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Development and validation of a high-performance liquid chromatography with tandem mass spectrometry method for quantification of tofacitinib in human plasma

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Abstract

Introduction. The use of tofacitinib as a pharmacological treatment for rheumatoid arthritis remains relevant in the light of the predicted increase in prevalence of the disease. At the same time, due to the withdrawal of several foreign pharmaceutical companies from the Russian pharmaceutical market, there has been a heightened demand for domestically produced medications, including generic formulations of tofacitinib. Registration of generic drugs necessitates the conduct of bioanalytical studies. Development and validation of a method for quantifying the analyte in biosamples remains to be a crucial part of the bioequivalence studies.

Aim. The aim of this research is to develop and validate a method for the quantitative determination of tofacitinib in human plasma using high-performance liquid chromatography as the separation system coupled with a tandem mass spectrometer for detection purposes.

Materials and methods. Biosample preparation was based on plasma proteins precipitation using acetonitrile. Baricitinib was selected as an internal standard. The analytical range of the method was 1.00 to 200.00 ng/mL and was further expanded to 0.30 to 200.00 ng/mL during the analytical phase of the study. The mobile phase consisted of water and acetonitrile, both acidified with formic acid (0.1 % v/v). The stationary phase was a Phenomenex Kinetex C_{18} column [100 × 3.0 mm, with a particle size of 5 μm (Phenomenex, USA)]. Sample separation and detection were carried out using high-performance liquid chromatography coupled with a tandem mass spectrometry (HPLC-MS/MS), operating in positive ion mode. The multiple reaction monitoring (MRM) transitions selected for the analyte and the internal standard were 313.30 to 173.00 m/z and 371.90 to 186.00 m/z, respectively.

Results and discussion. The developed assay was validated in accordance with the current requirements of regulatory documentation from the EAEU (Eurasian Economic Union), FDA (US Food and Drug Administration), and EMA (European Medicines Agency) with the following parameters being evaluated: selectivity, specificity, carry-over, matrix effect, recovery, calibration curve, lower limit of quantitation, accuracy, precision, stability. The validated method was applied in the analytical part of a bioequivalence study of domestically produced generic tofacitinib.

Conclusion. A method for the quantitative determination of tofacitinib in human blood plasma with an analytical range of 0.30–200.00 ng/mL was developed and validated. Application of the assay during the analytical phase of bioequivalence study of generic tofacitinib confirms the possibility of using the method in similar bioanalytical investigations.

Keywords: tofacitinib, validation, LC-MS/MS, bioequivalence, pharmacokinetics, bioanalytical study

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, Evgeniya S. Vetrova have participated in the development and validation of the bioanalytical method, as well as in conducting the analytical part of the study. Natalia S. Bagaeva, Kseniia K. Karnakova carried out statistical processing of the data. Mariia 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.

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Разработка и валидация методики количественного определения тофацитиниба в плазме крови человека методом высокоэффективной жидкостной хроматографии с тандемной масс-спектрометрией

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Резюме

Введение. Применение тофацитиниба в медикаментозном лечении ревматоидного артрита не теряет актуальности в связи с прогнозируемым ростом распространенности данного заболевания. Вместе с тем, в результате ухода многих зарубежных компаний с фармацевтического рынка России возник спрос на выпуск лекарственных препаратов отечественного производства, в том числе воспроизведенных препаратов тофацитиниба, для регистрации которых обязательно проведение биоаналитических исследований. Необходимой частью аналитического этапа изучения биоэквивалентности является разработка и валидация методики количественного определения анализируемого вещества.

Цель. Целью данного исследования является разработка и валидация методики количественного определения тофацитиниба в плазме крови человека с использованием высокоэффективной жидкостной хроматографии в качестве системы разделения и тандемного масс-спектрометра в качестве метода детектирования.

Материалы и методы. Пробоподготовка биообразцов была основана на осаждении белков плазмы крови ацетонитрилом. В качестве внутреннего стандарта был выбран барицитиниб. Аналитический диапазон методики составил 1,00–200,00 нг/мл и в дальнейшем при проведении аналитического этапа исследования был расширен до 0,30–200,00 нг/мл. Подвижная фаза включала воду и ацетонитрил, подкисленные муравьиной кислотой до значения 0,1 % об. Неподвижная фаза была представлена Phenomenex Kinetex C_{18}, 100 × 3,0 мм, 5 мкм (Phenomenex, США). Разделение и детектирование проб проводили в системе высокоэффективного жидкостного хроматографа с тандемным масс-спектрометрическим детектором (ВЭЖХ-МС/МС) в положительном режиме ионизации. Для аналита и внутреннего стандарта были подобраны следующие MRM-переходы: 313,30 → 173,00 m/z, 371,90 → 186,00 m/z соответственно.

Результаты и обсуждение. Разработанная методика была валидирована согласно действующим требованиям нормативной документации ЕАЭС, FDA, EMA по следующим параметрам: селективность, специфичность, эффект переноса, эффект матрицы, степень извлечения, градуировочная кривая, нижний предел количественного определения, точность, прецизионность, стабильность. Проведена апробация методики в рамках аналитического этапа исследования.

Заключение. Разработана и валидирована быстрая, нетрудоемкая и чувствительная методика количественного определения тофацитиниба в плазме крови человека с подтвержденным аналитическим диапазоном 0,30–200,00 нг/мл. Апробация методики при проведении аналитического этапа изучения биоэквивалентности препарата тофацитиниба подтверждает возможность использования методики в подобных биоаналитических исследованиях.

Ключевые слова: тофацитиниб, валидация, ВЭЖХ-МС/МС, биоэквивалентность, фармакокинетика, биоаналитические исследования

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, Е. С. Ветрова участвовали в разработке и валидации биоаналитической методики, а также в проведении аналитической части исследования. Н. С. Багаева, К. К. Карнакова проводили 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.

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INTRODUCTION

Rheumatoid arthritis is a type of autoimmune inflammatory rheumatic disease [1] that has seen an increase in global incidence over the past three decades by more than 120 %, reaching almost 18 million cases in 2020. According to estimates made by foreign researchers, the number of individuals suffering from various forms of this pathology, considering the prevalence of the condition and socio-demographic index, will exceed 25 million in the near future [2]. Despite the predicted increase in rheumatoid arthritis prevalence, there has been a

consistent reduction in disease severity over the last three decades. This is attributed to scientific discoveries in medicine that have allowed for changes in treatment approaches and improved patient support during the treatment process [3]. It is important to acknowledge that the risk group encompasses residents of industrialized nations, who are subject to certain controllable risk factors linked to lifestyle, environmental contamination, and other factors. Uncontrollable factors include gender (the incidence is higher in women), age (over sixty years)[1], and a genetic predisposition [3, 4]. Given these considerations, the issue of rheumatoid arthritis management remains pertinent today.

Tofacitinib is a drug that has been approved for use as a treatment of various diseases, including rheumatoid and psoriatic arthritis, as well as ulcerative colitis [1]. The efficacy of tofacitinib in treating these conditions is based on its ability to bind reversibly to the Janus kinase (JAK) site, thereby blocking the signaling pathways from cytokine receptors (such as those of the interleukin-6 (IL-6) family) and interferon to intracellular transcription factors [1, 4]. Rheumatoid and psoriatic arthritis involve inflammatory processes that activate these signaling pathways, leading to an increase in the levels of certain substances. For example, the concentration of interleukin-6 can increase from picogram levels in the healthy state to microgram levels during inflammation [5]. In addition to its effect on interferons, tofacitinib also inhibits the activity of T-lymphocytes. By affecting the transmission of interferon-mediated signals, it helps prevent the development of pathological changes in skin and bone tissue that are characteristic to rheumatoid and psoriatic arthritis [6]. Therefore, tofacitinib can be an effective and safe option for the treatment of these conditions in pharmacotherapy [1, 6].

Since the end of February 2022, several foreign pharmaceutical companies have withdrawn from the domestic market, along with the ceasing the international clinical trials and the reduction in the availability of raw materials and commodities essential for drug production in Russia[2]. Despite this, there has been an increase in demand for imported and locally produced drugs, with sales increasing by more than 20 % since October 2022[3]. Given this situation, it has become more significant to address the issue of expanding the market for domestic pharmaceutical products, as well as through the development and marketing of generic drugs. However, this process requires bioanalytical testing to ensure the safety and efficacy of these products.

The aim of this study was to develop and validate a rapid, non-labor-intensive, sufficiently sensitive method for the quantification of tofacitinib, using high-performance liquid chromatography (HPLC) with a tandem mass spectrometry (MS/MS) detector, based on literature data analysis to assess the feasibility of conducting a bioequivalence study.

MATERIALS AND METHODS

Reagents and Solutions

Acetonitrile (Biosolve Chimie, Netherlands) of «HPLC-S Gradient grade» purity was used to prepare the eluents. Mobile phase was composed of formic acid with a purity of 98 %, which was produced by PanReac, Germany and AppliChem, Spain. Additionally, demineralized water was used, which was obtained using the Hydrolab R5 water purification system from Poland. The use of ultrapure reagents is not required for sample preparation, so acetonitrile with a purity of «reagent grade» produced by Komponent-Reactiv, Russia was used as the precipitant.

The tofacitinib citrate reference standard with a purity of 100.40 %, used for the preparation of stock standard solutions (SSs), was provided by Metrochem Api Private Limited, India. The baricitinib reference standard (internal standard, IS) with a purity of 99.78 % was supplied by Shanghai Famo Biotechnology Co. Ltd., China. Quantities of these substances were transferred into 100.0 mL volumetric flask to prepare tofacitinib (TOF) SS and 50.00 mL volumetric flask to prepare baricitinib (BAR) SS. By dissolving 16.1 mg of tofacitinib citrate and 10.0 mg of baricitinib in appropriate volumes of pure methanol of «special purity grade» for gradient HPLC» (Himmed, Russia), solutions of TOF and BAR were prepared with concentrations of 100 000.00 ng/mL and 200 000.00 ng/mL, respectively. Analyte working standard solutions (WSs), applied for the preparation of calibration samples (CSs) and quality control samples (QCs), were obtained by transferring the appropriate volumes of TOF SS into volumetric flasks. The IS WS was prepared using the same methodology. The first level of CS concentrations corresponded to the lower limit of quantification (LLOQ), the last level of CS concentrations (8^{th} for validation cycles 1, 2, 3, 5; 9^{th} for validation cycle 4) corresponded to the upper limit of quantification (ULOQ). QCs included LLOQ, a low concentration level (L), three medium concentration levels (M1, M2, M3), and a high concentration level (H). CS and QC concentrations obtained by adding a specified volumes of WSs to blood plasma are presented in Table 1.

Eluents A and B for the mobile phase were prepared by combining 1000 µL of formic acid (FA) with 500 mL of solvent (demineralized water or acetonitrile, respectively), followed by adjusting the volume to 1000 mL.

[1] Rheumatoid arthritis. World Health Organization. Available at: https://www.who.int/ru/news-room/fact-sheets/detail/rheumatoid-arthritis. Accessed: 26.03.2024.

[2] Will we be left without medicinal products: what is happening to the Russian pharmaceutical market after the sanctions? Available at: https://fedpress.ru/article/3243884. Accessed: 26.03.2024.

[3] Pharmaceutical market in Russia: October 2023. DSM Group. Analytical reports. Available at: https://dsm.ru/docs/analytics/october23.pdf. Accessed: 26.03.2024.

Table 1. Concentration levels of calibration standards and quality control samples

Calibration standards for 1–3 validation cycles		1	2	3	4	5	6	7	8
Calibration standards for the 4th validation cycle	1	2	3	4	5	6	7	8	9
TOF concentration, ng/mL	0.30	1.00	2.50	5.00	10.00	25.00	50.00	100.00	200.00
BAR concentration, ng/mL	150.00								
Quality control samples	Number of the validation cycle								
	1, 2, 3, 5	4	1, 2, 3, 5	4	1–4				
	LLOQ	LLOQ	L	L	M1	M2	M3	H	
TOF concentration, ng/mL	1.00	0.30	3.00	0.90	6.00	30.00	120.00	150.00	
BAR concentration, ng/mL	150.00								

Procedure for processing plasma samples

Intact blood plasma (IBP), hemolytic intact blood plasma (HBP), and lipemic intact blood plasma (LBP) were used for validation purposes. The procedure for preparing plasma samples was carried out using a method that involves the precipitation of biological matrix proteins using acetonitrile (ACN). The first step of the sample preparation process involved transferring a specified amount of each matrix to Eppendorf tubes. The samples were then processed according to the steps described in Figure 1. After completing the sample preparation, chromatographic vials containing prepared samples were placed in the autosampler. Once the samples had reached the temperature set by the operator within the device's storage compartment, analysis could commence.

Apparatus and Chromatographic Conditions

The Nexera XR HPLC system (Shimadzu Corporation, Japan) was employed to separate substances in samples. The chromatographic system comprised the following components: a unit for the preparation and supply of a mobile phase with a specific composition, an autosampler integrated with an external sample loader (rack changer), a column module equipped with a thermostat, and a high-pressure switching valve.

The separation was achieved using a Phenomenex C_{18} pre-column (4 × 3.0 mm) and a Phenomenex Kinetex C_{18} analytical column (100 × 3.0 mm, 5 µm) (Phenomenex, USA). Target substances were eluted using a gradient mobile phase elution mode (GRAD) as shown in Figure 2, with a flow rate of 1 mL/min during the entire sample analysis period.

Mass spectrometric detection conditions

After elution, the substances were introduced into a Shimadzu LCMS-8040 triple quadrupole mass selective detector for electric field spray ionization (ESI) analysis. Detection parameters are summarized in Table 2 below.

LabSolutions software (Ver. 5.91) (Shimadzu Corporation, Japan) was used to process the raw data obtained from the detection results.

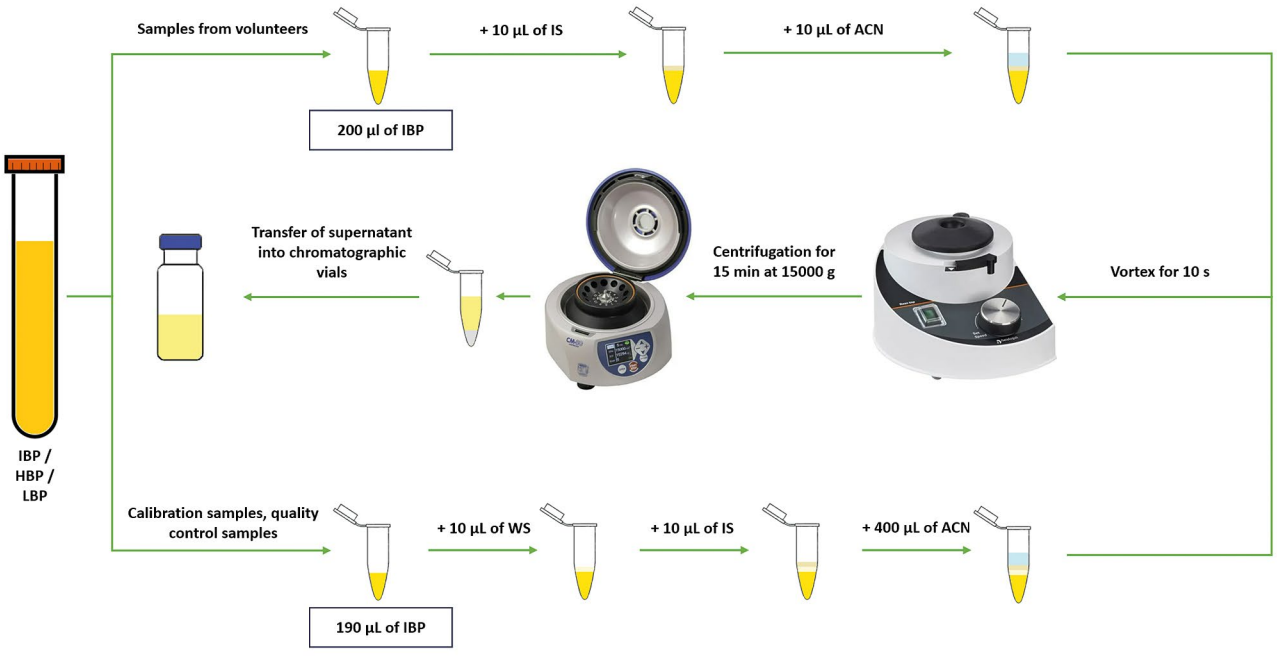


Figure 1. Sample preparation scheme

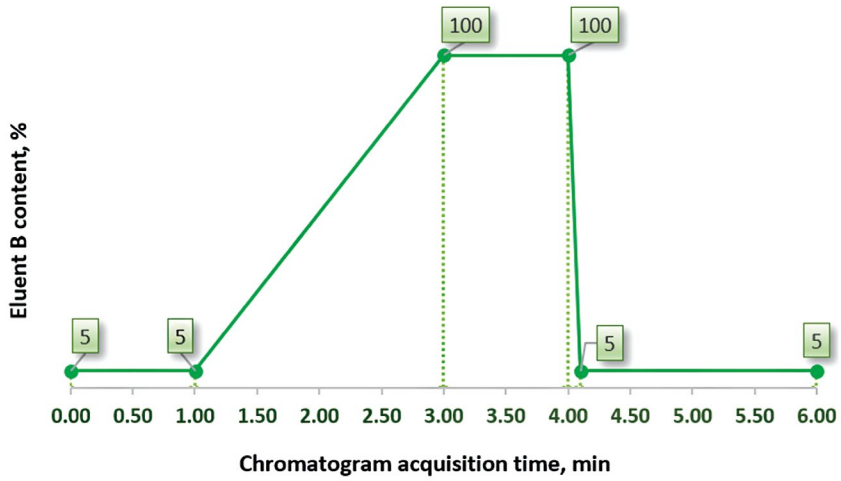


Figure 2. **Composition of the mobile phase during gradient elution**

Table 2. **Tofacitinib and baricitinib detection parameters**

Detection parameters	Ionization mode	Nebulizing gas, L/min	Desolvation gas, L/min	ESI temperature, °C	Parent ion, m/z	Product ion, m/z	Capillary voltage, kV
TOF	+	3	20	400	313.30	173.00	+4.5
BAR					371.90	186.00	

Validation of the Bioanalytical Method

Method validation was carried out in accordance with the regulations for bioequivalence studies of medicinal products approved within the EAEU[1], the guidelines for the validation of bioanalytical methods by FDA[2], and EMA[3].

The parameters that need to be evaluated in order to carry out a full validation of an analytical method in accordance with the listed documentation are shown in Figure 3, together with the samples and acceptance criteria (AC) required for the analysis.

When validating the parameter «Stability» it is necessary to assess several stability types. These forms of stability are evaluated by subjecting the samples that have been stored under different specific conditions to analysis, as shown in Figure 4.

[1] Rules for Conducting Bioequivalence Studies of Medicinal Products within the Eurasian Economic Union (Approved by Decision N 85 of the Council of the Eurasian Economic Commission of 03.11.2016). Available at: https://docs.cntd.ru/document/456026107/ Accessed: 26.03.2024.

[2] Bioanalytical Method Validation. Guidance for Industry. U.S. Food and Drug Administration, Center for Drug Evolution and Research (CDER). Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry/ Accessed: 26.03.2024.

[3] Guidline on bioanalytical method validation. European Medicines Agency. Committee for medicinal products for human use. Available at: https://www.ema.europa.eu/en/bioanalytical-method-validation/ Accessed: 26.03.2024.

RESULTS AND DISCUSSION

Literature review

Table 3 summarizes the methods employed for the quantitative determination of TOF in human biological fluids (blood plasma, blood serum, urine). Most of the reviewed studies utilized the HPLC-MS/MS method, with the analyte being ionized via electrospray or TurbolonSpray in positive mode. Additionally, a technique has been developed for the measurement of the target compound in plasma using ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) [7].

Literature analysis demonstrates that elution is typically conducted in an isocratic mode (ISO) [7-14]. The solvents in the mobile phase are most often modified with ammonium salts [7, 9–14] or acetic acid (AA) [7]. Additionally, there are two methods using classical eluents: water acidified with formic acid (FA) (eluent A) and acetonitrile acidified with FA (eluent B) [8, 15]. This composition of both eluents facilitates research and analysis.

In the methods presented, complex sample preparation procedures predominate, including solid-phase extraction (SPE) [9–12], which requires the use of a significant amount of expensive reagents and supplies. Liquid-liquid extraction (LLE) [7, 13, 14] also necessitates specialized equipment such as an evaporator. A modification of the precipitation method, which involves dilution of samples with ultrapure water at the end of the sample preparation process [15], should be noted. Pro-

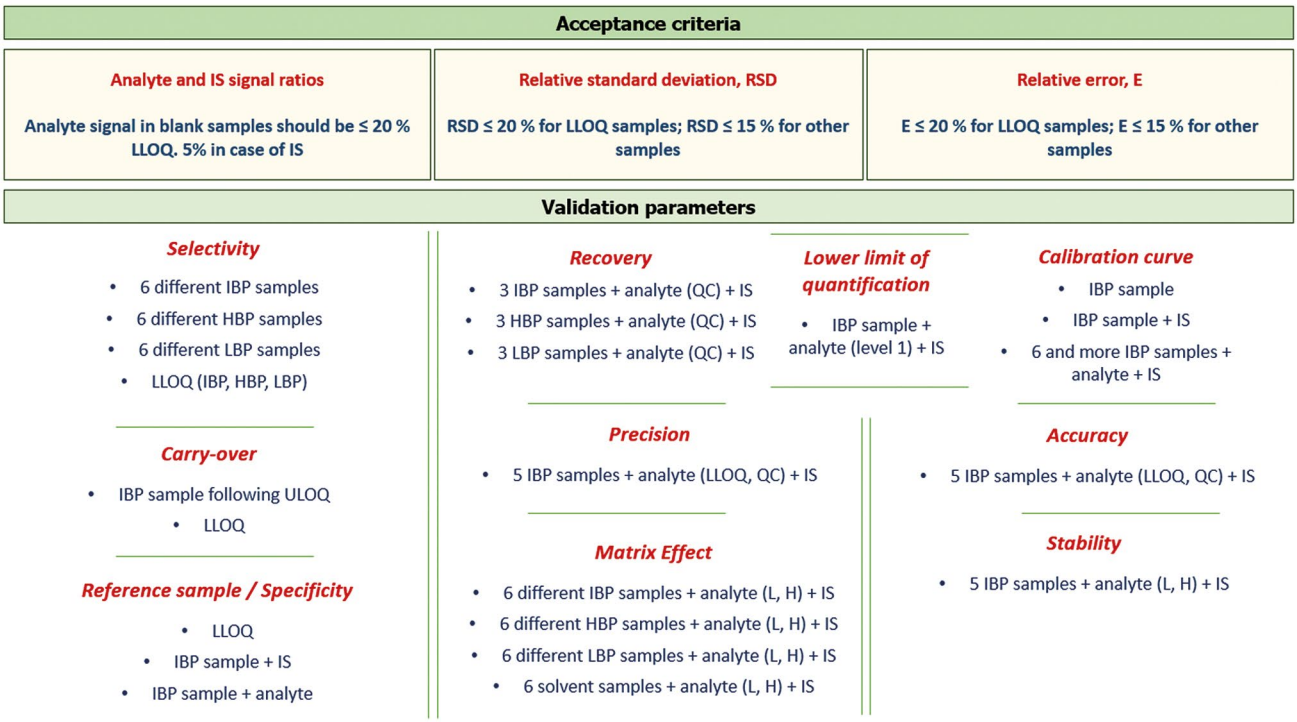


Figure 3. **Validation parameters and acceptance criteria**

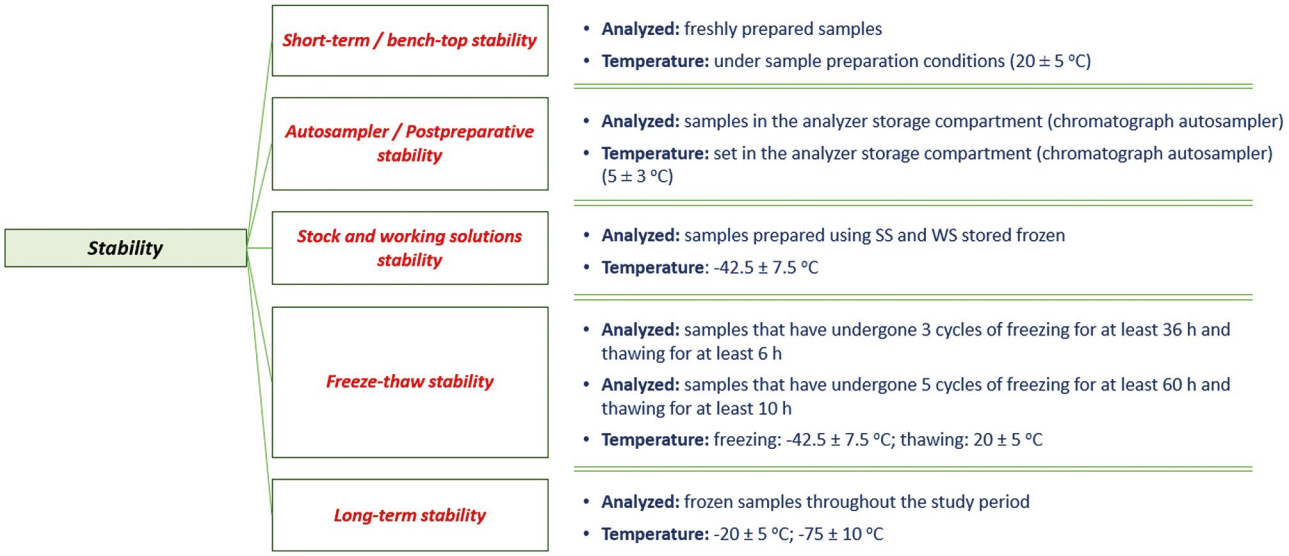


Figure 4. **Assessment conditions for different types of stability**

tein precipitation remains to be the simplest, fastest, and least expensive method for sample preparation using an organic solvent such as ACN or methanol (MeOH) [8].

The most sensitive method reported in literature for the determination of tofacitinib in human plasma is a UPLC-MS/MS-assay with a lower detection limit of 0.05 ng/mL. This assay is highly sensitive, but it requires specialized UPLC equipment that may not be available in most laboratories. Developing an alternative method that is more accessible and easier to use, while maintaining similar sensitivity, would be of significant interest. HPLC-MS/MS method has proven to be reliable and accurate and therefore could be a promising alternative.

Method development

HPLC-MS/MS method development involves the selection of mass spectrometry parameters and chromatographic conditions.

Table 3. **Bioanalytical methods for determination of tofacitinib in biological matrices**

Analysis method	Ionization conditions	Chromatogram register time, min	Mobile phase composition	Biological matrix	Sample preparation	Analytical range, ng/mL	Reference
UPLC-MS/MS	ESI (+)	1.40	Ammonium acetate + water + AA / ACN (ISO)	Blood plasma	LLE	0.05 – 100.00	[7]
HPLC-MS/MS	ESI (+)	4.10	FA + water / FA + ACN (ISO)	Blood serum	Precipitation with ACN	0.10 – 100.00	[8]
HPLC-MS/MS	TurboIonSpray (+)	45.00	Ammonium acetate + MeOH + FA (ISO)	Blood plasma, urine	SPE	1.00 – 100.00	[9]
HPLC-MS/MS	TurboIonSpray (+)	–	Ammonium acetate + MeOH + FA (ISO)	Blood plasma, urine	SPE	0.10 – 350.00	[10]
HPLC-MS/MS	TurboIonSpray (+)	–	Ammonium acetate + MeOH + FA (ISO)	Blood plasma	SPE	0.10 – 350.00	[11]
HPLC-MS/MS	ESI (+)	–	Ammonium acetate + MeOH + FA (ISO)	Blood plasma	SPE	0.10 – 350.00	[12]
HPLC-MS/MS	ESI (+)	6.00	Ammonium formate +FA + water + ACN (ISO)	Blood plasma	LLE	5.00 – 100.00	[13]
HPLC-MS/MS	ESI (+)	4.00	ACN / ammonium formate + water (ISO)	Blood plasma, urine	Dilution with glycine buffer, LLE, filtration	1.00 – 100.00	[14]
HPLC-MS/MS	ESI (+)	7.00	FA + water / FA + ACN (GRAD)	Blood plasma, serum	Precipitation with MeOH, dilution with water	1.00 – 400.00	[15]

In the first step, a suitable IS was selected based on the physicochemical properties of the analyte. Acid dissociation constant (pKa) values, octanol/water partition coefficients (logP) and the structure of the compounds were taken into the consideration when deciding on an IS that is similar to TOF (see Table 4). Baricitinib was opted for as a suitable IS for the current study.

The development of mass-spectrometry conditions was performed using the multiple reaction monitoring (MRM) method, which allows for the identification of a specific fragmentation pathway of the parent ion of a substance, resulting in products with different masses. The resulting MRM-transitions for TOF are the following: 313.30 to 173.00 m/z and for BAR: 371.90 to 186.00 m/z. In order to increase the signal intensity of the analyte and IS, the optimization of several mass spectrometric parameters was carried out, including MRM-transitions, collision energy, quadrupole, desolvation line and needle voltages.

After selecting the conditions that maximize peak intensities for mass spectrometry detection, it is essential to determine the chromatographic parameters that enable effective separation of a biological sample.

For stationary phase a Phenomenex Kinetex C_{18} column (100 × 3.0 mm, with a particle size of 5 μm) was employed. The mobile phase constituted of 0.1 % formic acid-water solution (eluent A) and 0.1 % formic acid-acetonitrile solution (eluent B). Elution was performed in gradient mode, which allowed for obtaining high peak intensities, satisfactory peak asymmetry factors for the analyte and IS (1.054 and 1.115, respectively), and a high signal-to-noise ratio (S/N) for the analyte at LLOQ for the first analytical range (25.56). After reducing the LLOQ to 0.30 ng/mL, the S/N for TOF was found to be 15.81. This indicates that the sensitivity of the analytical method is sufficient. To ensure accurate results, the analysis time for each injection was extended to 6 minutes to allow for adequate system washing and equilibration.

A suitable sample preparation method was determined by examining the samples obtained through the precipitation of plasma proteins using organic solvents: ACN and MeOH. Methanol-based samples revealed suspended solids that proved impossible to separate by centrifugation. The analysis of samples prepared with ACN as a precipitating agent showed satisfactory extraction of the desired substances, along with the acceptable data reproducibility. Therefore, the employment of the LLE method was not required.

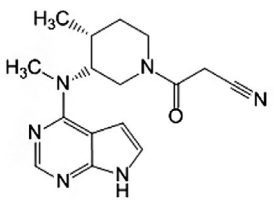
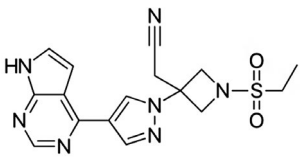
Validation

Full validation of the method for quantitative determination of tofacitinib in human plasma was carried out within validation cycles 1–3. Validated range was 1.00–200.00 ng/mL.

Analytical approbation of the method in terms of bioequivalence study revealed a 5 % excess of LLOQ over the lowest maximum concentration of TOF among all analyzed samples. Consequently, a partial validation was carried out to reduce the LLOQ by expanding the analytical range. The partial validation within the 4th validation cycle involved the assessment of the following parameters: selectivity, calibration curve, lower limit of quantification, accuracy and precision. Additionally, samples stability during five freeze-thaw cycles, as well as stock and working solutions stability was evaluated.

Long-term stability of the analyte in the biological matrix (human blood plasma) was assessed twice: within the 3rd cycle (the first stage) and the 5th cycle (the second stage), in order to extend the shelf life of biological samples during the analytical phase of the study. Consequently, the long-term stability of the analyte was reassessed within the 5th validation cycle with the following parameters: calibration curve, accuracy, precision, and stability.

Table 4. Properties of tofacitinib and baricitinib

Investigated substance	Tofacitinib	Baricitinib
Compound name (IUPAC)	3-[(3R,4R)-4-methyl-3-[methyl({7H-pyrrolo[2,3-d]pyrimidin-4-yl})amino]piperidin-1-yl]-3-oxopropanenitrile	2-[1-(ethanesulfonyl)-3-(4-{7H-pyrrolo[2,3-d]pyrimidin-4-yl}-1H-pyrazol-1-yl)azetidin-3-yl]acetonitrile
Structural formula		
pKa	9.15[1]	13.89[2]
logP	1.808[1]	1.10[2]

Note. [1] Tofacitinib. Drugbank. Available at: https://go.drugbank.com/drugs/DB08895. Accessed: 26.03.2024.
[2] Baricitinib. Drugbank. Available at: https://go.drugbank.com/drugs/DB11817. Accessed: 26.03.2024.

1. Selectivity, specificity, carry-over

The TOF and BAR signal levels in the blank samples were not more than 20 and 5 % of the arithmetic mean of TOF and BAR signal values in the LLOQ samples, respectively, which is consistent with the requirements specified in the regulatory documentation. Figure 5 illustrates an example of chromatograms obtained from the blank and LLOQ sample analysis.

The specificity of the method (standard sample suitability) was similarly assessed by comparing the signals from the sample without TOF and the sample without BAR to those at the LLOQ level. Sample test results met the AC.

2. Matrix effect

The parameter was estimated at two QC concentration levels (L and H) using IBP, HBP, and LBP samples. The quantitative results of the estimation of the matrix effect on TOF are presented in Table 5. The RSD values for all matrix types were less than 15 % for both concentration levels.

Table 5. Baricitinib-normalized matrix effect for tofacitinib, relative standard deviation

Biological matrix	IBP		HBP		LBP	
Concentration level	L	H	L	H	L	H
Average	1.34	1.25	1.29	1.20	1.31	1.16
RSD, %	9.69	6.42	12.93	6.00	9.98	3.93

3. Recovery

Table 6 presents the average recovery values for different matrix types. The RSD ranged from 1.77 to 12.36 %, which falls within the acceptable limits.

4. Calibration curve, accuracy, precision, lower limit of quantification

The simplest linear regression model was chosen for the calibration graph. For the five calibration curves obtained during the validation process, the correlation coefficients for the selected function type were not less than 0.99, while E for the measured CS concentrations remained within the specified tolerance limits (not exceeding ±20 % for the 1st concentration level, not exceeding ±15 % for the remaining concentration levels). A representative calibration graph from the 4th validation cycle is depicted in Figure 6. The corresponding correlation coefficient for this calibration curve is 0.9944968 and the linear regression equation is

$$f(x) = 0.707283x + 0.00276663.$$

For the selected TOF concentration ranges (1.00–200.00 ng/mL and 0.30–200.00 ng/mL), inter-and intra-

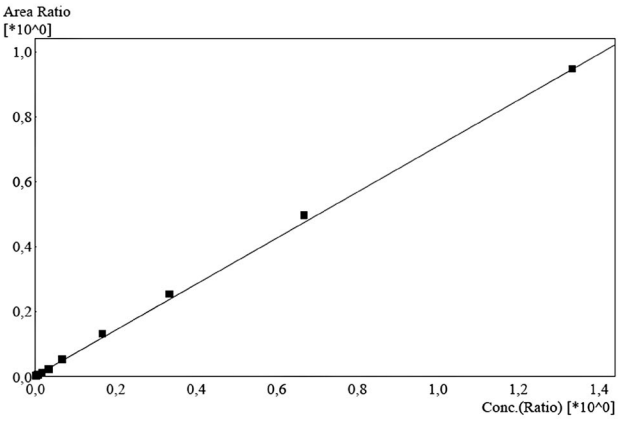
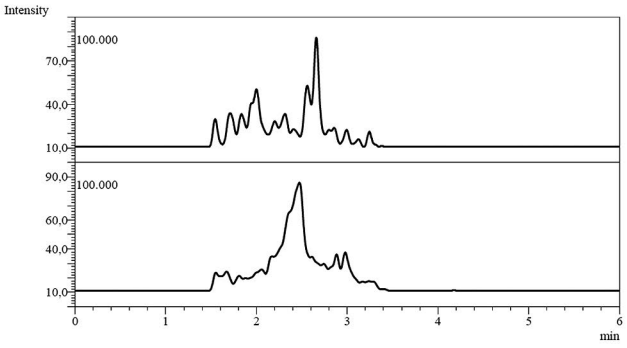


Figure 6. Calibration curve of the 4th validation cycle



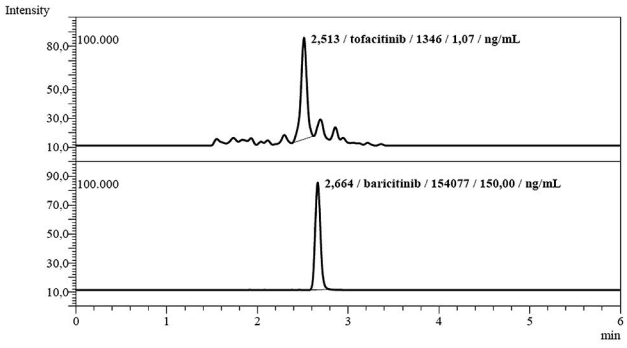


Figure 5. Example of chromatograms used to evaluate selectivity

Table 6. Baricitinib-normalized recovery for tofacitinib, relative standard deviation

Biological matrix	Average value of recovery					RSD, %				
	L	M1	M2	M3	H	L	M1	M2	M3	H
IBP	97.66	101.16	105.71	105.08	98.57	9.41	7.62	4.39	2.05	5.68
HBP	99.80	106.07	98.33	95.80	102.84	12.36	6.04	11.70	2.28	7.74
LBP	107.34	95.02	95.41	100.78	96.65	6.07	9.02	5.70	1.77	4.13

day accuracy and precision of the LLOQ and QC samples were evaluated for all validation cycles. Values for inter-day parameters are presented in Table 7.

The lower limit of quantification was determined based on the data from the calibration curve, accuracy, and precision assessment. For cycles 1 through 3 of the full validation study, the LLOQ was set at 1.00 ng/mL. After analyzing the data from cycle 4 of the partial validation study, a value of 0.30 ng/mL was determined for the LLOQ parameter. In both instances, obtained values corresponded to the first concentration level of TOF. The chromatograms for the LLOQs are presented in Figure 7.

5. Stability

By analyzing low (L) and high (H) QCs during stability estimation process, the resulting E values did not exceed ±15 %. Therefore, the following types of stability were demonstrated:

- short-term stability (BT-St.);
- post-preparative stability of samples (PP-St.) for not more than 41 hours;
- sample stability after 3 cycles of thawing and freezing (3-FT-St.) – within the 3rd validation cycle;
- sample stability after 5 cycles of thawing and freezing (5-FT-St.) – within the 4th validation cycle;
- stability of SSs (SS-St.) and WSs (WS-St.) for 3 days – within the 3rd validation cycle;
- stability of SSs (SS-St.) and WSs (WS-St.) for 5 days – within the 4th validation cycle;
- long-term stability (LT-St.) of analyte in biological matrix (plasma) at the extreme values of storage temperature range: from –25 to –15 °C and from –85 to –65 °C for 3 days – within the 3rd validation cycle;
- long-term stability (LT-St.) of the analyte in biological matrix (plasma) at the extreme values of storage temperature range: from –25 to –15 °C and from –85 to –65 °C for 66 days – within the 5th validation cycle (Table 8).

Method application

Validated method was applied for tofacitinib determination in a bioequivalence study of a domestic tofacitinib formulation and a reference drug: Jaquinus® modified-release, film-coated tablets containing 11 mg of tofacitinib (Pfizer Inc., USA). The pharmacokinetic properties of tofacitinib were investigated in this study following a single dose administration of the test drug (Test) and the reference drug (Ref.), both administered under fasting and fed conditions. Based on the analysis of blood plasma samples, individual pharmacokinetic profiles were generated, as illustrated in Figure 8.

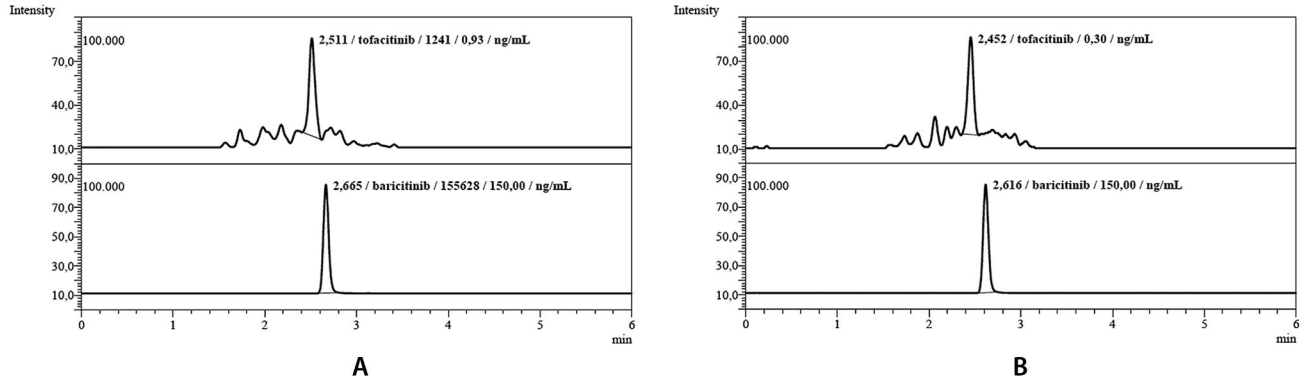


Figure 7. Chromatograms for the lower limit of quantification for validation cycles 1–3 (A) and 4 (B)

Table 7. Inter-day accuracy and precision for tofacitinib

Number of the validation cycle	1, 2, 3, 5	4	1, 2, 3, 5	4	1, 2, 3, 5			
Concentration level	LLOQ	LLOQ	L	L	M1	M2	M3	H
Nominal TOF concentration, ng/mL	1.00	0.30	3.00	0.90	6.00	30.00	120.00	150.00
Number of the validation cycle	Average value of TOF concentration in the cycle, ng/mL							
1	1.02	–	3.07	–	6.03	29.55	115.42	139.01
2	1.06	–	2.68	–	6.23	31.33	119.19	147.15
3	0.94	–	2.71	–	5.80	29.38	111.13	147.80
4	–	0.29	–	0.87	5.27	32.40	126.09	160.36
5	0.98		3.22	–	6.24	31.93	118.15	152.76
Inter-day average TOF concentration, ng/mL	1.00	0.29	2.92	0.87	5.92	30.92	118.00	149.42
E, %	–0.05	–2.00	–2.77	–3.56	–1.41	3.07	–1.67	–0.39
RSD, %	8.98	11.18	10.09	14.19	7.21	5.28	5.15	5.12

The following pharmacokinetic parameters were determined using specialized software:

- C_{max} – maximum plasma concentration of tofacitinib;
- t_{max} – time to reach C_{max};
- $t_{1/2}$ – plasma half-life of tofacitinib.

Table 9 presents average values for C_{max} and $t_{1/2}$, expressed as the arithmetic mean (M), along with the standard deviation (σ), for the sample of volunteers (n). For t_{max}, the data are reported as median (Median), with the range of values from minimum (Min) to maximum (Max), for the same sample (*n*).

An example of biosamples chromatograms from a volunteer participating in a clinical trial of domestically manufactured tofacitinib is shown in Figure 9.

CONCLUSION

An accessible, and sensitive method for the quantitative analysis of tofacitinib was developed. The method is based on sample separation using a high-performance liquid chromatography system. Detection is achieved using a tandem triple-quadrupole mass spectrometry detector. The range of this method is 0.30–200.00 ng/mL. The method was validated in accordance with the relevant regulatory documentation of the EAEU, FDA, EMA. After validation, the method was successfully applied in a bioequivalence study of a generic domestic formulation of tofacitinib. The results obtained

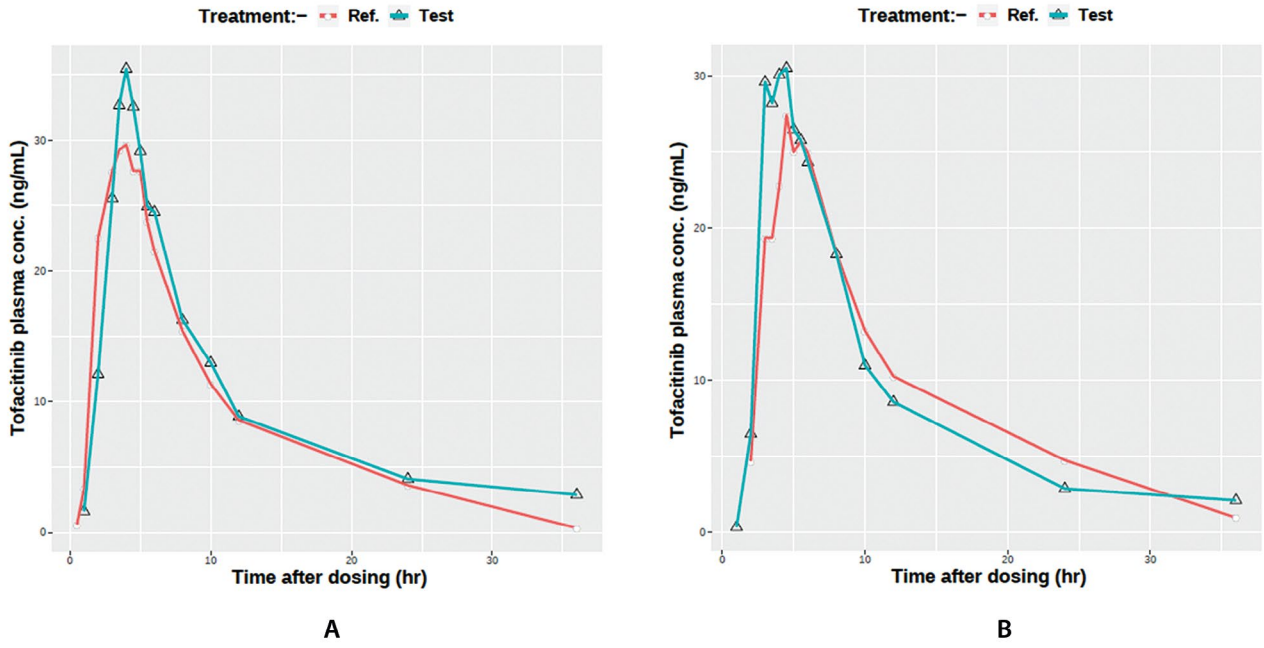


Figure 8. **Individual pharmacokinetic profiles of a volunteer with drugs administration under fasting (A) and fed (B) conditions**

Table 8. **Results of tofacitinib stability assessment**

Stability		Concentration level	L		H	
		Nominal TOF concentration, ng/mL	3.00		150.00	
		Number of the validation cycle	Average TOF concentration, ng/mL	E, %	Average TOF concentration, ng/mL	E, %
BT-St.		1	2.66	–11.40	148.26	–1.16
PP-St.		1	2.78	–7.27	129.37	–13.75
		2	2.63	–12.20	132.21	–11.86
		3	2.75	–8.20	136.37	–9.08
3-FT-St.		3	2.59	13.53	140.09	–6.60
5-FT-St.		4	2.98	–0.67	157.00	1.67
SS-St.		3	2.60	–13.47	145.37	–3.08
		4	3.05	1.67	151.23	0.82
WS-St.		3	2.60	–13.40	141.87	–5.42
		4	2.97	–0.87	159.25	6.17
LT-St.	–20 °C	3	2.82	–5.93	139.52	–6.99
	–80 °C	3	2.96	–1.20	141.04	–5.97
	–20 °C	5	3.04	1.20	151.50	1.00
	–80 °C	5	3.06	1.93	151.68	1.12

Table 9. **Summary data of tofacitinib pharmacokinetic parameters during single dose administration of the drugs**

Drug administration conditions	Fasting		Fed	
Pharmacokinetic parameter	Test drug	Reference drug	Test drug	Reference drug
	M (σ) [n] / Median (Min – Max) [n]			
C_{max}, ng/mL	36.97 (14.96) [18]	37.46 (12.72) [18]	49.35 (13.52) [19]	52.44 (17.66) [19]
t_{max}, h	3.75 (2–5) [18]	4 (2–5) [18]	5 (3.5–10) [19]	5 (3.5–8) [19]
$t_{1/2}$, h	5.77 (2.42) [18]	5.68 (1.46) [18]	7.14 (2.57) [19]	5.54 (2.33) [19]

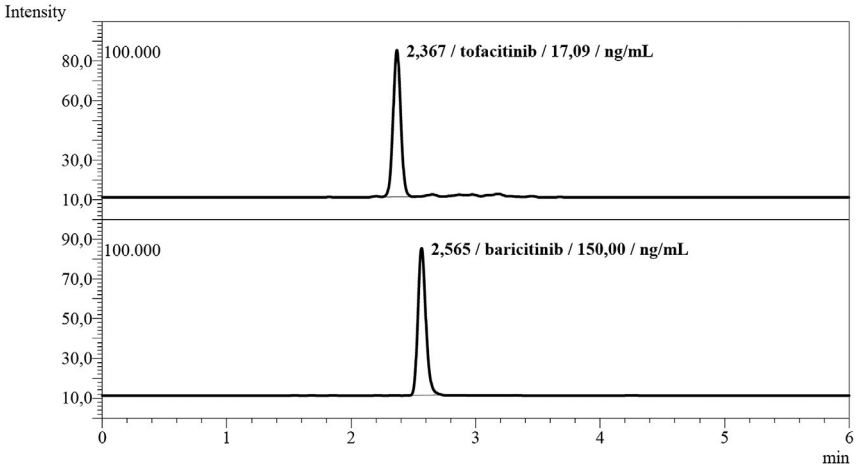


Figure 9. **Chromatograms of a volunteer blood plasma sample after 8 hours after administration of the drug**

during the validation confirm method suitability for the utilization in bioanalytical research of the pharmacokinetics and bioequivalence of tofacitinib formulations.

REFERENCES

1. Nasonov E. L., Avdeeva A. S., Lila A. M. Efficacy and safety of tofacitinib for immune-mediated inflammatory rheumatic diseases (Part I). *Rheumatology Science and Practice*. 2020;58(1):62–79. (In Russ.) DOI: 10.14412/1995-4484-2020-62-79.
2. GBD 2021 Rheumatoid Arthritis Collaborators. Global, regional, and national burden of rheumatoid arthritis, 1990–2020, and projections to 2050: a systematic analysis of the Global Burden of Disease Study 2021. *The Lancet Rheumatology*. 2023;5(10):e594–e610. DOI: 10.1016/S2665-9913(23)00211-4.
3. Finckh A., Gilbert B., Hodkinson B., Bae S.-C., Thomas R., Deane K. D., Alpizar-Rodriguez D., Lauper K. Global epidemiology of rheumatoid arthritis. *Nature Reviews Rheumatology*. 2022;18(10):591–602. DOI: 10.1038/s41584-022-00827-y.
4. Smolen J. S., Aletaha D., Barton A., Burmester G. R., Emery P., Firestein G. S., Kavanaugh A., McInnes I. B., Solomon D. H., Strand V., Yamamoto K. Rheumatoid arthritis. *Nature Reviews Disease Primers*. 2018;4:18001. DOI: 10.1038/nrdp.2018.1.
5. Nasonov E. L., Feist E. The prospects of interleukin-6 inhibition in rheumatoid arthritis: Olokizumab (novel monoclonal antibodies to IL-6). *Rheumatology Science and Practice*. 2022;60(5):505–518. (In Russ.) DOI: 10.47360/1995-4484-2022-505-518.
6. Korotaeva T. V., Loginova E. Yu. Results of studying the clinical efficacy and safety of tofacitinib in the treatment of psoriatic arthritis. *Modern Rheumatology Journal*. 2019;13(2):112–118. (In Russ.) DOI: 10.14412/1996-7012-2019-2-112-118.
7. Bharwad K. D., Shah P. A., Shrivastav P. S., Singhal P. Development and validation of a rapid and sensitive UPLC–MS/MS assay for the quantification of tofacitinib in human plasma. *Biomedical Chromatography*. 2019;33(4):e4458. DOI: 10.1002/bmc.4458.
8. Church S., Hyrich K. L., Ogungbenro K., Unwin R. D., Barton A., Bluett J. Development of a sensitive biochemical assay for the detection of tofacitinib adherence. *Analytical Methods*. 2023;15(14):1797–1801. DOI: 10.1039/D2AY01800D.
9. Dowty M. E., Lin J., Ryder T. F, Wang W., Walker G. S., Vaz A., Chan G. L., Krishnaswami S., Prakash C. The pharmacokinetics, metabolism, and clearance mechanisms of tofacitinib, a Janus kinase inhibitor, in humans. *Drug Metabolism and Disposition*. 2014;42(4):759–773. DOI: 10.1124/dmd.113.054940.
10. Krishnaswami S., Boy M., Chow V., Chan G. Safety, tolerability, and pharmacokinetics of single oral doses of tofacitinib, a Janus kinase inhibitor, in healthy volunteers. *Clinical Pharmacology in Drug Development*. 2015;4(2):83–88. DOI: 10.1002/cpdd.171.
11. Krishnaswami S., Wang T., Yuan Y., Alvey C. W., Checchio T., Peterson M., Shi H., Riese R. Single- and multiple-dose pharmacokinetics of tofacitinib in healthy Chinese volunteers. *Clinical Pharmacology in Drug Development*. 2015;4(5):395–399. DOI: 10.1002/cpdd.202.
12. Menon S., Riese R., Wang R., Alvey C. W., Shi H., Petit W., Krishnaswami S. Evaluation of the effect of tofacitinib on the pharmacokinetics of oral contraceptive steroids in healthy female volunteers. *Clinical Pharmacology in Drug Development*. 2016;5(5):336–342. DOI: 10.1002/cpdd.270.
13. Abdelhameed A. S., Attwa M. W., Kadi A. A. An LC–MS/MS method for rapid and sensitive high-throughput simultaneous determination of various protein kinase inhibitors in human plasma. *Biomedical Chromatography*. 2017;31(2):e3793. DOI: 10.1002/bmc.3793.
14. Kadi A. A., Abdelhameed A. S., Darwish H. W., Attwa M. W., Bakheit A. H. Liquid chromatographic-tandem mass spectrometric assay for simultaneous quantitation of tofacitinib, cabozantinib and afatinib in human plasma and urine. *Tropical Journal of Pharmaceutical Research*. 2016;15(12):2683–2692. DOI: 10.4314/tjpr.v15i12.21.
15. Tachet J., Versace F., Mercier T., Buclin T., Decosterd L. A., Choong E., Girardin F. R. Development and validation of a multiplex HPLC-MS/MS assay for the monitoring of JAK inhibitors in patient plasma. *Journal of Chromatography B*. 2023;1230:123917. DOI: 10.1016/j.jchromb.2023.123917.