Characterization of the binding properties of recombinant FVIII concentrates with von Willebrand Factor

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Background

The binding of factor VIII (FVIII) to von Willebrand factor (vWF) is essential for the protection of FVIII against proteolytic degradation in plasma. The FVIII concentrates Advate, Kogenate and ReFacto are produced using diverse manufacturing processes, raising the question if the various products show differing vWF-binding properties. The aim of the investigation was the characterization of the binding kinetics of recombinant FVIII concentrates with vWF.

Methods

First, we measured the ration of FVIII activity (FVIII:C) to factor VIII antigen (FVIII:Ag; i.e. factor VIII protein) of the concentrates. Table 1 shows the activity and the antigen results for the investigated batches of the FVIII concentrates (Advate, Kogenate and ReFacto).

The investigation of the complex formation of FVIII and vWF was performed by using an ELISA-based method. Concerning this matter, a polyclonal vWF-binding antibody was immobilized to the surface of a microwell plate. vWF was added to each well and later incubated with different FVIII concentrates. Bound FVIII was detected by activity measurement using a chromogenic assay (ELECTRACHROME™ Factor VIII, Instrumentation Laboratory) and by antigen level using an enzyme immunoassay (IMUBIND® Factor VIII ELISA, american diagnostica). Analysis of complex formation was accomplished by endpoint determination using multiple FVIII-concentrations and steady incubation time (Fig. 1) as well as by kinetic measurement, consistent concentration and various incubation times (Fig. 2).

Results obtained by the measurement of bound rFVIII activity, after incubation with steady activity levels, were used to determine the dissociation constant and the velocity constant of complex formation by applying the Langmuir model for independent binding sites and the equation of complex-concentrations of the balance reaction.

Results

The FVIII:C/FVIII:Ag ratio for our used batch of Kogenate and ReFacto were 0.75 and for Advate 0.5 (theoretically, the ratio should be 1 for FVIII molecules having full activity and complete antigen recognition by the FVIII-specific antibody).

Fig. 1 shows the binding curves of Advate, Kogenate and ReFacto to immobilized plasma vWF after steady incubation time of increasing concentrations of FVIII. In Fig. 1A, in which bound FVIII were quantified by activity, Advate and Kogenate showed identical binding curves while ReFacto differed in its binding behaviour. However, when we repeated these experiments and quantified bound FVIII by antigen level (FVIII:Ag) instead of by activity, Advate showed the highest binding capacity (Fig. 1B). This is in agreement with the determination of the FVIII:C/FVIII:Ag ratio showing that Advate (at least the used batch) contained the highest amount of inactive FVIII (Tab. 1).

The kinetic measurement also revealed similar binding properties for Advate and Kogenate (Fig. 2A). Furthermore, the steady rising curves of the kinetic measurements elucidated that along with the active FVIII, inactive proteins slowly bound to vWF as well (Fig. 2B). Again, ReFacto differed in its binding behaviour. Kinetic measurements of ReFacto always led to saturation curves and the activity determination showed a significantly increased amount of bound FVIII (Fig. 2A).

Tab. 2 provides information about the kinetics of FVIII binding to immobilized plasma vWF and shows that the rate constants of association (k_{on}) and dissociation (k_{off}) are similar for all 3 concentrates.

Fig.1: Binding curves of 3 rFVIII concentrates to vWF

Analysis of bound FVIII to immobilized vWF was accomplished by endpoint determination using increasing FVIII-concentrations and steady incubation time. Bound FVIII were quantified by:

A). Activity (chromogenic assay)

B). Antigen level (ELISA)

Fig. 1A

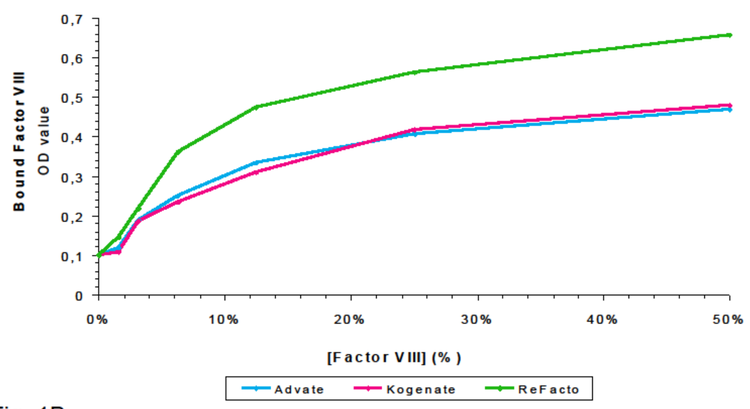

Fig. 1B

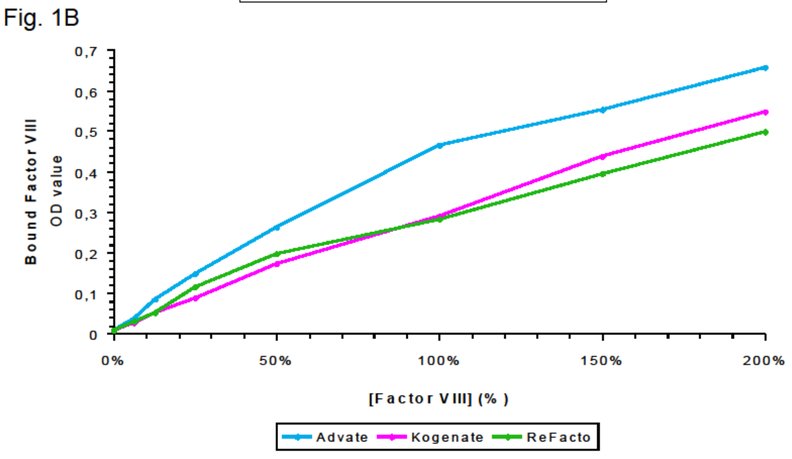

Fig. 2A

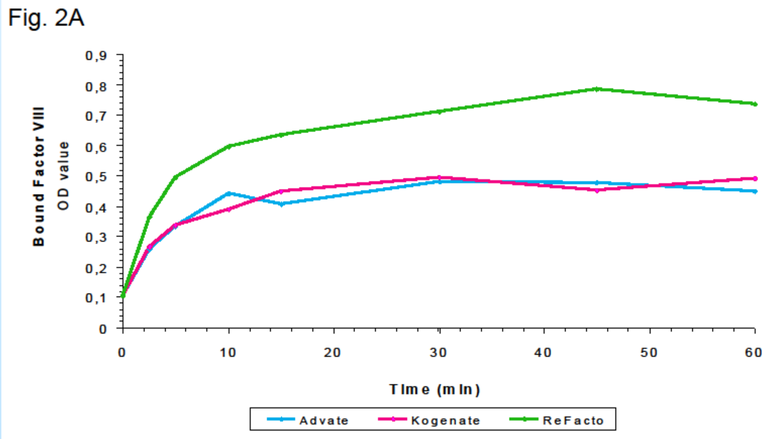

Fig. 2B

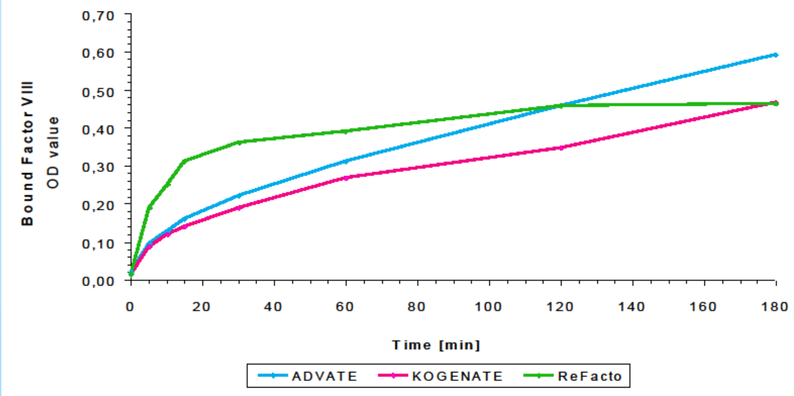

Fig. 2: Time-dependent complex formation of rFVIII with vWF

The rate of FVIII-vWF complex formation was determined by incubating a consistent FVIII concentration with vWF for various time intervals, and by assaying the concentration of bound FVIII by:

A). Activity (chromogenic assay)

B). Antigen level (ELISA)

Tab. 1: Ratio: Activity/Antigen

Factor VIII product	Ratio Activity/Antigen
Advate	0.5
Kogenate	0.75
ReFacto	0.75

Tab. 2: Kinetics of Factor VIII Binding to immobilized plasma vWF

	k_{on} [M^{-1} s^{-1}]	K_{off} [s^{-1}]	K_d [M]
Advate	1,44 ± 0,28 * 10^4	0,91 ± 0,17 * 10^{-6}	6,34 * 10^{-11}
Kogenate	1,55 ± 0,01 * 10^4	1,22 ± 0,01 * 10^{-6}	7,91 * 10^{-11}
ReFacto	1,88 ± 0,51 * 10^4	1,36 ± 0,37 * 10^{-6}	7,22 * 10^{-11}

Conclusions

In could be observed that Advate and Kogenate showed similar binding properties concerning vWF complex formation. Applying equal activity of FVIII and measurement of activity, led to identical binding graphs, regarding endpoint and kinetic measurement (Fig. 1A and 2A). ReFacto differed in its binding behaviour and showed an increased amount of bound FVIII, implying elevated binding possibilities of the much smaller protein, due to the deleted B-domain, to the steric hindered vWF concerning the binding assay. Despite significantly higher amounts of bound FVIII concerning ReFacto there are no significant differences in binding velocity, as proven by kinetic calculation (Tab. 2).

In conclusion, the investigation showed that the differences in binding properties, regarding the different rFVIII concentrates, were so little that it seems unlikely that the choice of the rFVIII product has any clinical implications for treatment of haemophilia patients. Likewise it could be shown that, in addition, inactive proteins also bind vWF.

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