Thrombin generation for the control of heparin treatment, comparison with the activated partial thromboplastin time

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Summary. Heparin can be quantified with antifactor Xa and IIa tests (aXa, aIIa) but the anticoagulant power of heparin depends upon plasma properties as well as upon heparin concentrations and thus differs between subjects. Measuring the effect, as with the activated partial thromboplastin time (APTT) therefore is clinically more relevant. Here we investigate the use of the endogenous thrombin potential (ETP) for this purpose. In 12 volunteers 9000 IU of four heparins of different mol. wt distributions were injected. Samples were taken at 11 time points between 0 and 24 h. With the exception of the 0 and 24-h time points, heparin could be demonstrated by its aIIa and aXa activity in virtually all samples. The APTT showed the effect of this heparin in 34% of the samples; the ETP in 80%. This is partly due to the wide margins of the normal values, caused by large interindividual variation [coefficient of variation (CV) approximately 12% for the APTT, approximately 17% for the ETP]. The intraindividual variation is much smaller (CV approximately 4% for the APTT, approximately 5% for the ETP). Relative to the baseline value of the individual, the heparin effect was recognized by the APTT in 55% of the cases and by the ETP in 98%. There were no large differences between the different types of heparin.

Keywords: APTT, heparin, thrombin generation.

Introduction

Heparin exerts its anticoagulant action through binding to antithrombin (AT) with a specific pentasaccharide structure, the A-domain [1]. For efficient anticoagulation a heparin should contain the C-domain, i.e. a stretch of 17 monosaccharides with the A-domain at its reducing end [2]. Antifactor Xa activity (aXa) reflects the concentration of A-domain, antithrombin activity (aIIa) that of the C-domain. With the aid of aXa and aIIa tests, intraindividual differences in heparin pharmacokinetics can be readily found. The activated partial thromboplastin time (APTT) measures the response of the patients’ plasma to the heparin present. There are important interindividual differences in heparin pharmacodynamics [3]. Plasmas vary in their content of pro- and anticoagulant factors [e.g. factor (F)VIII, tissue factor pathway inhibitor] and heparin binding proteins (albumin, histidin-rich glycoprotein, fibrinogen, platelet factor 4, lactoferrin and many others [4,5]). Important variations in the heparin response have been observed during pregnancy [6], in kidney disease [7], in deep vein thrombosis compared with coronary artery disease [8] in neonates [9] and in preschool ages [10] and in the obese [11]. In approximately 10% of the population the effect of heparin is so low as to justify the diagnosis of heparin resistance [12]. Measuring the heparin level, as with the aXa and the aIIa tests, therefore cannot replace assessing the effect of heparin action on the clotting system. The only generally available function test for this purpose is the APTT in one of its many varieties.

The sensitivity of the APTT to heparin is highly method and instrument dependent [13–17]. Standardization is fraught with problems [18,19,20–22]. There is bad correlation between the prolongation of the APTT and the heparin level or clinical course under heparin treatment [23]. The rationale behind the traditional therapeutic aim of a prolongation of the APTT to 1.5–2.5 times the normal value is questionable (see Kher [24] and references therein). One cannot but conclude that the APTT appears a poor means to assess heparin pharmacodynamics.

In practice heparins, notably low-molecular-weight heparins (LMWHs), are given in standard doses. Large clinical trials have proven the efficacy and safety of this approach [25]. It has been argued that for certain groups of patients adjusted dose therapy
would be preferable, especially for those who are not or badly represented in the clinical trials, such as children, aged people and patients with serious concomitant disease. In obese people weight-adapted doses have been shown to be preferable [11,26].

Given the poor performance of the APTT, the only means to decide whether, in what cases and how heparin therapy should be adjusted is clinical bleeding and rethrombosis. Although these remain the ultimate criteria, a reliable function test of the heparin effect would be a useful means for solving such questions.

In this study we investigate whether thrombin generation can serve this purpose. To this end we compare two tests that measure the heparin concentration in plasma, i.e. antifactor Xa (aXa) and antithrombin (aIIa) activity and two tests that measure the heparin effect, the APTT and the endogenous thrombin potential (ETP, i.e. the area under the thrombin generation curve). The samples were obtained from volunteers who received a subcutaneous injection of 9000 aXa-units of four different heparins of average molecular masses of 13, 10.5, 8.0 and 4.5 kDa in a study described previously [27]. It will appear that the APTT is indeed a poor indicator of the heparin effect compared with the ETP.

Materials and methods

Heparins

Three heparins (sodium salts) were commercially obtained: UFH [Liquemin® N; Hoffmann-La Roche AG, Basel, Switzerland; average molecular weight (AMr) 13 000, range 3000–30 000]; certoparin (Sandoparine®; Novartis, Nuerenberg, Germany; AMr 8000, range 6500–9500); and enoxaparin (Clexane®; Rhône Poulenc Rorer GmbH, Antony, France; AMr 4500, range 3500–5500). A medium-molecular-weight heparin (MMWH) was provided by Biochemie GmbH, Schaftenau, Austria (AMr 10 500, range 9500–11 500). Pre-filled glass syringes containing 9000 aXa-U of either heparin were used for s.c. injection.

Subjects

The subjects were a subgroup of those described previously [27], i.e. those from whom sufficient material was left for additional analysis. In short, they were males of around 25 years (range 18–30 years), mean body weight approximately 80 kg (range 60–95 kg), height approximately 1.8 m (range 1.67–1.98 m) who were healthy according to medical history, physical examination and routine biochemical, hematological and hemostatic tests. All subjects provided written informed consent.

No other medication was taken for 4 weeks before study entry and during the trial, notably no aspirin. Subjects were not allowed to consume alcohol or caffeine-containing products within 48 h of drug administration, were given a standard diet and were asked to avoid strenuous physical exercise.

Study design

The earlier study [27] was a randomized, double-blind, crossover trial. Four times in succession, at an interval of 7 days, each of the 12 volunteers was given a single s.c. injection of 9000 aXa-U of one of the heparins. Blood samples were taken at \( t = 0 \) and at \( t = 0.5, 1, 1.5, 2, 3, 4, 5, 8, 10, \) and 24 h after heparin administration. The study protocol was approved by the local Ethics Committee (Ethikkommission der Bayerischen Ärztekammer) and was performed in accordance with the Declaration of Helsinki and with European Community Guidelines for good clinical practice.

Veneupuncture

Venous blood was collected by clean venepuncture (discarding the first 2 mL of blood) through an 18-G indwelling canula (Vasofix®; Brauntüte® and Mandrin®; B. Braun Melsungen, Melsungen, Germany) into S-Monovettes® (Sarstedt, Nünbrrecht, Germany) containing 0.106 M sodium citrate (9 : 1 v/v). Platelet-poor plasma was prepared by centrifugation at 3000 \( \times \) g for 20 min at 15 °C, transferred into cryovials in aliquots of 0.5 mL, frozen in liquid nitrogen and stored at −70 °C until further analysis.

APTT, anti-FXa and antithrombin activity

Routine analyses were performed on an ACL 3000 coagulation automaton (Instrumentation Laboratory, Kirchheim, Germany). Duplicate values were generally determined. If the deviation was > 3%, the measurement was repeated. The validity of each determination was checked by parallel controls with control plasma and the 4th International Standard for heparin (1983) or the 1st International Standard for LMWH (National Institute for Biological Standards and Control, London, UK).

APTT activity was determined using a commercial kit according to the instructions of the manufacturer (APTT Micro Kieselgur; Instrumentation Laboratory).

Plasma aXa and aIIa activities were measured by amidolytic assays [28,29], using reagents from Chromogenix AB (Mölndal, Sweden). Plasma samples diluted with AT containing Tris buffer were incubated with bovine FXa and bovine thrombin, respectively. After mixing with the chromogenic substrates S2222 (FXa) and S2238 (thrombin) amidolytic activity was recorded at 405 nm for 120 s. The inhibition of the original activity by incubation with the sample was compared with that of known concentrations of the 4th international standard. It was checked that the aXa and aIIa activities obtained with this standard were identical. The optical density (OD) values measured with the zero-time samples (\( n = 48 \)) and the \( t = 24 \) samples (\( n = 48 \)) were compared and not significantly different. Together they were used to determine the normal range (mean ± 2 SD) of OD values corresponding to the absence of heparin activity.

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Chromogenic ETP determination

In order to allow measurement of OD changes, samples were defibrinated with Ancrod. Citrated plasma was mixed with a 1:50 volume of the Ancrod solution and incubated for 10 min at 37 °C, then kept on ice for 10 min, after which the clot was wound out on a plastic spatula.

Thrombin generation was measured in a Cobas Bio centrifugal analyzer (F. Hoffmann-La Roche) as described earlier [30]. To four parts of defibrinated plasma was added one part of a solution containing recombinant tissue factor (rTF, approximately 30 pm) and procoagulant phospholipid (PPL, 6 μM). In preliminary experiments we tested the influence of the concentration of rTF and PPL on the thrombin generation curve in the presence of different concentrations of the different heparins. It appeared that the influence of heparin on the lag phase before the thrombin burst was strongly influenced by variation in rTF and PPL. However, the influence of heparin on the ETP was not affected by changes in rTF and PPL (results not shown).

The reaction was started by adding one part buffer with the slow chromogenic substrate Msc-Val-Arg-pNA (0.5 mm) and CaCl₂ (100 mM). The course of the OD at 405 nm was recorded at 30-s intervals during 15 min. The analyzers were connected to a personal computer and the data were transformed to a standard format file which gave the OD as a function of time and from which the ETP was calculated off-line according to [30] (further details on website http://www.thrombin.com).

Results

Normal values

We first determined the normal limits of the APTT and the ETP. For this we used the four points obtained at t = 0 for each volunteer (n = 48). Then we determined whether these values were different from those obtained at the t = 24 point (n = 48), which they were not (Table 1). So no effect of the heparin injection could be demonstrated after 24 h. The normal boundaries (mean ± 2 × SD) of the APTT and the ETP were determined from these data (n = 96). For each volunteer the baseline values of APTT and ETP were determined from the combined t = 0 and t = 24 points (n = 8). The day-to-day variation per individual appeared to be much smaller than the variation between individuals (Table 1). We therefore also measured the heparin effect in relation to the individual zero time value. For the APTT we calculated the individual APTT ratio, i.e. the APTT under heparin treatment divided by the APTT at zero time. For the ETP we calculated the percentage of inhibition of the ETP under heparin relative to the ETP at zero time. At zero time these relative values were 1 (APTT prolongation) and 0 (% ETP inhibition) by definition. The normal boundaries of these relative values as well as those for the aIIa and aXa values were calculated on the basis of the values measured at t = 24 (n = 48).

Sensitivity of the APTT and the ETP to the heparin effect

In the samples taken between 0.5 and 10 h after s.c. injection, the aIIa and the aXa tests demonstrated the presence of heparin in 95% or more of the cases. The effect of this heparin on the clotting system caused a significant prolongation of the APTT, relative to the population mean, in 34% of the samples, whereas the ETP showed inhibition in 80%. In a restricted set of data, where the samples at t = 0.5, t = 8 and t = 10 that contain low concentrations of heparin were left out, heparin could be demonstrated by aXa and aIIa tests in 99–100% of the cases and the APTT was significantly prolonged in 43% of the samples, the ETP inhibited in 93%.

Table 1 Individual variation of the normal activated partial thromboplastin time (APTT) and endogenous thrombin potential (ETP)

<table>
<thead>
<tr>
<th>Subject</th>
<th>APTT zero time</th>
<th>APTT 24 h</th>
<th>APTT both</th>
<th>ETP zero time</th>
<th>ETP 24 h</th>
<th>ETP both</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>30.0 ± 1.5</td>
<td>29.9 ± 1.1</td>
<td>30.0 ± 1.2</td>
<td>702 ± 54</td>
<td>703 ± 20</td>
<td>703 ± 38</td>
</tr>
<tr>
<td>B</td>
<td>36.7 ± 0.8</td>
<td>36.7 ± 1.5</td>
<td>36.7 ± 1.1</td>
<td>758 ± 35</td>
<td>727 ± 95</td>
<td>742 ± 69</td>
</tr>
<tr>
<td>C</td>
<td>31.7 ± 1.1</td>
<td>31.6 ± 1.5</td>
<td>31.6 ± 1.2</td>
<td>712 ± 47</td>
<td>685 ± 24</td>
<td>699 ± 37</td>
</tr>
<tr>
<td>D</td>
<td>34.8 ± 1.5</td>
<td>34.0 ± 0.8</td>
<td>34.4 ± 1.2</td>
<td>708 ± 18</td>
<td>695 ± 19</td>
<td>701 ± 18</td>
</tr>
<tr>
<td>E</td>
<td>27.6 ± 1.4</td>
<td>28.3 ± 1.0</td>
<td>27.9 ± 1.2</td>
<td>783 ± 33</td>
<td>793 ± 36</td>
<td>788 ± 33</td>
</tr>
<tr>
<td>F</td>
<td>30.2 ± 1.6</td>
<td>31.0 ± 1.8</td>
<td>30.6 ± 1.7</td>
<td>985 ± 63</td>
<td>963 ± 65</td>
<td>974 ± 60</td>
</tr>
<tr>
<td>G</td>
<td>36.6 ± 2.2</td>
<td>37.5 ± 0.9</td>
<td>37.1 ± 1.6</td>
<td>821 ± 14</td>
<td>803 ± 24</td>
<td>812 ± 20</td>
</tr>
<tr>
<td>H</td>
<td>39.6 ± 2.5</td>
<td>39.7 ± 1.8</td>
<td>39.6 ± 2.0</td>
<td>688 ± 40</td>
<td>700 ± 24</td>
<td>694 ± 31</td>
</tr>
<tr>
<td>I</td>
<td>31.8 ± 1.4</td>
<td>32.5 ± 1.1</td>
<td>32.1 ± 1.2</td>
<td>614 ± 26</td>
<td>624 ± 13</td>
<td>619 ± 20</td>
</tr>
<tr>
<td>K</td>
<td>26.4 ± 1.1</td>
<td>26.9 ± 0.8</td>
<td>26.6 ± 0.9</td>
<td>503 ± 9</td>
<td>460 ± 93</td>
<td>481 ± 65</td>
</tr>
<tr>
<td>L</td>
<td>33.8 ± 1.1</td>
<td>34.2 ± 1.4</td>
<td>34.0 ± 1.2</td>
<td>846 ± 101</td>
<td>875 ± 85</td>
<td>861 ± 88</td>
</tr>
<tr>
<td>M</td>
<td>34.6 ± 1.5</td>
<td>37.3 ± 1.6</td>
<td>36.0 ± 2.1</td>
<td>617 ± 44</td>
<td>619 ± 10</td>
<td>618 ± 30</td>
</tr>
<tr>
<td>Average-A</td>
<td>32.8 ± 3.9</td>
<td>33.3 ± 4.0</td>
<td>33.0 ± 3.9</td>
<td>728 ± 125</td>
<td>725 ± 121</td>
<td>726 ± 123</td>
</tr>
<tr>
<td>SD-A</td>
<td>1.5 ± 0.5</td>
<td>1.3 ± 0.4</td>
<td>1.4 ± 0.4</td>
<td>40 ± 25</td>
<td>37 ± 29</td>
<td>38 ± 22</td>
</tr>
<tr>
<td>Total-CV</td>
<td>11.9</td>
<td>12.0</td>
<td>11.8</td>
<td>17.2</td>
<td>16.7</td>
<td>16.9</td>
</tr>
<tr>
<td>Intra-CV</td>
<td>4.6 ± 1.6</td>
<td>3.9 ± 0.12</td>
<td>4.2 ± 1.3</td>
<td>5.5 ± 3.4</td>
<td>5.1 ± 4.0</td>
<td>5.2 ± 3.0</td>
</tr>
</tbody>
</table>

CV, Coefficient of variation. The intraindividual CV is the mean ± SD of the individual CVs. The standard deviations are measured on n = 4 in the first two columns, on n = 8 in the third one and on the 12 averages in the bottom row. Average-A, Average of the 12 individual averages; SD-A, average of the 12 individual SDs.

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When related to the individual zero-time value, the APTT ratio showed the effect of heparin in 55% of the samples (74% in the restricted set) and the ETP was inhibited in 98% (100% in the restricted set).

**The effect of heparin on the function tests**

In view of the fact that the intraindividual coefficient of variation, i.e. the sum of the day-to-day variation and the experimental error, is of the same order of magnitude for the APTT and the ETP (Table 1), the essential difference in sensitivity to the heparin effect cannot be attributed to a difference in experimental variation. We therefore further investigated the relation between APTT and ETP on the one hand, and aIIa and aXa activities on the other.

Plotting APTT as such against aXa and aIIa activity resulted in extreme scattering (Fig. 1). This scattering was much reduced when the prolongation was related to the individual zero time value (Fig. 2). It is seen that the relation cannot be distinguished from linear. When the aXa activity was taken as the independent variable, two families of points could be clearly distinguished, one for the LMWHs and one for the other two preparations. If the aIIa activity is taken as the independent variable, such a distinction could not be made, although there was a trend for UFH to scatter around a steeper line than the others.

Plotting the ETP inhibition against aXa or aIIa activity (Fig. 3) showed a sharp increase of inhibition at low heparin activity that approached complete inhibition at higher values, which suggests a hyperbolic relationship. Again, if the aIIa activity was taken as the independent variable, the points come in two series, one for the LMWHs and one for UFH and MMWH.

**Discussion**

The dosage (9000 IU) and heparins (UFH and LMWHs) used in this study are representative of clinical practice. Heparin, as
assessed by its aIIa and aXa activity, could be demonstrated to be present in 95% or more of the samples. We first asked the question, in how many of these samples does the heparin present cause a significant prolongation of the APTT or significant inhibition of the ETP?

Normal limits were determined as the mean and 2·SD of the samples taken at \( t = 0 \) and \( t = 24 \) of the 12 volunteers. The APTT in the heparin-containing samples was significantly prolonged in 34% of the samples taken; the ETP in 80%. In the restricted set these values were 43% (APTT) and 93% (ETP) (Table 2).

The interindividual variation of baseline values of the APTT was approximately 12% and that of the ETP approximately 17%. This is more than twice as large as the day-to-day variation per individual (Table 1). It follows that the normal limits defined on the basis of a normal population are much wider than the individual normal limits and consequently a small increase of a naturally short APTT or a small decrease of a naturally high ETP will fall within the normal limits of the population.

We compensated for the large interindividual variation by expressing the prolongation of the APTT and the inhibition of the ETP relative to the individual baseline value. In this way the effect of heparin could be detected in 55% (APTT) and in 98% (ETP) of the samples [in the samples taken between 1 and 5 h after injection these values were 74% (APTT) and 100% (ETP), respectively]. There were no large differences between the different types of heparin; if anything, LMWH was more readily detectable than UFH and the MMWH was detected most often (Table 2).

It should be noted that the two mechanisms of action behind the two functional tests that are compared here are entirely different. In the APTT, and in general in all clotting tests, the lag time before explosive thrombin generation is measured, whereas the ETP quantifies the effect of heparin on the bulk of thrombin formation. During the lag time thrombin has an essential function as a feedback activator. Heparin interferes with the lag time primarily through inhibition of this feedback [31]. On the bulk phase heparin acts by draining formed thrombin more quickly into the antithrombin–thrombin complex.

The feedback mechanism involves factor (F)V and, depending upon the degree of contact activation and the amount of TF present, also FVIII and factor XI. FV can be activated by the membrane bound- and hence heparin-insensitive meizothrombin. FVIII is activated by soluble thrombin only and thereby subject to the action of heparin. It therefore is not surprising that the effect of a given amount of heparin on the lag time depends upon the trigger used. The effect upon the bulk of thrombin formation, i.e. on the ETP, however, is independent of the pathway triggered ([31] and results not shown). For this reason it may be surmised that the results from different types of ETP-based measurement [32–34] using different triggers will have less influence on the outcome of this test than on that of the APTT.

The reason for the better performance of the ETP is not to be found in a higher experimental accuracy or in a more stable normal value, because the intraindividual coefficients of

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Table 2: Sensitivity to heparin treatment

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>APTT</th>
<th>ETP</th>
<th>AIIa</th>
<th>AXa</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>432</td>
<td>34</td>
<td>55</td>
<td>80</td>
<td>98</td>
</tr>
<tr>
<td>R.S.</td>
<td>264</td>
<td>43</td>
<td>74</td>
<td>93</td>
<td>100</td>
</tr>
<tr>
<td>UFH</td>
<td>108</td>
<td>27</td>
<td>41</td>
<td>68</td>
<td>96</td>
</tr>
<tr>
<td>MMW</td>
<td>108</td>
<td>51</td>
<td>65</td>
<td>86</td>
<td>97</td>
</tr>
<tr>
<td>Certoparin</td>
<td>108</td>
<td>26</td>
<td>61</td>
<td>82</td>
<td>99</td>
</tr>
<tr>
<td>Enoxaparin</td>
<td>108</td>
<td>31</td>
<td>55</td>
<td>84</td>
<td>98</td>
</tr>
</tbody>
</table>

Each figure (except N) gives the percentage of samples in which the test in the column was significantly different from the relevant normal control (see text). All, All samples except the 0 and 24-h time points; R.S. (restricted set), the previous group with the 0.5, 8 and 10-h time points omitted. Lower rows: samples after injection of the heparin indicated.
variation of both methods are roughly identical and the ETP detects more interindividual difference than the APTT does (Table 1). The reason is rather in the different form of the dose–response curve. It is seen in Figs 2 and 3 that approximately 0.1 aIIa IU of any heparin will cause 50–80% of inhibition of the ETP against a prolongation of the APTT of 1.2–1.4 times.

In the plots of the APTT and the ETP as a function of aXa activity, two different families of points can be distinguished. The higher mol. wt heparins seem to be more potent than the LMWHs. This is readily explained by the fact that heparins act primarily via inhibition of thrombin (see [35]) and that, per unit aXa, the higher mol. wt heparins carry more aIIa activity. In other words: the excess aXa activity encountered in LMWHs does not significantly influence the outcome of either the APTT or the ETP.

That a relation is nevertheless found between aXa activity and prolongation of the APTT or inhibition of the ETP is caused by the fact that there is a strong correlation between the concentrations of circulating C- and A-domains. This correlation depends upon the heparin injected (hence the two hyperbolas in Figs 2b and 3b) and shifts with time after the injection, but not to such an extent as to abolish the correlation (experiments to be published). aXa activity that is not accompanied by aIIa activity, as encountered in idraparinux, for example, can cause adequate inhibition of thrombin generation (e.g. see [36]), but higher aXa activities are required than those achieved in this study.

In the plots of the APTT ratio as a function of the aIIa activity little, if any, difference was seen between the various types of heparin. This shows that the aIIa activity is the primary cause of APTT prolongation. The common observation that the APTT is less sensitive to the action of LMWHs than to that of UFH (e.g. [37]) must be primarily a consequence of using aXa units to express LMWH activity (Figs 1b and 2b).

The present results may contribute indirectly to the ongoing discussion of the necessity of controlling heparin therapy. The performance of aIIa and anti-FXa tests shows that they efficiently demonstrate—and therefore probably also reliably quantify—heparin in plasma (Table 2). The broad scattering in Figs 1–3 shows that the same heparin concentration may inhibit the clotting system much more in one individual than in another, even if they are biologically as similar as the healthy male volunteers of this study. We therefore think that the individual response of patients to a standard dose of heparin may differ significantly. However, using the APTT, dose adjustment appears of little avail [38]. Our data suggest that this may be due to the fact that concentrations of heparin that already significantly inhibit thrombin generation cause only marginal prolongation of the APTT (Fig. 4).

Prior to the question of the desirability of control is the question of whether adequate control is at all possible. Our results suggest that the nature of the response of the APTT to heparin makes it a poor means to assess the heparin effect, and that automated thrombin generation measurement might be a useful alternative [34].

References


