



**Abstract N° PP-MO-419**

**NEW ASSAYS FOR MEASURING DIRECT THROMBIN INHIBITORS IN PLASMA**

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Direct Thrombin Inhibitors (DTIs: Lepirudin, Bivalirudin, Argatroban, Dabigatran, etc...) have increasing applications in severe clinical situations associated with a high risk context, and for some of them have promising applications as prophylactic drugs (oral absorption). When used for curative applications, laboratory methods are required for drug efficacy adjustment and for avoiding overdosage. These methods must present the most limited impact to the progressive activity of plasma Anti-Thrombin (AT), which must not interfere in the assay. A clotting method and a chromogenic assay were developed for quantitating DTIs. For the clotting assay, a substrate normal plasma pool (or a procoagulant mixture containing purified fibrinogen) is mixed with diluted test plasma (1:8 to 1:20), and coagulation is initiated with human thrombin (in the alpha form) in presence of calcium, and clotting time (CT) is recorded. A linear dose-response curve is obtained between DTI concentration and CT (from about 30 seconds to 90 seconds;  $r^2 > 0.99$ ). This assay has no matrix effect and can be used for any DTI. In the chromogenic kinetics assay, tested specimen (1:10) is incubated with thrombin substrate, and human thrombin is then added. Colour development measured at 405 nm is an inverse relationship of DTIs' concentration. This assay has excellent performances with Hirudin and its analogues, but is not sensitive for Argatroban concentrations in the usual therapeutic range (this DTI has a potent anti-coagulant effect but its inhibitory potency on the thrombin chromogenic activity is low and "protected" by AT). These methods have a dynamic range for Hirudin from 0.1 to 2.0  $\mu\text{g/ml}$ , and this range can be extended from 0.25 to 5.0  $\mu\text{g/ml}$  in some applications such as ECC. Specific calibrations with the DTI used are required. The clotting method also offers a working range from 0.1 to 2.0  $\mu\text{g/ml}$  of Argatroban. Both methods offer safe, reliable and rapid tools for measuring DTIs' activity in plasma. They introduce appropriate tools for analytical and pre-clinical studies for emerging DTIs, such as dabigatran etexilate. Especially, the clotting method is of high practice and accuracy and can be used in any laboratory equipped with a coagulation instrument.

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## INTRODUCTION

- Direct Thrombin Inhibitors (DTIs) have increasing and promising curative, preventive or prophylactic applications in severe clinical situations at high risk context, and are candidates for substituting to long term oral anticoagulant therapies with vitamin K antagonists.
- Laboratory methods are required for adjustment of drug efficacy and for avoiding overdosage. They must present the most limited impact to other plasma factors (eg. Antithrombin, Prothrombin, Fibrinogen).
- Ecarin Clotting Time (ECT) and aPTT are useful but too sensitive, insufficiently reliable at high DTI therapeutic levels, and patient coagulation factors may interference.
- Specialized calibrated clotting and chromogenic assays, fully automatable, with no matrix effect, accurate and sensitive at low and high concentration ranges, were developed for quantitating various DTIs.

## METHODS

### Clotting assay ("Hemoclott Thrombin Inhibitors"):

Sensitized thrombin time, using a "substrate" normal plasma pool (R1) mixed with the diluted test plasma (1:8 to 1:20). Clotting time (CT) is recorded after addition of (h)- $\alpha$ -thrombin (R2) containing calcium.

### Chromogenic kinetics assay ("Biophen DTI"):

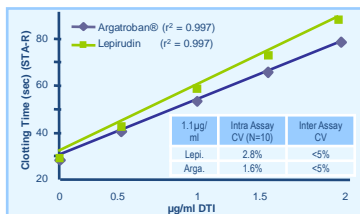
Tested specimen (1:10 to 1:30) is incubated with thrombin substrate (R1), and (h)- $\alpha$ -thrombin (R2) is added. Measured A405 is inversely proportional to DTI concentration.

### Aim:

- To evaluate dose response curves to various DTIs in plasma;
  - To establish accuracy, reproducibility;
  - To compare with a conventional aPTT assay.
- Direct Thrombin Inhibitors tested:
- Lepirudin (Refludan<sup>®</sup>)
  - Argatroban<sup>®</sup>

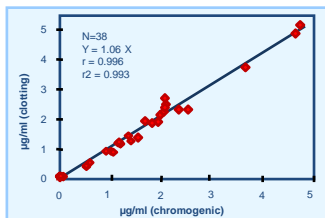
## RESULTS

Clotting assay: dose response curves with 2 DTIs



>Excellent linearity in the usual therapeutic range for any DTI (Lepirudin, Argatroban<sup>®</sup> ...).

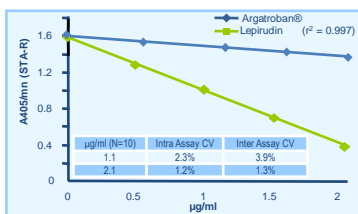
Linear regression analysis for measured Lepirudin with both methods (various levels added to normal plasmas)



>Excellent correlation and possible extended dynamic range up to 5µg/ml (usual eg. in ECT).

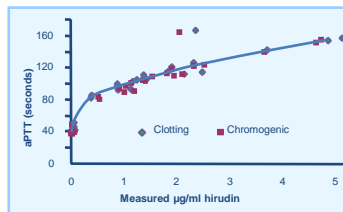
NB: For Argatroban<sup>®</sup>, the clotting assay offers a much higher sensitivity than the chromogenic assay, which is then not appropriate within the normal therapeutic range.

Chromogenic assay: dose response curve with 2 DTIs



>Excellent performance with Hirudin and analogues, but not suitable for the Argatroban<sup>®</sup> usual therapeutic range (with low inhibitory potency of thrombin chromogenic activity).

Measured aPTT on Lepirudin spiked plasma samples (clotting or chromogenic assays), normals or Argatroban<sup>®</sup> treated patients (clotting only).



Mean [Range]	µg/ml Argatroban (clotting assay)	ratio M/T (aPTT reagent 1)	ratio M/T (aPTT reagent 2)
Normals (N=20)	0 [-0.01]	1.0 [0.9-1.2]	1.0 [0.9-1.1]
Treated patients (N=33)	1.0 [0.3-2]	2.5 [1.0-4.9]	3.2 [1.2-6.1]

>aPTT (>1 µg/ml) lacks of linearity and reliability (clotting times too prolonged).

## CONCLUSIONS

- Clotting and chromogenic assays **simple and rapid, standardized, calibrated** with the DTIs used. Specific calibrators and controls available for Hirudin and Argatroban<sup>®</sup>.
- Clotting method (1:8 dilution): excellent linearity, sensitivity, and accuracy over the usual therapeutic range, from **0.1 to 2.0 µg/ml** (possibly 0.25 to 5.0 µg/ml) for **Lepirudin and Argatroban<sup>®</sup>**, and we predict that it could also be used with **Bivalirudin<sup>®</sup>** or new oral DTIs such as **dabigatran etexilate**.
- Reflects the patient "true anti-IIa potential".
- Well correlated methods ( $r^2 > 0.99$ ), consistent with aPTT results.
- Safe, highly stable, and reliable tools, easily performed with basic equipment or major coagulation analyzers**, for measuring DTIs' activity in plasma with **no matrix effect** or in purified milieu. Especially useful for monitoring DTIs in emergency or in curative applications, and in analytical and pre-clinical studies for emerging DTIs, for which methods are requested by both users and authorities.

## GENERAL REFERENCES

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