

Modification of Functional Serum Antithrombin III Assay for Fibrometer

Results of Examination of 50 Patients

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ABSTRACT

The functional antithrombin III assay of von Kaulla is described and modified for the fibrometer with substitution of readily available Owren's buffer for previously described buffers. The assay is compared with immunological techniques on 50 hospital patients and comparable discrimination of normals and patients with thrombotic disease are shown. Normal values are 51.1 ± 13.9 secs. Results in patients with thrombotic diseases are 16.7 ± 1.8 secs.

Introduction

Clotting may be initiated by the action of collagen on Factor XII or by the action of Factor VII on tissue thromboplastin. Through either mechanism, Factor X is activated and bound, and thrombin is rapidly evolved. Thrombin has a profound impact on the entire clotting mechanism. Apart from direct fibrinogen cleavage, thrombin accelerates the activity of Factors V, VIII, and XIII as well as potentiates the aggregation of platelets.³ The effect, then, of freely circulating thrombin is potentially catastrophic. Antithrombin III may be of critical importance in the control of thrombin both locally and freely circulating. Antithrombin III exerts its effect directly on thrombin¹ as well as inhibiting thrombin formation with the inhibition of activated Factors X,¹⁴ IX, XI, and XII.⁷

The laboratory detection of prethrombotic or hypercoagulable states depends upon the evaluation of the kinetics of thrombin formation as well as the activity of antithrombins present. Von Kaulla et al have described a panel of laboratory tests useful in the laboratory detection of hypercoagulable states. The panel includes a thrombin generation test, antithrombin III activity, a fibrinogen level, a Factor VIII level, a platelet count and platelet aggregation studies.¹³ The most valuable test in the panel is the thrombin generation test. It quantitates hypercoagulability. However, some intensive training in the performance of this test is required and this generally places the test beyond the capacity of smaller, non-specialized laboratories.

The second test of great value is the antithrombin III activity. A useful labora-

tory method modified from Zuck¹⁵ and adaptable to smaller, nonspecialized laboratories (such as in the author's 175 bed suburban general hospital) is reported. A group of 50 patients, a number with thrombotic complications owing to oral contraceptives, were examined with the modified functional antithrombin III assay and the results compared to an immunologic assay for antithrombin III.

Methods

Antithrombin III activity was assayed in two fashions. In the first, the functional assay was performed by the method of Von Kaulla and Von Kaulla,¹¹ modified for an electrical clot detection instrument* by Zuck.¹⁵ The assay is further modified in the laboratory by substitution of readily available buffers for those originally described. Additionally, the assay was also evaluated on an optical clot detection instrument.† In the second, the immunologic assay was performed by radial immunodiffusion.‡

Ten ml of blood were drawn into 12 × 75 mm nonsiliconized glass tubes. The blood was allowed to clot at room temperature for two hours. The specimen was then centrifuged for five min at 3500 × *g*. Serum was removed for immediate assay by the functional technique. If the assay were to be done at a later time, the serum was immediately frozen in polypropylene tubes at -30°. Freezing significantly prolonged the functional antithrombin III assay time but it was corrected by appropriate buffering in the test system.

Topical thrombin § was dissolved in 50 percent w/v glycerol in distilled water to reach a concentration of 200 units per ml.

This was mixed well and allowed to stand at room temperature for four hrs. Aliquots were frozen at -30° and remained stable for several weeks when not thawed. One ml thrombin solution was diluted with 1.3 ml buffered saline for use in the test system. Buffered saline was prepared by mixing one part Owren's buffer with four parts normal saline. The working thrombin solution was stable in ice for four hrs.

For the purposes of this study only, Michaelis barbital buffer and TRIS buffer were used in place of Owren's for evaluation. A 1.0 percent w/v fibrinogen reagent was also made with buffered saline. The fibrinogen¶ was dissolved in buffered saline and 1 mg reagent grade barium sulfate was added for each ml of final solution. The mixture was stirred for several minutes and double centrifuged until clear. This too was frozen at -30° and was stable for several weeks.

Testing was performed by placing 0.8 ml serum into a 12 × 75 mm glass tube and warming in a 37° heating well. Exactly 0.2 ml working thrombin solution was added and the interval timer started. After five min of incubation, 0.1 ml of the 1.0 percent fibrinogen reagent was placed into a cup suitable for clot detection and was warmed. After exactly six minutes of incubation, 0.2 ml of the generation mixture was transferred to the cup and the instrument timer started. A control was prepared by incubating 0.8 ml buffered saline and 0.2 ml working thrombin solution in a glass tube in a heating well at 37°. Exactly 0.1 ml fibrinogen solution was added into suitable cups and was warmed for 30 sec. The generation mixture was added as described. The instrument timer was started. Control times ran between four and seven sec.

Immunologic testing was performed on both plasma and serum. The immunodif-

* Bioquest fibrometer.

† Sherwood coagulyzer.

‡ Behring Diagnostics (Antithrombin III AC-CUPAK).

§ Parke-Davis.

¶ Sigma.

fusion plate was removed from the refrigerator where it was stored at 4°. The plate was allowed to stand open at room temperature for five min. Protein standard plasma supplied with the plate was reconstituted with 0.5 ml distilled water. Solutions of 1:1, 1:2 and 1:4 dilutions were prepared with distilled water for construction of a calibration curve. Patient specimens were diluted 1:2 with normal saline. Each well received either 5 λ of standard or specimen. The plates were allowed to stand in a horizontal position at room temperature and read at 48 hrs. The square of the diameters of the precipitin rings was graphically compared against the known concentrations of antithrombin III. A linear relation was seen at 48 hrs. Patient values were then obtained in the same fashion from the calibration curve.

Results

Fifty patients were examined with these methods. Buffering systems to bring the standardized thrombin solution to pH 7.6 (TRIS), pH 7.42 (Michaelis barbital buffer), and pH 7.35 (Owren's) were examined in the antithrombin III functional assay. Between run standard deviations of one second were seen. In run standard deviations of less than one second were also seen. Both types of clot detection instruments were used with equal ease and accuracy though occa-

sional failure of clot detection by optical methods was noted.

The 50 patients included seven males, 19 females who were well and not taking oral contraceptives and 20 females who were well and taking oral contraceptives. Each of the females had a history of migraine-like headaches at some point during the course of oral contraceptive therapy. Four females were not taking oral contraceptives and had deep vein thromboses; three males had deep vein thromboses. No patient was on coumadin.

Immunologic determination of antithrombin III activity after 48 hrs diffusion and the functional serum antithrombin III assay correlated well with clinical presentation. In patients with thrombotic disease, the functional assay revealed very low antithrombin III levels of 16.7 ± 1.8 secs (normals, 51.1 ± 13.9 secs). By immunodiffusion, normal values were 20.2 ± 2.4 mg per dl in serum and 26.3 ± 3.3 mg dl in plasma. In patients with thrombotic disease, the levels were 13.1 ± 2.5 mg per dl in serum and 16.7 ± 3.1 mg per dl in plasma (table I).

Discussion

Alteration of the balance between thrombin generation and its inactivation by antithrombin III may relate to clinical hypercoagulability. A low level of antithrombin III activity does not imply that

TABLE I
Determination of Antithrombin III Activity

Patient Groups		Functional Serum Assay Seconds			Immunologic Assay mg per dl	
		Owren	Michaelis	Tris	Plasma	Serum
No medications	mean	51.1	50.7	50.1	26.3	20.2
	S.D.	13.9	13.7	13.6	3.3	2.4
Oral contraceptives	mean	19.2	19.1	18.8	16.6	12.9
	S.D.	3.1	3.2	3.5	3.4	2.8
Thrombosis	mean	16.7	16.5	16.2	16.7	13.1
	S.D.	1.8	1.8	1.9	3.1	2.6

thrombin has actually been generated but rather indicates a loss of a protective mechanism against thrombin traces in the blood. The presumption is that once clotting is initiated, it proceeds rapidly. A low level of antithrombin III activity does not invariably lead to thromboembolism. Thromboembolic complications have not been reported in affected persons below the age of ten years.

The existence of people with low levels of antithrombin III activity, many of whom develop deep vein thrombosis and thromboembolism, supports the biological significance of antithrombin III.^{4,6,8,9,10,12} Acquired deficiency of antithrombin III with attendant risk of thrombotic manifestations has been described in women on oral contraceptives,^{5,13,16} in persons with cirrhosis² and in people of advancing age.² Additionally, where active clotting is proceeding, antithrombin III levels should be decreased.^{1,2}

Antithrombin III can be measured by both a functional and immunologic assay. The functional antithrombin III assay presented offers the advantages of semiautomation, easily available reagents, reliable clinical correlation and rapid performance times. The assay measures the capacity of serum to neutralize progressively a known amount of thrombin. The rate of clotting is dependent upon the residual thrombin of the incubation mixture and is inversely related to the antithrombin III activity present. A standardized fibrinogen solution is used to promote clotting in the test system. The functional assay shows excellent correlation with the immunologic assay. The findings described suggest that the test system may discriminate a symptomatic predisposition to thrombosis in the same manner as it discriminates patients with clinical thrombosis. The test system should be available in any laboratory.

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