

A new method for the assay of factor XI in plasma and the preparation and use of a new artificial substrate

R. E. BLEWITT

From the Oxford Haemophilia Centre, Churchill Hospital, Headington, Oxford

SYNOPSIS An improved method for the one-stage assay of factor XI in plasma has been proposed; it is reproducible and compares well with an old, established method.

A short investigation into the preparation and use of artificially prepared factor XI-deficient plasma has been carried out: this substrate can be used as an alternative to the congenitally deficient substrate.

Factor XI is defined by Nossel (1972) as 'that activity which is able to correct the prolonged clotting time of plasma deficient in factor XI but does not correct the clotting time of plasma congenitally deficient in factor XII or any other clotting factor'. It is activated by factor XIIa, which is formed when factor XII contacts certain specific surfaces, and, by way of factors XI, IX, VIII, and X in the intrinsic pathway, this leads to the formation of a fibrin clot.

Congenital deficiency of factor XI, as first described by Rosenthal *et al* (1953), gives rise to a mild haemorrhagic disease, in which there appears to be no obvious relationship between the severity of the clinical bleeding state and the level of factor XI (Nossel, 1972).

Methods for assaying the level of factor XI in plasma have been described by Rapaport, Schiffman, Patch, and Ware (1961), Egeberg (1961), and Nossel, Niemets, Mibashan, and Schulze (1966). Those of Rapaport and Egeberg are based on the ability of test and control plasmas to correct the prolonged clotting time of plasma congenitally deficient in factor XI. The method of Nossel *et al* uses a celite eluate containing the factor XI from a test or control plasma, and measures its ability to accelerate the clotting time of intact normal plasma. However, the poor slopes of the plasma dilution curves and the long clotting times encountered in these assay methods both contribute to their questionable accuracy. The work described in this paper is concerned with the development of a modified assay which corrects these faults and gives more

reliable results. The method described is based on a modification of that of Rapaport *et al* (1961). It makes use of a commercial reagent (activated Thrombofax) containing ellagic acid, the component which activates factor XII, and inosithin, the source of phospholipid which is required in the intrinsic pathway. The use of congenital factor-XI-deficient plasma is recommended, but as an alternative an improved method of preparing an artificial substrate is described. Results obtained using this artificial substrate are shown to be identical to those using the congenitally deficient material.

The Assay Method

MATERIALS

Imidazole buffer is prepared according to the method of Biggs and Macfarlane (1962).

0.025 Molar calcium chloride is prepared from a stock molar solution (British Drug Houses).

Activated Thrombofax reagent (Orthodiagnosics, Raritan, New Jersey 08369) is used as supplied.

For congenital factor-XI-deficient plasma fresh whole blood was collected from a 0% factor-XI-deficient patient into 3.8% trisodium citrate in the proportion of 1 volume anticoagulant to 9 volumes blood, centrifuged at 1400 *g* for 20 minutes, the plasma separated, and stored in 2 ml aliquots at -20°C in plastic, stoppered tubes.

Standard plasma consisted of a pool of 25 normal plasmas: whole blood was collected and separated as described for the congenital factor-XI-deficient plasma; the plasmas were pooled and stored in aliquots at -20°C .

TECHNIQUE FOR THE ASSAY OF FACTOR XI
 Test and standard plasmas are treated alike.

A set of three serial dilutions of plasma (1 in 10, 1 in 30, 1 in 100) are prepared in imidazole buffer in plastic tubes: dilutions of 1 in 100, 1 in 300, 1 in 1000 should be used for the standard plasma if a test plasma is known to have a factor XI level of less than 10%. When using the new artificial substrate plasma, it is necessary to use different dilutions to those mentioned above, when levels of factor XI below 15% are being assayed (see 'Assessment of the new substrate').

Congenital factor-XI-deficient plasma, 0.1 ml, and 0.1 ml freshly prepared plasma dilution are mixed in a clotting tube and warmed to 37°C. Activated Thrombofax reagent, 0.1 ml, is added and the mixture incubated for five minutes at 37°C. Warm 0.025M calcium chloride, 0.1 ml, is added and the clotting time recorded.

Each plasma dilution is tested in duplicate and the mean clotting time of the two results is calculated.

The mean clotting time of each dilution is plotted on a linear scale against the log of the plasma dilution as straight parallel lines: the test plasma line can then be compared with that of the standard plasma, and the percentage factor XI in the test plasma read from the standard line.

Assessment of the New Assay Method

PREPARATION OF A PLASMA DILUTION CURVE
 A dilution curve was prepared using the standard plasma, in the range 1 in 10 to 1 in 1000, for observation of the slope and the clotting times given by the new assay method. This is shown in figure 1a where clotting time is plotted against the log of the plasma dilution. It will be seen that the clotting times are conveniently short, ranging from 55.6 sec at the 1 in 10 dilution to 131.1 sec at the 1 in 1000 dilution, with a long blank time (imidazole buffer only) of greater than 160 seconds. The gradient of the curve is constant from the 1 in 10 dilution to the 0% level of factor XI, and sufficiently steep to allow for accurate reading of a test plasma from the standard plasma curve. In practice, the assay of very low levels of factor XI appears to result in the loss of the gradient of the test plasma curve, a phenomenon which has also been observed in the assays of factors VIII and IX. A 0% factor XI plasma when assayed gives clotting times equal to or greater than those of the blank, indicating that a true 0% level of factor XI can be detected by this method.

COMPARISON OF ASSAY RESULTS

Four known levels of factor XI (prepared by diluting

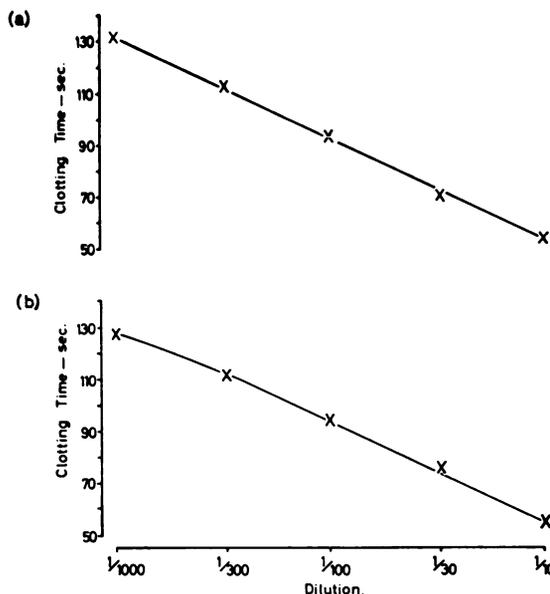


Fig 1 Standard plasma dilution curves. (a) Congenital factor-XI-deficient plasma as substrate; (b) artificially prepared factor-XI-deficient plasma as substrate.

the standard plasma in factor-XI-deficient plasma) were assayed four times each by the method described above and by a modification of the method described by Rapaport *et al* (1961) as reported by Austen and Rhymes (1974). A summary of the assay results is given in table I. These results were compared by statistical analysis using the 'analysis of variance' technique, which showed that there was no significant difference between the two sets of results.

REPRODUCIBILITY OF ASSAY RESULTS

Table II gives duplicate results at two levels of factor XI determined using the new assay technique. As will be seen, the standard deviation at the 25% level is 1.67 and that at the 75% level, 4.01. These analyses indicate that the 95% confidence limits for an assay result are $\pm 13\%$ of the value in question. This was of course for one operator under ideal conditions: for routine laboratory use it has been shown by experience that these can increase to $\pm 25\%$ of the value in question.

Preparation and Use in the New Assay System of Artificially Prepared Factor-XI-deficient Substrate Plasma

Nossel (1964) describes a method of preparing artificial factor-XI-deficient plasma by twice adsorbing

Expected Level of Factor XI	Method Austen and Rhymes (1974)					Activated Thrombofax Method				
	Assay Results (%)					Assay Results (%)				
	Mean Clotting Times for Dilution Range 1/10 to 1/100					Mean Clotting Times for Dilution Range 1/10 to 1/100				
5	4	3	5	4	165.5-245.0	6	4	5	4	111.4-153.7
10	10	11	11	10	146.5-207.3	11	7	9	9	98.9-142.0
25	24	23	28	27	125.6-192.4	16	22	24	22	83.7-122.5
75	78	70	65	75	99.3-163.1	72	86	63	71	66.1-100.5
Blank					217->240					175->180

Table I Comparison of assay results using the method of Austen and Rhymes (1974) and the activated Thrombofax method

Level of Factor XI (%)	Assayed Level (%)										Mean	SD					
25	24	23	21	25	23	19	25	23	23	23	22	22	25	25	23	23.1	1.67
75	75	78	76	70	75	68	73	73	73	73	73	66	76	71	65	72.7	4.01

Table II Reproducibility of results using the new assay technique

high-spun plasma with Celite 512 (John's Manville, USA) in the proportion of 6 mg Celite/ml plasma, followed by incubating the plasma at 37°C for 18 hours. Investigations carried out on this method revealed that the reduction in the level of factor XI (0.1%) was accompanied by a fall in the levels of factor V (5-10%) and factor VIII (40-50%). Although the slope of the standard plasma dilution curve was adequate when using this substrate plasma, the clotting times were greatly prolonged. This is due to the reduction in factor V and factor VIII content in the substrate plasma and suggests that assay results obtained using this material could be affected by the amount of factor VIII, and particularly factor V, in the test samples. By assaying the plasma for factors V, VIII, and XI at intervals during the preparation, it was found that the Celite adsorption removed more than 95% of the factor XI but very little of factors V and VIII: the long incubation period, however, had very little effect on removing the residual factor XI (0.1% remaining), but appeared to account for the large decreases in the levels of factors V and VIII. It was further shown that the level of factor XI reached a minimum in three to six hours, suggesting that incubation for 18 hours may be unnecessary. As a result of these findings, the possibility of using more Celite per ml plasma (to remove even more factor XI than the 95% removed by the above method without removing factor XII) and using a shorter incubation period (for removal of any residual factor XI and conservation of the levels of factors V and VIII) was considered and investigated.

The method of preparation of artificial factor-XI-deficient plasma which was found to be the most acceptable is described below; experiments to

ascertain its suitability for use as a substrate in the new factor XI assay method were carried out, and the results statistically analysed.

PREPARATION OF ARTIFICIAL FACTOR-XI-DEFICIENT PLASMA

Fresh plasma from a normal donor is prepared and adsorbed twice with Celite 512 in the proportion of 10 mg Celite/ml plasma for 10 minutes at 37°C; frequent inversion of the tubes to ensure continued suspension of the Celite is essential. The Celite is removed each time by centrifugation at 1400 g for 10 minutes. The plasma is then incubated for five hours at 37°C, and can be used fresh, or it can be stored in aliquots at -20°C before use.

ASSESSMENT OF THE NEW SUBSTRATE

Preparation of a plasma dilution curve

A standard curve was prepared using the standard plasma in the range 1 in 10 to 1 in 1000 for observation of the slope and clotting times given by the artificial substrate in the new assay method. This is shown in fig 1b where the clotting time is plotted against the log of the plasma dilution. Comparing it with the curve prepared using congenital substrate (fig 1a), it will be seen that the clotting times are directly comparable, except for the blank which was shorter using the artificial substrate (130 sec compared with >160 sec for the congenital substrate). The gradient of the curve remained constant and comparable to that of the congenital substrate up to a 1 in 1000 dilution, the residual factor XI being 0.1%. Levels of factors V and VIII were only slightly decreased to 60-70% after incubation for five hours (fig 2).

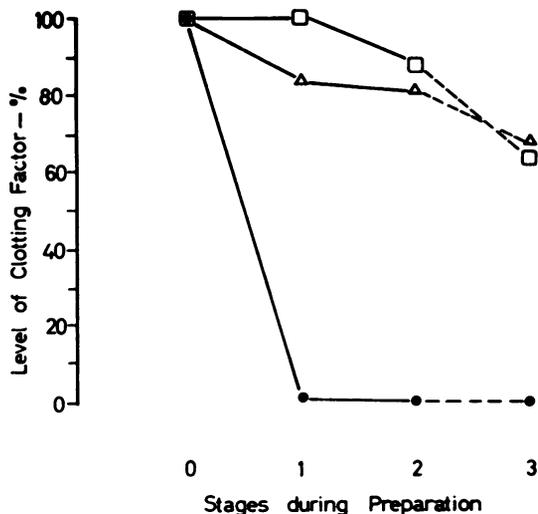


Fig 2 Changes in the levels of factors V (□), VIII (△), and XI (○) during the preparation of artificial factor-XI-deficient plasma. Stage 0, pretreatment; 1, after first adsorption; 2, after second adsorption; 3, after five-hour incubation.

Comparison of assay results

Four known levels of factor XI (prepared by diluting the standard plasma in congenital factor-XI-deficient plasma) were assayed four times each by the new assay method, first using congenital substrate and then the artificial substrate. For the assay of the 5% and 10% levels of factor XI using the artificial substrate, it was found that the normal range of dilutions, ie, 1 in 10, 1 in 30, and 1 in 100, was unsuitable, due to interference from the minute amount of factor XI remaining in the substrate: as a result, dilutions of 1 in 5 and 1 in 10 only were used to construct the sample curve; this produced results very similar to those obtained using the congenital substrate. A summary of the assay results is given in table III. The results were compared statistically using the analysis of variance technique: this showed that there was no significant difference between the results using the artificial substrate and those using the congenital material. The statistical

Expected Level of Factor XI (%)	Congenital Substrate Plasma				Artificial Substrate Plasma			
	Assay Results (%)				Assay Results (%)			
5	4	4	4	3	3	3	6	7
10	10	10	8	8	9	7	11	14
25	28	24	20	20	24	21	20	28
75	74	73	73	65	78	69	79	71

Table III Comparison of assay results using congenital and artificial substrate plasma in the new assay method

analyses also showed that there was no significant difference between the error of assay using artificial substrate and the error of assay using congenital substrate. However, it should be noted that to obtain this good accuracy and repeatability with samples of less than 15% a restricted number of dilutions only must be used.

EFFECT OF OTHER CLOTTING FACTOR DEFICIENCIES ON THE ASSAY OF FACTOR XI

To confirm that other factor deficiencies were not reflected as a deficiency in factor XI using both substrates, a series of factor XI assays were performed on congenital V-, VIII-, IX-, and XII- deficient plasmas and the results compared. The results are summarized in table IV. From these results it appears that complete deficiency of factor V does cause a slightly low factor XI result to be recorded using the artificial substrate: further investigations will need to be carried out to determine the exact degree of deficiency of factor V which produces this effect.

Deficient Factor	Congenital Substrate (%)	Artificial Substrate (%)
V	90	74
VIII	65	64
IX	70	70
XII	58	56

Table IV Comparison of results of factor XI assays on congenital factors V-, VIII-, IX-, and XII-deficient plasmas

Conclusion

In conclusion, this method could replace other methods already in use in laboratories for the assay of factor XI: it produces results which are statistically the same as those obtained using conventional methods, and which are readily reproducible; it is perfectly satisfactory using either congenital or artificial factor-XI-deficient substrate plasma. Furthermore, it has the advantage of potential use in automated coagulation equipment, since the reaction mixture is not opaque (change in opacity is generally recorded as the endpoint of coagulation in these machines).

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