Non-vitamin K dependent oral anticoagulants (NOACs) controls

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In recent years non-vitamin K dependent oral anticoagulants (NOACs) have started to replace warfarin for treatment and prevention of deep venous thrombosis (DVT), pulmonary embolism (PE) and stroke in patients with and without atrial fibrillation. There is a need for a simple and rapid method to detect the presence of these drugs in patient plasma. To meet these new demands, MediRox is developing a screening assay based on a novel prothrombin time (PT) method for rapid detection of NOACs in plasma. The assay is semi-quantitative and by dividing the International Normalised Index (INR) from a NOAC sensitive PT method with the INR from a NOAC insensitive PT method, NOAC containing samples be detected while plasma from normal donors and with warfarin are excluded.

The purpose of this project is to develop prototypes of assay quality controls for detection of NOACs in plasma.

The results show that the method used for the NOAC control prototypes is applicable and the PT ratio is comparable to patient samples for the low, medium and high concentrations of NOAC. The effect of lyophilisation indicates that the PT ratios for the NOAC control prototypes were nearly unaffected by the lyophiliisation. The in-use stability at room temperature (20-25°C) for all NOAC control prototypes were at least 24 hours.

The methodology for production needs to be further optimised to increase the commutability to patient samples with very high concentrations of NOAC. The data indicates that the effect of lyophilisation is minimal and the stability of the NOAC control prototypes are satisfying, which is promising for future product development of NOAC controls.
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Abstract
In recent years non-vitamin K dependent oral anticoagulants (NOACs) have started to replace warfarin for treatment and prevention of deep venous thrombosis (DVT), pulmonary embolism (PE) and stroke in patients with and without atrial fibrillation. There is a need for a simple and rapid method to detect the presence of these drugs in patient plasma. To meet these new demands, MediRox is developing a screening assay based on a novel prothrombin time (PT) method for rapid detection of NOACs in plasma. The assay is semi-quantitative and by dividing the International Normalised Index (INR) from a NOAC sensitive PT method with the INR from a NOAC insensitive PT method, NOAC containing samples can be detected while plasma from normal donors and with warfarin are excluded.

The purpose of this project is to develop prototypes of assay quality controls for detection of NOACs in plasma.

The results show that the method used for the NOAC control prototypes is applicable and the PT ratios are comparable to patient samples for low, medium and high concentrations of NOAC. The effect of lyophilisation indicates that the PT ratios for the NOAC control prototypes were nearly unaffected by the lyophilisation. The in-use stability at room temperature (20-25°C) for all NOAC control prototypes were at least 24 hours.

The methodology for production needs to be further optimised to increase the commutability to patient samples with very high concentrations of NOAC. The data indicates that the effect of lyophilisation is minimal and the stability of the NOAC control prototypes are satisfying, which is promising for future product development of NOAC controls.
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### Abbreviations

<table>
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<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>Activated Protein C</td>
</tr>
<tr>
<td>APTT</td>
<td>Activated partial thromboplastin time</td>
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<td>DVT</td>
<td>Deep Venous Thrombosis</td>
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<tr>
<td>F</td>
<td>Factor</td>
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<tr>
<td>INR</td>
<td>International Normalised Ratio</td>
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<td>ISI</td>
<td>International Sensitivity Index</td>
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<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
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<tr>
<td>LMWH</td>
<td>Low-molecular-weight Heparin</td>
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<tr>
<td>NOAC</td>
<td>Non-vitamin K dependent oral anticoagulants</td>
</tr>
<tr>
<td>PE</td>
<td>Pulmonary Embolism</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>pNA</td>
<td>Para nitroaniline</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin time</td>
</tr>
<tr>
<td>TAFI</td>
<td>Thrombin activatable fibrinolysis inhibitor</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>TM</td>
<td>Thrombomodulin</td>
</tr>
<tr>
<td>t-PA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>UFH</td>
<td>Unfractionated Heparin</td>
</tr>
<tr>
<td>VKA</td>
<td>Vitamin K antagonist</td>
</tr>
<tr>
<td>vWF</td>
<td>Von Willebrand factor</td>
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1 Introduction

1.1 Purpose of the project

For many years warfarin has been the dominated anticoagulant therapy for treatment and prevention of deep venous thrombosis (DVT), pulmonary embolism (PE) and stroke in patients with and without atrial fibrillation (Gosselin & Adcock, 2015). The prothrombin time (PT) assay has been used with accompanying normal and abnormal coagulation controls to monitor therapy. In recent years new oral anticoagulant drugs, non-vitamin K dependent oral anticoagulants (NOACs) have been introduced on the market. NOACs have shown to be as effective as warfarin and the side effects are similar (Plitt & Bansilal, 2017). Thus, many patients choose NOAC instead of warfarin.

MediRox develops, manufactures and markets reagents, controls and calibrators for coagulation diagnostics. The company is currently supplying approximately 50 percent of the PT tests and controls used in the clinical laboratories in Scandinavia. The introduction of NOACs in the clinical laboratories has been a challenge. NOACs have shown to interfere with routine as well as specialty coagulation assays potentially generating false positives and negatives, possibly resulting in patient misdiagnosis (Hillarp, et al., 2011) (Lindahl, et al., 2011) (Hillarp, et al., 2014) (Mani, 2014). Multiple coagulation assays are needed for detection and quantification of NOACs, which is impractical and costly. There is a need for a simple and rapid method to detect the presence of these drugs in patient plasma. To meet these new demands, MediRox is developing a screening assay based on a novel PT method for rapid detection of NOACs in plasma.

The purpose of this project is to develop prototypes of assay quality controls for detection of NOACs in plasma.

1.2 Boundary conditions

The project was conducted at the department of Research and Development at MediRox, during January to June 2018. MediRox is a ISO 13485:2012 certified organisation. All products are CE marked for in vitro diagnostic use and the products are developed in accordance to the regulations provided by the Swedish Medical Products Agency’s (LVFS:2001:7). The present project is performed under a confidentiality agreement. Information regarding suppliers, composition of products, chemical compounds and concentrations will be decoded and in some cases omitted. It will be done in the report as well as in presentations.

1.3 Expected impact of the project

MediRox is supplying clinical laboratories worldwide with a variety of reagents, controls and calibrators used for haemostatic screening, quantification of coagulation factors, determination of enzyme activity, to monitor warfarin therapy etc. The products are used in clinical investigations of suspected thrombotic events and increased bleeding propensity. MediRox’s products can be used as diagnostic tools, as a part of patient diagnosis.
Anticoagulant therapy, such as warfarin and NOACs, is frequently prescribed for treatment and prevention of DVT, PE and stroke in patients with and without atrial fibrillation (Lee, et al., 2017). Using NOAC instead of warfarin will make everyday life easier for patients as they do not need monitoring. Compared with warfarin there are no known diet and drug interaction (Plitt & Bansilal, 2017).

The recent years have shown an increase in receiving NOAC instead of warfarin as antithrombotic therapy (Lee, et al., 2017). Patients on warfarin need to be monitored and there is no need of monitoring NOACs because of the known mechanism (Leterre, et al., 2016). With the known mechanism and with less interaction the prescription of NOAC instead of warfarin have increased and there is no indication that it would not continue to increase.

The introduction of the new anticoagulants, NOACs in the clinical laboratories has been a challenge, due to the interfering propensity of NOACs to many routine and specialty coagulation assays (Hillarp, et al., 2011) (Lindahl, et al., 2011) (Hillarp, et al., 2014) (Mani, 2014). Multiple coagulation assays are needed for detection and quantification of these drugs, which is impractical and costly (Favaloro, 2015). There is a need for a simple and rapid method to detect the presence of these drugs in patient plasma, to minimise possible patient misdiagnosis due to interfering NOACs.

Screening for an unknown presence of NOACs in patient plasma are impractical and costly today, as several coagulation analyses are needed. Thus, MediRox is developing a screening assay based on a novel PT method, to rule out NOACs as interferents to other coagulation analysis in the clinical laboratories. To detect NOAC with the new method, reagents and accompanying controls are needed for quality control. This project is done as a pilot study of the NOAC controls to optimise the methodology for the future production process and to provide stability data.

1.4 Objectives of the work

The main goal with this project was to develop product prototypes of assay quality controls (called NOAC controls) for measurement of NOACs using MRX PT NOAC reagent prototype. The main goal was divided into smaller sub-goals, see Figure 1. The sub-goals are presented in the order they needed to be met from left to right to reach the main goal. Under each sub-goal, key activities are presented in white.
The first sub-goal was to determine a preliminary product specification for the NOAC controls. Consultations with a medical advisor and a representative at the marketing and sales office at MediRox were performed and specifications such as number of levels, stability requirements, etc. were discussed in order to meet the clinical and the customer demands. The NOAC controls should reflect patients treated with NOACs, and a three level control representing low, medium and high concentrations of NOAC were selected for the preliminary product specification. The second sub-goal comprises optimisation of the method used for production of NOAC controls, including choice of drug and effect of lyophilisation. The third sub-goal was a comparison of drugs from four suppliers, and the forth sub-goal was estimation of the stability of the NOAC controls.

Figure 1: The sub-goals of the project are marked in black and the related activities for each sub-goal in white.
2 Theory and methodology

2.1 Scientific background

2.1.1 Haemostasis

Under normal conditions the blood maintains as a fluid. The blood consists of erythrocytes, leukocytes, platelets and plasma. Platelets are small and can be found close to the endothelial walls in the bloodstream. The concentration of platelets are normally 150-350*10^9/L (Gardner & Tweedle, 2002). The walls of the blood vessels consist of endothelial cells which are negatively charged and repulse the negatively charged platelets to prevent spontaneous platelet aggregation (Periayah, et al., 2017). Other antithrombotic factors are present on the endothelial cells. The endothelial cells also produce endogen heparin sulphate which together with antithrombin inactivates active coagulation factors (Nilsson-Ehle, et al., 2012).

When a vessel is injured the physiological process of haemostasis starts to stop the bleeding. The haemostasis can be divided in three different steps, primary and secondary haemostasis and fibrinolysis (SBU, 2002). The primary haemostasis comprises of vasoconstriction and formation of a platelet plug followed by the secondary haemostasis including fibrin network formation for stabilisation of the platelet plug (Nilsson-Ehle, et al., 2012). The third step is fibrinolysis, comprising lysis of the coagula.

Coagulation cascade

Thirteen different coagulation factors circulate in the plasma. These are all inactivated except from FVII that occurs in 1-2% in its active form (Nilsson-Ehle, et al., 2012). These coagulation factors act during the coagulation cascade, see Figure 2.

![Figure 2: Coagulation cascade with the intrinsic and extrinsic pathway.](image-url)
Primary haemostasis

The pressure in the vessel is changed upon an injury and the autonomic nervous system together with local factors regulate the pressure, causing vasoconstriction (Nilsson-Ehle, et al., 2012). The vasoconstriction results in decreased blood flow in the vessel and thereby a decreased bleeding. Platelets are activated by the injured endothelia and substances enhancing the vasoconstriction are released (Nilsson-Ehle, et al., 2012).

The endothelium and platelets are both negatively charged and repulse each other, but this is changed when the vessel is injured. The platelets start to roll on the walls and finally reach the subendothelium (Periayah, et al., 2017). Collagen is present in the subendothelium which activates platelets. Activated platelets change conformation from discoid to irregular. Fibrinogen and von Willebrand factor (vWF) bind to the activated platelets. Dense bodies and α-granulae are released from the activated platelets where α-granulae consists of vWF, FV, FXIII and fibrinogen (Periayah, et al., 2017). Dense bodies mainly consist of ADP, Ca\(^{2+}\) and serotonin. Ca\(^{2+}\) is an important coagulation factor. Thrombin, generated by the extrinsic pathway of the coagulation cascade convert fibrinogen to fibrin leading to a stable platelet plug (Hangge, et al., 2017).

Secondary haemostasis

The coagulation cascade is divided in the intrinsic pathway and the extrinsic pathway which merges at the Factor Xa (FXa)/FVa complex also called prothrombinase complex (Monroe & Hoffman, 2005), see Figure 2. When tissue factor (TF) is exposed on the endothelial cell membrane, a complex is formed with FVII and Ca\(^{2+}\) and the extrinsic pathway is activated (Mackman, 2008). The TF complex activates either FIX or FX. Activated FIXa forms the tenase complex together with FVIIIa and Ca\(^{2+}\) on the surface of the platelet membrane (Nilsson-Ehle, et al., 2012). The tenase complex convert FX to FXa and a second complex is created by FXa together with FVa and Ca\(^{2+}\). This complex is called prothrombinase complex and cleaves prothrombin to thrombin (Nilsson-Ehle, et al., 2012). When a small amount of thrombin has been produced it triggers coagulation factors in the intrinsic pathway which initiate the coagulation cascade. Thrombin activates several coagulation factors as a positive amplification by activating FV, FVIII and FXI in the intrinsic pathway (Monroe & Hoffman, 2005). The intrinsic pathway is activated by contact of negatively charged surfaces. This can occur due to insertion of a prosthesis and a trauma. Activation of the intrinsic pathway leads to a conversion of FXII to FXIIa which in turn activates FXIa, and FXIa activates FIXa (Periayah, et al., 2017). The extrinsic and intrinsic pathway merge at the prothrombinase complex.

Thrombin activates several coagulation factors but it also converts fibrinogen to fibrin, i.e a clot is formed (Wheeler & Gailani, 2016). Furthermore, thrombin activates prothrombin activatable fibrinolysis inhibitor (TAFI) and activated protein C (APC). The endothelial receptor thrombomodulin (TM) is located on the healthy endothelial tissue, which thrombin binds to activating protein C and protein S. APC inactivates FVa and FVIIIa (Monroe & Hoffman, 2005). The excess of the coagulation factors Xa, IXa, XIa and thrombin are inactivated by antithrombin in the plasma (Nilsson-Ehle, et al., 2012). Antithrombin inhibits the coagulation cascade together with endogenous heparin by binding FIXa, FXa, FXIIa and thrombin.
**Fibrinolysis**

During the fibrinolysis, the clot will be degraded, see Figure 2. The endothelia produce the enzyme tissue plasminogen activator (t-PA) which binds to fibrin and the plasminogen activator inhibitor (PAI-1) (SBU, 2002). Plasminogen binds to t-PA and plasminogen is activated to plasmin. Plasmin degrades fibrin to several products and D-dimers are formed (Nilsson-Ehle, et al., 2012). The complex of t-PA and PAI-1 will be transported to the liver and degraded.

### 2.1.2 Haemostatic disorders

Cardiovascular diseases can be caused by an imbalance in the vascular system potentially leading to bleeding or thrombosis (Mackman, et al., 2007). Several risk factors have been associated with development of cardiovascular diseases such as high cholesterol levels, high blood pressure, smoking as well as genetic factors (Nilsson-Ehle, et al., 2012). Studies have indicated that a change in diet, exercise, reduction of stress etc. can prevent development of cardiovascular diseases (Nilsson-Ehle, et al., 2012).

Bleeds can be caused by genetic disorders such as haemophilia A and B, *i.e.* lack or heavily reduced levels of the coagulation factors VIII and IX, lack of platelets making it difficult/impossible to form a clot upon an injury (Nilsson-Ehle, et al., 2012). Thrombosis is formation of an unwanted blood clot and it can be divided into two types; atrial and venous thrombosis. Most of the heart attacks and strokes are caused by arterial thrombosis while cardiovascular death caused by venous thrombosis is the third leading cause (Mackman, 2008). Several factors have been associated with development of thrombosis such as alterations in the coagulation system, high viscosity and stasis of the blood as well as formation of atherosclerotic plaques (Mackman, et al., 2007).

### 2.1.3 Haemostasis screening assays

**Activated partial thromboplastin time (APTT)**

Activated partial thromboplastin time (APTT) is a screening assay measuring the function of the factors in the intrinsic pathway, FXII, FXI, FIX, FVIII and FV, fibrinogen and prothrombin (SBU, 2002). The method is also used to monitor heparin therapy with unfractionated heparin (UFH), but not low-molecular-weight heparin (LMWH) (Favaloro, 2015). Heparin inhibits the coagulation and causes a higher risk of bleeding. The APTT is measured in seconds and a long APTT indicates that the patient is treated with heparin and a slightly higher APTT indicates a possible factor deficiency, seen in haemophilia A and B (Nilsson-Ehle, et al., 2012). If the APTT is prolonged approximately 1.5 times from the normal range there is an increased risk of bleeding during an operation (SBU, 2002). Lupus anticoagulants and low levels of fibrinogen can also result in prolonged APTT (Nilsson-Ehle, et al., 2012).
**Prothrombin time (PT)**

PT measures the function of the vitamin K dependent factors, FVII, FX and prothrombin in the extrinsic pathway of the coagulation cascade (SBU, 2002). FIX is also a vitamin K dependent coagulation factor, but a PT result is not dependent on the function of FIX. There are two types of PT methods on the market, PT Quick which is frequently used worldwide and PT Owren’s which is the predominant PT method used in Scandinavia (Nilsson-Ehle, et al., 2012). A PT Quick result is also dependent on the FV and fibrinogen levels (Nilsson-Ehle, et al., 2012).

In the 1950’s, Paul Owren developed a method called PT Owren’s which is used as a screening test for acquired and inherited bleeding disorders and to monitor warfarin therapy (Hillarp, et al., 2004). The PT Owren’s reagent contains of phospholipids, thromboplastin from rabbit brain, calcium and bovine plasma lacking the vitamin K dependent factors (Hillarp, et al., 2004). The reagent initialises the coagulation cascade, when it is added to plasma samples, and a fibrin clot is formed. The time from adding the reagent until a clot is formed is measured and reported in seconds.

The clotting time is converted to an international normalised ratio (INR), describing the relationship between plasma from normal donors, marked as mean normal PT in equation (1) and the extended clotting time for plasma with warfarin (SBU, 2002). The calculation of INR can be seen in equation (1) (Poller, 1998).

\[
INR = \left( \frac{PT}{MNPT} \right)^{ISI} \tag{1}
\]

**Equation 1**

INR = International normalised ratio  
PT = Prothrombin time  
MNPT = Mean of PT of plasma samples from at least 20 persons  
ISI = International sensitivity index of thromboplastin

The ISI-value represent the sensitivity index of the thromboplastin used in the reagent. The clotting time will be extended if warfarin is present in the plasma as compared to normal donor plasma. Other reasons for an extended INR-value are vitamin K deficiency, reduced liver function or lack of coagulation factors.

**2.1.4 Oral anticoagulantia therapy**

Oral anticoagulants (OACs) are used for prevention of thrombosis and ischemic stroke in patients with atrial fibrillation. The probability of atrial fibrillation increases with age and are estimated to 3% in persons over 20 years (Hammersley & Signy, 2017).

Atrial fibrillation is one form of arrhythmia where the heart beats are rapid and irregular. Atrial fibrillation is one of the most common conditions at the emergency (Gudlaugsdottir, et al., 2018). Arrhythmia is caused when the sinus knot in the right atrium cannot control the beat rhythm, by that the heart can only work suboptimally (Gudlaugsdottir, et al., 2018). The heart cannot circulate
the blood optimally between the contractions and stagnant blood can appear in the heart. Stagnant blood can cause a thrombosis which is when blood is clotting locally. The thrombus can then be transported with the blood stream to the brain or lungs and get stocked which increases the risk of a stroke.

Patients with atrial fibrillation are treated with anticoagulantia to reduce the risk of thrombosis with either the traditionally used drug warfarin, which is a vitamin K antagonist (VKA), or with the recently introduced drugs of NOACs (Hammersley & Signy, 2017). In Sweden, the prescription of OACs have increased from 51% in 2011 to 74% in 2016 (Stockholms läns Landsting, 2017). Another study in Korea showed that an increase in the prescriptions of OACs instead of aspirin to patient with atrial fibrillation has been observed between 2008 and 2012 and in 2013 when NOACs were approved, the used of OACs increased even more (Lee, et al., 2017). The prescriptions of OACs increased from 32% in 2008 to 46% in 2015, and half of the patients choose NOACs instead of warfarin in 2015 (Lee, et al., 2017).

The elimination of NOACs occurs partially by the kidney and the liver or in the liver and the intestine (Plitt & Bansilal, 2017). The mechanism for elimination differ between NOACs. The kidney accounts for 20-80% of the elimination of NOACs depending on drug (Plitt & Bansilal, 2017). Therefore NOACs are not prescribed to patients with reduced renal function, they are prescribed warfarin. Patients with reduced liver function are not prescribed some of the NOACs because of the elimination by the liver. Before treating any patient with NOAC both renal and liver function are controlled (Plitt & Bansilal, 2017). Thus, NOACs will not completely replace warfarin as OACs.

**Warfarin**

Warfarin is a synthesised coumarin that was discovered in 1921 and warfarin has been used as rodenticide since 1940 (Wardrop & Keeling, 2008). Since 1954, warfarin has been used in patients with atrial fibrillation to reduce the risk of thrombosis and stroke (Knesek, et al., 2012). Studies have shown that warfarin therapy has reduced the incidence of stroke with two-thirds (Hammersley & Signy, 2017). Warfarin is a VKA and inhibits the synthesis of vitamin K by preventing carboxylation of glutamic acid. (SBU, 2002). The dose requirements are individual for each patient due to differences in dose response (Lindahl, et al., 2017). Warfarin therapy requires frequent follow ups with PT determinations and dose adjustments. The therapeutic range (PT INR 2-3) is narrow and warfarin has a slow onset of action (Knesek, et al., 2012).

The effectiveness of warfarin is influenced by several factors such as food and drugs, but also by genetic factors (Lee, et al., 2017). The metabolism of warfarin is affected by drug-drug interactions due to inhibition of cytochrome P450 leading to decreased coagulation due to increased clearance (Knesek, et al., 2012). Drug-food interactions occur between warfarin and vegetables containing vitamin K, leading to an increased propensity of coagulation and between warfarin and grapefruit leading to a decreased propensity of coagulation (Vranckx, et al., 2018).
There are four NOACs on the market today, the direct thrombin inhibitor dabigatran and the FXa inhibitors apixaban, rivaroxaban and edoxaban (Tripodi, et al., 2015). The onset and offset of action are rapid and there is no need to monitor therapy due to the known pharmacodynamic (Hammersley & Signy, 2017). Thus, using NOACs instead of warfarin will make life easier for patients as they do not have to visit the health care clinic every month for PT INR determination. NOAC drugs are orally distributed once or twice a day (Mani, 2014). Today there are no evidence of interaction between drug and food. Drug-drug interactions exists and drugs that leads to less coagulations and increase the bleeding risk because of higher concentration of NOAC in the blood are the ones that inhibits P-glycoprotein (P-gp) or CYP3A4 (Vranckx, et al., 2018). Other drugs leading to an increased propensity for coagulation and thereby higher risk of thrombosis because of less concentration of NOAC in the blood are the ones that induce cell transport P-gp or CYP450 (Vranckx, et al., 2018).

The concentration of NOAC in the circulation varies considerably from person to person and during the day depending on the duration of drug intake (Testa, 2016). The exposure interval for dabigatran is between 12-237 ng/ml where the interval is based on 73 patients and analysed with LC-MS/MS (Skeppholm, et al., 2014). Rivaroxaban analysed with LC-MS/MS on 71 patients had an exposure interval between 5-375 ng/ml (Al-Aieshy, et al., 2016). When analysing apixaban on 70 patients with LC-MS/MS the exposure interval was 15-186 ng/ml (Skeppholm, et al., 2015). The mean peak value of NOAC in treated patients are about 250 µg/L (Mani, 2014).

NOACs are specific and effective anticoagulants. Factor Xa inhibitors prevent the formation of the prothrombin complex where the intrinsic and extrinsic pathway merge. Normally the prothrombin complex converts prothrombin to thrombin in the coagulation cascade but when factor Xa is inhibited, the cascade is partly blocked. Only one factor Xa can generate over 1,000 thrombin molecules thereby illustrating the effectiveness of the drug (Zhou, et al., 2015).

The introduction of NOACs in the clinics and the laboratories has been a challenge. Conventional coagulation assays cannot be used to estimate the effect of the drugs on the coagulation. Quantitative assays for NOACs have been developed using chromogenic substrate methods and LC-MS/MS, but the concentration of the drug in the circulation do not translate to the degree of inhibition of the coagulation. Furthermore, NOACs have shown to interfere with routine as well as specialty coagulation assays potentially generating false positives and negatives, possibly resulting in patient misdiagnosis (Favaloro, 2015). Multiple coagulation assays are needed for detection and quantification of these drugs, which is impractical and costly. There is a need for a simple and rapid method to detect the presence of these drugs in patient plasma. To meet these new demands, MediRox are developing a screening assay based on a novel PT method for rapid detection of NOACs in plasma.
2.2 Methodology

Others have previously shown that plasma samples from patients treated with NOACs can be detected using two PT assays, one sensitive to NOACs and one insensitive NOACs. A ratio is calculated by dividing the INR generated from the PT method sensitive to NOACs (MRX PT NOAC reagent prototype) with the INR generated from an insensitive PT method (MRX PT Owren’s reagent), see equation (2).

\[
PT \text{ ratio} = \frac{PT \text{ NOAC (INR)}}{MRX \text{ Owren's PT (INR)}} \tag{2}
\]

Others have also used modified PT methods for detection of NOACs in plasma (Lindahl, et al., 2017) (Letertre, et al., 2016). Plasma pools spiked with NOACs showed a linear dose response for the PT ratio and the concentration of NOAC from 40 µg/L to 400 µg/L (Lindahl, et al., 2017) and from 32 µg/L to 475/457/572 for dabigatran, rivaroxaban and apixaban, respectively (Letertre, et al., 2016). The limit of detection ranged from 20-80 µg/L depending on drug (Lindahl, et al., 2017). Furthermore, a linear relationship between the PT ratio and the concentration of NOAC in patient plasma samples was observed (Lindahl, et al., 2017).

NOACs are relatively new on the market and there is, to the best of our knowledge no published studies on the effect of lyophilisation of plasma with NOAC. Coumarin is another anticoagulant, and the effect of lyophilisation on plasma pools with coumarin has been studied. The clotting time increased with 15.97% after lyophilisation as compared to before lyophilisation (Poller, et al., 1999).
3 Materials and Methods

3.1 Materials
The assay quality controls evaluated in this project are based on human plasma. The collection of plasma was done according to Swedish law on biobanking and research ethics. Further information regarding suppliers, composition of NOAC controls, chemical compounds and concentrations are omitted.

3.2 Methods

3.2.1 PT analysis using a coagulation analyser
The NOAC controls were analysed with two PT assays, one sensitive (MRX PT NOAC reagent prototype) and one insensitive (MRX PT Owren’s) to NOACs. The PT assays were calibrated using NOAC-free plasma samples with known INR-values. The PT NOAC reagent is under development and an assay calibration was performed at each run (with exception for the comparison between before and after lyophilisation, and the stability tests) to reduce lot to lot variation for the reagent and accompanying buffer. The samples were assayed in duplicates and two coagulation controls were included at each run.

PT ratios were calculated for the NOAC controls by dividing the INR values generated from the PT assay sensitive to NOACs with INR values from the PT assay insensitive to NOACs. These PT analyses were performed using an ACL Top 700 and an ACL Top 500 (Instrumentation Laboratory, Bedford, MA) which is highly automated. ACL Top 700 was used for all PT ratio analysis except from the stability study where ACL Top 500 was used. Two instruments were used in this project, as the old instrument was replaced with a new instrument during this time period. The machine measures the absorbance at 671 nm from the initiation of the reaction (when the reagent is added to the sample) to the formation of a clot. The PT results are presented in seconds and converted to INR, see section 2.3.2 Prothrombin time (PT).

3.2.2 Optimisation of the methodology
The method for production of the controls was optimised, including determination of the concentrations added with an external quantitative assay, comparison with patient plasma samples and the effect of lyophilisation, see below and Figure 1. PT NOAC ratios from patient plasmas were used as references, and compared with PT ratios generated from plasma pools spiked with drugs. The cut off for approval was +/- 25% from the defined target concentration for the NOAC controls. The limit for approval is identical to the limits established for a similar product by a competitor.

3.2.3 Effect of lyophilisation
The control material was lyophilised and dissolved in water before use. This means that the effect of the lyophilisation needs to be addressed. This was done by a comparison of the ratios generated before and after lyophilisation. This is a part of the method development, to evaluate if the lyophilisation process effects the PT ratio.
3.2.4 Stability study

The stability of the controls was evaluated for NOAC control prototypes with drugs from four suppliers using two methods. The specification used for the recovery is $<+/-10\%$ from the initial measurement, for both methods.

The first method comprises reconstitution of five controls in 1 ml of water, one for each time point, zero, three, six, 24 and 48 hours, see Figure 5. The controls were stored at room temperature (approximately 20-25 degrees) until analysis. All controls were reconstituted at the same occasion and the “reference point” marked as 0 h in Figure 3, considered as 100% represent a control analysed 30 minutes after reconstitution followed by three hours, six hours, 24 hours (new reagent), 48 hours (third reagent). The recovery of the PT ratio for controls reconstituted three, six, 24 and 48 hours before analysis was expressed in %. An % higher than 100% means that the INR is higher, i.e. the clotting time is extended and vice versa.

The second method includes measurement of the PT ratio at five different occasions, zero, three, six, 24 and 48 hours after reconstitution of the controls, see Figure 3. Thus, two vials of each NOAC control was reconstituted, pooled together and followed for 48 hours. Two vials from each supplier and level, the same controls as in Table 5, then pool the two of them together. Measurements at six and 24 hours are missing for method two due to technical problems with the ACL Top.

![Figure 3: Stability study with method 1 where five vials was reconstituted at same occasion then analysed at each time point and method two where two vials were pooled then the stability was measured in the same vial at each time point.](image)
3.2.5 Determination of the concentration of NOAC

The lyophilised NOAC controls were sent to an accredited laboratory to determine the concentrations. A chromogenic substrate method was used for quantification of NOAC. The chromogenic substrate method is described briefly, below. An excess of factor Xa or IIa is added to patient plasma. NOACs inhibits FXa or FIIa. A chromogenic substrate with para nitroaniline (pNA) is added and the excess of factor Xa or FIIa cleave the substrate leading to colour development. The colour development is measured at 405 nm and the intensity is inversely proportional to the concentration of NOAC in the plasma sample.
4 Results

4.1 Product specification
When consulting a medical advisor and a representative at the marketing and sales office at MediRox, we discussed whether a two or three level control is needed/competitive, as well as other critical specifications such as stability requirements, etc to meet the clinical and the customer demands. They recommended development of NOAC controls with three levels representing low, medium and high PT ratios. Each control level reflects patient groups with low, medium and high levels of NOACs. The stability of the NOAC controls is preferably the same or longer as compared to similar products produced by competitors. Upon addition of drugs to the controls, a deviation of +/- 25% from the target concentration was acceptable, see Table 1, as this deviation is similar to those reported for similar products produced by competitors. The decision of which supplier of NOAC to choose will be based on the assays done but also the delivery time and price. The future user of the controls will get an assay result presented in a PT ratio, and this ratio corresponds to a target concentration.

Table 1: Product specifications for the prototype controls. * Limit based on percentage deviation from target concentration.

<table>
<thead>
<tr>
<th>Target (ratio)</th>
<th>Limit (low) *</th>
<th>Limit (high) *</th>
<th>Verification</th>
</tr>
</thead>
</table>
| NOAC control LOW | MediRox internal data | -25% | +25% | Analysis 1  
MRX Owren’s PT  
Analysis 2  
PT NOAC (reagent prototype) |
| NOAC control MEDIUM | MediRox internal data | -25% | +25% | Analysis 1  
MRX Owren’s PT  
Analysis 2  
PT NOAC (reagent prototype) |
| NOAC control HIGH | MediRox internal data | -25% | +25% | Analysis 1  
MRX Owren’s PT  
Analysis 2  
PT NOAC (reagent prototype) |

4.2 Choice of drug
One part of the optimisation was to decide which NOAC to use. Previous studies internally at MediRox have shown different effects of NOACs on the selected PT assays. Based on these findings, two NOACs were selected for evaluation, named NOAC X and NOAC Y in the report. Plasma pools from normal donors were spiked with NOACs to simulate treated patients. The PT ratios generated by NOAC-spiked plasma pools were compared with PT ratios generated from authentic samples with corresponding concentrations, see Figure 4.

A chromogenic substrate method has been used by the accredited central hospital laboratory (Linköping, Sweden) for determination of the concentration of NOACs in patient plasma samples. The PT ratios for the patient samples were previously determined using a different reagent lot and a different coagulation analyser (Sysmex 2100).
The plasma pools spiked with NOAC X and Y have been prepared in the same way and the chemical used for dissolving the drugs are the same. The additive has been analysed separately by addition of the additive without NOAC to normal donor plasma and analysed using the ACL Top. The PT ratio generated by the plasma pool spiked with the chemical was 0.98 and without the chemical 1.0 indicating that the increased PT ratios observed in the plasma pools spiked with NOACs represent the effect of NOACs on the plasma coagulation, and not the additional chemical.

The PT ratios are higher for NOAC X than for NOAC Y, see Figure 4. A higher PT ratio means that less amount of NOAC can be added to the controls, which is one important factor to take into consideration when choosing NOAC to work with. Plasma from normal donors have a ratio of approximately 1 and the controls should reflect treated patients, not normal donors without NOACs. Therefore, the PT ratio of the low level control needs to be clearly separated from 1. A reference interval will be established during the validation of the reagent. Figure 4 shows that NOAC X generates the highest ratio in relation to the concentration added, and is therefore chosen for further analysis and the prototype production.

**Figure 4**: PT ratios of plasma from patients treated with NOAC X and Y, and plasma spiked with NOAC X and Y to simulate treated patients.

NOAC X has a sustainability of two years according to the supplier and the current stock solution has been stored for two years and three months at -20 degrees. To evaluate if the inhibiting effect has been reduced during the storage of two years and three months, a comparison was made between the old NOAC X and new NOAC X from the same supplier. The new stock solution of NOAC X has been dissolved in the same way as the old NOAC X stock solution. The old NOAC X drug was analysed twice, one time before ordering the new NOAC X, and one time together with the new NOAC X, see Figure 5. The first analysis with the NOAC X (stored in the freeze at
-20 degrees for two years and three months) is called NOAC 1X old and the second is called NOAC 2X old in Figure 5A. A similar comparison has been made between the old and new NOAC X, a plasma pool has been divided in two parts and spiked with the old and the new NOAC X, see Figure 5B.

The PT ratios were similar for NOAC X new and old, indicating that the stock solution can be stored at -20 degrees for two years and three months and used for production of NOAC controls. Furthermore, PT ratios generated from plasma pools spiked with NOAC X old at two separate occasions are also similar, indicating that the methodology might be reproducible.

![Figure 5: Plasma from normal donors spiked with A) old NOAC at two different times and B) new (stored two weeks) and old (stored 27months) NOAC X.](image)

4.3 Comparison to patient samples

NOAC X from four different suppliers was used for production of high level controls. The number after X represents which supplier it is. NOAC X4, a drug from the fourth supplier, arrived very late and all planned analyses have not been made. Therefore, NOAC X from three suppliers was added to plasma pools and compared with four selected patient samples, with known concentrations of NOACs for NOAC X1 and X2 and three patient samples for NOAC X3, see Figure 6. The methodology used is described more in detail in section 4.2 Choice of drug.

Plasma pools spiked with NOAC X2 and NOAC X3 show a linear relationship between the added amount and the PT ratio, shown in Figure 6B and 6C. The PT ratio for NOAC X2 is slightly higher than the PT ratio for NOAC X3. A comparison between the suppliers of NOACs shows that NOAC X1, see Figure 6A, generates the highest PT ratio for the sample with the highest concentration of drug (Figure 6D). Analysis of plasma pools spiked with NOAC X1 also indicate an exponential behaviour at high concentrations (Figure 6A) in contrast to NOAC X2 and X3 which indicate a linear relationship (Figure 6B and 6C). The data indicate a linear relationship for NOAC X from supplier 1, 2 and 3 up to 250 µg/L.
The PT ratios generated from the spiked plasma pools are comparable with the PT ratios generated from patient samples for the two lowest concentrations, but this is not true for the higher concentrations. The PT ratios generated from the spiked plasma pools are lower than PT ratios generated from patient samples for the two highest concentrations, indicating that the method is not yet fully optimal.

Figure 6: Plasma from normal donors spiked with NOAC X, light blue and grey dots) manufactured by three different suppliers (A, B, C) to evaluate dose response compared with patient samples (shown in dark blue dots). The assay results (A, B, C) are merged in figure 6D.
4.4 Reconstitution

A single NOAC control with a high concentration of NOAC X from each supplier was lyophilised and reconstituted in 1 ml water with two different methods. The first method included addition of 1 ml of water to the vial, an incubation for 30 minutes, followed by careful stirring and inversion for three times before analysis. The second method included addition of 1 ml water to the vial followed by an immediate careful stirring, an incubation for 30 minutes followed by careful stirring and inversion three times before analysis.

An ocular inspection of the solubility was done, and all NOAC X from the four suppliers were soluble after reconstitution, with an exception of NOAC X4 where a small thread or lump was observed. The two methods were similar and the first method was chosen for further use in the project, as MediRox’s other controls utilise the first method for reconstitution and it will be easier for the customer if the method for reconstitution is identical for all controls.

4.5 Concentration determination

The lyophilised controls were sent to an accredited laboratory to determine the concentrations. Table 2 shows the differences from the theoretical value for the high level controls with NOAC X from four suppliers. The concentration is the same for the four high level controls. To be just as good as the competitor with the narrowest acceptable difference in concentration, a difference of +/- 25% from the theoretical value was considered as acceptable. Each concentration was determined twice with a chromogenic substrate method and the average concentration have been used to recalculate the concentrations in the stock solutions.

Table 2 shows that the concentrations of NOAC X in the high level controls determined by a chromogenic substrate method are all within the specification (difference from the theoretical concentration < +/- 25%), indicating that the chosen method is applicable. The low, medium and high level controls were produced twice based on the recalculated concentration in the stock solution for NOAC X1, X2 and X3, but only once for NOAC X4 due to a delayed delivery of the drug from supplier 4.

Controls with low, medium and high levels of the drug were produced for supplier 1-3 based on the determined concentrations of NOACs in the stock solutions. The controls were sent to the same accredited laboratory to determine the concentrations of NOAC in the low, medium and high level controls. The differences from the theoretical concentration are presented in Table 3. All values are within the specification of +/- 25%.
After the quantification of NOAC X in the NOAC controls an overlap in the selected ranges for the medium and high control levels were noticed. This was a mistake in the design and the target concentration was adjusted for one of the levels. A new production of NOAC controls with low, medium and high levels were made using the recalculated concentrations in the stock solutions. NOAC X from four suppliers were included in this production. The concentrations were determined by the same accredited laboratory as described above for the first and second production of controls. All NOAC controls showed assay results within the acceptable limit of $\pm 25\%$ from the theoretical value. NOAC X4 from supplier 4 showed results closest to the theoretical concentration. The high level controls based on drugs from supplier 1-3 showed a higher difference between the determined concentration and the theoretical concentration at this production as compared to the previous one. In contrast, the difference between the determined concentration and the theoretical concentration for the low and medium level controls was reduced for the NOAC X from the same suppliers respectively.

Table 2: Differences in the determined concentration from the theoretical concentration for the high level NOAC control for NOACs from four suppliers.

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Level</th>
<th>Differences from theoretical value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>High</td>
<td>1%</td>
</tr>
<tr>
<td>2</td>
<td>High</td>
<td>9%</td>
</tr>
<tr>
<td>3</td>
<td>High</td>
<td>17%</td>
</tr>
<tr>
<td>4</td>
<td>High</td>
<td>-23%</td>
</tr>
</tbody>
</table>

Table 3: Differences in the determined concentration from the theoretical concentration for the low, medium and high level NOAC control for NOACs from three suppliers.

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Level</th>
<th>Differences from theoretical value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>21%</td>
</tr>
<tr>
<td>1</td>
<td>Medium</td>
<td>23%</td>
</tr>
<tr>
<td>1</td>
<td>High</td>
<td>9%</td>
</tr>
<tr>
<td>2</td>
<td>Low</td>
<td>21%</td>
</tr>
<tr>
<td>2</td>
<td>Medium</td>
<td>12%</td>
</tr>
<tr>
<td>2</td>
<td>High</td>
<td>0%</td>
</tr>
<tr>
<td>3</td>
<td>Low</td>
<td>17%</td>
</tr>
<tr>
<td>3</td>
<td>Medium</td>
<td>13%</td>
</tr>
<tr>
<td>3</td>
<td>High</td>
<td>-1%</td>
</tr>
</tbody>
</table>

Table 4: Differences in the determined concentration from the theoretical concentration for the low, medium and high NOAC control for NOACs from four suppliers.

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Level</th>
<th>Differences from theoretical value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Low</td>
<td>19%</td>
</tr>
<tr>
<td>1</td>
<td>Medium</td>
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<td>17%</td>
</tr>
<tr>
<td>2</td>
<td>Low</td>
<td>15%</td>
</tr>
<tr>
<td>2</td>
<td>Medium</td>
<td>9%</td>
</tr>
<tr>
<td>2</td>
<td>High</td>
<td>10%</td>
</tr>
<tr>
<td>3</td>
<td>Low</td>
<td>12%</td>
</tr>
<tr>
<td>3</td>
<td>Medium</td>
<td>1%</td>
</tr>
<tr>
<td>3</td>
<td>High</td>
<td>4%</td>
</tr>
<tr>
<td>4</td>
<td>Low</td>
<td>4%</td>
</tr>
<tr>
<td>4</td>
<td>Medium</td>
<td>0%</td>
</tr>
<tr>
<td>4</td>
<td>High</td>
<td>1%</td>
</tr>
</tbody>
</table>
4.6 Effect of lyophilisation

The effect of lyophilisation has been evaluated by comparing the PT ratio and seconds before and after lyophilisation. For the study of the effect of lyophilisation the controls showed in Table 3 are used. The PT ratios generated before and after lyophilisation for the low, medium and high level controls (Figure 7) indicate minor effects of lyophilisation for NOACs from supplier 1-3. The same calibration was used in order to study the effect of lyophilisation only. The effect of lyophilisation measured in seconds was <4.95% for the low, medium and high controls from supplier 1-3.

![Figure 7: Effect of lyophilisation by analysing the PT ratio before (light lines) and after (dark lines) for the low, medium and high level controls for suppliers 1-3.](image)

4.7 Stability

A stability study was done using two different methods, described in detail in the method section 3.2.4 Stability. The stability study using the first method, comprising several controls in different vials reconstituted at the same time analysed at five time points, indicates that all NOAC controls are stable at room temperature for 24 hours after reconstitution, see Table 5. The recovery of the PT ratio at zero, three, six, 24 and 48 hours are <+/- 10% for all NOAC controls tested, regardless of selected drug supplier, with one exception. The high level NOAC control from supplier 3 showed a recovery of 111% at 48 hours. NOAC controls produced using NOAC X4 showed a
recovery of +/-3% indicating that very stable controls can be produced using NOAC X from supplier 4.

Table 5: Stability of NOAC controls with 100% stability at time zero then the stability calculated based on the zero point with measurements on a different vial at each time point.

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Level</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>24</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>High</td>
<td>100%</td>
<td>100%</td>
<td>101%</td>
<td>100%</td>
<td>106%</td>
</tr>
<tr>
<td>1</td>
<td>Medium</td>
<td>100%</td>
<td>102%</td>
<td>102%</td>
<td>103%</td>
<td>106%</td>
</tr>
<tr>
<td>1</td>
<td>Low</td>
<td>100%</td>
<td>102%</td>
<td>100%</td>
<td>99%</td>
<td>104%</td>
</tr>
<tr>
<td>2</td>
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<td>100%</td>
<td>98%</td>
<td>99%</td>
<td>103%</td>
</tr>
<tr>
<td>2</td>
<td>Medium</td>
<td>100%</td>
<td>95%</td>
<td>97%</td>
<td>99%</td>
<td>101%</td>
</tr>
<tr>
<td>2</td>
<td>Low</td>
<td>100%</td>
<td>101%</td>
<td>99%</td>
<td>103%</td>
<td>103%</td>
</tr>
<tr>
<td>3</td>
<td>High</td>
<td>100%</td>
<td>99%</td>
<td>96%</td>
<td>98%</td>
<td>111%</td>
</tr>
<tr>
<td>3</td>
<td>Medium</td>
<td>100%</td>
<td>101%</td>
<td>99%</td>
<td>101%</td>
<td>104%</td>
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<tr>
<td>3</td>
<td>Low</td>
<td>100%</td>
<td>102%</td>
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<tr>
<td>4</td>
<td>High</td>
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<td>100%</td>
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<td>97%</td>
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<td>101%</td>
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<tr>
<td>4</td>
<td>Low</td>
<td>100%</td>
<td>97%</td>
<td>97%</td>
<td>98%</td>
<td>102%</td>
</tr>
</tbody>
</table>

The stability study using the second method, (measurement of the PT NOAC ratio in the same control material at five different occasions, zero, three, six, 24 and 48 hours after reconstitution of the controls) indicates that all NOAC controls are stable at room temperature for 48 hours after reconstitution (Table 6), with an exception for the low level NOAC control produced with NOAC X from supplier 3, where a recovery of 110% was observed. Measurements at six and 24 hours after reconstitution of the controls are missing for method two due to technical problems with the ACL Top.

Table 6: Stability of NOAC controls with 100% stability at point zero then the stability calculated based on the zero point with measurements on the same vial at each time point.

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Level</th>
<th>0</th>
<th>3</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>High</td>
<td>100%</td>
<td>101%</td>
<td>92%</td>
</tr>
<tr>
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<td>Medium</td>
<td>100%</td>
<td>101%</td>
<td>101%</td>
</tr>
<tr>
<td>1</td>
<td>Low</td>
<td>100%</td>
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<td>98%</td>
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<td>105%</td>
<td>102%</td>
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<td>100%</td>
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<td>2</td>
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<td>High</td>
<td>100%</td>
<td>103%</td>
<td>106%</td>
</tr>
<tr>
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<td>99%</td>
<td>101%</td>
</tr>
<tr>
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<td>100%</td>
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<td>110%</td>
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<td>100%</td>
<td>107%</td>
<td>101%</td>
</tr>
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<td>Medium</td>
<td>100%</td>
<td>101%</td>
<td>99%</td>
</tr>
<tr>
<td>4</td>
<td>Low</td>
<td>100%</td>
<td>97%</td>
<td>94%</td>
</tr>
</tbody>
</table>
4.8 Summary suppliers

A comparison between the NOAC controls produced with NOAC X from four suppliers are summarised in Table 7. A plus (+) represents an approval of the test and a minus (–) represents a disapproval of the test. Data for NOAC X from supplier four are not complete due to a late arrival of the product. Thus, supplier 4 got a – on delivery time in Table 7. Supplier 4 also got a – on the reconstitution due to the observed lump/thread in the control material at one occasion. From this summary, supplier 2 is the one that passes all tests without any doubts.

Table 7: Summary of suppliers with + for a positive test and – for a negative test. Supplier 4 misses result for two tests due to late arrival.

<table>
<thead>
<tr>
<th>Assays</th>
<th>X1</th>
<th>X2</th>
<th>X3</th>
<th>X4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delivery time</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Price</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Comparison to patient samples</td>
<td>+*</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Reconstitution</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Concentration determination</td>
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<td>+</td>
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<td>Effect of lyophilisation</td>
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<td>+</td>
<td></td>
</tr>
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<td>Stability method 1</td>
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<td>+</td>
<td>+**</td>
<td>+</td>
</tr>
<tr>
<td>Stability method 2</td>
<td>+</td>
<td>+</td>
<td>+**</td>
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* Showed similar results for the comparison between spiked plasma pools and patient samples. Results indicate an exponential graph instead of linear (as the patient samples).
** Recovery >10% 48h after reconstitution.
5 Discussion

The purpose of this project was to develop NOAC control prototypes to provide MediRox with knowledge of the methodology for production. The results indicate that the method for production of NOAC control prototypes is applicable for low, medium and high concentrations of NOAC, there is a minor effect of lyophilisation and the product is stable for 24 hours. Based on this data MediRox has decided to initiate a product development project to further develop the NOAC assay control prototypes.

After consulting a medical advisor and the representative at the marketing and sales office at MediRox, a product specification of a three level control was decided for the NOAC control prototypes in this project. The rationale is, that a three level control will reflect treated patient groups, with low, medium and high concentrations of NOACs. The design of the three control levels, based on patient plasma levels is quite complex, as NOACs do not have specific therapeutic intervals and the exposure interval vary from person to person (Al-Aieshy, et al., 2016) (Skeppholm, et al., 2015) (Skeppholm, et al., 2014). The concentration of NOAC, rivaroxaban, in plasma has shown to vary between 4-400 µg/L in a group of 60 patients (Al-Aieshy, et al., 2016). The key account manager at the marketing and sales office recommended a tree level control as the use of NOACs are predominant in Scandinavia, Europe and USA. In general, clinicians and laboratory technicians in these regions are prioritising high quality and a three level control will most likely be appreciated for assay control as compared to a two level control.

Another important aspect of the product design is the choice of limits for the control levels. Limits established for similar products by competitors are considered as a good comparison. The competitor with the narrowest limits had +/- 25% from the target concentration, which is why the NOAC controls have the same limit in this project, as MediRox’s product should be equally as good or better than competitor’s products. It would be desirable to adjust the limits in the future, to more narrow limits, to decrease the lot-to-lot variation. The results in this project indicate that this might be difficult, as the differences between the theoretical concentration and determined concentration ranged from -23 to + 23% (Table 2 and 3). It is important to remember that the amount of data is limited so far, and more research is needed.

The stability requirement for the NOAC controls has also been established after a comparison with the stability of similar products manufactured by other companies. The stability varied between 24-48 hours. Thus, the stability study is done for 48 hours but the stability target is 24 hours, which is identical to the stability claim for MediRox’s current coagulation controls.

As previously pointed out, the NOAC controls should reflect treated patients and a NOAC should preferably be added to the controls. Two different NOACs, NOAC X and NOAC Y, were evaluated when choosing a drug to use, see Figure 4. These two drugs were selected for evaluation, based on previous internal evaluations of these drugs in combination with findings in the literature. NOAC X has higher PT ratio compared to NOAC Y (Figure 4) and is therefore chosen for method optimisation and NOAC control prototype production. The advantage is clear, a smaller amount of drug is needed which is favourable for future production cost for the product.
Authentic plasma samples from normal donors were used in the design of the low level prototype to ensure that the PT ratio for the low level control is clearly separated from the untreated group, avoiding potential misinterpretations. Plasma samples from patients treated with NOACs were selected and used as a guideline for the PT ratios generated by the NOAC controls. The PT ratios for the NOAC-containing patient samples were analysed using a Sysmex 2100 and the spiked plasma pools/NOAC controls were analysed with ACL Top 700 and with different reagent lots. These differences might influence the MNPT and ISI-values, possibly influencing the PT ratios.

Lindahl and colleagues have shown a linear relationship between the PT ratio, using a similar PT method, and the added concentration of NOAC (Lindahl, et al., 2017). In accordance with this, a linear relationship was observed up to a limit for the PT ratio when NOAC X and NOAC Y were added to plasma pools, and analysed with MRX PT NOAC reagent prototype, see Figure 4.

The PT ratios in Lindahls study showed, for example, PT ratios between 1.1-1.3 for all NOACs at 100 µg/L and PT ratios between 1.3-2.0 for all NOACs at 400 µg/L analysed on the point of care instrument Simple Simon® (Lindahl, et al., 2017). Comparing the results from that study with this study, PT ratios generated from MRX PT NOAC reagent prototype were in general higher than PT ratios generated from the PT reagent used in Lindahls study, for samples with the same concentration of NOAC. This indicate that the sensitivity for NOACs is higher for MRX PT NOAC than the other studied PT reagent, but lot of factors can influence the assay sensitivity such as choice of instrument.

The PT ratios for the two lowest concentrations of NOAC X and NOAC Y in the spiking experiments are similar to the corresponding concentrations in patient plasmas with NOAC. The NOAC controls are designed to report PT ratios within this interval, where the method conformity is satisfying, but very high concentrations for NOAC X and NOAC Y spiked in plasma showed a rather high discrepancy. This indicate that further assay optimisation is needed. It is also important to remember that there is a variability in factor concentrations between individuals, possibly influencing the extrinsic pathway and the PT ratio.

A previous study has shown that plasma pools spiked with NOACs give a linear relationship between the added amount of NOAC and the PT ratio, with a similar method (Lindahl, et al., 2017). Even though the methods cannot be compared completely, a linear relationship is expected for NOAC drugs using PT ratio assay. NOAC X1, Figure 6A, did not show linearity which was unexpected due to previous study, it showed to be more exponential. NOAC X2, Figure 6B, and NOAC X3, Figure 6C, showed both linearity which was expected. They are all compared with each other, in Figure 6D, where NOAC X1 has the highest ratio, NOAC X3 has the lowest ratio and NOAC X2 showed a PT ratio between NOAC X1 and NOAC X3. Even though NOAC X1 is exponential it is the most similar graph in comparison with treated patients. A larger number of patient samples need to be analysed to determine if patient plasmas show linearity or an exponential behaviour. In this case the patient samples tend to be exponential but this can be a coincidence and it is a thin basis to draw any conclusions from. It would be optimal to analyse more patient samples to elucidate if they act exponentially or linear. Therefore, it would be an advantage to analyse a larger number of patient samples and calculate the equation from the straight line based on these samples. The spiked plasma pools can be compared with this line and a more accurate
comparison between the patient samples and the spiked samples would have been done. The mean peak value of NOAC in treated patients are about 250 µg/L and the PT ratios generated from all NOAC suppliers shows linearity for concentrations up to 250 µg/L (Mani, 2014). Thus, no major differences can be observed for PT ratios between the NOAC suppliers in this range.

NOAC X1 and NOAC X2 were analysed at the same time and NOAC X3 at another occasion. This means that NOAC X1 and NOAC X2 are analysed using the same plasma pool and reagent as compared to NOAC X3, which can have a minor impact. The differences observed between suppliers of the same NOAC X can occur due to several things. The drug arrived as a powder from all suppliers and stock solutions have been made from powder from each supplier. The differences can be caused by the weighting of the powder, different purity, additives, etc.

In summary, the method needs to be further optimised to increase the PT ratio in the plasma pools spiked with very high concentrations of NOAC X, regardless of the choice of supplier, in comparison to PT ratios generated from patient samples with corresponding NOAC concentration.

MediRox sells other controls today, all are lyophilised, which the NOAC controls also will be. After reconstitution with 1 ml water the solubility for each control was ocular inspected. All control material were soluble expect from NOAC X4 that had a thread or lump in it. Each time a NOAC X4 control was reconstituted special attention was paid to the control material in the vial. The thread or lump has only been observed once, so far. Thus, it could be a coincidence but it could also mean that the drug is not completely soluble after lyophilisation.

The concentration determination of the NOAC controls was done by an accredited laboratory. There is always an uncertainty for all methods and the concentrations for the NOAC controls are based on one single measurement. The concentration of NOAC X can be determined using LC/MS-MS, which is considered as the golden standard. Method comparisons have shown statistically significant correlations (with $R^2$-values>0.9) between chromogenic substrate methods and LC/MS-MS (Letertre, et al., 2016).

The determined concentrations were chosen to be the “true concentration” and the concentrations in the stock solutions were recalculated based on the determined concentrations. Thus, the concentrations presented in Table 2, based on the “true concentrations” were expected to show a large degree of similarity between the theoretical concentrations and the determined concentrations of NOAC controls produced by NOAC X with “true concentrations”, but they were not. When the concentrations were recalculated and determined for NOAC X1/2/3 more exact values were expected for all levels. The determined concentration for the low and medium level controls showed a larger difference from the theoretical concentration as compared to the high level controls. The differences can occur due to different purity of drugs, at the weighting of the drug, when dissolving it. The calculations and the laboratory protocol used at the production of low and medium controls have been carefully inspected and the pipettes used at the production of the controls have recently been calibrated. Thus, the differences in the determined concentration for the low and medium level controls from the theoretical concentration, are most likely not caused by these factors. The differences in the determined concentration from the theoretical
concentration can also be influenced by the method uncertainty of the chromogenic substrate method used in the accredited central hospital laboratory, with a more predominant uncertainty at low concentrations.

The comparison between similar products from different competitors showed that the competitor with the narrowest acceptable limits of the difference from the theoretical concentration is +/- 25\% using a comparable method. This indicates that it is difficult to produce products with narrow cut offs, reflected by a rather high variation in the estimated concentrations, which is possibly the reason for competitors’ high limit values. This indicates that it is difficult to achieve similar concentrations at repeated trials. One reason can be that the drug is hard to solve, possibly causing variation in concentrations. A pattern is observed between the high, medium and low level controls in Table 3, namely that the deviation in \% between the determined and theoretical concentration is smallest for the high control level, followed by the medium level control and last low level control. This means that the NOAC controls with the lowest concentration have greater variation. This trend of greater variation with lower concentration seen in Table 3 could not be observed in the following production of NOAC control prototypes, presented in Table 4. The variation was generally lower for all control levels from the second production (Table 4) as compared to the variation for controls from the first production (Table 3). Even though the concentrations vary, they are within the acceptable limit of +/- 25\%, indicating that the method used is applicable in the used range of concentrations. Further adjustments in the target concentrations, to achieve a more significant separation between the controls, method optimisation, etc are needed.

Lyophilisation can effect the PT in plasma (Poller, et al., 1999), but the effect of lyophilisation on PT in plasma with NOACs are, to the best of our knowledge, unknown. The effect of lyophilisation on plasma with and without coumarin has been studied using PT expressed in seconds. Coumarin is also an anticoagulant and the result showed an extended clotting time of 15.97\% and increased INR levels after lyophilisation for PT (Poller, et al., 1999). The INR values are increased due to a prolongation of the clotting time. This effect was used as an upper limit for the effect of lyophilisation for plasma spiked with NOAC X. To the best of our knowledge, there are no studies on the effect of lyophilisation in plasma pools from patients treated with NOACs. The effect of lyophilisation measured in seconds was <4.95\% for the low, medium and high controls from supplier 1-3. Furthermore, the results presented in Figure 7, indicate a minor effect on the PT ratio using plasma with NOAC. When taking these results in to consideration, the effect of lyophilisation using NOAC X from supplier 4 was assumed to show the same pattern as NOAC X from supplier 1-3.

The stability study was performed using two different methods, one including the effect of analysis in several control vials and the effect of usage of several different reagent vials (method 1), see Table 5, and one method excluding the effect of analysis in several control vials, excluding the effect of usage of several different reagent vials (method 2), see Table 6. There are advantages and disadvantages with both methods, as it is difficult to conduct a stability study without using several vials of either the reagent or the controls. The optimal method to reduce reagent variation is to reconstitute control vials of each level at each time point of the stability. Then analyse them all together at time zero with the same regent.
The results generated using method 1 and 2 are conclusive. All NOAC controls were stable (recovery % < +/- 10%) for 48 hours after reconstitution with both methods, with an exception for the high control level with NOAC X from supplier 3. One reason for the increased recovery at 48 hours for the NOAC control from supplier 3 can be a degradation of proteins in the plasma pool, a loss in their tertiary structure and thereby a reduction in activity causing prolonged clotting times. This phenomenon can not only be explained by a degradation of plasma proteins including coagulation factors, as the same plasma pool was used for production of NOAC controls for all suppliers.

Thus, the stability study was done for 48 hours but the stability target is 24 hours, which is identical to the stability claim for MediRox’s current coagulation controls. Thus, NOAC control prototypes using NOACs from all suppliers were within the specification.

It is also important to evaluate the stability of the stock solutions stored at -20°C. This comparison was possible using NOAC X from supplier 3. According to the supplier of NOAC X, it has a sustainability of two years in -20 °C. The old stock solution (>2 years) was compared with a new NOAC X stock solution and similar PT ratios were observed (Figure 5). This indicates that the stock solutions might be stored and used for control production, without a major decrease in effect. Further stability studies are needed for the stock solutions and NOAC controls regarding in use stability, shelf life, etc.

A comparison between the performance of NOAC X from the different suppliers are presented in Table 7. At the beginning of the project, it was assumed that it would be easier to distinguish between NOAC X from the suppliers from each other, but it was not. Even though the technical performance as well as prices were approximately the same, supplier 2 might be slightly favorable as it passed all the assays, Table 7. It is not considered as a disadvantage that a supplier could not be selected due to similar technical performance, other parameters as price and delivery time should be investigated further to make a final decision.
6 Conclusion

This project provides a good foundation for a future product design of NOAC controls. The methodology for production of NOAC control prototypes has been optimised, knowledge has been gained about the usage of additives, effect of lyophilisation and stability. The data indicates that the effect of lyophilisation is minimal and the stability of the controls are satisfying, \textit{i.e.} at least 24 hours, which is promising. MediRox has decided to initiate a product development project based on this data for further develop of NOAC assay controls.

The main goal of the project was to develop prototypes of controls which have been done. Now it is up to MediRox to decide which supplier to use. Except from that, all activities have been successfully fulfilled in this project.

One of the main results indicate that the method used for production of the NOAC control prototypes is applicable for low, medium and high concentrations but needs to be further optimised to generate PT ratios similar to patient samples with very high concentrations of NOAC. Analysis of a larger number of patient samples and further optimisation of the methodology are recommendations for further work to increase the conformity for the NOAC control prototypes. Furthermore, a minor adjustment in the concentrations added, are recommended to more clearly separate the control levels from each other. Lyophilisation of the NOAC control prototypes showed PT ratios nearly unaffected of the lyophilisation process. Further characterisation of the lyophilisation process is recommended, for example analysis of residual water, to ensure an optimal production process for the future product. The stability of the NOAC control prototypes should be more carefully studied, including both methods, several batches, etc.
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