Cutting Through the Matrix

High Throughput Analysis of Anticoagulants and Antiplatelets in Plasma using SPE-LC-MS/MS

By Patrick Wale, Application Scientist - Chromatographer

Introduction

Antithrombotic medications, which include anticoagulants and antiplatelets, are pharmaceutical agents that interfere with specific components within the blood coagulation process[1], reducing the formation of potentially harmful blood clots[2-9] (Figure 1). These medicines play a critical role in modern cardiovascular healthcare[4], where they are routinely prescribed for the prevention and treatment of thromboembolic disorders[10], including deep vein thrombosis (DVT)[3], pulmonary embolism (PE)[11], stroke prevention in atrial fibrillation (AF)[8,10,12-14,] and other conditions associated with clot formation[2,15-17]. While anticoagulants act primarily against fibrin clots within the veins and heart, antiplatelet medications inhibit the activation and aggregation of platelets[2] within high pressure arteries. Because these actions are complementary both medications may be used together to further reduce the risk of thrombotic cardiovascular events[6,16,18], although this substantially increases bleeding risk and requires careful clinical management[2,7,10,18].

Early anticoagulation strategies relied on heparin based therapies administered by injection[19], limiting their suitability for long-term outpatient treatment[4,9]. The introduction of vitamin K antagonists (VKAs), particularly warfarin, revolutionised anticoagulant therapy and subsequently became the standard of care for prevention and treatment for more than five decades[1,11,19]. Owing to its effectiveness, warfarin remains one of the most widely prescribed oral anticoagulants worldwide and continues to play an important role in clinical practice[11,20]. Despite its success, warfarin possesses a notoriously narrow therapeutic index[1] and exhibits substantial interpatient variability due to genetic, dietary, and environmental factors[4,21]. These significant limitations, combined with severe bleeding events[14,18] have historically made warfarin the second leading cause of drug related emergency room visits[22,23]. This often necessitates frequent laboratory monitoring and dose adjustments to minimise the risk[4,18,20,21]. This drove the development of direct oral anticoagulants (DOACs), also referred to as non-vitamin K antagonist oral anticoagulants (NOACs)[9,10,12,19,24]. Dabigatran[3,4,8,10,15,16] became the first approved DOAC in 2008, quickly followed by rivaroxaban, apixaban, and edoxaban. They provided improved efficacy without requiring the burdensome lifestyle modifications associated with VKAs[3,4,10-12,15,19,24].

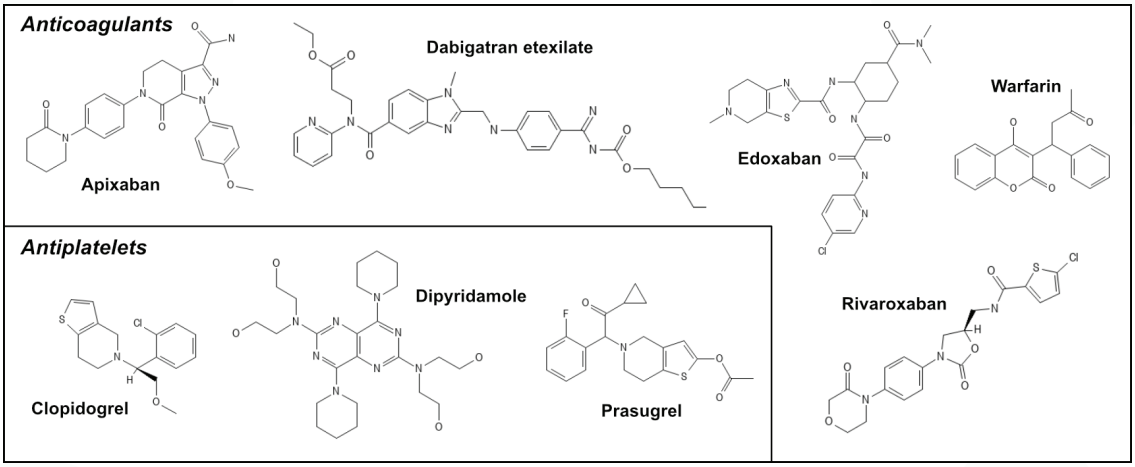


Figure 1 - Chemical structures of anticoagulants: apixaban, dabigatran etexilate, edoxaban, rivaroxaban and warfarin and antiplatelets: clopidogrel, dipyridamole and prasugrel.

Early antiplatelet treatment was dominated by acetylsalicylic acid (aspirin), introduced during the mid twentieth century[6,7,25], however, it was later associated with incomplete platelet inhibition and residual thrombotic risk. This led to the development of more selective antiplatelet agents, particularly the thienopyridine class represented by ticlopidine and its successor clopidogrel[6,7]. Further advances resulted in the introduction of prasugrel, which provided a more rapid, potent, and consistent platelet inhibition than clopidogrel[2,25]. Alongside these agents, dipyridamole emerged as an alternative therapy that inhibited phosphodiesterase activity and increased extracellular adenosine concentrations, producing both antiplatelet and vasodilatory effects[1,25].

Although DOACs and antiplatelet treatments do not require the routine laboratory monitoring associated with VKAs[16,21,26], testing and measuring drug concentrations remains essential in critical clinical situations[3,10,18,27,29]. Factors including advanced age, renal dysfunction, obesity, gastrointestinal absorption disorders[15], and concomitant medications can significantly alter drug exposure, hence therapeutic activity[2,10,30]. Furthermore, with the approval of specific DOAC reversal agents, such as idarucizumab for dabigatran[31] and andexanet alfa for rivaroxaban[13,28], determining the exact concentration of anticoagulants has become imperative for safe and effective treatment[21,32].

Monitoring these medications requires highly reliable, rapid, and sensitive analytical methods[3,18,27]. Several coagulation-based, phenotypic and platelet function assays have been developed[28,33], including diluted thrombin time, ecarin clotting time, and drug-specific anti-factor Xa assays[24,29,34,35]. These techniques exhibit several limitations[13,36,37] with many requiring individual drug calibration and possessing poor accuracy at low plasma concentrations[13,28,33,36]. Current assays lack the versatility to quantify multiple drugs simultaneously[3,24,34,36]. Such limitations can become particularly problematic in emergency[18,27,31] and high-throughput clinical environments where rapid[13], reliable, and comprehensive measurements are required[15,26,28,34].

Consequently, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has emerged as the gold-standard analytical technique[38-40] for anticoagulant and antiplatelet quantification in biological matrices[5,20,21,34,37]. Rather than measuring downstream clotting, LC-MS/MS directly and selectively quantifies the precise concentration and offers an unprecedented degree of specificity, selectivity, and sensitivity, making it the superior method for determining drug concentrations in plasma[5,34,41].

While several LC-MS/MS methods have been reported for separately quantifying anticoagulants and antiplatelets in plasma[5,34,42-44], few methods combine quantification of both into a single method. Plasma represents a highly complex biological matrix[34,42] containing proteins, phospholipids[5], salts, and endogenous metabolites. These are capable of causing ion suppression[43,45], reduced sensitivity[45], and contamination of LC-MS ion sources[5,45]. Effective sample preparation is therefore essential. Traditional approaches frequently employ protein precipitation or phospholipid removal sample preparation[5,43], which can leave behind interfering compounds reducing sensitivity and method flexibility. Solid phase extraction (SPE) provides a cleaner extract by effectively and selectively removing endogenous interferences while retaining target analytes. Additionally, as drug monitoring increasingly demands lower quantification limits and higher sample throughput, SPE-based workflows provide a robust and scalable solution for clinical bioanalysis.

The purpose of this application note was to utilise the novel Microlute® CP HLB SPE 96-well plate to develop and validate a sensitive, high-throughput SPE-LC-MS/MS method for the simultaneous quantification of major anticoagulant (apixaban, dabigatran, edoxaban, rivaroxaban, warfarin) and antiplatelet (prasugrel, clopidogrel, dipyridamole) medications in animal plasma. The European Medicines Agency (EMA) guidelines for bioanalytical methods[46] were referenced during method validation.

Materials and Methods

Chemicals and Reagents

Apixaban (99.9%) was purchased from Thermo Scientific Chemicals, clopidogrel (98.83%) was purchased from Targetmol Chemicals, edoxaban (99.69%) was purchased from Aladdin Scientific, dabigatran etexilate (99.4%), dipyridamole (99.7%), prasugrel (99.2%), rivaroxaban (99.2%), warfarin (91.3%), dimethyl sulfoxide (DMSO) (99%) were purchased from Tokyo Chemical Industry, hydrocortisone-21-acetate (99%) was purchased from Sigma Aldrich which was used as an internal standard (IS). LC-MS methanol (MeOH) (99.99%), formic acid (97.5%) and phosphoric acid (85%) were purchased from Fisher Scientific. Bovine plasma with sodium citrate was purchased from Biosera, Cynomolgus monkey plasma with K_2EDTA, Sprague Dawley rat plasma with K_2EDTA, Beagle plasma with K_2EDTA, CD-1 (ICR) mouse plasma with K_2EDTA and New Zealand White rabbit plasma with K_2EDTA were purchased from BioIVT. Ultrapure water (H_2O) was generated by an Avidity Solo S UVTOC purification system. Microlute® CP 10 mg HLB SPE 96-well plate, 96-well square 2 mL polypropylene plates, 15 mL centrifuge tubes, UltraPPM Lite positive pressure manifold and Ultravap® Mistral nitrogen blowdown evaporator were all manufactured by Porvair Sciences.

Stock & Standards

Individual stock solutions of each antithrombotic analyte (AA) were prepared at 500 µg/mL in DMSO. These stock solutions were combined and diluted with methanol/water (50:50, v/v) to create several working solutions (WAAS) from 10-5,000 ng/mL. A 2,500 µg/mL stock solution of hydrocortisone-21-acetate was prepared in methanol. This stock solution was diluted with DMSO to create a working internal standard (WIS) solution of 250 ng/mL

Instrumentation and LC-MS/MS Method

The multiple reaction monitoring (MRM) transitions, cone voltages and collision energies of all eight AAs and IS were tuned by directly infusing a 200 ng/mL working solution into the Waters Xevo TQ-S Micro triple quadrupole mass spectrometer with the following parameters: capillary voltage ±2.5 kV, ion source temperature 150°C, desolvation temperature 450°C, desolvation gas flow 1000 L/hr, infusion rate 10 µL/min.

The UHPLC method was developed with a biphenyl column and methanol/water mobile phases. Subsequently, the source parameters and physical source adjustments were optimised for each AA based on the corresponding mobile phase elution composition, resulting in the final method detailed in Table 1.

UHPLC & Mass Spectrometer Conditions:	
UHPLC: ACQUITY Premier UPLC	**Mass Spectrometer:** Xevo TQ-S Micro
Solvent A: Ultrapure Water	**Source Type:** ESI Positive/Negative
Solvent B: Methanol	**Capillary Voltage:** +2.0 kV/ -2.0 kV
Flow Rate 400 µL/min	**Desolvation Temperature:** 650°C
Seal Wash On (5 min)	**Source Temperature:** 150°C
Sample Temperature 13 °C	**Desolvation Gas Flow:** 1,200 L/hr
Syringe Draw Rate: 30 µL/min	**Cone Gas Flow:** 0 L/hr
Needle Placement: 4 mm	**Detector Gain:** 1.00
Injection Volume: 2 µL	**Minimum Points Per Peak:** 15
Needle Wash: 3 sec methanol/water (50:50, v/v)	**Collision Gas:** Argon (99.998%)
Column Temperature: 30°C	**Mass Resolution:** Unit (0.75 Da)
Column: Restek Raptor Biphenyl, 1.8 µm, 30 x 2.1 mm HPLC Column	

Gradient

Time (min)	A (%)	B (%)
0.00	80	20
0.50	80	20
1.50	50	50
5.00	0	100
6.00	0	100
6.01	80	20
7.50	80	20

Table 1a - UHPLC and mass spectrometer conditions used for analysis of AAs and IS.

MRM Transitions:

Analyte	Retention Time (min)	Polarity	Precursor ion (m/z)	Cone voltage (V)	Product ion (m/z)	Collision energy (V)
Apixaban	3.62	+	459.96	90	443.22 (Quan)	26
					199.10	40
Clopidogrel	4.12	+	321.90	10	211.99 (Quan)	17
					183.99	24
Dabigatran etexilate	4.36	+	628.28	10	289.16 (Quan)	42
					332.15	28
Dipyridamole	3.88	+	505.23	12	385.21 (Quan)	46
					429.30	48
Edoxaban	3.54	+	548.16	50	366.19 (Quan)	22
					152.12	36
Hydrocortisone-21-acetate (IS)	3.18	+	405.01	60	327.07 (Quan)	15
					309.16	15
Prasugrel	4.18	+	373.90	19	206.05 (Quan)	20
					109.05	58
Rivaroxaban	2.94	+	435.90	75	144.97 (Quan)	22
					231.07	16
Warfarin	3.10	-	306.93	15	161.01 (Quan)	20
					249.98	22

Table 1b - MRM transitions and source parameters used for analysis of AAs and IS.

Plasma Preparation

Working plasma samples (WPS), whether blank or spiked, were prepared immediately before SPE by diluting the samples 1:1 with phosphoric acid/water (4:96, v/v) in a centrifuge tube.

SPE Method

WPS were processed using a 10 mg HLB 96-well plate and a positive pressure manifold, following the SPE steps detailed below.

- **Condition:** 500 µL methanol
- **Equilibration:** 500 µL ultrapure water
- **Load:** 500 µL working plasma samples (WPS)
- **Wash:** 500 µL methanol/water (30:70, v/v) *(sorbent dried at 20 PSI for 2 minutes after wash with the positive pressure manifold)*
- **Pre-Elution:** 20 µL WIS added to the required wells of the elution collection plate, this also acted as a keeper during the evaporation step
- **Elution:** 2 x 500 µL methanol *(sorbent dried at 20 PSI for 2 minutes after each elution with the positive pressure manifold)*
- **Evaporation step:** The eluate was evaporated using a nitrogen blowdown evaporator at 30°C until only the keeper liquid remained
- **Reconstitution:** 205 µL ultrapure water and 25 µL diluent (water/methanol/DMSO - 46:46:8, v/v/v) was added to each well. For matrix-matched calibration standards spiked with WAAS, the diluent volume was adjusted toa final volume of 250 µL.

Results & Discussion

Method Development and LC-MS/MS optimisation

To develop the UHPLC method two columns; a Waters ACQUITY UPLC-BEH C18 (2.1 x 50 mm, 1.7 µm) and a Restek Raptor Biphenyl (2.1 x 30 mm, 1.8 µm) were evaluated alongside varying concentrations of formic acid in the mobile phase. Under identical conditions, the biphenyl column increased the average retention time of the AAs by 13% compared to the C18 column. This increased retention, driven by strong pi-pi interactions with the stationary phase, necessitated a higher methanol concentration for elution. The elevated methanol content consequently enhanced electrospray ionisation efficiency, resulting in larger peak areas and improved overall sensitivity.

While adding formic acid reduced tailing and improved peak shapes for dipyridamole, dabigatran etexilate, prasugrel, and clopidogrel, it caused up to a 4.5-fold drop in sensitivity for most analytes. The sole exception was dipyridamole, which exhibited a 6-fold increase in sensitivity. Formic acid was ultimately removed from the mobile phase, though doing so meant that initial starting conditions exceeding 20% methanol led to peak fronting and broadening.

SPE Method Optimisation

To maximise AA recovery and ensure sample cleanliness, the organic compositions of the wash and elution steps were optimised (Table 2). A 30:70 (v/v) methanol/water mixture proved to be the optimal wash solvent with 60:40 (v/v) resulting in an average of 30% AA breakthrough. The elution step required two 500 µL methanol elution passes for complete AA recovery, particularly for dabigatran etexilate (Table 2). When processing the unfiltered WPS samples no blocking or clogging was observed with a consistent and controllable liquid flow (1 drop every 2 seconds).

Recovery/ Breakthrough (%)	Wash/Elution Step Optimisation (methanol/water, v:v)						
	0:100	20:80	40:60	60:40	80:20	100:0	2x100:0
Apixaban	0.01	0.06	0.81	7.04	80.3	89.2	88.9
Clopidogrel	0.02	0.01	0.09	0.05	6.81	79.1	80.7
Dabigatran etexilate	0.13	0.01	0.05	0.04	6.56	55.8	94.2
Dipyridamole	0.01	0.02	0.18	0.71	60.0	73.4	107
Edoxaban	0.01	0.03	0.37	1.73	59.0	90.7	95.1
Prasugrel	0.01	0.01	0.09	0.06	7.77	81.2	86.6
Rivaroxaban	0.02	0.06	0.85	6.12	80.5	83.2	87.7
Warfarin	0.08	0.21	2.32	30.3	91.1	93.9	98.4

Table 2 - Average recovery/breakthrough (%) of AAs during the wash and elution optimisation process. 80 ng/mL of AAs were loaded onto the SPE plate in neat solvent after which 500 µL of the above compositions of methanol/water (v:v) were subsequently flowed through collected and analysed vs the nominal load concentration.

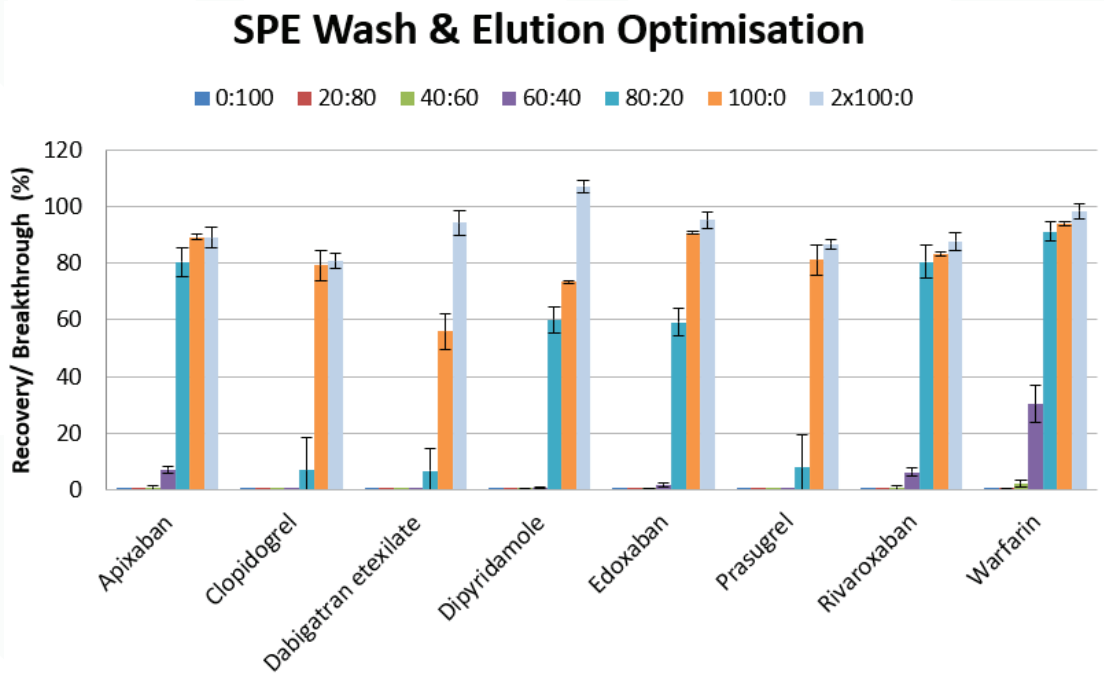


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Method Validation

Guided by the principles of the EMA guidelines, a targeted validation was performed to establish method reliability. Bovine plasma was utilised as the blank matrix to evaluate the following areas for all AAs: calibration curve and range, matrix effect, accuracy and precision, selectivity, specificity, carryover, dilution integrity, and reinjection reproducibility.

Calibration curve and range

Ten concentration levels, ranging from 0.5 - 500 ng/mL, were prepared by spiking blank bovine plasma post-SPE with WAAS. These standards were both prepared and analysed in triplicate alongside solvent and matrix blanks. Calibration curves were constructed by plotting the peak area ratios (analyte/IS response) against nominal concentrations, with a $1/x^2$ weighting factor. All AAs exhibited strong linearity over the concentration range (Table 3), yielding correlation coefficients (R^2) > 0.98. A six-point calibration curve spanning 10 - 500 ng/mL was created, which demonstrated good accuracy with residuals <±14.6% and precision <±12.3% in accordance with guidelines (<±15% and <±20% at LLOQ).

The limits of detection (LOD) and quantification (LOQ) were determined experimentally by comparing the signal-to-noise (S/N) ratios of low-concentration samples (0.05 – 10 ng/mL in post-SPE blank bovine plasma) to the baseline noise at the AA retention time. The LOD and LOQ were established at concentrations yielding S/N ratios of 3.3 and 10, respectively (Table 3).

Matrix Effect

Matrix effects (ME) were evaluated for each AA by comparing two calibration curves (10 - 500 ng/mL) – one was prepared in blank bovine plasma post-SPE and the other in neat solvent. The matrix effect was then determined by comparing the slopes of the two curves using Equation 1. While all AAs exhibited some degree of ME, the extent varied significantly, from mild ion suppression in the case of edoxaban (-2.8%) to pronounced ion enhancement for dabigatran etexilate (271.7%) (Table 3).

$$Matrix\ Effects\ (\%) = \left(\frac{Slope\ in\ matrix\ -Slope\ in\ neat\ solvent}{Slope\ in\ neat\ solvent}\right) \times 100\%$$

Equation 1 – Calculation of matrix effects using calibration curve slopes in matrix and neat solvent. Positive values indicate ion enhancement and negative values indicate ion suppression.

Analyte	Linearity	R^2	LOD (ng/mL)	LOQ (ng/mL)	Matrix Slope *(Solvent Slope)*	ME (%)
Apixaban	2.5 – 500	0.9937	0.5	0.8	5.77 *(6.26)*	-4.8
Clopidogrel	5.0 – 500	0.9885	0.06	0.1	50.7*(108)*	-51.6
Dabigatran etexilate	0.5 – 500	0.9948	6	10	31.4 *(7.19)*	271.7
Dipyridamole	1.0 – 500	0.9947	3	5	8.14 *(4.96)*	65.9
Edoxaban	0.5 – 500	0.9960	0.5	0.8	6.12 *(6.22)*	-2.8
Prasugrel	10 – 500	0.9904	0.1	0.5	133 *(156)*	-19.2
Rivaroxaban	2.5 – 500	0.9936	0.8	1	6.84 *(6.50)*	5.5
Warfarin	5.0 – 500	0.9875	0.06	0.1	18.6 *(18.8)*	-6.2

Table 3 – Linearity, correlation coefficients (R^2), limit of detection/quantification (LOD/LOQ), calibration curve slopes matrix-/neat and matrix effects (ME) for all AAs using SPE–LC–MS/MS method.

Accuracy and Precision

The accuracy and precision of the method were assessed by analysing quality control (QC) samples spiked at six concentration levels across and above the calibration range prior to SPE; 10 (LLOQ), 20, 80, 200, 400 and 750 ng/mL. These were evaluated within a single analytical run on one day (intra-day), and the 20 ng/mL and 400 ng/mL across two separate days (inter-day) with four replicates at each level. Precision was expressed as the relative standard deviation (%), while accuracy was calculated as a percentage of the nominal concentration and stated as a recovery (%).

The method showed recoveries >53% at the LLOQ with %RSD <12.1%. Between 20-400 ng/mL recoveries ranged from 70-120% with %RSD <15.0%. Despite being outside the calibration range, 750 ng/mL still showed good recoveries >70% with %RSD <4.8%. Inter-day testing yielded recoveries >60% with %RSD <14.8% at 20 ng/mL and >90% and <10.9% at 400 ng/mL. All results are acceptable according to the guidelines, with precision within ±20% for the LLOQ and within ±15% for higher levels (Table 4).

Analyte	Nominal Concentration (ng/mL)	Intra-day Recovery (% ± SD)	Intra-day (%RSD)	Inter-day Recovery (% ± SD)	Inter-day (%RSD)
Apixaban	10	61 ± 7.4	12.1	-	-
	20	99 ± 2.8	2.8	105 ± 6.4	6.1
	80	108 ± 7.3	6.8	-	-
	200	97 ± 1.2	1.2	-	-
	400	93 ± 4.2	4.6	96 ± 4.8	5.0
	750	89 ± 3.1	3.5	-	-
Clopidogrel	10	56 ± 4.7	8.4	-	-
	20	83 ± 2.3	2.8	75 ± 8.6	11.4
	80	84 ± 10.5	12.5	-	-
	200	111 ± 10.2	9.2	-	-
	400	108 ± 7.4	6.8	99 ± 10.9	11.0
	750	84 ± 1.4	1.7	-	-
Dabigatran etexilate	10	60 ± 5.0	8.2	-	-
	20	120 ± 8.8	7.3	108 ± 14.8	13.6
	80	111 ± 7.8	7.0	-	-
	200	106 ± 9.8	9.2	-	-
	400	96 ± 7.4	7.6	92 ± 7.6	8.2
	750	72 ± 3.5	4.8	-	-
Dipyridamole	10	53 ± 6.3	12.0	-	-
	20	70 ± 9.6	13.8	60 ± 13.0	21.6
	80	92 ± 14.0	15.0	-	-
	200	111 ± 8.6	7.7	-	-
	400	113 ± 2.2	1.9	106 ± 8.6	8.1
	750	88 ± 4.0	4.6	-	-
Edoxaban	10	62 ± 5.7	9.2	-	-
	20	89 ± 3.9	4.3	86 ± 4.4	5.1
	80	93 ± 9.1	9.8	-	-
	200	103 ± 2.1	2.1	-	-
	400	106 ± 2.3	2.2	101 ± 5.7	5.6
	750	94 ± 3.3	3.5	-	-
Prasugrel	10	60 ± 6.2	10.3	-	-
	20	92 ± 4.7	5.1	85 ± 7.9	9.3
	80	91 ± 9.0	10.0	-	-
	200	107 ± 4.2	4.0	-	-
	400	100 ± 2.7	2.7	92 ± 9.0	9.8
	750	70 ± 2.0	2.9	-	-
Rivaroxaban	10	63 ± 5.7	9.1	-	-
	20	94 ± 3.3	3.5	89 ± 5.7	6.3
	80	99 ± 9.8	9.9	-	-
	200	108 ± 1.0	0.9	-	-
	400	102 ± 2.9	2.8	96 ± 7.7	8.1
	750	77 ± 2.8	3.6	-	-
Warfarin	10	63 ± 5.1	8.2	-	-
	20	97 ± 3.9	4.0	93 ± 5.8	6.2
	80	101 ± 8.8	8.7	-	-
	200	106 ± 2.2	2.0	-	-
	400	95 ± 3.2	3.3	90 ± 5.9	6.6
	750	74 ± 2.3	3.2	-	-

Table 4 – Intra-day recovery and %RSD of AAs spiked at 10,20,80,200,400,750 ng/mL (n=4) and inter-day recovery and %RSD of AAs spiked at 20,400 ng/mL (n=8) in WPS analysed using SPE–LC–MS/MS method.

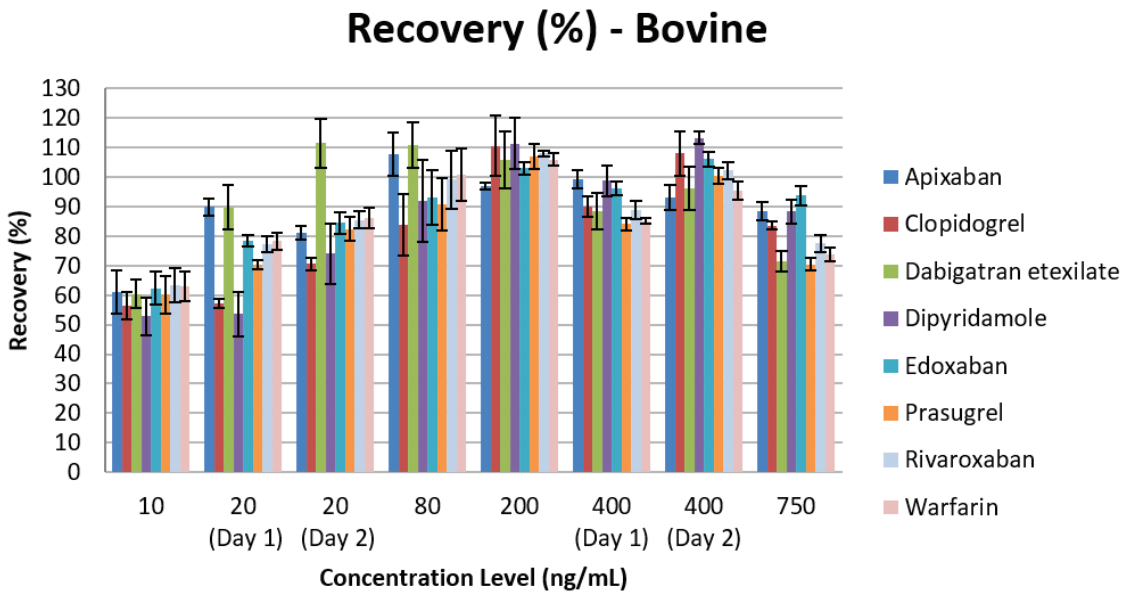


Figure 3 - Average recovery (%) of AAs at each concentration level (10, 20, 80, 200, 400, 750 ng/mL) in bovine plasma spiked prior to performing SPE–LC–MS/MS method.

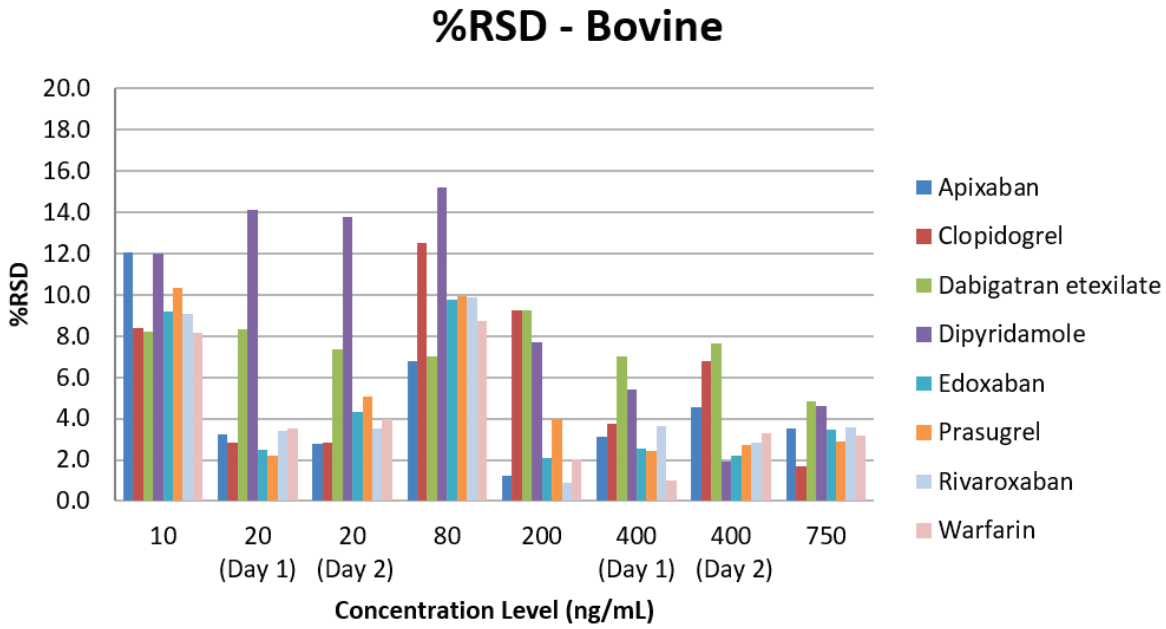


Figure 4 - %RSD of AAs at each concentration level (10, 20, 80, 200, 400, 750 ng/mL) in bovine plasma spiked prior to performing SPE–LC–MS/MS method.

Method Detection and Quantification Limits

Method detection and quantification limits (Table 5), which accounted for all stages of the analytical process, were established using Equation 2, combining LLOQ recovery data with the LOD, LOQ, and ME values (Table 3).

$$M_X = \frac{L_x}{Recovery \times ME} \; / \; concentration\ factor$$

Equation 2 – Calculation of MDL or MQL (M_x), where L_x is the LOD (for MDL) or LOQ (for MQL), recovery is the value at 10 ng/mL (LLOQ) (as a decimal), ME is the matrix effect (as a decimal), and the concentration factor is 1

Analyte	LLOQ Recovery (%)	MDL (ng/mL)	MQL (ng/mL)
Apixaban	61	0.89	1.42
Clopidogrel	56	0.23	0.38
Dabigatran etexilate	60	2.29	3.82
Dipyridamole	53	3.45	5.75
Edoxaban	62	0.82	1.31
Prasugrel	60	0.20	0.98
Rivaroxaban	63	1.21	1.51
Warfarin	63	0.10	0.16

Table 5 – SPE recovery of AAs spiked at 10 ng/mL (LLOQ) in WPS analysed using SPE–LC–MS/MS method. MDL, method detection limit; MQL, method quantification limit (calculated using Equation 2 and data from Table 3).

Specificity and Selectivity

The method achieved baseline chromatographic separation for all peaks, with a minimum resolution of 2.44 between clopidogrel and prasugrel (Figure 5). Furthermore, monitoring two distinct product ions ensured unambiguous identification.

To determine method selectivity, the quantification and qualification MRM transitions of each AA were compared between unspiked and 10 ng/mL (LLOQ) spiked post-SPE bovine plasma blanks. The blank plasma exhibited no interferences or cross talk at the retention times corresponding to the AAs or IS with blank responses measured at less than 0.35% and 0.62% respectively within <20% and <5% limits.

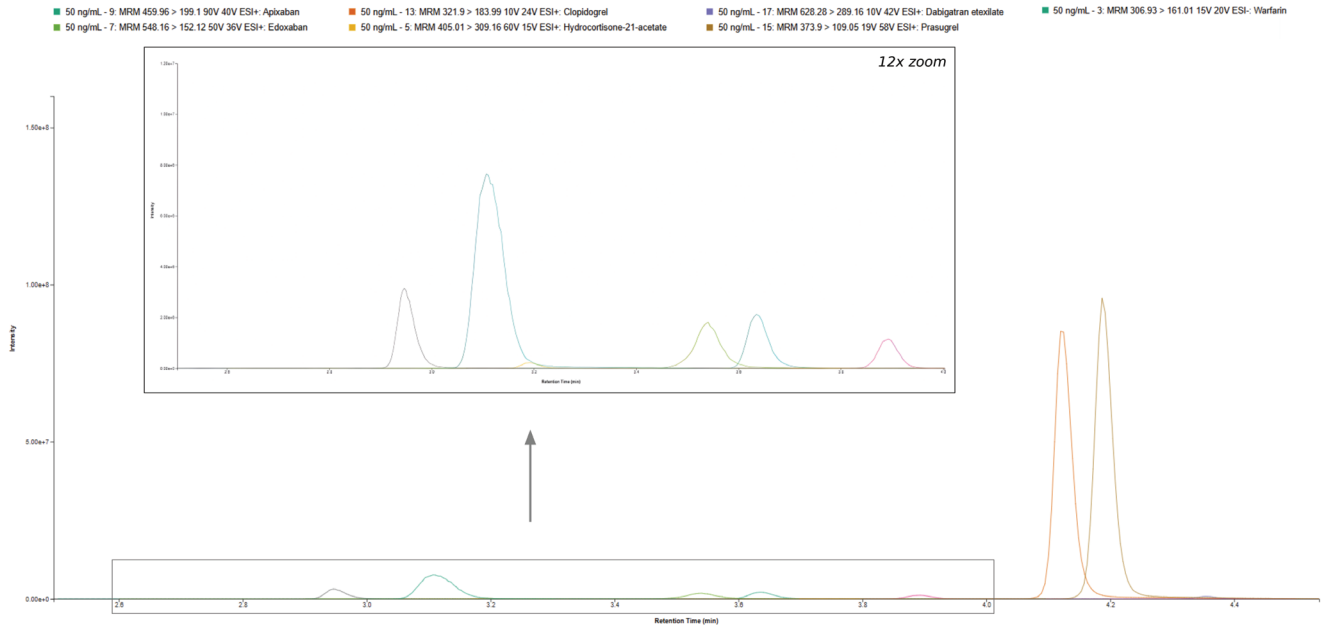


Figure 5 - Chromatographic separation of 50 ng/mL AAs QC sample with IS using LC–MS/MS method.

Carryover

Carryover was assessed by injecting the highest calibration standard, 500 ng/mL (ULOQ) in triplicate, followed immediately by the analysis of solvent blanks to monitor for any AA carryover. AA and IS responses in the solvent blanks were less than 1.94% and 1.02%, respectively, within the <20% and <5% limits.

Dilution integrity

To evaluate the suitability of the method for samples exceeding the calibration range, dilution integrity was assessed. A post-SPE bovine plasma blank was spiked at 750 ng/mL in triplicate and subsequently diluted 1:1 with an unspiked post-SPE plasma blank. For all AAs, precision was <2.5% and accuracy ranged from 72-115%.

Reinjection reproducibility

Finally, method reinjection stability was evaluated using two 80 ng/mL QC samples prepared for accuracy and precision testing. These samples were reinjected at 2, 6 and 12 hours following their initial injection. The results demonstrated a %RSD <6.5% and a percentage difference <±13.5%.

Phospholipid Removal

The efficiency of phospholipid removal during the SPE clean up step was evaluated by monitoring common phospholipids in post-SPE bovine plasma blanks. To quantify the extent of removal, the responses were compared against a solvent blank and a series of reference standards representing 95%, 98%, 99%, and 99.9% removal. The specific MRM transitions monitored for each phospholipid, alongside their calculated removal percentages, are summarised in Table 6.

Analyte	Precursor [Product] (m/z)	Core voltage [Collision energy] (V)	PL Removal Average (%)	PL Removal (%RSD)
16:0 LPC	496.30 [184.10]	80 [90]	91.8	1.16
18:0 LPC	524.30 [148.10]	23 [90]	93.6	0.71
34:1 SM	703.50 [184.10]	80 [90]	99.1	0.10
34:2 PC	758.60 [184.10]	80 [90]	99.5	0.03
36:2 PC	786.50 [184.10]	80 [90]	99.4	0.04
38:4 PC	810.70 [184.10]	80 [90]	99.4	0.04

Table 6 – MRM transition and source parameters used for phospholipids (PL) analysis with average removal and %RSD (n=12). LPC = Lysophosphatidylcholines, PC = Phosphatidylcholine, SM = Sphingomyelin

The total peak areas from all the phospholipid traces are presented against removal standards to show the total removal by the SPE method (Figure 6)

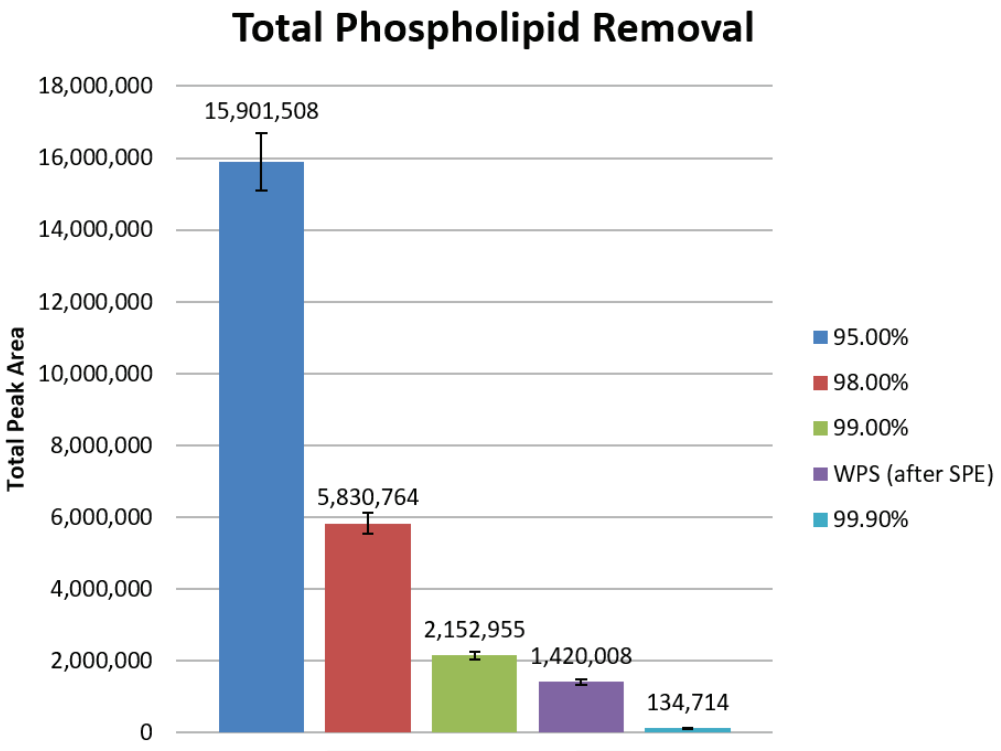


Figure 6 – Comparison of total phospholipid peak areas in WPS sample after SPE and 95% - 99.90% removal standards.

Animal Plasma Testing

The developed method was then tested on five different animal plasmas to assess the performance of the method and SPE sample preparation. The matrix effects of each animal plasma were assessed and most yielded similar results to bovine. Notable differences were dabigatran etexilate in mouse and monkey plasma, as well as warfarin in mouse plasma.

ME(%)	Bovine	Mouse	Rat	Rabbit	Beagle	Monkey
Apixaban	-4.8	-2.2	-34.2	-12.0	-45.4	-16.5
Clopidogrel	-51.6	-63.8	-78.9	-46.6	-54.4	-51.3
Dabigatran etexilate	271.7	1173	290.8	342.3	347.8	589.8
Dipyridamole	65.9	103.6	-65.1	38.5	-32.9	160.1
Edoxaban	-2.8	58.7	-7.7	-9.3	-31.0	15.9
Prasugrel	-19.2	1.9	-58.6	-3.8	-34.6	-0.6
Rivaroxaban	5.5	8.6	8.6	-1.4	-21.1	47.2
Warfarin	-6.2	133.0	6.4	-14.4	-36.2	22.3

Table 7 – Matrix effects (ME) for all AAs in each animal plasma using SPE–LC–MS/MS method calculated using Equation 1.

Recovery was generally consistent across all plasma matrices and concentration levels (Table 8), typically >70% from 20-750 ng/mL apart from dipyridamole in mouse and rat plasma. The lower recoveries may be attributed to binding of dipyridamole to proteins, specifically alpha-1-acid glycoproteins (AGP), within the mouse and rat plasma. The result of which causes the bound dipyridamole to pass through the SPE during the load/wash steps. Additionally, mouse and rat plasma possess a higher percentage of endogenous lipids per volume than the other plasmas leading to a more challenging and concentrated matrix, reflected in Table 7. Rabbit, beagle and monkey plasma showed the most consistent performance across the AAs, while rat plasma exhibited lower recovery for clopidogrel at ≥20 ng/mL.

Level (ng/mL)		Apixaban	Clopidogrel	Dabigatran etexilate	Dipyridamole	Edoxaban	Prasugrel	Rivaroxaban	Warfarin
20 (Day 1)	Bovine	90	57	90	54	78	70	77	78
	Mouse	91	75	87	6	70	78	76	84
	Rat	81	42	86	39	70	53	72	75
	Rabbit	67	55	71	63	59	62	62	67
	Beagle	76	69	78	69	70	72	65	74
	Monkey	77	80	79	74	79	77	77	78
20 (Day 2)	Bovine	81	71	111	74	84	82	86	86
	Mouse	71	74	76	3	64	73	73	73
	Rat	66	42	81	14	67	53	70	71
	Rabbit	71	73	68	72	67	69	70	72
	Beagle	65	64	69	41	65	65	67	67
80	Bovine	108	84	111	92	93	91	99	101
	Mouse	104	76	78	0	73	76	91	84
	Rat	104	84	91	2	90	78	92	91
	Rabbit	119	111	108	128	103	108	109	111
	Beagle	104	96	115	100	96	101	93	105
200	Bovine	97	111	106	111	103	107	108	106
	Mouse	97	131	82	24	66	108	95	103
	Rat	101	68	101	28	92	108	99	98
	Rabbit	98	114	90	96	92	100	98	92
	Beagle	112	126	97	80	99	109	102	110
	Monkey	96	94	94	77	84	88	90	87
400 (Day 1)	Bovine	99	90	88	99	96	84	89	85
	Mouse	120	120	93	12	88	101	103	105
	Rat	116	57	104	14	99	84	94	90
	Rabbit	97	98	86	99	94	92	89	83
	Beagle	126	124	99	108	108	109	99	111
	Monkey	124	114	123	101	118	112	117	109
400 (Day 2)	Bovine	93	108	96	113	106	100	102	95
	Mouse	112	133	114	0	104	120	110	120
	Rat	100	79	99	9	99	111	95	93
	Rabbit	97	112	88	100	102	100	96	88
	Beagle	113	114	104	78	108	106	104	108
750	Bovine	89	84	72	88	94	70	77	74
	Mouse	103	111	67	18	75	83	84	77
	Rat	105	71	80	47	91	85	76	70
	Rabbit	88	89	81	98	97	78	81	74
	Beagle	116	99	85	103	110	93	89	94

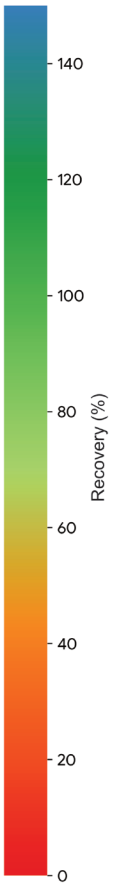


Table 8 – Intra-day recovery of AAs spiked at 10,20,80,200,400,750 ng/mL (n=3) and inter-day recovery of AAs spiked at 20,400 ng/mL (n=6) in WPS analysed using SPE–LC–MS/MS method for all 6 animal plasmas.

Apixaban, dabigatran etexilate, edoxaban, prasugrel, rivaroxaban and warfarin demonstrated comparable recoveries across all species, supporting the viability of the extraction procedure for a diverse range of animal plasma matrices. Precision remained acceptable throughout the study (Table 9).

Level (ng/mL)		Apixaban	Clopidogrel	Dabigatran etexilate	Dipyridamole	Edoxaban	Prasugrel	Rivaroxaban	Warfarin
20 (Day 1)	Bovine	3.2	2.9	8.3	14.1	2.5	2.2	3.4	3.5
	Mouse	1.7	3.7	10.0	40.5	5.4	4.2	5.6	6.8
	Rat	4.3	15.5	8.6	58.0	7.2	7.3	4.3	4.9
	Rabbit	9.3	5.9	3.2	5.5	6.9	4.8	5.0	6.7
	Beagle	6.4	3.7	28.6	20.3	8.2	12.2	8.8	9.7
	Monkey	9.1	24.4	7.2	38.2	6.0	12.8	7.3	6.6
20 (Day 2)	Bovine	2.8	2.8	7.3	13.8	4.3	5.1	3.5	4.0
	Mouse	2.9	3.9	9.6	86.5	0.6	3.1	1.8	3.5
	Rat	2.6	4.2	3.7	54.5	2.5	3.7	2.9	2.6
	Rabbit	9.3	11.6	14.4	9.1	9.3	7.1	8.5	7.4
	Beagle	2.3	10.8	2.5	39.7	4.5	2.8	1.5	3.5
80	Bovine	6.8	12.5	7.0	15.2	9.8	10.0	9.9	8.7
	Mouse	5.8	8.6	19.3	49.4	9.1	10.5	9.5	10.9
	Rat	3.8	29.2	24.3	56.7	6.0	6.7	5.9	9.1
	Rabbit	0.8	5.7	3.8	5.2	4.2	1.0	4.9	5.7
	Beagle	3.0	2.5	3.9	5.3	3.1	0.2	0.5	1.8
200	Bovine	1.2	9.2	9.2	7.7	2.1	4.0	0.9	2.0
	Mouse	0.8	1.7	10.7	0.0	9.6	4.8	4.6	8.3
	Rat	1.1	0.8	2.0	0.0	0.9	1.5	1.9	2.1
	Rabbit	6.8	8.8	8.2	6.9	6.5	9.1	7.1	7.4
	Beagle	6.0	7.4	11.3	8.3	7.9	7.9	4.1	4.5
	Monkey	12.8	7.6	16.2	16.2	23.4	13.7	15.6	15.7
400 (Day 1)	Bovine	3.1	3.7	7.0	5.4	2.6	2.5	3.6	1.0
	Mouse	8.9	8.2	6.2	0.2	7.2	7.2	7.7	4.0
	Rat	6.9	5.6	4.4	2.3	6.4	3.5	5.1	5.1
	Rabbit	11.0	11.2	10.8	9.8	10.8	12.1	9.4	10.0
	Beagle	13.5	13.3	13.2	14.5	14.4	13.4	13.0	12.2
	Monkey	11.6	30.9	9.1	20.5	12.7	16.7	13.9	13.6
400 (Day 2)	Bovine	4.6	6.8	7.6	1.9	2.2	2.7	2.8	3.3
	Mouse	1.6	23.8	8.6	15.9	3.1	7.8	3.8	1.6
	Rat	1.2	16.2	8.6	86.6	3.6	4.0	1.4	2.3
	Rabbit	4.8	10.7	17.0	4.5	4.5	4.7	4.1	3.7
	Beagle	3.9	7.2	5.9	5.8	5.3	5.8	3.6	3.6
750	Bovine	3.5	1.7	4.8	4.6	3.5	2.9	3.6	3.2
	Mouse	6.7	16.4	13.2	30.8	7.0	8.8	5.2	7.3
	Rat	4.3	30.9	13.0	2.0	0.1	11.5	0.9	0.8
	Rabbit	5.8	6.4	7.4	6.7	5.6	5.2	7.2	6.3
	Beagle	3.4	4.2	7.0	6.2	7.1	5.1	3.2	4.4

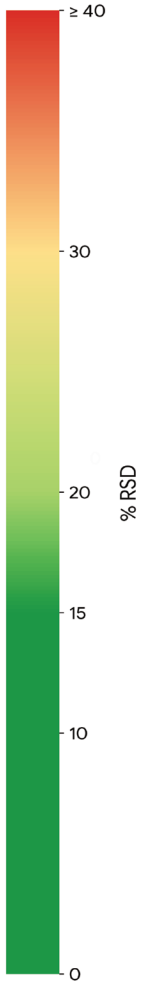


Table 9 – Intra-day %RSD of AAs spiked at 10,20,80,200,400,750 ng/mL (n=3) and inter-day %RSD of AAs spiked at 20,400 ng/mL (n=6) in WPS analysed using SPE–LC–MS/MS method for all 6 animal plasmas.

Conclusion

A sensitive SPE-LC-MS/MS method was developed and validated for the simultaneous quantification of major anticoagulant and antiplatelet medications in animal plasma. The method demonstrated excellent linearity (R^2>0.98), low MQLs (<5.75 ng/mL) and good reproducibility (<15% RSD), with recoveries exceeding 60% at the LLOQ and 70% at concentrations above 80 ng/mL. The Microlute® CP HLB 96-well plate provided reliable extraction and sample clean-up, including effective phospholipid removal (>91.8%) without clogging. Performance was generally good across the six animal plasma matrices. However, analyte and species dependent trends were observed, particularly for dabigatran etexilate and dipyridamole in mouse and rat plasma. The method combined anticoagulants and antiplatelets in one scalable workflow which offers significant advantages over assay-based methods, including a low sample volume (250 µL), moderate run time (7.5 min), and the flexibility to include additional compounds. Coupled with the simple sample preparation workflow these features make the method a promising platform for clinical antithrombotic monitoring, where robustness and sensitivity are essential. Further validation in human plasma, including stability assessments and analysis of clinical samples, is required before the method can be adopted for routine antithrombotic monitoring.

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