Prothrombinase-Induced Clotting Time Assay for Determination of the Anticoagulant Effects of Unfractionated and Low-Molecular-Weight Heparins, Fondaparinux, and Thrombin Inhibitors

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Abstract

The prothrombinase-induced clotting time assay (PiCT, Pentapharm, Basel, Switzerland) is a clotting assay sensitive to factor Xa and factor IIa inhibitors. It is based on the addition of factor Xa and snake venom RVV-V (Russell viper venom factor V activator) specifically activating factor V and phospholipids to platelet-poor plasma. Following an incubation time, the mixture is recalcified and the clotting time is determined. An almost linear dose-response and high sensitivity of the assay for unfractionated heparin (UFH), low-molecular-weight heparins (LMWHs), r-hirudin, and argatroban was found. Fondaparinux showed a nonlinear dose-response. By using ex vivo samples, the following Pearson correlation coefficients were found: $r = 0.85$ between amidolytic anti-Xa and PiCT for 120 LMWH and 24 control samples; $r = 0.86$ between amidolytic anti-Xa activity and PiCT for 68 UFH and 24 control samples; and $r = 0.94$ between ECT and PiCT for 38 hirudin samples. Thus, PiCT is a promising assay for the monitoring of anticoagulants inhibiting factor Xa and/or factor IIa.

Immediately acting anticoagulants such as heparins and direct thrombin inhibitors (DTIs) are among the most frequently applied drugs in any field of medicine.1-3 The common mode of action of these drugs is their inhibition of activated factor X (FX) and/or factor II. Elimination pathways are metabolic, renal, and/or hepatic.3,4 Various patient variables (eg, age and obesity)5,6 and comorbidities (eg, renal dysfunction)7-8 can affect the pharmacodynamics and/or pharmacokinetics of anticoagulants. Bleeding complications due to overdose or accumulation of anticoagulants have been described.9,10 The issue of monitoring, for individualization of therapy or for safety, has been discussed for decades.11-17

Low-molecular-weight heparins (LMWHs) and fondaparinux exert their main anticoagulant activity by promoting the inhibition of FXa by antithrombin. Conventional clotting assays such as the activated partial thromboplastin time (aPTT), prothrombin time, and thrombin clotting time are not sufficiently sensitive for monitoring of LMWH or fondaparinux concentrations in clinically applied concentrations. Therefore, 2 groups of specialized assays for the detection of these drugs have been introduced: (1) In the amidolytic assays,18 FXa is added to a diluted sample and specific peptide substrates are applied to detect the residual (noninhibited) portion of the added FXa. (2) In the chronometric assays (ie, clotting assays), added FXa is used to trigger the clotting of the sample, together with phospholipids and calcium chloride.19 In the Heptest assay (Haemachem, St Louis, MO),20 a bovine plasma fraction is also added, which is reported to be rich in factor V (FV) and fibrinogen.

In recent discussions in the literature,21-25 several authors expressed their opinion as to whether the anti-Xa activity of LMWH should be monitored. All authors agreed that monitoring...
of LMWH should be available for special patients or clinical situations such as patients with renal insufficiency, severely obese patients, children, and underweight persons. However, most authors criticized the limited predictivity of the results of the available chromogenic or chronometric tests with respect to the efficacy (ie, antithrombotic potential) and safety (ie, bleeding risk) of the tested substances.

Unfractionated heparin (UFH) requires monitoring and dose adaptation owing to its highly variable pharmacokinetics and pharmacodynamics.\(^{1,11}\) It is typically monitored using the aPTT or activated clotting time. Both assays have been repeatedly reported to depend on the reagents and the instruments used.\(^{12,26}\) There may also be a poor relationship between the results of clotting time and actual UFH concentrations in the sample.\(^{27}\) Therefore, specific target ranges of aPTT, eg, 1.5- to 2.5-fold prolongation for treatment of venous thromboembolism, may be associated with varying intensities of anticoagulation depending on the reagent used by the laboratory.\(^{13}\)

DTIs are usually monitored by the aPTT or activated clotting time test.\(^{28}\) As described for UFH, the results depend on the reagents and the instruments used. Moreover, with rising DTI levels, the aPTT is not adequately increased. In the search for more specific methods for the assessments of DTI, amidolytic assays (anti-IIa activity testing)\(^{29}\) and the ecarin clotting time (ECT)\(^{30}\) have been developed. However, their routine use in clinical situations has remained limited.

The aim of our study was to characterize the prothrombinase-induced clotting time assay (PiCT, Pentapharm, Basel, Switzerland) as a new monitoring method that is sensitive to the assessment of UFH, LMWHs, fondaparinux, and DTIs. Experimental evaluations elucidating the proof of principle and sensitivity of the method and comparisons with existing methods are shown.

Previously, PiCT has been briefly described in literature\(^{31}\) and was used in various examinations for the monitoring of heparins and DTI and FXa inhibitors.\(^{32-34}\) However, a detailed evaluation of its main components and an overview of its applications has not been published.

### Materials and Methods

Venous blood was collected into commercially available vials prefilled with 3.2% citrate in a volume ratio of 1:9 (citrate/blood) from healthy volunteers and anticoagulant-treated patients following ethical committee approval. In ex vivo plasma samples of patients, neither the anticoagulant dose nor the interval between drug application and blood collection were standardized but reflected normal clinical practice. There were no additional selection criteria for anticoagulant-treated patients. Plasma was obtained by centrifugation at 2,500g for 15 minutes and frozen at –70°C until analyzed.

### PiCT Assay

For this assay, 50 µL of platelet-poor plasma are added to 50 µL of a reagent containing a combination of FXa, the snake venom RVV-V\(^{35}\) (Russell viper venom factor V activator), and phospholipids. Types and concentrations of phospholipids are proprietary information of the manufacturer. During a specified incubation time (180 seconds), the RVV-V activates FV to FVa. In contrast with the RVV fraction used for the RVV test, the RVV-V used in the PiCT assay is devoid of FXa-activating properties, which makes the test independent of the FXa activity in the analyzed sample. The added FXa is inhibited by antithrombin and antithrombin-heparin complexes. Thereafter, 50 µL of calcium chloride (25 mmol/L) is added to reverse the calcium chelation of the citrated sample. Mediated by the free Ca\(^{++}\) ions, the prothrombinase complex is formed on the phospholipid surfaces and thrombin is generated. The generation of thrombin is faster when high amounts of active FXa are left in the solution, whereas the thrombin generation is slow when most FXa is bound by antithrombin. The formed thrombin is also bound by antithrombin, antithrombin-heparin, or other endogenous or exogenous thrombin inhibitors. As soon as free thrombin is formed, the sample clots and the clotting time is detected.

Unless specified differently, the PiCT was assessed by using the commercial PiCT reagent (Pefakit PiCT, Pentapharm) using the adaptations provided by the manufacturer. The costs of the PiCT assay are similar to or lower than the costs of standard anti-Xa assays.

The clotting times of PiCT can be converted to ratios by dividing the clotting time of the individual sample by the clotting time of control plasma prepared from a pool of plasma samples obtained from healthy people.

### Other Assays

In addition, the amidolytic anti-Xa methods Coamatic Heparin (IL, Milan, Italy; method 1) and Rotachrom Heparin (Stago, Asnières, France; method 2) and the chronometric test Heptest were performed according to the manufacturers’ instructions. The Coamatic test was performed on the Behring Coagulation System (Dade-Behring, Marburg, Germany), the Rotachrom assay on the STA compact (Stago), and the Heptest on the AMAX (Trinity Biotech, County Wicklow, Ireland). The ECT was performed on the Behring Coagulation System by adding 50 µL of plasma to 100 µL of ecarin solution (4 U/mL, Pentapharm) and recording the clotting time. The aPTT was performed by means of Parthromtin SL, Dade-Behring.

### In Vitro Experiments and Ex Vivo Studies

With the samples collected as described, in vitro experiments and ex vivo studies were performed in single determinations.
In Vitro Experiments

Since the study was done, PiCT assay kits that contain the activator reagent and all other components in fixed concentrations have become commercially available. To illustrate the function of each component, the concentrations of the components were varied, one at a time. A plasma pool was prepared from 3 individual samples from healthy volunteers and analyzed without addition of anticoagulant, as well as with the addition of 0.5 or 1 U of LMWH/mL. The concentration range tested was 0 to 1 µg/mL of FXa, 0 to 20 U/mL of RVV-V, and 0 to 200 µg/mL of phospholipid. FXa was obtained from Enzyme Research (South Bend, IN), RVV-V and phospholipids were obtained from Pentapharm.

For the assessment of the sensitivity of the PiCT toward different anticoagulants, serial dilutions of various anticoagulants (UFH, Roche, Basel, Switzerland; dalteparin, Pfizer, Karlsruhe, Germany; enoxaparin, Sanofi-Aventis, Bad Soden, Germany; fondaparinux, GlaxoSmithKline, Munich, Germany; r-hirudin, Pharmion, Hamburg, Germany; and argatroban, GlaxoSmithKline, Philadelphia, PA) were prepared in 5 individual platelet-poor plasma samples and the PiCT was determined.

Ex Vivo Studies

By using 120 samples from LMWH-treated patients, 68 samples from UFH-treated patients, and 24 samples from healthy untreated subjects, the following assays were done in parallel: PiCT ratio, Heptest (LMWH samples only), aPTT (UFH samples only), and amidolytic anti-Xa activity tests (Coamatic Heparin, Rotachrom Heparin).

To compare the sensitivity of the PiCT, ECT, and Heptest toward the direct inhibitor r-hirudin, 38 ex vivo samples from patients receiving this drug were analyzed.

To study the effects of different clotting detection techniques, 41 samples from UFH-treated patients and 93 samples from LMWH-treated patients were analyzed in parallel on the AMAX analyzer using optical and mechanical detection of clotting.

Statistical Analysis

Data analysis was performed with Statview 5.0 (SAS Institute, Cary, NC) and Microsoft Excel, Microsoft, Redmond, WA. Data are given as the mean ± SD for individual determinations. The Pearson correlation coefficient r was determined by using Microsoft Excel.

Results

In Vitro Experiments

In the first experiment, the concentrations of the key elements of the PiCT activator reagent were varied. (Figure 1) shows the clotting times of 3 plasma samples (0, 0.5, and 1 anti-Xa U/mL) following activation with the PiCT reagent in which one of the key components was varied at a time (FXa/ RVV-V or phospholipids). The results were expressed as clotting times (in seconds) and ratios (ratio of individual clotting time to the clotting time of a nonanticoagulated sample). As expected, rising amounts of FXa in the activator reagent led to shorter clotting times for all 3 samples and had the strongest influence on the clotting times. However, the ratio remained almost unchanged in the range of 0.1 to 1 anti-Xa U/mL. The concentration of RVV-V (the FV activating enzyme) did not alter clotting times in the range of 1 to 20 U/mL, revealing a complete activation of FV in the sample already using 1 U/mL of RVV. At concentrations higher than 50 µg/mL the phospholipid concentration had only a minor effect on the clotting times. Lower concentrations of phospholipids...
resulted in a concentration-dependent prolongation of PiCT clotting times.

Analysis of the PiCT in samples from 24 healthy volunteers not undergoing anticoagulation produced a PiCT ratio of $1.03 \pm 0.12$.

Figure 2 shows the dose-response ratios for PiCT toward different anticoagulants. At LMWH or UFH concentrations of 0.2 U/mL, a 2.0-fold and, at concentrations of 0.4 U/mL, a 3.0-fold prolongation of PiCT was found. At concentrations of 0.5 µg/mL hirudin and argatroban, a 2-fold prolongation of PiCT was determined, whereas concentrations higher than 1.0 µg/mL led to PiCT ratios higher than 3. Analysis of samples spiked with fondaparinux revealed a nonlinear dose response. A ratio of 2 was found at concentrations of 0.2 µg/mL of fondaparinux, whereas more than 1 µg/mL was required for a ratio of 3.

Ex Vivo Studies

In the tested ex vivo LMWH (n = 120) and control (n = 24) samples, the correlation of PiCT toward an amidolytic anti-Xa activity test revealed an almost linear correlation with a correlation coefficient of 0.85. The coefficient of correlation of Heptest values to amidolytic anti-Xa activity was 0.88, and the correlation of the 2 tested anti-Xa methods was 0.94 (Figure 3).

In the ex vivo UFH (n = 68) and control (n = 24) samples, the correlation coefficient of PiCT ratio vs anti-Xa activity was 0.86, anti-Xa activity vs aPTT was 0.65, and anti-Xa activity method 1 vs method 2 was 0.90 (Figure 4).

In the next experiment, ex vivo samples from patients receiving r-hirudin were analyzed by using the PiCT, Heptest, and ECT. The ECT and PiCT ratios correlated well ($r = 0.94$), whereas only a weak prolongation of the Heptest was found (Figure 5).

The comparison of PiCT determined by using optical and mechanical detection revealed an excellent correlation with a correlation coefficient of 0.988 (data not shown).

Discussion

The PiCT is an anticoagulant assay sensitive to anti-Xa and anti-IIa agents. It is based on a combination of phospholipids, calcium chloride, and 2 purified proteins: FXa and a specific FV activator from snake venom (RVV-V). During the incubation phase, the RVV-V activates the FV in the sample, which allows for the immediate formation of the prothrombinase complex after recalcification, without the need for FV activation by endogenous thrombin-mediated feedback reactions. The prothrombinase complex converts prothrombin to thrombin. As soon as free thrombin is formed, the clotting of the sample is detected. The pipetting procedure is simple and resembles the procedure of the aPTT. The optical signal is sharp, and an excellent correlation of optical and mechanical detection of clotting was found.

In this study, the results of PiCT were converted to ratios by dividing the clotting time of the sample by the clotting time of a plasma pool of plasma from healthy donors. It is also possible to express PiCT results in anti-Xa units, after calibration of PiCT against the LMWH WHO standard. However, this leads to the problem of how to express PiCT values obtained in samples containing anticoagulants other than LMWH (such as DTI) and how to handle overlapping anticoagulation with different drugs, which both may affect PiCT clotting times. Whether PiCT could be useful for monitoring overlapping anticoagulation is still under investigation.

Evaluation of the Sensitivity of PiCT Toward Various Anticoagulants

Analysis of spiked samples and ex vivo samples revealed a high sensitivity of PiCT toward LMWH, UFH, and DTIs. This offers the opportunity to use 1 coagulation assay for the monitoring of various anticoagulant drugs that are used clinically. Most anticoagulant drugs, approved and in development, are directed against FXa and/or thrombin. Because PiCT is sensitive to anti-Xa and anti-IIa, this assay might simplify the monitoring of established and new anticoagulant strategies in routine clinical use. Regarding specificity, however, a clotting assay is less specific when compared with the amidolytic assays for assessment of anti-Xa or anti-IIa activity. On the one hand, the lower sample dilution and the less artificial test environment may also lead to more clinically relevant results. On the other hand, minimal dilution of the patient sample introduces more influence of plasma factors like concentrations of antithrombin, fibrinogen, and lupus anticoagulants. This has to be shown by further comparative studies. The variations found between the assays correspond
well to findings reported in literature. Kitchen et al and Depasse et al reported considerable variation in different anticoagulant assays. According to our results, a relatively high variation between anti-Xa levels and aPTT can be found (Figure 4). This is in accordance with reports in literature concerning the usefulness of the aPTT as a monitoring method for UFH. Based on the better correlation of PiCT and anti-Xa levels (Figure 4), it can be expected that PiCT might permit more accurate monitoring of UFH than the aPTT.

A difference in the relationship between anti-Xa activity and the PiCT ratio between the in vitro dose response (Figure 2) and ex vivo clinical samples from patients receiving subcutaneous LMWH (Figure 3) was observed. LMWH added in vitro in a dose of 0.4 U/mL caused a 3-fold prolongation of the PiCT ratio. In contrast with this 3-fold PiCT prolongation in the in vitro samples, the amidolytic anti-Xa assay (method 1) revealed a PiCT ratio of 2 to 2.5 in ex vivo samples at 0.4 U/mL of anti-Xa activity. In the UFH samples, a comparable relationship between anti-Xa activity and the PiCT ratio was found in the spiked and ex vivo samples. This difference has to be taken into account when comparing PiCT results after in vitro spiking of plasma with ex vivo analysis of patient samples. This difference might be based on heparins (LMWH or UFH) present in the sample that have been inactivated by endogenous heparin antagonists such as platelet factor 4 (PF4). Amidolytic heparin assays have been reported to contain substances that liberate inactivated heparins in the sample from the protein binding (dextran sulfate).
PiCT reagent does not contain such substances, ie, only what is not bound to PF4 can inhibit the added FXa in the assay. Theoretically, differences between dextran sulfate–containing assays (eg, amidolytic heparin assays) and test principles that do not contain this compound (eg, PiCT) should be more pronounced for UFH than for LMWH because PF4 is known to bind more readily to UFH than to LMWH. Why in our data the observed difference was more obvious for LMWH than for UFH is unclear. Another difference of in vitro and ex vivo patient samples is the different composition of anti-Xa vs anti-IIa activity. The latter disappears much faster in the circulation, and samples are left containing mostly anti-Xa activity. These issues will be studied in further detail in future studies.

Fondaparinux did not reveal a linear dose response (Figure 2), suggesting that PiCT can be used for the detection of fondaparinux doses of 0 to 0.75 µg/mL, whereas the quantification of higher doses is not possible with the current setup.

**Evaluation of Assay Components of PiCT**

The analysis of the differential effect of the PiCT components shows the function of the 3 main constituents (Figure 1): FXa activates the clotting process independent of earlier stages of the coagulation cascade and also independent of the FX concentration in the sample. The FXa concentration applied had a strong influence on the clotting time of all 3 samples tested. RVV-V is used to preactivate
the latter, however, it may be advantageous to perform PiCT as sulfaminoheparosans also shown that PiCT may be used for newer compounds such as heparins well with published data for the sensitivity of PiCT toward neutralizes DTI (mechanism still under investigation).

It can be suggested that some component in the Heptest reagent was responsible for the variations that we found between the assays, especially in the UFH-treated samples. Further clinical trials on anticoagulant-treated patients with precisely defined anticoagulant dose, application, and blood sample collection time points are required to establish the peak and trough levels for PiCT using different drugs and anticoagulation intensities. The clinical predictivity of the assay with respect to safety and efficacy of anticoagulant treatments needs to be established.

Conclusion

PiCT is a new coagulation assay for monitoring various anticoagulants. It is sensitive to anti-Xa and anti-IIa agents and has a procedure similar to that of the aPTT.

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Conflict of interest: Dr Wilmer and Ms Rudin are employees of the manufacturer of the presented assay. Dr Calatzis is a coinventor of the presented method and a consultant to the manufacturer of the assay. Drs Peetz and Spannagl have received honoraria for participation in an advisory board meeting of Pentapharm.
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References


