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TECHNICAL INFORMATION

Catalog Number: 101141, 154163
Thrombin

Source: *Bovine plasma*
Unitage Definition: The unit defined by the U.S. standard and is approximately equal to the stated unitage. A U.S. unit is defined as the amount required to clot 1 ml of standardized fibrinogen solution in 15 seconds. Approximately 2 U.S. units are required to clot 1 ml of oxalated human plasma in the same period of time. U.S units = NIH units.
Recommended Storage: 0ºC or colder and is for *in vitro* diagnostics only
Physical Description: White powder
Description: Bovine Thrombin is prepared from fresh bovine plasma by an ion exchange method. Fibrinogen is removed from the prothrombin complex by precipitation. The concentrated prothrombin complex is then activated by beef thromboplastin and calcium. The thrombin solution is clarified by centrifugation and filtration and then lyophilized. The final product has an activity of approximately 100 NIH units (= U.S. units) per mg protein[8].
Inhibitors: AEBSF, PMSF, Antithrombin, TLCK, 3,4-dichlorocoumarin, leupeptin
Quantitative Determination of Plasma Fibrinogen using Thrombin
Summary:
Fibrinogen (Factor I) is a plasma protein which is synthesized in the liver. Conversion of fibrinogen, a soluble monomer, to fibrin which then polymerizes to form an insoluble clot, is the final step in the coagulation "cascade"[1]. This conversion is accomplished by the enzyme thrombin. Using this phenomenon, plasma fibrinogen can be isolated from plasma by thrombin and directly quantitated. Elevated levels of plasma fibrinogen occur in a variety of conditions associated with acute inflammation or tissue necrosis. This protein is thus one of the "acute phase reactants"[2]. Elevated levels also occur in pregnancy or during the administration of estrogen-containing oral contraceptives[3]. Depressed levels of plasma fibrinogen may occur in severe chronic liver disease[1] or with disseminated intravascular coagulation[4]. In this latter syndrome, fibrinogen concentration is severely depressed because coagulation factors and platelets are consumed due to widespread deposition of fibrin-platelet thrombi in arterioles and capillaries; a severe, life-threatening hemorrhagic diathesis results. Congenital afibrinogenemia is a rare inherited disorder in which fibrinogen is not synthesized. The concentration of plasma fibrinogen may be estimated indirectly by a modification of the thrombin clotting time[5]. However, direct measurement is accomplished using thrombin in the "clottable protein" assay[6].
Principle of the "Clottable Protein" Assay
Since the formation of a coagulum of insoluble fibrin is the end result of the action of thrombin on fibrinogen, this phenomenon has been used to determine fibrinogen concentration in plasma. Platelet-poor plasma is obtained from blood which has been anti-coagulated with citrate. The addition of thrombin and calcium begins the coagulation of fibrinogen to form fibrin which is wound on a glass rod. An excess of thrombin is used to assure complete conversion of fibrinogen to fibrin. The coagulum is then removed, thoroughly washed to remove soluble protein, dissolved in alkali, and the protein measured using the colorimetric biuret reaction[7]. A standard curve of protein concentration versus absorbance is constructed using serial dilutions of a standard solution of known protein concentration. The absorbance obtained from the dissolved fibrin clot is converted to protein concentration using the standard curve.
Specimen Collection, Preparation and Storage
Blood is collected aseptically by approved medical techniques into 12 x 75 mm test tubes containing citrate anticoagulant. Centrifugation at 2000 X g for 15 minutes at 5ºC will result in platelet-poor plasma. The specimen of platelet-poor plasma should then be tested promptly during the same working day. Endogenous anticoagulants, e.g. fibrin split products, or exogenous anticoagulants, e.g. heparin, may interfere with the test. These effects will usually be overcome by the use of excess thrombin which is incorporated into the test procedure.
Procedure
A. Thrombin: use bovine thrombin as a lyophilized powder.
B. Supplementary Materials Required

– Test tubes (12 x 75 mm)
– 37ºC water bath
– Boiling water bath
– 10% NaOH
– Biuret reagent
– Protein standard solution
– Glass stirring rods
– Calcium-Epsilon Amino Caproic Acid Buffer (2.5 g EACA, 5 ml of 0.075 M Barbital buffer pH 8.6, 2.4 g $CaCl_2$ Dihydrate brought to 100 ml in saline)
– Metal forceps

C. Treatment Required before Use

– Mixing:

Add distilled water to the thrombin for a final concentration of 1000 units/ml. Mix gently to avoid foaming.

D. Clottable Protein Assay
The following is one useful method that is a modification of referenced methods[1,6]. Other methods may also work and give satisfactory results. MP does not guarantee the success of this procedure.

– Add 0.5 ml of citrated plasma to 1.5 ml of Ca-EACA buffer in 12 x 75 mm test tubes in duplicate. Place tubes in a 37°C water bath.
– Add 0.1 ml of thrombin solution (0.2 ml if patient is on heparin) and a glass stirring rod. Twirl rod until strands of fibrin are seen. Let stand of 5 minutes.
– Remove the rod and gently squeeze it against the upper side of the tube to remove excess fluid.
– Wash the rod with attached clot under a gentle stream of saline (1 minute) followed by distilled water (1 minute).
– Peel the clott of the rod gently with metal forceps so that the clot comes off as a sleeve. Place the clot into a clean 12 x 75 mm test tube. Add 0.5 ml distilled water and 1.0 ml 10% NaOH. Place in boiling water bath for 3 minutes or until clot is digested. Let cool for at least 10 minutes.
– Prepare blank by adding 0.5 ml of distilled water to 1 ml of 10% NaOH; heating and cooling is performed as in Step 5.
– Prepare controls by adding 0.5 ml of the high (500 mg/dl) and 0.5 ml of the low (25 mg/dl) standards respectively to 1 ml of 10% NaOH; heating and cooling as in step 5. (See section E for standard protein solution preparation).
– Add 2.0 ml of Biuret reagent to all tubes. Let stand at room temperature for 30 minutes.
– Read absorbance of samples and controls against blank in a spectrophotometer at wavelength 540 nm. Concentration is then obtained from the standard curve.

E. Preparation of Standard Curve for Biuret Reaction

– A bovine albumin standard protein solution of known concentration (MP catalog number 810291) should be used.
– The standard should be serially diluted to obtain at least six concentrations ranging from 800 mg/dl to 25 mg/dl protein.
– At least five tubes should be run at each concentration. Each point on the standard curve is an average of these five determinations. Plot absorbance against protein concentration.
– The curve should approach linearity and intersect the ordinate at 0 concentration.

NOTE: A new standard curve should be prepared for each new batch of biuret reagents. High and low controls are included with each run as a check of the standard curve. Values obtained for controls should be ± 10% of expected values. Other controls should include normal fresh plasma from donors with known stable plasma fibrinogen levels. Determinations on these control specimens should be recorded on quality control charts so that the means and standard deviations may be calculated. The coefficient of variation should not exceed 10%.

Expected Results
Normal values for plasma fibrinogen vary greatly but the generally accepted normal range is 200-450 mg/dl of plasma[9,10].

Limitations of the Procedure
Accurate results are limited by the assay range of the standard curve (25 - 800 mg/dl). If unusually high levels are obtained, the digested clot may be diluted in half before biuret determination and the results multiplied by two. If abnormally low values are obtained, other tests should be used to determine whether disseminated intravascular coagulation is present, e.g., prothrombin time, partial thromboplastin time, detection of fibrin split products, Fastor V assay, platelet count, and erythrocyte morphology. If congenital afibrinogenemia is suspected, family studies should be instituted.
Circulating anti-coagulants or anti-thrombins may also cause falsely low values.
Diseases and drugs which alter the level of plasma fibrinogen have previously been outlined in the summary.

Specific Performance Characteristics
The clottable protein assay is a widely accepted clinical laboratory method for the direct quantitation of plasma fibrinogen. It has been in use in most coagulation laboratories for over two decades[1].
Although thrombin is a proteolytic enzyme which will cleave other proteins, its action in the final step of coagulation is specific for fibrinogen. The method is sensitive down to levels of 25 - 50 mg/dl; lower levels require further testing to rule out disseminated intravascular coagulation.
Since the method does not involve the prior purification or enrichment of fibrinogen from plasma prior to coagulation, some protein may be non-specifically trapped in the clot and cause falsely elevated levels. Thus, if other tests strongly suggest disseminated intravascular coagulation, the fibrinogen level may be indirectly estimated using a modified thrombin clotting time[5].
According to the literature, the coefficient of variation for samples with a normal level of fibrinogen may be 2.5%[11].

Availability:

Catalog Number	Description	Size

101141	Thrombin, activity > 100 NIH units/mg protein	2 KU 10 KU 25 KU
154163	Thrombin, activity > 1500 units/mg protein	1 KU 5 KU 10 KU 50 KU 100 KU

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