ประสิทธิภาพในการวินิจฉัยของการตรวจวิเคราะห์ร่วมของ Ischemia-Modified Albumin และ Protein Carbonyl ในภาวะกล้ามเนื้อหัวใจตายแบบ เอส ที่ ยก

สาระ คำานวณ*1 รูปภาพ แนะรุ่นเรียงวงศ์*2 เสาร์นี* เหลื่องว่ามิ อัจฉริยา ภูระพันธุ์*2 อธิชาติ ยาชะยั่ง*2 กิตติ อินทุธาส*2 เหมือนเพื่่ โภชน์ที่*3 ปาร์เรีย เสนาระรง*3 ทวิพร สำราญพัฒนา*2 และ อะซร ทองศรี*2

*1 ภาควิชาเทคนิคการแพทย์ คณะศิริราชศัลย มหาวิทยาลัยนวมินทราชินี
*2 ภาควิชาเทคนิคการแพทย์ศิริราชศัลย คณะศิริราชศัลย มหาวิทยาลัยนวมินทราชินี
*3 ภาควิชาการแพทย์ควบคุม คณะศิริราชศัลย มหาวิทยาลัยนวมินทราชินี

บทคัดย่อ
การศึกษาที่มีต่อประสิทธิภาพในการประเมินความสามารถในการวิเคราะห์ Ischemia Modified Albumin (IMA) และ Protein Carbonyl (PC) ในการวินิจฉัยภาวะกล้ามเนื้อหัวใจตายแบบเอส-ที่ยก และประสิทธิภาพในการวิเคราะห์ร่วมของสารวิเคราะห์ที่ 2 ชนิด ในชีวิตของผู้ป่วยเตียงนาน 30 ราย เปรียบเทียบข้อมูลของผู้ป่วยที่ได้รับการวินิจฉัยแต่ละวิธีการต่อเนื่องกันว่ามีการกล้ามเนื้อหัวใจตายแบบเอส-ที่ยกนาน 30 ราย ผลการค้นพบว่าระดับของ IMA ในชีวิตของผู้ป่วย สรุปว่าในผู้สูญเสียสุขภาพต่อเนื่องมีอัตราสัดส่วนตัวที่สูงสุดในระดับ PC ที่ชีวิตของผู้ป่วย 80.00% ทั้งในผู้ที่เป็นโรค IMA รวมกับ PC พบว่าพื้นที่มีผลบวกมั่นคงอยู่สูงสุด 80.00% และประสิทธิภาพของวิธีการตรวจ IMA รวมกับ PC พบว่ามีการกล้ามเนื้อหัวใจตายแบบเอส-ที่ยก 85.00%

*การทบทวน: กล้ามเนื้อหัวใจตายแบบเอส-ที่ยก, Ischemia Modified Albumin (IMA), Protein Carbonyl (PC)
*การติดต่อขอข้อมูล: ดร.สาระ คำานวณ ภาควิชาเทคนิคการแพทย์ คณะศิริราชศัลย มหาวิทยาลัยนวมินทราชินี 085-966-353 โทรสาร 085-966-300 E-mail address: sarawutk@nu.ac.th
Diagnostic Performance of Combinatorial Determination of Ischemia Modified Albumin and Protein Carbonyl in ST Elevation Myocardial Infarction

Sarawut Kumphune*, 1, Titiporn Mekrungruangwong², Saowanee Luangaram³, Ajchara Putaloon², Apichart Yathongchai³, Kitti Intuyot¹, Meunfun Opanun¹, Pajaree Sanorwong¹, Sivaporn Samranphat⁵ and Tomon Thongsri⁴

¹Department of Medical Technology
²Department of Cardiac Thoracic Technology
³Department of Physical Therapy, Faculty, of Allied Health Sciences, Naresuan University
⁴Department of Medicine, Buddhachinaraj Hospital

Abstract

The aims of this study were evaluation of the ability of using Ischemia Modified Albumin (IMA) and Protein Carbonyl (PC) for ST elevation myocardial infarction (STEMI) diagnosis and efficiency of combinatorial determination these two markers. Serum from 30 STEMI patients, and 30 healthy control donors were measured for the level of serum IMA by Albumin Cobalt Binding assay, and PC content by spectrophotometric 2,4-Dinitrophenylhydrazine (DNPH) assay. The cutoff level of IMA and PC content were analyzed, and the diagnostic sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and efficiency were analyzed. The serum IMA level in STEMI was significantly higher (p = 0.0007) than that of healthy controls with a sensitivity of 60%, a specificity of 96.67%, a PPV of 94.74%, a NPV of 70.73%, and a test efficiency of 78.33%. Similarly, the serum PC content level in STEMI was significantly higher (p < 0.0001) than that of healthy controls.
with a sensitivity of 63.33%, a specificity of 96.67%, a PPV of 95.00%, a NPV of 72.75%, and efficiency of 80.00%. Moreover, measuring serum IMA level in combination with PC content enhanced NPV to 80.00%, and test efficiency to 85.00%.

Keywords: ST Elevation Myocardial Infarction (STEMI), Ischemia Modified Albumin, Protein Carbonyl

*Corresponding author:* Dr. Sarawut Kumphune, PhD, Department of Medical Technology, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok, 65000 Thailand Tel.: +66(0)83-960 6006 Fax: +66(0) 55 966 300 E-mail address: sarawutk@nu.ac.th
Introduction

Cardiovascular diseases (CVD), principally heart disease and stroke, are a leading cause of global morbidity and mortality. The World Health Organization (WHO) statistic report 2008 predicted that, although non-communicable diseases are considered the leading killers worldwide currently, ischemic heart disease and cerebrovascular disease (stroke) will become the 2 diseases resulting in the majority of deaths in 2030.\(^{1}\) Most seriously, ischemic heart disease is still ranked on the top of the mortality by cause from 2004 to 2030. Although the annual mortality from this disease is decreasing in developed countries\(^{2}\) it is increasing in the more populace developing countries.\(^{3}\)

The diagnosis of Acute Myocardial Infarction (AMI) focus on clinical assessment such as history of chest pain associated with Electrocardiogram (ECG) changes such as ST-segment elevation (STEMI) and biochemical markers alterations.\(^{4}\) However, lack of specificity of symptom and low sensitivity of ECG result in misdiagnosis and delay essential intervention. Assessment of cardiac biomarker level is the one of the most essential and effective way to detect myocardial damages. Current conventional cardiac markers e.g. Creatine Kinase-MB (CK-MB), cardiac Troponin T or I (cTnT, cTnI) are released as a result of cardiomyocytes necrosis, which irreversibly damage and turn to cell death.\(^{5}\)

It has been known that myocardial ischemia and/or ischemia–reperfusion injury claimed as an oxidative stress, which can generate Reactive Oxygen Species (ROS), subsequently resulted in biochemical modifications of some biomolecules such as protein, lipid, and DNA.\(^{6-7}\) Ischemia-modified albumin (IMA) and Protein Carbonyl (PC) have known as proteins that are modified on the similar basis of oxidation–reduction reaction.\(^{6, 8, 9}\) Determination of IMA concentration has been approved by US FDA for a use marker for diagnosis of acute myocardial ischemia after the onset of the chest pain.\(^{10}\) Similar to PC, it has been reported that the level of serum protein carbonyl correlated to the severity of coronary artery disease\(^{6}\) and possibly used as a marker for myocardial ischemia. However, as lack of specificity of IMA, it is reported that the interpretation of a positive IMA finding combined with other clinical indices improve diagnostic power.\(^{11}\) Therefore, we aimed to evaluate the ability of using IMA and PC as cardiac markers for diagnosis of ST elevation myocardial infarction (STEMI) and the diagnostic performance when perform combinatorial measurement of these two markers.
Materials and Methods

Study Population

We assessed 30 patients who were admitted to the Coronary Care Unit of Buddhachinaraj Hospital, Phitsanulok, Thailand, with a final diagnosis of ST elevation myocardial infarction (STEMI) according to the pattern of ECG change with ST segment elevation. Patients who had diabetes, liver diseases, renal diseases, will not be recruited in this study. Similarly, smoking behavior, taking antioxidant supplements were considered exclusion criteria. Another thirty healthy controls were also recruited from healthy blood donors. In this control group, all volunteer subjects were screened for history of any heart diseases, liver diseases, renal diseases, diabetes, smoking behavior, and taking antioxidant supplements. All subjects gave written informed consent before enrolling to this study, and the study protocol was approved by the Naresuan University ethical clearance committee on human rights related to research involving human subjects.

Sample collection and preparation

The whole blood from healthy subjects and STEMI patients were collected and spin down for serum collection. All sera were collected within 2 hours after blood collection. The serum will be frozen at \(-20^\circ\text{C}\) or lower until the experiments were performed.

Spectrophotometric determination of serum Ischemia Modified Albumin

A rapid, spectrophotometric determination of serum IMA was developed from Bar-O et al.\(^{(12)}\) Two hundred microliter of human serum were incubated with 50 \(\mu\text{L}\) of 0.1% cobalt chloride (Sigma, CoCl\(_2\cdot6\text{H}_2\text{O}\)) in \(\text{H}_2\text{O}\) for 10 min at room temperature for adequate cobalt-albumin binding. Fifty microliters of dithiothreitol (DTT) (Sigma, 1.5 mg/ml \(\text{H}_2\text{O}\)) was added for colorizing the reaction for 2 min before quenching with 1.0 mL of 0.9% NaCl. Then, the absorbance were measured at 470 nm (Shimadzu, model UV160U), color development with DTT was compared to a serum-cobalt blank without DTT and reported in absorbance units (ABSU). The within-run coefficient of variation (CV) was 1.00% and between-day coefficient of variation (CV) was 4.75%. The linearity regression of the test in both standard albumin, and pooled serum were 0.9274 and 0.9524, respectively.

Spectrophotometric determination of serum Protein Carbonyl

Colorimetric measurement of protein carbonyl (PC) were performed on the basis the reaction between 2, 4- dinitrophenylhydra-
zone (DNPH) and protein carbonyls, producing a Schiff base to produce a corresponding hydrazone, which can be measured spectrophotometrically.\(^{13,14}\) The serum was diluted in 1:10 with phosphate-buffered saline (PBS) to the final concentration of total protein less than 10 mg/ml. Two hundred microliters of diluted serum were mixed with 800 μl of 10 mM DNPH in 2.5 M HCl. Sample control were also prepared by adding equal volume of diluted serum to 800 μl of 2.5 M HCl. Then, the reactions were incubated at room temperature for 1 hour, the dark environment with 15 min interval of vortexing. One milliliter of 20% (w/v) Trichloroacetic acid (TCA) was added before incubating on ice for 5 min. After centrifugation at 10,000 g for 10 min at 4°C, the supernatants were collected and mixed with 1 ml of 10% (w/v) TCA before spinning down at 10,000 g for 10 min at 4°C. The protein pellet was washed for 3 times with 1 ml of 1:1 (v/v) ethanol: ethyl acetate and centrifuged at 10,000 g for 10 min at 4°C. After final washing, the protein pellet was resuspended in 500 μl of 6 M guanidine hydrochloride and centrifuged at 10,000 g for 5 min at 4°C. The supernatant were collected and measured the absorbance at 370 nm using control as a blank. The protein carbonyl concentration (nmol/ml) was calculated from the equation 1. The total corrected protein amount (mg), in the final step of reaction, was calculated from a bovine serum albumin standard curve dissolved in 6 M guanidine hydrochloride and read at 280 nm. The protein amount was calculated using equation 2. Then the carbonyl content (nmol/mg) was calculated from protein carbonyl concentration per amount of corrected protein. The within-run coefficient of variation (CV) was 7.93% and between-day coefficient of variation (CV) was 7.47%. The linearity regression of the test using standard albumin was 0.9670.

Equation 1: Protein Carbonyl (nmol/ml)
\[
[A_{370}/0.011M^{-1}] \times [500 \mu l/200\mu l]
\]
Equation 2: Protein concentration (mg/ml) = \[(A_{280} - y \text{ intercept})/\text{slope}\] × 2.5 × 10

Statistical analysis

Data were analyzed by the GraphPad Prism version 5.00. Data were tested for normal distribution by Shapiro–Wilk test. Differences of IMA and PC level between groups were analyzed by unpaired t-test or Mann–Whitney U test. A p-value < 0.05 was considered to be statistically significant. The receiver operating characteristic (ROC) curve analysis and the area under the curve was performed for determination of serum IMA and PC in the all STEMI patients included in the
study population. The optimum cutoff values for determination of serum IMA and PC were selected from the ROC analysis. This optimum cutoff was used to dichotomously classify positive or negative serum IMA and PC level, and used for calculating of diagnostic sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic efficiency of the test.

**Results**

**Baseline characteristic**

The baseline characteristics of all subjects in the study were summarized as shown in Table 1. Of note, 70% of STEMI patients in this study were men. The serum CK-MB activity and cTnT in all of STEMI patients in this study was higher than reference level.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Healthy Controls (n=30)</th>
<th>STEMI (n=30)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>36.03 ± 11.04</td>
<td>60.20 ± 11.28</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gender (men/women)</td>
<td>14/16</td>
<td>21/9</td>
<td>-</td>
</tr>
<tr>
<td>CK-MB (u/L)</td>
<td>NA</td>
<td>102.3 ± 14.95</td>
<td>-</td>
</tr>
<tr>
<td>cTnT (ng/ml)</td>
<td>NA</td>
<td>4.81 ± 1.26</td>
<td>-</td>
</tr>
<tr>
<td>IMA (ABSU)</td>
<td>0.4693 ± 0.08</td>
<td>0.5779 ± 0.17</td>
<td>0.0007</td>
</tr>
<tr>
<td>Protein Carbonyl content (nmol/mg)</td>
<td>0.3946 ± 0.07</td>
<td>0.6749 ± 0.33</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Diagnostic efficiency of using IMA for diagnosis of myocardial infarction.

Serum Ischemia Modified Albumin (IMA) was spectrophotometrically determined, by albumin cobalt binding (ACB) test, from all STEMI patients and healthy controls. The results showed that mean of serum IMA level in STEMI was 0.5779 ± 0.17 ABSU, which was significantly higher than healthy controls (mean serum IMA = 0.4693 ± 0.08, $p = 0.0007$) Fig. 1. The area under ROC curve of ABC test for determining serum IMA level in STEMI patients was 0.7556 (95% CI, 0.6208–0.8903). The optimum decision level for IMA was determined to be 0.5495 ABSU (Fig. 2), and this decision limit was used in subsequent analyses for diagnostic performance, which was calculated for diagnostic sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and test efficiency. The results showed yielded a sensitivity of 60% (95% CI, 40.06%–77.34%), a specificity of 96.67% (95% CI, 82.78%–99.92%) (Table 2), a PPV of 94.74%, a NPV of 70.73%, and a test efficiency of 78.33% (Table 3).

![Fig. 1](image)

**Fig. 1** Level of serum ischemia modified albumin in STEMI compared with healthy control. Inside the scatter plot represent the mean and standard deviation. Comparison of serum IMA level is performed using Mann-Whitney test. The mean of serum IMA level in STEMI was 0.5779 ± 0.17 ABSU, which is significantly higher than that of healthy controls ($p = 0.0007$)
Fig. 2 Receiver Operator Characteristic curve (ROC) of ischemia modified albumin (IMA) for detecting acute myocardial infarction. The area under the ROC=0.7556 (95% Confidence Intervals: 0.6208-0.8903). Best IMA cut-off point: 0.5495 ABSU (sensitivity: 60.00%; specificity: 96.67%).

Table 2 Diagnostic sensitivity and specificity of measuring serum IMA and PC content

<table>
<thead>
<tr>
<th>Markers</th>
<th>Cutoff</th>
<th>Sensitivity</th>
<th>95% CI</th>
<th>Specificity</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMA</td>
<td>0.5495</td>
<td>60.00%</td>
<td>40.06% -77.34%</td>
<td>96.67%</td>
<td>82.78%-99.92%</td>
</tr>
<tr>
<td>PC</td>
<td>0.5140</td>
<td>63.33%</td>
<td>43.86%-80.07%</td>
<td>96.67%</td>
<td>82.78%-99.92%</td>
</tr>
</tbody>
</table>

Table 3 Diagnostic efficiency of measuring serum IMA, PC content, and combination of IMA and PC

<table>
<thead>
<tr>
<th>STEMI</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMA</td>
<td>94.74</td>
<td>70.73</td>
<td>78.33</td>
</tr>
<tr>
<td>PC</td>
<td>95.00</td>
<td>72.50</td>
<td>80.00</td>
</tr>
<tr>
<td>IMA + PC</td>
<td>92.00</td>
<td>80.00</td>
<td>85.00</td>
</tr>
</tbody>
</table>
Discussion

In the present study we demonstrated the ability of two oxidative modified proteins, ischemia modified albumin (IMA) and protein carbonyl (PC), as a potent cardiac biomarkers for diagnosis of acute myocardial infarction. We also aimed to examine the usefulness of combinatorial measurement of serum IMA and PC content to identify acute myocardial infarction.

Generation of reactive oxygen species resulted in modification of some biomolecules such as lipid, proteins and nucleic acid. The process of protein oxidation introduces new functional groups such as hydroxyls and carbonyls group\(^{15, 16}\), which generated by many different mechanisms, which can then indicate the degree of ROS mediated protein oxidation contribute to altered function and turnover.\(^{15, 16}\) The outcomes of protein oxidation are fragmentation, cross-linking and unfolding may accelerate or hinder proteolytic and proteosome-mediated turnover, according to the severity of oxidative damage. IMA and PC were found to be modified as a result of oxidative modification and also in many oxidative related disorders such as myocardial ischemia, renal ischemia, etc.\(^{5, 6, 12, 17-19}\)

According to this, there are many studies demonstrated the ability of these two proteins as the early biomarkers. For IMA, N-terminal modifications resulted in reduction to cobalt binding capacity of serum protein albumin.\(^{9}\) Therefore, the Albumin Cobalt Binding (ACB) test was developed to determine the level of serum IMA and was also approved by US Food and drug Administration (FDA) in 2003 as the diagnostic test for Acute Myocardial Infarction.\(^{20}\) However, the ACB test is not specific for myocardial ischemia. It also found that IMA can be increased in cerebral, gastrointestinal intestinal and skeletal muscular ischemia as well as myocardial ischemia.\(^{11}\) Therefore, it is recommended that the interpretation of a positive IMA finding should be combined with other clinical indices.\(^{11}\) So, it is hypothesized that determination of serum IMA, in combination with PC level may be useful for AMI diagnosis.

Our study showed the usefulness of determining serum IMA and PC content level to identify acute myocardial infarction. The level of both serum IMA and PC content were significantly higher in STEMI compared to healthy control. ROC analysis showed that measuring serum IMA level and PC content can be used as a diagnostic test for acute myocardial infarction. With the optimum cutoff, the sensitivity of measuring IMA and PC were around 60%, with NPV of 70–72%, which were not high enough. The efficiency of the test was 78–80%. However, the results
showed the high specificity of 96.67% and gave PPV of 94–65%. Moreover, determination of serum IMA level in combination of serum PC content level improved test performance by increase NPV to 80% and efficiency of the test to 85%.

Therefore the results suggested that combinatorial determination of these two biomarkers capable of increasing the diagnostic performance, similar to previous findings that combination of IMA with cTnT enhanced diagnostic efficiency for acute myocardial infarction.\(^{(21)}\) Although combinatorial determination of serum IMA and cTnT have benefit to enhance the efficiency of the test\(^{(21)}\), but cTnT is a necrosis marker used in assessing cellular necrosis and not early enough to assess an early phase of acute myocardial ischemia, which may occur without proceeding to infarction.\(^{(22)}\) Moreover, it has been reported that IMA assay can distinguished myocardial in serum within 30 minutes after ischemia\(^{(23)}\), but determination of cTnT or CK-MB, another classical cellular necrosis markers, need at least 3–6 hours to detect the significant level of these markers.\(^{(24)}\) Therefore, combining IMA measurement with cTnT or CK-MB, probably is not suitable and useful in case of diagnosis of early phrase of cardiac injury, such as in Non-ST elevation myocardial infarction (NSTEMI). Promising new markers that appear earlier than cTnT or CK-MB, which can be used in combination with IMA, is benefit in term of the earliness of detection and finally resulted in improvement of patient outcomes. Protein carbonyl is the most widely used oxidative modified marker, similar to IMA. PC increase rapidly in blood stream and still remain in at least 24 hours.\(^{(25)}\) Studied from Mutlu-Turkoglu et al. indicated that PC increase in coronary artery disease, atherosclerotic lesion in human and during ischemia-reperfusion.\(^{(26)}\) Generation of PC can be found following myocardial infarction suggested that diagnostic potential of PC may use as the marker of coronary artery disease.\(^{(27)}\) So, our study showed combination of measuring serum PC content has diagnostic usefulness for acute myocardial infarction.

This study has some limitations. In this study there was a significant different in age between STEMI and Healthy control. According to the source of specimens from healthy controls were collected some blood donors and the average age of the donors are less than 60 years old, unlike the STEMI population whom the average age is approximately 60 years old. It has been reported that oxidative modified forms of proteins accumulate during aging.\(^{(16)}\) For example, increases in protein carbonyls occur in rat hepatocytes, drosophila, brain, and kidney of mice and in brain tissue of gerbils.\(^{(28)}\)
In humans protein carbonyls increase with age in brain, muscle, and human eye lens. The carbonyl content of human fibroblasts also increases as a function of age of the donor. However, in our study, age differences between STEMI and healthy control were not affected with the interpretation of serum IMA level and PC content level. As shown in Fig. 5, there were no correlation between age and the level of serum IMA and PC content. It has been demonstrated by Aparci M. et al. that the relation of albumin cobalt binding capacity test results is independent to the existing of hypertension, diabetes, and advanced age.

![Protein Carbonyl Content](image)

**Fig. 3** Level of serum Protein Carbonyl (PC) content in STEMI compared with healthy control. Inside the scatter plot represent the mean and standard deviation. Comparison of serum PC content level is performed using unpaired t test. The mean of serum PC content level in STEMI was $0.6749 \pm 0.33$ nmol/mg, which is significantly higher than that of healthy controls ($p < 0.0001$).
Fig. 4 Receiver Operator Characteristic curve (ROC) of Protein Carbonyl (PC) content for detecting acute myocardial infarction. The area under the ROC = 0.7950 (95% Confidence Intervals: 0.6678-0.99). Best IMA cut-off point: 0.5140 nmol/mg (sensitivity: 63.33%; specificity: 96.67%)

Fig. 5 Correlation of age and serum IMA, PC content level in STEMI. There are no correlation between age and serum IMA, PC content level in STEMI, r = 0.2060 and 0.1675, respectively.
It has been reported that some of patients with angina pectoris have not shown elevation of ST segment on electrocardiogram (ECG) or so-called Non-ST elevation myocardial infarction (NSTEMI). Although NSTEMI are associated with a lower degree of 30-day mortality than STEMI, mortality rates are higher in those patients who survive to admission to hospital.\(^\text{30}\) Undetectable of ST-elevation of ECG lead to delay in final diagnosis and affect treatment and clinical outcome. Therefore, determination of high sensitivity, specificity and early ischemic biomarker is useful for diagnosis of acute myocardial ischemia, particularly in NSTEMI patients, and need to be further investigated.

**Conclusion**

Our finding demonstrated the benefit of measuring serum IMA level and PC content as a diagnostic marker for acute myocardial infarction. In addition, determination of IMA level in combination with PC content enhanced diagnostic efficiency. Further study will be investigated the usefulness and applicability of these early markers for early diagnosis in NSTEMI.

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**References**


