Comparison of three methods for the estimation of plasma antithrombin

DS McLELLAN, JD DEVLIN, GH HEYSE-MOORE,* AND A ARONSTAM

From the Treloar Haemophilia Centre, Lord Mayor Treloar Hospital, Alton, Hants, UK

SUMMARY Plasma antithrombin levels were measured by clotting, immunological, and amidolytic methods on two groups of subjects: 20 normal individuals and nine patients studied serially post-operatively (hip replacement). The postoperative patients were observed for the emergence of deep-vein thrombosis using ^{125}I -fibrinogen uptake measurements (FUT).

The three methods gave similar ranges for the normal subjects, were reproducible ($\text{cv} < 5\%$), and detected early postoperative reduction of antithrombin levels. All three methods failed to show any significant differences in preoperative antithrombin levels between the positive and negative FUT groups.

Correlation studies were performed on the pooled data from the normal and postoperative group (range 60-130% of normal; 100 samples). The best correlation ($r = 0.75$; $P < 0.01$) was achieved with the chromogenic kit assay method versus the Mancini immunoassay technique. The thrombin agarose (total antithrombin) gel diffusion technique correlated less well with the chromogenic ($r = 0.65$; $P < 0.01$) and Mancini immunoassay ($r = 0.45$; $P < 0.01$) methods. It is concluded that the chromogenic kit method gives a rapid, reproducible, and specific measurement of antithrombin III. The thrombin agarose diffusion method, though not specific for antithrombin III, is a cheap and simple method to perform. The potential of the three methods for detecting the prethrombotic stage and early thrombosis is discussed.

Current interest in the relationship between thrombosis and antithrombin levels is complexed by the variety of methods available for the measurement of this natural anticoagulant¹ and the variable reliability of methods for the detection of thrombosis.²

Comparisons of clotting methods with immunoassays for the measurement of plasma antithrombin levels have at times given conflicting results. Bounameaux *et al.*³ showed no significant correlation between two clotting methods and two immunoassay procedures. In contrast, Frigola⁴ and Abildgaard *et al.*⁵ showed good correlation ($r = 0.8$).

The emergent amidolytic methods claim to measure antithrombin III activity specifically, and have previously been shown to correlate well with both immunoassay and clotting procedures.⁶⁻⁸ Gaffney *et al.*⁹ have, however, shed some doubts on

the specificity of artificial chromogenic substrates in experiments with degraded thrombin preparations.

Variable loss of antithrombin components and altered method precision after defibrination—via heating, clotting, or Arvin treatment—have been reported.^{6-8,10} The validity of comparing 'defibrinated' clotting methods with 'plasma' antithrombin methods is therefore dubious.

In this study the total progressive antithrombin assay method of Lane *et al.*,¹¹ which does not require defibrination, was compared with the 'plasma' antithrombin III specific Mancini radial immunodiffusion¹² and Kabi chromogenic substrate (S2238) technique.^{13,14} Postoperative samples were chosen for part of the study to give as wide a range of antithrombin levels as possible and to compare the sensitivity of the three methods to the previously reported postoperative drop in antithrombin levels.^{15,16}

The emergence of postoperative thrombosis was monitored by ^{125}I -fibrinogen uptake testing (FUT).

*Present address: Princess Elizabeth Orthopaedic Hospital, Wonford Road, Exeter, Devon.

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Three plasma antithrombin methods

Antithrombin levels on the FUT positive and negative groups were compared using the three methods.

The antithrombin methods chosen rely on three different measurement principles: clotting, immunological, and amidolytic.

Material and methods

SAMPLING

Blood samples were collected with minimal stasis by venepuncture with a plastic syringe and 21 G needle into 1/10 volume 3.8% sodium citrate. The samples were centrifuged at 2000 *g* at 4°C for 20 minutes. The top two-thirds of the platelet-poor plasma was harvested into plastic tubes and stored immediately at -40°C until tested (within three months of sampling).

SUBJECTS

Two groups of individuals were tested:

Normal subjects

Twenty normal individuals of both sexes (12 men and 8 women) aged 18 to 50 years of age. To our knowledge, none of the women was taking high oestrogen level oral contraceptives.

Postoperative subjects

Nine patients undergoing major hip surgery tested before operation and daily for up to 10 days after operation.

STANDARDS

Equal aliquots of freshly collected, citrated plasma from 20 healthy individuals were pooled and deep frozen at -40°C. This pool of normal plasma was used as a 100% reference plasma for the three procedures used.

IMMUNOASSAY TECHNIQUE

Antithrombin III (α_2 globulin) was measured using the Mancini¹² radial immunodiffusion technique.

THROMBIN-AGAROSE GEL DIFFUSION TECHNIQUE (TAD)

A method based on the Lane *et al.*¹¹ two-stage technique for measuring total progressive antithrombin was used. In this technique antithrombin inhibition of thrombin clotting is measured using an agarose gel medium.

CHROMOGENIC SUBSTRATE TECHNIQUE

The method used was a two-stage assay kit (Coatest-Kabi Diagnostics) using the artificial substrate S-2238.^{13 14}

PROCEDURE STAGE I

A 1/60 dilution of test plasma and serial dilutions of reference plasma (spanning the range 25-125% of average normal) were made in pH 8.4 EDTA buffer containing excess heparin. The dilutions were mixed with thrombin and incubated for 30 seconds at 37°C.

PROCEDURE STAGE II

The chromogenic substrate S-2238, in the presence of polybrene, was used as the substrate for residual thrombin. *p*-Nitralinine (*p*-NA) released from the substrate H-D-Phe-Pip-Arg-*p*-NA was measured photometrically at 405 nm using the end-point procedure (ie, stopping the reaction at 30 seconds with 50% acetic acid).

The amount of thrombin inhibited by the antithrombin III-heparin complex is proportional to the amount of antithrombin III present.

DETECTION OF DEEP VEIN THROMBOSIS

- (1) One hundred milligrams of KI was given orally 24 hours preoperatively and 100 μ Ci of ¹²⁵I-fibrinogen was given intravenously at least 4 hours preoperatively. Radioactivity was measured over the operated leg from mid-thigh and over the other leg from groin to ankle at 2 in (5 cm) intervals, and readings were expressed as a percentage of the reading over the heart. Measurements were done on the day before operation and daily thereafter for at least five days except in the case of patient 4, in whom only four postoperative measurements were made. A positive FUT scan was recorded when the reading was raised by 25% over that of the previous day and maintained for more than 24 hours.
- (2) Doppler flow ultrasound scan of femoral vein was carried out daily.
- (3) Clinical examination of the lower limbs was carried out daily.

REAGENTS

Immunoassay technique

Partigen antithrombin plates were used (Boehringer Diagnostics).

Thrombin agarose gel diffusion method

- (1) Agarose (Miles Labs Ltd, Slough)
 - (a) 2.0% in isotonic saline
 - (b) 0.6% in distilled water
- (2) Human Thrombin (Ortho Diagnostics—Fibrin-dex) was reconstituted with 1 ml of isotonic saline to give 50 NIH units/ml, further diluted 1/10 to 5 U/ml in plastic tubes immediately before use.
- (3) Human Fibrinogen (Lister Institute, Elstree) was reconstituted and diluted in 0.85% saline to give

a concentration of approximately 2.5 mg/ml clottable fibrinogen.

S-2238 Chromogenic substrate method

The reagents used were as supplied in the kit (Coatest-Kabi Diagnostics).

- (1) Heparinised diluent buffer—A Tris/EDTA/NaCl buffer adjusted to pH 8.4 with HCl. Heparin added to make a final concentration of 3 IU/ml.
- (2) Thrombin—0.14 mol/l NaCl containing 5 g/l carbowax 6000 to give a final thrombin concentration of 10 NIH units/ml.
- (3) Substrate S-2238—D-D-Phe-Pip-Arg-pNA-2HCl 25 mg in 25 ml distilled water (0.75 mmol/l).
- (4) Polybrene—1.0 mg/ml in distilled water.
- (5) 50% acetic acid.

Results

NORMAL SUBJECTS

The mean, standard deviation, and range ($\bar{x} \pm 2$ SD) for the 20 normal subjects tested by the three methods are shown in Table 1. The results are in close agreement, and the ranges conform to the widely reported tight normal range for antithrombin III.

Table 1 Mean and normal range of antithrombin ($n = 20$) assay methods

Method	Mean	SD	Range ($\bar{x} \pm 2$ SD)
Mancini (% agarose)	99.1	8.4	82.3-115.9
S-2238 (% activity)	100.4	9.7	81.0-119.8
Chromogenic thrombin			
agarose (% activity) diffusion	99.2	9.5	80.2-118.2

METHOD CORRELATIONS

In order to gain the widest range of values for comparison, the results from the normal subjects and from the nine patients tested before operation and serially postoperatively were pooled. This resulted in 100 samples ranging from 60-130% of average normal. The scatter diagrams and regression lines for comparison of the three methods are shown in Figures 1-3. The best correlation was obtained between the S-2238 chromogenic substrate method and the Mancini immunoassay ($r = 0.75$; $P < 0.01$).

The thrombin agarose diffusion method, although correlating significantly ($P < 0.01$) with the other two methods, had a lower correlation coefficient ($r = 0.61$ and 0.45) with the chromogenic and immunoassay methods respectively. In order to test whether the inclusion of 'pathological' samples affected the method correlations, additional corre-

Fig. 1 Antithrombin correlation: chromogenic substrate against immunoassay ($n = 100$). Dashed line denotes identity.

Fig. 2 Antithrombin correlation: agarose diffusion against chromogenic substrate ($n = 100$). Dashed line denotes identity.

lations were performed on the normal subject group. Figure 4 shows that the correlation between the chromogenic substrate and immunoassay methods is very good ($r = 0.85$; $P < 0.01$). The TAD method correlated badly with both the immunoassay ($r = 0.013$, not significant) and chromogenic substrate ($r = 0.055$, not significant) methods.

METHOD PRECISION

A limited replication analysis was performed to examine whether the poorer correlation of the TAD method could be explained by differences in method precision. The results are shown in Table 2. These results indicate that all methods have an acceptable

Fig. 3 Antithrombin correlation: agarose diffusion against immunoassay ($n = 100$). Dashed line denotes identity.

Fig. 4 Antithrombin correlation: immunoassay against chromogenic substrate (normal samples $n = 20$).

Table 2 Precision of antithrombin assays

Method	Mean (x)	SD	Coefficient of variation	n
Mancini (% agarose)	99.4	4.4	4.4	10
S-2238 (% activity)	98.9	1.91	1.9	10
Chromogenic thrombin agarose (% activity) diffusion	102	3.0	2.9	24

coefficient of variation, although the data suggest that the chromogenic substrate method is the most precise.

The replicates on the chromogenic and immunoassay methods were day-to-day, while the data on the TAD method comprised eight replicates on each of three plates (ie, intra and inter assay).

POSTOPERATIVE STUDIES

Daily antithrombin values were measured before operation and serially postoperatively using the three methods. All of the patients had preoperative values within the normal range when measured by the chromogenic substrate and immunodiffusion methods. Two patients (1 and 9) had slightly elevated levels by the TAD method (see Table 3).

The mean changes in antithrombin from the baseline for each day, using each method, are shown in Figure 5. These results demonstrate a significant reduction in antithrombin by all three methods on the first day after operation. Student's paired t test gave $P < 0.01$ for both the chromogenic substrate and Mancini immunoassay methods and $P = 0.02$ for the TAD method.

POSITIVE V NEGATIVE FIBRINOGEN UPTAKE TESTS

Four of the nine patients who underwent hip surgery showed positive evidence of deep vein thrombosis within three days of surgery, as demonstrated by FUT.

The antithrombin results of the positive and negative FUT groups (see Table 3) were compared for all three methods. None of the methods showed a significant difference between the two groups for the first day postoperative drop in antithrombin values (Wilcoxon rank sum test). The TAD method showed the greatest group mean difference with a drop of 20% in the positive FUT antithrombin level compared with only 8% for the negative FUT group.

When the differences between the antithrombin levels on day 1 and days 3 and 4 were examined for the TAD method on the two graphs a significant difference ($P < 0.05$) was noted. All four patients in the positive FUT group showed $> 25\%$ increase in values between the first and fourth postoperative samples, with an average rise of 47%. By comparison, the negative FUT group had an average rise in antithrombin value of only 6% with a maximum difference of 22% (see Table 3).

Discussion

The above results show that the three methods tested are reproducible. The close correlation obtained between the S-2238 chromogenic substrate method and the Mancini immunoassay in both normal and pooled subjects ($r = 0.85$ and 0.75 respectively) agrees with the previously reported

Table 3 Plasma antithrombin levels on the preoperative, 1st day postoperative and 4th day postoperative samples of the FUT positive and negative groups of patients

FUT	Patient	Antithrombin assay								
		Chromogenic substrate			Mancini immunoassay			Thrombin agarose diffusion		
		Preop	Day 1 post	Day 4 post	Preop	Day 1 post	Day 4 post	Preop	Day 1 post	Day 4 post
Positive	1	115	84	106	94	74	94	124	91	118
	2	101	86	98	100	88	105	86	84	128
	3	106	93	109	105	88	100	92	70	126
	4	94	76	112	86	66	96	88	60	120
Negative	5	86	84	102	82	82	94	94	87	109
	6	82	64	91	88	68	88	94	94	104
	7	102	68	89	105	86	100	104	70	86
	8	115	113	121	110	105	105	120	120	120
	9	107	90	103	104	96	112	132	136	116

Fig. 5 Postoperative change in antithrombin values. Results were derived from observations in nine patients undergoing hip surgery.

close correlations for these two principles.^{7,8} In our hands, the precision experienced with the chromogenic method was better than has been previously reported for amidolytic methods by Blombäck *et al.*⁷ and Odegård.¹³

The significant but poorer correlation of the TAD method with the other two methods could result from reduced assay precision or specificity differences of the assay. In view of the demonstrated adequate precision of the TAD method (Table 2) the second mechanism appears the more likely. Lane *et al.*,¹¹

using the TAD method, showed that the method measures α_2 -macroglobulin and α_1 -antitrypsin in addition to the antithrombin III- α_2 -globulin component of total progressive antithrombin. Using immunoabsorption experiments, it was shown that total progressive antithrombin comprises 47% antithrombin III, 29% α_2 -macroglobulin, and 26% α_1 -antitrypsin. It must be pointed out that Lane's figures were produced using bovine thrombin, as have most of the reported studies on clotting procedures. In our experiments, human thrombin was used, which, although less sensitive than bovine thrombin in terms of inhibition zone size, appeared the more suitable regarding specificity. The use of human thrombin does not appear to have adversely affected the precision of the assay.

Previous studies of antithrombin levels in postoperative patients have given varying results. Stathakis *et al.*¹⁵ demonstrated significantly reduced levels in 39 patients undergoing major abdominal surgery. Conversely, Donati *et al.*¹⁷ reported no significant alteration in 17 patients undergoing hysterectomy. The consensus, however, indicates a significant drop in levels from day 1 after operation, returning by day 8 to baseline.¹⁶ Our findings confirm this view, although in our study baseline values were reattained by the fourth day after operation. It is possible that the differences in reported studies may, in part, be attributable to differences in the site and extent of the operative procedure.

Immunological methods for measuring antithrombin III have been shown to be insensitive to reductions in biological activity of the protein in certain situations. Sas *et al.*,¹⁸ investigating the variant hereditary antithrombin III variant, Budapest, which has a low biological activity and gives rise to a high thrombotic tendency, found normal antigenic levels by the Mancini immunodiffusion technique.

Bounameaux *et al.*³ demonstrated a similar discrepancy in a woman whose antithrombin III biological activity dropped when she was taking an ethinyl oestradiol contraceptive pill whereas the Mancini immunodiffusion antithrombin values remained unchanged. No discrepancies were noted using the Mancini method in our postoperative study. In fact the method gave a higher level of statistical significance for the initial drop in antithrombin levels than did the other two methods studied.

There is a strong association between reduced levels of antithrombin III and the development of thrombosis.¹⁹ This is thought to be related to the anti- $\bar{X}a$ activity of the protein.²⁰ None of the postoperative patients in the present study who developed positive FUT scans had preoperative baseline values below the normal range for any of the three methods used. Thus, although antithrombin levels < 80% are high risk for thromboses, the converse is not necessarily true.

Theoretically, the less specific total antithrombin method might be expected to give the better index of thrombosis in the postoperative situation. This is because the α_2 -macroglobulin component contributes significantly to antiplasmin activity, which has been reported to be reduced in the immediate postoperative period,²¹ and might therefore augment the antithrombin III drop in the thrombotic situation. Similarly, α_1 -antitrypsin, an acute phase reactant, might be expected to augment the secondary rise of antithrombin III. It is, therefore, interesting that a significant difference in postoperative values between day 1 and day 4 was noted for the positive FUT group using the TAD method. This significant finding is a compound of three trends: the apparent greater drop of antithrombin by day 1, the more rapid increase, and the 'rebound' effect, elements which in isolation failed to achieve significance.

The number of postoperative patients studied in this pilot project is too small for reliable statistical conclusions to be drawn. However, the limited findings generally support the conclusions of a recent major trial,²² which showed that antithrombin III estimations in individual patients are unlikely to be of great value in the early detection of thrombosis. The value of the total antithrombin method should be reassessed as part of a larger study.

In practical terms, the chromogenic substrate (S-2238) kit method has the advantages of rapid reproducible measurement of antithrombin III using a colorimetric end-point system, with the option of automating the method. However, the method would require routine regular usage to justify the costs of the labile reagents. The Mancini immunoassay is slightly cheaper to perform than the unmodified chromogenic method, although the

end-point takes longer to develop—not always a disadvantage. The possibility of measuring non-active antithrombin III proteins restricts the use of this method. The thrombin-agarose gel diffusion method is straightforward procedurally and the cheapest of the techniques to perform. It, too, has the debatable disadvantage of an overnight incubation delay in achieving an end-point. The method's lack of specificity for antithrombin III may have clinical advantages as outlined.

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- Requests for reprints to: DS McLellan, Haemophilia Laboratory, Lord Mayor Treloar Hospital, Alton, Hants, UK.