Monitoring of Anticoagulant Effects of Direct Thrombin Inhibitors

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ABSTRACT

Monitoring of direct thrombin inhibitors with the activated partial thromboplastin time (aPTT) is limited by poor linearity and reproducibility. Recently, direct prothrombin activation methods have been developed for coagulation analysis: ecarin clotting time (ECT) and prothrombinase-induced clotting time (PiCT). Laboratory monitoring of the direct thrombin inhibitors lepirudin, argatroban, and melagatran was analyzed and compared with monitoring unfractionated heparin (UFH).

Plasma samples of six healthy donors were spiked with lepirudin and argatroban extending to 3000 ng/mL, melagatran extending to 1000 ng/mL, and UFH up to 0.48 IU/mL. Clotting times of aPTT (two reagents), ECT, PiCT, and prothrombin time were determined in a KC 10a micro instrument.

At 3000 ng/mL ECT values were 339.1 ± 25.0 seconds with lepirudin and 457.5 ± 29.5 seconds with argatroban. ECT was 586.0 ± 38.2 seconds with 1000 ng/mL melagatran. The PiCT method provided clotting times of 137.0 ± 8.4 seconds with UFH, 128.0 ± 23.4 seconds with lepirudin, and 151 ± 23.9 seconds with argatroban, and 153.5 ± 9.9 seconds with melagatran, with the concentrations mentioned.

ECT is more sensitive to therapeutic drug concentration ranges than aPTT (prolongations of 3–7 versus 2–3). PiCT provides results that are comparable with direct thrombin inhibitors and UFH. This method could therefore be suitable for monitoring both drug groups.

KEYWORDS: Thrombin inhibitors, ecarin clotting time, prothrombinase-induced clotting time, hirudin, argatroban, melagatran

Objectives: Upon completion of this article, the reader should be able to (1) appreciate the rationale for developing direct thrombin inhibitors and (2) judge which laboratory tests are most useful in monitoring these compounds.

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In patients with venous thromboembolism, anticoagulation with heparin is well established and is the standard initial treatment. Unfractionated heparin (UFH) is administered intravenously or subcutaneously. It requires dose adjustment, usually based on the activated partial thromboplastin time (aPTT). Low-molecular-weight heparins (LMWHs) have been introduced as equally effective and safe alternatives to UFH in the treatment of deep-vein thrombosis and pulmonary embolism. LMWHs differ from standard UFH in that they have a higher ratio of anti-factor Xa to anti-factor IIa activity, greater bioavailability, longer plasma half-life, a more predictable anticoagulant response, and less adverse events such as heparin-induced thrombocytopenia. Recently, numerous new substances with directly acting inhibitory actions to one or more binding sites of thrombin have been discovered or systematically developed by analogue design. A few of them have made it into clinical practice or late clinical trial phases. Examples are argatroban and melagatran.

Since the introduction of the first direct thrombin inhibitor (DTI), hirudin, monitoring of direct inhibitory effects has been addressed as an issue. One of the possibilities is to extend the aPTT method, originally designed for heparin monitoring, to DTIs. This method is unsatisfactory for the same reasons as with heparins: poor linearity and reproducibility due to dependence on a large number of factors in the clotting cascade. The coagulation cascade is activated at a high stage involving influences of factors XII and VIII, V, and II, the entire intrinsic system, resulting in high inner noise. A plateau effect makes monitoring more difficult, especially in higher concentration ranges. Development of the novel ecarin clotting time (ECT) method provided a linear test for hirudin measurement. The method is based on specific activation of thrombin to meizothrombin alone, reducing interference with other factors, as compared with the aPTT.

The recently introduced prothrombinase complex-induced clotting test (PiCT) uses the entire prothrombinase complex for clot activation. The system is made up of factor X (FX), phospholipids, calcium, and FXa, preactivated by the activator RVV-Va from Russell’s viper venom. Cloot activation by the whole prothrombinase complex containing preactivated FV is about 30,000 times faster than that by FXa alone and 1000 times faster than by FXa together with calcium ions and phospholipids. This increased velocity of prothrombin activation decreases errors in test results and leads to increasing reproducibility and linearity. The coagulation cascade is activated at a lower stage; therefore, covering of feedback mechanisms is improved. The sensitivity of the method to fluctuation of plasma factor levels and other disturbance variables (inner noise) is reduced. The prothrombin time (PT) is a further routine method for laboratory coagulation testing. It is a complex test system activating higher stages (FVII, extrinsic system) of the coagulation cascade that covaries with but does not itself represent the essential parameter influenced by anticoagulation.

In the present study, concentration-effect relationships of UFH and DTIs, r-hirudin, argatroban, and melagatran on aPTT, ECT and PiCT, and PT were analyzed in normal plasma samples.

**MATERIALS AND METHODS**

Blood from six healthy volunteers was collected by clean cubital vein puncture into plastic vials containing 3.8% sodium citrate (plasma/citrate, 9:1 v/v). The centrifuged plasma samples (1800 g, 10 minutes) were shock frozen in liquid nitrogen, stored at –80°C, and analyzed within 4 weeks. After thawing, plasma samples were spiked with hirudin (molecular mass approximately 6500 Da, lepirudin; obtained from Aventis, Frankfurt am Main, Germany) and argatroban (molecular mass 526.7; kindly provided by Mitsubishi Chemical Corp., Tokyo, Japan) at concentrations ranging from 300 to 3000 ng/mL. Melagatran (molecular mass 473.6; courtesy of AstraZeneca, Mölndal, Sweden) was spiked in lower concentrations between 30 and 1000 ng/mL because of its higher gravimetric potency found in preliminary experiments. UFH was added within the PiCT method in the following concentrations: 0.048, 0.08, 0.16, 0.32, and 0.48 IU/mL. In the aPTT method, a concentration of 0.0016 IU/mL was also tested.

Clotting time measurements were carried out on a KC 10 a micro device from Amelung Comp. (Lemgo, Germany). PT was determined with a recombinant thromboplastin reagent (Aventis Behring, formerly Dade Behring, Marburg, Germany; ISI [international sensitivity index] value = 1.09; lot number: 526935). Fifty microliters of plasma was incubated at 37°C for 3 minutes (micromethod). To start the clotting time assay, 100 µL of PT reagent (dissolved according to the manufacturer’s instructions) was added. The ECT is also a one-stage test. Incubation of 50 µL of plasma was carried out for 2 minutes under conditions as described earlier. Ecarin reagent (Pentapharm, Basel, Switzerland, lot number 8303/116–08, 50 µL) was added to start the reaction. Normal ranges were 42.1 ± 0.9 seconds. The ECT is insensitive to heparins.

The aPTT testing was carried out with Pathromtin SL from Aventis Behring (lot 523751 A) and Plateletin LS from Biomerieux (Castres Cedex, France, lot 161419) as a two-step test (incubation of 50 µL plasma + 50 µL reagent for 2 minutes, start of clot formation by recalcification with 50 µL CaCl$_2$ (25 mM]). Normal values were 33.5 ± 4.0 seconds with Pathromtin SL and 34.1 ± 4.0 seconds with Plateletin LS. PiCT was carried out as a two-step test with PiCT reagent of
Pentapharm (Basel, Switzerland) in the same manner as the aPTT but with 3 minutes of incubation. Normal range for the PiCT was 29.9 ± 4.6 seconds. All data are given as mean value ± standard deviations.

RESULTS

Activated Partial Thromboplastin Time
For the aPTT test series, two reagents with different sensitivities have been used. Pathromtin SL® proved to be more sensitive to all inhibitors tested. Up to a concentration of 0.08 IU/mL, sensitivities of both reagents to UFH were equivalent. UFH prolonged clotting times at this concentration to 51.1 ± 14.0 seconds with Platelin LS® and to 51.0 ± 6.3 seconds with Pathromtin SL®. Above this concentration, the curves started diverging. Platelin LS developed a moderate plateau effect (Fig. 1A) with 68.5 ± 18.3 seconds at 0.16 IU/mL, 161.3 ± 104.3 seconds at 0.32 IU/mL, and 210.7 ± 106.9 seconds at 0.48 IU/mL (3000 ng/mL Liquemin®, MM =12,500). Pathromtin SL, in contrast, developed a linear course starting from 0.16 IU/mL (78.2 ± 12.6 seconds) up to the end of the concentration range tested (216.8 ± 98.1 seconds at 0.32 IU/mL and 357.7 ± 157.0 seconds at 0.48 IU/mL).

DTIs prolonged the aPTT to a lesser extent (Fig. 1B–D). With lepirudin a plateau effect occurred with both reagents in the concentration range above 2000 ng/mL. Addition of 3000 ng/mL lepirudin (equigravimetric to 0.48 IU/mL UFH) yielded clotting times of 108.1 ± 17.6 seconds with Platelin LS and 140.5 ± 26.8 seconds with Pathromtin SL (Fig. 1B). Similar results could be obtained with argatroban. Platelin LS delivered clotting times of 82.3 ± 8.6, 99.5 ± 13.5, and 114.3 ± 14.1 seconds with 1000, 2000, and 3000 ng/mL argatroban, respectively. At the same concentrations, aPTT values with Pathromtin SL were as follows: 97.1 ± 12.8, 113.5 ± 22.2, and 142.8 ± 13.4 seconds, respectively. Linearity of the curves was improved, however (Fig. 1C). A level of anticoagulation similar to that with 3000 ng/mL of the other inhibitors
could be obtained by addition of 1000 ng/mL melagatran (118.9 ± 14 seconds with Platelin LS and 160.8 ± 11.8 seconds with Pathromtin SL). The concentration-effect relationship was almost linear with Pathromtin SL. Also with Platelin LS, good linearity was achieved (Fig. 1D).

**Ecarin Clotting Time**
The ECT with lepirudin delivered an entirely linear concentration-effect relationship. A concentration of 3000 ng/mL prolonged clotting times to 339.1 ± 25.0 seconds. Details are shown in Figure 2A.

Argatroban influenced clotting times in the ECT method in a different manner. The concentration-effect curve was slightly sigmoidal with a steeper convex part up to 1000 ng/mL and 279.3 ± 24.5 seconds, respectively, and a flatter concave one up to 300 ng/mL and 457.5 ± 29.5 seconds (Fig. 2B).

Melagatran showed a convex, almost linear dose-effect relationship with a steeper part in lower concentration ranges and a slightly flatter part above (Fig. 2C). Melagatran again had the strongest effects among all inhibitors tested. Rather low concentrations, compared with the other inhibitors, led to distinct ECT prolongations. A concentration of 300 ng/mL resulted in clotting times of 311.6 ± 18.1 seconds. Addition of 1000 ng/mL prolonged the ECT to 586.0 ± 38.2 seconds. The ECT is insensitive to heparins (data not shown).

**Prothrombinase-Induced Clotting Time**
The PiCT developed a rather flat concentration-effect curve with UFH. Addition of 0.16 IU/mL prolonged coagulation times to 81.7 ± 2.9 seconds. Concentrations of 0.32 and 0.48 IU/mL resulted in 118.1 ± 6.7 and 137.0 ± 8.4 seconds, respectively. A slight plateau effect occurred (Fig. 3A).

The concentration-effect relationship of lepirudin was somewhat different from that of UFH on one hand and from the other two DTIs argatroban and melagatran. Concentrations of 300 and 1000 ng/mL prolonged clotting times to 47.8 ± 22.3 and 57.7 ± 11.5 seconds. A steep final part appeared above 2000 ng/mL (78.8 ± 12.5 seconds); 3000 ng/mL lepirudin yielded PiCT values of 128.0 ± 23.4 seconds (Fig. 3B).

Argatroban showed a concentration-effect relationship very similar to that of UFH. The sensitivity of the PiCT method to argatroban was somewhat higher than to UFH (Fig. 3C). Doses of 1000, 2000, and 3000

![Figure 2](image-url) Concentration-ECT (seconds) relationships of lepirudin (A), argatroban (B), and melagatran (C). Data are given as mean clotting times ± standard deviations.
ng/mL led to PiCT values of 95.9 ± 8.4, 130.0 ± 15.3, 151 ± 23.9 seconds, respectively.

The concentration-effect relationship of melagatran consisted of a very steep initial part up to a dosage of 100 ng/mL. This concentration elevated clotting times to 76.2 ± 3.1 seconds (Fig. 3D). Then the curve turned into a markedly flattened part but for the most part remained linear. No plateau effect occurred up to a concentration of 1000 ng/mL resulting in a clot formation time of 153.5 ± 9.9 seconds.

Prothrombin Time
The PT yielded a very flat concentration-effect relationship with lepirudin (Fig. 4A). At the highest concentration tested (3000 ng/mL), the PT was prolonged to 14.0 ± 1.3 seconds.

Argatroban prolonged clotting times in the PT method linearly (Fig. 4B). A medium therapeutic range concentration of 1000 ng/mL prolonged PT values to 11.5 ± 0.5 seconds. Addition of 2000 ng/mL led to clotting times of 37.5 ± 4.1 seconds. A dosage of 3000 ng/mL resulted in 63.2 ± 5.5 seconds.

Melagatran had a nonlinear dose-effect relationship with a plateau in the lower concentration range (up to 300 ng/mL and 16.8 ± 1.0 seconds, respectively) and a steep linear part in the range from 500 ng/mL (30.7 ± 3.5) to 1000 ng/mL (95.0 ± 6.8) (Fig. 4C). Due to the addition of heparin antagonists to the thromboplastin reagents, the PT was also insensitive to heparins (data not shown).

DISCUSSION
In the present study different methods for laboratory monitoring of anticoagulant effects of UFH and DTIs were compared. At present, a great diversity of aPTT reagents is available on the market. The test results vary with the different reagents and sometimes even with different charges of the same reagent to a large extent, as described in the literature since the late 1970s. For these reasons, in the present study, the aPTT was tested with two reagents with different sensitivities. Both contained silicon dioxide as a surface activator. According to the manufacturer’s data, Pathromtin SL contains purified vegetable phospholipids. The comparison of two reagents with different sensitivities showed that __ ls __ le __ ll
Figure 4 Concentration-PT (seconds) relationships of lepirudin (A), argatroban (B), and melagatran (C). Data are given as mean clotting times ± standard deviations.

accuracy of monitoring UFH strongly depends on the choice of the aPTT reagent. This conclusion is especially valid for upper concentration ranges. In the present study, the high-sensitivity reagent Pathromtin SL provided linear results with UFH, in contrast to the low-sensitivity Platelin LS, which showed a plateau effect above 0.32 IU/mL (≈ 2000 ng/mL, Fig. 1A). In summary, sensitivity differences between Pathromtin SL and Platelin LS were of quantitative as well as qualitative nature for UFH.

With DTIs, results were different. Sensitivity differences between Pathromtin SL and Platelin LS were of only a quantitative nature for DTIs. Higher sensitivity of Pathromtin SL compared with Platelin LS resulted in parallel shifts of the curves without affecting their shapes (Fig. 1B–D). Yet, the reagent with the higher sensitivity to all drugs tested is to be preferred. The need for standardization of aPTT reagents is further stressed by the results obtained. Also, the introduction of a normalized aPTT ratio analogous to the International Normalized Ratio (INR) for PT seems to be possible and suitable, as proposed for UFH by Kritikos and Melhem in 1993 and Bates and co-workers in 2001. Reed and co-workers, however, present the opposite opinion, namely that the use of the INR format for aPTT would give no confidence in any individual results and could be misleading. Still, they admit that this conclusion was based on analyses of only 5 out of more than 30 reagents available at that point in time.

Despite all its drawbacks, the aPTT is a suitable method for monitoring both heparins and DTIs, especially in smaller centers where alternative methods, such as ECT, are not available.

The novel ECT test provides a superior surveillance method for DTIs. As a final-stage method it activates the coagulation cascade at a low step: the cleavage of prothrombin to meizothrombin and other intermediates with thrombin activity. This principle stands in contrast to long-range methods such as aPTT and PT, which activate the coagulation cascade at a high stage by activation of FXII (intrinsic system) and FVII (extrinsic system), respectively. By accelerated clot system activation, covering of feedback mechanisms between different coagulation factors is improved. These feedback mechanisms appear from FIIa via FV and from FXa via FVII and FV. Therefore, fluctuations in clotting factor levels, such as during oral anticoagulation, affect a final-stage method such as the ECT less than the aPTT or PT. The results of quick activation of prothrombin have higher specificity, higher linearity, and less errors. For lepirudin, concentrations in the test samples can easily be calculated from a linear equation. Good linearity is also provided by the ECT for argatroban and melagatran. Therapeutic concentration ranges are narrower than those tested in the present study: 500 to 1500 ng/mL with argatroban and 100 to 300 ng/mL with melagatran. The concentration-effect curve of
melagatran is almost linear in the therapeutic range (Fig. 1C). In the case of argatroban, a simple linear equation can be obtained only by tangential approximation (Fig. 2B). According to the advantages described earlier, the ECT should be preferred wherever possible.

The novel, experimental PiCT method produced somewhat heterogeneous results. As it is a final-stage method too, some of the advantages found with the ECT could also be expected for the PiCT. Indeed, the standard deviation was strongly reduced compared with the aPTT. This finding occurred with all inhibitors with the exception of the highest concentration range of argatroban (2000–3000 ng/mL, Fig. 3A–D versus Fig. 1A–D). In contrast to the ECT, prothrombin is not cleaved directly, but the entire prothrombinase complex consisting of FXa, phospholipids, calcium ions, and FVa is activated. This might be the reason why less linearity was found with the PiCT than with the ECT. A great advantage of this principle is that the PiCT method is also sensitive to heparins, which cannot interact with meizothrombin in the ECT method. With UFH, the PiCT delivered a curve similar to that of the aPTT with Platelin LS. The main difference was lack of the flat initial part of the aPTT curve in the PiCT method (Fig. 3A versus 1A, black diamonds).

The DTIs lepirudin, argatroban, and melagatran exerted inhomogeneous effects on the PiCT. A common phenomenon with all three DTIs was a steep initial part of the concentration-effect curve. Then the curves turned to a flat linear part, which persisted with argatroban and melagatran. In the case of lepirudin, the highest part (above 2000 ng/mL) was turning more steeply again, resulting in a sigmoidal curve consisting of a steep onset, a flat plateau, and a steep final stage. A possible explanation could be the fact that the proteinous agent hirudin is a bivalent inhibitor for both the active catalytic site and the anion binding exosite of thrombin whereas the synthetic tripeptides argatroban and melagatran is almost ineffectual in normal plasma. Argatroban provided a moderate linear increase of clotting times. Melagatran showed a parabolic/hyperbolic concentration-effect relationship. The mechanistic backgrounds could be similar to those described earlier for the PiCT method. The PT is not a suitable method for monitoring DTIs. The effect of DTIs on the PT during concomitant administration with oral anticoagulants is a considerable source of error. In factor-deficient plasmas, as during use of vitamin K antagonists, hirudins also exerted stronger actions on the PT. These synergistic interferences between argatroban and warfarin in human plasma samples ex vivo and in vitro have also been described in the literature.

Clinical Implications
In many locations, especially in smaller clinical centers, the ECT method is still not available in daily clinical routine. In these cases, the aPTT remains the method of choice. The therapeutic range is a prolongation to 1.5- to 2-fold of the local normal value, as described in the literature. This range is based on long-term clinical experience. The experimental basis was described as “meager” for UFH and lacking for other anticoagulants by Kher and co-workers. In the present work, therapeutic concentrations of UFH (0.3 to 0.6 IU/mL) displayed aPTT prolongations of 5 to 6 times with Platelin LS and 6 to 10 times with Pathromtin SL. According to such discrepancies between in vivo and in vitro results, some authors doubt that in vitro results are transferable to in vivo situations for the aPTT.

Because of different dose-effect relationships, Kher and co-workers questioned the transferability of doubling the aPTT from heparins to DTIs. According to the results presented here, dosage up to a two- to threefold prolongation of the aPTT seems to be suitable to achieve therapeutic concentrations of lepirudin (1200 to 2800 ng/mL), argatroban (500 to 1500 ng/mL), and melagatran (200 to 500 ng/mL). However, melagatran does not require monitoring in normal clinical settings without complications such as severe renal insufficiency because of a broad therapeutic range and reproducibility of plasma drug levels and anticoagulant effects.

The ECT method makes it possible to establish a common normal range for lepirudin, argatroban, and melagatran. A three- to sevenfold prolongation seems to be suitable to achieve therapeutic concentrations of lepirudin (1200 to 2800 ng/mL), argatroban (500 to 1500 ng/mL), and melagatran (200 to 500 ng/mL). Further clinical data are required to investigate the applicability of this model to administration in vivo.

The PiCT method seems to have some promising advantages regarding the precision of the method. The low error described in the original article is consistent with the results of the present work. The linearity of the method, however, is little improved against the aPTT and inferior to the ECT.
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