Relative Stoichiometry and Affinity for Describing Von Willebrand Factor Binding of Coagulation Factor VIII

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Introduction

The von Willebrand Factor (vWF) is the biggest multimeric protein in the body and plays an important role in hemostasis. Its main functions are to bind to collagen, to platelet GPIb and to coagulation factor VIII (FVIII). Von Willebrand Disease (vWD) is the most common bleeding disorder in the Caucasian population. If, as a qualitative defect, a variant vWF protects FVIII less than normal, not only the adhesion but also the formation of the clot is affected. This type of diseases is called vWD type 2Normandie (2N) and it cannot be identified by an aberrant multimeric pattern. Using the published home made [1-7] ELISA assays on the vWD Type 2N as well as one commercially available assay [8-9], it is not possible to determine the stoichiometry or the affinity of the interaction, which would make it possible to classify and to correlate the variant regions of the vWF-molecule with functional features. Furthermore, it is complicated to perform the home-made assays, because two ELISA plates have to be processed in parallel. To replace the ill-defined term “binding capacity” by terms of classical biochemical meaning, namely the number of binding sites and the affinity of the binding, a simple method has therefore been developed combining the advantages of both techniques, namely biochemical clarity of the results and technical straightforwardness to determine the relative stoichiometry and affinity of the vWF interaction with FVIII, which can be carried out on one ELISA plate.

Methods

The vWF concentration of the samples was determined using BCS® (Siemens Healthcare). Normal plasma (PNP) was obtained by pooling 15 plasma samples from healthy donors. Human vWF was used as a control (Wilate®); it was kindly donated by Octapharma. Recombinant FVIII (Recombinate®) was obtained from Baxter Deutschland GmbH. The plasma samples and controls (PNP and Wilate®) were adjusted to a vWF concentration of 10 mU/ml by diluting them with a blocking buffer consisting of tris buffered saline (TBS) pH 8.0, supplemented with 2% bovine serum albumin (BSA). All the chemicals or reagents used were of the highest analytical grade. Each well of the high binding 96 well ELISA plates (Greiner Bio One) were coated with 150 µl of 0.05 M carbonate/bicarbonate buffer (coating buffer) pH 9.6, containing 1.5 µg/ml of capture antibody (Polyclonal Rabbit Anti-Human von Willebrand Factor, DAKO) and stored overnight at 4°C. After washing four times with TBS/BSA/Tween® washing buffer (TBS with 0.1% BSA and 0.05% Tween®20, pH 8.0) the wells were incubated for 1 h with a blocking buffer and washed again four times. Then 100 µl of TBS were added to the blanks, while 100 µl of the diluted controls and plasma samples containing 10 ng vWF were added to the other wells. After incubation periods of at least 2 h at room temperature (RT) or overnight at 4°C, the wells were washed three times with CaCl₂ elution buffer (TBS with 0.1% BSA and 0.05% Tween®20, containing 350 mM of CaCl₂, pH 8.0) and then incubated for 1 h at RT with CaCl₂ elution buffer in order to remove all the bound FVIII from the vWF. Then the wells were washed four times with CaCl₂ washing buffer (TBS with 0.1% BSA and 0.05% Tween®20, containing 10 mM of CaCl₂, pH 8.0). Afterwards, blanks were incubated with CaCl₂ washing buffer and the plasma probes and controls were incubated with three different concentrations 18 ng (108 mU), 6 ng (36 mU) and 2 ng (12 mU) of Recombinate® diluted in CaCl₂ washing buffer for 2 h at 37°C. After washing four times with TBS/BSA/Tween® washing buffer, the blanks were incubated with TBS; the controls and plasma probes were incubated with 100 µl of a 3 µg/ml solution of polyclonal Sheep anti-human Factor VIII antibody, horseradish peroxidase (HRP) conjugated (Affinity Biologicals) in blocking buffer for 1h at RT. Then the plates were washed 9 times with TBS/BSA/Tween® washing buffer. The color was developed by adding 100 µl of H₂O₂ (Perhydrol®, Merck, 0.42 µl/ml) activated ortho-phenylenediamine dihydrochloride (OPD, DAKO, 0.3 mg/ml) solution. After 10 minutes of incubation at 4°C, the reaction was discontinued by adding 50µl of 2N H₂SO₄ per well. Then the absorbance of 492nm light was determined in a microtitre plate reader (Lambda E, BioTek) as an
optical density (OD). According to the double reciprocal Lineweaver-Burk-plot [10], the relative stoichiometry and the relative affinity of the different plasma samples in relation to the PNP were determined. The gradient (a) and the point of intersection with the y-axis (b) were determined to calculate the stoichiometry of the binding sites \( (N = 1/b) \) and the affinity \( (K = a/b) \). In relation to PNP, the relative stoichiometry was calculated with \( N/N(PNP) \) and the relative affinity was calculated with \( K(PNP)/K \).

**Results**

Fourteen duplicates of a normal plasma pool ranged from 0.8 to 1.3 for the relative number of the binding sites and from 0.7 to 1.5 for the relative binding affinity. The reference range (confidence interval CI 95%) as derived from 30 non-related healthy donors was 0.34 – 1.98 for the relative affinity and 0.27 – 1.47 for the relative stoichiometry. The interaction of the pure vWF derived from Wilate® with the pure FVIII (Recombinate®) was within the reference range. Even after four days of plasma storage at 4°C ±2°C, the results did not differ significantly. Two pathological samples of patients showed a markedly reduced number of binding sites concomitant with the normal affinity per binding site.

**Discussion**

Whenever the percentage of FVIII and the vWF differ by more than a factor of two, the relative stoichiometry and affinity can help to distinguish between the vWD 2N and other causes of the irregularity, particularly in the many cases of the more rapid degradation of FVIII, due to preanalytical influences. Even the sequencing of the vWF gene cannot reveal all of the possible mechanisms that lead to an impaired stoichiometry and affinity between the vWF and FVIII. This new method involves less material and time, when compared to the previously described home-made methods. Additionally, it offers in contrast to all other assays an easier method for achieving a detailed insight into the biological interaction between the vWF and FVIII, as was recently proposed by Favaloro [11].

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**References**

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Illustrations

Illustration 1

Figure 1: Lineweaver-Burk-Plot of PNP, Wilate® and two different pathological samples to calculate the relative stoichiometry and the relative affinity of the vWF binding with FVIII.
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