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The “normal” factor VIII concentration in plasma

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Abstract

Introduction—The quantitation of factor (F)VIII by activity-based assays is influenced by the method, procedure, the quality and properties of reagents used and concentrations of other plasma proteins, including von Willebrand factor (VWF).

Objective—To compare FVIII concentrations measured by activity-based assays with those obtained by an immunoassay and to establish the influence of plasma dilution on the FVIII clotting activity (FVIIIc).

Methods—The APTT, a chromogenic assay (Coatest) and two in-house immunoassays were used. Albumin-free recombinant FVIII was used as the calibrator in all assays.

Results—For a group of 44 healthy individuals (HI), the mean value observed for FVIII antigen (FVIIIag; 1.22 ± 0.56 nM; S.D.) is substantially higher than that for FVIIIc (0.65 ± 0.29 nM) and the chromogenic assay (FVIIIch; 0.50 ± 0.23 nM). A positive correlation between FVIIIag and VWFag with $R^2=0.20$ was observed. Since plasma VWF has an inhibitory effect on FVIIIc, we evaluated the influence of plasma dilutions on FVIIIc in HI (n=105). At a 4-fold dilution, estimates of FVIIIc by clotting assay were much lower than FVIIIag (0.77 ± 0.31 vs. 1.14 ± 0.48 nM). At 10- and 25-fold dilutions, the estimated FVIIIc increased to 0.87 ± 0.36 and 0.94 ± 0.44 nM, respectively.

Conclusions—1) In plasma, FVIIIag is higher than FVIIIc and FVIIIch; and 2) Real FVIII concentrations in plasma can be estimated by measuring FVIIIag.

Keywords

Factor VIII antigen; Factor VIII activity; von Willebrand factor; APTT assay; Coatest assay; Immunoassays

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The factor (F) VIII concentration in plasma of healthy individuals and hemophilia A patients is routinely evaluated by activity-based assays. Clinical laboratories and manufacturers of FVIII concentrates use one-stage activated partial thromboplastin time (APTT) clotting assay (measures FVIIIc) and a chromogenic assay, which measures FXa generation by the intrinsic FXase (FVIIIa/FIXa/phospholipid) at saturating FIXa concentrations [1]. Despite their wide use, the results of both assays are heavily dependent upon procedure and the quality and properties of reagents used [2,3]. The concentrations of other plasma proteins also have a pronounced effect on FVIII clotting activity [4]. As a consequence, values for FVIII concentration observed in activity-based assays are often different from the antigen concentration [5].

Discrepancies in FVIII quantitation are also observed between the results of the APTT and chromogenic assays. These discrepancies are dependent upon the concentration of FVIII in test plasmas [6], standards used [7,8] and incubation times in the chromogenic assay [3,9]. The nature, purity and structure of natural and recombinant FVIII proteins are also confounders for these two assays as well [8,10,11].

FVIII forms a complex through its light chain with VWF, which protects FVIII from proteolysis and concentrates FVIII at the site of active hemostasis [12,13]. The *in vivo* half life of FVIII is dependent upon the presence and concentration of VWF and is as short as 1 hour in the absence of this carrier protein [14,15]. However, in the APTT clotting assay, VWF acts as an inhibitor of FVIIIc and becomes a confounder in this FVIII activity-based assay [4,16].

In this study, we compared the FVIII concentration in plasma from healthy individuals measured by two conventional activity-based assays and by an immunoassay and evaluated the influence of the dilution of test plasma in FVIII/VWF-immunodepleted substrate plasma on FVIIIc measurements. We also looked for correlations between FVIII and VWF antigen (ag) in healthy individuals.

Materials and methods

Materials

Albumin-free recombinant FVIII (rFVIII) produced in CHO cells was provided by Baxter Health Care Corp. (Duarte, CA), characterized extensively in our laboratory [17] and used as the calibrator (standard) in all assays. The concentration of this product (0.62 mg/ml) was established by the absorbance at 280 nm using an extinction coefficient $E^{0.1\%}$ value of 1.3. Purified human VWF and polyclonal goat anti-VWF antibody were provided as gifts by Dr. R. Jenny from Haematologic Technologies, Inc (Essex Junction, VT). FVIII/VWF-immunodepleted plasma (DDP; substrate plasma) was purchased from Precision Biologic (Dartmouth, Canada) and three lots of congenital FVIII-deficient plasma were purchased from George King Bio-Medical Inc. (Overland park, KS). The APTT reagent was purchased from Trinity Biotech PLC Bray (Wicklow, Ireland). Biotinylated monoclonal anti-FVIII heavy-chain antibody 24 (α -FVIII-24; IgG1, κ) and monoclonal anti-vWF antibody (α -vWF-99) were produced in house. Monoclonal anti-FVIII light-chain antibody 68 (α -FVIII-68, IgG2b, κ) was produced at Mayo Medical School, Rochester, MN [18]. HRP-streptavidin, bovine serum albumin (BSA), β -mercaptoethanol and Tween 20 were purchased from Sigma (St. Louis, MO), chromogenic substrate from KPL (Gaithersburg, MD) and HRP-goat anti-mouse Ig was purchased from Amersham Life Sciences (Arlington Heights, IL). R-phycoerythrin (PE) was purchased from SouthernBiotech (Birmingham, AL).

Plasma samples

Freshly frozen citrate plasma samples from healthy individuals were from Red Cross blood donors at the Fletcher Allen Health Center, Burlington, Vermont (n=44; 25 males, median age 32.4±6.7 years and 19 females, median age 29.3±9.9 years) and from the Institute of Cardiology, Jagiellonian University, Krakow, Poland (n=105; 72 females, median age 40.4±11.3 years and 33 males, median age 39.3±12.0). The donors were Caucasian, reflecting populations of Burlington, VT and Krakow, Poland. Donors' informed consent was obtained in accordance with the Declaration of Helsinki.

Luminex Fluorescence Immunoassay (FLI) of FVIII antigen

Microsphere beads with predefined 658:712 nm emission ratios (Luminex classification #38) were coupled to α -FVIII-68 according to the manufacturer's instructions (Luminex Corp., Austin, TX). The binding of FVIII to α -FVIII-68 beads was probed with biotinylated α -FVIII-24 and detected with PE-coupled streptavidin. 5×10^3 α -FVIII-68 beads in 50 μ l of PBS, pH7.4 were added to the wells of a microtiter plate. rFVIII standard was diluted in DDP substrate plasma containing 10 mM BME to 0.25-20 pM concentration and 50 μ l of rFVIII-containing plasma was added to the α -FVIII-68 beads-containing wells. Following 3 hr incubation at room temperature (r.t.), excess antigen was removed by 3 washes with PBS, 0.05% Tween 20. 100 μ l of PBS, 1% BSA, 0.05% Tween 20, pH7.4 containing 1 μ g of biotinylated α -FVIII-24 was added and incubated at r.t. for 1 hr. Following 3 washes with PBS, 0.05% Tween 20, the beads were treated for 30 min with 100 μ l PBS, 1% BSA, 0.05% Tween 20 containing 0.5 μ g PE-streptavidin. Non-specific binding controls were done using an isotype-matched mAb with specificity irrelevant to FVIII coupled to microsphere beads with a different emission ratio (658/712 nm; Luminex classification #027) than those used for α -FVIII-68. The Luminex technology permits the specific and non-specific binding to be determined simultaneously. The fluorescence intensity observed with non-specific mAb was subtracted from that recorded with biotinylated α -FVIII-24, and a FVIII calibration curve built. For the quantitation of FVIII antigen in plasma, test plasma samples were treated with 10 mM β -mercaptoethanol (BME) for 2 hr, diluted in substrate plasma and subjected to the FLI assay as described above. The detection limit of this assay is 3 pM.

Enzyme-linked immunosorbent assay (ELISA) for VWF

The double-sandwich assay was performed as described [4]. Polyclonal goat anti-VWF antibody was immobilized in the wells of a microtiter plate (50 μ l/well at 5 μ g/ml). Calibration curve was built by sequential dilutions (to 1.25-80 ng/ml) of purified VWF in PBS, 1% BSA, pH 7.4 followed by the addition of 50 μ l dilute sample to coated wells. 50 μ l of α -VWF-99 (10 μ g/ml) were added and incubated for an hour. Binding was detected with HRP-goat anti-mouse IgG and a chromogenic substrate. The detection limit of this assay is 2.5 ng/ml (0.025% of mean physiologic vWF concentration). For the evaluation of VWF concentration in test plasma, plasma was diluted 200- and 400-fold in PBS, 1% BSA, pH7.4 and applied to the assay.

APTT

Test plasma was diluted in DDP substrate plasma and incubated for 5 min at 37° with 100 μ l of the APTT reagent. Clotting was initiated by the addition of 100 μ l 25 mM CaCl_2 and clotting times were determined using the ST8 instrument (Diagnostica Stago, Parsippany, NJ). To build a calibration curve, DDP substrate plasma was reconstituted with various concentrations (21-1500 pM) of albumin-free rFVIII standard. For the titration of rFVIII standard in various FVIII-deficient plasmas, rFVIII was added at varying concentrations (0-1500 pM) to the corresponding plasma and incubated for 5 min at 37° prior to the addition of the APTT reagent.

Results

Evaluation of FVIII antigen FLI

The FVIII FLI relies upon the observation that the addition of 10 mM BME to plasma results in the dissociation of FVIII from VWF and exposure of FVIII epitopes for antibody binding [19,20]. Fig. 1 shows FLI data for rFVIII titration in our in-house FVIII-immunodepleted and FVIII/VWF-immunodepleted (DDP) plasma from a commercial source. The in-house FVIII-depleted plasma was reduced with 10 mM BME before depleting FVIII with an anti-FVIII monoclonal antibody and DDP was treated with 10 mM BME prior to rFVIII titration. The in-house FVIII-depleted plasma contained a physiologic concentration of VWF (10 µg/ml), whereas DDP contained less than 0.025% VWF (detectability limit of the assay). The data of the Fig. 1 indicate that 10 mM BME does not interfere with the quantitation of FVIII. Thus all FVIII FLI assays were performed in the presence of 10 mM BME.

Titration of rFVIII in FVIII-depleted plasma showed that the reliable detection limit of FVIII in the FLI assay is ≤ 2 pM ($<0.2\%$ of mean physiologic FVIII concentration in HI). The reliable FVIII concentration in the assay was defined as the concentration of FVIII that can be reproducibly measured with a coefficient of variation of less than 15%. The coefficient of variation for FVIII concentrations between 2 and 20 pM was 12-15% ($n=3$). Thus the FLI assay can measure FVIII concentrations significantly below 1% of mean physiologic, i.e. is more sensitive than activity-based assays for FVIII.

The influence of substrate plasma on the APTT results

rFVIII standard was titrated into three lots of congenital FVIII-deficient and one lot of DDP plasma (Fig. 2). None of plasmas had detectable anti-FVIII inhibitory antibody, although one of them (lot #884-711e1) had a relatively high concentration (106 nM) of a non-inhibitory anti-FVIII antibody [21]. Throughout the entire range of rFVIII concentrations used (0-1500 pM), the clotting times recorded were different for each lot of FVIII-deficient plasma. The shortest clotting times for all rFVIII concentrations used were observed for DDP plasma. Each lot of congenital FVIII-deficient plasma showed longer clotting times than those observed in DDP plasma. Moreover, each lot of congenital FVIII-deficient plasma was different from the two other lots with respect to clotting times. For example, upon the addition of 1000 pM rFVIII standard to DDP plasma, it clotted in 23.9 s. Similar addition of rFVIII to the congenital FVIII-deficient plasma lot #890-930x1 led to a clotting time of 35.6 s. Two other lots of congenital FVIII-deficient plasma clotted upon addition of 1000 pM rFVIII in 30.6 and 32.9 s. To achieve the clotting time of 35.6 s, observed in one lot of the congenital FVIII-deficient plasma upon addition of 1000 pM rFVIII (lot #890-930x1), 210 pM rFVIII had to be added to the DDP plasma and 500 pM rFVIII to another lot of the congenital FVIII-deficient plasma (lot #884-711e1), the lot containing non-inhibitor anti-FVIII antibody. These data indicate that the well-established response of each FVIII-deficient plasma to the same amount of rFVIII standard is quite different and is dependent upon the properties of that plasma.

The influence of the assay on FVIII quantitation in healthy individuals

The FVIII concentration in the plasma of 44 healthy individuals was measured by the two commonly used activity-based assays, i.e. the APTT (FVIIIc) and chromogenic (Coatest; FVIIIch) and in the FLI assay developed in our laboratory. The indicated concentrations of FVIII in all three assays varied over a relatively wide range Fig. 3). For example for FVIIIc, the range determined by the APTT varied from 0.24 nM to 1.58 nM, i.e. from 34% to 226% of the generally accepted mean physiologic concentration (0.7 nM)[22]. Similar variability was observed in another activity-based assay (FVIIIch) as well (from 0.18 nM to 1.03 nM).

The antigen concentration (FVIIIag) was higher than FVIIIc and FVIIIch for 41 of 44 plasma samples. The average values for the apparent normal FVIII concentrations were significantly different in all three assays, with the highest value of 1.26 ± 0.64 nM (S.D.) for the FVIIIag and the lowest for the FVIIIch (0.50 ± 0.23 nM). The FVIIIc value of 0.65 ± 0.29 nM was the closest to the accepted “normal” FVIII concentration of 0.7 nM.

Despite pronounced inter-assay differences for the apparent FVIII concentration, there were good correlations between assays (Fig. 4). The correlation coefficient (R^2) between the FVIIIag and FVIIIc values was 0.70 (panel A), between FVIIIch and FVIIIag was 0.72 (panel B) and between FVIIIc and FVIIIch was 0.84 (panel C).

The influence of plasma dilution on FVIIIc measurements

Based on our earlier observation that VWF inhibits the FVIII activity estimated by the APTT assay [4], we hypothesized that the APTT estimated FVIIIc should increase with increasing dilutions of test plasma with VWF-depleted, FVIII-deficient plasma. We diluted plasma samples from 105 healthy individuals (Krakow cohort) in FVIII/VWF-immunodepleted substrate plasma 4-, 10- and 25-fold and measured FVIIIc for each dilution. At 4-fold dilution, the average FVIIIc for this group of healthy individuals was 0.77 ± 0.31 nM, i.e. close to the accepted “mean physiologic concentration” (Fig. 5). A further dilution of test plasmas to 10-fold increased the average FVIIIc value to 0.87 ± 0.36 nM. At 25-fold dilution, FVIIIc was estimated to be 0.94 ± 0.44 nM, approaching that of the FVIIIag (1.14 ± 0.48 nM).

FVIII and VWF antigen concentrations in healthy individuals

The concentrations of FVIIIag and VWFag were measured using immunoassays (FLI and ELISA, respectively) in plasmas from healthy individuals ($n=105$). The FVIIIag varied over a wide range from 0.32 to 2.6 nM, with an average value of 1.14 ± 0.48 nM.

Similar to FVIIIag, the values of VWFag varied widely (between 3.5 and 26.6 $\mu\text{g/ml}$) with average value of 12.4 ± 4.6 $\mu\text{g/ml}$.

Plasma samples with the highest FVIIIag had either the highest or close to the highest values for VWFag. And *vice versa*, samples with the lowest FVIII antigen had either the lowest or one of the lowest concentrations of VWF. Thus, it is not surprising that there was direct correlation between the concentrations of FVIIIag and VWFag with the R^2 value of 0.20 (Fig. 6).

Discussion

Our data indicate that: 1) FVIIIag in plasma from healthy individuals is higher than the FVIII concentration estimated by activity-based assays (APTT and Coatest; 2) Plasma dilution influences FVIIIc due to an inhibitory effect of VWF; and 3) FVIIIag in plasma positively correlates with VWFag.

The two most commonly used activity-based assays for the quantitation of FVIII in both plasma and FVIII concentrates (APTT and chromogenic) are susceptible to several factors which influence estimates of FVIII concentration. These include the quality of the reagents, alterations in the procedures used in different laboratories and by the experience and precision of the personnel. Additionally, each of these assays has distinctive shortcomings.

In the chromogenic assay, FXa generation by the FVIIIa/FIXa complex over a selected time period is estimated by a single-point measurement of FXa formed. FVIIIa is the limiting component of the intrinsic FXase and FIXa is present at a saturating (nM) concentration.

The saturating FIXa concentration is chosen to stabilize the FVIIIa produced during the activation step [23]. Nonetheless, the maximum activity of the intrinsic FXase does not last for more than 1.5-2.0 min and is observed after a 0.5-1.0 min lag phase (S. Butenas, unpublished observation). Thus, it is not surprising that the results of this assay are influenced by the incubation time of FX with the intrinsic FXase [3] and that a longer incubation time gives lower results for FVIII activity in hemophilia A patient plasma [9].

Based on the data of this study and those published previously [4], it seems that the major confounder of the APTT assay is VWF. In the absence of VWF in FVIII-deficient plasma, the clotting activity of rFVIII was 2-5-fold higher than that observed in any congenital FVIII-deficient plasma. This observation, in an agreement with our previously published data, indicates that VWF acts as an inhibitor of FVIII clotting activity in the APTT assay [4]. All three congenital FVIII-deficient plasmas tested had VWF concentrations within a normal range (7.9-11.1 µg/ml) and there was no correlation between VWF levels and FVIII clotting activity. This lack of correlation is not surprising because at normal physiologic concentrations (or close to them) of VWF, variations in its concentration have no detectable effect on FVIII clotting activity [4]. However, at VWF concentrations below 25% of mean physiologic, the influence of VWF on FVIIIc decreases with decreasing concentration of it but remains detectable even at as low as 4% of physiologic concentration.

In addition to VWF, other components of plasma (not identified in this study) have an influence on the APTT assay. Each lot of congenital FVIII-deficient plasma, containing VWF within a normal range, showed different clotting times at identical rFVIII concentrations (or in the absence of it), although all of these plasmas had <1% of FVIII clotting activity. The differences were not trivial: to achieve the same clotting time in all three lots of congenital FVIII-deficient plasma, the requirement for rFVIII varied up to 2.5-fold.

It has been observed that FVIII and VWF levels are determined to a certain extent by the blood group of individuals and that there is a correlation between the concentration of these proteins [24,25]. VWF being a carrier protein for FVIII, which protects FVIII against proteolysis *in vivo* [12,13], it is not surprising that such a correlation exists, although, according to O'Donnell *et al.*, only approximately 50% of individuals with high FVIII levels have high VWF levels as well [26].

In summary, we confirmed the correlation between VWFag and FVIIIag in healthy individuals. We also showed that the APTT assay is compromised by the properties of plasma (both substrate and test), primarily by VWF, and that measurements of FVIII antigen in plasma represent an essential adjunct to activity-based assays, because they eliminate the influence of plasma properties (in FVIIIc) and shortcomings of the chromogenic assay. In general, activity-based assays will be necessary for the initial stratification of hemophilia patients, since certain mutations in FVIII gene lead to the expression of a non-functional protein at normal physiologic concentrations [27]. The antigen assay can then be used for monitoring FVIII replacement therapy in individuals who do not have non-functional protein. However, larger studies are needed to assess the clinical value of FVIIIag assay.

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Fig. 1.

Titration of rFVIII in the FLI assay. rFVIII was added either to FVIII/VWF-immunodepleted plasma (●) or to FVIII-immunodepleted plasma containing 10 mM BME (○).

Fig. 2. Titration of rFVIII standard in the APTT assay using DDP plasma (♦) and congenital FVIII-deficient plasma lot 884-711e1 (■), lot 893-014f1 (●) and lot 890-930x1 (▲) as substrate plasma.

Fig. 3. FVIII concentration in plasma from healthy individuals (n=44) determined by the immunoassay (FLI), clotting assay (APTT) and chromogenic assay (Coatest).

Fig. 4. Correlation between FVIII concentration (nM) measured in plasma from healthy individuals (n=44) using three assays: (A) APTT vs. FLI; (B) Coatest vs. FLI; and (C) APTT vs. Coatest.

Fig. 5. FVIIIag (FLI) and FVIIIc (APTT) concentration in plasma from healthy individuals (n=105); FVIIIc dependence on test plasma dilution in FVIII/VWF-immunodepleted substrate plasma.

Fig. 6.
Correlation between FVIIIag and VWFag in healthy individuals.