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ORIGINAL ARTICLE

Measurement of extended half-life recombinant FVIII molecules: *In vitro* and *ex vivo* evidence of relevant assay discrepancies

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Abstract

Background: Extended half-life recombinant FVIII products (EHL-rFVIIIs) have been engineered to improve the pharmacokinetic profile of FVIII, enabling better hemostatic protection with a reduced number of injections in persons with hemophilia. Previous studies showed several discrepancies in FVIII activity (FVIII:C) measurements for EHL-rFVIIIs comparing one-stage clotting assay (OSA) and chromogenic assay (CSA), although a systematic investigation of this phenomenon is still lacking.

Objective: Evaluation of the accuracy and precision of measurement of all available EHL-rFVIIIs with 5 different assays both *in vitro* and *ex vivo*.

Methods: Damoctocog alfa pegol, rurioctocog alfa pegol, turoctocog alfa pegol, and efmoroctocog alfa were tested with 3 OSA types: (1) aPTT-based commercial reagents with colloidal silica (Synthasil, Werfen-IL); (2) ellagic acid, Synthafax (Werfen-IL); and (3) OSA calibrated with each EHL-rFVIII product and colloidal silica. Measurements were also carried out with 2 different commercially available CSA reagents (Coamatic Factor VIII, Chromogenix-Werfen) and Trinichrom FVIII (Tcoag-Stago). A Bland–Altman analysis was performed to compare all assays.

Results: The simple OSA showed significant discrepancies between the expected and measured EHL-rFVIII concentrations as CSA methods, whereas the calibrated OSA assay was accurate and precise in determining the activity of all EHL-rFVIIIs in the *in vitro* setting. Comparable results were found using *ex vivo* plasma samples.

Conclusion: In this study, only OSA with a calibration curve constructed with each EHL-rFVIII product showed acceptable accuracy and precision in EHL-rFVIIIs measurements.

Stefano Lancellotti and Monica Sacco contributed equally to this study.

KEYWORDS
blood coagulation, coagulation factor VIII, congenital, extended half-life rFVIII, factor VIII deficiency, tests, therapeutic drug monitoring

Essentials

- The EHLFVIII products (EHL-rFVIIIs) improve the FVIII PK profile in persons with hemophilia.
- Levels of EHL-rFVIII significantly differ in coagulative and chromogenic assays.
- A Bland–Altman analysis of EHL-rFVIII levels measured with different assays was performed.
- The coagulative assay calibrated with the same EHL-rFVIIII showed the highest accuracy.

1 | INTRODUCTION

Hemophilia A (HA) is a congenital bleeding disorder caused by factor VIII (FVIII) deficiency and consequently reduced thrombin generation burst. The severity of the bleeding phenotype depends on the variation in residual factor activity levels in plasma. Replacement therapy with FVIII concentrates represents the cornerstone of hemophilia treatment able to both prevent and control acute bleeds by providing FVIII levels able to restore normal coagulation. In this light, the possibility of measure FVIII levels in plasma is relevant to make diagnosis and to monitor treatment efficacy. Indeed, measurement of the FVIII procoagulant activity (FVIII:C) requires highly sensitive and specific laboratory assays for the evaluation of the hemostatic competence of these patients both at diagnosis and during replacement therapy. The activated Partial Thromboplastin Time (aPTT)-derived assays are the gold standard tests, and in fact, FVIII levels are commonly measured by using the 1-stage clotting assay (OSA), which relies on the ability of a given plasma sample to correct the aPTT of FVIII Deficient Plasma. Several aPTT reagents are commercially available, and they display varied specificity and sensitivity, which might impinge upon the accuracy and precision of FVIII measurement. On the other hand, chromogenic substrate assays (CSA), which measure FVIII:C by using the amount of activated FX generated in the system, are highly specific but often underutilized. In the last decade, several recombinant FVIII (rFVIII) products have been engineered to improve the pharmacokinetic profile of FVIII through slower clearance, a greater area under the curve and, ultimately, 1.4- to 1.8-fold longer half-life than native FVIII molecule. Their use enables us to achieve and maintain higher FVIII levels with a reduced number of injections [1–5]. These products have been obtained by fusion with the Fc fragment of immunoglobulins (ie, efmoroctocog alfa) or through conjugation with polyethylene glycol (PEG) moieties (ie, rurioctocog alfa pegol, turoctocog alfa pegol, and damoctocog alfa pegol) [6,7]. The multiplicity of therapeutic FVIII molecules available and their increasing widespread use in clinical practice poses a question concerning the impact of their structural heterogeneity on laboratory measurement as already experienced in the past with the first B-domain–deleted product, moroctocog alfa, for which simple OSA showed relevant underestimation that could be corrected by using a product-specific laboratory standard [8,9]. Similarly, silica-based aPTT reagents were reported to underestimate the relative recovery of rFVIII-PEG products as compared to ellagic acid/polyphenolic acid reagents [10], whereas CSA overestimates rFVIII-PEG and rFVIII-Fc [11]. This complex scenario is further confounded by the different recommendations given by European and US regulatory agencies to assign product potency (ie, CSA recommended by EMA and OSA by the FDA) [12,6]. Discrepancies can be minimized by using a product-specific reference standard instead of a plasma standard for calibration [7,13]. Recently, the United Kingdom Haemophilia Centre Doctors' Organization guideline recommended including local validation of each new product to find the appropriate factor assay that yields recovery within 20% for FVIII activity above 0.30 IU/mL or 30% for FVIII activity between 0.10 and 0.30 IU/mL [14]. To monitor the replacement therapy with FVIII concentrates, the World Federation of Hemophilia (WFH) recommends that laboratories use an FVIII assay that has been validated with the specific concentrate used for treatment [8]. Hence, the aim of this study was to evaluate FVIII:C in plasma samples spiked *in vitro* with all available EHL-rFVIIIs and in *ex vivo* samples from 4 patients who received the 4 different EHL-rFVIIIs for PK assessment by comparing OSA performed with 3 different aPTT-based OSA types and 2 different chromogenic assays (CSA).

2 | MATERIALS AND METHODS

2.1 | EHL-rFVIII Products

The EHL-rFVIII products tested were efmoroctocog alfa, rurioctocog alfa pegol, turoctocog alfa pegol, and damoctocog alfa pegol. Moroctocog alfa has been used as a reference product, having a specific standard calibrator. All EHL-rFVIIIs were reconstituted according to the manufacturer's instructions and serial dilutions (ie, 1.0/0.5/0.1/0.05/0.025/0.0125/0.00625 IU/mL) were obtained by using HemosIL FVIII Deficient Plasma (PC8), Werfen. Because of the well-known variability of the standard error of FVIII-level determination at low and high FVIII concentration, the Bland–Altman analyses were performed for high EHL-rFVIII concentrations (1.0/0.5/0.1/0.05/0.025 IU/mL) and low concentration range (0.1/0.05/0.025/0.0125/0.00625 IU/mL). The main characteristics of the used EHL products used in this study are listed in Table 1. The lot numbers of each product used in the study were as follows:

TABLE 1 Main characteristics of EHL-rFVIII products approved for hemophilia A.

Molecule characteristics	Molecule name	Commercial name	Mean terminal half-life (t1/2)	Company
PEGylated FVIII 20-kDa PEG	Rurioctocog alfa pegol (formerly BAX-855)	Adynovi	14-16 h	Takeda
rBDD-FVIII-Fc	Efmoroctocog alfa	Elocta	19 h	Sobi
GlycoPEGylated rBDT-FVIII Site-specific 40-kDa PEG	Turoctocog alfa pegol	Esperoct	18-19 h	Novo Nordisk
PEGylated rBDD rFVIII 60 kDa	Damoctocog alfa pegol (formerly BAY94-9027)	Jivi	19 h	Bayer

rBDD-FVIII-Fc, recombinant B-domain–deleted factor VIII; Fc, fragment crystallizable; PEG, polyethylene glycol; rBDT-FVIII, recombinant B-domain truncated FVIII.

Efmoroctocog alfa 3000 IU → Lot# 33314-F (exp. 01/2022) and 250UI lot# 30882-1A (exp.10/2019)

Rurioctocog alfa pegol 250 IU → Lot# TDNW013A (exp. 04/2022)

Turoctocog alfa pegol 500 IU → Lot# KSAP32 (exp. 11/2021)

Damoctocog alfa pegol 1000 IU → Lot# BXJBTF1 (exp. 10/2020)

Moroctocog alfa → lot# 18/106 (exp. 10/2026)

All the EHL-rFVIII concentrates were generous gifts by the relative manufacturer companies (Table 1).

2.2 | FVIII activity measurements

2.2.1 | OSA

FVIII:C was tested through OSA (ACL TOP 750 LAS Coagulation Analyzer, Werfen) by using 2 different aPTT-based OSA: (1) aPTT-based commercial reagents with colloidal silica (Synthasil, Werfen-IL); (2) ellagic acid, Synthafax (Werfen-IL); The factor VIII OSA relies upon measuring the degree of correction of the aPTT when spiked test plasma is added to FVIII Deficient Plasma. To quantify the FVIII:C level, the clotting time obtained is compared to a standard curve on logarithmic/linear scale graph.

2.2.2 | Specific EHL-rFVIII-calibrated OSA

A drug-specific OSA calibration curve was constructed starting from 1.5 IU/mL (labeled concentration based on the manufacturer's certificate of potency) of each EHL-rFVIII product, as calibrator, using HemosIL FVIII Deficient Plasma (PC8) and the SynthASil reagent (both from Werfen). Preliminary measurements showed that using the specific EHL-rFVIII product calibration curve, the use of SynthAsil or SynthaFax as activator did not provide different results for the measurement of EHL-rFVIII level in samples prepared with the same nominal FVIII concentration. Hence, we decided to use only the SynthASil reagent to carry out EHL-rFVIII-calibrated OSA. All EHL products' levels were measured on serial dilutions for the 2 concentration ranges (ie, 1.0/0.5/0.1/0.05/0.025 and 0.1/0.05/0.025/0.0125/0.006125 IU/mL) by using the specific EHL-rFVIII-based calibration curve as a reference.

2.2.3 | CSA

All EHL-rFVIIIs were evaluated by using 2 different commercially available CSA, referred to as CSA1 (Coamatic FVIII, from Chromogenix-Werfen) and CSA2 (Trinichrom FVIII, from Tcoag-Stago), respectively. CSA1 employs bovine FIXa (0.3 U) and FX (2.7 IU), bovine thrombin (1 NIH-U ≅ 10 nM) colyophilized with $CaCl_2$ (40 mmol), chromogenic substrate (N-a-Z-D-Arg-Gly-Arg-pNA, 7.7 mg), and synthetic thrombin inhibitor (0.2 mg) with mannitol. Likewise, CSA 2 employs freeze-dried bovine FX, bovine FIXa, bovine thrombin, calcium, phospholipid cofactors, FX substrate (MeO-CO-D-CHG-Gly-Arg-pNA), and thrombin inhibitor (concentrations of each reagent is not reported in the technical sheet of this product).

2.3 | *Ex vivo* samples from persons with severe hemophilia A

Ex vivo plasma samples containing the aforementioned 4 EHL-rFVIIIs were obtained from 4 persons with severe HA without history of inhibitors, aged 30 to 36 years, with normal VWF levels (85-104 IU/dl) and non-O blood groups. These samples were obtained on the occasion of a PK assessment at the following time points: 0, 1, 4, 8, 24, 48, and 72 hours following the usual prophylactic dose of EHL-rFVIII. The PK assessment was performed in the absence of any reported bleed, trauma, and infection in the preceding 4 weeks. The first PK blood sample was obtained after a wash out of approximately 92 ± 5 hours from the last FVIII injection. The level of EHL-rFVIII in the sample taken at each time was measured with the 5 methods reported above. The PK profiles with the relative best-fit parameters were calculated using the WinNonlin program (Certara University), according to the appropriate equation of the noncompartmental model.

2.3.1 | Bland–Altman analysis

Bland–Altman plots were obtained by interpolating the means between 3 measurements (x values) and the differences estimated between the 2 methods (*y* values). Means (Xd) and 95% CI of the differences were also obtained from a 1-sample Student's *t*-test to determine whether

TABLE 2 EHL-rFVIII levels (IU/mL) recovery measured with different assays over a nominal concentration range 1.0 to 0.025 IU/mL.

Efmoroctocog alfa (nominal concentration)	OSA calibrated (IU/mL)	Silica OSA (IU/mL)	Ellagic acid-OSA (IU/mL)	CSA1 (IU/mL)	CSA2 (IU/mL)
1.00 IU/mL	**1.205 (+20.5)**	**2.250 (+125)**	0.984	0.832	**1.417 (+41.7)**
0.50 IU/mL	0.558	**0.966 (+93.2)**	0.411	**0.311 (−37.8)**	**0.613 (+22.6)**
0.100 IU/mL	0.105	**0.148 (+48)**	0.088	0.082	0.112
0.050 IU/mL	0.051	**0.069 (+38)**	0.046	**0.037 (−26)**	0.045
0.025 IU/mL	0.025	**0.033 (+32)**	**0.015 (−40)**	0.023	**0.015 (−40)**
Rurioctocog alfa pegol (nominal concentration)	**OSA calibrated (IU/mL)**	**Silica OSA (IU/mL)**	**Ellagic acid-OSA (IU/mL)**	**CSA1 (IU/mL)**	**CSA2 (IU/mL)**
1.00 IU/mL	0.940	1.179	**0.640 (−36)**	1.000	1.060
0.50 IU/mL	0.467	0.577	**0.289 (−42.2)**	0.406	0.500
0.100 IU/mL	0.096	0.098	**0.066 (−34)**	0.083	0.090
0.050 IU/mL	0.050	0.055	**0.030 (−40)**	0.041	0.050
0.025 IU/mL	0.023	0.022	**0.012 (−52)**	0.021	**0.020 (−20)**
Turoctocog alfa pegol (nominal concentration)	**OSA calibrated (IU/mL)**	**Silica OSA (IU/mL)**	**Ellagic acid-OSA (IU/mL)**	**CSA1 (IU/mL)**	**CSA2 (IU/mL)**
1.00 IU/mL	1.134	1.148	0.862	1.072	0.910
0.50 IU/mL	0.440	0.440	**0.343 (−31)**	**0.367 (−26.6)**	0.420
0.100 IU/mL	0.102	0.086	**0.079 (−21)**	0.091	**0.050 (−50)**
0.050 IU/mL	0.053	0.048	**0.035 (−30)**	0.048	**0.060 (+20)**
0.025 IU/mL	**0.031 (+24)**	0.027	**0.019 (−24)**	**0.033 (+32)**	**0.030 (+20)**
Damoctocog alfa pegol (nominal concentration)	**OSA calibrated (IU/mL)**	**Silica OSA (IU/mL)**	**Ellagic acid-OSA (IU/mL)**	**CSA1 (IU/mL)**	**CSA2 (IU/mL)**
1.00 IU/mL	1.037	**0.885 (−30.2)**	0.842	**0.765 (−23.50)**	**0.390 (−61)**
0.50 IU/mL	0.443	**0.349 (−21)**	**0.374 (−25.2)**	**0.237 (−52.60)**	**0.170 (−66)**
0.100 IU/mL	0.119	0.079	0.088	**0.075 (−25.00)**	**0.040 (−60)**
0.050 IU/mL	0.053	0.041	0.046	**0.039 (−22.00)**	**0.010 (−80)**
0.025 IU/mL	**0.031 (+24)**	0.023	**0.020 (−20)**	0.021	**0.010 (−60)**

All values represent the mean of duplicate measurements (SD: 5-10%). The values in bold express ≥20% level change (± percentage shown in parentheses) compared with the nominal concentration.

the difference of values obtained with the 2 methods under investigation was different from ideality, that is zero. The 95% CI values were determined by using SPSS software (version 21, Microsoft). Linear regression analysis and the correlation between the difference and the mean values were also calculated. Bias plots were also evaluated and an agreement interval of ±20% [9] was considered as acceptable comparing results from different measurement methods.

3 | RESULTS

3.1 | EHL-rFVIII recovery with different assays over a factor concentration range of 1.0 to 0.025 IU/mL

The drug recovery (FVIII:C) obtained from serial dilutions of FVIII Deficient Plasma (ie, 1.0/0.5/0.1/0.05/0.025 IU/mL) spiked with of EHL-rFVIIIs by using the 5 different laboratory methods (OSA calibrated, Silica- or Ellagic Acid-based OSA, CSA1, CSA2) are reported in Table 2 as mean values between 2 different drugs' batches measurements. Moroctocog alfa spiked samples were used as reference because of its well-known standardization procedure.

3.1.1 | Efmoroctocog alfa

Efmoroctocog alfa is a B-domain–deleted-Fc-fused recombinant FVIII molecule, with the same primary sequence of moroctocog alfa. The recovery of this product was correctly measured with the OSA calibrated method only, and in part with the simple OSA with ellagic acid, whereas silica OSA largely overestimated its level. At variance, using the CSA tests, we observed an inacceptable recovery of the drug (Table 2).

3.1.2 | Rurioctocog alfa pegol

Rurioctocog alfa pegol is a full-length recombinant FVIII molecule randomly PEGylated with 20-kDa PEG moieties. FVIII:C measurements in spiked samples were globally precise with all methods but the simple OSA with ellagic acid that severely underestimated the drug at any nominal concentration (Table 2).

3.1.3 | Turoctocog alfa pegol

Turoctocog alfa pegol is a B-domain truncated recombinant FVIII molecule conjugated with a 40-kDa PEG chain specifically linked to the glycans located on the residual B-domain aminoacidic sequence. The recovery FVIII:C data obtained on spiked samples showed similar accuracy with both OSA calibrated with the same product and with simple silica OSA. In contrast, both OSA with ellagic acid and the CSA tests showed an overestimated and underestimated level of this factor at different nominal concentrations, respectively, as shown in Table 2.

3.1.4 | Damoctocog alfa pegol

Damoctocog alfa pegol is a B-domain–deleted recombinant FVIII molecule in which a specific amino acid change has been introduced to allow a site-specific PEGylation with 2 30-kDa PEG branches. The recovery of damoctocog alfa pegol was correctly measured in practice by the calibrated OSA only, whereas the other methods did not show precise determinations of the factor level, as shown in Table 2.

3.2 | EHL-rFVIII recovery with different assays over a factor concentration range of 0.1 to 0.006125 IU/mL

This analysis was separately carried out because of the higher variance of the measured factor level at low concentrations. As expected, the recovery variance was significantly higher over the range of 0.006125 to 0.1 IU/mL than at higher concentrations. The data listed in Table 3 show that only calibrated OSA, together with the simple silica and ellagic OSA could provide reasonable recovery of the EHL-rFVIII, whereas both CSAs were characterized by a low reliability of measurement for damoctocog alfa pegol (underestimation) and Efmoroctocog alfa (overestimation). CSA1, calibrated OSA as well as silica and ellagic acid-OSA gave reliable recovery measurements of both rurioctocog alfa pegol and turoctocog alfa pegol at almost all low factor concentrations (Table 3).

3.2.1 | Bland–Altman plot method

The Bland–Altman plot method is a widely cited graphical approach to assess the equivalence of quantitative measurement techniques. The differences between 5 methods (silica OSA, ellagic acid-OSA, EHL-rFVIIIs calibrated OSA, CSA1, and CSA2) were compared in linear regression analysis by using Bland–Altman plots. For a comparative analysis, a calibration curve (n = 5 from 1 IU/mL to 0.025 IU/mL) constructed with the validated standard calibrator of moroctocog alfa was also used. All the graphs derived from the Bland–Altman analysis of all products and assays are shown in Supplementary Figure 1 for the 1.0 to 0.025 IU/mL concentration range and in Supplementary Figure 2 for 0.1 to 0.006125 IU/mL concentration range. Tables 4 and 5 summarize the obtained results and facilitating their interpretation. Globally, a significant disagreement in level assessment was observed by comparing each method with the specific EHL-rFVIII-calibrated OSA, taken as the most accurate method. Moreover, besides the disagreement of the measured levels, a frequent proportional bias (positive or negative) was observed both at higher and lower concentration ranges. Remarkably, even the comparison between CSA1 and CSA2 revealed for both damoctocog alfa pegol and efmoroctocog alfa a substantial disagreement with a proportionality bias: CSA1 provided levels higher than CSA2 for damoctocog alfa pegol, whereas the inverse occurred for efmoroctocog alfa, as shown in Figure 1. Finally, Table 6 reports the expected variations of the results obtained with the 2 simple OSA and the 2 CSA tests compared with the specific EHL-rFVIII-calibrated OSA.

3.3 | Pharmacokinetic analysis of *ex vivo* samples

These measurements were carried out to assess whether the EHL-rFVIII levels measured as a function of time could depend even for *ex vivo* samples on the specific assay used. The EHL-rFVIII levels obtained in clinical samples from persons with hemophilia qualitatively and in part quantitatively agreed with those obtained *in vitro* using the specific product. In particular, the highest differences were observed at higher EHL-rFVIII concentrations, namely for damoctocog alfa pegol and emforoctocog alfa, as shown in Figure 2. Similar to what had been observed *in vitro*, also *ex vivo* results showed that CSA1, CSA2, as well as simple OSA strongly underestimated levels of damoctocog alfa pegol and emforoctocog alfa. Such discrepancies, found by using different laboratory assays, could ultimately alter the calculated pharmacokinetic parameters of single products (Supplementary Table 2).

4 | DISCUSSIONS AND CONCLUSIONS

4.1 | Comparison of in vitro recovery of EHL-rFVIII concentrates between different assays

In this study, we evaluated the precision and accuracy of different OSA and CSA to estimate FVIII:C values of all currently available EHL-rFVIIIs in samples spiked *in vitro*. The observed discrepancies in laboratory measurements showed that currently available OSA and CSA do not provide consistent results. The results obtained in this study showed that only the OSA calibrated with the same EHL-rFVIII concentrate showed sufficient accuracy in the measurement of the

TABLE 3 EHL-rFVIII levels (IU/mL) recovery measured with different assay over a nominal concentration range of 0.1 to 0.006125 IU/mL.

Efmoroctocog alfa (nominal concentration)	OSA calibrated (IU/mL)	Silica OSA (IU/mL)	Ellagic acid-OSA (IU/mL)	CSA1 (IU/mL)	CSA2 (IU/mL)
0.100 IU/mL	0.112	**0.150 (+50)**	0.127	0.090	**0.160 (+60)**
0.050 IU/mL	0.061	0.063	0.062	0.045	0.040
0.025 IU/mL	**0.035 (+40)**	**0.033 (+32)**	0.030	0.025	0.030
0.0125 IU/mL	0.016	0.016	0.016	0.015	**0.030 (+140)**
0.006125 IU/mL	**0.008 (+30.61)**	**0.008 (+30.6)**	0.007	**0.010 (+63.3)**	**0.010 (+63.3)**
Rurioctocog alfa pegol (nominal concentration)	**OSA calibrated (IU/mL)**	**Silica OSA (IU/mL)**	**Ellagic acid-OSA (IU/mL)**	**CSA1 (IU/mL)**	**CSA2 (IU/mL)**
0.100 IU/mL	0.115	0.093	0.091	0.084	**0.150 (+50)**
0.050 IU/mL	0.064	0.045	0.050	0.044	0.045
0.025 IU/mL	0.027	0.031	0.027	0.026	**0.0375 (+50)**
0.0125 IU/mL	0.014	0.013	0.014	0.016	**0.025 (+100)**
0.006125 IU/mL	**0.0082 (+33.8)**	**0.008 (+30.6)**	**0.009 (+46.9)**	**0.011 (+79.6)**	**0.01 (+63.3)**
Turoctocog alfa pegol (nominal concentration)	**OSA calibrated (IU/mL)**	**Silica OSA (IU/mL)**	**Ellagic acid-OSA (IU/mL)**	**CSA1 (IU/mL)**	**CSA2 (IU/mL)**
0.100 IU/mL	0.124	0.084	0.097	0.093	**0.200 (+100)**
0.050 IU/mL	0.051	0.045	0.052	0.046	**0.105 (+110)**
0.025 IU/mL	0.029	0.025	0.028	0.024	**0.045 (+80)**
0.0125 IU/mL	0.012	0.013	0.015	0.015	**0.020 (+60)**
0.006125 IU/mL	0.007	0.006	**0.008 (+30.6)**	**0.011 (+79.6)**	**0.020 (+226.5)**
Damoctocog alfa pegol (nominal concentration)	**OSA calibrated (IU/mL)**	**Silica OSA (IU/mL)**	**Ellagic acid-OSA (IU/mL)**	**CSA1 (IU/mL)**	**CSA2 (IU/mL)**
0.100 IU/mL	0.098	**0.055 (−45)**	0.097	**0.068 (−32)**	**0.030 (−70)**
0.050 IU/mL	0.055	**0.032 (−36)**	0.046	**0.027 (−46)**	**0.020 (−60)**
0.025 IU/mL	**0.033 (+32)**	**0.016 (−36)**	0.023	0.018	**0.005 (−80)**
0.0125 IU/mL	0.016	**0.008 (−36)**	0.013	0.012	**0.005 (−60)**
0.006125 IU/mL	**0.012 (+95.9)**	0.005	0.007	**0.009 (+46.9)**	0.005

All values represent the mean of duplicate measurements (SD: 15%-20%). The values in bold express ≥30% level change (± percentage is shown in parentheses) compared with the nominal concentration

factor recovery, as also suggested by previous studies [15]. However, it must be outlined that this is only a pilot study not employing a validated internal standard. Hence, in analogy with what was done for moroctocog alfa, only a specific and validated standard calibrator should be employed in clinical laboratories to guarantee an accurate, precise, and standardized measurement of EHL-rFVIII concentration.

Our results showed that a better accuracy and precision of damoctocog alfa pegol measurement are obtained with silica OSA and calibrated OSA as compared with any CSA, although the manufacturer's datasheet reports that the product's potency is assigned by means of a CSA assay (not detailed). At variance with our data, Gu et al. [16] demonstrated an acceptable recovery of damoctocog alfa pegol by using CSA (Biophen FVIII:C; Aniara) and with ellagic acid-based aPTT reagents, SynthAFax (Werfen-IL), and Dade Actin (Siemens), but not with silica-based reagents APTT-SP (Werfen-IL) and STA PTT5 (STAGO), suggesting that the PEG moiety may interfere with the activation of FXII on the silica surface. In that study, however, samples had been spiked *in vitro* with nominal damoctocog alfa pegol concentrations but using the WHO-8 standard as a calibrator. In another study, Muller et al. [17] showed that: (1) CSA (Siemens) showed a concentration-dependent overestimation of efmoroctocog alfa recovery, whereas it severely underestimated (≅ halved value) damoctocog alfa pegol recovery. Furthermore, CSA could measure with acceptable accuracy and precision the recovery of both rurioctocog alfa pegol and turoctocog alfa pegol; (2) silica-based OSA-overestimated damoctocog alfa pegol recovery; (3) all OSA underestimated rurioctocog alfa pegol recovery at all concentrations, and turoctocog alfa pegol recovery at high and low concentrations. It has to be outlined that in the study by Müller et al. [17], both reagents and the plasma calibrator were from the same company (Siemens). In the present study, reagents from different companies were employed. This may explain the different results obtained in the present study.

TABLE 4 Results of the Bland–Altman analysis of the different assays used to assess the comparability of the calibrated OSA (reference assay) with the other tests over the 1.0-0.025 IU/mL concentration range.[a]

	OSAsil		OSAel		CSA1		CSA2	
FVIII product	Agreement (95% CI)	Proportional bias	Agreement (95% CI)	Proportional bias	Agreement (95% CI)	Proportional bias	Agreement (95% CI)	Proportional bias
Efmoroctocog alfa	No	Yes B = 0.98*, $P < .001$	No	Yes B = 0.7566, $P = .001$	No	Yes B = 0.753, $P < .001$	Yes	Yes B = −0.975, $P < .001$
Rurioctocog alfa pegol	No	Yes $B = -0.859$, $P < .001$*	No	Yes B = −0.946, $P < .001$	No	No B = −0.396, $P = .144$	No	Yes B = −0.975, $P < .001$
Turoctocog alfa pegol	Yes	No B = -0.23, $P = .41$	No	Yes B = 0.955, $P < .001$	Yes	No B = −0.249, $P = .371$	No	Yes B = −0.898 $P < .001$
Damoctocog alfa pegol	No	Yes B = 0.90, $P < .001$	No	Yes B = 0.744, $P = .001$	No	Yes B = 0.874, $P = .001$	No	Yes B = 0.991, $P < .001$

[a]All the Bland–Altman plots are reported in Supplementary Figure 1.
*The sign of the correlation coefficient of the linear regression expresses the positive or negative proportionality bias between methods.

4.2 | Bland–Altman analysis

The Bland–Altman regression analysis showed that only for turoctocog alfa pegol and in part for emforoctocog alfa different methods could provide statistically interchangeable results. Namely, levels of turoctocog alfa pegol measured with simple silica OSA and CSA1 (Werfen) were comparable to those measured with the EHL-calibrated OSA method (Tables 4 and 5 and Supplementary Figure 1). Likewise, efmoroctocog alfa levels were comparably measured by CSA2 and EHL-calibrated OSA (Supplementary Figure 1 and Table 4). The Bland–Altman regression analysis showed for all the remaining products a lack of interchangeability between methods with the

TABLE 5 Results of the Bland–Altman analysis of the different assays used to assess the comparability of the calibrated OSA (reference assay) with the other tests over the 0.1 to 0.006125 IU/mL concentration range[a]

	OSAsil		OSAel		CSA1		CSA2	
FVIII product	Agreement (95% CI)	Proportional bias	Agreement (95% CI)	Proportional bias	Agreement (95% CI)	Proportional bias	Agreement (95% CI)	Proportional bias
Efmoroctocog alfa	Yes	Yes B = −0.922, $P = .026$	Yes	No B = −0.877, $P = .051$	Yes	Yes B = 0.942, $P = .017$	Yes	No B = −0.718, $P = .172$
Rurioctocog alfa pegol	No	Yes B = 0.914, $P = .030$	No	Yes B = 0.994, $P = .001$	No	Yes B = 0.986, $P = .002$	Yes	Yes B = 0.718, $P = .172$
Turoctocog alfa pegol	Yes	Yes B = 0.996, $P < .0001$	No	Yes B = 0.986, $P = .002$	Yes	Yes B = 0.934, $P = .02$	Yes	Yes B = −0.981, $P = .003$
Damoctocog alfa pegol	No	Yes B = 0.995, $P < .0001$	No	No B = −0.357, $P = .555$	No	Yes B = 0.879, $P = .05$	Yes	Yes B = 0.982, $P = .003$

[a]All the Bland–Altman plots are reported in Supplementary Figure 1.
The sign of the correlation coefficient of the linear regression (B) expresses the positive or negative proportionality bias between methods.

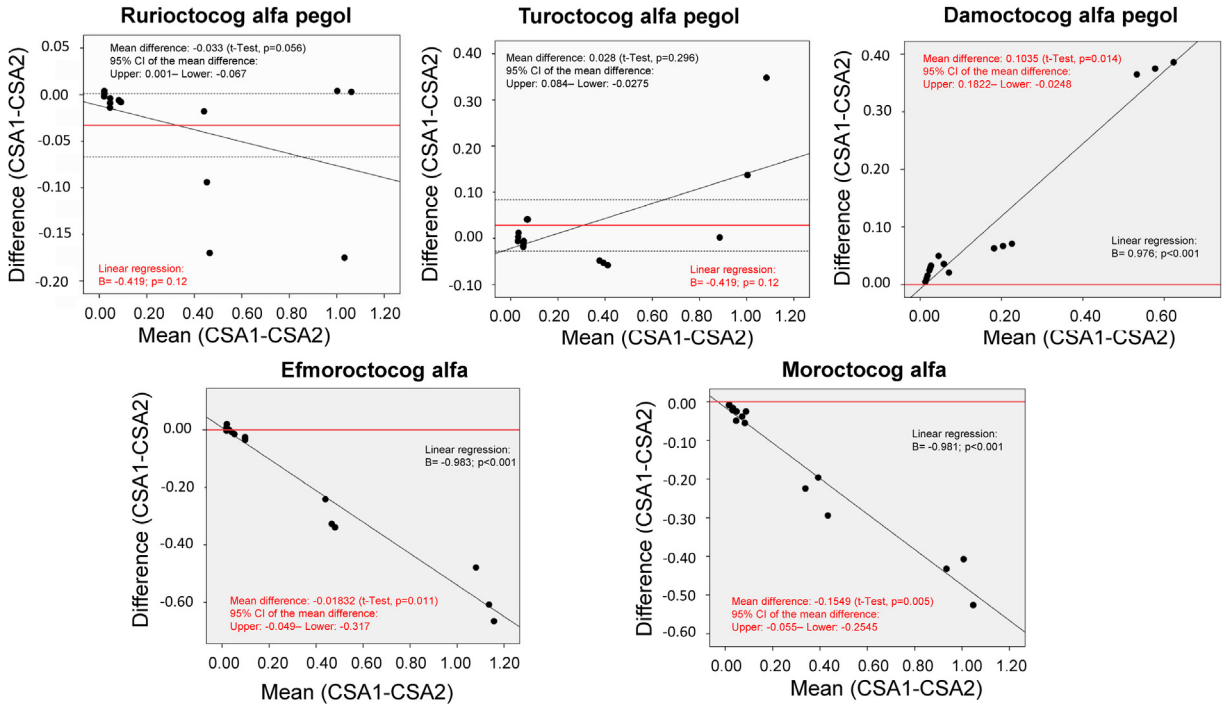

FIGURE 1 Bland–Altman plots for the comparative analysis of the results of FVIII activity by 2 CSA tests. In the inset of each plot, the results of the one-sample Student's *t*-test of the difference of the activity values and the best-fit linear regression parameters are also shown. In red characters, the results of the analytical procedures characterized by a $P < .05$ are considered statistically significant. When the one-sample Student's *t*-test of the difference values (y axis) was statistically significant ($P < .05$), showing the lack of congruent results, the zero value (that is the theoretical perfect congruence of the results obtained with the 2 analyzed tests) was shown with a red line. At variance, when the one-sample Student's *t*-test was not significant ($P > .05$) the calculated mean (red line) together with the upper and lower 95% CI (95%) values are shown (dotted line). The difference and the mean values are expressed as IU/mL.

presence of proportionality bias. The use of rigorous statistical methods, such as the Bland–Altman analysis, in the assessment and comparison of clinical laboratory assays to ensure the appropriate interpretation of the results is mandatory. The Bland–Altman plot system does not say if the agreement is sufficient or suitable to use a method or the other indifferently. It simply quantifies the bias and a range of agreement, within which 95% of the differences between 1 measurement and the other are included. It is possible to say that the bias is significant because the line of equality is not within the CI of the mean difference. In our experimental setting, we have assumed that the OSA method calibrated against each single product should be the most precise calibrator. This proposal agrees with the recent WFH guidelines that state as follows: "For monitoring replacement therapy with FVIII or FIX concentrates, the WFH recommends that laboratories use a FVIII/FIX assay that has been validated for use with the specific concentrate used for treatment. REMARK: This recommendation is particularly important for modified molecular forms of FVIII and FIX" [8]. The activity of all FVIII concentrates used in clinical practice is calibrated against the World Health Organization (WHO) International Standards (IS). Two types of WHO IS have been established to pursue harmonization in the diagnosis and treatment of hemophilia A: (1) NIBSC 07/316, and (2) NIBSC 07/350 [18]. On the other hand, the CSA nowadays is the international reference method of the European Pharmacopoeia for the assignment of factor VIII concentrate potency/recovery [19]. However, the European Pharmacopoeia recommendations do not give specific guidance for the new modified rFVIII products, both pegylated and Fc-fusion proteins (https://drive.google.com/file/d/1MjDijh2XkUW-ua3ZJ3M443bJspCZW4oA/view) [20]. At variance with European rules, the FDA recommends the one-stage assay for FVIII potency assignment [21]. Therefore, in the United States, most laboratories use the one-stage assay to monitor the treatment of persons with hemophilia [21].

TABLE 6 Expected variations of the EHL-rFVIII level measured by the different assays compared to the respective EHL-rFVIII calibrated OSA method.

EHL-rFVIII product	OSA-Sil	OSA-el	CSA1	CSA2
Efmoroctocog alfa	↑	↓	↓	=
Rurioctocog alfa pegol	↑	↓	↓	↑
Turoctocog alfa pegol	=	↓	=	↓
Damoctocog alfa pegol	↓	↓	↓	↓

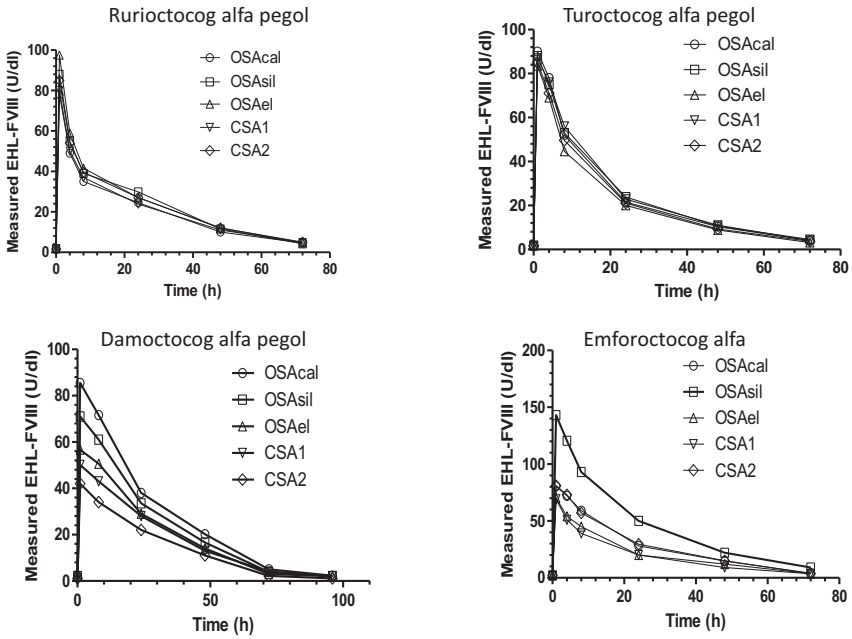

FIGURE 2 Pharmacokinetic profiles were obtained with the 4 EHL-rFVIII concentrates investigated in the study in 4 persons with severe hemophilia A. The relative best-fit pharmacokinetic parameters obtained by using a noncompartmental model are listed in Supplementary Table 2 in the Supplementary data file.

4.3 | How to set the clinical laboratories for EHL-rFVIIIs' monitoring

In clinical laboratories, the OSA is still the prevailing method for monitoring factor VIII levels in patients' plasma samples, mainly because of lower cost and because it was the first assay to be used in this diagnostic practice since the middle of the last century. The availability of specific Laboratory Standards could allow the use of any aPTT reagent (ie, Actin FS, Auto APTT, Cephascreen, CK Prest, Pathrombin SL, PTT-A, SynthASil) to measure FVIII:C by OSA. Our results, although requiring further clinical studies with a higher number of observations, also showed how the different laboratory tests could alter even the pharmacokinetic profile of the product. It must be outlined that this strategy would render the measurements independent from any aPTT reagent used in clinical laboratories, as the same factor would be used for both the standard calibration curve and *ex vivo* patient samples, differently from a setting where simple OSA methods are calibrated against plasma-derived FVIII for patients treated with EHL-rFVIII products. It was in fact hypothesized that PEG moiety may differently interact with aPTT reagents, influencing the response in aPTT-based tests [22–24]. A novel information emerging from the present study concerns the significative difference observed using CSAs distributed by different diagnostic manufacturers, reinforcing the opportunity to use a specific EHL-rFVIII product as a standard calibrator.

4.4 | Conclusive remarks

As to the possibility to use a standard calibrator for each product, we are aware of the difficulty to rapidly obtain the authorization by the regulatory agencies for the use of specific standard calibrators for diagnostics and the practical complications concerning their use in clinical laboratories. In the meantime, laboratories engaged in the management of persons with hemophilia under treatment with EHL-rFVIII products should consider the problematic nature of this situation. Engagement with the clinicians seems relevant because increasingly different tests may need to be undertaken for the various products, especially at this time, when several EHL-rFVIII products are entering the therapeutical armamentarium. Only regular internal monitoring and using of different laboratory methods as well as participation in external quality assurance exercises will ensure the continuously good performance of the used assay. Otherwise, inaccurate factor recovery values could lead to an incorrect and potentially dangerous management of persons with hemophilia, resulting in possible bleeding and thrombotic complications.

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AUTHOR CONTRIBUTIONS

M.S., M.T., and S.L. performed the research and revised the manuscript. M.E.M. designed some experimental measurements and critically revised the manuscript. R.D.C. designed the research study, analyzed the data, and wrote the manuscript.

RELATIONSHIP DISCLOSURE

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INFORMED PATIENT CONSENT

All patients signed informed consents before blood collection.

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SUPPLEMENTARY MATERIAL

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