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The Nijmegen Modification of the Bethesda Assay for Factor VIII:C Inhibitors: Improved Specificity and Reliability

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Summary

Antibodies against factor VIII coagulant activity can appear in haemophiliacs who are treated with factor VIII preparations but also spontaneously in non-haemophiliacs. The Bethesda assay is the most commonly used method to detect these antibodies, but it lacks specificity especially in the lower range resulting in unreliable data.

Two modifications are proposed and tested to resolve the imperfections:
1. Buffering the normal plasma used in the assay- and control mixture with 0.1 M imidazole to pH 7.4.
2. Replacing the imidazole buffer in the control mixture by immunodepleted factor VIII deficient plasma.

These modifications allow better discrimination between positive and negative samples and improve reliability.

Introduction

The development of factor VIII-inhibitors may complicate substitution treatment in haemophiliacs. Consequently, these patients are in danger of uncontrolled bleeding and need long-term treatment with expensive factor VIII preparations to induce immunotolerance.

Early reports concerning the incidence of inhibitors were controversial (1), but recent prospective studies confirmed their presence in the blood between 5% and 20% of haemophiliacs (2-8). Moreover, inhibitors were also detected in healthy individuals (9).

The reasons for the wide variation in results are manifold and include the type of concentrate used, the severity of haemophilia, exposure time as well as ethnic origin, family history and age at the start of substitution therapy. However, both over- and underestimation might originate from the use of the Bethesda assay used to detect inhibitors. There is no agreed cut-off value for the Bethesda Units (BU/ml) to discriminate between inhibitor-positive and negative patients which range from zero to 0.8 BU/ml (2-10) which indicates that the assay lacked specificity especially at the lower range of activity.

We recently drew attention to this anomaly and reported that insufficient buffering and variable protein concentration in the assay medium may produce false positive results (11). In this report, we describe two modifications of the original Bethesda assay which result in markedly increased specificity and allowed us to diagnose and monitor the presence of factor VIII:C inhibitors.

Materials and Methods

Plasma Preparation

Nine volumes of blood were drawn into one volume of 0.13 M sodium citrate using Vacutainers (Becton-Dickinson, New Jersey, USA). Plasma was prepared by centrifugation for 15 min at 4300 × g and 4° C, then stored at −70° C until required.

Normal pooled plasma (N-pool) was prepared by mixing plasma from at least 50 healthy blood donors aged 18-65 years selected to represent all blood groups and sexes equally.

Buffering was achieved by adding solid imidazole (Merck, Darmstadt, Germany) to yield a concentration of 0.1 M then adjusting the pH by slowly adding 1 N HCl while constantly stirring at 4° C.

Factor VIII:C Assay

Factor VIII:Coagulant activity (FVIII:C activity) was measured in a one stage APTT-based clotting assay (12) using PTT(a)-reagent (Stago, Asniere, France), Immunodepleted factor VIII deficient plasma (Organon Teknika, Durham, USA) and N-pool as a 100% reference.

Clotting times were recorded with a KC-10 coagulometer (Amelung, Lemgo, Germany).

One Bethesda Unit (BU) was defined as that amount of inhibitor that resulted in 50% residual FVIII:C activity.

The inhibitor activity was read in BU/ml from a semilogarithmic plot representing the correlation between residual FVIII:C activity (logarithmic) and inhibitor activity (linear) (13). When the residual FVIII:C activity in the test mixture was below 25%, retesting was performed by diluting with 0.1 M imidazole buffer pH 7.4 until a residual FVIII:C activity of 25% to 75% was obtained.

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Table 1  pH values and remaining FVIII:C activities in test- and control mixtures of the original and modified Bethesda assay

<table>
<thead>
<tr>
<th>Assay mixtures</th>
<th>pH before incubation</th>
<th>pH after 2 h incubation</th>
<th>Remaining FVIII:C activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-buffered N-pool and haemophilic plasma (inhibitor free) 1:1</td>
<td>7.7 ± 0.1</td>
<td>8.3 ± 0.1</td>
<td>68 ± 6</td>
</tr>
<tr>
<td>Non-buffered N-pool and 0.1 M imidazole buffer pH 7.4 1:1</td>
<td>7.6 ± 0.1</td>
<td>7.8 ± 0.1</td>
<td>83 ± 6</td>
</tr>
<tr>
<td>0.1 M imidazole buffered N-pool pH 7.4 and haemophilic plasma 1:1</td>
<td>7.4 ± 0.1</td>
<td>7.4 ± 0.1</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>0.1 M imidazole buffered N-pool and immunodepleted factor VIII deficient plasma 1:1</td>
<td>7.4 ± 0.1</td>
<td>7.4 ± 0.1</td>
<td>97 ± 4</td>
</tr>
<tr>
<td>0.1 M imidazole buffered N-pool and 0.9% saline 1:1</td>
<td>7.4 ± 0.1</td>
<td>7.4 ± 0.1</td>
<td>83 ± 8</td>
</tr>
</tbody>
</table>

All data are the means of 5 determinations ± the standard deviation.

Modified Bethesda Assay

Test plasma as well as immunodepleted factor VIII deficient control plasma (pool of at least 20 individuals, Organon Teknika, Durham, USA) were mixed with equal volumes of 0.1 M imidazole buffered at pH 7.0, 7.4, 7.7, 8.0, 8.3 and 8.6, and either citrated congenital FVIII deficient plasma (inhibitor free), immunodepleted FVIII deficient plasma (Organon Teknika, Durham, USA) or saline (0.9% NaCl). Patients

Samples of plasma were obtained from 4 groups of patients: 32 haemophiliacs without history of inhibitor: 10 with less than 1 percent FVIII:C activity (group Ia) and 22 with activity between 1 and 20% (group Ib), 5 patients with a history of factor VIII inhibitor but free of medication and symptoms of inhibitor at the time of sampling (group II), 6 patients who were being treated for an inhibitor at the time of sampling and still had evidence of inhibitor-activity such as bleeding complications and decreased recovery after factor VIII substitution (group III) and 8 patients with high levels of inhibitor activity in the initial stage of treatment with immunosuppressives (group IV).

Results

pH Changes and FVIII:C Inactivation

The pH of the mixture of non-buffered N-pool and inhibitor-free haemophilic plasma (test mixture in the original Bethesda assay) increased to about 8.3 during incubation whereas the pH of the original control mixture of N-pool with 0.1 M imidazole-buffer pH 7.4 only slightly increased to about 7.8 (Table 1). In addition, a lower amount of FVIII:C activity was remaining in the test mixture compared to the control mixture (p < 0.05). Buffering the N-pool with 0.1 M imidazole stabilized the pH in the mixtures with factor VIII deficient plasma, both congenital and immunodepleted, and 0.9% saline at the initial value of pH 7.4. The FVIII:C inactivation was only 3–5% in the buffered plas-
Table 2. Anti-FVIII:C activity in patient samples with the original and modified Bethesda assay

<table>
<thead>
<tr>
<th>Category</th>
<th>Type of patient</th>
<th>FVIII:C activity in plasma (%)</th>
<th>No. of patients</th>
<th>Inhibitor activity original assay (BU/ml)</th>
<th>Inhibitor activity modified assay (BU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>Severe haemophiliacs no history of inhibitor</td>
<td>&lt;1</td>
<td>10</td>
<td>0.5-0.9</td>
<td>0.0</td>
</tr>
<tr>
<td>Ib</td>
<td>Mild/sub-haemophiliacs no history of inhibitor</td>
<td>1-20</td>
<td>22</td>
<td>0.5-0.9</td>
<td>0.0</td>
</tr>
<tr>
<td>II</td>
<td>Haemophiliacs history of inhibitor free of symptoms and medication</td>
<td>2-8</td>
<td>5</td>
<td>0.1-0.7</td>
<td>0.0</td>
</tr>
<tr>
<td>III</td>
<td>Haemophiliacs under treatment for inhibitor</td>
<td>&lt;1-3</td>
<td>6</td>
<td>0.5-1.9</td>
<td>0.6-1.7</td>
</tr>
<tr>
<td>IV</td>
<td>Patients with high level of inhibitor in initial stage of treatment</td>
<td>&lt;1</td>
<td>7</td>
<td>13-315</td>
<td>10-320</td>
</tr>
</tbody>
</table>

Table 3. Inhibitor assays with the modified Bethesda method in samples with high inhibitor activities

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>(type of inhibitor)</td>
<td>Dilution factor</td>
<td>Residual FVIII:C activity</td>
</tr>
<tr>
<td>1 (I)</td>
<td>250</td>
<td>66</td>
</tr>
<tr>
<td>2 (I)</td>
<td>400</td>
<td>20</td>
</tr>
<tr>
<td>3 (I)</td>
<td>400</td>
<td>76</td>
</tr>
<tr>
<td>4 (I)</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>5 (I)</td>
<td>400</td>
<td>75</td>
</tr>
<tr>
<td>6 (II)</td>
<td>400</td>
<td>75</td>
</tr>
<tr>
<td>7 (II)</td>
<td>40</td>
<td>76</td>
</tr>
</tbody>
</table>

Column A: Plasma samples were assayed in two different dilutions and inhibitor activities were calculated from the correlation graph and multiplied with the dilution factor.

Column B: Plasma samples were assayed in at least 4 different dilutions and the activity was estimated by finding the dilution giving 50% inhibition.

Plasma from the patients in group II showed no activity in the modified assay whereas with the original assay the activity ranged from 0.1 to 0.7 BU/ml. With both methods increased inhibitor activities were detected in all samples from patients in group III, but all except one sample showed lower activities (0.2-0.4 BU) with the modified method. All samples from patients in group IV showed high activities with both methods. However, the calculated inhibitor activity in the latter varied with the degree of plasma dilution (Table 3, column A). In plasma of patients with type I inhibitor (samples 1-5) the activity increased with decreasing dilution whereas the empirically determined data were in between (Table 3, column B). In contrast, plasma of patients of type II inhibitor (6, 7) showed maximal activity at the highest dilution and the empirically determined inhibitor activities were even higher.

Plasma mixtures rising to approximately 17% in mixtures with saline. This relation between pH and FVIII:C inactivation was further investigated in mixtures of buffered N-pool at various pH levels and factor VIII deficient plasma or saline (Fig. 1). FVIII:C inactivation increased with increasing pH but was only marginal at physiological pH. FVIII:C inactivation in mixtures with half the protein concentration was higher at all pH levels compared to mixtures of N-pool and deficient plasma.

Patients

No inhibitor activity was detected with the modified method in the samples obtained from the 32 haemophiliacs of groups Ia and Ib whereas with the original assay inhibitor activities ranging from 0.5 to 0.9 BU/ml were found (Table 2).
Residual FVIII: C activity

Typical curves representing the relation between plasma dilution and residual FVIII: C activity in type I and type II patients are shown in Fig. 2. None of the samples exhibited a linear correlation between concentration and the logarithm of the residual FVIII: C activity.

Discussion

The Bethesda assay is the most widely used method for detection of FVIII: C inhibitors. FVIII: C inactivation in both a test mixture (normal plasma/patient plasma) and a control mixture (normal plasma/0.1 M imidazole buffer pH 7.4) is measured and expressed in inhibitor activity. As was implied but not specifically noted by Kasper (13), reported by Biggs (14) and confirmed by our findings. Data above the hypothetical inactivation curve between 50% and 100% residual FVIII: C activity of each individual sample will result in an underestimation of inhibitor activity whereas data below the curve will result in the converse which explains that reduced protein concentration leads to further inactivation of FVIII: C activity. These phenomena may be the source of falsely positive inhibitor activities in patient samples when assayed with the original Bethesda assay. We therefore introduced two substitutions in order to circumvent these imperfections.

Firstly, the N-pool plasma used in test and control mixture was buffered with 0.1 M imidazole to stabilize the pH at 7.4. Secondly, the imidazole buffer in the control mixture was replaced by immuno-depleted factor VIII deficient plasma to attain comparable protein concentrations in both mixtures. As a result, FVIII: C inactivation was virtually identical in both control and test mixtures being marginal in both.

Plasma-samples of 32 haemophiliacs which were free of inhibitor activity yielded values of 0.0 BU/ml with the modified method, whereas activities between 0.5 and 0.9 BU/ml were obtained with the original method. Plasma-samples from patients with a history of inhibitor activity that was absent at the time of sampling gave identical results.

Samples from patients that were receiving immunosuppressive medication and who still had clinical evidence that an inhibitor was present, showed elevated activities with both methods but the activities with the modified method were lower than with the original method.

These data indicate that the specificity of the modified method was improved while the sensitivity remained unchanged.

The specificity for detection of high inhibitor activities was similar with both methods but the calculated activities were dependent on the degree of dilution. This appears to be due to the aberrant kinetics of factor VIII and its inhibitor which is not linearly correlated but is complex as was implied but not specifically noted by Kasper (13), reported by Biggs (14) and confirmed by our findings. Data above the hypothetical linear curve between 50% and 100% residual FVIII: C activity of each individual sample will result in an underestimation of inhibitor activity whereas data below the curve will result in the converse which explains the anomalies shown in Table 3. Quantification of high inhibitor activities will be erroneous unless a concentration-independent assay is devised.

If there is a need for more uniform quantification, e.g. follow-up of treatment, it may be more relevant to estimate empirically the plasma dilution that gives 50% inhibition.

In conclusion, we present two modifications of the original Bethesda assay which improve the specificity and reliability and suggest a method for the optimal quantification of high inhibitor activities.

We suggest to define this method the “Nijmegen assay” for detection and quantification of FVIII: C inhibitors.

Acknowledgements

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References