Using reagents specific to Japan to measure turoctocog alfa pegol in hemophilia A plasma: A two-site study

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Abstract: Turoctocog alfa pegol (ESPEROCT®; N8-GP; Novo Nordisk A/S, Bagsvaerd, Denmark) is an extended half-life recombinant factor VIII (FVIII) shown to be efficacious for the prevention and treatment of bleeding episodes in patients with hemophilia A. Post-administration monitoring of patients on factor replacement products may be necessary during the course of treatment. However, many reagents currently available in Japan for the measurement of FVIII activity have never been tested for accuracy when measuring turoctocog alfa pegol. Here, we evaluated FVIII activity measurements of hemophilia A plasma spiked with turoctocog alfa pegol using both the ACL TOP® 550 and CS-5100 coagulation analyzers and activated partial thromboplastin time (aPTT) reagents, a chromogenic kit and calibrators specific to Japan. Ten of 13 aPTT reagents and the chromogenic kit (Revohem®FVIII Chromogenic [Sysmex, Kobe, Japan]) recovered turoctocog alfa pegol within ±30% of target concentration. Three aPTT reagents that use silica as a contact activator underestimated turoctocog alfa pegol recovery in spiked samples and therefore should not be used to measure FVIII activity in patients treated with turoctocog alfa pegol. Overall, turoctocog alfa pegol can be accurately measured with reagents and kits available in and widely used throughout Japan.

Key words: Post-infusion monitoring, Hemophilia A, Factor VIII activity assay, One-stage assay, aPTT reagent

1. Introduction

Turoctocog alfa pegol (ESPEROCT®; N8-GP; Novo Nordisk A/S, Bagsvaerd, Denmark) is a recombinant factor VIII (FVIII) replacement product created by the glycoPEGylation of the truncated B domain of turoctocog alfa (NovoEight®, Novo Nordisk A/S), leading to a half-life extension of 1.6 fold1 in adult patients with hemophilia A (1.9 fold in children)2.

The efficacy and tolerability of turoctocog alfa pegol for fixed-dose prophylactic treatment and use during surgical intervention in patients with hemophilia A has been confirmed in the pathfinder clinical trial program2–5, and turoctocog alfa pegol has been recently approved in Japan. Turoctocog alfa pegol potency is determined using a two-stage chromogenic kit (Coamatic®; Chromogenix; Instrumentation Laboratory, Bedford, MA, USA) and subsequently has been verified using six other chromogenic kits6. Although the simple, fixed-dose turoctocog alfa pegol regimen raises patient trough level to an estimated mean of 3 IU/dL in adults7, accurate FVIII activity measurement may be necessary in a number of clinical situations, including surgical procedures, emergencies and to determine individual pharmacokinetics. Two methods are currently used to measure FVIII activity in patients with hemophilia A: two-stage chromogenic assays and activated partial thromboplastin time (aPTT)-based one-stage clotting assays.

The most common method used for FVIII activity measurements worldwide is the aPTT-based one-stage clotting assay8. Methods for performing FVIII activity
measurements may vary across laboratories, depending on the instruments, calibration method and aPTT reagent used. Furthermore, aPTT reagents can contain various contact activators, and there are many different aPTT reagents currently available on the market. Usage and availability of these aPTT reagents also varies from region to region. Local methods and reagents used to measure FVIII activity should therefore be tested prior to introduction of FVIII replacement products in a new geographical region.

A recent international field study among 67 clinical laboratories in 25 different countries found that turoctocog alfa pegol could be accurately measured using routine methodology in most of the participating clinical laboratories\(^9\). However, three aPTT reagents (APTT-SP, TriniCLOT\(^{TM}\) and STA\(^{®}\) PTT-Automate, all which contain a silica-based contact activator) underestimated the recovery of turoctocog alfa pegol and were deemed unsuitable for monitoring patients treated with turoctocog alfa pegol\(^9\). Four Japanese laboratories participated in the international field study, using three different aPTT reagents and one chromogenic kit. However, many widely used aPTT reagents available on the Japanese market were not included in the field study\(^{10}\). In the present two-center study, we evaluated FVIII activity measurements of hemophilia A plasma spiked with turoctocog alfa pegol using instruments, aPTT reagents, a chromogenic kit and calibrators specific to Japan.

### 2. Materials and methods

#### 1) Sample measurement at site 1 (Hemophilia Research, Novo Nordisk A/S, Maaloev, Denmark)

Congenital hemophilia A plasma (Batch-6256; George King Biomedical Inc., Overland Park, USA) was spiked with 0.2 IU/mL, 0.6 IU/mL or 0.9 IU/mL turoctocog alfa pegol or 0.6 IU/mL rFVIII (Advate\(^{®}\), Lot-LE011T532 AS, Takeda Pharmaceutical Company Ltd., Tokyo, Japan) according to potency label and frozen at –80°C until analysis.

At site 1, 25 μL sample, 25 μL FVIII-deficient plasma, 50 μL aPTT reagent and 50 μL CaCl\(_2\) were mixed together prior to determination of clotting time. Samples were measured using the ACL TOP\(^{®}\) 550 coagulation analyzer (Instrumentation Laboratory, Holliston, USA) and a modified pre-programmed FVIII activity analysis identical for all eight aPTT reagents tested (listed in Table 1) except for the contact activation time. Contact activation time was adjusted for individual aPTT reagent according to the manufacturer recommendations.

Calibration curves were determined using triplicate values of eight different concentrations (0–150%) of HemosIL\(^{®}\) Calibration Plasma (Instrumentation Laboratory). Normal and low control samples (Instrumentation Laboratory) were used to verify the calibration curve. New calibration curves were prepared for each day of analysis. Samples were thawed at 37°C for 5 minutes, aliquoted and measured in triplicate on three separate days, leading to a maximum of nine activity determinations for each sample.

#### 2) Sample measurement at site 2 (Sysmex Corporation, Kobe, Japan)

Congenital hemophilia A plasma (Batch-547680A; George King Biomedical Inc.) was spiked with 0.2 IU/mL, 0.6 IU/mL or 0.9 IU/mL turoctocog alfa pegol or 0.6 IU/mL rFVIII (Advate\(^{®}\), Lot-LE011T532 AS, Takeda Pharmaceutical Company Ltd., Tokyo, Japan) according to actual potency and frozen at –80°C until analysis.

At site 2, 40 μL of 20x diluted sample, 40 μL FVIII-deficient plasma, 40 μL aPTT reagent and 40 μL CaCl\(_2\) were mixed together prior to determination of clotting time. Samples were measured using the CS-5100 coagulation analyzer (Sysmex Corporation) and a modified pre-programmed FVIII activity analysis identical for all twelve aPTT reagents tested (listed in Table 1) except for the contact activation time. Contact activation time was adjusted for individual aPTT reagents according to the manufacturer recommendations. The only chromogenic kit available for in vitro diagnostic measurement of FVIII activity in Japan, Revohem\(^{TM}\) FVIII Chromogenic\(^{11}\) (Sysmex, Kobe, Japan; also marketed as Factor VIII Chromogenic Assay [Siemens, Marburg, Germany])
was used for chromogenic determination of FVIII activity. Calibration curves were determined using duplicate values of seven different concentrations (0–150%) of Standard Human Plasma (Siemens). Normal and pathological control samples (Siemens) were used to verify the calibration curve.

3) Statistics
All results are presented as mean percent target concentration. Error is presented as standard deviation. As previously published, values within ±30% of the target concentration were considered to be within the acceptable range.

3. Results

1) Reagents that measured turoctocog alfa pegol within the acceptable range
Overall, 10 of 13 aPTT reagents specific to Japan and tested as part of this investigation measured turoctocog alfa pegol within the acceptable range (Fig. 1). Use of the ACL TOP® 550 coagulation analyzer (site 1) resulted in a slight overestimation of turoctocog alfa pegol at the 0.2 IU/mL concentration for the aPTT reagents Thrombocheck APTT-SLA (134%), Thrombocheck APTT(S) (149%) and Coagpia® APTT-N (141%) (Fig. 1A). This slight overestimation of the 0.2 IU/mL sample was not observed using the CS-5100 coagulation analyzer (site 2) (Fig. 1A). All aPTT reagents tested at a single site recovered within ±30% acceptable range at all concentrations (Fig. 1B).

All measurements of FVIII activity performed using the Revohem™ FVIII Chromogenic kit at site 2 were within the upper bound of the acceptable range. Measurements of samples spiked with turoctocog alfa pegol resulted in percent target concentrations that were similar to the rFVIII control (turoctocog alfa pegol: 0.2 IU/mL, 118% target concentration; 0.6 IU/mL, 128% target concentration; 0.9 IU/mL, 126% target concentration; rFVIII: 0.6 IU/mL, 122% target concentration).

2) Reagents that underestimated turoctocog alfa pegol
The use of aPTT reagents PTT-LA, STA®-PTT and Platelin-LII aPTT resulted in an underestimation of turoctocog alfa pegol recovery, regardless of sample concentration or coagulation analyzer (Fig. 2). Sample

Table 1 Overview of aPTT one-stage clotting assay reagents used to measure turoctocog alfa pegol*

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Manufacturer</th>
<th>Activator</th>
<th>Phospholipid origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ThromboCheck APTT-SLA</td>
<td>Sysmex Corporation</td>
<td>Ellagic acid</td>
<td>Synthetic</td>
</tr>
<tr>
<td>ThromboCheck APTT(S)</td>
<td>Sysmex Corporation</td>
<td>Ellagic acid</td>
<td>Soybean cephalin</td>
</tr>
<tr>
<td>ThromboCheck APTT</td>
<td>Sysmex Corporation</td>
<td>Ellagic acid</td>
<td>Cephalin of rabbit brain</td>
</tr>
<tr>
<td>Coagpia® APTT-N</td>
<td>Sekisui Medical Co. Ltd.</td>
<td>Ellagic acid</td>
<td>Rabbit brain</td>
</tr>
<tr>
<td>PTT LA FR [PTT-LA]</td>
<td>Fujirebio Inc.</td>
<td>Silica</td>
<td>Rabbit brain</td>
</tr>
<tr>
<td>Platelin LII [TrimiCLOT™ aPTT S]</td>
<td>Kyowa Medex</td>
<td>Micronized silica</td>
<td>Rabbit brain</td>
</tr>
<tr>
<td>Coagsearch® APTT</td>
<td>A&amp;T</td>
<td>Ellagic acid</td>
<td>Cephalin of rabbit brain</td>
</tr>
<tr>
<td>Data-fi APTT (Actin)</td>
<td>Sysmex Corporation</td>
<td>Ellagic acid</td>
<td>Rabbit brain</td>
</tr>
<tr>
<td>Data-fi APTT (FSL)</td>
<td>Sysmex Corporation</td>
<td>Ellagic acid</td>
<td>Soybean and Rabbit brain</td>
</tr>
<tr>
<td>Data-fi APTT (FS)</td>
<td>Sysmex Corporation</td>
<td>Ellagic acid</td>
<td>Soybean</td>
</tr>
<tr>
<td>STA®-Cephascreen</td>
<td>Diagnostica Stago</td>
<td>Polyphenolic activator</td>
<td>Cephalin of rabbit brain</td>
</tr>
<tr>
<td>Revohem™ APTT-SLA</td>
<td>Sysmex Corporation</td>
<td>Ellagic acid</td>
<td>Synthetic</td>
</tr>
</tbody>
</table>

*Based on translations of package insert; reagent names outside of Japan are included in brackets.
Recovery of turoctocog alfa pegol in spiked samples using various aPTT reagents

Mean FVIII activity of hemophilia A plasma spiked with 0.2 IU/mL, 0.6 IU/mL or 0.9 IU/mL turoctocog alfa pegol or 0.6 IU/mL rFVIII. A) aPTT reagents measured at both site 1 and site 2. B) aPTT reagents only measured at a single site. Dashed lines represent ±30% of target concentration (the acceptable range of recovery). Error bars represent standard deviation. aPTT, activated partial thromboplastin time; FVIII, factor VIII; rFVIII (Advate®).

aPTT reagents that recovered turoctocog alfa pegol below the acceptable range

Mean FVIII activity of hemophilia A plasma spiked with 0.2 IU/mL, 0.6 IU/mL or 0.9 IU/mL turoctocog alfa pegol or 0.6 IU/mL rFVIII. Dashed lines represent ±30% of target concentration (the acceptable range of recovery). Error bars represent standard deviation. aPTT, activated partial thromboplastin time; FVIII, factor VIII; rFVIII (Advate®).
measurements ranged from 18% to 67% of target concentration. The use of the CS-5100 coagulation analyzer (site 2) combined with one of these three aPTT reagents generally resulted in a greater underestimation of turoctocog alfa pegol recovery.

4. Discussion

Accurate measurement of FVIII activity is necessary for the clinical management of hemophilia A patients in certain clinical scenarios. However, reagents and methods used to measure FVIII activity can vary depending on routine clinical laboratory practice and geographical location. Ten of 13 aPTT reagents recovered turoctocog alfa pegol within the acceptable range. There was a slight overestimation of the 0.2 IU/mL sample using some aPTT reagents at site 1. All measurements were within the acceptable range at site 2. Site 1 used the ACL TOP® 500 coagulation analyzer, whereas site 2 used a CS-5100 coagulation analyzer to measure the samples. The ACL TOP® determines coagulation point by the maximum acceleration of the velocity curve and change in absorbance. CS series analyzers, on the other hand, determine coagulation point using 50% light transmittance. This difference in determination method may account for differences in the measurement of the 0.2 IU/mL sample.

According to a 2015 survey of Japanese hospitals conducted by the Japan Medical Association, only 3% of hospitals in Japan use ACL series coagulation analyzers. In comparison, 23% of hospitals use CS series analyzers. Furthermore, 38% of hospitals use Sysmex CA series analyzers, which employ similar methods to make measurements as CS series analyzers.

Moreover, of the 10 aPTT reagents that accurately recovered turoctocog alfa pegol in this study, many are among the most commonly used aPTT reagents in the 2015 survey. Thrombocheck aPTT-SLA, Data-fi Actin, Thrombocheck APTT, Coagpia® and STA®-Cephascreen account for approximately 85% of aPTT reagents used at Japanese hospitals. However, the survey of Japanese hospitals was limited to aPTT reagents used in hospitals and did not address specific methods used to measure FVIII activity in samples from patients with hemophilia A.

Overall, three of 13 aPTT reagents (STA®-PTT Automate, Platelin-LII, and PTT-LA) underestimated turoctocog alfa pegol recovery. Therefore, results from FVIII activity measurements determined using these reagents in patients treated with turoctocog alfa pegol should be interpreted with caution. All three of these aPTT reagents contain silica as the contact activator and both STA®-PTT Automate and Platelin-LII (TriniCLOT™ aPTT S) have been previously found to underestimate turoctocog alfa pegol recovery. In the presence of specific aPTT reagents that contain silica-based contact activators, a direct comparison of turoctocog alfa pegol and turoctocog alfa (NovoEight®) showed that thrombin activates turoctocog alfa pegol more slowly. A similar phenomenon was also described in the PEGylated recombinant (r)FVIII product BAY-94-9027 and was hypothesized to be caused by an interaction between PEG and the silica surface.

For the single chromogenic kit tested, turoctocog alfa pegol and rFVIII recoveries were within the upper bound of the acceptable range. This slight overestimation when measuring FVIII replacement products using chromogenic kits was also observed in the international field study for both turoctocog alfa pegol and rFVIII samples and was consistent across five different chromogenic kits. Results from the present study align with results from the international field study for Factor VIII Chromogenic Assay (Siemens), a kit with identical reagents to Revohem™ FVIII chromogenic. Furthermore, slight over-recovery of FVIII activity in samples containing extended half-life or standard rFVIII products when measured using chromogenic assays is common when using a normal plasma calibrator traceable to the World Health Organization 6th international standard. Accuracy of FVIII recovery is increased when using a plasma calibrator traceable to the World Health Organization 8th international standard. Thus, the slight over-recovery...
seen in the present study might be due to use of a normal pooled plasma calibrator traceable to the World Health Organization 6th international standard. Further research into this phenomenon is necessary.

In summary, reliable and accurate measurements of FVIII activity in samples from patients with hemophilia A are indispensable for good healthcare. In the present study, we found that most aPTT reagents widely used throughout Japan and the only chromogenic kit currently available on the Japanese market could accurately measure turoctocog alfa pegol in spiked samples. However, three aPTT reagents that use silica as a contact activator (PTT-LA, STA®-PTT and Platelin-LII) underestimate turoctocog alfa pegol recovery in spiked samples, and therefore should not be used to measure FVIII activity in patients treated with turoctocog alfa pegol.

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Disclosures

M. Ezban and M. Hansen are employees or were former employees and shareholders of Novo Nordisk A/S. A. Deguchi and H. Terano are employees of Novo Nordisk Pharma Ltd.

References