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MYOGLOBIN DETERMINATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Urine and serum myoglobin have been separated on an anion-exchange column, packed by the slurry technique. Urine or serum was injected directly into the column and eluted isocratically with Tris buffer.

Freshly prepared myoglobin from human muscle gives two peaks in the chromatogram. Upon storage, one peak slowly decreases while the other increases in size and has the same capacity factor as horse myoglobin. In myoglobinuria, the latter peak is usually detected in urine, while in serum both peaks are detected. For routine assays, horse myoglobin is used as a standard.

The minimum detectable level is 2 mg/l. Urine myoglobin from patients with myoglobinuria ranged from 20 to 3000 mg/l, while normal subjects had undetectable levels. Patients with myoglobinuria also had detectable levels of myoglobin in their serum. Urine myoglobin was found to be unstable; it should be analyzed immediately.

Although the present method is not sensitive enough to detect myoglobin in the urine of normal subjects, it is clinically useful for confirming and determining myoglobin in patients with myoglobinuria. It has the advantage of speed and simplicity. Using more sensitive detectors would enhance the usefulness of this method.

INTRODUCTION

Myoglobin is a low-molecular-weight protein present in high concentration in muscle and in low concentration in serum and urine. However, in myoglobinuria, after different kinds of muscle injury, myoglobin increases up to hundred-fold in urine. Such levels cause acute renal damage[1]. For this reason, it is important to detect and quantitate myoglobin in urine and serum rapidly to prevent renal damage. The only other method available at the present time for quantitating myoglobin is radioimmunoassay. It is sensitive, but time-consuming and in general not suitable for emergency work.

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Here, we describe the quantitation of myoglobin in urine and serum on an anion-exchange column by high-performance liquid chromatography without sample pretreatment. We demonstrate that myoglobin from both muscle tissue and serum is eluted in two peaks. The method is very simple and rapid.

MATERIALS AND METHODS

Equipment

A Constametric I pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) was used to deliver the solvent at a flow-rate of 1.0 ml/min through a 250 × 4.6 mm I.D. column, packed with AX-300 6.5 μm average particle size (Synchrom, Linden, IN, U.S.A.) in the mobile phase by the slurry technique. The effluent was monitored at 405 nm, 0.005 a.u.f.s. with a fixed-wavelength detector, Model 153, Beckman, Fullerton, CA, U.S.A.

Homogenates

Skeletal muscle (10 mg), obtained from autopsy was homogenized in 1.0 ml distilled water and centrifuged at 10,000 *g* for 2 min.

Peroxidase-like activity

The effluent of the column was collected and the different fractions were tested qualitatively with Ames Hemastix (Ames Division, Miles Labs., Elkhart, IN, U.S.A.).

Procedure

After direct injection into the column of 10 μl urine or 50 μl serum, the column is eluted with Tris buffer (pH 7.5, 22 m*M*).

RESULTS AND DISCUSSION

Homogenates of human skeletal muscle, when freshly prepared, show two peaks, MI and MII, Fig. 1. Both of these peaks have peroxidase-like activity, which is characteristic of myoglobin and hemoglobin. After 24 h of storage of the homogenates at 37°C, MII slowly disappears with a concomitant increase in MI. The two peaks are stable if the homogenates are stored refrigerated or frozen.

It is difficult to speculate on the difference, the mechanism of conversion, and the importance of these two peaks. Injection of the homogenates into a column of Spherogel 2000 SW (Toyo Soda, Japan) produced a single peak (Fig. 2). This favors the argument that the difference involves a charge difference rather than dimerization. It would be of interest clinically to determine whether myoglobin from patients with different skeletal muscle disease and from other tissues would show these two peaks in the same ratio.

In serum from patients with myoglobinuria both MI and MII are detected (Fig. 3) and both exhibit peroxidase-like activity. On the other hand, urine of these patients contains mainly MI with an occasional trace of MII (Fig. 4).

Under the assay conditions, horse myoglobin was eluted with the same capacity factor as MI; thus, it was subsequently used as a standard. This simplified the routine assays, since human myoglobin is difficult to obtain commercially and to purify[2].

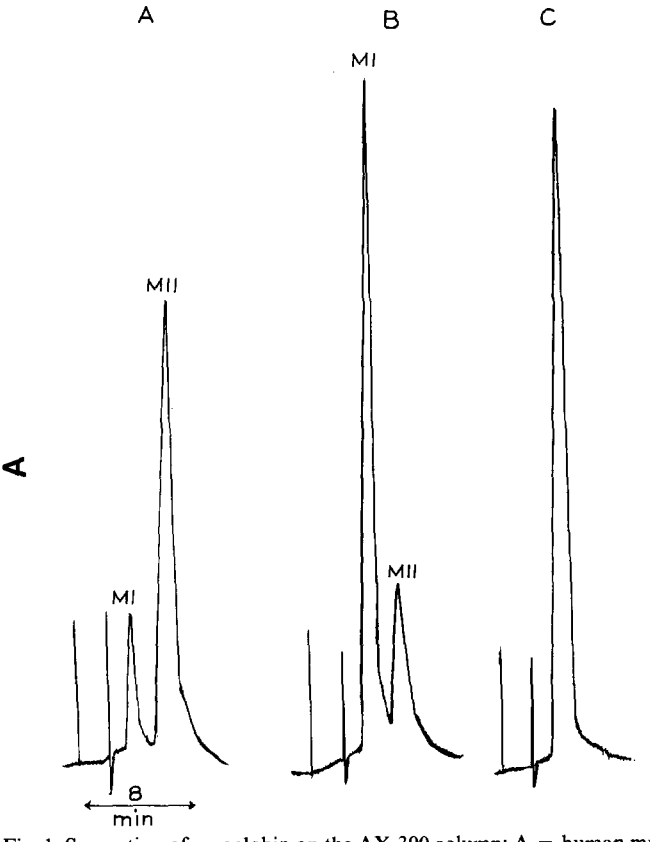

Fig. 1. Separation of myoglobin on the AX-300 column: A = human muscle myoglobin immediately after homogenization; B = human muscle myoglobin after 2 h at 37°C; C = horse myoglobin.

In general, human serum gave cleaner chromatograms than urine. Bilirubin and different hemoglobin variants (A, F, C and S) dit not interfere with the myoglobin peak.

The recovery of 5 mg/l myoglobin, added to serum, was 94% (n = 7), and for 50 mg/l myoglobin added to urine, it was 95% (n = 10). The reproducibility (coefficient of variation) for ten replicate injections of serum is 5% and of urine 8%. The linearity of the test is summarized in Fig. 5.

The normal range for myoglobin in serum is below 0.1 mg/l (ref. 3) and in urine it is below 2 mg/l (ref. 4). After severe exercise, levels up to 6 mg/l (ref. 4) can be detected in urine. With 50-μl injections the minimum detectable level is 2 mg/l (signal-to-noise ratio 5). Although values in the normal range cannot be detected with this method, elevated values, as seen in myoglobinuria, can be detected and quantitated. Table I illustrates that in myoglobinuria myoglobin levels range between 20 and 3000 mg/l. We also demonstrate that in these cases there is a concomitant increase in the serum myoglobin, though the increase in serum is less dramatic than in urine. Unfortunately, myoglobin is not as stable in urine as in serum, even when the samples are stored frozen. Thus, it is important to assay the urine as soon as

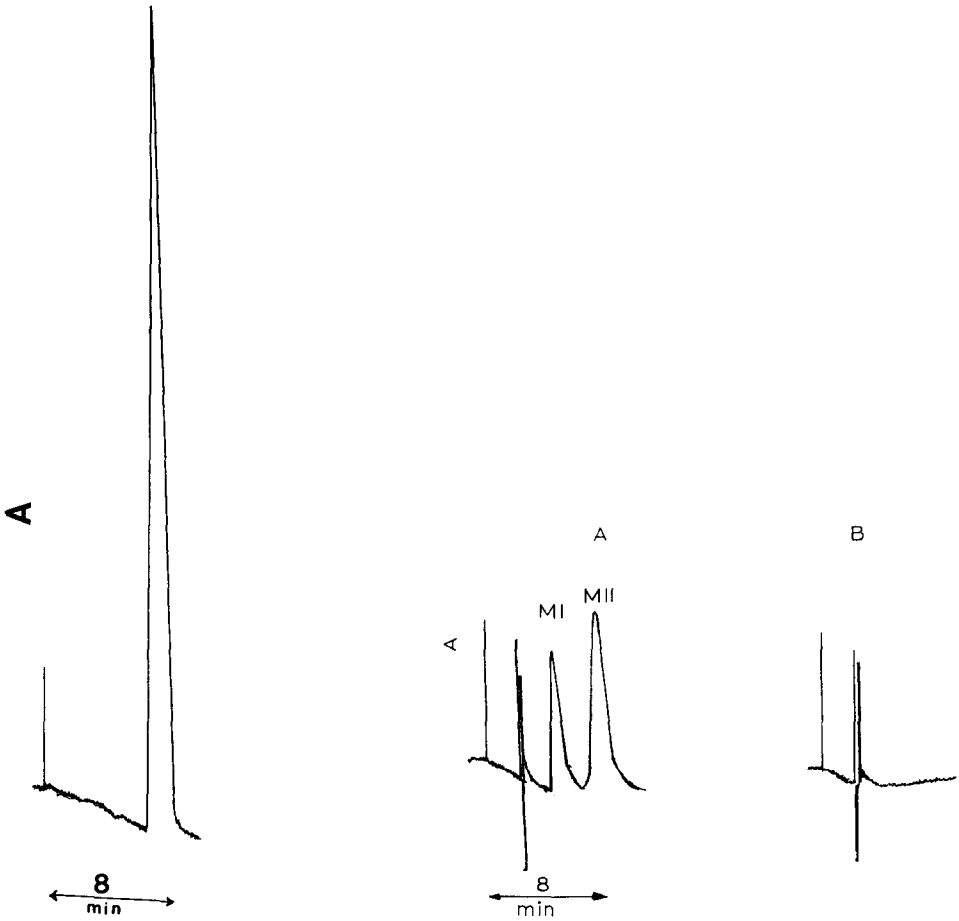

Fig. 2. Chromatogram of skeletal muscle myoglobin on a Spherogel 2000 SW column (300 × 7.5 mm I.D.). Eluent: phosphate buffer 10 m*M*, pH 7.5.

Fig. 3. Chromatogram of human serum myoglobin from: A = patient with myoglobinuria; B = normal subject.

possible. It is also recommended to assay both the serum and urine to confirm myoglobinuria.

Decreasing the pH of the mobile phase buffer (Fig. 6) or increasing its ionic strength decreased the capacity factor. We tried several other types of columns, in-

TABLE I

SERUM AND URINE MYOGLOBIN IN CASES OF MYOGLOBINURIA

	Patient number	*Mean (mg/l)*	*Range (mg/l)*
Serum	8	6.1	2–25
Urine	7	461	20–3000

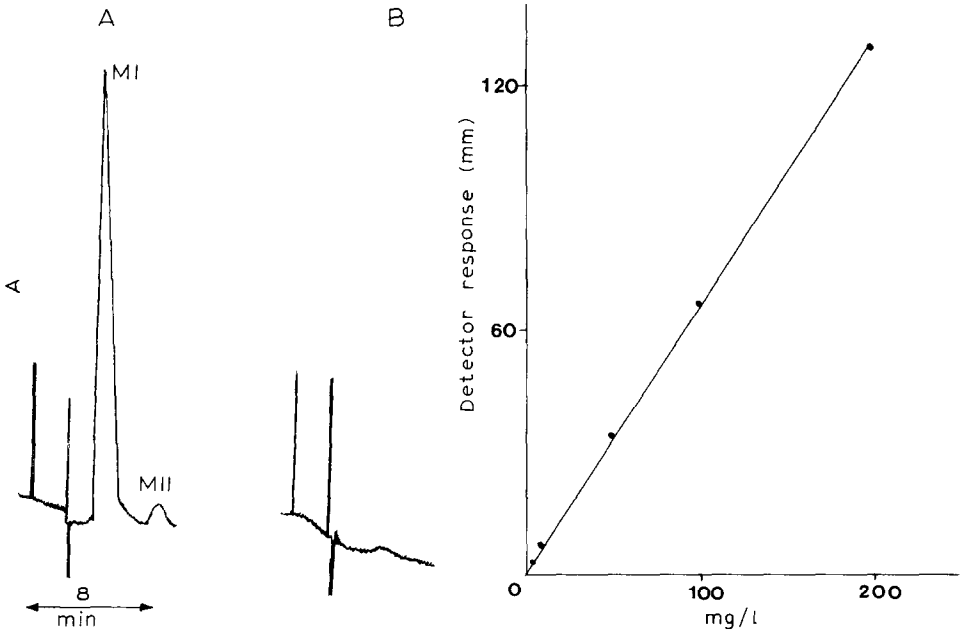

Fig. 4. Urine myoglobin from: A = patient with myoglobinuria; B = normal subject.

Fig. 5. Detector response of myoglobin plotted against concentration.

cluding reversed-phase C_{18} and Spherogel 2000 SW columns. Neither of these gave separations comparable to the AX-300. When the column lost resolution, it was emptied and repacked with the same old resin.

Since sample pretreatment is not required, the method is quite rapid (less than 8 min) and suitable for emergency work. The method is not sufficiently sensitive compared to radioimmunoassay to measure normal or slightly elevated levels. However, the sensitivity of the method could be increased by using more sensitive detectors and narrower columns or by measuring the peroxidase-like activity. This method

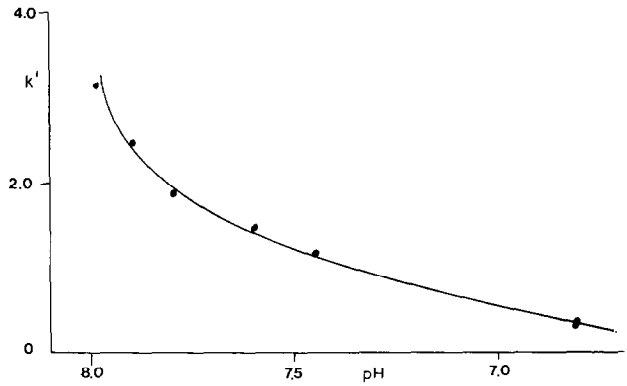

Fig. 6. Effect of pH of the Tris buffer on the capacity factor (k') of myoglobin.

has the advantage over radioimmunoassays that it can measure slight molecular differences of variants, which might be clinically important. Also, it can be used for preparing small amounts of purified myoglobin.

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