Evaluation of Human Serum Albumin Cobalt Binding Assay for the Assessment of Myocardial Ischemia and Myocardial Infarction

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Background: Clinical diagnoses were correlated with results of a Co(II)–albumin binding assay in 167 patients treated at an emergency department of a health maintenance organization.

Methods: Patients were evaluated as being nonischemic or potentially ischemic through standard coronary disease indicators [creatine kinase (CK), CK-MB, cardiac troponin I, and electrocardiographic findings] and were tested by a Co(II)–albumin binding assay. Samples were tested anonymously, and the study was double-blinded. The sensitivity and specificity of this assay for the detection of ischemia were evaluated by ROC curve analysis. Known Co(II) binding sites on albumin were analyzed by N-terminal amino acid sequencing.

Results: The mean absorbance units (ABSU) ± 2 SD for non-myocardial ischemic and myocardial ischemic individuals measured at 470 nm were 0.43 ± 0.10 and 0.63 ± 0.25, respectively (P < 0.0001). The area under the ROC curve was 0.95 [95% confidence interval (CI), 0.92–0.99], and at a cutoff value of 0.50 ABSU, sensitivity and specificity were 88% (78–94%) and 94% (86–98%), respectively, suggesting a high distinction between the two groups. When we compared non-acute myocardial infarction (AMI) and AMI ischemic individuals, the area under the ROC curve was 0.66 (95% CI, 0.53–0.79) and was considered a poor discriminator between these two groups. N-Terminal amino acid sequencing data for purified albumin showed normal amino acid residues for six of seven high-ABSU (≥0.70) individuals and one nonischemic individual tested. However, only one individual with a high ABSU (0.80) had two missing amino acid residues (DA) from the N-terminal region. Clinical diagnosis for this patient did not reveal an ischemic event.

Conclusions: The Co(II)–albumin binding test may serve as a useful diagnostic tool in emergency facilities for the assessment of myocardial ischemia. High and low ABSU were associated with myocardial ischemic individuals and non-myocardial ischemic individuals, respectively. However, the Co(II)–albumin binding was a poor discriminator between ischemic individuals with and without MI.

Myocardial ischemia results from the lack of adequate blood perfusion to the myocytes, leading to a deficiency of oxygen and nutrients, eventually compromising their vital functions. In a clinical setting, myocardial ischemia is assessed by an individual’s symptoms and electrocardiographic (ECG)5 studies. The ECG changes may include ST-T segment wave alterations (1). Myocardial ischemic manifestations are vague and multiple. Symptoms may include chest pain (angina), epigastric and arm discomfort with exertion or at rest, shortness of breath, nausea, and vomiting (1). However, these symptoms may be subtle and are not easily recognized.

Prolonged ischemia can lead to myocardial cell death...
(necrosis), which is known as myocardial infarction (MI). Acute MI (AMI) or an evolving MI is diagnosed by measuring myocardial proteins in the serum [e.g., creatine kinase MB (CK-MB), troponin I or T] along with ECG studies and imaging procedures. Ideally, it is essential to identify myocardial ischemia before the onset of irreparable myocardial cell damage. Thus, identification of a biochemical marker that is sensitive and specific for myocardial ischemia and can be rapidly measured in serum would be clinically valuable. Recently, a serum-based biochemical test has been found to be useful in the diagnosis of acute myocardial ischemia (2). The basic principle of this test involves the N-terminal region of human serum albumin (HSA) and its inherent affinity for the metal ion, Co(II). Serum albumin of individuals with myocardial ischemia exhibits reduced binding to Co(II) compared with serum albumin of nonischemic individuals. This reduced binding of Co(II) to serum albumin has also been observed in individuals with transient myocardial ischemia after elective percutaneous transluminal coronary angioplasty (3). The value of the Co(II)–albumin binding assay was further validated in a multicenter study of acute coronary syndrome patients (4).

We undertook this study to evaluate whether the Co(II)–albumin binding assay could be clinically useful in patients with suspected myocardial injury in the setting of an emergency department of a health maintenance organization. Because the N-terminal amino acid sequence DAHK provides the binding site for Co(II) and other transitional metals (5, 6), we examined the N-terminal amino acid sequence of the purified albumin obtained from arbitrarily selected individuals with high absorbance units (ABSU) to understand the mechanism of reduced Co(II) binding to serum albumin.

Materials and Methods
Stored serum samples that were originally obtained from the emergency department at Kaiser Foundation Hospital (Honolulu, HI) and maintained at −20 °C were used. The interval between sample collection and analysis was <8 weeks. On the basis of results obtained for five randomly chosen specimens, we determined that storage at −20 °C did not significantly affect the values measured by the assay. The mean between-run CV was 6.3% for tests at 3-week intervals, which was comparable to the mean within-run CV (4.3%). Blood had been collected in red-top Vacutainer Tubes in the absence of any anticoagulants, and serum had been harvested by centrifugation for 10 min at 1000g within 30 min of collection. The final discharge diagnosis was taken for each of the individuals as noted in the medical record chart. After the diagnosis was recorded, specimens were arbitrarily numbered and all patient identifiers were removed to retain anonymity. The study was of a double-blinded and masked design. When all of the assays were completed, diagnostic information was merged with the Co(II)–albumin assay data. The protocol of this study was approved by the Institutional Review Board of Kaiser Foundation Hospital.

Nonischemic Individuals
Specimens were designated as nonischemic if the chart-reviewed diagnosis showed no evidence of myocardial ischemia or the individual was generally healthy. Individuals in this group consisted of 39 (42%) males and 53 (58%) females 17–88 years of age.

Ischemic (Non-MI and MI) Individuals
Patients with renal diseases were excluded from this study. Clinical assessment of myocardial ischemia included several objective clinical indices, imaging studies, ECG studies, and serum cardiac biochemical markers, namely CK-MB and cardiac troponin I (cTnI). The diagnosis of MI was based on criteria defined by the Joint European Society of Cardiology/American College of Cardiology Committee (1), which are as follows: “Criteria for acute, evolving or recent MI...Typical rise and gradual fall (troponin) or more rapid rise and fall (CK-MB) of biochemical markers of myocardial necrosis with at least one of the following: a) ischemic symptoms; b) development of pathologic Q waves on the ECG; c) ECG changes indicative of ischemia (ST segment elevation or depression); or d) coronary artery intervention (e.g., coronary angioplasty).” The ischemic group consisted of 46 (61%) males and 29 (39%) females 43–100 years of age.

Study Protocol
Spectrophotometric Co(II)–albumin binding assay. The assay is based on the premise that myocardial ischemia causes changes in HSA that are demonstrated by reduced exogenous Co(II) binding (2). The concentration of ischemia-modified serum albumin can be determined by addition of a known amount of Co(II) to a serum specimen and measurement of the unbound Co(II) by colorimetric assay using dithiothreitol (DTT). An inverse relationship thus exists between the amount of albumin-bound cobalt and the intensity of the color formation. All reactions were carried out in 1.5-mL Eppendorf tubes at standard room temperature and in duplicate. Preparations for the Co(II)–albumin binding protocol involved the addition of 200 μL of patient serum to 50 μL of a solution of 1 g/L cobalt chloride, followed by vigorous mixing, and a 10-min incubation. DTT (50 μL of a 1.5 g/L solution) was then added and mixed. After a 2-min incubation, 1.0 mL of a 9.0 g/L solution of NaCl was added. The absorbance of assay mixtures was read at 470 nm with a Hewlett Packard 8452A Diode Array Spectrophotometer. The blank was prepared similarly with the exclusion of DTT. All chemicals, including cobalt chloride and DTT, were purchased from Sigma-Aldrich.

Linearity of the assay. The linearity of the albumin–Co(II) binding assay was examined with use of commercial HSA
over a concentration range of 20–60 g/L and was analyzed by linear regression.

**Precision of the assay.** Within-run precision was assessed by analyzing 10 replicates of a pooled serum with ABSU close to 0.50. The same sample was also used for the between-day precision study over 5 consecutive days.

**Purification and sequence determination of the NH₂ terminus.** We evaluated the N-terminal sequence of HSA from one nonischemic individual and seven individuals with myocardial ischemia with ABSU ≥0.70, using the Applied Biosystems Procise Sequencer 494 automated sequencer. The sequencer cleaved the 11 amino acid residues at the NH₂ terminus by rapid liquid-phase Edman degradation as part of the analytical process.

**Purification of HSA from blood specimens.** Approximately 200 µL of human serum was diluted 10-fold with phosphate-buffered saline. The solution was loaded on a column of Cibacron Blue immobilized on Sepharose 6B (Amersham). After the column was washed with 10 bed volumes of phosphate-buffered saline, HSA was eluted with 3 mol/L NaCl. The eluate was dialyzed against phosphate-buffered saline and passed over a column of Lipidex-1000 (Packard Instruments) to remove hydrophobic ligands possibly bound to HSA (7). The resulting protein exhibited only one band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentrations were determined by the bicinchoninic acid method (BCA; Pierce).

**Measurement of cardiac markers.** CK was measured by an enzymatic rate method (8) on a Beckman LX20 Synchron instrument. CK-MB and cTnI were measured on a Bayer ACS180, and the results were expressed in mass units (9–11). The reference intervals and the cutoff values for cardiac markers used in this study were as follows. CK-MB >5.0 µg/L is considered suggestive for AMI. We also used the CK index [(CK-MB/CK) × 100, where CK-MB is in µg/L and CK is in U/L] to differentiate CK-MB arising from cardiac and noncardiac tissues. CK index values ≥2.5 are considered suggestive for AMI. A cTnI value ≥1.0 µg/L is suggestive for AMI. The minimum detectable concentration was 0.2 µg/L for both cTnI and CK-MB. The CK reference interval was 61–224 U/L for males and 38–173 U/L for females. For CK-MB measurements, the between-day CV was 3.5% (n = 24) and 3.3% (n = 24) at mean concentrations of 4.3 and 88.5 µg/L, respectively. For cTnI measurements, the between-day CV was 6.3% (n = 24) and 4.6% (n = 24) at mean concentrations of 0.88 and 36.4 µg/L, respectively. For the CK index, the between-day CV was 1.7% at a mean concentration of 57 U/L.

**STATISTICAL ANALYSIS**

Study groups were analyzed using ROC curves with cutoff points ranging from 0.30 to 1.00 ABSU with 0.05-ABSU intervals, which gave rise to a 15-category scale. Sensitivity [(true positive)/(true positive + false negative)] and specificity [(true negative)/(true negative + false positive)] were obtained for all cutoff points. The positive predictive value [(true positive)/(true positive + false positive)] and negative predictive value [(true negative)/(true negative + false negative)] were obtained for the optimal cutoff point. The area under the curve was obtained by the trapezoidal rule (12). Binomial distribution statistics were used to calculate the 95% confidence intervals (95% CIs). The Wilcoxon test was used to determine the P values for group comparisons; P values <0.05 considered significant.

**Results**

The corresponding ABSU for the linearity study ranged from 0.74 to 0.30. The regression equation was as follows: ABSU = 0.9257 – 0.0100(albumin, in g/L). The square of the correlation coefficient (r²) was 0.9719. The ranges for absorbance values for the Co(II)–albumin binding assay were 0.30–0.60 and 0.45–1.00 for the nonischemic and ischemic individuals, respectively. The nonischemic group had a mean ± 2 SD of 0.43 ± 0.10 ABSU, whereas the ischemic populations had a higher mean, 0.63 ± 0.25 ABSU (P <0.0001). The within-run CV was 2.5–6.0% for five repeats of 10 replicates with a mean CV of 4.1% at a mean ABSU of 0.526. The between-day CV was 3.8% at a mean ABSU of 0.536. Fig. 1 shows a scatter plot distribution of the results for the 167 individuals tested. ROC curve analysis was used to determine performance characteristics and/or the optimal cutoff of this assay for identifying individuals with myocardial ischemia from nonischemic individuals and AMI individuals from non-AMI ischemic individuals. For nonischemic and ischemic
suitable for assessing myocardial ischemia. The Co(II) and myoglobin, used in assessing cellular necrosis, are not individuals. Biochemical markers such as CK-MB, cTnI, currently, there are no well-defined biochemical markers ing to the trapezoidal rule (12) to be 0.95 (95% CI, 0.92–0.99). At a cutoff point of 0.50 ABSU, sensitivity and specificity were 88% (95% CI, 78–94%) and 94% (86–98%), respectively (Fig. 2). The positive predictive value was 92% (95% CI, 83–97%), and the negative predictive value was 91% (83–96%). ROC curve analysis revealed an area of 0.66 (0.53–0.79; Fig. 2) for non-AMI vs AMI ischemic individuals, suggesting a poor discrimination between the two groups. N-Terminal amino acid sequence analysis for six of the seven individuals with high ABSU (patients C–H) showed a wild-type N-terminal sequence of DAHKSEVAHRF. However, in one individual who was non-ischemic and had a high ABSU (patient B), the sequence observed was XXHKSEVAHRF, in which the two amino acids (DA) were absent (Table 1). The control, nonischemic individual had a wild-type sequence.

**Discussion**

Currently, there are no well-defined biochemical markers for identification of myocardial ischemia in non-AMI individuals. Biochemical markers such as CK-MB, cTnl, and myoglobin, used in assessing cellular necrosis, are not suitable for assessing myocardial ischemia. The Co(II)–albumin binding assay has been reported to be an early marker for myocardial ischemia (2, 4, 5).

Our results confirm previous studies (2–4) that reported that the Co(II)–albumin colorimetric assay distinguishes myocardial ischemic patients from nonischemic patients (P <0.0001). However, the test is a poor discriminator between ischemic patients with and without MI. ROC curve analysis of the Co(II)–albumin assay was consistent with these observations, and at a cutoff value of 0.50, the sensitivity and specificity were 88% and 94%, respectively, for identifying individuals with myocardial ischemia from nonischemic individuals. Because it is known that Co(II) binding occurs at the N-terminal region of HSA (5, 6), we examined the N-terminal amino acid sequence of purified albumin from randomly selected sera with high ABSU values (≥0.70) as well as one with a low ABSU value (0.35). All of the albumin specimens had a normal N-terminal amino acid sequence up to the 11th amino acid residue, with the exception of one that lacked the N-terminal DA dipeptide. This individual with serum albumin lacking N-terminal DA residues and a high ABSU did not have the diagnosis of myocardial ischemia; hence, this was considered a false-positive test. All of the remaining specimens with high ABSU values were from patients with a diagnosed myocardial ischemia except the one with a low ABSU value that was obtained from a nonischemic individual. Therefore, the DA deletion is probably not correlated with myocardial ischemia. A previous study (13) showed the presence of albumin lacking the Asp (D) or Asp-Ala (DA) residues in the N-terminal region in normal human serum. These species are presumably present because of a defect in hepatic intracellular processing of proalbumin and do not bind nickel. The prevalence of the deletions of the N-terminal residues of HSA is not known.

**Table 1. Sequence data for individuals with high ABSU and one individual with low ABSU.**

<table>
<thead>
<tr>
<th>Individual</th>
<th>ABSU (470 nm)</th>
<th>N-Terminal sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (control)*</td>
<td>0.35</td>
<td>DAHKSEVAHRF&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.80</td>
<td>--HKSEVAHRF&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.76</td>
<td>DAHKSEVAHRF</td>
</tr>
<tr>
<td>D&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.76</td>
<td>DAHKSEVAHRF</td>
</tr>
<tr>
<td>E&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.74</td>
<td>DAHKSEVAHRF</td>
</tr>
<tr>
<td>F&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.70</td>
<td>DAHKSEVAHRF</td>
</tr>
<tr>
<td>G&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.93</td>
<td>DAHKSEVAHRF</td>
</tr>
<tr>
<td>H&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.73</td>
<td>DAHKSEVAHRF</td>
</tr>
</tbody>
</table>

*<sup>a</sup>Clinical diagnosis: *nonischemia; †ischemia.
<sup>b</sup>Wild-type sequence.
<sup>c</sup>Sequence missing DA residues.

HSA is the most abundant multifunctional protein in blood, with a mean concentration of 0.63 mmol/L; it consists of 585 amino acid residues (66.5 kDa), is synthesized in the liver, and has a half-life of ~19 days. The binding of transition metals to the N-terminal region of albumin has been studied. However, the biochemical mechanism that causes altered Co(II) binding to albumin during ischemia is not understood; it appears to be reversible, as shown in a study of patients with transient ischemia induced during elective percutaneous transluminal angioplasty (3). Co(II)–albumin assay results were abnormal during transient ischemia and returned to baseline values by 6 h after percutaneous transluminal angioplasty. In vivo structural modifications that alter the binding capacity of albumin for Co(II) can potentially occur as the result of acidosis, reduced oxygen tension,
various ion-pump disruptions, and generation of free radicals. In vitro N-terminal auto cleavage of the DA residues from the NH$_2$ terminus of albumin stored at 30 °C has also been reported (14). The physiologic relevance of this observation is yet to be understood.

In conclusion, understanding the modifications that may potentially occur in vivo that affect Co(II)–albumin interactions (e.g., methylation, N-acetylation, and Cu$^{2+}$ mobilization) may provide better methods for the early diagnosis of myocardial ischemia.

References