

FACTORS AFFECTING THE FACTOR VIII:C REACTIVITY WHEN TESTED IN PURIFIED SYSTEMS WITH CHROMOGENIC ASSAYS

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Introduction

- Highly purified Factor VIII:C (FVIII:C) concentrates, extracted from plasma or recombinant, are available or being developed.
- Potency of these concentrates is usually measured with chromogenic assays, which present a high variability according to the diluent (FVIII:C deficient plasma or buffer with albumin) or the reference material used.
- Highly purified FVIII:C concentrates, whether extracted from plasma or recombinant (B-domainless), give a much more reactive dose response curve when diluted in FVIII:C deficient plasma than in the assay buffer, which contains Bovine Serum Albumin (BSA) as carrier.
- Our goal was to identify factors affecting this variability for rendering the assays more reliable.

Material and Methods

- FVIII:C concentrates extracted from plasma or recombinant and B-domainless.
- Chromogenic assay **BIOPHEN Factor VIII:C*** (Xa generation).
- FVIII:C deficient plasma, immunodepleted (FVIII:C < 0.1%).
- Assay buffer: Tris-Saline buffer supplemented with 1% BSA and PEG.
- Concentrates: tested pre-diluted in FVIII:C deficient plasma or in the assay buffer (1 unit/ml), then diluted **1:40** (standard assay dilution) with the assay diluent.
- Method calibrated with the **NIBSC** secondary plasma standard, lot 3.
- Isolation of plasma fractions: normal plasma or FVIII:C deficient plasma were fractionated in order to identify the fractions required for fully expressing the FVIII:C activity. Three fractionation methods were used: Ethanol precipitation, Ammonium Sulfate Salt fractionation or PEG fractionation. Fractions were then added to the FVIII:C diluent in order to analyse which ones support the FVIII:C activity.

- The most reactive fraction was then further purified using ion exchange chromatography and gel filtration.

* Patent pending

Results

- Assay calibration curves yielded A405 values from about 2.00 (200% FVIII:C) to 0.020 (0%).
- When highly purified extracted or recombinant FVIII:C were tested diluted in plasma, recovery was close to 100%. When these fractions were diluted in buffers, recoveries were respectively of 30% and <10%.
- We showed that a dialysable plasma fraction was required along with 2 protein plasma fractions. The first one contained ceruloplasmin and the second one α -1-Acid-GlycoProtein or AGP. We confirmed the role of these two proteins by testing purified ceruloplasmin or AGP from Sigma in the diluent. The combination of the dialysable fraction with AGP was the most active for FVIII:C activity recovery.
- By testing the different divalent ions (Ca^{++} , Li^{++} , Ni^{++} , Cu^{++} , Mg^{++} , Mn^{++} , Zn^{++} ,...) we showed that adding Cu^{++} , or AGP in the diluent was able to restore most of the FVIII:C reactivity. Using Cu^{++} and AGP, was still more effective and slightly enhanced by trace amounts of Zn^{++} .
- This observation allowed us to develop a diluent containing Cu^{++} , Zn^{++} , and AGP.
- Recovery of both concentrates was then restored to about 100% and 85%. The supplemented diluent did not change the FVIII:C concentrations measured in plasma.

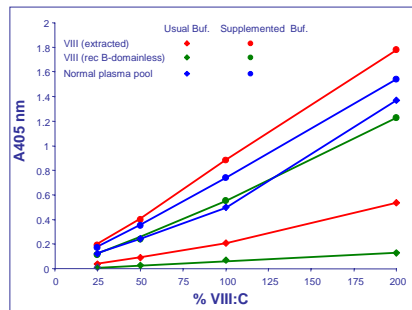
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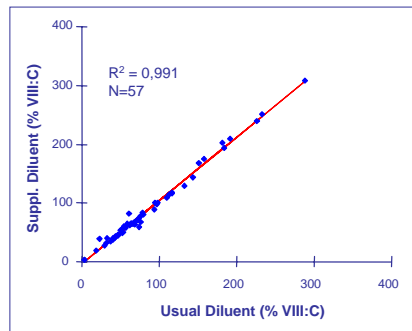
Conclusions

- Presence of Cu^{++} , and of Cu^{++} , or Zn^{++} -binding proteins is required for allowing a full expression of FVIII:C activity in chromogenic assays.
- An optimized diluent containing Cu^{++} (the most relevant), potentiated by Zn^{++} (trace amounts) and AGP (which some isoforms can bind Cu^{++}), was developed for testing FVIII:C concentrates.
- Using this diluent, highly purified FVIII:C concentrates or recombinant Factor VIII:C preparations (B-domainless) yield the same reactivity than when diluted in FVIII:C deficient plasma. FVIII:C measurements in plasma remain unchanged.

Effect of diluent used on FVIII:C concentrates reactivity



Comparison of diluents for testing FVIII:C in plasma



Purified AGP



Recovery of 2 Purified FVIII:C concentrates in different diluents

