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ORIGINAL ARTICLE

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Reagent-specific underestimation of turoctocog alfa pegol (N8-GP) clotting activity owing to decelerated activation by thrombin

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Abstract

Background: Factor VIII (FVIII) procoagulant activity is commonly assessed by measuring the activated partial thromboplastin time (APTT) to clot formation using one of the many available but differently composed reagents. The majority of APTT reagents also accurately recover the activity of the extended half-life molecule N-glycoPEGylated FVIII (N8-GP; turoctocog alfa pegol), while a few silica-based reagents give a low recovery.

Objective: To identify the cause of N8-GP activity underestimation in the presence of certain silica-based APTT reagents.

Methods: Development of FVIIIa-dependent tenase activity and appearance of FVIIIa from N8-GP and its non-PEGylated counterpart (N8) were compared using clotting assays, a factor Xa (FXa) activity assay mimic thereof, and thrombin activation time courses.

Results: A strong correlation was demonstrated between clotting and FXa activity assays based on similar recoveries of N8-GP activity and equal responses to an altered duration of the contact activation phase, validating the latter as a useful clotting assay mimic. Contact activation phase duration influenced, and could even eliminate, N8-GP activity underestimation. Thrombin-catalyzed conversion of N8-GP to FVIIIa was considerably slower than that of N8 despite similar extents of adsorption to silica particles in APTT-SP, suggesting different modes and/or orientations of adsorption.

Conclusions: Some contact activators reduce thrombin's ability to cleave N8-GP more than native FVIII. Decelerated thrombin activation of N8-GP is reflected in delayed FVIIIa-dependent appearance of FXa activity in plasma, in turn leading to prolonged clotting time. This forms the basis for underestimation of N8-GP activity as measured by one-stage clotting assay against a FVIII standard.

KEYWORDS

blood coagulation tests, coagulation factor VIII, hemophilia A, silicon dioxide, turoctocog alfa pegol

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Essentials

- Certain silica-based reagents underestimate N8-GP (turoctocog alfa pegol) clotting activity.
- Compared with N8, thrombin activation of N8-GP is decelerated and causing the underestimation.
- The duration of the contact activation phase also influences the recovery of N8-GP activity.
- Clinical laboratory awareness of the importance of the choice of N8-GP assay reagent warranted.

1 | INTRODUCTION

Hemophilia drugs with prolonged duration of action, permitting longer intervals between infusions, offer more convenient prophylactic regimens for the patients. Hemostatic molecules with an extended half-life (EHL) have therefore been a focus area in the biopharmaceutical industry for the past decade. Recent and imminent launches include modified long-acting factor VIII (FVIII) and factor IX (FIX) products in the form of the pro-cofactor and zymogen, respectively, conjugated to polyethylene glycol (PEG) or fused with albumin or the Fc portion of immunoglobulin G. Successful concomitant extension of a molecule's residence time in circulation and retention of its hemostatic potency is unfortunately sometimes accompanied by discrepancies when attempting to assess the level of biological activity using the native protein counterpart as the reference.[1,2] In other words, the modification may alter the functional assay behavior compared with the parent factor. A majority of the activated partial thromboplastin time (APTT) reagents accurately, or acceptably, estimate the activity of PEGylated FVIII molecules (including N-glycoPEGylated FVIII [N8-GP]; turoctocog alfa pegol) in one-stage (OS) clotting assays. However, discrepant recoveries have been observed using certain APTT reagents, as well as when comparing OS and chromogenic assays.[3-7] This complicates post-infusion monitoring of EHL factors in hemophilia A or B patients.[8]

N8-GP carries a 40-kDa PEG moiety covalently attached to an *O*-linked glycan in the truncated B domain and represents a PEGylated form of turoctocog alfa (NovoEight, N8).[9] A significant degree of underestimation of N8-GP clotting activity has been observed with some silica-based APTT reagents.[4,5] Considering that thrombin cleavage of N8-GP yields an activated cofactor identical to native FVIIIa, the reason for the underestimation is to be found in the N8-GP pro-cofactor and could for instance be related to its activation kinetics. The present study employed, and partly dissected, the OS clotting method complemented by measurements of factor Xa (FXa) chromogenic activity (ie, FVIIIa cofactor activity) generated under conditions mimicking the clotting assay and thrombin activation studies to gather appealing evidence for a principal mechanism underlying N8-GP activity underestimation when using certain silica-based reagents in the OS FVIII clotting assay.

2 | MATERIALS AND METHODS

2.1 | Materials

N8,[10] N8-GP,[9] and hirudin were produced in-house at Novo Nordisk A/S (Måløv, Denmark). HemosIL APTT-SP (liquid) was from Instrumentation Laboratory (Bedford, MA), Pathromtin SL and FVIII-deficient plasma were from Siemens (Marburg, Germany), STA-PTT Automate from Diagnostica Stago (Asnières sur Seine, France), and TriniCLOT aPTT HS from Tcoag (Bray, Ireland). The SSC/ISTH secondary coagulation standard lot #4 (SSCLOT4) was from NIBSC (Potters Bar, UK) and used as normal human plasma. Von Willebrand factor was from American Diagnostica (Stamford, CT), factor IXa (FIXa) from Sekisui Diagnostics (Lexington, MA), factor X (FX) and factor XIa (FXIa) from Enzyme Research Laboratories (South Bend, IN), and thrombin from Roche Diagnostics (Penzberg, Germany). The fibrin polymerization inhibitor H-Gly-Pro-Arg-Pro-NH_2 (GPRP) was from Bachem (Bubendorf, Switzerland), and the chromogenic substrate S-2765 obtained from Chromogenix (Milan, Italy).

2.2 | Preparation of FVIII samples for activation studies

N8-GP and N8 had specific activities of 9974 and 8963 IU/mg FVIII protein, respectively, as determined by COAMATIC Factor VIII (Chromogenix, Milan, Italy), and were dissolved by adding 0.9% (w/v) NaCl. The FVIII solutions, possessing an activity level between 400 and 800 IU/mL, were aliquoted and frozen at −80°C. An aliquot was thawed before use and diluted to 30-100 IU/mL in 20 mmol/L Hepes, pH 7.4, containing 0.15 mol/L NaCl and 10 mg/mL bovine serum albumin. The sample was further diluted to 3-10 IU/mL in FVIII-deficient plasma and finally diluted to 0.3-1 IU/mL in Hepes buffer for assay use (except thrombin activation studies [direct dilution in Hepes buffer to 10 IU/mL]).

2.3 | Modified COATEST SP FVIII chromogenic assay

The COATEST solutions were prepared as instructed by the manufacturer (Chromogenix, Milan, Italy) in the package insert. Fifty microliters (μL) of FVIII sample (N8 or N8-GP; 0.6 IU/mL) was then mixed with 50 μL FVIII-deficient plasma and 50 μL APTT reagent to resemble the APTT assay contact phase. Controls were included in which Hepes buffer replaced FVIII and/or plasma and/or APTT reagent. A sample of 25 μL was immediately withdrawn from these mixtures and added to 50 μL of the prepared COATEST solution containing FIXa, FX, phospholipid (and thrombin activity), followed by 5 minutes of incubation at 37°C. COATEST $CaCl_2$ solution (25 μL) was added followed by 3 minutes of incubation. FXa formation was terminated and quantified by adding 50 μL of the COATEST solution containing S-2765/I-2581 (and excess EDTA), after which the microtiter plate was

placed in a SpectraMax 340PC spectrophotometer and the absorbance increase at 405 nm monitored for 2 minutes.

2.4 | FXa generation assay mimicking one-stage FVIII clotting assay

Twenty-five microliters (μL) of FVIII sample (N8, N8-GP; 0.3-1 IU/mL) was mixed with 25 μL FVIII-deficient plasma (containing 10 mmol/L GPRP) and 25 μL APTT reagent, and incubated at 37°C for the time period recommended in the APTT reagent inserts for OS clotting assay. This step thus represents the APTT assay contact phase. Fifty microliters (μL) of 0.375 mmol/L S-2765 in 20 mmol/L Hepes buffer, pH 7.4, containing 0.15 mol/L NaCl and 8.75 mmol/L $CaCl_2$, was added followed by continuous measurement of the increase in absorbance at 405 nm for 10 minutes. These two steps combined represent an entire APTT assay with its contact and clotting phases. The sum of any pre-existing enzymatic activity in the plasma and activity generated during the contact phase was assessed in a sample where Hepes buffer replaced FVIII. In addition, a separate experiment was conducted where excess EDTA alone or EDTA solution containing 100 nmol/L hirudin was added at a time point when the absorbance in a mixture containing N8 had become significantly greater than in the buffer blank, showing that the FVIII-dependent activity measured only stemmed from FXa without detectable contribution from thrombin.

2.5 | One-stage clotting assay

FVIII samples to be used in this assay were prepared by dilution to 10 IU/mL in Hepes buffer containing 10 mg/mL bovine serum albumin, followed by dilution to 1 IU/mL in FVIII-deficient plasma before being placed in the instrument. Alternatively, normal human plasma (SSCLOT4) was used as the sample. The clotting assay was performed on an ACL TOP 550 instrument, using APTT reagents according to the manufacturer's instructions and adhering to the variable duration of the contact activation phase. With APTT-SP and Pathromtin SL, contact activation phases of shorter and longer duration than recommended were also employed.

2.6 | FXIa activity measurements

Diluent (the one used in the ACL TOP instrument; 37°C), ice-cold SSCLOT4 plasma and APTT reagent (37°C) were mixed and incubated at 37°C, thus mimicking the contact activation phase of the OS clotting assay. Samples were withdrawn half way into the contact activation phase, at the end of it (duration as recommended by the manufacturer; see Table 1), as well as after twice the duration. Sample quenching and quantification of FXIa activity content according to a modified Rox Factor XIa method were performed as described,[11] except that the samples from FXIa generation in plasma were diluted only 100-fold in MES buffer. The FXIa concentrations in the samples were derived from a standard curve made from the purchased FXIa (using the specific activity provided on data sheet from Enzyme Research Laboratories).

TABLE 1 Factor XIa accumulation in normal human plasma (SSCLOT4) during a contact activation phase of recommended duration (CAP-RD) and the ensuing recorded clotting time after recalcification (mean ± SD, n = 3)

APTT reagent (CAP-RD)	Factor XIa (mU/mL)	Clotting time (s)
APTT-SP (180 s)	97 ± 26	47.2 ± 0.3
TriniCLOT aPTT HS (300 s)	82 ± 9	45.9 ± 0.8
STA-PTT automate (240 s)	96 ± 18	49.5 ± 1.2
Pathromtin SL (120 s)	15 ± 5	57.3 ± 0.3

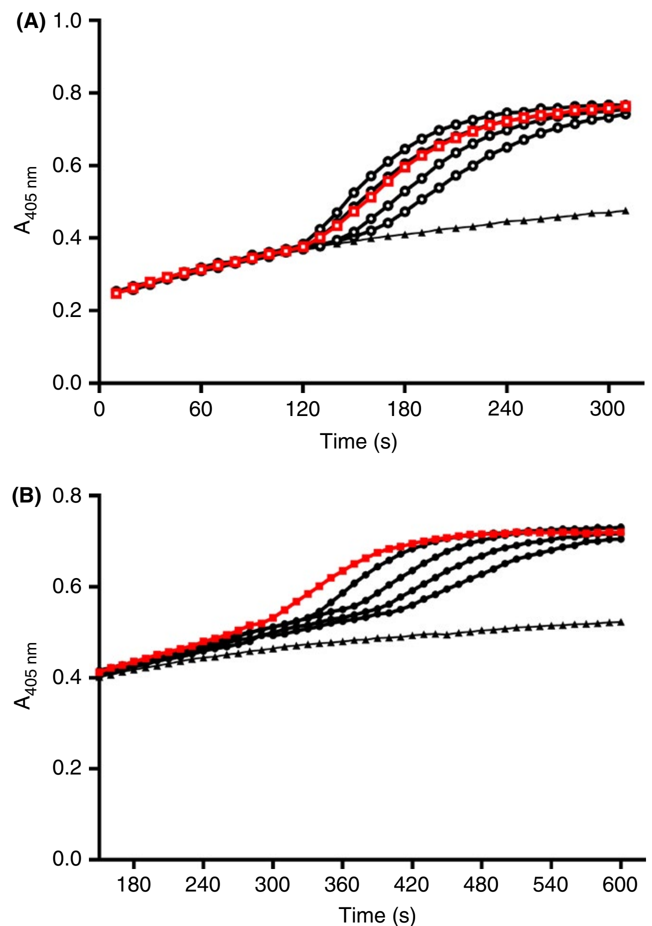

FIGURE 1 Real-time factor Xa (FXa) activity after recalcification in a system mimicking OS clotting assay. (A) Measurements of FXa activity in the presence of APTT-SP. The red curve represents a sample containing 1.0 IU/mL of N8-GP and the black curves, from left to right, show 1.0, 0.7, 0.5, and 0.3 IU/mL of N8. The curves obtained with 1.0 IU/mL of N8-GP and 0.7 IU/mL of N8 overlay. The flattening out of the curves towards the end of the measurements is owing to S-2765 depletion. The straight, bottom curve (triangles) represents background hydrolysis of the FXa substrate by plasma component(s) in the absence of added factor VIII. (B) Measurements of FXa activity in the presence of Pathromtin SL. Curve assignments are as described in panel A

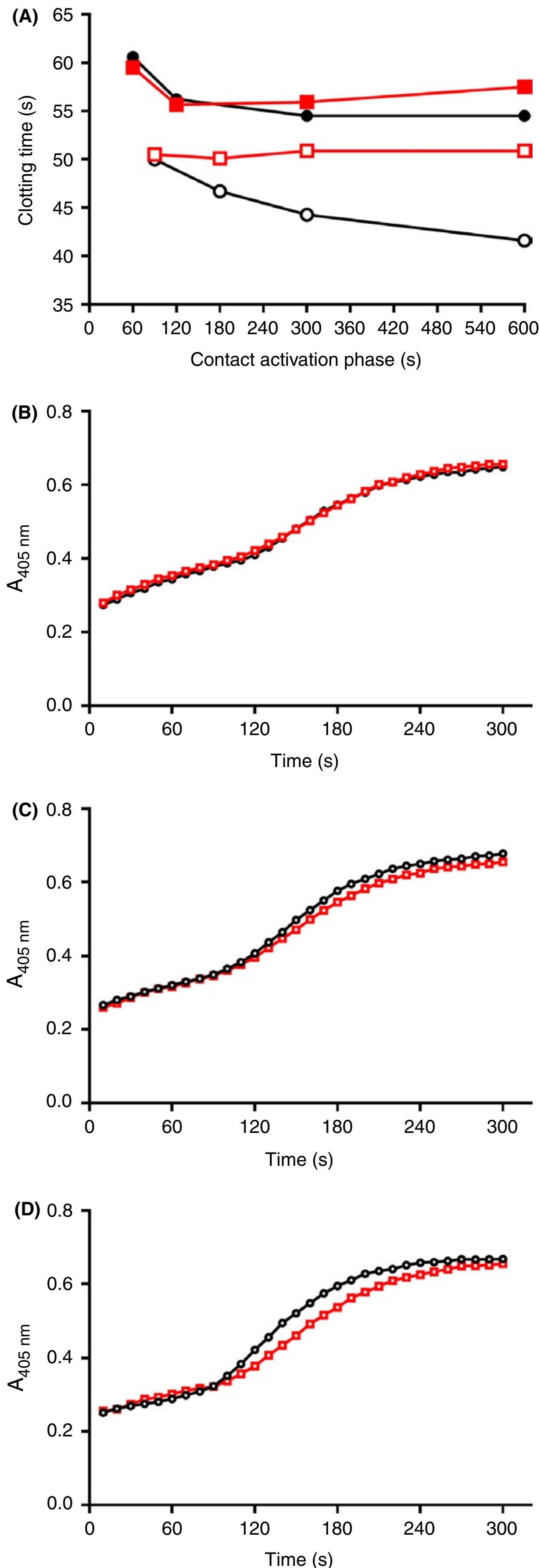

FIGURE 2 Effect of the duration of the contact activation phase on N8-GP recovery in OS clotting and factor Xa (FXa) generation assays. (A) Clotting times obtained with 1 IU/mL N8-GP (red squares) or N8 (black circles) spiked into FVIII-deficient plasma in the presence of APTT-SP (open symbols) or Pathromtin SL (filled symbols) are shown. The duration range of the contact activation phase embraced the recommended incubation times of 180-220 and 120 s, respectively. (B-D) Real-time FXa activity measurements with 1.0 IU/mL of N8-GP (red curves) or N8 (black curves) after a contact activation phase in the presence of APTT-SP lasting for 90 s (B), 180 s (C), and 300 s (D)

2.7 | FVIII activation by thrombin

Thrombin activation of N8 and N8-GP was studied by mixing 40 µL FVIII sample (10 IU/mL in Hepes buffer containing 20 mg/mL bovine serum albumin), 10 µL von Willebrand factor solution (50 µg/mL), 50 µL APTT reagent or buffer and finally 50 µL of a thrombin solution (20-250 pmol/L [depending on the APTT reagent] in Hepes buffer containing 20 mg/mL bovine serum albumin and 25 mmol/L $CaCl_2$). After 30, 60, 90, 120, and 150 seconds at 37°C, 25 µL of this mixture was transferred to 25 µL of a solution containing hirudin (100 nmol/L), FIXa (5 nmol/L), and phospholipids (30 µmol/L). FX (25 µL 300 nmol/L) was then added, followed by 3 minutes of incubation. FX activation was terminated and FXa formation quantified by the addition of 50 µL Hepes buffer containing 25 mmol/L EDTA and 2 mmol/L S-2765 and immediate initiation of a continuous measurement of the absorbance increase at 405 nm.

2.8 | Assessment of silica adsorption

Ten units of N8-GP or N8 in Hepes buffer was incubated with half the volume of APTT-SP or APTT-SP diluted to various degrees with APTT-SP supernatant. For this purpose, APTT-SP supernatant was collected after spinning APTT-SP for 10 minutes at 10 000 *g*. After 5 minutes of incubation at 37°C, the mixture was centrifuged for 10 minutes at 10 000 *g*. A sample of the supernatant (65 µL) was transferred to 35 µL reducing sample buffer, and 18 µL (~1.4 U of N8-GP/N8) was eventually applied on a polyacrylamide gel. SDS-PAGE was performed using a 7% NuPAGE Novex Tris-acetate gel and Tris-acetate running buffer (Invitrogen, Carlsbad, CA), and proteins were silver-stained.

3 | RESULTS AND DISCUSSION

Initial tests in a modified chromogenic assay using samples comprising parts of or the complete OS assay activation phase mixture confirmed two prerequisites for the subsequent investigations. First, using samples comprising either FVIII alone or FVIII plus FVIII-deficient plasma, we demonstrated the presence of the same amount of activatable FVIII protein in the N8 and N8-GP preparations as judged from the parallel yields of FXa activity. Second, using samples containing FVIII, FVIII-deficient plasma plus an APTT reagent (APTT-SP, TriniCLOT aPTT HS or STA-PTT Automate) known

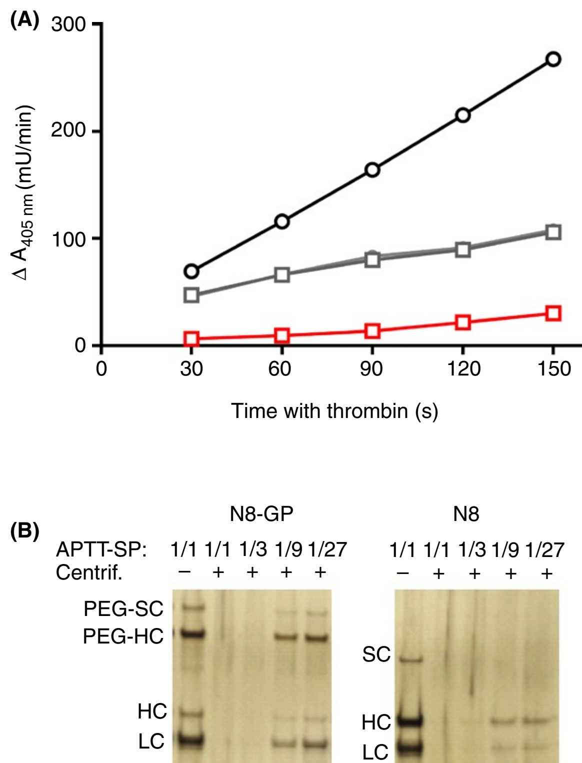

FIGURE 3 N8-GP and N8 activation by thrombin and adsorption to silica. (A) Development of factor Xa (FXa) activity resulting from the activation of N8-GP (red squares) or N8 (black circles) to factor VIIIa (FVIIIa) by 30 pmol/L thrombin in the presence of APTT-SP. The amount of FXa activity, reflecting the amount of FVIIIa cofactor activity, increased linearly with the exposure time to thrombin. The indistinguishable grey curves show FXa activity arising from N8-GP and N8 activation by 20 pmol/L thrombin with buffer replacing APTT-SP. (B) Adsorption of N8-GP and N8 to silica particles in APTT-SP. The amounts of protein remaining in solution after incubation with undiluted (1/1) or indicated dilutions of APTT-SP without (–) or after centrifugation (+) are visualized by SDS-PAGE (see section 2 for details). HC, heavy chain; LC, light chain; SC, single-chain

TABLE 2 Relative rate of thrombin activation of N8-GP compared to N8 in the presence of silica-based APTT reagents

APTT reagent ([IIa])	N8-GP rate/N8 rate*
APTT-SP (30 pmol/L)	0.14
TriniCLOT aPTT HS (30 pmol/L)	0.09
STA-PTT automate (75 pmol/L)	0.14
Pathromtin SL (250 pmol/L)	0.30
None (20 pmol/L)	0.96

*Individual rates are defined as the increase in FVIIIa cofactor activity (measured as generated FXa chromogenic activity) over 90 s (activity after 150 s exposure to thrombin subtracted by that after 60 s [depicted in Figure 3A]).

to underestimate N8-GP activity when measured against a plasma FVIII standard,[3-7] a relative reduction of FXa formation was demonstrated in the case of N8-GP. Based on measured FXa activity, the underestimating reagents gave ~60% recovery of N8-GP cofactor activity when compared with the N8 activity (arbitrarily defined as 100%). In the presence of Pathromtin SL, which returns the expected recovery of N8-GP activity,[4,5] we observed indistinguishable FXa activity levels with N8 and N8-GP. Overall, the FXa generation profiles were in agreement with the OS clotting assay findings, indicating that N8-GP activation kinetics was relatively slower in the presence of an underestimating APTT reagent. However, the outcome of this particular assay is conditional and depends on the (unknown) concentration of thrombin present and the time thrombin is allowed to act on FVIII.

Next we employed an experimental setup more closely mimicking the OS FVIII clotting assay to see whether the low (with APTT-SP) or anticipated recovery (with Pathromtin SL) of N8-GP activity were reflected in real-time FXa activity profiles. Measurements of FXa formation are warranted because a delayed FX activation would strongly indicate decelerated generation of FVIIIa cofactor activity and form a link to prolonged clotting times. In the presence of APTT-SP, FXa activity above background level indeed appeared with a delay for N8-GP compared with N8 (Figure 1A). The data indicated approximately 70% recovery of N8-GP activity with APTT-SP, and similar values were obtained with the other underestimating reagents TriniCLOT aPTT HS and STA-PTT Automate (data not shown). Using Pathromtin SL, the development of FXa activity was not delayed for N8-GP, rather slightly the opposite, which is in agreement with the anticipated full recovery as seen in the OS assay (Figure 1B).

Detectable FXa activity appeared later than would be expected based on the recorded clotting times in the OS assay. This may to some extent be due to the different parameters measured in the two assays, ie, chromogenic FXa activity vs plasma clot formation, but the presence of a competing FXa substrate (S-2765) definitely contributes to the delay. The presence of enzymatic activity in the contact-activated plasma capable of hydrolyzing S-2765 (the slope of the buffer sample in Figure 1) suggests that S-2765 may delay FIX activation and/or other event(s) occurring after recalcification, including cleavage mediated by FXa. Supportive of such a delay, we demonstrated that the OS clotting time was prolonged in the presence of S-2765, in this case most likely due to competition between S-2765 and prothrombin for FXa. For instance, a sample containing 1 IU/mL N8 produced clotting times of approximately 45 and 65 seconds in the absence and presence of 0.15 mmol/L S-2765, respectively.

The influence of the duration of the contact activation phase on N8-GP activity assessment was studied with APTT-SP and Pathromtin SL using three incubation times; the recommended time (see Table 1), plus a reduced and an extended duration. It turned out that N8-GP activity estimation in the OS clot assay depended on the duration with both reagents. The recommended incubation times resulted in a longer clotting time for N8-GP than for N8 when using APTT-SP (underestimating) and indistinguishable clotting times with

Pathromtin SL (returns the expected recovery of N8-GP activity) (Figure 2A). Underestimation of N8-GP activity with APTT-SP could be avoided by using a shorter contact activation phase of 90 seconds, whereas contact activation times extended beyond 120 seconds with Pathromtin SL began to lead to underestimation. Assessment of N8-GP activity using STA-PTT Automate displayed a similar pattern as APTT-SP in response to the duration of the activation phase, although the underestimation did not disappear entirely (not shown). When using APTT-SP in the FXa generation assay, the activity curves obtained with N8 and N8-GP were well-separated after 300 seconds incubation, moved closer at 180 seconds, and became indistinguishable after an incubation time of 90 seconds (Figure 2B-D), closely resembling the clotting time pattern (Figure 2A). The identical patterns demonstrated a strong correlation between the OS clotting and FXa generation assays.

How to explain a recovery varying with the duration of the contact activation phase? It is reasonable to assume that a longer contact activation phase results in a larger amount of FXIa being generated, and the concentration of FXIa dictates the rate by which FIXa is subsequently formed after recalcification. After a short contact activation phase, the rate of N8-GP activation apparently suffices to keep up with the relatively slow formation of FIXa to give the same FXa generation and clotting time as those obtained with N8. With an extended contact activation phase, more FXIa is formed and the subsequent generation of FIXa occurs more rapidly. If N8-GP exhibits relatively slower activation kinetics by thrombin, N8-GP eventually fails, before N8 does, to provide FVIIIa molecules at a sufficient rate to satisfy the growing pool of FIXa and consequently N8-GP activity gets underestimated. In support of this hypothesis, we documented a more productive contact activation phase in the presence of an underestimating APTT reagent as compared with Pathromtin SL, both in terms of higher level of FXIa accumulation and shorter clotting time of normal human plasma (Table 1).

Being central in the above hypothesis, the kinetics of activation by thrombin was assessed for N8-GP and N8. After different incubation times with thrombin, the resulting FVIIIa cofactor (tenase) activity was quantified as FXa formation. In the presence of APTT-SP, FVIIIa developed significantly slower from N8-GP than N8 (Figure 3A). If buffer replaced APTT-SP, identical processing rates were observed for the two FVIII forms. The rates of N8-GP activation in the absence and presence of APTT-SP were similar, whereas the rate of N8 activation was apparently enhanced by APTT-SP, plausibly owing to local accumulation of favorably oriented N8 molecules on the silica. Ratios between the rates of N8-GP and N8 activation by thrombin are summarized in Table 2. For the data to be useful, the activation of both FVIII forms needed to be detectable and linear over the time period, a requirement that necessitated the use of different thrombin concentrations for different reagents (see Table 2). The ratios obtained were generally below what would be expected based on the N8-GP activity recoveries obtained in the FXa and OS clot assays. This includes a ratio for Pathromtin SL significantly below one (0.30), albeit considerably higher than for the closely grouped underestimating APTT reagents (~0.1). The low ratios might be due to very different concentrations of thrombin generated in the FXa and OS clot assays compared with those used in the thrombin activation assay, as well as owing to other differences in experimental conditions. Nonetheless, a ratio clearly below 1 for Pathromtin SL would explain why N8-GP eventually becomes underestimated when the contact activation phase lasts sufficiently long (Figure 2A).

The decreased activation rate of N8-GP in the presence of APTT-SP was not evidently owing to PEG-mediated adsorption, since N8-GP and N8 appeared to stick to similar degrees to the surface of the silica particles (Figure 3B). However, the two FVIII forms may adsorb in, or distribute in a unique manner between, different preferred orientations or conformations on the silica, resulting in less accessibility of N8-GP for thrombin cleavage.

In summary, OS FVIII clotting assays are generally used to monitor hemophilia A treatment, making it very important that they estimate N8-GP clotting activity accurately.[12] Although a majority of APTT reagents return an acceptable recovery of N8-GP activity, the choice of reagent can matter. The duration of the contact activation phase also influences the recovery. Our data provide a potential simple remedy for N8-GP activity underestimation when using APTT-SP, namely shortening of the contact activation phase (to 90 seconds). This option to totally eliminate the underestimation of N8-GP activity may however be unique for APTT-SP. In a broad sense, our findings infer that whenever thrombin activates modified FVIII with slower kinetics than native FVIII, underestimation of EHL FVIII activity will occur when FXIa accumulates to above a certain threshold level. Above this reagent-specific level, subsequent FIXa formation occurs fast enough to reveal attenuated activation of EHL FVIII.

Finally, our compiled findings with N8-GP (herein) and N9-GP[11,13] illustrate how a common type of modification ("activation peptide" glycoPEGylation) can elicit various mechanisms in the presence of the contact activator of an APTT reagent. These mechanisms influence APTT-based procoagulant activity estimations, whereas measurements of N8-GP and N9-GP activity triggered directly by FXIa are unaffected by the PEGylations.[13,14]

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RELATIONSHIP DISCLOSURE

The authors are employees of Novo Nordisk A/S, the manufacturer of N8-GP (turoctocog alfa pegol).

AUTHOR CONTRIBUTIONS

E. Persson designed the research, analyzed data, and wrote the manuscript. T. Foscolo contributed to the research design,

performed experiments, analyzed data, and revised the manuscript. M. Hansen provided input to the research design and revised the manuscript.

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