Micellar Liquid Chromatography Determination of Rivaroxaban in Plasma and Urine. Validation and Theoretical Aspects

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Abstract

A Micellar Chromatographic method to determine rivaroxaban in plasma and urine has been developed. The samples were dissolved in the mobile phase (SDS 0.05 M – 1-propanol 12.5 %, phosphate buffered at pH 7) and 20 μL directly injected, avoiding the extraction and purification steps. Using a C18 column and running under isocratic mode at 1 mL/min, analyte was eluted without interference from the matrix in less than 6.0 min. The detection absorbance wavelength was set to 250 nm. The procedure was validated by Food and Drug Administration guidelines in terms of: system suitability, calibration range (0.05 – 5 mg/L), linearity, sensitivity, robustness, carry-over effect, specificity, accuracy (-11.1 to 4.2%), precision (<19.9%), stability and analysis of incurred samples. The method was found reliable, practical, easy-to-conduct, rapid, relatively eco-friendly, safe, inexpensive, widely available and with a high sample throughput. The method was applied to the analysis of incurred samples, including incurred sample reanalysis, to verify that the instrumentation works correctly. In addition, the constants of the different partition equilibria occurring in the column were elucidated in order to have a better comprehension of the theoretical aspects of the retention mechanism. A moderately strong association between rivaroxaban and the stationary phase and the micelles was found, weakened by short chain alcohol.

Keywords: Anticoagulant; Biological Fluid; Direct injection; Micellar Chromatography; Partition equilibrium; Validation.

1. Introduction

Rivaroxaban (RIV, Fig. 1) is an oxazolidinone belonging to the group of the direct or novel oral anticoagulants (DOAC or NOAC, respectively) commonly used to prevent blood clots. Its activity is attributed to a potent, irreversible and selective inhibition of free and clot bound Factor Xa [1-10], thus blocking thrombin generation [11-15]. RIV was the first DOAC and was approved by FDA in 2011. It is currently one of the most prescribed anticoagulants [11]. RIV is indicated to treat and/or prevent thromboembolic disorders, like venous tromboembolism (VTE), deep vein thrombosis (DVT), pulmonary embolism, *etc*. It is used for the prophylaxis of VTE after total orthopedic hip or knee replacement therapy and stroke or systemic embolism in patients with atrial fibrillation [1-15]. It is not recommended for prescription in patients under 18 years old, pregnancy and those with severe renal or liver failure [2,10,11]. RIV may cause excessive bleeding at overdose [11]. Its therapeutic and adverse effects are closely related to the plasmatic concentration [2,4,10,12,15].

RIV displays a more favorable benefits-to-risk profile than conventional anticoagulants. It is administered orally and rapidly absorbed by the gastrointestinal system. It has high efficacy and tolerability, a rapid onset of action, predictable pharmacokinetics (Table SM1) and pharmacodynamics, large therapeutic window (nearly 6 to 491 μg/L [2,12]), a low risk of interaction with food, absence of accumulation in patients without co-morbidities, low likelihood of adverse effects and rapid elimination. Clinical effects are dose-dependent and no patient-adapted dosage is usually required. In fact, RIV can be prescribed at a fixed daily dose for the most patients [1-12,16,17].

Therapeutic Drug Monitoring (TDM) of RIV in a given individual may be relevant in certain circumstances in order to guide the decision making about the treatment ensuring the optimum efficacy. Plasma levels might be influenced by several factors altering the drug metabolism exposing the patients to a significant risk of under- or over anticoagulation. The direct quantification of RIV in plasma is preferred for drug testing [1-5,7-9,13-17], although in urine is

also feasible [3,9,14,15] and has the advantage of being non-invasive. The level of RIV in plasma reflects the presence of the drug at a specific point in time, since in urine infers the exposure for an extended period [9]. Therefore, clinical laboratories require rapid and sensitive analytical assays able to identify and quantify RIV in both biological fluids to implement an effective TDM approach.

Micellar liquid chromatography (MLC) using a C18 column, and anionic sodium dodecyl sulfate as surfactant, and short-chained monoalcohol as organic modifier has been proven a reliable tool for the determination of drugs in plasma and urine [18], avoiding the major drawbacks of hydro-organic HPLC: use of sophisticated/expensive detectors and devices, need of large, cumbersome and variable-recovery extraction or purification steps, use of a large quantity of hazardous chemicals, extensive manipulation, *etc*. Indeed, micellar solutions are able to solubilize small drugs [18]. Besides, micelles and monomers strongly interact with proteins, lipids, glycosides and other macromolecules, usually present in the biological fluids, provoking its denaturing, solubilization, releasing bounded drugs and preventing further interaction with the analytes [19]. In the column, these biopolymers remain solubilized in the micellar mobile phase without risk of precipitation, and are barely retained, being harmless eluted at the front of the chromatogram. Therefore, after a sample dilution, the biological fluids, can be directly injected in the chromatographic system [18]. Hybrid micellar solutions contain innocuous and biodegradable salts, and only up to 12.5% of organic solvent [20]. The retention mechanism in MLC depends on two partition equilibria for moderately hydrophobic solutes (bulk hybrid micellar mobile phase – micellar pseudo phase; bulk hybrid micellar mobile phase - monomer-modified stationary phase). Besides, the chromatographic behavior of a solute can easily be predicted from the composition of the mobile phase, using equations constructed from the three-phase model [21].

The aim of the work is develop a reliable, rapid, practical, easy-to-handle and inexpensive procedure to determine RIV in plasma and urine by MLC. The method will be fully validated to evaluate its analytical performance and used in incurred plasma samples and commercial

pharmaceutical formulations to ensure its suitability for routine analysis. Another goal was to investigate the interaction of RIV with the different chemical environments occurring in the column, for a better comprehension about the theoretical aspects of the retention mechanism and the role of the main components of the mobile phase on the retention factor, by calculating the physicochemical constants of the corresponding partition equilibria.

2. Experimental

2.1 Standard and chemicals

Powdered standard of Rivaroxaban (purity>99.4%) was purchased from InterQuim (Barcelona, Spain)**.** Sodium dodecyl sulfate (>99%), dimethyl sulfoxide (DMSO, >99.6%) and sodium dihydrogen phosphate dihydrate (>99%) were bought from Scharlab (Barcelona, Spain). The solvent 1-propanol (HPLC grade) came from Merck (Darmstadt, Germany). Hydrochloric acid 37%) was from J.T. Baker (Phillipsburg, NJ, USA). Sodium hydroxide (>98%) was purchased from Riedel-deHaën (Hannover, Germany). Ultrapure water was *in-lab* produced using an ultrapure water generator device Simplicity UV (Millipore S.A.S., Molsheim, France), from deionized water, provided as tap water by the university. All aqueous solutions were prepared using this water.

2.2 Preparation of solutions and mobile phases

To prepare the micellar solutions, the adequate amount of SDS and $NaH_2PO_4 \cdot H_2O$ (according to the desired concentration) was solved in ultrapure water and the pH was fixed to the seleted value by adding drops of HCl or NaOH solutions. Then, the appropriate volume of the organic solvent (depending on the chosen proportion) was introduced, and the volumetric flask was filled up with ultrapure water. Finally, this solution was ultrasonicated for 5 min to attain a complete solubilization and filtered through a 0.45 µm-Nylon membrane filters (Micron Separations, Westboro, MA, USA), with the aid of a vacuum pump, to remove solid particles. These solutions were kept in amber bottles (to avoid bacterial growth) at room temperature.

A stock solution of rivaroxaban of 100 mg/L was prepared by weighing the proper weight and dissolving it in DMSO. The working solutions were made by dilution of the stock solution in mobile phase. These solutions were stored in a fridge at 4ºC.

2.3 Chromatographic conditions

The chromatographic system was a HP1100 (Agilent Technologies, Palo Alto, CA, USA) equipped with an isocratic pump, a degasser, an autosampler and an UV-Visible absorbance diode array detector (DAD), connected to a PC. The software Agilent Chemstation (Rev. B.04.03(16) 2010) was used to control the instrument, visualize and register the signal, and the measurement of the chromatographic parameters (dead time, t_0, retention time, t_R and peak area, PA). The experimental dead time was 1.0 min. Retention factor (*k*), efficiency (number of theoretical plates, N) and tailing factor (T) were calculated as in [22].

The stationary phase was in a C18 Kromasil C18 column (AkzoNobel, Amsterdam, The Netherlands) with the following parameters: 150 x 4.6 mm; 5 µm particle size; 10 nm pore size. Injection volume and absorbance wavelength were 20 µL and 250 nm, respectively. The mobile phase was an aqueous solution of 0.05 M SDS - 12.5% 1-propanol buffered at pH 7 with a phosphate salt. It runs under isocratic mode at 1 mL/min. All the analyzed solutions were filtered through a 0.45-µm Nylon membrane filter (Micron Separations) with the aid of a 3 mL syringe, and then introduced in the chromatographic vial. The special care of the chromatographic instrumentation required when working with micellar mobile phases can be seen in [23]. Under these conditions, the column has a lifespan of at least 1000 injections [23].

2.4 Sample collection and processing

The study was approved by two local Ethics Committees, Hospital and the University Ethic Committee for Analysis of Research Projects. Written informed consent was obtained from all participants and all research was performed in accordance with the 2013 Helsinki Declaration

157 principles. Plasma and urine samples from patients and healthy volunteers were provided by a local
158 Hospital, after consent of doctors and patients. The “healthy volunteers” do not take any medication
159 and their blood is free of drugs. They were six: three men and three women, and, for each gender,
160 one in his/her twenties, another in his/her thirties and the last one in his/her forties. For
161 confidentiality reasons, the samples from the patients were sent unlabeled. No personal and clinical
162 information about the patients or the healthy volunteers (except that indicated above) was provided
163 from the Hospital. The laboratory undertakes to do not transmit any information to other institutions
164 (except that here indicated).

165 The blood was collected using a BD SST tube (SBD Vacutainer Systems, Plymouth, UK),
166 and centrifuged at +4ºC at 3000 rpm for 10 min (3000 x *g*), to get the non-cellular fraction, which
167 was immediately frozen and stored at -20ºC. The reference validated and accredited standard
168 method used by the Hospital includes this kind of tubes, then no loss of RIV at this step is expected.
169 Urine samples were collected in glass tubes and kept at the same temperature. Afterwards, these
170 samples were sent to the laboratory, where they were kept under the same conditions. A matrix-
171 matched blank was made by mixing equivalent volumes of the plasma or urine, respectively, of the
172 six healthy volunteers.

173 Plasma and urine, either blank or patient samples, were treated in the same way. The
174 samples were thawed until the onset of melting, the same day of the analysis. Afterwards, an aliquot
175 was 1/5-dissolved in mobile phase, filtered and directly injected [21]. The fortification was
176 performed by adding the appropriate volume of a standard solution of Rivaroxaban, before the
177 dilution. All the validation studies “in matrix”, for these biological fluids, were performed using
178 spiked blank samples.

179

180 **3. Results and discussion**

181 *3.1 Optimization of the chromatographic conditions*

182 The main chromatographic conditions to optimize in MLC method [18,21] are: surfactant,
183 modifier concentrations and pH. Using C18 and SDS as column and surfactant, respectively, the
184 stationary phase is saturated with SDS monomers adsorbed on its surface, with the sulfate group
185 oriented to the mobile phase, at concentrations > 10 mM, slightly over the critical micellar
186 concentration (CMC) of 8 mM. Working above this concentration, SDS monomers in the mobile
187 phase are organized as micelles, which number per volume unit is proportional to the initial
188 concentration of SDS. The amount of free monomers remains nearly constant and equal to the
189 CMC. Therefore, we can work at relatively low concentration of surfactant (recommended working
190 concentrations 0.05 - 0.15 M) in which the mobile phase has a low viscosity and low background
191 signal.
192 According to the moderate hydrophobicity (log Po/w = 1.7) of RIV [11], a pure micellar
193 mobile phase would provide too long capacity factors. Therefore, 1-propanol was added to reduce
194 the retention time and, additionally, enhance the efficiency. The studied interval of concentrations
195 for 1-propanol was that recommended in MLC, 2.5 to 12.5% [18].
196 The effect of the pH was studied at 3, 5 and 7, all of them inside the working range of the
197 C18 column (2.5 - 7.5), by the analysis of a working solution of 0.5 mg/L of RIV. At pH 3, no peak
198 was observed, and at pH 7 the best peak appeared with a nearly Gaussian shape at reasonable
199 retention time. Therefore, these values were taken as optimal. According to the acid/basic activity
200 of RIV (pKa 1.0 and 13.4), the molecule is neutral under the working conditions [11].
201 The qualitative influence of the SDS and 1-propanol on the elution parameters was
202 examined. A working solution of RIV (0.2 mg/L) was analyzed using nine mobile phases selected
203 following an experimental design based on a face-centered composite design plus the central point.
204 We take the combination of the minimum (-1) and maximum (+1) levels for both factors ($2^2 = 4$
205 assays), the combination of the average value (0) of one of them and the minimal and maximal of
206 the other one (2 x 2 = 4 assays), and the combination of the average values (1 assay). The -1/0/+1
207 values were the minimal, average and maximal concentration of each component recommended in

MLC, respectively [24]. The composition of the assayed mobile phases and the results are shown in Table 1. From these results, we deduce that RIV has a binding behavior face to the micelles in the mobile phase, as the retention factor and the efficiency decrease at increasing values of SDS-micelles. By the way, the elution strength of the mobile phase and the efficiency grow up at higher values of 1-propanol, as usual in reverse phase MLC. The optimal mobile phase composition was set to 0.05 M SDS – 12.5 % 1-propanol – pH 7. The analyte elutes at a short time, but far from the expected front of the chromatogram for biological fluids [18], and the efficiency is maximal. Therefore, the chromatographic analysis of a diluted sample can be carried out in only 6.0 min, using a low amount of organic solvent and a simple isocratic program.

Detection conditions were optimized by Agilent ChemStation Rev. B.04.03(16) 2010 software. Absorbance spectrum of rivaroxaban (200 – 500 nm) was on-line measured at the maximum height during the chromatographic run, in order to obtain its spectrophotometric properties in the same micellar environment as used for the analysis. The maximum of absorbance was found at 250 nm, and then this value was selected to maximize the signal-to-noise ratio. For peak purity studies, the absorbance spectra were taken at different points of the peak (at half-height and 0.1-height, both fronting and tailing), and they show the same shape as above.

3.2 Determination of the partition physicochemical constants

According to the three-phase model, in MLC using pure micellar solutions, the solutes are partitioned between three phases: surfactant-modified stationary phase, bulk aqueous solvent, and micellar pseudo-phase. The equilibria are described by two partition constants: K_{AS} (between the bulk mobile phase and stationary phase; thus increasing the retention) and K_{AM} (between the mobile phase and the micelles, thus decreasing the retention). Therefore, K_{AS} and K_{AM} quantify the degree of interaction of the solute with the modified stationary phase and the micelles, respectively. In hybrid micellar mobile phases, we assume that this model is also applicable, but the organic solvent displaces the equilibria. This is only applicable at low concentration of organic solvents (<22 % for

1-propanol), as higher values causes the disaggregation of the micelles. We introduce two constants, K_{AD} and K_{MD}, which evaluates the shift in the equilibrium to the solvent and the micelle, respectively, with respect to the pure micellar solution (thus decreasing the retention in both cases). According to this three-phase theory, in MLC the retention factor of a moderately hydrophobic solute can be modeled from the concentration of surfactant and alcohol by the following mechanistic equation [18,20]:

$$k = \frac{\Phi K_{AS} \frac{1}{1 + K_{AD}\varphi}}{1 + K_{AM} \frac{1 + K_{MD}\varphi}{1 + K_{AD\varphi}} [SDS]} \qquad \text{Eq. 1}$$

where Φ is the phase ratio (volume of stationary phase divided by the volume of mobile phase in the column), φ is the proportion of 1-propanol in %, v/v and [SDS] in (M). If φ = 0, the constants K_{AD} and K_{MS} are removed from the equation, and if [SDS], the retention is Φ K_{AS} (as in hydroorganic HPLC). The equation may be reorganized to:

$$\frac{1}{k} = \frac{1}{\Phi K_{AS}} + \frac{K_{AD}}{\Phi K_{As}}\varphi + \frac{K_{AM}}{\Phi K_{AS}}[SDS] + \frac{K_{AM}K_{MD}}{\Phi K_{AS}}[SDS]\varphi \qquad \text{Eq. 2}$$

Eq. 2 was fitted using the experimental values of *k* obtained from the experimental design (Table 1), by curve-fitting non-linear least-square regression (5 freedom degrees) [25], in order to calculate the physicochemical constants. An adequate goodness-of-fit was reached (R^2>0.991). The values of the constants were: Φ K_{AS} = 45.3; K_{AM} = 44.9; K_{AD} = 3,9 and K_{MD} = 0.15.

A moderate/strong interaction was found between both the stationary phase and the micelles with RIV, probably by hydrophobic interactions. The presence of 1-propanol significantly modifies the two equilibria towards the bulk mobile phase, by the resulting increase of the hydrophobic interactions in the bulk mobile phase. However, the occurrence of 1-propanol barely affects the

interaction of the analyte with the micelles.

3.3 Method validation

The method was fully validated under guidelines of the FDA guidance for Industry, Bioanalytical Method Validation 2018 (developed for analysis of biological fluids) [26] and several guides about validation of chromatographic methods [18,27]. The general parameters were determined in plasma samples: system suitability, linearity, calibration range, sensitivity, robustness (as the results are similar for both biological fluids [28]), while specificity, carry-over effect, accuracy and precision were separately determined for plasma and urine. Stability was determined in stock solution, diluted plasma and urine, and plasma and urine.

3.3.1 Specificity

The ability of the procedure to distinguish the analyte from endogenous compounds of the biological fluids was evaluated. The following analyses were performed: plasma (Fig. 2A and Fig. 2C, respectively) and urine (Fig. 2B and 2D) were analyzed before and after fortification at 1 mg/L. Additionally, the absorbance spectrum was measured at five points as in 3.1.

In blank samples, a broad band (<3.5 min) was observed at the beginning of the chromatogram, corresponding to proteins and other macromolecules, strongly associated to the micelles, thus enough far from the analyte [18]. The baseline was quite stable, and no peaks were visualized close or at the window time of the analyte. In the fortified samples, no peaks were observed near or partially overlapping the analyte.

The following experiments were performed to assess the peak purity:

- For plasma and urine, the spiked samples were compared to those non-fortified. The chromatograms show similar shape, except the peak corresponding to RIV.
- The chromatograms from processed samples were overlaid to that obtained in 3.1, and the peaks assigned to the analyte were alike.

281 - The absorbance spectra were compared and were found rather similar, regardless of the time
282 taken, and were comparable to that obtained in 3.1.
283 These results point to the absence of compounds co-eluting with RIV, in the different
284 matrices. Therefore, the method provides reliable qualitative and quantitative signals about the
285 analyte.
286
287 *3.3.2 System suitability testing (SST)*
288 The main chromatographic parameters and their variability were determined, in order to
289 dispose of reference values to further check the normal running of the instrumentation and evaluate
290 the reproducibility of the chromatographic response. A blank plasma sample fortified at 2.5 mg/L of
291 drug was injected by n = 6. The evaluated parameters and acceptance criteria were: tailing factor,
292 1.41, <0.8-1.6; retention factor, 3.64, >2.0; number of theoretical plates, 2325 >2000; RSD of the
293 retention time 1.3%, <1.5% and RSD of the peak area 1.4%, <1.5%. According to the results, the
294 chromatographic response is enough consistent and useful for a reliable qualitative/quantitative
295 analysis.
296
297 *3.3.3 Calibration range and linearity*
298 Several blank plasma spiked samples containing increasing concentrations of RIV were
299 prepared in the range 0.025 – 5 mg/L, and analyzed by triplicate. The average peak area (PA) values
300 (response variable) and the corresponding concentration (independent variable) were processed by
301 least-square linear regression [29]. The slope was 119.8±0.6 and the y-intercept was 0.7±0.6. The
302 reliability of the model was verified by the following tests:
303 - The residuals were homoscedastic, as the variances of the observed variable at the different
304 concentration levels (calculated from the PA replicates) were found equal (p = 4.1%< 5%).
305 - No trend was detected for the residuals, by visual examination of a plot residual *v.s.* concentration.
306 - No outliers were found in the regression model: the relative residuals were <1.66% (acceptance

criteria <3) and the Cook's squared Distances were <0.5 (acceptance criteria <1) for all points.
- The y-intercept confidence (0.7±0.7) interval includes zero, then no systematic error was noticed.
- A significant correlation was found by a t-test on *r* (p=0.000%, acceptance criteria <5%) and comparing the model and residual variance by a F test (p=0.000%, acceptance criteria <5%).
- The determination coefficient (r^2 = 0.9997) and the relative residual standard deviation (<1.3%) meet the acceptance criteria (> 0.990 and 5%, respectively) and then an adequate linearity was found between the two variables in the studied interval. The lower limit of quantification (LLOQ) was the lowest concentration fitting the acceptance criteria for accuracy and precision, 0.025 mg/L, and the upper limit of detection (ULOQ) was 5 mg/L.

3.3.3 Sensitivity

The limit of detection is the smallest concentration of RIV injected that provides a peak clearly differentiable from the baseline noise, whereas the limit of quantification was the minimal concentration of analyte that can be reliably determined. These parameters were calculated as 3.3 and 10 the standard deviation of the y-intercept; 0.008 and 0.025 mg/L. The calibration range covers the expected range of concentrations in patient's incurred sample (Table SM1), except the lower range, between 8 and 25 µg/L [3,8,13]. However, the no-detection of RIV points to the absence of therapeutic concentrations of the drug (under 6-8 g/L).

3.3.4 Carry over effect

The possible increase of the signal due to the presence of analyte remaining from previous injections was investigated. For each biological fluid, two blank samples, fortified at the ULOQ (5 mg/L) and unfortified, were consecutively analyzed. No peak was observed at the window time of RIV in the chromatogram obtained from the blank. Therefore, the occurrence of cross contamination was considered negligible.

3.3.5 Accuracy and precision

These parameters were determined in matrix at four levels: LLOQ, 0.1; 0.25 and 2 mg/L. Six samples were successively prepared and analyzed in the same run. The accuracy was the closeness of agreement (in %) between the average value of the found concentrations and the true value, while the within-run precision (repeatability) was the dispersion (RSD) of the obtained concentrations. The effect of time in the variability was studied. The same experimental protocol was conducted five different days (using renewed solutions) over a three-month period. The between-run precision (intermediate precision) was the relative standard deviation of the five average values of found concentration. The results are shown in Table 2.

All the values were under the acceptance criteria (for LLOQ <20% and for higher levels, <15% [26]), the method provides adequate quantitative values for bioanalysis. These performances were reached by the use of an easy-to-handle dilution as sample preparation, where the sample is quantitatively introduced in the column with the absence of extraction / purification steps, decreasing the subsequent sources of variance and the risk of analyte loss.

3.3.6 Robustness

The variation of the main chromatographic responses (retention factor, peak area and efficiency) was examined at small, but deliberate, changes in the main operational parameters. These were changed from their optimal value, and the tested oscillations were that we considered that may occur during the laboratory work and chromatographic run, in a usual situation. The studied factors and the studied interval were: A) detection wavelength ± 5 nm; B) SDS in mobile phase ±0.05 M; C) 1-propanol in mobile phase ± 0.2 %; D) pH in mobile phase ±0.2; E) flow-rate ±0.05 mL/min and F) injection volume ±2µL.

A blank plasma sample spiked with 2.5 mg/L was analyzed by testing eight different set of experimental conditions, which value was fixed by an experimental design following a Youden approach [30]. Differences >8.0% were judged significant, meaning that they may be especially

controlled during the analysis. The retention factor was significantly affected by the concentration of SDS in mobile phase and the flow-rate, the peak area by the concentration of SDS and the injection volume, and the efficiency by the flow and the injection volume. Therefore, these factors operational parameters must be especially controlled during analysis to achieve adequate analytical results.

3.3.7 Stability

The degradation of RIV was studied in the below mentioned chemical environments and external conditions. In each case, the tested conditions were those likely to be encountered during the actual storage, handling and analysis, either in a Hospital or a clinical laboratory. The stability was examined by the monitoring of the concentration of RIV/nominal value (0.2 mg/L in the injected solution, in all cases) and the emergence of peaks from the decomposition products. The degradation was considered significant if >5% for standard solutions and 15% for processed and unprocessed biological fluids.

The experiments were conducted as follows. For all the cases, no new peaks were detected and the concentration of RIV remains quite invariant, and then the drug was found stable during the studied periods.

a) Stock solutions: A standard solution of 0.2 mg/L was prepared and stored in amber vials in a fridge at +4ºC. Each day, for two months, the solution was thawed, analyzed and re-introduced in the fridge. Therefore, the analyte is stable during this period, and the working standard solutions can be safely used without introducing a systematic error in the results.

b) Bench top Processed Sample Stability: Samples from both biological fluids were diluted and placed in the autosampler. For one day, the samples were injected each hour (six replicate injections). Standard Operating Procedure indicate that, each day, all the samples received by the laboratory can be diluted and afterward injected in the chromatograph successively in the same sequence run, up to 240 analyses. Could be used in QC (quality control) and system suitability

385 solutions to-be-analyze. This optimization of time management permits to increase the number of
386 samples that can be analyzed in a day and the productivity of the laboratory.
387 c) Long term storage of the biological fluids: a set of 15 spiked samples of plasma and urine were
388 stored at -20ºC in the darkness. Each day, a sample was analyzed (six replicate injections).
389 Therefore, the biological fluids should be analyzed up to 15 days after sampling.
390 d) Long term freeze-and-thaw for the biological fluids: a fortified sample of plasma and another of
391 urine were kept at -20ºC in the darkness. Each day (15 days), they were thawed, an aliquot was
392 analyzed (six replicate injections), and reintroduced in the freezer. Therefore, an individual sample
393 can be stored and analyzed several times at least during this time, albeit undergoing abrupt
394 temperature changes, without affecting the reliability of the results.
395
396 *3.4 Analysis of incurred samples*
397 The suitability of the assay was evaluated by the analysis of biological samples provided by
398 a local Hospital. QCs samples were incorporated, using spiked samples of plasma and urine. System
399 Suitability Test, blank samples, quality control (QCs) and Incurred Sample Reanalysis were also
400 performed. All the samples were analyzed in the same run. The results can be seen in Table 3.
401 Samples of plasma and urine with the same number correspond to the same patient. The
402 chromatogram obtained from the analysis of plasma and urine samples from patient 4 is shown in
403 Fig. 2E and 2F, respectively.
404 The results of the SST comply with acceptance criteria, indicating that the instrument
405 components are roadworthy and ready to perform the analyses. In all the incurred samples, RIV
406 peak was observed without overlapping from endogenous compounds. No unknown peaks or cross
407 contamination were detected in the blank QCs. The quantitative results of the other QCs (same
408 acceptance criteria as accuracy in 3.3.5) and the reanalyzed incurred samples (difference <20%)
409 were close to the nominal and the previous found concentration, respectively, thus pointing to the
410 absence of drift in the measurements. Despite the high number of analysis (44 injections), the entire

411 batch was examined in a short period, with a minimal participation of an operator, and using
412 available instrumentation, laboratory material and reagents. Besides, the volume of hazardous
413 chemicals manipulated and wasted, as well as the charge associated to waste treatment, is rather
414 limited. Therefore, the practical performances of the method allow its implementation in a clinical
415 laboratory.
416
417 *3.5 Application to drug monitoring*
418 The procedure was applied to determine the pharmacokinetics of RIV in a healthy subject, in
419 order to prove its suitability to this sort of clinical studies. Following oral administration of a single
420 20-mg tablet to a healthy volunteer, the plasma was extracted each hour over 12 h and analyzed.
421 The chromatograms were similar to those obtained in 3.4. The peak corresponding to the analyte
422 was observed and none metabolite or interfering compounds were detected. Figure 3 depicts the
423 curve RIV concentration *v.s.* time after ingestion. Peak-plasma was reached at nearly 2 h, and the
424 Cmax was 0.2 mg/L. The elimination half-life was 4.5 h.
425
426
427
428 **4. Conclusions**
429 The use of micellar liquid chromatography with direct injection of the samples has been
430 demonstrated as a valuable alternative for the determination of RIV in plasma and urine. The main
431 advantage is, without doubt, the shortening and simplification of the sample preparation. In
432 addition, the analyte was eluted in less than 6.0 min and clearly identifiable and quantifiable. In
433 spite of the occurrence of macromolecules in the matrices, analyte was detected without
434 interferences and kept up stability in the column. These advantages were achieved because of the
435 remarkable properties of micellar solutions to bind and solubilize biopolymers and drugs. The
436 method was rapid, easy-to-handle, semi-automated, economic, ecofriendly, safe, with a high sample

throughput, and useful for a laboratory with a large workload, like a clinical one. The procedure was rigorously and fully validated in terms of system suitability, specificity, linearity, calibration range, sensitivity, carry-over effect, accuracy, precision, robustness, stability and analysis of incurred samples (including blanks, QCs and ISR in the same batch); and the results fit the acceptance criteria. The procedure was found suitable for determination of RIV in the two studied matrices for clinical purposes. Finally, the retention equilibria in the column were investigated, from the data obtained using an experimental design of only 9 assays, by chemometrics. The binding between RIV and both micelle and stationary phase were moderately strong and essentially by hydrophobic interaction. The binding was weakened by the occurrence of 1-propanol in the bulk mobile phase.

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6. Conflict of interest disclosure

The authors declare that they have no conflicts of interest.

7. References

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FIGURES

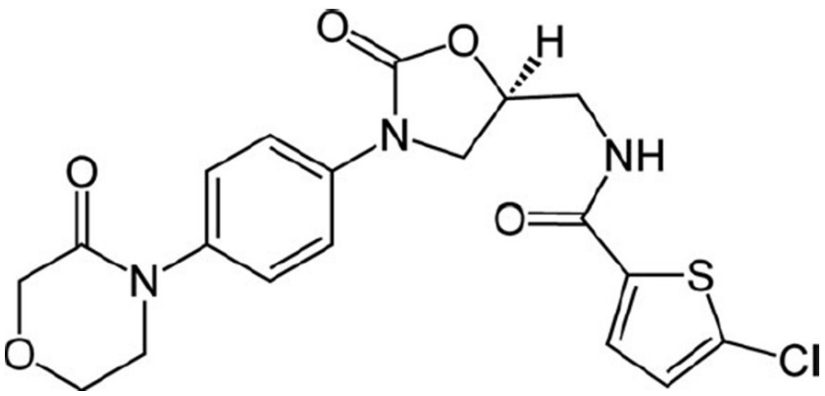


Figure 1. Structure of rivaroxaban or (S)-5-chloro-N-{[2-oxo-3-[4-(3-oxomorpholin-4-yl) phenyl] oxazolidin-5-yl] methyl} thiophene-2-carboxamide.

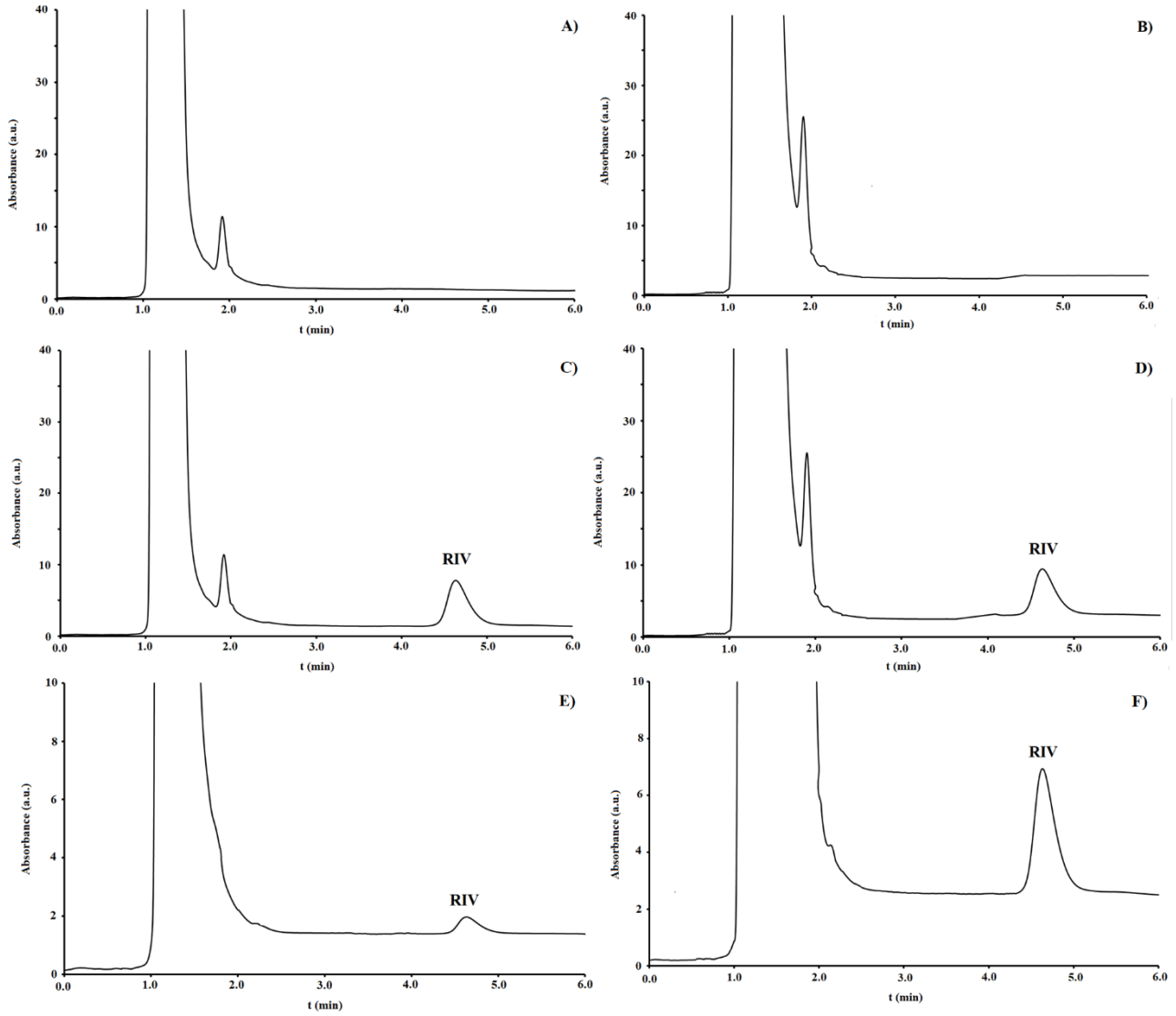
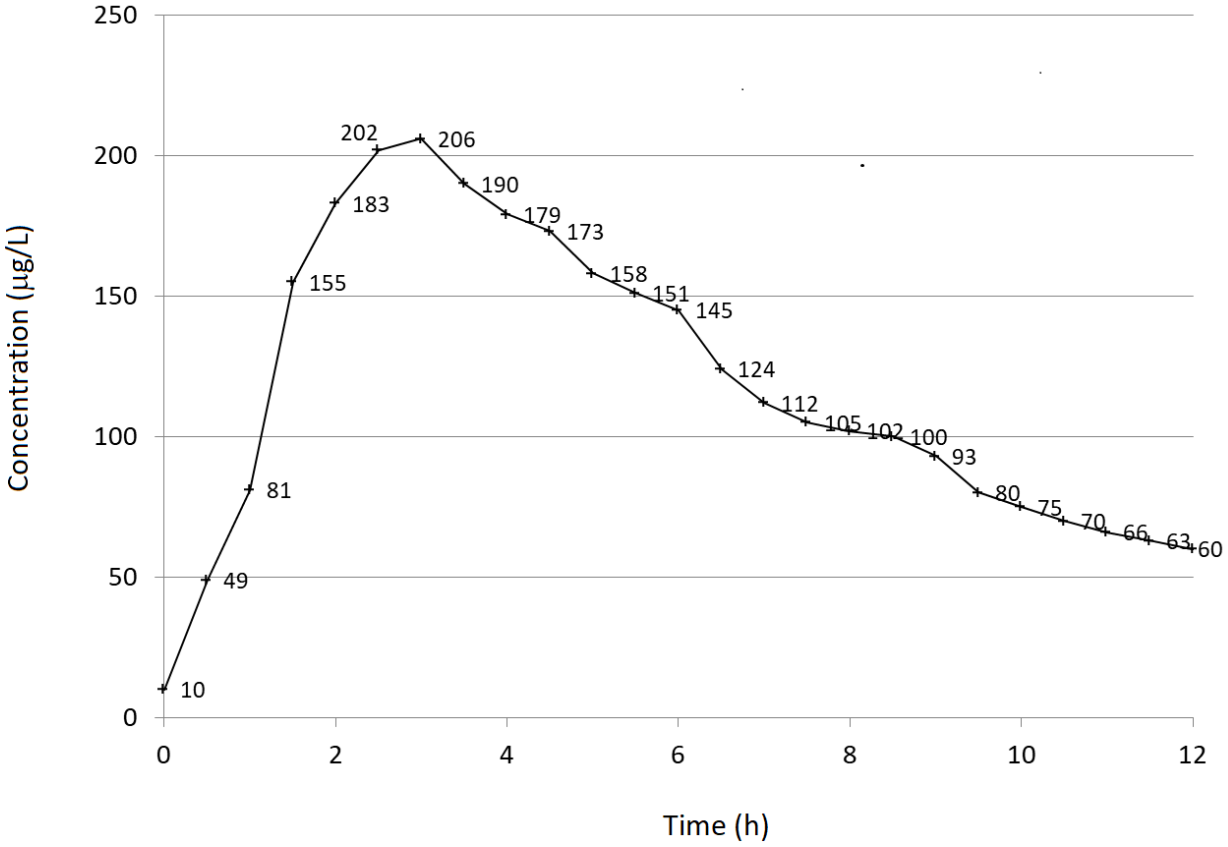


Figure 2. Chromatograms obtained by the analysis of: plasma A) blank, C) fortified at 1 mg/L with RIV and E) from patient 4; and urine B) blank, D) spiked at 1 mg/L of RIV and F) from patient 4.



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600 **Figure 3**. Plasmatic concentration-time profile of RIV after single ingestion of a 20-mg tablet by a

601 healthy volunteer (the measured concentrations are shown with the same unity as the y-axis).

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613 **TABLES**

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615 **Table 1**. Chromatographic parameters of rivaroxaban depending on the composition of the mobile phase (Experimental
616 dead time was 1.0 min)

[SDS] (M)	1-propanol (%)	t_R (min)	Efficiency (number of theoretical plates)
0.05	2.5	10.03	1362
0.05	7.5	6.71	1418
0.05	12.5	4.64	2029
0.10	2.5	5.92	1104
0.10	7.5	4.34	1275
0.10	12.5	3.28	1912
0.15	2.5	5.08	913
0.15	7.5	3.53	1189
0.15	12.5	2.78	1763

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619 **Table 2.** Accuracy and precision studies (%)

Matrix	Level	Accuracy[a]	Within-run Precision[a] (repeatability)	Between-run (intermediate) Precision, %
Plasma	0.025 mg/L	-11.1	19.9	18.5
	0.1 mg/L	+5.6	7.4	6.9
	0.25 mg/L	+3.8	5.5	6.1
	2 mg/L	+0.0	1.2	1.4
Urine	0.025 mg/L	-9.5	17.4	16.2
	0.1 mgL	+5.0	9.1	8.4
	0.25 mg/L	+4.2	7.0	5.8
	2 mg/L	+0.2	1.5	2.3

620 **[a]n=6; [b]n=5**

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627 **Table 3**. Analysis of incurred samples (Px indicated the patient's number).

Sample	[Rivaroxaban]
Blank plasma sample (1 mg/L) x6	Fit the acceptance criteria (see section 3.3.2)
Plasma QC (0.075 mg/L)	0.069 mg/L
Plasma P1	0.125 mg/L
Plasma P2	Not detected
Plasma QC (0.2 mg/L)	0.190 mg/L
Plasma P3	0.420 mg/L
Plasma P4	0.090 mg/L
Blank plasma QC	Not detected
Plasma QC (0.5 mg/L)	0.470 mg/L
Plasma P5	0.321 mg/L
Plasma P6	0.259 mg/L
Plasma QC (0.075 mg/L)	0.070 mg/L
Plasma P7	0.128 mg/L
Plasma P8	0.032 mg/L
Plasma QC (0.2 mg/L)	0.185 mg/L
Plasma P9	0.059 mg/L
Plasma P10	0.226 mg/L
Plasma QC (0.5 mg/L)	0.523 mg/L
Plasma P3 reanalyzed	0.392 mg/L
Plasma 6 reanalyzed	0.228 mg/L
Urine QC (0.075 mg/L)	0.072 mg/L
Urine P1	0.821 mg/L
Urine P2	0.059 mg/L
Urine QC (1 mg/L)	1.123 mg/L
Urine P3	4.826 mg/L
Urine P4	0.685 mg/L
Blank urine	Not detected
Urine P5	3.325 mg/L
Urine P6	2.394 mg/L
Urine QC (2.5 mg/L)	2.387 mg/L

Urine P7	0.995 mg/L
Urine P8	0.325 mg/L
Urine QC (0.075 mg/L)	0.083 mg/L
Urine P9	0.587 mg/L
Urine P10	1.715 mg/L
Urine QC (1 mg/L)	0.926 mg/L
Urine 2 reanalyzed	0.318 mg/L
Urine 5 reanalyzed	3.654 mg/L
Urine QC (2.5 mg/L)	2.323 mg/L

SUPPLEMENTARY MATERIAL

Table SM1. Chemical and pharmacokinetic characteristics of Rivaroxaban [2,5,8,10-13]

Characteristic	Value	Reference
Solubility in water	Almost insoluble	[5,13]
Molecular weight	436 g/mol (small molecule)	[5,13]
Bioavalilability	Doses up to 10 mg: 80-100%, regardless of the food intake. For 15 and 20 mg doses, it has a lower bioavailability in the fasted state, and then must be taken with food	[11]
Time to peak-plasma	2-4	[11]
Half-life	5-9 adults; 11-13 elderly	[11]
Protein binding	92-95%	[11]
C_{max} for doses 5 - 60 mg	40 to 400 μg/L	[12]
C_{though} for doses 5-60 mg	8 to 160 μg/L	[12]
Elimination	Urine (66%): 36 % as unchanged drug and 30% as metabolites Faeces (34%): 7 % unchanged and 21% as metabolites	[15]
Major or active circulating metabolite	None	[2,8,10, 11,12]