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# Determination of Apixaban Levels in Human Plasma by a High-Throughput Liquid Chromatographic Tandem Mass Spectrometry Assay

## Determinarea rapidă a apixabanului în plasma umană prin cromatografie de lichide de înaltă performanță cuplată cu spectrometrie de masă în tandem

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### Abstract

A high-throughput liquid chromatography method with detection by tandem mass spectrometry (LC-MS/MS) was developed and validated for the quantification of apixaban in human plasma. The separation was performed on a Gemini-NX column under isocratic conditions using a 33:67 (v/v) mixture of acetonitrile and 1 mM ammonium formate in water at 40 °C with a flow rate of 0.5 mL/min. The detection of apixaban was performed in multiple reaction monitoring mode ( $m/z$  417.2 from  $m/z$  460.2) with electrospray positive ionization. A single-step protein precipitation with methanol was used for plasma sample preparation. The method was validated with respect to selectivity, linearity ( $r > 0.994$ ), intra-day and inter-day precision ( $CV < 14.4\%$ ) and accuracy (bias  $< 9.5\%$ ) over the range of 9.70 - 970.00 ng/mL plasma. The lower limit of quantification (LLOQ) was 9.70 ng/mL and the recovery was between 97.4 - 104.5 %. The method is fast, efficient, requires the processing of a small volume of plasma (50  $\mu$ L), a short run-time (1 min) for chromatographic analysis, and a simple and rapid preparation of samples. It is very well suited for clinical therapeutic drug monitoring and pharmacokinetic studies.

**Keywords:** apixaban; LC-MS/MS; therapeutic drug monitoring

### Rezumat

S-a elaborat și s-a validat o metodă de cromatografie de lichide de înaltă performanță cuplată cu spectrometrie de masă în tandem (LC-MS/MS) pentru cuantificarea apixabanului în plasma umană. Separarea s-a realizat pe

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o coloană Gemini-NX în condiții izocratice folosind un amestec de acetonitril și formiat de amoniu 1 mM în apă (33:67, v/v) la 40 °C, cu un debit de 0,5 mL/min. Detecția apixabanului s-a realizat prin monitorizarea reacțiilor multiple ( $m/z$  417,2 din  $m/z$  460,2) cu ionizare pozitivă prin electrospray. Pentru pregătirea probelor de plasmă s-a folosit o singură etapă de precipitare a proteinelor cu metanol. Metoda s-a validat cu respectarea selectivității, linearității ( $r > 0,994$ ), preciziei intrazilnice și interzilnice ( $CV < 14,4\%$ ) și a acurateții ( $bias < 9,5\%$ ) pe domeniul de concentrații 9,70 – 970,00 ng/mL plasmă. Cea mai mică limită de cuantificare (LLOQ) a fost de 9,70 ng/mL, iar randamentul de recuperare din plasmă a avut valori cuprinse între 97,4 – 104,5 %. Metoda este rapidă, eficientă, necesită prelucrarea unui volum mic de plasmă (50  $\mu$ L), un timp de lucru scurt (1 min) pentru analiza cromatografică și o pregătire simplă și rapidă a probelor. Se pretează foarte bine pentru monitorizarea concentrațiilor apixabanului în timpul tratamentului clinic și pentru studii de farmacocinetică.

**Cuvinte cheie:** apixaban, LC-MS/MS, monitorizarea terapeutică a medicamentelor

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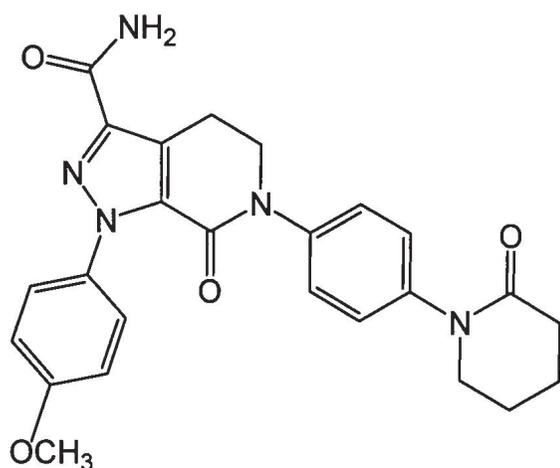
## Introduction

Treatment and long-term prevention of venous and arterial thromboembolism is still a therapeutic domain in need for safe, effective, easy-to-use and monitor drugs, as it remains a common cause of mortality and morbidity [1].

Apixaban (Fig.1) is a pyrazole derivative small-molecule, designed as a potent, oral, reversible agent which selective blocks the active site of Xa (apparent dissociation constant [Kd] 0.08 nmol/L) [2]. It represents a new oral anti-coagulant molecule, and it received the authorization for European use in 2011, being approved in prevention of stroke and systemic embolism in adults with non-valvular atrial fibrillation, for

treatment of deep venous thrombosis, pulmonary embolism and for the prevention of recurrence of DVT and PE after elective hip or knee replacement surgery [3]. The available evidence provided by the pre- approval studies (AVERROES, ADVANCE, ARISTOTLE, AMPLIFY) and various analysis of emerging clinical experience suggest that apixaban is non-inferior to existing standard anti-thrombotic therapies and that it has improved safety, expressed as reduced hemorrhagic risk [4-6].

At therapeutic doses, the absolute bioavailability of apixaban is approximately 50%, with low to moderate within subject and inter-subject exposure variability and with linear pharmacokinetics. Cmax is reached approximately 3 h post-dose in healthy volunteers [2]. Apixaban's plasma protein binding is approximately 87% and it has a half-life of approximately 12 hours. It has multiple routes of elimination, with 27% of the total clearance being attributed to the renal excretion. The main metabolic pathway is through CYP3A4/5, P-glycoprotein and breast cancer resistance protein (BCRP) being involved in its transport [3]. Because Apixaban is metabolized by the liver (partially by CYP 3A4) there are few recommendations to prescribe it to patients with hepatic impairment. No dosage adjustment of Apixaban is necessary in patient with mild hepatic impairment, but it should be used with



**Figure 1. Chemical structure of apixaban**

cautions in patients with moderate liver disease (Child class Pugh A or B) and a discontinuation period up to 5 days can be considered before elective surgery in such patients [7]. The present clinical experience allows its administration in renal impairment with a creatinine clearance  $\geq$  15 ml/min. This is best done by calculating creatinine clearance using Cockcroft-Gault formula (some calculators are also available online), but with some adjustments regarding body-mass index [7]. Strong inhibitors or inducers of both CYP3A4 and P-glycoprotein can markedly influence the plasmatic concentrations of apixaban if co-administered [3]. There are no interactions between food and pharmacokinetics and pharmacodynamics of apixaban [8].

Apixaban has high oral bioavailability and it is absorbed throughout the gastrointestinal tract. Despite this feature, currently there are no warnings or recommendations regarding its use in special populations at high risk for developing venous thromboembolism (after gastric bypass surgery, lap-band weight loss surgery or extended resection of the small bowel) [7].

Thrombin has a major role in hemostasis. When thrombin is activated from prothrombin, it converts soluble fibrinogen in an insoluble compound (fibrin); on the other hand thrombin activates coagulation factors V, VIII and XI and activates platelets. Factor Xa is another important factor for anticoagulant drugs (generates the Xa complex, that converts prothrombin in thrombin).

Apixaban inhibits free and clot-bound factor-Xa, and prothrombinase activity; it has no direct effects on platelet aggregation, but indirectly inhibits platelet aggregation induced by thrombin. By inhibiting Factor-Xa, apixaban prevents thrombin generation and thrombus development. [8].

The main therapeutic advantage for apixaban is the lack of the regular coagulation monitoring requirements specific to the coumarin deriva-

tives, while having predictable pharmacokinetics and pharmacodynamic effects. Moreover, it involves the administration of standard, indication-dependent, twice daily doses; it has a rapid onset of action (in 3 to 4 hours after oral intake maximum concentration  $C_{max}$  is achieved) and decline of the anticoagulant effect (after intravenous administration a half-life of approximately 12 hours), with considerable fewer clinically significant drug interactions [3,5,11].

Therefore, routine monitoring seems unnecessary in most clinical circumstances, although some exceptions could be easily anticipated. Firstly, the hemorrhagic risk associated with an eventual overdose could become a serious concern, as its influence on clotting tests is small and highly variable, making irrelevant the clinical monitoring through usual laboratory assessment tests [12].

In this context, the plasma Anti-Factor Xa chromogenic assays started to be used to evaluate the anti-Xa activity of apixaban, but results proved to be variable using different commercial kits [13]. On the other hand, apixaban overdose does not have an antidote and various algorithms for managing hemorrhage have been suggested to this end, using nonspecific reversal agents [14]. Assays to measure drug levels would also be necessary in various clinical situations as those involving unavoidable co-prescription with interacting drugs or those which need differentiation between therapy inefficacy and poor adherence [2,5,11]. Moreover, unplanned surgery or postoperative periods would benefit from precise evaluations of hemorrhagic risk, as apixaban plasma concentrations proved to be linearly correlated to its anti-Xa activity, over a wide dose range of apixaban (0.5 – 50 mg), for both patients and healthy subjects [3]. Furthermore, for dabigatran and rivaroxaban, two other novel oral anticoagulants, several algorithms are available for the management of the bleeding risk associated with emergency surgery and invasive procedures, considering the drugs plasma

concentrations, obtained through the use of sensitive analytical methods [15,16].

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the reference method used in therapeutic monitoring of novel direct oral anticoagulants due its precision, accuracy, sensitivity and robustness [14-17]. There are several pharmacokinetics studies of apixaban [7, 18-23] and assessments of different laboratory tests [17,24] that used LC-MS/MS for quantification of apixaban in human plasma.

The main objective of our study was to develop and validate a specific and quantitative high throughput LC-MS/MS method for the analysis of apixaban in human plasma with applicability in clinical therapeutic drug monitoring, pharmacokinetics, as well as in bioequivalence studies. The protocol study was reviewed and validated by the Ethics Committee of the University of Medicine and Pharmacy of Tirgu Mures and has been performed in the accordance with standards of the Declaration of Helsinki and local regulations. All subjects provided written informed consent.

## Material and methods

### *Reagents*

Acetonitrile of isocratic grade for liquid chromatography, methanol, ammonium formate of analytical-reagent grade were from Merck (Darmstadt, Germany). Water was deionised using a Milli-Q Water purification system (Millipore, Milford, MA, USA). The human blank plasma was supplied by the Regional Blood Transfusion Centre of Cluj-Napoca (Romania) from healthy volunteers, men and women.

### *Apparatus*

HPLC separations were carried out on a system from Agilent Technologies (Darmstadt, Germany), an 1100 series model consisting of a

G1312A binary pump, an in-line G1379A degasser, an G1329A autosampler, a G1316A column oven and an Agilent Ion Trap Detector 1100 VL. Other apparatus used in the experiment were:

a centrifuge 204 Sigma (Osterode am Harz, Germany); Analytical Plus and Precision Standard balances (Mettler-Toledo, Switzerland); a Vortex Genie 2 mixer (Scientific Industries, New York, USA); an ultrasonic bath Elma Transsonic 700/H (Singen, Germany).

### *LC-MS/MS conditions*

Apixaban separation was achieved at 40°C on a Gemini-NX (5 mm x 2 mm i.d., 3 µm) chromatographic column (Agilent Technologies) with a mobile phase consisting of acetonitrile and 1 mM ammonium formate in water (33:67, v/v) in isocratic conditions, with a flow of 0.5 mL/min. The detection of apixaban was in the multiple reaction monitoring (MRM) mode (monitored transition: m/z 417.2 from m/z 460.2) using an electrospray ion source (polarity: positive, nebulizer 60 psi (nitrogen), dry gas nitrogen at 12 L/min, dry gas temperature 350°C). The run-time was 1 minute.

### *Standard solutions*

A stock solution of apixaban in methanol (0.485 mg/mL) was diluted in the same solvent (48.5 µg/mL) or in drug-free human plasma (0.970 µg/mL) to obtained the working solutions. These solutions were subsequently used both for preparing plasma calibration standards with the concentrations of 9.70, 19.40, 38.80, 77.60, 194.00, 388.00, 582.00 and 970.00 ng/mL, and for quality control (QC) plasma samples of 38.80 ng/mL (lower), 194.00 ng/mL (medium) and 582.00 ng/mL (higher). All prepared solutions were stored at -20°C until analysis.

### *Pretreatment of samples*

Standards and plasma samples (50 µL) were deproteinized with methanol (150 µL), mixed (10 s) and centrifuged (6 min at 6000 rpm). Vol-

umes of 1  $\mu$ L from supernatants were analyzed in HPLC system.

### **Method validation**

The selectivity and the specificity of the method were evaluated. The chromatograms of plasma samples marked with apixaban were compared with those obtained from different plasma blank samples (n=6). Six aliquots of spiked plasma with the analyte at the lowest level of the calibration curve were analyzed to determine the sensitivity.

The levels of apixaban were automatically calculated by external standard method. The linearity was studied by the least squares analysis:  $y = b + ax$ , weighted (1/y) linear regression, where y - peak area and x - analyte concentration (ng/mL).

Five different aliquots (n = 5) from lower, medium and, respectively, higher QC plasma samples were analyzed on the same day to determine intra-day precision (expressed as coefficient of variation, CV %) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias %). One sample from each QC plasma samples (at lower, medium and higher levels) was analyzed on five different days (n = 5) to determine inter-day precision and accuracy.

The lowest calibration standard with an accuracy and precision below 20% was considered to be the lower limit of quantification (LLOQ).

The absolute recoveries (at LLOQ, lower, medium and higher levels) were calculated by comparing the response of apixaban in labeled plasma samples with his response in standard solutions with the same concentration, prepared in mobile phase and processed in the same manner with plasma sample (n = 5).

### **Results**

Electrospray (ESI) positive mode yielded a better spectrometer response for apixaban than

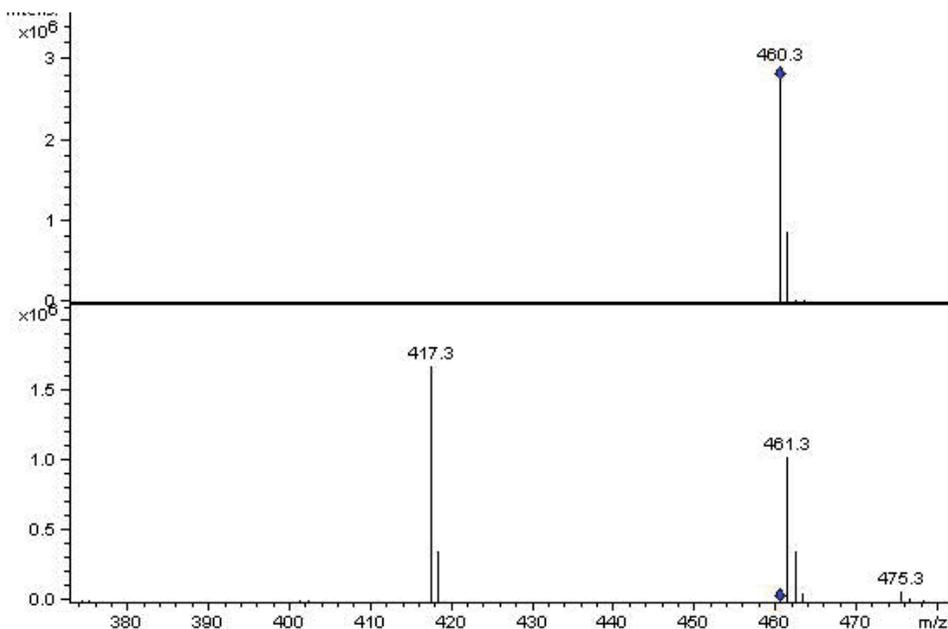
the negative mode. Apixaban forms the molecular ion  $[M+H]^+$  (m/z 460.2) by accepting a proton in the acidic mobile phase. The use of tandem MS detection allows to obtain a better selectivity and sensitivity by the fragmentation of the molecular ion. This was fragmented at the optimum collision energy of 0.9 V, and the ion transition m/z 460.3  $\rightarrow$  417.3 was monitored and analysed (Fig.2).

To achieve symmetrical chromatographic peaks, good resolution and a short retention time for apixaban, an absolutely necessary feature of a high-throughput analysis, the developed LC-MS/MS method was optimized. The best results were obtained with a Gemini-NX column and a mixture of acetonitrile and 1 mM ammonium formate in water (33:67, v/v) under isocratic conditions at 40 °C and a flow rate of 0.5 mL/min. The method is rapid, with a total run time of instrumental analysis of 1 min and a retention time of apixaban of 0.82 min. Sextuple analysis of blank samples showed no interfering endogenous peaks at the retention time of apixaban in human plasma. (Fig.3).

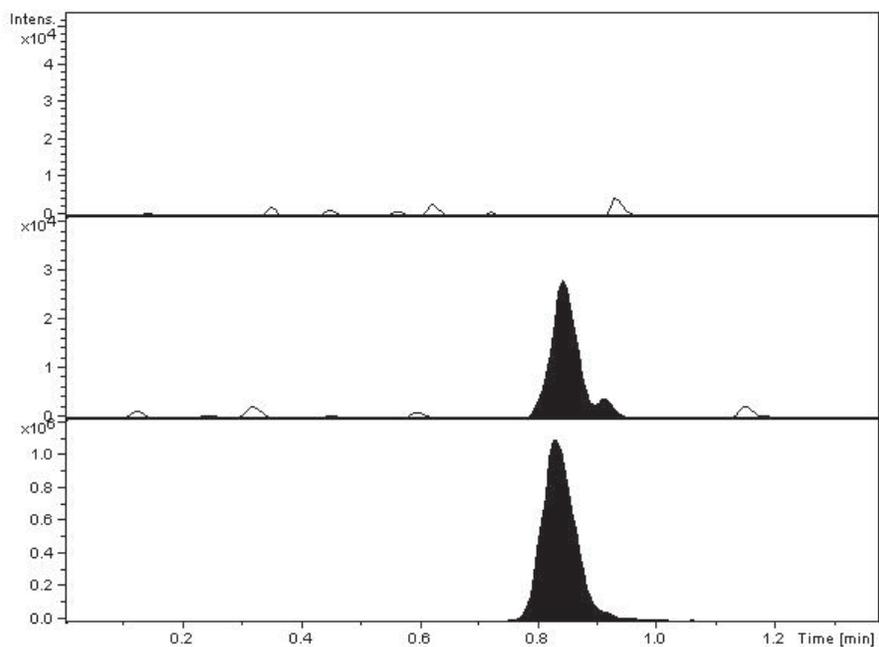
The linearity of the method was maintained throughout the range of concentration studied (9.70 - 970.00 ng/mL), with a correlation coefficient higher than 0.994 ( $r^2 > 0.9885$ ). The values obtained for intra-day and inter-day precision and accuracy during the validation for plasma are shown in Table 1 and Table 2, respectively. The lower limit of quantification (LLOQ) was established at 9.70 ng/mL with an acceptable accuracy and precision (Table I and Table II).

### **Discussion**

There are few method described for the quantification of apixaban in human plasma, alone or simultaneously with other novel oral anticoagulants. Gous et al. measured plasma levels of apixaban, dabigatran, edoxaban, and rivaroxaban in human plasma by turbulent flow liquid chromatography with high resolution mass



**Figure 2. Mass spectra of apixaban obtained by electrospray ionisation in positive ion mode: (up) full-scan spectrum with pseudo-molecular ion  $[M+H]^+$  ( $m/z$  460.3); (down) MS/MS reactive (fragmentation) spectra, obtained with a collision energy of 0.7 V**



**Figure 3. Representative chromatograms of (up) drug-free plasma, (middle) plasma spiked with apixaban at LLOQ (9.70 ng/mL) and (down) plasma sample obtained from a patient 2 h after the administration of 5 mg apixaban (concentration found: 587 ng/mL).**

**Table I. The intra-day precision (CV %) and accuracy (bias %) and recovery data for the measurement of apixaban in human plasma (n = 5)**

Nominal concentration (ng/mL)	Found concentration mean		CV (%)	Bias (%)	Recovery	
	ng/mL	± SD			(%)	± SD
9.70	10.49	0.95	9.0	8.2	104.5	5.4
38.80	42.50	5.32	12.5	9.5	101.9	1.1
194.00	210.69	9.49	4.5	8.6	97.4	0.8
582.00	603.20	29.06	4.8	3.6	98.6	0.8

**Table II. The inter-day precision (CV %) and accuracy (bias %) and recovery data for the measurement of apixaban in human plasma (n = 5)**

Nominal concentration (ng/mL)	Found concentration mean		CV (%)	Bias (%)	Recovery	
	ng/mL	± SD			(%)	± SD
9.70	9.91	1.11	11.2	2.2	103.2	2.1
38.80	39.08	5.63	14.4	0.7	99.7	1.5
194.00	205.57	12.45	6.1	6.0	97.6	1.0
582.00	594.43	27.06	4.6	2.1	98.5	3.1

spectrometry. Turbo flow column allows online extraction (protein precipitation step), but this procedure is not routinely available in most laboratories. The range of calibration was 1-500 ng/mL, but our clinical practice demonstrated higher plasma levels of apixaban in patients after repeated administration than this range of concentrations (Fig.4). Processed volume plasma was 100 µL and total time of analysis was 6 min [18].

Schmitz et al. quantified dabigatran, rivaroxaban and apixaban in plasma by ultra performance liquid chromatography (UPLC) coupled with tandem MS/MS as reference method to evaluate the analytical performance of several coagulation assays. The calibration range was 23-750 ng/mL, and LLOQ < 1 ng/mL [20].

Delavenne et al. developed and validate a LC-MS/MS method to quantified apixaban in human plasma after protein precipitation with methanol. Chromatographic separation was performed on a Luna MercuryMS C18 column with gradient of the mobil phase consisting of

0.1% formic acid and acetonitrile. The run time of instrumental analysis was 1.3 min, and the retention time of apixaban was 0.92 min. They processed 0.1 mL plasma and studied the calibration on the concentration range of 0-500 ng/mL. Therefore, concentrate sample dilution is required. LLOQ was estimated of 5 ng/mL [24].

Raghavan et al. developed a LC-MS/MS method to evaluate metabolism and pharmacokinetics of apixaban after oral administration to humans. They processed 0.2 mL of plasma samples and used <sup>13</sup>CD<sub>3</sub>-labeled apixaban as the internal standard. The separation was performed isocratically on Phenomenex Luna C18 column with a mixture of 0.1% formic acid in acetonitrile and water (60:40, v/v). The detection used a positive electrospray ionization source and monitored the mass transition of m/z 460 to 443. LLOQ was 1 ng/mL [21].

A few studies of metabolism and/or pharmacokinetics briefly presents the LC-MS/MS methods applied to determine apixaban plasma lev-

els. Thereby, Upreti et al. [25], and Wang et al. [26] determined pharmacokinetics of apixaban in healthy subjects using a LC-MS/MS method validated by Intertek Pharmaceutical Services with a LLOQ of 1 ng/mL. Frost et al. studied pharmacokinetics of apixaban after single dose in healthy subjects by a LC-MS/MS method with LLOQ of 1 ng/mL and quantification range of 1 - 1000 ng/mL [7]. In another study, Frost et al. applied a LC-MS/MS method after solid phase extraction to determine pharmacokinetics of multiple oral doses of apixaban in healthy subjects, LLOQ being also of 1 ng/mL [22]. Cui et al. determined pharmacokinetics profile of apixaban after single- and multiple-dose in healthy Chinese subjects using a validated LC-MS/MS method with atmospheric pressure ionization and protein precipitation of samples before the chromatographic analysis (LLOQ of 1 ng/mL) [23].

Our developed and validate method is very simple and rapid, requires a smaller plasma volume (50 µL) and a shorter time of analysis than other methods reported in the scientific literature [18,20,21,24]. The instrumental analysis is performed in 1 minute and a set of 24 samples requires a preparation time of about 15 minutes. The large studied calibration range (9.70 – 970.00 ng/mL) allows direct analysis of plasma samples after protein precipitation and centrifugation without additional dilution.

Because the method has not been the subject of the audit of National Agency for Medicines and Medical Devices, this was partially validated, in accordance with international regulations [28-32]. But the validation performed covers the most important parameters of the developed analytical method.

The obtained results proved a good linearity, sensitivity (LLOQ of 9.70 ng/mL), accuracy and precision over the studied concentration range. All values for accuracy and precision were within recommended limits (Table 1 and Table 2). The recovery values were between 97.4 - 104.5

%, which means no analyte loss during sample preparation due to adsorption on precipitated proteins.

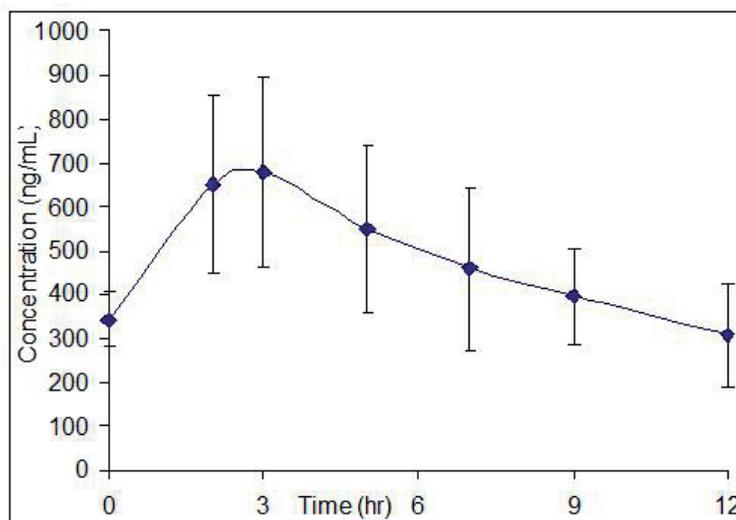
Despite the safety and efficacy of apixaban, there is limited information on possible strategies for reversal of its antithrombotic effects, in patients presenting with medical and surgical emergencies [9].

Before discontinuing anticoagulation with apixaban for reasons other than bleeding, the risks of thrombotic events must be carefully assessed. Once stopped, treatment should be restarted according to the hemorrhagic events risk factors (including antiplatelet therapy, treatment with other anticoagulants, fibrinolytic therapy, chronic NSAIDs).

The safety of neuraxial anesthesia for patients treated with oral anticoagulants should be considered regarding the potential benefit versus the risk before neuraxial intervention [8]. When this manoeuvre is performed, patients are at risk for developing an epidural or spinal hematoma that may result in long-term or permanent neurological sequels.

Another medical practice problem arises from the lack of antidotes for oral anticoagulants. In patients presenting major bleedings, is vital to assess the cause of bleeding: is there a local source (e.g. gastrointestinal anatomic site, coagulopathy resulting from liver disease, disseminate intravascular coagulation) or an over dose of the oral anticoagulant? Appropriate standard treatment should be immediately initiated; if bleeding persists the use of specific procoagulants (activated prothrombin complex concentrate, prothrombin complex concentrate and recombinant factor VIIa) can be considered, but currently only very limited experience is described with the use of these products.

Apixaban is highly protein bound and therefore cannot be removed by hemodialysis like dabigatran does (hemodialysis is effective in re-



**Figure 4. Plasma profile of apixaban to six hospitalized patients after administration of repeated doses (5 mg) of apixaban (mean concentration  $\pm$  SD).**

moving 60% of the dabigatran in the blood in 2-3 hours in case of drug toxicity) [7].

Despite the fact that activated charcoal is frequently used to manage overdose or accidental ingestion of different chemicals, just few data suggest that it may be useful in a perioperative setting in particular overdoses of apixaban.

There are only few reasons why practical medicine needs a simple, accurate and fast method to quantify the oral anticoagulant concentration in blood.

This high-throughput method was successfully used for therapeutic drug monitoring and pharmacokinetic parameter estimation in hospitalized patients being under treatment of apixaban for anti-thrombotic therapy. A typical plasma profile of apixaban obtained from six patients after repeated administration of apixaban in doses of 5 mg is showed in Fig.4.

In conclusion, the developed and validated LC-MS/MS method stands out through simplicity, accuracy and accessibility. In contrast to other LC-MS/MS methods reported in the scientific literature for quantification of apixaban in

plasma it is faster (both for sample preparation and as instrumental analysis) and inexpensive, essential features for high-throughput methods used in routine analysis. It has wide applicability in clinical level monitoring of apixaban as well as in pharmacokinetics or bioequivalence studies.

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### Conflict of interest

I.T. has received funds for research, lecture fees, educational presentations from Abbott, Astra Zeneca, Bayer, Boehringer Ingelheim, Pfizer and Sanofi. All other authors declare that they have no conflict of interest regarding this study. There were no implications of marketing authorization holder or manufacturer in this study.

### Abbreviations

CV - coefficient of variation  
ESI – electrospray

QC - quality control  
 LC-MS/MS - liquid chromatography tandem mass spectrometry  
 LLOQ - lower limit of quantification  
 MRM - multiple reaction monitoring  
 UPLC - ultra performance liquid chromatography  
 LMWH-low molecular weight heparin

## References

1. Nutescu E. Apixaban: a novel oral inhibitor of factor Xa. *Am J Health Syst Pharm.* 2012;69(13):1113-26. DOI: 10.2146/ajhp110418
2. Scaglione F. New oral anticoagulants: comparative pharmacology with vitamin K antagonists. *Clin Pharmacokinet.* 2013;52(2):69-82. DOI: 10.1007/s40262-012-0030-9
3. European Medicine Agency, Eliquis: Summary of Product Characteristics. Available at [http://www.ema.europa.eu/docs/en\\_GB/document\\_library/EPAR\\_-\\_Product\\_Information/human/002148/WC500107728.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/002148/WC500107728.pdf). Accessed October 23rd, 2014.
4. Agnelli G, Buller HR, Cohen A, Curto M, Gallus AS, Johnson M et al. Oral apixaban for the treatment of acute venous thromboembolism. *N Engl J Med.* 2013 Aug 29;369(9):799-808. DOI: 10.1056/NEJMoa1302507
5. Cheng JW, Barillari G. Non-vitamin K antagonist oral anticoagulants in cardiovascular disease management: evidence and unanswered questions. *J Clin Pharm Ther.* 2014;39(2):118-35. DOI: 10.1111/jcpt.12122
6. Bruins Slot KM, Berge E. Factor Xa inhibitors versus vitamin K antagonists for preventing cerebral or systemic embolism in patients with atrial fibrillation. *Cochrane Database Syst Rev.* 2013 Aug 8;8:CD008980. DOI: 10.1002/14651858.CD008980.pub2
7. Bauer KA. Pros and cons of new oral anticoagulants. Management of Thromboembolic Disease. *Hematology Am Soc Hematol Educ Program.* 2013;2013:464-70. DOI: 10.1182/asheducation-2013.1.464
8. Levy JH, Faraoni D, Spring JL, Douketis JD, Samama CM. Managing New Oral Anticoagulants in the Perioperative and Intensive Care Unit Setting. *Anesthesiology.* 2013 Jun;118(6):1466-74. DOI: 10.1097/ALN.0b013e318289bcb
9. Gogarten W, Vandermeulen E, Van Aken H, Kozek S, Llau JV, Samama CM. Regional anesthesia and antithrombotic agents: recommendations of the European society of Anaesthesiology. *Eur J Anaesthesiol.* 2010 Dec;27(12):999-1015. DOI: 10.1097/EJA.0b013e32833f6f6f
10. Frost C, Wang J, Nepal S, Schuster A, Barrett YC, Mosqueda-Garcia R, et al. Apixaban, an oral, direct factor Xa inhibitor: single dose safety, pharmacokinetics, pharmacodynamics and food effect in healthy subjects. *Br J Clin Pharmacol.* 2013 Feb;75(2):476-87. DOI: 10.1111/j.1365-2125.2012.04369.x
11. Bauer KA. Pros and cons of new oral anticoagulants. *Hematology Am Soc Hematol Educ Program.* 2013;2013:464-70. DOI: 10.1182/asheducation-2013.1.464
12. Brieger D. Anticoagulation: a GP primer on the new oral anticoagulants. *Aust Fam Physician.* 2014;43(5):254-9.
13. Douxfils J, Chatelain C, Chatelain B, Dogné JM, Mullier F. Impact of apixaban on routine and specific coagulation assays: a practical laboratory guide. [Abstract] *Thromb Haemost.* 2013;110(2):283-94. DOI: 10.1160/TH12-12-0898
14. Vilchez JA, Gallego P, Lip GYH. Safety of new oral anticoagulant drugs: a perspective. *Ther Adv Drug Saf.* 2014;5(1):8-20. DOI: 10.1177/2042098613507945
15. Pernod G, Albaladejo P, Godier A, Samama CM, Susen S, Gruel Y, et al. Management of major bleeding complications and emergency surgery in patients on long-term treatment with direct oral anticoagulants, thrombin or factor-Xa inhibitors: proposals of the working group on perioperative haemostasis (GIHP) - March 2013. *Arch Cardiovasc Dis.* 2013;106(6-7):382-93. DOI: 10.1016/j.acvd.2013.04.009
16. Dincq AS, Lessire S, Douxfils J, Dogné JM, Gourdin M, Mullier F. Management of Non-Vitamin K Antagonist Oral Anticoagulants in the Perioperative Setting. *Biomed Res Int.* 2014;2014:385014. DOI: 10.1155/2014/385014
17. Eby C. Novel anticoagulants and laboratory testing. *Int J Lab Hematol.* 2013 Jun;35(3):262-8. DOI: 10.1111/ijlh.12065
18. Gous T, Couchman L, Patel JP, Paradzai C, Arya R, Flanagan RJ. Measurement of the direct oral anticoagulants apixaban, dabigatran, edoxaban, and rivaroxaban in human plasma using turbulent flow liquid chromatography with high-resolution mass spectrometry. *Ther Drug Monit.* 2014 Oct;36(5):597-605. DOI: 10.1097/FTD.0000000000000059

19. Korostelev M, Bihan K, Ferreol L, Tissot N, Hulot JS, Funck-Brentano C, et al. Simultaneous determination of rivaroxaban and dabigatran levels in human plasma by high-performance liquid chromatography-tandem mass spectrometry. *J Pharm Biomed Anal.* 2014 Nov;100:230-5. DOI: 10.1016/j.jpba.2014.08.011
20. Schmitz EM, Boonen K, van den Heuvel DJ, van Dongen JL, Schellings MW, Emmen JM, et al. Determination of dabigatran, rivaroxaban and apixaban by ultra-performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS) and coagulation assays for therapy monitoring of novel direct oral anticoagulants. *J Thromb Haemost.* 2014 Oct;12(10):1636-46. DOI: 10.1111/jth.12702
21. Raghavan N, Frost CE, Yu Z, He K, Zhang H, Humphreys WG, et al. Apixaban metabolism and pharmacokinetics after oral administration to humans. *Drug Metab Dispos.* 2009 Jan;37(1):74-81. DOI: 10.1124/dmd.108.023143
22. Frost C, Nepal S, Wang J, Schuster A, Byon W, Boyd RA, et al. Safety, pharmacokinetics and pharmacodynamics of multiple oral doses of apixaban, a factor Xa inhibitor, in healthy subjects. *Br J Clin Pharmacol.* 2013 Nov;76(5):776-86. DOI: 10.1111/bcp.12106
23. Cui Y, Song Y, Wang J, Yu Z, Schuster A, Barrett YC, et al. Single- and multiple-dose pharmacokinetics, pharmacodynamics, and safety of apixaban in healthy Chinese subjects. *Clin Pharmacol.* 2013 Dec 6;5:177-84. DOI: 10.2147/CPAA.S51981
24. Delavenne X, Mismetti P, Basset T. Rapid determination of apixaban concentration in human plasma by liquid chromatography/tandem mass spectrometry: application to pharmacokinetic study. *J Pharm Biomed Anal.* 2013 May 5;78-79:150-3. DOI: 10.1016/j.jpba.2013.02.007
25. Upreti VV, Wang J, Barrett YC, Byon W, Boyd RA, Pursley J, et al. Effect of extremes of body weight on the pharmacokinetics, pharmacodynamics, safety and tolerability of apixaban in healthy subjects. *Br J Clin Pharmacol.* 2013 Dec;76(6):908-16. DOI: 10.1111/bcp.12114
26. Wang X, Mondal S, Wang J, Tirucherai G, Zhang D, Boyd RA, et al. Effect of activated charcoal on apixaban pharmacokinetics in healthy subjects. *Am J Cardiovasc Drugs.* 2014 Apr;14(2):147-54. DOI: 10.1007/s40256-013-0055-y
27. Gouin-Thibault I, Flaujac C, Delavenne X, Quenet S, Horellou MH, Laporte S, et al. Assessment of apixaban plasma levels by laboratory tests: suitability of three anti-Xa assays. A multicentre French GEHT study. *Thromb Haemost.* 2014 Feb;111(2):240-8. DOI: 10.1160/TH13-06-0470
28. Guidance for Industry, Bioanalytical Method Validation. U.S. Department of Health and Human Services, Food and Drug Administration. Federal Register. 2001;66.
29. Guidance on the Investigation of Bioavailability and Bioequivalence. The European Agency for the Evaluation of Medicinal Products, Committee for Proprietary Medicinal Products. 2001; CPMP/EWP/QWP/1401/98.
30. Imre S, Vlase L, Muntean DL. Bioanalytical method validation. *Rev Romana Med Lab.* 2008;10(1):13-21.
31. Guideline on bioanalytical method validation. European Medicines Agency. Committee for Medicinal Products for Human Use (CHMP). 275542 / 2014. [http://www.ema.europa.eu/docs/en\\_GB/document\\_library/Scientific\\_guideline/2011/08/WC500109686.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf)
32. Guidance for Industry. Bioanalytical Method Validation. U.S. Department of Health and Human Services. Food and Drug Administration. Center for Drug Evaluation and Research (CDER). Center for Veterinary Medicine (CVM). September 2013 <http://www.fda.gov/downloads/drugs/guidancecomplianceregulatory-information/guidances/ucm368107.pdf>