Title: A METHOD FOR REDUCING HEPARIN INTERFERENCE IN DIAGNOSTIC TESTS FOR CARDIAC TROPONIN

Effect of two Heparin Antagonists on the Immunomatrix cTNI Immunoassay

![Graph showing effect of Polybrene and Protamine on cTNI concentration]

Abstract: The present invention is directed to a process for reducing heparin anticoagulant interference binding in antibody-antigen diagnostic assay, such as a diagnostic assay for cardiac Troponin I. The process utilizes a moiety, such as a highly charged peptide, which competes with heparin at the antibody-antigen binding site. Preferably, the moiety is a heparin antagonist such as a protamine salt or hexadimethrine bromide (polybrene).
A METHOD FOR REDUCING HEPARIN INTERFERENCE IN DIAGNOSTIC TESTS FOR CARDIAC TROTONIN

[0001] This application derives priority from U.S. Provisional Application No. 60/266,892, filed on February 7, 2001, and incorporated herein.

FIELD OF THE INVENTION

[0002] The present invention relates to assays for diagnostic testing. More particularly, the invention is directed to processes for reducing anticoagulant interferences in diagnostic tests, such as diagnostic immunoassays for cardiac Troponin I.

BACKGROUND OF THE INVENTION

[0003] Cardiac Troponin is a complex of three proteins of the thin filament of muscle. It is a muscle protein which functions to regulate muscle contraction.

[0004] Cardiac Troponin I measurement has become the method of choice in detection of cardiac muscle damage because its structure in heart muscle is unique muscle found in other parts of the body. The measurement has been facilitated by the generation of antibodies directed to the cardiac specific regions of Troponin I and incorporating these into commercially available assays. The major issue with these assays is that there is a lack of both standardization and correlation among methods. (Wu, A.H.B. Laboratory and Near Patient Testing for Cardiac Markers. J. Clin Ligand Assay, 22: 32-27 (1999)).
The standardization issue is further complicated by the fact that Troponin I appears as free or complexed moieties which are associated through charge interaction. The literature also suggests that the binding reactivity of the free and complexed forms differs in different assays. (Mockel, M. et al The acute coronary syndrome diagnosis and prognostic evaluation by troponin I is influenced by the test system affinito to different troponin complexes. Clinica Chimica Acta 293: 139-155 (2000).

It is believed that heparin binds to cardiac Troponin I (cTNI) via a charge-charge interaction. Heparin is frequently found in blood derived as a compound in samples because it is used as an anti-coagulant for sample collection or it is being used as a treatment in cardiac patients. The interaction of heparin with both free and complexed Troponin I can further complicate the assay results. It is reported by one manufacturer that the bias seen with heparin samples is 30% lower than in serum or other plasma samples (Dade Behring Opus plus Troponin I instructions for use). Consequently, it would be desirable if such interference could be overcome in order to provide a more accurate result. It would also be desirable if the correlation of different assay methods could be improved with the elimination of an interfering factor.

SUMMARY OF THE INVENTION

In one aspect, the present invention is directed to a process for reducing heparin anticoagulant interference binding in an antibody-antigen diagnostic assay, such as a diagnostic assay for cardiac Troponin I. The process utilizes a moiety, such as a highly charged peptide, which competes with heparin at the antibody-antigen
binding site. Preferably, the moiety is a heparin antagonist such as a protamine salt or hexadimethrine bromide (polybrene).

[0008] In another aspect, the invention relates to a process for increasing the sensitivity of an antibody-antigen diagnostic assay for cardiac Troponin I. The process utilizes a moiety such as a highly charged peptide which competes with heparin at the antibody-antigen binding site. Preferably, the moiety is a charged peptide with a sequence similar to the native sequence but sufficient different to allow for a specific antibody-antigen reaction. More preferably, the moiety is a heparin antagonist, and most preferably, the moiety is a protamine salt such as protamine sulfate or polybrene.

[0009] In a further aspect, the present invention is directed to a quantitative strip base immunoassay such as a quantitative strip base luminescent immunoassay to be used in the point of care environment. The immunoassay is useful for the testing for indications of acute myocardial infarction. The immunoassay can be utilized in conjunction with a moiety which competes with heparin at the antibody-antigen binding site or the moiety can be included in strip based immunoassay, such as with a solubilized substrate, etc.

[0010] The invention accordingly comprises several components or steps and the relation of one or more of such components or steps with respect to each of the others and the article possessing the features, properties, and the relation of elements exemplified in the following detailed disclosure.
BRIEF DESCRIPTION OF THE DRAWINGS

[00011] The invention may take form in various components and arrangements of components and in various steps and arrangements of steps. The drawings are only for purposes of illustrating preferred embodiments and are not to be construed as limiting the invention.

[00012] Fig. 1 is a listing of the skeletal and cardiac amino acid sequences for human Troponin I;

[00013] Fig. 2 is a chart illustrating the kinetics of cardiac Troponin I in three human matrices;

[00014] Fig. 3 is a graph showing the effect of protamine against heparine using an immunoassay for cardiac Troponin I;

[00015] Fig. 4 is a chart demonstrating the effect produced by the heparin antagonists, polybrene and protamine, on an immunoassay for cardiac Troponin I;

[00016] Fig. 5 is an illustration of quantitative strip based luminescent immunoassay containing, or utilized in conjunction with, a heparin antagonist.

DETAILED DESCRIPTION OF THE INVENTION

[00017] It has previously been thought that heparin binds to positively charged areas of the cTNI molecule. (Uettwiller-Geiger, D. et al/ Analytical performance of Beckman Coulter's Access AccuTnl (troponin I) in a multicenter evaluation. Clin. Chem. 47: A204 number 670,20001). When the sequence is examined, applicants noted that the critical antibody binding domains required for specificity contain highly charged amino acids. In order to verify that these regions were responsible for heparin
association, applicants attempted to compete the binding of heparin to cTNI with synthetic peptides with sequences and charges similar to sequences and charges in the native molecule but with sufficient differences to not cause interference with the specific antibody binding. The specific region is in the amino acid N terminus 7 to 38 positions. (see Figure 1) These studies supported the hypothesis that the heparin was binding to the highly charged sequences associated with the antigen binding site.

[00018] Subsequently, a series of charged moieties were evaluated for their ability to compete for the binding of heparin to cTNI. All of the molecules tested that had a strong positive charge were found to be effective in eliminating the interference by heparin that had been previously observed. In particular, protamine sulfate and polybrene were evaluated and found to be particularly effective.

[00019] The present invention is further illustrated by the following examples. It is to be understood that the present invention is not limited to the examples, and various changes and modifications may be made in the invention without departing from the spirit and scope thereof.

**EXAMPLES**

Example 1:

[00020] Fresh frozen human plasma and heparinized human plasma (both from SeraCare, San Diego CA) were compared in spike and recovery experiments using human cTNI (Hytest, Finland) preparations at estimated values of 25, 50 and 75 ng/ml concentrations. Recovery in a prototype cTNI assay (Immunomatrix, Gaithersburg, MD) showed between 50 and 91% lower values in the heparinized plasma than the fresh
frozen plasma. Based on these findings, an examination of the kinetics of binding was performed using heparinized plasma (SeraCare, San Diego), heparinized plasma with the addition of 2mg/ml protamine sulfate salt (Sigma, St. Louis MO) and serum (in house pool) all spiked with 20ng/ml of human cTNI (Hytest, Finland). The results showed that the serum kinetics were at completion after 10 minutes with a 50% Bmax reached in 1 minute. Heparinized plasma showed much slower kinetics reaching completion after 40 minutes with a 50% Bmax after 10 minutes. Heparinized plasma with the addition of 2 mg/ml of protamine showed kinetics similar to those of serum with a 50% Bmax after 1 minute and a 10 minute completion time (Figure 2). The results show that a low bias will result in a cTNI assay of a fixed time if heparinized plasma is compared to serum but that this bias can be removed with the addition of the highly charged cationic protamine protein.

Example 2:

[00021] Heparinized plasma aliquots (SeraCare, San Diego, CA) with or without the addition of 2 mg/ml of protamine sulfate were spiked with various concentrations of cTNI (Hytest, Finland) ranging from 0.5 ng/ml to 100 ng/ml. These were then tested for cTNI concentration as blinded samples on both the Opus Plus system (Dade Behring, Newark, DE) and the Vitros Immunoassay System (Ortho Diagnostic Systems, Raritan, NJ). The results showed a remarkable under recovery without protamine but significant increase in samples in both systems with the addition of protamine (see table 1 below). Although there is over-recovery with the Protamine in both systems, it was unclear as to how the original material was calibrated, its composition of free and complexed Troponin and overall purity. (Wagner, TL et al On the Interaction of Cardiac Troponin I
(cTNI) and Heparin. A Possible Solution. Clin. Chem. 47: A212, Number 696, 2001.) It did however demonstrate the suppression of reactivity by heparin in the two systems examined and the removal of suppression with the addition of protamine.

Table 1: Evaluation of Heparin and Heparin / Protamine samples by Opus and Vitros Troponin I Immunoassay Systems

<table>
<thead>
<tr>
<th>Gravimetric values*(ng/ml)</th>
<th>Heparin</th>
<th>Heparin / Protamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Opus</td>
<td>Vitros</td>
</tr>
<tr>
<td>0</td>
<td>&lt;0.5</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>&lt;0.5</td>
<td>0.16</td>
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<td>2</td>
<td>&lt;0.5</td>
<td>0.562</td>
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<td>5</td>
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<td>1.6</td>
</tr>
<tr>
<td>10</td>
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<td>3.29</td>
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<tr>
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<tr>
<td>100</td>
<td>16.9</td>
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</tr>
</tbody>
</table>

* Based on values provided with the cTNI from Hytest

Example 3:

[00022] Heparin samples with or without protamine were prepared at levels of 0, 2.5 and 5 ng/ml of cTNI using the gravimetric values provided by the manufacturer (Hytest, Finland). These samples were then used in the Immunomatrix prototype cTNI assay which uses two antibodies in a sandwich format. The antibodies (BiosPacific, Emeryville, CA) are directed to the peptide 3 and peptide 4 sequences (see figure 1). Peptide 3 was suspected to have a possible heparin interference site due to the amino acid sequence information. The samples were tested and the results showed a shallow curve with the heparin samples but a much steeper curve with the samples that contained 2mg/ml protamine. (Figure 3) The zero values were similar regardless of the
presence or absence of protamine. Therefore, we conclude that the addition of protamine will increase the sensitivity of the cTNI assay.

Example 4:

[00023] There are other heparin antagonists available commercially. We evaluated hexadimethrine bromide (polybrene) (Sigma, St. Louis, MO) as an alternative to protamine in the Immunomatrix prototype cTNI assay as described in example 3. Using samples prepared from heparinized plasma with either 2 mg/ml protamine or 1 mg/ml polybrene and spiked with levels of cTNI at 0, a low concentration (1ng/ml) or a high concentration (50 ng/ml) an evaluation of the effect of these two heparin antagonists was made. The results (figure 4) show that either of these two antagonists gives a similar increase in the RLU readings obtained in the cTNI immunoassay. We therefore conclude that either of these heparin antagonists would be suitable for use in the cTNI assay to reduce the interference observed in heparinized samples.

[00024] The examples indicate that protamine and other heparin antagonists appear to complex heparin and therefore prevent heparin from binding to Cardiac Troponin I (cTNI). This in turn produces more accurate readings for plasma patient samples and/or diminishes the bias observed in cTNI assays.

[00025] The invention has been described with reference to the preferred embodiments. Obviously, modifications and alterations will occur to others upon reading and understanding the proceeding detailed description. It is intended that the
invention be construed as including all such modifications and alterations insofar as they come within the scope of the appended claims or the equivalents thereof.
WHAT IS CLAIMED IS:

1. A method of reducing heparin anticoagulant interference binding in an antibody-antigen diagnostic test for cardiac Troponin I comprising the step of adding to the test a moiety which competes with heparin at the antibody-antigen binding site.

2. The method of claim 1, wherein the antigen-antibody binding site comprises binding sites on the human Troponin I molecule which are known to be specific for cardiac Troponin I.

3. The method of claim 1, wherein the antigen-antibody binding site comprises amino acids found between the 7 and 84 positions.

4. The method of claim 1, wherein the moiety is a highly charged peptide.

5. The method of claim 1, wherein the moiety is a charged peptide with a sequence similar to the native sequence but sufficiently different to allow for a specific antibody-antigen reaction.

6. The method of claim 1, wherein the moiety is a heparin antagonist.

7. The method of claim 1, wherein the moiety is a protamine salt.
8. The method of claim 7, wherein the protamine salt is protamine sulfate.

9. The method of claim 6, wherein the antagonist is hexadimethrine bromide (polybrene).

10. A method of reducing interference produced by an anticoagulant in a diagnostic test for cardiac Troponin I utilizing antibody-antigen binding for cardiac Troponin I comprising the step of increasing the kinetics of the binding pair in the presence of an interfering anticoagulant by the addition of a specific antagonist.

11. The method of claim 10, wherein the anticoagulant is heparin.

12. The method of claim 11, wherein the antagonist is specific for heparin.

13. The method of claim 12, wherein the antagonist is a protamine salt.

14. The method of claim 12, wherein the antagonist is protamine sulfate.

15. The method of claim 12, wherein the antagonist is hexadimethrine bromide (polybrene).

16. A quantitative strip based immunoassay for determining the presence of cardiac Troponin I, comprising a sandwich immunoassay containing anti-Troponin I antibodies
linked to a solid support; solubilized anti-Troponin I antibodies bond to an indicator substrate; and a solubilized antagonist for heparin.

17. The immunoassay of claim 16, wherein the antagonist is protamine salt.

18. The immunoassay of claim 16, wherein the antagonist is hexadimethrine bromide.
Figure 1.

Human Troponin I amino acid sequences.

Peptide 1 = 1 to 15
Peptide 2 = 16 to 25
Peptide 3 = 26 to 39
Peptide 4 = 40 to 65

Skeletal TnI
Cardiac TnI

Stated areas are differences between skeletal and cardiac TnI sequences.
Figure 2. Kinetics of cTNI in three human matrices

![Graph showing kinetics of cTNI in different matrices.](image)

- Serum
- Plasma
- Plasma/Protamine

Incubation Time (min.)

%B$_{0 max}$
Figure 3: Effect of Protamine in Heparin Matrix Using the Immunomatrix cTnI Assay
Figure 4: Effect of two Heparin Antagonists on the Immunomatrix cTNI Immunoassay

Relative cTNI concentration
Immunomatrix cTnI Assay

Protocol

10ul of Sample Applied
60ul of chase Applied
Allow test to run for 4 minutes
Read test in Luminometer for 1 minute

Figure 5.

Heparin Antagonists
Protamine Sulfate

Hexadimethrine Bromide - Polybrene

\[
\text{HGPRRRSSSRVRPRRRRRR-GGGRR-OH} \quad \text{(20 H}_2\text{SO}_4)\]

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_3 \\
\text{CH}_3 & \quad \text{CH}_3 \\
\text{N} - (\text{CH}_2)_n - (\text{CH}_2)_m - & \quad \text{2Br}.
\end{align*}
\]
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : G01N 33/53, 33/68, 33/533
US CL : 435/6, 7.1, 7.2, 7.5, 7.71, 7.72, 7.95, 288.5, 288.7, 960, 971, 973; 436/518, 523, 528, 535, 538, 819

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 7.2, 7.5, 7.71, 7.72, 7.95, 288.5, 288.7, 960, 971, 973; 436/518, 523, 528, 535, 538, 819

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practicable, search terms used)

MEDLINE, EMBASE, SCISEARCH, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>X</td>
<td>WO 99/40442 A1 (PASTEUR SANOFI DIAGNOSTICS) 12 August 1999, see Abstract.</td>
<td>1-6, 9-12, 15-16, 18</td>
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<td>Y</td>
<td>US 5,677,133 A (OBERHARDT) 14 October 1997, see entire document.</td>
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<td>A</td>
<td>US 5,320,812 A (HARPER) 14 June 1994, see entire document.</td>
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See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"X" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"Y" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"A" document member of the same patent family

Date of the actual completion of the international search

06 May 2002 (06.05.2002)

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12 JUN 2002

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