Appendix D

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COMPARATIVE IN VITRO STUDY ON THE EFFECT OF ENOXAPARIN DERIVED COMPOUNDS ON FVIIa ACTIVITY IN PURIFIED RECONSTITUTED EXPERIMENTAL SYSTEM.

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Mai 2006

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Introduction and background

The tissue factor (TF) pathway is preponderant in the initiation of blood coagulation in normal haemostasis and in thrombotic states ([1]). Factor VII (FVII) is a serine protease zymogene that is converted by limited proteolysis to activated factor VII (FVIIa) - a two chain serine protease. In steady state conditions, traces of FVIIa are present in normal plasma whereas FVIIa levels increase in thrombotic states ([2], [3]). The blood coagulation process is initiated when cryptic tissue factor is exposed to circulating blood and binds to plasma factor VIIa triggering the thrombin generation process ([4]). Thrombin generation may be described as occurring in two consecutive phases (reviewed in [5] and [6]). In the *initiation phase*, FVIIa/TF complex activates factor IX (FIX) and factor X (FX) ([7], [8]). The free factor Xa that is initially produced generates picomolar amounts of thrombin which induce an initial platelet activation. Additionally, FXa cleaves FIX and generates FIXα, which is also the intermediate product following the proteolytic cleavage of FIX by either FVIIa/TF complex or FXIa ([9],[10]). The *propagation phase* is characterized by further activation of platelets by thrombin, which also activates factors VIII, V and XI. Formation of the enzymatic complex composed by FIXa, FVIIIa, negatively charged phospholipids and calcium ions (intrinsic tenase) leads to further activation of FX, which in the presence of FVa, phospholipids and calcium ions forms the prothrombinase complex. Prothrombinase is the activator of prothrombin and induces a burst of thrombin generation. During the coagulation process, feedback activation of FVII zymogene is induced principally by FXa, FIXa and FVIIa/TF complex as well as by FXIa and FXIIa ([11], [12], [13]). After triggering the TF pathway the contribution of FX, FIX and FXI to the activation of FVII was significantly more important than that of any other serine protease of blood coagulation ([14]).

Since the amount of generated serine-protease is amplified at each step downstream of the coagulation process, inhibition of the generation and the activity of serine proteases located at the initial steps of blood coagulation may result in a diminution of thrombin generation. Factor Xa is located at a critical point of the coagulation process, but upstream as compared to thrombin.

In steady state conditions approximately 1% of total plasma factor VII protein is present as the cleaved enzymatic form of the protein, factor VIIa ([15]). The extent of the thrombin production is largely dependent on the preexistent concentration of factor VIIa in plasma and the concentration of tissue factor exposed at the vascular lesion ([16]).

Recently it has been shown that FVIIa is in competition with the zymogene FVII which functions as an inhibitor of thrombin generation. An increase of FVIIa saturates the available TF molecules and accelerates thrombin generation ([17]).

The activation process of FVII by coagulation proteases which are essential components of blood coagulation mechanism (i.e. FXa, FIXa, FXIIa and thrombin) is well studied and feed-back activation loops of FVIIa after initiation of blood coagulation have been described in details (see previous paragraphs). At present, no adequate description exists for the mechanism by which resident plasma factor VIIa is produced and circulates.

Recently published studies stress the importance of distinct FVII activation pathways from proteases that are not directly implicated in blood coagulation mechanisms. Such an alternative pathway is the Factor VII activating protease (FSAP) ([18],[19]).FSAP is a potent activator of the coagulation zymogen factor VII ([20]) and was also found to

be an activator of single-chain pro-urokinase-type plasminogen activator ([21]). Genetic studies observed relatively common polymorphisms in this protein, (i.e Marburg polymorphisms) are associated with progressive stages of stenotic vessel disease originating from atherothrombosis ([22]).
In a prior study we showed that a LMWH (enoxaparin), unfractionated heparin and the synthetic pentasaccharide, significantly inhibited FVIIa generation and prothrombin activation during *in vitro* clotting of human plasma ([23]). More recently we showed that the synthetic pentasaccharide (as well as enoxaparin) enhances the AT-mediated inhibition of FXa and FIXa, resulting in a significant diminution of FVIIa generation (14). Inhibition of FIXa by the synthetic pentasaccharide (fondaparinux) has been recently shown by the group of J Weitz ([24]).

Clinical issues: FVIIa levels in plasma, hypercoagulable state and association with thrombotic risk

It is established, though some issues yet have not been fully clarified, that increased FVIIa levels are associated with high risk for ischemic coronary events (3,[25,26,27,28,29]). In addition, before antithrombotic treatment initiation plasma levels of FVIIa are about 3 - 4-fold increased as compared to the normal values ([30]). Myocardial ischemia and cardiopulmonary bypass surgery induce activation of FVII and increase thrombin generation ([31]). Moreover increased levels of FVIIa have been observed in hypercoagulable states associated with lupus, idiopathic thrombocytopenic purpura, disseminated intravascular coagulation and in diabetic patients ([32,33,34,35]).

From a therapeutical point of view increasing data show that antithrombotic treatment down-regulates in vivo the generation and the activity of FVIIa. A study published from our group showed that administration of enoxaparin in patients with acute unstable angina results in rapid reduction (about 60%) of both FVIIa and prothrombin F_{1+2} (30). In addition phase II and III clinical trials show that specific inhibitors of FVIIa (i.e. NAPc2 and ASIS) have a significant antithrombotic effect in the prophylaxis and treatment of VTE and arterial thrombosis as well as in the treatment of disseminated intravascular coagulation ([36, 37]).
The ensemble of the studies presented herein allows to conclude that factor FVIIa is generated by at least two pathways: proteolytic cleavage of FVII by serine proteases generated after TF pathway activation (such as FXa, FIXa, FXIIa and thrombin) by proteases which are not directly involved in blood coagulation process.
In addition high levels of FVIIa in plasma are associated with increased in vivo thrombin generation, hypercoagulable state and a high risk of ischemic coronary syndromes.
As we mentioned above several studies from our group showed that UFH, LMWH (enoxaparin) and the synthetic pentasaccharide down-regulate FVIIa generation via the inhibition of FXa and FIXa and inhibit the activity of FVIIa in an antithrombin dependent manner.

Heparins (UFH and LMWHs) are heterogeneous mixtures of oligosaccharides and polysaccharides with different degree of sulphatation. Some of them possess the pentasaccharide domain, and are defined as High Affinity Material for the binding to antithrombin, whereas others does not possess the pentasaccharide domain, are defines as Low Affinity Material and they do not manifest any antithrombin mediated

antithrombotic activity. In addition the affinity for antithrombin depends on the methylation of the pentasaccharide chain.
We have already shown *(Gerotziafas Doctoral Thesis, Paris VI)* that the inhibition of FVIIa by heparins (UFH and enoxaparin) and the synthetic pentasaccharide is a phenomenon composed by two distinct processes: The inhibition of FVIIa generation, mediated principally by FXa and FIXa and secondly by thrombin and FXIIa and the inhibition of FVIIa activity.
In a previous study, we demonstrated that a series of fractions or oligosaccharides isolated from Enoxaparin, were able to inhibit the Factor VIIa generation with different potenties. So, we classified these compounds according to their high, intermediate or low inhibitory effect; However, one cannot exclude that the action of these compounds could also be related to direct inhibition of Factor VIIa itself. This is the aim of this project

Aim of the Project

The primary aim of the present study is to compare the effect of 15 synthetic compounds on FVIIa activity in order to determine their relative inhibitory activity, expressed on a gravimetric basis in a purified experimental system.

Experimental procedure

The study was done by measuring FVIIa levels in a purified experimental system in presence and in absence of physiologically relevant concentrations of TF and antithrombin (AT). The experimental protocol used in the present study is based on the previously published one ([38]).

Inhibition of FVIIa activity.

In purified experimental system

The effect of the studied compounds on the activity of FVIIa was studied in the following experimental system

- rFVIIa at concentration similar to that observed in human plasma after triggering TF pathway,
- purified human AT at concentration yielding 100% of normal activity in human plasma
- physiologically relevant concentration of recombinant human TF
- the studied compounds at increasing concentrations
- $CaCl_2$

Purified experimental system

Modified experimental system :			
Reagents	Final Concentration	Volume	Initial concentration
FVIIa (NovoSeven)	0.5 µg/ml	50	1.75 µg/ml
AT	1.42 µg/ml	50	5 µg/ml
TF (Recombiplastin)	6 pmole/L	25	42 pM
Compounds	0.1µg/ml	25	0.7 µg/ml
CaCl2	0.1 M	25	0.7
Total volume		175	

Incubation time: 60 min

Deep freezing and measurement of FVIIa with the FVIIa Staclot assay in defrozen sample.

FVIIa assay

FVIIa levels was measured with the one-stage clotting assay using recombinant thromboplastin (TF1–218) truncated to interact only with FVIIa (Staclot FVIIa-rTF; Diagnostica Stago, Asnières, France), with clotting times determined by spectrophotometry.

All measurements of FVIIa were done in a single time point, one hour after the addition of $CaCl_2$.

The compounds were studied in parallel. In the purified system the experiments were performed three times.
The concentrations of the compounds are expressed on a gravimetric basis.

Results

Quality control of the experimental conditions and the assay for FVIIa measurement

Influence of the studied compounds on the assay for FVIIa measurement

The assay for the measurement of FVIIa was not influenced by the presence of the compounds. Figure 1 shows that the calibration curve for FVIIa measurement was not modified when the Oligo P2 (2.5 µg/ml) was added in the substrate FVII deficient plasma.

The addition of the 15 compounds at the same concentration (2.5 µg/ml) in the control plasma for FVIIa (included in the FVIIa-rTF Stactot assay) did not change the reference values.

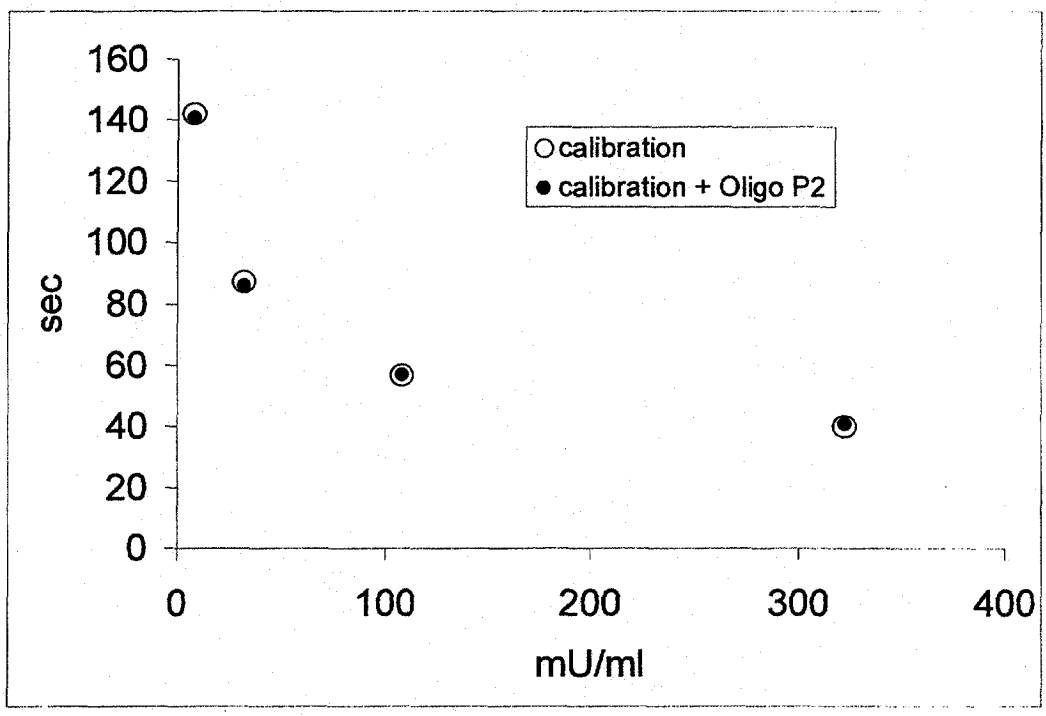


Figure 1. Representative calibration curve of FVIIa assay (Staclot VIIa-rTF) constructed according the manufacturer's instruction (open cycles) and in the presence of the Oligo P2 (2.5 µg/ml added in FVII deficient plasma).

Repeatability of the purified experimental system

The purified reconstituted system is composed by TF, AT, $CaCl_2$ and rFVIIa. The nominal final concentration of rFVIIa is 500 ng/ml (the calculation is based on the concentration of rFVIIa given by NovoNordisc for the clinical use of NovoSeven).

- The concentration of FVIIa measured in the control experiment (TF, AT, rFVIIa and Buffer) was about 30% lower to its nominal concentration because the assay measures FVIIa activity.
- During the study period we used two different lots of NovoSeven. This resulted in two different baseline values of FVIIa in the control experiments. The activity of FVIIa from the second lot was lower to that of the first one. For this reason and in order to make the necessary comparisons, the results of compounds activity of on FVIIa are not given in absolute values (residual FVIIa ng/ml) but as a percentage (%) of inhibition versus the respective control experiment, calculated by the formula:

*% of inhibition = {1-(FVIIa concentration in the presence of oligo/FVIIa concentration in control)}*100*

Intra- and inter-assay coefficients of variation (CV)

- Measurement of FVIIa in control experiment (in the absence of compounds) was repeated ten times in the same series and the CV was 2%.
- FVIIa from aliquots of the same control sample was measured in 5 different days and the coefficient of variation was 5%.
- Measurements of FVIIa in the presence of compounds (final concentration 0.5 µg/ml) were done in three different days and the CV was 2% to 5% for all compounds.
- The experimental system was reconstituted 5 times where all the compounds were studied and the CV of the percentage of inhibition for the same Oligo in the same concentration was 10% for the concentrations higher than 0.1 µg/mll. The CV were higher (15%) when the compounds were studied in lower concentrations (0.01 to 0.06 µg/ml).

Effect of compounds on FVIIa activity

First part of the Study

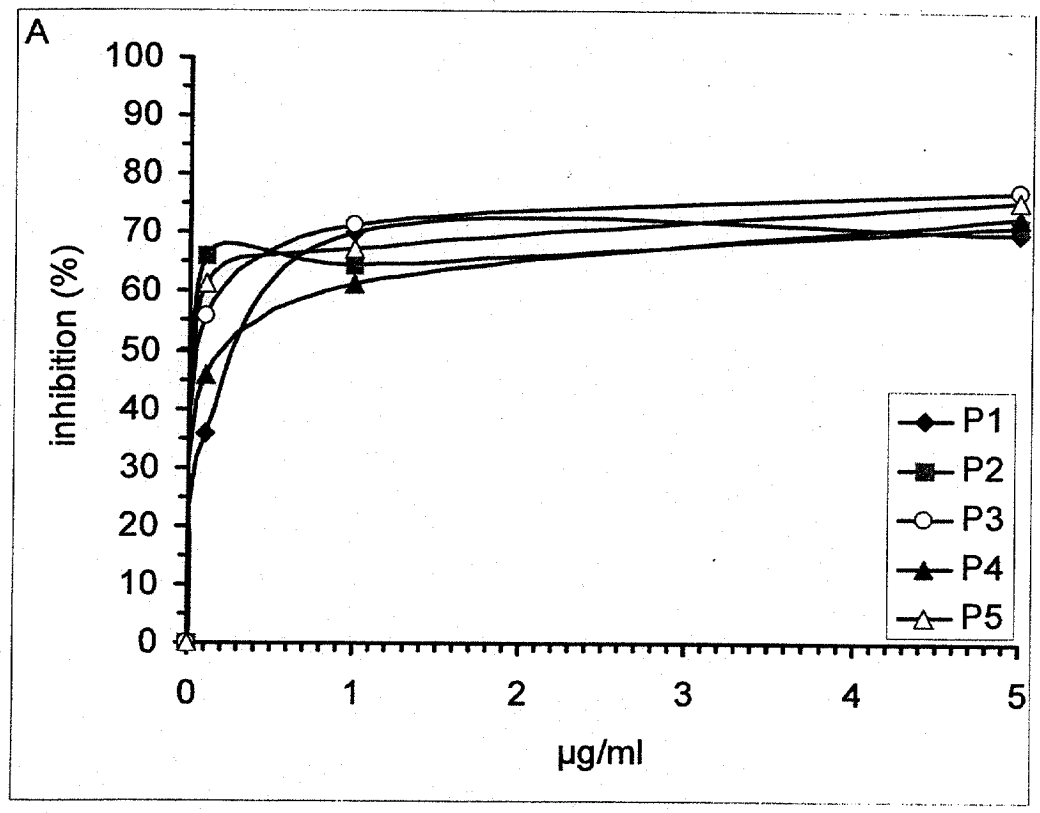
Influence of increasing concentrations of compounds on FVIIa activity

In the first part of the study the 15 compounds were studied at a concentration ranging from 0.1 μg/ml to 5 μg/ml in which they manifested a significant inhibition of FVIIa generation in the previous study. The 15 compounds were added in the experimental system containing already AT, FVIIa, TF and after the addition of $CaCl_2$ samples were incubated for 1 hour at 37°C and then they were freezed.

As it is shown in Figure 1 all the compounds significantly inhibit the activity of FVIIa. The maximum inhibition of FVIIa is 70% to 80%. For all the compounds except the P1 and P9, the maximum inhibition of FVIIa is observed in the presence of concentrations equal or higher to 0.1 μg/ml.

Thus, at the concentrations ranging between 0.1 μg/ml and 5 μg/ml there is a plateau of the inhibitory activity of the studied compounds.

Analysing the data obtained when compounds were at the concentration of 0.1 μg/ml it was possible to differentiate their effect on FVIIa. As it is shown in Figure 2 two compounds are less active than the others (P1 and P9) inducing 36% inhibition of FVIIa and three compounds (P2, P6 and P12) are the most active inducing a 66% inhibition of FVIIa. All the other compounds (P3, P4, P5, P7, P8, P10, P11, P14, P13 and P15,) inhibited FVIIa by 46% to 60%. Further classification of the compounds which have intermediate activity is probably biased by the increased inter-assay CV when the compounds are in low concentrations.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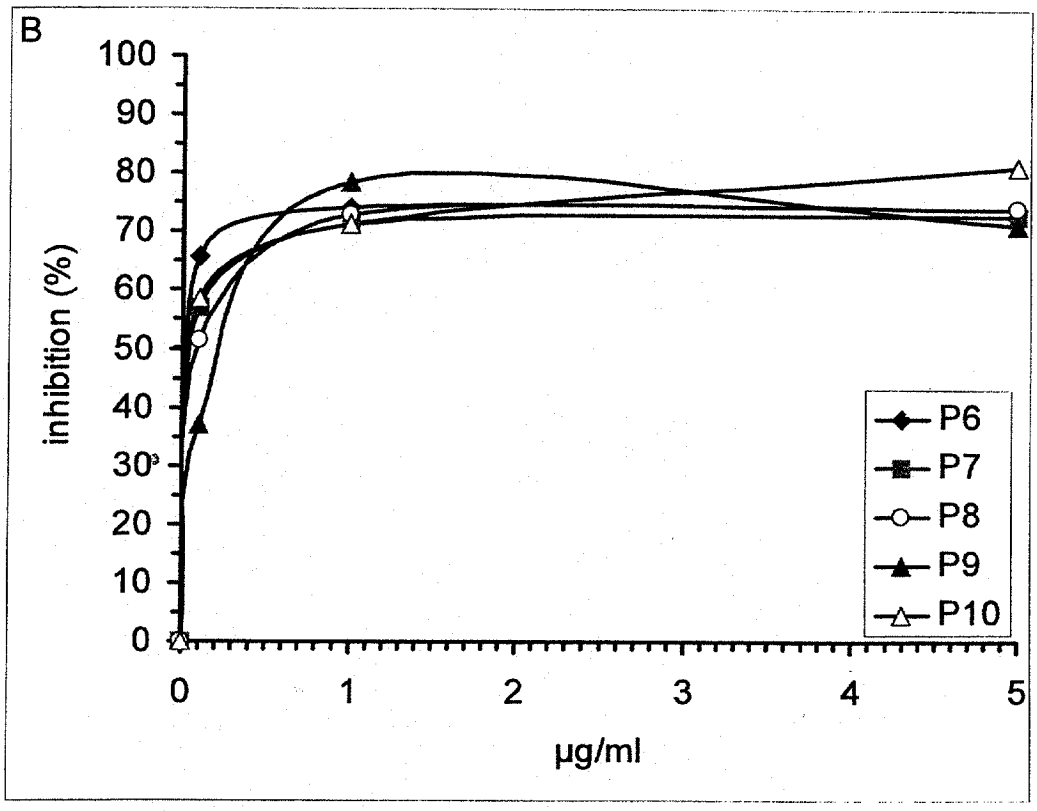
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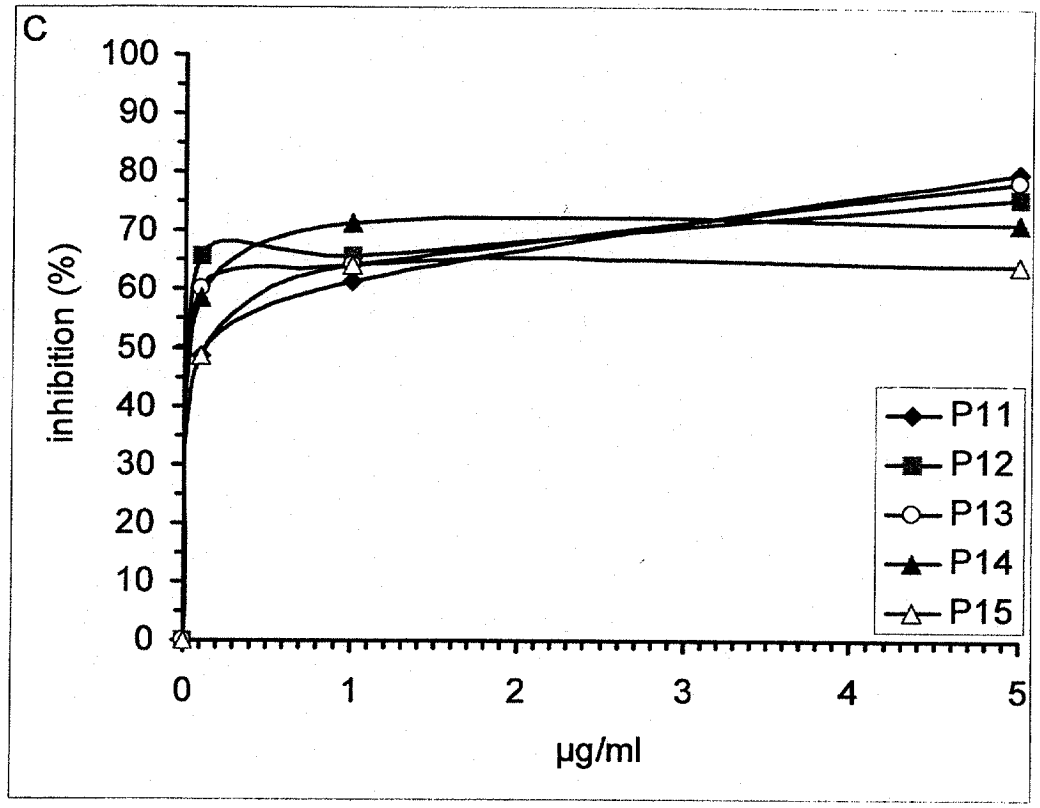


Figure 1. Inhibitory effect of increasing concentrations (0.1 µg/ml to 5 µg/ml) of the compounds on the activity of FVIIa. For better presentation of the curves the 15 compounds are presented in each frame (A, B, C) by group of five. Values are means of three experiments.

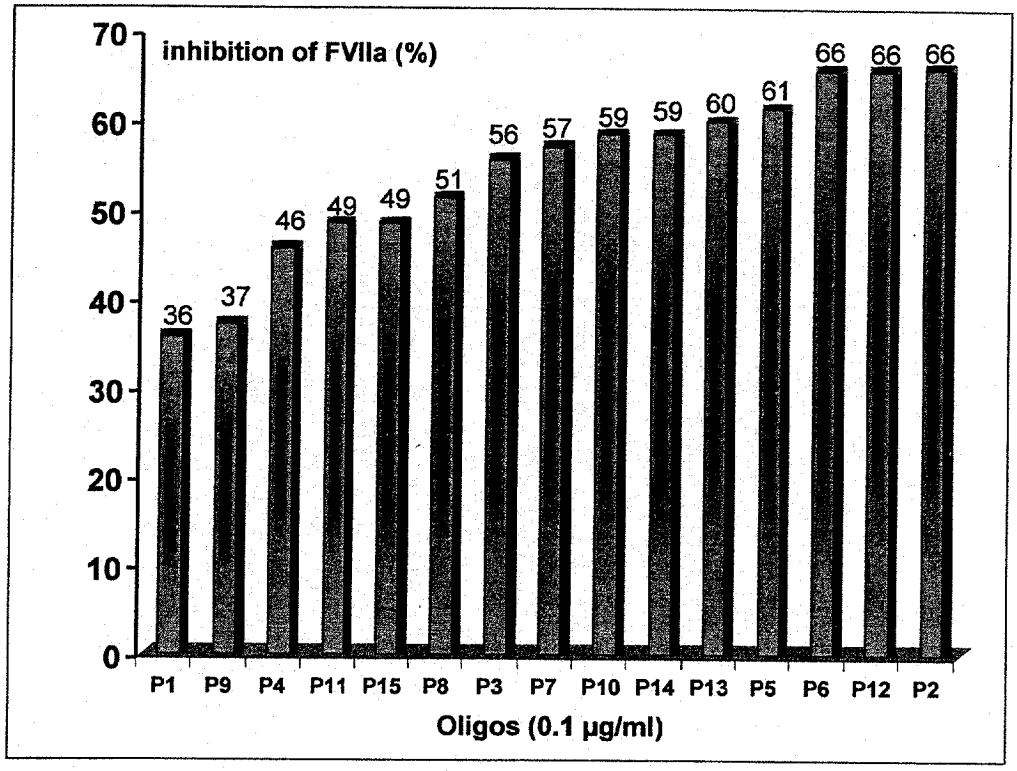


Figure 2. Inhibition of FVIIa activity by the same concentration of the 15 compounds (0.1 µg/ml) presented in increasing order according to their inhibitory activity. Values are means of three experiments

In conclusion, these data show that:

- All the studied compounds have some inhibitory activity against FVIIa.
- The plateau of their inhibitory activity is observed at concentrations higher than 0.1 µg/ml.
- All the compounds have not the same inhibitory activity against FVIIa but there is an important variability among them.
- The compouunds can be arbitrary classified as having
  - "lower" activity : P1 and P9 with 36% inhibition of FVII
  - "intermediate" activity P3, P4, P5, P7, P8, P10, P11, P13, P14, P15 with inhibition of FVIIa between 46% to 61%
  - high activity : P2, P6 and P12 inducing a 66% inhibition of FVIIa.

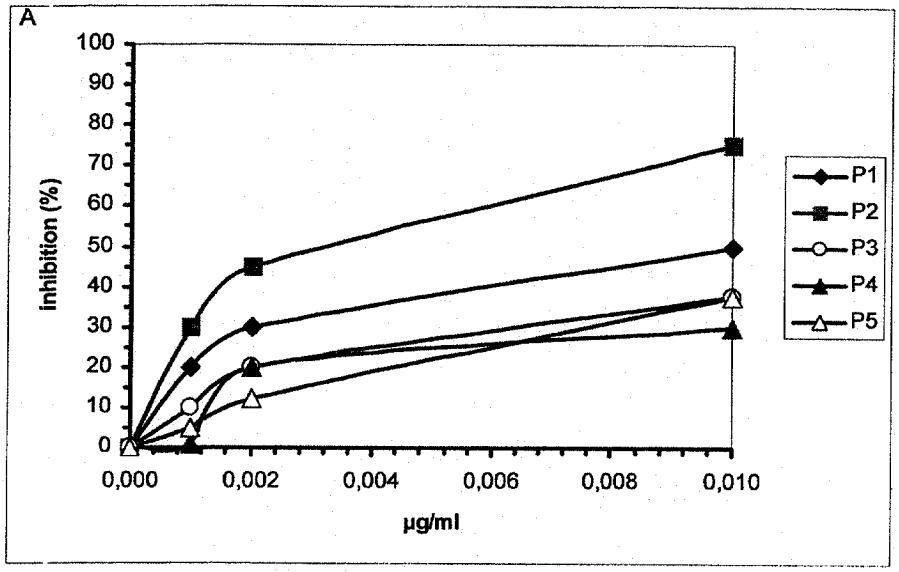
Second part of the Study

Lowest active concentration of the compounds against VIIa

Since all compounds at the concentration of 0.1 μg/ml manifested a significant inhibitory effect (higher than 36%) against FVIIa, in the second part of the study the compounds were studied in lower concentrations in order to determine the minimal active concentration. The studied concentration range was 0, 0.001, 0.002, 0.01, 0.024, 0.067 μg/ml. The concentration/effect curves of the compounds are shown in Figure 3. A significant inhibitory activity of all compounds persisted when they were studied at concentrations as low as 0.01 μg/ml. At this concentration a variability of their effect against FVIIa was also observed. Among them the compound P2 was shown to be more active than the others, inducing about 60% inhibition of FVIIa.

In order to compare the compounds, they were studied in even lower concentrations (0.002 μg/ml and 0.001 μg/ml or 2 ng/ml and 1 ng/ml). The lower active concentrations were estimated with curve fit. In the presence of low concentration the CV of the assay was 15%. For this reason, the 17% inhibition was determined as threshold for significant inhibition. All values inferior to 17% were considered as not significantly different compared to the control experiment. Figure 4 depicts the lower active concentrations of the compounds against FVIIa. The data presented in Figure 4 allow to classify the compounds in at least 3 groups

P2 is the most active since it inhibits FVIIa at 2 ng/ml. P12 and P14 are the less active since the minimal active concentration is 10 ng/ml. An intermediate activity is shown by P1, P7, P8 which have 3 ng/ml minimal active concentration (MAC), P3, P5, P6, P10 with 4 ng/ml MAC, P13, P14 with 4.5 ng/ml MAC and P4, P9 with 5 ng/ml MAC. The MAC for P11 is 7 ng/ml.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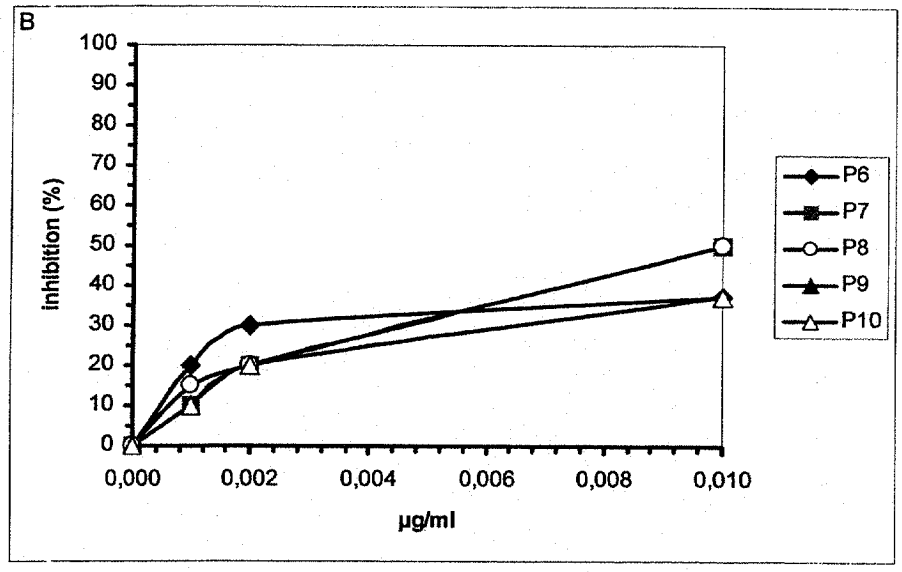
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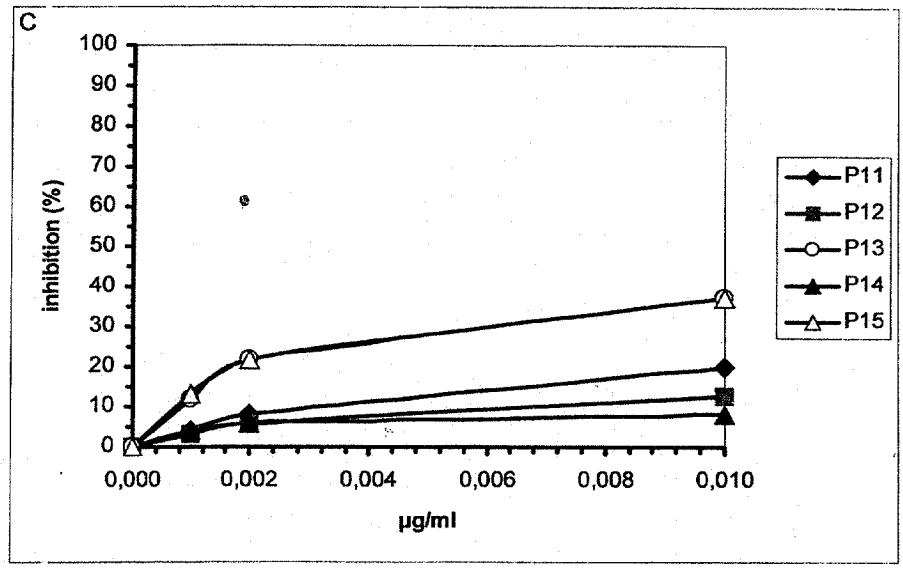
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P14
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Figure 3. Inhibitory activity of the compounds at concentrations lower to 0.01 µg/ml. For better presentation of the curves the 15 compounds are presented in each frame (A, B, C) by group of five. Values are means of three experiments.

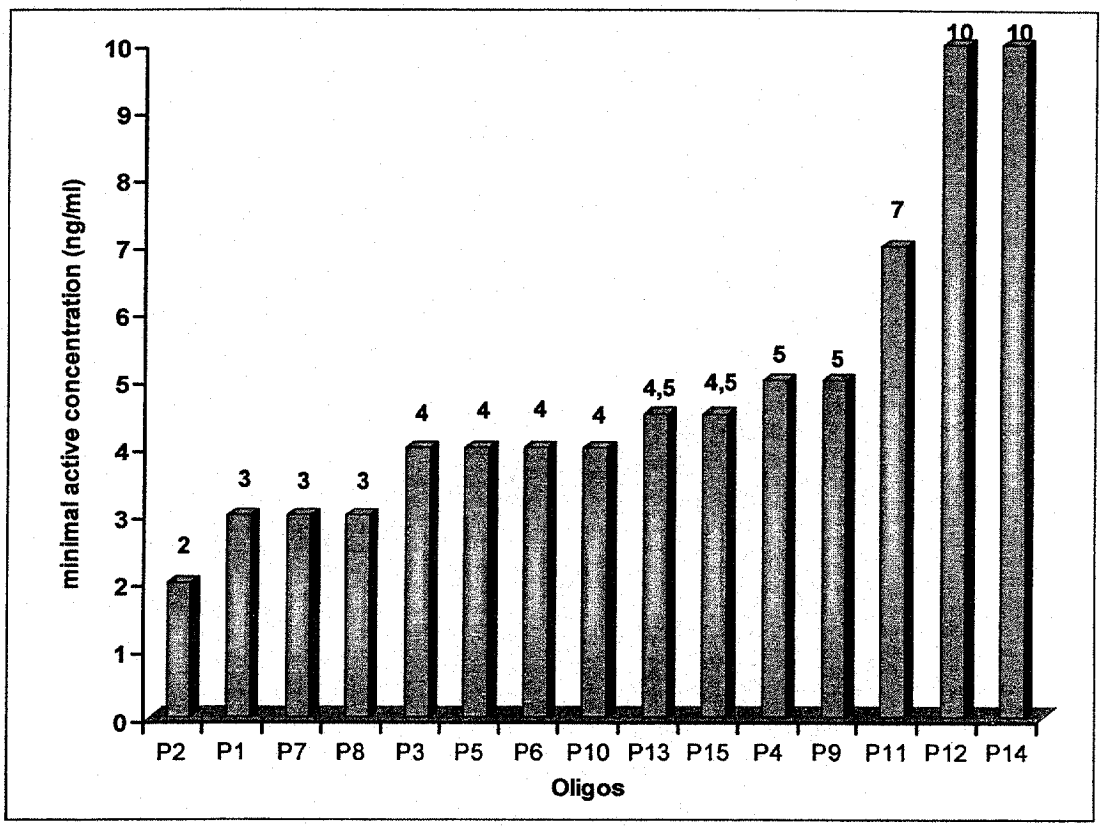

Figure 4. Lowest active concentrations of the compounds calculated from the data shown in Figure 3.

In conclusion these data show that

- The inhibitory activity against FVIIa persists for all compounds at very low concentrations (level of ng/ml)
- The variability of the activity of the compounds is shown when they are tested in the basis of the *minimal active concentration*
- Based on the *minimal active concentration*, the compounds can be classified in three groups
    - "lower" activity: P7, P12, and P14 with 7 ng/ml to 10 ng/ml MAC
    - "intermediate" activity: P3, P4, P5, P6, P9, P10, P13, P15 with 4 ng/ml to 5 ng/ml MAC
    - "higher" activity: P2, P1, P7 and P8 with 2 ng/ml to 3 ng/ml MAC.

- These active concentrations correspond in fact to very low anti-Xa activities

Comparison of the activity of the compounds against FVIIa activity, FVIIa generation and prothrombin activation

Classification	Inhibition of FVIIa activity		Inhibition of FVIIa generation	Inhibition of prothrombin activation
	MAC	Max Effect		
High activity	P1, P2, P7, P8	P2, P6, P12	P12, P13, P14	P9, P12, P13, P14
Intermediate activity	P3, P4, P5, P6, P9, P10, P13, P15	P3, P4, P5, P7, P8, P10, P11, P13, P14, P15	P9, P10, P11	P1, P2, P4, P5, P6, P8, P10, P11
Low activity	P11, P12, P14	P1, P9	P15	P15

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