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Development, Validation, and Application of a Novel Ligand-Binding Assay to Selectively Measure PEGylated Recombinant Human Coagulation Factor VIII (BAX 855)

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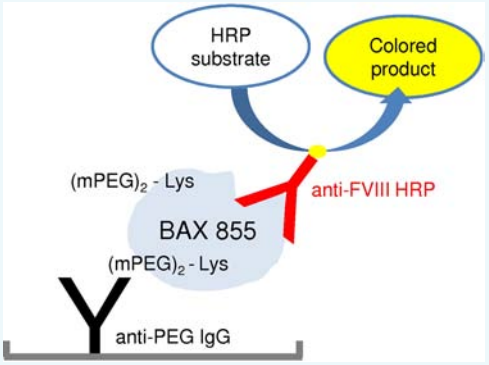
ABSTRACT: BAX 855 is a PEGylated recombinant factor VIII preparation that showed prolonged circulatory half-life in nonclinical and clinical studies. This paper describes the development, validation, and application of a novel ligand-binding assay (LBA) to selectively measure BAX 855 in plasma. The LBA is based on PEG-specific capture of BAX 855, followed by immunological factor VIII (FVIII)-specific detection of the antibody-bound BAX 855. This assay principle enabled sensitive measurement of BAX 855 down to the low nanomolar range without interference from non-PEGylated FVIII as demonstrated by validation data for plasma from animals typically used for nonclinical characterization of FVIII. The selectivity of an in-house-developed anti-PEG and a commercially available preparation, shown by competition studies to primarily target the terminating methoxy group of PEG, also allowed assessment of the intactness of the attached PEG chains. Altogether, this new LBA adds to the group of methods to selectively, accurately, and precisely measure a PEGylated drug in complex biological matrices. The feasibility and convenience of using this method was demonstrated during extensive nonclinical characterization of BAX 855.



INTRODUCTION

Factor VIII (FVIII) concentrates (plasma derived or recombinant) are used to normalize hemostasis and to prevent bleeding episodes in patients with hemophilia A. A longer-acting FVIII concentrate would reduce the frequency of infusions, and thus improve patient convenience and compliance with enhanced clinical outcomes. Modification with polyethylene glycol (PEGylation)[1−3] is a well-known method to improve the pharmacokinetic profile and prolong the half-life of therapeutic proteins by reducing renal clearance.[4] Renal clearance of FVIII appears to be of minor importance; however, chemical modification of FVIII may interfere with binding to low density lipoprotein receptor-related protein most likely involved in FVIII clearance by the liver.[5]

PEG-modified conjugates are used in several therapeutic fields. PEGylated drugs approved for human use include enzymes such as PEG-adenosine deaminase (Adagen, Enzon),[6] PEGasparaginase (Oncaspar, Enzon),[7] interferons,[8−10] antibodies, and antibody fragments.[11] PEGylation can be performed with several functional approaches that use different activation groups to attach the PEG to the protein. *N*-Hydroxysuccinimide (NHS)-activated PEG has often been applied to establish a covalent linkage to the ε-amino group of a lysine, but other functional groups are also in use.

PEGylated proteins require analytical methods capable of specifically measuring the protein-bound PEG moiety. PEG itself is a relatively inert chemical compound that is usually measured using its refraction index, but also by means of a colorimetric assay.[12] These analytical methods, however, require PEG concentrations of at least 10 μg/mL, are not sufficiently robust to work in complex protein mixtures such as plasma, and have limited selectivity. We therefore set out to develop an LBA that applied the selectivity of an anti-PEG antibody. Rabbit anti-PEG IgG antibodies were described in 1983.[13] Recently, monoclonal antibodies were developed for the sensitive measurement of PEG-modified proteins.[14−16] One of these monoclonal anti-PEG antibodies, developed in rabbits, has been reported to bind to the terminal methoxy group of PEG, unlike the others which bind to repeated units within the PEG chain.

This paper describes the development and validation of this new LBA to selectively measure PEGylated human recombinant FVIII (rFVIII) BAX 855 in plasma from laboratory animals. The LBA uses a combination of an in-house-developed and purified rabbit anti-PEG IgG antibody and a polyclonal, commercially available anti-human FVIII-peroxidase conjugate. BAX 855 is captured by the plate-bound anti-PEG antibody and then detected with the anti-FVIII antibody. The LBA was validated for use in plasma from FVIII-deficient mice, from rats and macaques, according to the EMA guideline on bioanalytical assay validation.[17]

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Table 1. Antibody Development in Rabbits Using Different Antigens[a]

animal plasma pool (n = 12)					individual animal plasma samples			
	hSA		PEG-hSA		PEG-VWF			
	Day 0	Day 21	Day 0	Day 21	dilution 1/50			
dilution	OD	OD	OD	OD	rabbit	Day 0	Day 36	Day 50
10	0.000	0.847	0.004	1.140	#1	0.000	0.669	0.651
100	0.007	0.951	0.008	1.215	#2	0.000	0.420	0.329
1000	0.000	0.578	0.005	0.989	#3	0.000	0.440	0.343
5000	0.003	0.473	0.005	0.743	#4	0.000	0.423	0.408

[a]The table shows the blank-corrected optical densities measured for the different samples in direct ELISAs using human serum albumin, PEGylated human serum albumin, and PEGylated VWF (PEG-VWF) as capturing antigens. Binding of rabbit IgG was detected by a peroxidase-labeled anti-rabbit IgG preparation.

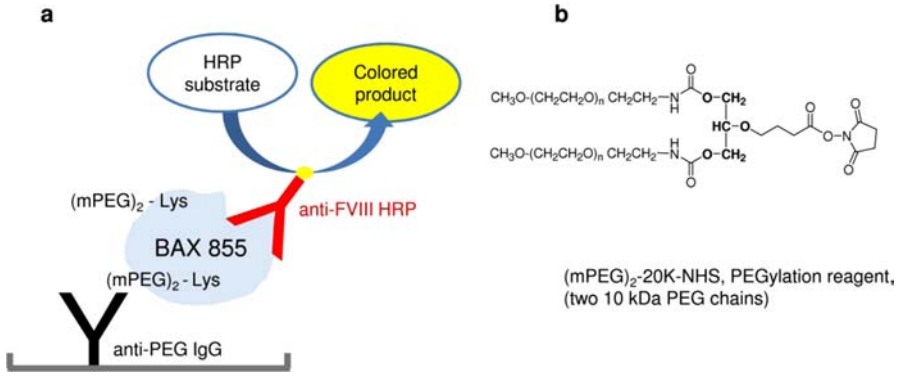

Figure 1. Schematic representation of the PEG-FVIII LBA (a) and chemical structure of the PEGylation reagent (b). The NHS coupling chemistry predominantly targets the ε-amino group of lysine.

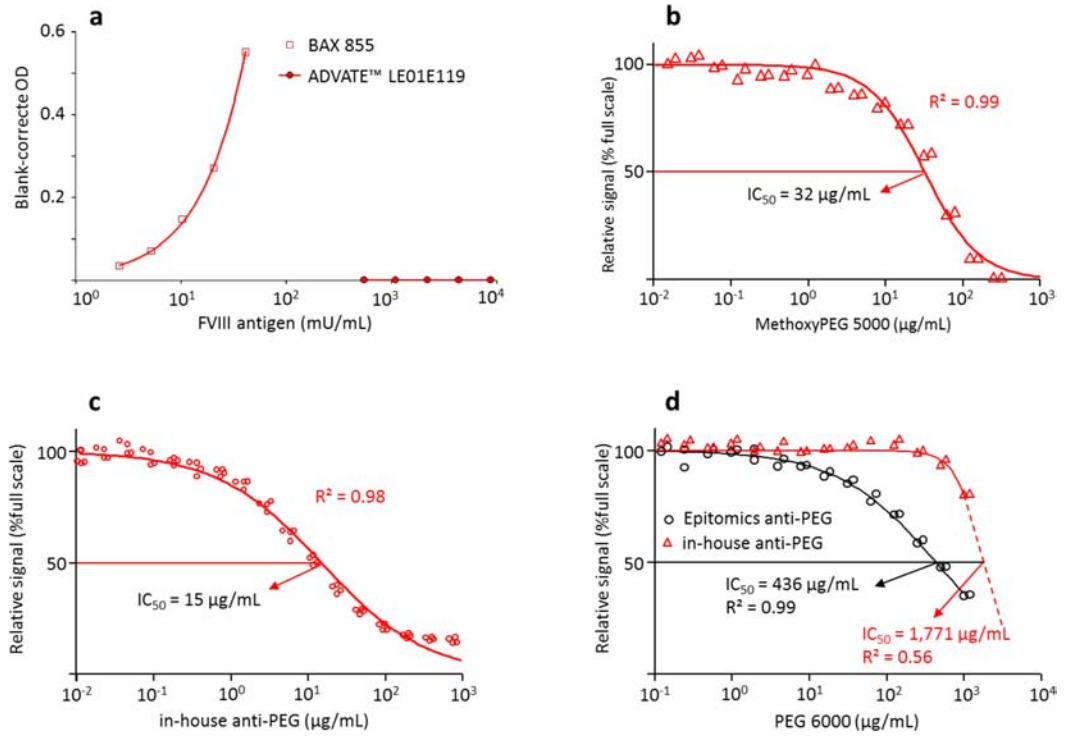

Figure 2. Demonstration of the selectivity of the PEG-FVIII LBA (a) shows the signals obtained in the PEG-rFVIII LBA for BAX 855 and the starting material ADVATE, comparing dilutions with known FVIII antigen concentrations; (b) shows the competition curve obtained for the PEG-rFVIII LBA with the in-house developed anti-PEG IgG, BAX 855, and mPEG 5000 as competitor; (c) shows the competition curve obtained by adding increasing concentrations of the in-house anti-PEG IgG to the PEG-rFVIII LBA, using commercially available anti-PEG IgG from Epitomics as the capturing antibody; (d) shows the competition curves obtained for the Epitomics and the in-house anti-PEG IgG using PEG 6000 as competitor.

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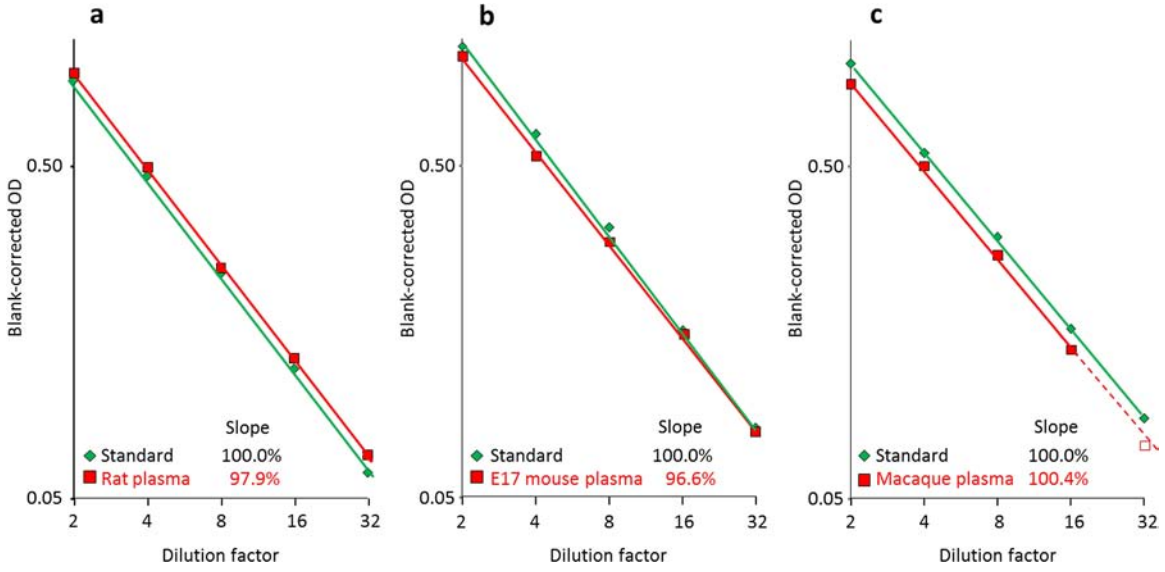

Figure 3. Investigation of parallelism in the citrated plasma of laboratory animals. (a), (b), and (c) show the dilution-response curves of BAX 855 diluted in buffer (=standard) and in citrated rat, FVIII-deficient mouse (E17), and macaque plasma. The relative slopes of the dilution curves are shown.

RESULTS

Preparation and Characterization of the anti-PEG IgG Antibody. The PEGylated hSA immunization antigen had a molar degree of PEGylation of 4.6. A direct ELISA with the immunization antigen was used to determine the presence of anti-PEG IgG antibodies (Table 1). To allow sensitive binding of the anti-PEG IgGs to their target, the commonly used detergents polysorbate 20 and Triton X-100 were not included in the washing/dilution buffers as both contain PEG groups.

As expected, the results obtained for the plasma pool sample taken 21 days after immunization were biased by the fact that hSA represents a foreign protein for the immunized rabbits. Binding to non-PEGylated hSA showed antibody development against native hSA. In contrast, the plasma pool sample taken before immunization did not show any binding to hSA or PEGylated hSA. Using a PEGylated VWF preparation for the direct ELISA, however, demonstrated successful specific anti-PEG IgG formation. Clear binding to PEGylated VWF was shown for four individual rabbit plasma samples, with no binding to the native protein.

Overall, the data showed that at least a portion of the rabbit IgG generated by immunization with PEGylated hSA specifically bound to the PEG moiety of a PEGylated protein conjugate irrespective of protein moiety.

The selectivity of the anti-PEG IgG was demonstrated by adding mPEG 5000 when running the direct ELISA with PEGylated hSA. Thus, mPEG 5000 at a concentration of 10 mg/mL reduced the binding to PEGylated hSA by 84%. Residual binding of rabbit IgG in the presence of mPEG reflected the presence of IgG directed toward native hSA epitopes and was in line with the data obtained when native hSA was used for the direct ELISA.

In a separate approach, 20 kDa-PEGylated rVWF was bound to the plate via an anti-human VWF antibody and detected by the binding of an anti-PEG IgG peroxidase conjugate prepared from the anti-PEG antiserum after IgG purification and depletion of anti-hSA IgGs. A mPEG 5000 serial dilution series from 50 mg/mL to 24 μg/mL was used to determine the competition and to calculate the 50% inhibition level (IC_{50}), which was 0.18 mg/mL.

Altogether, these data demonstrated the rabbit antisera to contain rabbit IgG that specifically bound to mPEG regardless of chain length, as shown for conjugates prepared with 5 and 20 kDa mPEGs and the protein to which they were attached.

Development of the LBA to Measure PEGylated rFVIII. The anti-PEG IgG preparation obtained can theoretically be applied with an anti-FVIII antibody for three different LBA formats, two of which have recently been described:[18] (1) A "PEG−PEG" LBA, using the anti-PEG antibody as capturing and as detection antibody after labeling, and (2) a "FVIII-PEG" LBA, using an anti-FVIII antibody as capturing and the labeled anti-PEG IgG as detection antibody. According to our best knowledge, the third, a "PEG-rFVIII" LBA, using the anti-PEG antibody for capturing and the labeled anti-FVIII IgG as detection antibody (Figure 1) has not been described so far.

For reasons including the masking of epitopes by PEGylation, the PEG-rFVIII format was selected for measurement of rFVIII. The suitability and selectivity of this design was determined by direct comparison of the binding curves obtained for ADVATE and BAX 855 (Figure 2a). The design proved to be specific for the presence of rFVIII-bound PEG, as ADVATE showed no response even when loaded at a 20-fold higher concentration of FVIII antigen. Furthermore, the assay signal clearly showed a dose-dependent relation to the FVIII antigen concentration in the low mU/mL range (40 to ~2 mU/mL).

Next, the feasibility of using two commercially available anti-FVIII peroxidase conjugates to detect anti-PEG captured BAX 855 was compared. Although both detection antibodies provided calibration curves with a high linearity ($R^2 \geq 0.997$) of 41 to 2.6 mU/mL FVIII antigen, there were clear differences in their signal intensities. The ASSERACHROM conjugate yielded more than 2-fold lower ODs than Cedarlane, due either to a lower specific peroxidase activity or to the binding to epitopes on the rFVIII molecule that had been shielded by PEGylation.

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Table 2. Results of the Bioanalytical Method Validations Using Citrated Rat, Macaque (Mac.), and FVIII-Deficient Mouse (E17) Plasma[a]

	inter-run precision[b] (n = 6)			intrarun precision (n = 6)			recovery (%)			total error[c]		
PEG ng/mL	Rat	E17	Mac.	Rat	E17	Mac.	Rat	E17	Mac.	Rat	E17	Mac.
5	10.1	17.5	17.6	8.5	5.7	8.7	87.8	84.3	86.1	22.3	33.2	31.5
10	12.5	12.2	9.7	ND[d]	ND	ND	96.7	81.0	80.6	15.8	31.2	29.1
50	9.6	6.8	7.7	ND	ND	ND	99.5	95.0	97.4	10.1	11.8	10.3
100	10.5	10.1	9.9	ND	ND	ND	100.4	97.6	96.0	10.1	12.5	13.9
400	5.6	10.3	6.7	5.9	5.0	3.4	97.8	100.4	99.2	7.8	9.9	7.5
Mean	9.6	9.9	8.5	ND	ND	ND	98.6	93.5	93.3	11.0	16.4	15.2

[a]The column "PEG" gives the concentrations of rFVIII-bound PEG. BAX 855 was spiked to the citrated animal plasma samples to obtain the target concentrations. [b]Inter-run and intrarun precision data are given as the relative standard deviations of the means. [c]Total errors were obtained as the sum of accuracy, calculated as the absolute difference 100−recovery and precision, given as RSDs. [d]ND stands for not determined.

These data clearly advocated the use of the Cedarlane reagent, as the higher measuring signals obtained are of advantage for the assay's performance.

Specificity of the PEG-rFVIII LBA Demonstrated by Competition Experiments. All competition experiments were carried out with the BAX 855 preparation VNH5K002A at a FVIII concentration of 138 mU/mL. MethoxyPEG 5000 efficiently competed with the binding of BAX 855 to the plate-bound in-house developed and purified anti-PEG IgG (Figure 2b) with an IC_{50} of 32 μg/mL (R^2 = 0.99). Under these conditions, the commercially available anti-PEG antibody from Epitomics, used in a 5-fold lower coating concentration, had an IC_{50} of 2.4 μg/mL. Such obvious inhibition confirmed the selective interaction between the PEG moiety and the two anti-PEG antibodies.

Another competition experiment verified the specificity of the in-house anti-PEG IgG. Figure 2c shows the competition curve obtained when the in-house anti-PEG IgG was used to inhibit the binding of BAX 855 to the plate-bound commercial anti-PEG IgG from Epitomics. An IC_{50} of 15 μg/mL (R^2 = 0.98) demonstrated that the in-house anti-PEG IgG efficiently competed for the binding of BAX 855 to the plate-bound commercial anti-PEG IgG.

The last competition experiment (Figure 2d), using dihydroxy PEG 6000 as competitor, revealed the methoxy group of PEG to be important for the binding of both anti-PEG IgG preparations. In both cases, much higher concentrations of PEG 6000 were required to achieve measurable inhibition of the binding of BAX 855 to the plate-bound anti-PEG antibody. Nevertheless, the competition provoked by dihydroxy PEG 6000 demonstrated that, apart from the methoxy group, other epitope(s) contributed to the binding.

Influence of Citrated Animal Plasma on the PEG-rFVIII LBA. No obvious influence on the assay was detected when the anti-PEG IgG-coated wells were incubated with high concentrations of animal protein as present in 1/20-diluted samples of citrated E17 mouse, rat, or macaque plasma spiked with BAX 855. The dilution-response curves obtained (Figure 3) were as linear and parallel as those found when BAX 855 was diluted in buffer only. A high degree of parallelism was apparent when comparing their slopes: For the three animal plasma samples spiked with BAX 855, these slopes differed by less than 4% from that of the calibration curve, prepared in buffer. Such a small difference may be deemed irrelevant as it falls clearly within the acceptable assay variability for LBAs. The native plasma samples did not elicit any response in the PEG-rFVIII LBA when loaded in the minimal dilution of 1/20.

These data demonstrated that the PEG-rFVIII LBA can measure low concentrations of rFVIII-bound PEG in the citrated plasma of laboratory animals, applied for nonclinical characterization of biological drugs. Furthermore, these findings supported the construction of assay calibration curves in buffer matrix instead of plasma, thus eliminating the need for different calibration curves depending on sample matrix.

Validation Data for the PEG-rFVIII LBA. The recent EMA guideline on bioanalytical method validation[17] sets the defaults for validation of an LBA when using the assay for drug measurement in a biological matrix. The key data of the three assay validations in measuring BAX 855 in E17 mouse, rat, and macaque plasma are summarized in Table 2.

Using the combination of anti-PEG capturing and polyclonal FVIII-specific detection antibody facilitated the specific measurement of human PEGylated rFVIII even in monkey plasma where a high degree of cross-reactivity usually interferes with the specific immunological measurement of human FVIII. The validation results demonstrated the LBA to be suitable for its intended use in plasma from all three species. Mean accuracy, expressed as the recovery of BAX 855 spiked to the plasma of macaques, rats, and E17 mice at five concentrations, was 93.2%, 96.4%, and 91.7%, respectively, with all individual values within a ±25% range, even at the lower limit of quantification (LLOQ).

The intra- and inter-run precision of this assay also complied with recommendations for LBAs, i.e., the relative standard deviations for the means of six repetitions did not exceed 20% and 25% at the LLOQ. Intrarun precision was determined for the validation samples with the concentration close to the assay's LLOQ (4 ng/mL) and for the sample with the rFVIII-bound PEG concentration; inter-run precision was determined for all five validation samples with rFVIII-bound PEG concentrations of 5, 10, 50, 100, and 400 μg rFVIII-bound PEG/mL.

Combining the results for accuracy and inter-run precision yielded the total error, an assay characteristic coined by the EMA guideline. For LBAs, this total error should not exceed 30%, or 40% at the LLOQ. In line with these recommendations, the total errors were 22.3% and 33.2% for rat and E17 mouse plasma, and 31.5% for macaque plasma. Similarly, the mean total errors, determined for 10 to 400 μg/mL rFVIII-bound PEG were 11.0% and 16.4% for rat and E17 mouse plasma, and 15.2% for macaque plasma. The mean total error determined for BAX 855 samples in buffer matrix, however, was as low as 6.9%. The buffer sample's less complex matrix and lower protein concentration than the animal plasma samples may explain the better assay performance.

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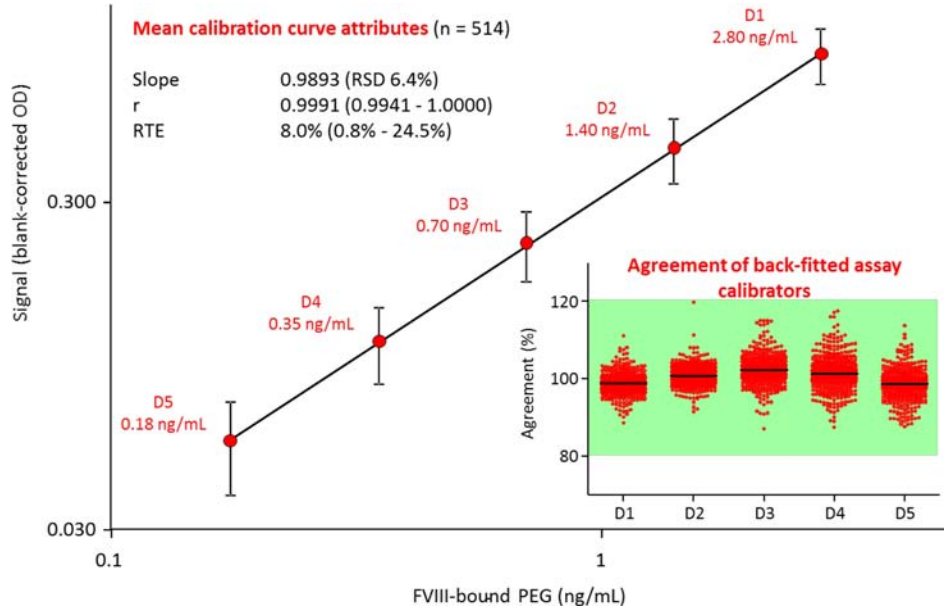

Figure 4. Mean calibration curve and back-fitting results for calibration curves constructed during nonclinical characterization of BAX 855. The rFVIII-bound PEG concentrations of the five assay standards D1 to D5 are shown. The inset shows the agreement of the back-fitted assay calibrators with their respective nominal concentrations. The 100 ± 20% range, representing the EMA acceptance criterion for this performance attribute for assay calibration curves, is highlighted.

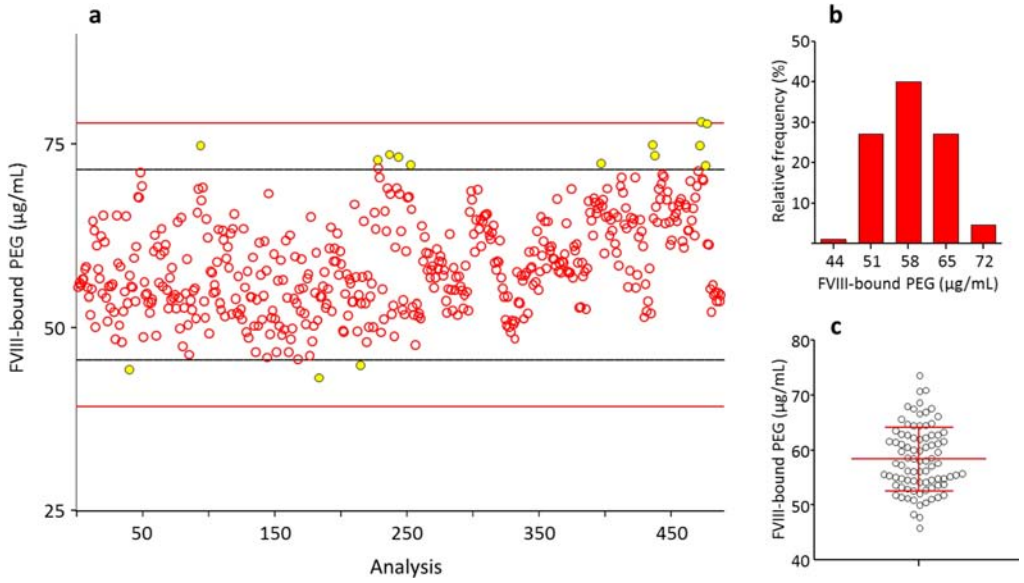

Figure 5. Results for the assay control during nonclinical characterization of BAX 855 (a) show the average control chart with full circles indicating results outside the double SD-range determined for the assay control, while (b) and (c) show the distribution of results.

Use of the PEG-rFVIII LBA in Nonclinical BAX 855 Studies. The nonclinical development of the PEGylated rFVIII preparation BAX 855 was supported by measurement of rFVIII-bound PEG. An overview of all studies carried out is provided in ref 19. The LBA's adequate performance over time is demonstrated by the assay's calibration curve data shown in Figure 4. The 514 five-point log–log calibration curves, which ranged from 2.80 to 0.18 ng rFVIII-bound PEG (or 26.1 to 1.63 ng/mL expressed as BAX 855 concentration), had a mean correlation coefficient of 0.9993 with all individual values higher than 0.9941, and a low mean relative total error RTE (8.0%) with individual values ranging from 0.8% to 24.5%. The back-fitted assay concentrations of the assay calibrators were within 100 ± 20%, thus clearly meeting the recently published acceptance criterion for LBA calibration curves.[17] Furthermore, the RSD of 6.4% determined for the mean slope of the calibration curves demonstrates their high similarity.

The LBA's precision over time is illustrated by a graphical summary on the data obtained for the assay control in more than 500 runs, carried out within one year (Figure 5). This control was also measured in a serial dilution series averaging the concentrations determined for four serial dilutions to obtain its mean concentration. The RSD of this mean was lower than 15%, a value commonly regarded to demonstrate an adequate level of parallelism in the multipoint assay format.[20] The overall mean concentration of 58.4 µg rFVIII-bound PEG/mL had an

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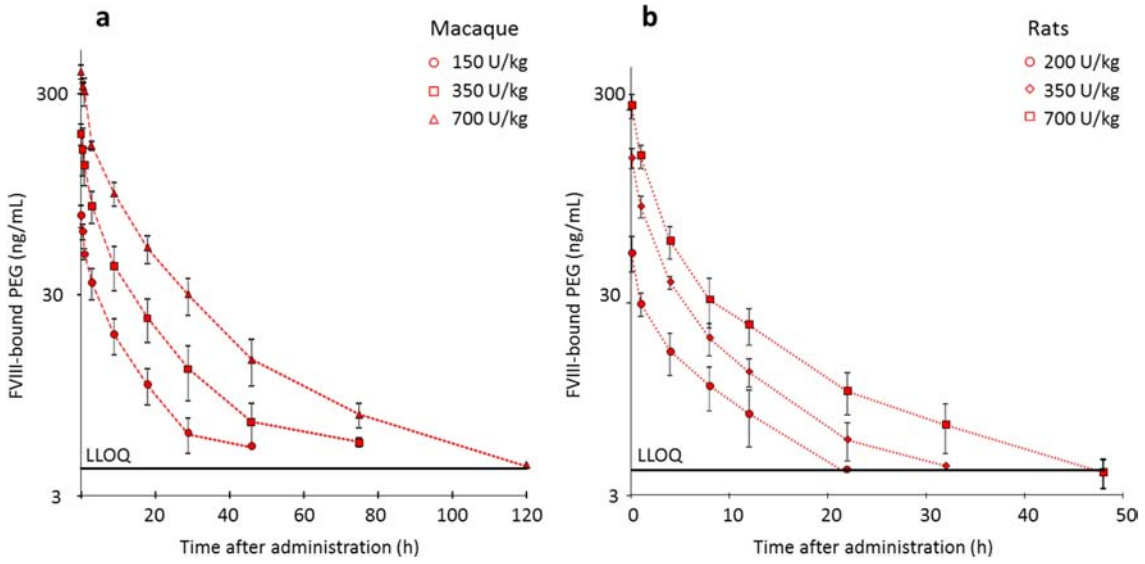

Figure 6. Use of PEG-FVIII in animal studies for BAX 855 (a) and (b) show the mean concentration−response curves obtained in macaque and rat studies, respectively, using a semilogarithmic plot; error bars mark the single standard deviation of the mean (n = 6), while the line represents the LBA's lower limit of quantification (LLOQ).

RSD of 10.1%, with only 15 of 487 individual values outside the ±2 SD range but within ±3 SD. The mean concentrations were normally distributed as shown by a D'Agostino and Pearson omnibus normality test, calculated by using GraphPad Prism 5.00.

Finally, to demonstrate the applicability of the PEG-FVIII LBA, Figure 6 shows time vs rFVIII-bound PEG concentration curves, determined with the PEG-rFVIII LBA in the samples derived from macaques (Figure 6a) and rats (Figure 6b). BAX 855 was administered as a single dose at one of three dose levels. The results obtained with the PEG-rFVIII LBA clearly reflected the BAX 855 doses administered and allowed valid statistical data evaluation, resulting in the common descriptive pharmacokinetic parameters.

DISCUSSION

In a recent review of methods to measure PEGylated compounds, Cheng et al.[18] discussed several assays including (1) direct ELISA, where the PEGylated compound is coated to the well and detected by binding of an anti-PEG antibody, (2) sandwich PEG ELISA, based on using an anti-PEG antibody for both capture and detection, and (3) combination sandwich ELISA, in which the PEGylated protein is captured by an anti-protein antibody and detected by the binding of an anti-PEG antibody.

These methods, however, appear to exclude the specific and to the best of our knowledge new assay based on the PEG-specific capture of the PEGylated compound followed by a protein-specific detection, despite its apparent advantages. PEGylation, especially when carried out so as to preserve as much as possible the biological activity of a protein, addresses accessible, solvent-exposed parts of the protein, rendering the attached PEG moieties themselves accessible for interaction with the capturing anti-PEG antibody. Subsequently, this interaction further aligns the PEGylated protein such that epitopes not shielded by the attached PEG residues become available for binding to the antiprotein antibody. Both events contribute to creating conditions necessary to measure PEGylated protein with adequate sensitivity and selectivity.

The data presented here for the presented PEG-rFVIII LBA confirmed the validity of these considerations in terms of the assay's sensitivity. Full-length rFVIII ADVATE, the protein modified by PEGylation, has a molecular weight of 280 kDa.[21] Consequently, the full-length PEGylated rFVIII encoded BAX 855, modified with two 20-kDa PEG residues per mol FVIII[19] can be assigned a molecular weight of 320 kDa.

Conventionally, FVIII activity is expressed in plasma units with about 200 ng FVIII corresponding to one unit FVIII.[21] Since extensive characterization data showed that PEGylation of ADVATE, resulting in the PEGylated rFVIII BAX 855, can be performed without a loss in activity,[19] a nearly unchanged mass-activity ratio applied for BAX 855 as only the addition of PEG has to be considered. Thus, 229 ng BAX 855 corresponded to the activity of 1 U FVIII. The LBA's calibration curve ranged from 2.80 to 0.18 ng/mL ng rFVIII-bound PEG/mL, translating to BAX 855 concentrations of 26.1 to 1.6 ng/mL (0.082 to 0.005 nM) or 117 mU/mL to 7.3 mU FVIII activity/mL. Plasma samples had to be diluted at least 1/20, so the LLOQ was 32 ng/mL BAX 855 (0.1 nM). The validation samples covered a BAX 855 concentration range from 0.15 to 12 nM.

These data confirmed the adequate sensitivity of the LBA, presenting the method as a complementary approach to the two-dimensional electrophoresis recently described for structural analysis of PEGylated rFVIII.[22] While the 2D-DIGE approach has been shown to provide a high resolution on the molecular level down to subtle differences caused by the number of sialic acids attached to the N-glycans of FVIII, the combined LBA detects the conjugate as the entire molecule. Clearly, this reduction in resolution was a prerequisite for the quantitative data evaluation, being the primary objective of this assay.

The basis for the PEG-rFVIII LBA was an in-house generated polyclonal rabbit anti-PEG IgG antibody preparation. In line with other reports,[13−15,23,24] immunization of rabbits with a multiple 5-kDa PEGylated hSA preparation also induced the generation of anti-PEG IgG. All rabbits developed measurable, stable titers of anti-PEG IgG, despite using only PEGylated

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human serum albumin and not a highly immunogenic protein such as keyhole limpet hemocyanin, as for the commercially available anti-PEG IgG.[16] Similar results would likely be obtained by using monoclonal anti-PEG IgG antibodies, as supported by our competition experiments. The reason is the quite simple homogeneous and linear structure of PEG that limits the number of available epitopes.

The PEGylated hSA, obtained by applying a NHS-based coupling chemistry, had a molar degree of PEGylation of 4.6, which appeared optimal for achieving the desired anti-PEG immune response. Thus, it had been reported that PEGylated ovalbumin conjugated with six 11-kDa PEG chains elicited the production of antibodies that were able to precipitate both PEG and ovalbumin. In contrast, higher PEGylated ovalbumin with a molar PEGylation degree of 20 had predominantly non-immunogenic properties both regarding the anti-ovalbumin and anti-PEG responses.[13] Similar findings were reported with other carrier molecules and haptens, where an increasing degree of substitution resulted first in an increase and then in a decrease in immunogenicity.[25]

Human serum albumin, being a foreign protein for rabbits, also induced anti-hSA IgG in all rabbits, which was successfully removed by affinity chromatography on immobilized hSA. As intended, the anti-PEG capture step, based mainly on the specificity of the anti-PEG IgG, provided the specificity for PEGylated rFVIII with no signs of binding when the unmodified full-length rFVIII preparation ADVATE or animal plasma samples, containing high levels of animal albumin, were analyzed. This enabled monitoring of the dose-concentration curves of PEGylated rFVIII in FVIII-competent animal models without the usual interference by endogenous FVIII when using conventional immunological or activity assays.

The assay setup with the anti-PEG capturing antibody and the polyclonal FVIII-specific detection antibody allowed selective measurement of PEGylated rFVIII in the presence of nonmodified endogenous animal or even human FVIII. This selectivity distinguishes the LBA from generic commercial FVIII ELISA systems (e.g., Asserachrom or Visulize), as the applied antibody combinations are not exclusively selective for human FVIII, so that the sum of endogenous and administered FVIII rather than the human FVIII administered is measured. Likewise, the FVIII activity assays cannot differentiate and provide the sum of endogenous and administered FVIII activity present in the sample. In contrast, the PEG-rFVIII LBA provides concentrations of PEGylated rFVIII, independent of the presence of unmodified FVIII, expressed as different units of measure. Per definition, we used "ng rFVIII-bound PEG/mL", but "ng BAX 855/mL" or "U FVIII/mL" are also applicable.

The PEG-rFVIII LBA's layout followed the typical requirements for sandwich ELISAs,[26] except that the use of PEG-containing detergents such as Tween or Triton X-100, often included to increase the efficiency of washing steps and to limit protein adsorption, was avoided as these detergents were likely to reduce the assay's sensitivity. Instead, the high concentration of 3% nonfat dry milk was successfully used in the dilution buffer, and the number of washing steps was increased.

Characterization of the binding characteristics of the in-house developed anti-PEG IgG demonstrated that binding occurred independently of PEG chain length. This outcome was due to the fact that the anti-PEG IgG preparation, generated by immunization with multiple 5 kDa-PEGylated human serum albumin, showed high binding to various PEGylated rFVIII preparations although these conjugates contained two branched 10 kDa PEG chains. Thus, neither the protein nor the length of the PEG chain influenced the binding.

There are only sparse data on the epitope characterization of anti-PEG IgG antibodies. Thus, the binding site of two rabbit anti-PEG antibodies was identified as complementary to six to seven repeating $-CH_2-CH_2O-$ units.[13] Clearly, such an epitope would be in line with the binding characteristics of the in-house developed rabbit IgG anti-PEG preparation. The results of our competition studies, however, made the exclusive participation of such an epitope unlikely as the data highlighted the importance of the terminal methoxy group for efficient interaction.

Thus, methoxyPEG showed much higher competition efficiency than dihydroxy PEG. This finding is in line with the observations recently reported by Saifer et al.[24] where the authors ascribed the PEG-terminating methoxygroup an essential quantitative and qualitative role for anti-PEG antibody development. Similar competition behavior was found for the commercially available anti-PEG IgG preparation, while at least quantitative equivalence between the two preparations was shown by the direct competition experiment, where the in-house developed preparation efficiently competed with the commercial one.

The specific binding characteristic of the anti-PEG antibody, recognizing the terminal part of the PEG chain, enabled the PEG-rFVIII LBA to demonstrate not only the molecular integrity of the entire conjugate, but also more of the PEG chain attached. This is important since breaking the PEG chain can metabolically be accompanied by the generation of low-molecular-weight acidic fragments.[27,28]

To address the integrity of a PEGylated insulin, Elliott et al.[29] described two independent approaches: (1) gel electrophoresis and immunoblotting with an anti-PEG and an anti-insulin antibody and (2) nuclear magnetic resonance spectroscopy. The LBA described here may provide additional data regarding the circulatory fate of a PEGylated compound, targeting measurement of the intact PEGylated compound or at least of PEGylated fragments thereof that are still recognized by the polyclonal detection antibody. By selecting appropriate monoclonal antibodies that bind to known epitopes on the PEGylated protein antibody, the PEG-rFVIII LBA allows tailoring of specific assay formats to obtain further insight into the fate of PEGylated fragments.

Recently, an LC-MS method was described by Xu et al.[30] to quantify a PEGylated peptide in human plasma. The authors applied an immunoaffinity purification method using an anti-PEG antibody followed by two-dimensional LC-MS for an investigational peptide containing 38 amino acids with a 40 kDa branched PEG. In their intraday validation, the authors demonstrated an accuracy of 94.8% to 105.8%, with <9.8% RSD for the immunoaffinity purification method over a dynamic range of 2 to 200 nM. The LBA presented here demonstrated similar accuracy and precision, but at a more than 30-fold higher sensitivity. It should be noted, however, that immunoassay can never achieve the selectivity of an LC-MS method.

Bioanalytical method validation of the LBA was carried out essentially according to the recent EMA guideline. Citrated plasma from FVIII-deficient mice and from rats and macaques was identified as required to demonstrate the adequate performance of the assay. The validation samples covered a

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range of rFVIII-bound PEG of 5 to 400 ng/mL, corresponding to BAX 855 concentrations of 23.7 to 1.898 ng/mL (0.07 to 5.93 nM). Within this range, accuracy and precision data were acceptable. Most importantly, the newly introduced total error of the LBA fully met the criterion defined in the EMA guideline for validation. The data obtained using this LBA were part of the nonclinical data package that formed the basis for approval by European authorities of the phase 1 clinical study initiated in 2011.

In summary, a selective and sensitive LBA was developed and successfully validated to measure PEGylated rFVIII BAX 855 in plasma from FVIII-deficient mice and from rats and macaques. All results obtained during the validation complied with the specifications defined in the EMA guideline for bioanalytical method validation. The assay's mean total errors, giving the sum of absolute accuracy and precision, were 11.0%, 16.4%, and 15.2% for rat, E17 mouse, and macaque plasma samples spiked with rFVIII-bound PEG concentrations of 10 to 400 ng/mL, and were <35% at the assay's LLOQ of 4 ng/mL using a minimum dilution of 1/20 for the plasma samples. This concentration of rFVIII-bound PEG corresponded to 0.05 nM BAX 855 (17 ng/mL).

The selectivity of the LBA enabled measurement of BAX 855 without interference by endogenous FVIII in wild-type animal models, and the multipoint approach selected as the assay's format, provided a robust performance during nonclinical characterization of BAX 855. It is plausible that tailoring the PEG-rFVIII LBA would allow measurement of any PEGylated protein, simply by using the specific enzyme-labeled antibody for detection after capture by the plate-immobilized anti-PEG IgG.

EXPERIMENTAL PROCEDURES

Materials. Four PEGylated rFVIII preparations were used to set up and characterize the assay performance (see Table 3).

Table 3. PEGylated rFVIII Preparations Used in the Study

sample code/lot no.	bound PEG[a] [μg/mL]	FVIII activity [U/mL]	DP [mol/mol]
BAX 855 ORHLUFB09003 PSR[b]	56.2	2333	2
BAX 855 #F8−855−09.022	5.3	161	2
BAX 855 #F8−855−09.024	4.5	133	2
BAX 855 #VNH5K002A	4.2	138	2

[a]Bound PEG was determined by an HPLC-based method, FVIII activity by a chromogenic method. The degree of PEGylation (DP) is given in mol PEG per mol FVIII, using a molecular weight of 280 kDa to calculate the molar FVIII concentrations. [b]BAX 855 preparation ORHLUFB09003 PSR was used to construct the assay calibration curve of the final PEG-rFVIII LBA.

PEGylation of rFVIII was carried out in collaboration with Nektar Therapeutics (Huntsville, AL, USA), using a branched 20 kDa methoxypolyethylene glycol (mPEG) NHS reagent (see Figure 1). The full-length rFVIII preparation ADVATE was used for the conjugation process, yielding the PEGylated rFVIII preparation BAX 855 with a low degree of PEGylation: ~2 mol PEG per rFVIII molecule. This conjugate showed an improved pharmacokinetic profile without compromising its specific activity. mPEG (5 kDa) and PEG 6000 were from VWR (Vienna, Austria). Further materials and reagents are described in the methods.

Preparation and Purification of a Rabbit anti-PEG IgG. Rabbit anti-PEG antiserum was obtained after immunization of rabbits (New Zealand White) using the adjuvant TiterMax Classic (Sigma, Vienna, Austria) and administering 5 kDa-PEGylated human serum albumin (hSA) as the immunization antigen.[31] Serum samples were taken before and after immunization. Direct ELISAs with hSA, PEGylated hSA, and PEGylated recombinant von Willebrand factor (VWF), were used to detect the formation of anti-PEG IgG. Finally, a purified rabbit IgG fraction was obtained by Protein G affinity chromatography (GE Healthcare, Vienna, Austria). The IgG fraction obtained by acid elution (0.2 M glycine, pH 2.5) was immediately neutralized and stored at +4 °C after addition of 0.02% NaN_3. Since a portion of the rabbit IgG fraction was shown to bind to nonmodified hSA, affinity chromatography on immobilized hSA was used for further purification.

Description of the LBA for BAX 855. Rabbit anti-PEG IgG (20 μg/mL in 0.1 M carbonate buffer, pH 9.5; 100 μL/well) was incubated with a Maxisorp F96 plate (VWR; Vienna, Austria) overnight at +4 °C. Blocking was carried out at room temperature (RT) with PBS (DB; PBS with 3% (w/v) nonfat dry milk [Maresi, Vienna, Austria], and 2 mM benzamidine [Sigma, Vienna, Austria], pH 6.1) for 2 h. PBS was used as washing buffer between the individual steps. Serial dilution series of the standard/samples were incubated at RT for 1 h. Then, sheep anti-human FVIII-peroxidase (Cedarlane CL20035HP, Szabo; Vienna, Austria; 1/250 in DB) was added and incubated at RT for 1 h, before measuring the peroxidase activity with SureBlue (KPL, Hamburg, Germany) at 450 nm. Quantitative evaluation was based on a calibration curve obtained by a log−log fitting. This five-point calibration curve defined a range of 2.8 to 0.18 ng/mL rFVIII-bound PEG or 26.1 to 1.63 ng/mL (0.082 to 0.005 nM) when expressed as BAX 855 concentrations.

Additional Methods. Protein-bound PEG concentrations and free PEG were measured with HPLC-based methods, FVIII antigen with the ASSERACHROM VIII:Ag kit (Diagnostica STAGO), and FVIII activity with the chromogenic assay (Baxalta). ELISAs to characterize the anti-PEG antisera were carried out as follows: The respective protein (hSA, PEGylated hSA or PEGylated VWF) was coated to Maxisorp F96 plates by incubation in carbonate buffer, pH 9.5. Blocking and dilution of the samples was carried out with DB (see above). Bound rabbit IgG was detected by binding of a goat anti-rabbit IgG peroxidase conjugate (BioRad, Vienna, Austria). Peroxidase activity was measured with SureBlue (KPL).

Selectivity Check of the PEG-rFVIII LBA. BAX 855 #VNH5K002A was used for the competition studies. Serial dilution series of mPEG 5000 and PEG 6000 were incubated with 1/1000-diluted BAX 855 before performing the LBA. Either the in-house purified anti-PEG IgG or the commercially available rabbit monoclonal anti-PEG antibody PEG-B-47 from Epitomics (ABCAM) was used as capturing antibody. Competition curves were calculated with GraphPad Prism 5.00 using the log (inhibitor) vs normalized response function with variable slope. Finally, serial dilution series of the in-house purified anti-PEG IgG, mixed with BAX 855, were used to compete with the binding of BAX 855 to wells coated with the Epitomics antibody. The competition curves obtained provided data to test equivalence of the two preparations' binding characteristics.

Assay Validation. Assay validation was carried out in citrated FVIII-deficient mouse (E17), rat, and macaque plasma

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according to the EMA guideline for bioanalytical method validation.[17] Rat and E17 plasma samples came from Baxalta (Vienna, Austria), citrated plasma from macaques was provided by Covance (Münster, BRD). Accuracy was addressed by spike-recovery of BAX 855 in these three matrices and using the back-fitting approach for the assay calibration curves. Assay precision was determined as intra- and inter-run precision at five rFVIII-bound PEG levels (5 to 400 ng/mL, corresponding to BAX 855 concentrations of 47 to 3795 ng/mL) and for the assay control over an extended period. Finally, the influence of the citrated E17 mouse, rat, and macaque plasma on the assay's linearity was investigated.

Measurement of Plasma Samples from Animal Studies. All animal studies were conducted under GLP at AAALAC-certified facilities following the relevant laws and regulations. During nonclinical characterization, the BAX 855 concentrations, expressed as the levels of rFVIII-bound PEG, were measured with the PEG-rFVIII LBA in the citrated plasma samples from rats (CD, obtained from Charles River, Germany) and macaques (*Macaca fascicularis* of Mauritian origin). Samples for BAX 855 measurement were taken before dosing and 0.08, 0.5, 2, 6, 12, 24, 36, and 48 h after dosing for the rat study, and before dosing and 0.08, 0.5, 1, 3, 9, 18, 29, 46, 75, and 120 h after dosing for the macaque study. Frozen aliquots were received for measurement.

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Notes

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ABBREVIATIONS

rFVIII, recombinant coagulation factor VIII; LBA, ligand-binding assay; NHS, *N*-hydroxysuccinmide; hSA, human serum albumin; VWF, von Willebrand factor

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