Improved Estimation of Urinary Myoglobin by Counterimmunoelectrophoresis, as Compared with the Double Immunodiffusion Technique

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We describe a simple, sensitive, and relatively inexpensive counterimmunoelectrophoretic technique for detection and estimation of myoglobin. The test can be completed within 90 min, compared with 12–48 h required by double immunodiffusion. With the counterimmunoelectrophoretic technique we could detect myoglobin in concentrations of 2–3 mg/liter, while the detection limit by double immunodiffusion was about 9 mg/liter.

Additional Keyphrases: assessment of muscle damage • emergency tests

Myoglobin is a normal constituent of skeletal and cardiac muscle. It has been found in the urine of patients after various types of muscle damage (1–11), for which it is a specific, sensitive, and diagnostic indicator of serious complications of myoglobinuric states such as renal failure, hyperkalemia, respiratory failure, and aspiration of nasopharyngeal or esophageal contents into the lungs as a result of weakened muscles of deglutition and respiration (12).

Several investigators have demonstrated myoglobin in serum and urine in varying amounts after injury to heart muscle, particularly after acute myocardial infarction (13–15). The largest amounts of myoglobin measured in urine appear in samples taken within hours of onset of clinical symptoms of myocardial infarction (11).

The possibility that this determination may become very widely used in the diagnosis of myocardial infarct makes methodological improvement particularly important. Present techniques for detecting or quantitating urinary myoglobin include electrophoresis (16–19), hemagglutination inhibition (20), immunodiffusion (20–23), chromatography (24, 25), precipitation with ammonium sulfate (17), immunofluorescence (26), filtration (27), ultracentrifugation (21), spectrophotometry (28), and isoelectric focusing (29). Although useful and reliable, these techniques may be subject to difficulties of interpretation, require prolonged test times, or are difficult and expensive to perform. Immunological methods are the most sensitive and precise. Specific antiserum that do not react with hemoglobin or other constituents of serum, urine, or muscle tissue allow myoglobin to be detected more sensitively than with other techniques (12).

The present paper describes a simple, sensitive, inexpensive and rapid immunologic technique, in which a monospecific antiserum is used in a counterimmunoelectrophoretic (CIE) system.

Materials and Methods

Myoglobin was purified from the urine of myoglobinuric patients. A 100-ml urine pool containing about 1 g of myoglobin per liter was concentrated to about 10 ml in a Diaflo ultrafiltration cell (UM-10). A 1-ml aliquot of the concentrate was applied onto a column containing Sephadex G-75 (2.6 cm i.d., 90 cm gel height) previously equilibrated with potassium phosphate buffer (0.1 mol/liter, pH 6.6) (30) and
the myoglobin was eluted at ambient room temperature with the same phosphate buffer, pumped through the gel with a peristaltic pump (20 ml/h flow rate). The column effluent was monitored at 280 nm and 5-ml fractions were collected. The preparation was not contaminated with detectable hemoglobin or other constituents of serum or urine, as determined immunologically and spectrophotometrically. The total myoglobin concentration of this preparation was 0.15 mg/liter. Monospecific antimonyoglobin antiserum that showed one precipitin arc by immunoelectrophoresis, similar to reported data (12, 14, 31), was purchased from Cappel Laboratories, Inc., Downingtown, Pa. 19335.

Counterimmunoelectrophoresis

Kodak lantern slides (Eastman Kodak Co., Rochester, N.Y. 14650) 3/4 x 4 inches, are each coated with 13 ml of Seakem agarose (Marine Colloids, Inc., Rockland, Maine 04841), 8.5 g/liter of barbital buffer (pH 8.3, 50 mmol/liter). A pattern of 30 wells per lantern slide is cut, the distance between the wells in each pair being 6 mm, center to center, and the diameter of the wells being 1.5 mm. The pattern of the 60 wells can be seen in Figure 1, which shows a Plexiglass punch designed in this laboratory for convenience in making these CIE slides.

Two slides can be tested simultaneously in an electrophoresis chamber (Shandon Vokam Model SAE 2761; Shandon Scientific Co., Ltd., London, England) filled with 800 ml of the same barbital buffer containing 4 g of trisodium citrate per liter (32) used to prepare the slides and run at 10 mA per slide for 90 min (constant current). Specimens or myoglobin standard are diluted with the same barbital buffer containing 3.6 ml of formaldehyde per liter (33). Specimens and their dilutions, or myoglobin standards are placed in the wells on the cathode side, while the antiserum is placed in the wells on the anode side of each pair of wells, each well being filled completely with about 8 μl of either the specimens or the antiserum.

Table 1. Comparison of Results for Double Immunodiffusion and Counterimmunoelectrophoresis (Reciprocal Titer for 35 Specimens)

<table>
<thead>
<tr>
<th>DID</th>
<th>CIE</th>
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<td>Mb Std</td>
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Abbreviations: ND, none detected; U, detectable in undiluted specimen only; Mb, myoglobin.

Double Immunodiffusion

For the double-immunodiffusion (DID) technique (34), precleaned microscope slides (Scientific Products, Evanston, Ill. 60065), 25 × 75 mm, are each layered with 3 ml of Ion Agar (Colab Laboratories, Inc., Glenwood, Ill. 60425), 10 g/liter of barbital buffer. A trough 5.6 cm long and 1 mm wide was cut in the middle of the slide, and seven wells (each well center is 6 mm from the edge of the trough) 8 mm apart, were cut on each side of the trough. The trough is filled with 0.2 ml of the myoglobin antiserum, while the wells are filled with about 10 μl of either the specimens or the myoglobin standard (or dilutions of these).

Slides are kept at room temperature in a moisture chamber for 48 h before the final results are read. The positive results for either CIE or DID are read as precipitin lines at the optimum concentration of antigen and antibody between the antiserum trough and the myoglobin-containing wells.

Thirty-five specimens were tested by both CIE and DID at room temperature. Purified myoglobin and normal human urine were the positive and negative controls, respectively.
Results

Table 1 shows the results, expressed as the reciprocal of the titer obtained for both DID and CIE. The myoglobin standard had a concentration of 150 mg/liter. With the CIE technique we could detect myoglobin in dilutions of this standard as great as 64-fold (about 2.3 mg/liter), while the end point of detection by DID was a 16-fold dilution (about 9.2 mg/liter). This latter figure corresponds to the sensitivity of DID as reported by various investigators (20, 31).

CIE is evidently more sensitive than DID (Table 1), particularly for samples of low concentration, 2-3 mg/liter. Five of the 10 specimens that showed a fourfold sensitivity difference were at these concentrations. For 23 specimens CIE was twice as sensitive, while two specimens gave similar results for both CIE and DID. In no case was DID more sensitive than CIE.

Even at the detection limit of the CIE procedure, precipitin lines were readily visible at the end of the run and the results could be read without staining the gel. Moreover, identical results were invariably obtained in replicate runs on any given specimen.

Discussion

DID is widely used for detection of myoglobin because of its simplicity. CIE is even more simple, and also more reliable, faster, more sensitive, and more economical. Precipitin lines are far easier to read in CIE. CIE can be done in 90 min, whereas 48 h is required for DID. The sensitivity of CIE is two to four times that of DID, and the cost (in terms of materials and labor) of CIE is about half that of DID. We did not find that the results were improved by use of gel scanning.

The technique described offers advantages that are lacking in other methods for detecting myoglobin. The more sensitive techniques, hemagglutination inhibition and Sephadex chromatography, require more sample and reagent preparation, and results are obtained only after 48–72 h. Techniques that yield results in 12–24 h, such as immunodiffusion and paper electrophoresis, are not sensitive enough to detect concentrations in smaller than 0.5–2 mg/liter.

A recent report by Saranchak and Bernstein (11) emphasizes the value of myoglobin determination in myocardial infarction. They noted that increased amounts of myoglobin appear in urine within hours after the onset of clinical symptoms. Measuring myoglobin in the urine is being increasingly stressed as an important indicator of muscle damage in a variety of disease situations such as trauma-crush syndrome, excess physical exertion, alcoholic myopathy, renal failures, and myositis syndromes (12), giving new urgency to the need for a better test for myoglobinuria.

This technique is suitable for use in the laboratory diagnosis of such medical emergency conditions as myocardial infarction, open heart surgery, overdose of heroin, electrical injury, and other situations where muscle damage and myoglobin leakage into the urine could lead to acute tubular necrosis, which may progress to renal failure (35). Our technique could also be used to monitor daily myoglobin concentration in the urine of myoglobinuric patients.

Addendum

After submission of this manuscript, we found that formaldehyde and sodium citrate incorporation could be eliminated by using barbital-boric acid buffer (pH 8.6, 153 mmol/liter) in preparation of the agarose slides and in the electrophoresis chamber.

We thank Drs. H. J. Smith and R. E. Thiers for their continual encouragement, and Miss R. Bovis and Mrs. P. Purdy for their technical assistance. The technique for preparing myoglobin, described under Materials and Methods, is the previously unpublished work of J. M. Beattie and J. A. Demetriou.

References

We describe a modified method for determining serum triglycerides (triacylglycerols), which is based on the heptane extraction procedure of Gottfried and Rosenberg [Clin. Chem. 19, 1077 (1973)] with the stable saponification, oxidation, and color development reagents of Neri and Frings [Clin. Chem. 19, 1201 (1973)]. This modified method eliminates one heating step, reduces saponification time to 5 min, absorbances are read at room temperature, and the calibration curve is linear to 3.0 g/liter. A sample comparison between the proposed method and the automated Block and Jarrett [Am. J. Med. Technol. 35, 1 (1969)] procedure showed no significant difference ($r = 0.98$). The coefficient of variation (47 duplicate samples) for the modified method was 8.3%. Further validation was obtained from analysis of quality-control samples; the proposed method gave equivalent values.

## Materials and Methods

### Reagents

**Saponification reagent.** Dissolve 10.0 g of KOH in 75 ml of water and 25 ml of isopropanol. This solution is stable for at least two months at room temperature in a brown glass bottle.

**Sodium metaperiodate reagent.** Dissolve 77 g of anhydrous ammonium acetate in 700 ml of distilled water. Add 60 ml of glacial acetic acid and 850 mg of sodium metaperiodate. Dilute to 1 liter with distilled water and mix thoroughly. The resulting solution is stable for at least two months at room temperature if stored in a brown glass bottle.

**Acetylacetone reagent.** Add 0.4 ml of 2,4-pentanedione to 100 ml of isopropanol. This reagent is stable for at least two months at room temperature in a brown glass bottle.

**Triolein stock standard solution.** 1 g/dl of isopropanol. The triolein (trioleoylglycerol) was purchased from Applied Science Laboratories, State College, Pa. 16801.

**Triolein working standards.** Working standards of 100, 200, and 300 mg/dl are prepared by diluting 2.5, 5.0, and 7.5 ml of the stock standard to 25 ml with isopropanol.