INVITED REVIEW

The role of the laboratory in treatment with new oral anticoagulants

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Summary. Orally active small molecules that selectively and specifically inhibit coagulation serine proteases have been developed for clinical use. Dabigatran etexilate, rivaroxaban and apixaban are given at fixed doses and do not require monitoring. In most circumstances, these drugs have predictable bioavailability, pharmacokinetic effects, and pharmacodynamic effects. However, there will be clinical circumstances when assessment of the anticoagulant effect of these drugs will be required. The effect of these drugs on laboratory tests has been determined in vitro by spiking normal samples with a known concentration of active compound, or ex vivo by using plasma samples from volunteers and patients. Data on the sensitivity of different reagents are now available, and so guidance as to the effect and interpretation of a test result is now possible. Laboratories should be aware of the sensitivity of their own assays to each drug. This may be achieved by using appropriate calibrated plasma samples.

Keywords: anticoagulants, apixaban, dabigatran, laboratory, rivaroxaban.

Oral direct inhibitors of serine proteases have been produced by structure-guided design [1], and inhibitors of both thrombin and factor Xa have now been shown to be selective, orally active, safe, and at least as effective as low-dose low molecular weight heparin for thromboprophylaxis, and as effective as, if not superior to, warfarin for long-term prevention of thromboembolism. Dabigatran [2,3], rivaroxaban [4–6] and apixaban [7] are the first thrombin and FXa inhibitors to be licensed for the preven-

tion of thromboembolism in patients with atrial fibrillation. For some patients, these drugs offer substantial benefits over oral vitamin K antagonists (VKAs). For the majority of patients, these drugs are prescribed at fixed doses without the need for monitoring or dose adjustment. There are no food interactions and very limited drug interactions. The rapid onset of anticoagulation and short half-life make the initiation and interruption of anticoagulant therapy considerably easier than with VKAs. As with all anticoagulants produced so far, there is a correlation between intensity of anticoagulation and bleeding. Consequently, the need to consider the balance of benefit and risk in each individual patient is no less important than with VKA therapy.

Measurement of the intensity of anticoagulation caused by an oral direct inhibitor (ODI)

There are specific situations when clinical management will require knowledge of the intensity of anticoagulation. Situations in which measurement will be required include the following:

1 before surgery or an invasive procedure, such as spinal anesthesia, when a patient has taken a drug in the previous 24 h (or longer if the patient has renal impairment);
2 when a patient is bleeding;
3 when a patient has taken an overdose;
4 when a patient has deteriorating renal function.

In these situations, a test must be readily available in a short time frame, and so, for many laboratories, this will be a qualitative test, such as the prothrombin time (PT) assay or the activated partial thromboplastin time (APTT) assay. The result of a qualitative test can indicate whether anticoagulation is supratherapeutic, therapeutic, or subtherapeutic, but, as it is not quantitative, it cannot be used to determine the plasma concentration of the drug. Interpretation of the test result will be dependent on:

1 which ODI the patient has taken;
2 the dose;
3 when the drug was last taken;
4 the expected half-life;

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5 factors that influence pharmacokinetics.

Interpretation of the 'coagulation screen'

Measurement of the PT and APTT (and, in some hospitals, the fibrinogen level) is a routine procedure in the emergency room and when patients are hospitalized. As these tests are affected by ODIs, 'abnormal results' may be misinterpreted by non-expert clinicians. Laboratories need to be actively involved in the education of all clinical staff, and be available to interpret test results. For example, in a patient with sepsis, prolongation of the PT and APTT may be caused by an ODI rather than the sepsis itself. In this situation, reference to the drug history, platelet count and D-dimer level will help in the interpretation of the results. All acute-care clinicians are going to have to develop knowledge of how routine coagulation tests are affected, as many patients having a 'coagulation screen' will be taking these drugs. They are also going to have to learn whether and how the degree of anticoagulation caused by ODIs can be assessed with routine coagulation tests. The biggest educational challenge is not going to be with clinicians who prescribe and manage anticoagulant therapy, but with the many clinicians who are not routinely involved in anticoagulant care but who will nevertheless encounter an increasing number of patients incidentally taking ODIs.

Identification of 'treatment failure'

The long pharmacodynamic half-life of VKAs and the reliability of the International Normalized Ratio (INR) as a measure of anticoagulant intensity have enabled clinicians to determine whether thromboembolic events during treatment are likely to result from anticoagulant failure (an event occurring in the presence of therapeutic anticoagulation) or from subtherapeutic anticoagulation. This allows the intensity of anticoagulation to be revised, and issues with concordance and compliance to be addressed. This may not be feasible with ODIs. As a result of their short half-life, it is unlikely that measurement of the intensity of anticoagulation after thromboembolism has occurred will reliably indicate whether the event was the result of a treatment failure despite adequate anticoagulation, a treatment failure resulting from inadequate anticoagulation, or a treatment failure resulting from inadequate compliance.

Is there a case for 'monitoring' and dose adjustment of ODIs?

The exclusion criteria for clinical trials of ODIs mean that patients who would not have been eligible for inclusion in the clinical drug development programs will be treated postlicensing. Consequently, it is uncertain whether the pharmacokinetic and pharmacodynamic effect of ODIs will be the same in these patients, and therefore whether

efficacy and safety will be the same. It is likely that clinicians will consider 'monitoring' treatment with a view to dose adjustment in these patients. Examples might include establishing the dose required to achieve average therapeutic levels:

1 in patients at extremes of body weight;
2 in specific patients with severe renal impairment when an ODI might be considered to be potentially beneficial;
3 very occasionally in patients taking other drugs that are known to significantly affect pharmacokinetics.

This course of action assumes that there is an optimum therapeutic level that is associated with maximum benefit and minimum risk. Furthermore, even if this concept is correct, the average therapeutic level and dose required to achieve this in clinical trials may not be applicable to patient groups who were not included in the clinical trials. Until studies are performed to address these issues, or, at the very least, substantial observational data become available, there is no evidence base for monitoring with a view to dose adjustment in these situations. Nevertheless, it is likely that, in specific patients, some clinicians will choose to monitor or at least measure the anticoagulant effect, and/or drug level, with a view to dose adjustment.

The potential relationship between dose adjustment and patient-specific outcomes as compared with fixed-dose group-specific outcomes might also be considered in the context of trial inclusivity. It is often assumed that the overall results of a clinical trial are generalizable to all patients in the trial and all similar future patients; in other words, that the relative treatment effect in individual patients is similar to the overall trial result. Although this assumption underpins the application of trial results to clinical practice, it has rarely been tested. Where it has been tested, there has been significant heterogeneity of treatment effects [8]. On the basis of estimates that the same dose of an ODI can result in a 30% difference in thrombin generation inhibition, it has been suggested that bleeding may be more likely in high responders and thrombosis more likely in low responders [9]. However, the implication that dose adjustment would improve individual patient clinical outcomes has not been tested. As the group-specific outcome of patients treated with ODIs in clinical trials is at least non-inferior to that with warfarin treatment, any move towards monitoring and dose adjustment for routine patients (i.e. who would have been eligible for trial inclusion) is not advisable until a strong evidence base for such a practice is provided.

Operating characteristics of laboratory tests of coagulation

Data on the effects of ODIs on laboratory tests of coagulation are now available, and so guidance on the interpretation of a test result is now possible [10]. Laboratories should be aware of the sensitivity of their own assays to

each drug. This can be achieved by using appropriate calibrated plasma samples. Some commercial calibrants are not designed for use in clotting assays, and may not contain normal plasma. Even in those that do, the PT and APTT may be prolonged independently of the drug effect. In this case, the calibrants could give misleading results.

Dabigatran

Peak dabigatran plasma levels are reached 2 h after ingestion, although this may be delayed for up to 6 h after the first postoperative dose. The steady-state mean dabigatran peak plasma concentration, measured ~ 2 h after administration of 150 mg of dabigatran etexilate twice daily, was 175 ng mL^{-1}, with a range of 117–275 ng mL^{-1} (25–75th percentile range). The mean trough concentration, measured 12 h after a 150-mg evening dose, was 91 ng mL^{-1}, with a range of 61–143 ng mL^{-1} (25–75th percentile range). Plasma concentrations of dabigatran showed a biexponential decline, with a mean terminal half-life of 11 h in healthy elderly subjects. After multiple doses, a terminal half-life of approximately 12–14 h was observed [11].

The APTT is more sensitive than the PT to the anticoagulant effect of dabigatran. The APTT shows a curvilinear dose–response relationship with dabigatran, with a steep increase at low concentrations and linearity above a dabigatran concentration of 200 ng mL^{-1} [12–15]. Above 100 ng mL^{-1}, the APTT is invariably prolonged [14]. APTT results are influenced by coagulometers and reagents, and APTT results need to be interpreted with caution, as there is variable sensitivity [12,14,15]. However, a normal APTT ratio is likely to exclude a therapeutic intensity of anticoagulation caused by dabigatran. Although a degree of anticoagulation caused by dabigatran cannot be excluded by a normal APTT, the intensity might be regarded as no more than that achieved with a prophylactic dose of low molecular weight heparin. Further studies are required to determine the relative sensitivity of APTT reagents to dabigatran, in order to give more specific recommendations regarding choice of APTT reagent. In the April 2012 Supplementary Exercise from the UK National External Quality Assessment Service (NEQAS), there was a 40% variance in responsiveness to dabigatran of the 10 most frequently used APTT reagents. This variance between reagents was consistent up to a dabigatran concentration of 500 ng mL^{-1} (S. Kitchen, personal communication).

The effect of dabigatran on the activated clotting time is similar to that on the APTT, but with a flatter dose–response curve, which limits its utility [15]. In comparison with the APTT, the PT is relatively insensitive to dabigatran [14,15]. Even at 200 ng mL^{-1}, the PT is not prolonged, or is prolonged by < 3 s, with some reagents [14]. There is extreme variation in PT reagent sensitivity [14,15]. In the NEQAS exercise, there was a 60% variance

between the most frequently used thromboplastins. The INR/International Sensitivity Index (ISI) methodology used for VKAs is not suitable for measurement of dabigatran, and the discrepancy between PT results is compounded by point of care-derived INRs [16,17].

A standard thrombin time (TT) shows a linear concentration response to dabigatran [14,15], but results are highly dependent on reagents and coagulometers. Most TT assays will be too sensitive, with high variability above a dabigatran concentration of 25 ng mL^{-1} [12]. The TT might be used as a sensitive method for determining whether any dabigatran is present, which will be excluded by a normal TT. The Hemoclot Thrombin Inhibitors (Hyphen Biomed, Neuville-sur-Oise, France) assay is a quantitative measurement of direct thrombin inhibitors for use with calibrators including a dabigatran kit. This is a diluted-plasma TT. Test plasma is diluted 1 : 8 in buffered saline, and added to an equal volume of thrombin solution (1.5 NIH units mL^{-1} α-thrombin). The dilution of the inhibitor results in a linear dose–response curve up to and beyond 1000 ng mL^{-1}, with a correlation coefficient of 0.99 [12,15,18,19]. A therapeutic level of dabigatran is in the range of 40–100 s, but results vary considerably between laboratories, in large part because of the use of different coagulometers [12,18,19].

Measurement of fibrinogen in patients taking dabigatran can give falsely low results, but with marked variation with different reagents. When plasma was spiked with dabigatran, the fibrinogen level was significantly underestimated in Clauss fibrinogen assays using HemosIL C (Instrumentation Laboratories, Bedford, MA, USA) and Multifibren U (Siemens, Erlangen, Germany) [14]. The effect was dose-dependent, and this phenomenon was confirmed in the NEQAS Supplementary Exercise in April 2012 with dabigatran-spiked pooled plasmas. Alternative reagents from the manufacturers of HemosIL C and Multifibren U (HemosIL Quantitative Fibrinogen Assay and Siemens Thrombin) do not underestimate fibrinogen [14] (confirmed in the NEQAS exercise).

Dabigatran has no effect on D-dimer assays [20], but suppression of D-dimer levels because of inhibition of thrombin in patients treated with dabigatran would be expected. Ecarin is an enzyme derived from the venom of the viper *Echis carinatus*. It converts prothrombin into meizothrombin, and, as dabigatran inhibits meizothrombin, the ecarin clotting time (ECT) can be used to measure the activity of dabigatran [12,15]. There is a linear dose-dependent prolongation of the ECT up to 500 ng mL^{-1} dabigatran [12]. Batroxobin is a serine protease derived from the venom of *Bothrops atrox*. It has thrombin-like proteolytic activity, leading to clot formation by aggregation of fibrin I monomer, which is not affected by dabigatran (reptilase time) [20].

Dabigatran produces a concentration-dependent prolongation of the lag time of tissue factor-induced thrombin generation. At a dabigatran concentration of

$< 100 \; \mu g \; L^{-1}$ there is a paradoxical increase in the peak thrombin level [12,21,22]. A proposed explanation is that the algorithm used to calculate the thrombin concentration, when measured by Calibrated Automated Thrombography, introduces errors when a small molecule direct thrombin inhibitor such as dabigtran is present in the plasma sample. This error results in an erroneously high reported thrombin activity [22]. This artefact is apparent following orthopedic surgery when patients are given dabigatran as thromboprophylaxis. There are prolongations of the lag time and time to peak thrombin level, but increases in the peak thrombin level and the area under the TT curve (endogenous thrombin potential [ETP]) [23].

Measurements of coagulation factors and natural anticoagulants are significantly affected by thrombin inhibition, and such measurements are therefore not reliable in patients receiving dabigatran [14,20]. Measurement of antithrombin with an FXa-based assay is not affected by dabigatran [14,20]. Measurement of antithrombin with a thrombin-based assay could result in a false-normal antithrombin level in a patient with antithrombin deficiency [14,20]. Clot-based protein C assays could result in a false-normal level in a patient with deficiency. This is avoided with a chromogenic assay [20].

Rivaroxaban

Peak rivaroxaban plasma levels are reached within 3 h of ingestion. At the dose of 20 mg once daily used for stroke prevention in atrial fibrillation, the peak rivaroxaban level is approximately 290 ng mL (range, 177–409 ng mL; 5–95th percentiles), with a trough of $\sim 32 \; ng \; mL^{-1}$ (range, $5–155 \; ng \; mL^{-1}$). Similar drug levels were found in patients receiving rivaroxaban 20 mg daily for the treatment of deep vein thrombosis [24]. In patients undergoing total hip replacement, the peak level after 10 mg was $125 \; ng \; mL^{-1}$ (range, $91–196 \; ng \; mL^{-1}$), with a trough of $9 \; ng \; mL^{-1}$ (range, $1–38 \; ng \; mL^{-1}$) [25]. Only approximately one-third of active rivaroxaban is cleared by the kidneys, and there is no accumulation of drug when the creatinine clearance is above $15 \; mL \; min^{-1}$ [26].

The PT is more suitable than the APTT for assessing the intensity of anticoagulation in patients taking rivaroxaban, owing to its linearity over a broad range of concentrations and its higher sensitivity [27]. There is a linear concentration–response relationship, but there is marked variability between PT reagents, with an up to three-fold difference between them [27–31]. This emphasizes the need for each laboratory to determine the sensitivity of their own PT assay to rivaroxaban, or other FXa inhibitors, and this can be achieved by using appropriate commercially available calibrants. Calculation of the INR with a VKA-derived ISI increases the discrepancy between PT results, and should not be used [29]. The accuracy and imprecision of point of care measurement of the PT in patients taking rivaroxaban requires evaluation. The PT is useful as a readily available method for determining the relative degree of anticoagulation in patients taking rivaroxaban, if a reagent with known sensitivity is used. A normal PT ratio with most reagents excludes a therapeutic intensity of anticoagulation caused by rivaroxaban Although some degree of anticoagulation cannot be excluded by a normal PT, the intensity could be regarded as no more than that achieved with a prophylactic dose of low molecular weight heparin.

The TT (including Hemolcot; Hyphen Biomed) and ECT are not affected by rivaroxaban [27,28]. The Clauss fibrinogen assay is not affected by therapeutic levels of rivaroxaban [27,30]. The reptilase time is not affected [27]. Russell's viper venom activates FX to FXa, and the dilute Russell's viper venom time assay is sensitive to low concentrations of rivaroxaban, with a curvilinear response [32].

Rivaroxaban has no effect on D-dimer assays [28], but suppression of D-dimer levels because of inhibition of thrombin generation in patients treated with rivaroxaban would be expected.

Rivaroxaban can be quantified with calibrated anti-FXa assays by the use of chromogenic substrates [27–30,33,34]. Assays should be calibrated with rivaroxaban calibrants, and not low molecular weight heparin standards, as different assays have different dynamic ranges for rivaroxaban [29,34] and other FXa inhibitors [29]. Although it has been proposed that the PT assay, which is readily available in most laboratories, in conjunction with rivaroxaban calibrators may be useful for the measurement of peak plasma levels of rivaroxaban [35], the stronger correlation between rivaroxaban levels and anti-FXa activity in a chromogenic assay favors its use, when available, for quantitation of rivaroxaban and other FXa inhibitors, such as apixaban [29].

Thrombin generation is reduced by rivaroxaban, with prolongations of the lag time and time to peak thrombin level, and a decrease in the peak thrombin level and ETP [36–38]. However, the effect on lag time is greater than that on the amount of thrombin generated. The concentration of rivaroxaban required to reduce the ETP by 50% is 3.5-fold higher than that required to double the lag time [37].

The effects of rivaroxaban on thrombin generation and the APTT are similar in neonatal and adult plasma [39], as are the effects on the PT, APTT, thrombin generation and anti-FXa assays in infants, children, and adults [40].

Apixaban

Apixaban is a direct inhibitor of FXa, with predictable pharmacokinetic and pharmacodynamic activity [41]. As with rivaroxaban, the PT sensitivity varies considerably between thromboplastins, and conversion to the INR increases the variability [29]. Different anti-FXa assays show variable dose responsiveness, and so calibration is required in order for them to be used to quantify apixaban levels.

Assessment of effects of 'reversal agents'

As with all anticoagulant drugs produced so far, there is a correlation between intensity of anticoagulation and bleeding. As yet, specific antidotes to ODIs are not available for clinical use, but these are in development as rapid reversal agents. Management of bleeding in a patient taking an ODI depends on the severity of bleeding, recognizing that:

1 urgent measurement of the intensity of anticoagulation can help to determine the material contribution of an ODI to bleeding;
2 laboratory tests and their interpretation will also be required to recognize contributory coagulopathy unrelated to an ODI.

When there is major bleeding, particularly life-threatening bleeding, clinicians will probably elect to consider administration of prothrombin complex concentrate (PCC) or recombinant FVIIa (rFVIIa) [42–44]. The effects of PCC and rFVIIa on bleeding have not been studied in clinical trials in patients with bleeding. The effect of PCC on laboratory tests of coagulation in healthy volunteers taking ODIs has been studied. Prolongation of coagulation test results caused by rivaroxaban is reversed by PCC (50 IU kg^{-1} of four-factor concentrate), but the effect of dabigatran is not [45] In this *in vitro* spiking study, FEIBA appeared to have the greatest ability to restore the parameters of thrombin generation, but it remains to be determined whether this translates into superior clinical efficacy.

The effects of PCC and rFVIIa in animal bleeding models indicate that there is an apparent dissociation of the effects on laboratory tests as compared with the bleeding tendency that is class-specific (or drug-specific). In a mouse tail bleeding model, administration of 1.5 mg kg^{-1} oral dabigatran etexilate achieved a mean plasma dabigatran level of ~ 200 ng mL^{-1}. Administration of PCC at 14.3 µg kg^{-1} plus rFVIIa at 3 mg kg^{-1} corrected the prolonged APTT but did not reduce blood loss [46]. A higher dose of PCC alone up to 50 µg kg^{-1} had no effect on the APTT, but increased thrombin generation in a dose-dependent manner and reduced blood loss in a rabbit kidney incision model [47]. In a rabbit hepato-splenal incision model, animals given 5 mg kg^{-1} rivaroxaban were treated before injury with 40 µg kg^{-1} PCC or 150 µg kg^{-1} rFVIIa. Both PCC and rFVIIa partially corrected the PT, APTT, and ETP, and rFVIIa corrected the prolonged lag time, but neither reduced rivaroxaban-induced bleeding [48].

Table 1 General effects of thrombin and factor Xa inhibition by drugs such as dabigatran and rivaroxaban (and expected with other FXa inhibitors)

Test	Thrombin inhibition	Clinical utility	FXa inhibition	Clinical utility
PT	++	No	+++	Qualitative
INR	Not applicable	No	Not applicable	No
POC PT	Not applicable	No	++	Uncertain
APTT	+++	Qualitative	+	Uncertain
ACT	++	No	+	No
TT	++++	Limited	Not affected	No
Dilute plasma TT (e.g. Hemoclot)	+++	Quantitative	Not affected	No
Fibrinogen	Not affected by high thrombin concentration reagents		Not affected	No
D-dimer	Not affected	No	Not affected	No
Ecarin	++	Quantitative	Not affected	No
Reptilase	Not affected	No	Not affected	No
Russel's viper venom	++	No	++	No
Chromogenic anti-FXa	Not affected	No	+++	Quantitative
Thrombin generation lag time	Prolonged	Uncertain	Prolonged	Uncertain
Thrombin generation peak thrombin level	Initial increase	Uncertain	Reduced	Uncertain
Thrombin generation ETP	Reduced at higher concentrations	Uncertain	Reduced	Uncertain
Clotting factors	False low with clot-based assays. Testing not recommended. Positive inhibitor screen and Bethesda assay		False low with clot-based assays. Testing not recommended. Positive inhibitor screen and Bethesda assay	
Antithrombin	FXa inhibition assay not affected Elevated in FIIa inhibition assay		FIIa inhibition assay not affected Elevated in FXa inhibition assay	
Protein C	Chromogenic assay not affected. Elevated in clot-based assay		Chromogenic assay not affected. Elevated in clot-based assay	

ACT, activated clotting time; APTT, activated partial thromboplastin time; ETP, endogenous thrombin potential (area under the TT curve in thrombin generation assay); POC, point of care; PT, prothrombin time; TT, thrombin time.

Conclusion

The general effects of thrombin and FXa inhibition by drugs such as dabigatran and rivaroxaban are summarized in Table 1. Detailed information is required in relation to the sensitivity and assay performance of different PT and APTT tests. The complexity of responsiveness of thrombin kinetic measurements requires further understanding before any clinical utility can be considered. It is not yet known which, if any, coagulation tests might be used as surrogate markers of reversal of anticoagulation, and therefore as an indication of a reduction of bleeding tendency in response to treatment with PCCs, rFVIIa, or FEIBA.

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