Critical role of factors II and X during coumarin anticoagulation and their combined measurement with a new Fiix-prothrombin time

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Vitamin K antagonists (VKA) are monitored with prothrombin time (PT) based assays that are equally sensitive to reductions in factors II, VII or X. We compared the effect of vitamin K dependent (VKD) coagulation factors on PT and also on rotational thromboelastometric (ROTEM) parameters. The PT was equally sensitive to reductions in factors II, VII or X but ROTEM parameters correlated poorly with the PT in anticoagulated patients’ plasmas. ROTEM parameters were more affected by mild and moderate reductions in FII or FX than by FVII or FIX which had little influence except at very low coagulant activity. We developed a modified PT that was sensitive only to reductions in factors II and X. The Fiix-PT (Fiix-INR) correlated well with PT (INR) but the Fiix-INR fluctuated less than the INR in an anticoagulated patient reflecting its insensitivity to FVII. The ROTEM results suggest that mild to moderate reductions in factors II or X are more important during clot formation than factors VII or IX. Reductions in FII and X may better reflect anticoagulation with VKA than FVII or DX. The new Fiix-PT may more accurately reflect the degree of therapeutic anticoagulation in patients treated with VKA than the current PT which is subject to a confounding variation caused by FVII.

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Introduction

During initiation of treatment with VKA, the coagulant activity (activity) of each VKD coagulation factor declines at different rates reflecting their half-lives which vary from 3.5 hours for FVII to 72 hours for FII [1,2]. The degree of anticoagulation is monitored with prothrombin time (PT)-based tests such as the Quick PT [3] or the Owren PT (prothrombin-proconvertin time, P&P-test) [4], the latter test being mainly used for monitoring anticoagulation in the Nordic countries, Holland and Japan. The international normalized ratio (INR) based on either PT variant will lead to practically identical results [5]. Both PT assays use undiluted thromboplastin and recalification to activate coagulation in citrated platelet poor plasma (PPP). The Owren PT differs in that it is done on diluted test plasma which makes it less sensitive to heparin and lupus anticoagulant than the PT [6]. Since absorbed bovine plasma is added, it corrects for all factor deficiencies other than those of vitamin K dependent factors making the test insensitive to environmental temperature which can affect FV. Both PT variants are equally sensitive to reduced activity of factors (F) II, VII and X but are not sensitive to reductions in activity of FIX. Additionally, the Quick PT is sensitive to reduced concentrations of factors V and fibrinogen that are not affected by VKA but may be a source of variation for other reasons.

The PT is presumed to accurately reflect the antithrombotic effect of VKA. However, prior studies have indicated that the antithrombotic effect of FVII may be of minor importance compared to the activity of FX and, in particular prothrombin [7,8]. Also, factor VII does not start to prevent thrombin generation until levels are well below 5% [7,9]. Since FVII has the shortest half-life, fluctuations in the PT may simply reflect FVII activity changes rather than a true change in antithrombotic effect. This may have therapeutic implications, as studies have suggested that measuring prothrombin alone as native prothrombin antigen by ELISA more accurately reflects the antithrombotic effect of VKA than does the PT [8,10–12].

We evaluated the roles of each VKD coagulation factor on clotting in vitro using PT assays and also rotational thromboelastography (ROTEM), a method that perhaps more accurately reflects the complex in vitro clotting process by measuring as well the rate of the following bulk clot formation and the final clot strength [13]. In the ROTEM experiments coagulation was activated with highly dilute thromboplastin as described previously [13]. The findings suggested that FII and FX play a dominant role in clotting, and we, therefore, developed a new PT variant, Fiix-PT, which measures the combined effects of only FII and FX on fibrin formation.

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Materials and methods

Plasma

Blood was drawn from patients on warfarin anticoagulation and from twenty healthy donors (10 men, 10 women). Using a minimal stasis technique, the blood was drawn through a 21 G needle into 1/10 vol/vol 3.2% (0.109 mol/L) buffered sodium citrate Vacutte® tubes (Greiner, Kremsmunster, Austria). Platelet poor plasma (PPP) was prepared by centrifugation of the blood at 4 °C for 15 minutes at 2,500 g, removal of supernatant and recentrifugation for 15 minutes at 2,500 g. The pooled normal plasma (PNP) was prepared by pooling the recentrifuged normal plasma and stored in 0.5 mL aliquots at −80 °C. Immunodepleted plasmas were selectively depleted of coagulation factors II, VII, IX or X of both FII and X (Haematologic Technologies Inc. (Essex Junction, Vermont, USA), and there was no residual inhibitory activity in the plasmas. Adsorbed plasma was made by incubating 9 volumes of PNP with 1 volume aluminium hydroxide gel (Al(OH)₃ 1 g in 4 ml H₂O) (Sigma Alderich Chemie GmbH product number A8222, Steinheim, Germany).

Frozen-thawed platelets and washed platelets were made from freshly donated blood bank platelet concentrate in acid-citrate-dextrose (ACD). For both, the platelet rich plasma (PRP) was centrifuged for 15 minutes at 150 g and the platelet-rich pellet was then resuspended twice. The supernatant was removed and the pellet was resuspended twice. The final suspension was counted automatically using Sysmex 4000 (Sysmex Corp., Kobe, Japan), and the final solution was adjusted to a platelet count of 1000x10⁹/L. To make platelet phospholipids the final solution was frozen at −80 °C, thawed again and then aliquoted and frozen again. Alternatively, the washed platelets were stored at room temperature and used for experiments within two days.

Reagents

Factor deficient plasmas used for assaying coagulation factors were obtained from Diagnostica Stago (Asnieres, France). Hepes buffer (Hepes 20 mM, NaCl, 150 mM, with and without CaCl₂, 200 mM, pH 7.4) was obtained from Bie & Berntsen Inc. (Herlev, Denmark). Recombinant thromboplastin (Innovin®) was obtained from Dade Behring (Marburg, Germany). The Innovin was reconstituted and stored at −720° centigrades in Eppendorf tubes. One tube of Innovin was reconstituted for each experiment and 10 μl Innovin were mixed into 990 μl of Hepes CaCl₂ to make a 1:100 dilution which was further diluted by mixing 100 μl of the 1:100 dilution with 500 μl of Hepes CaCl₂ to make a 1:600 working solution.

ROTEM experiments

Dynamic blood clot formation profiles were recorded by a ROTEM® Thromboelastometry Coagulation Analyzer (Pentapharm, Munich, Germany) as described in detail elsewhere [13–15]. However, instead of whole blood we used citrated PPP. In brief, the reaction mixture contained 280 μl of citrated plasma + 20 μl of buffer + 20 μl dilute thromboplastin and CaCl₂. All results shown are based on quadruplicate experiments. The ROTEM measurements were based on activation of coagulation with highly diluted thromboplastin (final reaction mixture dilution of TF 1:17,000) [13]. Assessment of plasma clot formation was based on standard thromboelastometry parameters such as the clotting time (CT), maximum velocity of clot formation (MaxVel) and the maximum clot firmness (MCF). The CT characterizes the initiation phase of clot formation, the MaxVel shows the propagation phase of clotting and the MCF reflects the stabilization phase.

ROTEM experiments were done with factor deficient plasma (deficient in FII, FVII, FIX or FX) mixed with varying proportions of PNP to make final VKD coagulation factor concentrations of 1, 2, 4, 8, 16, 32 and 50%. The experiments were done without and with added platelet phospholipid or washed blood bank platelets. The experimental cuvettes were mounted in an aluminium cup holder that was thermostatically controlled at 37 °C. A mixture of 270 μl of each plasma dilution and 30 μl of buffer, washed platelets (final count 720x10⁹/L) or platelet phospholipid solution were pipetted into the measuring cups. The measurement was started by adding 20 μl of the recombinant thromboplastin 1:600 dilution and placing the cup on a 6 mm diameter pin attached to the lower end of a vertical axis.

Quick- and Owren-prothrombin time

The Quick PT and the Owren PT used the STA-R coagulation analyzer (Diagnostica Stago, Asnieres, France). The Quick PT was performed on undiluted PPP using the STA-Néoplastine Cl plus® with an international sensitivity index (ISI) of 1.3. The Owren PT was done on diluted plasma (1:7 in buffer) using the STA-SPA 50 reagent with ISI of 1.01 (both from Diagnostica Stago, Asnieres, France). The Owren PT reagent is similar to the PT reagent but also contains absorbed bovine plasma as a source of coagulation factor V and fibrinogen and, thus, is only sensitive to the activity of coagulation factors II, VII and X. The Owren PT coagulant activity percentage was calculated based on a standard curve made from the clotting times of serially diluted pooled plasma in Owren buffer [5]. The INR was calculated as previously described [5,16–18]. The Quick PT and Owren PT result in equivalent INR's [5].

Factor II and X prothrombin time (Fiix-PT, Stuart-prothrombin time)

PPP immunodepleted of both FII and FX (Fiix-depleted plasma) was specially made by double immunodepletion of PNP (Haematologic Technologies Inc., Essex Junction, Vermont, USA). In different experiments, the Fiix-depleted plasma was mixed with test plasma in various proportions and with different test plasma pre-dilutions in Owren buffer as detailed in Results. Standard PT reagent (thromboplastin and calcium, Néoplastine Cl plus®, as above) was then added and clotting times were measured.

Statistical analysis

The GraphPad Prism 5.0 statistical software (GraphPad Inc, CA, USA) was used for graphing and for statistical calculations including non-linear regression analysis. Experimental data is shown graphically as mean ± standard error of the mean (SEM).

Results

Dependence of PT on activity of individual of VKD factors

The relationship between PT and reduced activity of factors II, VII, IX and X in PPP is shown in Fig. 1 using experiments in which PNP was mixed in varying amounts with plasma completely immunodepleted of a single VKD factor. Both PT methods were equally sensitive to the activity of factors II, VII and X throughout the activity range.

ROTEM parameters in relation to Owren’s PT in patients on warfarin

The relationship between Owren PT and ROTEM parameters were compared using samples obtained from 65 patients on VKA (Fig. 2). There was only a moderate correlation between the coagulant activity percentage and the ROTEM CT (R² = 0.27) with considerable
variation in CT at a similar coagulant activity. Also, there was poor correlation between the coagulant activity and MaxVel ($R^2 = 0.08$) and MCF ($R^2 = 0.11$). Identical findings were present with the Quick PT (data not shown). ROTEM parameters were then also measured in PPP that had been depleted of all VKD factors by absorption with Al(OH)$_3$. Absorbed plasma was gradually repleted with VKD factors by adding PNP, and the results showed that there was a good correlation between the PT coagulant activity and the ROTEM parameters ($R^2 = 0.79$ for CT, 0.64 for MaxVel and 0.77 for MCF) (curves not shown). This difference in results may indicate that VKD factors are unequally reduced in the anticoagulated patient plasmas.

**Dependence of ROTEM parameters on individual VKD factor coagulant activity**

We evaluated the influence of each VKD factor on coagulation using ROTEM with PPP selectively immunodepleted of each VKD factor which was then gradually repleted by mixing with PNP. The CT was prolonged more by mild and moderate selective reduction in factors II or X than by similar reduction in factors VII or FIX. The results were similar in PPP (panel A), PPP with added frozen-thawed platelets (panel B) and in PPP with added washed blood bank platelets (panel C). The MaxVel (Fig. 4) was also more dependent on factors II and X than on VII or IX. Finally, MCF (Fig. 5) in PPP (panel A) and plasma with frozen thawed platelets (panel B) was more dependent on factors II and X than on VII or IX, but results with washed platelets (panel C) showed a greater dependence on factor II than X. Taken together, the results showed that factors II and X influenced ROTEM results similarly and much more than factors VII and IX.

**Fix - prothrombin time**

Since factors II and X have the greatest effect on coagulation in the ROTEM experiments at VKD factor activity similar to that during therapy, we developed a PPP based test that is sensitive only to reduced activity of factors II and X and applicable to routine coagulation instruments. This was done by correcting all deficiencies other than those of factors II and X by mixing factor II and X-depleted plasma with the test sample prior to measuring the PT.

Plasma standards with decreasing activity of coagulation factors for experimental standard curves were made as follows: Normal PNP was serially diluted in vitro in Owren’s buffer with added 3.2% sodium citrate solution in a method similar to the preparation test plasmas and standard curve for Owren’s PT. Thus, serial standards were made representing coagulant activity ranging from 1.5-50 percent. Each standard was diluted further 1:7 with Owren’s buffer. In order to identify test conditions allowing the measurement of coagulant activity as low as 1.5% different experiments were done. For each experimental standard curve the serial dilutions were mixed with undiluted FII and X - depleted plasma (Fixx depleted plasma) in proportions shown in Fig. 6 in order to correct for all factor deficiencies other than factors II and X. The clotting times of the test plasma dilutions were subsequently measured by adding undiluted thromboplastin and calcium as with the PT. The results are shown in Fig. 6 which demonstrates the clotting times obtained in relation to the combined coagulant activity of FII and FX. In order to be able to measure coagulant activity as low as 3.1% the proportion of (undiluted) test plasma to Fixx depleted plasma had to be over 0.17 and for activity as low as 1.6% over 0.28.

We decided to use the Fixx-PT composition identified in the experiment shown with a solid bold line in Fig. 6 (ie proportion of test plasma to Fixx-depleted plasma 0.31) since this allows the measurement of Fixx-coagulant activity as low as 1.6%. Using this curve we subsequently tested samples from 38 patients on stable warfarin therapy (Fig. 7). The Fixx-INR correlated well with the traditional INR ($R^2 = 0.87$, $y = 0.98x - 0.01$). The intra-assay variation of the Fixx-PT was 0.8 – 1.6 percent on fresh samples with INR between 1.4 and 4.6. The same samples were frozen in aliquots and once thawed, the intra-assay variation was 0.4-0.8 and inter-assay variation was 0.4-0.6 percent.

We also compared results using the traditional INR and the Fixx-INR as well as coagulant activity of factors II, VII and X during warfarin initiation in patients. This figure shows that the fluctuation of traditional INR is faster and larger than that of the Fixx-INR. Fig. 8A and 8B show that the fluctuation of INR with the traditional INR is highly dependent on FVII activity. This is best evident during the first days when INR rises to levels over target whereas the Fixx-INR hits target with a slower response. In light of the ROTEM experiment this may indicate that the Fixx-INR better reflects the patients’ coagulability. Interestingly, as anticoagulation stabilizes the FX levels is lower (10-20%) than the FII level (20-30%).

**Discussion**

The results show that factors II and X play a more predominant role in comparison to factors VII and IX in global clot formation as observed in vitro with ROTEM. The poor correlation of ROTEM parameters with the INR in patient plasmas likely reflects a discrepancy between activity of factor VII and that of the more stable factors II and X as has been previously suggested based on thrombin
generation measurements [8,19]. The results suggest the possibility that rapid changes in factor VII activity during initiation and following dose changes of VKA may exaggerate a fluctuation in prothrombin time (INR) that actually does not influence the antithrombotic effect or risk of bleeding. This may instead mainly lead to unnecessary dose changes, too frequent monitoring tests and further fluctuation.

This confounding effect of FVII can possibly be circumvented by monitoring coumarins with the Fiix-PT which is not influenced by factor VII. A randomized double blinded single center clinical study powered to compare the efficacy (thromboembolic events) and safety (bleeding) of dosing VKA based on Fixx-PT (Fixx-INR) in comparison to the PT (INR) is now underway.

ROTEM is a global coagulation assay usually done using whole blood. Whole blood ROTEM results are a function not only of coagulation factors but also of platelet function, proteases, inhibitors and fibrinolysis [20]. In addition to measuring the time to initiation of fibrin polymerization (CT, initiation phase) it also measures the
subsequent aspects of coagulation such as the bulk clot formation or propagation phase (alpha angle or MaxVel) and the maximum clot firmness reflecting the stabilization phase [13,21]. Therefore, ROTEM may better describe in vitro clot formation than traditional clotting time assays such as PT or PTT. ROTEM is still under investigation but may have utility in predicting bleeding, e.g. in hepatic and cardiopulmonary bypass surgery or as a bedside tool to tailor prohemostatic therapy in patients with congenital and acquired coagulopathies [13,22–25].

In our ROTEM experiments, we used highly dilute thromboplastin (1:17,000) to more closely reflect the physiological concentration of tissue factor than does the high concentration used in PT assays [13,19]. We were unable to use whole blood for the ROTEM experiments since we had to work with blood deficient in one or...
more of the four VKD coagulation factors. For this purpose only frozen citrated plasma could be obtained by us. However, despite the use of plasma all three ROTEM coagulation phases were readily identified and identical to the pattern observed in whole blood. In order to partially mimic clotting in whole blood we also repeated the ROTEM experiments by enriching PPP with platelet phospholipid or with added washed blood bank platelets. Since results were similar we consider the experimental conditions to be adequate.

We are not aware of prior studies using ROTEM to evaluate the relationship of VKD coagulation factors on clot formation. Our experimental ROTEM results are based on highly diluted thromboplastin as opposed to the undiluted thromboplastin in the PT and this may explain how the ROTEM test differs from the PT. However, our results and conclusions are supported by others that have come to the similar conclusions as we have using different in vitro methods and animal models. Firstly, a study using anticoagulated plasma from patients on warfarin demonstrated that a poor relationship existed between the INR and thrombin generation in response to dilute tissue factor [19]. Also, Xi et al. showed that prothrombinase activity in anticoagulated samples from patients on VKA was dependent on both factors II and X but less on factor VII concentration which demonstrated a threshold relationship first evident at concentrations below 5% [8]. Third, Zivelin et al. found that the antithrombotic effect of VKD factor deficiency in rabbits depended on the activity of factors II and X, but not on the activity of factors VII, and these authors concluded that monitoring of VKA therapy might be more accurate using assays that principally measure factors II or X [7]. Fourth, it was shown in clinical studies, that monitoring VKA using the native prothrombin antigen by ELISA better predicted adverse events than monitoring with the PT [10–12]. Taken together, all these studies, using different methods, suggest that monitoring that focuses on the activity of factors II and X and ignoring the influence of factors VII and IX could be an improvement that might remove confounding fluctuations in PT assays caused by changes in factors VII.

VKA therapy even including monitoring cost is relatively cheap compared to the new oral anticoagulant agents such as dabigatran and rivaroxaban. The coumarin effect is well studied and can immediately be reversed using prothrombin complex concentrates and more slowly by administering plasma and vitamin K. On the other hand the dose effect varies markedly due to genetically determined metabolic variations [26]. Also, the anticoagulant effect measured by traditional INR fluctuates considerably in many patients, sometimes due to known drug and food interactions but often for unknown reasons [27]. This leads to a need for frequent monitoring in order to ensure efficacy and safety. It is hoped that new oral anticoagulant agents can be administered safely without monitoring [28–30]. This remains to be confirmed in practice, especially in the elderly which are over half of all anticoagulated patients [31]. Many of the elderly may have decreased renal function which may limit the use of renally excreted anticoagulants such as dabigatran and rivaroxaban.

We conclude that at VKD factor activity relevant to therapeutic VKA therapy, factors II and X have a predominant role compared to factors IX and VII. The results suggest further that rapid changes in factors VII activity during initiation and following dose changes of VKA may exaggerate fluctuations in the INR that do not translate into an antithrombotic affect, but lead to unnecessary dose changes and too frequent monitoring. This confounding effect of factor VII could be circumvented by monitoring VKA therapy with a prothrombin time variant such as the Fixx-PT, which is sensitive to reductions only in factors II and X and not factor VII as the Fixx-PT may more accurately reflect the anticoagulant effect of VKA than do the currently applied INR tests.

**Conflict of Interest Statement**

Brynja R. Gudmundsdottir and Pall T. Onundarson have invented the Fixx-PT and have applied for a patent on the invention.
Fig. 8. Four examples of sequential INR and Fixx-INR measurements in patients dosed based on INR. In two patients (panels A and B) VKD factor coagulant activity is also shown illustrating the marked changes in INR and not Fixx-INR related to fluctuations in FVII.

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Disclosure:

Pall T. Onundarson and Brynja R. Gudmundsdottir have filed a patent application IS 050010 for the Fixx method.

References


