



Manufactured By: HYPHEN BioMed

BIOPHEN HEPARIN

ANALYSIS OF THE AT III EFFECT IN THE BIOPHEN HEPARIN DEVICE

(Biophen Heparin 6 and Biophen Heparin 3)

(Ref. A221003 & A221006)

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BIOPHEN HEPARIN

Effect of ATIII concentration in the tested plasma on the measurement of Heparin concentrations.

1. Aim of the study:

The BIOPHEN Heparin assay is an anti-Xa chromogenic method for measuring Heparin (UFH) or Low Molecular Weight Heparin (LMWH) in plasma. In this assay, Heparin can form complexes with endogenous ATIII, and then inhibits bovine Factor Xa, which is present at a constant concentration and in excess.

The residual amount of Factor Xa is then measured by its activity on a specific substrate for factor Xa, SXa-11.

The assay requires ATIII, in excess, respectively to the amount of Heparin or LMWH to be measured. This ATIII is supplied by the tested plasma itself. The aim of the study is:

- To determine the lowest concentration of ATIII necessary for allowing an accurate measurement on the overall working range (0 to 2 IU/ml).
- To propose an alternative method when the ATIII is a limiting Factor.
- To check the effect of high ATIII concentrations in plasma, as it can be the case during the use of therapeutic ATIII concentrates, on the measurements of UFH or LMWH.

2. Background:

ATIII is a "serpin" (serine esterase protein inhibitor), with a molecular weight of 58 Kd, (58 000 Daltons), about 150 µg/ml (i.e. 2:5 µM/L).

It forms a stoichiometric complex with UFH or LMWH, and is then a potent inhibitor of Factor Xa.

If ATIII is at a molar concentration below that of UFH or LMWH, then the Heparin measurement can be underestimated.

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3. Materials and reagents:

- BIOPHEN Heparin
- Human plasma, made deficient in ATIII by depletion on Heparin Sepharose.
- Purified Human ATIII, from HYPHEN BioMed: vial of 1.5 mg or 10 Units (Reference APP004B), or vial of 3.75 mg or 25 Units (Reference APP004D).
- Unfractionated Heparin: Sodium Heparin from Choay (Sanofi-Aventis), vial of 5 ml (5000 IU/ml).
- Low Molecular Weight Heparin, Fragmin at 10 000 IU/ml, Lot 96072A51, exp 04-2003.
- BIOPHEN Heparin Calibrator.

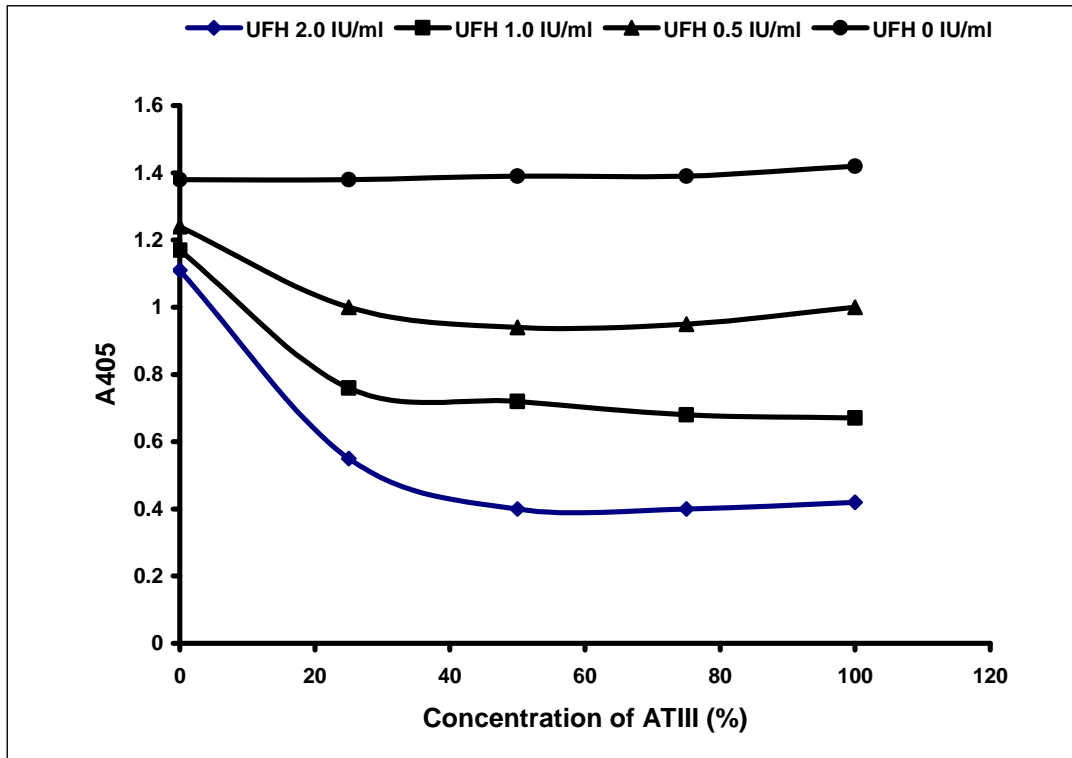
4. Protocol:

- A concentration range of UFH or LMWH is spiked in human citrated plasma, at different ATIII concentrations. These plasmas, containing different ATIII concentrations, are obtained by mixing variable amounts of ATIII deficient plasma with normal human plasma, in order to achieve the ATIII concentrations of: 0 % ; 25% ; 50% ; 75% and 100%. The UFH or LMWH concentrations tested range from 0 to 2 IU/ml (0 ; 0.5 ; 1 ; and 2 IU/ml). The heparin is then measured respectively to a calibration curve obtained with the BIOPHEN Heparin Calibrator. This study allows determining the lowest ATIII concentration required for accurately measuring UFH and LMWH over the dynamic assay range.
The BIOPHEN Heparin assay is used according strictly to the insert protocol. The method where reagents are restored with 6 ml of distilled water, for Biophen Heparin 6, or 3 ml of distilled water for Biophen Heparin 3, is used.
- A protocol variant is then designed for compensating the lack of ATIII, when this inhibitor is at a concentration below the one required.
ATIII is directly diluted in water (or in a solution of 1% BSA in distilled water), and added in the assay as a replacement of distilled water. As the assay involves one volume of plasma and 3 volumes of distilled water, ATIII is tested at concentrations of about 20%, 30%, 40% and 50% (i.e. 30, 45, 60, and 75 µg/ml), which corresponds to a plasma supplementation with ATIII of 60%, 90%, 120% and 150%.
- The same variant protocol is then used for testing the effect of high ATIII concentrations in plasma, by replacing distilled water in the assay by ATIII at 35%, 70% and 100%, corresponding to an actual ATIII supplementation in plasma of about 105% , 210% and 300%

5. Results:

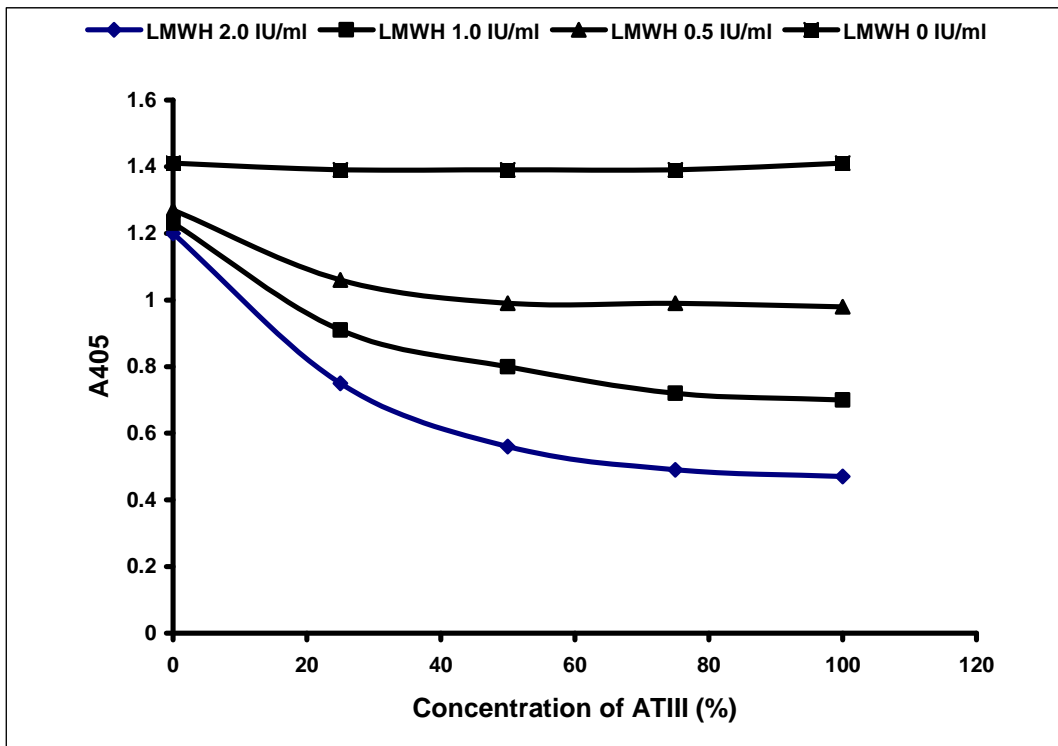
1.1 ATIII Defect:

The here below graphs show the A405 obtained for 4 concentrations of UFH or LMWH spiked in plasma with ATIII concentrations ranging from 0 to 100 %.



There is no effect of AT III concentrations, when this inhibitor is present at least at a 50 % concentration.

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Same comment than here above.

These data show that a concentration of at least 50% ATIII is required for ensuring the right measurement of UFH or LMWH in plasma.

1.2 Supplementation of ATIII:

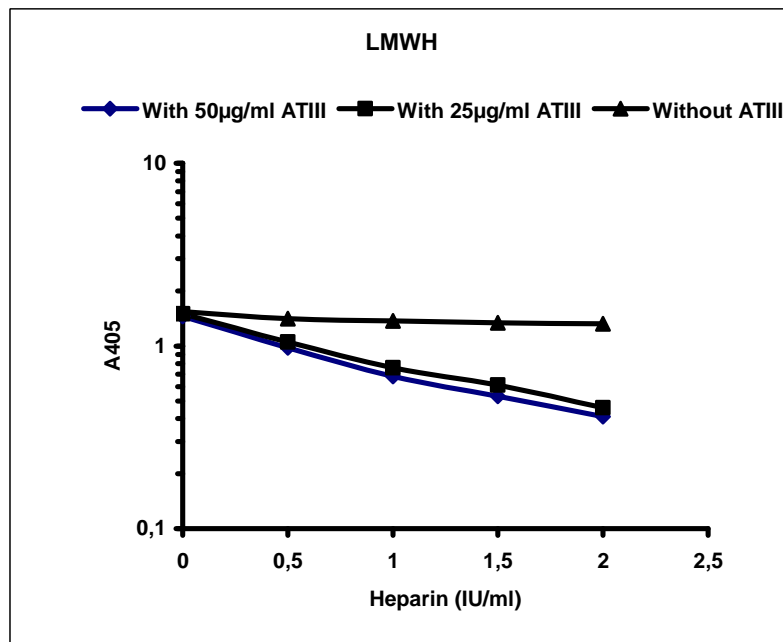
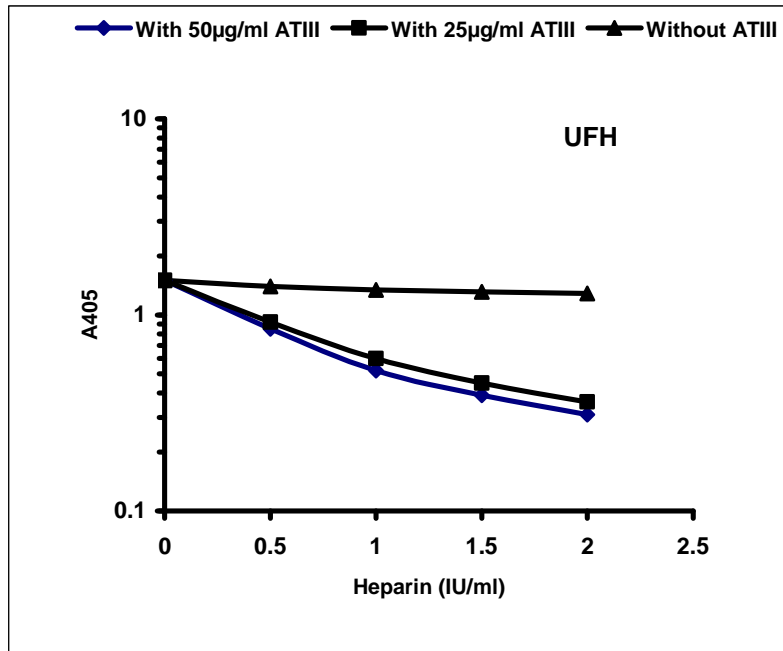
When low concentrations of ATIII are expected or present, an exogenous supply of ATIII in the BIOPHEN Heparin assay must be considered.

In the BIOPHEN Heparin protocol, distilled water is then replaced by a purified ATIII preparation in water, or, preferentially, in 1% Bovine Serum Albumin solution (1% BSA), in distilled water.

The ATIII concentration to be used is of 25 to 50 µg/ml, achieving a corresponding concentration in the assayed plasma of 50 to 100%.

The here below graphs show the correcting effect of ATIII, added exogenously, on the BIOPHEN Heparin assay. Dose response curves UFH or LMWH are prepared in an ATIII deficient plasma, and tested in the assay without or with addition of exogenous ATIII.

These data show that a concentration of ATIII of 50% in the tested plasma allows an accurate measurement of LMWH or UFH.

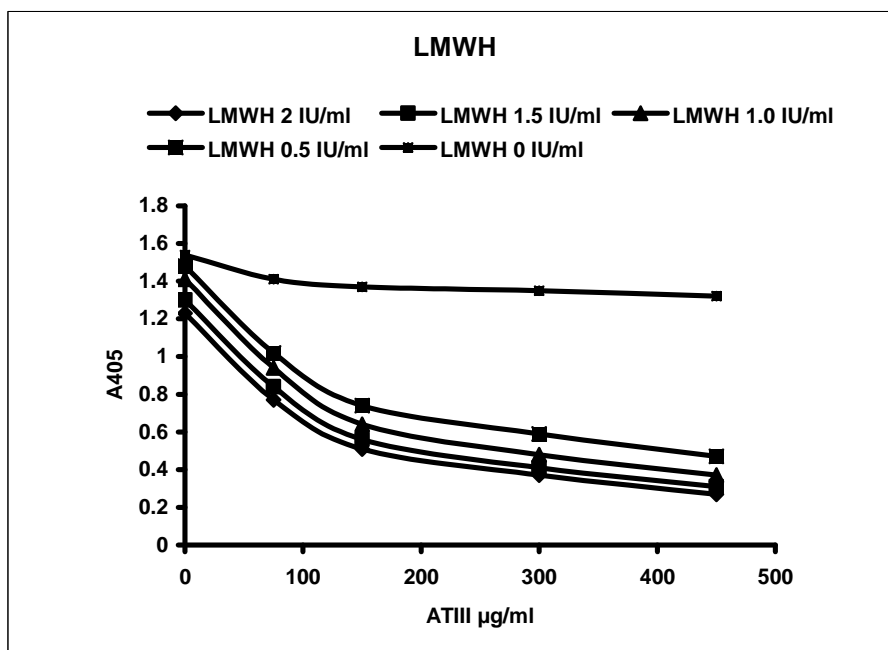
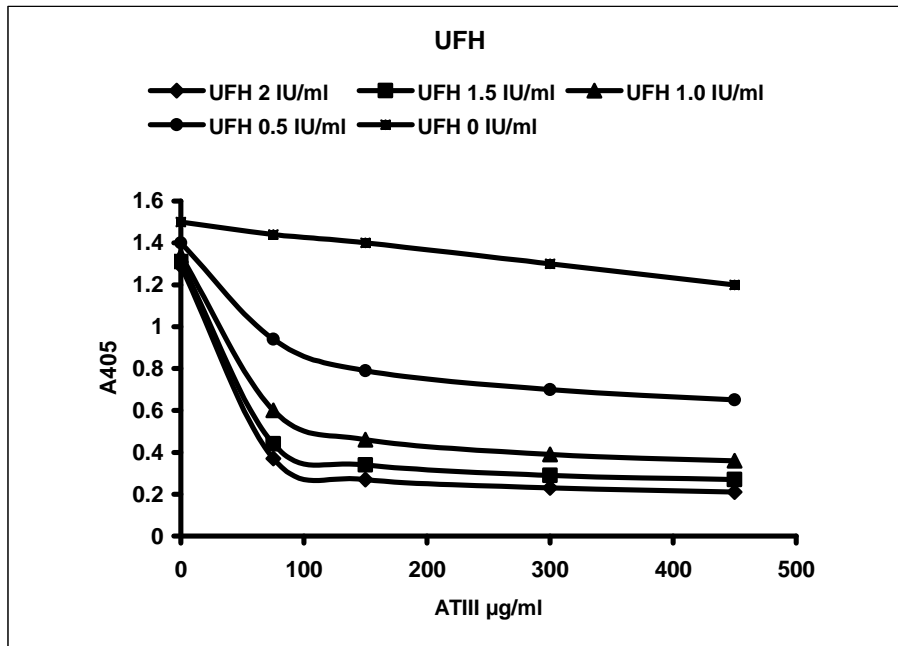


These data show that supplementing the assay with 25 µg/ml of purified human AT III is sufficient for ensuring the right reactivity, and measuring correctly the UFH or LMWH concentrations.

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1.3 Effect of High ATIII concentrations:

The assay of BIOPHEN Heparin was performed using increasing ATIII concentrations, instead of distilled water, in order to check the effect of ATIII levels, on measurements of UFH or LMWH. ATIII is expressed, on the here below graphs, as the corresponding concentration in plasma. It is reminded that 100% of ATIII corresponds to about 150 µg/ml.



These data show that there is already a progressive inhibition (but very slow) of Bovine Factor Xa with increasing concentrations of ATIII in the chromogenic assay of Heparin (UFH) or LMWH. This inhibition is evidenced by a decreased A405, already for the 0 IU/ml Heparin concentration. However, when 25µg/ml of heparin are added in the assay (corresponding to 75 µg/ml in tested plasma or 50 %), a right dose response curve is obtained with UFH or LMWH. When increasing ATIII concentrations are used, there is a parallel translation of the calibration curve. The assay is valid, provided that the same ATIII concentration is present throughout the assay.

When a plasma with high ATIII concentrations is present in a test series, this could mimic presence of heparin, as the result of the progressive ATIII inhibitory activity on Factor Xa.

6. Conclusions:

- The assay is insensitive to ATIII concentrations, provided that this endogenous ATIII is present at a concentration > 50 %.
- If low ATIII concentrations are present (<50%), an assay variant can be designed by replacing the addition of distilled water by a 25µg/ml ATIII concentration in 1% BSA in distilled water.
- If high ATIII concentrations are present, (>150%), this can interfere in the assay and mimic presence of low amounts of heparin.

NOTA: Variant method (7.5 ml or 3.75 ml)

- The Biophen Heparin assay practice has been “improved” by proposing a new variant method. Reagents R1 and R2 are restored with 7.5 ml of distilled water, for Biophen Heparin 6, or with 3.75 ml for Biophen Heparin 3.
- Plasma is diluted with AT III at 75 µg/ml concentration in physiological saline (9g/L NaCl) containing 1 % BSA, instead of AT III at 25 µg/ml in distilled water containing 1 % BSA, and the assay is performed as follows, at 37°C:

25 µl Plasma (Tested or calibrator)

25 µl AT III in Physiological saline containing 1 % BSA

125 µl of R1 (Substrate)

- Incubation of 2 – 5 min at 37°C

125 µl of R2 (Factor Xa)

- Incubation of 120 seconds (test tube) at 37 °C (or kinetics mode)

Stop the reaction with 300 µl of 2 % Citric Acid (or 20 % Acetic Acid); this step is not required for the kinetics mode.

Read at 405 nm versus the corresponding blank (blank is not required for the kinetics mode).

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