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ORIGINAL RESEARCH PAPER

Development and validation of LC-MS/MS method for determination of plasma apixaban

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ABSTRACT

Oral anticoagulants are a group of drugs used for the prevention and treatment of venous thrombosis and venous thromboembolism. For the last ten years, direct oral anticoagulants (DOAC) have been available and are equally effective, but significantly safer than vitamin K antagonists. In the case of an overdose, their most important side effect is still bleeding. Due to their widespread use, as well as increased toxicological importance there is a need to develop an analytical method for the determination of DOAC in biological material.

The aim of this paper was to establish a method for the quantification of apixaban as one of the representatives of DOAC. The methodology of the study included the measurement of apixaban in the plasma of patients treated in the intensive care unit. Plasma apixaban concentrations were determined by LC-MS/MS technique using carbamazepine as an internal standard. Obtained validation parameters indicate that the introduced method is sensitive, reliable, precise and accurate. Using this method, apixaban can be quickly and easily detected and quantified in plasma in patients who are suspected of overdosing with this drug.

KEYWORDS

anticoagulants, DOAC, apixaban, LC-MS/MS

INTRODUCTION

Apixaban (*Eliquis*®, Fig. 1) is an oral anticoagulant, direct, reversible and highly selective inhibitor of factor Xa, whether it is free in plasma, bound to a clot, or a part of prothrombinase complex [1, 2]. Since 2012, it has been approved for the prevention of stroke and systemic embolism in adult patients with non-valvar atrial fibrillation, as well as for the prevention and treatment of deep vein thrombosis and pulmonary thromboembolism [3].

Apixaban is rapidly absorbed after oral administration, and reaches maximum plasma concentrations after 3–4 h. The bioavailability of apixaban does not depend on food intake and is 50% due to incomplete resorption and the effect of the first passage through the liver. The elimination half-life of apixaban in healthy volunteers is 8–14 h [4, 5]. The metabolism of apixaban takes place in the liver, and part of the drug is excreted unchanged by the biliary

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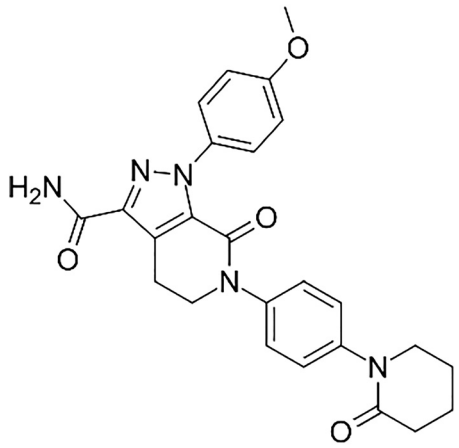

Fig. 1. Structure of apixaban

and renal routes [6, 7]. It is metabolized by O-demethylation, hydroxylation and sulfation of hydroxylated O-demethyl metabolite via cytochrome P450 (predominantly CYP3A4/5) enzyme. In addition to CYP3A4/5, apixaban is a substrate for the P-gp transporter [8, 3, 9].

Besides predictable pharmacokinetics, poor interaction with other drugs and reduced need for continuous laboratory monitoring are the main advantages of direct oral anticoagulants (DOAC) over the vitamin K antagonists. However, there is no anticoagulant that reduces the risk of thrombosis without a concomitant risk of bleeding. In situations such as overdose, sudden bleeding, preparation for emergency surgery, compliance assessment, evaluation of drug interaction, or assessment of drug accumulation in the case of renal or hepatic insufficiency, identifying and determining DOAC concentration in the patient's blood is of great benefit to the physician. It is not uncommon that the doctor has no information on which anticoagulant the patient uses in therapy or whether it is any of the drugs from the DOAC group [10].

Nowadays, apixaban is in a focus because of its use in patients with diagnosed COVID-19.

Conventional hemostasis tests are inexpensive, readily available, and rapid, but cannot be used to quantify DOAC in plasma, which is necessary in certain clinical situations [10]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is one of the most sensitive analytical methods for the detection, identification and quantification of analyzed compounds on biological samples.

Literature survey reveals that there are several methods for determination of apixaban using preferably liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) because this technique ensures precision, accuracy, sensitivity and robustness [11–16]. Protein precipitation method was used as a reference method for sample preparation.

The aim of this study was to introduce and validate a fast, simple and sensitive method for the determination of apixaban in human plasma by the LC-MS/MS technique after preparation of plasma samples by the protein precipitation method which can be applied in clinical therapeutic drug monitoring, pharmacokinetic and bioequivalence studies.

EXPERIMENTAL

Chemicals and reagents

Apixaban (Toronto Research Chemicals, Toronto, Canada), was used as the reference standard. Carbamazepine, (Sigma-Aldrich, St. Louis, Missouri, USA), was used as an internal standard.

Organic solvents used for sample preparation and determination were acetonitrile (HPLC grade, Sigma-Aldrich, St. Louis, Missouri, USA) and methanol (HPLC grade, Merck, Darmstadt, Germany). For the preparation of the mobile phase, formic acid LC-MS/MS of purity was used (Sigma-Aldrich, St. Louis, Missouri, USA).

Deionized water was obtained using a TKA GenPure device (Thermo Fisher Scientific).

Stock solutions, calibration standards and quality control samples

Primary stock solution of apixaban (1 mg/mL) was prepared by dissolving 5.0 mg (±0.1 mg) of standard in 5 mL of methanol and then stored at −20 °C. Stock solution of carbamazepine was prepared at a concentration of 100 mg L^{-1}.

Working solutions were prepared freshly on each day of analysis as serial dilutions in deionized water in an appropriate ratio. The internal standard (IS) at a concentration of 100 ng/mL in acetonitrile was prepared from a stock solution.

Quality control (QC) and method validation (MV) samples were prepared by diluting the appropriate working solutions with human citrate plasma ("pool"), so that the following concentrations were obtained: 5 ng/mL, 400 ng/mL and 750 µg/mL as and 1,500 ng/mL to test the sample dilution effect. The calibration curve was constructed based on the results from seven standards, after diluting the appropriate working solutions with human plasma, so as to obtain final concentrations: 3 ng/mL, 10 ng/mL, 50 ng/mL, 100 ng/mL, 250 ng/mL, 500 ng/mL and 1,000 ng/mL. Calibration curve and QC samples were prepared from different working solutions.

Blood samples for obtaining "pool" plasma were collected from volunteers who were not on anticoagulant therapy at the time of donation.

Sample preparation

Plasma samples for quantitative analysis of apixaban were obtained from patients from the Clinic of Cardiology and Emergency Internal Medicine at Military Medical Academy in Belgrade, Republic of Serbia. All patients had previously signed informed consent. Blood samples were delivered in sterile polypropylene vacuum tubes, 3.5 mL volume, with 3.2% sodium citrate as anticoagulant. Immediately upon

receipt, they were centrifuged, and the separated plasma was stored at −80 °C until the time of analysis. All samples (calibrators, QC and patient samples) were prepared by the method of protein precipitation with acetonitrile according to the following procedure: 400 µL of plasma sample and 800 µL of solvent (acetonitrile and internal standard) were added to each plastic tube. Such prepared plasma samples were shaken for 1 min on a vortex mixer and then centrifuged for 10 min at 13,000 rpm and 4 °C. After centrifugation, the supernatant was filtered through a nylon filter into an LC-MS/MS assay vial.

Liquid chromatography

Chromatographic analysis was performed on a liquid chromatograph (ACELLA, Thermo Fisher Scientific Inc., Madison, WI, USA), and chromatographic separation was achieved on an Infinity Lab Poroshell 120 EC-C18 column, 4.6 × 100 mm, 2.7 µm by isocratic elution at constant flow of mobile phase. A mixture of acetonitrile and 0.1% formic acid in water (50:50 v/v) was used as the mobile phase, which was previously filtered through a 0.45 µm membrane filter. The column temperature was maintained at 30 °C and the injector sample racks at 4 °C. The flow rate of the mobile phase was 400 µL/min, and the injection volume was 10 µL.

Mass spectrometry

A liquid chromatograph was coupled to a triple quadrupole mass detector (TSQ Quantum Access MAX, Thermo Fisher Scientific Inc., Madison, WI, USA).

Ion identification and quantification was performed in positive mode (ESI +). Detection of apixaban and internal standard was performed in multireaction monitoring mode (MRM). The following transitions were monitored: *m/z* 460.3 > 443.2 for apixaban and *m/z* 237.1 > 193.0 for carbamazepine. Optimization of the mass spectrometer conditions was performed by injecting apixaban solution and carbamazepine solution directly into the mass spectrometer. The following parameters were obtained: collision gas: argon, voltage in the detector: 5500 V, source temperature: 250 °C, capillary temperature: 250 ºC, spray voltage: 4500 V, evaporator temperature: 350 ºC, capillary transfer temperature: 290 ºC, scan time: 0.01 s, carrier gas pressure: 50 arbitrary units, auxiliary gas pressure: 10 arbitrary units, collision pressure: 1.0 mTorr. The system control and data acquisition were performed using software TSQ Tune and XCalibur.

Method validation

Assay validation was performed according to European medicines agency – EMA and International council for harmonisation - ICH Guidelines [17, 18]. The specificity was determined by analyzing blank plasma samples from five different subjects. The linearity, accuracy and precision were evaluated using 7 calibrators and 3 levels of quality control (QC). The appearance of the analyte signal remaining from the previously analyzed sample (Carry Over) was tested during the performing the analytical sequence, by injecting the solvent after the calibration standard of the highest concentration. The plasma sample spiked with apixaban in a concentration of 400 ng mL^{-1} was used for stability testing of samples. All samples were analyzed in triplicate. Short-term stability was estimated by comparison of freshly prepared samples with ones kept at room temperature for 4 h. Stability during frosting (−20°C) and thawing was estimated after three cycles with the time range between cycles 12–24 h. Stability in autosampler was estimated by analyzing samples at the beginning and after 24 h in autosampler at 10 ºC. The stability of the stock solution at 4° C was evaluated by assaying samples after two months. The long-term, short-term stability and the stability during three successive freeze–thaw cycles were also assessed for each QC level at −20° C ($n = 3$). Dilution effect for QC/MV was tested for a sample of concentration 1,500 ng mL^{-1}. QC/MV sample was prepared in a duplicate and then diluted using plasma pool in ratio 1:1 in order to adjust concentration to the linearity range of the method. Testing was performed in 5 reruns.

RESULTS AND DISCUSSION

Liquid chromatography

Representative chromatograms of drug free human plasma and plasma sample spiked with apixaban (3 ng mL^{-1}) and carbamazepine (100 ng mL^{-1}) are shown in Figs 1 and 2.

The full method analysis time was 5.50 min. Retention time for apixaban was 3.51 min and for carbamazepine was 4.37 min.

Method validation

Accuracy, precision and linearity. The accuracy, intra and inter day precision shown in Table 1 remained within acceptance criteria. Intra and inter day precision were less than 14% and accuracy shows a deviation less than 10% from target concentration at each tested level.

The method showed to be linear in the tested range. The typical equation of the calibration curve was $y = 0.04x - 0.0002$ for apixaban (Fig. 1), where y represents the ratio of apixaban peak area to that of carbamazepine and x the plasma concentration. Least square regression with 1/concentration weighting was used to calculate calibration curve. The relative standard deviation of the slopes and intercept of calibration curves were below 6% for apixaban. The mean r^2 of the calibration curve was 0.998 and residual analysis (data not shown) confirmed the linearity of the method (Figs. 3 and 4).

Limits of quantification and detection. In human plasma, calculated LOQ was 0.880 ng mL^{-1} and the LOD was 0.264 ng mL^{-1}.

Stability

Short-term, three freeze–thaw cycles, indicated that apixaban was stable in human plasma (Table 2).

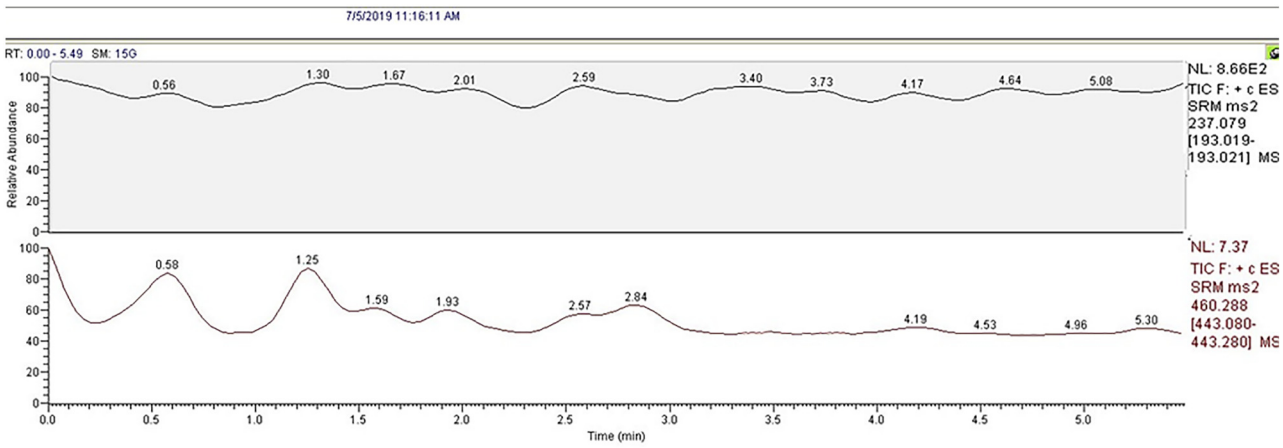

Fig. 2. Chromatogram of blank plasma sample

Table 1. Accuracy and precision within analytical sequence and among different analytical sequences

QC/MV sample	Accuracy within analytical sequence (*Recovery* %)	Accuracy among analytical sequences (*Recovery* %)	Precision within analytical sequence (%RSD)	Precision among analytical sequences (%RSD)
QC/MV1 (5 ng mL^{-1})	106.0	105.0	0.5	1.2
QC/MV2 (400 ng mL^{-1})	99.0	101.0	11.5	13.8
QC/MV3 (750 ng mL^{-1})	106.0	103.0	9.6	10.3

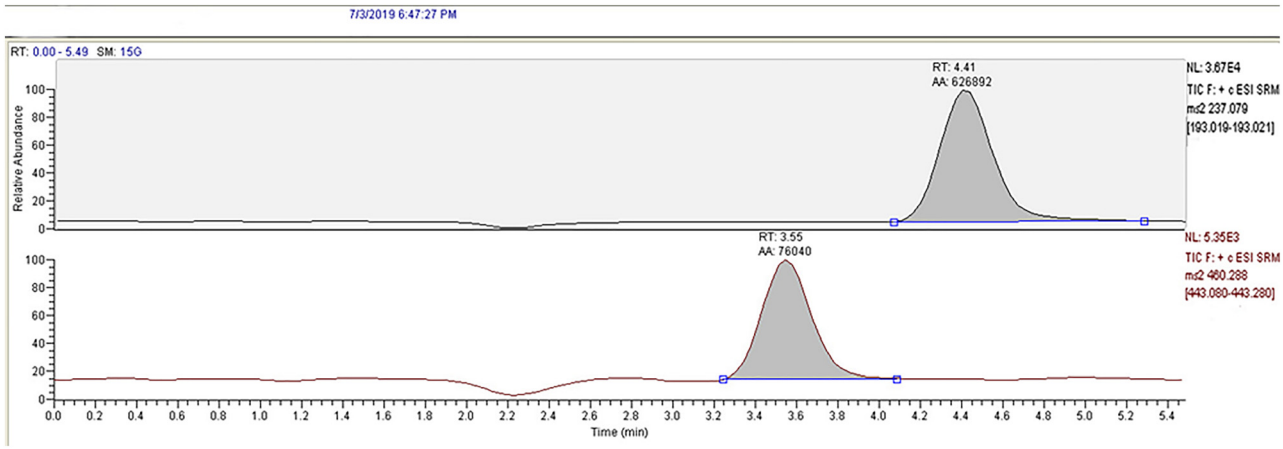

Fig. 3. Chromatogram of plasma sample spiked with apixaban (3 ng mL^{-1}) and carbamazepine (100 ng mL^{-1})

Discussion of validated method

In this study we presented a LC-MS/MS validation method for determination of apixaban in human plasma. Several methods of tandem liquid chromatography/mass spectrometry (LC-MS/MS) for determination of apixaban in biological samples were described in literature so far [11–16]. Routine application of these methods for the analysis of biological samples is available only in specialized laboratories within large hospital centers. In addition to sophisticated equipment and highly educated staff, such laboratories must work 24 h a day, because emergency patients such as those diagnosed with pulmonary embolism, can be admitted at any time of the day. These conditions are not available to most smaller hospitals that also encounter such patients. Furthermore, these analyses are also expensive. The use of expensive internal standards such as deuterated analogs or [13C, 2H7]-apixaban is not available for many laboratories. That is the reason why we decided to use carbamazepine. The advantages of the established method compared to previous methods described in the literature are greater sensitivity, usage of an isocratic elution, and HPLC column which can be found in every laboratory. The clinical use of apixaban is emerging (especially since it is a part of some COVID-19 protocols), and since the most important side effect of this drug is life-threatening acute hemorrhage, the need for the development

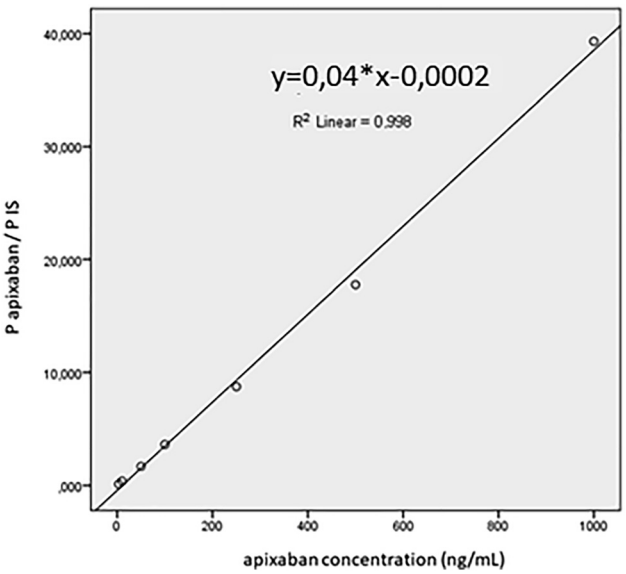

Fig. 4. Calibration curve for apixaban in plasma

of identification and quantification of this drug in plasma is evident. Qualitative and quantitative analysis of apixaban using LC-MS/MS method in this study was conducted through several phases: preparation of samples using protein precipitation method, method validation and application of validated LC-MS/MS method for determination of concentration of apixaban in patient plasma.

The advantage of sample preparation using protein precipitation method is that relatively small amounts of sample (400 µL) are required. In addition it is fast, simple, economic and suitable for urgent clinical-toxicological analyses. According to literature data, method of the protein precipitation is the most often used for preparation of samples containing apixaban [11–13, 15, 16]. The amount of sample is of great significance in laboratory practice. The small amount of sample injected enables longer life of chromatographic column. Given that there it is not always possible to get any larger amount of plasma from patient, 400 µ*L* of sample is an optimal amount. Deuterated standard analogues of apixaban are very often used as internal standards, but in our analysis carbamazepine was chosen as an internal standard because of its similar physico-chemical characteristics (e.g. lipophilicity which enabled IS to elute near apixaban). Isocratic elution was applied for chromatographic separation of analytes, and good peak resolution was achieved by mass detector optimization. Retention times of apixaban and carbamazepine were 3.51 and 4.37 min respectively. The total time of preparation and analysis of one sample is 25 min which makes this method suitable in a situation when the clinician requests fast information. Analysis of blank samples showed that there are no interferences at retention times of analytes which proved that the method is specific and that there are no interferences with other compounds.

Method validation showed that method is sensitive, precise, accurate and linear in wide range of concentrations (3–1,000 ng mL^{-1}) which is confirmed with high correlation ($r = 0.999$). Achieved linearity range completely fulfils needs of clinical-toxicological analysis and complies with current literature data. Literature data for apixaban concentrations in plasma are in range 20–500 ng mL^{-1}, while data of accurate concentrations are not available due to limitations of clinical data [19, 20].

In cases of acute poisoning, apixaban concentrations were up to 2,700 ng mL^{-1} [21, 22]. Using this method one sample with apixaban concentrations above linearity range was analyzed (1,500 ng mL^{-1}) while among analyzed patients' samples there were no apixaban concentrations out of linearity range.

Parameters of sample stability were tested. Stability of samples was examined under the different conditions and obtained results were acceptable and in the range of allowed variations (±15%) compared to freshly prepared solutions (Table 2). If it is not possible to analyze samples right after the reception, the samples should be centrifuged and separated plasma should be kept at 2–8 °C. If samples are kept for a longer period, freezing at –20 °C is recommended.

Validated method was used for assay determination of apixaban in five patient plasma samples. Plasma samples were delivered from internal medicine intensive care unit. Two out of five patients received apixaban for the treatment of lung embolism while the rest hospitalized patients were treated with this drug for atrial fibrillation. All obtained results were in therapeutic dosage range because the treatment was according to the recommendations and under the controlled medical supervision. In the sample 2 the obtained the lowest concentration (62 ng L^{-1}) was in the therapeutic range and can be explained with the time of blood sampling and taking the last dose of apixaban which must be considered when measuring drug concentration in patients on controlled anticoagulant therapy.

If implemented in regular laboratory practice, this method would improve care of patients in which there is a doubt of apixaban overdose. Patients in coma or unable to communicate from distinct reasons are often admitted in intensive care units with data of DOAC usage. If bleeding would be found (e.g. intracranial hemorrhage which may cause disturbance of consciousness), then it would be significant for doctor to get the information in the shortest period of time which anticoagulant was used and what is its blood concentration. This method enables both information in case of apixaban in therapy: identification of drug and

Table 2. Stability of tested compounds

Compound	Short-time stability of sample (% of variation)	Freeze-thaw stability (% of variation)	Stability of samples in autosampler (% of variation)	Long term stability of stock solution (% of variation)
Apixaban	3.1	5.9	4	4.2

determination of its plasma concentration. Each information is very important for clinician because knowledge of used anticoagulant is required for administration of specific antidote (andexanet in the case of apixaban) while the concentration of anticoagulant enables assessment of degree of intoxication and monitoring the effect of the antidote [23]. Sometimes within smaller hemorrhages which are not life threatening it is found that drug concentration in blood is slightly above therapeutic range and drug effect can pass in time and by using supportive treatment measurements. If this analytical method is applied the dilemma weather supportive treatment is sufficient or expensive antidote should be administered is less stressful.

Application of developed and validated LC-MS/MS method can be for scientific purposes. Conventional hemostasis tests are nonspecific, and because of that unreliable in determination of concentration of DOACs, and particularly apixaban [24]. Comparison of concentration change of apixaban to a different homeostasis tests could provide completely picture of concentration-effect relationship.

CONCLUSION

Because of increasingly widespread application and increasingly large toxicological significance, the need for development of analytical method for assay determination of apixaban in biological material is evident. Based on set goals and obtained results it can be concluded:

Method of tandem liquid chromatography mass spectrometry for assay determination of apixaban in patients' plasma was set. Sample preparation procedure was fast and simple which is a significant contribution to the development of urgent analyses and used HPLC column is very common for most laboratories.

Statistical data analysis showed that method is linear within a wide range of concentrations, precise, accurate, specific, sensitive and suitable for routine apixaban assay determination in patients' plasma.

Apixaban concentration was determined in all analyzed plasma samples and obtained results were in therapeutic range.

All obtained results showed that with this method concentration of apixaban in patients' plasma can be fast and reliable determined within therapeutic monitoring, but also in poisoning cases.

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