Mechanism of Interference by Hemolysis in Immunoassays and Requirements for Sample Quality

To the Editor:
Cardiac troponin I (cTnI) is considered organ-specific, and few circumstances other than heart muscle injury produce false increases in plasma. We encountered unexpectedly high cTnI in a two-site, enzyme-linked immunoassay using polyclonal goat antibody (1) when by mistake, two visually clear but grossly hemolyzed samples from one patient were not rejected by the laboratory.

Hemoglobin (Hb) reportedly is not an interferent in the test: “. . . levels of the following do not appear to interfere . . . hemoglobin 2,000 mg/dL” (2). Elsewhere, the manufacturer states that: “Plasma samples should be clear to straw in color” (3) and “. . . strongly hemolyzed . . . samples should be avoided” (4). No data are presented that hemolysis interferes. These statements suggested that “hemolysis”, rather than “hemoglobin”, interfered.

Off-label tests were carried out in which freeze-thawed plasma was added to a plasma sample that had a measured cTnI concentration of <0.5 mg/L before freezing, showed <0.5 mg/L as well.

Estimates of hemolysis were made by determining a hemolysis index (HI) for each sample using a Hitachi 911 instrument: “HI is reported in hemolysis units corresponding to hemoglobin concentration in mg/dL. These units are linear and semiquantitative. A hemolysis index of 500 is equivalent to a known hemoglobin concentration of approximately 500 mg/dL” (5).

When hemolysate was added to plasma, cTnI was increased from reference values to high values in a dose-related way (Table 1). The manufacturer’s interference study was performed by adding high concentration Hb stock into samples. This confirmed that the addition of human methemoglobin (Sigma Diagnostics, cat. no. 525-18) in concentrations of up to 30 g/L (3000 mg/dL) did not affect cTnI results. Recentrifugation (10 min at 2000g) of hemolyzed specimens that were adversely affected eliminated interference in 18 samples, but not in 2 (which produced cTnI values of 3.9 and 11.2 mg/L).

Our results raise several points and questions: (a) “Hemoglobin” is not equivalent to “hemolysate”. The latter term implies the presence of debris (e.g., cell membranes), protein, and other potential interferents from cells. (b) Interference is minimal below HI values of 300 (moderate visual hemolysis), but borderline increases may occur and confound diagnosis. (c) Is interference by hemolysis limited to only the method tested? Future TnI assays are likely to use other reagents, antibodies, and principles.

I believe that hemolysis, not Hb, is an interferent in one immunoassay method for cTnI. Although manufacturers are aware of this issue, users must be alert to the importance of sample quality and must understand the mechanism of interference, especially in settings where hemolysis is an increasing problem because phlebotomy services are decentralized.

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References

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Urine Concentration of Biopyrrins: A New Marker for Oxidative Stress in Vivo

To the Editor:
Currently interest in reactive oxygen species (ROSs) is heightened because of their involvement in cardiovascular, neurodegenerative, and chronic inflammatory diseases and cancer. The high reactivity of ROSs coupled with their very short life span is a stumbling block in the direct measurement of these species in human subjects (1).

Bilirubin, a metabolite of heme, apparently functions as an antioxi-

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**Table 1. HI and cTnI concentrations in plasma samples.**

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Hemolysate/plasma volume ratio, %</th>
<th>HI of specimen used in analysis</th>
<th>cTnI concentration measured, µg/L</th>
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The oxidative metabolites of bilirubin could be a biological marker for in vivo ROS production. Recently Yamaguchi et al. (3) identified novel oxidative metabolites of bilirubin in human urine as substances that are recognized by an anti-bilirubin monoclonal antibody, 24G7, but are negative in the diazo reaction. These metabolites are tripyrrole biocompounds and are designated biopyrrins. The production of biopyrrins increases in oxidatively insulated rats subjected to endotoxin treatment (4) and ischemia-reperfusion (5). Increased excretion of biopyrrins in urine has been reported in patients who have undergone resection of esophageal cancer (6). Thus, the production of biopyrrins appears to reflect the degree of oxidative stress in vivo, and the measurement of biopyrrins may be useful for estimating oxidative stress. To date, however, the production and excretion of biopyrrins in healthy human subjects have not been critically examined.

The first urine in the morning was collected from 33 healthy volunteers (33 ± 18 years old) and from 14 patients (56 ± 13 years old) who had undergone abdominal operations lasting >5 h. Subjects gave informed consent according to the ethics rules of Kyushu University Hospital. The biopyrrin concentration in urine was corrected by the concentration of creatinine in the same sample. The samples from healthy subjects were collected periodically, 5 to 25 times per person (average, 18), for 1 month. The total number of samples collected was 600. For each individual, the mean (M) of the urine concentration of biopyrrins and its standard deviation (SD) were determined. The CV (defined as SD/M) was used to assess day-to-day variation, i.e., the degree of change of the biopyrrin concentration between days in a given individual. Subsequently, a mean, SD, and CV were calculated for each group of the healthy subjects.

The biopyrrin concentration was determined by subtracting the concentration of the diazo reaction-positive substances (i.e., bilirubin) from the concentration of the anti-bilirubin monoclonal antibody 24G7-reactive substances. The production of the anti-bilirubin monoclonal antibody 24G7-reactive substances was measured with a biopyrrin measuring kit (Shino-test), using bilirubin as a calibrator according to the manufacturer’s instructions.

The concentrations (± SD) of biopyrrins in healthy male and female subjects and in the total number of healthy subjects were 0.82 ± 0.29, 0.74 ± 0.11, and 0.79 ± 0.24 μmol per gram of creatinine, respectively. The mean values of the day-to-day CV and SDs of the means in healthy male and female subjects and in the total number of healthy subjects were 0.33 ± 0.08, 0.32 ± 0.06, and 0.33 ± 0.08, respectively. The physiological change of the urine concentration of biopyrrins between days was small, and the concentration seldom fluctuated more than twice the mean value in a given individual.

The concentration of biopyrrins increased markedly from 0.74 ± 0.44 μmol/g creatinine before the operation to 3.37 ± 6.24, 3.66 ± 6.88, and 2.59 ± 2.71 μmol/g creatinine at 1, 3, and 5 days after the operation, respectively. The value decreased to 0.83 ± 0.61 μmol per gram of creatinine at 14 days after the operation. The increase was not simply related to the increase in serum bilirubin because the concentrations of serum bilirubin and urine biopyrrins were not correlated significantly.

We conclude that biopyrrins may prove useful as a practical clinical test for the estimation of oxidative stress in vivo.

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**References**


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**Vitamin B<sub>6</sub> Deficiency May Also Be Important**

To the Editor:

Takahashi et al. (1) described no changes in bone metabolism markers and a nonsignificant decrease in vitamin K concentrations after low-energy neck or femur fractures in a group of 28 elderly women. Their theory was that vitamin K is required for γ-carboxylation of osteocalcin (2) and that the acute fracture should