Maintenance control of oral anticoagulant therapy by a chromogenic substrate assay for factor X

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SUMMARY An amidolytic assay employing the chromogenic substrate S 2337 (Kabi Diagnostica) was used to assay factor X in 35 healthy controls and in 100 outpatients receiving oral anticoagulant therapy. This method correlated well with a coagulation assay of factor X in the control group \( r = 0.88 \). When compared with two routine tests for the control of anticoagulant therapy (Thrombotest and prothrombin ratio) good correlation was obtained between the methods, \( r = 0.84 \) and \( r = -0.74 \) respectively. These results suggest that a chromogenic substrate assay for factor X might be a suitable method for the maintenance control of oral anticoagulant therapy.

The recent development of amidolytic assays using chromogenic peptide substrates has been rapidly applied to a number of aspects of haemostasis and thrombosis, and there have been reports\(^1\)\(^2\) that tests employing chromogenic substrates might be used to control oral anticoagulant therapy. Although the prothrombin time\(^3\) or similar tests based on the 'extrinsic' clotting system have been almost universally accepted for many years as the most suitable method for the laboratory control of oral anticoagulant therapy, difficulties are still experienced with this apparently simple procedure. Chromogenic peptide substrate assays have several advantages which might overcome some of these difficulties.\(^4\)\(^5\)

It was therefore decided to examine whether a chromogenic substrate assay of factor X might offer a possible alternative to current tests.

Material and methods

CLINICAL MATERIAL
One hundred patients aged 21-69 years were selected randomly from an anticoagulant clinic along with 35 healthy controls aged 18-61 years. None of the patients had undergone surgery within the previous four weeks or received recent blood products. All patients had been taking Warfarin BP tablets as an anticoagulant for a minimum time of seven days.

REAGENTS
Plasma samples were prepared from venous blood collected into one-tenth volume of 3.8% sodium citrate. All samples were tested in duplicate within 2 hours of collection.

The thromboplastin used for measuring prothrombin times was the British Comparative Thromboplastin Reagent (BCT.\(^6\)) but for coagulation assays a saline suspension of rabbit brain was used (Dade Activated Thromboplastin).

Normal human plasma was prepared from a pool of 10 healthy adults. Factor X deficient substrate plasma for clotting assays was a lyophilised human plasma congenitally deficient in factor X (Dade).

Chromogenic substrate S 2337 and all reagents for the amidolytic assay of factor X, including Russell's Viper Venom, human normal and factor X deficient substrate plasmas and buffers, were obtained from Kabi Diagnostica (Stockholm, Sweden).

ANTICOAGULANT CONTROL
The Thrombotest\(^7\) (Nyggaard & Co, Oslo, Norway) was used in all patients for the routine laboratory control of anticoagulant therapy. Venous samples were tested immediately in duplicate according to the manufacturer's instructions. Ninety-one patients also had the prothrombin time\(^3\) measured, and results were expressed as the prothrombin ratio.\(^6\)

FACTOR X ASSAYS
Factor X coagulant activity was measured in both patients and controls by a one-stage method\(^8\) and expressed as percent activity of the normal plasma pool. All samples were tested in duplicate using an automated coagulometer, Coag-a-Pet\(^\text{TM}\) (General Diagnostics).

Factor X amidolytic activity was measured according to the method of Aurell et al.\(^9\) but using...
the chromogenic substrate S 2337. Adsorbance in all patients and controls (diluted 1:2 in normal saline) at 405 nm was recorded using a Unicam SP 1800 spectrophotometer (Pye Unicam, Cambridge). Results were expressed as percent activity of normal human plasma (Kabi Diagnostica).

Results

The results of the factor X assays performed by both methods on patients and controls are given in the Table. The control group range (mean ± 2 SD) with

<table>
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<tr>
<th>Controls (n = 35)</th>
<th>Patients (n = 100)</th>
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<tr>
<td></td>
<td>Coagulation</td>
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<tr>
<td>Mean</td>
<td>98.8</td>
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<tr>
<td>SD</td>
<td>17.5</td>
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<td>CV</td>
<td>4.97</td>
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Mean factor X levels (% normal), standard deviation (SD) and coefficient of variation (CV) in controls and patients by coagulation and chromogenic substrate assays

The amidolytic assay using the S 2337 substrate was 92.0 ± 28.8%. When this method was compared with the conventional clotting assay where the control group range was 98.8 ± 35% a good correlation was evident between the two methods, with a correlation coefficient (r) of 0.88. The coefficient of variation at this level of factor X calculated from the difference between duplicates was much less for the chromogenic substrate assay, being 1.14% compared to 4.97% for the coagulation assay. The factor X levels for the 100 patients taking oral anticoagulants showed an actual range of 6-70% (mean 19.1%) for the clotting assay and 6-65% (mean 23.6%) for the amidolytic assay. Once again there was good correlation (r = 0.89) between the two methods, and the coefficients of variation were also less at this level of factor X, being 1.86% and 0.99% respectively (Table).

Thrombotest results for the patients ranged from less than 5% to 40%, and so not all were within the therapeutic range for this laboratory of 5-15%. Seventy-two patients were within the therapeutic range while 11 were overanticoagulated (less than 5%) and 17 were underanticoagulated (more than 15%). When the Thrombotest results were compared to the chromogenic substrate assay of factor X (Fig. 1) a good correlation was found between tests, r = 0.84. A good correlation coefficient (r = -0.74) was also obtained when the prothrombin ratio was compared to the amidolytic assay of factor X (Fig. 2). These results are very comparable to those of other workers who have made similar correlations, although using a different chromogenic substrate, S 2222 (Kabi Diagnostica), for the factor X amidolytic assay.
Discussion

The routine laboratory control of oral anticoagulant therapy has been performed for many years using a prothrombin time technique or similar test. Despite the apparent simplicity of these methods several problems still exist, principally relating to standardisation and choice of thromboplastin. The establishment of a uniform system of reporting results and use of national thromboplastin reagents has improved standardisation but the method of comparative ratios requires a large amount of data and technical expertise and still produces variation in results. Different thromboplastins show variation in sensitivity to the coumarin-induced anticoagulant defect, to activation of plasma samples before testing, and to the inhibitory effect of PIVKA. Thromboplastin of human brain origin shows interbatch variation, and occasional preparations can be almost completely insensitive. Widespread availability of human brain may become more difficult in the future because some doubts have been raised regarding its long-term safety as a laboratory reagent. It would therefore be advantageous to have tests that were not dependent on human tissue as a reagent.

The introduction of chromogenic substrates into the field of haemostasis investigation provides several potential advantages which might overcome some of the problems associated with anticoagulant control. Improved standardisation should result from clearer definition of test symptoms used and parameters measured. Greater precision should also be possible with these methods compared to clotting tests, as the low coefficients of variation in this study support (Table). Also amidolytic assays employing chromogenic substrates can be adapted for use with automated enzyme analysers, which should certainly save manpower and improve the efficiency of handling large numbers of specimens from anticoagulant clinics.

Chromogenic substrate S 2337 has potential advantages over the S 2222 substrate used in other similar studies to assay factor X. It is a highly specific and sensitive substrate for factor Xa, reacts very rapidly, and requires only half the concentration of substrate, thereby hopefully reducing costs. Like the S 2222 assay, this method does not measure PIVKA X, and activity is not inhibited by antithrombin III. Moreover the addition of polybrene to the buffers results in minimal inhibition of factor Xa, thus allowing the use of this test in patients concomitantly receiving heparin. This assay is therefore potentially very suitable for use in patients receiving oral anticoagulants.

From the regression line of factor X on Thrombotest (Fig. 1), a therapeutic range suitable for control-
of patients either overanticoagulated or underanticoagulated in this study is rather small for accurate correlations to be made, but a poorer correlation coefficient was certainly noted for the latter group of patients (r = 0.71). Much further work will be required to establish precise therapeutic values and the relative risks from bleeding and thrombosis, and meticulous standardisation of methods and reagents will also be necessary.

Amidolytic assays have many advantages over conventional clotting methods that would be of benefit for controlling oral anticoagulant therapy, and the S 2337 substrate appears to be a particularly promising development. The encouraging results obtained in this study indicate that this chromogenic substrate warrants further investigation as a potential method for the laboratory control of maintenance oral anticoagulant therapy.

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