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BIOPHEN
HEPARIN ANTI-Xa (2 stages)
REF: 221010

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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INTENDED USE:

This Heparin Anti-Xa method is a two-stage chromogenic assay for measuring the concentrations of heparins (UFH, LMWH), for heparin concentration ranges from about 0.005 to 0.1 IU/ml (*) in the tested dilution (or from 0 to 1 IU/ml in plasma tested diluted 1:10) using the manual method. This method can be used for testing heparin in human citrated plasma, or in purified solutions.
This kit is for research use only and should not be used for patient diagnosis or treatment.

TEST PRINCIPLE:

The Heparin Anti-Xa (2 stages) assay is a chromogenic anti-Xa method developed for measuring homogeneously heparins in plasma or in purified solutions, for their Anti-Xa activity.
Heparin is a sulphated polysaccharide with a high affinity for antithrombin. When complexed with heparin, antithrombin exhibits a fast acting and potent inhibitory activity for coagulant serine esterases: IXa, Xa and thrombin. LMWH, and heparin analogues, such as Sodium Danaparoïd, inhibit more efficiently Factor Xa than thrombin. Pentasaccharide (Arixtra®) inhibits more specifically Factor Xa. Anti-Xa assays are then the methods of choice for measuring heparins and their analogues.
The Heparin Anti-Xa assay is a two-step chromogenic method based on the inhibition of a constant amount of factor Xa, by the tested heparin in presence of exogenous antithrombin (stage 1), and hydrolysis of a Factor Xa specific chromogenic substrate (CS11(65)), by the factor Xa in excess (stage 2). pNA is then released from the substrate. The amount of pNA released is then a relation of the residual factor Xa activity. There is an inverse relationship between the concentration of heparin and color development, measured at 405 nm.

Heparin + AT ➔[AT Hep.]
[AT Hep.] + [FXa (excess)] ➔ [FXa-AT-Hep.] + [residual FXa]
[residual FXa] + Substrate ➔ Peptide + pNA

REAGENTS SUPPLIED:

The Heparin Anti-Xa (2 stages) kit contains 2 vials of human ATIII, 2 vials of Bovine FXa, 2 vials of a specific Factor Xa substrate, and 4 vials of assay reaction buffer.

Reagent 1 (R1): ATIII (h)
Human Antithrombin (AT), lyophilized vial containing about 5 IU/ml:
2 vials (each vial to be restored with **1 mL** of distilled water, then diluted **1:5** in R4 buffer before use, in order to obtain a solution at about 1 IU/ml).

Reagent 2 (R2): FXa (b)
Purified bovine Factor Xa, lyophilized vial containing about 40 µg (i.e. about 90nkats, when determined in optimized conditions with CS-11(22) specific substrate)). FXa concentration is exactly adjusted from lot to lot for offering an optimized assay reactivity and linearity:
2 vials (each vial to be restored with **1 mL** of distilled water, then diluted **1:5** in R4 buffer before use, in order to obtain a solution at about 8 µg/ml or 18nkats/ml).

Reagent 3 (R3): Substrate
Chromogenic substrate specific for FXa (CS-11(65)), lyophilized vial of about 4 mg (about 6 µmol), in presence of mannitol.
2 vials (each vial to be restored with **5 mL** of distilled water, in order to obtain a concentration of about 1.2 mM).

Reagent 4 (R4): Buffer
Assay reaction buffer: Tris 0.05M, NaCl 0.175M, EDTA 0.0075M, at pH 8.40, containing PEG at 0.1%, and sodium azide as preservative.
4 vials of 25 ml, ready to use.

Note:

- ***Warning:*** The assay reaction buffer contains sodium azide (0.9g/L NaN_3), which may react with lead and copper plumbing to form highly explosive metal azides. Flush with large volumes of water when discarding into a sink.
- The Human plasma used for the purification of Antithrombin was tested and found negative for HIV antibodies, HBs Ag and HVC antibodies. However, no assay may warrant the total absence of infectious agents. Any product of human origin must then be handled with all the required cautions, as being potentially infectious.
- BSA and Factor Xa were prepared from bovine plasma, which was tested for the absence of infectious agents, and collected from animals free from BSE. However, no test may totally exclude the absence of infectious agents. As any product of bovine origin, these BSA and thrombin must be used with all the cautions required for handling a material potentially infectious.
- The Factor Xa and AT concentrations are adjusted if required for each lot for providing the right reactivity in the assay.

STORAGE CONDITIONS:

Unopened reagents, must be stored at 2–8 °C, in their original packaging box. They are then stable until the expiration date printed on the label

Note: Stability studies for 3 weeks at 30°C show that the reagents can be shipped at room temperature for a short period without damage.

PREPARATION AND STABILITY OF REAGENTS:

Note: Reconstitution volumes can vary according to the automate used. Refer to each specific instrument adaptation.

REAGENT 1: Human Antithrombin (AT)
Reconstitute each vial with exactly **1 mL** of distilled water. Shake thoroughly until complete dissolution of the contents (vortex). Let to homogenize for 30 minutes at room temperature (18-25 °C), while shaking the vial from time to time. Just before use, dilute **1:5** with **R4** buffer (if all the vial is used, add 4 ml of R4 buffer to the 1 ml of restored AT). Homogenize the contents before each use.
Stability of AT, restored with 1 ml, provided that any contamination or evaporation is avoided, kept in its original vial:

- 15 days at 2-8°C.
- 7 days at room temperature (18-25°C).
- 2 months frozen at -20°C or below. (

Cautions: freezing conditions and stability of the thawed product should be checked in the working conditions of the laboratory user.

ATIII diluted 1:5 in R4 buffer is stable for about 8 hours at RT (18-25°C) or at 2-8°C.

REAGENT 2: Factor Xa
Reconstitute each vial with exactly **1 mL** of distilled water. Shake thoroughly until complete dissolution of the contents (vortex). Let to homogenize for 30 minutes at room temperature (18-25 °C), while shaking the vial from time to time. Just before use, dilute **1:5** with **R4** buffer (if all the vial is used, add 4 ml of R4 buffer to the 1 ml of restored FXa). Homogenize the contents before each use.
Stability of FXa, restored with 1 ml, provided that any contamination or evaporation is avoided, kept in its original vial:

- 15 days at 2-8°C.
- 7 days at room temperature (18-25°C).
- 2 months frozen at -20°C or below.

Cautions : freezing conditions and stability of the thawed product should be checked in the working conditions of the laboratory user.

FXa diluted 1:5 in R4 buffer is stable for about 8 hours at RT (18-25°C) or at 2-8°C.

REAGENT 3: Factor Xa specific chromogenic substrate
Reconstitute each vial with exactly **5 mL** of distilled water. Shake thoroughly until complete dissolution of the contents (vortex). Let to homogenize for 30 minutes at room temperature (18-25 °C), while shaking the vial from time to time (vortex). Check that all the substrate is dissolved before use.
Homogenize the contents before each use.
Stability of restored substrate, provided that any contamination or evaporation is avoided, kept in its original vial:

- 2 months at 2-8°C.
- 7 days at room temperature (18-25°C).
- 2 months frozen at -20°C or below.

Cautions: freezing conditions and stability of the thawed product should be checked in the working conditions of the laboratory user.

REAGENT 4: Assay Reaction Buffer at pH 8.40
Ready to use vial of 25 ml. Shake before use.
Stability of opened original vial provided that no contamination of buffer occurs:

- 2 months at 2-8°C.
- 7 days at room temperature (18-25°C).

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Cautions: In order to improve stability, reagents must be closed with their original stoppers and screw caps following each use. Reagents must be handled with care, in order to avoid any contamination during use. If the substrate becomes yellow, this indicates presence of a contaminant. It must be rejected, and a new vial must be used. Incubating the reconstituted vials at RT allows stabilizing the reagents, and obtaining a homogeneous reactivity. Take care to limit as much as possible any evaporation of the reagents during use, e.g. by using chimneys.

Note:

- The lyophilized vials (Reagents 1, 2 and 3) are closed under vacuum. Remove carefully the stopper, in order to avoid any lost of powder when opening the vials.
- According to the automated method used, the reagents can be reconstituted with volumes different from those recommended. In any case, the established reactive ratios (respective reagent concentrations in the reactive milieu) between the reagents must be strictly respected.
- Use only reagents from kits with a same lot number. Do not use reagents from kits with different lots when running the assay. Reagents are optimized for each lot of kits.

REAGENTS REQUIRED BUT NOT PROVIDED:

Reagents:

- Distilled water, Acetic acid (20%) or 2% citric acid (end point method).
- Normal citrated plasma pool, obtained in order to avoid any platelet activation, for preparing the heparin calibration range.
- Heparin Reference Material (BIOPHEN® Heparin Calibrator #222001, BIOPHEN® UFH Calibrator #222301, USP, EP or International Standards from NIBSC, Internal References, etc.)

Materials:

- Spectrophotometer or automatic instrument for chromogenic assays.
- Stopwatch; Calibrated pipettes.

TESTED SPECIMEN:

Purified: dilute the heparin preparation with R4 buffer in order to bring it at a concentration within the assay working range (and preferably 0.01 to 0.1 IU/ml).

Plasma:

Blood (9 volumes) must be collected on 0.109 M citrate anticoagulant (1 volume), with great care, in order to avoid activation and PF4 release. Sampling must be performed through a net venipuncture, and the first drops must be discarded. Specific collection tubes for heparin testing, such as the CTAD (Citrate, Theophylline, Adenosine and Dipyridamole) tubes, can be used. They improve specimen stability by limiting platelet activation and liberation of anti-heparin proteins.

- Within 1 hour, blood must be centrifuged at 3,000 g for 20 min at 18°C or below, and plasma decanted into a plastic tube, using a plastic pipette.
- Storage of plasma:
  - Up to 2 hours at 20°C
  - Up to 1 month frozen at –20°C or below (before use, thaw for 15 min. in a water bath at 37°C).

Note: Refer to GEHT or CLSI/NCCLS recommendations for further instructions on specimen collection, handling and storage. Discard any plasma presenting an unusual aspect (haemolysed, lipaemic aspect….).

CALIBRATION CURVE:

Prepare the calibration curve with commercial titrated preparations or by preparing your own calibration.

Using the Heparin reference material, prepare a calibration curve of Heparin, in a normal human citrated plasma pool collected in order to avoid any platelet activation (for assaying plasma samples) or in R4 buffer (for assaying samples in purified milieu), as follows:

Heparin (IU/ml):	0.0	0.25	0.50	0.75	1.0

For calibrations in plasma, it is also possible to use commercially available calibrators (ex: BIOPHEN® Heparin Calibrator #222001 and BIOPHEN® UFH Calibrator #222301).

Then prepare a **1:10** dilution (i.e. 0.1 ml + 0.9 ml) of each point with **R4** buffer for the assay.

In order to get the full assay performances, the calibration curve must be prepared just before running the assay.

SAMPLES AND CONTROLS:

Samples (expected level ≤ 1 IU/ml) will be tested at **1/10** dilution with R4 **buffer**.

To obtain expected level between 0.01 – 0.10 IU/ml in tested solution, purified samples will be tested in appropriate dilution with R4 buffer

TEST PROCEDURE:

The Heparin Anti-Xa assay is specifically designed for two-stage methods, automated on instruments, or can also be used manually with end point methods.

The assay is performed **at 37°C** and the color developed is measured **at 405 nm**.

Whether the method used, the assay must be performed according to the scheme reported for the manual method in order to keep a homogeneous reactivity to Heparin.

- ***Manual method:***

Into the microwell or the plastic test tube, incubated **at 37°C**, introduce:

	Microwell	Test Tube
Calibrator or tested Heparinized sample (At the 1:10 or appropriate dilution).	40 µl*	200 µl
Antithrombin (R1)	40 µl*	200 µl
Mix and incubate at 37°C, for 2 minutes then introduce:		
Factor Xa (R2) preincubated at **37°C**	40 µl*	200µl
Mix and incubate at **37°C,** for **exactly 2 minutes (stage 1),** then introduce:		
Factor Xa Substrate (R3) preincubated at **37°C**	40 µl*	200 µl
Mix and incubate at **37°C** for **exactly 2 minutes (stage 2).**		
Then stop the reaction by introducing		
Citric Acid (20g/L)	80 µl**	400 µl
Mix and measure the absorbance **at 405nm** against the corresponding blank.		

*(Or *50µl and **100µl if preferred).*

The yellow color is stable for 2 hours.

The sample blank, when required, is obtained by mixing the reagents in the reverse order from that of the test i.e. citric acid (20g/l)**,** Factor Xa substrate, Factor Xa, Antithrombin and heparinized sample.

Measure the absorbance **at 405 nm**. The sample blank value must be deduced from the absorbance measured for the corresponding assay.

- ***Automated methods:***

Adaptations to the various analysers (STA-R, etc.) are available upon request. Reconstitution volumes are susceptible to vary according to the automate used**. Refer to each specific adaptation and specific cautions for each instrument.**

Note:

- Unless an adaptation is duly validated, if higher or lower reactive volumes are required for the method used, the same respective proportions for each reagent concentration, and for the overall reactive volume, must be strictly respected, in order to keep a homogeneous reactivity.
- The stage 1 time must be strictly respected because heparin catalyzes antithrombin activity (2). If this time has to be modified for automation, assay parameters must be adjusted accordingly, and are taken into account in the proposed adaptation on instruments.

QUALITY CONTROL:

Use of suitable quality controls allows validating the calibration curve, as well as the homogeneous reactivity of the assay to UFH or LMWH, from run to run, when using a same lot of reagents. Various plasma controls are available:

BIOPHEN UFH Control Plasma (#223101).
BIOPHEN LMWH Control Plasma (#223001).
BIOPHEN LMWH Control Low (#223701).

Note: A new calibration curve must be carried out for each new batch of reagents, after an important maintenance of the instrument, or if measured values are not in compliance with the one expected. Each laboratory can define its own acceptance range, according to the protocols and instruments used.

RESULTS:

For the manual end point method, using a linear graph paper, plot the heparin concentrations (0 to 0.1 IU/ml) on abscissa, and the corresponding A405 on ordinates. Alternatively, statistics software can be used for establishing the dose response calibration curve. An inverse linear relationship is obtained between heparin concentrations and Absorbances (A405). Draw the calibration curve obtained. Calculate the "r²" value. Calibration is acceptable if: **$r^2 \geq 0.98$**

Usually, when using the manual test tube method, the A405 values range from about 1.80 (1.80 ± 0.20) for the 0 IU/ml Heparin concentration, to about 0.40 (0.40 ± 0.20) for the 0.1 IU/ml Heparin concentration in the tested dilution.

Indicatively, for the microplate method, A405 is expected from about 1.2 (1.2 ± 0.20) for the 0 IU/ml Heparin concentration, to about 0.3 (0.3± 0.20) for the 0.1 IU/ml Heparin concentration in the tested dilution. A405 values can differ according to the instrument application used.

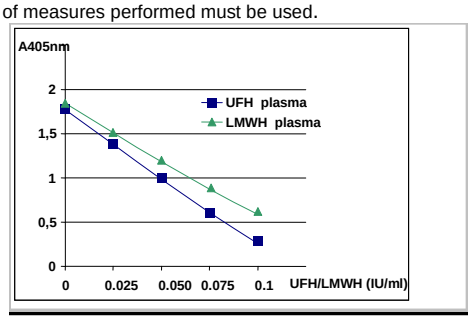
Deduce the heparin concentration for the tested specimen directly from the calibration curve (concentration corresponding to the measured A405), or by using the software.

Multiply the concentration measured by the specimen dilution factor (i.e. x10 for plasma).

The results obtained should be for research purposes only and not used for patient diagnosis or treatment.

EXAMPLE OF CALIBRATION CURVE:

The calibration curves below, obtained using the water bath method, is indicated for UFH or LMWH in plasma as an example only. Only the calibration curve generated for the series of measures performed must be used.



ASSAY PERFORMANCES:

Dynamic range: 0.005 to 0.1 IU/ml in the tested dilution (i.e. 0 to 1 IU/ml in plasma)

Detection threshold: ~ 0.005 IU/ml in the tested dilution.

Standardization: International (NIBSC) or Internal reference for Unfractionated Heparin (UFH) or Low Molecular Weight Heparin (LMWH), Pharmacopoeia preparations spiked in plasma.

In order to get the optimal assay performances, the working instructions must be carefully observed.

CHARACTERISTICS:

This heparin assay is a two-stage Anti-Xa assay for measuring accurately and sensitively heparin concentrations in plasma or in purified systems. Tested plasma needs to be diluted before assaying it.

This assay, using a predilution of Antithrombin and FXa reagents in R4 buffer, is in compliance with the US Pharmacopoeia (USP36:2013) and European Pharmacopoeia (EP:2013) Anti-Factor Xa assay for unfractionated heparin.

VARIANT PROTOCOL:

If a higher working range for heparin is required, the standard assay dilution (d=1:10) can be adjusted accordingly. For example, use a 1:20 dilution (i.e. d: 2) for a working range from 0 to 2 IU/ml in plasma. The heparin concentrations measured must then be multiplied by the dilution factor used.

(*) For UFH, 1 International Unit (IU) is equivalent to 1 USP Unit (4).

REFERENCE:

1. Lyon SG et al. Modification of an Amidolytic Heparin Assay to Express Protein-Bound Heparin and to Correct for the effect of Antithrombin III concentration. Thromb Haemost, 58(3), 884-887. 1987
2. Beeck H et al, Measurement of antithrombin activity by thrombin-based and factor Xa-based chromogenic assays. Blood Coagul Fibrinolysis, 11(2), 127-135, 2000.
3. Holmer et al, Studies of the Mechanism of the Rate-Enhancing Effect of Heparin on the Thrombin-Antithrombin III Reaction. Eur. J. Biochem., 93, 1-5, 1979.
4. USP Statement on Heparin Potency Unit Assignment and Harmonization with the International Standard for Unfractionated Heparin (21 Aug 2009)