

***In Vitro* Characterization of the Neutralization of Unfractionated Heparin and Low Molecular Weight Heparin by Novel Salicylamide Derivatives**

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Abstract

Introduction: Unfractionated heparin and the low molecular weight heparins (LMWHs) are commonly used in the treatment of acute coronary syndromes, as prophylaxis against deep vein thrombosis and pulmonary embolism and to prevent clotting during interventional and surgical procedures. The neutralization of unfractionated heparin is critical following the completion of coronary bypass surgery to avoid excessive blood loss. Unfractionated heparin can be neutralized by protamine sulfate, a highly cationic peptide that binds to heparin in a charge-dependent manner. However, the use of protamine can be associated with serious side-effects such as hypotension, bronchoconstriction, or pulmonary hypertension, possibly due to the release of histamine. Additionally, large doses of protamine can produce an anticoagulant effect. This study characterizes the ability of a series of low molecular weight, homogeneous, synthetic, polycationic salicylamide derivatives (PolyMedix, Radnor, PA) to neutralize the anticoagulant actions of unfractionated heparin and enoxaparin.

Methods: Human plasma was supplemented with unfractionated heparin or enoxaparin (Sanofi-Aventis, Paris, France) at a concentration of 10 µg/ml. Protamine sulfate or one of six of the structurally distinct salicylamide derivatives was added to aliquots of heparinized plasma to achieve final concentrations of 50, 25 and 12.5 µg/ml. The supplemented plasmas were immediately analyzed using clotting (aPTT, Heptest, thrombin time) and amidolytic (anti-Xa, anti-IIa) assays.

Results: Using the *in vitro* assays, protamine sulfate was shown to concentration-dependently neutralize the actions of unfractionated heparin in all of the assays. Two of the salicylamide derivatives tested produced an effect comparable to protamine, while three derivatives exhibited a relatively stronger neutralization of unfractionated heparin. The extent of neutralization measured by anti-Xa and anti-IIa assays was also greater with the derivatives. While residual anti-Xa and anti-IIa activities (20% and 10%, respectively) were observed even with a 5-fold gravimetric excess of protamine, complete neutralization was observed with the salicylamide derivatives. Protamine is known to be less effective at neutralizing LMWHs. In this study, the anticoagulant activity of enoxaparin as measured by aPTT and Heptest was neutralized approximately 50% by protamine even at a 5:1 protamine to enoxaparin ratio. The derivatives were able to completely neutralize the anticoagulant effects of enoxaparin. A similar pattern was observed with the amidolytic assays. While protamine was unable to neutralize the anti-Xa activity of enoxaparin, 5 of the 6 salicylamide derivatives concentration-dependently inhibited the anti-Xa activity.

Discussion: These studies demonstrate that the PolyMedix series of salicylamide derivatives can effectively neutralize the anticoagulant and anti-protease actions of unfractionated heparin and LMWHs such as enoxaparin. Initial results suggest that such agents are more effective than protamine at neutralizing other LMWHs. Future studies are designed to characterize the compounds PK/PD profiles. These results warrant further studies on the neutralization profile of PolyMedix series of salicylamide derivatives in animal models of bleeding and thrombosis.

Background

Unfractionated heparin and the low molecular weight heparins (LMWHs) are commonly used in the treatment of acute coronary syndromes, as prophylaxis against deep vein thrombosis and pulmonary embolism and to prevent clotting during interventional and surgical procedures. The neutralization of unfractionated heparin is critical following the completion of coronary bypass surgery to avoid excessive blood loss. Unfractionated heparin can be neutralized by protamine sulfate, a highly cationic peptide that binds to heparin in a charge-dependent manner. However, the use of protamine can be associated with serious side-effects such as hypotension, bronchoconstriction, or pulmonary hypertension, possibly due to the release of histamine. Additionally, large doses of protamine can produce an anticoagulant effect on its own and LMWHs are only partially inhibited by protamine. In the past, other agents have been evaluated for use as heparin antagonists. These include recombinant platelet factor 4, polybrene (hexadimethrine bromide) and heparinase. None of these were determined to be clinically useful. This study characterizes the ability of a series of low molecular weight, homogeneous, synthetic, polycationic salicylamide derivatives (PolyMedix, Radnor, PA) to neutralize the anticoagulant actions of unfractionated heparin and enoxaparin.

Purpose

To characterize the ability of a series of salicylamide derivatives to neutralize the anticoagulant and anti-protease activities of unfractionated and low molecular weight heparin using *in vitro* assays.

Test Agents

Lovenox - Sanofi-Aventis, Paris, France

Unfractionated heparin – Choay, Paris, France

Protamine sulfate – Institute Choay, Paris France

Salicylamide derivatives – PMX 60054, PMX 60056, PMX 60065, PMX 60077, PMX 60079, PMX 60087, PolyMedix, Radnor, PA

Methods

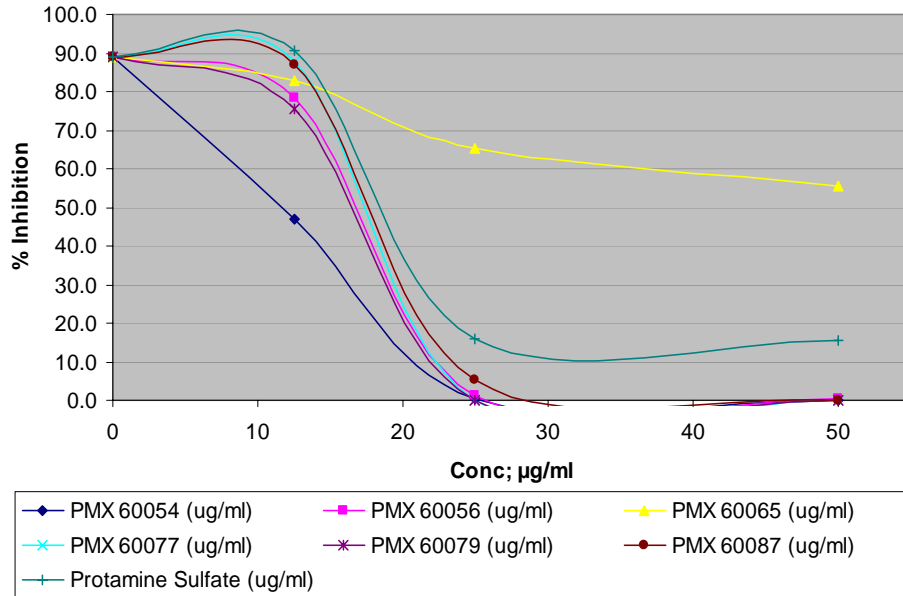
Anticoagulant activity assays

Anticoagulant activity was measured in terms of prolongation of the aPTT (bioMérieux, Durham, NC), Heptest (Haemachem, St. Louis, MO) and thrombin time (Enzyme Research Laboratories, South Bend, IN). Heparin (or LMWH) and protamine sulfate (or PMX 600xx) were supplemented to normal human plasma and all assays were performed according to the manufacturer's recommendations.

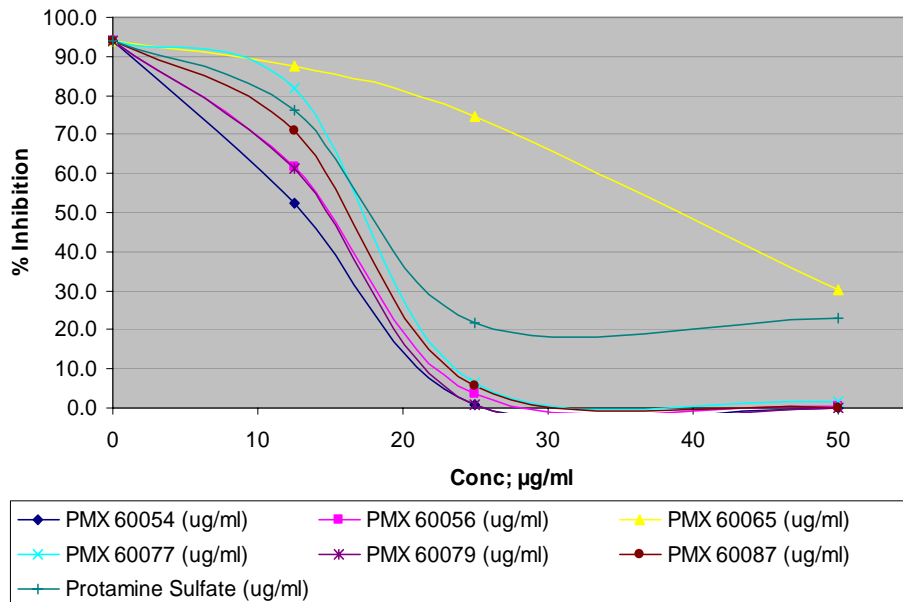
Antiprotease assays

The effect of LMWHs on factor Xa and thrombin activity was assessed using amidolytic assays as previously described (Hoppensteadt, D *et al* Semin. Thromb. Hemost. 11(2):112-20, 1985).

Neutralization of Heparin as Measured by Anti-IIa Assay

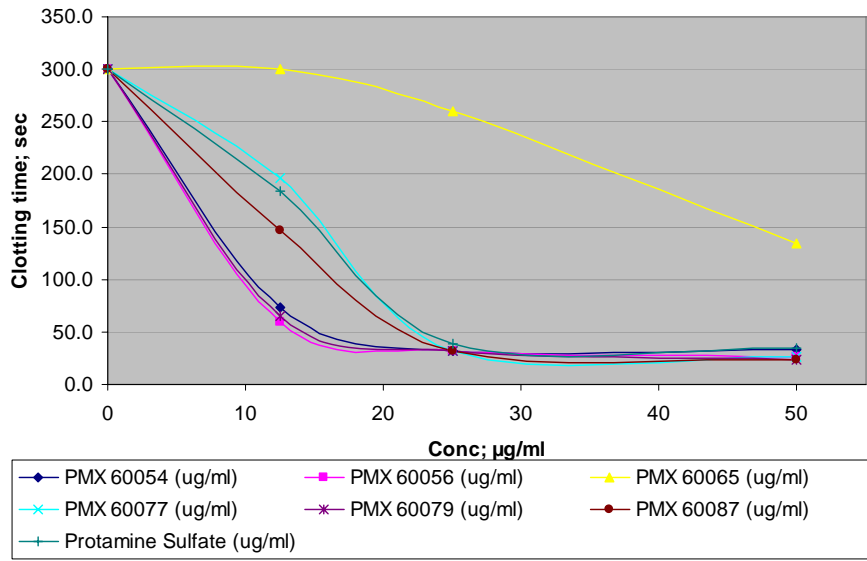


Neutralization of Heparin as Measured by Anti-Xa Assay

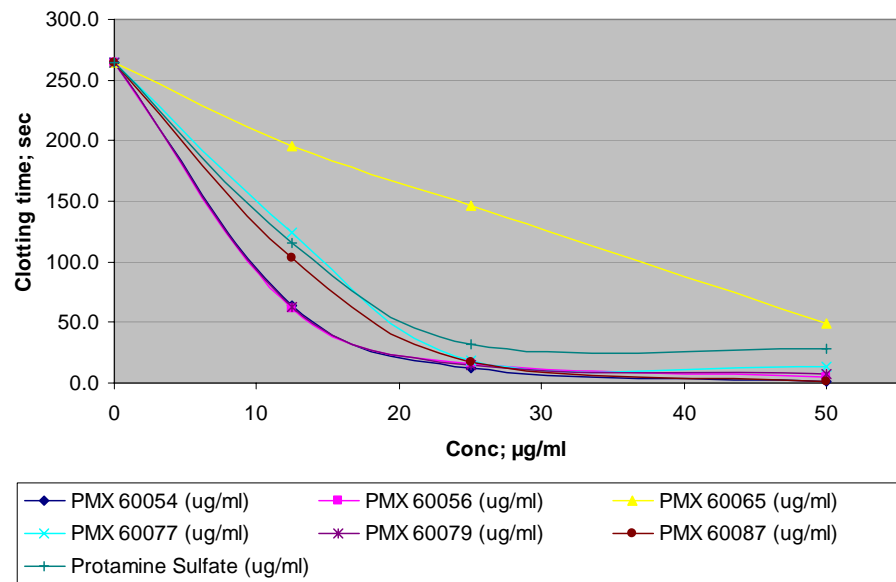


Neutralization of the antiprotease activity of heparin. With the exception of PMX 60065, all of the PMX compounds were able to neutralize both the anti-Xa and anti-IIa activities of heparin with a similar potency. Protamine sulfate was unable to completely inhibit either the anti-IIa or anti-Xa activities of heparin.

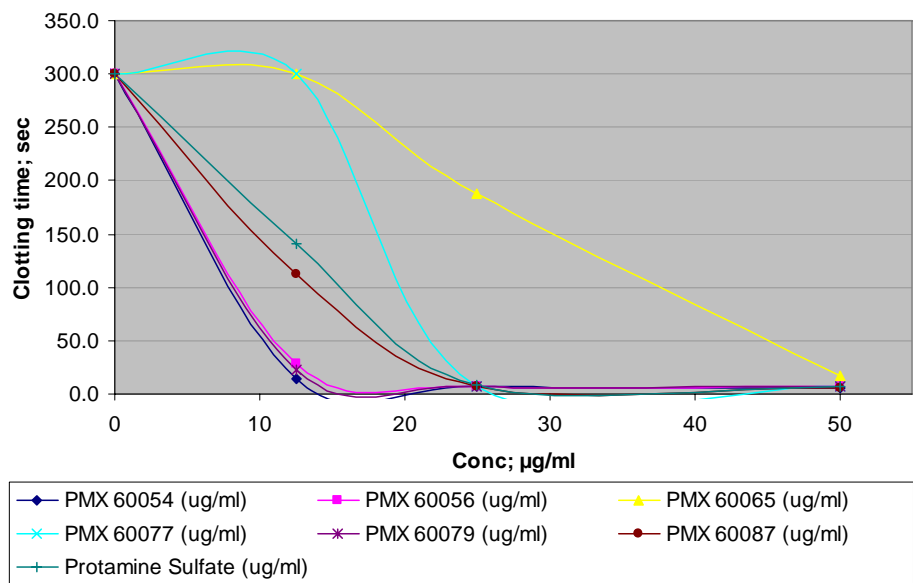
Neutralization of Heparin as Measured by aPTT



Neutralization of Heparin as Measured by Heptest

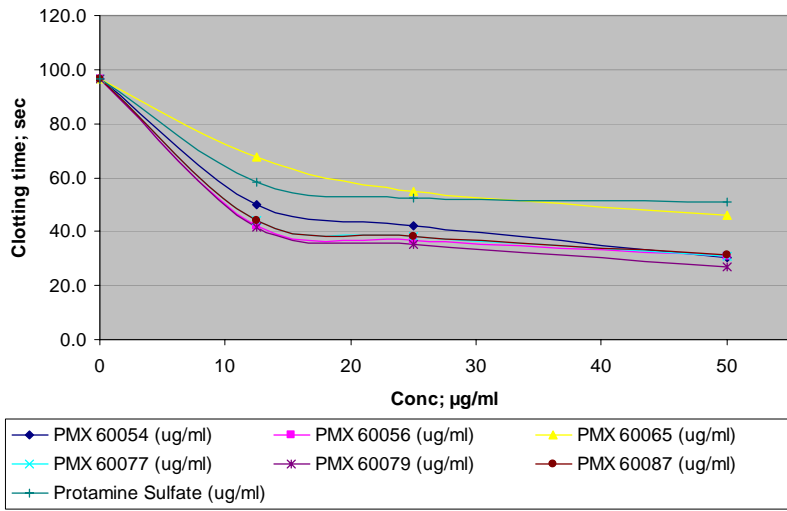


Neutralization of Heparin as Measured by Thrombin Time

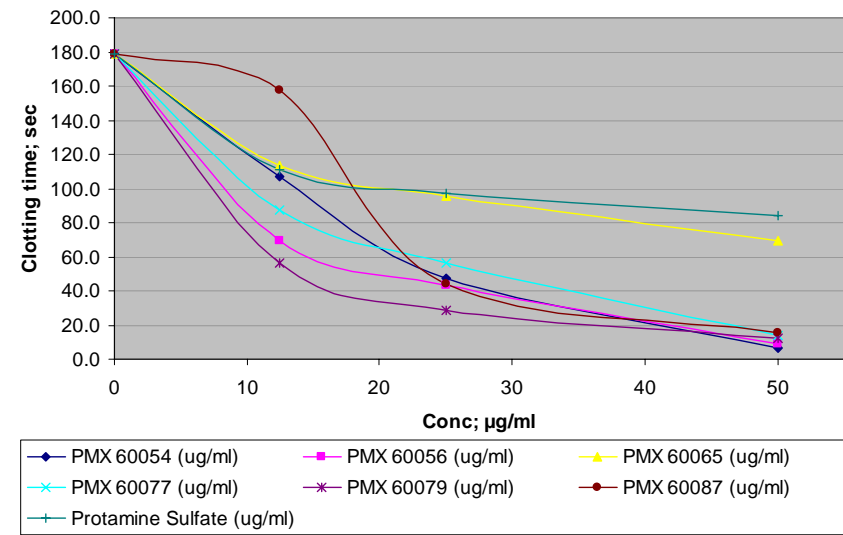


Neutralization of the anticoagulant activity of heparin. PMX 60056, PMX 60054 and PMX 60079 produced a more potent neutralization of heparin than protamine sulfate.

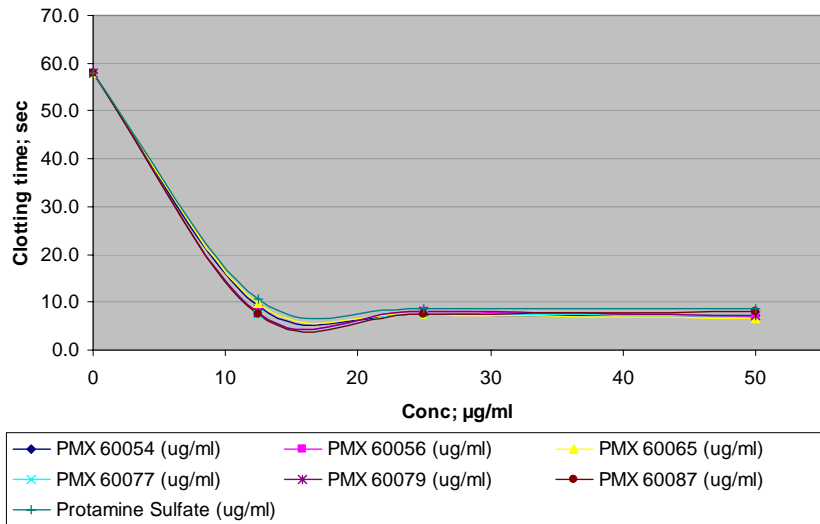
Neutralization of Enoxaparin as Measured by aPTT



Neutralization of Enoxaparin as Measured by Heptest

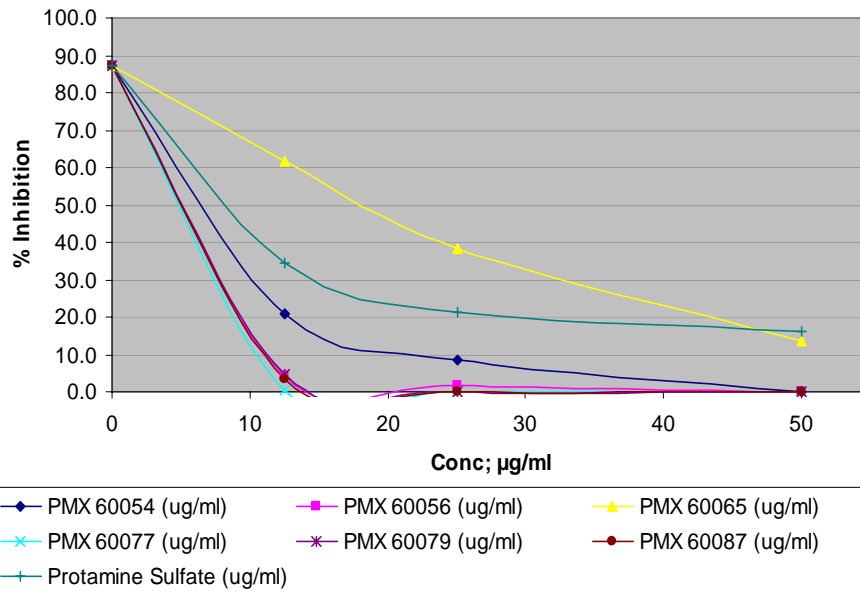


Neutralization of Enoxaparin as Measured by Thrombin Time

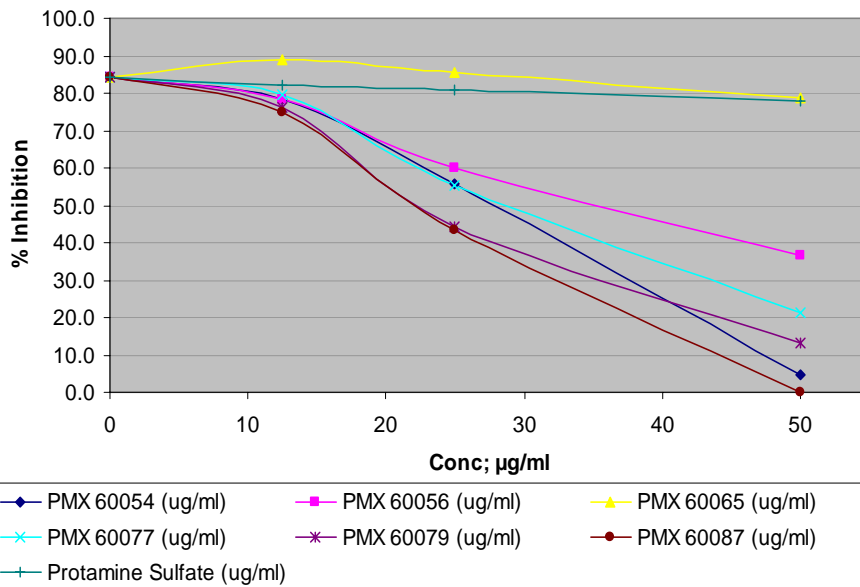


Neutralization of the anticoagulant activity of enoxaparin. Protamine sulfate was less effective at neutralizing the anticoagulant effects of the LMWH enoxaparin (10 µg/ml) when measured using the aPTT and Heptest assays. The PMX compounds, with the exception of PMX 60065, exhibited a larger degree of neutralization of aPTT prolongation, particularly at higher ratios of PMX compound to enoxaparin. A similar pattern was observed when anticoagulant activity was measured using the Heptest.

Neutralization of Enoxaparin as Measured by Anti-IIa Assay



Neutralization of Enoxaparin as Measured by Anti-Xa Assay



Neutralization of the anti-protease activity of enoxaparin. Protamine sulfate was unable to neutralize enoxaparin's anti-Xa activity, even at a ratio of 5:1. A similar lack of effect was observed with PMX 60065. All other PMX compounds concentration-dependently neutralized enoxaparin's anti-Xa activity, though with differing potencies. Protamine sulfate was able to neutralize most of the anti-IIa activity of enoxaparin, with ~20% residual anti-IIa activity at concentrations >25 µg/ml. The PMX compounds, with the exception of PMX 60065, were at least as effective as protamine sulfate and in most cases were better able to neutralize anti-IIa activity of enoxaparin.

Summary

- Using the *in vitro* assays, protamine sulfate was shown to concentration-dependently neutralize the actions of unfractionated heparin in all of the assays.
- Two of the salicylamide derivatives tested produced an effect comparable to protamine, while three derivatives exhibited a relatively stronger neutralization of unfractionated heparin.
- The salicylamide derivatives more effectively neutralized the anticoagulant and antiprotease actions of the low molecular weight heparin, enoxaparin.

Conclusions

These studies demonstrate that the PolyMedix series of salicylamide derivatives can effectively neutralize the anticoagulant and anti-protease actions of unfractionated heparin and LMWHs such as enoxaparin. Initial results suggest that such agents are more effective than protamine at neutralizing other LMWHs. Future studies are designed to characterize the compounds' PK/PD profiles. These results warrant further studies on the neutralization profile of the PolyMedix series of salicylamide derivatives in animal models of bleeding and thrombosis.