Factors Affecting Urinary Myoglobin Stability In Vitro

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Abstract

Urine myoglobin concentrations are measured clinically to assess rhabdomyolysis and the related risk of renal damage. We studied urine myoglobin concentrations in vitro to explore the factors affecting stability.

Myoglobin was very unstable in urine specimens, especially below pH 6.5, and its immunoreactivity deteriorated rapidly with increasing temperatures. The deterioration rate was influenced greatly by urine myoglobin concentration, suggesting rate-limiting kinetics. Myoglobin in acidic phosphate-buffered saline was significantly more stable than in acidic urine, indicating that urinary factors in addition to pH are involved in myoglobin instability. These unidentified urinary factors had a molecular weight of less than 10 kd.

Our results provide additional insight into the mechanism involved in the instability of the urine myoglobin concentration. Understanding the stability of myoglobin in the preanalytic in vitro phase and its potential in vivo instability is essential in ensuring the reliability and clinical usefulness of urine myoglobin measurements.

Myoglobin, an 18-kd oxygen-carrying heme protein, is found in the cytoplasm of cardiac and striated muscles. It is released into the circulation following muscle injury and myocardial infarction and is cleared almost exclusively by the kidneys. Myoglobin is, however, absent or present in very low concentrations in the urine of healthy people.1 In rhabdomyolysis with severe muscle damage, myoglobin appears in the patient’s urine, sometimes at extremely high levels.2,3 Myoglobinuria might be visible—reddish brown, dark-colored urine—when myoglobin concentrations are very high, ie, more than 300,000 µg/L, but in less extreme cases, myoglobinuria is not visible.2,4

Myoglobinuria is considered nephrotoxic and is believed to cause acute tubular necrosis.4,5 There are several theories about the mechanism for the toxic effects of urinary myoglobin. According to one theory, acute tubular necrosis develops as a result of kidney tubule depositions of myoglobin in which the concentration exceeds the renal tubular reabsorption threshold.6,8 Also, heme proteins are considered to exacerbate ischemic renal injury through multiple and potentially overlapping mechanisms.9 Accumulating data provide evidence that renal injury is caused directly by lipid peroxidation, generated primarily through redox cycling of the iron in the myoglobin heme groups from ferrous to ferric and ferryl oxidation states.6,10 More recent reports demonstrate that kidney damage through lipid peroxidation could be initiated by the heme center of the ferryl myoglobin complex without the release of free iron.11-14 It is plausible that a combination of such processes contributes to the overall renal damage. Because acute tubular necrosis might lead to life-threatening renal failure, it is important to identify patients at high risk and treat them promptly.15,16 Thus, urine myoglobin determination, by qualitative or
been documented.1,22-24 Because myoglobin immunoassays are quantification of urine myoglobin by immunologic methods has apparent analytic adequacy. Instability and inaccuracy in the been approved for the analysis of urine myoglobin despite their globin tests. Nevertheless, myoglobin immunoassays have not been approved for the analysis of urine myoglobin despite their apparent analytic adequacy. Instability and inaccuracy in the quantification of urine myoglobin by immunologic methods has been documented.1,22-24 Because myoglobin immunoassays are being recommended and used for the assessment of myoglobin-uric patients,18,25 myoglobin instability and the clinical correlates of myoglobinuria measurement remain significant, and, therefore, the issue deserves more thorough study.

We describe our study of urinary myoglobin stability. By using radioimmunoassay, nephelometric immunoassay, and microparticle enzyme immunoassay, we demonstrated that immunoreactive myoglobin deteriorates rapidly in urine specimens. We analyzed some of the major underlying factors implicated in urine myoglobin instability and specified the preferred preanalytic handling and storage conditions of urine specimens, starting at the patient’s bedside.

**Materials and Methods**

**Myoglobin Assays**

N Latex Myoglobin (Dade Behring Diagnostic, Somerville, NJ), a nephelometric immunoassay, measures the agglutination that results from binding of polystyrene-coupled specific antibodies to human myoglobin. The kit is approved by the US Food and Drug Administration for serum and plasma samples only and has good (<5%) precision in such specimens.20 It also has been shown to perform well with no modifications for the analysis of urine myoglobin.1 The assay’s range is 6 to 2,000 µg/L of myoglobin, with automatic dilution capability. The N Latex Myoglobin assay was used throughout this study, unless otherwise stated.

AxSYM myoglobin (Abbott Laboratories Diagnostics Division, Abbott Park, IL), a microparticle enzyme immunoassay, is based on a fluorometric detection. The substrate for the antibody-conjugated alkaline phosphatase is 4-methylumbelliferyl phosphate, and the fluorescent product is methylumbelliferone. The analytic measurement range of the AxSYM myoglobin is 1 to 1,000 µg/L, extended up to 10,000 µg/L by automatic dilution. The kit is approved by the US Food and Drug Administration for serum and plasma samples only and has a precision of less than 9% in such specimens.

Radioimmunoassay (RIA), using an in-house method that used rabbit antihuman myoglobin, was described previously.20

**Human Myoglobin Standard**

The standard was isolated from human skeletal muscle (code No. X555, DAKO, Carpinteria, CA).

**Phosphate-Buffered Saline**

The phosphate-buffered saline (PBS) was composed of the following, unless adjusted otherwise: 0.01-mol/L concentration of phosphate and 0.15-mol/L concentration of sodium chloride at a pH of 7.4.

**Urine Pretreatment**

Several treatments were designed to denature, deplete, and/or inhibit enzymatic activity within urine specimens. Each was done using a urine specimen from a healthy person and carried out separately followed by adjusting the pH as desired, all before the addition of myoglobin to the analyzed specimen [Table II].

**Urine Samples**

Urine specimens were collected from healthy volunteers and patients with rhabdomyolysis. Specimens were obtained and used in accordance with protocols approved by the University of Washington (Seattle) Human Subjects Committee.

**Protocol for Specimen Collection From Healthy Subjects**

For determination of the urine myoglobin reference range, urine specimens from 25 healthy laboratory volunteers were obtained following different levels of physical activity as described herein.

Two spot urine samples were obtained from each volunteer on the same day as follows: (1) an early morning urine specimen, without previous physical activity; (2) a second urine specimen 3 to 5 hours later following some level of physical activity, ranging from sedentary activities (such as sitting at the desk, working in the laboratory, walking to and from the bus) to intense workout (such as a low-impact, 45-minute workout; 6-mile bike ride to work; 16-mile run). Complete emptying of the bladder was requested at both times.

Urine specimens were kept refrigerated or on ice at all times. They were analyzed for myoglobin on the day of collection.

One blood specimen (red-top tube, about 10 mL) was obtained from each volunteer before the second urine specimen was given. These blood samples were used for establishing the myoglobin serum reference range.

**Protocol for Urine Myoglobin Method Comparison**

Urine specimens from patients were used for myoglobin method comparison performed at 2 periods during the study. During the first comparison study, specimens were divided into aliquots and frozen at –20°C as soon as they arrived in the laboratory. Before analysis, sodium bicarbonate was added to
To ensure stability of myoglobin in urine, a protocol was established that involved adding sodium bicarbonate to achieve a final concentration of 0.1% for high myoglobin concentrations, and 2% for very acidic urine specimens. This protocol was used for the correlation of the RIA with the Dade Behring N Latex Myoglobin (BN) method and the first comparison of the myoglobin AxSYM assay with the nephelometric N Latex Myoglobin (BN) method.

In late stages of the study, when knowledge of the pH effect on urine myoglobin stability was better established, we found that addition of sodium bicarbonate was insufficient for very acidic urine specimens. We thus instituted a modified protocol requesting urine samples to be transferred immediately at the time of collection into preprepared transport tubes containing bicarbonate to allow a final concentration of 2%. Specimens were frozen at –20°C on arrival in the laboratory and thawed only before myoglobin analysis. When dilutions were required, they were done with 1% sodium bicarbonate solution.

The comparison of the myoglobin AxSYM assay with the nephelometric N Latex Myoglobin (BN) method was carried out twice during the study. During the earlier study period, urine specimens from 41 patients with myoglobin concentrations ranging from 6 to 25,000 µg/L produced a correlation coefficient of $r = 0.990$ and the following regression equation: $y = 1.299x - 105$, where $x$ is the AxSYM myoglobin concentration and $y$ is the concentration by the BN method. During the later comparison study, 17 urine specimens with myoglobin concentration ranging from 1 to 38,000 µg/L produced a correlation coefficient of $r = 0.999$ and the following regression equation: $y = 1.324x + 13.5$, where $x$ is the AxSYM myoglobin concentration and $y$ is the myoglobin concentration by the BN method.

The comparison of RIA with N Latex Myoglobin (BN) results was done at early stages of the study and included 90 urine samples with myoglobin concentrations ranging from 6 to 5,000 µg/L (a relatively narrower range). The analysis produced the following results:

$$r = 0.985$$
$$y = 0.98x - 2.1$$

where $x$ is the RIA myoglobin concentration and $y$ is the concentration by the N Latex Myoglobin (BN) method.

### Parameters Involved in Urine Myoglobin Instability In Vitro

#### pH

Fresh urine specimens were obtained from 9 healthy subjects. All had undetectable myoglobin levels of less than 6 µg/L. Urine pH values were measured, and the specimens were modified by addition of purified human myoglobin to levels of about 250 µg/L. Table 2 shows the changes in urinary
myoglobin immunoreactivity after 100 minutes at room temperature. When the urine specimens were slightly acidic, pH less than 6, a decrease of 37% to 53% in the myoglobin level was detected. In contrast, urinary myoglobin was relatively stable during the same period when the urine pH was 6.5 or more. Similar results were obtained when the myoglobin concentration was measured by RIA (data not shown).

The effect of pH on urine myoglobin stability during a 24-hour incubation is shown in Figure 1. Multiple aliquots of a single urine specimen were adjusted to different pH values and modified by the addition of human myoglobin to levels between 200 and 300 µg/L. Myoglobin concentrations were measured at 0, 1.5, and 3 hours at room temperature and after refrigeration for 24 hours. At an acidic pH of 4.5, there was a dramatic decline in urine myoglobin immunoreactivity during the first 1.5 hours at room temperature, with nearly complete disappearance of measurable myoglobin after 24 hours of refrigeration. At a pH of 6.5 or more, the urinary myoglobin concentration was stable during the first 3 hours and decreased by 20% or less after 24 hours in the refrigerator. A similar pH effect was observed with urine specimens from other healthy subjects. Increasing the pH could not reverse the effect of acidic pH on urine myoglobin instability after the measurable concentration had declined.

Temperature

The effect of temperature on urinary myoglobin was studied in urine samples from a healthy subject (myoglobin concentration, <6 µg/L) spiked with human myoglobin to an initial level of 111 µg/L. The urine pH was adjusted to 5.3 before myoglobin addition. The myoglobin concentration was measured at 0, 2, 4, 6, and 24 hours. Figure 2 shows the changes of myoglobin levels during a 24-hour period and at different temperatures (–20°C, 8°C, 22°C, and 37°C). Urine myoglobin concentrations declined rapidly with time at all studied temperatures. The magnitude of decrease, however, was smaller at the lower temperatures. At 22°C and at 8°C, myoglobin was very unstable and lost 64% and 40% of the initial immunoreactivity, respectively, within the first 2 hours. At ~20°C, urine myoglobin was better protected against such deterioration, but still lost 40% of its original concentration after 24 hours. Urinary myoglobin was very unstable at 37°C, with 95% of its original concentration lost after only 2 hours.

Concentration

We next studied the relationship between urinary myoglobin instability and initial urinary myoglobin concentration. Urine specimens were obtained from 6 patients with rhabdomyolysis; the myoglobin levels, determined by the Dade Behring nephelometer (2 specimens) or the AxSYM immunoanalyzer (4 specimens), were greatly elevated (>20,000 µg/L). Urine samples were diluted, if required, with urine from healthy subjects (myoglobin concentration, <6 µg/L) and adjusted to a pH of 4.5 to 5.2.

Urine samples from patients demonstrated temperature and pH effects on myoglobin stability similar to those shown in urine samples from healthy subjects that were spiked with human myoglobin. In 4 patients’ specimens analyzed by the AxSYM assay, myoglobin levels decreased rapidly within the first 30 minutes at room temperature, by 30% to 75%, and continued to decrease during the next 2 hours (data not shown). The proportion of decline, though, was not constant and seemed dependent on the initial myoglobin concentration. As shown in Table 3 (results from 1 representative patient; samples diluted, then analyzed by the Dade Behring nephelometric assay), the percentage decrease of myoglobin concentrations after 2 and 24 hours was smaller at higher myoglobin concentrations and seemed to approach saturation, indicating the possibility of rate-limiting kinetics.

Table 2: Stability of Urine Myoglobin in Nine Subjects

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>pH</th>
<th>Myoglobin Concentration (µg/L)</th>
<th>0 Minutes</th>
<th>100 Minutes</th>
<th>Change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.4</td>
<td>248</td>
<td>208</td>
<td>–16</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7.3</td>
<td>264</td>
<td>252</td>
<td>–5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7.2</td>
<td>270</td>
<td>291</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6.5</td>
<td>231</td>
<td>237</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.7</td>
<td>228</td>
<td>141</td>
<td>–38</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5.7</td>
<td>219</td>
<td>118</td>
<td>–46</td>
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<td>152</td>
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<tr>
<td>9</td>
<td>5.5</td>
<td>212</td>
<td>100</td>
<td>–53</td>
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</table>

Studying the Underlying Mechanism of Urinary Myoglobin Instability

In an attempt to understand the mechanism of urinary myoglobin instability and to search for the urinary factors that control it, we carried out further studies. All steps following addition of myoglobin to the studied samples were done at room temperature.

We first isolated the influence of pH in the absence of urine by examining the effect of pH on myoglobin stability in PBS at a pH of 7.4 and adjusted to 4.9. Table 4 summarizes the effects of acidic and slightly alkaline pH on spiked myoglobin concentrations in PBS and urine. As demonstrated in our previous experiments, urine myoglobin stability was strongly pH-dependent on pH, with a 23% decrease at pH 7.4 and more than a 97% decrease at pH 4.9 after 23 hours. In contrast, myoglobin immunoreactivity in PBS was almost unaffected by pH. At pH 4.9 and pH 7.4, myoglobin added to PBS was stable for the first 2 hours and decreased only slightly (≤16%) after 23 hours. The relative stability of myoglobin in PBS and the lack of pH effect indicated that low urine pH was not by itself a cause for instability in the myoglobin concentration.
To assess the possible contribution of an enzyme to the instability of myoglobin in urine, we determined myoglobin stability in urine specimens (from healthy subjects) that were treated before the addition of myoglobin (Table 1). Human myoglobin was added to the pretreated samples and to untreated original urine samples. Pretreatment procedures designed to denature or remove urine proteins or to inhibit urine proteases had no effect on myoglobin instability (data not shown). Myoglobin remained unstable at acid pH as it was in the original untreated urine. The results of these studies suggested that myoglobin stability in urine is not protein-dependent.

In our final study, we measured myoglobin stability in urine depleted of its low-molecular-weight compounds of less than 10 kd. As shown in Table 5, myoglobin added to a urine sample from a healthy subject that was dialyzed earlier (24 hours against PBS at 4°C) was very stable for 24 hours at all studied pH values (pH 5.1, 6.05, and 7.5). Myoglobin added to the same nondialyzed urine was unstable at all studied pH values, especially at acidic pH values of 5.1 and 6.05, as expected from our previous study results. The dialysis studies demonstrated that the urinary factors involved in urinary myoglobin instability are smaller than 10 kd and that in their absence, urinary myoglobin was stable.

Discussion

Serum myoglobin immunoassays are used for the quantitation of urine myoglobin in the diagnosis and monitoring of rhabdomyolysis and myoglobinuric renal damage. Our goals were to study the immunologic stability of myoglobin in urine...
and to examine the mechanism responsible for its instability. Myoglobin immunoreactivity in urine rapidly deteriorated at an acidic pH in a temperature-dependent process. We found that the percentage of decline was significantly lower at higher initial myoglobin concentrations. Our results further indicated that the loss of immunoreactivity is not likely due to a proteolytic degradation process and is dependent on one or more unidentified urinary factors. These factors are unlikely to be proteins but seem to be relatively small molecules, smaller than 10 kd.

Our study demonstrated that myoglobin was absent or present in very low levels (<6 µg/L) in the urine of healthy subjects despite its relatively broad range in the corresponding blood samples. Similar results were obtained in another study.1

Analysis of the parameters involved in urine myoglobin stability in vitro revealed some major influences, ie, urine pH, storage length and temperature, and urinary myoglobin concentration. We found that urine myoglobin immunoreactivity was very unstable at even mildly acidic pH (<6.5), a pH well within the range observed in clinical specimens and very common in patients with rhabdomyolysis in whom metabolic acidosis often develops.27 Significant deterioration starts within 100 minutes of urine specimen collection, depending on the specimen storage temperature and pH. Similar findings regarding pH and temperature effects on urine myoglobin concentrations were reported by another group.1 However, they reported urine myoglobin stability measurements during a period of days, not hours.

Our data, indicating that urine myoglobin deteriorates significantly during a short period, have important implications for the preanalytic phase of urine specimen handling and processing. Although the magnitude of myoglobin instability increased at higher temperature (ie, 37°C), even at a temperature as low as –20°C, a depletion of 40% in the myoglobin concentration was detected after 24 hours. In contrast, the urine myoglobin concentration was relatively stable for at least 3 hours in neutral or basic pH (6.5-8.8), with some noticeable deterioration after 24 hours at room temperature. These data indicate that measurements that purport to reflect in vivo myoglobin concentrations require rapid adjustment of urine to a basic pH. In our institution, we transport urine specimens for myoglobin determination in standard 10-mL tubes containing 200 mg of sodium bicarbonate (2% final concentration). This amount of sodium bicarbonate was found to adjust the pH of even very acidic urine samples to 8.0. Lower temperatures (<–20°C) slow the loss of immunoreactivity but are insufficient to completely stabilize the protein.

The findings on the relative stability of myoglobin in PBS and the lack of acid effect in such a buffer indicated that the low pH is not by itself a cause of urine myoglobin instability. The documented urine myoglobin deterioration probably is affected by unidentified urinary factors, and it is enhanced but not caused directly by low pH. Our study demonstrated that the urinary factors involved in urinary myoglobin instability can be removed by dialysis and have a molecular size smaller than 10 kd. These factors did not seem to be proteins, as evidenced by the findings that boiling the urine to denature proteins, removing macromolecules larger than 25 kd, and using chemical reduction to cleave disulfide bonds did not affect myoglobin instability. Furthermore, enzyme inhibitors did not influence stability.

The extent of instability depended not only on urine pH, temperature, and the unidentified urinary factors but also on the initial urinary myoglobin concentration, with relatively faster deterioration at lower concentrations. Myoglobin levels continued to drop with time at all concentrations studied, but the absolute decrease seemed to approach a saturation limiting rate, a process that suggests rate-limiting kinetics. As a result, the percentage rate of decline in a specific patient was lower at high myoglobin concentrations than in urine samples from the same patient that were diluted with urine from a healthy subject. These findings seem to imply that urine myoglobin instability might not be as important clinically in cases with massively elevated levels of myoglobinuria, situations in which the percentage decrease in the urine myoglobin concentration would be less significant than in cases with marginal levels of myoglobinuria. However, in some of the patients’ specimens starting at myoglobin concentrations of 20,000 µg/L, a decrease of up to 75% was detected within the first 30 minutes, suggesting that all patients’ samples should be adjusted to a basic pH at collection time.

Our findings have several important research implications. One of the possible reasons that there is no established or consensus threshold of urine myoglobin concentration for the risk of acute renal failure is that previous studies might be inaccurately reporting the in vivo urine myoglobin concentration. Despite the availability of quantitatively precise serum myoglobin immunoassays, the instability of myoglobin in urine still might result in inaccurate determinations of urinary myoglobin concentrations.

The excessive myoglobin instability in acidic urine at 37°C raises the possibility that such a phenomenon might exist in vivo. Instability of urine myoglobin while in the kidneys or in the bladder at 37°C could lead to an in vivo fall in the measurable urine myoglobin concentration, which could be similar to or greater than the problem of preanalytic instability in acidic urine ex vivo. The phenomenon of urine myoglobin instability might explain the failure to detect myoglobinuria in patients who have had myocardial infarction and had elevated levels of serum myoglobin.24 Both in vivo and in vitro instability also can explain some reported inconsistencies occasionally found between urine myoglobin level and the degree of damage to the kidneys, as reflected by serum creatinine concentrations.17

The mechanisms of myoglobin-induced injury to the kidney probably are multifactorial. Nephrotoxic effects might involve oxidant redox cycling between ferrous, ferric, and ferryl forms of iron complexed to myoglobin, which in turn initiates
l lipid peroxidation.\textsuperscript{11-14} It was shown that this process is pH-dependent and that alkaline conditions prevent myoglobin-induced lipid peroxidation by stabilizing the reactive ferryl-myoglobin complex.\textsuperscript{11-14} This pH-dependent oxidation mechanism and enhanced myoglobin solubility at basic pH explain in part why patients treated for rhabdomyolysis-induced acute renal failure by alkalization with sodium bicarbonate are better protected from permanent renal injury.\textsuperscript{12,15,17,27} It is plausible that in vivo instability of urine myoglobin at acidic pH provides a clue to the pathogenesis of myoglobin-induced acute renal failure.

The immune reactivity of the various iron-oxidized myoglobin moieties is unknown. The observed analytic instability of urine myoglobin,\textsuperscript{2,22} mediated by certain urine factors, might well be a result of myoglobin oxidation. Our data, demonstrating that the urinary factors involved in myoglobin instability are not proteins or proteolytic enzymes, together with the known pH-dependent change in myoglobin iron, support the hypothesis that the change in myoglobin reactivity in immunoassays depends on its heme moiety. Better understanding of the processes involving urine myoglobin stability in vivo and in vitro should improve the contribution of its laboratory measurement to the diagnosis and assessment of patients with rhabdomyolysis and patients with other types of muscle damage.

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