

A New Chromogenic Method for Potency Assignment of Factor IX Concentrates.

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Introduction

Factor IX (FIX) clotting methods demonstrate a limited resolution in FIX concentrate potency determinations. It has also been reported that FIX concentrates may contain activated FIX (FIXa) [1] and that trace amounts of FIXa levels may lead to a higher potency assignment when assayed with one-stage clotting method [2].

A chromogenic method has now been developed for determination of FIX potency in FIX concentrates. FIX is activated by FXIa and the FIX activity is determined from the formation of FXa. Similar to clotting methods, thrombin is generated during the assay.

This study presents data from an evaluation of the method as regards resolution, stability, imprecision and correlation with a clotting method. Furthermore, the influence of FIXa and FX has been investigated.

Materials

Chromogenic method reagents:

Reagent A, lyophilized (contains human FVIII, human FX)
Reagent B, lyophilized (contains bovine FXIa, phospholipids, Ca²⁺)
Sample Dilution Buffer
FXa substrate S-2765
Chromogenix

Clotting method reagents:

FIX deficiency plasma
APTT SP
Laboratory
DadeBehring, Hyphen
Instrumentation

Tested materials:

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

Four FIX concentrates denoted A, B, C and D.

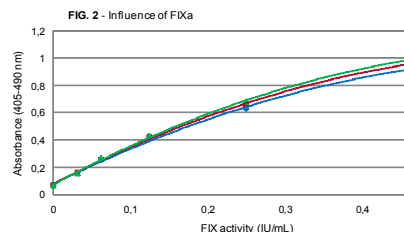
Method

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.

Quadratic and log-log curve-fitting models were used and potency assignments were made using parallel line analysis.

TABLE 1 – Microplate Assay

Sample	RT	25 µL
Reagent A	RT	25 µL
<i>Incubation 4 min. 37 °C</i>		
Reagent B	RT	150 µL
<i>Activation 14 min. 37 °C</i>		
S-2765		50 µL
<i>Hydrolysis 7 min. 37 °C</i>		
Citric Acid (2%)		50 µL



2 and 10 IU FIXa / 1000 IU FIX (2 and 10 %) was added to the WHO FIX standard to evaluate the effect of preactivation. No significant interference was found for 1% FIXa, whereas 2 and 10 % FIXa resulted in a FIX potency overestimation of ≤ 5 % and ca 10 %, respectively.

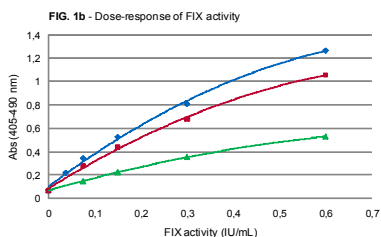
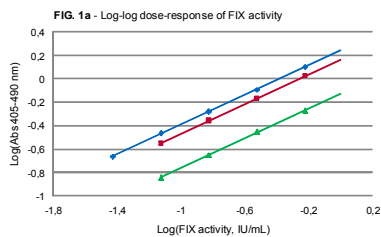
References

- Gray E et al. J Thromb Haemost 1995; 73 (4): 657-9
- Pickering WM, Gray E. J Thromb Haemost 2007; 8 Supplement 2: P-1156

Conclusions

1. A robust method for potency assignment of FIX concentrates has been developed with a resolution of about 0.8 absorbance units between 0.04 – 0.6 IU FIX/ml in the sample dilution.
2. All assayed FIX concentrates demonstrated a parallel line relationship when evaluated in a log-log graph.
3. Estimation of the imprecision indicated within and between series CV of 3% and 7%, respectively.
4. Assigned FIX potencies with the chromogenic method were in agreement with a FIX clotting method.
5. The method has a low FIXa interference as illustrated by ≤ 5 % overestimation of the FIX potency at up to 2 % FIXa.
6. Addition of 2 IU FX/IU FIX resulted in an interference of < 2%.
7. The reagents are stable for at least 8 hours at 4°C.

Results



Log-log and lin-lin dose-response graphs of the WHO FIX standard and two different concentrates.

In the log-log graph all assayed FIX concentrates demonstrated a parallel line relationship at a 95% confidence level. A quadratic curve fit was applicable for all concentrates in the lin-lin graph.

TABLE 2 – Potency assignment with chromogenic and clotting methods

Method	Concentrate B	Concentrate C	Concentrate D
Chromogenic	8.4 ± 0.5 (n = 4)	9.3 ± 0.7 (n = 4)	7.3 ± 0.7 (n = 4)
Clotting	9.2 ± 1.6 (n = 5)	8.5 ± 0.5 (n = 3)	7.2 ± 1.3 (n = 5)

Each concentrate was assayed in 3 - 5 series with chromogenic and clotting methods. Parallel line analysis was used for potency assignment vs the WHO FIX Standard.

FX interference study

Addition of 2 IU FX/IU FIX resulted in an interference of < 2%.

Imprecision

Within series imprecision was estimated from analysis of three FIX concentrates at 0.3 IU/ml using four independent assay series with triplicate testing of each sample. Between series imprecision was estimated from four FIX potency determinations of three FIX concentrates using parallel line analysis.

Within series CV: 3 %

Between series CV: 7 %

Stability

The stability of Reagent A and Reagent B has been estimated in a preliminary study. An evaluation of dose-response curves after storage at 4 °C for 0 - 8 hours showed essentially no change in slope and reagent blank values.

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