. For an overdetermined system of n >3, this set of equations can be written according to Eq. 4 for each wavelength at which **e** is the error:

$$\mathbf{A}' + \mathbf{e} = \varepsilon^{\lambda}_{O_2Hb} \cdot \boldsymbol{c}_{O_2Hb} + \varepsilon^{\lambda}_{COHb} \cdot \boldsymbol{c}_{COHb} + \varepsilon^{\lambda}_{MetHb} \cdot \boldsymbol{c}_{MetHb} \qquad (4)$$

Eq. 4 for all wavelengths used can be summarized as Eq. 5, in which $\Sigma\mathbf{e}^2$ (a measure for total error) is minimal when $\partial(\Sigma\mathbf{e}^2)/\partial\boldsymbol{c}_{O2Hb}$ is 0, $\partial(\Sigma\mathbf{e}^2)/\partial\boldsymbol{c}_{COHb}$ is 0, and $\partial(\Sigma\mathbf{e}^2)/$

$\partial c_{MetHb}$ is 0:

$$\sum \mathbf{e}^2 = \sum [\varepsilon^{\lambda}_{O_2Hb} \cdot c_{O_2Hb} + \varepsilon^{\lambda}_{COHb} \cdot c_{COHb} + \varepsilon^{\lambda}_{MetHb} \cdot c_{MetHb} - \mathbf{A}']^2 \quad (5)$$

This yields a determined system of three equations with three unknown concentrations of $O_2Hb$, COHb, and MetHb that can be solved with standard mathematical procedures.

The program has been used with a set of absorbance data for wavelengths from 480 to 650 nm with intervals of 2 nm (general model) and specific sets of absorbance data for wavelengths as applied in the various types of multiwavelength hemoglobin photometers (specific models).

### MULTICOMPONENT ANALYSIS

We carried out a series of experiments with the four mixtures of LBH derivatives to determine the following: (*a*), the influence of the use of different absorptivities (LBH, bovine, and human); (*b*), the influence of different sets of wavelengths (general model or various specific models); and (*c*), the combined influence of different absorptivities and different sets of wavelengths. Each of the specific models when used in combination with the absorptivities of human hemoglobin was the closest simulation of a particular multiwavelength hemoglobin photometer we could make. The results obtained by these simulations were then compared with those obtained by measurements with the corresponding multiwavelength hemoglobin photometers in various hospital laboratories.

The absorbance of the four mixtures of LBH derivatives was measured by a similar procedure on the same HP8450A spectrophotometer as had been used for the determination of the $O_2Hb$, COHb, and MetHb absorptivities of LBH.

The influence of differences in the absorptivities on the results of MCA of mixtures of LBH derivatives was established by calculations with the general model. The $O_2Hb$, COHb, and MetHb fractions of the four different mixtures of LBH derivatives were determined with the general model and the absorptivities of LBH, bovine hemoglobin, and human hemoglobin, respectively. The results obtained with the LBH absorptivities were designated the true values of the LBH mixtures.

To determine the influence of the specific models on the results of MCA of mixtures of LBH derivatives, we compared the true values of the LBH mixtures with the results of calculations with the specific models based on LBH absorptivities. The following specific models, corresponding as far as the wavelengths were concerned with several common multiwavelength hemoglobin photometers *(7)*, were used: a 4-wavelength model (corresponding to the CO-Oximeter IL282/482, Instrumentation Laboratory), a 6-wavelength model (corresponding to the Hemoximeter OSM3/ABL520, Radiometer), an 8-wavelength model (corresponding to the CO-Oximeter CCD270, Chiron Diagnostics), and a 17-wavelength model (corresponding to the CO-Oxylite AVL912).

The combined influence of specific models and the absorptivities of human hemoglobin was determined by calculating the $O_2Hb$, COHb, and MetHb fractions of the four different mixtures of LBH derivatives with the specific models based on human absorptivities. Thus, we applied to the LBH mixtures the methods as implemented in the common multiwavelength hemoglobin photometers. The results were compared with the true values of the LBH mixtures.

Finally, a comparison was made of the true values of the LBH mixtures with the results obtained with multiwavelength hemoglobin photometers at various hospital

**Table 1. Intervial precision of four mixtures of LBH.**

| | Reconstitution with demineralized water, $P_{O_2}$ ≈20 kPa | | | Reconstitution with demineralized water, $P_{O_2}$ >100 kPa | | |
|---|---|---|---|---|---|---|
| | Mean (n = 5) | SD | CV | Mean (n = 5) | SD | CV |
| $FO_2Hb$, % | | | | | | |
| | 90.8 | 0.16 | 0.2% | 90.8 | 0.22 | 0.2% |
| | 68.1 | 0.04 | 0.1% | 68.3 | 0.07 | 0.1%[a] |
| | 60.0 | 0.12 | 0.2% | 60.1 | 0.07 | 0.1% |
| | 40.2 | 0.08 | 0.2% | 40.8 | 0.11 | 0.3%[a] |
| *F*COHb, % | | | | | | |
| | 4.8 | 0.11 | 2.3% | 4.8 | 0.04 | 0.9% |
| | 10.4 | 0.11 | 1.1% | 10.2 | 0.19 | 1.9% |
| | 12.8 | 0.13 | 1.0% | 13.0 | 0.08 | 0.6% |
| | 19.3 | 0.04 | 0.2% | 19.0 | 0.07 | 0.4%[a] |
| *F*MetHb, % | | | | | | |
| | 4.8 | 0.11 | 2.4% | 4.6 | 0.12 | 2.7% |
| | 23.1 | 0.12 | 0.5% | 23.0 | 0.08 | 0.4% |
| | 28.8 | 0.09 | 0.3% | 28.9 | 0.09 | 0.3% |
| | 42.8 | 0.15 | 0.3% | 42.6 | 0.11 | 0.3% |

[a] Significantly different from reconstitution with $P_{O_2}$ ≈20 kPa, according to the unpaired Student *t*-test ($P < 0.01$).

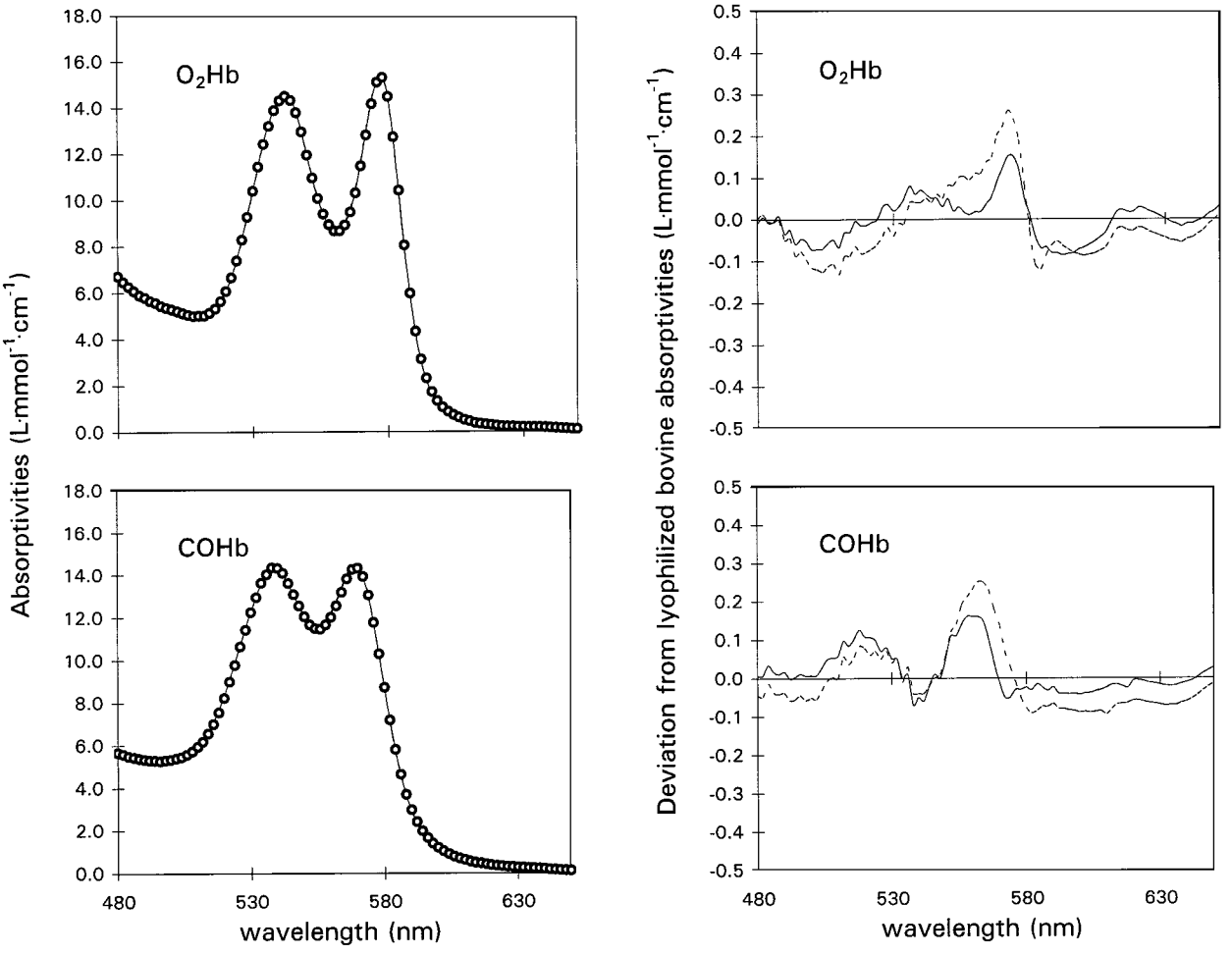


Fig. 1. Absorptivities of bovine, human, and lyophilized bovine hemoglobin as a function of wavelength for $O_2Hb$ and COHb (*left*) and deviation of absorptivities of bovine and human hemoglobins from absorptivity of LBH (*right*).
(○), LBH; (———), bovine hemoglobin; (- - - -), human hemoglobin.

laboratories. To this end, a set of vials containing the four mixtures of LBH derivatives was sent to these laboratories as unknown test material in a quality assessment trial. The mean values of the results obtained for each LBH derivative were grouped for the various types of instruments: 14 IL282/482, 13 OSM3/ABL520, 17 CCD270, and 4 AVL912 multiwavelength hemoglobin photometers.

## Results

The intervial precision of the four LBH mixtures, reconstituted with demineralized water with $Po_2$ ≈20 kPa, is shown in Table 1. The SD of each hemoglobin derivative of each LBH mixture of all samples was ~0.2%, which corresponds to a coefficient of variation (CV) of 0.1–0.2% in $FO_2Hb$, 0.2–2.3% in *F*COHb, and 0.2–2.4% in *F*MetHb.[6]

[6] Fractions of the hemoglobin derivatives are given in percentages: $F$ = 0.3 = 30%. When the spread in this value is given as 2%, this means that $F$ is between 28% and 32%. The percentage is not used as a relative measure unless it is explicitly stated, as in the case of CVs.

Some minute differences were obtained when the reconstitution was carried out with demineralized water with $Po_2$ >100 kPa (Table 1), showing the presence of traces of HHb in some of the LBH mixtures.

The left panels of Fig. 1 show the absorptivities of bovine, human, and lyophilized bovine hemoglobin as a function of wavelength for $O_2Hb$ and COHb. The right panels of Fig. 1 show the deviation the absorptivities of bovine and human hemoglobin from the LBH. The absorptivities of MetHb as a function of wavelengths are shown in Fig. 2. In the upper panel, the spectrum of LBH MetHb at pH 7.06 is compared with that of native bovine MetHb at pH 7.28, which is within the range of pH values of freshly hemolyzed bovine blood. The lower panel shows a comparison of the absorption spectrum of LBH MetHb at pH 7.26 with that of human MetHb at pH 7.21, which is within the range of pH values of freshly hemolyzed human blood.

In Table 2, the absorptivities of the various derivatives of LBH and of bovine and human hemoglobin at the

**Table 2. Absorptivities ($L \cdot mmol^{-1} \cdot cm^{-1}$) at room temperature of lyophylized bovine, bovine, and human $O_2Hb$, COHb, and MetHb at wavelengths (nm) used in specific models corresponding with the wavelengths used in particular multiwavelength hemoglobin photometers.**

| | Lyophilized bovine | | | Bovine | | | Human | | |
|---|---|---|---|---|---|---|---|---|---|
| | $O_2Hb$ | COHb | MetHb pH 7.26 25 °C | $O_2Hb$ | COHb | MetHb pH 7.28 25 °C | $O_2Hb$ | COHb | MetHb pH 7.21 25 °C |
| IL282/482 CO-Oximeter | | | | | | | | | |
| 535.0 | 12.80 | 13.82 | 6.72 | 12.86 | 13.83 | 6.57 | 12.81 | 13.83 | 6.67 |
| 585.2 | 8.97 | 5.09 | 3.86 | 8.91 | 5.07 | 3.67 | 8.87 | 5.01 | 3.78 |
| 594.5 | 2.14 | 1.90 | 3.51 | 2.06 | 1.86 | 3.43 | 2.07 | 1.82 | 3.37 |
| 626.6 | 0.20 | 0.30 | 3.87 | 0.21 | 0.28 | 3.92 | 0.16 | 0.23 | 3.82 |
| OSM3 Hemoximeter | | | | | | | | | |
| 535.0 | 12.80 | 13.82 | 6.72 | 12.86 | 13.83 | 6.57 | 12.81 | 13.83 | 6.67 |
| 560.0 | 8.66 | 12.02 | 4.14 | 8.68 | 12.18 | 3.98 | 8.77 | 12.25 | 4.18 |
| 577.0 | 15.18 | 11.03 | 4.01 | 15.26 | 11.00 | 3.78 | 15.31 | 11.00 | 4.00 |
| 622.0 | 0.22 | 0.35 | 3.69 | 0.25 | 0.34 | 3.78 | 0.20 | 0.29 | 3.64 |
| 636.0 | 0.17 | 0.22 | 3.81 | 0.15 | 0.20 | 3.81 | 0.11 | 0.15 | 3.77 |
| 670.0 | 0.10 | 0.05 | 0.46 | 0.10 | 0.08 | 0.47 | 0.10 | 0.05 | 0.46 |
| CCD270 CO-Oximeter | | | | | | | | | |
| 556.96 | 9.16 | 11.56 | 4.35 | 9.17 | 11.71 | 4.18 | 9.26 | 11.76 | 4.38 |
| 564.91[a] | 9.16 | 13.47 | 3.98 | 9.20 | 13.59 | 3.81 | 9.30 | 13.71 | 4.03 |
| 577.56 | 15.23 | 10.62 | 4.01 | 15.30 | 10.59 | 3.78 | 15.34 | 10.57 | 3.99 |
| 597.38 | 1.44 | 1.47 | 3.44 | 1.37 | 1.43 | 3.39 | 1.36 | 1.39 | 3.28 |
| 605.42 | 0.63 | 0.82 | 3.33 | 0.58 | 0.78 | 3.37 | 0.55 | 0.73 | 3.19 |
| 624.56 | 0.21 | 0.32 | 3.80 | 0.22 | 0.31 | 3.86 | 0.18 | 0.25 | 3.75 |
| 635.72 | 0.17 | 0.22 | 3.82 | 0.15 | 0.20 | 3.82 | 0.11 | 0.15 | 3.78 |
| 650.23 | 0.09 | 0.11 | 1.90 | 0.12 | 0.13 | 2.04 | 0.10 | 0.09 | 1.94 |
| AVL912 CO-Oxylite | | | | | | | | | |
| 530.0 | 10.39 | 12.25 | 7.02 | 10.41 | 12.30 | 6.95 | 10.35 | 12.29 | 6.94 |
| 536.0 | 13.18 | 14.02 | 6.65 | 13.26 | 14.03 | 6.49 | 13.22 | 14.05 | 6.61 |
| 542.0 | 14.47 | 14.08 | 6.17 | 14.53 | 14.02 | 5.98 | 14.52 | 14.04 | 6.17 |
| 548.0 | 12.94 | 12.54 | 5.40 | 12.97 | 12.54 | 5.23 | 12.99 | 12.55 | 5.44 |
| 554.0 | 10.07 | 11.49 | 4.63 | 10.10 | 11.60 | 4.47 | 10.17 | 11.63 | 4.66 |
| 560.0 | 8.66 | 12.02 | 4.14 | 8.68 | 12.18 | 3.98 | 8.77 | 12.25 | 4.18 |
| 566.0 | 9.47 | 13.81 | 3.97 | 9.52 | 13.91 | 3.79 | 9.61 | 14.04 | 4.02 |
| 572.0 | 12.79 | 13.92 | 3.98 | 12.94 | 13.87 | 3.78 | 13.05 | 13.99 | 4.01 |
| 578.0 | 15.28 | 10.29 | 4.02 | 15.33 | 10.26 | 3.78 | 15.36 | 10.24 | 3.99 |
| 584.0 | 10.40 | 5.79 | 3.91 | 10.33 | 5.78 | 3.70 | 10.28 | 5.71 | 3.83 |
| 590.0 | 4.32 | 2.96 | 3.67 | 4.23 | 2.94 | 3.54 | 4.26 | 2.89 | 3.56 |
| 604.0 | 0.71 | 0.90 | 3.34 | 0.65 | 0.86 | 3.36 | 0.63 | 0.81 | 3.19 |
| 612.0 | 0.37 | 0.54 | 3.34 | 0.39 | 0.53 | 3.42 | 0.35 | 0.46 | 3.25 |
| 622.0 | 0.22 | 0.35 | 3.69 | 0.25 | 0.34 | 3.78 | 0.20 | 0.29 | 3.64 |
| 630.0 | 0.18 | 0.27 | 3.94 | 0.18 | 0.25 | 3.97 | 0.14 | 0.20 | 3.90 |
| 640.0 | 0.15 | 0.19 | 3.45 | 0.14 | 0.17 | 3.46 | 0.10 | 0.13 | 3.42 |
| 648.0 | 0.10 | 0.12 | 2.23 | 0.12 | 0.14 | 2.36 | 0.10 | 0.10 | 2.24 |

[a] Obtained from Chiron Diagnostics; other wavelengths from Mahoney et al. *(7)*. Absorptivity values for LBH were measured with a HP8450A spectrophotometer. The bovine hemoglobin absorptivities are from Zijlstra and Buursma *(12)*, and the human hemoglobin absorptivities are from Zijlstra et al. *(11)*.

wavelengths used in common multiwavelength hemoglobin photometers and in the corresponding specific models are consolidated.

Fig. 3 shows the stability after reconstitution of a mixture of LBH derivatives when stored for 1 h at 25 °C. When vials containing lyophilized LBH derivatives were stored at 2–8 °C and measured after reconstitution several times over 1.5 years, no changes in the composition of the different LBH mixtures were observed.

The second column of Fig. 4 shows the true values of the LBH mixtures, i.e., the results of calculating the fractions of $O_2Hb$, COHb, and MetHb of the four LBH mixtures with the help of the general model and the absorptivities of LBH. The results of the same calculations with the absorptivities of either bovine or human hemoglobin are shown as the differences between these values and the corresponding true values. When the absorptivities of bovine hemoglobin are used, the differences from

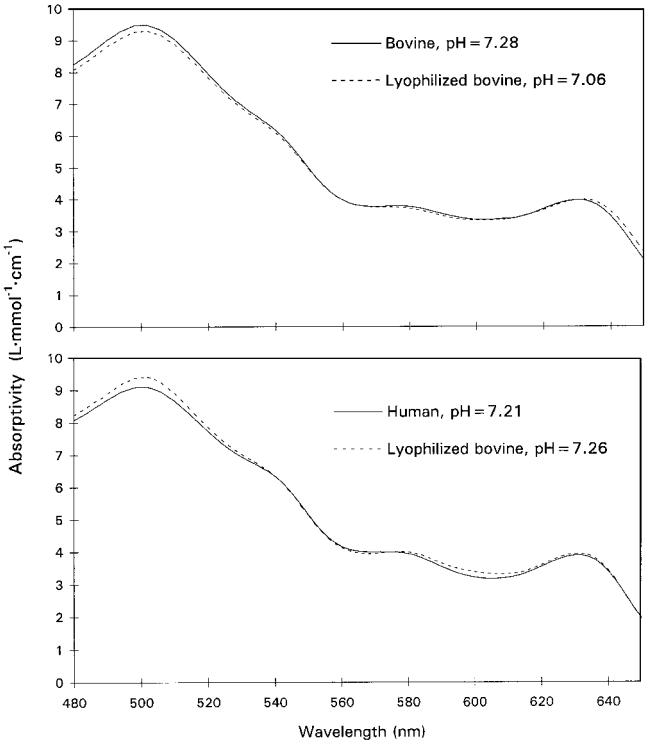


Fig. 2. Absorptivities of lyophilized bovine MetHb at various pH values in comparison with those of bovine and human MetHb.
The pH of the various solutions was measured at 25 °C.

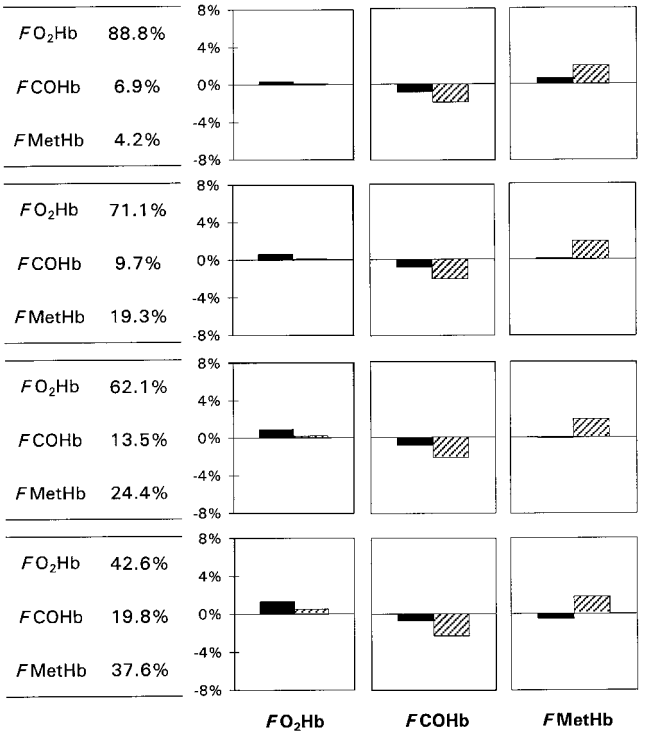


Fig. 4. Fractions of $O_2$Hb (*FO2Hb*), COHb (*FCOHb*), and MetHb (*FMetHb*) of four LBH mixtures as calculated with the general model and absorptivities of LBH (true values, *second column*) and with the general model with absorptivities of bovine and human hemoglobin.
The *solid bars* represent the values obtained with absorptivities of bovine hemoglobin minus the true values. The *shaded bars* represent the values obtained with absorptivities of human hemoglobin minus the true values.

the true values are slight (from −0.8% to 1.3%), which is in line with the expectation based on Figs. 1 and 2. The values obtained with the absorptivities of human hemoglobin deviate slightly more (from −2.3% to 1.9%).

The open bars in Fig. 5 represent the results obtained when the specific models and the absorptivities of LBH were used. The bars represent the differences with respect to the true values of the four LBH mixtures in the same manner as those in Fig. 4. It seems that the more overdetermined systems give a somewhat better agreement. The shaded bars in Fig. 5 show the results obtained with the specific models based on the absorptivities of human hemoglobin, again with respect to the true values. In addition, the results obtained with various multiwavelength hemoglobin photometers at different laboratories are presented by the solid bars.

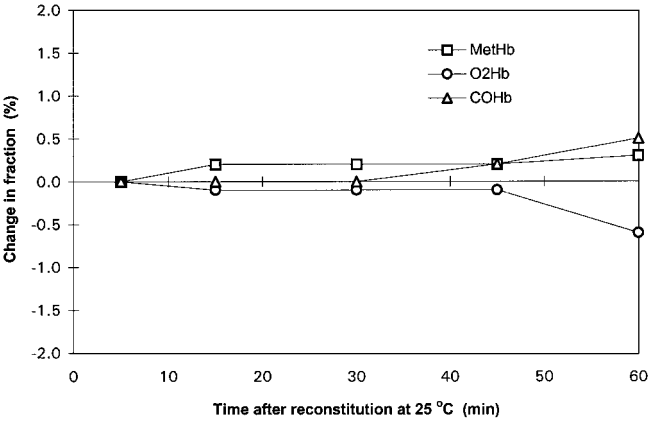


Fig. 3. Changes in the fractions of $O_2$Hb, COHb, and MetHb during first hour after reconstitution of an LBH mixture.

It should be noted that in the calculations underlying the open bars, the only difference with the determination of the true values is the use of a limited set of wavelengths. The shaded bars show a greater deviation from the true values because in these calculations the use of the absorptivities of human hemoglobin instead of LBH absorptivities comes in addition to the selection of a limited set of wavelengths. These bars are the result of the closest simulation of the multiwavelength hemoglobin photometers, for which the actual outcome is presented by the solid bars. The differences between the simulated multiwavelength hemoglobin photometers, as given by the shaded bars, and the true values are often, but not in all cases, in agreement with the differences between the results of the actual multiwavelength photometers and the true values, as presented by the solid bars.

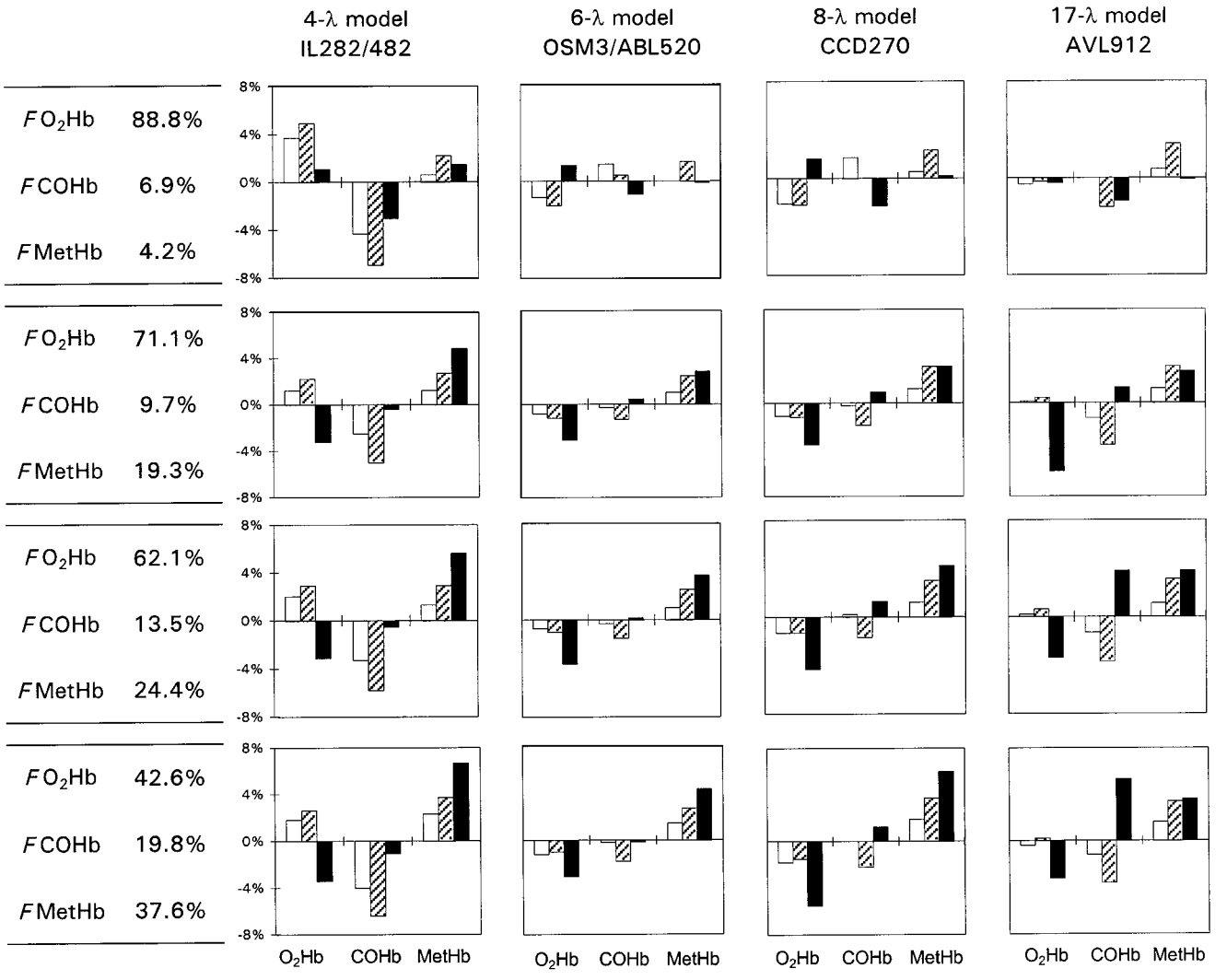


Fig. 5. Fractions of $O_2Hb$ (*$FO_2Hb$*), COHb (*FCOHb*), and MetHb (*FMetHb*) of four LBH mixtures as calculated with the general model and absorptivities of LBH (true values, *second column*), as calculated with the various specific models and absorptivities of LBH and human hemoglobin, respectively, and as measured with various multiwavelength hemoglobin photometers.

The *open bars* represent the values calculated with the specific models and the LBH absorptivities minus the true values. The *shaded bars* represent the values calculated with the specific models and the human absorptivities minus the true values. The *solid bars* represent the values measured with the various multiwavelength hemoglobin photometers minus the true values. λ, wavelength.

## Discussion

Although a stable solution of a single hemoglobin derivative (MetHb cyanide) has been in general use for at least the last 30 years as an international reference solution *(13, 14)*, the preparation of a stable solution containing known concentrations of various hemoglobin derivatives proved to be beset with difficulties. The work of Sprokholt and co-workers *(9, 10)*, however, in which hemoglobin was used primarily as an oxygen buffer in blood gas quality-control material, led to a technique for the preparation of an SFH solution containing fractions of $O_2Hb$, COHb, and MetHb, which remained constant for >400 days. On the basis of this experience, we developed the present material and tested its potential for quality assessment in spectrophotometric MCA of hemoglobin derivatives.

For practical reasons we preferred bovine blood as starting material. It can be obtained easily, can be processed without the precautionary measures necessary in handling human blood, and its spectrophotometric properties are only slightly different from those of human blood *(12)*. Because we chose a dry material, which must be redissolved by adding water, we had to restrict the mixture to the three components $O_2Hb$, COHb, and MetHb. Ideally, a four-component mixture including HHb, which would have allowed us to check the oxygen saturation measurement simultaneously, would have been preferable; however, the strict control of $Po_2$ necessary for the presence of a constant fraction of HHb has, to date, proven to be an insurmountable problem.

As is shown in Table 1, there is hardly any difference between the solutions obtained after reconstitution of

different vials containing the same mixture of LBH derivatives. Reconstitution with water with a very high $Po_2$ shows that there is indeed practically no HHb present in the mixtures. Although part of the differences found between the two reconstitution procedures is statistically significant, the amount of HHb actually present in the mixtures is negligible for all practical purposes. The long-term stability of the LBH preparations warrants a shelf-life of >1 year when the vials are kept at 2–8 °C. After reconstitution, there is ample time for the measurements, as shown by Fig. 3.

Comparison of the absorptivities of LBH with those of bovine and human hemoglobin, as presented in Figs. 1 and 2 and in Table 2, shows that processing of bovine hemoglobin, i.e., preparation of the stroma-free solution and lyophilization, storage, and reconstitution of the solution, has little influence on the absorption spectra of the hemoglobin derivatives. However, it appears that the affinity of LBH MetHb for the $OH^-$ ion is different from that of bovine hemoglobin, as demonstrated by the upper panel of Fig. 2; the best coincidence between the absorptivity spectra of LBH MetHb and bovine MetHb is obtained at different pH values. The pH that ensues after reconstitution of the LBH solution is 7.26, which is within the range of pH values of freshly hemolyzed bovine blood, i.e., the pH at which the absorptivities of bovine hemoglobin have been determined *(12)*. The pH of freshly hemolyzed human blood is ~7.21; the absorptivities of human blood have therefore been measured at this pH value *(11)*. The fairly good agreement between the absorptivities of LBH MetHb at pH 7.26 and human MetHb at pH 7.21, as shown in the lower panel of Fig. 2, was a good reason to keep the pH of reconstituted LBH at 7.26, instead of lowering it to the value that gave best agreement of the absorption spectrum with that of bovine hemoglobin.

We termed the $O_2$Hb, COHb, and MetHb fractions, determined with the general model and with the absorptivities of the pure LBH solutions, the true values. These values have been used as baseline values in Figs. 4 and 5. We realize that this term is only justified within the perspective of what is presently attainable with spectrophotometric methods. Because the method used to obtain the true values is not different from the one that had been checked thoroughly earlier by means of other, as far as possible, nonspectrophotometric methods *(2)*, the uncertainty in the true $O_2$Hb, COHb, and MetHb fractions may be assumed not to exceed ± 2%.[6]

As shown in Fig. 4, the influence of using the absorptivities of bovine hemoglobin instead of those determined for LBH is negligible for all practical purposes, as could be expected on the basis of the absorptivity spectra shown in Figs. 1 and 2. This confirms that the reconstituted LBH solution is spectrophotometrically equivalent with fresh bovine hemoglobin. Fig. 4 also shows that the error introduced by using the absorptivities of human hemoglobin is slight enough to consider LBH a fair substitute for human hemoglobin in spectrophotometric MCA.

As with the present methods, the results of MCA of hemoglobin derivatives are not completely independent of the set of wavelengths used; we therefore investigated the relevance of these differences with the help of our computer model, by varying the set of wavelengths while keeping absorbances and absorptivities constant. The difference in the results obtained with the specific models in comparison with the general model, as illustrated by the open bars in Fig. 5, seems to confirm the theoretical expectation that measuring at more wavelengths allows better analysis of a multicomponent mixture. However, it also appears that the selection of particular wavelengths has a distinct influence on the results.

Each of the specific models used in combination with the absorptivities of human hemoglobin was the closest simulation of a particular multiwavelength hemoglobin photometer we could make. As shown by the shaded bars in Fig. 5, the results thus calculated for the four LBH mixtures are in fairly good agreement with the true values, with the exception of the four-wavelength model, in which the results sometimes differ >5% from the true values. Fig. 5 also shows that the deviation of the results obtained with the various multiwavelength hemoglobin photometers from the true values (solid bars) is in quite a few cases not in agreement with the deviation of the results of the corresponding specific model from the true values. Several properties of the multiwavelength hemoglobin photometers are obviously not taken into account in the specific models or are considered in a different way. There may be differences in spectral band width, in the absorptivities used, and in the way the fractions of the hemoglobin derivatives are calculated from the absorbances. Moreover, the difference in temperature between the calculations, which were based on absorbances and absorptivities measured at room temperature, and the multiwavelength hemoglobin photometers, which usually are at 37 °C, may have played a part. As shown by Steinke and Shepherd *(15)*, temperature has a small but appreciable influence on the absorption spectra of $O_2$Hb, COHb, and HHb. This influence, however, can hardly account for much of the difference between calculated and measured values, because the measured absorbances and the absorptivities used in the calculations will be similarly influenced by the differences in temperature. For MetHb the situation is more complex, because the higher temperature in the hemoglobin photometers will lower the pH, and any difference in $OH^-$ affinity between LBH MetHb and human MetHb might have yielded a less perfect agreement at 37 °C between the spectra of human and LBH MetHb than the one shown in the lower panel of Fig. 2.

Possible differences in the mathematical processing of the absorbance data even within the same type of multiwavelength hemoglobin photometer have been suggested by the work of Gourlain et al. *(16)* who, in their investi-

gation of the interference of methylene blue in MCA of hemoglobin by various instruments, found that methylene blue affected the OSM3 and the Radiometer ABL520 differently. The two instruments contain the same photometric system; therefore, differences in data processing must be responsible for the difference in methylene blue interference. In the present investigation we could not analyze any differences between these instruments because we did not receive the results of the field trial with LBH separately for the OSM3 and the ABL520.

Our attempt to establish more accurate target values for the LBH preparations in the various multiwavelength hemoglobin photometers by computer simulation of the measuring systems implemented in these instruments was only partly successful. However, this approach may be useful for studying the influence of factors such as wavelength selection or differences in absorptivities on MCA of hemoglobin derivatives. The possible interference by contaminating dyes, as described by Gourlain et al. *(16)*, probably can be studied more easily in this way.

The data for Fig. 5 suggest that our simulation is best for the OSM3 and the CCD270, although certainly not perfect. In these cases, when the results obtained with the corresponding model had been taken as the target values instead of our true values for the four LBH preparations, the agreement with the results obtained with the actual instruments would have been better.

Apart from these considerations, the potential of LBH as a reference material is shown by the fact that the fractions as measured with the multiwavelength hemoglobin photometers are generally within 6% of the true values and that often the agreement is much better. This is a promising result in light of the present limitations of spectrophotometric MCA, which include a high sensitivity to small differences in wavelength, the varying influence of turbidity as a result of different procedures used for hemolysis and turbidity control, and the uncertainty in the true values.

In summary, it has been demonstrated that stable LBH can be prepared containing constant mixtures of $O_2$Hb, COHb, and MetHb, which after reconstitution yields a hemoglobin solution that is spectrophotometrically equivalent with a solution of fresh bovine hemoglobin. The results obtained thus far with an LBH preparation show its suitability as a quality-control material for photometric MCA of hemoglobin. Using a computer model of a particular multiwavelength hemoglobin photometer may become a suitable method for more accurately assigning target values to a particular LBH mixture.

## References


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