MINISTRY OF HEALTH OF THE RUSSIAN FEDERATION

GENERAL PHARMACOPOEIA MONOGRAPH

Determination of blood coagulation factor activity	**GPM**.1.8.2.0003.15 **First Edition**

The present Pharmacopoeia Monograph applies to methods used to determine activity of human blood coagulation factors I, II, VII, VIII, IX, X, XI, von Willebrand factor, and antithrombin III in plasma and blood preparations.

GENERAL PROVISIONS

Coagulation factor activity testing is based on two approaches:

1. One-step method. Blood coagulation recovered in factor deficient plasma after addition of the respective factor (Clot-based method).

2. Two-step method. At the first stage, the proteolytic activity of factor II or factor X is intensified using a specific co-factor, leading to production of activated factor IIa or Xa, respectively. At the second stage, the amount of the produced activated factor is determined by the reaction in which it breaks down a specific chromogenic peptide (Chromogenic method).

The chromogenic method has two variations: by the kinetics of the chromogen production induced by the activated factor or by the endpoint of chromogen accumulation over a certain incubation time.

Both methods may be performed using plastic test tubes, microtitre plates, optical-mechanical, mechanical semi-automatic, and fully automated coagulometers.

In all the methods, factor activity is determined by comparing the activity of a tested sample with the activity of the NIBSC International Standard or a working standard sample of the coagulation factor calibrated versus the International

Standard expressed in International Units of activity (IU). The IU equivalence of an International Standard is established by the WHO. One IU (100 %) is the activity of a coagulation factor in 1.0 mL of fresh normal pooled plasma obtained from 300 donors. Activity may be expressed in IU/mL, IU/vial, IU/mg protein, and as a percentage of the manufacturer's label claim.

FACTORS

Factor I (Fibrinogen)

Clot-based method

Fibrinogen is assayed using the Clauss method.

Principle of the method

Addition of thrombin to a fibrinogen-containing sample results in the formation of a fibrin clot. A linear dependence may be obtained with high-activity thrombin (approximately 10 IU/mL) and samples with low fibrinogen concentrations (below 100 mg/dL).

Fibrinogen is assayed using commercial test systems that contain a thrombin reagent containing a heparin inhibitor and a buffer to dilute samples or a commercial thrombin reagent.

Fibrinogen concentrations are expressed in mg/dL. A calibration diagram is obtained using a fibrinogen standard sample or an International Standard-certified calibrator plasma. The standard sample or calibrator plasma sample is dissolved in distilled water according to the Instructions for Use. Five successive dilutions of the standard sample are prepared, beginning from a concentration of approximately 100 mg/dL, using the sample dilution buffer (pH = 7.3 ± 0.1). The test is carried out at 37 ℃ in accordance with the Instructions for Use of the kit. The clotting time is determined for each clot three times. Obtained results are used to plot a calibration graph in log-log coordinates to demonstrate the dependence between clotting time and fibrinogen concentration.

Two dilutions of the tested sample with a concentration below 100 mg/dL are prepared. The clotting time is determined for each dilution three times. The amount of fibrinogen in each dilution is determined using the calibration diagram.

Factor II (Thrombin)

1. Clot-based method.

Factor II is determined using human factor II-deficient plasma as the substrate. The coagulation process is activated by addition of a calcium thromboplastin mixture.

Sodium chloride – imidazole buffer, pH 7.3 ± 0.1, with 0.1 % bovine or human albumin added, is used for dilution of the standard and tested samples. A series of successive dilutions of the factor II standard sample over the activity range from 0.3 IU/mL to 1 IU/mL is prepared to plot a calibration graph. The tested sample is diluted to a concentration below 1 IU/mL. Three dilutions of the tested sample are analyzed. For each sample, the coagulation time should be determined at least twice. Measurement is performed within 1 hour after the tested sample is diluted.

The test is carried out at 37 ± 0.5 °C. Transfer 50 μL of the factor II-deficient plasma and 50 μL of the standard or tested sample dilution into a plastic test tube. Incubate this mixture at 37 ± 0.5 °C over 120 to 240 seconds. Coagulation time starts from the moment 200 μL of calcium thromboplastin pre-heated to 37 ± 0.5 °C is added to the mixture. Depending on analytical technique, reagent volumes may vary proportionately.

The calibration graph is charted in semi-log coordinates. Factor II activity values are plotted along the logarithmic axis of abscissas, whereas respective coagulation times of the standard sample dilution are plotted along the axis of ordinates. The activity of factor II for each dilution of the tested sample is determined by the calibration graph. The FII activity (A) of the tested sample is calculated according to the following equation:

$$A = A_x \cdot k$$

where A_x is the activity of the respective dilution of the tested sample determined by the calibration graph;

k is the dilution of the tested sample.

2. Chromogenic method.

This method uses comparison of the enzymatic activity of Factor IIa produced after specific factor II activation towards a specific chromogenic peptide substrate with the same standard sample activity (international sample or an equivalent).

Factor II is activated with ecarin isolated from the venom of the saw-scaled viper. Activated factor II (thrombin) selectively breaks down the chromogenic substrate (H-D-phenylalanine-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride, 4-toluenesulfonyl-glycyl-prolyl-L-arginine-4-nitroanilide, H - D-cyclohexylglycyl-α-aminobutyryl-L-arginine-4-nitroanilide, D-cyclohexylglycyl-L-alanyl-L-arginine-4-nitroanilide diacetate), leading to production of p-nitroaniline. The kinetics of this reaction are studied by photometry at 405 nm. The optical density value obtained is proportionate to the factor II activity.

Factor II is determined in the chromogenic method using special test kits. This analysis is performed in accordance with the Instructions for Use of the kit. The standard sample and the tested sample are first diluted with factor II-deficient plasma to a concentration of approximately 1 IU/mL (stock dilution). The stock dilution is used to prepare three dilutions of the standard sample and three dilutions of the tested sample using Tris-buffered saline, pH 8.4. Each dilution of the standard sample is tested twice, and the obtained values are used to chart a calibration graph. The tested samples are analyzed three times.

Testing is performed manually, using microtitre plates and a spectrophotometer maintaining temperature within the range of 37 ± 0.5 °C or in automated mode, by means of a coagulometer.

To perform the manual test, transfer a 25 μL portion of each dilution of the tested sample or standard sample into a microtitre plate well. Add 125 μL of the dilution buffer and 25 μL of ecarin into each well, and incubate at 37 ± 0.5 °C for 2

minutes. After the incubation time is through, transfer 25 μL of the factor IIa chromogenic substrate into each well.

Determine the optical density change rate at wavelength 405 nm continuously over 3 minutes, and calculate the average optical density change rate (ΔA/min).

If continuous optical density measurement is unfeasible, determine the optical density at wavelength 405 nm at equal intervals (e. g., 40 seconds) and chart a graph representing the optical density – time dependency; then calculate the ΔA/min as the inclination of the line. The ΔA/min values obtained for each individual dilution of the standard sample and the tested sample are used to calculate the activity of the tested sample.

3. Thrombin absence test.

This test is performed by coagulometry.

A solution of the tested sample reconstituted in accordance with the Instructions for Use is prepared for this test. If the sample contains heparin, it should be neutralized by addition of protamine sulfate, 10 μg of protamine sulfate per IU of heparin.

Preparation of the fibrinogen solution.

Dissolve 0.3 g of fibrinogen in 100 mL, stir, and leave to stand for 15 to 20 minutes at room temperature.

The shelf-life of this solution is 1 month if stored in the temperature range of 2 to 8 ^{0}C.

Preparation of the thrombin solution.

The freeze-dried powder is dissolved in accordance with the manufacturer's Instructions for Use, left to stand for 15 to 20 minutes at room temperature, and diluted with 0.9 % sodium chloride solution to a thrombin concentration of 1 IU/mL.

The shelf-life of this solution is 6 months if stored at minus 20 °C. The solution should not be refrozen after thawing.

Procedure description

Transfer equal volumes of the reconstituted tested sample and the fibrinogen solution into two test tubes. Transfer equal volumes of the thrombin solution and the fibrinogen solution into a third test tube (control sample), then stir the contents of the test tubes with a rotational movement. Incubate one test tube with the reconstituted tested sample in a water bath at 37 °C for 6 hours, and the other test tube with the reconstituted tested sample at room temperature for 24 hours. Report the presence or absence of coagulation (clotting).

Incubate the control sample in a water bath at 37 °C, and report the clotting time.

Coagulation at 30 seconds in the control sample is the acceptance criterion.

Factor VII

1. Clot-based method.

Factor VII is determined using human factor VII-deficient plasma as the substrate. The coagulation process is activated by addition of a calcium thromboplastin mixture.

Sodium chloride – imidazole buffer, pH 7.3, with 0.1 % bovine or human albumin added, is used for dilution of the standard and tested samples. A series of successive dilutions of the factor VII standard sample over the activity range from 0.3 IU/mL to 1 IU/mL is prepared to plot a calibration graph. The tested sample is diluted to a concentration below 1 IU/mL. Three dilutions of the tested sample are analyzed. For each sample, the coagulation time should be determined at least twice. Measurement is performed immediately after the tested sample is diluted.

The test is carried out at 37 ± 0.5 °C. Transfer 50 µL of the factor VII-deficient plasma and 50 µL of the standard or tested sample dilution into a plastic test tube. Incubate this mixture at 37 ± 0.5 °C over 120 to 240 seconds. Coagulation time starts from the moment 200 µL of calcium thromboplastin pre-heated to 37 ± 0.5 °C is added to the mixture. Depending on analytical technique, reagent volumes may vary proportionately.

The calibration graph is charted in semi-log coordinates. Factor VII activity values are plotted along the logarithmic axis of abscissas, whereas respective coagulation times of the standard sample dilution are plotted along the axis of ordinates. The activity of factor VII for each dilution of the tested sample is determined by the calibration graph. The FVII activity (A) of the tested sample is calculated according to the following equation:

$$A = A_x \cdot k$$

where A_x is the activity of the respective dilution of the tested sample determined by the calibration graph;

k is the dilution of the tested sample.

2. Chromogenic method.

Factor VII is activated (FVIIa is produced) in the presence of tissue factor and Ca^{2+} ions. The complex consisting of FVIIa, TF, Ca^{2+}, and phospholipid activates factor X. Activated factor X (FXa) selectively breaks down the FXa-1 chromogenic substrate Methoxycarbonyl-D-cyclohexylglycyl-glycyl-L-arginine-n-nitroanilide acetate, leading to production of n-nitroaniline. The sample is tested by photometry at 405 nm. The optical density (or absorption increment) value obtained is proportionate to the factor VII amount.

Factor VII is determined in the chromogenic method using special test kits. This analysis is performed in accordance with the Instructions for Use of the kit. The standard sample and the tested sample are first diluted with factor VII-deficient plasma to a concentration of approximately 1 IU/mL (stock dilution). The stock dilution is used to prepare three dilutions of the standard sample and three dilutions of the tested sample using Tris-buffered saline, pH 7.3 – 8.0, with 0.1 % human or bovine albumin added. Each dilution of the standard sample is tested twice, and the obtained values are used to chart a calibration graph. The tested samples are analyzed three times.

Testing is performed manually, using plastic tubes or microtitre plates, at 37 ± 0.5 °C, or in automated mode, by means of a coagulometer.

Transfer the dilutions of the tested sample or standard sample into the microtitre plate wells, and add the calcium – thromboplastin mixture and the factor X solution; then incubate at 37 ± 0.5 °C over 2 to 5 minutes, and then add the factor Xa chromogenic substrate solution.

Determine the optical density change rate at wavelength 405 nm or in the kinetic mode, then interrupt the hydrolysis reaction in 3 to 15 minutes by addition of 20 % (volume / volume) glacial acetic acid solution, and determine the optical density.

Factor VIII

1. Clot-based method.

Factor VIII is determined using human factor VIII-deficient plasma. The APTT reagent serves as the source of phospholipids necessary for inducing coagulation.

Sodium chloride – imidazole buffer with human or bovine albumin added (0.1 %, pH 7.3 ± 0.1) is used for dilution of the standard and tested samples. A series of successive dilutions of the standard sample, beginning from the 2 IU/mL concentration, is prepared to plot a calibration graph. The tested sample is diluted to a concentration of approximately 0.5 – 2 IU/mL. Three dilutions of the tested sample are analyzed. For each sample, the coagulation time should be determined at least twice. Measurement is performed within 1 hour after the tested sample is diluted.

The test is carried out at 37 ± 0.5 °C. Transfer 100 µL of the factor VIII-deficient plasma, 100 µL of the standard or tested sample dilution, and 100 µL of the APTT reagent into a plastic test tube, and incubate this mixture at 37 ± 0.5 °C over 2 minutes. Coagulation time starts from the moment 100 µL of 0.025 M calcium chloride solution pre-heated to 37 ± 0.5 °C is added to the mixture. Depending on analytical technique, reagent volumes may vary proportionately.

The calibration graph is charted in semi-log coordinates. Factor VIII activity values are plotted along the logarithmic axis of abscissas, whereas respective

coagulation times of the standard sample dilution are plotted along the axis of ordinates. The activity of factor VIII for each dilution of the tested sample is determined by the calibration graph. The FVIII activity (A) of the tested sample is calculated according to the following equation:

$$A = A_x \cdot k$$

where A_x is the activity of the respective dilution of the tested sample determined by the calibration graph;

k is the dilution of the tested sample.

2. Chromogenic method.

Factor VIII is assayed using a set of reagents in which factor VIII serves as a co-factor for factor IXa during activation of factor X into Xa, which breaks down the chromogenic substrate.

Principle of the method

Factor X is activated by factor IXa into Xa in the presence of calcium ions and phospholipids. If the amount of factor X is excessive and the quantities of calcium, phospholipids, and factor IXa are optimal, the factor X activation rate is in a linear dependency on the amount of factor VIII. Factor Xa hydrolyzes the chromogenic substrate S-2765 (N-a-Z-DArg-Gly-Arg-pNA), leading to release of the chromogenic moiety whose colour is registered by spectrophotometry at 405 nm. The produced factor Xa amount and, accordingly, colour intensity is proportionate to the factor VIII activity of the sample.

The reagent set for the factor VIII activity test remains stable over the period specified by the manufacturer, if stored in the temperature range of 2 to 8 ^{o}C, and contains the following:

1. 7.7 mg with S-2765, with the synthetic inhibitor thrombin I-2581 added. The reagent is reconstituted in 6.0 mL of sterile Water for Injections. The reconstituted solution remains stable over 1 month in the temperature range of 2 to 8 ^{o}C. Heat to 37 ^{o}C before use.
2. Bovine factor reagent: 0.3 unit of IX, 2.6 IU unit of factor X, and 1 NIH unit of thrombin lyophilized in the presence of 40 mmol of calcium

chloride and 0.2 mmol of phospholipids. The reagent is reconstituted in 2.0 mL of sterile Water for Injections. The reconstituted solution remains stable over 12 hours in the temperature range of 2 to 8 °C, over 2 weeks at 30 °C, and over 1 month at minus 80 °C. Do not store at minus 20 °C. Heat to 37 °C before use.

3. x10 Tris-buffered saline concentrate. Remains stable over 1 month in the temperature range of 2 to 8 °C. Dilute 1:10 with sterile Water for Injections before use.

Additional reagents:

1. Standard sample – human factor VIII concentrate solution (NIBSC, Eur. Pharm. Ref. Std. BRP H 0920000) or factor VIII international standard-calibrated plasma.
2. Control normal or pathological factor VIII international standard-calibrated plasma.
3. 0.9 % sodium chloride solution.
4. 20 % acetic acid or 2 % citric acid (used in the chromogen accumulation endpoint method).
5. Milli-Q deionized laboratory water.

Equipment:

1. Plastic test tubes;
2. Microtitre plates;
3. Thermostat 37 °C;
4. Spectrophotometer, 405 nm, or microtitre plate reader, 490 nm;
5. Calibrated pipettes;
6. Vortex;
7. Stopwatch.

The tested sample should be diluted to the expected activity of 1 IU/mL prior to the determination.

The determination may be carried out in the kinetic method or by the endpoint, using test tubes (macro-method) or microtitre plates (micro-method).

Calibration

A calibration curve is obtained for each determination. The standard sample is diluted in two steps: preliminary dilution to an activity of 1 - 2 IU/mL and final dilutions to obtain a calibration dependency over the range of 0 – 2 IU/mL. The determination should be performed within 30 minutes after dilution.

Procedure description:

Test tube variant:

Transfer 200 µL of the diluted standard, control or tested sample into test tubes, incubate for 3 to 4 minutes at 37 °C, add 50 µL of the pre-heated factor reagent, incubate for 2 to 4 minutes at 37 °C, and add 50 µL of the chromogen solution.

Microtitre plate variant:

Transfer 50 µL of the diluted standard, control or tested sample into microtitre plate wells, incubate for 3 to 4 minutes at 37 °C, add 50 µL of the pre-heated factor reagent, incubate for 2 to 4 minutes at 37 °C, and add 50 µL of the chromogen solution.

Kinetic method:

After adding the chromogen solution, determine the optical density change for 2 to 10 minutes at 405 nm.

Endpoint method:

After adding the chromogen solution, continue incubation of the mixture at 37 °C for 2 to 10 minutes, and after that add 50 µL of 20 % acetic acid or 2 % citric acid to interrupt the reaction. Determine the optical density of the solution in comparison with the buffer at 405 nm.

Calculations

Plot the dependency of optical density change per minute (for the kinetic method) or optical density (for the endpoint method) of the standard solution dilutions and factor VIII concentration. The activity of the tested sample is determined by the calibration curve, taking into consideration the preliminary dilution of the sample.

Factor IX

1. Clot-based method.

Factor IX is determined using human factor IX-deficient plasma. The APTT reagent serves as the source of phospholipids necessary for inducing coagulation.

Sodium chloride – imidazole buffer, pH 7.3, with 0.1 % bovine or human albumin added, is used for dilution of the standard and tested samples. A series of successive dilutions of the standard sample over the activity range from 0.3 IU/mL to 1 IU/mL is prepared to plot a calibration graph. The tested sample is diluted to a concentration below 1 IU/mL. Three dilutions of the tested sample are analyzed. For each sample, the coagulation time should be determined at least twice. Measurement is performed within 1 hour after the tested sample is diluted.

The test is carried out at 37 ± 0.5 °C. Transfer 100 µL of the factor IX-deficient plasma, 100 µL of the standard or tested sample dilution, and 100 µL of the APTT reagent into a plastic test tube, and incubate this mixture at 37 ± 0.5 °C over 2 minutes. Coagulation time starts from the moment 100 µL of 0.025 M calcium chloride solution pre-heated to 37 ± 0.5 °C is added to the mixture. Depending on analytical technique, reagent volumes may vary proportionately.

The calibration graph is charted in semi-log coordinates. Factor IX activity values are plotted along the logarithmic axis of abscissas, whereas respective coagulation times of the standard sample dilution are plotted along the axis of ordinates. The activity of factor IX for each dilution of the tested sample is determined by the calibration graph. The FIX activity (A) of the tested sample is calculated according to the following equation:

$$A = A_x \cdot k$$

where A_x is the activity of the respective dilution of the tested sample determined by the calibration graph;

k is the dilution of the tested sample.

Factor X

1. Clot-based method.

Factor X is determined using human factor X-deficient plasma as the substrate. The coagulation process is activated by addition of a calcium thromboplastin mixture.

Sodium chloride – imidazole buffer, pH 7.3, with 0.1 % bovine or human albumin added, is used for dilution of the standard and tested samples. A series of successive dilutions of the factor X standard sample over the activity range from 0.3 IU/mL to 1 IU/mL is prepared to plot a calibration graph. The tested sample is diluted to a concentration below 1 IU/mL. Three dilutions of the tested sample are analyzed. For each sample, the coagulation time should be determined at least twice. Measurement is performed immediately after the tested sample is diluted.

The test is carried out at 37 ± 0.5 °C. Transfer 50 µL of the factor X-deficient plasma and 50 µL of the standard or tested sample dilution into a plastic test tube. Incubate this mixture at 37 ± 0.5 °C over 120 to 240 seconds. Coagulation time starts from the moment 200 µL of calcium thromboplastin pre-heated to 37 ± 0.5 °C is added to the mixture. Depending on analytical technique, reagent volumes may vary proportionately.

The calibration graph is charted in semi-log coordinates. Factor IX activity values are plotted along the logarithmic axis of abscissas, whereas respective coagulation times of the standard sample dilution are plotted along the axis of ordinates. The activity of factor IX for each dilution of the tested sample is determined by the calibration graph. The FIX activity (A) of the tested sample is calculated according to the following equation:

$$A = A_x \cdot k$$

where A_x is the activity of the respective dilution of the tested sample determined by the calibration graph;

k is the dilution of the tested sample.

2. Chromogenic method.

Factor X is activated with FX-activator isolated from serpentine venom. Activated factor X (FXa) selectively breaks down the chromogenic substrate FXa-

1 N-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride, N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide hydrochloride, methanesulfonyl-D-leuyl-glycyl-L-arginine-4-nitroanilide, methoxycarbonyl-D-cyclohexylalanyl-glycyl-L-arginine-4-nitroanilide acetate, leading to production of n-nitroaniline. The samples are tested studied by photometry at 405 nm. The factor X amount is proportionate to the increase in the optical density of the solution.

Factor X is determined in the chromogenic method using special test kits. This analysis is performed in accordance with the Instructions for Use of the kit. The standard sample and the tested sample are first diluted with factor X-deficient plasma to a concentration of approximately 1 IU/mL (stock dilution). The stock dilution is used to prepare three dilutions of the standard sample (according to the Instructions for Use) and three dilutions of the tested sample. Each dilution of the standard sample is tested twice, and the obtained values are used to chart a calibration graph. The tested samples are analyzed three times.

Testing is performed manually, using plastic test tubes or microtitre plates at 37 ± 0.5 °C or in automated mode, by means of a coagulometer.

To perform the manual test, transfer a 12.5 µL portion of each dilution of the tested sample or standard sample into a microtitre plate well. Add 25 µL of the specific factor X activator obtained from the venom of Russell's viper into each well, and incubate at 37 ± 0.5 °C for 90 seconds. After that, transfer 150 µL of the working dilution of the factor X chromogenic substrate into each well.

Determine the optical density change rate at wavelength 405 nm continuously over 3 minutes, and calculate the average optical density change rate (ΔA/min), or determine the optical density at wavelength 405 nm at equal intervals (e. g., 30 seconds) and chart a graph representing the optical density – time dependency; then calculate the ΔA/min as the inclination of the line. The ΔA/min values obtained for each individual dilution of the standard sample and the tested sample are used to calculate the activity of the tested sample.

Von Willebrand factor

The test is carried out using the agglutination method or enzyme immunoassay.

Von Willebrand factor activity is determined by comparing the activity of this factor with the activity of the standard sample.

Agglutination method

This method is based on determination of the co-enzyme activity of von Willebrand factor during agglutination of a platelet suspension in the presence of ristocetin A.

The test may be performed with quantitative methods, using automated equipment, or with a semi-quantitative method, by visual examination of the agglutination in a series of dilutions.

Semi-quantitative method.

Dilute the standard sample and the reconstituted solution of the tested sample in a series of dilutions in 0.9 % sodium chloride solution containing 1 – 5 % human albumin to calculated von Willebrand factor concentrations of 0.5 IU/mL, 1.0 IU/mL, and 2.0 IU/mL. Apply 0.1 mL of each dilution of the standard sample and the tested sample on an object plate, add 0.1 mL of the ristocetin-containing platelet suspension to each sample, and mix over 1 minute. Use the dilution solution as the negative control. Following 1-minute incubation at room temperature, examine the platelet agglutination visually. The maximum dilution at which platelet agglutination occurs is the ristocetin co-enzyme activity titre of the tested sample.

Quantitative method.

Prepare not less than 2 series of consecutive dilutions of the standard sample and the tested sample in the dilution buffer to calculated von Willebrand factor concentrations of 0.5 IU/mL, 1.0 IU/mL, and 2.0 IU/mL.

The determination is performed in accordance with the Instructions for Use of the automatic equipment’s manufacturer. Obtain the optical density change (solution turbidity) to von Willebrand factor activity dependency.

The content of von Willebrand factor in the tested sample is determined using the coefficients of the linear equation representing the dependency between the optical density of the solution and the content of von Willebrand factor in the standard sample.

Enzyme immunoassay.

This method is based on determination of the collagen-binding activity of von Willebrand factor. Upon specific binding of von Willebrand factor to collagen fibrils and subsequent binding of enzyme-conjugated polyclonal antibodies to von Willebrand factor, the addition of the chromogenic substrate results in production of a chromophore that can be quantified by spectrophotometry. There is a linear dependence between the collagen – von Willebrand factor binding and the optical density.

The determination is performed using test systems approved for use in Russian health care practice, in accordance with the Instructions for Use.

Prepare not less than three consecutive dilution series of the standard sample and the tested sample in the dilution buffer to a calculated von Willebrand factor concentration of 1.0 IU/mL. After that, carry out the test as described in the Instructions for Use supplied by manufacturer of the test system used.

Activated blood coagulation factors.

The determination is performed by coagulometry.

A solution of the tested sample is reconstituted for this test. If the sample contains heparin, it should be neutralized by addition of protamine sulfate, 10 μg of protamine sulfate per IU of heparin. Then prepare 1:10 and 1:100 dilutions of the tested sample with Tris-buffered saline, pH 7.5.

Transfer 0.1 mL portions of the standard human plasma and 0.1 mL portions of the phospholipids solution into three test tubes, place in a water bath at 37 ± 0.5 °C for 60 seconds, and after that add 0.1 mL of the Tris-buffered saline into the first test tube (control sample), 0.1 mL of the 1:10 dilution of the tested sample into the second test tube, and 0.1 mL of the 1:100 dilution of the tested

sample into the third test tube. Afterwards, immediately add 0.1 mL of 3.7 g/L calcium chloride solution pre-heated to 37 ^{o}C to the contents of each test tube. Determine the clotting time – from the calcium chloride solution is added in.

A coagulation time of the control sample in the range of 200 to 350 seconds is the acceptance criterion.

Specific coagulation factor activity

Specific coagulation factor activity is calculated according to the following equation:

$$\text{Specific activity} = \frac{\text{Coagulation factor activity, IU/mL}}{\text{Protein content, mg/mL}}$$

Antithrombin III activity determination

Chromogenic method

The ATIII determination method is based on the ability of antithrombin III (ATIII) to neutralize thrombin in the presence of heparin. Heparin and thrombin excesses are added to the ATIII-containing sample. The resultant ATIII-heparin complex neutralize a thrombin amount that is proportionate to the ATIII amount. The remaining thrombin selectively breaks down the chromogenic substrate, leading to production of n-nitroaniline, and the absorption of the latter is determined at 405 nm. Therefore, the ATIII amount is in inverse proportion to the absorption of free n-nitroaniline in the tested sample.

ATIII activity is determined using commercial test systems. A calibration diagram is obtained using an ATIII standard sample or an International Standard-certified calibrator plasma. The standard sample or calibrator plasma sample is dissolved in distilled water according to the Instructions for Use. The optical density to ATIII activity dependency is linear over the ATIII activity range from 0.1 to 1.0 IU/mL. Using the heparin-containing buffer, prepare 4 dilutions of the standard sample or calibrator plasma with ATIII activities ranging from 0.1 to 1.0 IU/mL. The analysis is carried out at 37 °C, in accordance with the procedure

described in the Instructions for Use of the kit. The optical density value is determined for each dilution at 405 nm three times, and a calibration graph representing the absorption to ATIII activity dependency is plotted in linear coordinates.

Two dilutions of the tested sample with an approximate ATIII activity of less than 1.0 IU/mL are prepared. ATIII activity is determined in the tested samples values at 37 °C, in accordance with the procedure described in the Instructions for Use of the kit. The ATIII activity of each tested dilution is determined by the calibration graph. The ATIII activity of the tested sample is calculated according to the following equation:

$$A = A_x \cdot k$$

where A_x is the ATIII activity of the respective dilution;

k is the dilution of the tested sample.

Heparin assay

1. Clot-based method

This method is based on the ability of heparin to prolong the coagulation time of normal plasma through inhibition of a number of factors.

Normal human plasma, a heparin standard sample, the APTT reagent, and 0.025 M calcium chloride solution are used for this test. Isotonic 0.9 % sodium chloride solution is used as the diluent of the standard sample and tested samples. The heparin standard sample is dissolved in distilled water in accordance with directions included in the Instructions for Use. Three dilutions of the standard sample with a heparin activity of 0.3 IU/mL, 0.4 IU/mL, and 0.5 IU/mL are prepared. The standard samples with these activities should prolong the coagulation time of normal plasma at least 1.5-fold, otherwise dilutions with greater heparin activities should be employed. Simultaneously, three dilutions of the tested sample should be prepared so that the approximate heparin activities of these dilutions lie within the heparin activity range of the standard sample dilutions.

The test is carried out with an automatic or semi-automatic coagulometer, in plastic test tubes, at 37 °C. Transfer 100 μL of the normal human plasma, 100 μL of the standard or tested sample dilution or 100 μL of isotonic 0.9 % sodium chloride solution into a test tube (blank experiment), add 100 μL of the APTT reagent, and incubate the mixture for 120 to 240 seconds at 37 ± 0.1 °C. After that, transfer 100 μL of 0.025 M calcium chloride solution pre-heated to 37 °C into the test tube and record the sample coagulation time. Depending on analytical technique, reagent volumes may be altered proportionately. The coagulation time of normal plasma (blank experiment) should be in the range of 25 to 40 seconds. The coagulation time should be determined three times for each dilution of the standard sample and the tested sample.

2. Chromogenic method

This method is based on degradation of a chromogenic substrate specific for activated factor X (FXa). Addition of ATIII and FXa excesses to a heparin-containing sample results in inhibition of an Fxa amount proportionate to the heparin amount. The remaining FXa cleaves n-nitroaniline from the specific chromogenic substrate, and the absorption of n-nitroaniline is determined at 405 nm. Therefore, the absorption value is in inverse proportion to the heparin amount. This method is used to determine the content of both unfractioned and low-molecular weight heparin in anti-Xa units.

In the chromogenic method, the heparin amount is determined using commercial test systems. A calibration diagram is obtained using a heparin standard sample or an International Standard-certified calibrator plasma. The standard sample or calibrator plasma sample is diluted in distilled water according to the Instructions for Use. Four dilutions of the standard sample with a heparin concentration of less than 1 anti-Xa unit/mL are prepared using the dilution buffer, pH 8.4. The analysis is carried out at 37 °C, in accordance with the procedure described in the Instructions for Use of the kit. The absorption value is determined for each dilution at 405 nm three times, and a calibration graph representing the heparin concentration to absorption dependency is plotted in linear coordinates.

This dependency is linear over the heparin concentration range of 0 to 1.0 anti-Xa unit/mL.

For the tested sample, prepare two dilutions with a heparin concentration of less than 1 anti-Xa unit/mL. The analysis is carried out at 37 °C, in accordance with the procedure described in the Instructions for Use of the kit. The absorption value is determined for each dilution three times.

Testing is performed manually, using plastic test tubes or microtitre plates, or in automated mode, by means of a coagulometer.

To perform the manual test, transfer 20 µL portion of the human standard plasma and 20 µL of the antithrombin III solution into the microtitre plate wells. After that, transfer 20 µL, 60 µL, 100 µL, and 140 µL, respectively, of the tested sample or the standard sample into these wells and bring the volume of the solution in each well to 200 µL with the buffer (the heparin activity of the final reagent mixture is 0.02 to 0.08 IU/mL).

Transfer 40 µL from each well of the microtitre plate into a second series of wells and add 20 µL of the bovine factor Xa solution into each of them; then incubate for 30 seconds at 37 ± 0.5 °C and then add 40 µL of the factor Xa chromogenic substrate into each well and incubate again at 37 ± 0.5 °C for 3 to 15 minutes, determining the substrate degradation rate by continuous optical density measurement at wavelength 405 nm (kinetic mode) or after the reaction is stopped by addition of 20 % (volume / volume) glacial acetic acid solution (final measurement point).

The content of heparin in the tested dilution is determined by the calibration graph, taking into consideration the dilution.

During automatic-mode coagulometer measurements, the dependency between optical density change and heparin concentration is obtained.