A new chromogenic assay for the specific determination of prothrombin

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SUMMARY A new method for the specific assay of prothrombin is described which utilises Taipan snake venom and the chromogenic substrate Tos-Gly-Pro-Arg-pNA (Chromozym TH). The method is sensitive and reproducible and shows good correlation with a conventional specific assay for prothrombin. The reaction is dependent on calcium ions and phospholipid, and is therefore sensitive to the coumarin-induced prothrombin defect. The assay is rapid and well suited for use in the routine coagulation laboratory.

The recent introduction of chromogenic substrates has led to the development of assay techniques for most coagulation factors, fibrinolytic components, and their respective inhibitors (Stormorken, 1976). These substrates are synthetic tri- or tetra-peptides having amino acid sequences analogous to the site of cleavage of their natural counterparts. The peptide is coupled to a chromophore, p-nitroaniline (pNA), through an amide bond, which when bound to the peptide is colourless, absorbing light only in the near-UV region. After enzymatic cleavage of the substrate, however, pNA is released from the peptide and may be measured by its absorbance at 405 nm. The increase in absorbance is directly proportional to the enzymatic activity.

Several workers have developed assays for the estimation of prothrombin and combined measurement of the vitamin K-dependent coagulation factors based on these synthetic substrates. Various prothrombin-activating principles have formed the basis of these methods.

Russel viper venom (RVV) has been employed to measure the combined activity of factors II, V, and X using the substrate S-2238 (Kabi AB, Stockholm) in the presence of phospholipid and calcium ions (Bergström and Egberg, 1978). The same workers also used the venom from Echis carinatus (Ecarin) in a specific assay of prothrombin. This venom activates prothrombin directly in the absence of calcium ions (Franza et al., 1975), and the method is consequently insensitive to the coumarin-induced prothrombin defect.

Bergström and Blombäck (1974) used a mixture of partially purified clotting factors V, VIII, X, XI, XII, calcium ions, and phospholipid (Protram) to activate prothrombin. The thrombin produced was measured with the early chromogenic substrate S-2160 (Svendsen et al., 1972). This method gave good agreement with conventional prothrombin determinations in normal individuals and in dicoumarol-treated patients. Commercial tissue thromboplastins have not been found useful in chromogenic assays of factors II, VII, and X (Bergström and Blombäck, 1974).

The venom of the Taipan snake (Oxyuranus scutellatus) is able to convert prothrombin to thrombin in the presence of calcium ions and phospholipid (Denson, 1969) and may be used in a one-stage specific clotting assay of prothrombin (Denson et al., 1971).

The present work was performed to adapt this principle to a rapid specific prothrombin assay utilising the thrombin-specific substrate Chromozym TH. The principle of the chromogenic assay is shown in Figure 1.

Material and methods

Tos-Gly-Pro-Arg-pNA (Chromozym TH) was obtained from the Boehringer Corporation and dissolved in distilled water to a concentration of 1·5 mmol/l. The solution is stable for six months at +4°C.

Taipan venom/calcium chloride reagent and Bell and Alton platelet substitute were obtained from Diagnostic Reagents Ltd. Both reagents were reconstituted in distilled water, as directed by the manufacturer, and were stable during a working day at room temperature.

Tris-imidazole buffer 0·2M, pH 8·2, was prepared

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Specific assay of prothrombin by a clotting technique based on the one-stage prothrombin time was performed using a kit obtained from the Boehringer Corporation.

ASSAY PROCEDURE
After some initial experiments the following assay conditions were chosen: One millilitre of a 1/400 dilution of citrated plasma in buffer was warmed at 37°C; 0.2 ml of phospholipid immediately followed by 0.2 ml Taipan venom/calcium chloride reagent were added, and the mixture was incubated at 37°C for 5 minutes; 0.2 ml Chromozym TH was added, and the mixture was further incubated for exactly 5 minutes. The reaction was then terminated by blowing in 0.5 ml of 50% acetic acid, mixing immediately by means of a vortex mixer. The colour produced was stable for at least 18 hours. The optical density was read at 405 nm in plastic disposable cuvettes. A blank was prepared in exactly the same way, except that the additions of substrate and acetic acid were reversed so that colour formation due to thrombin production did not occur. Blanks for each test sample were not found to be necessary unless the plasma was unduly turbid or jaundiced.

Calibration curves were prepared by diluting pooled normal citrated plasma in buffer to give concentrations equivalent to 125%, 100%, 75%, 50%, 25%, and 10% prothrombin.

ADDITIONAL METHODS
Normotest and Thrombotest (Nyegaard and Co, Oslo, Norway) were performed on citrated venous blood according to the manufacturer.

**Fig. 1 Principle of the chromogenic Taipan assay.** The site of cleavage of the substrate is shown.

Acetic acid 50% was prepared by dilution of glacial acetic acid in distilled water.

**Fig. 2 (A)** Effect of activation time on thrombin generation. (B) Effect of phospholipid and Taipan concentrations on thrombin generation. The reagents were reconstituted as in the text, and then further diluted as required in distilled water. (C) Effect of substrate concentration. (D) Effect of initial plasma dilution on the linearity of the assay.

**Results**
Figure 2 shows the evaluation of the optimum conditions for the assay. Prothrombin activation was maximal after 5 minutes, longer periods (up to 15 minutes) producing no further thrombin generation. The effect of phospholipid was maximal at a dilution of 1/10, and that of the Taipan venom at a dilution of 1/2. Decreasing substrate concentration gave a linear decrease in final optical density and was eventually used at a concentration of 1.5 mmol/l. Decreasing plasma dilution resulted in increasing fibrin formation with consequent absorption of liberated thrombin. This resulted in deviation from linearity of the calibration curve. An initial plasma dilution of 1/400 was chosen as being the lowest dilution which gave both linearity to 125% prothrombin and acceptably high optical density readings in the range 10-25% prothrombin. An example of a calibration curve is shown in Figure 3. It was
shown that as the reaction was consistently linear in the range 0-125\% prothrombin, the test samples could be assayed with accuracy using the spectrophotometer (a Pye-Unicam SP30 UV) in the 'concentration' mode by direct comparison with the 100\% standard. For spectrophotometers without this facility the calculation $A^{0.05\%}$ Test/$A^{0.05\%}$ Standard $\times$ 100\% could be applied. The normal range obtained for 35 normal individuals was 96.5 $\pm$ 24.5\% (mean $\pm$ 2 SD).

The method was compared to a conventional clotting technique for the specific assay of prothrombin, and a correlation coefficient of 0.97 was obtained (Fig. 4). The correlation coefficients between the chromogenic method and Normotest and Thrombotest were 0.89 and 0.71 respectively. There was low within-batch variation between repeated analysis of the same samples. The coefficients of variation for the ranges 80-100\% and 20-40\% were 2.2\% and 2.9\% respectively.

Fig. 3 A typical calibration curve.

The addition of heparin to the test plasma reduced the thrombin activity generated. The addition of Polybrene to the buffer at a concentration of 0.05 g/l prevented the inhibition but was also associated with a slight decrease in final optical density (Fig. 5).

Fig. 5 Effects of heparin and Polybrene on the assay.

Discussion

Conventional clotting assays for the specific determination of prothrombin have been of the one- or two-stage types. The one-stage methods have been based either on the classical one-stage prothrombin (Quick) time using prothrombin-free plasma as substrate or on the snake venoms Notechis scutatus (Tiger snake), Echis carinatus (Ecarin), and Oxyuranus scutellatus (Taipan snake). The Tiger snake assay is also sensitive to factor V, but the Taipan (Denson et al., 1971) and Ecarin (Franza and Aronson, 1976) methods are specific for prothrombin. The two-stage assay (Biggs and Douglas, 1953) is laborious and is not suited to multiple testing.

The more recently introduced chromogenic assays for prothrombin have utilised physiological (Protram) prothrombin activation (Bergström and Blombäck, 1974) or the venom from Echis carinatus directly to activate prothrombin (Bergström and Egberg, 1978). These assays had advantages over the more conventional techniques, being adaptable to automation and having greater reproducibility and speed. The Ecarin method, however, is relatively insensitive to the absence of $\gamma$-carboxyglutamic acid residues in the coumarin-induced prothrombin, although there is some evidence that Ecarin does slowly activate coumarin prothrombin (Egberg and Bergström, 1978). The Protram method is sensitive to the coumarin-induced defect and was considered to be suitable for the control of oral anticoagulant therapy (Norén, 1970).

Fig. 4 Correlation between a conventional clotting assay and the Taipan chromogenic method.
The present method, based on activation of prothrombin by the venom of the Taipan snake, combines the specificity of the corresponding clotting technique with the speed and reproducibility of the chromogenic methods. Being dependent on calcium ions and phospholipid, the assay is also sensitive to the coumarin-induced prothrombin and shows good correlation with a conventional clotting technique. The method is linear in the range 0-125%, and may be rendered insensitive to heparin by the addition of Polybrene to the buffer.

The method is not intended to be used for the control of oral anticoagulant therapy, but it is probably as suitable as the Protram method and is less sensitive to heparin. The correlation between the new method and Thrombotest was not expected to be very high as the latter is also sensitive to factors VII and X. The correlation was, however, better than that obtained by Bergström and Egberg (1978) for the Ecarin method, demonstrating the greater sensitivity of the Taipan assay for the coumarin-induced abnormal prothrombin.

The Taipan chromogenic assay is offered as an alternative technique for the specific assay of prothrombin in the diagnosis of bleeding disorders and evaluation of prothrombin depression in liver disease. The assay is less expensive than the Ecarin technique and, being based on an end-point method, offers an advantage to those haematology departments not equipped with reaction-rate analysers.

References


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