THROMBOLYSIS IN MYOCARDIAL ISCHEMIA

MANUAL OF OPERATIONS

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CHAPTER 22

PROCEDURES FOR COAGULATION STUDIES

22.1 INTRODUCTION

The T3 study utilizes the resources of several discrete units. The Principal Investigators and Research Nurse/Coordinators of the Clinical Centers involved in the T3 trial are responsible for drawing and processing the appropriate blood samples from their patients. These samples will be sent to the Coagulation Core Laboratory (CCL) at the University of Vermont which will be responsible for performing selected assays within a short period after receipt. The CCL will also develop a sample repository so that future testing may be done on this important patient group. The samples will be accessioned from the repository for approved analysis.

22.2 TIMING OF BLOOD SAMPLES

Blood samples will be collected at the following times:

1. Before infusion of t-PA;
2. Fifty minutes after the initiation of infusion of t-PA;
3. Twelve hours after the initiation of infusion of t-PA;
4. Twenty-four hours after the initiation of infusion of t-PA;
5. Forty-eight hours after the initiation of infusion of t-PA; and
6. Ninety-six hours after the initiation of infusion of t-PA. (The 96-hour blood samples should be obtained, if possible. However, if a patient is discharged prior to 96 hours, obtain a sample on the day of discharge, if feasible. It is not necessary for a patient to return as an outpatient to obtain the 96-hour sample unless the patient is returning for another reason.)

Two tubes (5 ml and 10 ml lavender) will be collected at all time points. In addition, a 4.5 ml sodium citrate tube will be collected at 12, 24, 48, and 96 hours. The pre, 12, 24 and 48 hour times correspond to protocol draw time for enzymes as shown on T3 Form 19.

The blue-top citrate tube is stable at room temperature and at 4°C.

Ideally, when a patient is entered into the study, the draw tube packets (to be used for blood collection during the first 96 hours after entry) should be removed from cold storage for that patient.

22.3 COLLECTION, PROCESSING, AND LABELING

All blood samples for the basic assays (fibrinogen, FDP, and heparin) and for the sample repository will be collected in three tubes (5 ml and 10 ml lavender containing EDTA/PPACK/Aprotinin and 4.5 ml blue containing sodium citrate). Both "draw tubes" and "mailing tubes" will be provided by the Coagulation Core Laboratory.

No other lavender tube should be substituted for the lavender top tube supplied by the Coagulation Core Laboratory. The tube supplied by the Core Laboratory contains a special anticoagulant that is not available for commercial use and is critical to the T3 samples. Tubes from the Core Laboratory should be stored in the refrigerator prior to use.

After drawing the blood, make every effort to mix the tubes, especially the lavender tube, by gentle inversion for at least 30 seconds before putting it on ice to insure proper mixing of the anticoagulant with the blood to avoid clotting.

All samples collected except the preinfusion sample should be spun down as soon as possible and no later than 30 minutes after collection. The only exception is the preinfusion sample which may be left on ice for processing with the second set of specimens.

If it is not feasible to fill all collection tubes, the priority for filling tubes will be the two 5 ml tubes first and then the

10 ml tube second. At times, it may be necessary to substitute two 5 ml lavender tubes for the one 10 ml lavender tube.

22.4 PROCESSING

All tubes are spun at 2,000 - 3,000 G for 10 minutes at either room temperature or at 4°C.

The plasma is transferred by pipet to the plastic mailing tubes as follows:

1. Plasma from the blue citrate draw tube is transferred to the green mailing tube.
2. Plasma from the lavender draw tubes (either three 5 ml tubes or one 5 ml and one 10 ml tube) is aliquoted into three lavender mailing tubes. First pipet approximately one ml plasma (from any of the lavender draw tubes) into one lavender mailing tube. Then split the remainder of the plasma from all the lavender draw tubes into two lavender mailing tubes (approximately 3 mls in each).

Make sure all mailing tubes are correctly labeled with T3 patient ID Number, Name Code, date and time of draw. It is also helpful to include time point such as '12 Hr' or '50 Min.'

The samples are frozen upright at -20°C or preferably at -70°C.

22.5 MAILING OF THE BLOOD SAMPLES

Each Clinical Center should store blood samples until specimens for four to five patients have been accumulated but for no more than one month before shipping to the Coagulation Core Laboratory.

The Coagulation Core Lab will supply the Clinical Centers with mailing containers. These containers can hold approximately 80 tubes (samples for four to five patients) and will need approximately five pounds of dry ice for each shipment. This may vary depending on the distance of the Clinical Center from Vermont.

Shipments should be sent by Federal Express with overnight delivery specified. Tubes should be grouped by time point with a rubber

band and all samples from one patient put into a ziplock bag. Half the dry ice should be placed in the bottom of the container. The samples in ziplock bags should be placed on top of the dry ice in the container, and the remainder of the dry ice put on top of the samples.

The blood samples record form (Form 20), one for each patient, and transmittal form (Form 51) should be enclosed with each shipment.

The correct mailing address for the Coagulation Core Laboratory is given in the T3 Address Directory.

Samples should be mailed on Monday, Tuesday, or Wednesdays ONLY.

22.6 COAGULATION CORE LABORATORY FORMS

22.6.1 Blood Sample Record Form (Form 20)

This form provides information regarding the samples obtained on each patient. It should be mailed to the Coagulation Core Laboratory at the same time as the samples, preferably in a separate envelope contained in the shipping container. A copy is also sent to the Data Coordinating Center.

22.6.2 Blood Samples Transmittal Form

Along with the blood samples, the Transmittal Form should be mailed to the Coagulation Core Laboratory. This form lists all samples enclosed in the shipment. A copy should be sent to the Data Coordinating Center.

22.7 RECEIVING SAMPLES AT COAGULATION CORE LABORATORY

Staff at the Coagulation Core Laboratory (CCL) ensure that all samples are still frozen by visual inspection. If not, they are immediately placed in the 70°C freezer to refreeze. The time and date the shipment arrived, whether samples were frozen, and the presence of dry ice are noted on the Blood Sample Transmittal Form.

Each patient is assigned a VTC number according to a Master List using the next available consecutive number. This number is

written on the Blood Sample Transmittal Form and on the top of the Blood Sample Record Form along with the date received.

Blood Sample Record Forms are filed numerically by VTC number with a corresponding master data sheet attached. Assay results are entered on this data sheet for entry into the computer data base.

Samples are removed from the shipping box one patient at a time and identified according to ID Number and Name Code. The VTC number is written on all tubes. Any problems (mislabeling, hemolysis, etc.) are written directly on the appropriate Blood Record Form. Care is taken that the samples remain frozen during this process.

For each shipment received from a Clinical Center, an acknowledgement form will be completed and returned to the Clinical Center. The top of the acknowledgement form is completed noting the Clinical Center, person who sent the shipment, number of boxes received and date received. A list of samples received for each patient is prepared. Any problems encountered with the samples are transferred to this acknowledgement form from the Blood Sample Record Form. The original acknowledgement form is sent with the shipping box to the Clinical Center and a copy is kept in the CCL files.

The styrofoam shipping boxes including new draw tube packets are repackaged in new cardboard outer sleeves and are returned to the Clinical Center by UPS.

Patient information is entered into the computer data base when samples are received. An inventory of samples received is electronically transmitted every two weeks to the Data Coordinating Center. Errors concerning patient identification are flagged at the Data Coordinating Center. A listing of any errors found and possible corrections to the patient identification are sent to the CCL. After reviewing possible corrections from the Data Coordinating Center in conjunction with the original material sent from the Clinical Center and if necessary, contacting the Clinical Center staff, the data base is updated with the correct information.

All data from assays are entered into the computer after samples are analyzed. After review and final approval of data, data are transmitted electronically to the Data Coordinating Center followed by hard copy of the assay data sent by mail. Periodically, a hard copy of the data file from the Data Coordinating Center's data base is sent to the CCL for review.

22.8 ALIQUOTING PROCEDURES

Before samples stored in the mailing tubes can be used for assays, they are thawed and centrifuged at 10,500 RPM for 15 minutes at 4°C then transferred by pipette to four, 1 ml cryogenic vials (eight vials for the 10 ml lavender tubes).

These vials are identified by VTC #/time point drawn/type of tube. For example, 435/12/L would be patient # 435, the 12-hour time point, lavender tube.

Vials are stored in a specially-made freezer inventory system which consists of cryogenic boxes approximately 4x4x2 inches each holding 81 vials, and steel inventory towers each holding nine boxes. Twenty-one towers fit into a -135°C freezer.

Each vial is assigned a matrix location within the box and tower which is noted in special files and on the computer. Any sample can be easily located from any of the six freezers with this method.

22.9 FIBRINOGEN ASSAY

22.9.1 Principle

Fibrinogen, a soluble plasma protein, is converted to fibrin, an insoluble polymer of fibrinogen, in the presence of thrombin. The thrombin clotting time of diluted plasma is inversely proportional to the fibrinogen concentration of the plasma. Based on Clauss' theory, the Dade method for determining functional fibrinogen concentration is based on the clotting time of plasma using 100 NIH units/ml of thrombin. A BBL fibrometer is used.

2.9.2 General Procedure

Samples are diluted 1:10 with assay buffer and prewarmed for 2-5 minutes at 37°C. Thrombin (.1 ml) is added while activating the timer/probe of the BBL fibrometer. The probe senses the formation of a fibrin thread, and the timer stops. Each sample is run in duplicate; the average number of seconds from the two laboratory measurements is used to convert to mg/dl fibrinogen concentration from the standard curve.

22.9.3 Standard

A standard curve is made using Dade Fibrinogen Calibration Reference at three different dilutions. These samples are run over a period of three days to obtain a good mean value. Standards are rerun when a new lot of thrombin or control is used, or controls are consistently out of range when plotted on a Levy-Jennings plot.

22.9.4 Controls

Controls are run approximately after every ten samples. Controls used are normal, pooled plasma and Data Ci-Trol Level 1. Two different dilutions are used and run in duplicate. Controls are acceptable if they fall within a pre-defined range.

22.9.5 Special Notes

Lavender tubes require pre-incubation of one hour at 37°C to hydrolyze excess PPack. Calcium must be added to the assay buffer when assaying lavender tubes.

22.10 DETERMINATION OF FIBRINOGEN DEGRADATION PRODUCTS (FDP)

For the T3 study, the CCL staff have developed an ELISA assay for the measurement of FDPs. Like the other assays, it does not discriminate crosslinked from fibrinogen-based products, but rather acts as a screening assay for fibrin(ogen) breakdown. The advantages of this assay are two-fold: first, results are obtained as a continuous variable. The other FDP assays are dilutional analyses yielding a "titer" which is interpreted as fixed, discrete values. The ELISA assay

uses a standard curve allowing results to be obtained in a continuous manner. Second, the ELISA format allows the assay to be adapted to the Micro Assay System Robots, greatly increasing through-put and efficiency.

The basic design of the ELISA assay is simple. Microtiter plates are coated with fibrinogen, and a fixed amount of anti-fibrinogen polyclonal antibody (Sigma, St. Louis, MO) is added. This antibody has been conjugated to horseradish-peroxidase by standard methods. After incubation of three hours at room temperature, the excess solution is washed off and substrate for peroxidase added. In the absence of added standard FDPs (prepared by lysis of plasma with a known amount of fibrinogen) or unknown thrombin-clotted plasma (to remove all clottable fibrinogen reactivity), concentrations of solid phase fibrinogen and anti-fibrinogen have been adjusted such that an absorbance at 405 nm of approximately 1.5 is reached in 30 minutes. The presence of competing fibrinogen reactivity from standards or clotted plasma results in a concentration-dependent decrease in absorbance, as less antibody binds to the solid phase.

22.11 HEPARIN PHOTOMETRIC ASSAY

22.11.1 Principle

Heparin forms a complex in the presence of antithrombin-III. This complex exerts an inhibitory effect on various coagulation proteins such as Xa and IIa. When an excess of Xa and IIa is added to the complex, there is a certain amount of the serine protease that is not inhibited. The residual protease is then mixed with a chromogenic substrate specific for Xa and IIa. The intensity of color development (e.g., paranitroanaline formation) is inversely proportional to heparin concentration.

22.11.2 General Procedure

Citrated platelet-poor plasma is used for these assays. Plasma is diluted in a buffer from which AT-III has been added. Fifty

(50) uL of this mixture is incubated with Xa (two minutes) or IIa (one minute) in a microtiter plate. Then 50 uL of substrate is added, and the reaction is stopped with acetic acid (after one minute with Xa, or after three minutes with IIa). The plate is then read in a plate reader at 405 nm.

22.11.3 Standard

Standard (Hepanorm) which are furnished by Diagnostica Stago are used to make up the standard curve. There are four points: 0, 1, 2, and 6 U/ml of heparin. Standards are run in triplicate.

22.11.4 Controls

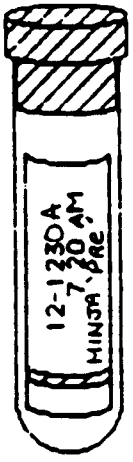
Controls are made up using platelet-poor plasma supplied by the Red Cross. Heparin is added to give heparin concentration of 0.5 U/ml. This in turn is diluted to give heparin concentrations of 0.25 and 0.125 U/ml. These controls are run in duplicate on each plate.

22.11.5 Special Notes

AT-III, enzymes, and chromogenic substrate are supplied by Diagnostica Stago.

EXHIBIT 22-1

BLOOD COLLECTION INSTRUCTIONS



MOST IMPORTANT !!!!

LABEL EVERY TUBE WITH:

* T3 PATIENT # 12-123
* T3 NAMECODE
* TIME OF COLLECTION
* DATE OF COLLECTION

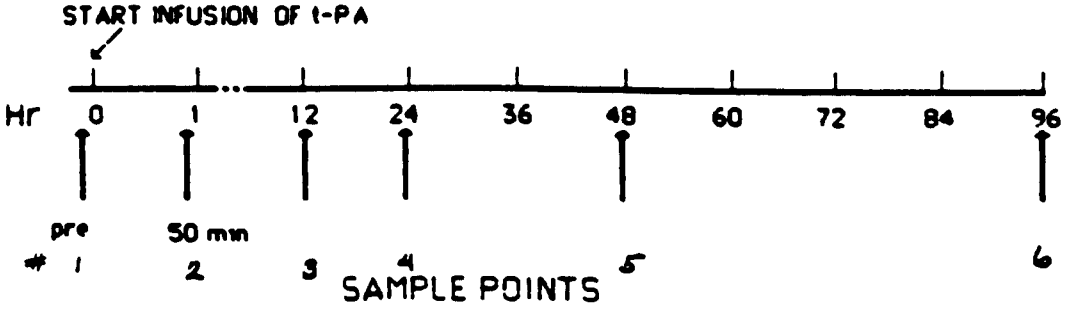


EXHIBIT 22-2

SEPARATE "DRAW TUBES" AND "MAILING TUBES" IMMEDIATELY, TO AVOID CONFUSION