Quantitative Determination of Methemoglobin by Measuring the Solvent-Water Proton-Nuclear Magnetic Resonance Relaxation Rate

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We report a new method for the quantitative determination of human methemoglobin (methHb) based on the measurement of the solvent-water proton-nuclear magnetic resonance (NMR) relaxation rate $R_1$ [normalized to 1 mmol/L hemoglobin (Hb) concentration]. MethHb (%) is estimated from the linear dependence of $R_1$ on the methHb concentration, taking into account the simple relationship

$$[\text{MethHb}] = \frac{(R_1 - R^\text{HbO}_2)(R^\text{methHb} - R^\text{HbO}_2)}{100},$$

where $R^\text{HbO}_2$ and $R^\text{methHb}$ are values for the solvent-water relaxation rate of standard 1.0 mmol/L solutions of the oxygenated derivative of human hemoglobin (HbO$_2$) and of methHb, respectively. The minimum methHb that may be determined from the analysis of the experimental data is 0.5 ± 0.4%.

Additional Keyphrases: hemoglobin · intermethod comparison

All reactions involving the heme group in hemoglobin (Hb) cause a detectable change in the visible absorption spectrum of the hemoprotein. This applies not only to the reversible binding of ligands such as oxygen, but also to the oxidation and reduction of the heme iron (I, 2). Thus, oxidation of ferrous Hb is accompanied by a marked increase in absorbance in the red region of the visible spectrum (i.e., between 600 and 650 nm), which gives rise to a mahogany-brown color (I, 2). Oxidized ferric Hb (i.e., methemoglobin (methHb)) in erythrocytes is readily reduced to ferrous Hb by the methHb reducing system (2). Oxidation of Hb has clear-cut clinical relevance: accumulation of significant amounts of methHb in circulating erythrocytes leads to cyanosis because of the impairment of oxygen transport. Thus, when the amount of oxidized heme increases, the oxygen affinity for Hb increases and the heme–heme cooperativity decreases (I–3).

Normal erythrocytes circulate in vivo for 120 days, during which they are exposed to a variety of endogenous and exogenous agents capable of inducing the formation of methHb. In the absence of an efficient methHb reducing system in the erythrocytes, it has been estimated that methHb may accumulate at a rate of 2–3% per day. However, when the reducing system is operating and there is no unusual exogenous oxidant exposure, erythrocytes contain <1% methHb (2).

Several surveys of methHb concentrations in various normal populations have been reported. MethHb may be somewhat increased in the erythrocytes of normal infants (2). Most mammals that have been tested have <1% methHb. In contrast, high concentrations of methHb are observed in reptiles and fish; up to 50% methHb has been reported in turtles (2).

Although several spectrophotometric methods for methHb determination in blood have been described, "the available techniques are suitable for clinical diagnosis of hereditary methemoglobinemia or severe intoxication, but give scant precision in the normal range and, at any rate, below 10% values" (4, 5).

We report a new method for the quantitative determination of methHb based on the measurement of the solvent-water proton-nuclear magnetic resonance (NMR) relaxation rate $R_1$ (s$^{-1}$). Around neutral pH and in the acidic pH region, methHb binds a water molecule at the sixth coordination position of the heme–iron paramagnetic center (I, 2). This displays a very different relaxation behavior from that of the solvent water and water molecules interacting directly with the surface of the macromolecule (6–8). For a given nucleus, the relaxation between nuclear-spin energy values primarily depends on its proximity to local magnetic fields (8). In diamagnetic systems, proton nuclei (on the same or on neighboring molecules) represent the most common source for such magnetic fields. The marked enhancement of the relaxation efficiency observed in the presence of a paramagnetic species is simply the result of the magnetic moment of the unpaired electron's being almost 1000-fold higher than that of the proton. This means that if a water molecule is in the primary coordination sphere of a paramagnetic metal ion for a brief time, a reduction of the relaxation time of the bulk water is expected to occur to an extent proportional to the concentration of the paramagnetic species (8).

Materials and Methods

Human oxygenated Hb (HbO$_2$) was prepared as a standard from blood samples as previously reported (I, 2, 9). HbO$_2$ concentration was determined from its
absorptivity values, $e$, of 13.8 and 14.6 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 541 nm and 577 nm, respectively (1, 2, 10). As expected for HbO$_2$ samples free of other Hb derivatives (i.e., metHb), the values of HbO concentration so obtained are in excellent agreement (>99%) with those obtained from the absorptivities of the carboxylated, deoxygenated, and ferric cyanide derivatives (respectively 13.4 and 13.4 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 540 nm and 569 nm; 12.5 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 555 nm; and 11.0 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 540 nm); as well as by the pyridine–hemochromogen method (16.0 and 32.0 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 525 nm and 557 nm, respectively) (1, 2, 4, 5, 10).

The measured HbO$_2$ concentration was unaffected by preincubation of the hemoprotein solution with the enzymic system for metHb reduction (11).

Human carboxyHb (HbCO) was prepared as a standard from HbO by carboxylation as previously reported (1, 2, 8, 10). HbCO concentration was determined on the basis of $e = 13.4$ and 13.4 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ (at 540 nm and 569 nm, respectively) (1, 2, 10). As expected for HbCO samples free of other Hb derivatives (i.e., HbO$_2$), values of HbCO concentration so obtained agree excellently (>99%) with those obtained from the absorptivities of the oxygenated, deoxygenated, and ferric cyanide derivatives (respectively 13.8 and 14.6 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 541 nm and 577 nm; 12.5 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 555 nm; and 11.0 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 540 nm); as well as by the pyridine–hemochromogen method (16.0 and 32.0 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 525 nm and 557 nm, respectively) (1, 2, 4, 5, 10).

Human metHb was prepared as a standard from HbO$_2$ by treatment with sodium nitrite as previously reported (1, 2, 10). MetHb concentration was determined on the basis of $e = 10.0$ and 4.4 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ (at 500 nm and 631 nm, respectively; pH 6.5) (1, 2, 10). As expected for metHb samples free of other Hb derivatives (i.e., HbO$_2$), values of metHb concentration so obtained agree excellently (>99%) with those obtained from the absorptivities of the ferric cyanide, carboxylated, and deoxygenated derivatives (respectively 11.0 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 540 nm; 13.4 and 13.4 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 540 nm and 569 nm; and 12.5 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 555 nm); as well as by the pyridine–hemochromogen method (16.0 and 32.0 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 525 nm and 557 nm, respectively) (1, 2, 4, 5, 10).

The standard for human sulfhemoglobin (sulfHb) was prepared from metHb by treatment with hydrogen peroxide and ammonium sulfide, as previously reported (2). SulfHb concentration was determined on the basis of $e = 21.4$ mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 620 nm (2). As expected for sulfHb samples free of other Hb derivatives (i.e., metHb), values of sulfHb concentration so obtained agree excellently (>99%) with those obtained by the pyridine–hemochromogen method ($e = 16.0$ and 32.0 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 525 nm and 557 nm, respectively) (2, 4, 5).

Hb from normal control subjects as well as from patients with various diseases or being treated with nitroprusside was prepared from blood samples (1, 2, 9). The total Hb concentration in blood samples from normal control sub-

jects and from patients was determined from the absorptivities of the deoxygenated, carboxylated, and ferric cyanide derivatives (respectively 12.5 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 555 nm; 13.4 and 13.4 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 540 nm and 569 nm; and 11.0 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 540 nm) as well as by the pyridine–hemochromogen method (16.0 and 32.0 mmol$^{-1}$·L$^{-1}$·cm$^{-1}$ at 525 nm and 557 nm, respectively) (1, 2, 4, 5, 10). Values of total Hb concentration of normal control subjects' and patients' blood samples obtained by different methods agree very well (>99%). The concentration of metHb present in the normal control subjects' and patients' blood samples was quantified by the multiple-wavelength spectrophotometric method, metHb being converted to its cyanide derivative (2, 4, 5). Values of metHb concentration are the average of at least five independent determinations; the calculated SD was ±5% (2, 4, 5).

Hb concentration in the text is expressed on the basis of heme content. Hb concentration was determined at pH 6.5 (0.1 mol/L phosphate buffer) and 25.0 °C, with a Cary 219 double-beam spectrophotometer (Varian Associates, Palo Alto, CA) (1, 2, 4, 5, 10). The characterization of Hb was previously reported (1, 2, 9).

The components of the metHb reducing system (11)—catalase, ferrodoxin, ferrodoxin-NADP$^+$ reductase, NADP$, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase—were obtained from Sigma Chemical Co. (St. Louis, MO). Carbon monoxide was obtained from Caraccioloossigeno Srl (Rome, Italy). All the other products were from Merck AG (Darmstadt, Germany). All chemicals were of analytical grade and used without further purification.

Spin-lattice relaxation times $T_1$ (s) for HbO$_2$, HbCO, metHb, sulfHb, and HbO$_2$/metHb mixtures were measured by the usual (180° − τ − 90°) inversion-recovery pulse sequence method, at 25.0 °C, with a Stelar SpinMaster Spectrometer (Stelar, Mede (PV), Italy) operating at variable field strength to allow measurement at the proton Larmor frequencies of 8.0, 10.0, 20.0, and 60.0 MHz. The inversion-recovery experiment consists of a 180° pulse applied to the sample to completely invert the magnetization, so that $M_z = -M_o$, and $M_y = 0$. The system is then allowed to relax for some time after the initial pulse, after which $M_z(t) = M_o(1 - 2 \cdot e^{-\tau/T_1})$. A 90° pulse is then applied to tip the magnetization into the xy plane, so that now $M_x = M_z(t)$. The pulse width corresponding to the 90° flip angle was 3.3 μs. The instrument detects $M_{xy}$ as it decays and converts it into a digital form as a free induction decay for storage. Next, the graph of free-induction-decay intensities vs time is a direct analog to a plot of $M_x$ vs τ. The analysis of the latter plot by a simple curve-fitting procedure allows evaluation of the best value for the free-induction-decay intensity ($S_x$) and $T_1$ (s). For further details, see Martin et al. (8). A reproducibility check (20 measurements) gave an SD of ±0.4% in the experimentally determined $T_1$ values. Reported $T_1$ values are the average of at least five independent measurements.

Values of $T_1$ of solvent-water protons in the presence of HbO$_2$ (free of metHb), HbCO (free of HbO$_2$), metHb (free of HbO$_2$), and sulfHb (free of metHb) as well as of
Hb from the normal control subjects and the patients were obtained as follows: 0.05 mL of the buffered (pH 6.5, 0.1 mol/L phosphate buffer) Hb solution (1.0 mmol/L) was put in an NMR tube (φ = 5 mm), and the proton spin-lattice relaxation time T1 was determined.

Values of T1 of solvent-water protons in the presence of an artificial mixture of HbO2 and metHb were obtained as follows: a buffered (pH 6.5, 0.1 mol/L phosphate buffer) solution of HbO2 (free of metHb; 1.0 mmol/L) was mixed with a buffered (pH 6.5, 0.1 mol/L phosphate buffer) solution of metHb (free of HbO2; 1.0 mmol/L) to give a final volume of 0.1 mL. Then, 0.05 mL of the 1.0 mmol/L HbO2/metHb mixture was transferred to an NMR tube (φ = 5 mm) for the T1 measurement.

Measurements were carried out at pH 6.5 (0.1 mol/L phosphate buffer) to avoid altering the metHb content i.e., by forming the hydroxy-met derivative of Hb, which occurs at alkaline pH values (1).

Results and Discussion

The solvent-water relaxation rate R1 (= T1⁻¹; s⁻¹) may be strongly affected by the solubilized species. Either dia- or paramagnetic proteins enhance the relaxation rate, which is usually much higher for the latter. Because the relaxation rates are additive, the contribution of noninteracting species to the observed relaxation rate is dependent on their relative molar fractions (6–8). For the system considered here, the observed relaxation rate (R1; s⁻¹) may be represented as follows:

\[ R_1 = x_{\text{HbO}_2} \cdot R_{1,\text{HbO}_2} + x_{\text{metHb}} \cdot R_{1,\text{metHb}} \]  

(1)

where \(x_{\text{HbO}_2}\) and \(x_{\text{metHb}}\) are the molar fractions of HbO2 and metHb, respectively. Next, \(R_{1,\text{HbO}_2} (= R_{1,\text{HbO}_2} - [\text{HbO}_2]) + R_{1,\text{HbO}_2} = 0.462\) s⁻¹) and \(R_{1,\text{metHb}} (= R_{1,\text{metHb}} - [\text{metHb}] + R_{1,\text{metHb}} = 0.1068\) s⁻¹) represent values of the solvent-water relaxation rate of 1.0 mmol/L standard solutions of HbO2 and metHb, respectively (Figure 1). \(R_{1,\text{HbO}_2} = 0.034\) s⁻¹) and \(R_{1,\text{metHb}} = 0.030\) s⁻¹) indicate the paramagnetic contribution to the solvent-water relaxation rate of standard 1.0 mmol/L HbO2 and metHb solutions, respectively, with \(R_{1,\text{HbO}_2} = 0.338\) s⁻¹) being the proton relaxation rate of the pure water solvent (Figure 1).

Values of R1 for HbO2, HbCO, metHb, and sulfHb are only slightly dependent on the magnetic field strength. Next, the slope of the line shown in Figure 1 is very similar at 20 and 60 MHz. Values of R1 for HbO2, HbCO, metHb, and sulfHb determined here agree well with published values (8, 12–15).

As expected (8), R1 values depend linearly on the molar fraction of HbO2 and metHb, ranging from 0.462 s⁻¹ (for 100% HbO2) to 1.068 s⁻¹ (for 100% metHb) (Figure 1). Thus, metHb (as well as HbO2) concentration (in %) may be easily determined according to equation 2, derived from equation 1, once the total Hb concentration is independently evaluated:

\[ \text{[MetHb]} = \frac{[(R_1 - R_{1,\text{HbO}_2})/(R_{1,\text{metHb}} - R_{1,\text{HbO}_2})] \cdot 100\]  

(2)

Because the accuracy of the determination depends on the slope of the straight line shown in Figure 1, we suggest carrying out the R1 measurements at the highest possible concentration of Hb. Furthermore, the temperature has to be controlled carefully during the acquisition of the data (~5–10 min) because of its well-known effect on relaxation in such systems (6). On the basis of the instrumental reproducibility factor (<0.5%) for the measured R1 values, the method reported here may allow for the determination of metHb (as well as HbO2) at concentrations as low as 0.5 ± 0.4%.

In principle, the determination of metHb concentration may be affected by the presence of minor Hb derivatives (i.e., HbCO and sulfHb) in the sample. However, because R1 values for sulfHb and HbCO are 0.460 and 0.473 s⁻¹, respectively (i.e., very close to that observed for HbO2; R1 = 0.462 s⁻¹), measurements of metHb concentration are essentially unaffected by the presence of sulfHb and HbCO in the sample. HbCO, possibly present in the sample, may be easily converted to HbO2 by gentle stirring of the Hb solution under white-light irradiation at 4.0 °C (1, 2, 10).

To check the applicability of the NMR method reported here, we determined metHb concentration in blood samples of normal control subjects and patients with different diseases and undergoing treatment with nitroprusside and compared the concentrations with those obtained from the multiple-wavelength spectrophotometric method (2, 4, 5). The metHb concentration obtained by the two methods agree excellently (Table 1).

The use of the NMR spectroscopy for the determination of metHb (as well as HbO2) concentration appears to offer several advantages over other reported methods (2, 4, 5). From the practical standpoint, the large (and almost constant) difference between values of R1 for
metHb and HbO₂ at any magnetic field strength between 20 and 60 MHz allows a rapid and direct determination of physical indexes with commercial NMR instruments. Next, values of R₁ can be obtained directly, thereby avoiding the cumbersome and dangerous spectrophotometric procedure that is based on the conversion of Hb to its cyanide derivative. Once the total Hb concentration is known, the NMR method reported here allows determination of metHb (as well as HbO₂) concentrations as low as 0.5 ± 0.4%. In addition to its reliability, the NMR method is simple and rapid because no separation steps are required.

Finally, these findings show that determination of the solvent-water proton relaxation rate in the presence of paramagnetic substrates may significantly broaden the analytical applications of NMR spectroscopy.

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References