PERIÓDICO TCHÊ QUÍMICA
ARTIGO ORIGINAL

DETERMINAÇÃO DA TERIFLUNOMIDA ATRAVÉS DE UMA AMPLA GAMA DE CONCENTRAÇÕES DINÂMICAS EM PLASMA HUMANO POR LC-MS/MS

DETERMINATION OF TERIFLUNOMIDE ACROSS A WIDE DYNAMIC CONCENTRATION RANGE IN HUMAN PLASMA BY LC–MS/MS

ВЭЖХ–МС/МС МЕТОД АНАЛИЗА ТЕРИФЛУНОМИДА В ПЛАЗМЕ КРОВИ ЧЕЛОВЕКА В ШИРОКОМ ДИАПАЗОНЕ КОНЦЕНТРАЦИЙ

MOSKALEVA Natalia E.[1], MESONZHNIK Natalia V.[2], KUZNETSOV Roman M.[3], MARKIN Pavel A.[4], APPOLONOVA Svetlana A.[5 *]

[1,2,3,4,5] Institute of Translational Medicine and Biotechnology, I. M. Sechenov First Moscow State Medical University

** Correspondence author*
e-mail: appolosa@yandex.ru

Received 06 February 2019; received in revised form 05 June 2019; accepted 03 July 2019

RESUMO

A leflunomida é uma droga antirreumática com propriedades anti-inflamatórias e antirreumáticas, que é rapidamente metabolizada no organismo pelo metabólito ativo teriflunomida, que causa sua atividade farmacológica. Na dose diária habitual de 20 mg de leflunomida, a concentração plasmática esperada de teriflunomida é de cerca de 35 µg/ml. A farmacocinética do fármaco é caracterizada por uma grande variabilidade interindividual e uma longa semi-vida, que, em combinação com possíveis efeitos secundários, cria a necessidade de controlar a concentração de teriflunomida no plasma sanguíneo. A teriflunomida pode aumentar o risco de morte fetal ou efeitos teratogênicos quando administrada a mulheres grávidas. As concentrações plasmáticas de teriflunomida inferiores a 0,02 µg/ml são preferidas para os doentes que antecipam a gravidez. Neste estudo, um método sensível e de alto desempenho para análise de teriflunomida no plasma sangüíneo humano em uma ampla gama de concentrações foi desenvolvido e validado usando um espectrômetro de massa de cromatografia líquida de quadrupolo triplo (HPLC-MS / MS). A preparação da amostra foi realizada por precipitação de protea com acetonitrilo, seguida de separaçãoo cromatográfica utilizando uma coluna Acquity UPLC BEN C8 1,7 mm, 2,1 x 50 mm (Waters, EUA). A D4-teriflunomida foi usada como padrão interno. O método desenvolvido foi validado na faixa de concentração de 0,001 µg/ml a 200 µg/ml de teriflunomida no plasma. A precisão (%), definida como a diferença entre a concentração nominal e medida e a reprodutibilidade (coeficiente de variação CV) variou de -5,02% a 5,00% e de 0,47% a 9,30%, respectivamente, dentro da série e entre séries de amostras. O método desenvolvido foi utilizado com sucesso para analisar amostras voluntárias de plasma sanguíneo após tomar 20 mg de leflunomida.

Palavras-chave: *cromatografia-espectrometria de massa, teriflunomida, farmacocinética, monitoramento terapêutica*.

ABSTRACT

Leflunomide is an antirheumatic drug with anti-inflammatory and antirheumatic properties, it is rapidly metabolized in the body to the active metabolite teriflunomide, which causes its pharmacological activity. At the usual 20-mg daily dosage of leflunomide, the expected teriflunomide plasma concentration is about 35 µg/ml. The pharmacokinetics of the drug is characterized by a large interindividual variability and a long half-life, which in combination with possible side effects creates the need to control the concentration of teriflunomide in the blood plasma. Teriflunomide may increase the risk of fetal death or teratogenic effects when administered to pregnant women. Teriflunomide plasma concentrations less than 0.02 µg/ml are preferred for patients anticipating pregnancy. In this study, a sensitive and high-performance method for analyzing teriflunomide in human blood plasma in a wide range of concentrations was developed and validated using a triple quadrupole liquid chromatography-mass spectrometer (HPLC-MS/MS). Sample preparation was performed by protein precipitation with acetonitrile, followed by chromatographic separation using an Acquity UPLC BEN C8 1.7 mm, 2.1 × 50 mm column (Waters, USA). D4-teriflunomide was used as an internal standard. The developed method was validated in the concentration range from 0.001 µg/ml to 200 µg/ml teriflunomide in plasma. Accuracy (%),

DOI: 10.52571/PTQ.v16.n32.2019.626_Periodico32_pgs_608_620.pdf

defined as the difference between the nominal and measured concentration and reproducibility (coefficient of variation CV) ranged from -5.02% to 5.00% and from 0.47% to 9.30%, respectively, within the series and between series of samples. The developed method was successfully used to analyze volunteer blood plasma samples after taking 20 mg of leflunomide.

Keywords: *chromatography-mass spectrometry, teriflunomide, pharmacokinetics, therapeutic monitoring.*

АННОТАЦИЯ

Лефлуномид - противоревматический препарат с противовоспалительными и противоревматическими свойствами, в организме быстро метаболизируется до активного метаболита терифлуномида, обуславливающего его фармакологическую активность. При обычной суточной дозе лефлуномида 20 мг ожидаемая концентрация терифлуномида в плазме составляет около 35 мкг/мл. Фармакокинетика препарата характеризуется большой межиндивидуальной вариабельностью и длительным периодом полувыведения, что в сочетании с возможными побочными эффектами создает необходимость контроля концентрации терифлуномида в плазме крови. Терифлуномид может увеличить риск смерти плода или тератогенных эффектов при назначении беременным женщинам, поэтому при планировании беременности прием препарата заранее отменяют, концентрация терифлуномида в плазме должна быть менее 0,02 мкг/мл. В данном исследовании разработан и валидирован чувствительный и высокопроизводительный метод анализа терифлуномида в плазме крови человека в широком диапазоне концентраций при помощи жидкостного хромато-масс-спектрометра на основе тройного квадруполя (ВЭЖХ-МС/МС). Подготовку пробы проводили простым осаждением белка ацетонитрилом с последующим хроматографическим разделением при помощи колонки Acquity UPLC BENC8 1.7 mm, 2.1 × 50 mm (Waters, USA). В качестве внутреннего стандарта использовали d4-терифлуномид (IS). Разработанный метод валидировали в диапазоне концентраций от 0.001 мкг/мл до 200 мкг/мл терефлуномида в плазме крови. Точность (%), определяемая как разница между номинальной и измеренной концентрацией и воспроизводимость (коэффициент вариации CV) варьировали от -5.02% до 5.00% и от 0.47% до 9.30% соответственно внутри одной серии и между сериями образцов. Разработанный метод был успешно использовали для анализа образцов плазмы крови добровольцев после приема 20 мг лефлуномида.

Ключевые слова: *хромато-масс-спектрометрия, терифлуномид, фармакокинетика, терапевтический мониторинг.*

1. INTRODUCTION

Teriflunomide (also known as A77-1726) is the active metabolite of leflunomide – an immunosuppressive disease-modifying antirheumatic drug (DMARD), developed by Sanofi-Aventis to treat active moderate to severe rheumatoid arthritis (RA) and psoriatic arthritis. Teriflunomide noncompetitively inhibits dihydroorotate dehydrogenase, an essential enzyme for de novo synthesis of pyrimidines. As a consequence, the division of activated T lymphocytes, which plays an important role in RA pathology, is blocked (Fox, 1998). The chemical structure features the unique mechanism of action, as well as the low price; both are of the key considerations for leflunomide therapy, especially when other DMARDs are not effective or not tolerated (Strand *et al.*, 1999). It is also used as an adjunct therapy to methotrexate, sulfasalazine, and other biological DMARDs (Weinblatt *et al*., 1999; Dougados *et al.*, 2005; Hopkins *et al*., 2014). Leflunomide is approved in the US and EU and in 43 other states all over the world for the treatment of adult patients with early or late active rheumatoid arthritis as a disease-modifying antirheumatic drug (DMARD).

Leflunomide is a prodrug available for oral administration in solid forms (tablets) containing 10, 20, and 100 mg of the active drug. It is well absorbed orally with an estimated bioavailability around 80%, and almost completely metabolized to an active metabolite - teriflunomide (A77-1726) (Grabar *et al.*, 2009), the structure showed in Figure 1. Plasma levels of leflunomide are observed only occasionally and at very low concentrations. Teriflunomide biotransformation occurs by a non-enzymatic reaction in the submucosal wall of the intestinal tract (Rozman, 2002). More than 99% of teriflunomide is bound to proteins; its half-life is approximately 15 days in RA patients (14-18 days) (Rozman, 2002; Rakhila *et al.*, 2011). Teriflunomide is excreted both in urine and in the feces. Its long half-life is thought to be the result of low hepatic clearance and of enterohepatic recirculation. Unfortunately, metabolic pathways for leflunomide and teriflunomide are not yet completely elucidated.

Major metabolites found in urine are 4-(trifluoromethyl)aniline oxanilate, methylhydroxyteriflunomide, and leflunomide glucuronide. At the usual 20-mg daily dosage of leflunomide, the expected teriflunomide plasma concentration is about 35 mg/L (Rozman, 2002).

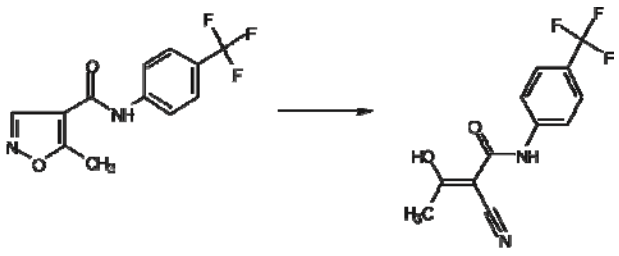

Figure 1. *Chemical structure of teriflunomide and leflunomide*

Being an effective disease-modifying antirheumatic drug, leflunomide treatment is associated with high interindividual variability in pharmacokinetics, as well as with adverse reactions such as diarrhea, hypertension, and liver toxicity. According to animal studies, leflunomide may increase the risk of fetal death or teratogenic effects when administered to pregnant women. Enhanced elimination of the drug may be required in patients who are or who wish to become pregnant, and also in patients who are high-profiled to toxicity. Moreover, teriflunomide can persist up to 2 years after ceasing therapy unless elimination is accelerated. This is usually accomplished through the use of activated charcoal or a bile acid sequestrant as cholestyramine, reducing the half-life of teriflunomide to approximately 1 day. Teriflunomide plasma concentrations less than 20 ng/ml of 2 independent tests at least 2 weeks apart are preferred for the patients with anticipated pregnancy to minimize the potential risk of teratogenesis associated with the drug (Cassina *et al.*, 2012). Due to antiviral effects, teriflunomide is actively used in clinical applications with renal transplant recipients, assisting in clearing infections, such as BK polyomavirus (BKV) and cytomegalovirus. Serum trough levels of 40 µg/mL (148 µmol/L) or greater have been associated with progressive clearance of BKV. However, concentrations significantly above those needed to control viral proliferation can induce hepatotoxicity and may be related to the development of thrombotic microangiopathies (Sobhani *et al.*, 2010). Hence, therapeutic drug monitoring over a wide range of concentrations of teriflunomide (from low potentially teratogenic up to high therapeutic and hepatotoxic concentrations) is important in optimizing drug therapy or drug elimination.

A variety of analytical methods have been reported to determine teriflunomide in different biological matrices (Dias *et al.*, 1995; Moskaleva *et al*., 2017; Li *et al*., 2002; van Roon *et al.*, 2004; Schmidt *et al*., 2003). Most sample preparation methods are based on liquid-liquid extraction of teriflunomide from the biological matrix with following evaporation of organic phase and its reconstitution in the mobile phase or suitable solvent. Dias *et al.* (1995) have tested several organic solvents for extraction recovery for both leflunomide and teriflunomide from a pool of whole human blood and concluded that ethyl acetate was the most suitable. The recoveries obtained for teriflunomide were in the range of 78 – 108% in human blood for concentration ranging from 400 to 100000 ng/mL (Dias *et al.*, 1995).

The sensitivity of the analytical method plays an important role in its application to pharmacokinetic as well as to therapeutic drug monitoring and elimination studies (Moskaleva et al., 2017). Traditional UV-detectors are recently replaced by highly selective and sensitive mass-spectrometry detection methods. The sensitivity of reported UV-methods for teriflunomide determination in serum samples is commonly limited to 0.5 – 1.0 µg/mL (Li *et al.*, 2002; van Roon *et al*., 2004; Schmidt *et al.*, 2003). Moreover, UV-methods are less specific and require additional sample preparation procedures, especially for complex biological matrixes. For example, a method for the simultaneous determination of leflunomide and teriflunomide in human plasma is described by Schmidt *et al.* (2003). Plasma samples were prepared by extraction with ethyl acetate using warfarin as an internal standard. Such method included a low plasma volume requirement (250 µL). However, the chromatographic analysis time was very high (22 min) and thus is not suitable for high throughput analysis. Similarly, van Roon et al. presented a simple and rapid method for teriflunomide by HPLC-UV in human serum and discussed its application for the optimization of leflunomide therapy. The assay was linear over the concentration range of 500 – 100000 ng/mL with a total chromatographic run time of 13 min (van Roon *et al*., 2004).

The use of mass-spectrometry allows to get LLOQ values for teriflunomide in human plasma all the way to 10 ng/mL and less, as well as to reduce the total chromatographic run time. A rapid and sensitive LC-MS/MS assay has been proposed for the determination of teriflunomide in

human plasma by Parekh et al. (Parekh *et al.*, 2010). The chromatographic separation was achieved on an Inertsil ODS-3 C18 (50 mm× 4.6 mm, 3 µm) analytical column following triple quadrupole mass spectrometry detection in MRM negative ion mode. The method was validated over a wide dynamic concentration range of 10.1 – 4001 ng/mL with a total run time of 2 min. Another one interesting LC-MS/MS method for the determination of teriflunomide over a 40 000-fold dynamic range using overlapping calibrators has been published by Rule et al. (Rule *et al.*, 2015). The method required a 40 000-fold dynamic range, which was achieved by dividing the curve range into 2 separate regions, but with a single extraction procedure used for both. The method was validated for use in therapeutic monitoring of leflunomide in human serum and plasma. Whereas the chromatographic run time for the proposed method is about 4 minutes, the method cannot be considered a high-throughput as it requires the preparation of 2 calibration curves and reanalysis of samples falling outside the calibration range.

The paper presents a simple, rapid, sensitive, and high throughput HPLC-MS/MS method for the determination of teriflunomide over a wide dynamic concentration range in human plasma. During the method development the authors assumed that: (a) a simple sample preparation should be used; (b) a wide dynamic concentration range should be covered without using additional calibration curves or separate calibration regions; (c) the method must be precise, robust and suitable for clinical trials applications and/or therapeutic drug monitoring purposes.

2. EXPERIMENTAL

2.1. Chemicals and materials

Teriflunomide ((Z)-2-Cyano-3-hydroxy-N-[4-(trifluoromethyl) phenyl]-2-butenamide) was purchased from Sigma Aldrich (St Louis, MO, USA). The stable isotope labeled internal standard (IS) d4-teriflunomide was purchased from Toronto Research Chemicals (Ontario, Canada). High purity water was prepared in-house using a Milli-Q water purification system obtained from Merck KGaA (Darmstadt, Germany). HPLC grade methanol, 2-propanol, and acetonitrile were purchased from Thermo Fisher Scientific Inc. (Madrid, Spain) and Panreac (Barcelona, Spain) respectively. Extra pure formic acid and ammonium formate were purchased from Fluka (Steinheim, Germany). Drug-free (blank) heparinized human plasma was obtained from healthy volunteers that had previously signed a consent form; it was stored at - 70ºC prior to use. The subject samples obtained post study conduction also kept stored at - 70ºC to maintain the integrity of the matrix.

2.2. Stock and working solutions

Stock solutions of teriflunomide and d4-teriflunomide were prepared in water: methanol (50:50, v/v) at a free base concentration of 10 mg/mL. Aliquots of all stock solutions were kept stored in brown glass vials under refrigeration at 2-4ºC before use. Primary dilutions were prepared from stock solutions by dilution with methanol: water(2:1, v/v). The secondary dilutions and subsequent working solutions were prepared as and when required using the same diluent as those for the primary dilutions. Thus, these working standard solutions prepared were used for the calibration curve and quality control samples.

2.3. Calibration and quality control samples

Teriflunomide working standard solution was used for bulk spiking of the calibration curve and quality control samples for the method validation, as well as the subject sample analysis. Screened blank plasma from six different lots with least interference and the retention time of the analyte and internal standard was used for bulk spiking. Standard calibration curves were prepared by spiking the previously screened blank plasma samples with the appropriate amount of teriflunomide standard solutions. Six quality control (QC) samples were prepared from working standard solution in a manner similar to calibration curves (Table 1).

***Table 1**. Concentration levels for quality control (QC) samples*

#	QC level	Teriflunomide nominal concentration
1	A	1.0 ng/mL
2	B	3.0 ng/mL
3	C	500 ng/mL
4	D	30 µg/mL
5	E	100 µg/mL
6	F	175 µg/mL

2.4. Sample preparation

A 10 µL aliquot of human plasma sample was mixed with 2 µL of internal standard working solution (5 µg/mL final concentration) and 988 µL of acetonitrile. The sample mixture was gently stirred for 3 min using a Vortex-Genie 2 mixer and then centrifuged at 14 000 for 5 min. The supernatant was directly transferred to vials, and 0.5 µL was injected into the HPLC-MS/MS system.

2.5. HPLC-MS/MS analysis

The analysis was performed with the Nexera X2 LC system (Shimadzu Corporation, Japan) coupled with the LCMS-8050 triple quadrupole system (Shimadzu Corporation, Japan). Sample introduction and ionization was performed in negative ion mode. The Nexera X2 LC system consisted of two LC020AD pumps, DGU-20A5R degasser, SIL-20ACXR autosampler, CTO-20AC column oven, and CBM-20A control module.

Chromatographic separation was carried out at 40ºC using an Acquity BEN C8 column 1.7 µm, 2.1 × 50 mm (Waters, USA). The mobile phases consisted of 5mM of ammonium formate aqueous solution with 0.1 % formic acid (solvent A) and acetonitrile (solvent B). The elution was performed with the following gradient program: 0 min (15 % B), 1.5 min (99 % B), 2.5 min (99 % B) and 2.6 min (15 % B). Total run time for each sample analysis was 3.0 min with a constant flow rate of 0.6 mL/min. The injection volume of the sample was 0.5 µL.

Mass spectra were obtained using negative electrospray ionization and the multiple reaction-monitoring modes (MRM). Nitrogen was used as the collision gas. MS operating parameters were optimized as follows: desolvation temperature: 250ºC; heat block temperature: 400ºC; interface temperature: 300ºC; nebulizer gas flow rate: 3 L/min; heating gas flow rate: 10 L/min and drying gas flow rate: 10 L/min.

2.6. Method validation

The proposed method was validated for selectivity, linearity, precision, accuracy, matrix effect and stability according to US FDA and EMA guidelines for bioanalytical method validation (Food and Drug Administration, 2001; EMA, 2012). Selectivity was verified by analyzing the blank plasma samples obtained from six different sources to test for interference at the retention time of teriflunomide, as well as the standard internal d4-teriflunomide. These six sources included normal controlled plasma lots and two controlled plasma lots each of haemolysed and lipemic plasma containing heparin as the anticoagulant.

A method with a wide dynamic concentration range requires accurate and precise calibration curves. Six calibration concentration levels were used in addition to the blank sample and zero samples. All calibrators were distributed evenly over an analytical range of 1 ng/mL – 200 µg/mL. Each calibrator was analyzed in triplicate for three consecutive days. Regression analysis, including the goodness of fit test and lack of fit test, was performed for each calibration curve. All calibration curves were verified using the back-calculated concentrations of the calibration standards.

Six replicates of quality control samples (QCs) were analyzed within one batch for within-day accuracy and precision and on three different days for between-day accuracy and precision evaluation. Accuracy was defined as the percent relative error (%RE) and precision as the relative standard deviation (RSD).

The matrix factor (MF) was calculated both for teriflunomide and the internal standard (IS) using at least six lots of blank matrix from individual donors. MF was calculated as the ratio of the peak in the presence of matrix to the peak area in the absence of matrix (pure solution of the analyte). Additionally, the IS normalized MF was calculated by dividing the MF of teriflunomide by the MR of the IS. The determination was performed for all QCs levels.

A stability study was performed. Teriflunomide was considered stable if the recovery of the mean test responses were within 15% of appropriate controls. The stability of spiked human plasma kept at room temperature of about 25ºC (bench-top stability) was evaluated for 24 h. The sample stability of the process was evaluated by comparing the extracted (precipitated) plasma samples that were injected immediately with the same samples that were re-injected after being kept in the autosampler at 6 ºC for 12 h. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen and thawed five times with freshly spiked quality control samples. Six aliquots of all QCs levels were used for stability studies evaluation.

2.7. Method application to plasma samples derived from patients under leflunomide treatment

The developed method was applied to determine the plasma concentrations of teriflunomide within the framework of a clinical trial study, where each of the 24 healthy male volunteers under fasting conditions received a tablet containing 20 mg of leflunomide orally. Five milliliters of venous blood samples were withdrawn from each volunteer and transferred into heparinized tubes according to the following time schedule: prior dosing (0) and at 2h, 4h, 8h, 12h, 24h, 48h, 72h, 96h, 120h, 168h, 216h, 264h, 336h, 432h, 528h, 624h and 720h post-dose. All blood samples were centrifuged immediately at 5000 rpm for 10 min to obtain plasma. Plasma samples were labeled and kept frozen at -35ºC until analysis.

All volunteers were adult Caucasian men aged from 18 to 45 years who were selected after completing a thorough medical, biochemical, and physical examination. Informed consent was obtained from all subjects after explaining the aims and risks of the study. The study protocol was approved by the Human Investigation Ethics Committee at the Sechenov First Moscow State Medical University, Moscow, Russia.

2.8. Standardization

Each batch of the human leflunomide treatment study samples was analyzed along with one blank plasma sample (matrix processed without IS), one zero sample (matrix processed with IS), calibration standards, and QCs in triplicates worked up the same way as study samples. HPLC–MS/MS data analysis was conducted using Lab Solutions version 5.8.6 software (Shimadzu Corporation, Japan).

For quantification, the peak area ratio of the target ion of the teriflunomide to those of the internal standard was compared with weighted ($1/x^2$) least squares calibration curve in which the peak area ratios of the calibration standards were plotted versus their concentrations.

3. RESULTS AND DISCUSSION

3.1. Method development

The goal of this work was to develop and validate a simple, rapid, and sensitive bioanalytical method for teriflunomide determination in human plasma over a wide dynamic concentration range. In order to achieve this goal, the sample preparation, detection parameters, and chromatographic conditions were optimized. Electrospray ionization (ESI) was conducted in negative ionization mode as teriflunomide has high electron affinity due to the presence of the trifluoromethyl group. Full scan spectra for teriflunomide and IS predominantly contained deprotonated precursor $[M-H]^-$ ions at *m/z* 269 and *m/z* 273 respectively. The most abundant and consistent product ions for teriflunomide were observed at m/z 82 and m/z 160 while the most abundant and consistent product ions for IS were observed at m/z 80 and m/z 164.

Furthermore, optimization of chromatographic conditions by using the combination of an Acquity UPLC BEN C8 column and a mobile phase containing 5 mM of ammonium formate aqueous solution with 0.1% formic acid allowed achieving better separation of analytes and provided a low background chromatogram. The flow-rate was optimized at 0.6 mL/min to reduce the total run time for each sample up to 3.0 min. The retention time for teriflunomide was1.9 min. MRM transitions, fragmentor voltage, collision energy (CE), and retention times of teriflunomide, and internal standard are summarized in Table 2. The product ion spectrum of teriflunomide is presented in Figure 2.

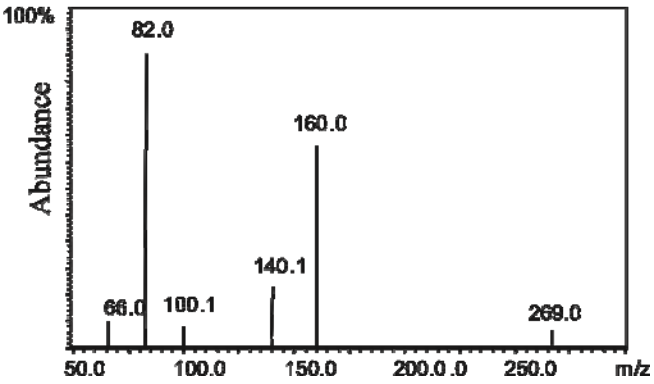

Figure 2. *Product ion spectrum (negative ESI) of teriflunomide*

3.2. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank plasma with the chromatograms from the same plasma samples spiked with teriflunomide (1.0 ng/mL) and IS (5 µg/mL). The proxy measure

of chromatograms obtained from blank plasma, plasma spiked at LLOQ, and the real clinical trial sample is presented in Figure 3. No interfering peaks of endogenous compounds were observed at the retention time of teriflunomide or the internal standard in blank human plasma containing heparin as anticoagulant from six different lots of normal controlled plasma, including lipemic and hemolyzed ones.

3.3. Carry-over effect

The carry-over effect is among the most problematic issues in bioanalysis. It can be observed due to the adherence of plasma phospholipids with the sampling device and the subsequent absorption of the drug. In order to eliminate the possible carry-over effect, three types of needle wash solvents were used. 50 % of 2-propanol was used as the primary needle rising solvent. The following rising was performed using the mixture of acetonitrile:2-propanol: water: formic acid (700/200/100/2, v/v). And finally, the needle was equilibrated with the mobile phase solution.

Carry-over was assessed by injecting blank samples after a high concentration sample (calibration standard at the upper limit of quantification). Carry-over in blank samples following the high concentration standard was not higher than 20% of the lower limit of quantification (LLOQ) and 5% for the internal standard.

3.4. Linearity and calibration

The peak area ratios of calibration standards were proportional to the concentration of teriflunomide in each assay over the analytical range of 1.0 ng/mL – 200 µg/mL. Calibration curves obtained at three consecutive days were linear and well described by least squares lines. A weighting factor of $1/x^2$ was applied to achieve the homogeneity of variance. The correlation coefficients (r^2) for teriflunomide were above 0.99. The back-calculated concentrations for calibration standards were within 15% of their nominal values. Moreover, additional calibration curves for teriflunomide concentrations (1.0 – 1000 ng/mL) and (1.0 – 200 µg/mL) were constructed. Precision and accuracy for all calibration curves were evaluated and compared using QCs. Calibration curve parameters are summarized in Table 3.

3.5. The lower limit of quantification

The lower limit of quantification (LLOQ) was determined as the lowest standard of the calibration curve that can be quantitatively determined with acceptable precision and accuracy. Thereby, the LLOQ for teriflunomide was 1.0 ng/mL. The response of analyte at the LLOQ was at least 5 times the response compared to blank response, and the peak of analyte was identifiable, discrete and reproducible with a precision of 20% and accuracy of 80–120%.

3.6. Precision and Accuracy

Precision and accuracy of the assay were determined by performing the complete analytical runs of replicate QC samples (n = 6) on the same day and within the three consecutive days. The data from these QC samples were examined by one-way analysis of variance (ANOVA). Within and between-run variability data are summarized in Table 4 and Table 5.

The mean concentration of teriflunomide was within 15% of the nominal values; the RSD values did not exceed 15% for the QC samples, respectively both for within- and between-run. The above results demonstrated that the method was accurate and precise, and this data were well in line with the FDA guidance requirements. Finally, the full-scale calibration curve (1.0 ng/mL – 200 µg/mL) was used for the developed method, since no significant difference between the accuracy and precision for QA samples analysis was observed using additional calibration curves.

3.7. Matrix effect

To evaluate the matrix effect in the experiment, chromatographic peak areas of teriflunomide from the spike after extraction samples were compared to the neat standards at the same concentrations. Percent nominal concentrations estimated were within the acceptable limits (99.2 –101.2%) after evaluating six different lots of plasma (Table 6). The same evaluation was performed on IS, and no significant peak area differences were observed. Thus, ion suppression or enhancement from plasma matrix was negligible for this method.

Table 6. *Matrix effect in human plasma (n = 6)*

Concentration	Ion suppression (%)	
	Mean	RSD (%)
1.0 ng/mL	101.2	1.2
3.0 ng/mL	99.2	1.1
500 ng/mL	100.1	2.1
30 µg/mL	99.5	0.9
100 µg/mL	99.4	3.1
175 µg/mL	99.8	2.5

3.8. Stabilities

The results of stability experiments showed the absence of significant degradation during chromatography, extraction, and sample storage processes for teriflunomide plasma samples. Benchtop and process stabilities were investigated for all QC levels. The results revealed that the analyte remained stable in plasma for 24 h at room temperature of about 25ºC and 12 h in the autosampler at 6ºC. It was also confirmed that repeated freeze and thawing (five cycles) of plasma samples spiked with analyte did not affect the stability of teriflunomide. Stability data are summarized in Table 7.

3.9. Method application to plasma samples derived from patients under leflunomide treatment

The validated HPLC-MS/MS method was applied to plasma samples derived from patients under leflunomide treatment. 24 healthy male volunteers received orally a tablet containing 20 mg of leflunomide. The mean plasma concentration-time profile is presented in Figure 4. Plasma concentrations of teriflunomide could be determined up to 720 h after a single dose administration of 20 mg teriflunomide. This simple and selective method for the determination of teriflunomide in human plasma was applicable for drug monitoring and pharmacokinetic purposes. The pharmacokinetic curve parameters are shown in Table 8.

4. CONCLUSION

A simple, rapid, sensitive, and high-throughput HPLC-MS/MS method for the determination of teriflunomide over a wide dynamic concentration range in human plasma has been developed and validated. Simple sample preparation and short (3.0 min) sample analysis run time allow applying the proposed method for teriflunomide therapeutic drug monitoring as well as for pharmacokinetic studies. The proposed method was proven to be a robust tool; it also has been successfully applied to plasma samples derived from patients under leflunomide treatment.

5. ACKNOWLEDGEMENTS

The study was supported by the "Russian Academic Excellence Project 5-100".

6. REFERENCES

1. Fox, R. I. Mechanism of action of leflunomide in rheumatoid arthritis. *Journal of Rheumatology*, **1998,** 25, 20–26. https://doi.org/10.1006/clim.1999.4777/
2. Cassina, M., Johnson, D. L., Robinson, L. K., Braddock, S. R., Xu, R., Jimenez, J. L., Chambers, C. D. Pregnancy outcome in women exposed to leflunomide before or during pregnancy. *Arthritis and Rheumatism*, **2012,** 64(7), 2085–2094. https://doi.org/10.1002/art.34419
3. Dias, V. C., Lucien, J., LeGatt, D. F., Yatscoff, R. W. Measurement of the active leflunomide metabolite (A77 1726) by reverse-phase high-performance liquid chromatography. *Therapeutic Drug Monitoring*, **1995**, 17(1), 84–88.
4. Dougados, M., Emery, P., Lemmel, E. M., Zerbini, C. F., Brin, S., Riel, P. van. When a DMARD fails, should patients switch to sulfasalazine or add sulfasalazine to continuing leflunomide? *Annals of the Rheumatic Diseases*, **2005,** 64(1), 44–51. https://doi.org/10.1136/ard.2003.016709
5. EMA. Guideline on bioanalytical method validation. *EMA Guideline*, **2012,** 3–23. https://doi.org/EMEA/CHMP/EWP/192217/2009
6. Food and Drug Administration. FDA Bioanalytical Method Validation. U.S. *Department of Health and Human Services*, **2001**, May, 4–10. https://doi.org/http://www.labcompliance.de/documents/FDA/FDA-Others/Laboratory/f-507-bioanalytical-4252fnl.pdf
7. Grabar, P. B., Grabnar, I., Rozman, B., Logar, D., Tomšič, M., Šuput, D., Dolžan, V. Investigation of the influence of CYP1A2 and CYP2C19 genetic polymorphism on 2-

cyano-3-hydroxy-N-[4-(trifluoromethyl)phenyl]-2-butenamide (A77 1726) pharmacokinetics in leflunomide-treated patients with rheumatoid arthritis. Drug Metabolism and Disposition, **2009,** 37(10), 2061–2068. https://doi.org/10.1124/dmd.109.027482

8. Hopkins, A. M., O'Doherty C. E, Foster D. JR, Upton R. N, Proudman S. M, Wiese M. D. Individualization of leflunomide dosing in rheumatoid arthritis patients. *Personalized Medicine*, **2014,** 11(4), 449-461. https://doi.org/10.2217/pme.14.23
9. Li, J., Yao, H. W., Jin, Y., Zhang, Y. F., Li, C. Y., Li, Y. H., Xu, S. Y. Pharmacokinetics of leflunomide in Chinese healthy volunteers. *ActaPharmacol Sin*, **2002**, 23(6), 551–555. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12060531
10. Moskaleva, N. E., Baranov, P. A., Mesonzhnik, N. V., Appolonova, S. A. HPLC-MS/MS method for the simultaneous quantification of desmethylmebeverine acid, mebeverine acid and mebeverine alcohol in human plasma along with its application to a pharmacokinetics study. *Journal of Pharmaceutical and Biomedical Analysis*, **2017**, 138, 118–125. https://doi.org/10.1016/j.jpba.2017.02.006
11. Parekh, J. M., Vaghela, R. N., Sutariya, D. K., Sanyal, M., Yadav, M., Shrivastav, P. S. Chromatographic separation and sensitive determination of teriflunomide, an active metabolite of leflunomide in human plasma by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B*, **2010**, 878(24), 2217–2225. https://doi.org/10.1016/j.jchromb.2010.06.028
12. Rakhila, H., Rozek, T., Hopkins, A., Proudman, S., Cleland, L., James, M., Wiese, M. Quantitation of total and free teriflunomide (A77 1726) in human plasma by LC-MS/MS. *Journal of Pharmaceutical and Biomedical Analysis*, **2011,** 55(2), 325–331. https://doi.org/10.1016/j.jpba.2011.01.034
13. Rozman, B. Clinical pharmacokinetics of leflunomide. Clinical Pharmacokinetics, **2002,** 41(6), 421–30. https://doi.org/10.2165/00003088-200241060-00003
14. Rule, G. S., Rockwood, A. L., Johnson-Davis, K. L. LC–MS/MS Method for Determination of Teriflunomide, Over a 40,000-Fold Dynamic Range Using Overlapping Calibrators. *Therapeutic Drug Monitoring*, **2015**, 37(4), 472–478. https://doi.org/10.1097/FTD.0000000000000166
15. Schmidt, A., Schwind, B., Gillich, M., Brune, K., Hinz, B. Simultaneous determination of leflunomide and its active metabolite, A77 1726, in human plasma by high-performance liquid chromatography. *Biomed Chromatogr*, **2003,** 17(4), 276–281. https://doi.org/10.1002/bmc.244
16. Sobhani, K., Garrett, D. A., Liu, D. P., Rainey, P. M. A rapid and simple high-performance liquid chromatography assay for the leflunomide metabolite, teriflunomide (A77 1726), in renal transplant recipients. *American Journal of Clinical Pathology*, **2010**, 133(3), 454–457. https://doi.org/10.1309/AJCPR23YAOYFSZTX
17. Strand, V., Cohen, S., Schiff, M., Weaver, A., Fleischmann, R., Cannon, G., Loew-Friedrich, I. Treatment of active rheumatoid arthritis with leflunomide compared with placebo and methotrexate. Leflunomide Rheumatoid Arthritis Investigators Group. *Arch Intern Med*, **1999**, 159(21), 2542–2550. https://doi.org/10.1001/archinte.159.21.2542
18. van Roon, E. N., Yska, J. P., Raemaekers, J., Jansen, T. L. T. A., Van Wanrooy, M., Brouwers, J. R. B. J. A rapid and simple determination of A77 1726 in human serum by high-performance liquid chromatography and its application for optimization of leflunomide therapy. *Journal of Pharmaceutical and Biomedical Analysis*, **2004**, 36(1), 17–22. https://doi.org/10.1016/j.jpba.2004.05.019
19. Weinblatt, M. E., Kremer, J. M., Coblyn, J. S., Maier, a L., Helfgott, S. M., Morrell, M., Strand, V. Pharmacokinetics, safety, and efficacy of combination treatment with methotrexate and leflunomide in patients with active rheumatoid arthritis. *Arthritis and Rheumatism*, **1999,** 42(7), 1322–8. https://doi.org/10.1002/1529-0131(199907)42:7<1322::AID-ANR4>3.0.CO;2-P

Table 2. *HPLC and MS parameters*

Analyte	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)
Teriflunomide	1.9	269	160 82	11 11	25 21	15 30
D4-teriflunomide	1.9	273	164 80	11 11	25 21	15 30

Table 3. *Calibration curve parameters for teriflunomide.*

Linear range	Slope (Mean ± SD)	Intercept (Mean ± SD)	R2 (Mean ± SD)
1.0 ng/mL – 1000 ng/mL	0.994 ± 0.012	0.0001 ± 0.004	0.9988 ± 0.0007
1.0 µg/mL – 200 µg/mL	0.988 ± 0.002	0.0005 ± 0.016	0.9992 ± 0.0005
1.0 ng/mL – 200 µg/mL	0.988 ± 0.001	0.0002 ± 0.063	0.9968 ± 0.0004

Table 4. *Within-run (n = 6) precision and accuracy evaluated using low-range and full-scale calibration curves.*

QC	Spiked conc.	Calibration curve 1.0 ng/mL – 200 µg/mL			Calibration curve 1000 ng/mL - 200 µg/mL			Calibration curve 1.0 ng/mL – 1000 ng/mL		
		Calc. conc. (Mean ± SD)	RSD (%)	Relative error (%)	Calc. conc. (Mean ± SD)	RSD (%)	Relative error (%)	Calc. conc. (Mean ± SD)	RSD (%)	Relative error (%)
A	1.0 ng/mL	1.02 ± 0.04	4.32	2.25	---	---	---	0.95 ± 0.02	2.04	-5.02
B	3.0 ng/mL	3.04 ± 0.11	3.37	1.42	---	---	---	2.96 ± 0.04	1.28	-1.00
C	500 ng/mL	496.77 ± 3.82	0.77	-0.64	---	---	---	496.47 ± 2.32	0.47	-0.71
D	30 µg/mL	30.49 ± 0.43	1.41	1.66	29.85 ± 1.27	4.27	-0.49	---	---	---
E	100 µg/mL	99.63 ± 2.34	2.35	-0.37	100.58 ± 1.96	1.94	0.58	---	---	---
F	175 µg/mL	175.15 ± 2.62	1.50	0.08	174.21 ± 1.25	0.71	-0.45	---	---	---

Table 5. *Between-run (n = 18) precision and accuracy evaluated using low-range and full-scale calibration curves*

QC	Spiked conc.	Calibration curve 1.0 ng/mL – 200 µg/mL			Calibration curve 1000 ng/mL - 200 µg/mL			Calibration curve 1.0 ng/mL – 1000 ng/mL		
		Calc. conc. (Mean ± SD)	RSD (%)	Relative error (%)	Calc. conc. (Mean ± SD)	RSD (%)	Relative error (%)	Calc. conc. (Mean ± SD)	RSD (%)	Relative error (%)
A	1.0ng/mL	1.01 ± 0.07	6.31	1.58	---	---	---	1.05 ± 0.07	6.35	5.00
B	3.0 ng/mL	3.03 ± 0.10	3.30	0.83	---	---	---	2.98 ± 0.30	9.30	-0.47
C	500 ng/mL	498.43 ± 6.01	1.21	-0.31	---	---	---	502.36 ± 6.02	1.19	0.47
D	30 µg/mL	29.92 ± 2.64	8.80	-0.26	30.39 ± .46	4.81	1.31	---	---	---
E	100 µg/mL	101.16 ± 3.05	3.02	1.16	101.11 ± 4.30	4.24	1.11	---	---	---
F	175 µg/mL	174.79 ± 2.31	1.32	-0.12	176.26 ± 2.63	1.49	0.72	---	---	---

Table 7. *Stability in human plasma*

Five freeze-thaw cycles (n = 6), human plasma samples			
QC	Concentration	Bias (%)	RSD (%)
A	1.0 ng/mL	-0.56	6.15
B	3.0 ng/mL	2.28	5.46
C	500 ng/mL	-2.11	4.53
D	30 µg/mL	1.57	4.70
E	100 µg/mL	-6.58	4.15
F	175 µg/mL	-5.80	4.86
Stored at room temperature for 24 h (n = 6), human plasma samples			
QC	Concentration	Bias (%)	RSD (%)
A	1.0 ng/mL	-3.89	5.10
B	3.0 ng/mL	-1.67	5.96
C	500 ng/mL	-6.78	1.76
D	30 µg/mL	4.90	1.73
E	100 µg/mL	-9.62	0.86
F	175 µg/mL	-10.79	6.55
12 h at 6ºC in the auto sampler (repeated analysis, n = 6)			
QC	**Concentration**	**Bias (%)**	**RSD (%)**
A	1.0 ng/mL	-1.12	3.17
B	3.0 ng/mL	-0.97	4.55
C	500 ng/mL	1.31	4.79
D	30 µg/mL	-2.61	5.05
E	100 µg/mL	-4.18	3.42
F	175 µg/mL	-0.54	3.47

Table 8. *Pharmacokinetic parameters of teriflunomide in human plasma (data are Mean ± SD, n = 24)*

Parameter	Teriflunomide
C_{max} (ng/ml)	1911.19 ± 401.3
t_{max} (h)	2.52 ±0.9
$AUC_{0\rightarrow t}$ (ng×h/ml)	331822 ± 51607
$AUC_{0\rightarrow \infty}$ (ng×h/ml)	402292 ± 70359
Kel (h^{-1})	0.0022 ± 0.0005
MRT (h)	416 ± 55
$T_{1/2}$	326.98 ± 73.35
$C_{max}/AUC_{0\rightarrow t}$ (h^{-1})	0.0057±0.0005
$C_{max}/AUC_{0\rightarrow \infty}$ (h^{-1})	0.0047± 0.0004

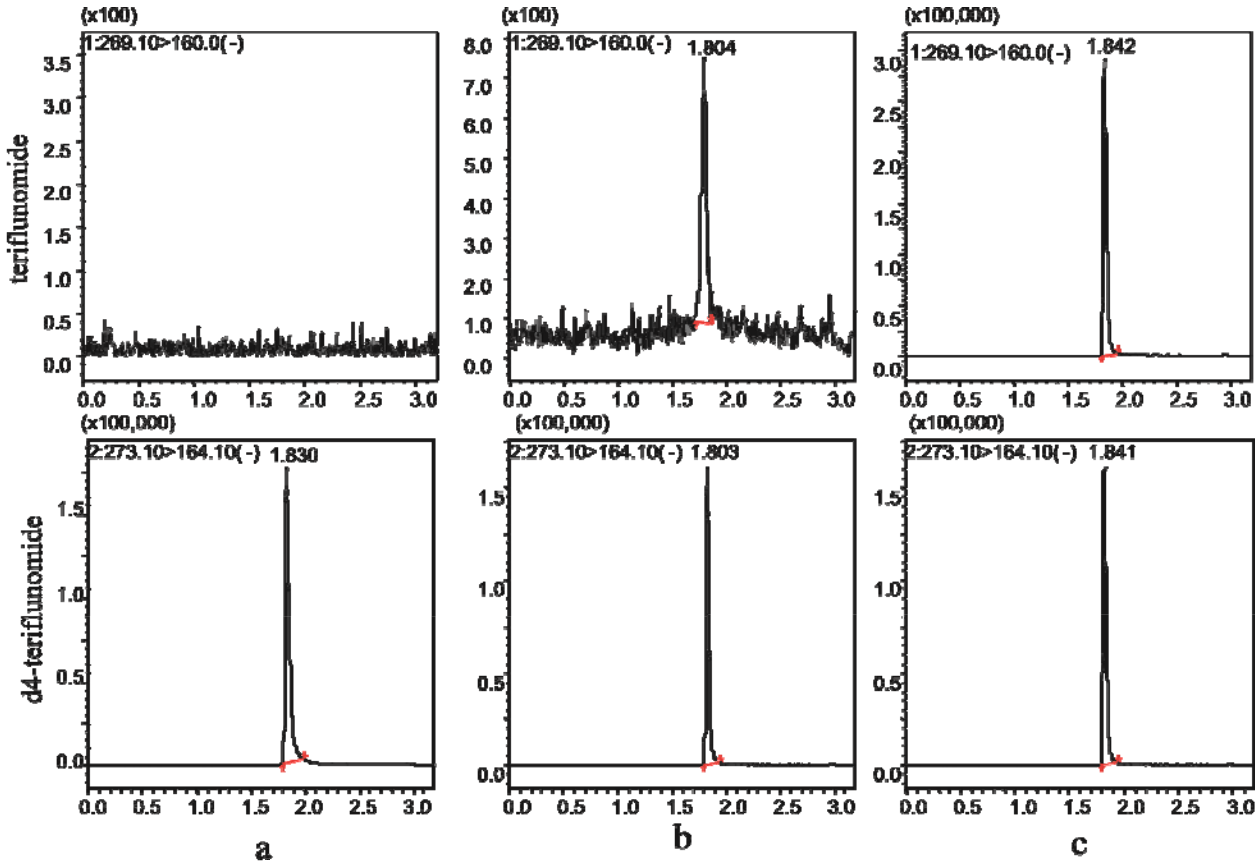

Figure 3. *Typical MRM chromatograms of teriflunomide and d4-teriflunomide (internal standard): (a) blank plasma; (b) the lowest calibration sample (LLOQ); and (c) a plasma sample obtained from a patient after leflunomide therapy*

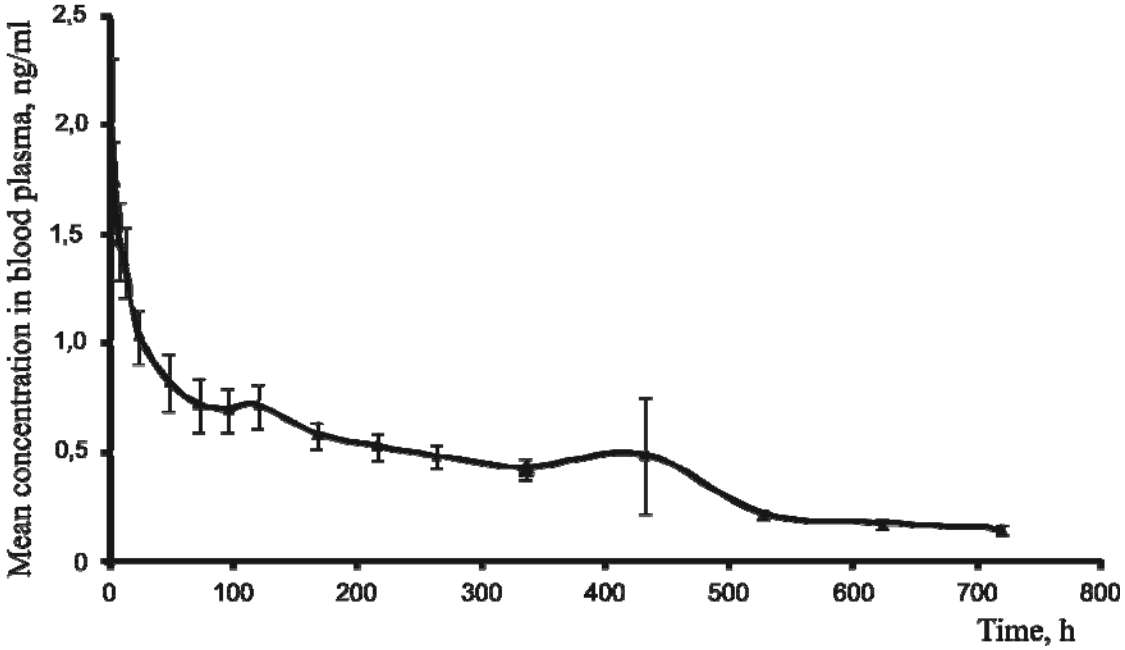

Figure 4. *The mean plasma concentration-time profile for teriflunomide after oral administration of a tablet containing leflunomide (20 mg) to 24 healthy volunteers (the data means ± SD)*