Estimation of Plasma and Urinary Hemoglobin by a Rate Spectrophotometric Method

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A method is described for estimating plasma and urinary hemoglobin concentrations as low as 3 mg/L. The assay measures at 528 nm the rate of peroxidation of chlorpromazine by hemoglobin and is not affected by ascorbate and bilirubin concentrations up to 500 μmol/L. Results by this method (mean ± SD: 54.4 ± 41.6 mg/L; n = 19) correlated well with those by a scanning spectrophotometric method (52.5 ± 41.6 mg/L; r = 0.98) and a Coulter Instrument method (r = 0.99; Coulter method: 125 ± 15 g/L; rate method: 122 ± 15 g/L; n = 10, r = 0.99). The correlation for assays of 20 plasma samples by our method and a tetramethylbenzidine method was also good (r = 0.95) though the latter gave lower results (31.1 ± 31.6 mg/L) than the chlorpromazine method (50.9 ± 41.1 mg/L). The chlorpromazine rate method gave an intra- and interday CV of 7.9% and 9.7%, respectively, at a hemoglobin concentration of 31 mg/L.

Indexing Term: chlorpromazine peroxidation

High concentrations of plasma hemoglobin (Hb), i.e., >50 mg/L (1), and the presence of urinary Hb are symptomatic of intravascular hemolysis.² Current methods for measuring plasma and urinary Hb use the peroxidative activity of heme on various substrates, some of which are potentially carcinogenic and therefore unsuitable for general laboratory use (1). Also, most peroxidative methods are inhibited by unknown plasma constituents (1). Other methods that measure plasma Hb require high concentrations of Hb (50 mg/L (2)), measure only oxy-Hb, are affected by plasma constituents (3, 4) or by bilirubin (5, 6), or require complex absorbance scans and manipulations. A recent comparison of eight methods for measuring plasma Hb highlighted these problems (7).

In one potentially useful method, a noncarcinogenic peroxidase substrate, chlorpromazine, was used with an endpoint spectrophotometric measurement of Hb (8, 9). Using this method (8), we found that higher concentrations of Hb reached the endpoint earlier and the maximal absorbance at the endpoint lasted only 30 to 60 s. To overcome this problem, we tried a rate kinetic method to estimate Hb. The results of this study are presented here.

Materials and Methods

Materials

Acetic acid/phosphoric acid solution. To 28 mL of glacial acetic acid add 4 mL of concentrated H₃PO₄; the final concentration of H₃PO₄ is 1.9 mol/L.

Chlorpromazine solution. Dissolve 250 mg of chlorpromazine (Sigma Chemical Co., St. Louis, MO; cat. no. C 8138) in 25 mL of water. This solution is stable for 1 week when stored in the dark at 4 °C.

Hydrogen peroxide solution. To 9.0 mL of water add 1.0 mL of 300 μL/mL H₂O₂. This solution is prepared just before use and is stable for 6 h at 4 °C.

Chicken egg albumin (CA) solution. Prepare on the day of use 100 mg of chicken egg albumin (Sigma; cat. no. A 5503) in 5 mL of water.

Procedures

Blood collection. We tested the following blood-collection tubes as to their suitability for estimation of plasma Hb. To do this, we assessed intra- and intertube variability in relation to plasma Hb concentration. Blood-collection tubes (1-mL, containing EDTA, cat. no. 24182; 1- and 10-mL, containing lithium heparin, cat. no. 24121 and 24184, respectively) were purchased from Disposable Products (Adelaide, Australia), 5-mL EDTA-containing Exetainer tubes from Labco Ltd. (High Wycombe, UK; cat. no. EX5KE5), and 4-mL Vacutette tubes containing EDTA (cat. no. 454036) or heparin (cat. no. 454084) from Greiner (Kremmenuster, Austria). Blood was collected from a healthy donor through a 21-gauge needle (Terumo, Melbourne, Australia) and dispensed carefully into the various collection tubes after removal of the needle. Blood was not collected directly into evacuated tubes, and the evacuated tubes used (Exetainer and Vacuette) were opened to atmosphere before filling to minimize trauma to the blood. On one occasion, blood was collected into a syringe heparinated with lithium heparin (1000 IU/mL) and dispensed as 1-mL aliquots into 10 separate plastic tubes.

Routine outpatient clinical blood samples collected into Exetainers for a complete profile were used for comparison with three other methods.

All blood samples were processed within 4 h after collection by centrifugation at 350 × g for 3 min. The plasma was then removed and centrifuged at 15 000 × g in the Beckman (Beckman Instruments, Fullerton, CA) Microfuge E for 3 min; the cell-free supernate was separated and analyzed for Hb.

All procedures were in accordance with the ethical standards of the Flinders Medical Centre.

Hemoglobin standards. We diluted the Coulter 4C-plus Hematology Reference Control blood (Coulter Electronics, Herts, UK; cat. no. 7546845) in CA solution to give Hb concentrations ranging from 160 to 10 mg/L. These solutions, used as Hb standards, were prepared on the day of the assay in glass or plastic tubes.

Interference studies. Ascorbic acid (Ajax Chemical Co., Sydney, Australia), unconjugated bilirubin (Sigma; cat.

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³ Nonstandard abbreviations: Hb, hemoglobin; CA, chicken egg albumin; and TMB, tetramethylbenzidine.
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no. B4126), and bilirubin (from two icteric plasmas from patients with obstructive jaundice) were tested as possible interferents in the assay. We dissolved ascorbic acid and bilirubin in water and NaOH (0.1 mol/L), respectively, to give 50 mmol/L concentrations. A 100-fold dilution of these in plasma gave the highest concentration (0.5 mmol/L) at which these possible interfering metabolites were tested. Lower concentrations were obtained by serial twofold dilutions in plasma not supplemented with these compounds. Bilirubin interference was also tested by serial twofold dilutions into CA solution of two icteric plasma samples with bilirubin concentrations of 503 and 306 μmol/L (reference range <20 μmol/L).

Because certain methods measure only specific forms of Hb such as oxy-Hb (7), we tested the effect of different forms of Hb on the analytical result. An EDTA-treated blood sample was diluted 1000-fold in water to give a sample with Hb ~100 mg/L. Two portions of this diluted blood were quantitatively converted to carboxy- and oxy-Hb by extensive gassing with carbon monoxide and oxygen, respectively (10). A further portion was converted within 1 min to cyannmethemoglobin (cyanmeth-Hb) by treating 10 mL of lysate with 100 μL of Zap-O-Globin (a ferricyanide/lyzing reagent from Coulter Electronics).

Human myoglobin (Dako, Glostrup, Denmark; cat. no. X555) was tested at 40 mg/L in CA solution for interference with the plasma Hb method.

Other procedures. We used a Beckman DU 7500 diode-array spectrophotometer to measure rates. All gases used were of highest purity and purchased from CIG Industries (Adelaide, Australia). Other reagents used were AR grade.

In the recovery experiments, the Coulter 4C-plus Reference Control was diluted to an Hb content of 1.6 g/L in CA solution. Fifty and 10 μL of this solution were diluted to 1 mL with plasma or urine to give added concentrations of Hb of 80 and 16 mg/L, respectively.

In correlating data we computed means, SDs, and product moment correlation coefficients and used statistical tables to evaluate the significance of the r values (11).

**Assay Methods**

The chlorpromazine rate method is done at room temperature. To 3-mL glass cuvettes add, in the following order, 0.8 mL of the acetic acid/phosphoric acid solution; 10 μL of the 0 (CA solution; blank) and 10–160 mg/L Hb standards or unknowns; 0.25 mL of chlorpromazine reagent; and 0.5 mL of the H2O2 solution. Mix and measure the rate of absorbance change at 528 nm for 3 min. Plot the rate of absorbance change at 528 nm against the Hb concentrations of the standards to calculate the unknown concentrations.

Plasma Hb measured by the chlorpromazine rate method was compared with measurements by the tetramethylbenzidine (TMB) (3), Coulter S plus VI (Coulter Electronics), and direct spectrophotometric methods (1, 6). The Coulter instrument method measures Hb in whole blood; for comparison, these whole-blood samples were diluted 1000-fold in CA solution for estimation by the rate method because of the greater sensitivity of the latter method. Accordingly, we multiplied the chlorpromazine assay results by 1000. All other method comparisons used undiluted plasma.

**Results and Discussion**

The effect of increasing the concentration of H3PO4 in glacial acetic acid when chlorpromazine is at the assay concentration of 1.6 g/L is shown in Figure 1A. Maximal activity was obtained at about 1.9 mol/L H3PO4 in the acetic acid/phosphoric acid solution (final assay concentration 1 mol/L). The effect of doing the assay in the...
presence of increasing amounts of chlorpromazine in a 1.9 mol/L H₃PO₄ solution in glacial acetic acid is shown in Figure 1B. Maximal activity when the chlorpromazine reagent was -10 g/L (assay concentration 1.6 g/L), so we used this concentration for our assay. The rate obtained was linear over the 3-min measuring period at an assay concentration of H₂O₂ of 0.28 mol/L. Higher concentrations of H₂O₂ yielded faster rates but they were not linear for more than 1-2 min. The current chlorpromazine rate assay used about two- to fourfold more phosphoric acid, H₂O₂, and chlorpromazine than the original endpoint assay (8). These concentrations of reactants gave rates that allowed the sensitivity required for clinical specimens.

The assay is performed at room temperature (23 °C). At 37 °C the rate is faster but nonlinear before 3 min at higher Hb concentrations (>100 mg/L). To overcome this problem, we halve the H₂O₂ assay concentrations to 0.14 mol/L for reactions performed at 37 °C.

Unlike one endpoint chlorpromazine method (9), ascorbate did not influence the rate assay at plasma ascorbate concentrations ranging from 0 to 500 μmol/L. Normal plasma ascorbate values are <120 μmol/L (12). Bilirubin at concentrations of 60 to 500 μmol/L in both standards and plasma samples did not affect the assay result. Similarly, two plasma samples with bilirubin exceeding 500 and 300 μmol/L, after serial twofold dilutions in CA solution to an overall eightfold dilution, gave plasma Hb results that were constant after allowing for dilution, thus indicating no detectable interference. We found that myoglobin has the same peroxidative activity as Hb per gram of protein and therefore will interfere if present in the plasma.

The Hb results obtained for the various Hb forms in treated blood samples compared with the untreated blood were: 89 mg/L, untreated; 104 mg/L, cyanmet-Hb; and 93 mg/L each for oxy- and carboxy-Hb. These results are within the 2 SD precision for the assay and therefore indicate that the forms of Hb present do not influence the rate assay significantly.

A standard curve obtained with the rate method is shown in Figure 2. The Hb detection limit of the assay was 3 mg/L, equivalent to five times the blank rate. This level of sensitivity was achieved by using chicken egg albumin, which prevented Hb from sticking to the glassware (see Figure 2) and gave a blank of 0.002 A/min when the CA concentration in the assay was 0.128 g/L. The upper concentration of Hb for linearity in the standard curve was 200 mg/L.

The mean Hb by the rate spectrophotometric method for whole-blood samples collected from 10 donors was 122 (15 SD) g/L, compared with 125 (15 SD) g/L by the Coulter S plus VI instrument (r = 0.99, P <0.01). For 20 plasma specimens, the rate method gave a mean of 50.9 (41.1 SD) mg/L compared with the mean of the TMB method of 31.1 (31.6 SD) mg/L (r = 0.95, P <0.01). Lower results were expected with the TMB method because inhibitors of peroxidase present in plasma affect that method (2, 3). The chlorpromazine rate method also correlated well (r = 0.96; P <0.01) with the direct spectrophotometric assay (6) for 19 plasma samples: 54.4 ± 41.6 vs 52.5 ± 41.6 mg/L, respectively.

Assay of 10 separate plasma specimens supplemented with standards to give added Hb concentrations of 16 and 80 mg/L gave recoveries ranging from 95% to 110%. In 10 fresh urine specimens from healthy persons Hb was not detected. Adding Hb to these urines at 16 and 80 mg/L gave recoveries ranging from 95% to 109%. These results taken together indicate the lack of effect of possible inhibitors on the rate method and good correlation with reliable assay methods.

A plasma sample assayed 30 times in one day gave a mean Hb of 31 mg/L, with a CV of 7.9%. This same sample stored frozen at -20 °C and assayed 12 times over the next month gave a mean of 34 mg Hb/L with a CV of 9.7%.

Intra- and interblood collection-tube comparisons for the plasma Hb rate method were performed with blood from a healthy donor, as described in Materials and Methods. The results showed considerable intra- and intertube variation in the plasma Hb concentrations (Figure 3). This same blood collected into 1-mL EDTA-containing tubes gave plasma Hb concentrations that were 2.5- to 20-fold higher than the 1-mL heparin-containing tubes (Hb >300 mg/L; data not shown). Obviously, therefore, the choice of specimen tube used for blood collection is very critical for analysis of plasma Hb: We found that the heparin Vacuette tube (Greiner) was the most suitable. In this tube the heparin is mist-sprayed into the tube; in contrast, it is pipetted into the larger 10-mL tube, which may explain in part the variations in the results obtained with different collection tubes. These results are consistent with the recommended method for blood collection in plasma Hb assays, which suggests that tubes be mist-sprayed with heparin (1000 IU/mL) before blood collection (1).
In conclusion, we present a rate kinetic method for the analysis of plasma Hb that is an adaptation of previous assays based on endpoint measurement (8, 9). The current assay is not subject to ascorbate interference or transient absorbance changes inherent in the endpoint assays that use chlorpromazine (8, 9).

References


Fast, Manual, Nonradioactive Method for DNA Sequencing

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We describe a protocol that allows nonradioactive detection of sequencing products after manual, direct, solid-phase sequencing of polymerase chain reaction-amplified DNA. The amplified DNA fragment to be studied is biotinylated at the 5' end of one of the two oligonucleotide primers used for amplification, allowing coupling to streptavidin-coated magnetic beads. The immobilized double-stranded DNA is then separated into single strands by alkaline treatment. A 5'-biotinylated sequencing primer is used after saturating with a biotin solution any possible remaining affinity sites on the streptavidin-coated magnetic beads. Sequencing is performed by using T7 DNA polymerase, and the sequencing products are electrophoresed in denaturing polyacrylamide sequencing gel. After transfer of the products to a nylon membrane, the sequencing pattern is revealed by chemiluminescence. Biotinylated alkaline phosphatase is bound to the 5' end of the sequencing primer via a streptavidin bridge and catalyzes the reaction by cleaving a phosphate group from a chemiluminescent substrate. The emitted photons are detected by exposing the membrane to x-ray film. This method is simple, rapid, and consistently successful and reproducible.

Indexing Terms: polymerase chain reaction · biotin-streptavidin interaction · chemiluminescence · enzymatic methods · electrophoresis, polyacrylamide gel