⟨208⟩ ANTI-FACTOR Xa AND ANTI-FACTOR IIa ASSAYS FOR UNFRACTIONATED AND LOW MOLECULAR WEIGHT HEPARINS

This chapter provides information and procedures to determine factor Xa inhibitory activity and factor IIa (thrombin) inhibitory activity for unfractionated heparin (UFH) and low molecular weight heparins (LMWH).

INTRODUCTION

Unfractionated heparin and LMWH exert their anticoagulant effect by potentiating the activity of plasma coagulation inhibitors. Of all the commonly known glycosaminoglycans, only UFH, LMWH, and heparan sulfate (hereafter referred to generally as heparin) contain a specific pentasaccharide sequence that can bind to the plasma coagulation inhibitor, antithrombin (AT). AT-dependent assays, therefore, are developed to ensure the specificity of the methods for measuring the anticoagulant activity of heparin. Binding of heparin to AT induces a conformational change, thereby increasing AT's binding to and subsequent inactivation of activated blood coagulation factors. The major coagulation targets for the AT–heparin complex are factor IIa (thrombin) and coagulation factor Xa. Once AT is activated by the pentasaccharide sequence of heparin, it interacts with factors Xa and IIa via its reactive center loop. For efficient inhibition of thrombin, the heparin molecule also must bind to both AT and IIa. This interaction requires an extra length of approximately 13 monosaccharides attached at the nonreducing end of the AT-binding heparin pentasaccharide sequence. This minimum heparin motif for AT inhibition of thrombin, known as the C-domain, has an approximate molecular weight of 5400 Da. Although the potentiation of AT inactivation of factor Xa also depends on molecular weight, the additional saccharide units of the C-domain are not essential, and heparin with a molecular weight less than 5400 Da can potentiate AT to inactivate factor Xa. By convention, the potency ratio of anti-factor Xa to anti-factor IIa for UFH is 1. Unfractionated heparin is heterogenous and polydisperse but contains little or no material of molecular weight less than 5400 Da. The mean molecular weights of LMWH products are lower than those of UFH, and they contain a higher proportion of material that weighs less than 5400 Da. The potency ratio of anti-factor Xa to anti-factor IIa for LMWH products is greater than 1.5. This chapter describes assay procedures for the measurement of anti-factor Xa and anti-factor IIa activity of LMWH heparin in the presence of AT.

In the test system, heparin is bound to AT, and factor IIa or factor Xa added to the mixture binds to the heparin–AT complex. The residual factor IIa or factor Xa not inhibited by the heparin–AT complex is quantified by a chromogenic substrate that is specific for either factor IIa or factor Xa and is added in the final step. Analysts note an inverse relationship wherein more color is produced by more residual enzyme, which equates to less heparin activity.

As for any enzymatic assay, temperature and timing of the reaction, proper handling of the reagents, and the order in which the reagents are added are critical to the optimal performance of the assay.

Anti-Factor Xa and Anti-Factor IIa Assays for Unfractionated Heparin

- **ANTI-FACTOR Xa ACTIVITY FOR UFH**

The following procedure is used where specified in the individual monographs. This assay can be performed manually in plastic tubes utilizing heated block stations or water bath. Microtiter plate equipment with a reader and automated coagulometer can improve reproducibility and throughput.

pH 8.4 buffer: Dissolve amounts of tris(hydroxymethyl)aminomethane, edetic acid or edetate sodium, and sodium chloride in water containing 0.1% of polyethylene glycol 6000 to obtain solutions having concentrations of 0.050 M, 0.0075 M, and 0.175 M, respectively. Adjust, if necessary, with hydrochloric acid or sodium hydroxide solution to a pH of 8.4.

Antithrombin solution: Reconstitute a vial of antithrombin (see *Reagents, Indicators, and Solutions—Reagent Specifications*) as directed by the manufacturer, and further dilute with *pH 8.4 buffer* to obtain a solution having a concentration of 1.0 Antithrombin IU/mL.

Factor Xa solution: Reconstitute bovine factor Xa as directed by the manufacturer (see *Factor Xa* in *Reagents, Indicators, and Solutions—Reagent Specifications*), and further dilute in *pH 8.4 buffer* to obtain a solution that gives an absorbance value of 0.65–1.25 at 405 nm when assayed as described below but using 30 µL of *pH 8.4 buffer* instead of 30 µL of the *Standard solutions* or the *Sample solutions*. [NOTE—*Factor Xa solution* contains about 3 nanokatalytic units/mL but can vary depending on the manufacturer of factor Xa or the substrate used.]

Chromogenic substrate solution: Prepare a solution of a suitable chromogenic substrate for amidolytic test (see *Reagents, Indicators, and Solutions—Reagent Specifications*) specific for factor Xa in water to obtain a concentration of 1 mM.

Stopping solution: 20% (v/v) solution of acetic acid

Standard solutions: Reconstitute the entire contents of an ampule of USP Heparin Sodium for Assays RS with water, and dilute with *pH 8.4 buffer* to obtain at least 5 dilutions in the concentration range of 0.03–0.375 USP Heparin Units/mL.

Sample solutions: Dissolve or dilute an accurately measured quantity of Heparin Sodium in *pH 8.4 buffer*, and dilute with the same buffer to obtain solutions having activities approximately equal to those of the *Standard solutions*.

Analysis: [NOTE—The procedure also can be performed using alternative platforms. Perform the test with each *Standard solution* and *Sample solution* in duplicate.]

To each of a series of suitable plastic tubes placed in a water bath set at 37°, transfer 120 µL of *pH 8.4 buffer*. Then separately transfer 30 µL of the different dilutions of the *Standard solutions* or the *Sample solutions* to the tubes. Add 150 µL of *Antithrombin solution*, prewarmed at 37° for 15 min, to each tube, mix, and incubate for 2 min. Add 300 µL of *Factor Xa solution*, prewarmed at 37° for 15 min, to each tube, mix, and incubate for 2 min. Add 300 µL of *Chromogenic substrate solution*, prewarmed at 37° for 15 min, to each tube, mix, and incubate for exactly 2 min. Add 150 µL of *Stopping solution* to each tube, and mix. Prepare a blank for zeroing the spectrophotometer by adding the reagents in reverse order, starting with the *Stopping solution* and ending with the addition of 150 µL of *pH 8.4 buffer*, and excluding the *Standard solutions* or the *Sample solutions*. Record the absorbance at 405 nm against the blank. The volume of the reactants can be increased or decreased to suit the assay format provided that the proportions of the reference sample or the test sample and the reagents are kept the same.

Calculations: Plot the log of the absorbance values of the *Standard solutions* and the *Sample solutions* vs. heparin concentrations in USP Units. Calculate the activity of heparin sodium in USP Units/mg using statistical methods for slope ratio assays. Calculate the anti-factor Xa activity of heparin sodium:

$$\text{Result} = A \times (S_T/S_S)$$

A = the potency of USP Heparin Sodium for Assays RS
S_T = slope of the line for the *Sample solutions*
S_S = slope of the line for the *Standard solutions*

Express the anti-factor Xa activity of the *Sample solution* as USP Heparin Units/mg, calculated on the dried basis. Calculate the ratio of anti-factor Xa activity against anti-factor IIa potency (see *Assay* below):

anti-factor Xa activity/anti-factor IIa potency

Acceptance criteria: 0.9–1.1

• **ANTI-FACTOR IIa ACTIVITY FOR UNFRACTIONATED HEPARIN**

pH 8.4 buffer: Dissolve 6.10 g of tris(hydroxymethyl)aminomethane, 10.20 g of sodium chloride, 2.80 g of edetate sodium, and, if suitable, 0–10.00 g of polyethylene glycol 6000 and/or 2.00 g of bovine serum albumin in 800 mL of water. [NOTE—2.00 g of human albumin may be substituted for 2.00 g of bovine serum albumin.] Adjust with hydrochloric acid to a pH of 8.4, and dilute with water to 1000 mL.

Antithrombin solution: Reconstitute a vial of antithrombin (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in water to obtain a solution of 5 Antithrombin IU/mL. Dilute this solution with *pH 8.4 buffer* to obtain a solution having a concentration of 0.125 Antithrombin IU/mL.

Thrombin human solution: Reconstitute thrombin human (factor IIa) (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in water to obtain a solution of 20 Thrombin IU/mL, and dilute with *pH 8.4 buffer* to obtain a solution having a concentration of 5 Thrombin IU/mL. [NOTE—The thrombin should have a specific activity of NLT 750 IU/mg.]

Chromogenic substrate solution: Prepare a solution of a suitable chromogenic thrombin substrate for amidolytic test (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in water to obtain a concentration of 1.25 mM.

Stopping solution: 20% (v/v) solution of acetic acid

Standard solutions: Reconstitute the entire contents of an ampule of USP Heparin Sodium for Assays RS with water, and dilute with *pH 8.4 buffer* to obtain at least four dilutions in the concentration range of 0.005–0.03 USP Heparin Unit/mL.

Sample solutions: Proceed as directed for *Standard solutions* to obtain concentrations of Heparin Sodium similar to those obtained for the *Standard solutions*.

Analysis: [NOTE—The procedure also can be performed using alternative platforms.] For each dilution of the *Standard solutions* and the *Sample solutions*, at least duplicate samples should be tested. Label a suitable number of tubes depending on the number of replicates that will be tested. For example, if five blanks will be used: B1, B2, B3, B4, and B5 for the blanks; T1, T2, T3, and T4 each at least in duplicate for the dilutions of the *Sample solutions*; and S1, S2, S3, and S4 each at least in duplicate for the dilutions of the *Standard solutions*. Distribute the blanks over the series in such a way that they accurately represent the behavior of the reagents during the experiments. [NOTE—Treat the tubes in the order B1, S1, S2, S3, S4, B2, T1, T2, T3, T4, B3, T1, T2, T3, T4, B4, S1, S2, S3, S4, B5.] Note that after each addition of a reagent, the incubation mixture should be mixed without allowing bubbles to form. Add twice the volume (100–200 µL) of *Antithrombin solution* to each tube containing one volume (50–100 µL) of either the *pH 8.4 buffer* or an appropriate dilution of the *Sample solutions* or the *Standard solutions*. Mix, but do not allow bubbles to form. Incubate at 37° for at least 1 min. Add to each tube 25–50 µL of *Thrombin human solution*, and incubate for at least 1 min. Add 50–100 µL of *Chromogenic substrate solution*. Note that all reagents, *Standard solutions*, and *Sample solutions* should be prewarmed to 37° just before use. The volume of the reactants can be increased or decreased to suit the assay format provided that the proportions of the reference sample or the test sample and the reagents are kept the same.

Two different types of measurements can be recorded:

1. Endpoint Measurement: Stop the reaction after at least 1 min with 50–100 µL of *Stopping solution*. Measure the absorbance of each solution at 405 nm using a suitable spectrophotometer (see *Ultraviolet-Visible Spectroscopy* ⟨857⟩). The RSD over the blank readings is less than 10%.
2. Kinetic Measurement: Follow the change in absorbance for each solution over 1 min at 405 nm using a suitable spectrophotometer (see ⟨857⟩). Calculate the change in absorbance/min (ΔOD/min). The blanks for kinetic measurement are also expressed as ΔOD/min and should give the highest values because they are carried out in the absence of heparin. The RSD over the blank readings is less than 10%.

Calculations: The statistical models for *Slope ratio assay* or *Parallel-line assay* can be used depending on which model best describes the correlation between concentration and response.

Slope ratio assay: For each series, calculate the regression of the log absorbance or the log change in absorbance/min against concentrations of the *Sample solutions* and of the *Standard solutions*, and calculate the potency of heparin sodium in USP Units/mL using statistical methods for slope ratio assays. Express the potency of heparin sodium/mg, calculated on the dried basis.

Parallel-line assay: For each series, calculate the regression of the absorbance or change in absorbance/min against log concentrations of the *Sample solutions* and the *Standard solutions*, and calculate the potency of heparin sodium in USP Units/mL using statistical methods for parallel-line assays. Express the potency of heparin sodium/mg, calculated on the dried basis.

Acceptance criteria: The potency of heparin sodium, calculated on the dried basis, is NLT 180 USP Heparin Units in each mg.

Change to read:

Anti-Factor Xa and Anti-Factor IIa Assays for Low Molecular Weight Heparins

▲The following procedure is used where specified in the individual monographs. This assay can be performed manually in plastic tubes utilizing heated block stations or water bath. Microtiter plate equipment with a reader and automated coagulometer can improve reproducibility and throughput. Acetic acid solution (stopping solution) is used for manual and microtiter plate assay. Automated coagulometers measure initial kinetic rate, and because of that, stopping of the reaction is not needed.▲ (ERR 1-Aug-2020)

• **Anti-Factor Xa Activity for Low Molecular Weight Heparin**

Acetic acid solution: Glacial acetic acid and water (42:58)

pH 7.4 polyethylene glycol 6000 buffer: Dissolve 6.08 g of tris(hydroxymethyl)aminomethane and 8.77 g of sodium chloride in 500 mL of water. Add 1.0 g of polyethylene glycol 6000 or 10.0 g of bovine serum albumin, adjust with hydrochloric acid to pH 7.4, and dilute with water to 1000 mL.

pH 7.4 buffer: Dissolve 6.08 g of tris(hydroxymethyl)aminomethane and 8.77 g of sodium chloride in 500 mL of water. Adjust with hydrochloric acid to pH 7.4, and dilute with water to 1000 mL.

pH 8.4 buffer: Dissolve 3.03 g of tris(hydroxymethyl)aminomethane, 5.12 g of sodium chloride, and 1.40 g of edetate sodium in 250 mL of water. Adjust with hydrochloric acid to a pH of 8.4, and dilute with water to 500 mL.

Human antithrombin solution: Reconstitute a vial of human antithrombin (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in water to obtain a solution containing 5 Antithrombin Units per mL. Dilute this solution with *pH 7.4 polyethylene glycol 6000 buffer* to obtain a solution having a concentration of 1.0 Antithrombin Unit/mL.

Factor Xa solution: Reconstitute an accurately weighed quantity of bovine factor Xa (see *Reagents, Indicators, and Solutions—Reagent Specifications*) as directed by the manufacturer's instructions. Further dilute the stock solution with *pH 7.4 polyethylene glycol 6000 buffer* to obtain a solution that gives an increase in absorbance value at 405 nm of NMT 0.20 absorbance units/min or 0.8 absorbance units after 4 min of incubation with the chromogenic substrate when assayed as described below but using as an appropriate volume, *V*, the volume in µL of *pH 7.4 buffer* instead of *V* µL of the standard or the sample solution.

Chromogenic substrate solution: Prepare a solution of a suitable chromogenic substrate for amidolytic test (see *Reagents, Indicators, and Solutions—Reagent Specifications*) for factor Xa in water to obtain a concentration of about 3 mM. Dilute with substrate *pH 8.4 buffer* to obtain a solution having a concentration of 0.5 mM. [Note—Preheat reagents to 37 ± 1° 15 min before use.]

Standard solutions: Reconstitute and dilute the entire contents of an ampule of USP Low Molecular Weight Heparin for Bioassays RS with distilled water and then further dilute with *pH 7.4 buffer* to obtain at least four dilutions in the concentration range of 0.025–0.2 USP anti-factor Xa Units/mL.

Sample solutions: Proceed as directed for *Standard solutions* to obtain concentrations of LMWH similar to those obtained for the *Standard solutions.*

Analysis

Samples: *Standard solutions, Sample solutions, Human antithrombin solution, pH 7.4 buffer, Factor Xa solution, Chromogenic substrate solution,* and *Acetic acid solution*

Label 18 suitable tubes: B1 and B2 for blanks; T1, T2, T3, and T4 each in duplicate for the dilutions of the *Sample solutions;* and S1, S2, S3, and S4 each in duplicate for the dilutions of the *Standard solutions.* [Note—Treat the tubes in the order B1, S1, S2, S3, S4, T1, T2, T3, T4, T1, T2, T3, T4, S1, S2, S3, S4, B2.] To each tube add 50 µL of *Human antithrombin solution* and an equal volume, *V,* of either the blank, *pH 7.4 buffer*, or an appropriate dilution of the *Sample solutions* or the *Standard solutions.* Mix, but do not allow bubbles to form. Incubate at 37° for 1.0 min. Add to each tube 100 µL of *Factor Xa solution,* and incubate for 1.0 min. Add 250 µL of *Chromogenic substrate solution.* Stop the reaction after about 4.0 min with 250 µL of *Acetic acid solution.* Measure the absorbance of each solution at 405 nm using a suitable spectrophotometer (see ⟨857⟩). Use quartz or disposable polystyrene cuvettes. The volume of the reactants can be increased or decreased to suit the assay format provided that the proportions of the reference sample or the test sample and the reagents are kept the same.

System suitability: The assay is valid if the following requirements are met:

1. A blank solution gives an increase in absorbance value at 405 nm of NMT 0.20 absorbance units/min (or 0.8 absorbance units in total) when assayed using an appropriate volume (50 µL) of *pH 7.4 buffer* instead of 50 µL of the *Standard solution* or the *Sample solution*.
2. The reading of the blank B2 is not more than ±0.05 absorbance units against blank B1.

Calculations: For this bioassay, parallel-line or slope ratio analysis can be applied.

Calculate the potency of the test sample in USP anti-factor Xa Unit/mL, using a statistical model for parallel assays, plotting absorbance against log concentrations of the *Sample solutions* and of the *Standard solutions.* In some cases, log transformation of absorbance may be needed to obtain linearity for the dose–response curves. The assay is valid when the data fulfill the acceptance criteria for regression, linearity, and parallelism as required for parallel line assay. For slope ratio analysis, plot absorbance against concentrations of *Sample solutions* and of the *Standard solutions.* In some cases, log transformation of absorbance may be needed to obtain linearity for the dose–response curves. The assay is valid when the data fulfill the acceptance criteria for regression, linearity, and common intercept as required for slope ratio assay (see *Design and Analysis of Biological Assays* ⟨111⟩ and *Analysis of Biological Assays* ⟨1034⟩).

Express the anti-factor Xa activity of sample/mg, calculated on the dried basis:

$$\text{Anti-factor Xa USP Unit/mg} = [\text{standard potency (USP Unit/mL)} \times \text{potency ratio}]/[\text{sample concentration (mg/mL)}]$$

• **ANTI-FACTOR IIa ACTIVITY FOR LOW MOLECULAR WEIGHT HEPARIN**

Acetic acid solution, pH 7.4 polyethylene glycol 6000 buffer, pH 7.4 buffer, pH 8.4 buffer, and **Human antithrombin solution:** Proceed as directed in the *Anti-Factor Xa Activity*, except that the concentration of the *Human antithrombin solution* is 0.5 Antithrombin Unit/mL.

Thrombin human solution: Reconstitute thrombin (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in water, and dilute in *pH 7.4 polyethylene glycol 6000 buffer* to obtain a solution having a concentration of 5 Thrombin Units/mL. Use the *Thrombin human solution* immediately after preparation.

Chromogenic substrate solution: Prepare a solution of a suitable chromogenic substrate for the amidolytic test (see *Reagents, Indicators, and Solutions—Reagent Specifications*) for thrombin in water to obtain a concentration of about 3 mM. Dilute immediately before use with *pH 8.4 buffer* to 0.5 mM.

Standard solutions: Reconstitute and dilute the entire contents of an ampule of USP Low Molecular Weight Heparin for Bioassays RS with distilled water, and further dilute with *pH 7.4 buffer* to obtain at least four dilutions having concentrations in the range of 0.015–0.075 USP Unit of anti-factor IIa activity/mL.

Sample solutions: Proceed as directed for *Standard solutions* to obtain concentrations of LMWH similar to those obtained for the *Standard solutions*.

Procedure: Proceed as directed under *Anti-Factor Xa activity*, but use *Thrombin human solution* instead of *Factor Xa solution* and use the *Human antithrombin solution* as described above.

System suitability: The assay is valid if the following requirement is met:

1. The reading of blank B2 is NMT ±0.05 absorbance units against blank B1.

Calculations: Proceed as directed for calculation of *Anti-Factor Xa Activity*.

ADDITIONAL REQUIREMENTS

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Heparin Sodium for Assays RS
USP Low Molecular Weight Heparin for Bioassays RS