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Development and Validation of a Rapid, ICH M10-Compliant LC-MS/MS Method for Simultaneous Estimation of Rivaroxaban and Aspirin in Human Plasma: Application to Pharmacokinetic Studies of the COMPASS Regimen

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Abstract: *The combination of rivaroxaban (a direct Factor Xa inhibitor) and low-dose aspirin (dual pathway inhibition, the "COMPASS regimen") is increasingly used for secondary prevention of atherothrombotic events, creating a need for a sensitive, specific bioanalytical method capable of quantifying both agents simultaneously despite the marked instability of aspirin in biological matrices. This study aimed to develop and validate a rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous estimation of rivaroxaban and aspirin in human K2EDTA plasma, fully compliant with the ICH M10 (2022) bioanalytical method validation guideline.*

Chromatographic separation was achieved on a Phenomenex Gemini C18 column (50 × 4.6 mm, 5 µm) using an isocratic mobile phase of 10 mM ammonium acetate (pH 4.5) and acetonitrile (30:70, v/v) at 0.8 mL/min. Detection used a Shimadzu LCMS-8050 triple quadrupole mass spectrometer operating in polarity-switching electrospray ionisation mode (positive for rivaroxaban, negative for aspirin) with rivaroxaban-d4 and aspirin-d4 as internal standards. Plasma samples (20 µL) were processed by protein precipitation with acetonitrile containing 0.1% formic acid. The method was validated for selectivity, linearity, accuracy, precision, recovery, matrix effect, carryover, dilution integrity and stability according to ICH M10 (2022)..

Keywords: Rivaroxaban; Aspirin; LC-MS/MS; ICH M10; Bioanalytical method validation; Simultaneous estimation; COMPASS regimen; Human plasma

I. INTRODUCTION

Thromboembolic disorders — including deep vein thrombosis, pulmonary embolism, atrial fibrillation-related stroke and acute coronary syndrome — remain a leading cause of morbidity and mortality worldwide, arising from inappropriate activation of the coagulation cascade and pathological thrombus formation [1,2]. For decades, vitamin K antagonists and heparins were the mainstay of anticoagulant therapy, but their narrow therapeutic windows, variable pharmacokinetics and requirement for routine coagulation monitoring prompted the development of direct oral anticoagulants (DOACs) [3]. Rivaroxaban, the first oral direct Factor Xa inhibitor, selectively and reversibly inhibits Factor Xa (Ki = 0.4 nM), interrupting the coagulation cascade at a point of convergence between the intrinsic and extrinsic pathways [4,5].

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In parallel, low-dose aspirin (acetylsalicylic acid) continues to be a cornerstone of antiplatelet therapy for secondary cardiovascular prevention. The combination of rivaroxaban (2.5 mg twice daily) and aspirin (100 mg once daily) — dual pathway inhibition, evaluated in the COMPASS trial — has demonstrated a significant reduction in major adverse cardiovascular events in patients with stable atherosclerotic disease [6]. Reliable quantification of both agents in the same plasma sample is therefore required to support pharmacokinetic characterisation, bioequivalence assessment of generic combinations, and therapeutic drug monitoring.

Bioanalysis of this combination presents two distinct challenges. First, rivaroxaban and aspirin ionise most efficiently in opposite electrospray polarities (positive and negative, respectively), historically necessitating separate chromatographic runs. Second, aspirin undergoes rapid, temperature- and pH-dependent esterase-mediated hydrolysis to salicylic acid (plasma half-life of only 15–20 minutes at 37 °C), making it one of the most unstable analytes routinely encountered in bioanalysis [7,8]. A systematic literature search identified only a small number of published methods that attempt simultaneous LC-MS/MS estimation of rivaroxaban and aspirin, and none was fully compliant with the final ICH M10 (2022) guideline: limitations included analyte derivatisation, incomplete long-term stability data for aspirin, and matrix-effect evaluation using fewer than the six to eight plasma lots recommended by ICH M10 [9–11].

The present work therefore aimed to develop and fully validate, in accordance with ICH M10 (2022), a rapid polarity-switching LC-MS/MS method for the simultaneous estimation of native (non-derivatised) rivaroxaban and aspirin in human K2EDTA plasma, with particular emphasis on characterising and controlling aspirin instability, so that the method would be suitable for application in pharmacokinetic studies of the rivaroxaban–aspirin (COMPASS) combination.

II. MATERIALS AND METHODS

2.1 Chemicals, reagents and reference standards

Reference standards of rivaroxaban (99.87%) and aspirin (99.92%), and the deuterated internal standards rivaroxaban-d4 and aspirin-d4 (isotopic purity ≥99%), were procured with certificates of analysis (Table 1). LC-MS grade acetonitrile and methanol, ammonium acetate, formic acid, acetic acid and ammonium hydroxide were used throughout. Pooled and individual-lot human K2EDTA plasma (containing 1.5 mg/mL sodium fluoride to inhibit esterase activity), including hemolyzed and lipemic lots, was sourced commercially and screened to confirm the absence of rivaroxaban, aspirin and common interfering co-medications prior to use.

Table 1. Reference standards used in the study

Compound	CAS No.	Molecular formula	MW (g/mol)	Purity (%)
Rivaroxaban (RIV)	366789-02-8	C19H18ClN3O5S	435.88	99.87
Aspirin (ASA)	50-78-2	C9H8O4	180.16	99.92
Rivaroxaban-d4 (IS)	1261394-13-9	C19H14D4ClN3O5S	439.90	99.3 (isotopic)
Aspirin-d4 (IS)	76061-79-5	C9H4D4O4	184.18	99.1 (isotopic)

2.2 Instrumentation

Chromatography was performed on a Shimadzu Nexera X2 LC-30AD system with SIL-30AC autosampler (10 °C) and CTO-20AC column oven, coupled to a Shimadzu LCMS-8050 triple quadrupole mass spectrometer (ESI source, polarity-switching time 15 msec), controlled by LabSolutions software (v5.97). Separation used a Phenomenex Gemini C18 analytical column (50 × 4.6 mm, 5 µm, 110 Å) with a matching SecurityGuard C18 pre-column.

Table 2. Final chromatographic and mass spectrometric conditions

Parameter	Setting
Analytical column	Phenomenex Gemini C18, 50 × 4.6 mm, 5 µm
Mobile phase	10 mM ammonium acetate (pH 4.5) : acetonitrile (30:70, v/v)
Flow rate	0.8 mL/min

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Column temperature	40 °C
Injection volume	5 µL
Total run time	3.0 minutes
Ionisation source	ESI, polarity switching (positive for RIV; negative for ASA)
Retention time – aspirin / RIV	0.85 min / 1.52 min

2.2.1 MRM transitions

Table 3. Optimised multiple reaction monitoring (MRM) parameters

Analyte	Precursor (m/z)	Product, quantifier (m/z)	DP (V)	CE (V)	Polarity
Rivaroxaban	436.20	145.10	−32	−28	Positive
Rivaroxaban-d4 (IS)	440.20	149.10	−32	−28	Positive
Aspirin	179.10	137.10	+25	+16	Negative
Aspirin-d4 (IS)	183.10	141.10	+25	+16	Negative

2.3 Preparation of calibration standards and quality control samples

Calibration standards were prepared in blank human K2EDTA plasma at seven non-zero concentrations spanning 1–500 ng/mL (rivaroxaban) and 10–5000 ng/mL (aspirin). Quality control (QC) samples were prepared independently at the lower limit of quantification (LLOQ), low (LQC), medium (MQC) and high (HQC) concentrations (Table 4).

Table 4. Calibration range and quality control concentrations

Level	Rivaroxaban (ng/mL)	Aspirin (ng/mL)
LLOQ	1.0	10
LQC	3.0	30
MQC	150.0	1500
HQC	400.0	4000
ULOQ	500.0	5000

2.4 Sample preparation

Analytes were extracted from plasma by protein precipitation, selected over liquid-liquid extraction and solid-phase extraction after comparative evaluation (Section 3.1.3) on the basis of speed, simplicity, and — critically — minimal risk of aspirin hydrolysis during processing. Briefly, 20 µL of plasma was mixed with 180 µL of ice-cold internal standard working solution (rivaroxaban-d4 50 ng/mL and aspirin-d4 500 ng/mL in acetonitrile containing 0.1% formic acid), vortexed (2500 rpm, 2 min) and centrifuged (14,000 rpm, 10 min, 4 °C). A 150 µL aliquot of supernatant was transferred to an autosampler vial maintained at 10 °C, and 5 µL was injected. All processing steps were performed under ice-cold, acidified conditions to minimise ex vivo aspirin hydrolysis.

2.5 Method validation

The method was validated according to ICH M10 (2022) for system suitability, specificity/selectivity (six individual plasma lots plus one hemolyzed and one lipemic lot), sensitivity (LLOQ), linearity (three batches, $1/x^2$-weighted regression), intra- and inter-batch accuracy and precision (six replicates × four QC levels × three batches), extraction recovery, matrix effect (eight plasma lots), carryover, dilution integrity (2-fold and 10-fold), and stability (bench-top, autosampler, freeze-thaw, long-term frozen, and stock solution). Acceptance criteria followed ICH M10 throughout (accuracy ±15%, precision ≤15% for QC levels; ±20%/≤20% at LLOQ; $r^2 \geq 0.98$; IS-normalised matrix factor CV ≤15%).

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III. RESULTS AND DISCUSSION

3.1 Method development

3.1.1 Ionisation mode and precursor ion selection

Direct infusion experiments showed that rivaroxaban produced an intense [M+H]+ signal at m/z 436.20 in positive ESI mode, with negative-mode response below 10% of positive; aspirin, bearing a carboxylic acid group (pKa 3.5), readily formed [M–H]− at m/z 179.10 in negative mode with a weak, unstable positive-mode signal (Table 5). Positive mode was therefore selected for rivaroxaban and negative mode for aspirin, requiring polarity switching within a single chromatographic run.

Table 5. Precursor ion response by ionisation polarity

Compound	ESI+ intensity (cps)	ESI− intensity (cps)	Selected mode	Precursor (m/z)
Rivaroxaban	2.8×10^6	2.1×10^5	Positive	436.20
Aspirin	3.2×10^4	1.9×10^6	Negative	179.10

Polarity-switch timing was optimised by scanning the switch point from immediately after aspirin elution (1.0 min) to 1.5 min; a switch at 1.3 min — after aspirin (0.85 min) but with a 100 msec stabilisation delay before rivaroxaban elution (1.52 min) — gave stable peak areas for both analytes without truncating the rivaroxaban peak, allowing both analytes to be quantified in a single 3-minute run instead of two separate 6-minute injections.

3.1.2 Chromatographic optimisation

Four C18 columns were screened (Table 6). The Phenomenex Gemini C18 (bidentate bonding, pH stable 1–12) gave the best peak symmetry for both the weakly basic rivaroxaban and the acidic aspirin, and was selected for further development.

Table 6. Column screening results

Column	ASA RT (min)	RIV RT (min)	Tailing (ASA/RIV)	Outcome
Phenomenex Gemini C18	0.85	1.52	1.12 / 1.08	Selected
Waters XBridge C18	0.92	1.68	1.25 / 1.18	Rejected (broader peaks)
Agilent Zorbax Eclipse Plus C18	0.78	1.45	1.45 / 1.22	Rejected (ASA tailing)
Thermo Hypersil GOLD C18	0.81	1.40	1.18 / 1.15	Rejected (low RIV retention)

Mobile phase pH, buffer strength and acetonitrile content were optimised sequentially. A mobile phase pH of 4.5 (10 mM ammonium acetate) gave the best compromise between aspirin peak shape (partially ionised, ~90% at pH 4.5) and rivaroxaban symmetry; lower pH increased aspirin tailing and higher pH increased tailing for both analytes. A buffer concentration of 10 mM and an acetonitrile content of 70% (v/v) were selected as they gave the sharpest peaks with acceptable backpressure (~1800 psi) and adequate resolution (2.8) within a 3.0-minute run; a flow rate of 0.8 mL/min gave the best signal-to-noise ratio for rivaroxaban without excessive backpressure (Table 7).

Table 7. Effect of flow rate on peak width, backpressure and sensitivity

Flow rate (mL/min)	RIV peak width (min)	Backpressure (psi)	S/N (RIV, 50 ng/mL)
0.6	0.22	1350	450
0.7	0.18	1580	520
0.8 (selected)	0.14	1800	580
0.9	0.12	2050	550
1.0	0.10	2300	500

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3.1.3 Sample-preparation optimisation

Protein precipitation, liquid-liquid extraction (LLE) and solid-phase extraction (SPE) were compared. LLE (MTBE extraction with evaporation/reconstitution) gave poor aspirin recovery (65%) owing to hydrolysis during evaporation; SPE (Oasis HLB) produced the cleanest extracts but aspirin recovery remained only 72% with a longer, costlier workflow. Protein precipitation with acetonitrile containing 0.1% formic acid (9:1, solvent:plasma) gave the best combination of speed, recovery (RIV 96%, ASA 92%) and aspirin stability, and was selected as the final method (Table 8).

Table 8. Protein-precipitation solvent screening (recovery and aspirin bench-top stability)

Precipitating solvent	Recovery RIV (%)	Recovery ASA (%)	ASA remaining at 4 h RT (%)
Acetonitrile (ACN)	94	89	85
ACN + 0.1% formic acid (selected)	96	92	92
ACN + 0.2% formic acid	95	91	93
Methanol	88	82	78
Methanol + 0.1% formic acid	90	85	88

3.2 System suitability

Six consecutive injections of the MQC extract met all pre-defined system-suitability criteria: retention-time %CV ≤ 0.71%, peak-area %CV ≤ 1.50%, tailing factor ≤ 1.12, theoretical plates ≥ 3245, and signal-to-noise ratio at LLOQ ≥ 12.8 for all analytes and internal standards (Table 9), confirming the system was fit for validation.

Table 9. System suitability data (n = 6 injections of MQC)

Parameter	RIV	RIV-d4	ASA	ASA-d4	Acceptance
Mean RT (min)	1.521	1.520	0.851	0.850	–
%CV RT	0.53%	0.46%	0.71%	0.59%	≤ 2.0%
%CV Peak area	1.40%	1.50%	1.34%	1.40%	≤ 2.0%
Tailing factor	1.08	1.07	1.12	1.10	≤ 1.5
Theoretical plates	4,521	4,589	3,245	3,298	≥ 2000
S/N at LLOQ	18.5	22.1	12.8	15.2	≥ 10

3.3 Specificity and selectivity

No relevant endogenous interference was observed at the retention times of either analyte or internal standard across six normal plasma lots and one hemolyzed and one lipemic lot. Peak areas attributable to interference at the analyte retention times ranged from 0.3–2.3% (RIV) and 0.8–4.2% (ASA) of the corresponding LLOQ response — all well below the 20% acceptance limit — while IS interference in double-blank plasma did not exceed 0.2% of the IS response in any lot (limit ≤ 5%). As expected, the hemolyzed and lipemic lots showed the highest, though still passing, background (Table 10).

Table 10. Selectivity summary – interference as % of LLOQ response (8 plasma lots)

Plasma lot	RIV interference (%LLOQ)	ASA interference (%LLOQ)	Result
Lots 1–6 (normal donors)	0.3–0.8%	0.8–1.2%	Pass
Lot 7 (hemolyzed)	2.3%	4.2%	Pass
Lot 8 (lipemic, TG 380 mg/dL)	1.4%	2.5%	Pass

3.4 Linearity and calibration

Calibration curves were linear over 1–500 ng/mL (rivaroxaban) and 10–5000 ng/mL (aspirin) using $1/x^2$-weighted linear regression, which gave markedly better accuracy at the LLOQ (mean absolute %RE 0.9%) than unweighted regression (3.8%) or 1/x or 1/y weighting (Table 11, Fig. 1). Mean correlation coefficients across three batches were

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0.9987 ± 0.0002 (RIV) and 0.9991 ± 0.0001 (ASA), and all back-calculated calibration standards, including LLOQ and ULOQ, met the ±15% (±20% at LLOQ) acceptance criterion in every batch (Table 12).

Table 11. Effect of regression weighting on rivaroxaban calibration accuracy (Batch A)

Weighting scheme	%RE at LLOQ	%RE at mid-range	%RE at ULOQ	Mean absolute %RE
None (unweighted)	−12.5%	−1.2%	+0.3%	3.8%
1/x	−5.8%	−0.8%	+0.1%	2.1%
1/x² (selected)	−2.0%	−0.3%	−0.3%	0.9%
1/y	−8.2%	−1.0%	+0.5%	2.7%

Table 12. Inter-batch calibration curve summary (3 batches, 1/x² weighting)

Analyte	Range (ng/mL)	Mean slope ± SD	Mean r² ± SD
Rivaroxaban	1–500	0.004388 ± 0.000019	0.9987 ± 0.0002
Aspirin	10–5000	0.000925 ± 0.000005	0.9991 ± 0.0001

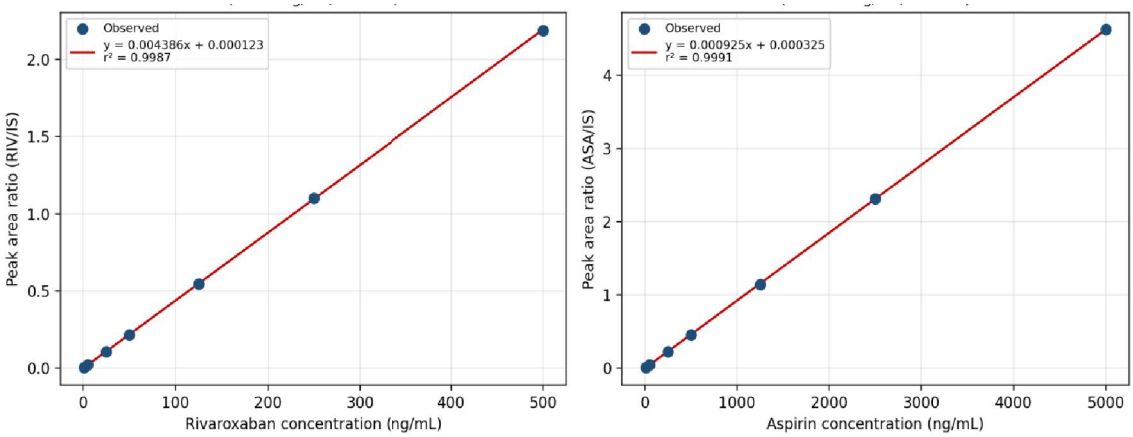

Figure 1. Representative calibration curves (Batch A) for (A) rivaroxaban (1–500 ng/mL) and (B) aspirin (10–5000 ng/mL) in human K2EDTA plasma, using 1/x²-weighted linear regression.

3.5 Accuracy and precision

Intra-batch precision (%CV, n = 6) ranged from 2.6–8.4% for rivaroxaban and 2.8–11.5% for aspirin across LLOQ, LQC, MQC and HQC levels, with accuracy (%RE) within −5.0% to +2.7% (RIV) and −4.0% to +4.0% (ASA). Inter-batch precision (n = 18, three batches) was slightly higher, as expected, but remained within 3.4–10.4% (RIV) and 3.6–12.4% (ASA), with accuracy within ±4.0% for both analytes at all levels (Table 13). All results met the ICH M10 acceptance criteria (±15%/≤15% for QC levels; ±20%/≤20% at LLOQ).

Table 13. Intra- and inter-batch accuracy and precision

QC level	Nominal RIV (ng/mL)	Intra %CV / %RE (RIV)	Inter %CV / %RE (RIV)	Nominal ASA (ng/mL)	Intra %CV / %RE (ASA)	Inter %CV / %RE (ASA)
LLOQ	1.0	8.4% / −5.0%	10.4% / −4.0%	10	11.5% / −4.0%	12.4% / −3.0%
LQC	3.0	4.5% / +2.7%	5.8% / +3.3%	30	6.4% / +4.0%	7.1% / +2.7%
MQC	150.0	3.5% / −1.0%	4.7% / −1.5%	1500	4.6% / −0.5%	5.2% / −0.8%
HQC	400.0	2.6% / +0.6%	3.4% / +0.8%	4000	2.8% / −0.4%	3.6% / −0.2%

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3.6 Extraction recovery

Mean absolute recovery, determined by comparing extracted and post-extraction-spiked samples at three QC levels (n = 6), was 92.9% for rivaroxaban (range 92.4–93.9%, CV 3.0–3.8%) and 88.7% for aspirin (range 88.6–88.9%, CV 4.7–5.4%) (Table 14, Fig. 2). Recovery of the internal standards (94.6% RIV-d4; 93.8% ASA-d4) closely matched that of the corresponding analytes, so that any residual extraction losses are normalised by the IS ratio during quantification. The marginally lower and more variable recovery of aspirin, relative to rivaroxaban, is consistent with partial ex vivo hydrolysis during processing despite cold, acidified conditions, and with the comparatively higher polarity and protein affinity of aspirin and its hydrolysis product.

Table 14. Absolute extraction recovery of analytes and internal standards (n = 6 per level)

QC level	Recovery RIV (%, CV)	Recovery ASA (%, CV)	Recovery RIV-d4 (%, CV)	Recovery ASA-d4 (%, CV)
LQC	92.5% (3.5%)	88.6% (5.1%)	–	–
MQC	93.9% (3.0%)	88.9% (4.7%)	94.6% (2.9%)	93.8% (3.2%)
HQC	92.4% (3.8%)	88.7% (5.4%)	–	–
Mean	92.9%	88.7%	94.6%	93.8%

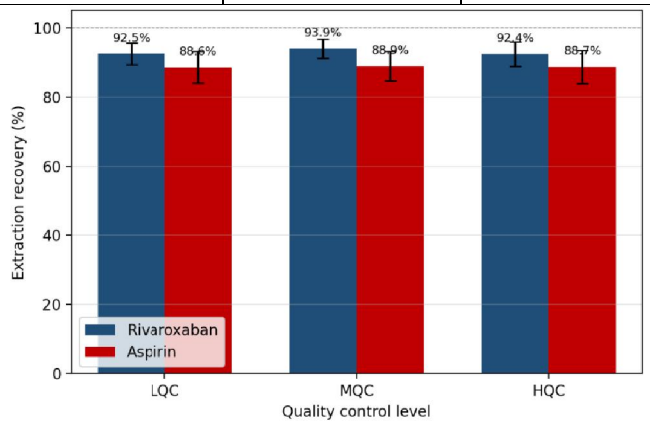

Figure 2. Absolute extraction recovery (mean ± SD, n = 6) of rivaroxaban and aspirin from human K2EDTA plasma at low, medium and high QC levels.

3.7 Matrix effect

IS-normalised matrix factors (MF), assessed across eight individual plasma lots — including hemolyzed and lipemic matrices — at LQC and HQC levels, were tightly clustered around 1.00 (range 0.95–1.02) for both analytes, with %CV of 1.11–1.82% (well within the 15% acceptance limit) (Table 15, Fig. 3). Hemolyzed and lipemic lots showed greater absolute ion suppression (MF 0.84–0.94) than normal plasma, but because the deuterated internal standards were suppressed to a similar degree, the IS-normalised MF for these challenging matrices remained within acceptance (0.95–0.98), confirming that the internal standards adequately compensate for matrix-related ion suppression across a range of donor plasma qualities.

Table 15. IS-normalised matrix factor summary across 8 plasma lots

Analyte	QC level	Mean IS-norm. MF	%CV (n = 8)	Acceptance
Rivaroxaban	LQC (3 ng/mL)	0.99	1.41%	≤ 15%
Rivaroxaban	HQC (400 ng/mL)	0.99	1.11%	≤ 15%
Aspirin	LQC (30 ng/mL)	0.99	1.82%	≤ 15%
Aspirin	HQC (4000 ng/mL)	0.99	1.31%	≤ 15%

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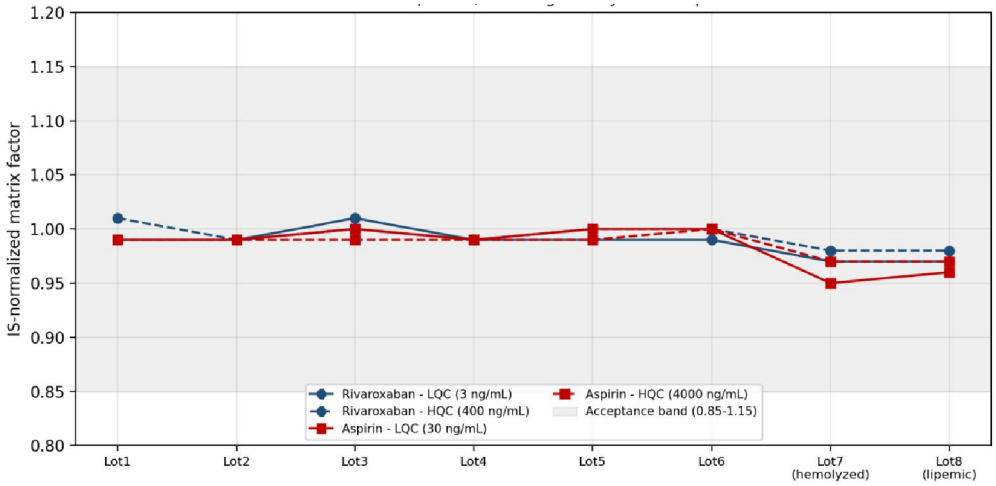

Figure 3. IS-normalised matrix factor for rivaroxaban and aspirin across eight individual plasma lots, including one hemolyzed and one lipemic lot, at LQC and HQC levels. Shaded band indicates the acceptable range (0.85–1.15).

3.8 Carryover

Carryover, assessed as the analyte peak area in blank plasma injected immediately after the ULOQ sample, was 15.9% of the LLOQ peak area for rivaroxaban and 15.2% for aspirin — both within the 20% acceptance limit — falling to below 5% by the second subsequent blank injection. IS carryover did not exceed 3.5% of the LLOQ IS response for either internal standard (limit ≤ 5%). While within specification, the observed carryover approaches the upper end of the acceptable range and comparable to some published methods (Table 16); routine analytical runs should therefore include a blank injection after any sample expected to exceed the ULOQ.

Table 16. Carryover assessment (blank injected immediately after ULOQ)

Analyte	Carryover, first blank (% of LLOQ)	Carryover, second blank (%)	IS carryover (% of LLOQ IS)	Acceptance
Rivaroxaban	15.9%	3.6%	3.5%	≤ 20% (analyte); ≤ 5% (IS)
Aspirin	15.2%	3.8%	3.8%	≤ 20% (analyte); ≤ 5% (IS)

3.9 Dilution integrity

Samples prepared at twice the ULOQ (1000 ng/mL RIV, 10000 ng/mL ASA) and diluted 2-fold or 10-fold with blank plasma before processing gave back-calculated concentrations within −1.5% of nominal for both analytes and both dilution factors, with %CV ≤ 3.5% (n = 5) (Table 17), confirming that plasma samples with concentrations exceeding the calibration range (e.g., Cmax samples) can be reliably diluted and reanalysed.

Table 17. Dilution integrity for samples above the ULOQ (n = 5 per dilution factor)

Analyte	Dilution factor	Accuracy (%RE)	Precision (%CV)
Rivaroxaban	2-fold	−0.3%	3.2%
Rivaroxaban	10-fold	−1.3%	2.8%
Aspirin	2-fold	−0.3%	3.5%
Aspirin	10-fold	−1.5%	3.1%

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3.10 Stability

Rivaroxaban proved stable under all conditions tested: ≤ 3.2% loss on the bench-top for 6 hours, ≤ 2.6% in the autosampler for 24 hours, ≤ 1.9% over three freeze-thaw cycles, and ≤ 4.2% after 60 days of frozen storage at −70 °C. Aspirin, consistent with its known lability, showed progressively greater degradation with time and temperature: 12.8% loss on the bench-top at 6 hours, 9.7% loss in the autosampler at 24 hours, 7.5% loss over three freeze-thaw cycles, and, critically, 13.0% loss by day 45 and 17.2% loss by day 60 of frozen storage — the latter exceeding the 15% acceptance limit at the low QC level (Table 18, Fig. 4). Aspirin stock solution in methanol at −70 °C likewise showed acceptable stability only up to 14 days (96.2% remaining); by 30 days, recovery fell to 92.5%, outside the acceptance limit.

Table 18. Stability summary (LQC, % change from time zero unless stated)

Stability type	Rivaroxaban	Aspirin	Recommended handling limit
Bench-top, 25 °C	6 h (≤3.2%)	6 h (≤12.8%)	Process within 4 h
Autosampler, 10 °C	24 h (≤2.6%)	24 h (≤9.7%)	Inject within 24 h
Freeze-thaw (3 cycles)	≤1.9%	≤7.5%	Maximum 3 cycles
Long-term frozen, −70 °C	60 d (≤4.2%)	45 d (≤13.0%); fails at 60 d (17.2%)	Analyse aspirin within 45 d
Stock solution	30 d at −20 °C (≤99.5%)	14 d at −70 °C (96.2%); fails at 30 d	Prepare aspirin stock fresh every 14 d

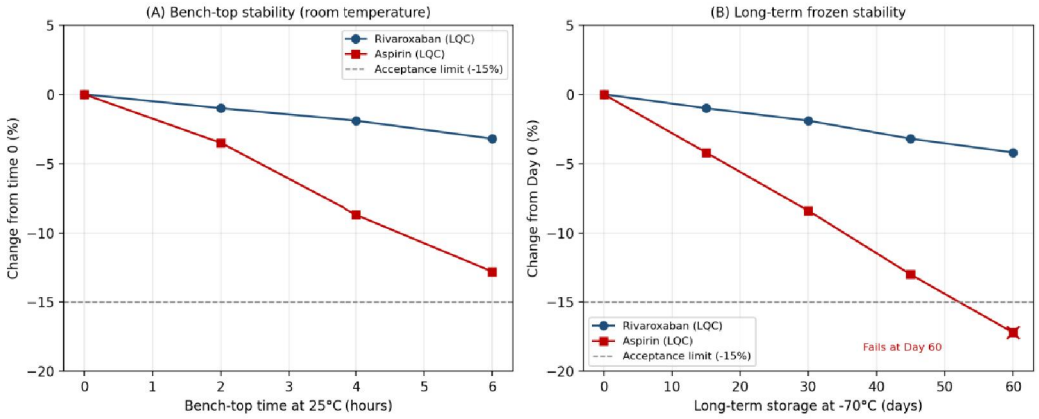

Figure 4. (A) Bench-top stability at room temperature and (B) long-term frozen stability at −70 °C for rivaroxaban and aspirin at the low QC level. The dashed line indicates the ±15% ICH M10 acceptance limit; aspirin exceeds this limit beyond 45 days of frozen storage.

IV. CONCLUSION

A sensitive, rapid and fully ICH M10 (2022)-compliant LC-MS/MS method was developed and validated for the simultaneous quantification of rivaroxaban and aspirin in human K2EDTA plasma using polarity-switching electrospray ionisation and a simple protein-precipitation extraction from only 20 µL of plasma. The method demonstrated excellent linearity ($r^2 \geq 0.998$), acceptable accuracy and precision at all quality-control levels, high and consistent extraction recovery, negligible IS-normalised matrix effect across eight plasma lots (including hemolyzed and lipemic matrices), and a rapid 3.0-minute run time suitable for high-throughput pharmacokinetic studies. Comprehensive stability testing identified aspirin — rather than rivaroxaban — as the critical determinant of sample-handling requirements, with frozen storage limited to 45 days at −70 °C and bench-top processing recommended within 4 hours. With these handling precautions observed, the method is considered suitable for pharmacokinetic, bioequivalence and therapeutic drug monitoring studies of the rivaroxaban–aspirin (COMPASS) combination.

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