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Reagent substitutions in the Centers for Disease Control and Prevention Nijmegen-Bethesda assay for factor VIII inhibitors

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The Nijmegen-Bethesda assay (NBA), considered the "gold standard" for measurement of factor VIII (FVIII) inhibitors in haemophilia A,[1] introduced two modifications to the traditional Bethesda assay (BA) for stabilization during the 2-hour incubation at 37°C: (i) buffering of normal pooled plasma (NPP) in the test and control mixtures with imidazole and (ii) substitution of FVIII-deficient plasma (FVIIIDP) for imidazole buffer (IB) in the control mixture and for specimen predilution.[2] The NBA has not been widely adopted in the United States, because of the increased cost incurred by use of FVIIIDP rather than buffer and the lack of FDA-approved commercial reagents.[3] Surveys of North American coagulation laboratories have shown that only 20% use the NBA, 70% use buffered NPP in a "hybrid" of the NBA and BA, and one-third use diluents other than those recommended in published methods.[3] This lack of methodological uniformity may partially account for poor interlaboratory reproducibility, a well-known problem with FVIII inhibitor testing.[3]

Verbruggen et al[4] proposed that a 4% bovine serum albumin solution (BSA) could be substituted for FVIIIDP in the NBA based on the study of 6 inhibitor-positive specimens; however, Kershaw and colleagues[5] found FVIIIDP and BSA results to be significantly different among 72 specimens. In a study of four specimens of various titres in 6 laboratories, imidazole buffer alone as diluent was reported to show equivalent results to FVIIIDP.[6] We performed this study to clarify whether reagents could be successfully substituted in the Centers for Disease Control (CDC)-modified Nijmegen-Bethesda assay (CDC- NBA)[7] and to document the performance characteristics of the assay with these changes.

FVIII coagulant activity (VIII:C) in International Units per decilitre (IU/dL) was measured by one-stage assay using PTT-A reagent (Diagnostica Stago, Parsippany, NJ, USA), 0.05 M imidazole buffer pH 7.4 (Siemens, Marburg, Germany) as analyser diluent, FVIIIDP from haemophilic subjects (George King Biomedical, Overland Park, KS, USA) as substrate and normal reference plasma (CCNRP; Precision Biologic, Dartmouth, Nova Scotia, Canada)

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diluted in 0.05 M imidazole buffer pH 7.4 (Siemens) as calibrator. Specimens from haemophilia A patients enrolled in the Registry for Bleeding Disorder Surveillance were processed and shipped to CDC as previously described.[7] FVIII inhibitors in Nijmegen-Bethesda units (NBU) were measured by NBA,[2] modified as previously described [7] with heating of specimens to 56°C for 30 minutes and then centrifuging at 2700 *g* for 5 minutes at room temperature prior to testing. Imidazole-buffered NPP (Precision Biologic) (IB-NPP) was used in control and test mixtures, and naturally deficient FVIIIDP (George King Biomedical) was used in control mixtures and for predilution of those specimens requiring dilution (<25% residual activity, >2.0 NBU). For comparison, assays were run concurrently with other diluents substituted for FVIIIDP both in the control mixture with IB-NPP and for predilution of positive specimens requiring dilution. Diluents tested were 4% bovine serum albumin (BSA) (Equitech, Kerrville, TX, USA; A-3059, Sigma-Aldrich Corp., St. Louis, MO, USA), 4% bovine serum albumin (Sigma) in 100 mM imidazole buffer (IB-BSA) and 0.05 M imidazole buffer (Siemens) (IB). Positive controls were inhibitor-positive patient plasmas (George King Biomedical) diluted in FVIIIDP to approximately 1.0 NBU. Statistical analyses were conducted using GRAPHPAD Prism version 6.0 and SAS version 9.3. A significance level of $P < .05$ was used.

The effect of the NPP preparation used in the control mixture on its stability was evaluated using NPP (Precision Biologic) unbuffered and buffered with imidazole (Sigma-Aldrich) or with 4-(2-hydroxyethy l)-1-piperazineethanesulfonic acid (HEPES) (Acros Organics, Fairfield, NJ, USA) to pH 7.4 and mixed 1:1 with FVIIIDP, BSA or an inhibitor- negative patient plasma in duplicate. VIII:C and pH were measured in duplicate before and after 2 hours of incubation at 37°C (Figure 1A). Both VIII:C and pH showed the least change in the imidazole-buffered NPP and BSA mixture.

The effect of the diluent used in the control mixture on its stability was examined using a 1:1 mixture of IB-NPP with BSA, IB-BSA or IB prepared in triplicate and tested for VIII:C in duplicate after 0, 30, 60 and 120 minutes of incubation at 37°C (Figure 1B). The FVIIIDP mixture decreased to 85% of its original value of 49 IU/dL. BSA and IB-BSA mixtures showed a slight increase to 107% of the original means of 42 and 43 IU/dL. The IB mixture maintained 97% of its original 45 IU/dL. Using ANOVA, the BSA, IB-BSA and IB curves were not significantly different from each other; each showed significantly less change during incubation than the FVIIIDP mixture. This is in contrast to the original observation of maintenance of 95% of pre-incubation activity using FVIIIDP in the NBA.[2]

Specimens not requiring predilution (<2.0 NBU) were tested with BSA, IB-BSA and IB substituted for FVIIIDP in the control mixture (Table 1A). Results for 15 positive specimens (0.5–1.9 NBU with FVIIIDP) were not significantly different; 311 negative specimens (<0.5 NBU with FVIIIDP) gave slightly but significantly higher NBU when the nonplasma diluents were used. Using FVIIIDP as the “gold standard,” the ability to correctly assign inhibitor status was compared for each diluent by evaluating the area under the receiver operating characteristic (ROC) curve. The diluents were not significantly different ($P = 0.9$).

For further comparisons, BSA was substituted in paired testing of 1239 specimens of 0–747.3 NBU. Median FVIIIDP and BSA results on 33 positive (≥0.5 NBU) specimens were

not significantly different by Wilcoxon matched-pairs signed-rank test ($P = .57$). Results differed significantly ($P < .0001$) on specimens with negative inhibitor titres (<0.5 NBU) with median for FVIIIDP of 0.0 NBU and for BSA of 0.2 NBU and a median of differences of 0.20 NBU. The 1.0 NBU positive control was also higher with BSA than with FVIIIDP, with medians of 1.2 and 1.0 NBU, respectively ($P < .0001$), and a median of differences of 0.20 NBU. Using BSA and a threshold for positivity of ≥0.5 NBU, 62 (5.1%) of the 1206 specimens that were negative with FVIIIDP were misclassified as positive, 48 of them at 0.5 NBU. When ≥0.6 NBU was used as the threshold for positivity for BSA, 14 specimens (1.2%) negative with FVIIIDP were classified as positive with BSA. A single specimen positive with FVIIIDP was negative with BSA using both cut-offs. This specimen was negative by chromogenic Bethesda assay and may represent a false positive with FVIIIDP. Comparison of the performance characteristics of the CDC-NBA using FVIIIDP and BSA is shown in Table 1B.

The limit of detection (LOD) for the standard CDC-NBA was previously calculated to be 0.2 NBU.[8] Using the methods described,[8] with 4 negative specimens prepared from equal parts NPP and FVIIIDP and 4 positive specimens prepared by diluting a known inhibitor in FVIIIDP to 1.0 NBU tested over 3 days, the LOD with BSA was calculated to be 0.30 NBU. For 30 specimens from healthy paid donors with no history of a bleeding disorder, mean ± SD was 0.060 ± 0.069, and LOD calculated as mean ± 3 SD was 0.267. The LOD of the CDC-NBA with BSA thus estimated to be 0.3 NBU is consistent with other findings presented here indicating that the CDC-NBA with BSA gives slightly higher values than the CDC-NBA with FVIIIDP at low levels.

This study demonstrates that substitution of BSA for FVIIIDP in the CDC-NBA is satisfactory, as reported for the original NBA.[5–7] As previously noted,[6,7] von Willebrand factor is not required in the diluent if present in the assay substrate. Among the nonplasma diluents tested, differences were small; however, diluents containing BSA may be preferable for specimens requiring extensive predilution to maintain protein concentration. Use of nonplasma diluents actually improved stability and could reduce both cost and the variability introduced by adding different FVIIIDP reagents. IB-NPP, as recommended in the original NBA,[3] showed greater stability than unbuffered or HEPES- buffered NPP. The consistent use of imidazole buffer throughout the assay may be advantageous.

The threshold for positivity of ≥0.5 NBU for the CDC- NBA with FVIIIDP previously established[7] has been validated by measurement of IgG_4 anti-FVIII antibodies,[8] which have been shown to correlate with the presence of a functional inhibitor.[9] BSA substitution required adjustment of the threshold to achieve the same results, a finding not previously reported. The observed drop in VIII:C during incubation accounted for the difference and was not an aberration in a single experiment, as it was seen in paired specimens throughout the study. Postincubation, the control mixture with FVIIIDP was consistently lower than with BSA. Because some types of immunodepleted FVIIIDP produce aberrant results,[10] only FVIIIDP from congenitally deficient patients, confirmed to be inhibitor-negative, was used. Additional buffering of FVIIIDP might resolve the difference; however, it seems unnecessary to add that cost if nonplasma diluents can be used.

In practice, few laboratories have enough specimens to set reference ranges for FVIII inhibitors. The ISTH has defined a positive inhibitor based on clinical practice[1]; however, our findings show that method modifications can change the results. Multilaboratory studies using standardized test protocols and recommended reagents are needed to validate this important parameter and to produce guidelines to reduce interlaboratory variability.

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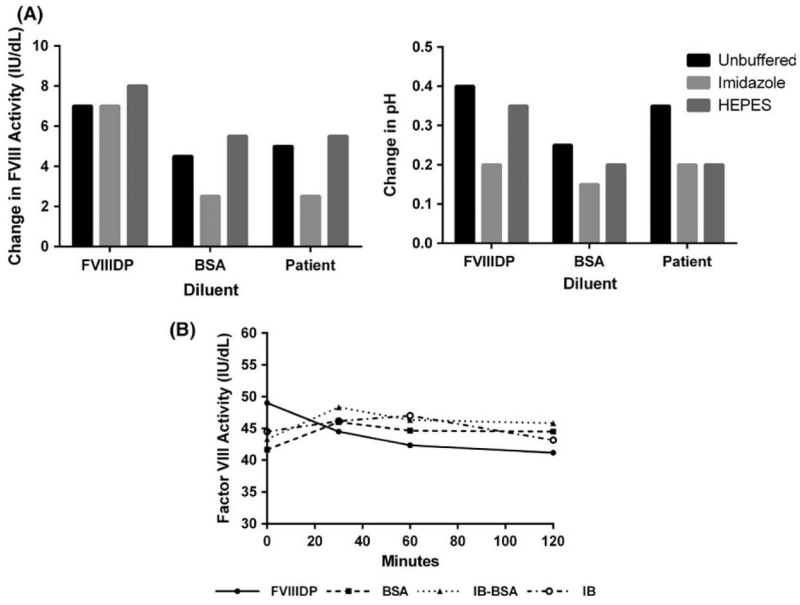

FIGURE 1.

Stability over 2-h incubation at 37°C of a 1:1 mixture of normal pool plasma (NPP) and diluent. (A). Change in factor VIII (FVIII) activity and pH of NPP unbuffered or buffered with imidazole or HEPES with factor VIII-deficient plasma (FVIIIDP), 4% bovine serum albumin (BSA) or an inhibitor-negative patient plasma. (B). Imidazole-buffered NPP with FVIIIDP, BSA, imidazole-buffered BSA (IB-BSA) and imidazole buffer (IB). Mean of duplicate determinations on triplicate specimens

TABLE 1

(A) Comparison of results with different diluents in the control mixture: factor VIII-deficient plasma (FVIIIDP), 4% bovine serum albumin (BSA), imidazole- buffered BSA (IB-BSA) or imidazole buffer (IB). (B) Summary of performance characteristics of the CDC-modified Nijmegen-Bethesda assay (CDC-NBA) using FVIIIDP or BSA in the control mixture and for predilution

A.	FVIIIDP	BSA	IB-BSA	IB
All Data (n = 326)				
Range	0–1.6	0–1.7	0–1.9	0–1.8
Median (IQR)	0 (0–0.2)	0.2 (0–0.3)*	0.2 (0.1–0.3)*	0.2 (0.1–0.3)*
Positives (n = 15)[a]				
Range	0.5–1.6	0.3–1.7	0.4–1.9	0.4–1.8
Median (IQR)	0.6 (0.5–1.0)	0.6 (0.6–1.0)	0.7 (0.5–0.9)	0.8 (0.6–1.1)
Negatives (n = 311)[a]				
Range	0–0.4	0–0.6	0–0.6	0–0.7
Median (IQR)	0 (0–0.2)	0.2 (0–0.3)*	0.2 (0.1–0.3)*	0.2 (0.1–0.3)*

B.	FVIIIDP		BSA	
	n	%	n	%
Between runs				
Positive Control	114	CV: 10.3	76	CV: 12.8
Negative Control	117	CV: 9.8	72	CV: 9.7
Within runs				
Positive Control	10	CV: 4.8	10	CV: 6.6
Negative Control	10	CV: 5.3	10	CV: 6.7
Sensitivity				
Positive patients	51[b]	100	33[a]	97
Negative patients	581[b]	98.8	1206[a]	98.8
Specificity				
Healthy subjects	30	100	30	100

*Significantly different from FVIIIDP results by Kruskal-Wallis test at $P < .05$.

[a]Defined as positive if ≥0.5 NBU and negative if <0.5 NBU using FVIIIDP.

[b]Specimens from patients with or without clinical history of inhibitor.