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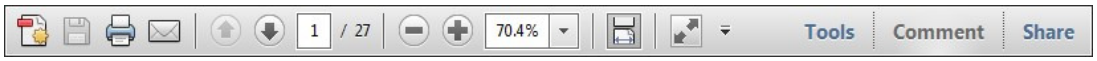

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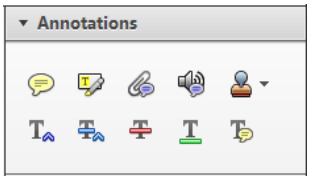

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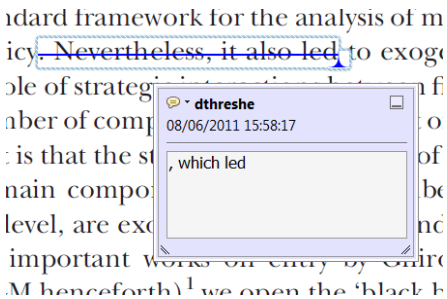

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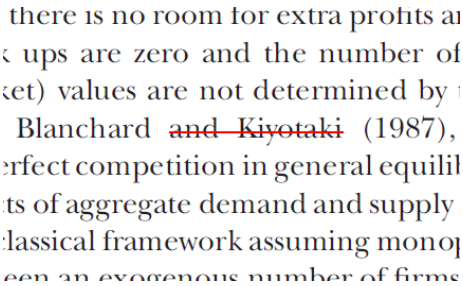

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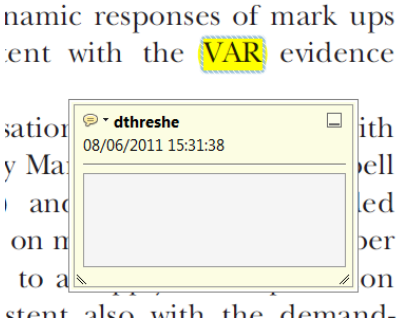

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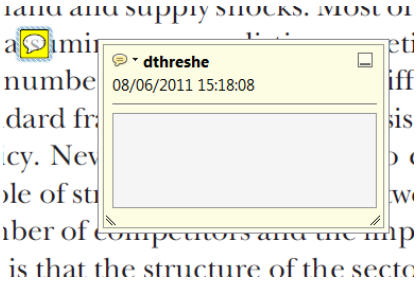

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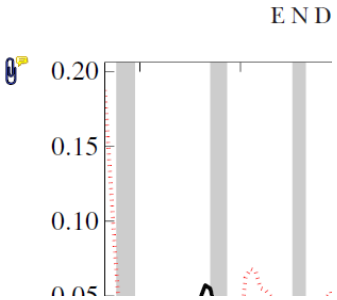

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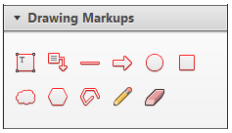

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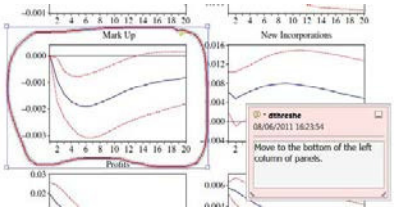

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REVIEW ARTICLE

A critical appraisal of one-stage and chromogenic assays of factor VIII activity

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Summary: Accurate and precise potency determination by manufacturers of different types of factor VIII product (plasma-derived and recombinant FVIII [rFVIII]) is vital to clinicians and patients using FVIII concentrates. A separate, but related, requirement is ascertaining the FVIII activity levels in clinical samples for diagnosing and treating hemophilia A. The one-stage clotting assay (OSA) and the chromogenic substrate assay (CSA) are the main assays used for these measurements, with both assays being used for potency assignments, and the OSA also being widely used for clinical monitoring. Although the assays can produce concordant results, discrepancies often occur, e.g. when measuring FVIII levels in patients with mild or moderate hemophilia A, or when assaying high-purity FVIII products. Modifications to rFVIII proteins, such as B-domain deletion (BDD), and technologies for improving the pharmacokinetic profile of rFVIII may exacerbate assay discrepancies. The CSA appears to be essentially unaffected by these modifications. However, the OSA underestimates the FVIII activity levels and therapeutic potential of some further modified BDD rFVIII products, especially those conjugated to poly(ethylene glycol); the extent of the effects is dependent on the specific OSA reagents used. Although the OSA remains the preferred choice for clinical monitoring in Europe and the USA, an awareness of the limitations of that assay has prompted more laboratories to adopt the CSA.

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Introduction

Adequate functioning of the coagulation cascade, which involves a series of sequential proteolytic reactions that activate coagulation factors and eventually result in the formation of a fibrin clot, is needed for effective hemostasis. Factor VIII is a 2332 amino acid glycoprotein with a discrete domain structure (A1–a1–A2–a2–B–a3–A3–C1–C2) [1–3] that functions as a cofactor for activated FIX (FIXa) in the pathway of the coagulation cascade. On secretion, FVIII is predominantly processed into a heterodimer containing a heavy chain (A1–a1–A2–a2–B) and a light chain (a3–A3–C1–C2) that are non-covalently bound via a metal ion through the A1 domain and the A3 domain [1–5]. This heterodimer circulates in a complex with von Willebrand factor (VWF) [1]. Activation of FVIII through proteolytic cleavage by thrombin generates an activated FVIII (FVIIIa) heterotrimer (A1a1/A2a2/A3–C1–C2) [6], which is released from VWF. FVIIIa binds to the phospholipid bilayer of activated platelets, and forms the tenase complex with FIXa in the presence of calcium ions to activate FX [1,7].

Hemophilia A is a congenital disorder in which different types of defect in the FVIII gene (*F8*) lead to a qualitative or quantitative deficiency of FVIII protein and subsequent activity, resulting in prolonged bleeding after trauma and recurrent spontaneous bleeding episodes. The mainstay of treatment for hemophilia A is replacement therapy with plasma-derived FVIII (pdFVIII) or recombinant FVIII (rFVIII) concentrates, which include full-length rFVIII proteins and modified rFVIII proteins with deletion or truncation of the B domain. New further modified rFVIII proteins, including a B-domain-truncated single-chain construct composed of covalently bonded heavy and light chains, and two-chain rFVIII proteins

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incorporating the fragment crystallizable (Fc) region of immunoglobulin (Fc fusion) or covalent attachment of poly(ethylene glycol) (PEG) molecules, have been developed.

In this review, we discuss the history and current situation relating to the use of the one-stage clotting assay (OSA) and the chromogenic substrate assay (CSA) for both manufacturers' potency assignment and the diagnosis and clinical monitoring of FVIII:C measurements in patients with hemophilia A.

FVIII activity assays

OSA

The one-stage activated partial thromboplastin time (APTT) clotting assay measures activity of the intrinsic and common pathways of the coagulation cascade. The first diagnostic test for hemophilia A was proposed in 1953, in which evidence of hemophilia A was provided if the patient's plasma sample (test sample) showed a normal prothrombin time and a prolonged partial thromboplastin time as compared with 'normal' plasma (reference sample) [8]. FVIII activity was quantified by measuring the ability of 'normal' plasma to shorten/correct the partial thromboplastin time when added to hemophilic plasma. The fundamental methodology of the OSA has not changed over time. Serial dilutions of the test plasma sample are mixed with equal volumes of FVIII-deficient plasma plus phospholipids and surface activator reagents; calcium ions are then added to start the coagulation reaction, and the APTT is recorded (Fig. 1A) [9]. The OSA is used with naturally or artificially depleted FVIII-deficient plasma. Technical developments include the introduction of combined phospholipid/activator reagents to aid automation [10].

CSA

The CSA is based on the same principle as the two-stage clotting assay [11]. The common first stage involves incubation of the plasma sample with FIXa, FX in excess, thrombin (optional), calcium ions and phospholipids to generate activated FX (FXa). The FVIII is activated, and the resulting FVIIIa acts as a cofactor for FIXa to convert FX to FXa. This is followed by a second stage to determine the amount of FXa produced. The CSA was developed after the synthesis of FXa-selective chromogenic substrates [12,13]. In the second step, FXa hydrolyzes the chromogenic substrate, and the color intensity of the resulting product (generally, *p*-nitroaniline released enzymatically) is directly proportional to the amount of FXa, which in turn is directly proportional to the amount of FVIII in the sample (Fig. 1B); therefore, the composition of assay components ensures quantitative assessment of FVIII activity [14].

Comparison of OSA and CSA

Both assays must fulfill three main requirements: they must provide a robust measure of FVIII activity, be sensitive to changes in pharmacokinetics, and be representative of the *in vivo* situation. In common with the OSA, the CSA is an indirect method of measuring FVIII activity, with the FVIII level being determined by comparison with a reference curve. Key features, advantages and limitations of the OSA and the CSA are shown in Table 1.

Historically, the CSA has been technically more complex to perform and more difficult to automate than the OSA, although protocols are available for the CSA on most modern automated equipment and are easy to perform. The CSA is also perceived as the slightly more expensive option, although this may depend on how the assay is implemented [15,16]. A further difference is that clotting factors other than FVIII are present at physiologic concentrations, whereas, in the ~~chromogenic assay~~, the initial FXa generation step is performed with concentrations of exogenous FX and FIXa as optimized by the kit manufacturer, and a low concentration of bovine thrombin may be present in order to accomplish activation of FVIII. Preactivation of FVIII increases activity in the OSA but not in the CSA (provided that FXa production is measured after a sufficiently long activation period), and the OSA is more likely to be affected by impurities such as lipids or heparin [17]. In both the OSA and the CSA performed on samples collected from patients receiving direct-acting oral anticoagulants, artefacts may appear, with the observed FVIII concentration being reduced [18]. Discussion continues over which assay is best and provides a better indication of 'true' FVIII activity [19–21]. Whichever assay is used, coagulant activity may be expressed in IU dL^{-1}, in IU mL^{-1}, or as a percentage. The FVIII reference range is 50–150 IU dL^{-1}, 0.5–1.5 IU mL^{-1}, or 50–150%; however, there is now a preference for avoiding the reporting of activity as a percentage. It is worth nothing that both the OSA and the CSA can be used in inhibitor assays, and it appears that the CSA produces fewer false-positive results; however such conclusions must be drawn cautiously [22].

Use of the CSA and OSA: potency assignment of FVIII

Potency assignments of both pdFVIII and rFVIII concentrates are required before release of an FVIII product by regulatory authorities. FVIII potency measurement must be accurate, as the two main purposes of potency labeling of products are to define the quantity of active substance in a vial and to guide dosing for treatment [23]. For example, potency assignments are determined relative to the current World Health Organization (WHO) International Standards (ISs) for FVIII [23] and are reported in international units. These potency assignments must be accurate to enable comparison between different products

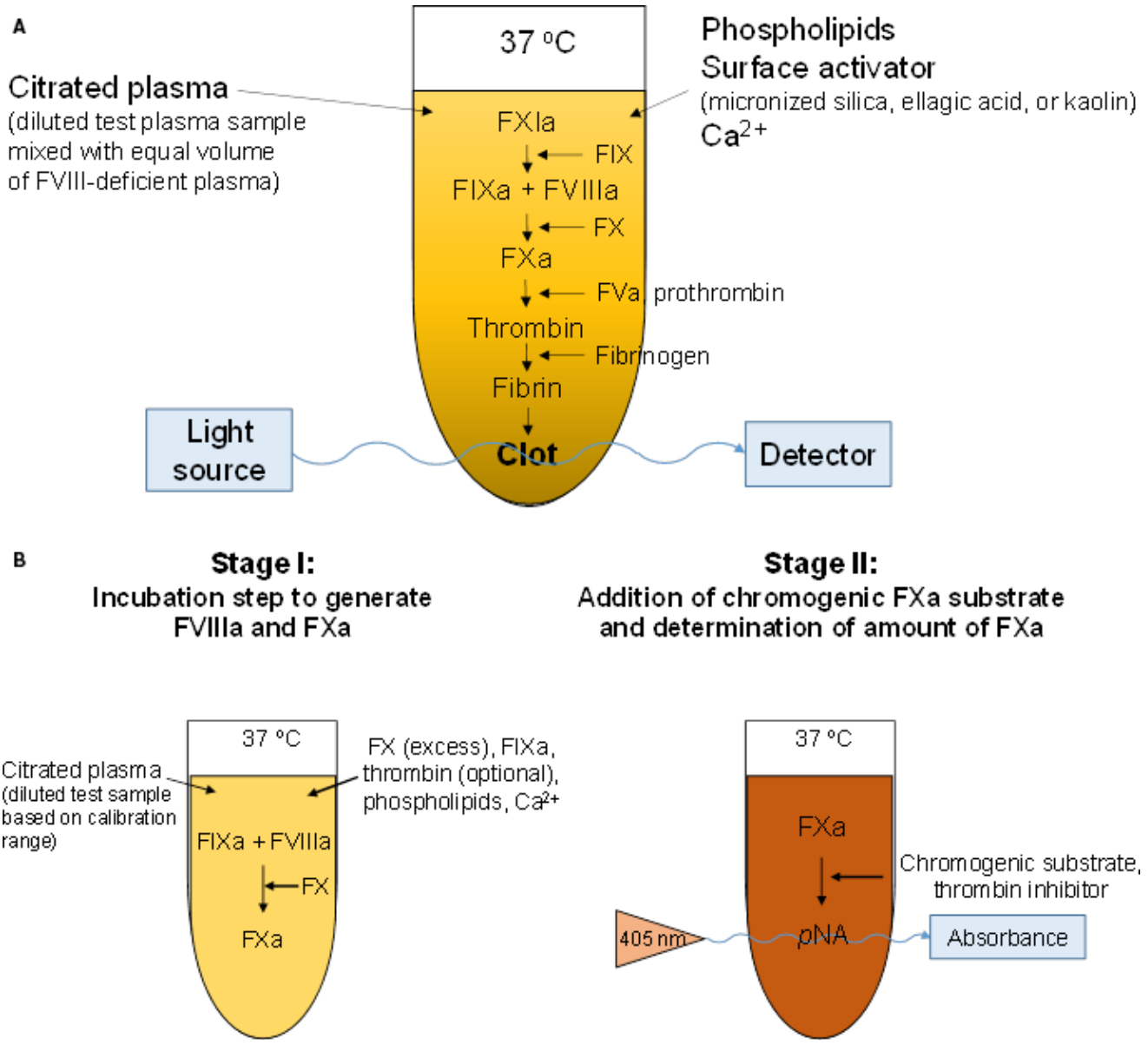

Fig. 1. Schematics of FVIII activity assays. (A) One-stage clotting assay. (B) Chromogenic substrate assay. FIXa, activated FIX; FVa, activated FVa; FVIIIa, activated FVIII; FXa, activated FX; FXIa, activated factor XI; *p*NA, *para*-nitroaniline.

(pdFVIII and rFVIII) and to ensure that products labeled in international units contain the correct amount of FVIII in the vial versus the labeled potency, and are therefore not overpriced or underpriced. Furthermore, accurate potency determination is vital to ensure optimal safety and efficacy for patients, and to uphold the integrity of the international unit. The dose based on the labeled potency of a product and the expected FVIII recovery in the patient should be established with data from clinical pharmacokinetic studies [23]. A dosing guideline may then be derived from which clinicians should be able to determine the optimal efficacious and safe dose for a specific patient. If manufacturers accurately and precisely determine the amount of FVIII in a drug product, a clinician can be confident in the international unit assignment and the appropriate dose based on standard clinical practice parameters, established pharmacokinetic data from clinical trials, and product labeling guidance.

Currently, the OSA and the CSA are used to assign potency. The European Medicines Agency (EMA) requires the CSA to be used. However, although the EMA stipulates that the CSA should be used, approval of the OSA for potency labeling by regulators is possible, albeit infrequently sought, provided that equivalence of results obtained with both methods is shown [24]. The US Food and Drug Administration (FDA) has no such guidance; currently all but two FVIII products are labeled by use of the OSA. Because the majority of laboratories

Table 1 Main features, advantages and limitations of the one-stage clotting assay (OSA) and chromogenic substrate assay (CSA) for determination of FVIII activity

	OSA	CSA
Main features	Measures the ability of reference plasma to shorten the APTT of hemophilic plasma One-stage procedure Physiologic concentrations of clotting factors other than FVIII Recommended by the US FDA as the reference method for FVIII potency assignment	Measures the ability of FVIII to act as a cofactor for FIX to activate FX Two-stage procedure: generation of FXa followed by reaction of FXa with a chromogenic substrate and photometric measurement Highly diluted concentrations of clotting factors Recommended by the EMA as the reference method for FVIII potency assignment
Advantages	Simple and rapid Inexpensive Easy to automate Widely used for clinical monitoring Detects some mild hemophilia variants where the OSA result is lower than the CSA result	Not sensitive to FVIII activation FVIII-deficient plasma not required Suitable across all FVIII concentrations Insensitive to lupus AC Lower interlaboratory variability than the OSA Detects some mild hemophilia variants where the CSA result is lower than the OSA result
Limitations (particularly with respect to new modified rFVIII products)	Sensitive to FVIII activation Large variety of assay kits, reagents, assay conditions and analyzers may result in high interlaboratory variability Sensitive to lupus AC, heparin, DOAC drugs, and lipid impurities Less sensitive at low FVIII concentrations Overestimates FVIII activity in some patients with a mild hemophilia A phenotype Higher interlaboratory variability than the chromogenic assay for rFVIII products Underestimates FVIII activity for BDD rFVIII products Some reagents may not be suitable for measuring the FVIII activity of new modified rFVIII products	More expensive than the OSA Not as widely used as the OSA, particularly for diagnosis and monitoring Perceived to be more difficult to automate than the OSA Perceived to be technically complex Overestimates FVIII activity in some patients with a mild hemophilia A phenotype Sensitive to DOAC drugs

AC, anticoagulant; APTT, activated partial thromboplastin time; BDD, B-domain deletion; DOAC, direct oral anticoagulant; EMA, European Medicines Agency; FDA, Food and Drug Administration; FXa, activated FX; rFVIII, recombinant FVIII.

in the USA use the OSA to monitor, the FDA's preference has been to label by use of the OSA, but decisions on potency labeling should not be driven by a desire to adapt to standard clinical laboratory practice; rather, it should be supported by thorough characterization of the *in vitro* properties of the new product and data on *in vivo* efficacy. The Subcommittee of the Scientific and Standardization Committee of the ISTH (SSC ISTH) has published recommendations for potency labeling as a guidance to manufacturers; these state that harmonization of the approach for assigning potency is vital, particularly for the new modified rFVIII products [23]. The method of choice for labeling is irrelevant when both the OSA and CSA agree, but it is crucial when there are significant discrepancies. An example of potency labeling issues occurred with ReFacto (Pfizer, Sandwich, UK). The results from a different study revealed that the CSA-assigned potency for the product-specific ReFacto laboratory standard (RLS) was ~ 20% too high [25]. Subsequently, the RLS was recalibrated, and this resulted in a 20% increase in the ReFacto protein level in each vial for the same labeled potency. Consequently, the therapeutic potential of ReFacto may have been overestimated by the previous potency label [26], and this may explain early reports of poorer efficacy of B-domain deletion (BDD) products than would be expected on the basis of the labeled potency [27–29]. Owing to the differences in potencies assigned by the OSA and the CSA, the successor products have different labeled potencies in the USA and the European Union (EU), with 1 IU of Xyntha (Wyeth Pharmaceuticals, Collegeville, PA, USA) (USA; OSA) being equivalent to 1.38 IU of ReFacto AF (Pfizer, Sandwich, UK) (EU; CSA) [30].

Use of the CSA and the OSA: clinical monitoring

The diagnosis of hemophilia A, monitoring of FVIII target levels during treatment, determination of the

pharmacokinetics of FVIII replacement therapies and the successful perioperative management of FVIII levels in patients with hemophilia A require estimates of FVIII:C with acceptable levels of accuracy and precision. In practice, variability of approximately 10–20% is tolerated for clinical monitoring, owing to the inherent differences in pharmacokinetic and clinical efficacy responses of individual patients to FVIII replacement products. The impact of discrepancies between the OSA and the CSA in clinical practice has recently been reviewed, with an emphasis on diagnosis and the management of inhibitors [11]. Overall, the OSA remains widely used for the diagnosis of hemophilia A and clinical monitoring of FVIII replacement therapy, because it is simple, rapid, inexpensive, and easy to automate.

Although the OSA is widely used, there are numerous assay kits, reagents, assay conditions, analyzers and combinations thereof available for the OSA, leaving the method riddled with variation. The interlaboratory variability of the OSA increases if the recommended technical standardization procedures are not followed [17]; these include the use of FVIII-deficient plasma containing a normal level of VWF, inclusion of albumin (1% w/v) in all assay buffers, and predilution with hemophilic plasma (or its equivalent) for the assay of all rFVIII or high-purity pdFVIII concentrates [17,31]. Standardization of assay procedures has reduced the variations observed for the OSA in collaborative studies to some extent [32–34]. Although the OSA accurately assesses FVIII activity for pdFVIII, it has a tendency to underestimate FVIII activity for some rFVIII products, particularly BDD rFVIII proteins, with OSA-based estimates often being 20–50% below chromogenically determined values [34–38]. Manufacturers will need to provide guidance to clinicians whose laboratories are using the OSA to measure FVIII:C of products that have been clinically validated and then labeled by use of the CSA, in order to allow appropriate interpretation of ~~one-stage~~ data.

The CSA is currently in use in many specialty clinical laboratories [39], and validation of the CSA for routine clinical laboratory environments is currently under investigation [40]. In Europe, it is accepted that the CSA shows lower variation than the OSA [33,34]. At low FVIII:C levels (3–5%), the CSA is considered to be more sensitive than the OSA [24], and may show greater precision [41], but the latter has not been consistently demonstrated [42,43]. The CSA is generally more reproducible across laboratories (Table 2). The improvements in sensitivity and precision obtained with the CSA over the OSA are clear advantages, but its complexity adds to the operator burden, and the additional step and reagents introduce a hypothetical risk of increased errors. The lower number of commercially available CSA kits than ~~of~~ OSA kits may limit interlaboratory variation, but, conversely, reduced competition between manufacturers may sustain elevated prices. It is also possible that the effective cost of the CSA is increased by the limited use of the kit, as it is only suitable for assaying FVIII, unlike the OSA, which is a modification of the APTT assay used for routine coagulation monitoring, and by the lack of kits designed for assaying a small number of samples. The majority of CSA kits are formulated such that the whole CSA kit must be used for assaying, which may result in wastage in centers with few patients with hemophilia A. This limitation may be circumvented by aliquoting and freezing reagents, but utilization of stored frozen aliquots should be validated by any laboratory embarking on such a protocol.

Discrepancies between the OSA and the CSA

Measurement of FVIII activity with the OSA and the CSA can provide comparable results, but discrepancies between the two types of assay occur in a number of circumstances. These include measurements of FVIII activity in patients with specific hemophilia A phenotypes or genetic mutations [21,44], and when high-purity pdFVIII concentrates and rFVIII products are assayed [34–38]. The discrepancies may be exacerbated by modifications to rFVIII, such as ~~B-domain deletion~~, [36], the length of the B-domain linker [45], and technologies designed to improve the pharmacokinetics of rFVIII, including PEGylation [41,42,46,47]. The factors known to result in assay discrepancies are detailed in Table 2.

Patients with non-severe hemophilia A

Assay discrepancies have been observed in approximately one-third of patients with non-severe hemophilia A [21,44]. The FVIII activity level may be higher when determined with the OSA than when determined with the chromogenic/two-stage assay [21,48–57] or vice versa [21,55,58,59].

Lower FVIII activity (≥ 2-fold difference) when ~~it is~~ measured with the CSA ~~than when it is measured~~ with the OSA is associated with the presence of missense mutations localized in the A1–A2–A3 domain interfaces, e.g. p.Arg546Trp, p.Arg550Gly and p.Arg550His [21,54,55,60], p.Asn713Ile [52], and p.His1973Leu [56]. These mutations are associated with reductions in the stability of the FVIII heterodimer and A2 domain in the activated heterotrimer (FVIIIa), and disruption of interdomain protein–protein interactions [21,54]. As FVIIIa is only generated once calcium ions have been added during the final rapid clotting stage of the OSA, the effect of instability of the FVIIIa heterotrimer is minimized. In contrast, during the incubation of the CSA first step, in which FVIII is proteolytically activated to form FVIIIa, a higher rate of A2 dissociation is favored, and would thus tend to reduce the observed FVIII activity [21,54].

Patients with mutations such as p.Tyr365Cys and p.Glu739Lys show higher levels of FVIII activity with the

Table 2 Factors resulting in discrepancies between the one-stage clotting assay (OSA) and chromogenic substrate assay (CSA) for determination of FVIII activity

Factor	CSA/OSA	Causes of discrepancy
Missense mutations in *F8*		
Localized in the A1–A2–A3 domain interfaces	≤ 0.5	These mutations are associated with reduced stability of the FVIII heterodimer and FVIIIa heterotrimer The effect is minimized in the OSA, ~~wheras~~ the incubation performed during the first step of the CSA favors a higher rate of A2 dissociation, leading to a reduction in observed FVIII activity
Located close to or within thrombin cleavage, FIX-binding or VWF-binding sites	≥ 2.0	These mutations affect thrombin activation or FVIII binding to FIXa or VWF The OSA is sensitive to alterations in thrombin binding or cleavage of FVIII, whereas the CSA is not
rFVIII products	Approximately 1.1–1.3*	To be determined
Modifications to rFVIII		
BDD/B-domain truncation	> 1†	For ReFacto, the difference can be corrected by the type and concentration of the phospholipid used in the OSA Some other B-domain-modified rFVIII products do not show significant discrepancies
PEGylation	> 1†	Interaction of the PEG moiety with silica-based reagents in the OSA results in prolonged APTT and underestimation of FVIII activity

APTT, activated partial thromboplastin time; BDD, B-domain deletion; FIXa, activated FIX; FVIIIa, activated FVIII; PEG, poly(ethylene glycol); rFVIII, recombinant FVIII; VWF, von Willebrand factor. †Values of the CSA/OSA ratio vary but, in general, FVIII activities obtained with the CSA are higher than those obtained with the OSA.

CSA than with the OSA [21,44,58,59]. The majority of mutations associated with this type of discrepancy are located close to or within thrombin cleavage sites, or FIX-binding or VWF-binding sites, and thus affect thrombin activation or FVIII binding to FIXa or VWF [21]. In the OSA, the physiologic thrombin concentrations initially formed activate FVIII in a short period of time, making the assay sensitive to any alterations to thrombin binding or cleavage of FVIII. In contrast, the requirement for incubation in the CSA means that changes in the affinity of thrombin for FVIII and/or changes in the FVIII activation kinetics will be overcome by the excess thrombin activating diluted FVIII [21].

In the majority of cases of hemophilia A, FVIII activity measured with either assay is low enough that the correct diagnosis will be made. However, approximately 5–10% of patients with mild hemophilia A show FVIII activity within the normal range when it is measured with one type of assay, but lower FVIII activity when it is measured with the other [21,61]. In these patients, diagnosis may be missed if only one assay is used. In most cases, the clinical phenotype correlates more closely with the lower FVIII activity obtained from either of these assays [21,61,62], but exceptions have been documented [63]. Thus, combined use of the OSA and the CSA is recommended for the diagnosis of mild hemophilia A [21,44,61,62,64,65].

Existing rFVIII products

Assay discrepancies have been noted for full-length rFVIII products, with higher FVIII activity levels being measured with the CSA than with the OSA [33–38]. Generally, the discrepancies are 8–20% [33,34,36], and thus not clinically significant (i.e. within the accepted variation of ≤ 20%), although reports of differences of ~ 40% exist [37]. However, the assay discrepancy for BDD rFVIII can be much larger, with the OSA giving values 20–50% lower than the CSA [34–38]. Plasma levels of the BDD rFVIII product ReFacto were up to ~ 50% lower when measured with the OSA than when measured with the CSA [36]. Laboratories using the OSA assay to measure ReFacto postinfusion plasma samples were provided with a product-specific reference standard to correct the underestimation.

Assay discrepancies have been observed for postinfusion samples of FVIII concentrates, with evidence that the difference is greater for rFVIII than for pdFVIII products. In the collaborative studies to calibrate the 5th and 6th WHO IS FVIII concentrates, the chromogenic/one-stage potencies of a rFVIII concentrate versus the WHO plasma standard were 1.48 and 1.26, respectively [30]. In a UK survey of postinfusion samples, the CSA gave significantly higher results than the OSA for Kogenate FS (Bayer Pharma AG, Berlin, Germany) (32% difference; $P < 0.0001$) but not for Advate (Baxter AG, Vienna, Austria) or ReFacto AF, when calibrated with a plasma standard [66]. The APTT reagent type markedly affected one-stage FVIII activities for ReFacto AF and Advate, but not for Kogenate FS [66]. For plasma-derived products, the situation depends on the test systems and product type. A mAb-purified

concentrate (Hemofil M; Baxter Healthcare Corporation, Westlake Village, CA, USA) showed 17–28% higher recovery when measured with the CSA than whenmeasured with the OSA [67], whereas mean CSA/OSA ratios of 0.97–1.11 have been observed for Hemofil M, Emoclot (Kedrion S.p.A., Barga, Italy) and Fanhdi (Grifols UK, Cambridge, UK) in clinical samples [68]. Postinfusion samples of recombinant and high-purity plasma-derived products are therefore regarded as concentrates diluted in a patient's plasma. To provide a 'like versus like' comparison, a concentrate standard (similar to the administered product) is proposed to be diluted in hemophilic plasma and used as the reference instead of a plasma standard [30].

New modified rFVIII products

The advent of rFVIII products with additional modifications designed to improve the pharmacokinetic properties of FVIII presents further challenges for the determination of FVIII activity [66]. Potential assay discrepancies are under investigation for these new products, and the currently reported data are shown in Table 3. It appears that BDD/truncation on its own does not necessarily lead to significant assay discrepancies. This is supported by the similar results for both Advate (full-length rFVIII) and NovoEight (Novo Nordisk A/S, Bagsværd, Denmark) (B-domain-truncated rFVIII) in a comparative field study [41], in which, despite discrepancies between the OSA and CSA, their magnitude was similar. In addition, the comparative field study for Eloctate (Biogen, Cambridge, MA, USA) (rVIII–Fc fusion protein) concluded that clinically insignificant assay discrepancies occurred as compared with a full-length FVIII product [42]. In common with the NovoEight comparative field study, the OSA overestimated FVIII activity at low concentrations, and better dose linearity was observed for the CSA [41,42]. Assay discrepancies have been noted for FVIII activity levels for the B-domain-truncated single-chain construct rVIII–SingleChain [69], but data are not yet available on the extent of the discrepancies. The underlying reason why some B-domain modifications result in assay discrepancies remains to be elucidated, although it may be that the length of the B-domain linker is important [45].

Assay discrepancies that are likely to be clinically relevant are apparent for the N8-GP (Novo Nordisk A/S) and BAY 94-9027 (Bayer Pharma AG) PEGylated rFVIII products. Although the PEG moiety does not appear to affect the CSA, it does seem to interact with silica-based APTT reagents, resulting in prolonged APTT and underestimation of FVIII activity in the OSA [46,47,70]. Ellagic acid and polyphenol APTT reagents do not appear to cause these issues, and it may therefore still be possible to monitor PEGylated rFVIII products with the OSA [46,47,70]. For BAX 855 (Baxter Healthcare Corporation), a PEGylated full-length rFVIII, data have not been reported on assay discrepancies. However, a modified assay specific for PEGylated rFVIII that combines the use of an anti-PEG antibody with a CSA has recently been developed and validated for the measurement of BAX 855 [71]. Although the mechanism for interference of the PEG moiety with particular APTT reagents has not been elucidated, the size and position of the PEG molecule on the rFVIII protein may play a role [70,72].

Options for resolving FVIII assay discrepancies

When the product manufacturers' preferred method of clinical monitoring, such as the CSA, is not available there are several options for resolving FVIII activity assay discrepancies with the available clinical laboratory methods.

Product-specific reference standard

This method was used with ReFacto and its successor ReFacto AF, when the CSA was not available. Accurate FVIII activities could be determined for ReFacto and ReFacto AF with the OSA, as long as the RLS or RLS-AF standard was used, respectively, instead of a plasma standard [35,73,74]. There are several advantages to this approach: it facilitates the standardization of results between assays; it is consistent with the 'like versus like' principle recommended by the SSC ISTH; and it provides greater reliability for critical determination of FVIII activity (e.g. in clinical monitoring). However, disadvantages must also be considered. Using a product-specific standard can be difficult to implement in routine laboratory procedures. Each assay and product standard may need to be validated on specific analyzers, so switching between standards would be time-consuming. Additionally, as the number of rFVIII products increases, the number of accompanying reference standards will probably also increase. Integrating further multiple reference standards into standard laboratory practice may prove challenging, requiring good communication between the hemophilia treatment center and the laboratory. Not only would clinical laboratories that support patient monitoring need to be informed of the specific rFVIII product that the patient is receiving, but they would also have to indicate which product-specific standard was used to obtain the reported result. In summary, although a product-specific standard provides a method for accurately determining FVIII activity with the OSA, in reality it is unlikely that most clinical laboratories would embrace this change; rather, they would continue to use their own internal procedures. For PEGylated rFVIII products, product-specific standards may also be necessary for potency measurements; a product-specific standard for N8-GP is currently under evaluation [46,47].

Table 3 Chromogenic substrate assay (CSA) versus one-stage clotting assay (OSA) FVIII activity ratios for modified recombinant FVIII (rFVIII) concentrates

Product	Modification	Comparison product	Type of study	CSA/OSA ratio/ FVIII recovery	Interlaboratory variation (CV%)	APTT reagent	One-stage APTT reagent effect	WHO IS reference	Postulated cause of discrepancy
NovoEight (turoctocog alfa) [41,68]	B-domain truncation	Advate	Comparative international field study (spiked plasma samples) [41]	0.68 (0.03 IU mL^{-1}) 1.01 (0.2 IU mL^{-1}) 1.23 (0.6 IU mL^{-1}) 1.30 (0.9 IU mL^{-1}) (comparable results to Advate)	OSA: 13–14% at medium/high concentrations; 35% at very low concentrations CSA: 7–8% at medium/high concentrations; 14% at very low concentrations	Wide variety of reagents used	NA	In-house standard	Authors conclude that assay discrepancy not clinically relevant
		Advate/Kogenate Bayer/Hemofil M/ Emoclot/Fanhdi	PK parameters in clinical trial samples and spiked plasma [68]	Clinical trial samples: 1.26 (NovoEight) 1.22 (Advate) 1.65 (Kogenate) 1.11 (Hemofil M) 0.97 (Emoclot) 1.11 (Fanhdi)	NA	Silica (SynthASil)	NA	Standard human plasma (clinical samples) 6th WHO IS (spiked samples)	All rFVIII products showed discrepancy irrespective of B-domain length
Nuwiq (Octapharma AG, Lachen, Switzerland) (simoctocog alfa) [75–77]	~~B-domain deletion~~	Advate/ Kogenate FS/ ReFacto AF	Potency comparison [77]	1.14 (human-cl rFVIII) 1.15 (Advate) 1.12 (Kogenate/ Kogenate FS) 1.33 (ReFacto/ ReFacto AF/ Xyntha)	NA	Ellagic acid (Dade Actin)	NA	7th/8th WHO IS	No significant assay discrepancy observed
		NA	Pediatric PTPs with PK evaluation [75]	PK parameters comparable with either method	NA (central laboratory measurement)	NA	NA	NA	–
		Kogenate FS	Phase II PK study [76]	PK parameters comparable with either method and with Kogenate FS	NA (central laboratory measurement)	NA	NA	NA	–

Table 3 (Continued)

Product	Modification	Comparison product	Type of study	CSA/OSA ratio/ FVIII recovery	Interlaboratory variation (CV%)	APTT reagent	One-stage APTT reagent effect	WHO IS reference	Postulated cause of discrepancy
rFVIII–Fc [42,78]	~~B-domain deletion;~~ Fc fusion protein	Advate	Comparative international field study (spiked plasma samples) [42]	1.04 (0.054 IU mL^{-1}) 1.26 (0.22 IU mL^{-1}) 1.26 (0.87 IU mL^{-1}) (comparable results to Advate)	OSA: rFVIII–Fc: 16% at high concentrations; 31% at low concentrations Advate: 10% at high concentrations; 34% at low concentrations CSA: ~~rFVIIIFc~~: 19% at high concentrations; 31% at low concentrations Advate: 18% at high concentrations; 38% at low concentrations	Wide variety of reagents used	NA	In-house standard	No significant assay discrepancy observed
		Advate	Phase I PK study [78]	32% higher (rFVIII –Fc) versus 21% higher (Advate) for CSA versus OSA (P = NS)	NA	Ellagic acid (Dade Actin FSL)	NA	5th WHO IS	–

Table 3 (Continued)

Product	Modification	Comparison product	Type of study	CSA/OSA ratio/ FVIII recovery	Interlaboratory variation (CV%)	APTT reagent	One-stage APTT reagent effect	WHO IS reference	Postulated cause of discrepancy
N8-GP [46,47]	B-domain truncation; site-specific O-glycoPEGylation (40 kDa)	Advate/Haemate (CSL Behring, Marburg, Germany)/ NovoEight	*In vitro* study [46]	CSA: comparable FVIII activity for NovoEight and N8-GP OSA: Advate, Haemate, and NovoEight: recovery 100% ± 25% of labeled value irrespective of APTT reagent type N8-GP: recovery 100% ± 25% with ellagic acid reagents; recovery < 75% with silica-based APTT reagents Full recovery with all APTT reagents with the use of N8-GP standard	NA	Ellagic acid (Actin FS, STA Cephascreen)/silica	Silica-based APTT reagents decreased measured N8-GP FVIII activities	NA	Interaction between silica-based APTT reagents and PEG moiety N8-GP standard allowed activity measurement in the normal range with all APTT reagents CSA not affected by PEG moiety
		Advate	Spiked FVIII-deficient plasma [47]	OSA: activity underestimated with silica-based reagents; normal range with kaolin-based and ellagic acid-based reagents Activity in the normal range irrespective of reagent type when N8-GP standard used CSA: activity in the normal range	NA	Ellagic acid (STA Cephascreen)/ kaolin (STA CK Prest)/silica (STA-PTT A, TriniCLOT Automated aPTT, TriniCLOT aPTT HS, TriniCLOT aPTT S)	Silica-based APTT reagents underestimated N8-GP FVIII activities	NA	–

Table 3 (Continued)

Product	Modification	Comparison product	Type of study	CSA/OSA ratio/ FVIII recovery	Interlaboratory variation (CV%)	APTT reagent	One-stage APTT reagent effect	WHO IS reference	Postulated cause of discrepancy
BAY 94-9027 [70]	~~B-domain deletion~~; site-specific PEGylation (60 kDa branched)	N/A	Spiked plasma samples	0.78–0.98 (APTT reagent Cephascreen)	NA	Ellagic acid (SynthAFax, Dade Actin)/polyphenol Cephascreen)/silica (APTT-SP, STA PTT 5)	Prolonged APTT clotting time observed with silica-based reagents Normal APTT clotting times with ellagic acid-based and polyphenol-based reagents	8th WHO IS	Impairment of FXII activation on the silica surface activator by PEG moiety

APTT, activated partial thromboplastin time; CV, coefficient of variation; IS, international standard; NA, not available; NS, not significant; PEG, poly(ethylene glycol); PK, pharmacokinetic; PTP, previously treated patient; WHO, World Health Organization.

Recommendation to use/not use specific reagents/kits

As the assay discrepancies for PEGylated rFVIII products are often related to particular OSA reagents/activators giving erroneous results, e.g. silica-based APTT reagents [46,47,70], clinical monitoring may be possible with the OSA as long as only manufacturer-recommended assay reagents (i.e. ellagic acid activators) are used. This approach may force clinical laboratories to adjust their choice of APTT reagents according to the rFVIII product prescribed by clinicians; however, this depends on the freedom that individual laboratories may have regarding the purchasing of kits. Alternatively, product-specific standards may be used to overcome the limitations of the OSA with respect to these modified rFVIII products [46,47]. The situation concerning other rFVIII modifications – BDD, B-domain truncation, and Fc fusion – is less clear. For these products, FVIII activity determination with the OSA without specifically recommended reagents may be feasible, but is likely to be product-dependent.

Manufacturer-guided clinical interpretation

Acceptable clinical interpretation of the FVIII activity of products that produce systematic discrepancies across reagents may be possible if well characterized, and if a correction factor is applied according to the manufacturer's guidelines. However, the feasibility of this approach would need to be demonstrated for individual products, and would be dependent on the OSA underestimation being predictable across the entire range of FVIII: C values observed in clinical practice (the relationship may be non-linear) and the variability being consistent with other rFVIII full-length products.

Conclusions

New modified rFVIII products pose challenges for FVIII potency and activity measurement, and their behavior in both the OSA the CSA must be fully characterized [23]. Despite being commonly used worldwide, because of its simplicity and widespread dissemination, the OSA has several limitations with respect to determination of FVIII activity levels for some BDD and other modified rFVIII products, particularly those that contain a PEG moiety. However, as it remains the method of choice in a number of countries, including the USA, options to convert inaccurate OSA results should be implemented and guided by the manufacturers, e.g. product-specific standards, avoidance of certain APTT reagents, or generalized guidance if there is systematic underestimation or overestimation of activity.

Clinical interpretation of all patient laboratory data is necessary for appropriate clinical decision-making. As the laboratory practice adjusts to fully support clinical moni-

toring of rFVIII activity in patients, the onus will be on clinicians. As manufacturers fully characterize the rFVIII products coming to market, appropriate guidance should be given regarding recommended laboratory methods and variance noted across methods used in current clinical practice. This will allow emergent interpretation of clinical results and permit clinicians to use alternative reference laboratories if needed in non-emergent settings. When reporting FVIII activity, laboratories will need to note, at a minimum, the current methods used in the laboratory, i.e. OSA or CSA, in addition to the activators used. Acceptable clinical interpretation of FVIII activity of products that produce systematic discrepancies across reagents may be possible if well characterized. This will be especially useful for ease of local laboratory evaluations when the local laboratory is unable to integrate multiple methods into practice.

Currently, the CSA is used less frequently than the OSA for clinical monitoring. However, its precision and suitability across all FVIII concentrations make it an attractive option. The evolution of CSA use may be accelerated if it is used to perform potency measurements and clinical monitoring during phase III studies of new modified rFVIII products, but considerable barriers remain. Less expensive CSA kits and reagents, and kits designed for lower sample throughput, will be needed if clinical laboratories are to consider their routine use. Overall, the ability of the CSA to accurately measure the FVIII activity levels of new modified rFVIII products will increase its role in potency assignment and probably also in clinical monitoring in the future.

Addendum

F. Peyvandi, J. Oldenburg, and K. Friedman: contributed to the design of this review, and identified the priorities and needs of the scientific community. F. Peyvandi: coordinated the review. All authors: critically reviewed the manuscript, providing substantial input, and gave final approval of the version to be published.

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F. Peyvandi has received honoraria for participating as speaker at satellite symposia and educational meetings organized by ~~Alexion, Baxter,~~ Bayer, Biotest, CSL Behring, Grifols, Novo Nordisk, and Sobi. She ~~has received~~ research grant funding from Alexion, Biotest, Kedrion Biopharma, and Novo Nordisk, and consulting fees from ~~Bio Products Laboratory Ltd, Grifols,~~ Kedrion, and LFB. She is member of the Ablynx scientific advisory board. Outside of the submitted work, J. Oldenburg reports receiving grants and personal fees from Baxter, Bayer, Biotest, CSL Behring, Grifols, Novo Nordisk, Octapharma, and Pfizer. He also reports receiving personal fees from Biogen Idec, Chugai, Roche, and Swedish Orphan Biovitrum. K. D. Friedman reports receiving personal fees from CSL Behring, during the conduct of the study. Outside of the submitted work, he reports receiving personal fees from Novo Nordisk, Instrumentation Laboratories, Octaphama, Baxter, and Biogen Idec.

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10

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