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BIOPHEN HEPARIN (AT+)
Ref 221007

Measurement of heparin, and heparin-like anticoagulants, using an anti-Xa kinetics chromogenic method, with addition of exogenous ATIII

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

Last revision: 23/06/2014

INTENDED USE:

Biophen Heparin (AT+) is a kinetics chromogenic assay for measuring the concentration of heparin, and heparin-like anticoagulants, in human citrated plasma or in any other biological fluid, using automatic or manual anti-Xa method, with addition of Antithrombin III (AT). Addition of exogenous AT allows measuring high concentrations of heparin, without lacking of endogenous AT, in vitro exclusively.

This kit is for research use only and should not be used for patient diagnosis or treatment.

TEST PRINCIPLE:

Biophen Heparin (AT+) is a kinetics chromogenic anti-Xa method developed for measuring homogeneously heparin (UFH) and Low Molecular Weight Heparin (LMWH), using the same calibration curve, provided that the adaptation used allows this superimposition.

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. Anti-Xa assays are then the methods of choice for measuring heparins and their analogues.

Biophen Heparin (AT+) is a kinetics method based on the inhibition of a constant amount of factor Xa, by the tested heparin in presence of exogenous antithrombin, and hydrolysis of a Factor Xa specific chromogenic substrate (SXa-11), by the factor Xa in excess. 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]
[FXa (residual)] + SXa-11 ➔ Peptide + pNA

SAMPLES:

Human citrated plasma or other sample to be assayed.

REAGENTS SUPPLIED:

R1*: Reagent 1:* Chromogenic substrate specific for factor Xa (**SXa-11**), lyophilized in presence of mannitol: 2 vials of 15 mg (to be restored with **7.5 mL** of distilled water).

R2: *Reagent 2:* Bovine Factor Xa, lyophilised: 2 vials of about 15 µg (to be restored with **7.5 mL** of distilled water).

R3: *Reagent 3:* Human Antithrombin III (AT), lyophilised: 2 vials of about 200 µg (to be restored with **2.5 mL** of distilled water).

Note:
- This assay was designed for minimizing the interference of anti-heparin substances in plasma, and especially that of PF4.
- Bovine Factor Xa was prepared from bovine plasma, which was tested for the absence of infectious agents, and collected from animals free from BSE. Purified human AT was prepared from human plasma, which was tested with registered methods and found negative for HIV antibodies, HBs Ag and HVC antibodies. However, no test may totally exclude the absence of infectious agents. As any product of biological origin, the reagents must be used with all the cautions required for handling a material potentially infectious.
- The bovine Factor Xa concentration is adjusted for each lot to provide the optimal reactivity in the assay.

REAGENTS REQUIRED BUT NOT PROVIDED:

Reagents:
- Distilled water, preferentially sterile.
- Acetic acid (20%) or 2% citric acid (end point method).
- Normal citrated human plasma pool, obtained in order to avoid any platelet activation, for preparing the heparin calibration range.
- Heparin Reference Material (USP, International Standards from NIBSC, Internal References, etc…) or other analogue to be assayed.
- Alternatively, plasma calibrators with a known concentration of UFH (e.g., # 222301), LMWH (e.g., 222001) (duly validated against the corresponding International Standard (NIBSC)) or other analogue to be assayed (e.g. Arixtra #222501).
- Control plasmas for LMWH (e.g., #223001, 223701), UFH (e.g., #223101) or other analogue to be assayed (e.g., Arixtra #224001).

- ***Materials:***
- Spectrophotometer or automatic instrument for chromogenic assays, at 405nm.
- Stopwatch; Calibrated pipettes.

TRACEABILITY TO THE REFERENCE MATERIAL:

UFH or LMWH calibrators are calibrated against the corresponding International Standards (NIBSC).

STORAGE CONDITIONS:

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

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: Factor Xa specific chromogenic substrate SXa-11

Reconstitute each vial with exactly **7.5 mL** of distilled water. Shake thoroughly (vortex). Let homogenize for 30 minutes at room temperature (18-25°C), while shaking the vial from time to time (vortex), until complete dissolution of the contents. Check the absence of any solid at the bottom of the vial.

Note: In all cases, before use, check the absence of solids at the bottom of the vial, which confirms that dissolution is complete. If necessary, incubate for 1 hour at RT or better at 37°C, while shaking (vortex) from time to time. If required, then additionally incubate overnight at RT.

Stabilize at room temperature and homogenize the contents before each use (vortex).

Stability of restored substrate, kept in its original vial, and provided any contaminaton or evaporation is avoided:
- 3 months at 2-8°C.
- 7 days at room temperature (18-25°C).
- Do not freeze.

REAGENT 2: Bovine Factor Xa

Reconstitute each vial with exactly **7.5 mL** of distilled water. Shake thoroughly until complete dissolution of the contents (vortex). Let homogenize for 30 minutes at room temperature (18-25 °C), while shaking the vial from time to time. Homogenize the contents before each use.

Stability of restored factor Xa, kept in its original vial, and provided any contaminaton or evaporation is avoided:
- 3 months at 2-8°C.
- 7 days at room temperature (18-25°C).
- Do not freeze.

REAGENT 3: Human Antithrombin III (AT))

Reconstitute each vial with exactly **2.5 mL** of distilled water. Shake thoroughly until complete dissolution of the contents (vortex). Let homogenize for 30 minutes at room temperature (18-25 °C), while shaking the vial from time to time. Homogenize the contents before each use. Stability of restored ATIII, kept in its original vial, and provided any contaminaton or evaporation is avoided:
- 3 weeks at 2-8°C.
- 7 days at room temperature (18-25°C).
- Do not freeze.

Cautions:
- In order to improve stability, reagents must be closed with their original screw cap following each use (white caps for factor Xa and AT, yellow cap for SXa-11).
- 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 allows stabilizing the reagents, and obtaining a homogeneous reactivity.

Note:
- R1, R2 and R3 vials are closed under vacuum. Remove carefully the stopper, in order to avoid any loss 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, unless the adaptation is duly validated, the established reactive ratios (respective reagent concentrations in the reactive milieu) 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

TESTED SPECIMEN:

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, Adénosine and Dipyridanole) tubes, can be used. They improve specimen stability. 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 NCCLS/CLSI recommendations for further instructions on specimen collection, handling and storage. Discard any plasma presenting an unusual aspect.

TEST PROCEDURE:

The Biophen Heparin (AT+) kit is specifically designed for Kinetics methods, automated on instruments, and can also be used for end point methods. Adaptations on automates are available upon request. The assay is performed **at 37°C** and the color developed is measured **at 405 nm**.

Whatever 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 UFH and LMWH.

CALIBRATION CURVE:
Using the Heparin reference material, and the standard dilution for the assay, the dynamic range is from 0 to 2 IU/ml heparin (UFH or LMWH). Prepare a calibration curve of Heparin, in a normal citrated human plasma pool, as follows:

Heparin (IU/ml):	0.0	0.5	1.0	1.5	2.0

Biophen Heparin (AT+) offering an homogeneous reactivity to UFH and LMWH, the assay can then be calibrated with the **Biophen Heparin calibrator (#222001**) for measuring UFH or LMWH (5 concentrations from 0 to 1.6 IU/mL).
When a specific calibrator for UFH is required, **Biophen UFH calibrator (#222301)** is available.

If a higher working range for heparin is required, prepare suitable calibrators covering the expected measuring range, and adjust the assay dilution accordingly (for both calibration and samples). For example, use a 1:2 dilution in physiological saline for a working range from 0 to 4 IU/ml, or a 1:5 dilution in physiological saline for a working range from 0 to 10 IU/ml in the tested specimen.
In order to get the full assay performances, the calibration curve can be prepared just before running the assay.

TESTED SAMPLES AND CONTROLS:
Samples and controls are assayed undiluted in the standard protocol.

TEST PROCEDURE:

- ***Manual method:***

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

Reagent	Microwell	Test Tube
Plasma(*)	15 µl	50 µl
ATIII reagent (**R3**)	15 µl	50 µl
R1: Substrate SXa-11 Preincubated **at 37°C**	75 µl	250 µl
Mix and incubate at **37°C**, for 2 minutes, then introduce:		
R2 : Factor Xa Preincubated **at 37°C**	75 µl	250 µl
Mix and incubate at **37°C** for **exactly**,	90 sec.	120 sec.
Then stop the reaction by introducing		
Citric Acid (20g/L)	100 µl	500 µl
Mix and measure the absorbance **at 405nm** against the corresponding blank.		

(*) *The dilution used can vary for assaying high concentrations of heparin, or for other assayed molecules using a specific calibration (e.g., Arixtra).*

The yellow color is stable for 2 hours.

The sample blank is obtained by mixing the reagents in the reverse order from that of the test i.e.: Citric acid (20g/l), substrate SXa-11, sample, ATIII reagent, factor Xa.
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 are available upon request. The assay is then performed kinetically. The reaction does not require to be stopped and sample blanks are automatically subtracted. **Reconstitution volumes can vary according to the automate used. Refer to each specific adaptation and specific cautions for each instrument.**

Note: If higher or lower reactive volumes than those indicated here above are required for the method used, the same respective proportions between reagent concentrations and volumes used, must be adhered to, in order to maintain the assay performances. Run a sample blank in presence of highly lipemic, icteric or haemolysed plasmas, or if the plasmas has a "colour" different from the usual one.

QUALITY CONTROL:

Use of quality control plasmas allows validating the calibration curve, as well as the homogeneous reactivity of the assay, e.g., to UFH and LMWH, from run to run, when using a same lot of reagents. The calibration curve is acceptable when the concentrations measured for controls are within the acceptance range. Various types of controls are available:
Biophen UFH Control: (low range) for UFH (#223101).
Biophen LMWH Control: (high range) for LMWH (#223001).
Biophen LMWH Control Low: (low range) for LMWH (# 223701).
Or **Biophen Arixtra Control**: for Arixtra (# 224001).
Each laboratory should verify its own target value and acceptance range, in the exact working conditions, for each new lot of controls.

Note:
- A new calibration curve must be carried out for each new lot of reagents, after each important maintenance of the analyzer, or when measured values for the quality controls are out of the acceptance range determined for the method.
- Each laboratory can establish its own acceptance ranges, according to the instruments and protocols used.
- Include at least one quality control (at different levels) in each test series.

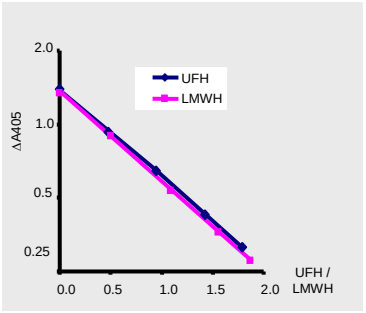
RESULTS:

For the end point manual method, using a semi-logarithmic graph paper, trace the calibration curve by plotting the absorbance at 405nm (**A405**) on ordinates, and the corresponding heparin concentration (0 to 2 IU/ml) on abscissae. Alternatively, statistics software can be used for establishing the dose response calibration curve. An inverse relationship is obtained between heparin concentrations and Absorbances (A405). Draw the calibration curve obtained.
Calculate the "**r²**" value: calibration is acceptable if **r² ≥ 0.98** for automated methods, and if measured values for controls are in compliance for the method used.
The heparin (or analogue) concentration in the tested specimen is directly deduced from the calibration curve (concentration corresponding to the measured A405 value), or by using the software.
Results are expressed in anti-Xa International Units per mL (IU/mL).

- Using automated methods, the concentrations are directly calculated by the analyser, respectively to the calibration curve.

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

EXAMPLE OF CALIBRATION CURVE: The calibration curve below is an example only, using the STAR method. Only the calibration curve generated for the series of assays performed must be used for calculating the concentrations.



PERFORMANCE CHARACTERISTICS:

- The enzymatic reaction is rapid, and allows obtaining a high sensitivity for this heparin (or analogue) assay.
- The **detection threshold** for the assay is evaluated on the calibration curve by measuring the "apparent" heparin concentration, which corresponds to the mean A405 value obtained for a sample free of heparin less 3 Standard Deviations (SD). This detection threshold is of about 0.05 IU/mL for heparin and 0.05 µg/ml for Arixtra.
- The **dynamic range** and working dilution, as well as associated performances, must be checked and validated in the exact laboratory working conditions (combination reagents and instrument) for each assayed anti-Xa molecule, depending on the obtained slope and linearity ($r^2 \geq 0.98$ is expected for automated methods).
The dilution of the calibration **and** assayed plasma must be identical, and can vary from 1:1 (undiluted) to 1:5 in physiological saline (or more) according to the assayed molecule and the expected range.
For concentrations ranging from 0 to 2 IU/ml "anti-Xa equivalent" (Eq.) in the tested dilution, the measuring range is from 0 to 2 Eq. for a 1:1 working dilution, from 0 to 4 Eq. for a 1:2 dilution, or from 0 to 10 Eq. for a 1:5 dilution (in physiological saline).
E.g., for Arixtra in the range 0 to about 1.5µg/ml, it is recommended for the manual method to test both the calibration and the plasma samples diluted 1:2 in physiological saline.
- Each kit allows running 2 series of about 50 tests using the manual method (test tube) or of about 100 tests by automated method.
- Indicatively, using a semi logarithmic scale on STA-R, the assay is linear up to about 2 IU/ml anti-Xa for UFH and LMWH using the standard protocol (1:1); or up to about 1.5µg/ml Arixtra when using the protocol "1:2 working dilution in physiological saline" (managed by the instrument).
- Example of **reproducibility** results obtained for heparins using the STAR instrument, on N=10:

Sample	IU/ml or µg/ml	Intra assay CV (%)
C1/UFH	0.21	3.6
C2/UFH	0.51	2.0
C3/LMWH	0.70	4.1
C4/LMWH	1.13	3.0

- Example of **reproducibility** results obtained for Arixtra using the STAR instrument:

	µg/ml	Intra assay CV (%) (N=10)	Inter assay CV (%) (%) (N=7)
C1/Arixtra	0.44	2.1	1.6
C2/Arixtra	1.18	2.3	3.3

- Results are well correlated with those obtained using Biophen Heparin (#221006) on STAR, on plasmas samples spiked with Arixtra: N=31 Y = 0.99X r2 = 0.994

Limitations of the procedure:

- Blood activation, during specimen collection and plasma preparation, may release platelet factor 4, which can inhibit heparin.
- No significant interference is observed for bilirubin concentrations <0.1 mg/ml, haemoglobin concentrations <2 mg/ml and triglycerides concentrations <1.25 mg/ml added to plasma. High levels of haemoglobin or of triglycerides may affect the results.
- For each assayed molecule, check that the AT concentration added in the assay is sufficient, as the measured concentration could be underestimated as the result of lack of AT.
- In order to get the optimal assay performances, comply strictly to the procedural instructions.
- Biophen Heparin (AT+) reagents are developed for measuring homogeneously Unfractionated Heparin (UFH) and Low Molecular Weight Heparin (LMWH), using the same calibration curve. The superimposition conditions are susceptible to slightly vary according to the combination of reagents and lots used, and the technical characteristics and specific adjustment of each instrument. Consequently, this superimposition has to be verified and validated in the exact laboratory working conditions and for each machine. Should this superimposition not be obtained, a separate calibration curve must be used for each type of heparin, and validated by assaying the homogeneous quality control system.

REFERENCES:

1. Leslie B et al. Investigation of the anticoagulant mechanism of a covalent antithrombin-heparin complex. J Biol Chem 52 (273): 34730-34736 (1999).
2. Charles K M et al. Design synthesis and structure activity relationship of a series of arginine aldehydes factor Xa Inhibitors. Part 1: structure based on the (D)-Arg-Gly-Arg tripeptide sequence. Bioorganics Med chem Letter 10 (2000): 13-16 (1999).