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Fibrinolytic and Anticoagulant Activity After a Single Subcutaneous Administration of a Low Dose of Heparin or a Low Molecular Weight Heparin-Dihydroergotamine Combination

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Key words

Heparin – Fibrinolytic activity – Anti-Xa activity – Bioavailability

Summary

The anticoagulant and potential profibrinolytic effect of a combination of low molecular weight heparin with dihydroergotamine (LMWH-DHE) and of unfractionated heparin was studied in eight healthy volunteers. Each volunteer received a subcutaneous injection of either LMWH-DHE (1,500 U anti-Xa of LMWH + 0.5 mg DHE), unfractionated heparin (5,000 IU) or of placebo (saline) between 7 and 8 h in the morning on three different occasions. Anti-Xa activity, and fibrinolytic activity measured by the euglobulin clot lysis time (ECLT) and by the fibrin plate assay were determined before and at different times after administration of the three substances. Anti-Xa activity in plasma reached a maximum four hours after injection of both LMWH-DHE and unfractionated heparin. LMWH-DHE showed a better bioavailability when compared to unfractionated heparin; the anti-Xa activity peak was two and a half fold higher after LMWH-DHE despite injection of a three fold lower dose of anti-Xa units. The half-life of anti-Xa activity was approximately 4 hours for LMWH-DHE but only 90 min for unfractionated heparin. The fibrinolytic activity measured by ECLT as well as by fibrin plate assay, showed a significant increase during the day reaching a peak 8–12 h after injection regardless of the product administered (including the placebo). The profile of the diurnal fibrinolytic activity curve was identical for all three substances. The increase in fibrinolytic activity, observed after administration of LMWH-DHE or unfractionated heparin, was therefore not due to these drugs but reflected the circadian physiological fluctuation of fibrinolysis.

Introduction

Heparin is used for the treatment and prevention of a variety of acute thromboembolic disorders. Its antithrombotic effect is generally considered to be mediated through the enhancement of the inhibitory activity of antithrombin III against many key enzymes in the coagulation cascade (1, 2).

Although the antithrombotic effect of heparin may be explained by its anticoagulant activity, it is still unclear whether heparin can prevent thrombosis by other mechanisms as well such as via interaction with platelets (3–7) or vascular tissue (8, 9). Furthermore, a profibrinolytic effect of heparin was noted in *in vitro* experiments (11–13), in *in vivo* human studies (10, 12–19) and in animal models of experimental thrombosis (15, 16). Nevertheless, the question whether heparin stimulates the fibrinolytic activity in man remains controversial (20–22) because most of the studies did not take into account the diurnal fluctuation of fibrinolytic activity (23, 24). The potential usefulness of low molecular weight heparin (LMWH) in the prevention of thromboembolic disease is currently being investigated (25–27). A combination of heparin with dihydroergotamine (DHE) seems to offer an advantage over heparin alone (28–30). In the present study, therefore, the in vivo effect on anti-Xa and fibrinolytic activity of a single dose of a combination of LMWH and DHE was compared to that of a single low dose of unfractionated heparin. To take into account the diurnal fluctuation of the fibrinolytic activity, we have compared fibrinolytic activity of eight volunteers before and at different times after injection of LMWH-DHE, unfractionated heparin or saline as placebo.

Materials and Methods

All chemicals used were of analytical grade. Two types of heparin (Sandoz-Wander Pharma AG, Berne, Switzerland) were used: 1) a LMW heparin preparation (average molecular weight of approximately 7,000 daltons) in combination with dihydroergotamine (LMWH-DHE) and 2) unfractionated heparin.

Volunteers

Eight healthy male volunteers aged 18–30 years, weighing between 65–75 kg, were selected for this study. All had given written consent after having been informed of the aims, procedures and risks of the study. Each volunteer underwent a careful physical examination. The medical history and various haematological and haemostatic laboratory examinations including bleeding time, thromboplastin time, activated partial thromboplastin time, fibrinogen, thrombin time and blood counts revealed no abnormalities. No volunteer was taking medication in the month before and during the study.

Study Design

On three occasions each fasting volunteer received either 1) 1,500 U anti-Xa of LMWH + 0.5 mg DHE, 2) 5,000 IU of unfractioned heparin or 3) an equal volume of 0.9% NaCl. The two heparins were administered randomly in the first two occasions with an interval of 12 days, while the placebo was given three months later. The products were injected subcutaneously between 7 and 8 h in the morning into the anterolateral abdominal wall. Blood samples were taken before injection and 1, 2, 3, 4, 6, 8, 12 and 24 hours after injection. The blood was mixed in precooled tubes with one tenth volume of 0.1 M citrate buffer, pH 4.5 (Behring, Marburg, Federal Republic of Germany). The tubes were immediately transferred to ice, and then centrifuged for 20 min at 2,000 g and 4° C. The resulting platelet poor plasma was fractionated into aliquots and frozen at −70° C.

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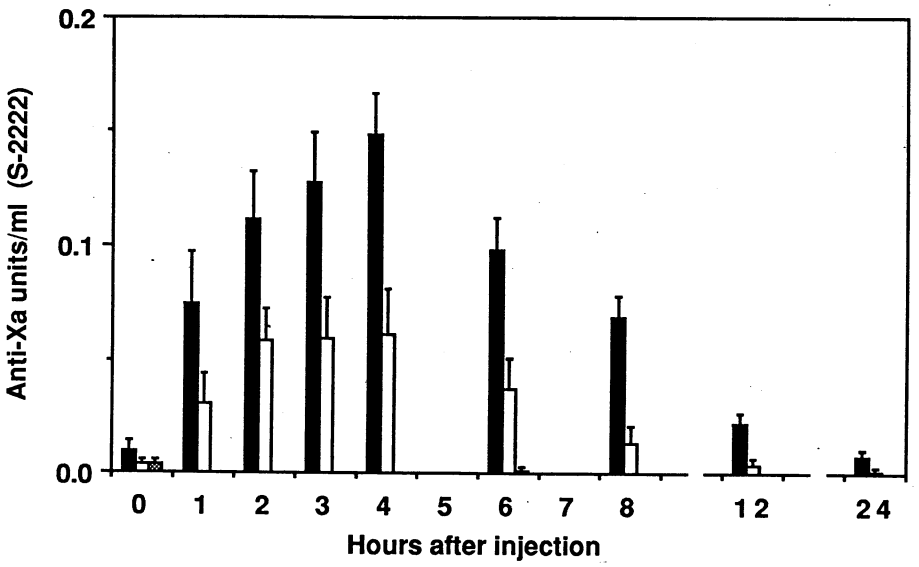

Fig. 1 Anti-Xa activity after subcutaneous injection, in eight healthy volunteers, of LMWH-DHE (solid bars), unfractionated heparin (open bars) and placebo (dotted bars). LMWH-DHE was administered at a dosage of 1,500 anti-Xa U of LMW heparin in combination with 0.5 mg of dihydroergotamine; unfractionated heparin was administered at a dosage of 5,000 IU. After injection of saline the anti-Xa activity was always below 0.01 U/ml. Bars at time 0 indicate the activity values before injection of the substances (7–8 a.m.). Values are means ± standard error

Anti-Xa Assays

Anti-Xa activity was determined by amidolytic and chronometric assays using the Coatest Heparin kit (Kabi Vitrum, Stockholm, Sweden) and the Hepaclot Stago kit (Diagnostica Stago, Asnières-Sur-Seine, France). The lower limit of sensitivity of these assays was 0.01 anti-Xa units/ml. For both assays, anti-Xa activity observed after LMWH-DHE or unfractionated heparin was compared to calibration curves established with the respective agents. Half-life of anti-Xa activity for both heparin preparations were derived using the formula $t_{1/2} = -(\ln 2)/b$, where b is the slope of the exponential regression curve of the anti-Xa activity in the descending phase.

Fibrinolytic Activity Determinations

Global fibrinolytic activity was measured by the euglobulin clot lysis time (ECLT) and by the fibrin plate assay.

The euglobulins were prepared as follows: 0.8 ml of plasma was diluted 10-fold with ice-cold deionized water and the pH adjusted to 5.9 by addition of 0.6 ml of 0.25% (v/v) acetic acid. The tubes were kept on ice for 30 min, after which they were centrifuged for 10 min at 1,300 g and 4° C. The supernatant was discarded and the tube walls carefully dried with an absorbent paper. The euglobulin precipitate was redissolved in 0.4 ml of 0.1 M Tris-HCl buffer, pH 7.5 and tested immediately.

Euglobulin Clot Lysis Time Assay

100 µl of the euglobulin solution were mixed with 100 µl of 0.1 M Tris-HCl, pH 7.5, containing 0.2% Tween 80, and then clotted with 100 µl of a solution of 10 U/ml thrombin and 0.025 M $CaCl_2$ followed by incubation at 37° C. Lysis times were recorded in minutes. Activity was expressed in arbitrary units (AU) using the formula: AU = 300/lysis time in minutes.

Fibrin Plate Assay

Fibrin plates (using bovine plasminogen-rich fibrinogen from Poviet, Boxtel, The Netherlands) were prepared as described (31). Aliquots of 30 µl euglobulin solution and 5 µl of 17.5 mM sodium flufenamate (Aldrich, Beerse, Belgium) were applied in quadruplicate on the plates. After 24 hours of incubation at 37° C, lysis zones were converted to international units of t-PA by comparison of the lysis diameters with those of a laboratory standard preparation of pure human t-PA (32) that had been standardized against the International Reference Preparation for t-PA (NIBSC 83/517) kindly provided by Dr. P. J. Gaffney (National Institute for Biological Standards and Control, South Mimms, Hertfordshire, UK). Fibrinolytic activity was then expressed, for each volunteer, as percent of the 7 to 8 a.m. values (basal level).

Statistical Analysis

The statistical significance of differences with time and between groups was determined by a two way analysis of variance (ANOVA) (33).

Results

Effect of LMWH-DHE and Unfractionated Heparin on Anti-Xa Activity

After administration of either LMWH-DHE or unfractionated heparin, the amidolytic anti-Xa activity became maximal for both agents (0.15 ± 0.02 U/ml and 0.06 ± 0.02 U/ml, respectively; mean ± SE) four hours after injection (Fig. 1). The average anti-Xa activity half-life was approximately 4 h for the LMWH-DHE and 1 h ½ for the unfractionated heparin. After the placebo

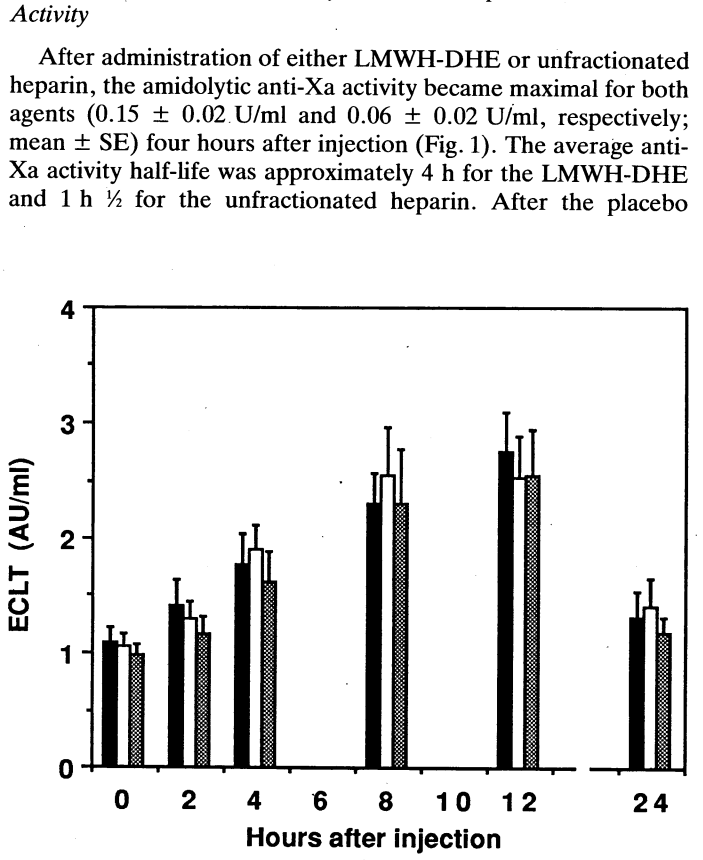

Fig. 2 Euglobulin clot lysis time (ECLT) after subcutaneous injection, in eight healthy volunteers, of LMWH-DHE (solid bars), unfractionated heparin (open bars) and placebo (dotted bars). ECLTs are expressed in arbitrary units (AU) of activity (see methods). Bars at time 0 indicate the activity values before injection of the substances (7–8 a.m.). Values are means ± standard error

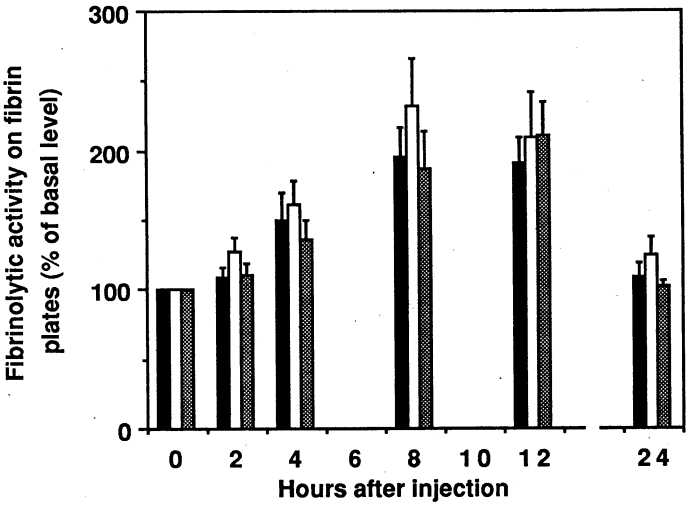

Fig. 3 Fibrinolytic activity in eight healthy volunteers, measured by the fibrin plate assay, after subcutaneous injection of LMWH-DHE (solid bars), unfractionated heparin (open bars) and placebo (dotted bars). Activities are expressed as a percent of the 7 to 8 a.m. values (time 0) measured before injection of the substances. Values are means ± standard error

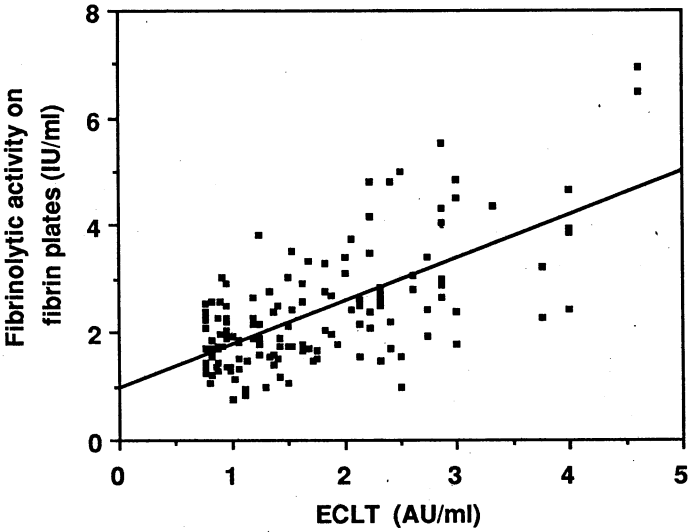

Fig. 4 Linear correlation between fibrinolytic activities determined by fibrin plate assay and by euglobulin clot lysis time (ECLT) assay. The correlation was performed using the values measured before and at different times after subcutaneous injection of either LMWH-DHE, or unfractionated heparin or placebo in eight healthy volunteers

injection, the anti-Xa activity remained constantly below 0.01 U/ml. Similar results were obtained using the anti-Xa chronometric assay (data not shown).

Effect of LMWH-DHE and Unfractionated Heparin on Fibrinolytic Activity

Fibrinolytic activity, as measured by euglobulin clot lysis time (Fig. 2) or fibrin plate assays (Fig. 3), showed a progressive significant increase during the day ($p < 0.001$ ANOVA) reaching its maximum 8–12 hours after injection whatever the product administered (including the placebo). On each time point the activity observed after injection of LMWH-DHE or unfractionated heparin was not significantly different from that observed after injection of saline. Fibrinolytic activity determinations of euglobulins on fibrin plates showed a fairly good correlation ($r = 0.66$) with those measured by the euglobulin clot lysis time (Fig. 4). Again, no differences were observed between the two heparins and placebo.

Discussion

In a variety of surgical procedures, the combination of heparin with dihydroergotamine was reported to provide a better antithrombotic prophylaxis than heparin given alone (28–30). LMW heparin fractions were shown to be effective in preventing postoperative deep vein thrombosis (25–27). It thus seemed to be worthwhile to study whether a combination of LMW heparin and DHE would offer further advantages as an antithrombotic agent. As a first approach we investigated in healthy volunteers the anti-Xa effect of a single subcutaneous dose of a combination of LMWH-DHE (1,500 anti-Xa units) in comparison to a single dose of unfractionated heparin (5,000 IU) or a saline placebo. Anti-Xa activity was observed after injection of the combination of LMWH with DHE or of unfractionated heparin but not after saline injection. Maximal anti-Xa activity was reached four hours post-injection for both heparin preparations. The anti-Xa activity peak observed after injection of LMWH-DHE was two to three times greater than after unfractionated heparin, despite the fact that the latter was administered at a three time higher dose. In part, this is due to the longer half-life of anti-Xa activity after injection of LMWH-DHE (4 hours) than of the unfractionated heparin (1 h ½), in part to better resorption from subcutaneous tissue. Thus, the bioavailability of the LMWH-DHE combination administered subcutaneously was far superior to that of unfractionated heparin injected by the same route. These results are in agreement with recently published findings (34). Several reports have suggested that heparin may also act via a stimulation of fibrinolytic activity (10, 12–19). To determine possible effects of the two heparin regimens on fibrinolysis we therefore also measured fibrinolytic activity. After injection of both the LMWH-DHE combination and the unfractionated heparin, a significant and progressive increase in fibrinolytic activity (determined by the both ECLT assay and fibrin plate assay) was observed ($p < 0.001$ ANOVA) reaching its maximum at the end of the afternoon i.e. 8–12 hours post injection. However the profile of the three diurnal fibrinolytic activity curves following injection of either LMWH-DHE, unfractionated heparin or placebo was identical, indicating that the fibrinolytic activity increases observed are not heparin induced but reflect the circadian physiological fluctuation of the fibrinolytic system.

Thus, the two heparins used in this study, administered as single subcutaneous injections and at the above mentioned dosage, have no influence on fibrinolytic activity. It cannot be excluded that larger amounts or repeated injections of unfractionated heparin, or of LMWH without DHE may have an effect on fibrinolysis. To establish such an effect, however, it will be necessary to take into account the physiological diurnal fluctuation of fibrinolytic activity.

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