

A Chromogenic Method for Detection of Activated Factor IX in Factor IX Concentrates.

Rosén Per R, Rosén Pia M, Andersson Margareta, Rosén Steffen
Rossix, Mölndal, Sweden

Introduction

The detection of activated Factor IX (FIXa) in Factor IX (FIX) concentrates is of importance since it can affect thrombogenicity [1] and might also result in an overestimation of the assigned FIX potency of FIX concentrates [2]. A recently developed chromogenic method for detection of FIXa in FIX concentrates [3] has now been further improved.

FIXa is determined from its ability to activate FX in the presence of FVIII, thrombin, phospholipids (PL) and calcium ions. The generated FXa is measured through hydrolysis of a chromogenic FXa substrate.

This study presents data from an evaluation of the method with regard to linearity, stability of reagents, imprecision, recovery of FIXa and interference of FX and FII.

The method has been applied to different FIX concentrates for determination of their content of FIXa.

Materials

Chromogenic method reagents:

Reagent 1, lyophilized (contains human FVIII, human FX)
Reagent 2, lyophilized (contains, human FIIa, phospholipids, Ca²⁺)
Sample Dilution Buffer
FXa-substrate S-2765 Chromogenix

Tested materials:

3rd WHO IS of Human FIX Concentrate (97/562) NIBSC
1st WHO IS of Human FIXa Concentrate (96/854) NIBSC
Human FX In House
Human FII Enzyme Research

Three FIX concentrates denoted A, B and C.

Method

The analyses were performed manually in 96 well microplates (611F96, Bibby Sterilin Ltd, UK) and absorbance readings were made in a V-Max microplate reader (Molecular Devices, USA) at 405-490 nm.

The level of FIXa was determined from linear dose-response curves using slope-ratio comparison vs. the WHO FIXa standard.

TABLE 1 – Microplate Assay

Sample	RT	50 µl
Reagent 1	RT	25 µl
	Incubation 4 min. 37°C	
Reagent 2	37°C	100 µl
	Activation 25 min. 37°C	
FXa Substrate	37°C	50 µl
	Hydrolysis 10 min 37°C	
Citric Acid (2%)		50 µl

Interference studies:

Addition of 1 IU/ml FX to 1 IU/ml of a FIX concentrate decreased the assigned FIXa potency with about 15% when the concentrate was assayed at 1 IU FIX / ml. However, further dilution to 0.3 IU FIX/ml did not influence the assigned FIXa level.

Addition of 0.4 IU/ml (≈50 µg/ml) FII to 1 IU/ml of a FIX concentrate decreased the assigned FIXa potency with about 10%, when the concentrate was assayed at 1 IU FIX / ml. As for FX, higher dilutions of the sample (0.3 IU FIX/ml) showed no influence of the added FII.

The interference studies show the importance of utilizing the linear part of the dose-response curves to avoid effects of contaminants and other media effects.

Imprecision:

A preliminary estimation of imprecision of FIXa assignments was made from dose-response analysis of a FIX concentrate in four independent assay series, using duplicates of each sample dilution.

Within series CV: < 5%
Between series CV: < 5%

Stability of reconstituted reagents:

Reagent 1: no indication of a decrease in activity after storage at 4°C for up to 24 h.
Reagent 2: no indication of a decrease in activity after storage at 4°C for up to 24 h.

References

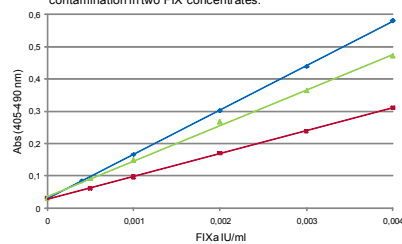
1. Gray E et al. J Thromb Haemost 1995; 73 (4): 657-9
2. Pickering WM, Gray E. J Thromb Haemost 2007; 8 Supplement 2: P-T-156
3. Rosén P et al. J Thromb Haemost 2007; 8 Supplement 2: P-M-050

Conclusions

1. A chromogenic FIXa method based upon FXa generation has been developed which appears to be suitable for measurement of FIXa in FIX concentrates.
2. The method provides a proper linear dose-response and the ability to discriminate between FIX concentrates with a difference in preactivation of 0.1 IU FIXa / 1000 IU FIX (0.1 ‰).
3. By utilizing the linear range of the dose-response curves, any media effects of the FIX concentrates are minimized.
4. Analysis of four FIX concentrates including the WHO FIX standard vs. the WHO FIXa standard indicated a preactivation level of 0.8 – 0.9 IU FIXa / 1000 IU FIX (0.8 – 0.9 ‰) in three of the concentrates, and ca 4,5 IU FIXa / 1000 IU FIX (4,5‰) in one concentrate.
5. Complete recovery of FIXa was obtained
 - a) when adding 0.13 and 1.3 ‰ of FIXa to a FIX concentrate.
 - b) of the assigned FIXa level in a FIX concentrate when added in constant amount to the FIXa standard dilutions.
6. Reconstituted reagents are stable for at least 24 h at 4°C.

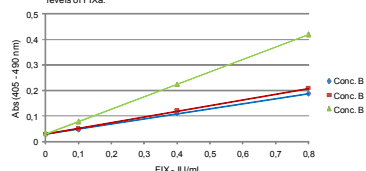
Results

FIG. 1 - Typical dose-response curves for the FIXa std and for the F contamination in two FIX concentrates.



Generated FXa was linearly related to FIXa over a ten fold range. The highest FIXa concentration at which linearity is obtained needs to be determined for each concentrate (typically 2 – 0.5 IU/ml FIX).

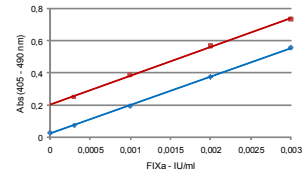
FIG. 2 - Dose-response curves of a FIX concentrate after addition of two diff levels of FIXa.



Recovery of FIXa added to FIX concentrate B, derived from the WHO FIXa standard calibration curve (not shown in figure):

0.13 ‰ FIXa Recovery: 97%
1.3 ‰ FIXa Recovery: 104%

FIG. 3 - Dose-response curves of FIXa in buffer and in the presence of FIX



The incremental increase of FXa is constant over the assayed FIXa range (slope-ratio = 0.99) and corresponds to 100% recovery of the previously assigned FIXa level (0.9 ‰) in concentrate B.

TABLE 2 – Preactivation levels in FIX concentrates

Sample	IU FIXa / 1000 IU FIX	CV, %
WHO FIX Standard	0.9	2.1
Concentrate A	4.5	4.2
Concentrate B	0.9	2.1
Concentrate C	0.8	2.0

Assigned preactivation levels in three FIX concentrates and the WHO FIX standard as determined from slope ratio comparison vs. the WHO FIXa standard. The table shows mean values and CV from three independent assay series.

Rossix