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FACTOR VIII ASSAY VARIABILITY IN POST INFUSION SAMPLES CONTAINING FULL LENGTH AND B-DOMAIN DELETED FVIII

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Abstract

Introduction

Although the variability in FVIII:C measurement is well recognised this has not been widely reported for post FVIII infusion samples.

Aim/Methods

Three samples from haemophilia A patients were distributed in a UK National External Quality Assessment Scheme (NEQAS) survey, each after treatment with either ReFacto AF, Kogenate FS or Advate. Fifty-two UK haemophilia centres performed FVIII assays using one stage (n = 46) and chromogenic (n= 10) assays. Centres calibrated assays with the local plasma standard and with ReFacto AF laboratory standard (RAFLS) for the ReFacto AF sample.

Results/Conclusions

Chromogenic assays gave significantly higher results than one stage assays ($p<0.0001$, 32% difference) in the post Kogenate sample but not in the post ReFacto AF (11% higher by chromogenic assay, ns) or post Advate samples (3% lower by chromogenic, ns) when assays were calibrated with plasma standards. Twenty centres used all Instrumentation Laboratory (IL) APTT reagents (Synthasil)/ IL deficient plasma/reference plasma) in the one stage assay and 15 used all Siemens reagents (Actin FS/Siemens deficient plasma/reference plasma); this made a significant difference to results post ReFacto AF (41% higher by IL reagents, $p<0.0001$) and Advate (39% higher by IL reagents, $p<0.0001$), but not Kogenate (7% higher by IL, ns) when calibrated with plasma standards. Differences between results obtained with different one stage assay reagents for monitoring Advate have implications for dosing patients. Furthermore there was considerable inter-laboratory variation as indicated by CVs in the range 15-26% for chromogenic assay and 12-19% for one stage assay results. This study suggests that external quality assessment schemes should offer participation in post FVIII infusion schemes where haemophilic patients are monitored.

Introduction

Replacement of FVIII in subjects with haemophilia A is an important component of successful management (1). Products containing recombinant FVIII are widely used and are the recommended treatment of choice in some countries (2). In some clinical settings, replacement therapy may be more cost effective if laboratory monitoring is utilised. This commonly involves determination of FVIII activity in post infusion samples. The assay used for potency labelling of FVIII concentrates is important since the labelled potency is used during clinical trials to establish efficacious dosing recommendations. Such recommendations are appropriate if a clinical laboratory uses an assay which gives similar results to that used for potency assignment, so awareness of any assay differences is important for patient management. Potency assignment for FVIII products is performed using the chromogenic assay in Europe and one stage assay in some other regions.

It is well known that the results of FVIII assays continue to vary between different centres worldwide (3, 4). There are additional issues related to the assay of post infusion samples containing full length recombinant FVIII since results of chromogenic FVIII assays may be 20 – 40% higher than results of one stage FVIII assays in this setting (5 – 8). It may be possible to obtain better agreement between results with different assays by the use of a concentrate standard for assay calibration (9). A similar relationship occurs in relation to samples containing one particular B-domain deleted FVIII (ReFacto, Pfizer) with results of chromogenic assays being approximately 20-50% higher than one stage assay results for an early formulation (7, 10-12) and for a later formulation (ReFacto AF) (13-15) of this concentrate.

The assay differences relate in part to the source of phospholipids present in the laboratory reagents that are used in the one stage assay (10, 16). It has been demonstrated that, for some test systems, one stage assay results are in agreement with results obtained by chromogenic assays if the one stage assay is calibrated using a concentrate standard prepared from the same product, both with ReFacto and ReFacto AF (8,11-15). An assessment of FVIII assay performance in samples containing a B-domain deleted FVIII product from a different manufacturer reported that chromogenic FVIII assay

results were up to 30% higher than results obtained by one stage techniques based on various reagent combinations and depending on the level of FVIII in the sample (17). The authors concluded that plasma containing this concentrate could be reliably assayed without using a product specific concentrate standard.

Recent guidance from SSC of ISTH, focusing largely on potency labelling of concentrates, stated that the optimal approach to post infusion testing of FVIII and FIX concentrates involves assay against a product reference material composed of the same material as that which is utilised for treatment (18) but recognised that this may be difficult to implement in the routine laboratory.

The ReFacto AF laboratory standard is available in countries where this concentrate is in use but the authors are unaware of any published data on how frequently this is used in routine practice. Data related to its impact on results of one stage FVIII assays involve only a few types of assay reagent in a small number of centres (14, 15). We therefore decided to survey UK Haemophilia centres on their current practice in relation to monitoring infusions of recombinant FVIII and at the same time centres were invited to assay FVIII in 3 samples which had been obtained from patients infused with one of 2 different widely used full length recombinant FVIII products (Kogenate FS, Bayer or Advate, Baxter) or a B-domain deleted FVIII (ReFacto AF), using either a plasma standard or the ReFacto AF laboratory standard (RAFLS) for assay calibration. All 52 centres who returned results were participants in the UK NEQAS Blood Coagulation Programme.

Methods

This exercise comprised 3 samples obtained from patients with haemophilia A following treatment with FVIII concentrates and after giving written informed consent in accordance with regulations.
The samples used and the details of infused products are shown in Table 1.
All samples were collected into 0.109M citrate; plasma was buffered with 0.8 g% HEPES and 1.0 g% glycine, and lyophilised prior to distribution through the post at room temperature. Stability of such samples prepared and distributed in this way is excellent (19). Samples were sent to a total of 57 UK

Haemophilia centres in 2011. Participants were asked to perform FVIII:C assays on all three samples using their routine method for post-concentrate samples and using their routine plasma calibrant. In the case of the ReFacto AF sample participants were invited to run the assay with two separate calibration curves – one prepared using their routine plasma calibrant, and one using a ReFacto AF Laboratory Standard (RAFLS), provided by Pfizer to UK NEQAS (Blood Coagulation) and distributed with the samples. Centres performing both one-stage and chromogenic assays were asked to perform both assays and in the case of the post ReFacto AF sample, with both calibrants.

Participants were also asked to provide information with respect to the FVIII concentrates used in their centres, and for details of their FVIII assay procedure for patients receiving ReFacto AF and other FVIII products.

Statistical analysis.

Results obtained by chromogenic or one stage assay were compared using an unpaired t test as were one stage FVIII assay results obtained by the two most commonly used commercial reagent sets

Results

Responses and factor VIII assay results were received from a total of 52 centres.
Details of practice described below related to concentrates used and the way in which FVIII assays were routinely performed on samples from patients receiving each particular concentrate.

Concentrates in use

Details of which concentrates were in use are shown in Table 2 for the 4 most commonly used brands together with details of whether one stage or chromogenic FVIII assays were usually performed and whether assays were routinely calibrated with a plasma or concentrate standard.

Other products listed by participants included the following 8Y (n=6), Alphanate (n=5), Wilate (n=5), Fandhi (n=4), Octanate (n=3), Optivate (n=2). Most centres routinely used more than one brand of concentrate and 3 or more concentrate brands were used in 36 centres with 8 centres using 5 or more brands.

Assay design for analysis of post infusion samples containing ReFacto AF

Of those responding to questions about assay calibration the majority routinely used the RAFLS when assaying post infusion samples from patients treated with ReFacto AF (Table 2). A fresh calibration curve was prepared with each assay in 14/32 centres (44%) using between 3 and 8 different dilutions (median 5). All but 1 centre diluted the RAFLS in buffer after making the initial pre-dilution in FVIII deficient plasma. The initial dilution is included to reduce the concentration of FVIII to around 100 IU/dl. A stored calibration curve was used by 18 centres (56%), using between 4 and 8 dilutions (median 7). All but 2 of these centres diluted the calibrant in buffer.

Thirty of the 32 centres providing details diluted test samples in buffer, using between 1 and 3 test sample dilutions (median 3) with 4 centres including only a single dilution of test plasma. The other 2 centres used FVIII deficient plasma to construct test sample dilutions

Assay design for analysis of post infusion samples containing Kogenate FS or Advate concentrates

A fresh calibration curve was prepared with each assay in 16/43 centres (37%), using between three and eight different dilutions (median six). All but two centres diluted the calibrant in buffer. A stored calibration curve was used by 27 centres (63%) using between five and eight dilutions (median eight). All but two of these centres diluted the calibrant in buffer.

All but five of the centres providing details diluted test samples in buffer, using between one and three test sample dilutions (median three) with four centres including only a single dilution of test plasma. The other five centres used FVIII deficient plasma to construct test sample dilutions

Relationship between results obtained by one stage and chromogenic FVIII assay

Results of FVIII assay by one stage or chromogenic assay are summarised in Table 3. Results obtained by chromogenic assay were significantly greater than those obtained by one stage assay for the post-Kogenate FS sample (difference of 32%, $p < 0.0001$). There was no significant difference between chromogenic and one stage FVIII assay results in either the post-Advate sample (3% higher by one stage assay) or the post- ReFacto AF sample for assays calibrated using plasma standards (11% higher by chromogenic assay). Analysis of the impact of using the ReFacto laboratory standard for assay calibration is given in other sections below.

One stage FVIII Assay results with different reagents (plasma standards as calibrants).

Two APTT reagent sets were used in FVIII assays in sufficient numbers for analysis. Twenty centres used Synthasil APTT reagent in combination with calibration plasma and FVIII deficient plasma from Instrumentation Laboratory (IL, Bedford, USA) in their one stage FVIII assay testing. At the time of the survey this source of FVIII deficient plasma typically contained <5 IU/dl von Willebrand Factor. There were 15 users of Actin FS APTT reagent in combination with calibration plasma and FVIII deficient plasma from Siemens (Marburg, Germany). At the time of the survey this deficient plasma typically contained normal or near normal levels of VWF. Results obtained using these two reagent sets are shown in Table 4. Results were significantly higher with IL reagents than with Siemens reagents for the post- ReFacto AF and post Advate samples but not for the post Kogenate FS sample.

Effect of ReFacto AF laboratory standard on one stage FVIII Assay results

Use of the ReFacto AF laboratory standard in place of the usual plasma standard for assay calibration made no significant difference to one stage FVIII assay results obtained on the post- ReFacto AF sample when all one stage assay data were combined irrespective of reagents used. However there were important differences between the impact of using the RAFLS for assay calibration depending on the commercial source of reagents used for the one stage assay (Table 4). Use of RAFLS in place of the usual plasma standard for calibration made a 42% difference to FVIII assay results obtained using the Siemens reagent set but only a 3% difference to results obtained with the IL reagent set. There was a 41 % difference between one stage results obtained with the 2 reagent sets when assays were calibrated with plasma standards which was reduced to only 5% when assays were calibrated with RAFLS

Chromogenic FVIII Assay results with different kits.

Use of the ReFacto AF laboratory standard in place of plasma standards made no significant difference to chromogenic FVIII assay results obtained on the post- ReFacto AF sample.

Five different kits were used amongst the 10 centres who returned chromogenic FVIII assay results. Results are shown in Table 5. Numbers were too small for statistical assessment.

Discussion.

Many haemophilia centres in the UK use both full length recombinant and B domain deleted (BDD) FVIII products in management of haemophilia A. Both one stage and chromogenic FVIII assays are in use and most centres use RAFLS , a product specific concentrate standard, to calibrate FVIII assays when patients are receiving ReFacto AF. We report here a survey of practice in UK haemophilia centres addressing which products are in use, which type of FVIII assay is performed and how these FVIII assays are calibrated. We also report on FVIII assays performed in 52 UK haemophilia centres on samples from moderate or severe haemophilia A subjects who had been infused with either Advate, Kogenate FS, or ReFacto AF.

At the time of the survey (2011) all centres were using more than one brand of FVIII concentrate and 36/42 respondents included ReFacto AF amongst these. In the present study all but 4 of 52 returned results by one stage assay. Ten of the 52 centres (19%) returned results by chromogenic FVIII assay. This was similar to the 23% of laboratory scientists reporting frequent use of chromogenic FVIII assays in their haemophilia centres in one international survey of 210 scientists from 7 countries (20). In another survey chromogenic FVIII assays were used in 7/13 centres whereas one stage assays were used in 12/13 (21).

The mean Factor VIII activity obtained by chromogenic assay was significantly greater than that obtained by one stage techniques for the sample collected after Kogenate FS infusion (32% higher by chromogenic assay, $p<0.0001$) but not for the sample collected post- Advate infusion where the difference was only 3% (higher by one stage assay, not significant). For the sample collected after ReFacto AF infusion there was no significant difference between one stage and chromogenic assay results irrespective of whether the assay was calibrated with the conventional plasma standard in local use (11% higher by chromogenic , not significant) or with RAFLS as one stage assay calibrator (4% difference, not significant).

A number of other studies assessing full length recombinant FVIII in a number of different formulations have also reported higher results by chromogenic assay in spiked samples (10, 17) and in post infusion

samples from patients (5, 8, 15). Lusher et al (5) reported that chromogenic assay results were on average approximately 35-40% higher than one stage. Three different APTT reagents were used in one stage assays and the discrepancy was greatest for the reagent containing Kaolin as activator (Lusher 1998). Another study reported one stage FVIII assay results to be 75-80% of those obtained by chromogenic assays for samples containing full length recombinant FVIII (10). A similar effect was reported by Hubbard and colleagues (9) for two full length recombinant materials. Results obtained with chromogenic assays were 45-53% higher than those obtained with a one stage assay . This study also demonstrated that the difference between results obtained by chromogenic and one stage assay could be abolished by use of a concentrate standard for assay calibration (9).

Samples containing Advate spiked into severe haemophilic plasma have been included in multi -centre studies of FVIII assays in relation to newer concentrates (17, 22). Both studies identified higher chromogenic assay results compared to one stage results when FVIII was in the 60-90 IU/dl range (by 12-19%) but either no difference (22) or lower (17) chromogenic assay results for samples containing 3-5 IU/dl FVIII.

There was considerable inter-laboratory variation for all samples irrespective of which concentrate had been infused or which standard had been used for assay calibration. This variability was higher for chromogenic results as indicated by CVs in the range 15-26%, compared to CVs of 12-19% for one stage assay results. Higher inter laboratory variability amongst chromogenic FVIII assay results compared to one stage data also occurred in samples containing advate in one inter-laboratory field study (22) though not in another (17). High inter- laboratory variation in FVIII assays remains a consistent finding in external quality assessment/proficiency testing surveys (3, 4, 8) and field studies (12, 17,) indicating that improvements in standardisation of FVIII assays are needed.

Overall our finding of higher chromogenic assay results compared to one stage for the sample containing Kogenate FS concurs with published data whereas our finding of similar one stage and chromogenic result for the sample containing Advate or ReFacto AF differs to some extent from previous studies. This may relate to the level of FVIII present in the test material but may also be partly a consequence of the pattern of reagents/kits used by the centres in our study since we noted one stage assay results were

around 40% higher for users of Instrumentation laboratory reagents compared to results obtained by Siemens reagent users for both Advate and ReFacto AF. Possible reasons for this in relation to ReFacto AF are discussed below. The finding of around 40% difference between results of one stage assays performed with different reagents has not been previously reported for samples containing Advate to the best of our knowledge. The cause of this difference is unknown but could relate to the nature of the APTT reagent (phospholipid/ and activator), the different deficient plasmas used (with and without VWF) or error in the potency assigned to the one or both of the plasma standards. If confirmed on a larger series of samples this has could impact on patient management decisions so further studies are required to investigate this.

There are a number of studies reporting higher results for chromogenic assay compared to one stage assay in samples containing ReFacto (8, 10, 12, 16) and ReFacto AF (14, 15). This relationship is affected by the nature of the laboratory reagents used in one stage assays. For example one stage assay results were shown to be approximately half of those obtained by chromogenic assay using unmodified APTT reagents in the one stage but there was no difference when phosphatidyl serine content was reduced to more physiologic levels or when platelets were used as source of phospholipid to support clotting reactions during one stage assays (10). In our study there was no difference between one stage assay results (calibrated with plasma standard) and chromogenic assay results when one stage assay was performed with IL reagents, in contrast to a 40% difference (lower by one stage) when one stage assays were performed with reagents from Siemens. There are 3 main differences between the one stage reagent sets - namely the reference plasma, the FVIII deficient plasma, and the APTT reagent. Any, or all of these 3 components could have contributed to the observed pattern of results. In relation to reference plasma it could in theory be a consequence of inaccuracy of potency assignment to one or both of the commercial standards . For the 2 FVIII deficient plasmas the most obvious difference at the time of the survey was the absence of VWF from IL FVIII deficient plasma compared to normal concentrations in the Siemens material. The two APTT reagents in question differ in their activator (Ellagic acid in Actin FS or silica in SynthASil) and in their phospholipid content. The phospholipid content of Actin FS is strikingly different from most other APTT reagents in that is lacks phosphatidyl serine and has an unusually high total concentration of phospholipid (23). The lower one stage FVIII

assay results obtained with Actin FS in the present study are consistent with the findings of Mikaelsson (10) who reported that higher phospholipid concentration in the one stage assay is associated with larger discrepancy compared to chromogenic assay results, and with a study by Caron and co workers (24) who restored agreement between one stage and chromogenic assay results by dilution of the APTT reagent and therefore the phospholipid content.

An alternative and more widely used approach to restoring agreement between chromogenic and one stage assay in samples containing ReFactois to calibrate one stage assays with a product specific standard and it has been proposed that concentrate specific standards for post infusion monitoring should be used when recommended by the concentrate manufacturer (25). This approach has been used to successfully abolish differences between one stage and chromogenic assay results of around 30-40% (lower by one stage) in some studies (8, 11-15). Recalibration of both ReFacto and the RLS reduced the differences between one stage and chromogenic assay results (13), though discrepancies have persisted following the reformulation of this particular BDD FVIII as ReFacto AF (15). In the present study there was good agreement between one stage assay and chromogenic when one stage assays were performed with IL reagents but not when Siemens reagent were used. Thus ReFacto AF lab standard was needed to deliver agreement with chromogenic assay results when using Siemens one stage reagents but not when using the IL reagent set.

Concentrates containing FVIII or FIX which has been modified to extend half life after infusion are in development and in use with recommendations about potency labelling (18) and deliberations about discrepancies between results obtained by different assays already published (26). Some have suggested that the use of product specific standards may be the optimal approach (18) whilst recognising that this may be difficult to implement in the routine laboratory. Responses to our questionnaire about current practice are therefore of interest, not just for current use of RAFLS, but as an indication of whether service laboratories can routinely handle such an approach in relation to future developments. We asked centres to indicate what material was used for calibration of FVIII assays. Most centres routinely used more than one brand of concentrate and many were using 3 or more and including ReFacto AF amongst these Approximately 90% of the centres where ReFacto AF was in use

for treatment of patients calibrated their FVIII assays with RAFLS if the patient was receiving this brand of concentrate . One centre used the 8th WHO international concentrate standard for assay calibration when monitoring samples collected after Kogenate FS or Advate infusions. The remainder all used plasma standards for assay calibration. Most participants in the present exercise were therefore regularly using different calibrants depending on which concentrate the patient had received.

In conclusion our study shows that results of FVIII assays performed after infusion of FVIII concentrate depend on the assay and reagent used. After infusion of Kogenate FS results were higher for chromogenic assay compared to one stage assay. After infusion of Advate or ReFacto AF one stage results were close to chromogenic but in both cases were affected by the type of one stage assay reagents used. Many centres were routinely performing factor VIII assays using different calibrants depending on which product was being used. We believe that regular proficiency testing/external quality assessment of post infusion monitoring is needed and further exercises will be undertaken.

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Authors Contributions

IDW,MM, SK, IJ, DPK and TALW designed the study. MM consented and recruited donors. IJ,SK and DPK analysed the data. SK drafted the manuscript. IDW,MM,IJ,DPK and TALW contributed to review and finalisation of the manuscript.

Disclosures

SK has received speaker and consultancy fees from Baxter, Bayer and Pfizer. IJ received payment as a speaker at a Pfizer meeting. MM has received support from Baxter and Bayer to attend scientific meetings and is the project leader for EUHANET which receives funding form Baxter, Bayer and Pfizer. The authors have indicated that they have no other conflicts of interest regarding the content of this article.

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Tables

Table 1. Post Infusion samples and concentrates used.

Sample	Baseline FVIII before infusion	Concentrate infused	Collection Time and dose
1	3 IU/dl	ReFacto AF	20 min post 2000U
2	<1 IU/dl	Kogenate FS	120 min post 2000U
3	<1 IU/dl	Advate	240 min post 2000U

Table 2. Concentrates and FVIII assays used in different centres

Product		**Assay type in routine use for monitoring each product**				**Material used for assay Calibration**	
	n*	**One Stage**	**Chromogenic**	**Two Stage clotting**	**Both One Stage & Chromogenic**	**Plasma**	**Concentrate**
Kogenate FS	31	28	1	1	1	29	1**
Helixate	26	24	1	1		24	1**
ReFacto AF	36	31	3	-	2	4	32***
Advate	39	35	2	1	1	33	-

- n = number of centres ** Full details not provided

*** ReFacto AF laboratory standard

(Apparent numerical discrepancies are a consequence of incomplete returns)

Table 3. Summary of FVIII assay results from all centres

	ReFacto AF sample		Kogenate FS	Advate
	Plasma	**RAFLS**	**Plasma**	**Plasma**
1 stage assays (n=46)				
Median (IU/dl)	**58.3**	**62.6**	**52.0***	**43.0**
CV (%)	17	19	12	17**
Range IU/dl)	40.0-73.9	36.5-97.6	42.2-72	31.6-63.6**
Chromogenic assays (n=10)				
Median (IU/dl)	**64.9**	**55.0**	**68.6***	**41.6**
CV (%)	22	26	15	19
Range (IU/dl)	37.9-77	29.2-86.5	44.0-78	23.2-49

* Significantly different, p <0.0001

** 1 centre reported results "<1u/dl" for sample containing Advate which was excluded from calculations of CV

RAFLS – ReFacto AF laboratory standard

Table 4. One stage FVIII assay results calibrated with plasma standards as determined using the 2 most widely used reagent sets

	Post- ReFacto AF Plasma std	Post- ReFacto AF ReFacto Lab std	Post- Kogenate FS Plasma std	Post- Advate Plasma std
Reagents from IL				
Median FVIII (IU/dl)	63.3	61.3	53.4	47.1
Range (IU/dl)	50 – 73	36 – 97	42 – 59	35 – 55
CV	9%	25%	9%	10%
Reagents from Siemens				
Median FVIII (IU/dl)	45.0	64.1	49.7	34.0
Range (IU/dl)	40 – 55	56 - 77	15 – 58	32 – 40
CV	11%	9%	9%	8%
Difference	41%	5%	7%	39%
Unpaired t test	p<0.0001	ns	ns	p<0.0001

Table 5. Factor VIIII results obtained with different Chromogenic assay kits

Kit/Manufacturer	N*	**FVIII (IU/dl) results Sample 1 ReFacto AF**		**FVIII (IU/dl) Sample 2 Kogenate FS**	**FVIII (IU/dl) Sample 3 Advate**
		Plasma standard	**RAFLS**	**Plasma standard**	**Plasma standard**
Biophen	2	43,77	29,56	55,73	23,49
Coamatic	1	67	86	70	45
Coatest	1	61	54	64	39
Electrachrome/IL	2	72,74	50,60	68,78	44,47
Siemens	4	38,52,63,72	49,54,58,66	44,64,69,71	36,37,37,48

*One of the 11 chromogenic assay users in table 2 did not return results

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