Enzyme linked immunosorbent assay for measuring antithrombin III

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SUMMARY A competitive ELISA for the measurement of antithrombin III antigen was developed using a pure preparation of antithrombin. This two step immunoassay requires only one antibody binding step and avoids the binding of anti-antithrombin antibody to the solid phase. Plasma samples measured by this ELISA correlated well with measurements taken with immune electrophoresis measurements ($r = 0.933$); the ELISA was also shown to be both sensitive and precise.

Antithrombin III (ATIII) is the major inhibitor of the coagulation serine enzymes which act primarily by inhibiting thrombin.¹ Its role is of vital physiological importance to maintaining a balance between the process of coagulation and anticoagulation. This is clearly shown by the greatly increased thrombotic tendency seen in patients with congenital antithrombin III deficiency.² Seen more frequently, however, is an acquired deficiency of antithrombin III,³ which occurs in liver disease, nephrotic syndrome,⁴ DIC⁵ and after major surgery and transplantation.⁶ Antithrombin III deficiency predisposes such patients to thrombosis and consumptive coagulopathy. In an attempt to overcome these problems, antithrombin replacement therapy is increasingly being used. The control of antithrombin supplementation depends largely on the measurement of antithrombin activity, but the antigenic titres are a useful adjunct to management. In the investigation of inherited antithrombin deficiency, however, measurement of both activity and antigenic titres is essential to diagnose antithrombin variants. Many plasma proteins are quantitated by immune electrophoresis (IEA) or by immune radiometric assay (IRMA), but lately these techniques have been superseded by enzyme linked immunosorbent assays (ELISA) which are cheap, rapid, and sensitive. The development of a successful antithrombin ELISA, however, has proved difficult. This is possibly due to poor binding of anti-antithrombin antibody to the solid phase. We present a sensitive ELISA method using commercially available materials which avoids this problem and compare measurements of antigenic antithrombin in patients by this ELISA and IEA.

Material and methods

Peroxidase conjugated rabbit anti-human antithrombin III immunoglobulin (cAb) was obtained from Dakopatts. Lyophilised antithrombin concentrate with purity of greater than 90% was provided by the Plasma Fractionation Laboratory, Oxford. Antithrombin concentrate ran as a single band on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) both in the native and reduced state. Two dimensional electrophoresis of the concentrate in the presence of heparin showed that about 20% of the antithrombin had reduced heparin binding; this is probably due to denaturation following heat treatment. Microtitre plates were supplied by Dynatech Immunolon and chemicals used were obtained from BDH.

Plasma was collected by centrifuging venous blood in tri-sodium citrate (0.38%) at $2500 \times g$ for five minutes and freezing it at -20°C for subsequent assay.

Coated ELISA plates were produced by adding $100\ \mu\text{l}$ of an antithrombin coating solution to each well and incubating for eight to 12 hours at 20°C . Coating solution was lyophilised antithrombin concentrate made to $3\ \mu\text{g}$ protein/ml with carbonate buffer (0.005 M, pH 9.6). Plate wells were then washed three times in PBS (0.01 M phosphate buffered saline, Tween 20 0.05% vol/vol, pH 7.2).

Samples of plasma for measurement were diluted 1/40 in conjugated antibody mix (PBS, 0.1% wt/vol BSA, 0.33% vol/vol cAb) and incubated for one hour at 37°C before being applied to a coated plate ($100\ \mu\text{l}$ /well) and incubated for one hour at 20°C . Plates were then washed twice with PBS and once in citrate phosphate buffer (0.1 M, pH 5.0) before detecting bound cAb by the addition of peroxidase substrate

solution (100 μ l/well, citrate phosphate buffer pH 5.0, orthophenylenediamine 6 mg/ml, H_2O_2 0.012% vol/vol). The reaction was stopped after three minutes with sulphuric acid (1 M, 150 μ l/well) and the absorbance read at 492 nm using a Titertek multiscan MCC plate reader.

A normal plasma pool was prepared using plasma from 20 subjects in whom there was no previous history of thrombosis. Test sample antithrombin values were obtained by interpolation from a standard curve created by assaying dilutions of a normal pool of plasma.

IEA was carried out according to the method of Laurell.⁷

Results

This ELISA was sensitive, exhibiting linearity to a 1/256 dilution of plasma (fig 1), which is equivalent to 0.5 μ g/ml antithrombin, assuming a normal plasma antithrombin concentration of 120 μ g/ml.⁸

The coefficient of variance for 14 measurements of an identical sample on one plate was 4% while the coefficient of variance for an identical sample measured on seven separate plates was 6%. Thus for both interbatch and intrabatch measurements the assay was precise.

A normal range (mean $\pm$ 2 SD) for this method was determined by assaying plasma samples from a group of normal subjects. The range extended from 78%–124%, where the mean is 100%. This normal pool was calibrated using a commercial antithrombin standard. Twenty thrombotic patients were assayed for antigenic antithrombin by IEA and ELISA and the

Fig 1 Dose response curve of ATIII obtained with ELISA.

Fig 2 Correlation between immunoelectrophoretic assay and ELISA ($r = 0.933$, $y = 1.40x - 39$).

correlation was 0.933, indicating that the ELISA was as accurate as IEA measurements (fig 2), over a wide range of antithrombin concentrations.

Discussion

Many plasma proteins are measured by a sandwich ELISA, where two similar antibodies bind selectively to the antigen.⁹ One antibody is bound to a solid phase while the other detector antibody is conjugated to a suitable label. The sensitivity of the ELISA is largely determined by the amount of the primary antibody bound to the solid phase (unpublished observations), and even when this is optimised it may prove insufficient to provide a sensitive assay.

In an attempt to produce a standard sandwich ELISA to measure plasma antithrombin, we were unable to achieve adequate primary antibody binding with commercial antibodies. Therefore, to overcome this problem we established an assay which avoids binding the anti-antithrombin antibody at the solid phase. This approach was made possible by the recent availability of a pure preparation of antithrombin.

In our two step immunoassay only one antibody binds selectively to the antigen. A constant amount of excess peroxidase conjugated anti-antithrombin antibody is incubated with the plasma sample and this leads to the formation of an antigen antibody complex. The concentration of the residual excess conjugated antibody is inversely proportional to the amount of antithrombin in the sample.

To determine the concentration of residual

Table Precision studies for ELISA performance

	Mean (SD) ATIII(%)	CV%
Intrabatch (n = 14)	126.1 (5.4)	4
Interbatch (n = 7)	88.6 (5.3)	6

antibody, aliquots of the incubation mixture are transferred into microtitre wells pre-coated with excess antithrombin for further incubation. Solid phase bound antigen conjugated antibody complexes provide a direct measure of the antithrombin concentration in the original sample.

Based on this principle we produced an ELISA which is sensitive (fig 1), precise (table), and which correlates well with IEA measurements of plasma antithrombin (fig 2).

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