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Determination of rivaroxaban – a novel, oral, direct Factor Xa inhibitor – in human plasma by high-performance liquid chromatography–tandem mass spectrometry

G. Rohde*

Drug Metabolism and Pharmacokinetics – Bioanalytics, Bayer HealthCare AG, Aprather Weg, 42096 Wuppertal, Germany

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ABSTRACT

A high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method allowing the sensitive and specific quantification of rivaroxaban (BAY 59-7939), a Factor Xa inhibitor in advanced development for the prevention and treatment of thromboembolic disorders, in human plasma is described. After precipitation of plasma proteins with methanol containing the internal standard followed by centrifugation, the plasma supernatant was injected directly onto the HPLC–MS/MS system. Concentrations could be determined between 0.50 and 500 μg/L. Inter-assay precision was ≤7.4% and inter-assay accuracy was between 96.3 and 102.9% throughout the entire working range. The method was applied successfully in several clinical studies, which allowed an accurate determination of rivaroxaban pharmacokinetics in human plasma.

1. Introduction

Factor Xa (FXa) is a key component of the blood coagulation cascade, which leads to thrombin activation and blood clotting [1]. In fact, FXa is the primary site of amplification of thrombin generation; one molecule of FXa leads to the generation of more than 1000 thrombin molecules [1]. Therefore, FXa is an important target for the prevention and treatment of thromboembolic disorders. Low molecular weight heparins (LMWHs), which are small- to medium-chain heparin molecules, are indirect FXa and thrombin inhibitors that are administered subcutaneously. They are the current standard of care for thromboprophylaxis after major orthopaedic surgery. However, a major drawback associated with these drugs, in addition to their subcutaneous administration, is the inability to measure their presence directly and, thus, to establish their pharmacokinetic profile in human plasma. LMWHs are 'measured' by assessing their pharmacodynamic effects (anti-FXa test).

Rivaroxaban (5-chloro-*N*-({(5S)-2-oxo-3-[4-(3-oxo-4-morpholinyl)phenyl]-1,3-oxazolidin-5-yl}methyl)-2-thiophenecarboxamide) ($C_{19}H_{18}ClN_3O_5S$, MW 435.9 g/mol, Fig. 1A) [2] is a novel, oral, selective, highly potent, direct FXa inhibitor. In preclinical studies, rivaroxaban demonstrated consistent and potent anticoagulant and antithrombotic effects [3,4]. Furthermore, the efficacy and safety of rivaroxaban have been demonstrated in clinical phase I–III trials [5–11].

A sensitive and specific analytical assay was required to assess the human pharmacokinetics of rivaroxaban, which should allow quantification of the unchanged drug, alone and during concomitant administration with other medications (for example, in drug–drug interaction studies and in clinical phase II–III studies).

In clinical pharmacology and clinical studies (especially in the dose-finding studies), rivaroxaban plasma concentrations ranging from 0.50 to 500 μg/L are considered as relevant. The assay described was validated over this concentration range and according to current guidelines on bioanalytical assay validation [12,13]. Protein precipitation in plasma samples followed by high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) was the assay technology of choice to achieve a robust, high throughput of samples.

2. Experimental

2.1. Chemicals

The reagents used were of analytical grade. Rivaroxaban and its structurally analogous internal standard (Fig. 1) were obtained as certified reference compounds (Bayer HealthCare AG, Wuppertal, Germany).

HPLC solvents in gradient-grade quality and methanol were obtained from Riedel-de-Haen (Seelze, Germany). Ammonium acetate and formic acid were obtained from Merck (Darmstadt, Germany). Water was purified by the Milli Q system (Millipore Waters, Eschborn, Germany). Blank plasma was obtained from healthy subjects.

* Tel.: +49 202 36 4566; fax: +49 202 36 4224.
E-mail address: gabriele.rohde@bayerhealthcare.com.

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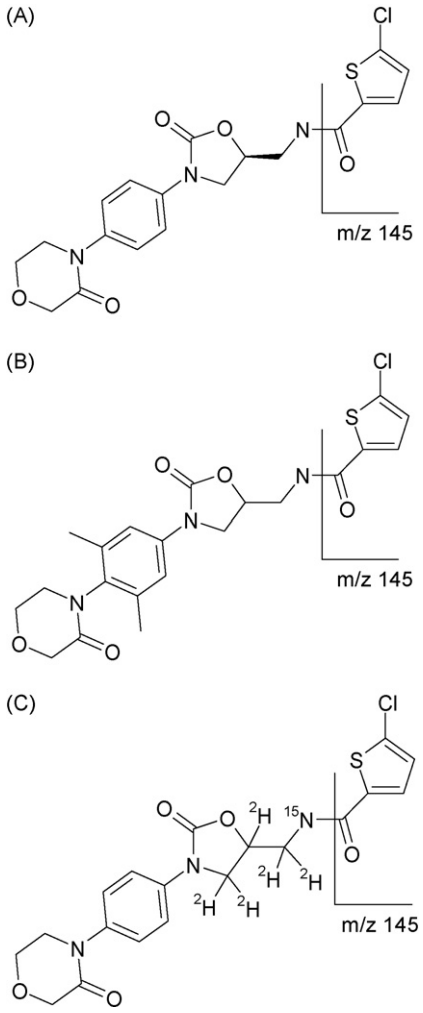

Fig. 1. Chemical structure of (A) rivaroxaban (BAY 59-7939), (B) internal standard (structural analogue) and (C) internal standard ([$^{2}H_5$, ^{15}N]) rivaroxaban, and their CID fragmentation patterns.

2.2. Assay technology

Separation by liquid chromatography on a RP-18 stationary phase, followed by ultraviolet (UV) detection at 270 nm, was the first approach investigated, based on the chemical structure and physico-chemical properties of rivaroxaban. A solid-phase extraction procedure (SPE) was used for sample clean up; the lower limit of quantification (LLOQ) was 2.0 μg/L. However, in most clinical pharmacology and clinical studies (especially in phase II, dose-finding studies), rivaroxaban plasma concentrations ranging from 0.50 to 500 μg/L are considered as relevant. Therefore, an HPLC–MS/MS assay validated over this concentration range was investigated. A simple and inexpensive precipitation of plasma proteins in autosampler glass vials, followed by centrifugation and direct injection of the plasma supernatant onto the HPLC–MS/MS system, was used instead of SPE for sample preparation. This assay technology allowed for a robust, high throughput of samples as well as a lower LLOQ compared with the HPLC–UV method.

2.3. Instrumentation and operating conditions

2.3.1. Instrumentation

An Applied Biosystems (Darmstadt, Germany) Sciex API 3000 tandem mass spectrometer equipped with a Turboionspray® interface, a CTC-PAL autosampler (Zwingen, Switzerland) and an Agilent 1100 system (Waldbronn, Germany) were used for the HPLC–MS/MS analyses. The data were processed using the validated PC software Concalc for Windows (CCW; INTEG Labordatensysteme GmbH, Remchingen, in co-operation with Bayer HealthCare AG, Wuppertal).

2.3.2. Chromatographic conditions

The autosampler temperature was kept at 10 °C. A Purosphere RP18e (5 μm) particle size, 125 mm × 4 mm internal diameter (ID) column preceded by a guard column (4 mm × 4 mm ID) of the same material (Merck, Darmstadt, Germany) was used for separation. The columns were operated at ambient temperature.

The mobile phase consisted of acetonitrile and 0.01 mol/L ammonium acetate buffer adjusted to pH 3.0 by addition of formic acid. The flow rate was set at 1.0 mL/min. The gradient for elution is given in Table 1. The stop time was after 6 min.

2.3.3. HPLC–MS/MS conditions

An Applied Biosystems triple quadrupole mass spectrometer (Sciex API 3000) was interfaced via a Sciex Turboionspray® probe with the HPLC system. The Turboionspray® device was maintained at 350 °C with an ionization voltage of 5 kV and an ion spray gas flow of 8 L/min. The nebulizing gas (N_2) and curtain gas flows (N_2) were set at 13 units and the declustering potential at 61 V for rivaroxaban and 66 V for the internal standard.

The dwell time was 300 ms, and mass analysers Q1 and Q3 were operated at unit (approximately 0.8 atomic mass units [amu]) and low (approximately 2 amu) mass resolution. The mass spectrometer was programmed to admit the protonated parent ion masses $[M+H]^+$ at m/z 436.1 for rivaroxaban and at m/z 464.2 for the internal standard via the first quadrupole filter (Q1). Collision-induced fragmentation at Q2 (collision energy 37 eV for rivaroxaban and 45 eV for the internal standard) yielded the product ions at Q3 of m/z 144.9 for rivaroxaban and the internal standard (Fig. 1). Peak height ratios of rivaroxaban and the internal standard obtained from selective reaction monitoring (SRM) of the analytes (m/z 436.1 → 144.9)/(m/z 464.2 → 144.9) were utilized for the construction of calibration lines, using log/log linear least-squares regression of the plasma concentrations and measured peak height ratios.

2.3.4. Sample preparation

An aliquot of 0.2 mL plasma was transferred to an autosampler glass vial and 0.5 mL of methanol containing the internal standard was added. The resulting suspension was vortexed for 10 s on a laboratory vortex followed by centrifugation for 10 min at 20 °C and 1500 × g. Finally, 40 μL of the above supernatant was directly injected onto the HPLC–MS/MS system.

2.3.5. Calibration (CAL) and quality control (QC)

Calibration samples were obtained by spiking aliquots of acetonitrile working solutions into blank plasma to give 10 concentrations (0.50, 1.0, 2.0, 5.0, 10, 20, 50, 100, 200 and 500 μg/L) in the range 0.50–500 μg/L, which were analysed as replicates. Quality

Table 1
Gradient elution conditions for determination of rivaroxaban concentrations in human plasma

Time (mins)	Eluent composition (% acetonitrile)
0	20
0.5	20
1.5	80
4.0	80
4.1	20
5.0	20

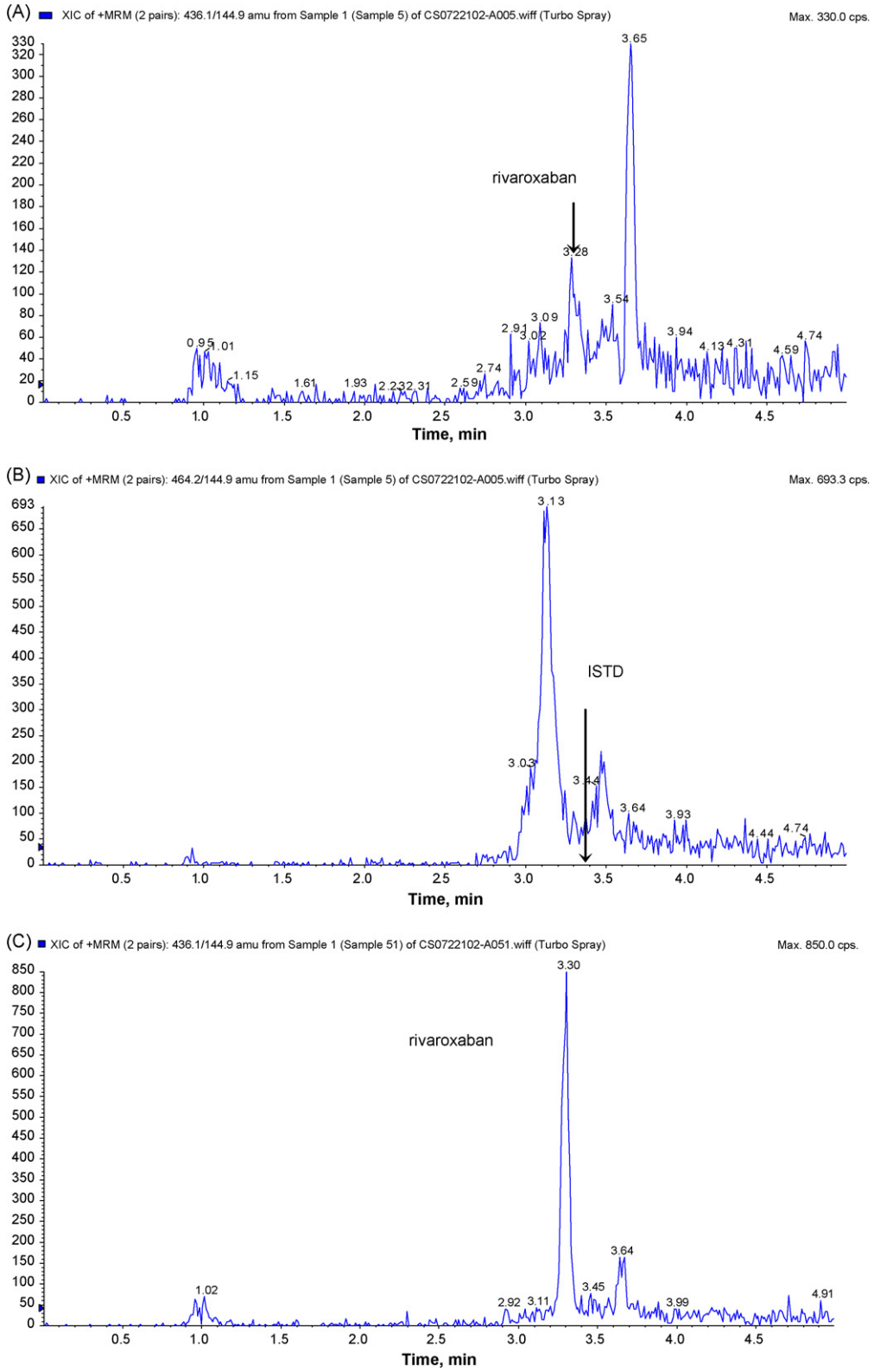

Fig. 2. Chromatograms of blank human plasma sample: (A) SRM (rivaroxaban): m/z 436.1 → 144.9; (B) SRM (ISTD) m/z 464.2 → 144.9, human plasma sample with 0.50 μg/L (lower limit of quantification) rivaroxaban and 29.5 μg/L internal standard; (C) SRM (rivaroxaban): m/z 436.1 → 144.9; (D) SRM (ISTD) m/z 464.2 → 144.9, patient plasma sample with 2.3 μg/L rivaroxaban and 29.5 μg/L internal standard; (E) SRM (rivaroxaban): m/z 436.1 → 144.9; (F) SRM (ISTD): m/z 464.2 → 144.9. x-axis: time (min), y-axis: intensity (cps), ISTD = internal standard.

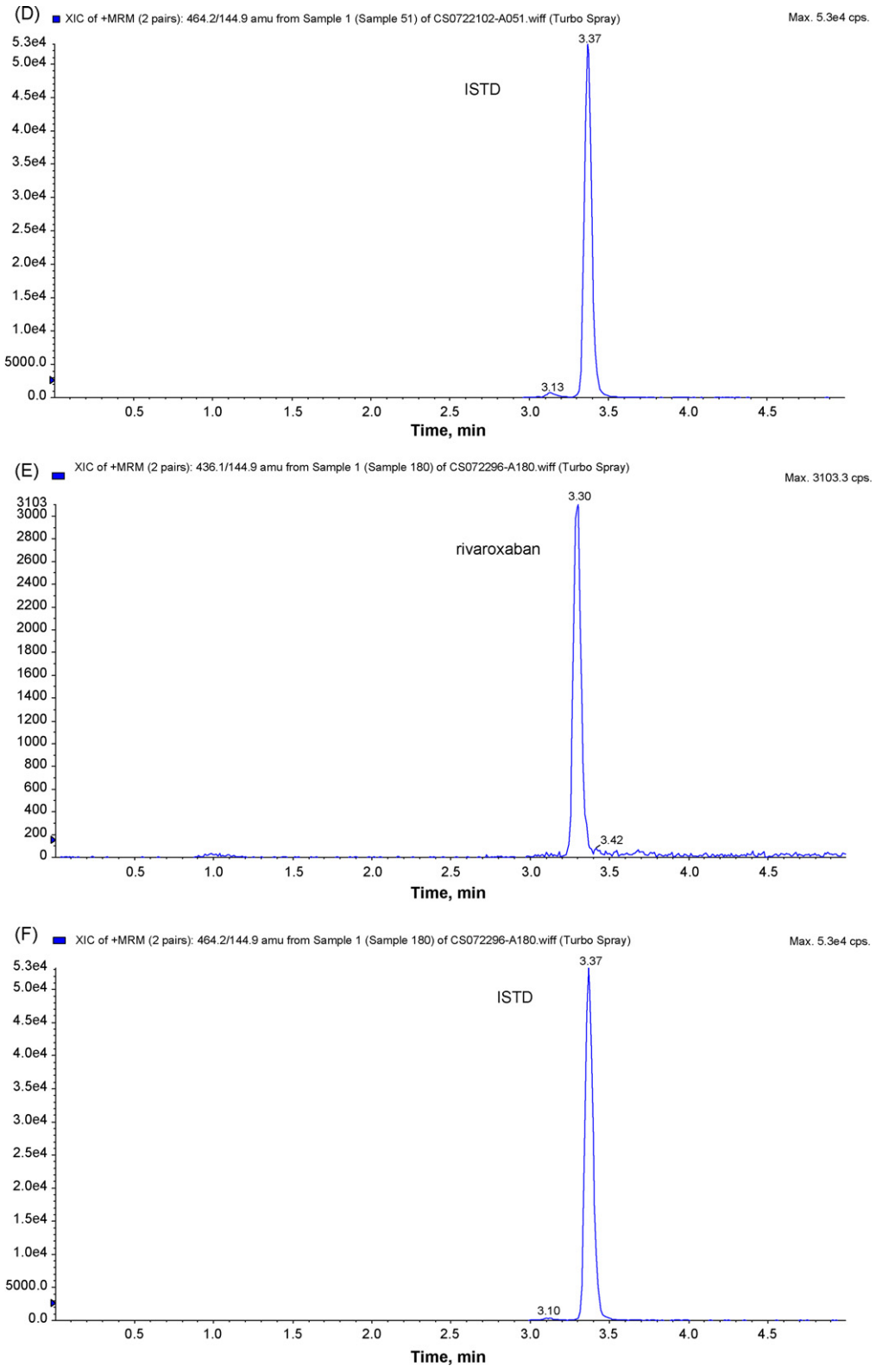

Fig. 2. (*Continued*).

control samples were prepared at three or four levels, depending on the range of concentrations expected in unknown samples. QC samples usually contained rivaroxaban in concentrations of 1.35, 26.6, 266 and 398 μg/L. At least two replicates of each QC sample (depending on the amount of samples within a sequence) were analysed together with the CAL and unknown samples in one sequence.

CAL samples were freshly spiked prior to a sequence. QC samples were stored together with samples of the respective study at <15 °C. CAL, QC and unknown samples were processed further as described

in Section 2.3.4 and calibration curves were obtained, as per Section 2.3.3.

3. Assay validation

Validation was performed according to the guidelines for bioanalytical assays in biomatrices [12,13]. Extensive stability investigations were performed in whole blood, plasma, and solutions for injection to guarantee valid bioanalytical data for rivaroxaban in human biological matrices.

Stability of rivaroxaban in the respective matrices and solvents was assessed by means of spiked samples stored under different conditions with regard to temperature, light, and storage container material. The investigated intervals were chosen in order to reflect the relevant time frame encountered with the actual study samples from sampling to analysis.

3.1. Selectivity and specificity

To investigate whether endogenous compounds in plasma interfere with the assay, plasma samples from six different healthy subjects were analysed. In addition, to determine whether co-administered medications interfered with the assay, QC samples with rivaroxaban were spiked with the maximum concentration of co-medications expected under study conditions in interaction studies: ketoconazole (3.5 mg/L), erythromycin (250 μg/L), warfarin racemate (4 mg/L), ranitidine (80 μg/L), midazolam (60 μg/L), rifampicin (17 mg/L), atorvastatin (5 μg/L), acetylsalicylic acid (4 mg/L) and naproxen (77 mg/L). These QC samples were compared with QC samples only containing rivaroxaban.

3.2. Accuracy and precision

QC samples (1.35, 26.6, 266 and 398 μg/L) were processed and analysed five to six times in the same run (intra-run precision and accuracy) in three different sequences (inter-run precision and accuracy). The accuracy was calculated at each concentration as the ratio of the measured concentration to the nominal concentration multiplied by 100%.

Table 3
Intra- and inter-run precision and accuracy (arithmetic means) of determination of rivaroxaban concentrations in human plasma

Nominal concentration (μg/L)		0.50 = LLOQ	1.35	26.6	266	398
Intra-run precision						
Run 1	*N*	5	5	5	5	6
	Accuracy (%)	94.0	105.0	102.4	104.6	99.5
	Precision (%)	5.5	2.8	2.9	2.6	4.3
Run 2	*N*	5	5	5	5	6
	Accuracy (%)	98.5	99.6	98.8	103.0	96.4
	Precision (%)	5.4	7.6	4.4	11.5	3.0
Run 3	*N*	5	5	5	5	6
	Accuracy (%)	96.5	97.0	99.2	101.2	101.7
	Precision (%)	11.2	11.0	2.0	2.9	2.4
Inter-run precision						
All samples	*N*	15	15	15	15	18
	Accuracy (%)	96.3	100.5	100.1	102.9	99.2
	Precision (%)	7.4	7.1	3.1	5.7	3.9

3.3. Lower limit of quantification

The LLOQ of the method was defined as the lowest concentration of rivaroxaban that could be quantitatively determined with acceptable precision and accuracy. Acceptance limits were defined as accuracy of 80–120% and precision of ≤20%.

3.4. Matrix effect

The effect of the lithium–heparin human plasma matrix on positive ionization with the Turboionspray® device was investigated by the analysis of pooled blank plasma extracts of 10 individuals spiked after protein precipitation with rivaroxaban at two concentration levels (2 and 500 μg/L). These samples were compared with rivaroxaban spiked in 200 μL water/500 μL methanol as reference containing the same concentrations (2 and 500 μg/L). The matrix effect of the internal standard was determined at its concentration in plasma after sample preparation (29.5 μg/L).

Table 2
Rivaroxaban stability in spiked samples

Matrix	Temperature	Storage conditions	Period	Concentration[a] (μg/L)	Result
Plasma supernatant protein precipitation	Approx. 9 °C	In autosampler	7 days	1.4; 267[a]	Stable
Stock solution in acetonitrile	≤8 °C		5 months	275 mg/L[b]	Stable
Working solution in acetonitrile	≤8 °C		5 months	5; 500	Stable
Whole blood (citrate)	Ambient	Daylight (window sill)	24 h	10; 100	Stable
	≤8 °C		24 h	10; 100	Stable
Whole blood (heparin)	Ambient	Daylight (window sill)	24 h	10; 100	Stable
	≤8 °C		24 h	10; 100	Stable
Plasma (citrate plasma)	Ambient	Daylight (window sill)	24 h	12; 720	Stable
	≤8 °C		24 h	12; 720	Stable
	≤15 °C		12 months	25; 750	Stable
	+37 °C	Yellow light	2 h	12; 720	Stable
Plasma (heparin plasma)	Ambient	Daylight (window sill)	24 h	12; 720	Stable
	≤8 °C		24 h	12; 720	Stable
	≤15 °C		39 months	25; 750	Stable
	37 °C	Yellow light	2 h	12; 720	Stable
Plasma (citrate plasma)	Freeze/thaw		3 cycles	19; 475	Stable
Plasma (heparin plasma)	Freeze/thaw		3 cycles	19; 475	Stable

[a] Two concentrations are presented to demonstrated stability over the working range.
[b] For stock solution, only one concentration is available.

Table 4
Measured signal intensities for the assessment of matrix effect and extraction recovery of rivaroxaban (2.00 and 500 μg/L) and internal standard (29.5 μg/L)

Sample concentration	Rivaroxaban (2.00 μg/L)	Rivaroxaban (500 μg/L)	Internal standard (29.5 μg/L)
(A) Pure solvent standard	2759.628	620017.015	39661.541
	2725.510	636787.813	38799.679
	2800.238	646971.511	40407.669
Mean (arithmetic)	2761.792	634592.113	39622.963
(B) Matrix extract, spiked after extraction	2568.868	512525.570	35944.008
	2392.569	538456.589	35871.479
	2537.548	559570.101	35654.362
Mean (arithmetic)	2499.662	536850.753	35823.283
(C) Matrix, spiked before extraction	2610.067	584507.690	38591.778
	2600.010	586865.295	39681.583
	2530.020	596677.007	40443.855
Mean (arithmetic)	2580.032	589349.997	39572.405
Extraction–recovery = (C)/(B) × 100	103.2	109.8	110.5
Matrix effect (%) [(B/A) × 100] − 100	9.5	15.4	9.6

In addition, incurred samples (n = 81) from a clinical pharmacology study in healthy subjects were analysed with the internal standard (structural analogue; Fig. 1B), and re-analysed with a labelled [$^{2}H_5$, ^{15}N]rivaroxaban internal standard (Fig. 1C).

3.5. Extraction recovery

Recovery was determined by spiking known amounts of rivaroxaban and internal standard into pooled blank matrix (C) and into

Table 5
Incurred sample re-analysis with structure analogue and labelled internal standard for the assessment of matrix effect

Sample no.	First measurement	Second measurement	Deviation of second measurement compared with first measurement (%)	Sample no.	First measurement	Second measurement	Deviation of second measurement compared with first measurement (%)
1	14.8	15.8	6.8	41	12.7	14.0	10.2
2	124	132	6.5	42	50.7	53.8	6.1
3	259	275	6.2	43	159	167	5.0
4	196	216	10.2	44	213	239	12.2
5	138	143	3.6	45	268	294	9.7
6	110	126	14.5	46	272	273	0.4
7	110	120	9.1	47	278	298	7.2
8	29.9	33.2	11.0	48	174	180	3.4
9	4.05	4.44	9.6	49	90.8	93.7	3.2
10	1.50	1.99	32.7	50	40.4	42.7	5.7
11	0.766	1.01	31.9	51	25.6	28.4	10.9
12	2.27	2.50	10.1	52	9.13	9.86	8.0
13	6.09	5.99	−1.6	53	2.91	3.29	13.1
14	26.1	27.6	5.7	54	1.45	1.67	15.2
15	60.7	61.9	2.0	55	1.03	1.36	32.0
16	209	206	−1.4	56	18.4	19.3	4.9
17	396	419	5.8	57	68.5	74.1	8.2
18	224	221	−1.3	58	114	131	14.9
19	116	122	5.2	59	128	133	3.9
20	45.6	47.8	4.8	60	138	140	1.4
21	24.2	26.6	9.9	61	135	145	7.4
22	9.84	9.85	0.1	62	116	122	5.2
23	3.35	4.31	28.7	63	80.4	92.6	15.2
24	1.50	1.65	10.0	64	63.3	65.2	3.0
25	17.4	19.1	9.8	65	33.2	38.4	15.7
26	149	149	0.0	66	23.0	25.9	12.6
27	225	220	−2.2	67	9.38	10.5	11.9
28	211	208	−1.4	68	5.65	6.04	6.9
29	211	219	3.8	69	2.80	3.32	18.6
30	215	221	2.8	70	0.535	0.712	33.1
31	227	225	−0.9	71	0.868	0.935	7.7
32	209	201	−3.8	72	1.46	1.68	15.1
33	180	186	3.3	73	20.1	19.1	−5.0
34	116	120	3.4	74	76.7	77.7	1.3
35	79.3	78.9	−0.5	75	93.2	88.0	−5.6
36	39.0	41.3	5.9	76	233	233	0.0
37	25.3	27.7	9.5	77	134	134	0.0
38	12.4	13.2	6.5	78	94.5	88.9	−5.9
39	3.82	4.06	6.3	79	77.5	74.5	−3.9
40	1.13	1.51	33.6	80	21.4	21.8	1.9
				81	2.52	2.48	−1.6

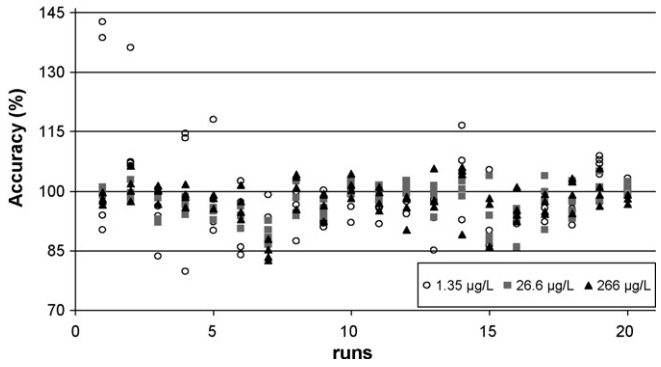

Fig. 3. Quality control (QC) samples of rivaroxaban in plasma assessing assay performance over 3 months (four QCs per concentration value per sample run). Analysis based on plasma samples from 1589 patients enrolled in a phase II study [14].

the extracted pooled blank matrix (B) as reference, respectively, and subjecting the spiked blank matrix samples to the extraction procedure. Peak heights in the matrix extracts were compared with the signal intensities derived from the reference, and recovery was calculated as quotients of signal intensities C:B in percent. The experiment was conducted at two concentrations (2 and 500 μg/L) throughout the working range.

4. Application

The method was applied in several clinical pharmacology and clinical studies within the development programme for rivaroxaban.

5. Results

5.1. Stability

Rivaroxaban was stable in all different matrices and under different conditions (Table 2). Stability was confirmed if the change in concentration during the observation period was less than ±15% in the case of biological matrix samples, and less than ±5% in the case of both stock and working solutions. All matrices were stored in polypropylene tubes except plasma supernatant, stock solutions, and working solutions, which were stored in glass vials or tubes.

5.2. Selectivity and specificity

The assay selectivity was determined by analysing extracts from six lots of blank heparin plasma from different sources. Relevant amounts of endogenous peaks at the retention times (t_R) of rivaroxaban (t_R = 3.30) and the internal standard (t_R = 3.37) were not observed (Fig. 2).

In addition, QC samples (1.35, 26.6, 266 and 808 μg/L) of rivaroxaban were spiked with the maximum concentrations of relevant co-medications expected under study conditions in drug interaction studies: ketoconazole, erythromycin, warfarin racemate, ranitidine, midazolam, rifampicin, atorvastatin, acetylsalicylic acid and naproxen. In all cases, the respective nominal concentration of rivaroxaban was confirmed in the presence of the co-medications.

5.3. Accuracy and precision

A formal validation experiment was carried out (Table 3). Inter-run precision was ≤7.4% and accuracy was between 96.3% and 102.9% throughout the entire working range. Based on these results, the LLOQ was 0.50 μg/L.

Data on the long-term performance of the assay, covering the analysis of 1589 unknown samples from patients randomized in the EINSTEIN phase II dose-finding study [14] over a period of 3 months, were analysed (Fig. 3). QC samples ran concurrently with study samples confirmed the values for precision and accuracy that were determined during the 3-run validation experiment. In the QC samples containing rivaroxaban at 1.35–266 μg/L, inter-run precision was ≤11.2% and accuracy was between 97.3% and 98.4% (n = 80 per concentration). Overall, 4.2% of the QC samples were out of specification, with a deviation from the nominal concentrations of more than ±15%. The long-term performance of the assay was confirmed by the analysis of more than 20,000 unknown patient plasma samples from an ongoing phase II study [15]. Over 138 analytical runs, inter-run precision of the QC samples (1.35, 26.6, 266 and 398 μg/L) was ≤7.8% and accuracy was between 98.4% and 98.9% (n = 537 per concentration). Only 2.4% of the QC samples were out of specification. HPLC columns were used for at least 1000 consecutive injections without significant deterioration of separation efficiency or peak shape.

5.4. Assessment of matrix effect and extraction recovery

Small but non-relevant matrix effects (<15%) for rivaroxaban (9.5–15.4%) and the internal standard (9.6%) were observed (Table 4). These matrix effects, which were within the predefined limit of ±15%, have no impact on quantification results because the matrix effect of rivaroxaban is compensated for by the internal standard.

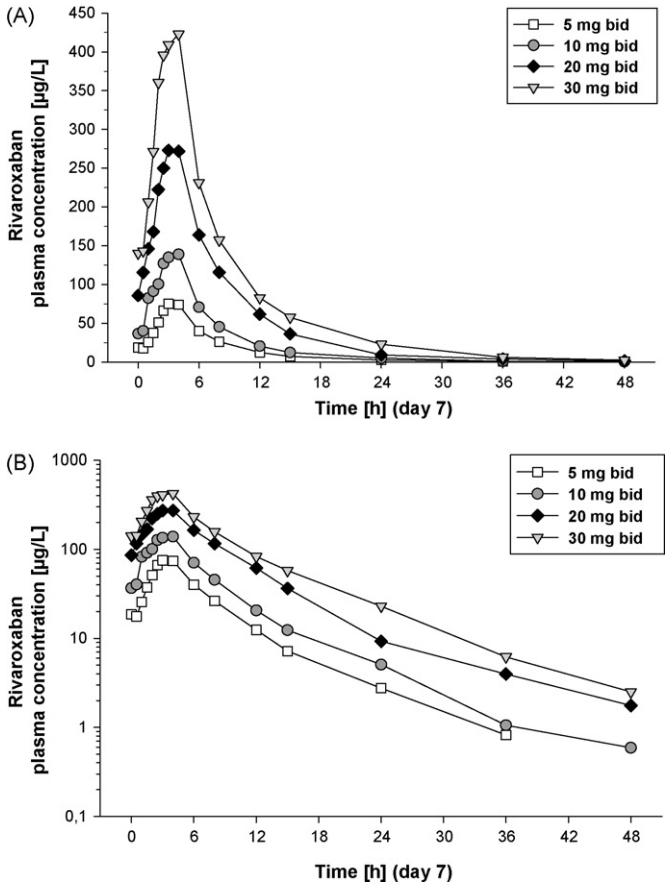

Fig. 4. Plasma concentration–time profiles of rivaroxaban after multiple dose administration in healthy subjects receiving 5–30 mg tablets twice daily (geometric mean; n = 7 each per dose group; profiles after last tablet intake at steady state) [6]. (A) Linear scale and (B) semi-log scale.

Incurred sample re-analyses were performed with the non-labelled and the labelled internal standard (Table 5). Similar concentration values (within biological variability) were determined with both internal standards, with a mean deviation of 7.5% for the second measurement, compared with the first measurement. Only six out of 81 samples had a deviation >20% (deviation range: −5.9 to 33.6%). These results and the results of the investigation of the matrix effect on the pooled plasma samples demonstrate that the unlabelled internal standard compensates well throughout the validated range of the rivaroxaban method for possible ion suppression effects. The extraction recovery after protein precipitation was 100%.

5.5. Application

The described assay has been applied to plasma obtained after administration of rivaroxaban as tablets in several thousands of patients and healthy subjects [5–10]. In healthy subjects, concentrations above the LLOQ could be measured 48–72 h post-administration, depending on the administered dose (Fig. 4) [6]. Concentrations reached a maximum after 2–4 h (mean t_{max}) with subsequent biphasic decay. The terminal elimination half-life was 5–9 h in healthy young subjects. The pharmacokinetics of rivaroxaban were dose proportional. No relevant accumulation was observed at any dose after multiple dose administration.

6. Discussion and conclusions

Formal validation according to the recommendations of the European Community (CPMP guidelines) and Food and Drug Administration [12,13] showed that the rivaroxaban plasma assay is appropriate for use in rivaroxaban clinical studies. Precision and accuracy were less than ±15% across the whole working range of the method. The LLOQ enabled a full description of the human pharmacokinetics of rivaroxaban in clinical studies and the assay was selective and specific, without interference from endogenous substances or concomitant medications. In addition, the high-throughput analysis achieved using HPLC–MS/MS will make this assay particularly applicable during pharmacokinetic investigations within phase II and III trials, where large numbers of samples are being analysed.

Sample stability was demonstrated in different matrices and under different conditions. No significant degradation of rivaroxaban or other interference due to a possible contribution of matrix constituents was detected in the stability samples. A small but non-relevant matrix effect of rivaroxaban in human plasma was observed. However, the internal standard compensated for this effect—a matrix effect in the same order of magnitude was detected with the internal standard. The extraction recovery after protein precipitation was complete.

FXa is an important target for the prevention and treatment of thromboembolic disorders. Although the use of LMWHs is the current standard of treatment, their pharmacokinetic profiles are poorly understood, because LMWHs exist as a conglomerate of small- to medium-chain heparin molecules in plasma. Direct FXa inhibitors, such as rivaroxaban, offer the potential for potent and selective anticoagulation. The assay described here demonstrates that rivaroxaban can be detected accurately and consistently in human plasma, allowing its pharmacokinetic profile to be elucidated directly.

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