

Comparative field study: impact of laboratory assay variability on the assessment of recombinant factor IX Fc fusion protein (rFIXFc) activity

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Summary

Due to variability in the one-stage clotting assay, the performance of new factor IX (FIX) products should be assessed in this assay. The objective of this field study was to evaluate the accuracy of measuring recombinant FIX Fc fusion protein (rFIXFc) activity in clinical haemostasis laboratories using the one-stage clotting assay. Human haemophilic donor plasma was spiked with rFIXFc or BeneFIX[®] at 0.80, 0.20, or 0.05 IU/ml based on label potency. Laboratories tested blinded samples using their routine one-stage assay and in-house FIX plasma standard. The mean spike recoveries for BeneFIX (n=30 laboratories) were 121 %, 144 %, and 168 % of expected at nominal 0.80, 0.20, and 0.05 IU/ml concentrations, respectively. Corresponding rFIXFc spike recoveries were 88 %, 107 %, and 132 % of expected, respectively. All BeneFIX concentrations were consistently overestimated by most laboratories. rFIXFc activity was reagent-dependent; ellagic acid and silica gave higher values than kaolin, which underestimated rFIXFc. BeneFIX demonstrated significantly reduced chromogenic assay activ-

ity relative to one-stage assay results and nominal activity, while rFIXFc activity was close to nominal activity at three concentrations with better dilution linearity than the typical one-stage assay. In conclusion, laboratory- and reagent-specific assay variabilities were revealed, with progressively higher variability at lower FIX concentrations. Non-parallelism against the FIX plasma standard was observed in all one-stage assays with rFIXFc and BeneFIX, leading to significant overestimation of FIX activity at lower levels and generally high inter-laboratory variability. Compared to the accuracy currently achieved in clinical laboratories when measuring other rFIX products, most laboratories measured rFIXFc activity with acceptable accuracy and reliability using routine one-stage assay methods and commercially available plasma standards.

Keywords

Activated partial thromboplastin time (aPTT), coagulation factor IX, haemophilia B, coagulation tests, blood, standardisation

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Introduction

Intravenous injection of replacement factor IX (FIX) is the mainstay of treatment for haemophilia B worldwide (1). BeneFIX[®] (BeneFIX[®] [package insert]. Pfizer Inc., Philadelphia, PA; 2011) is a recombinant factor IX (rFIX) product that is approved for the treatment of haemophilia B. Measurement of FIX levels for diagnosis of haemophilia B, or for the assessment of FIX activity in post-injection samples, is typically performed in clinical haemostasis laboratories using the FIX one-stage clotting assay (2) with commercially available pooled normal plasma standards calibrated against the World Health Organization (WHO) FIX plasma International Standard (3).

The FIX one-stage clotting assay is associated with significant variability, in particular when measuring recombinant FIX products (4–7). A number of elements contribute to the observed variability in one-stage assay performance. The lower limit of quantitation for most one-stage clotting assays is approximately 1 % factor activity levels (nearly equivalent to the cutoff for severe haemophilia), making accurate assessment of low activity levels difficult (8). Moreover, the wide selection of commercially available reagents and instruments leads to inter-laboratory variability (5–7). Differences between the characteristics of FIX International Standards (plasma or concentrates) and recombinant products further complicate activity determinations (9).

As new factor products are developed for the treatment of haemophilia, the accuracy and precision of commonly used assays with these products will need to be determined. While many clinicians currently prefer to treat empirically rather than based on laboratory results, adoption of longer-acting products may be accompanied by increased interest in individualised pharmacokinetic (PK) assessments to guide development of an initial dosing regimen (10). Failure to identify and recognise analytical variability can potentially lead to misinterpretation of PK parameters and inaccurate dosing (11).

The use of conventional rFIX products in the management of haemophilia B is limited by their short half-life, ranging from 14–36 hours (h) depending on the length of the sampling time

period (12–15). Fc fusion technology is a well-established technology for extending the half-life of therapeutic proteins (16), and seven Fc fusion proteins are currently approved by the US Food and Drug Administration (FDA) (17). This technology has been used for the development of a long-acting recombinant factor IX Fc fusion protein (rFIXFc) approved for the treatment of haemophilia B (18, 31). rFIXFc is a recombinant protein composed of a single molecule of rFIX covalently fused to the Fc domain of human immunoglobulin G1 (IgG₁) with no intervening sequence (19–21). Fc fusion technology utilises an endogenous pathway that is responsible for the long half-life of human IgG. During circulation, rFIXFc is internalised by endothelial cells lining the vasculature via pinocytosis. Upon binding to the neonatal Fc receptor (FcRn), rFIXFc is transported out of the cell and cycled back into the bloodstream. This interaction delays lysosomal degradation, thus prolonging the circulating half-life of the protein (19, 22).

Biochemical characterisation of rFIXFc demonstrated appropriate gamma-carboxylation, which is required for full activity, and indicates that other post-translational modifications were similar to those of BeneFIX and plasma-derived FIX (20). However, non-human glycan structures (i.e. N-glycolylneuraminic acid, galactose- α -1,3-galactose), which were detected in Chinese hamster ovary-derived BeneFIX (23), were not found in rFIXFc. This was as expected, since rFIXFc is produced in a human cell line, HEK 293H. Furthermore, the kinetics of in vitro tenase complex assembly and activated factor X (FXa) generation using purified components appear unaffected by the Fc fusion (24).

The objective of this field study was to evaluate the suitability of a variety of commercially available one-stage clotting assay reagents and instruments for the measurement of rFIXFc activity in plasma samples. Haemophilia B plasma samples spiked with rFIXFc were tested by clinical haemostasis laboratories using their routine procedures and plasma calibrators traceable to the WHO FIX plasma International Standard. A rFIX comparator (BeneFIX) was included to evaluate general laboratory variability when measuring conventional rFIX products.

Materials and methods

Field study kits

Field study kits were prepared and distributed to the participating laboratories (see Suppl. Information, available online at www.thrombosis-online.com). Human haemophilic donor plasma with no detectable FIX activity (<0.5%) was spiked with either rFIXFc or a rFIX comparator, BeneFIX, at nominal concentrations of 0.80 IU/ml, 0.20 IU/ml, or 0.05 IU/ml, based on the label potency for each product. The potency of rFIXFc was assigned using the Actin[®] (Siemens, Erlangen, Germany) activated partial thromboplastin time (aPTT) reagent against an rFIXFc working standard that was calibrated to the WHO FIX concentrate standard by the same method. Kits contained 3 frozen 1 ml aliquots of each of the three concentrations for both BeneFIX- and rFIXFc-spiked plasma to permit assays to be conducted in triplicate.

Study design

Field study kits were distributed to 30 laboratories in seven countries in North America, Europe, South America, and Australia. All clinical laboratories were blinded with respect to the drug product and concentration in each vial, and were instructed to assay three sets of samples on different occasions using their routine one-stage assay procedure and their in-house FIX plasma standard. Each laboratory provided procedural data on the type and source of reagents and substrate plasma used, instrument employed, number of dilutions performed in each assay, source of calibrators, and laboratory certification and type of proficiency testing conducted. Laboratories provided raw data and FIX activities calculated from in-house standard curves, and supplied the final results to Biogen Idec for statistical analysis.

In-house verification of field study results

To discriminate between reagent variability and inter-laboratory variability, the one-stage assay was performed in-house on samples spiked with either 0.80 IU/ml BeneFIX or 0.80 IU/ml rFIXFc, based on label potency. The aPTT reagents PTT-A[®] (Stago, Parsippany, NJ, USA) and C.K. Prest[®] (Stago) were evaluated on a Compact[®] coagulation analyser (Stago), while Actin and Actin FSL (both from Siemens), SynthAFax[®] (Instrumentation Laboratory, Bedford, MA, USA), SynthASil[®] (Instrumentation Laboratory), APTT-SP[®] (Instrumentation Laboratory), and Auto-APTT[®] (Tcoag, Parsippany, NJ, USA) were tested on a Sysmex[®] CA-1500 system (Siemens), each in three independent runs. The Sysmex instrument and the Stago analyser were calibrated using the WHO International Standard for FIX plasma (07/192), except for the evaluations of APTT-SP and Auto-APTT, which used reference plasma from Precision Biologic (Dartmouth, NS, Canada) for calibration.

In-house chromogenic assay

The field study samples were also assayed for chromogenic activity using a Biophen FIX chromogenic kit (Hyphen Biomed, Neuville, France). The WHO plasma standard (07/192) was used as the reference standard. All field study samples were tested with multiple dilutions in duplicate in a 96-well plate on a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) using an endpoint reading method.

Comparison of WHO 4th FIX concentrate and plasma standards

Potency assignment for FIX products typically employs a standard that is calibrated against the WHO FIX concentrate standard, while clinical haemostasis laboratories generally use a normal pooled plasma standard, which has an activity assigned against the WHO FIX plasma standard. To assess potential differences in FIX activity between the concentrate and plasma standards and determine whether this may have contributed to differences between measured and nominal FIX activity observed in the field study, the

rFIX product	Label activity (IU/ml)	Mean spike recovery (IU/ml)	% of expected (label activity)	Intra-laboratory CV (n=3)		Inter-laboratory CV (n=30)
				Median	Range	
BeneFIX	0.80	0.966	121	5.6 %	0 %-25.0 %	12.1 %
	0.20	0.289	144	3.6 %	0 %-21.4 %	19.7 %
	0.05	0.084	168	7.3 %	0 %-39.8 %	29.8 %
rFIXFc	0.80	0.707	88	6.7 %	0 %-18.4 %	26.3 %
	0.20	0.214	107	6.7 %	1.4 %-20.9 %	35.5 %
	0.05	0.066	132	8.4 %	0 %-36.7 %	44.1 %

rFIX, recombinant factor IX; IU, International Units; CV, coefficient of variation; rFIXFc, recombinant factor IX Fc fusion protein.

Table 1: rFIX one-stage clotting assay results.

WHO 4th FIX concentrate standard was directly compared against the WHO 4th FIX plasma standard using six different aPTT reagents at three dilutions in triplicate experiments. The aPTT reagents included Actin, Actin FSL, SynthAFax, SynthASil, PTT-A, and C.K. Prest.

Data and statistical analysis

Manual data entries were cross-checked against source documents. The 0.80 IU/ml and 0.20 IU/ml BeneFIX samples were apparently swapped in one laboratory, resulting in outliers for the corresponding results, and these two test results were excluded from the analysis. Two results from different laboratories for the 0.05 IU/ml rFIXFc sample were excluded as outliers because the results were reported as ≤ 0.01 IU/ml. Results were analysed for intra-laboratory and inter-laboratory variation and accuracy measurements were compared with nominal or consensus, i.e. laboratory mean, activity. Relative variation and accuracy for rFIXFc compared with BeneFIX was evaluated based on type of activating reagent (e.g. silica, kaolin, ellagic acid) and instrumentation used.

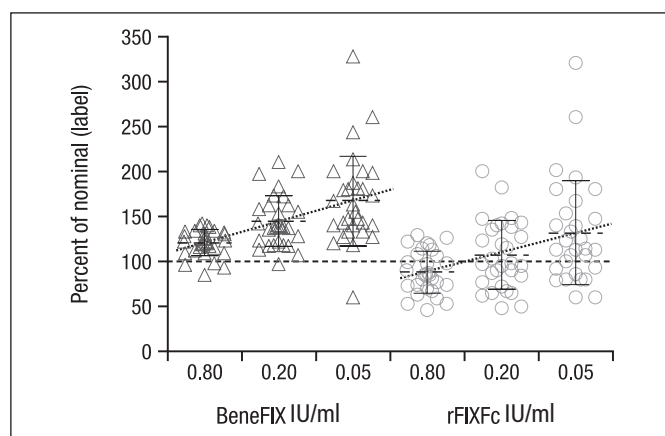


Figure 1: Mean (SD) FIX activity by one-stage clotting assay (n = 30 laboratories). SD, standard deviation; FIX, factor IX; rFIXFc, recombinant factor IX Fc fusion protein; IU, International Units. Dashed line represents the expected recovery. Dotted lines indicate the degree of non-linearity observed by the average laboratory across the three concentrations of BeneFIX or rFIXFc.

Statistical evaluations were performed by unpaired t-test or analysis of variance (ANOVA) methods to assess whether assay-related differences, including particular assay reagents, standards, instrumentation, or methodology, affected FIX activity measurements.

Results

Reagent and instrument use

An array of one-stage assay reagents and critical steps in methodology were employed by the 30 participating laboratories (Suppl. Table 1, available online at www.thrombosis-online.com). aPTT activating reagents used included ellagic acid (8 laboratories), silica (17 laboratories), kaolin (4 laboratories), and polyphenols (1 laboratory). Assay instrumentation varied among laboratories, but typically a combination of analyser and reagents from the same vendor was used. Overall, the range of aPTT reagents and instruments used by the 30 participating laboratories was representative of the distribution of reagents and coagulation analysers used among all major specialty haemostasis laboratories, based on the 2012 FIX proficiency testing administered by the College of American Pathologists (CAP; Northfield, IL, USA, <http://www.cap.org>) and the External Quality Control of Diagnostic Assays and Tests (ECAT) foundation (Leiden, The Netherlands, <http://www.ecat.nl>).

One-stage clotting assay

The FIX activity in the BeneFIX or rFIXFc samples was calculated as a percentage of nominal (label) activity (► Table 1, ► Figure 1). There were 90 test results per dose level of BeneFIX or rFIXFc across the 30 clinical laboratories. At 0.80 IU/ml, the mean spike recovery by the one-stage clotting assay was 0.966 IU/ml for BeneFIX and 0.707 IU/ml for rFIXFc. This corresponds with 121 % of expected for BeneFIX and 88 % of expected for rFIXFc. At the lower concentrations of 0.20 IU/ml and 0.05 IU/ml, the mean spike recovery for BeneFIX was 0.289 IU/ml and 0.084 IU/ml, respectively, which corresponds with 144 % and 168 % of expected. For rFIXFc, the mean spike recovery at 0.20 IU/ml and 0.05 IU/ml was 0.214 IU/ml and 0.066 IU/ml, respectively, which corresponds with 107 % and 132 % of expected. The higher degree of overesti-

mation of FIX activity at the 0.20 IU/ml and 0.05 IU/ml levels relative to the 0.80 IU/ml results was observed for both BeneFIX and rFIXFc in nearly all participating laboratories (► Figure 1). A small number of laboratories observed good dilution linearity for the three samples; however, there was no obvious trend in the methodology that correlated with laboratories that performed well in this respect. The median intra-laboratory coefficient of variation (CV) for the one-stage assay ($n=3$ independent tests per level) was below 10% for both products (► Table 1). Comparing mean results between laboratories, the inter-laboratory CV ranged from 12% to 30% for BeneFIX and from 26% to 44% for rFIXFc (► Table 1).

Differences were observed between laboratories, depending on the activating reagent used in the clotting assay (► Figure 2). Ellagic acid (8 laboratories) generally resulted in the highest observed activities, with mean \pm SD percent spike recovery and inter-laboratory variation (%CV) of $124 \pm 15\%$ (12% CV), $162 \pm 31\%$ (19% CV), and $193 \pm 67\%$ (35% CV) for BeneFIX and $115 \pm 14\%$ (12% CV), $150 \pm 27\%$ (18% CV), and $182 \pm 75\%$ (41% CV) for rFIXFc at the 0.80 IU/ml, 0.20 IU/ml, and 0.05 IU/ml concentrations, respectively. Silica (17 laboratories) typically resulted in lower observed recoveries. For silica, mean \pm SD percent spike recovery and %CV were $122 \pm 15\%$ (13% CV), $142 \pm 27\%$ (19% CV), and $162 \pm 44\%$ (27% CV) for BeneFIX and $85 \pm 15\%$ (17% CV), $98 \pm 27\%$ (27% CV), and $120 \pm 39\%$ (32% CV) for rFIXFc at the 0.80 IU/ml, 0.20 IU/ml, and 0.05 IU/ml concentrations, respectively. Observed recoveries were also generally lower with kaolin (4 laboratories). For kaolin, mean \pm SD percent spike recovery and %CV were $115 \pm 7\%$ (6% CV), $125 \pm 16\%$ (13% CV), and $148 \pm 22\%$ (15% CV) for BeneFIX and $53 \pm 5\%$ (10% CV), $61 \pm 8\%$ (14% CV), and $80 \pm 14\%$ (18% CV) for rFIXFc at the 0.80 IU/ml, 0.20 IU/ml, and 0.05 IU/ml concentrations, respectively. For BeneFIX activity in this field study, these differences, based on the type of aPTT reagent, were not statistically significant ($p > 0.05$). However, there were also no obvious correlations between other methodological differences (e.g. instrument, source of calibration plasma, calibration frequency, and number of dilutions per test) and relative accuracy among the 30 laboratories for BeneFIX or rFIXFc. Laboratories that trended high or low for rFIXFc also tended to report correspondingly higher or lower than expected results for BeneFIX at all concentration levels studied (► Figure 3).

In-house verification of field study results

The results of the in-house evaluation of BeneFIX and rFIXFc are shown in ► Figure 4. BeneFIX spike recovery with the eight reagents ranged from 83% (SynthAFax) to 125% (PTT-A); the spike recovery of rFIXFc ranged from 51% (C.K. Prest) to 121% (SynthAFax). The percent spike recovery of rFIXFc averaged $105.5 \pm 3.4\%$ of expected with the Actin reagent. For the Actin FSL reagent, the percent spike recovery of rFIXFc averaged $104.1 \pm 1.5\%$ of expected. SynthAFax, an aPTT reagent composed of ellagic acid and a synthetic mix of phospholipids, resulted in underestimation of BeneFIX with a spike recovery of 83.4% and an overestimation of rFIXFc with a spike recovery of 120.7%. Of note, SynthAFax was not used by any of the 30 laboratories in the

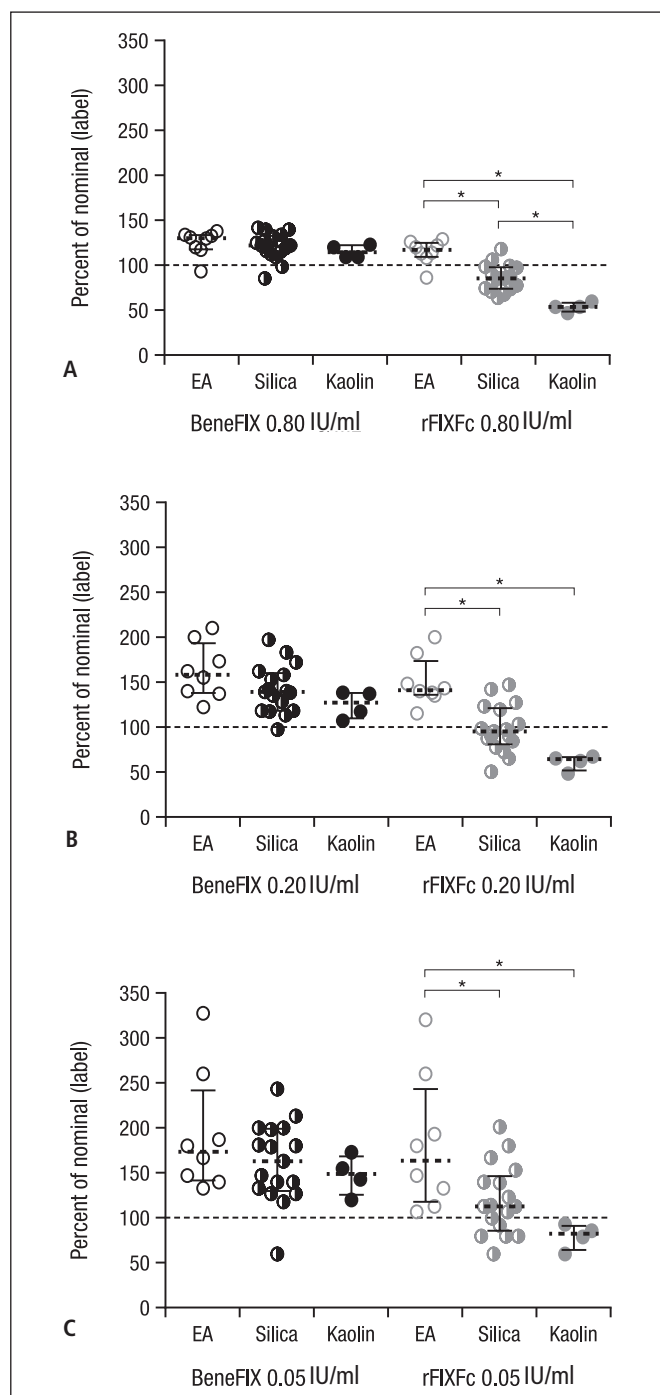


Figure 2: Median (25%/75%) FIX activity in the one-stage clotting assay by type of aPTT activating reagent for BeneFIX and rFIXFc at concentrations of 0.80 IU/ml (A), 0.20 IU/ml (B), and 0.05 IU/ml (C). FIX, factor IX; aPTT, activated partial thromboplastin time; rFIXFc, recombinant factor IX Fc fusion protein; IU, International Units; EA, ellagic acid. * $p < 0.05$.

field study. The four silica-based aPTT reagents evaluated in the in-house study averaged $81.0 \pm 3.3\%$ spike recovery of rFIXFc. The evaluation using the kaolin reagent C.K. Prest and the Stago coagulation analyser showed a spike recovery of $51 \pm 0.7\%$ for

rFIXFc. Overall, the in-house results agreed well with the trends observed in the field study.

The in-house chromogenic substrate assay results for BeneFIX and rFIXFc are shown in ► Table 2. When calibrated using the WHO International Standard for FIX plasma (07/192), the chro-

mogenic assay recovered 101%, 93.5%, and 84% of the nominal rFIXFc activity at the 0.80 IU/ml, 0.20 IU/ml, and 0.05 IU/ml levels, while BeneFIX activity ranged from 95% to 62% at the three concentrations.

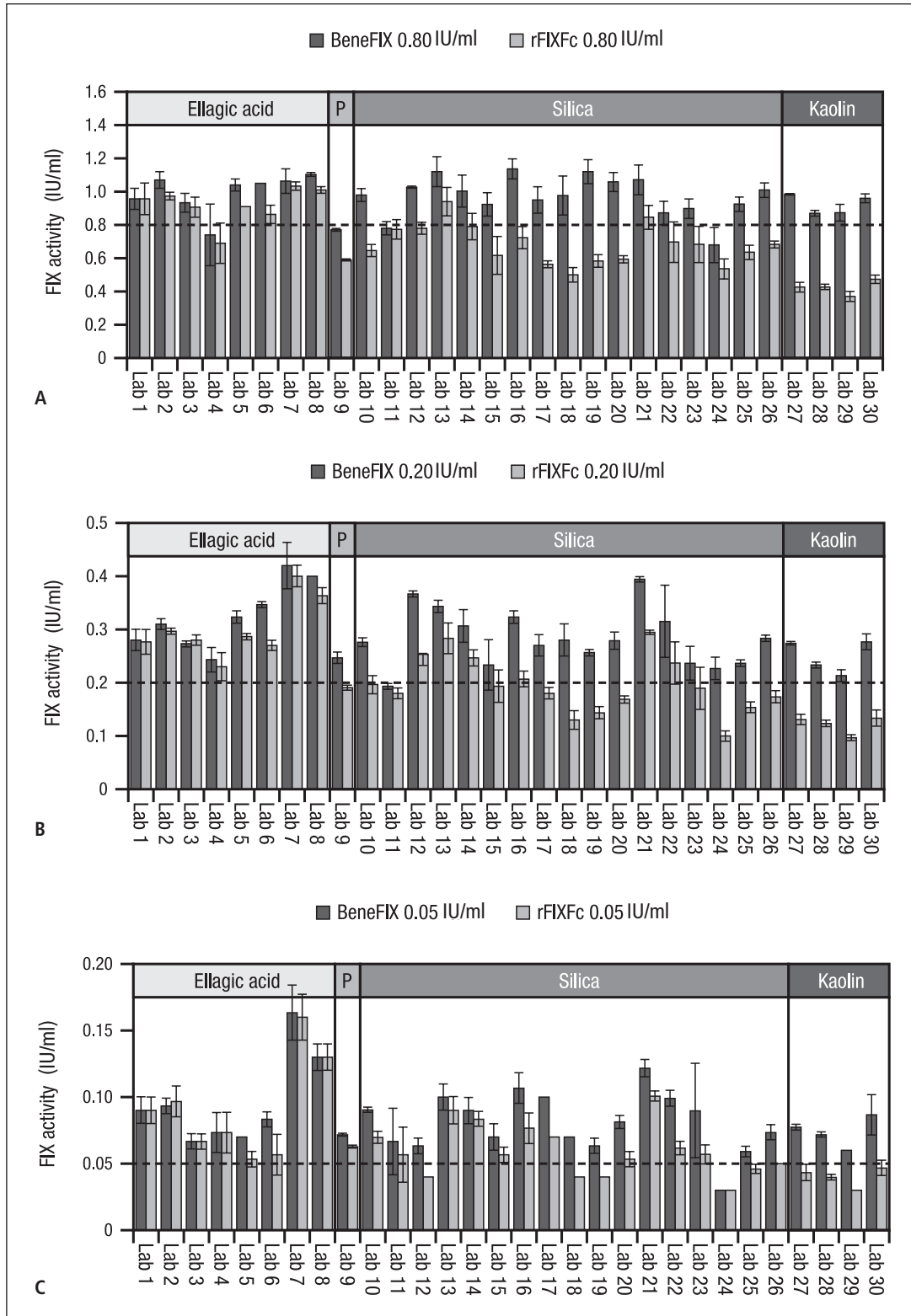


Figure 3: Individual laboratory one-stage assay results for BeneFIX and rFIXFc at concentrations of 0.80 IU/ml (A), 0.20 IU/ml (B), and 0.05 IU/ml (C). rFIXFc, recombinant factor IX Fc fusion protein; IU, International Units; FIX, factor IX; P, polyphe-nols. Dashed line represents the expected recovery.

Comparison of WHO 4th FIX concentrate and plasma standards

In the field study, BeneFIX activity was overestimated by an average of 121% at the 0.80 IU/ml concentration. The potency of the BeneFIX product used in this study was evaluated against the WHO 4th FIX concentrate standard and was demonstrated to have an average FIX activity within 5% of the label claim (data not shown). Thus, the higher apparent FIX activity for BeneFIX observed in the field study was not due to overfill of the BeneFIX vials. Results of the evaluation of the WHO 4th FIX concentrate standard directly against the WHO 4th FIX plasma standard demonstrated that the concentrate standard had 10-20% higher FIX activity than the plasma standard with most aPTT reagents used (Suppl. Figure 1, available online at www.thrombosis-online.com). At the 1.0 IU/ml concentration, the exact difference ranged from -0.6% to +18%, depending on the aPTT reagent used. The SynthAFax reagent produced different results from the other five reagents in that it showed comparable activity or slightly lower activity for the concentrate standard compared with the plasma standard. The SynthAFax results are also consistent with the lower apparent activity observed for BeneFIX in the in-house assays when using this reagent compared to the other reagents evaluated (► Figure 4). These differences between the two standards may in part account for the higher than expected activity seen for BeneFIX, and for rFIXFc using ellagic acid activators, in the field study and the in-house assays (► Figure 2 and ► Figure 4).

Discussion

The primary aim of this field study was to determine whether clinical haemostasis laboratories will be able to accurately monitor the activity of rFIXFc in people with haemophilia using their routine one-stage assay procedures and standards. Thirty laboratories in seven countries participated in the study, which demonstrated considerable inter-laboratory variability for both products in the

assessment of FIX activity by the one-stage assay. Elements contributing to this finding include the large range of reagents and instrumentation used as well as differences in laboratory technique (5-7).

Most laboratories in the field study overestimated the FIX activity of BeneFIX at all concentrations. rFIXFc was similarly overestimated in laboratories that used ellagic acid activators in the one-stage clotting assay. At the 0.80 IU/ml level, the overestimation may in part be explained by a difference in the standards used for potency assignment and clinical testing. The cause for the progressively higher overestimation of activities at the two lower FIX levels in the majority of laboratories is, however, unclear and was not observed in all laboratories. Accurate preparation of the test samples was confirmed by an in-house chromogenic FIX assay, which showed excellent dilution linearity for the three test concentrations against the WHO FIX plasma standard.

The measurement of rFIXFc activity was found to be dependent on the aPTT reagent used in the one-stage assay. In the eight laboratories using ellagic acid-based aPTT reagents, the measured rFIXFc activity averaged 115% (12% CV) of the label activity at the 0.80 IU/ml level. This modest overestimation of rFIXFc activity could be due to higher activity in the concentrate standard used for potency assignment compared with the plasma standard used in clinical haemostasis laboratories, as discussed above. Compared to the results with ellagic acid-based aPTT reagents and compared to label potency, rFIXFc activity was sometimes modestly underestimated by silica-based reagents. For the 17 laboratories using silica reagents, on average there was a 15% underestimation of rFIXFc activity at 0.80 IU/ml versus the nominal value. However, compared with the average ellagic acid result for rFIXFc, this resulted in an apparent difference of 30%. Approximately one-fourth of all laboratories performing silica-based assays produced results for rFIXFc that were in the same range as the results from laboratories using ellagic acid. At the lower concentrations, the underestimation of rFIXFc by silica-based reagents was compensated by a consistent overestimation of FIX activity by an average of 20% to 30% relative to the 0.80 IU/ml sample. Thus, it is not

Figure 4: In-house confirmation of one-stage assay reagent-specific differences. rFIXFc, recombinant factor IX Fc fusion protein; IU, International Units. Dashed line represents the expected recovery. The Stago reagents PTT-A and C.K. Prest were evaluated on a Stago Compact analyser, while all other reagents were evaluated on a Sysmex CA-1500 system.

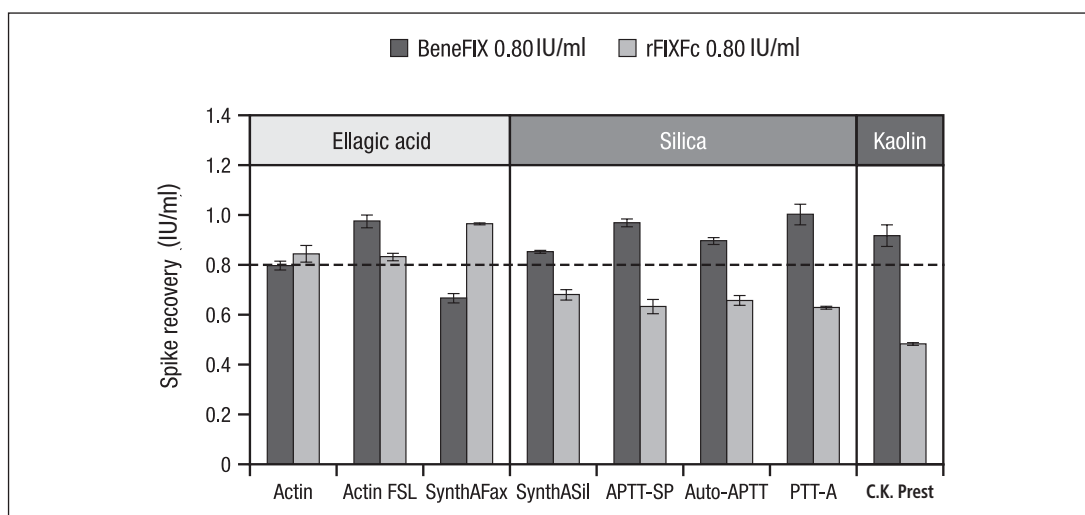


Table 2: In-house chromogenic substrate assay results (n = 3 independent assays).

rFIX product	Nominal concentration (IU/ml)	Mean \pm SD FIX activity (IU/ml)
BeneFIX	0.80	0.763 \pm 0.031
	0.20	0.154 \pm 0.012
	0.05	0.031 \pm 0.007
rFIXFc	0.80	0.808 \pm 0.072
	0.20	0.187 \pm 0.020
	0.05	0.042 \pm 0.005

rFIX, recombinant factor IX; IU, International Units; SD, standard deviation; rFIXFc, recombinant factor IX Fc fusion protein.

possible to conclude that silica-based FIX assays will necessarily produce less accurate results for rFIXFc than ellagic acid reagents, since this may depend largely on the particular laboratory, as clearly shown with BeneFIX.

Four of 30 laboratories in this field study used a kaolin-based aPTT reagent (C. K. Prest), with average results of 53% (10% CV) of the label activity for the 0.80 IU/ml rFIXFc samples. Similar results were found with the in-house studies using the same kaolin-based reagent on a Stago coagulation analyser, which showed a spike recovery of $51 \pm 0.7\%$ for the 0.80 IU/ml rFIXFc sample. The use of the kaolin reagent thus appears to result in more substantial and consistent underestimation of rFIXFc activity than the silica reagents. The underestimation of rFIXFc by the kaolin reagent versus the nominal 0.05 IU/ml was on average only 20%, which

What is known about this topic?

- The FIX one-stage clotting assay is associated with significant variability, in particular when measuring recombinant FIX products.
- The wide selection of commercially available reagents and instruments leads to inter-laboratory variability.
- Modified FIX products may encounter additional assay-related variabilities when activity is measured in the one-stage clotting assay at different laboratories.

What does this paper add?

- This paper describes results of a field study to evaluate the suitability of commercially available one-stage clotting assay reagents and instruments for the measurement of rFIXFc activity in plasma samples.
- Results revealed generally high inter-laboratory variability for both BeneFIX and rFIXFc.
- Despite these assay-related issues, the activity of rFIXFc was measured with acceptable accuracy and reliability in the majority of laboratories using their existing methods and reagents.

would not be expected to be clinically relevant. However, when compared to the average result obtained by ellagic acid at 0.05 IU/ml, there was still a 50% discrepancy. These points should be taken into account by treaters of haemophilia when using the kaolin reagent to measure rFIXFc activity.

Potency assignments for most FIX products are derived from a standard calibrated against the WHO FIX concentrate standard, whereas most clinical laboratories use a standard calibrated against the WHO FIX plasma standard. Potential differences between the concentrate standard and the plasma standard were investigated in the collaborative study that established the 4th International Standard for FIX concentrate (25). In that study, the 3rd International Standard for FIX plasma was found to be 6% lower than its assigned value when assayed against the 3rd International Standard for FIX concentrate. The 4th International Standard for FIX plasma was calibrated against the 3rd International Standard for FIX plasma, and this discrepancy between the plasma and concentrate standards was likely carried forward. When the WHO 4th FIX concentrate and plasma standards were compared in this study, a 10% to 20% lower FIX activity was seen with the plasma standard when compared to the concentrate standard for most of the six aPTT reagents tested. However, despite the differences between the two standards, the distribution of reagent-specific effects was similar when rFIXFc activity was assayed against the concentrate standard (data not shown). Therefore, discrepancies between the nominal and measured activities could not be entirely explained by the differences in the calibration standard.

The clinical significance of the observed differences in assaying FIX activity has not been fully established. In the phase 3 clinical study, B-LONG, all measurements were performed utilising a central laboratory (15). The central laboratory assay was developed and validated for plasma FIX, BeneFIX, and rFIXFc prior to conducting this field study, and used a particular silica-based aPTT reagent and a commercially available plasma standard, which proved to accurately recover BeneFIX and rFIXFc at all concentrations when included in the blinded field study (Lab 11 in ► Figure 3).

Inaccurate FIX activity measurements are a concern during surgical procedures, when local laboratory measurements are utilised in order to maintain sufficient FIX activity to allow for adequate recovery without increased risk of thrombosis (26). Marked variability in the measurement of FIX activity during the perioperative period might lead to unnecessary dose corrections and, in extreme cases, potential safety issues. However, it is unlikely that dose adjustments based on the range of laboratory measurements seen in this field study would be large enough to result in risk to the person with haemophilia from either underdosing or overdosing rFIXFc.

Overall, the results of this field study confirmed that the large variety of aPTT reagents used for FIX determinations by the one-stage assay and differences in methodologies and reference standards continue to produce large inter-laboratory variability, with considerable inaccuracies in many cases (27). The results of this field study suggest that, at some sites, physicians may not be able to rely on local laboratory results for accurate PK determinations

for either drug because the high assay variability and non-linearity observed in measurements for both BeneFIX and rFIXFc may render PK calculations misleading. In particular, the relative overestimation at lower FIX activity levels may significantly impact the prediction of terminal half-life and projected trough levels, resulting in overestimation of the amount of circulating BeneFIX by many laboratories and subsequent risk of undertreatment of the person with haemophilia. While current procedures in the majority of laboratories appear adequate for routine monitoring of BeneFIX and rFIXFc, further progress toward harmonisation of reference standards and method and reagent uniformity could potentially improve the overall accuracy when measuring recombinant FIX products.

A limitation of this study is the use of human haemophilic donor plasma spiked with rFIXFc or BeneFIX to evaluate FIX activity levels. It is possible that assay results using samples taken from people with haemophilia receiving treatment with recombinant FIX products would be different. However, haemophilic samples spiked with concentrate have been utilised by previous field studies comparing the accuracy and consistency of the one-stage clotting assay across clinical laboratories (28–30). In addition, it would not be feasible to conduct a field study on this scale using blood samples from people with haemophilia due to the volume of sample that would be required.

Conclusions

In this field study, significant inter-laboratory variability was observed for both BeneFIX and rFIXFc at 30 representative clinical haemostasis laboratories. Specific reagent dependency was observed for rFIXFc, with ellagic acid-based aPTT reagents yielding comparable results to BeneFIX at equivalent nominal concentrations. While BeneFIX was consistently overestimated in most laboratories, marginally lower FIX measurements were observed for rFIXFc in some laboratories using silica-based activators, and notably lower results were observed for rFIXFc in the four laboratories using kaolin-based activators. Overall, 80% of laboratories measured rFIXFc within an accuracy of $\pm 30\%$ at the 0.80 IU/ml level, compared to 70% of laboratories for BeneFIX. Not including the laboratories that used the kaolin-based activator, over 90% of laboratories reported rFIXFc results within $\pm 30\%$ of the expected 0.80 IU/ml value. We thus conclude that the activity of rFIXFc was measured with acceptable accuracy and reliability in the majority of laboratories using existing one-stage assay methods and current plasma standards.

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Author contributions

J. M. Sommer, Y. Buyue, H. Jiang, G. D. Kamphaus, and E. Gray designed the research study. Y. Buyue and S. Bardan performed the in-house laboratory analysis. J. M. Sommer, Y. Buyue, R. T. Peters, H. Jiang, E. Gray, and G. F. Pierce analysed the data. All authors reviewed the results, contributed to writing the paper, and approved the final version to be published.

Conflicts of interest

J. M. Sommer, Y. Buyue, S. Bardan, R. T. Peters, H. Jiang, G. D. Kamphaus, and G. F. Pierce are employees of Biogen Idec. E. Gray is an advisor for Biogen Idec, but received no material compensation.

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