Determination of Antithrombin-Dependent Factor Xa Inhibitors by Prothrombin-Induced Clotting Time

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ABSTRACT

Prothrombinase-induced clotting time (PiCT) determines the anticoagulant effects of heparins, low molecular weight heparins (LMWHs), and direct thrombin inhibitors. At present, this is the only method that measures the effects of all of these inhibitors, in contrast to the prothrombin time, activated partial thromboplastin time (aPTT), Heptest, ecarin clotting time, and the chromogenic assays. The antithrombin-dependent direct factor (F) Xa inhibitors fondaparinux and idraparinux were compared with the LMWH dalteparin on PiCT, aPTT, Heptest, and chromogenic anti-FXa assays in pooled human normal plasma samples. Fondaparinux and idraparinux prolonged the coagulation times in the PiCT, Heptest, and chromogenic FXa assays in a dose-dependent manner, in contrast to the aPTT. We conclude that PiCT is a suitable assay to determine the anticoagulant effects of these two new FXa inhibitors in patients receiving treatment with these compounds.

KEYWORDS: Prothrombinase-induced clotting time, fondaparinux, idraparinux, hirudin, argatroban, and melagatran

In clinical use, heparins and direct thrombin inhibitors are monitored mostly by activated partial thromboplastin time assays (aPTT). Limitations of aPTT methods include nonlinear dose–effect relationships with a plateau effect; variability between different testing instruments, reagents, and different lots of the same reagent; and the low sensitivity for low molecular weight heparins (LMWHs). These limitations promoted the development of new anticoagulant assays with more specific determination of the factor (F) Xa and thrombin inhibition. However, at present, no test is available for the determination of the anticoagulant effects of all inhibitors. Such an assay would facilitate and simplify the routine clinical work as well as an overlapping therapy between these agents.

Monitoring of direct thrombin inhibitors with aPTT suffers from insensitivity at high concentrations of the inhibitors. This results in a poor linearity and reproducibility of the assay and limits the clinical utility. Recently, direct prothrombin activation methods have been developed for coagulation analysis: ecarin clotting time (ECT) and prothrombinase-induced clotting time (PiCT). The reagents of PiCT consist of Russell viper venom-V (RVV-V), FXa, phospholipids, and Ca²⁺. RVV-V specifically activates FVa in the patient’s plasma. FVa is an essential component of the prothrombinase complex, in contrast to FXa. It is the major target of antithrombin (AT) and hence a suitable substrate for antithrombin-dependent assays. PiCT is more sensitive to AT-dependent FVa inhibition than aPTT. Both assays are rapid and simple to perform and do not require expensive reagents or equipment. The PiCT may be a useful addition to the monitoring of direct thrombin inhibitors.
cofactor in the prothrombinase complex and speeds up the activation of FX. FVa, FXa, and phospholipids of the reagent are built and compelled in the first incubation step of the assay. After addition of Ca$^{2+}$, prothrombin is converted to thrombin, cleaving fibrinogen to fibrin, which is determined by mechanical or optical detection. Laboratory monitoring of the direct thrombin inhibitors lepirudin, argatroban, and melagatran was analyzed and compared with monitoring of unfractionated heparin (UFH). Plasma samples of six healthy volunteers 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, ECT, PiCT, and prothrombin time (PT) were determined in a KC 10a micro instrument (Amelung Co., Lemgo, Germany).

In patients with venous thromboembolism, anticoagulation with heparin, LMWH, and recombinant hirudin (r-hirudin) is well established and is the standard initial treatment. UFH is administered intravenously or subcutaneously. It requires dose adjustment, usually based on the aPTT. 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-FXa to anti-FIIa 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 developed systematically by analog design. A few of them have made it into clinical practice or are in late phases of clinical trials. Examples are argatroban and melagatran. Since the introduction of the first direct thrombin inhibitor (DTI), hirudin, monitoring of direct inhibitory effects have been addressed. 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 lower stage involving influences of FXII, FXIII, FV, and FII—the entire intrinsic system. A plateau effect makes monitoring more difficult, especially in higher concentration ranges. Development of a novel ECT method provided a linear test for hirudin measurement. The method is based on specific activation of prothrombin to meizothrombin alone, reducing interference with other factors, as compared with the aPTT.

The recently introduced PiCT uses the entire prothrombinase complex for clot activation. The system is made up of FXa, phospholipids, calcium, and FVa, preactivated by the activator RVV-V from RVV included in reagent 1 of the test. Clot activation by the whole prothrombinase complex containing preactivated FVa is ~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, feedback mechanisms of thrombin might disturb this assay to a lower degree, if at all. In this study, concentration-effect relationships of the LMWH dalteparin and of the modified synthetic pentasaccharides fondaparinux and idraparinux on PiCT, aPTT, Heptest (Amelung Co., Lemgo, Germany), and the chromogenic S2222 anti-FXa assays 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 vol/vol). The centrifuged plasma samples (1800 × g for 10 minutes) were shock frozen in liquid nitrogen, stored at −80°C, and analyzed within 4 weeks. After plasma samples were thawed, they were spiked with dalteparin (lepirudin; molecular mass ~6500 d, obtained from Pfizer, Karlsruhe, Germany), fondaparinux, and idraparinux (both molecular mass ~1250 d; kindly provided by MSD, Munich, and Sanofi-Aventis, Berlin, Germany, respectively) at concentrations ranging from 0.0016 to 1.0 IU/mL (dalteparin) and 0.01 to 1.0 μg/mL (fondaparinux and idraparinux). Clotting time measurements were performed on a KC 10a micro device (Amelung Co., Lemgo, Germany). Fifty microliters of plasma was incubated at 37°C for 3 minutes (micromethod). To start the clotting time assay, 50 μL of the reagent (dissolved according to the manufacturer’s instructions) was added. PiCT was performed as a two-step test with PiCT reagent from Pentapharm (Basel, Switzerland) as a two-step test (incubation of 50 μL plasma + 50 μL reagent for 3 minutes, start of clot formation by recalcification with 50 μL CaCl$_2$ [25 mM]). The normal range for the PiCT was 29.9 ± 4.6 seconds. Heptest coagulation assay was done in the same manner as the PiCT but with an incubation time of 2 instead of 3 minutes (reagents from Laborservice Kappes, Augsburg, Germany; originally from St. Louis, MO). Normal values were < 22 seconds. The aPTT testing was performed with Pathromtin SL from Dade Behring (Munich, Germany) in the same manner as the PiCT but with 2 minutes of incubation. Normal values were 33.5 ± 4.0 seconds. The S2222 chromogenic assay was determined with reagents form Haemochrom Diagnostica using purified human FXa (Essen, Germany). Normal values were below 0.016 IU/mL for dalteparin or 0.016 mg/mL for fondaparinux and idraparinux. All data are provided as mean value ± standard deviation.
RESULTS

Prothrombin-Induced Clotting Time
For the PiCT test series, a concentration as low as 0.016 IU/mL (dalteparin) or 0.016 μg/mL (fondaparinux and idraparinux) was determined. Up to 0.12 IU/mL (or the equivalent value in micrograms per milliliter Q14), all three anticoagulants prolonged the PiCT to a similar extent. At higher concentrations, dalteparin exhibited a stronger anticoagulant effect measured with the PiCT test compared with fondaparinux and idraparinux. At 1 IU/mL, dalteparin PiCT was prolonged ~2.5-fold. At 1 μg/mL, fondaparinux and idraparinux prolonged the PiCT assay ~2-fold without differences between the two compounds (Fig. 1).

Heptest Coagulation Assay
The Heptest delivered an entirely linear concentration-effect relationship for all three anticoagulants. A concentration of 1 IU/mL prolonged clotting times from ~20 to 140 seconds (i.e., 7-fold). The lowest concentration of 0.016 IU/mL prolonged the clotting time to ~26 seconds with all three anticoagulants (Fig. 2).

Activated Partial Thromboplastin Time
The aPTT developed a flat concentration-effect curve with concentrations of 0.016 and 0.25 IU/mL using all three anticoagulants Q15. The addition of higher concentrations of dalteparin prolonged coagulation times to 200 seconds. Increasing concentrations up to 1.0 μg/mL of fondaparinux and idraparinux did not result in a prolongation of the aPTT (Fig. 3).

FXa Inhibition
Using the S2222 chromogenic FXa inhibition assay, 0.016 IU/mL inhibited FXa generation in the assay. There were only minor differences in the concentration-dependent inhibition curve up to 1.0 IU/mL between dalteparin and fondaparinux. Idraparinux exhibited an ~2-fold stronger inhibition of FXa in this assay compared with dalteparin and fondaparinux (Fig. 4). Data for the concentrations between 0.16 and 1.0 IU/mL are not shown.

DISCUSSION
In the present study, different methods for laboratory monitoring of anticoagulant effects of antithrombin-dependent FXa Q16 dalteparin, fondaparinux, and...
idraparinux were compared. aPTT and (since 2004) PiCT are available as a coagulation assay to determine FXa as well as thrombin inhibition. However, aPTT is rather insensitive toward LMWH preparations. Therefore, specific chromogenic assays as well as some coagulation assays have been developed to overcome this disadvantage of aPTT reagents. Heptest determines quite specifically the FXa inhibition and to some extent antithrombin-mediated thrombin inhibition. ECT determines the direct meizothrombin and thrombin inhibition by the specific inhibitors. The disadvantage for clinical routine and as a screening assay in research is that all methods have to be used to determine or screen the anticoagulant effect.

The PiCT has been shown to determine sensitively and specifically both antithrombin-dependent and antithrombin-independent FXa and thrombin inhibition. In this study, we showed that also the antithrombin-dependent inhibitors fondaparinux and idraparinux prolong the PiCT in a dose-dependent manner. There were no differences in the lower concentration range compared with the LMW dalteparin. At higher concentrations, fondaparinux and idraparinux prolonged the PiCT in a dose-dependent manner, but to a smaller extent compared with dalteparin.

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 ECT also provides good linearity 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.

In contrast to the ECT, prothrombin in the PiCT is not cleaved directly, but the entire prothrombinase complex consisting of FXa, phospholipids, calcium ions, and FVa is activated. An advantage of this principle is that the PiCT method is also sensitive to heparin, LMWH, fondaparinux, and idraparinux, which do not interact with meizothrombin in the ECT method. With UFH, the PiCT delivered a curve similar to that of the aPTT. LMWH prolongs the PiCT in contrast to aPTT, probably as a result of activated FXa in the PiCT reagent. Another explanation is that the RVV in the PiCT assay activated free FXa via activation of FV by this reagent in the assay. Recently, a one-step PiCT assay was described to determine the anticoagulant effect of oral direct FXa inhibitors. The mechanism of activation may be similar to the one-stage coagulation assay PT, which is also prolonged by this group of agents.

Clinical Implications

In all clinical centers, a global coagulation assay will be needed to determine the anticoagulant effect of the conventional as well as of the new coagulation inhibitors. At present, FXa and thrombin inhibitors are in clinical development. Some of the inhibitors were developed as subcutaneous or oral compounds. The effect of systemic and oral DTIs prolongs the PiCT in a dose-dependent manner. The subcutaneous, parenterally administered, indirect antithrombin-dependent FXa inhibitors fondaparinux and idraparinux have been shown in this study to prolong the PiCT in a dose-dependent manner.

The anticoagulant effect of the oral direct FXa inhibitor rivaroxaban can be determined by a modified one-stage PiCT.

The specific FXa or thrombin inhibitor assays, at present Heptest and chromogenic assays for the FXa inhibitors and the ECT-based assays for DTIs, may be more accurate and therefore used for specific clinical situations and for research purposes.

The aPTT remains the method of choice due to its clinical validation for the therapeutic ranges of 1.5- to 2-fold of the local normal value for patients treated with UFH, r-hirudin, and argatroban.

The PiCT method seems to have some promising advantages regarding the precision of the method. Additional clinical data are required to investigate the applicability and validity of this method in patients receiving treatment with FXa or thrombin inhibitors.

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