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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.

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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 Yersey, 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, Asnière, 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).

Original Bethesda Assay

Anti-FVIII:C activity was measured with the Bethesda assay according to Kasper (13).

N-pool was mixed with either an equal volume of patient plasma (test mixture) or with 0.1 M imidazole buffer pH 7.4 (control mixture). After 2 h of incubation at 37° C, the relative percentage FVIII:C activity of the test mixture compared to the control mixture (residual FVIII:C activity) was determined. The FVIII:C activity in the test mixture was corrected for intrinsic residual FVIII:C activity (e. g. at mild or sub-haemophilia).

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 of undiluted sample 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.

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

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

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 N-pool pH 7.4 and incubated for 2 h at 37° C. The FVIII:C activity was measured in both mixtures and corrected for the intrinsic activity. The inhibitor activity was quantified as described for the original Bethesda assay. If necessary, plasma samples were diluted before testing with immunodepleted factor VIII deficient plasma.

In addition, samples with inhibitor activity of more than 2 BU/ml were also assayed by empirically determining the plasma concentration which gives 50% inhibition. Therefore, a number of dilutions were tested and the residual FVIII:C activity of at least 4 assays within the range of 25% to 75% were plotted versus the plasma dilution semilogarithmically or arithmetically. The concentration that gave 50% residual FVIII:C activity was estimated by extrapolation and the reciprocal value of it equals the inhibitor activity in BU/ml.

pH Instability in Assay Mixtures and pH-dependent FVIII:C Inactivation

pH values and FVIII:C activities were measured in test and control mixtures of the original and the modified Bethesda assay before and after 2 h of incubation at 37° C.

pH related FVIII:C inactivation was studied by measuring both pH and FVIII:C activity before and after 2 h of incubation at 37° C in mixtures of N-pool containing 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).

The remaining FVIII:C activity was defined as the activity obtained after incubation compared to the preincubation value.

All pH measurements were carried out with a PHM-92-meter (Radiometer, Copenhagen, Denmark).

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).

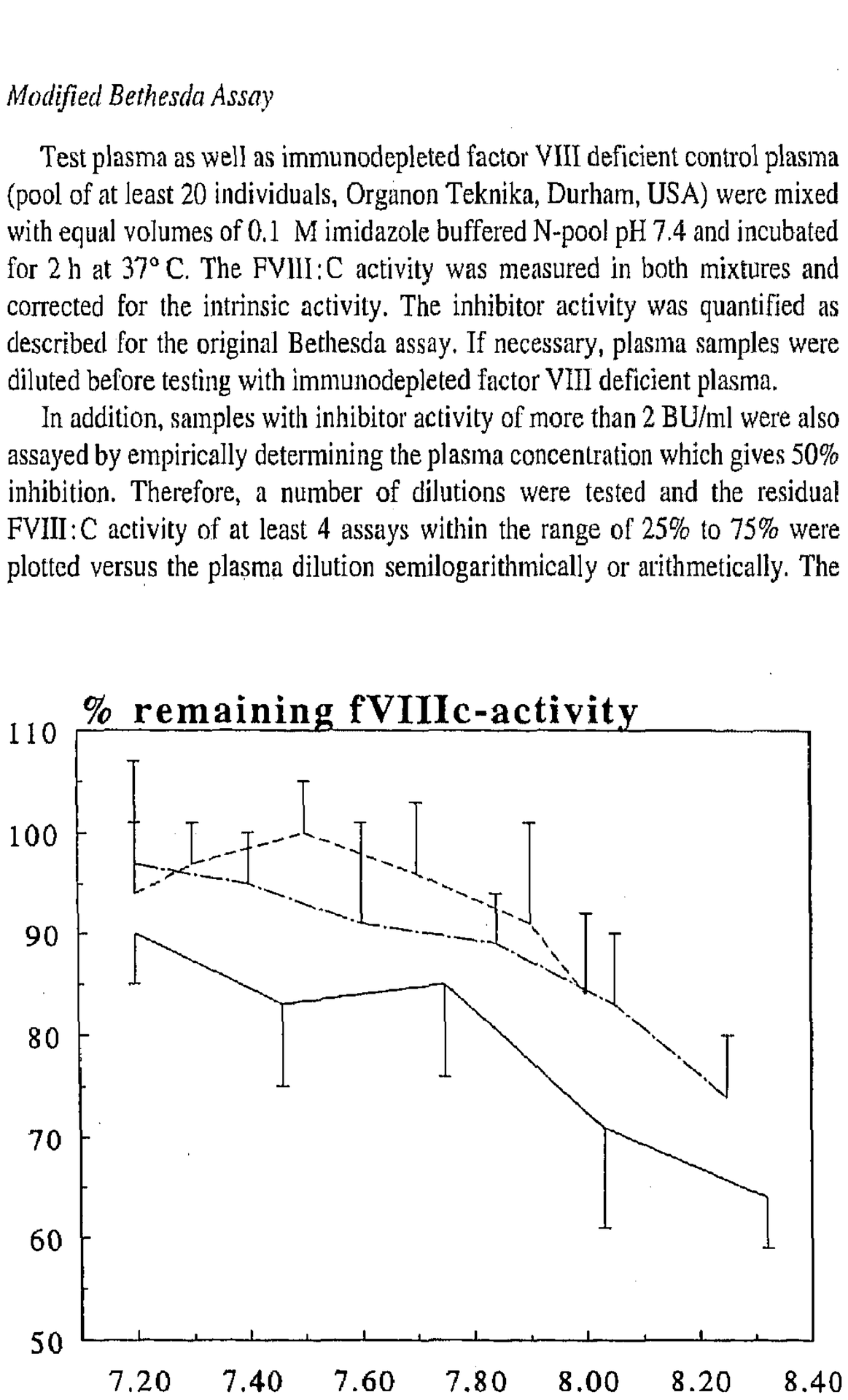

Fig. 1 pH dependent FVIII:C inactivation. Remaining FVIII:C activity is measured in mixtures of 0.1 M imidazole buffered N-pool and either haemophilic plasma (–·–·–), immunodepleted factor VIII deficient plasma (– – –) or saline 0.9% (——) after 2 h incubation at 37° C. Data are the mean of 5 assays ± the standard deviation. The plotted pH represents the actual pH measured after incubation and differs from that of the preset value of the buffered N-pool because of the intrinsic buffering capacity of the other constituents in the mixtures

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

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

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

Patient no.	A			B
(type of inhibitor)	Dilution factor	Residual FVIII:C activity	Calculated inhibitor activity (BU/ml)	Empirically estimated inhibitor activity (BU/ml)
1 (I)	250	66	150	180
	100	24	205	
2 (I)	200	76	80	85
	40	20	116	
3 (I)	120	74	54	60
	40	26	78	
4 (I)	200	75	84	105
	75	33	120	
5 (I)	40	84	10	17
	10	29	18	
6 (II)	400	71	200	220
	50	29	90	
7 (II)	800	76	320	320
	100	38	140	

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.

ma 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).

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.

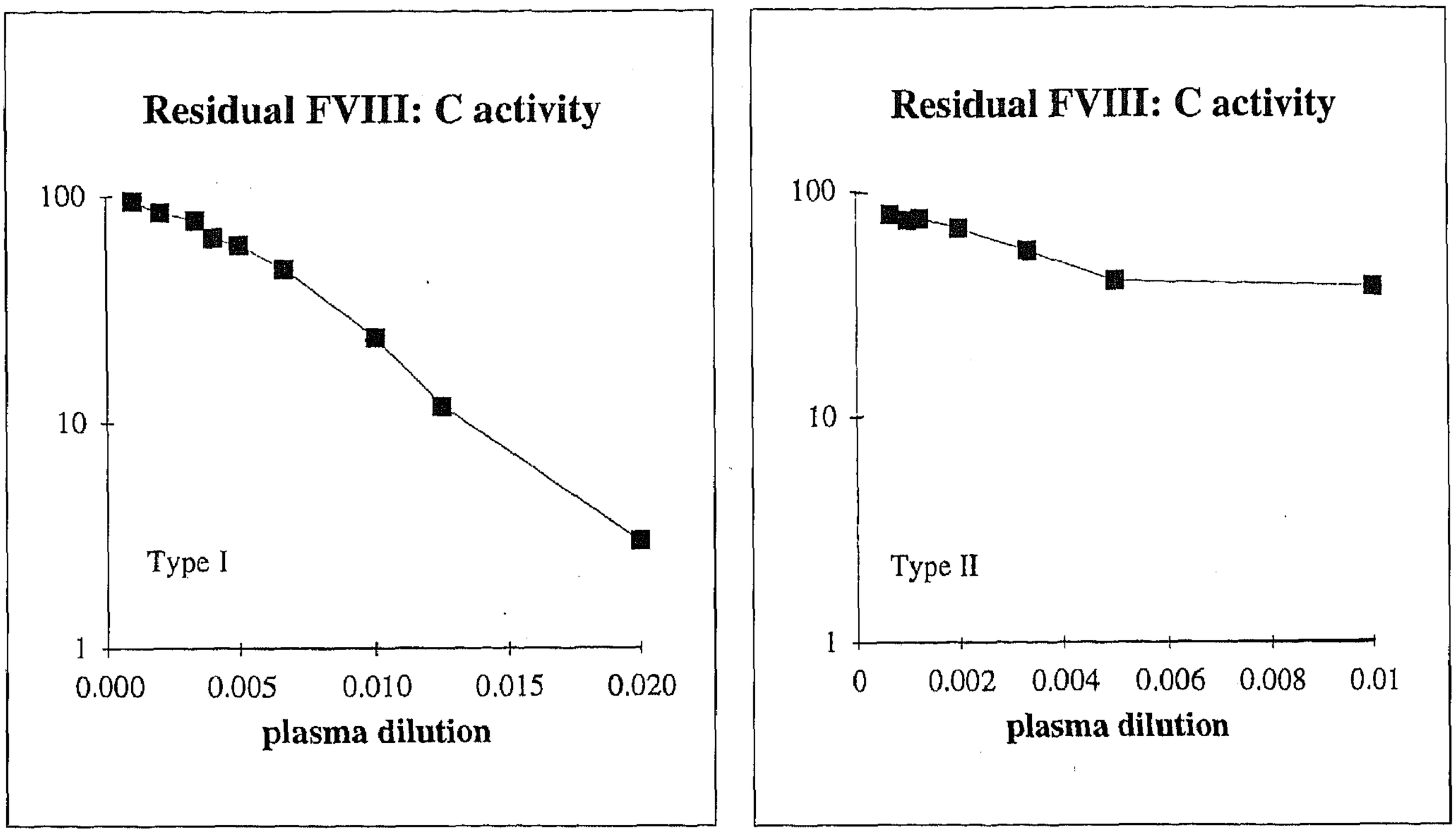

Fig. 2 Correlation between logarithm of residual FVIII : C activity and plasma concentration of a sample with high inhibitor activity type I and type II

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.

We observed a significant increase in pH *and* FVIII : C inactivation in mixtures of N-pool and inhibitor-free factor VIII deficient plasma. In experiments with imidazole-buffered N-pool (pH ranging from 7.2 to 8.3) we showed that FVIII : C inactivation increases with pH and 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 immunodepleted 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.

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References

1. Aledort L. World registry on factor VIII inhibitor patients: Why? Seminars in Hematology 1993; 30 (2) (Suppl 1): 7–9.
2. Addiego J, Gomperts E, Liu S-L, Bailey P, Courter S, Lee M, Neslund G, Kingdon H, Griffith M. Treatment of hemophilia A with a highly purified factor VIII concentrate prepared by anti-FVIIIc immunoaffinity chromatography. Thromb Haemost 1992; 67 (1): 19–27.
3. Ciavarella N, Schiavoni M. Frequency of inhibitors in haemophiliacs: letter to the editor. The Lancet 1992; 339: 1301.
4. Lusher J, Arkin S, Abildgaard C, Schwartz R, and the Kogenate Previously Untreated Patient Study Group: Recombinant factor VIII for the treatment of previously untreated patients with hemophilia A – Safety, efficacy, and development of inhibitors. N Engl J Med 1993; 328: 453–9.
5. Bray G, Courter S, Lynes M, Lee M, Gomperts E, and the Recombinate Study Group: Safety, efficacy, and inhibitor risk of recombinant factor VIII (Recombinate) in a cohort of previously untreated patients (PUPs) with severe hemophilia. Thromb Haemost 1993; 69: 1205 (Abstract).
6. Peerlinck K, Arnout J, Gilles J, Saint-Remy J, Vermylen J. A higher than expected incidence of factor VIII inhibitors in multitransfused haemophilia A patients treated with an intermediate purity pasteurized factor VIII concentrate. Thromb Haemost 1993; 69: 115–8.
7. Rosendaal F, Nieuwenhuis H, Van den Berg H, Heijboer H, Mauser-Bunschoten E, Van der Meer J, Smit C, Strangers P, Briët E, and the Dutch Hemophilia Study Group. A sudden increase in factor VIII inhibitor development in multitransfused hemophilia A patients in The Netherlands. Blood 1993; 81: 2180–6.
8. Briët E, Rosendaal F, Kreuz W, Rasi V, Peerlinck K, Vermylen J, Ljung R, Rocino A, Addiego J, Lorenzo J, Pabinger I. High titer inhibitors in severe Haemophilia A. A meta-analysis based on eight long-term follow-up studies concerning inhibitors associated with crude or intermediate purity factor VIII products: letter to the editor. Thromb Haemost 1994; 72 (1): 162–4.
9. Algiman M, Dietrich G, Nydegger U, Boieldieu D, Sultan Y, Kazatchkine M. Natural antibodies to factor VIII (anti-hemophilic factor) in healthy individuals. Proc Natl Acad Sci USA 1992; 89: 3795–9.
10. Ehrenforth S, Kreuz W, Scharrer I, Linde R, Funk M, Gungor T, Krackhardt B, Kornhuber B. Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. The Lancet 1992; 339: 594–8.
11. Verbruggen H, Novakova I, Wessels J, Verbeek K, Polenewen R. Frequency of inhibitors in haemophiliacs: letter to the editor. The Lancet 1992; 339: 1301.
12. Landell R, Wagner R, Brinkhous K. Effect of antihaemophilic factor on one-stage-clotting test: a presumptive test for haemophilia and a simple one-stage antihaemophilic factor assay procedure. J Lab Clin Med 1953; 41: 637–45.
13. Kasper C, Aledort L, Counts R, Edson J, Fratantoni J, Green D, Hampton J, Hilgartner M, Lazerson J, Levine P, MacMillan C, Pool J, Shapiro S, Shulman N, Eys J. A more uniform measurement of factor VIII inhibitors. Thrombos Diathes Haemorrh 1975; 34: 869–72.
14. Biggs R. Antibodies to factor VIII clotting activity (VIII:C). In: Human Blood Coagulation, Haemostasis and Thrombosis (third edition). Biggs R, Rizza CR, eds. Blackwell Scientific Publications, Oxford 1984; pp 205–41.

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