https://doi.org/10.33380/2305-2066-2024-13-1-1684
UDC 615.03



***Research article / Оригинальная статья***

# Development and Validation of an HPLC-MS/MS Method for Quantification of Apixaban in Human Plasma

**Ulyana D. Filonova[1, 2]✉, Polina K. Karnakova[1], Kseniia K. Karnakova[1], Mariia O. Popova[1], Anna A. Popova[1], Olga A. Archakova[1], Timofey N. Komarov[1, 3], Igor E. Shohin[1, 2]**

[1] LLC "Center of Pharmaceutical Analytics" (LLC "CPHA"). 8, Simferopol Boulevard, Moscow, 117246, Russia
[2] National Research Nuclear University MEPhI (Moscow Engineering Physics Institute). 31, Kashirskoe highway, Moscow, 115409, Russia
[3] Peoples' Friendship University of Russia named after Patrice Lumumba (RUDN University). 6, Mikluho-Maklaya str., Moscow, 117198, Russia

✉ **Corresponding author:** Ulyana D. Filonova. **E-mail:** u.filonova@cpha.ru

**ORCID:** Ulyana D. Filonova – https://orcid.org/0009-0009-7266-3296; Polina K. Karnakova – https://orcid.org/0000-0002-7518-0777; Kseniia K. Karnakova – https://orcid.org/0000-0002-4010-1231; Mariia O. Popova– https://orcid.org/0009-0008-1688-1902; Anna A. Popova – https://orcid.org/0000-0002-3874-8626; Olga A. Archakova – https://orcid.org/0000-0001-6621-1060; Timofey N. Komarov – https://orcid.org/0000-0001-8354-7877; Igor E. Shohin – https://orcid.org/0000-0002-1185-8630.

**Received:** 22.12.2023 **Revised:** 19.02.2024 **Published:** 20.02.2024

**Abstract**

**Introduction.** Apixaban is an anticoagulant used in a number of thromboembolic diseases with an improved benefit-to-risk ratio, according to multiple clinical studies. Due to the prescription of apixaban as antithrombotic therapy in patients with COVID-19, an increase in its use has been observed. Thus, due to the widespread use of apixaban and the need to conduct pharmacokinetic and bioequivalence studies of the drug, it is important to develop and validate a simple and sensitive method for the quantitative determination of apixaban in human blood plasma.

**Aim.** The aim of the study is to develop and validate a method for the determination of apixaban in human blood plasma using high-performance liquid chromatography with tandem mass selective detection (HPLC-MS/MS) for the subsequent bioanalytical study.

**Materials and methods.** The determination of apixaban in human plasma was carried out by HPLC-MS/MS with rivaroxaban as an internal standard. The method of protein precipitation with acetonitrile was used as sample preparation. Mobile phase: 0.1 % solution of formic acid in water (eluent A); 0.1 % solution of formic acid in acetonitrile (eluent B). The total run time was 3.00 min. Column: Shim-pack Velox Biphenyl; 2.7 μm; 50 × 2.1 mm. Ionization source: electrospray with positive ionization mode. MRM transitions: 460.15 → 443.10 m/z (apixaban); 436.05 → 144.95 m/z (rivaroxaban).

**Results and discussion.** The developed method was validated in accordance with the EAEU requirements for the following parameters: selectivity, calibration curve, accuracy and precision, lower limit of quantitation, suitability of standard samples, matrix effect, recovery, stability, carry-over, dilution effects. The parameters met the acceptance criteria.

**Conclusion.** The confirmed analytical range of the developed and validated method was 1.00–300.00 ng/mL in blood plasma. The method for determining apixaban in blood plasma is simple and sensitive. This method was tested during the analytical part of the bioanalytical study and can be used to conduct other pharmacokinetic studies of apixaban drugs.

**Keywords:** anticoagulants, DOAC, apixaban, HPLC-MS/MS, validation, COVID-19, pharmacokinetics

**Conflict of interest.** The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

**Contribution of the authors.** Timofey N. Komarov, Olga A. Archakova, Polina K. Karnakova, Ulyana D. Filonova have participated in the development and validation of the bioanalytical method, as well as in conducting the analytical part of the study. Kseniia K. Karnakova carried out statistical processing of the data. Maria O. Popova, Anna A. Popova were responsible for data quality and organization of the quality management system. Igor E. Shohin was responsible for the organizational part of the study. All of the above authors participated in the discussion of the results obtained in the form of a scientific discussion.

**For citation:** Filonova U. D., Karnakova P. K., Karnakova K. K., Popova M. O., Popova A. A., Archakova O. A., Komarov T. N., Shohin I. E. Development and validation of an HPLC-MS/MS method for quantification of apixaban in human plasma. *Drug development & registration*. 2024;13(1):224–240. (In Russ.) https://doi.org/10.33380/2305-2066-2024-13-1-1684

# Разработка и валидация методики количественного определения апиксабана в плазме крови с помощью метода ВЭЖХ-МС/МС

**У. Д. Филонова[1, 2]✉, П. К. Карнакова[1], К. К. Карнакова[1], М. О. Попова[1], А. А. Попова[1], О. А. Арчакова[1], Т. Н. Комаров[1, 3], И. Е. Шохин[1, 2]**

[1] Общество с ограниченной ответственностью «Центр фармацевтической аналитики» (ООО «ЦФА»). 117638, Россия, г. Москва, Симферопольский бульвар, д. 8
[2] Федерального государственного автономного образовательного учреждения высшего образования «Национальный исследовательский ядерный университет «МИФИ» (НИЯУ МИФИ). 115409, Россия, г. Москва, Каширское шоссе, д. 31
[3] Федеральное государственное автономное образовательное учреждение высшего образования «Российский университет дружбы народов имени Патриса Лумумбы» (РУДН), 117198,Россия, г. Москва, ул. Миклухо-Маклая, д. 6

✉ **Контактное лицо:** Филонова Ульяна Денисовна. **E-mail:** u.filonova@cpha.ru

**ORCID:** У. Д. Филонова – https://orcid.org/0009-0009-7266-3296; П. К. Карнакова – https://orcid.org/0000-0002-7518-0777;
К. К. Карнакова – https://orcid.org/0000-0002-4010-1231; М. О. Попова – https://orcid.org/0009-0008-1688-1902;
А. А. Попова – https://orcid.org/0000-0002-3874-8626; О. А. Арчакова – https://orcid.org/0000-0001-6621-1060;
Т. Н. Комаров – https://orcid.org/0000-0001-8354-7877; И. Е. Шохин – https://orcid.org/0000-0002-1185-8630.

**Статья поступила:** 22.12.2023 **Статья принята в печать:** 19.02.2024 **Статья опубликована:** 20.02.2024

**Резюме**

**Введение.** Апиксабан – антикоагулянт, применяющийся при ряде тромбоэмболических заболеваний и обладающий улучшенным соотношением пользы от приема препарата и рисков развития кровотечения согласно многочисленным клиническим исследованиям. В связи с назначением апиксабана в качестве антитромботической терапии пациентам с COVID-19 наблюдается рост его применения. Таким образом, из-за большого распространения апиксабана и необходимости проведения фармакокинетических исследований и исследований биоэквивалентности препаратов апиксабана важно разработать и валидировать простую и чувствительную методику количественного определения апиксабана в плазме крови.

**Цель.** Целью исследования является разработка и валидация методики определения апиксабана в плазме крови человека с помощью метода высокоэффективной жидкостной хроматографии с тандемным масс-селективным детектированием (ВЭЖХ-МС/МС) для последующего проведения фармакокинетического исследования.

**Материалы и методы.** Определение апиксабана в плазме крови человека проводили с помощью ВЭЖХ-МС/МС, используя ривароксабан как внутренний стандарт. Пробоподготовка проводилась путем осаждения белков плазмы крови ацетонитрилом. Подвижная фаза: 0,1%-й раствор муравьиной кислоты в воде (элюент А); 0,1%-й раствор муравьиной кислоты в ацетонитриле (элюент В). Время анализа: 3,00 мин. Колонка: Shim-pack Velox Biphenyl; 2,7 мкм; 50 × 2,1 мм. Источник ионизации: электроспрей с положительным режимом ионизации. MRM-переходы: 460,15 → 443,10 m/z (апиксабан); 436,05 → 144,95 m/z (ривароксабан).

**Результаты и обсуждение.** Валидация проводилась согласно действующим требованиям ЕАЭС по следующим валидационным параметрам: селективность, калибровочная кривая, точность и прецизионность, нижний предел количественного определения, пригодность стандартных образцов, эффект матрицы, степень извлечения, стабильность, перенос пробы, отсутствие влияния разбавления образцов. Результаты оценки всех параметров соответствовали установленным критериям приемлемости.

**Заключение.** Подтвержденный аналитический диапазон разработанной и валидированной методики составил 1,00–300,00 нг/мл в плазме крови. Методика определения апиксабана в плазме крови является простой и чувствительной. Данная методика была апробирована во время аналитической части исследования биоэквивалентности и может применяться для других фармакокинетических исследований препаратов апиксабана.

**Ключевые слова:** антикоагулянты, НОАК, апиксабан, ВЭЖХ-МС/МС, валидация, COVID-19, фармакокинетика

**Конфликт интересов.** Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

**Вклад авторов.** Т. Н. Комаров, О. А. Арчакова, П. К. Карнакова, У. Д. Филонова участвовали в разработке и валидации биоаналитической методики, а также в проведении аналитической части исследования. К. К. Карнакова проводила статистическую обработку данных. М. О. Попова, А. А. Попова отвечали за качество данных и организацию системы менеджмента качества. И. Е. Шохин отвечал за организационную часть исследования. Все вышеуказанные авторы участвовали в обсуждении полученных результатов в форме научной дискуссии.

**Для цитирования:** Филонова У. Д., Карнакова П. К., Карнакова К. К., Попова М. О., Попова А. А., Арчакова О. А., Комаров Т. Н., Шохин И. Е. Разработка и валидация методики количественного определения апиксабана в плазме крови с помощью метода ВЭЖХ-МС/МС. *Разработка и регистрация лекарственных средств.* 2024;13(1):224–240. https://doi.org/10.33380/2305-2066-2024-13-1-1684

## INTRODUCTION

Currently, anticoagulants are widely used in clinical practice – drugs that block plasma hemostasis reactions. Warfarin and other vitamin K antagonists are highly effective oral *anticoagulants*, but their administration is limited by a narrow therapeutic window, interaction with other drugs and foods, as well as the need for constant laboratory control and monitoring of the calculated value of the international normalized ratio [1, 2]. To overcome these limitations, the development of new oral anticoagulants (NOACs) began in 2004: apixaban, rivaroxaban, dabigatran etexilate, and edoxaban [3]. These drugs are not inferior in efficacy to warfarin, reduce overall mortality, are highly safe, and also allow patients to receive fixed doses without routine therapeutic monitoring [2, 4, 5].

Despite the fact that warfarin, registered in the Russian Federation in 2001, is still the most commonly used anticoagulant and is quite widely represented in the pharmaceutical market [6], recently NOACs have also become widespread in the Russian Federation, and there has been an increase in their prescriptions to patients. At the end of 2018, rivaroxaban took the leading position among the NOACs in the domestic pharmaceutical market, however, given the dynamics of growth in apixaban sales, it is expected that in the near future this drug may rise to first place [7]

According to a pharmacoeconomic analysis conducted in 2022, the use of apixaban for the prevention of ischemic stroke or systemic embolism in patients with nonvalvular atrial fibrillation is more cost-effective and requires less cost from the healthcare system compared to other NOACs [8]. The use of apixaban is also of great relevance due to its prescription to patients diagnosed with the coronavirus infection [Coronavirus Disease 2019 (COVID-19)] caused by the SARS-CoV-2 virus (Severe acute respiratory syndrome-related coronavirus 2) [9]. Although the SARS-CoV-2 virus primarily affects the respiratory system, in many patients the virus causes cardiovascular damage and hypercoagulability, manifested in most cases by venous thromboembolism, including pulmonary embolism (PE) and deep vein thrombosis (DVT) [10]. NOACs are included in the WHO guideline "Clinical management of COVID-19: Living guideline"[1], as well as in the guidelines of the Ministry of Health of the Russian Federation for the prevention, diagnosis and treatment of a new coronavirus infection[2] as antithrombotic therapy. According to the available data, anticoagulant therapy in patients with COVID-19 improves clinical outcomes and has a beneficial effect on the disease course [11–13].

***Apixaban*** (1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidine-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide) is a structurally novel neutral bicyclic pyrazole. It is a direct factor Xa inhibitor, which inhibits both free and bound factor Xa and does not require the presence of antithrombin III to manifest antithrombotic activity. By inhibiting factor Xa, apixaban reduces thrombin formation and blood clots, not affecting directly platelet aggregation, but inhibiting indirectly thrombin-induced platelet aggregation [14, 15].

Apixaban is approved in many countries for clinical use in a number of thromboembolic diseases, is used to reduce the risk of stroke in nonvalvular atrial fibrillation, thromboprophylaxis after hip or knee replacement, and the treatment of DVT or PE [16]. The results of key clinical trials have demonstrated that apixaban is an important alternative to existing anticoagulant drugs, such as vitamin K antagonists, such as warfarin, and low-molecular-weight heparins, with an improved benefit-to-bleeding risk ratio [16–18].

Thus, apixaban is a modern drug with high efficacy and proven safety [19–23]. In this regard, in order to conduct the analytical part of pharmacokinetic studies and bioequivalence studies of apixaban preparations, it is important to develop and validate a method for the quantitative determination of apixaban in blood plasma that would meet all our requirements.

This article presents the development and validation of a new sensitive and rapid method for the quantitative determination of apixaban in human blood plasma with the HPLC-MS/MS method using the gradient elution mode. This method is characterized not only by its short analysis duration and high sensitivity, but also has the simplest sample preparation method, which ensures optimal extraction of the active ingredient, and is easily accessible internal standard.

## MATERIALS AND METHODS

### *Reagents*

During the study, the following reagents were used: methanol (HPLC gradient grade, LLC TH "CHIMMED", Russia), acetonitrile (HPLC-S Gradient Grade, Biosolve Chimie, France; class chemically pure, LLC "Component-Reaktiv", Russia), formic acid (99–100 % grade, VWR Chemicals, France), demineralized water of class I (water purification system (demineralizer), Hydrolab R5, Poland).

### *Stock and working solutions*

Reference stock solution (RSS) of apixaban was prepared by dissolving the substance of apixaban reference sample (RS) (SynZeal Research Private Limited, India, assay of 100.00 %) in methanol until a concentration of 100 000.00 ng/ml was obtained in the solution. Working reference solutions of rivaroxaban internal standard (IS) was prepared by dissolving the weight of rivaroxaban reference stock solution (KINSY, S.L., Spain, assay of 100.00 %) in acetonitrile until the concentration in the solution reached 100 000.00 ng/ml. Reference stock solutions and working reference solutions of apixaban and rivaroxaban, as well as blank blood plasma samples, were stored in a freezer at a temperature of –42.5 ± 7.5 °C.

[1] Clinical management of COVID-19: living guideline, 18 August 2023. Geneva: World Health Organization; 2023 (WHO/2019-nCoV/clinical/2023.2). Licence: CC BY-NC-SA 3.0 IGO. Available at: https://www.who.int/publications/i/item/WHO-2019-nCoV-clinical-2023.2. Accessed: 20.12.2023.

[2] Timely methodological recommendations of the Ministry of Health of the Russian Federation. Prevention, diagnosis and treatment of new coronavirus infection (COVID-19). Version 18 (10/26/2023). Available at: https://static-0.minzdrav.gov.ru/system/attachments/attaches/000/064/610/original/%D0%92%D0%9C%D0%A0_COVID-19_V18.pdf. Accessed: 20.12.2023.

**Table 1.** **Concentrations of apixaban at calibration levels and quality control samples**

| Calibration levels | | Apixaban concentration, ng/mL | Rivaroxaban concentration, ng/mL |
|---|---|---|---|
| 1 | | 1.00 | 7500.00 |
| 2 | | 10.00 | 7500.00 |
| 3 | | 50.00 | 7500.00 |
| 4 | | 100.00 | 7500.00 |
| 5 | | 150.00 | 7500.00 |
| 6 | | 200.00 | 7500.00 |
| 7 | | 250.00 | 7500.00 |
| 8 | | 300.00 | 7500.00 |
| Quality control samples | | | |
| LLOQ | Lower limit of quantification (LLOQ) | 1.00 | 7500.00 |
| L | Low | 3.00 | 7500.00 |
| M1 | Middle 1 | 75.00 | 7500.00 |
| M2 | Middle 2 | 180.00 | 7500.00 |
| H | High | 240.00 | 7500.00 |

## *Sample preparation*

The sample preparation scheme is shown in Figure 1. 2-ml Eppendorf microcentrifuge tubes, chromatographic vials were used, which were placed in the chromatograph autosampler after sample preparation.

## *Chromatographic separation and detection*

For chromatographic separation, a high-performance liquid chromatograph Nexera XR (Shimadzu Corporation, Japan) was used. For mass spectrometry detection, a tandem mass-selective detector with a triple quadrupole LCMS-8040 (Shimadzu Corporation, Japan) was used. The chromatograph was equipped with a grading pump, a column and sample thermostat, a degasser, an autosampler, an automatic samples feeder into the autosampler, a high-pressure switching valve. Source data were processed using the LabSolutions software ver. 5.91 (Shimadzu Corporation, Japan).

For chromatographic separation, a Shim-pack Velox Biphenyl column, 2.7 μm, 50 × 2.1 mm and a Velox EXP Guard Biphenyl precolumn, 2.7 μm, 2.1 × 5.0 mm, were used. The following eluents were used as the mobile phase:

- Eluent A: 0.1 % solution of formic acid in water (v/v).
- Eluent B: 0.1 % solution of formic acid in acetonitrile (v/v).

The mobile phase composition gradient and its flow rate are shown in Table 2.

**Table 2.** **Mobile phase composition and flow rate**

| Time, min | Eluent A, % | Eluent B, % | Flow rate, mL/min |
|---|---|---|---|
| 0.00 | 80.00 | 20.00 | 1.00 |
| 0.45 | 20.00 | 80.00 | |
| 1.10 | 13.00 | 87.00 | |
| 1.20 | 0.00 | 100.00 | |
| 1.60 | 0.00 | 100.00 | |
| 1.61 | 80.00 | 20.00 | |
| 3.00 | 80.00 | 20.00 | |

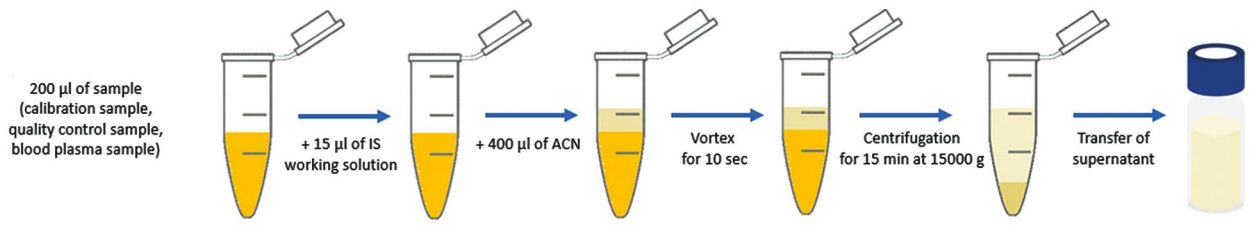


**Figure 1.** **Sample preparation**

The injection volume was 10 µl. Total run time: 0.00–3.00 min. Apixaban retention time was about 0.9 min, rivaroxaban retention time was about 0.9 min.

For mass spectrometry detection, an electrospray ionization (ESI) source was chosen. Ionization was carried out in a positive mode. Detection was carried out by monitoring MRM transitions (Multiple-Reaction Monitoring). The following MRM transitions were selected: 460.15 → 443.10 m/z (apixaban), 436.05 → 144.95 m/z (rivaroxaban). Parameters of the ionization source: drying gas 20 l/min, atomizing gas 3 l/min, desolvation line 250 °C, heating unit 400 °C, ESI needle voltage +4.9 kV.

## *Validation of the analytical method*

To validate the bioanalytical method, the rules for conducting bioequivalence drug studies within the Eurasian Economic Union[1], as well as the EMA[2] and FDA[3] guidelines, which included the validation parameters considered below, were taken as a basis.

### *Selectivity*

Six samples of blank blood plasma (BBP), hyperlipidemic blank blood plasma (HLBBP), and hemolyzed blank blood plasma (HBBP) were analyzed. Samples at the LLOQ level for BBP, HLBBP and HBBP were also analyzed. The analyte and IS signal should not exceed 20 % of the analyte signal at the LLOQ level and 5 % of the IS signal.

### *Calibration curve*

The calibration curve included levels № 1–8 in the analytical concentration range of 1.00–300.00 ng/mL (see Table 1). Relative error values (E, %) for assessing accuracy should be in the range from –20 to 20 % for concentration at the LLOQ level and from –15 to 15 % for other points. At least 75 % of calibration samples in at least six different concentrations should meet this acceptance criterion.

### *Accuracy and precision*

Blood plasma samples corresponding to LLOQ, L, M1, M2, H levels were analyzed (see Table 1). The analysis was performed in five consecutive cycles of five sample injections for each level of analyte concentrations. The study was conducted during the first to fifth sequences, both within and between cycles.

The calculated values of relative error (E, %) for the assessment of accuracy should be in range from –20 to 20 % for concentration at the LLOQ level and from –15 to 15 % for other points. To assess precision, the RSD should not exceed 20 % for the concentration at the LLOQ level, 15 % for the concentrations of other points.

### *Lower limit of quantification*

Based on the calibration curve, accuracy and precision, the lower limit of the quantification of the method was estimated as the minimum concentration of the analyte in blood plasma, which can be quantified in the analytical range with RSD and E values not exceeding 20 %.

### *Suitability of Reference Sample*

The analysis on BBP samples was carried out with the addition of IS solution without analyte, for which the analyte signal should not exceed 20 % of the LLOQ signal, and the BBP sample with the addition of analyte № 8 working solution without IS, for which the IS signal should not exceed 5 % of the IS signal. Samples were also analyzed at the LLOQ level.

### *Matrix effect*

Six samples were analyzed with the addition of working reference solutions of the analyte and the IS reference solution without the influence of the biological matrix and six samples prepared on BBP, HLBBP and HBBP, without taking into account the effect of recovery of the analytes and IS.

[1] Council of the Eurasian Economic Commission decision No. 85 dated November 3, 2016 "On approval of the Rules for conducting bioequivalence studies of medicinal products within the framework of the Eurasian Economic Union". Available at: https://docs.cntd.ru/document/456026107/ Accessed: 20.12.2023.

[2] European Medicines Agency. Available at: https://www.ema.europa.eu/en/bioanalytical-method-validation/ Accessed: 20.12.2023.

[3] Food and Drug Administration. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry/ Accessed: 20.12.2023.

*Recovery*

Three samples were analyzed each for levels L, M1, M2 and H prepared from BBP, HLBBP and HBBP without the effect of the recovery rate, and three corresponding samples each with the effect of the recovery rate. The RSD of the recovery rate of the analyte from biological matrices should not exceed 15 %.

*Stability*

Five samples were analyzed to assess each type of stability at the L and H levels. The obtained values of relative error should fit into the range of values from –15 to 15 %.

*Sample transfer*

Calibration sample № 8 with the maximum concentration and blank blood plasma samples were consequently analyzed. The analyte signal in blank blood plasma samples should not exceed 20 % of the signal at the LLOQ level, the IS signal of the sample should not exceed 5 % of the IS signal.

*Absence of effect of sample dilution*

Blood plasma samples were analyzed with two-fold dilution with blank blood plasma to obtain concentrations corresponding to the levels L and H. For the concentrations obtained, the RSD and E values should not exceed 15 %.

## RESULTS AND DISCUSSION

### *Development of the method*

A review of the literature data shows that there are several methods for the determination of apixabab using preferably high performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS)), as well as using high-performance liquid chromatography with diode-array detection (HPLC-DAD), high performance liquid chromatography with UV-detection (HPLC-UV)] and ultra-high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS)] (Table 3).

Labor intensive methods, such as solid-phase extraction (SPE) [26, 29] and liquid-liquid extraction (LLE) [27], were often used as sample preparation to improve the extraction of the analyte from biological matrices, and these methods had a rather low sensitivity. Some methods used a faster and simpler method of sample preparation by precipitating plasma proteins with various organic solvents, methanol or acetonitrile (see Table 3) [9, 24, 25, 28].

Based on the results of the analysis of the methods for apixaban quantification presented in the literature, we did not find any methods simultaneously with a simple method of sample preparation using the precipitation of blood plasma proteins with acetonitrile and high sensitivity, as well as a short analysis time and an available internal standard. Based on this, it was concluded that it was necessary to develop independently and validate a method that meets these requirements.

HPLC-MS/MS was selected as the preferred analysis method as one of the most sensitive and selective methods for the quantification of apixaban in human plasma. Rivaroxaban was selected as the internal standard for apixaban. This substance has a chemical structure similar to apixaban (Figure 2) and similar physical and chemical characteristics (Table 4).

When developing the method, such conditions of mass spectrometry detection were selected, which make it possible to obtain the peaks of apixaban and rivaroxaban with the highest intensity. An analysis of daughter ions obtained at different impact energies was carried out. For each of the substances, several MRM transitions were obtained, then the most intense MRM transitions were selected: 460.15 → 443.10 m/z (apixaban); 436.05 → 144.95 m/z (rivaroxaban). The highest intensity was obtained at an ESI needle voltage of +4.9 kV. Auto-optimization and voltage matching on the desolvation line and pre-quadrupole of the Q-array helped to increase the intensity of the signals by several times.

Chromatographic separation conditions were used to obtain apixaban and rivaroxaban peaks with optimal chromatographic parameters. For chromatographic separation, a Shim-pack Velox Biphenyl column (2.7 μm; 50 × 2.1 mm) was selected. 0.1 % solution of formic acid in water (eluent A, v/v) and a 0.1 % solution of formic acid in acetonitrile (eluent B, v/v) were selected as eluents of the mobile phase. Owing to the selected mobile phase gradient and flow velocity, it was possible to achieve a peak shape close to the shape of the Gaussian curve.

**Table 3.** **Bioanalytical methods of quantitative determination of apixaban**

| Analytical method | Ionization source; ionization (+/-) if applicable | Analysed sample | Sample preparation | Internal standard | Analytical range | Time of analysis, min | Reference |
|---|---|---|---|---|---|---|---|
| HPLC-MS/MS | Electrospray; + | Human plasma | Protein precipitation with acetonitrile | Carbamazepine | 3.00–1000.00 ng/mL | 5.5 | [9] |
| HPLC-MS/MS | Electrospray; + | Human plasma | Protein precipitation with methanol | Apixaban-d3 | 1.00–500.00 ng/mL | 3 | [24] |
| HPLC-MS/MS | Electrospray; + | Human serum | Protein precipitation with methanol | Promethazine | 1.00–1000.00 ng/mL | 4.5 | [25] |
| HPLC-DAD | – | Human plasma | Solid phase extraction | Chloramphenicol | 17.00–5280.00 ng/mL | 6 | [26] |
| HPLC-UV | – | Human plasma | Liquid-liquid extraction | Rivaroxaban | 10.00–500.00 ng/mL | 7.6 | [27] |
| UHPLC-MS/MS | Electrospray; + | Human plasma | Protein precipitation with acetonitrile with 1 % formic acid | Apixaban-$^{13}Cd_7$ | 5.00–800.00 nmol/L | 3 | [28] |
| HPLC-MS/MS | Electrospray; + | Human plasma | Solid phase extraction | Apixaban-$^{13}CD_3$ | 1.00–500.00 ng/mL | 70 | [29] |

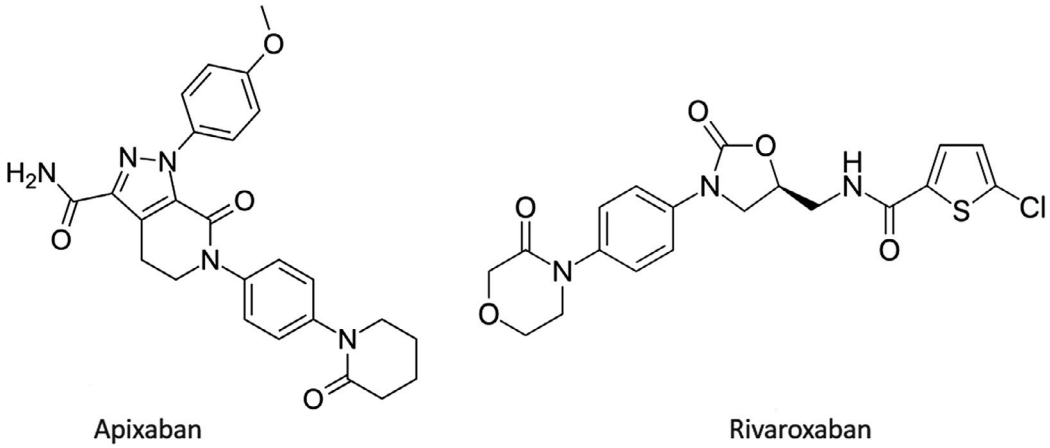


**Figure 2. Molecular formulas of apixaban and rivaroxaban**

**Table 4. Chemical and physical characteristics of apixaban and rivaroxaban**

| | pKa | logP | Molecular weight |
|---|---|---|---|
| Apixaban[1] | 13.07 | 2.22 | 459.497 |
| Rivaroxaban[2] | 13.60 | 1.74 | 435.881 |

**Note.** [1] Apixaban. Drugbank. Available at: https://go.drugbank.com/drugs/DB06605. Accessed: 20.12.2023.

[2] Rivaroxaban. Drugbank. Available at: https://go.drugbank.com/drugs/DB06228. Accessed: 20.12.2023.

For the extraction of apixaban from blood plasma, the simplest method of sample preparation was selected: precipitation of plasma proteins with acetonitrile in a ratio of 1:2. The selection of acetonitrile as a precipitant is justified by the fact that when using it, it is possible to achieve the most complete precipitation of blood plasma proteins and obtain the optimal shape of chromatographic peaks. As well, acetonitrile as a precipitant is superior to methanol, since its use does not require special conditions for storing samples in the chromatograph autosampler, which makes it possible to increase the period of "post-preparative" stability.

## *Validation of the method*

### *Selectivity*

Apixaban and rivaroxaban signals on blood plasma samples did not exceed 20 % of the LLOQ signal and 5 % of the IS signal, which meets the acceptance criteria. Figure 3 shows a chromatogram of a sample of blank human blood plasma.

### *Calibration curve*

Based on the data obtained, graphs of calibration curves were plotted in the coordinates "ratio of apixaban peak area to rivaroxaban peak area" and "ratio of apixaban concentration to rivaroxaban concentration in blood plasma". All calibration graphs were linear. A calibration graph of one of the cycles is shown in Figure 4. Equations of Gauge curves and the correlation coefficients (*r*) for calibration plots in validation cycles № 1–5 are shown in Table 5. The obtained correlation coefficients met the eligibility criteria and exceeded 0.99. The chromatogram of the calibration sample at level 8 (see Table 1) is shown in Figure 5.

**Table 5. Calibration equation and correlation coefficients**

| Cycle | Calibration equation | Correlation coefficient (*r*) |
|---|---|---|
| 1 | $y = 54.0977 \cdot x + 0.00293715$ | 0.9983996 |
| 2 | $y = 54.0349 \cdot x + 0.00257416$ | 0.9990636 |
| 3 | $y = 53.3817 \cdot x + 0.00257242$ | 0.9985084 |
| 4 | $y = 44.1182 \cdot x + 0.000185783$ | 0.9978233 |
| 5 | $y = 39.1754 \cdot x + 0.00152476$ | 0.9966636 |

### *Accuracy and precision*

The calculated values of relative standard deviation and relative error for the evaluation of precision and accuracy meet the acceptance criteria. Data on accuracy and precision between validation cycles № 1–5 are presented in Table 6.

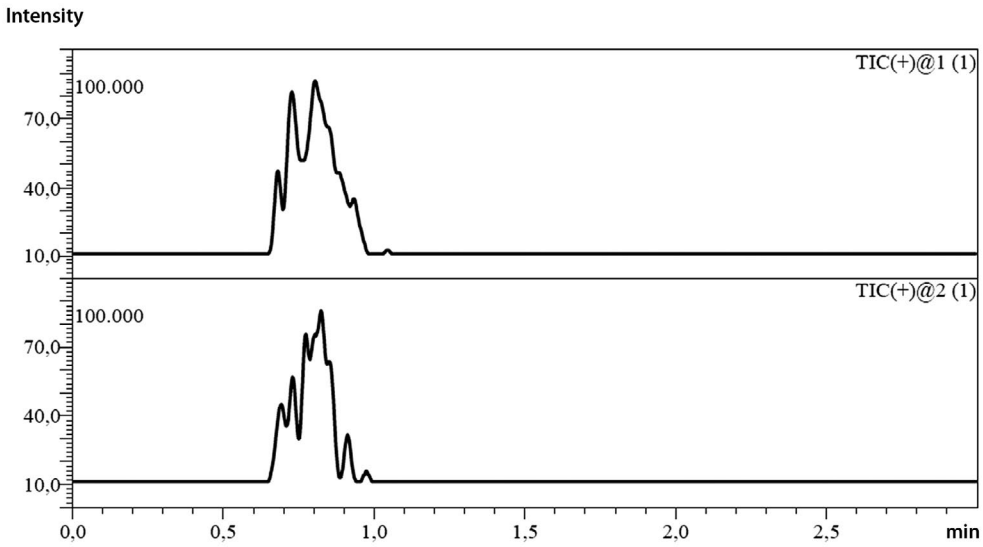


**Figure 3.** **Chromatogram of blank human plasma sample**

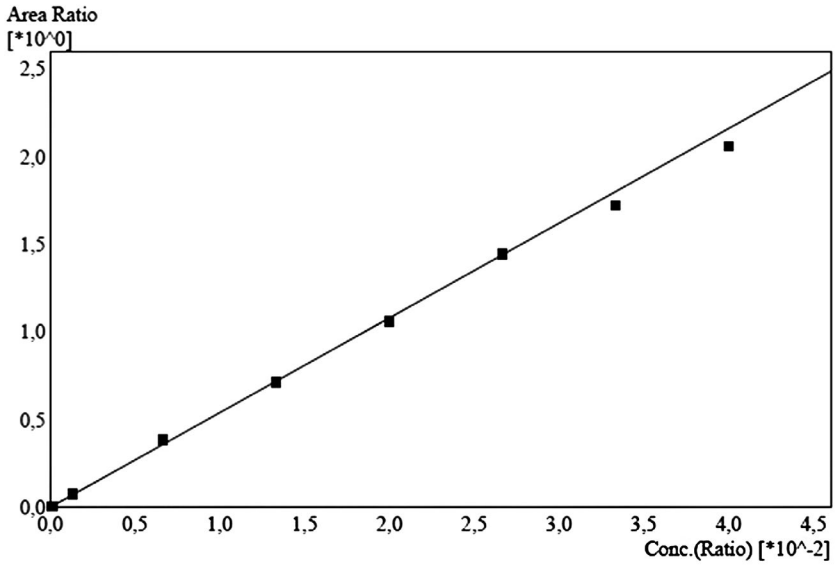


**Figure 4.** **Calibration curve**

**Table 6.** **Intra-day accuracy and precision in cycles № 1–5 (*n* = 25)**

| Sample | Injected (ng/mL) | Average | RSD, % | E, % |
|---|---|---|---|---|
| LLOQ | 1.00 | 0.97 | 12.39 | –3.44 |
| L | 3.00 | 2.96 | 7.02 | –1.31 |
| M1 | 75.00 | 75.78 | 5.33 | 1.05 |
| M2 | 180.00 | 185.02 | 8.61 | 2.79 |
| H | 240.00 | 240.20 | 6.99 | 0.08 |

*Lower limit of quantification*

Figure 6 shows a chromatogram of blood plasma containing apixaban at the level of the lower limit of quantitative determination, which was 1.00 ng/ml.

*Suitability of RS*

On the analyzed sample, prepared without the addition of an analyte solution, the apixaban signal did not exceed 20 % of the LLOQ signal. On the sample prepared without the IS, the rivaroxaban signal did not

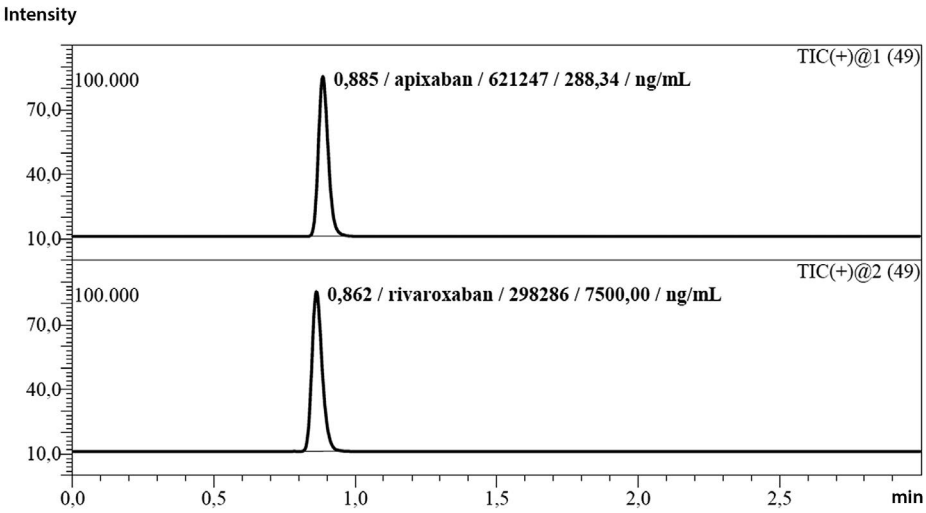


**Figure 5.** **Chromatogram of plasma sample at level 8**

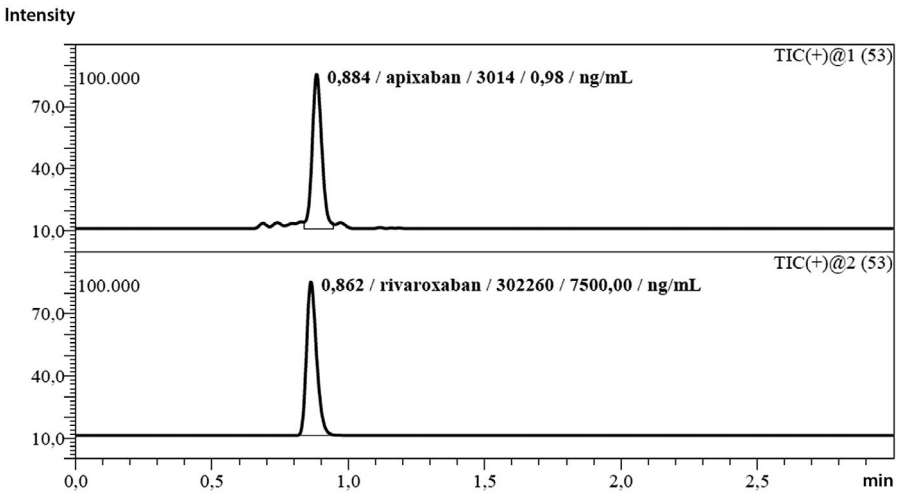


**Figure 6.** **Chromatogram of LLOQ plasma sample**

exceed 5 % of the IS signal. The absence of the influence of the internal standard and its impurities on the analyte was confirmed.

*Matrix effect*

The effect of the matrix was assessed at levels L and H. The results of this assessment are shown in Table 7. For rivaroxaban IS, the effect of the matrix was determined at the level of 7500.00 ng/ml. For the calculated values of the matrix factor normalized by IS, RSD was determined, the value of which did not exceed 15 %.

*Recovery*

The relative standard deviation of the estimated api-xaban recovery rate was 8.14 %, which does not exceed 15 % and meets the eligibility criteria. The data are presented in Table 8.

*Stability*

The stability of apixaban was assessed under different conditions, the results of which are presented in Table 9. The obtained values of relative error meet the acceptance criteria.

**Table 7.** **Calculation of IS-normalized matrix factors**

| Level | L | | | H | | |
|---|---|---|---|---|---|---|
| **Biological matrix** | **Blank blood plasma** | **Hemolyzed blank blood plasma** | **Hyperlipidemic blank blood plasma** | **Blank blood plasma** | **Hemolyzed blank blood plasma** | **Hyperlipidemic blank blood plasma** |
| Normalized Mf | 0.90 | 0.86 | 0.91 | 0.87 | 0.79 | 0.83 |
| | 0.76 | 0.82 | 0.80 | 0.88 | 0.82 | 0.79 |
| | 0.85 | 1.01 | 0.94 | 0.84 | 0.84 | 0.83 |
| | 0.75 | 0.77 | 0.75 | 0.76 | 0.82 | 0.81 |
| | 0.74 | 0.71 | 0.80 | 0.82 | 0.82 | 0.81 |
| | 0.82 | 0.83 | 0.78 | 0.78 | 0.90 | 0.87 |
| Average | 0.82 | | | 0.83 | | |
| RSD, % | 9.53 | | | 4.44 | | |

**Table 8.** **Calculation of apixaban recovery**

| Sample | | Biological matrix | | |
|---|---|---|---|---|
| | | **Blank blood plasma** | **Hemolyzed blank blood plasma** | **Hyperlipidemic blank blood plasma** |
| Recovery of apixaban, % | L | 108.38 | 99.19 | 90.80 |
| | | 108.92 | 86.24 | 100.89 |
| | | 102.84 | 82.13 | 89.64 |
| | M1 | 73.05 | 91.58 | 92.09 |
| | | 79.31 | 82.85 | 93.30 |
| | | 83.68 | 80.99 | 96.79 |
| | M2 | 90.54 | 86.92 | 96.79 |
| | | 88.99 | 95.55 | 91.43 |
| | | 91.68 | 94.44 | 94.38 |
| | H | 87.53 | 90.01 | 91.08 |
| | | 87.54 | 89,04 | 95.25 |
| | | 87.20 | 89.42 | 95.45 |
| Average | | 91.28 | | |
| RSD, % | | 8.14 | | |

*Sample transfer*

On blank blood plasma samples, the apixaban signal did not exceed 20 % of the LLOQ signal, the IS signal did not exceed 5 % of the IS signal. The results of the sample transfer assessment are presented in Table 10.

*Absence of effect of sample dilution*

The obtained RSD values for the precision assessment and E for the accuracy assessment met the acceptance criteria. If apixaban concentrations exceeding the values of the highest limit of quantitative determination are obtained within the analytical stage, it is possible to dilute the samples by 2 times. The data are shown in Table 11.

## *Use of the developed method*

The developed and validated procedure can be used for pharmacokinetic studies of apixaban preparations. Based on this method, the analytical stage of the bioequivalence study of the new national drug apixaban was successfully carried out. A representative chromatogram of a blood plasma sample from a volunteer participating in this study is shown in Figure 7.

**Table 9.** **Stability assessment of apixaban**

| Type of stability | | Bench-top | Postpreparative | Freeze-thaw | Stock solution | Work solution | Long-term 1 | | Long-term 2 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Time and storage conditions | | Analyzed freshly prepared; stored at 20 ± 5 °C | Analyzed stored in a chromatograph autosampler for at least 31 hours at 5 ± 3 °C | Analyzed after 5 freeze-thaw cycles: at least 60 hours at –42,5 ± 7,5 °C (freeze) and at least 10 hours at 20 ± 5 °C (thaw) | Analyzed after storage for 67 days at –42,5 ± 7,5 °C | | Analyzed at levels L and H when stored for 67 days | | Analyzed at levels L and H when stored for 95 days | |
| | | | | | | | at –20 ± 5 °C | at –75 ± 10 °C | at –20 ± 5 °C | at –75 ± 10 °C |
| Average | L | 2.74 | 2.79 | 3.22 | 3.41 | 3.40 | 3.40 | 3.41 | 3.07 | 2.70 |
| | H | 233.39 | 222.17 | 258.46 | 262.63 | 271.64 | 269.61 | 269.55 | 230.17 | 249.81 |
| E, % | L | –8.60 | –7.00 | 7.47 | 13.80 | 13.47 | 13.20 | 13.60 | 2.20 | –9.87 |
| | H | –2.75 | –7.43 | 7.69 | 9.43 | 13.19 | 12.34 | 12.31 | –4.10 | 4.09 |

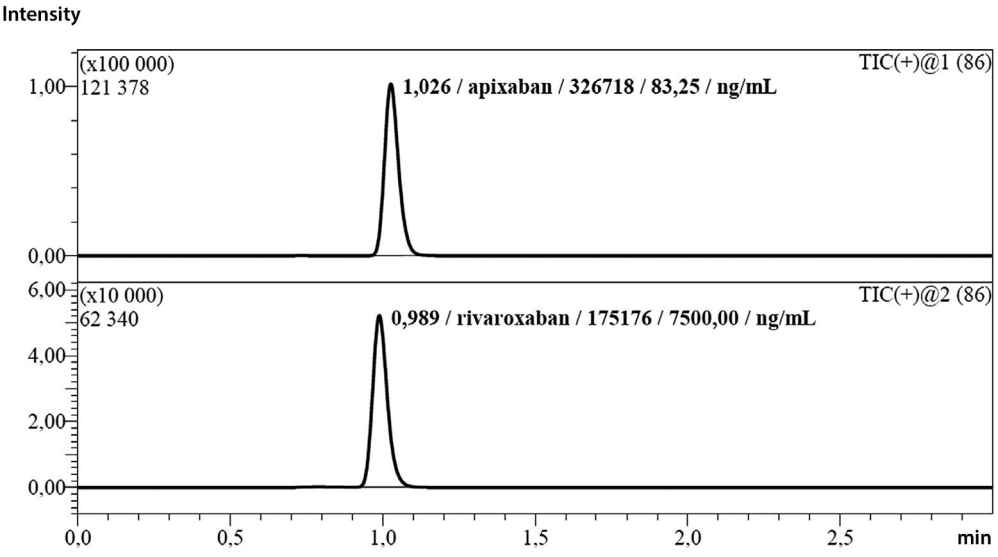


**Figure 7.** **Chromatogram of volunteer plasma sample**

**Table 10.** **Assessment of carry-over effect**

| № | Sample | Area value | | Area ratio, % | |
|---|---|---|---|---|---|
| | | Apixaban | Rivaroxaban | Apixaban | Rivaroxaban |
| 1 | LLOQ | 2733 | 285441 | – | – |
| | Blank blood plasma | 0 | 270 | 0.00 | 0.09 |
| 2 | LLOQ | 2356 | 252589 | – | – |
| | Blank blood plasma | 0 | 0 | 0.00 | 0.00 |
| 3 | LLOQ | 2431 | 276251 | – | – |
| | Blank blood plasma | 0 | 0 | 0.00 | 0.00 |

**Table 11.** **Inter-day accuracy and precision of sample dilution**

| Sample | Injected (ng/mL) | Dilution | Average | SD, % | RSD, % | E, % |
|---|---|---|---|---|---|---|
| L | 3.00 | 1:1 | 2.85 | 0.33 | 0.11 | –5.19 |
| H | 240.00 | 1:1 | 212.93 | 2.59 | 0.01 | –12.72 |

After taking the study drug and the comparison drug, individual profiles of changes in the values of apixaban concentrations in human plasma over time were recorded. These profiles were characterized by maximum drug concentration ($C_{max}$), area under "the concentration – time" curve from the moment of drug administration to the last detectable concentration at the time point *t* ($AUC_{(0-t)}$), area under "the concentration – time" curve from the moment of drug administration to infinity ($AUC_{(0-\infty)}$).

**Table 12.** **Values of pharmacokinetic parameters of apixaban**

| Pharmacokinetic parameters | Test drug | | | | Reference drug | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean | Geom Mean | SD | CV, % | Mean | Geom Mean | SD | CV, % |
| $AUC_{(0-t)}$, ng · h/mL | 2190.19 | 2097.50 | 592.65 | 27.06 | 2065.97 | 1976.97 | 563.69 | 27.28 |
| $AUC_{(0-\infty)}$, ng · h/mL | 2220.23 | 2129.65 | 589.84 | 26.57 | 2107.05 | 2021.57 | 561.91 | 26.67 |
| $C_{max}$, ng/mL | 265.35 | 254.48 | 71.82 | 27.07 | 235.82 | 226.40 | 63.40 | 26.89 |

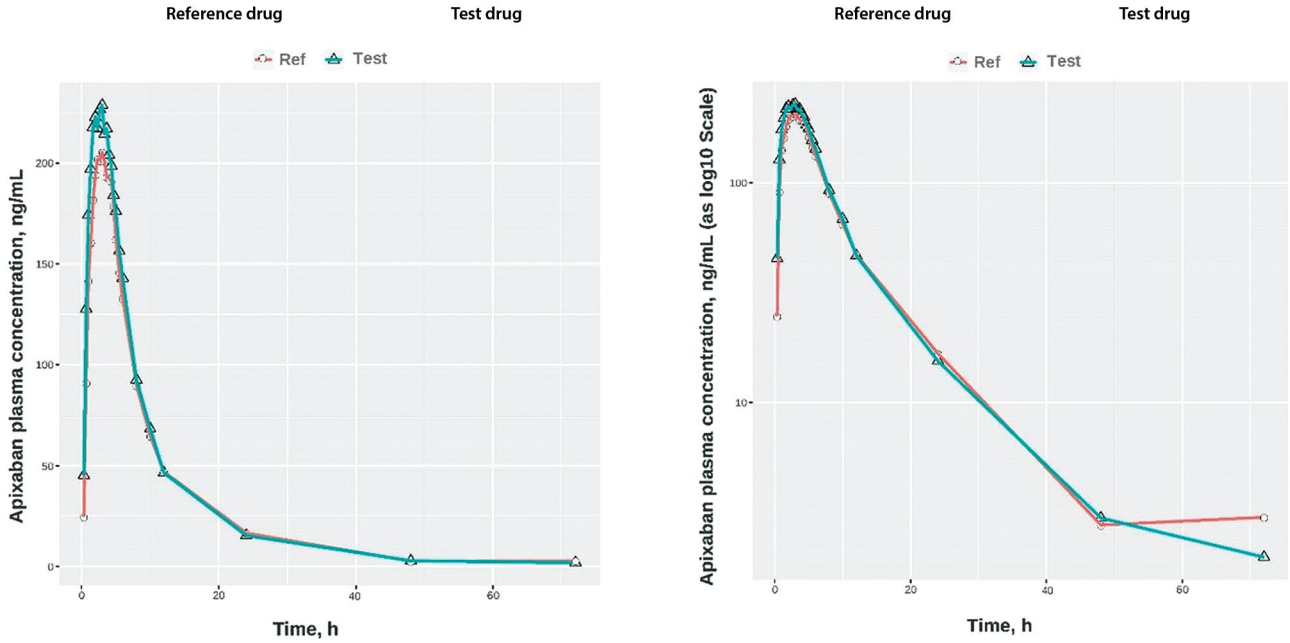


**Figure 8.** **Average pharmacokinetic profiles of apixaban (in linear and log-linear scale)**

The values of pharmacokinetic parameters, including the arithmetic mean (Mean) and geometric mean (Geom Mean), as well as the values of standard deviations (SD) and coefficients of variation (CV, %) are given in Table 12.

The time-averaged pharmacokinetic profiles of the test drug (Test) and the reference drug (Ref.) are shown in Figures 8 and 9. These profiles are presented in linear and loglinear transformation: in Figure 8 without standard deviations, in Figure 9 with standard deviations.

## CONCLUSION

A sensitive method for the quantification of apixaban in human blood plasma was developed and validated using the method of HPLC-MS/MS with a confirmed analytical range of 1.00–300.00 ng/ml. This method is characterized by a quick and simple way of sample preparation, a short analysis time, which significantly accelerates the study, as well as the use of an easily available substance as an internal standard. This me-

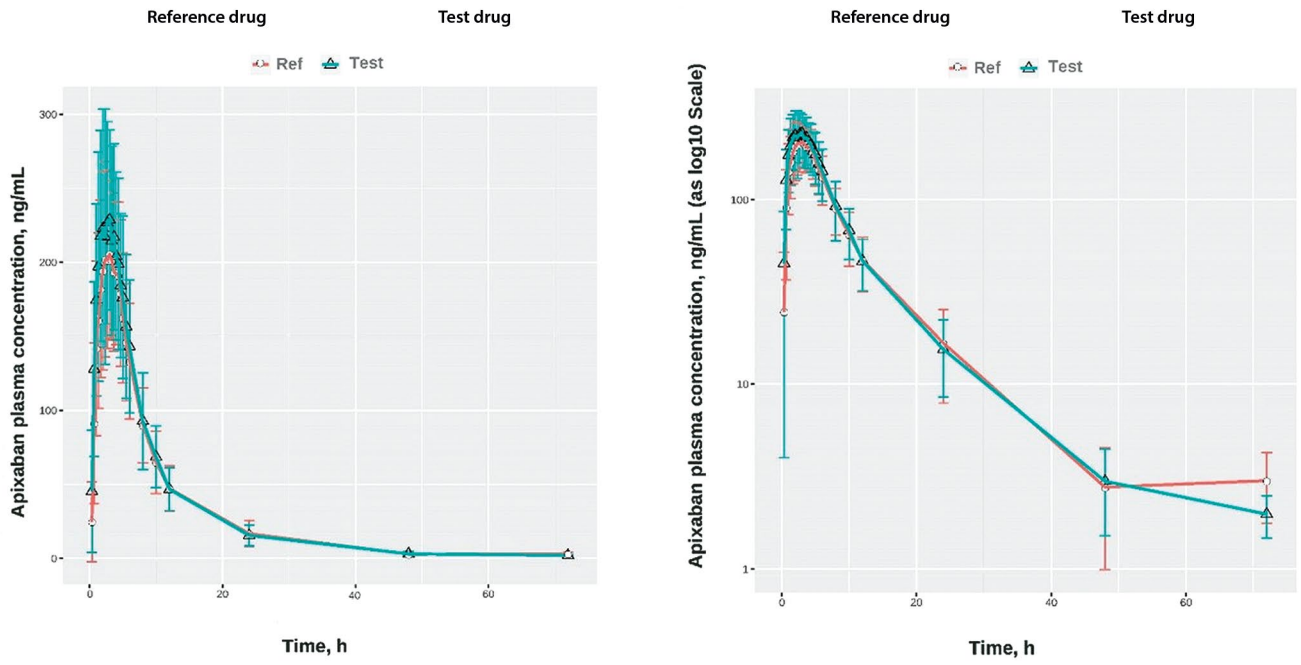


**Figure 9. Average pharmacokinetic profiles of apixaban (in linear and log-linear scale with standard deviations)**

thod has been successfully tested during the analytical part of the bioequivalence study of apixaban and can be used for other pharmacokinetic studies of apixaban preparations.

## REFERENCES

1. Vorobyeva N. M., Tkachyova O. N. Anticoagulant therapy in elderly patients: dilemma of choice between efficacy and safety. *Medical alphabet*. 2018;2(16):6–14. (In Russ.)
2. Panchenko E. P. Antithrombotic therapy of coronary heart disease: time-tested essential principles. *Therapeutic Archive*. 2019;91(6):19–24. (In Russ.) DOI: 10.26442/00403660.2019.06.000296.
3. Bokarev I. N., Kondrat'eva T. B. New anticoagulants in clinical practice. Effectiveness and problems. *Clinical Medicine*. 2016;94(5):383–387. (In Russ.) DOI: 10.18821/0023-2149-2016-94-5-383-387.
4. Van Rein N., Heide-Jørgensen U., Lijfering W. M., Dekkers O. M., Sørensen H. T., Cannegieter S. C. Major bleeding rates in atrial fibrillation patients on single, dual, or triple antithrombotic therapy: results from a Nationwide Danish Cohort Study. *Circulation*. 2019;139(6):775–786. DOI: 10.1161/CIRCULATIONAHA.118.036248.
5. Cools F., Wollaert B., Vervoort G., Verstraete S., Voet J., Hermans K., Heyse A., De Wolf A., Hollanders G., Boussy T., Anné W., Vercammen J., Faes D., Beutels M., Mairesse G., Purnode Ph., Blankoff I., Vandergoten P., Capiau L., Allu J., Bassand J., Kayani G. Treatment patterns in anticoagulant therapy in patients with newly diagnosed atrial fibrillation in Belgium: results from the GARFIELD-AF registry. *Acta Cardiologica*. 2018;74(4):309–318. DOI: 10.1080/00015385.2018.1494089.
6. Melekhov A. V., Gendlin G. E., Dadashova E. F., Alekseeva E. M., Zaigraev I. A., Nikitin I. G. The dynamics of application of anti-thrombotic in patients fibrillation of atriums: original data and review of national registers. *Medical Journal of the Russian Federation*. 2017;23(3):116–126. (In Russ.) DOI: 10.18821/0869-2106-2017-23-3-116-126.
7. Gruzdeva A. A., Khokhlov A. L., Ilyin M. V. Oral anticoagulants in the prevention of thromboembolic complications in cardiac patients: analysis of use in the Russian Federation. *Good Clinical Practice*. 2020;1:69–79. (In Russ) DOI: 10.37489/2588-0519-2020-1-69-79.
8. Kolbin A. S, Kasimova A. R, Gilyarevskiy S. R. Comparative pharmacoeconomic analysis of the use of apixaban, rivaroxaban and dabigatran for prevention of stroke and embolism in the vessels of the systemic circulation in patients with non-valvular atrial fibrillation. *Good Clinical Practice*. 2022;(4):50–59. (In Russ). DOI: 10.37489/2588-0519-2022-4-50-59.
9. Dzudovic J., Sakac M. C., Antunovic M., Repic A., Obradovic S., Djordjevic S., Dzudovic B. Development and validation of LC-MS/MS method for determination of plasma apixaban. *Acta Chromatographica*. 2022;34(3):332–337. DOI: 10.1556/1326.2021.00948.

10. Levi M., Thachil J., Iba T., Levy J. H. Coagulation abnormalities and thrombosis in patients with COVID-19. *Lancet Haematol*. 2020;7:e438–e440. DOI: 10.1016/S2352-3026(20)30145-9.
11. Wenzler E., Engineer M. H., Yaqoob M., Benken S. T. Safety and efficacy of apixaban for therapeutic anticoagulation in critically Ill ICU patients with severe COVID-19 respiratory disease. *TH Open: Companion Journal to Thrombosis and Haemostasis*. 2020;4(04):e376–e382. DOI: 10.1055/s-0040-1720962.
12. Billett H. H., Reyes-Gil M., Szymanski J., Ikemura K., Stahl L. R., Lo Y., Rahman S., Gonzalez-Lugo J. D., Kushnir M., Barouqa M., Golestaneh L., Bellin E. Anticoagulation in COVID-19: effect of enoxaparin, heparin, and apixaban on mortality. *Thrombosis and haemostasis*. 2020;120(12):1691–1699. DOI: 10.1055/s-0040-1720978.
13. Appiah D., Quinn N. J., Messing E. G., Veltri K. T. Safety and efficacy analysis of apixaban compared to heparins in hospitalized non-critically ill COVID-19 patients. *Hospital Pharmacy*. 2022;57(6):727–733. DOI: 10.1177/00185787221095764.
14. Pinto D. J., Orwat M. J., Koch S., Rossi K. A., Alexander R. S., Smallwood A., Wong P. C., Rendina A. R., Luettgen J. M., Knabb R. M., He K., Xin B., Wexler R. R., Lam P. Y. Discovery of 1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-*c*]pyridine-3-carboxamide (Apixaban, BMS-562247), a highly potent, selective, efficacious, and orally bioavailable inhibitor of blood coagulation factor Xa. *Journal of medicinal chemistry*. 2007;50(22):5339–5356. DOI: 10.1021/jm070245n.
15. Luettgen J. M., Knabb R. M., He K., Pinto D. J., Rendina A. R. Apixaban inhibition of factor Xa: microscopic rate constants and inhibition mechanism in purified protein systems and in human plasma. *Journal of Enzyme Inhibition and Medicinal Chemistry*. 2011;26(4):514–526. DOI: 10.3109/14756366.2010.535793.
16. Byon W., Garonzik S., Boyd R. A., Frost C. E. Apixaban: a clinical pharmacokinetic and pharmacodynamic review. *Clinical pharmacokinetics*. 2019;58(10):1265–1279. DOI: 10.1007/s40262-019-00775-z.
17. Granger C. B, Alexander J. H, McMurray J. J. V, Lopes R. D., Hylek E. M., Hanna M., Al-Khalidi H. R., Ansell J., Atar D., Avezum A., Bahit M. C., Diaz R., Easton J. D., Ezekowitz J. A., Flaker G., Garcia D., Geraldes M., Gersh B. J., Golitsyn S., Goto S., Hermosillo A. G., Hohnloser S. H., Horowitz J., Mohan P., Jansky P., Lewis B. S., Lopez-Sendon J. L., Pais P., Parkhomenko A., Verheugt F. W. A., Zhu J., Wallentin L. Apixaban versus Warfarin in Patients with Atrial Fibrillation. *New England Journal of Medicine*. 2011;365(11):981–992. DOI: 10.1056/NEJMoa1107039.
18. Romanova M. V., Puzenko D. V. Aspects of anticoagulant therapy in venous thromboembolism, pulmonary embolism. Focus on elderly patients. *Russian Journal of Cardiology*. 2022;27(3):97–106. (In Russ.) DOI: 10.15829/1560-4071-2022-4944.
19. Hylek E. M., Held C., Alexander J. H., Lopes R. D., De Caterina R., Wojdyla D. M., Huber K., Jansky P., Steg Ph. G., Hanna M., Thomas L., Wallentin L., Granger C. B. Major bleeding in patients with atrial fibrillation receiving apixaban or warfarin: The ARISTOTLE Trial (Apixaban for Reduction in Stroke and Other Thromboembolic Events in Atrial Fibrillation): Predictors, Characteristics, and Clinical Outcomes. *Journal of the American College of Cardiology*. 2014;63(20):2141–2147. DOI: 10.1016/j.jacc.2014.02.549.
20. Avezum A., Lopes R. D., Schulte P. J., Lanas F., Gersh B. J., Hanna M., Pais P., Erol C., Diaz R., Bahit M. C., Bartunek J., De Caterina R., Goto S., Ruzyllo W., Zhu J., Granger C. B., Alexander J. H. Apixaban in comparison with warfarin in patients with atrial fibrillation and valvular heart disease: findings from the Apixaban for Reduction in Stroke and Other Thromboembolic Events in Atrial Fibrillation (ARISTOTLE) Trial. *Circulation*. 2015;132(8):624–632. DOI: 10.1161/CIRCULATIONAHA.114.014807.
21. Lip G. Y., Keshishian A., Li X., Hamilton M., Masseria C., Gupta K., Luo X., Mardekian J., Friend K., Nadkarni A., Pan X., Baser O., Deitelzweig S. Effectiveness and safety of oral anticoagulants among nonvalvular atrial fibrillation patients: the ARISTOPHANES study. *Stroke*. 2018;49(12):2933–2944. DOI: 10.1161/STROKEAHA.118.020232.
22. Fralick M., Colacci M., Schneeweiss S., Huybrechts K. F., Lin K. J., Gagne J. J. Effectiveness and safety of apixaban compared with rivaroxaban for patients with atrial fibrillation in routine practice: a cohort study. *Annals of internal medicine*. 2020;172(7):463–473. DOI: 10.7326/M19-2522.
23. Rutherford O. C. W., Jonasson C., Ghanima W., Söderdahl F., Halvorsen S. Comparison of dabigatran, rivaroxaban, and apixaban for effectiveness and safety in atrial fibrillation: a nationwide cohort study. *European Heart Journal-Cardiovascular Pharmacotherapy*. 2020;6(2):75–85. DOI: 10.1093/ehjcvp/pvz086.
24. Jeong H.-C., Kim T.-E., Shin K.-H. Quantification of apixaban in human plasma using ultra performance liquid chromatography coupled with tandem mass spectrometry. *Translational and Clinical Pharmacology*. 2019;27(1):33–41. DOI: 10.12793/tcp.2019.27.1.33.
25. Rodina T. A., Melnikov E. S., Aksenov A. A., Belkov S. A., Sokolov A. V., Prokof'ev A. B., Ramenskaya G. V. Simultaneous Determination of Dabigatran, Rivaroxaban, Apixaban, and Warfarin in Human Blood Serum by HPLC-MS/MS for Therapeutic Drug Monitoring. *Pharmaceutical Chemistry Journal*. 2022;56(2):289–293. DOI: 10.1007/s11094-022-02633-y.
26. Gouveia F., Bicker J., Santos J., Rocha M., Alves G., Falcão A., Fortuna A. Development, validation and application of a new

HPLC-DAD method for simultaneous quantification of apixaban, dabigatran, edoxaban and rivaroxaban in human plasma. *Journal of Pharmaceutical and Biomedical Analysis*. 2020;181:113109. DOI: 10.1016/j.jpba.2020.113109.

27. Cini M., Legnani C., Padrini R., Cosmi B., Dellanoce C., De Rosa G., Palareti G., Marcucci R., Pengo V., Poli D., Testa S. DOAC plasma levels measured by chromogenic anti-Xa assays and HPLC-UV in apixaban-and rivaroxaban-treated patients from the START-Register. *International Journal of Laboratory Hematology*. 2020;42(2):214–222. DOI: 10.1111/ijlh.13159.
28. Lindahl S., Dyrkorn R., Spigset O., Hegstad S. Quantification of apixaban, dabigatran, edoxaban, and rivaroxaban in human serum by UHPLC-MS/MS – method development, validation, and application. *Therapeutic drug monitoring*. 2018;40(3):369. DOI: 10.1097/FTD.0000000000000509.
29. Raghavan N., Frost C. E., Yu Z., He K., Zhang H., Humphreys W. G., Zhang D., Pinto D., Chen S., Bonacorsi S., Wong P. C. Apixaban metabolism and pharmacokinetics after oral administration to humans. *Drug metabolism and disposition*. 2009;37(1):74–81. DOI: 10.1124/dmd.108.023143.