Elevated Factor VIII measured by FXa Activity

Method Sheet

Background

Historically, factor VIII (FVIII) has always been associated with bleeding, since the well known pathological condition Haemophilia A is a consequence of very low plasma levels of FVIII.
On the other hand, FVIII is also an acute phase reactant, and hence elevated levels are associated with conditions such as trauma, infection and exercise.
Common to many other coagulation factors, FVIII also rises during pregnancy.
FVIII is a key pro-coagulant factor and recent studies have shown the association between elevated levels of FVIII activity and an increased risk of venous and seemingly also arterial thrombosis [1-4].
A concomitant increase was also noticed for von Willebrand factor [1-3]. Importantly, FVIII activity shows a high correlation to FVIII antigen (FVIII:Ag), thereby attributing the increased activity to an increased FVIII synthesis [2, 5].
So far the increased plasma FVIII:Ag has not been linked to any polymorphism of the FVIII gene promoter [5], but the search for a genetic contribution is still under investigation. FVIII activity in thrombotic patients is often above 1.5 IU/ml and might reach levels of 4-5 IU/ml, sometime in connection with highly inflammatory conditions.
Therefore, a specific adaptation of Coamatic® Factor VIII has been developed to allow accurate determination of elevated FVIII activity.
The advantages in using a chromogenic method as compared to one-stage clotting methods are numerous.
In particular, the chromogenic method is not sensitive to pre-activation of FVIII[6], thereby avoiding overestimation of FVIII activity.
Furthermore, due to its linear dose-response, it has a higher resolution at elevated levels and also a high precision.
These features make Coamatic Factor VIII ideal as a tool for thrombophilia screening in addition to its established use for diagnosis of haemophilia and FVIII potency estimation of concentrates.

Reagents: Coamatic® Factor VIII

Kit configuration

S-2765 + I-2581	1 vial
Factor reagent	2 vials
Buffer, stock solution	1 vial

Measurement Principle

Factor VIII acts as enzymatic cofactor of factor IXa during the activation of factor X to factor Xa in the presence of calcium ions and phospholipids. Factor Xa hydrolyses the chromogenic substrate S-2765 thus liberating the chromophore pNA.
The colour is then read spectrophotometrically at 405 nm. The generated factor Xa and thus the intensity of colour is proportional to the factor VIII activity in the sample. Thrombin, that is contained in the factor reagent, brings about a rapid and complete activation of the FVIII present in the sample.

1. Factor X —(Factor IXa, Ca2-, Phospholipid; Factor VIII)→ Factor Xa
2. S-2765 —(Factor Xa)→ pNA + peptide

Specimen Collection

Follow the instructions described in the Coamatic Factor VIII package insert.

Determination of elevated levels of Factor VIII activity

The applications of the Coamatic Factor VIII kit are currently referred to a low assay range and to a normal assay range. The upper measuring limit using the procedure for the normal assay range is 1.42 IU/ml for the microplate method and 1 IU/ml for the ACL method.
The determination of FVIII activities higher than these limits, can be performed by pre-diluting the plasma samples 1:4 and assaying the diluted samples following the protocol described for the normal assay range but restricting this range to 0-1 IU/ml. The results should be multiplied by 4 to obtain the final value of FVIII activity.

- Pre-dilute the samples using the buffer contained in the Coamatic FVIII kit as follows: 1 vol plasma sample + 3 vol diluted buffer
- Dilute further as detailed in the package insert
- Follow the instructions contained in the Coamatic FVIII package insert (microplate procedure) or in the instrument application sheet (automated instruments).

Microplate method

Reagent preparation

Factor reagent:	reconstitute with 3.0 ml of sterile water
Substrate:	reconstitute with 6.0 ml of sterile water
Buffer:	dilute 1:10 with sterile water

Standard curve

The standard curve 0-1 IU/ml is prepared by using a human normal plasma calibrated against an International Standard for plasma FVIII. In case the normal plasma does not contain exactly 1 IU/ml FVIII, the values of the standard must be recalculated accordingly.

	Pre-dilution		*Final Dilution*	
FVIII IU/ml	Plasma µl	Buffer µl	Diluted Plasma µl	Buffer µl
1.00	-	-	25	2000
0.70	100	100	25	1400
0.50	100	100	25	2000
0.25	50	150	25	2000
0.00	-	-	-	2000

Sample dilution

Pre-dilute the sample by mixing 1 vol plasma with 3 vol of Coamatic Factor VIII Buffer.

Dilute further as follows:

Samples	25 µl
Buffer	2000 µl

Assay procedure

Diluted samples / controls/ standards	50 µl
Incubate at 37°C	*3-4 min*
Factor reagent (37°C)	50 µl
Incubate at 37°C	*2 min*
Substrate (37°C)	50 µl
Incubate at 37°C	*2 min*
Acetic acid 20%	50 µl

Read the absorbance at 405 nm, using a reference wavelength of 490 nm.

ACL method

This method is applicable to the ACL™ 200 / 300 / 3000 / 6000 / 7000.

Reagent preparation

Factor reagent:	reconstitute with 3.0 ml of sterile water
Substrate:	reconstitute with 5.25 ml of sterile water
Buffer:	dilute 1:10 with sterile water

Standard curve

The standard curve is prepared by using a human normal plasma calibrated against an International Standard for plasma FVIII.
Dilute the standard as follows:
25 µl plasma + 2000 µl buffer

Sample dilution

Pre-dilute the sample by mixing 1 vol plasma with 3 vol of Coamatic Factor VIII Buffer.
Dilute further as follows:

Samples	25 µl
Buffer	2000 µl

Assay procedure

Select the test Plasminogen (channel).
Place diluted normal plasma in POOL position.
Place buffer working solution in DIL position.
Place factor reagent in position 2.
Place substrate in position 3.
Place sample cups with diluted plasmas.

Measuring range

With pre-dilution of the sample the measuring range is 1-4 IU/ml with both the microplate and the ACL method.

Results

The evaluation of Coamatic Factor VIII with samples from thrombotic patients has been performed both with the microplate and the ACL applications. The standard curves are shown in figures 1 and 2 respectively.
The upper limit of the standard curve is 1 IU/ml in both methods resulting in an upper measurement limit of 4 IU/ml, with plasma samples diluted 1:4. The precision of the method has been evaluated by using plasma samples diluted according to the protocol described above.

FVIII	*Within series*		*Between series*		
IU/ml	CV%	n	CV%	n	N
1	3.0	35	6.0	5	7
4	3.0	35	6.0	5	7

The FVIII activity of 130 patient samples has been determined with Coamatic Factor VIII on ACL, by pre-diluting or not the plasma samples. The samples have been obtained from patients about three months after the thrombotic episode. The following results were obtained from linear regression analysis (figure 3):

Slope = 1.52 Range (x) = 0.45-3.28 IU/ml FVIII
Intercept = -0.57 Range (y) = 0.33-4.50 IU/ml FVIII
R = 0.96

For FVIII activities higher than 1 IU/ml, the samples can be under-estimated if the pre-dilution is not performed.

Coamatic Factor VIII has been compared with a one-stage clotting method on the ACL analyser. For the Coamatic Factor VIII assay, the samples were pre-diluted 1:4 as recommended in the protocol described above. For the clotting method the plasma samples were pre-diluted 1:4 (with 0.05 mol/l imidazol, 0.1 mol/l NaCl, pH 7.3; buffer recommended by the clotting reagent manufacturer) followed by the prescribed sample dilution 1:5. 71 plasma samples from thrombotic patients were analysed. The results are shown in figure 4.
The following results were obtained from linear regression analysis:

Slope = 1.28 Range (x) = 0.50-2.32 IU/ml FVIII
Intercept = -0.43 Range (y) = 0.18-2.51 IU/ml FVIII
R = 0.92

Conclusions

The results described in this method sheet represent a preliminary evaluation of Coamatic Factor VIII applied for the screening of samples from thrombotic patients. From the population of samples tested, about 25% had a FVIII activity higher than 1.4 IU/ml, thus confirming earlier published data [1, 2]. These results have been obtained by a simple modification of the existing applications and protocols, consisting in the pre-dilution 1:4 of the plasma samples. Coamatic Factor VIII is a kit suitable for use on a number of automated instruments as well as on microplates. In case the pre-dilution is done manually, the current application notes for automated instruments can then be adhered to, with the only exception of restricting the assay range to 0-1 IU/ml. Indeed, some instruments offer the possibility of also performing the pre-dilution step.

References

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