BIOPHEN HEPARIN 6
Ref 221006
Measurement of Unfractionated Heparin (UFH), using an anti-Xa chromogenic method

For in vitro diagnostic use only

INTENDED USE:
The Biophen Heparin 6 kit is a chromogenic assay for the quantitative determination of Unfractionated Heparin (UFH) in human citrated plasma using automated or manual method.

CLINICAL BACKGROUND:
Unfractionated Heparin (UFH) is currently used for curative or preventive indications. Measuring the heparin concentration in patients’ plasma allows monitoring the therapy and adjusting drug dosage.

TEST PRINCIPLE:
Biophen heparin 6 is a chromogenic anti-Xa method developed for measuring homogeneously heparin (UFH), in plasma.

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.

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

Biophen Heparin 6 is a kinetics method based on the inhibition of a constant amount of factor Xa, by the tested heparin in presence of endogenous 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

REAGENTS REQUIRED BUT NOT PROVIDED:

Reagents:

- Bovine Factor Xa was 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, this factor Xa must be used with all the cautions required for handling a material potentially infectious.
- The bovine Factor Xa concentration is adjusted for each lot for providing the right reactivity in the assay.

Note: This assay was designed for minimizing the interference of anti-heparin substances in plasma, and especially that of PF4.
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REAGENTS SUPPLIED:

Biophen Heparin 6 kit contains 4 vials of a specific Factor Xa substrate, and 4 vials of bovine Factor Xa:

R1: Reagent 1:
Chromogenic substrate specific for factor Xa (SXa-11), lyophilized in presence of mannitol:
4 vials of 15 mg (to be restored with 6 mL of distilled water).

R2: Reagent 2:
Bovine Factor Xa, Lyophilized:
4 vials of about 15 µg (to be restored with 6 mL of distilled water).

Note:
- Reconstitute each vial with 6 mL of distilled water. Shake thoroughly (vortex), let to homogenize for 30 minutes at room temperature (18-25°C), while shaking the vial from time to time (vortex), until complete dissolution of the content. Check the absence of any solid at the bottom of the vial.

REAGENT 1: Factor Xa specific chromogenic substrate SXa-11
Reconstitute each vial with exactly 6 mL of distilled water. Shake thoroughly (vortex). Let to homogenize for 30 minutes at room temperature (18-25°C), while shaking the vial from time to time (vortex), until complete dissolution of the content. 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 room temperature.

Stabilize at room temperature and homogenize the content before each use (vortex). Stability of restored substrate, kept in its original vial:
- 3 months at 2-8°C.
- 7 days at room temperature.
- Do not freeze.

REAGENT 2: Factor Xa
Reconstitute each vial with exactly 6 mL of distilled water. Shake thoroughly until complete dissolution of the content (vortex). Let to homogenize for 30 minutes at room temperature (18-25°C), while shaking the vial from time to time. Homogenize the content before each use.

Stability of restored factor Xa, kept in its original vial:
- 3 months at 2-8°C.
- 7 days at room temperature.
- 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, yellow caps 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 stabilising the reagents, and obtaining a homogeneous reactivity.

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.

PREPARATION AND STABILITY OF REAGENTS:
Note: Reconstitution volumes can vary according to the automate used. Refer to each specific instrument adaptation and specific cautions on each instrument.

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PREPARATION OF 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.

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).

Refer to NCCLS document H21-A2 for further instructions on specimen collection, handling and storage.
TEST PROCEDURE:
The Biophen Heparin 6 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.

Manual method:
Into the microwell or the test tube, incubate at 37°C, introduce:

<table>
<thead>
<tr>
<th>Microwell</th>
<th>Test Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted plasma</td>
<td>12 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>36 µl</td>
</tr>
<tr>
<td>R1: Substrate SXa-11 Preincubated at 37°C</td>
<td>80 µl</td>
</tr>
</tbody>
</table>

Mix and incubate at 37°C, for 2-5 minutes then introduce:

<table>
<thead>
<tr>
<th>R2 : Factor Xa Preincubated at 37°C</th>
<th>Mix and incubate at 37°C for exactly,</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 µl</td>
<td>90 sec.</td>
</tr>
<tr>
<td>200 µl</td>
<td>120 sec.</td>
</tr>
</tbody>
</table>

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 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, undiluted plasma, distilled water, factor Xa. Measure the absorbance at 405 nm. The sample blank value must be deducted from the absorbance measured for the corresponding assay.

Note:
- 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 an homogeneous reactivity.

CALIBRATION:
Biophen Heparin 6, can then be calibrated with the Biophen UFH calibrator (#223101), set of 5 calibrators at various UFH concentrations, covering the assay dynamic range.

QUALITY CONTROL:
Use of quality control plasmas allows validating the calibration curve, from run to run, when using a same lot of reagents. Quality control plasmas (lyophilized) are available:

Biophen UFH Control: (low range) for UFH (#223101).

RESULTS:
The heparin concentration in the tested specimen is directly deduced from the calibration curve. Results are expressed in anti-Xa International Units/mL (IU/mL), by reference to the International Standards (NIBSC).

Using a semi-logarithmic scale:
The assay is linear up to 1.0 IU/mL anti-Xa for UFH.

EXAMPLE OF CALIBRATION CURVE AND QUALITY CONTROL:
The following calibration curve, obtained with UFH, is indicated as an example only. The calibration curve generated for the series of measures performed must be used.

Quality Control: The calibration curve is acceptable when the concentrations measured for controls are within the acceptance range.

Note:
- Include at least one quality control (at different levels) in each series, in order to validate it.
- 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.

SPECIFIC PERFORMANCE CHARACTERISTICS:
- The enzymatic reaction is rapid, and allows obtaining a high sensitivity for this heparin assay.
- The detection threshold is of 0.05 IU/mL.
- Example of reproducibility obtained with plasmas supplemented within UFH, when using tACL 7000 instrument (IL).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intra Assay CV%</th>
<th>N</th>
<th>Inter Assay CV%</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>UFH Level 1 (0.38 IU/mL)</td>
<td>2.1</td>
<td>15</td>
<td>2.0</td>
<td>20</td>
</tr>
<tr>
<td>UFH Level 2 (0.74 IU/mL)</td>
<td>1.0</td>
<td>15</td>
<td>2.3</td>
<td>20</td>
</tr>
</tbody>
</table>

- Correlations: The BIOPHEN Heparin assay shows good correlation with Coamatic® Heparin performed on BCS and STA instruments, and with Rotachrom Heparin performed on STA-R instrument:
  - Coamatic® Heparin versus BIOPHEN Heparin (on BCS): Y = 0.91 X - 0.03 r = 0.99
  - Coamatic® Heparin (on BCS) versus BIOPHEN Heparin (on STA): Y = 0.87 X -0.06 r = 0.98
  - BIOPHEN Heparin versus Rotachrom Heparin (on STA-R): Y = 1.07 X -0.06 r = 0.97

All the studies were conducted outside the US.

Another study compared Biophen Heparin with Rotachrom Heparin in US. The following data were generated:

UFH: N = 40; y = 0.95 x – 0.0207; r = 0.976; r² = 0.952

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 <1.25mg/ml. High levels of haemoglobin or of triglycerides may affect the results. In order to get the full assay performances, the working instructions must be carefully observed.
- If the ATIII concentration in the tested plasma is >50%, heparin can be underestimated as the result of lack of ATIII. A variant protocol, with an exogenous source of ATIII, must then be used.
- High ATIII concentrations (> 150%) could interfere with the assay and mimic presence of low amounts of heparin.
- Underestimation of heparin concentration and heparin resistance has been reported in some patients with amyloidosis (6).
- In order to get the optimal assay performances, comply strictly to the procedural instructions.

EXPECTED VALUES:
For obtaining the right efficacy along with the lowest bleeding risk, heparin dosage must be within the therapeutic range recommended by each drug manufacturer, and for each specific indication.

REFERENCES: