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HEPTEST®

Product No. 830

Clotting Procedures for the Quantitative Determination of Heparin in Plasma and Whole Blood

For *In Vitro* Diagnostic Use

INTENDED USE

The HEPTEST® assays provide a sensitive, simple, rapid, and convenient in vitro quantitation of heparin, low molecular weight heparins, and heparinoids in plasma and whole blood.

BACKGROUND

Heparins are a group of highly sulfated mucopolysaccharides commercially isolated from porcine intestinal mucosa, and beef lung. Commercial heparin preparations are chemically heterogeneous, with molecular weight ranging from 4000 to greater than 25,000. Heparin by itself exhibits little anticoagulant activity. It requires a plasma cofactor, antithrombin III, for its anticoagulant activity. Specifically, heparin is a catalyst that accelerates the neutralization of activated clotting factors such as factor Xa and thrombin.

PRINCIPLE

The fundamental principle of the HEPTEST assays is the ability of heparin to catalyze the inactivation of exogenous bovine factor Xa by antithrombin III in the presence of naturally occurring plasma antagonist(s). The rate of factor Xa inhibition is directly proportional to the concentration of heparin present. This is indirectly measured by the prolongation of the recalcification time of the plasma sample. The HEPTEST assay consists of incubating an undiluted plasma or whole blood sample with an equal volume of factor Xa for a fixed time period at 37° C. This reaction mixture is then recalcified by the addition of RECALMIX® a reagent containing optimal concentrations of calcium chloride and brain cephalin in a bovine plasma fraction rich in factor V and fibrinogen. The time it takes the plasma mixture to clot (in seconds) is then converted to units of heparin per mL/ plasma using a previously constructed standard calibration curve.

REAGENTS SUPPLIED

Unopened vials are stable until the expiration date on the kit when stored at +2° - +8°C. Each HEPTEST kit contains sufficient reagents to perform the number of tests stated on the package label. The kit consists of factor Xa, lyophilized and stabilized in a buffer containing glycine, mannitol, PEG, NaCl and Tris-maleate, at pH 7.5, and RECALMIX, lyophilized and buffered, pH 7.5, which contains calcium chloride, brain cephalin, factor V and fibrinogen. **WARNING:** Do not mix different lots of any one reagent from kit to kit. Use only the reagent lots specific for each HEPTEST.

REAGENTS REQUIRED, BUT NOT SUPPLIED

Normal Human Plasma (NHP, Product No. 8306): To be used exclusively in the preparation of a standard heparin calibration curve for the HEPTEST assay.

Normal Control Plasma (NCP, Product No. 8300): To be used as a daily normal control in the HEPTEST assay.

Abnormal Control Plasma (ACP, Product No. 8301): To be used as a daily abnormal control in the HEPTEST assay.

Heparin Solution: To be used in establishing a standard calibration curve. Use the same brand, and if possible, the same lot number of pharmaceutical heparin (USP) as that employed in your institution, with a potency of no less than 1000 units per mL.

REAGENT PREPARATION AND STABILITY

factor Xa

Reconstitute with 2.0 mL of distilled water. Due to the trace amount factor Xa in the vial, inject the water through the rubber stopper with a sterile needle and syringe. Invert or vortex the vial to dissolve

the contents, and allow to equilibrate for 5 to 10 minutes at room temperature before use. Reconstituted reagent is stable for 8 hours at +25°C, one week at +5°C, and up to two weeks at -20°C. Once frozen, the reagent should be thawed only once. When thawed, allow to equilibrate at +25°C for 5 to 10 minutes.

RECALMIX

Reconstitute with 2.0 mL of distilled water. Mix gently or vortex to dissolve the contents. Reconstituted reagent remains stable for two hours at 37°C, 8 hours at 25°C and up to one week at -20°C. Once frozen, the reagent should be thawed only once. If a precipitate appears upon thawing at 37°C, do not centrifuge the reagent. The precipitate is largely due to the presence of insoluble fibrinogen which will not interfere with the assay.

SAMPLE COLLECTION AND PREPARATION

Plasma Sample

Mix nine parts of blood with one part of 3.8% sodium citrate. Citrated blood should be centrifuged within two hours of collection at a minimum of 2500 x g for 20 minutes, at +2° - +8°C. Remove the platelet poor plasma and either store at +2° - +8°C for up to 24 hours or at -20°C if it is not to be tested immediately.

Whole Blood Sample

Whole blood collected in sodium citrate or sodium oxalate may be used. The samples must be well mixed either manually (by gentle inversion 12 times) or on a rotator/ mixer prior to testing. Samples may be stored at +2° - +8°C for up to 24 hours. **Do not freeze.**

NOTE: HEPTTEST cannot be performed on serum samples unless they are diluted in NHP to supplement prothrombin requirements.

QUALITY CONTROL

A normal and abnormal control test should be performed daily with each test run. Each laboratory must establish its own control ranges to account for the inherent variability in reagents and instrumentation among laboratories (see OTHER REAGENTS REQUIRED, NOT SUPPLIED). Once an initial standard calibration curve is established, daily curve recalibrations are not necessary. This is provided that kit, control or reagent lot numbers have not been changed, and that daily controls are satisfactory.

PROCEDURE

Materials required but not provided

Manual method: 12 x 75 mm glass test tubes, 0.1 mL pipettes and tips, two stopwatches, and a 37°C water bath. If a clot detecting instrument is used, follow the manufacturer's instructions for use.

TEST PROCEDURES

NOTE: All assays are to be performed at 37°C ± 0.5°C. Prior to testing, pre-warm a convenient volume of RECALMIX for at least five minutes at 37°C. Pre-warm test plasma or whole blood for two minutes at 37°C. Factor Xa is used at 25°C.

HEPTTEST Assay (0.015 -1.0 U/ mL)

1. Pipette 0.1 mL undiluted test plasma or whole blood sample into a clotting tube.
2. Deliver 0.1 mL factor Xa to the same tube and immediately start the first stopwatch.
3. After exactly two minutes, add 0.1mL RECALMIX to the above mixture, and immediately start the

second stopwatch. Record the clotting time in seconds. If desired, convert the clotting time to units of heparin per mL of plasma using a standard heparin calibration curve (see Figure 1).

HEPTTEST HI Assay (0.15 - 5 U/ mL)

1. Pipette 0.1 mL undiluted test plasma or whole blood into a clotting tube,
2. Deliver 0.1 mL factor Xa to the same tube, and immediately start the first stopwatch.
3. After exactly 30 seconds, add 0.1 mL RECALMIX to the above mixture, and immediately start the second stop-watch. Record the clotting time in seconds. If desired, convert the clotting time to units of heparin per mL of plasma using a standard heparin calibration curve (see Figure 2).

NOTE: If the heparin level in the sample is found to be higher than the highest point on your standard curve, dilute the sample in NHP or unheparinized whole blood only, and retest it. To calculate the heparin concentration in such a sample, multiply the initial reading by the dilution factor. Do not dilute any plasma or whole blood sample in buffer. Use NHP, or unheparinized whole blood respectively.

Some automated applications are available. Please inquire.

STANDARD CURVE PREPARATION

Prepare the standard curves for HEPTTEST and HEPTTEST - HI assays using commercial heparin no less than 1000 units per mL. Initially prepare serial ten-fold dilutions of the heparin to obtain a potency of 1 unit per mL plasma or whole blood. At this point make serial two-fold dilutions down to at least 0.031 unit per mL or lower. The NHP or whole blood is considered to contain zero heparin and is to be used in place of test plasma or whole blood in the assay.

See Table 1 and Table 2 for HEPTTEST and HEPTTEST - HI dilution schemes.

Due to excessively long clotting times, the highest point on the curve should not exceed two units of heparin per mL.

Table 1

Standard Curve Dilutions for HEPTTEST ASSAY

Initial heparin (unit per mL)	Volume (mL)	NHP or whole Blood (mL)	Final Heparin (unit per mL)
1000	0.1	0.9	100
100	0.1	0.9	10
10	0.1	0.9	1.0
1.0	0.4	0.4	0.5
0.5	0.4	0.4	0.25
0.25	0.4	0.4	0.125
0.125	0.4	0.4	0.062
0.062	0.4	0.4	0.031
0.031	0.4	0.4	0.015
zero = NHP or whole blood	-	-	zero

Table 2
Standard Curve Dilutions for HEPTEST - HI ASSAY

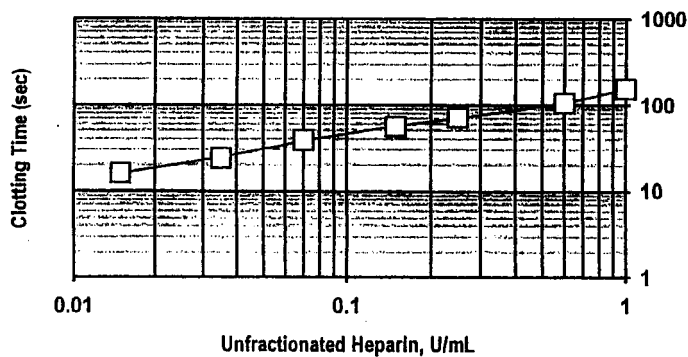
Initial heparin (unit per mL)	Volume (mL)	NHP or whole Blood mL	Final heparin (unit per mL)
1000	0.10	0.90	100
100	0.10	0.90	10
10	0.75	0.25	7.5
7.5	0.40	0.40	5.0
5	0.40	0.40	2.5
2.5	0.40	0.40	1.25
1.25	0.40	0.40	0.62
0.62	0.40	0.40	0.31
0.31	0.40	0.40	0.15
zero = NHP or whole blood	-	-	zero

PLOTTING CURVES

Standard Heparin (Unfractionated commercial heparin)

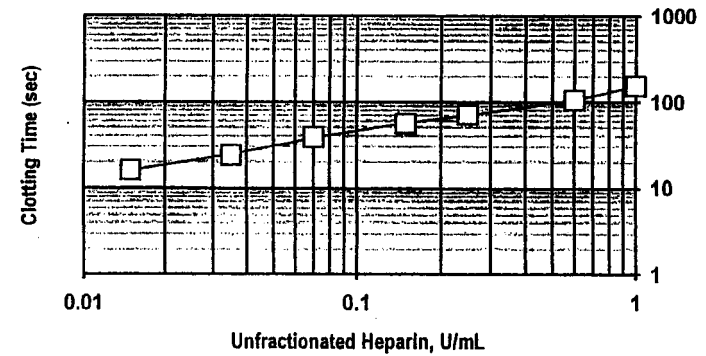
Clinical heparins are chemically heterogeneous, and there are considerable batch and brand variations. In most instances, a typical calibration curve as depicted in Figure 1 is obtainable, using a log-log plot

Heptest Assay
Figure 1



PLOTTING CURVE – continued

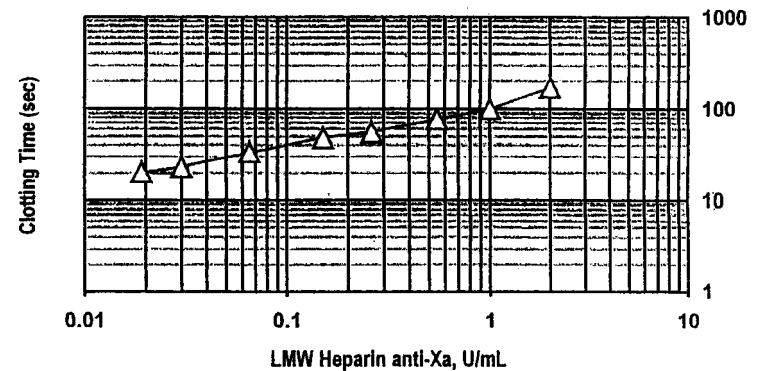
Heptest – HI Assay
Figure 2



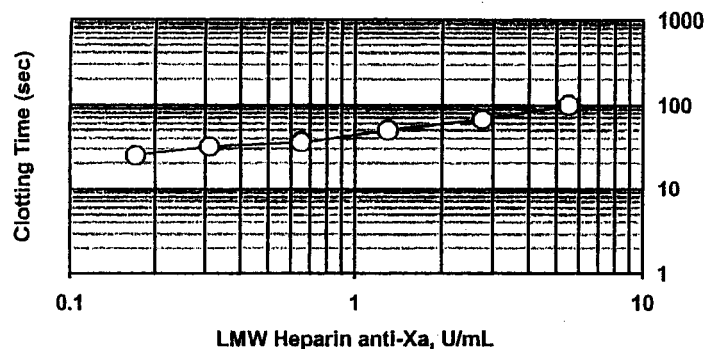
Low Molecular Weight Heparin Fractions, Fragments, and Heparinoids

The commercially available brands of low molecular weight heparins (LMWH) for clinical use are chemically heterogeneous and differ in clinical efficacy so that each compound should be considered as a separate drug. Because potency labeling practices vary from manufacturer to manufacturer, the First World Health Organization (WHO) Standard for LMWH was established in 1988. It is hoped that the availability of this standard would very soon eliminate discrepant labeling practices among LMWH manufacturers. Figure 3 depicts a calibration curve for WHO Standard for LMWH in the HEPTEST assay, and Figure 4 for the HEPTEST - HI assay. Log-log plots should be used. NOTE: The USP anticoagulant unit is not equivalent to the first International LMWH anti-Xa unit. Compare Figure 1 with Figure 4.

Heptest Assay
Figure 3



Hepetest – HI Assay
Figure 4



INTERPRETATION OF RESULTS

Results can be obtained directly from the standard calibration curve and expressed in unit of heparin per mL of plasma. For example, if a patient's plasma sample gives a clotting time of 118 seconds, it contains 0.5 unit of heparin per mL of plasma. see Figure 1.

HEPTEST results may also be reported in a) seconds clotting time and b) patient's clotting time (seconds) divided by the control to give a ratio. The latter is preferable, as it would eliminate variability in the control value when different instruments are employed. Furthermore, this would also eliminate performing a standard calibration curve for each type of heparin.

NOTE: The calibration curves depicted in Figures 1-4 are shown for illustrative purpose only, and should not be used for any other purpose. The slope of the curve may vary between laboratories using different techniques, different batches of reagents and heparin preparations. A new calibration curve must be established whenever a different lot number of reagents or controls are employed.

LIMITATIONS OF PROCEDURE

Effect of FDP, heparin antagonists and heparin enhancers: Test results on undiluted patient plasma will be influenced by a high titer of fibrinogen degradation products, heparin antagonists and heparin enhancers.

Concomitant heparin and oral anticoagulant therapies: Since the HEPTEST reaction mixture supplies all the necessary coagulation factors with the exception of factor II, a severe lowering of factor II has been shown to cause a prolongation of the HEPTEST clotting time. During the first 3 - 7 days of treatment with oral anticoagulants, factor II concentrations rarely fall below 40 %. This level of factor II would be sufficient to give a normal HEPTEST value in the presence of heparin, but would be falsely elevated when it falls below this critical concentration. THEREFORE, IT IS HIGHLY SUGGESTED THAT PLASMA SAMPLES FROM PATIENTS TREATED CONCOMITANTLY WITH HEPARIN AND ORAL ANTICOAGULANT (REGARDLESS OF THEIR PROTHROMBIN TIMES), BE TESTED AT A 1:2 OR 1:3 DILUTION IN NORMAL HUMAN PLASMA. To obtain the true heparin concentration in unit/ mL plasma, multiply the resulting value from the calibration curve by the dilution factor.

Neonates: The levels of vitamin K-dependent clotting proteins and antithrombin III are low in neonates. If they are put on heparin, treat the samples in the same manner as for those patients

receiving oral anticoagulant therapies concomitantly with heparin, in order to measure the heparin level more accurately (see above).

Anti-thrombin III deficiency: If the level of anti-thrombin III in the patient's plasma falls below the critical level of 30% of normal, a shorter, erroneous HEPTEST clotting time will result. Dilute such specimens 1:2 or 1:3 in normal human plasma prior to testing.

Heparinoids: a) Dermatans: Since the HEPTEST assay measures both anti-Xa and anti-IIa activities, the assay is sensitive to dermatans due to its anti-IIa action concomitant with heparin co-factor II. b) ORG-10172 (Organon Teknika): Patients treated with ORG-10172 have been monitored reliably with HEPTEST. The ex-vivo correlation between the anti-Xa activity and the HEPTEST is not ideal, as some of the activity initially detected by HEPTEST is rapidly metabolized and causes a lowering of the overall anticoagulant effect.

Use in Animals: HEPTEST has been employed to monitor pharmacodynamics and pharmacokinetics of low molecular weight heparins and dermatan sulfate in animals such as monkeys, rats, rabbits, and pigs. The HEPTEST clotting time of monkey plasma in the absence of heparin is comparable to the clotting time of normal human plasma, whereas the HEPTEST clotting times of rats, rabbits and pigs are considerably longer.

EXPECTED VALUES

Factor Xa and RECALMIX reagents in the HEPTEST are matched to provide a spread of over 150 seconds between the 0 and 1 Unit heparin/ mL (for porcine heparin). The zero heparin value clotting time is set between 14 and 18 seconds for normal human plasma, and depends on the assay technique. The response to *in vitro* addition of heparin varies from individual to individual. For example, when one unit of heparin is added to one mL plasma samples of two normal individuals, one sample may give a HEPTEST clotting time of 195 seconds, while the other may be only 150 seconds. This phenomenon may be age related, and certainly some normal individuals are either good or poor "heparin respondents". Following is a summary of the prophylactic and therapeutic ranges of Low Molecular Weight Heparin with the HEPTEST Assay:

Prophylactic*: 2.5 - 4.0 x normal control
Therapeutic*: 4.25 - 6.25 x normal control

*Harenberg J. Am J Hematol 1988; 29: 233-240.

SENSITIVITY

The heparin assay by the HEPTEST method is not influenced by plasma deficient in factor V, VII, VIII, IX, XI and XII. However, HEPTEST results are influenced by plasma samples containing less than 40 % of factor II and less than 30 % of anti-thrombin III. The test is not influenced by low fibrinogen content in the test plasma, as RECALMIX contains this factor.

PERFORMANCE CHARACTERISTICS

In one experiment, a plasma sample was spiked with three different concentrations of porcine heparin. Each plasma sample, including a control, was assayed 15 times using the BBL Fibrometer. The results for CV are as follows:

Plasma Sample Heparin, USP U/ mL	Mean clotting time (Seconds)	S.D. (+/-)	C.V. (%)
Control	16.2	0.17	1.05
0.1	39.8	0.35	0.88
0.5	117.9	0.75	0.64_
1.0	195.2	1.53	0.78

* HEPTTEST® and RECALMIX® are manufactured by and are registered trademarks of Haemachem, Inc., St. Louis, Missouri.

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