A Radioimmunoassay for Human Serum Myoglobin: Method Development and Normal Values

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We describe a radioimmunoassay that will measure both normal and above-normal concentrations of myoglobin in serum. Myoglobin isolated from human pectoralis muscle was purified by (NH₄)₂SO₄ fractionation and Sephadex gel filtration and injected into rabbits to elicit antisera. Myoglobin was radiolabeled by an acylation with [¹²⁵I]-3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester. With the purified myoglobin and antisera, we then developed a radioimmunoassay that involves simultaneous reagent addition, a 3.5-h incubation at 37 °C, and separation of the antibody-bound fraction by precipitation with polyethylene glycol. Information is given on detection limit, precision, linearity, accuracy, and specimen preservation. Cross-reactivity to human hemoglobin is negligible. Finally, we investigated the possible relationship between serum myoglobin concentration and muscle mass.

Additional Keyphrases: myocardial infarction • normal values • trauma management • sex-related differences • myoglobin purification, labeling, use as an antigen

We developed a radioimmunoassay for serum myoglobin because this technique offers greater sensitivity and specificity than do current widely used methods for serum and urinary myoglobin, many of which are based on the physical characteristics of myoglobin, such as the 16 500-dalton molecular weight and its heme-containing muscle protein. Such techniques include salt fractionation, membrane filtration, column chromatography, or electrophoresis to isolate the myoglobin, followed by colorimetric or spectrophotometric quantitation of the heme component (1).

Because these composite techniques lack sensitivity, they require either a large volume of sample or a sample in which myoglobin is present in high concentration. Sensitivity and specificity are improved by use of various immunological methods, including immunodiffusion, immunoelectrophoresis, hemagglutination inhibition, and complement fixation. However, even these methods cannot detect normal concentrations in serum or even very mildly increased concentrations of myoglobin in serum and urine (2–3). Indeed, the only technique with such sensitivity is the radioimmunoassay reported by Stone et al. (4) while our research was in progress. Their radioimmunoassay, although a major improvement in current methodology, is nevertheless limited in its clinical applicability by a 24-h incubation period.

We present here a detailed description of myoglobin purification from human muscle, of antibody induction in rabbits, and of ¹²⁵I incorporation into myoglobin. We report how these components are used in a rapid, specific, and sensitive radioimmunoassay. Factors that affect and limit the assay are delineated. Finally, we present our assessment of a normal or reference population.

Materials and Methods

Myoglobin Purification

Human pectoralis muscle obtained at autopsy less than 15 h postmortem is homogenized and extracted according to the method of Lugnabuhl (5). The myoglobin crystals are redissolved in tris(hydroxymethyl)-aminomethane buffer (50 mmol/liter, pH 7.5) for application to a Sephadex G-75 column (medium grade, Pharmacia Fine Chemicals, Inc., Piscataway, N. J. 08854). The column (2.5 x 30 cm) is eluted with more
of the buffer, at 25 °C. Aliquots of red-brown myoglobin-containing eluate are assayed for purity by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (6). The myoglobin concentration in column fractions is measured by first converting it to cyanometmyoglobin with potassium ferricyanide, measuring its absorbance at 540 nm, and comparing results with the absorbance of human cyanmethemoglobin standards (Hyceel, Inc., Houston, Tex. 77036) (7). The purified myoglobin (1 g/liter, in 1-ml aliquots) is stored at −70 °C until needed for radiolabeling, rabbit immunization, or radioimmunoassay experiments.

Myoglobin Iodination

To remove any of the tris(hydroxymethyl)aminomethane buffer, the purified myoglobin is dialyzed against phosphate buffer (0.1 mol/liter pH 7.5) for 24 h at 4 °C. It is then labeled with 125I by the method of Bolton and Hunter (8), with these modifications: 0.7 nmol of [125I]-3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester (500 KCi/mol; New England Nuclear, Boston, Mass. 02118) is transferred to the reaction vessel, a cone-shaped glass 1.5-ml vial, in approximately 35 μl of its solvent. For maximum safety, a charcoal trap is used to capture volatile radioactivity while the solvent is driven off under nitrogen. Myoglobin (4.5 μg) is 8 μl of phosphate buffer (0.1 mol/liter, pH 7.5) is added to the vessel and reacts for 1 h at 4 °C. After iodination, the labeled protein is separated from reaction products of lower molecular weight by chromatography at 25 °C on a 1 x 10-cm column containing Sephadex G-50. The column is eluted with the phosphate buffer containing added gelatin (2.5 g/liter). One-milliliter fractions are collected into tubes containing 200 μl of bovine albumin (60 g/liter, Fraction V), to obtain a final albumin concentration of 10 g/liter. Thus protected by protein, the labeled myoglobin can be stored at −20 °C for at least six weeks.

Antibody Generation

In our laboratories, antiserum to myoglobin was generated in five young New Zealand white rabbits by repeated subcutaneous injections of the purified antigen. Each rabbit was initially injected with a 1-ml mixture of 250 μg of myoglobin emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich. 48232). Three booster injections of myoglobin emulsified in Freund's incomplete adjuvant were given at days 21, 31, and 111 after the initial injection. The rabbits were bled at days 41 and 121. Sepharose-bound hemoglobin, prepared according to Porath et al. (9), was mixed with the rabbit serum for 24 h at 4 °C. After centrifugation, aliquots of the clear serum were removed from the sediment of Sepharose/protein complex and stored at −20 °C. Antibodies were successfully induced in all five animals.

Radioimmunoassay Solutions and Standards

Phosphate buffer solution contains sodium phosphate (0.1 mol/liter, pH 7.5), bovine serum albumin (10 g/liter, Fraction V), sodium chloride (0.1 mol/liter), and sodium azide (1 g/liter). The solution is stable for at least a week at 4 °C.

Myoglobin standards contain purified myoglobin diluted with phosphate buffer (Solution 1), to prepare working standards having concentrations of 25, 50, 75, 100, 150, 200, 300, 400, and 500 μg/liter. The working standards are stable for at least two weeks at 4 °C.

Antiserum from only one of the rabbits was used in the present study. Aliquots of the frozen serum were thawed and diluted 200-fold with the phosphate buffer solution before use. Unless otherwise stated, the final dilution of antiserum in the radioimmunoassay is 975-fold. Diluted antiserum is stable for at least two weeks at 4 °C.

125I-labeled myoglobin. An aliquot of the radiolabeled myoglobin is thawed and diluted with the phosphate buffer solution to a myoglobin concentration of 2.2 μg/liter. When freshly labeled, its radioactivity is about 60 μCi/liter. The labeled antigen is diluted on the day of analysis.

Gamma-globulin solution contains bovine gammaglobulin (10 g/liter, fraction II; Miles Laboratories, Inc., Research Division, Elkhart, Ind. 46514) and sodium azide (1 g/liter) in sodium phosphate buffer (0.1 mol/liter, pH 7.4). This solution is the source of carrier protein during the separation of antibody-bound myoglobin. It is stable for at least a week at 4 °C.

Polyethylene glycol solution contains polyethylene glycol (200 g/liter, average mw 6000–7500) in sodium barbital buffer (70 mmol/liter, pH 9.0). The solution must be mixed vigorously before use and is stable for at least eight weeks at 4 °C.

Radioimmunoassay Procedure

The six steps in the radioimmunoassay protocol are summarized in Figure 1. In step 1, 50 μl of sample (standard or serum), 100 μl of labeled antigen, 400 μl of phosphate buffer solution, and 100 μl of antiserum are added to appropriate tubes and gently vortex-mixed. Duplicate tubes are assayed for each standard or patient serum. In addition, duplicate nonspecific binding tubes are prepared for the 0-ng myoglobin standard and for every patient's serum. These tubes contain the same
reagent additions as described above except that an additional 100 μl of phosphate buffer (solution 1) is used in place of antiserum. In step 2, all tubes are incubated at 37 °C for 3.5 h, unless otherwise noted. Next, 200 μl of gamma globulin solution and 1 ml of cold polyethylene glycol solution are added to each tube with Cornwall side-arm syringes (Becton, Dickinson and Co., Rutherford, N. J. 07070). The tubes are then vortex-mixed. After 15 min of precipitate formation at 4 °C, the tubes are centrifuged for another 15 min (1500 × g) at 0 °C. In the final step, supernatant solutions are decanted by inverting the entire batch of tubes and then blotting the rims. The precipitates are counted for 1 to 2 min per tube in a gamma counter.

The data are corrected to net binding by subtracting the appropriate nonspecific binding counts from the complete reaction mixture tubes. The percent binding is then derived by dividing these corrected counts by the total count added to each tube. Concentrations of myoglobin in the unknown and in quality-control samples are determined by comparison to the standard curve of percent binding vs. myoglobin concentration.

Thirty-five patients’ specimens can be analyzed in a single batch within 6 h. Quality-control samples with normal and above-normal myoglobin concentrations are included in every run. To achieve maximum precision, we used an automatic pipette and a dispenser (Micromedic Systems, Inc., Philadelphia, Pa. 19106) to distribute the reagents and samples into aliquots.

Additional Materials and Procedures

We tested specimens from two populations to assess normal values. Group I was composed of serum samples from 50 healthy hospital employees. Group II was composed ethylenediaminetetraacetate-preserved plasma from 51 industrial workers. These workers had comprised part of a control group whose specimens were made available to us by the Northwest Lipid Research Center. Specimens from both populations were kept at −20 °C until assayed.

Creatinine was determined by the automated method of Chasson et al. (10).

The horse myoglobin and human hemoglobin used during the development work were obtained from Sigma Chemical Co., St. Louis, Mo. 63178). Hyland Q-Pak and Hyland Chemistry Control Serum (unassayed; Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif. 92626) were used as quality-control samples for the normal and above-normal concentration ranges, respectively. We found it most convenient to freeze these materials in small aliquots and thaw only the needed volume on the day of assay.

Results

Myoglobin Purification and Iodination

The myoglobin was judged suitably pure when, after Sephadex column chromatography, it migrated as a single component during electrophoresis on sodium dodecyl sulfate/polyacrylamide gel. Figure 2 shows such gels. The log of migration distance (in millimeters) is directly and linearly proportional to the molecular weight of purified protein standards and of the isolated human myoglobin. Migration distances for human and horse myoglobins were 13.60 and 13.65 cm, respectively. Thus, the size of our purified protein is consistent with that of human myoglobin. Further purification was unnecessary.

The myoglobin was radiolabeled as described above. In five successive experiments, the iodination efficiency varied from 40 to 44% incorporation, and the resulting specific activities of myoglobin varied from 23 to 29 Ci/g. A typical Sephadex elution pattern of the radioactive products in the iodinated antigen purification procedure is shown in Figure 3. There are two separate peaks of radioactivity; peak 1 contains labeled protein, peak 2

![Fig. 2. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis pattern of purified human myoglobin and of protein standards](image)

- (a) bovine serum albumin; (b) ovalbumin; (c) bovine carbonic anhydrase; and (d) horse myoglobin. The polyacrylamide gels (100 g/liter) are stained with Coomassie Brilliant Blue R

![Fig. 3. Elution pattern of iodination products during chromatography on G-50 Sephadex column](image)

Fractions contain 1 ml of eluent. The percentage of immunological reactivity found in selected fractions (percentage of 125I bound in antibody excess) is shown in circles

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iodinated ester. To test the immunological reactivity of iodinated materials, we diluted aliquots of eluted fractions to 60 μCi/liter and we incubated these diluted fractions with a 1300-fold dilution of human myoglobin antiserum. Other assay conditions were the same as described in the Materials and Methods section. We measured maximum binding \( (B_0) \) with the myoglobin standard. The circled percentages in Figure 3 represent maximum binding of labeled material observed in each fraction. In this experiment, net binding of the zero standard \( (B_0) \) ranged from 46 to 53%. We also determined binding depression for each elution fraction by testing an aliquot in the presence of the 25-ng myoglobin standard. The depressions ranged from 25 to 31% of maximum possible binding. Therefore the fractions of labeled myoglobin from peak 1 are reasonably homogeneous in their binding to antihuman myoglobin. The fractions from peak 2 did not show any binding to antihuman myoglobin, as was expected.

The dose/response data indicated that all fractions could be pooled, because their contents would react with comparable kinetics under the assay conditions being used. Aggregation and degradation products were either homogeneously dispersed in peak 1 or not present.

Optimization and Evaluation of the Standard Curve

We first studied the effect of incubation time on the standard curve (Figure 4). We used a 1300-fold antibody dilution, a 4 °C incubation temperature, and myoglobin standard concentrations ranging from 0 to 30 ng per assay tube. The 72-h incubation produced maximum sensitivity (percentage binding depression per myoglobin dose) over the range of standards. The 3.5-h incubation produced the least sensitivity. These data show that establishing equilibrium requires more than 24 h.

Next, we studied incubation temperature as a reaction variable (Figure 5). The incubation time was held at 3.5 h and the analyses were performed at 4, 25, and 37 °C. By increasing the incubation temperature, we increased the sensitivity of the assay. At 4 °C, there was only a 16% depression of radioactive binding over the range of 0 to 30 ng myoglobin. At 37 °C, however, there was a 28% binding depression for the same range of standards.

Maintaining the 3.5-h incubation time at 37 °C, we varied antiserum dilutions from 1/325 to 1/2600 (Figure 6). The greatest sensitivity over the entire standard range, 0 to 30 ng, was obtained with a 650-fold dilution of antiserum. In the range from 0 to 5 ng, however, the 975-fold dilution of antiserum gave the greatest sensitivity. For the 50-μl sample size, the 0 to 5 ng range includes both the normal and above-normal myoglobin concentrations in serum. Because of the possible importance of this clinical-decision range, we chose to use 975-fold dilution of antiserum for the assay.

Incubation buffer concentration, incubation volume, and polyethylene glycol concentration were all studied separately. The actual conditions reported here were selected for maximum test sensitivity and precision. Under the optimal conditions described in the Methods and Materials section, we obtained the standard curve shown in Figure 7. There is a net 35% depression in radioactive antigen binding over the standard range of 0 to 25 ng. The greatest sensitivity was in the range of 0 to 5 ng of myoglobin. Nonspecific binding of \(^{125}\)I-labeled myoglobin in the absence of antiserum ranged from 4 to 6% of the total counts added. The detection limit of
the method was determined by performing 20 replicate determinations of $B_0$. The smallest amount of myoglobin that could be discriminated from zero with 95% confidence was 0.5 ng. Therefore, the detection limit for myoglobin in a 50-µl sample is 10 µg/liter.

Assay Validation

We studied between-day precision of the method by measuring myoglobin concentration of commercial serum controls during four months under routine service conditions in our radioimmunoassay laboratory. The mean ±1 SD of the Hyland Chemistry control serum pool was 104 ± 12.9 µg/liter ($n = 24$). Corresponding results for the Hyland Q-Pak were 25 ± 4.4 µg/liter ($n = 26$).

The accuracy of the assay was judged by serum dilution and recovery studies. Figure 8, which shows the results obtained after dilution of a serum sample that had an abnormally high concentration of myoglobin, is representative of several dilution studies that demonstrated that the assay is linear from 0 to at least 450 µg/liter. The results of a recovery study are shown in Table 1. The average recovery was 104%. Two additional experiments gave similar results. The average recovery for all the studies was 99%.

Cross-reactivity with human hemoglobin was studied by analyzing a sample containing purified hemoglobin (22 g/liter) in phosphate buffer (0.1 mol/liter, pH 7.5). The average apparent myoglobin concentration found in three duplicate determinations was 11 µg/liter, which was at the limit of detection of the method.

![Fig. 6. Effect of antibody concentration on the standard curve for myoglobin](image)

Antiserum dilutions in the incubation mixture are (a) 1/325, (b) 1/650, (c) 1/975, (d) 1/1300, (e) 1/1950, and (f) 1/2600.

![Fig. 7. Typical standard curve for serum myoglobin under optimal assay conditions](image)

The average and range of duplicate determinations are plotted.

![Fig. 8. Myoglobin assay of serially diluted human serum](image)

Data points represent the average and range of duplicate determinations. Diluent is phosphate buffer.

<table>
<thead>
<tr>
<th>Added</th>
<th>Expected</th>
<th>Observed*</th>
<th>Recovered</th>
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<tr>
<td>0</td>
<td>—</td>
<td>2.1 ± 0.1</td>
<td>—</td>
</tr>
<tr>
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* Average and range of duplicate determinations.
Table 2. Normal Values for Myoglobin in Serum and Plasma

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<th>Mean (µg/liter)</th>
<th>SD</th>
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<td>10</td>
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<table>
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</tr>
<tr>
<td>men</td>
<td>39</td>
<td>16</td>
<td>26</td>
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</tbody>
</table>

*Donors were hospital employees.

*Specimens are from a control group of healthy industrial workers phlebotomized for studies at the Northwest Lipid Research Center, Seattle.

To test whether serum and ethylenediaminetetraacetate-containing plasma specimens gave comparable results, a specimen of each type was collected from a single venipuncture by standard protocol. The subjects were six apparently healthy volunteers. Our tests showed that the results were indeed the same, and we concluded that, for healthy donors at least, either specimen type yields a similar result. In addition, freezing serum at −20 °C before analysis had no effect on the detectable concentration of myoglobin.

**Normal Values**

The normal values for myoglobin in serum and plasma are shown in Table 2. The observed range was 10 to 68 µg/liter. There was no significant difference between the two sets of results, but the mean circulating myoglobin concentrations were significantly higher in men than in women.

To test the hypothesis that serum myoglobin concentration is directly related to muscle mass, we measured the creatinine concentration in 90 of the normal-study samples, and plotted the correlation in Figure 9. The regression line has a correlation coefficient of 0.74 and a standard error of about 0.12.

**Discussion**

Until the report of Stone et al. (4), the preparation of a suitably labeled antigen was the biggest obstacle to the development of a myoglobin radioimmunoassay. Kagen and Freedman (13) used a [14C]lysine-labeled myoglobin in what was essentially a radioimmunoassay system. Their objective, however, was to monitor lysine incorporation into myoglobin during tissue culture manipulations. They did not use standards and they did not quantitate cold antigen. Reichlin et al. reported in an abstract3 that they were unsuccessful in attempts to incorporate 125I into human myoglobin by the classic Chloramine T reaction. Instead, by using a Chloramine T reagent, they labeled horse myoglobin with 131I and then developed a heterologous assay. Stone et al. (4) described a Bolton and Hunter method for labeling human myoglobin, in which they synthesize the 125I-containing succinimide ester and react it with myoglobin in a continuous process. The pH, buffer type, reagent concentration, and temperature that they used for labeling differ from conditions reported here. Because they do not report labeling efficiency and labeled antigen-specific activity or labeling reproducibility, it is impossible to compare our experience in using commercially prepared succinimide ester with theirs. However, we find the Bolton and Hunter method to be very satisfactory.

In the only experiment in which 125I incorporation was lower than we routinely expect (90% lower), we discovered that the iodinated reagent had been decomposed or defective when received from the manufacturer.

Analysis time is the second factor that heretofore has limited the usefulness of immunological myoglobin assays. When applied either to diagnosis of myocardial infarction or to trauma-patient evaluation, complement fixation and the previously reported radioimmunoassay become clinically impractical because of their long incubation periods (12 and 24 h, respectively, excluding the manipulation time).

Myocardial infarction causes a significant increase in serum myoglobin 10 to 12 h after the onset of pain. However, if that information is not available to the clinician until 24 to 38 h after the infarction, it seemed to us that few clinicians would feel justified in ordering myoglobin determinations. Measurement of creatine kinase (EC 2.7.3.2) activity in serum, an index with which all cardiologists are now familiar, would provide similar diagnostic information in about the same amount of time that the myoglobin values were being ascertained with these immunological methods. The same considerations apply in trauma management. Although serum myoglobin is reported to increase before renal failure in some cases, we have concluded that serum myoglobin information should be very timely if it is to affect the course of patient management.

Brevity was therefore a major goal of our method development. With the 3.5-h incubation reported here, the complete assay can be performed on standards,

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controls, and a patient's serum in 5 to 5.5 h. Attempts to shorten the incubation further were unsuccessful because there was too little sensitivity. The polyethylene glycol precipitation is used in the separation step because it can be rapidly and accurately dispensed to individual tubes, and, after centrifugation, all supernates can be poured off simultaneously.

Our normal range for the whole group studied is quite similar to that reported by Stone et al. (4). The apparent difference in serum myoglobin concentrations between healthy men and women that we observed in this investigation has not previously been reported. Moreover, a histogram of our data showing frequency vs. concentration illustrates the nongaussian, right-skewed distribution for each sex and for the whole group. The median for the whole population was 4 μg/liter lower than the mean. Larger groups are needed to confirm these right-skewed distribution patterns. Because myoglobin is a major protein of striated muscle, we postulated that the lower "normal" range for healthy women was a reflection of their smaller muscle mass. Serum creatinine concentrations are similarly related to muscle mass (11, 12), so we examined the correlation between myoglobin and creatinine in serum. There was significant positive correlation, which leads us to recommend that muscle mass be considered when evaluating normal values for children and small women.

The clinical usefulness of this test rests on the availability of sensitive and accurate analysis. The method reported here demonstrates these characteristics in a clinical laboratory setting. It should be helpful in further investigations of possible diagnostic and prognostic applications of serum myoglobin determinations.

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References