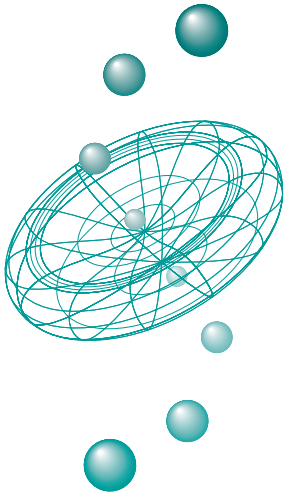
REVIEW ARTICLE



Diagnosis of Antithrombin Deficiency

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INTRODUCTION

Venous thromboembolism is a common and potentially lethal disease. Thrombosis and associated pulmonary embolism has a worldwide annual incidence of around 1 in 1,000 individuals, and represents a major cause of morbidity and mortality, contributing towards more than 50,000 deaths each year in the United States[1]. These authors showed that risk of recurrence at 12 months was 12.9%, and was 30.4%, ten years post first thrombosis. There is no internationally accepted definition for the term 'thrombophilia', but it is generally used to describe subjects with a predisposition to (usually venous) thrombosis. Thrombophilia may be an inherited or acquired condition – an inherited defect may be identified in approximately 25-30% of unselected patients with thromboembolic episode[2]. In 1965, antithrombin deficiency was diagnosed as the first condition to cause inherited thrombosis, subsequently a wide variety of defects have been associated with heritable thrombophilia[3].

ANTITHROMBIN

In 1939, when the anticoagulant activity of plasma was being investigated, Brinkhous, et al. speculated "….the substance in question might be defined as one which together with heparin not only prevents the formation of thrombin but also destroys any thrombin which may be present."[4]. The first subject diagnosed with familial thrombosis had antithrombin deficiency[5], and the author provided evidence that antithrombin III and antithrombin II (heparin cofactor) were the same substance. Antithrombin III is now simply named antithrombin[6] and it is recognised to be a serine protease inhibitor which inhibits thrombin, factors Xa, IXa, XIa, XIIa, kallikrein, plasmin, urokinase, trypsin[7], and factor VIIa is when in complex with tissue factor[8]. The majority of carriers of antithrombin gene mutations have venous thrombosis by middle age[6].

Antithrombin is a glycoprotein with molecular-weight 58, 200, the plasma concentration is approximately 125mg/L (2.3µM), and half-life is around 65 hours[9], although half-life of antithrombin in infused concentrate may be considerably shorter than this. Antithrombin comprises 432 amino acids and has four glycosylation sites: at Asn96, Asn135, Asn155 and Asn192, which may have variable sialic acid content, and a minor proportion of antithrombin (antithrombin beta) is not glycosylated at Asn135, having 25-30% less carbohydrate content, compared to the major fraction, antithrombin alpha[9, 10]. Antithrombin beta comprises 5-10% of the total, and has increased heparin-binding, which makes this isoform bind very strongly to the vessel wall, resulting in it being

the primary inhibitor of thrombosis in the circulation[11]. Antithrombin displays numerous major and minor peaks when investigated using crossed isoelectric focusing[9].
Antithrombin binds to specific sulphate groups on the pentasaccharide structure of heparin and becomes activated by more than a thousand-fold. The activated clotting factor then binds to antithrombin and cleaves the reactive site of antithrombin, which is termed P_1-P_1', and is the peptide bond between Arg 393 and Ser 394[12]. Antithrombin and enzyme then form a stable 1:1 proteolytically inactive complex.

ANTITHROMBIN DEFICIENCY

Antithrombin deficiency can be congenital, or acquired. Causes of acquired deficiency include thrombosis (including disseminated intravascular coagulation), liver disease, nephrotic syndrome[13], heparin therapy, and treatment with L-asparaginase[14]. *In vitro* studies suggest that antithrombin functions as a negative acute phase reactant protein, and levels may fall during infection[15]. Antithrombin activity level but not antigen concentration is moderately decreased in insulin dependent diabetics, compared to normal controls, and antithrombin activity correlates inversely with glycosylated plasma proteins[16]. Inherited deficiency of antithrombin has a prevalence of around 1/3000, although this prevalence could be higher if assays with greater sensitivity to functional variants were used[6]. Two major types of deficiency are recognised: type I is characterised by parallel reduction in activity and antigen levels in plasma whilst qualitative type II is defined by discrepant activity and antigen levels. Three subtypes of qualitative defects are recognised depending on whether the mutation affects the heparin binding site (HBS), reactive site (RS) or has pleiotropic [multiple] effects (PE). Unlike the other variants, heterozygous type II HBS antithrombin deficiency has reduced risk, possibly even no increased risk of thromboembolic episodes[6]; whereas the homozygous state is associated with both venous and arterial thrombosis; other types of homozygous antithrombin deficiency are believed to be incompatible with life. The Antithrombin Mutation Database (www.med.ic.ac.uk/divisions/7/antithrombin) currently identifies 80 distinct point mutations and 12 partial/whole deletions resulting in type I antithrombin deficiency, whilst distinct point mutations causing types RS, HBS and PE, presently number 12, 12 and 11, respectively.

MEASUREMENT OF ANTITHROMBIN ACTIVITY

Assays of antithrombin must measure the protein's function, as many deficient patients have type II deficiency, where antithrombin is quantitatively normal by antigen assay but activity level is significantly reduced. Choice of functional assay must take into account best practise, where precision and sensitivity to all functional defects is maximised. The type of assay in general use is chromogenic assay, as these assays can be very precise, a necessity for antithrombin measurement where the normal range is very narrow, the lower limit of normal is only around 20% lower than the population mean level. Accuracy is aided by use of standards which have been properly calibrated in international units per millilitre/decilitre. Designing an assay sensitive to all defects is not a simple achievement, as antithrombin function is measured after activation by heparin, which is followed by an initial bonding to and then enzymatic cleavage of antithrombin and formation of a stable inactive antithrombin-enzyme complex. Heparin differentially enhances the activity of antithrombin, thus reducing the effect of other inhibitors. Heparin co-factor II inhibits thrombin and is an additional (confounding) factor in antithrombin assays, especially when human thrombin is used as enzyme and incubation times with plasma are more than 30 seconds[17]. To significantly reduce this interference, which can cause over-estimation of antithrombin concentration, bovine thrombin can be used in place of human thrombin, or factor Xa can replace thrombin altogether. Bovine thrombin reacts minimally with heparin co-factor II, and heparin co-factor II does not react with factor Xa at all, resulting in more reliable detection of antithrombin deficiency, compared to an assay incorporating human thrombin[18]. Thrombin-based antithrombin assays must not be used in patients receiving hirudin, or other direct thrombin inhibitors, since antithrombin levels can be substantially overestimated[19].
In a U.K. National External Quality Assurance Scheme (U.K. NEQAS) survey, a sample from a patient with antithrombin Wobble[20] showed a mean antithrombin level of 50.2iu/dL using bovine thrombin-based assays (n=60) and 48.0iu/dL with factor Xa-based assay (n=110); in contrast, the mean level for the 30 laboratories using human thrombin was significantly higher, and was 61.8iu/dL (personal communication). In contrast, a sample from a subject with protein S deficiency but with normal antithrombin level gave comparable results by all three assays (***Table 1***).
Factor Xa-based assays of antithrombin, may be normal in the presence of two type II RS defects. The first is antithrombin Cambridge II[12], where the variant antithrombin (Ala384Ser) is cleaved by thrombin and fac tor Xa, but only factor Xa is successfully trapped by

Table 1 *Antithrombin Level (iu/dL) by Enzyme*

	Bovine Thrombin	Human Thrombin	Factor Xa
PS deficiency	102.0	102.8	105.5
AT Wobble (Thr85Lys)	50.2	61.8	48.0
n	60	30	110

Results from U.K. NEQAS survey. A sample from a subject with antithrombin deficiency (Antithrombin Wobble), and a sample from a subject with protein S deficiency were distributed to participant laboratories and results analysed according to which enzyme was used in the assay. Antithrombin level was significantly higher in plasma from the subject with AT Wobble when measured using human thrombin, when compared to assays using bovine thrombin and factor Xa, concordant results were seen in the subject with protein S deficiency and normal antithrombin level.

antithrombin as an inactive complex. The second, antithrombin Denver (Ser394Leu) is a P1´ reactive site variant of antithrombin that has slightly reduced inhibition rate constant for factor Xa (3.8 fold), but greatly reduced inhibition rate constant for thrombin (310 fold), when heparin is present[21]. Our experience using Sysmex CA-6000 analyser, is that both variants have significantly reduced antithrombin levels when measured using bovine thrombin based assay (Dade Behring assay, Sysmex, Milton Keynes, UK), compared to an assay based on factor Xa (Coamatic antithrombin kit, Quadratech, Epson, UK) and antigen assay, which are concordant. To maximise sensitivity to heparin binding site variants, a short, 30 second incubation of sample dilution with enzyme (in the presence of low concentration of heparin) has been recommended for antithrombin assays, as heparin binding site variants may not be detected with longer incubations[3, 22, 23]. ***Fig. 1*** shows antithrombin assays carried out on Sysmex CA-6000 analyser and Dade Behring kit, with thrombin incubation time from 1 to 5 minutes, tests were performed on four normal subjects plasma and from three subjects with genetically confirmed type II HBS antithrombin deficiency (two with antithrombin Toyama – Arg47Cys, and one with antithrombin Geneva - Arg129Gln). These results demonstrate the requirement for a short incubation time of antithrombin with thrombin to allow detection of heparin-binding variants.

MEASUREMENT OF ANTITHROMBIN ANTIGEN

Functional antithrombin assays must be used to initially detect deficiency, but immunological assays may assist in sub classification of antithrombin defects. Functional assay must be used to monitor treatment of patients receiving antithrombin concentrates, where the presence of degraded antithrombin molecules with reduced heparin-binding may be associated with discrepancies between functional and immunological levels[24]. Many immunological assays for antithrombin are in use, including Laurell rocket immunoelectrophoresis, enzyme linked immunosorbant assay and radial immunodiffusion, and recently an automated immuno-turbidimetric assay has become available (Diagnostica Stago). Although antithrombin activity levels are generally measured as a relative concentration (in iu/mL or iu/dL related to the amount of antithrombin in a pooled normal plasma), immunological levels of antithrombin are sometimes reported as a relative or an absolute concentration (mg/dL or mg/L); for comparability and consistency between different thrombophilia screening tests, relative concentrations are recommended

Heparin-binding studies and crossed immuno-electrophoresis with and without heparin may be used to identify molecular variants, particularly to help distinguish type II HBS and RS variants[25, 26].

QUALITY CONTROL PROCEDURES AND REFERENCE RANGE FOR ANTITHROMBIN ACTIVITY ASSAYS

Good assay design should be employed for all investigations. The most precise results are likely to be obtained using a 'live' antithrombin standard curve when diagnostic assays are being performed, although a stored curve may be used to monitor therapy, as long as quality control values are within acceptable limits. Calibration curves typically comprise four dilutions of reference plasma, three of the dilutions being in the range 0.4-0.9iu/mL. Drift in calibration curve data may occur over relatively short time periods. Assay precision should be evaluated to determine whether duplicate or replicate testing is required, although duplicate testing is not required on Sysmex analysers, abnormal results should

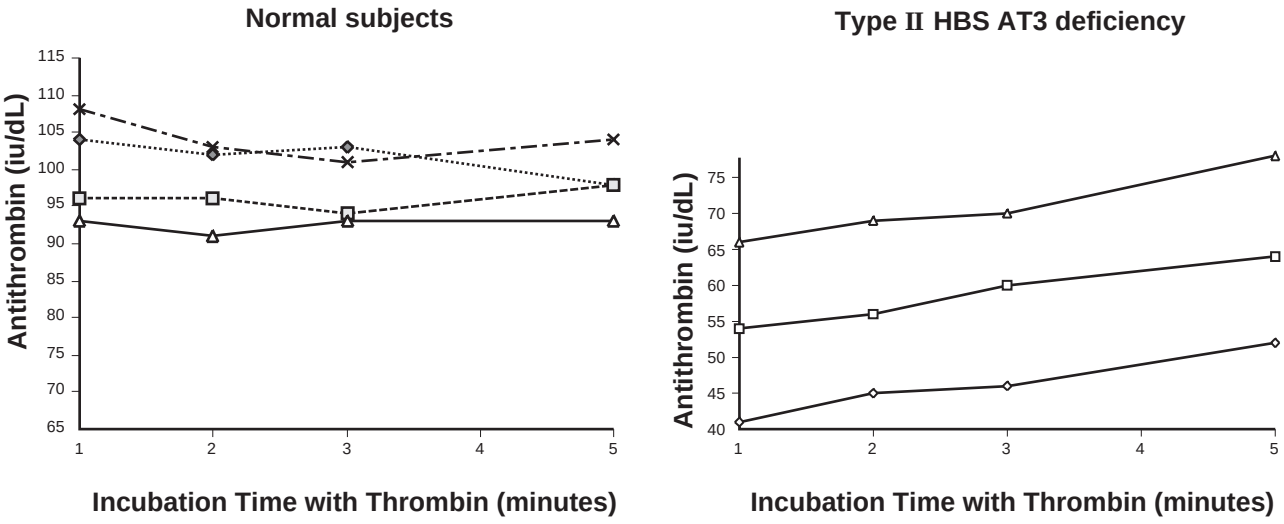

Fig. 1 *Antithrombin assays carried out on Sysmex CA-6000 analyser and Dade Behring kit, with thrombin incubation time varied from 1 to 5 minutes, tests performed on four normal subjects and three subjects with genetically confirmed type II HBS antithrombin deficiency.*

always be checked. Reference plasmas should either be obtained from carefully calibrated commercial sources, or from institutions such as the U.K. National Institute for Biological Standards and Controls (NIBS&C), and in either case, results must be reported in International Units per mL, or, dL. It is important to be aware that the assigned potency may only be accurate for the method(s) used to calibrate the standard plasma. Quality assurance should include internal quality control to ensure within and between-run precision; normal and abnormal level controls should be employed for each group of tests and results should be plotted to identify trends, we always include quality controls both at the beginning and end of each run, and within the run when testing more than twenty patient samples. Participation in external quality assessment schemes helps assess accuracy and allows between-laboratory comparison, and may identify method-related differences. The reference range for antithrombin should be based on the 95th percentile as distribution does not follow a normal, or, log-normal pattern[27]. The author's adult reference range includes assayed levels from approximately eighty healthy volunteers, although an absolute minimum of 40 donors has been recommended to construct a reference range, and a group of more than 120 donors is preferable[28]. Age, sex, oral contraceptive use, circadian variation may all influence antithrombin level. However, for practical purposes, it is acceptable to use a single (adult) reference range which includes similar numbers of men and women, taking no account of circadian rhythm, but hormone use should be taken into account when interpreting results. Very low levels antithrombin levels are found in neonates, and we refer to data from the literature to interpret these results[29]. Antithrombin activity is consumed during coagulation, and low levels are found in clotted samples. Interpretation of patient's results should be made with due regard to analytical and clinical variables to ensure inappropriate diagnoses are avoided.

A ROUTINE APPROACH TO ANTITHROMBIN TESTING

The following approach to the measurement of antithrombin is applied in the author's laboratory. We routinely use a Sysmex CA-6000 analyser with Dade Behring antithrombin assay kit. Sample dilutions are prepared using Sysmex System Buffer (Owren's veronal buffer), which may be sampled from a bottle outside the main instrument, but on newer analysers (CA-1500 and CA-7000), buffer is taken directly from an internal reagent position. To increase sensitivity to heparin binding site defects, we use a (shortened) one-minute incubation of sample dilution with bovine thrombin reagent.
Our current normal range for antithrombin activity is 0.84iu/mL to 1.16iu/mL, based on assay of levels in 79 normal subjects, tested over a number of days, and reference range set between 2.5th and 97.5th percentiles. We avoid testing plasma from subjects with on-going or recent thrombosis. We test citrated plasma, which has been stored in small aliquots below –35°C, and thawed at 37°C in a waterbath (once, only) for no more than 5 minutes. The following guidelines are used for issuing chromogenic antithrombin levels: the calibration curve must be observably linear and quality control results within limits (limits set at +/- 2 standard deviations from the mean of at least 12 runs); then results greater than 0.90iu/mL are issued without comment; results less than 0.90iu/mL are checked by re-introducing the sample to the analyser; antithrombin less than 0.80iu/mL are checked to confirm that the sample is plasma, not serum. Samples with antithrombin less than 0.90iu/mL are assayed for antithrombin antigen level and results issued if activity and antigen agree; if activity and antigen levels are discrepant, both are checked and results issued if in agreement, re-checked if there is an anomaly (***Fig. 2***).

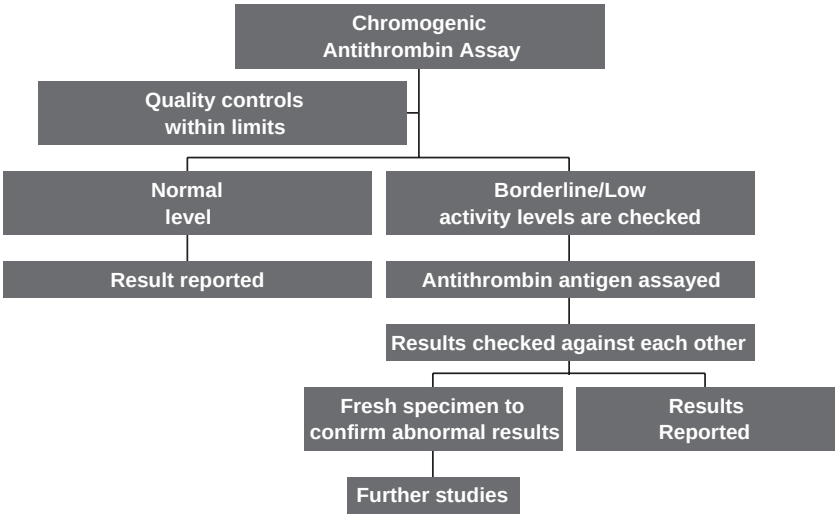

Fig. 2 *Procedure in the author's laboratory for the acceptance of antithrombin assay results*
Antithrombin activity results on patients are considered valid when quality control values are within acceptable limits, and borderline and low levels are checked and compared with antigen level (ELISA) – discrepancies are followed up by re-assay of both activity and antigen level and then results can be reported.

This approach of checking and re-checking is rigidly adhered to and has picked up analytical errors on a number of occasions. Complex analysers make highly accurate analysis of large numbers of samples possible, but analysers are prone to complex problems. We consider that duplicate antithrombin levels should be no more than around 5% apart, if excess imprecision is suspected, we assay antithrombin 10 times on plasma with normal antithrombin level and consider that a lack of precision is present when the coefficient of variation is greater than 2% in this run. Typically, we observe a coefficient of variation of around 1.3-1.9%, a normal sample showed levels varying by no more than 0.03iu/mL when the coefficient of variation was 1.3%, and results varied by as much as 0.06iu/mL when the coefficient of variation was 1.9%. An unacceptable coefficient of variation of 2.5% was associated with duplicate results varying from 0.83iu/mL to 0.90iu/mL. We consider it to be advantageous to analyse runs of at least twenty patient samples, with daily calibration. Large batches are economical in terms of time and reagents, and a trend to higher or lower results on an individual day can detect errors of accuracy even when values on the quality control plasmas are within 'acceptable' limits.

CONCLUSIONS

Antithrombin is activated by heparin, and binds to serine protease, which cleaves antithrombin and then becomes trapped in a stable 1 to 1 inactive complex. Type I antithrombin deficiency results from lack of production, or increased turnover, whereas type II deficiency arises from mutations causing failures of function. In order to screen for all types of antithrombin deficiency, the assay must be functional, specific, precise and sensitive. Assays using human thrombin may lack specificity, being influenced by heparin cofactor II, and can overestimate antithrombin concentration. Thrombin-based assays must not be used to test plasmas from subjects treated with direct thrombin inhibitors, as they have been shown to overestimate antithrombin concentration, whereas factor Xa-based assays are specific. Sensitivity to antithrombin Cambridge II and Denver is an issue with factor Xa-based assays, as they may not be detected using this type of assay, but are detected using bovine thrombin-based assay, in the presence of heparin. Antithrombin deficiency, with the exception of heterozygous type II HBS, is associated with a greatly increased risk of venous thrombosis. Patients receive counselling, anticoagulation at times of heightened thrombotic risk, and may be treated with antithrombin concentrate. Specific, precise and sensitive techniques to measure antithrombin are therefore essential in order to reliably detect all forms of antithrombin deficiency. Clinical and therapeutic conditions, along with other pre-analytical variables must be taken into account when interpreting results.

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