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Importance of measuring pharmacologically active metabolites of edoxaban: development and validation of an ultra-high-performance liquid chromatography coupled with a tandem mass spectrometry method.

Running short title: Simultaneous quantification of edoxaban and edoxaban-M4 metabolite by ultra-high-performance liquid chromatography-tandem mass spectrometry method in human plasma.

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Keywords: Edoxaban, Edoxaban-M4, Ultra-High-Performance Liquid Chromatography/tandem mass spectrometry, Metabolite, Direct Oral Anticoagulant

Abstract

Introduction: Although DOACs do not require regular measurements of their blood concentrations, clinical situations may require an assessment of their concentration. Among the factor Xa inhibitors, edoxaban is the only compound for which some metabolites (e.g. edoxaban-M4) are reported to be pharmacologically active. Therefore, their contribution could interfere with assays used for the estimation of edoxaban concentration. In addition, drug interactions may alter the metabolite/parent compound ratio making the sole estimation of edoxaban concentration, a poor assessment of the overall anticoagulation.

Aims: To develop a validated UHPLC-MS/MS method to quantify simultaneously edoxaban and its more relevant M4-metabolite in human plasma.

Methods: Electrospray ionization and chromatographic separation were optimized for the simultaneous dosage of edoxaban and edoxaban-M4. The method was validated according to regulatory guidelines for bioanalytical method validation.

Results: The total run time was 6 minutes. The method was validated for calibration curves, precision, accuracy, carry-over, selectivity, matrix effect and short-time stability.

Conclusion: This method permits quantification of edoxaban and edoxaban-M4 providing complementary information about the inhibitory effect of this active metabolite in chrometric or chromogenic assays. Although patients treated with edoxaban exhibits usually low concentrations of active metabolites, the measurement of edoxaban-M4 is interesting; especially in case of drug interactions. Indeed, concomitant prescriptions of edoxaban and carbamazepine or rifampicin is frequent and may lead to disturbance of the estimations of edoxaban concentration by chromogenic anti-Xa assays. Therefore, patients are at risk of having inadequate control of anticoagulation supporting the need of measuring the most representative edoxaban metabolite concomitantly to the parent compound.

Abbreviations

%Dev	Percentage of relative standard deviation
CV	Coefficient of variation
DMSO	Dimethyl sulfoxide
DOACs	Direct oral anticoagulants
EMA	European Medicines Agency
ESI	Electro spray ionization
ESI+	Electro spray on positive ionization mode
FDA	Food and Drug Administration
ICH	International Council of Harmonization
IS	Internal standard
LC	Liquid chromatography
LLOQ	Lower limit of quantification
ME	Matrix effect
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NPP	Normal pooled plasma
PBS	Phosphate buffer saline
PPP	Platelet poor plasma
QCs	Quality controls
UHPLC-MS/MS	Ultra-high-performance liquid chromatography coupled with mass spectrometry
ULOQ	Upper limit of quantification
VKAs	Vitamin K antagonists
VTE	Venous thromboembolisms

1. Introduction

To date, ultra-high-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS/MS) is quite expensive and time consuming, and thus measurements of DOACs have been performed with chromogenic FXa- or FIIa-based assays [1-8]. These tests initially responded to the clinical need by providing good sensitivity, high reproducibility and a short turn-around time [3]. Nevertheless, such tests are not able to discriminate between the inhibitory effect of the parent compounds and its metabolite which can become clinically relevant as already demonstrated with dabigatran and its acylglucuronide metabolite [9]. While apixaban and rivaroxaban do not have active metabolites, this is not the case for edoxaban [10]. Edoxaban received its market authorisation in Europe in June 2015 under the brand name of Lixiana[®] for the prevention of stroke and systemic embolism in patients with non-valvular atrial fibrillation as well as for the treatment of venous thromboembolisms (VTE) and pulmonary embolism (PE) [5]. Edoxaban has the particularity to release active metabolites which could be present at a sufficient level in the blood to have an impact on haemostasis. These metabolites are named edoxaban-M4, -M6 and -M8 [11]. The metabolism occurs through carboxylesterase-1 (CES-1), cytochrome P450 3A4/5 (CYP3A4/5) and non-enzymatic hydrolysis [12]. Although DOACs do not require regular measurements of their blood concentrations, clinical situations may require an assessment of their concentration (detection of drug accumulation in acute renal or hepatic failure; planning the timing of urgent invasive procedure; recurrence of stroke or bleedings). Currently, prothrombin time (PT) or activated partial thromboplastin time (aPTT) measurement are proposed for the estimation of the intensity of some DOAC. Nevertheless, these assays are strongly impacted by inter-reagent and inter-individual variability and must not be used in first intention to assess the intensity of anticoagulation with DOACs [5]. Current chromogenic tests for edoxaban are calibrated with standards containing 3 or 4 different levels of edoxaban. However, these standards do not include or consider active metabolites. This approach is questioning since, according to the Food and Drug Administration (FDA) guideline on Bioanalytical Validation [13], a validated method should meet the acceptance criteria regarding selectivity and specificity. Chromogenic anti-Xa assays have been reported to be sensitive to the presence of the M4-metabolite [14] and thus, the specificity criteria is certainly an issue. Normally, the assay should be free of potential interfering substance including metabolites [13]. This lack of specificity could lead to overestimation or underestimation of edoxaban concentrations depending on the clinical situation. Namely, the inhibitory constant (K_i) of these metabolites is not the same as the parent compound and thus, depending on the parent/metabolite ratio, the inhibitory effect on functional assay, like chromogenic anti-Xa assay, for a same initial amount of edoxaban may differ. Therefore, there is a need to develop sensitive and specific assays for edoxaban [3, 5].

In this context, we developed a validated UHPLC-MS/MS method to measure concentration of edoxaban and its active major metabolite-M4 in human plasma [13, 15].

2. Material and Methods

2.1. Preparation of the Normal Pooled Plasma (NPP)

Sixty individuals were included in the study. The exclusion criteria were thrombotic and/or hemorrhagic events, antiplatelet and/or anticoagulant medication, pregnancy and uptake of drugs potentially affecting the platelet and/or coagulation factor functions during the two weeks prior to the blood drawn. The study protocol is in accordance with the Declaration of Helsinki and has been approved by the Ethical Committee of the CHU UCL Namur, Yvoir, Namur, Belgium (B03920096633). Blood was taken by venipuncture in the antecubital vein and collected into 0.109M sodium citrate (9:1 v/v) tubes (Vacuette[®], Greiner Bio-One International, Kremsmünster, Austria) using a 21-gauge needle (Terumo, Leuven). The platelet poor plasma (PPP) was obtained from the supernatant fraction of blood tubes after a double centrifugation for 15 minutes at 2,500g at room temperature. Immediately after centrifugation, PPP from the 60 donors were brought together to obtain the NPP which was frozen at -80°C without any delay. Treatment of plasmas by proteins precipitation was performed within 4 hours after thawing [5].

2.2. Instrumentation and analytical conditions

All optimized parameters and protocol are provided in supplementary material (► **Supplementary material and Supplementary figures 1-2**).

2.3. Method validation

2.3.1. Calibration curve

Calibration curves were constructed from 3 to 500ng/mL for edoxaban and from 3 to 150ng/mL for edoxaban-M4 with six levels of non-zero concentration standards [13, 15]. Calibration ranges were chosen to cover (supra)-therapeutic ranges for edoxaban and edoxaban-M4. Calibration standards were run in triplicate in consecutive runs. The calibration curves were fitted using a weighted least-squares non-linear regression method by measuring the peak-area ratio of the analytes to the internal standard (IS). The acceptance criterion for each back-calculated standard concentration was $\pm 15\%$ deviation from the nominal value, except for the lower limit of quantification (LLOQ), for which a deviation of $\pm 20\%$ was permitted.

2.3.2. Precision and accuracy

The intra-day precision and accuracy of the assay were estimated by analysing in a single run of 3 replicates of each QC within 1 day. The inter-day precision and accuracy were assessed by repeating the analysis of three concentration levels of QC samples on 5 consecutive days. Accuracy was expressed as a percentage of relative standard deviation (%Dev), while the precision was expressed as the coefficient of variation (%CV). The accuracy and precision were required to be within a $\pm 20\%$ range for LLOQ and within a $\pm 15\%$ for higher concentrations of edoxaban. For edoxaban-M4 measurement, accuracy and precision were required to be within a $\pm 25\%$ range for LLOQ and within a $\pm 20\%$ for higher concentrations [13, 15].

2.3.3. *Carry-over*

The carry-over of analytes and IS were evaluated by injecting a blank sample immediately after a sample of upper limit of quantification. Carry-over should not exceed 20% of the signal of the LLOQ for analytes and 5% for IS [13, 15].

2.3.4. *Lower limit of quantification*

The LLOQ was defined as the lowest concentration of edoxaban or edoxaban-M4 that could be quantitatively determined with acceptable precision and accuracy. Acceptance limits were defined as accuracy of 80-120% and precision $\leq 20\%$.

2.3.5. *Selectivity*

Selectivity was evaluated using six independent blank human plasmas to confirm the absence of potential interfering substances in the matrix [13, 15]. Chromatograms were visually compared with the corresponding spiked plasma.

2.3.6. *Specificity*

Specificity was assessed to ensure that the method is free of interference due potential residual molecules (e.g. other anticoagulants) [13, 15]. Measurements of spiked samples with 100ng/mL apixaban, edoxaban, edoxaban-M4, rivaroxaban and dabigatran were performed in a single run to assess the potential interference by cross-reacting molecules.

2.3.7. *Matrix effect (ME%)*

To evaluate ME%, intermediate solutions were post-spiked in water or serum and compare with intermediate solutions post-spiked in NPP. The use of serum as alternative matrix is often encountered in clinical trials and the assessment of this matrix effect may inform on the possibility to perform the measurement in serum if needed.

2.3.8. Extraction recovery

Extraction recovery was determined by spiking known amounts of edoxaban, edoxaban-M4 and IS into NPP and into the extracted NPP and exposing the spiked NPP to the extraction procedure.

2.3.9. Short time stability

Edoxaban and edoxaban-M4 stability was assessed using QCs samples. Intermediate solutions of QCs were stored at -20°C and 4-7°C and plasmatic solutions were stored at -80°C and analysed on day 0 and after one month. Solutions were considered to be stable when average measured concentrations were within the limit of 80-120% of the nominal concentrations [13, 15].

2.4. Statistical analysis

The UHPLC-MS/MS calibration curve was performed using a non-linear regression with a weighting function of 1/x. Statistical analyses and graphics were computed using GraphPad Prism 5.0D[®] for Mac OS X.

3. Results

3.1. Optimization of the UHPLC-MS/MS method

MS detection parameters were optimized by direct infusion of the reference compounds using the electrospray interface in the positive mode for edoxaban and edoxaban-M4 (► **Table 1**). Selection of chromatographic conditions was optimized to suit an effective chromatographic resolution of analytes with symmetrical peak shapes, short retention time and overall short run time.

3.2. Method validation of the UHPLC-MS/MS method

3.2.1. Calibration curves, linearity and lower limit of quantification

According to results reported in ► **Supplementary figure 3**, non-zero calibrators were between ±15% of nominal concentrations and LLOQ were between ±20% of the nominal concentrations in each validation run. Although one calibration point is out of the range, 75% and a minimum of six non-zero calibrator levels meet the above criteria in each validation run [13, 15]. For routine purpose, the LLOQ was set at 3ng/mL for edoxaban and edoxaban-M4.

3.2.2. Precision and accuracy

Accuracy and precision obtained for inter-day and intra-day assays were within the limits of guidelines with calibration curves performed in NPP (►Supplementary table 1 & Supplementary figures 4-7).

3.2.3. Carry-over

There was no detectable carry-over for analytes and IS even at high concentration (500ng/mL).

3.2.4. Selectivity and specificity

No co-eluting components peaks from endogenous or exogenous compounds were observed at the retention time of edoxaban, edoxaban-M4 and [²H₆]-edoxaban (►Supplementary figure 8 & 9).

3.2.5. Matrix effect (ME%)

Non-relevant ME% (<15%) for edoxaban and IS were observed contrary to edoxaban-M4 measurements when these molecules were spiked in water. Serum matrix did not impact edoxaban and edoxaban-M4 measurements with our analytical method.

3.2.6. Stability

Stability of edoxaban and edoxaban-M4 was assessed for 28 days (-20°C) and for 155 days (4-7°C and -80°C). These compounds remained stable for the tested period and accuracy at each level was ±20% (some points are outside the range but represent less than 20% of all tested concentration levels) [13, 15].

4. Discussion

Although the scientific literature contains articles about liquid chromatography measurement of DOACs, few of them address the problem of the active metabolites and propose a simultaneous quantification of edoxaban and edoxaban-M4 in the same analytical run meeting criteria of the International Council of Harmonization (ICH), the European Medicines Agency (EMA) and FDA guidelines for ME, carry-over and stability [14, 16-18]. Our validation provides the pharmaceutical industries, the manufacturers and the physicians with a robust, sensitive and specific method for the measurement of edoxaban and its M4 metabolite.

Despite this must-needed validation, some limitations of have to be recognized. This method allows the measurement of edoxaban and edoxaban-M4 concentration without considering edoxaban-M6. Nevertheless, regarding the IC₅₀ (6.9nM) and the C_{max} of edoxaban-M6 (8.55ng/mL), the impact on chromogenic assays should

be negligible contrary to the impact of the edoxaban-M4 which has a lower IC_{50} (1.8nM) and a higher C_{max} (23.1ng/mL), in the absence of CYP inducers [10, 11, 19] in patients treated with edoxaban at 60mg once daily. Currently, no manufacturer has been able to offer us the edoxaban-M6 in sufficient quantity and with acceptable quality. Another limitation is that we only reported short time stability. Further investigations are underway to confirm the long-term stability of intermediate solutions of edoxaban/edoxaban-M4 and plasmatic solutions of analytes.

The different pharmacodynamic profiles and the interest of measuring the active metabolites

Among the factor Xa inhibitors, edoxaban is the only compound with pharmacologically active metabolites (edoxaban-M4, -M6 and -M8). These metabolites may contribute to the global anticoagulant activity and could interfere with chromogenic assays usually used for the estimation of the plasma level of edoxaban [5, 14]. Taking into consideration their respective IC_{50} towards human factor Xa, these metabolites would inhibit factor Xa at different degree. Namely the IC_{50} of edoxaban, and the metabolites-4 (M4; D21-2393), -6 (M6; D21-1402) and -8 (M8; D21-2135) are 3.0, 1.8, 6.9 and 2.7nM, respectively (►**Figures 1 & 2**) [10]. While these differences may only have a small impact in a population where the metabolic pathways are not inhibited or induced (the relative presence of edoxaban-M4 due to CES1 and edoxaban-M6 and -M8 due to CYP3A4/5 are less than 10% each [10]), the metabolite to parent ratio is increased in presence of inducers of CYP3A4/5 and P-gp. A potential interest of synchronously measuring edoxaban and edoxaban-M4 is to obtain complementary information about the impact of the active metabolite in chronometric or chromogenic assays.

This validated method could be applicable in common clinical situation or situations requiring a close monitoring of edoxaban and its most active metabolite. This is especially important since at low concentration (<30ng/mL) a deviation of more than 50% has been observed (anti-Xa vs LC-MS/MS) [14], suggesting that anti-Xa assays are not able to provide reliable results in these low values. This becomes of clinical interest in light of the low edoxaban concentrations observed at C_{Trough} in the various indications (►**Figure 3**) [3, 20-22]. Also, such low concentrations (i.e. 30 or 50ng/mL) are threshold for decision making (i.e. before urgent surgery with high bleeding risk or to guide antidote administration) and any inaccuracy may lead to mismanagement or inappropriate clinical judgments [23].

The issue of drug interactions

In addition, this technique could be interesting for adult population with drug-drug interactions which are frequently reported in the daily routine (e.g. co-treatment with quinidine, verapamil, ketoconazole, rifampin, ...)

[10, 12]. Indeed, pharmacokinetic interactions with edoxaban have already been highlighted, attesting that exposure to edoxaban and its active metabolites is significantly disturbed when edoxaban is administered with CYP3A4/5 inducers (e.g. rifampin) or inhibitors (e.g. cyclosporine, erythromycin) [10, 12, 14, 19, 24, 25] (►Table 2). For example, co-administration of rifampin and edoxaban leads to an increased concentration of edoxaban-M4 (23.1 ng/mL without rifampin vs 108ng/mL with rifampin) which is not formed through the CYP3A4/5 pathway. According to Mendell et al. the increased concentration is linked to a potential induction of CES1 through upregulation of the pregnane X receptor gene and to the inhibitory effect of rifampin on organic anion transporting polypeptide 1B1 (OATP1B1) [19].

Anti-epileptic agents are also frequently administered concomitantly with anticoagulant in the NVA setting [26]. Indeed, the cause of symptomatic epilepsy due to strokes and cerebrovascular diseases is estimated to be at 30-40% [27, 28]. With the exception of lamotrigine, pregabalin, zonisamide and lacosamide which have a safer pharmacokinetic profile, phenobarbital, phenytoin and carbamazepine may cause pharmacological interactions due to their impact on CYP3A4 and P-glycoprotein (P-gp) [28-30]. Although such drugs should be avoided, concomitant prescriptions of edoxaban and carbamazepine, first-intention treatment, still remains. Therefore, patients are at risk of exhibiting drug-drug interactions leading to inadequate control of the degree of anticoagulation or disturbed measurement of edoxaban concentration. This supports the need of measuring the most representative active metabolites according to special circumstances to know the real contribution of each compound in order to highlight abnormal edoxaban or metabolite levels [28-30].

To note, an interim analysis of our ongoing study (data not shown) evaluating the impact of co-administration of cytochrome 3A4 inducers and edoxaban on edoxaban-M4/edoxaban ratios, demonstrates that the concentration of edoxaban-M4 is increased compared to patients without such interactions. This increase is more pronounced at low concentrations of edoxaban and leads to a significant increase of the ratio edoxaban-M4/edoxaban. Deviations between chromogenic anti-Xa assessments and mass spectrometry measurements appears to be high in those with drug interactions (i.e. > 20%). Investigations are planned to confirm and compare these results to other chromatometric and chromogenic assays.

To conclude, the evidence from the literature and the data reported in regulatory documents that have led to the marketing authorization of edoxaban [31, 32] questions about the suitability of calibrators that do not consider the usual proportion of the active metabolites when reporting results as edoxaban equivalent.

5. **Conclusion**

In conclusion, this validated UHPLC-MS/MS method allows an accurate quantification of edoxaban and its active-metabolite, edoxaban-M4, in human plasma according to the recommendations of the ICH, EMA and FDA. Calibration ranges permit simultaneous estimations of edoxaban and edoxaban-M4 concentrations in patient's plasma. Although edoxaban treated patients exhibit usually low levels of active metabolites, some clinical situations may require an accurate estimation of the exposure for decision making. In addition, drug-drug interactions or hepatic insufficiency should stimulate investigations to explore abnormal levels of metabolites with different pharmacodynamic profile than edoxaban. Additional studies are needed to compared results obtained by this validated UHPLC-MS/MS method vs current coagulation/chromogenic assays in plasma from patients treated with edoxaban including those with drug interactions or with levels close to decision making thresholds.

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Conflict of Interest Disclosures

Among the authors, J. Douxfils is CEO and founder of QUALIblood s.a. and reports personal fees from Portola Pharmaceuticals, Stago, Roche, Roche Diagnostics and Daiichi-Sankyo, outside the submitted work. The other authors have no conflicts of interest to disclose.

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Table legends

Table 1: MS/MS parameters for edoxaban, edoxaban-M4 and corresponding internal standard

Table 2: Non-exhaustive list of drug-drug interactions with edoxaban

Figure legends

Figure 1: Postulated edoxaban metabolism for active metabolites

Figure 2: Chemical structure of edoxaban (DU-176b), edoxaban-M4 and internal standard ($[^2\text{H}_6]$ -edoxaban)

Figure 3: Laboratory testing of edoxaban and expected plasma concentrations after therapeutic doses

Tables

Table 1: MS/MS parameters for edoxaban, edoxaban-M4 and corresponding internal standard¹

Compound	Ion mode	Transition type	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)	Dwell time (s)
Edoxaban	ESI+	Quantification	548.212	152.169	40	32	0.035
	ESI+	Confirmation	548.212	366.19	40	20	0.035
Edoxaban-M4	ESI+	Quantification	521.162	321.176	38	24	0.035
	ESI+	Confirmation	521.162	339.12	38	18	0.035
[² H ₆]-edoxaban	ESI+	Quantification	554.316	158.160	32	30	0.035
	ESI+	Confirmation	554.316	372.27	32	18	0.035

¹ ESI+: Electrospray positive ionization mode

Table 2: Non-exhaustive list of drug-drug interactions with edoxaban²

	<i>CYP3A4/5 inducers</i>	<i>CYP3A4/5 inhibitors</i>	<i>Transporters inducers (P-gp, OATP, BCRP, OCT, ...)</i>	<i>Transporters inhibitors (P-gp, OATP, BCRP, OCT, ...)</i>
<i>Interactions</i>	Bosentan, carbamazepine, dabrafenib, efavirenz, enzalutamide, etravirine, lesinurad, perforate St John's wort, mitotane, modafinil, nevirapine, phenobarbital, phenytoin, pitolisant, primidone, rifabutin, rifampicin, rufinamide, telotristat, vandetanib	Amiodarone, aprepitant, atazanavir, ceritinib, clarithromycin, cobicistat, crizotinib, darunavir, diltiazem, erythromycin, fluconazole, fluoxetine, fluvoxamine, fosamprenavir, fosaprepitant, idelalisib, imatinib, itraconazole, ivacaftor, ketoconazole, lapatinib, lopinavir, netupitant, nilotinib, olaparid, pazopanib, grapefruit / pomelo, piperazine, posaconazole, ribociclib, ritonavir, roxithromycin, saquinavir, stiripentol, telaprevir, ticagrelor, tipranavir, verapamil, voriconazole	Carbamazepine, dabrafenib, perforate St John's wort, rifampicin, tipranavir	Alectinib, amiodarone, atorvastatin, azithromycin, cabozantinib, carfilzomib, ceritinib, ciclosporin, clarithromycin, cobicistat, crizotinib, daclatasvir, diltiazem, eliglustat, erythromycin, glecaprevir, itraconazole, ivacaftor, ketoconazole, lapatinib, nicardipine, pibrentasvir, ponatinib, propafenone, quinidine, ribociclib, ritonavir, saquinavir, ticagrelor, vandetanib, velpatasvir, venetoclax, verapamil, voxilaprévir
<i>Postulated impacts on edoxaban and metabolite concentrations</i>	The induction of cytochrome P450 isoenzymes 3A4/5 leads to a decrease of edoxaban concentration vs an increase of edoxaban-M6 and -M8 metabolites concentrations.	The inhibition of cytochrome P450 isoenzymes 3A4/5 leads to a stable of edoxaban concentration vs a decrease of normal edoxaban-M6 and -M8 metabolites concentrations. Edoxaban-M4 metabolite concentration tends to increase.	The induction of transporters leads to an increase of edoxaban concentration and an increase of edoxaban-M4, -M6 and -M8 metabolites concentrations.	The inhibition of transporters leads to a decrease of edoxaban concentration and a decrease of edoxaban-M4, -M6 and -M8 metabolites concentrations.

² 10. Parasrampurua, D.A. and K.E. Truitt, *Pharmacokinetics and Pharmacodynamics of Edoxaban, a Non-Vitamin K Antagonist Oral Anticoagulant that Inhibits Clotting Factor Xa*. Clin Pharmacokinet, 2016. **55**(6): p. 641-55. 24. CBIP, *Centre Belge d'Information Pharmacothérapeutique - Bon usage des médicaments - Interactions des médicaments*. 2018.

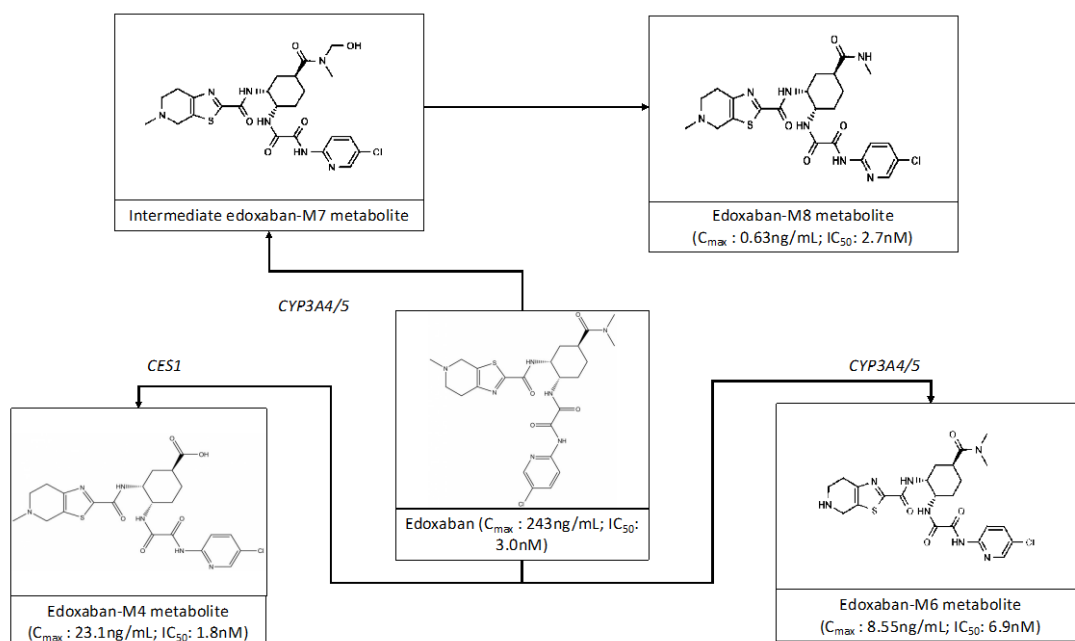
Supplementary table 1: Accuracy and precision for edoxaban and edoxaban-M4³

	Nominal concentration (ng/mL)	Within-Run Accuracy (%) - Day 1	Within-Run Accuracy (%) - Day 2	Within-Run Accuracy (%) - Day 3	Within-Run Accuracy (%) - Day 4	Within-Run Accuracy (%) - Day 5
<i>Edoxaban</i>	3	-9	16	-1	5	-3
	10	-14	2	1	-4	4
	75	-2	7	5	1	9
	250	-3	1	13	-1	-2
	500	-7	-2	-1	3	-5
<i>Edoxaban-M4</i>	3	-22	7	4	6	2
	10	-13	-7	0	-3	-13
	75	-16	-4	-4	8	-3
	125	-11	-7	9	12	-6
	150	-13	-7	-4	7	-6
	Nominal concentration (ng/mL)	Within-Run Precision (%) - Day 1	Within-Run Precision (%) - Day 2	Within-Run Precision (%) - Day 3	Within-Run Precision (%) - Day 4	Within-Run Precision (%) - Day 5
<i>Edoxaban</i>	3	4	0	2	2	3
	10	0	3	2	2	2
	75	3	3	1	2	1
	250	1	2	0	1	2
	500	2	2	0	1	2
<i>Edoxaban-M4</i>	3	4	1	6	4	6
	10	0	4	3	2	3
	75	6	3	2	1	3
	125	1	1	0	0	1
	150	0	2	0	0	2
	Nominal concentration (ng/mL)	Between-Run Accuracy (%) - Edoxaban in NPP	Within-Run Precision (%) - Edoxaban in NPP	Between-Run Accuracy (%) - Edoxaban-M4 in NPP	Within-Run Precision (%) - Edoxaban-M4 in NPP	-
<i>Edoxaban</i>	3	2	2	-	-	-
	10	0	2	-	-	-
	75	4	2	-	-	-
	250	2	1	-	-	-
	500	-2	1	-	-	-
<i>Edoxaban-M4</i>	3	-	-	0	4	-
	10	-	-	-7	3	-
	75	-	-	-3	3	-
	125	-	-	-1	1	-
	150	-	-	-5	1	-

³ Results are expressed as the mean of measured concentrations.

Figures

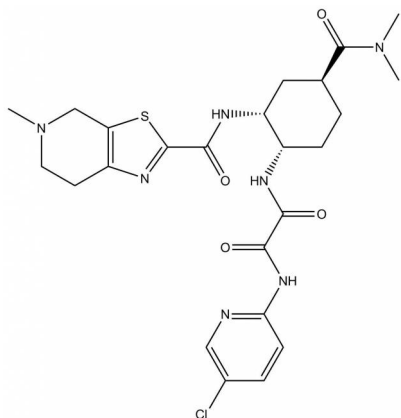
Figure 1: Postulated edoxaban metabolism for active metabolites ⁴



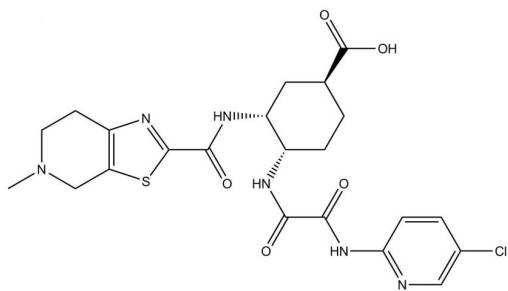
⁴ CES1: carboxylesterase-1; CYP3A4/5: Cytochrome P450 isoenzyme 3A4/5 ; IC_{50} : half-maximal inhibitory concentration; C_{max} : maximum observed plasma drug concentration

Figure 2: Chemical structure of (A) edoxaban (DU-176b), (B) edoxaban-M4 and (C) internal standard ($^{2}\text{H}_6$ -edoxaban)

(A)



(B)



(C)

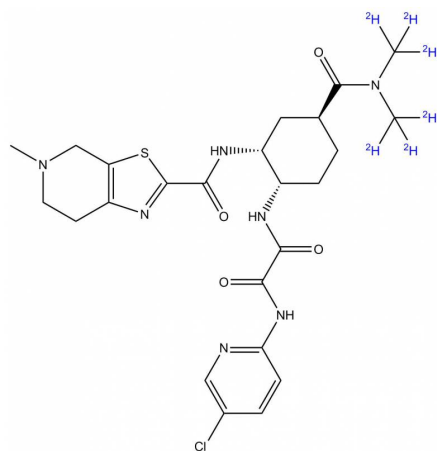
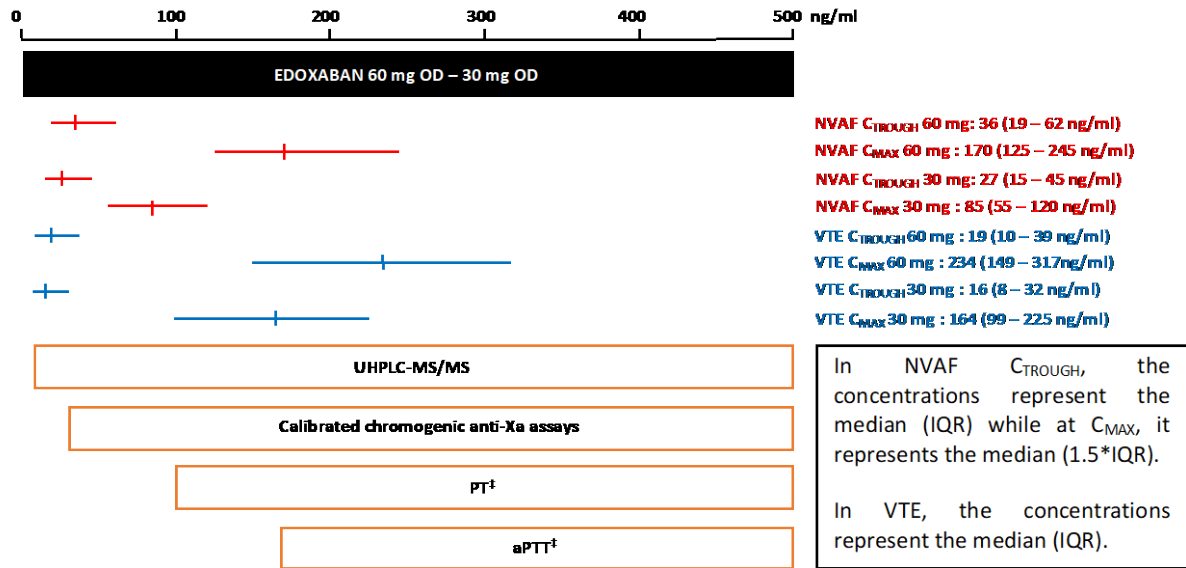


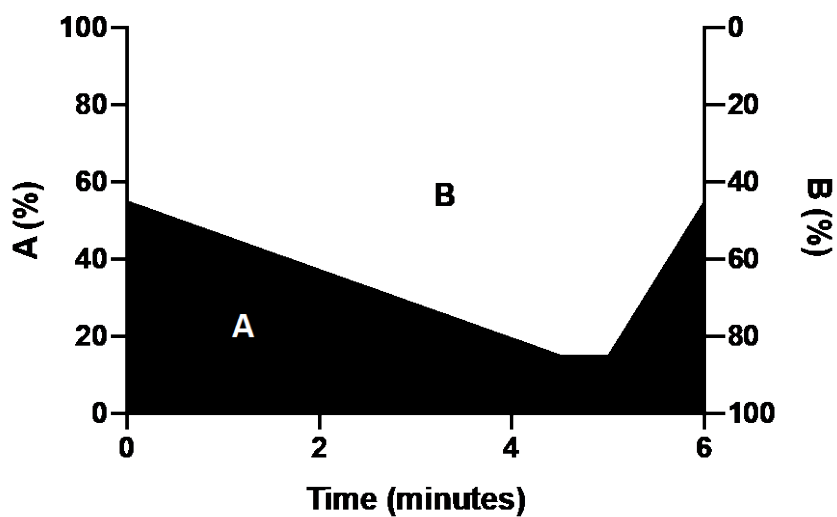
Figure 3: Laboratory testing of edoxaban and expected plasma concentrations after therapeutic doses⁵



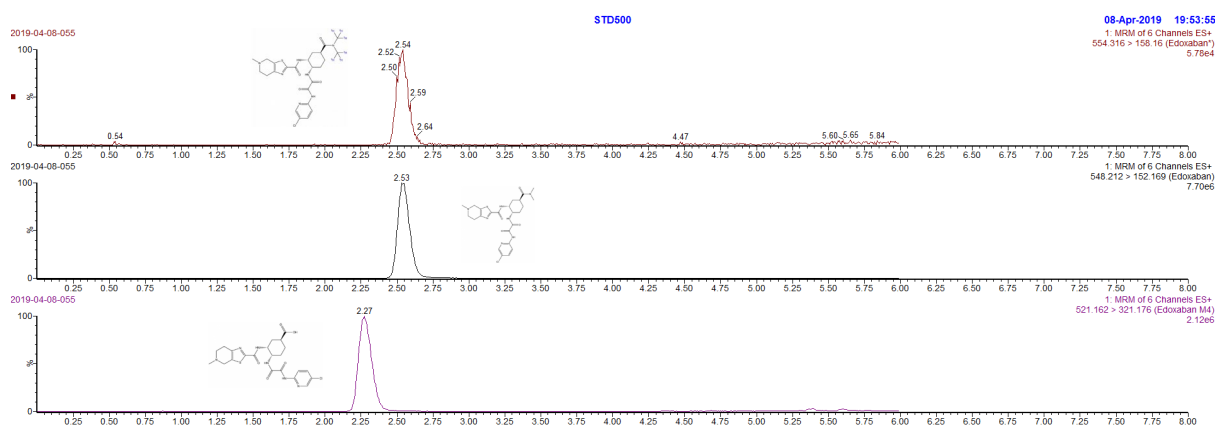
⁵ Red and blue lines represent plasma concentrations at peak and trough in NVAF and VTE, respectively. Orange boxes represent ranges of applicability of the corresponding test. †This represents the range of quantitation for sensitive reagents. Depending on the reagent, the sensitivity may be lower. aPTT, activated partial thromboplastin time; C_{MAX}, maximum plasma concentration during the dosing interval; C_{TROUGH}, minimum plasma concentration during the dosing interval; IQR, interquartile range; NVAF, non-valvular atrial fibrillation; PT, prothrombin time; VTE, venous thromboembolism. Notes: (i) Data on plasma concentration were extracted from Ruff et al. 20. Ruff, C.T., et al., *Association between edoxaban dose, concentration, anti-Factor Xa activity, and outcomes: an analysis of data from the randomised, double-blind ENGAGE AF-TIMI 48 trial*. The Lancet, 2015. **385**(9984): p. 2288-2295. , Weitz et al. 22. Weitz, J.I., et al., *Randomised, parallel-group, multicentre, multinational phase 2 study comparing edoxaban, an oral factor Xa inhibitor, with warfarin for stroke prevention in patients with atrial fibrillation*. Thromb Haemost, 2010. **104**(3): p. 633-41. and Verhamme et al. 21. Verhamme, P., et al., *Dose reduction of edoxaban preserves efficacy and safety for the treatment of venous thromboembolism. An analysis of the randomised, double-blind HOKUSAI VTE trial*. Thromb Haemost, 2016. **116**(4): p. 747-53..

Supplementary Figures

Supplementary figure 1: Gradient program for the mobile phase, with UPLC-grade water with 0.1% formic acid (A) and UPLC-grade methanol with 0.1% formic acid (B)



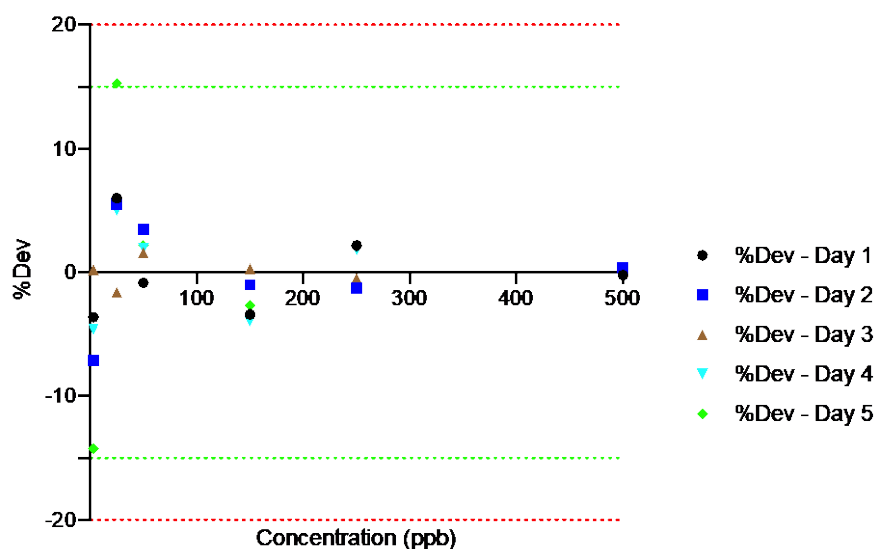
Supplementary figure 2: MRM chromatograms of edoxaban, edoxaban-M4 and [2H6]-edoxaban



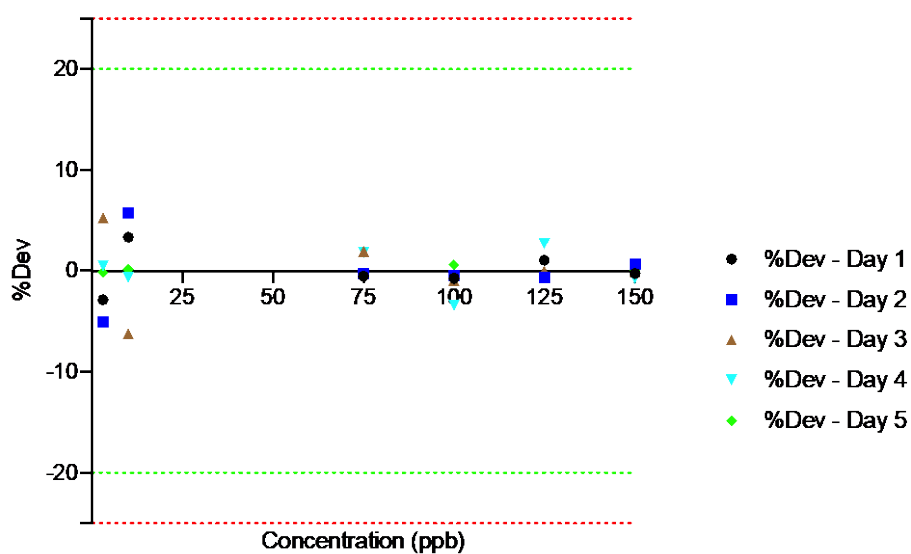
MRM chromatograms of [2H6]-edoxaban (12ng/mL), edoxaban (500ng/mL) and edoxaban-M4 (150ng/mL) using optimized MS and chromatographic conditions.

Supplementary figure 3: Calibration Curve – Edoxaban and edoxaban-M4

(A)



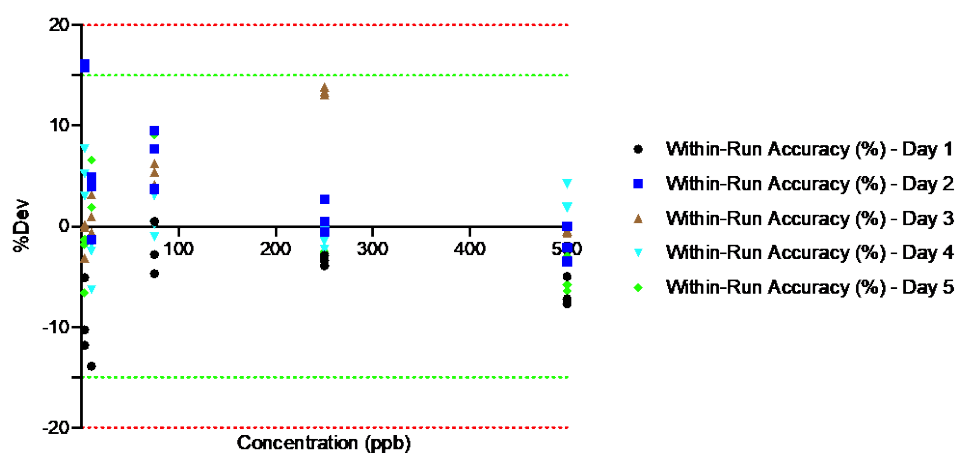
(B)



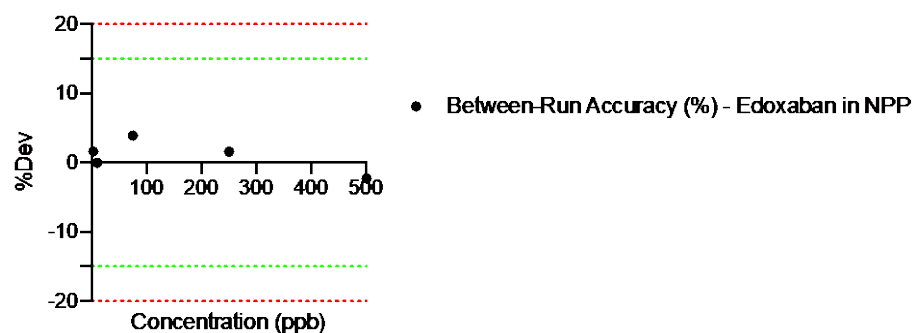
%Dev - Quadratic calibration curve of (A) edoxaban and (B) edoxaban-M4 in NPP with non-linear ponderation $1/x$. For edoxaban, red and green lines represent ranges of $\pm 20\%$ or $\pm 15\%$, respectively. For edoxaban-M4, red and green lines represent ranges of $\pm 25\%$ or $\pm 20\%$, respectively.

Supplementary figure 4: Accuracy – Within and between run for edoxaban

(A)



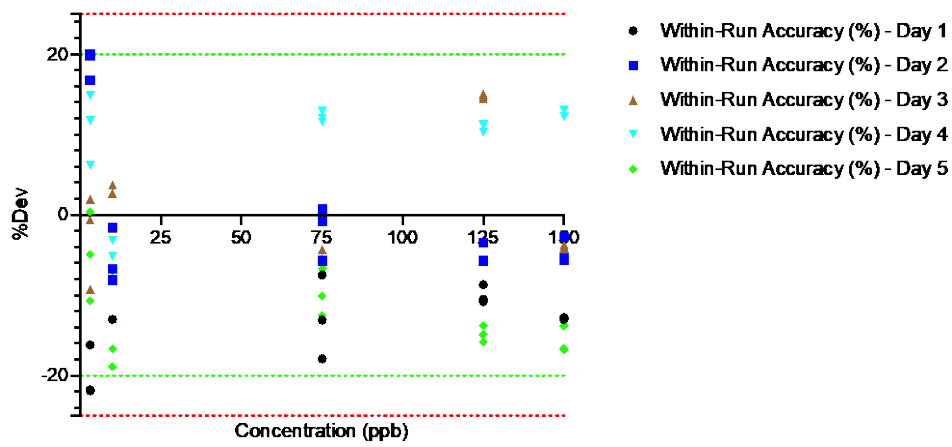
(B)



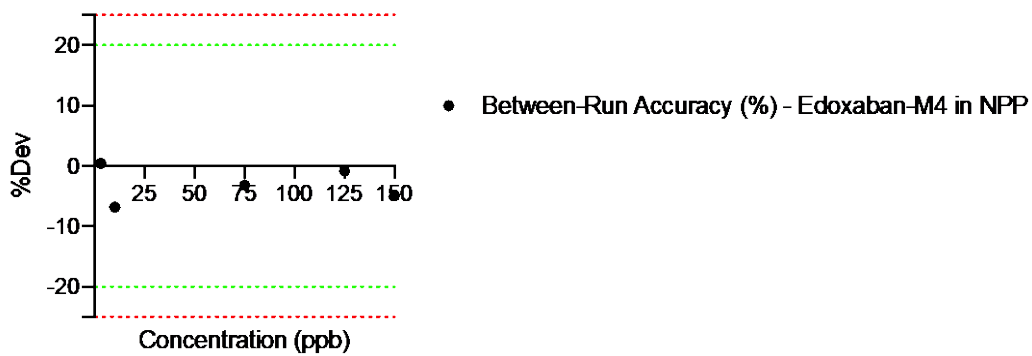
(A) Within-Run Accuracy (%) - Calibration curve of edoxaban in NPP. Red and green lines represent ranges of $\pm 20\%$ or $\pm 15\%$, respectively. (B) Between-Run Accuracy (%) - Calibration curve of edoxaban in NPP. Red and green lines represent ranges of $\pm 20\%$ or $\pm 15\%$, respectively.

Supplementary figure 5: Accuracy – Within and between run for edoxaban-M4

(A)



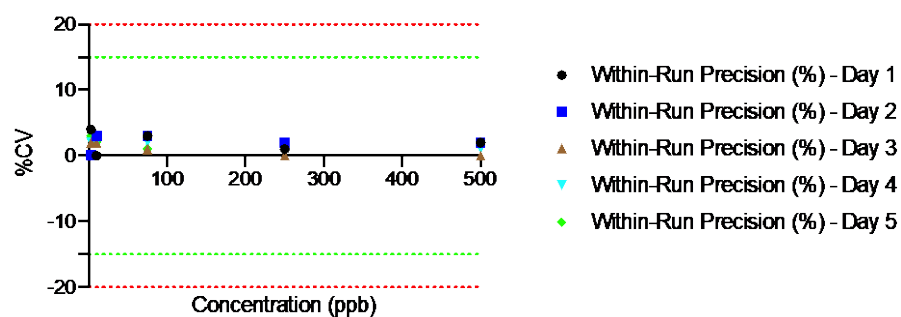
(B)



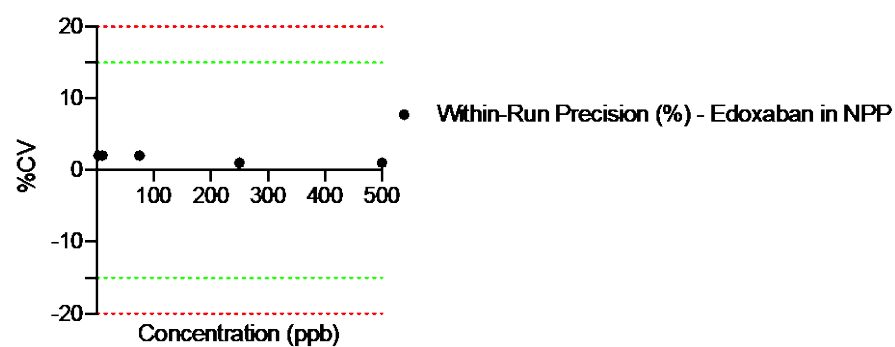
(A) Within-Run Accuracy (%) - Calibration curve of edoxaban-M4 in NPP. Red and green lines represent ranges of $\pm 25\%$ or $\pm 20\%$, respectively. (B) Between-Run Accuracy (%) - Calibration curve of edoxaban-M4 in NPP. Red and green lines represent ranges of $\pm 25\%$ or $\pm 20\%$, respectively.

Supplementary figure 6: Precision – Within and between run for edoxaban

(A)



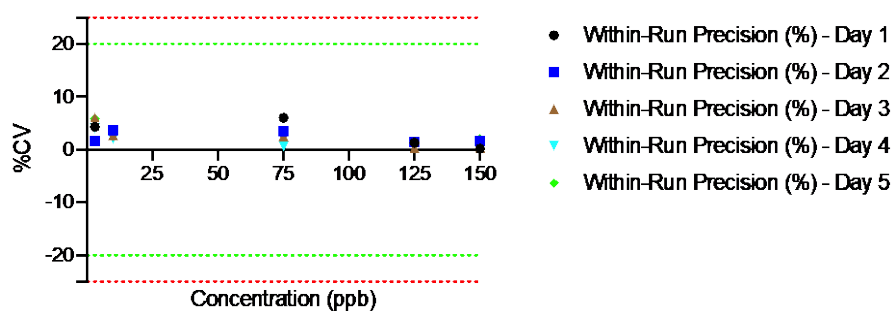
(B)



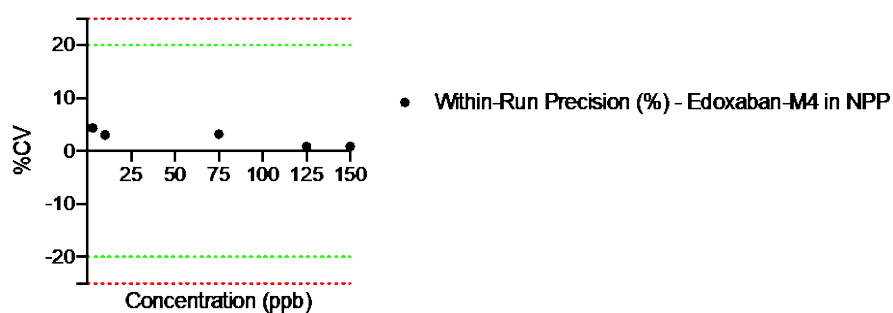
(A) Within-Run Precision (%) - Calibration curve of edoxaban in NPP. Red and green lines represent ranges of $\pm 20\%$ or $\pm 15\%$, respectively. (B) Between-Run Precision (%) - Calibration curve of edoxaban in NPP. Red and green lines represent ranges of $\pm 20\%$ or $\pm 15\%$, respectively.

Supplementary figure 7: Precision – Within and between run for edoxaban-M4

(A)



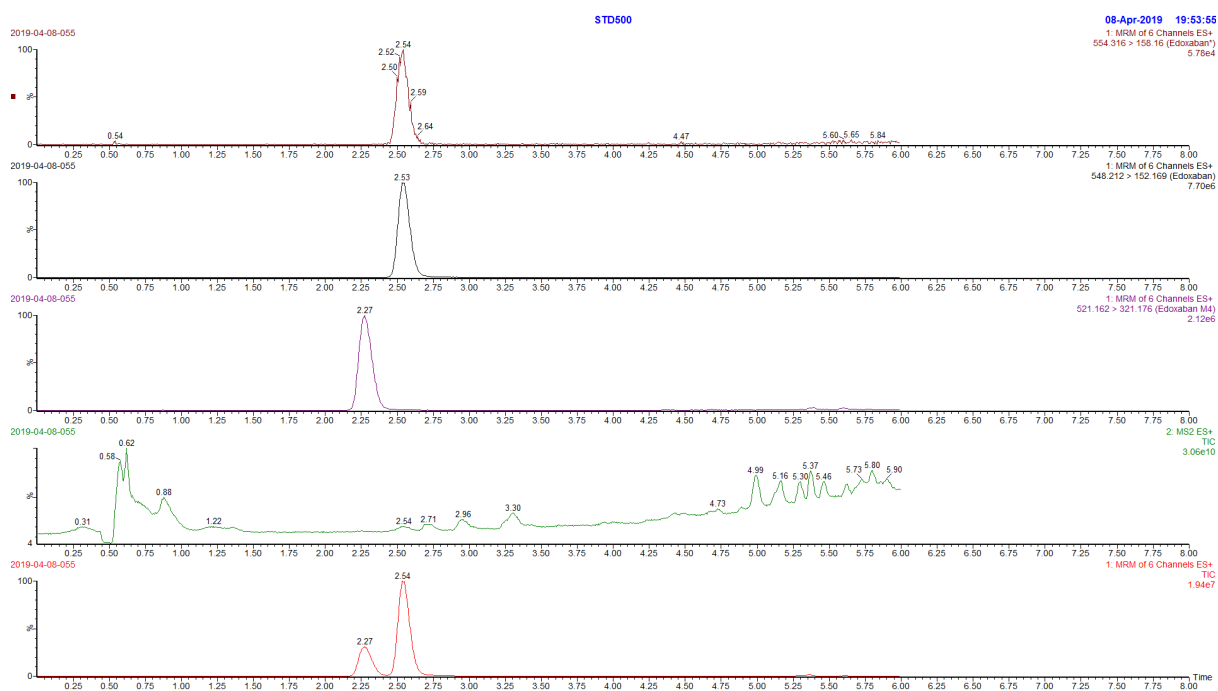
(B)



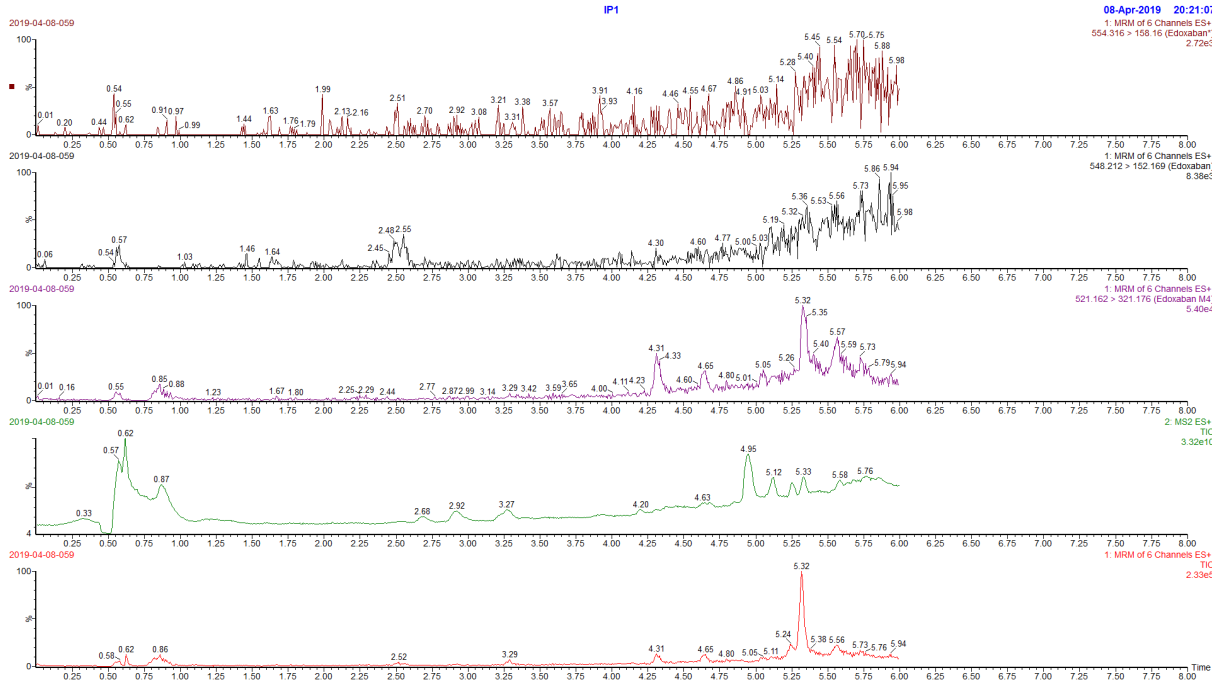
(A) Within-Run Precision (%) - Calibration curve of edoxaban-M4 in NPP. Red and green lines represent ranges of $\pm 25\%$ or $\pm 20\%$, respectively. (B) Between-Run Precision (%) - Calibration curve of edoxaban in NPP. Red and green lines represent ranges of $\pm 25\%$ or $\pm 20\%$, respectively.

Supplementary figure 8: Selectivity

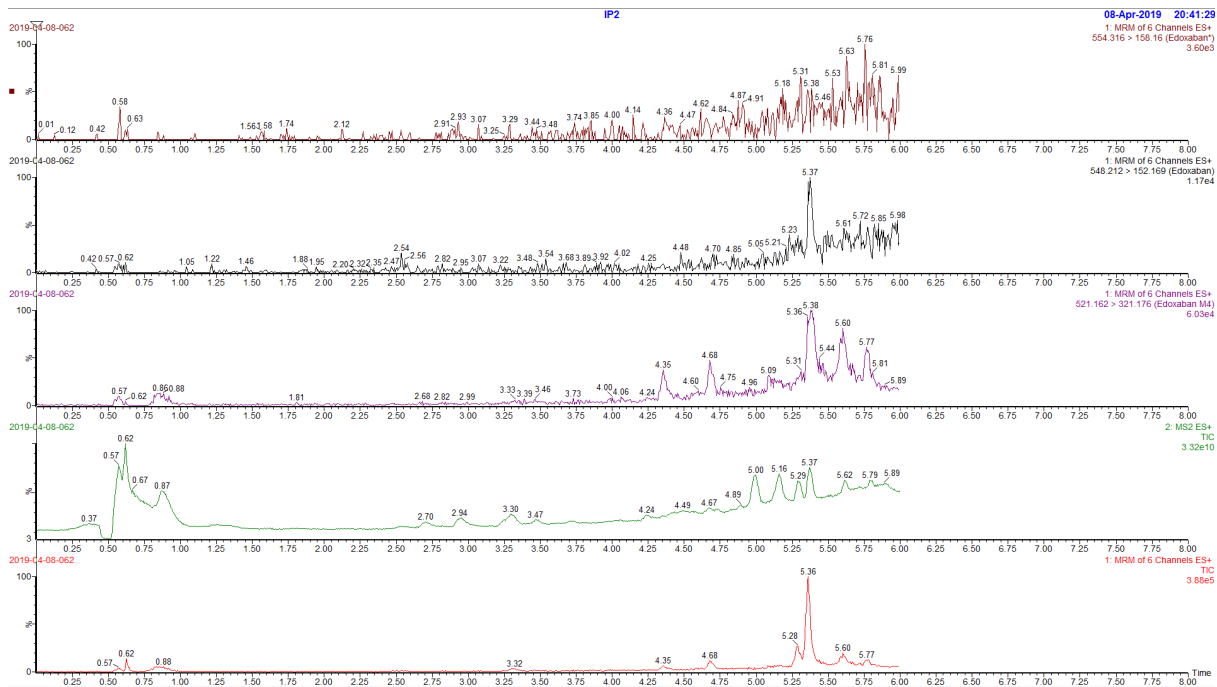
Selectivity assessment aims to verify that the substance being measured is the intended analyte to minimize or avoid interference. The analyze of blank samples of the appropriate biological matrix from six individual sources did not highlight any interference at retention time of edoxaban, edoxaban-M4 and the internal standard (chromatograms were visually checked to assess interferences). According to the visual interpretation of these chromatograms, no interference at predicted retention times of edoxaban, M4 or IS occurs in individual plasma without these molecules of interest.



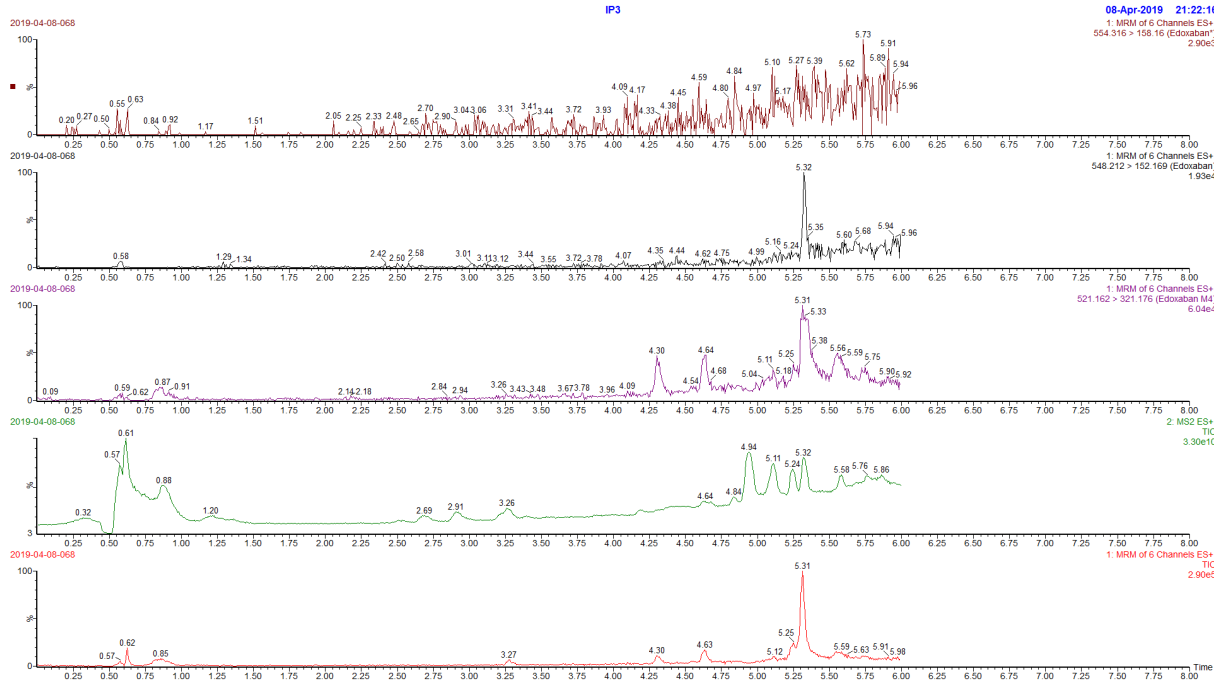
Spiked sample – 500ppb edoxaban – 150ppb edoxaban-M4 – 4ppb edoxaban.*



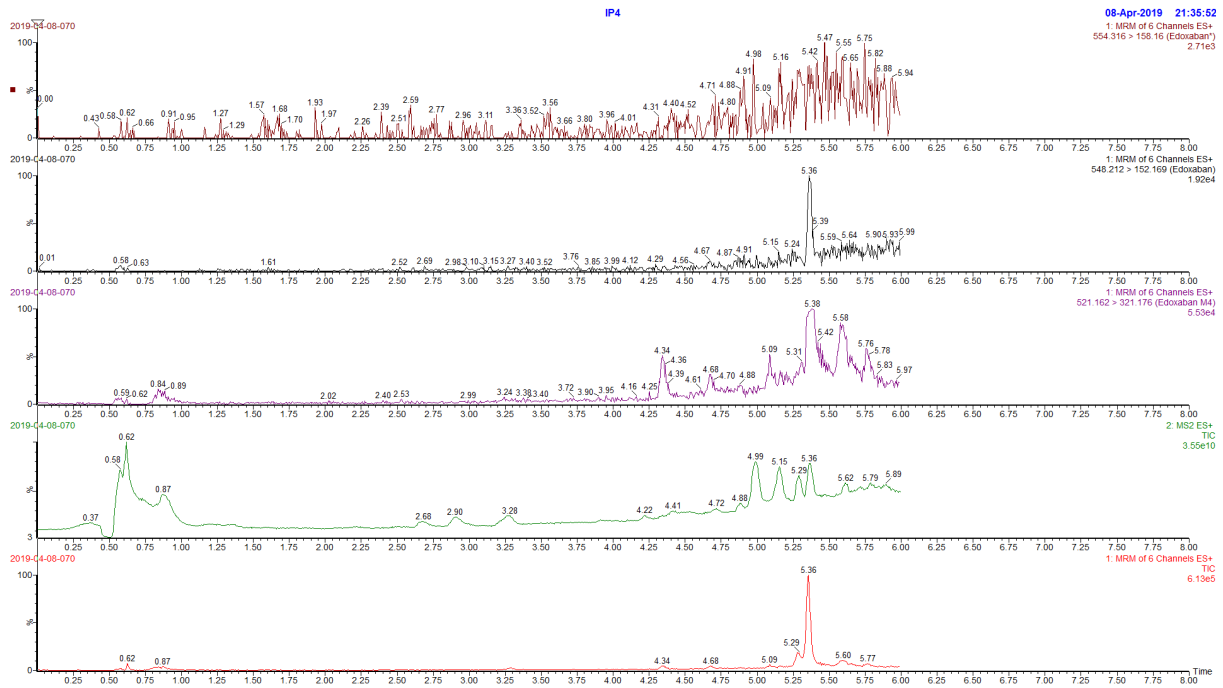
Individual plasma 1 – No edoxaban – No edoxaban-M4 – No internal standard.



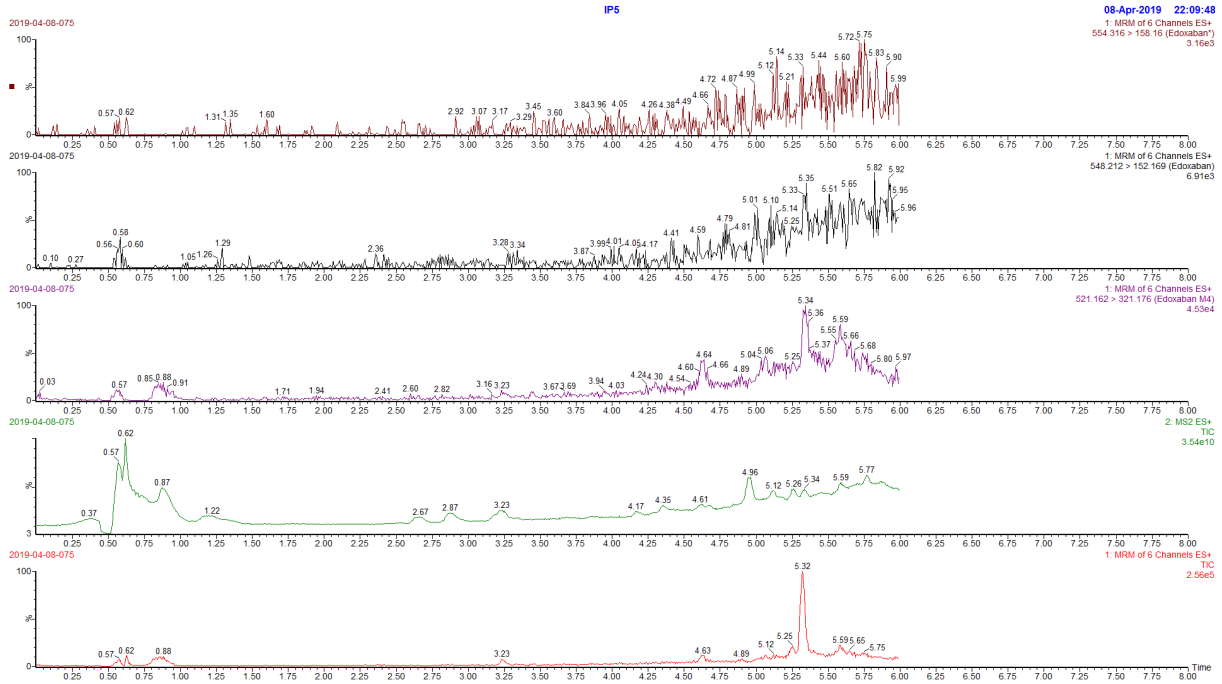
Individual plasma 2 – No edoxaban – No edoxaban-M4 – No internal standard.



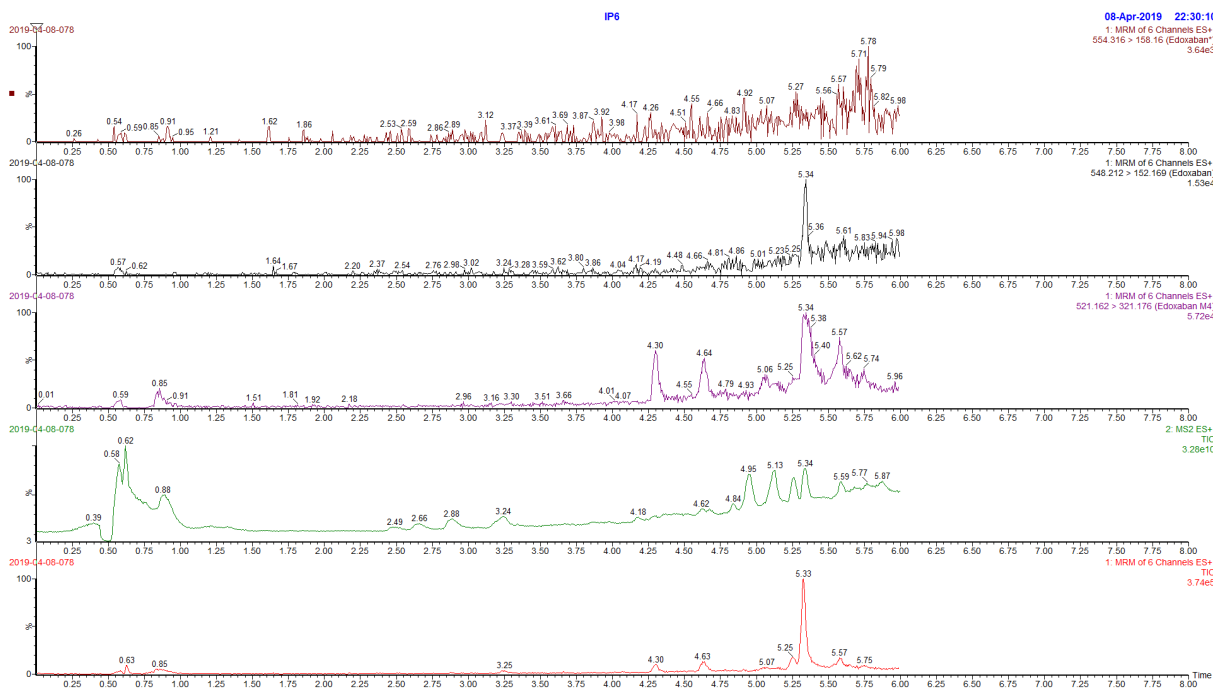
Individual plasma 3 – No edoxaban – No edoxaban-M4 – No internal standard.



Individual plasma 4 – No edoxaban – No edoxaban-M4 – No internal standard.



Individual plasma 5 – No edoxaban – No edoxaban-M4 – No internal standard.



Individual plasma 6 – No edoxaban – No edoxaban-M4 – No internal standard.

Supplementary figure 9: Representative chromatograms with retention times of apixaban, rivaroxaban, edoxaban, edoxaban-M4 and dabigatran

