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ARTICLES

A Procedure for Determining Dexketoprofen Trometamol in Human Plasma and Its Validation

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Abstract—A procedure for the determination of dexketoprofen trometamol in human plasma by liquid chromatography–mass spectrometry (HPLC–MS/MS) using ibuprofen as an internal standard was proposed and validated. The matrix effect on the analyte response value was estimated; its short-term and long-term stability in a biological matrix and an aqueous acetonitrile solution was investigated. In addition, the stability of the analyte after freezing and thawing of samples was studied. It was shown that the dilution of samples by one half does not affect the accuracy and precision of the analysis. The limit of detection and the lower limit of quantification were 0.01 μg/mL; the linearity range was 0.01–8.50 μg/mL ($R^2 = 0.9974$); the total analysis time was 3.5 min. Intra- and interday accuracy values were in the ranges 96.66–100.00% and 94.97–97.92%, respectively. Sample preparation, including liquid–liquid extraction with ethyl acetate in an acidic medium, is simple and fast. The developed procedure was successfully tested on real plasma samples from healthy volunteers in the framework of a comparative study of the pharmacokinetics and bioequivalence of a generic drug.

Keywords: dexketoprofen trometamol, ibuprofen, HPLC–MS/MS, validation, bioanalytical technique, human plasma

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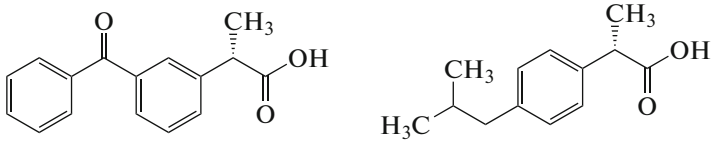
Nonsteroidal anti-inflammatory drugs (NSAIDs) form a group of pharmaceuticals exhibiting pronounced analgesic, antipyretic, and anti-inflammatory properties. The mechanism of their action is associated with the nonselective inhibition of enzymes of the cyclooxygenase group, involved in the biosynthesis of prostaglandins PGE1, PGE2, PGF1, and PGF2 together with thromboxanes A2 and B2 from arachidonic acid [1]. The most numerous representatives of NSAIDs are derivatives of various organic acids, in particular, propionic acid. This group includes drugs containing dexketoprofen (DKP, (2*S*)-2-(3-benzoylphenyl)propanoic acid, Scheme 1, structure I) as an active ingredient, ibuprofen (IBP, (*RS*)-2-(4-isobutylphenyl)propanoic acid, Scheme 1, structure II), ketoprofen ((*RS*)-2-(3-benzoylphenyl)propanoic acid), and several other compounds.



I II

Scheme 1. Chemical structures of (I) dexketoprofen and (II) ibuprofen.

Ketoprofen is a racemic mixture of two enantiomers of 2-(3-benzoylphenyl)propanoic acid. However, only the *S*(+) enantiomer, dexketoprofen, has an inhibitory effect on the isoenzymes COX-1 and COX-2. There is currently a trend to replacing racemic drugs with their pure enantiomeric forms. It helps to reduce the dose of the drug required for the achievement of the desired therapeutic effect, take down the incidence of adverse reactions, and also avoid the negative effect caused by the *R*(–) enantiomer and its metabolites [2].

The pure biologically active form isolated from the racemate also has a higher bioavailability [3].

Chemoreactomic analysis shows [4] that dexketoprofen is accumulated mainly in adipose tissue, muscles, and adrenal glands. The anti-inflammatory and analgesic effect of the drug can be due to the modulation of the metabolism of not only prostaglandins but also leukotrienes and enkephalins as well as the inhibition of several matrix metalloproteinases and glutamate receptors. The authors [4] point to the possible antiplatelet, vasodilatory, antitumor, and antidiabetic properties of DKP.

The elimination of DKP includes processes of its biotransformation, occurring in the liver, by conjugation with glucuronic acid (mainly, acylglucuronide) and subsequent excretion from the body by the kidneys in the form of various metabolites, mainly represented by hydroxyl derivatives [5]. However, in humans, hydroxylation plays a minor role [6]. The main metabolic pathways of DKP involve at least two isoenzymes of cytochrome P450: CYP2C8 and CYP2C9 [7]. It was reported that the half-life and clearance of the drug in healthy people after a single oral administration were 1.05 ± 0.04 h and 0.089 ± 0.004 L^{-1} h^{-1} kg^{-1}, respectively [8]; binding to plasma proteins was 99.2% [6].

In dosage forms, DKP is used as a water-soluble trometamol salt (dexketoprofen trometamol), available as coated tablets or solutions for parenteral (intramuscular and intravenous) administration.

Data were published on the identification of DKP by reversed-phase HPLC (RP–HPLC) with UV detection. However, most of the papers were devoted to the determination of the drug in nonbiological matrices [2, 9–15]; only a few publications reported the determination of DKP in urine or plasma of animals and humans [16–18]. The described methods of sample preparation are rather laborious, and the information given on the analytical procedures used is insufficient for their complete validation according to the requirements of the European Medicines Agency (EMA) [19] and the State Pharmacopoeia of the Russian Federation XIV [20]. Barbanoj et al. [16] determined the lower limit of quantification (LLQ) for DKP trometamol in human plasma and urine samples as 0.01 μg/mL by RP–HPLC/UV. The obtained LLQ value is comparable to the one in this paper. However, the validation parameters of the procedure were not reported, and the used method of sample preparation (liquid–liquid extraction (LLE) with ethyl acetate in an acidic medium followed by evaporating the organic fraction under nitrogen) was rather laborious [16]. We have shown that the LLE with ethyl acetate with addition of 0.6 M H_2SO_4 is sufficient for the achievement of the indicated LLQ value. This significantly shortens the analysis time in working with a large number of samples. Also, mass spectrometric (MS) detection offers many advantages over spectrometric detection in the UV region. In particular, it is characterized by higher selectivity. This is essential since proteins of the studied matrix are often coextracted along with the analyte during the biosamples preparation. Fengci et al. [17] validated the procedure for determining dexketoprofen trometamol using rabbit plasma, which has different biochemical parameters compared to the human plasma. Song et al. [18] achieved the LLQ value of 0.01 μg/mL using the HPLC–MS method in the selected ion monitoring mode of negative ions. This mode is characterized by insufficient specificity for analytes in complex biological matrices. This problem can be solved using the multiple reaction monitoring (MRM) mode, which ensures acceptable background cutoff efficiency.

The aim of this work was to develop and validate a procedure for the determination of dexketoprofen trometamol in human plasma by RP–HPLC coupled with tandem mass spectrometry (MS/MS), including the optimization of sample preparation conditions according to the criteria of rapidity and sensitivity.

EXPERIMENTAL

Reagents. We used standard reference samples of dexketoprofen trometamol (Zhejiang Raybow Pharmaceutical, China) and ibuprofen (Sigma-Aldrich, United States). Ibuprofen was used as an internal standard (IS). Both substances had a purity of at least 99.96%. Ethyl acetate (cp grade, EKOS-1, Russia) was used to extract the analytes from plasma. Chromatographic analysis was carried out using acetonitrile (grade 0, high-purity grade, Kriokhrom, Russia) and formic acid (98%, Sigma-Aldrich, United States). The solutions were prepared using twice-distilled water obtained by purification in a Fistreem Cyclon 044 system (United Kingdom) followed by double distillation with the addition of sulfuric acid (cp grade) and potassium permanganate (cp grade). To construct calibration curves and test the procedure, we used samples of intact plasma (chemically untreated and free of any foreign substances, except for anticoagulants) of volunteers, which were stored at –32°C.

Equipment. The analytical system included a complex based on an LC-20 Prominence liquid chromatograph (Shimadzu, Japan), a SIL-20A autosampler, an CTO-20A column oven, an AB Sciex QTRAP 3200 tandem mass spectrometer (AB Sciex, United States) with a heated electrospray ionization source, and a Phenomenex Luna $C_{18(2)}$ chromatographic column (50 × 2 mm, 3 μm, 100 Å) with a PerfectSil Target ODS-3 HD precolumn cartridge (10 × 4.6 mm, 3 μm). Sample preparation was carried out using a Microlab STARlet robotic system (Hamilton, Switzerland). For mixing the samples, we used an MSV-3500 universal tube vortex (Biosan, Latvia) with the further separation of the samples into precipitate and supernatant phases in an SL 16R multifunctional cen-

trifuge (Thermo Scientific, United States). Analyst 1.6.3 and MultiQuant 2.1 software was used to collect and process chromatographic data, respectively.

Preparation of stock solutions. Stock solutions of DKP (c = 1000 μg/mL) and IBP (c = 150 μg/mL) were prepared by dissolving their accurately weighed portions in specified volumes of a $CH_3CN–H_2O$ mixture (1 : 1, by volume). Then, the solutions were transferred into dark glass vials and stored in refrigerating chambers at –32 and 4°C, respectively. Working solutions of DKP with concentrations of 500, 50, and 5 μg/mL were prepared by serial dilutions of the stock solution with 50% acetonitrile. From them, ten calibration solutions were prepared in the concentration range 0.2–170.0 μg/mL. The solutions were stored at 4°C in dark glassware.

To characterize the precision of analysis, we prepared several quality control solutions with high (150.0 μg/mL), medium (15.0 μg/mL), and low (0.6 μg/mL) concentrations of DKP. Solutions for determining the upper and lower limits of quantification with the concentrations 0.2 and 170.0 μg/mL, respectively, were also prepared. The concentration of the dexketoprofen solution used to assess the effect of dilution was 300.0 μg/mL.

Preparation of calibration, quality control, zero, and blank samples in plasma. Before analysis, samples of intact plasma were thawed at room temperature. Portions of 285 μL were then placed in 2-mL Eppendorf tubes, and 15 μL of a standard calibration or quality control solution of DKP with the known concentration was added. To prepare zero (contain only internal standard) and blank (do not contain analyte or internal standard) samples, 15 μL of 50% acetonitrile was added to the plasma. The concentration range of DKP in the calibration samples was 0.01–8.50 μg/mL. The concentrations of dexketoprofen in quality control (QC) samples were 7.50 μg/mL for high QC, 0.75 μg/mL for medium QC, 0.03 μg/mL for low QC; 0.01 μg/mL for the LLQ, and 8.50 μg/mL for the upper limit of quantification (ULQ). The concentration of DKP used to evaluate the effect of sample dilution in plasma was 15.00 μg/mL.

Sample preparation. In the course of sample preparation, 100 μL of the IS (IBP, c = 150 μg/mL), 100 μL of 0.6 M H_2SO_4, and 800 μL of ethyl acetate were added to the calibration, QC, and zero samples in plasma with a total volume of 300 μL; 100 μL of 50% acetonitrile was added to blank samples instead of IBP. The mixture was stirred in a vortex shaker at 2500 rpm for 10 min and centrifuged at 10 000g for 8 min. After that, 500 μL of the upper organic phase was collected for further analysis under the conditions described below.

The procedure was validated according to the recommendations of [19, 20].

The recovery of an analyte from the biological matrix was preliminarily assessed using calibration samples with concentrations of 0.05, 1.00, and 5.00 μg/mL. For this purpose, 285 μL of twice-distilled water (simulating the volume of added plasma), 100 μL of 0.6 M H_2SO_4, 100 μL of IBP, and 800 μL of ethyl acetate were added to 15 μL of the corresponding calibration sample. After thorough mixing and centrifugation, the aqueous and organic phases were separated, and the latter was analyzed by HPLC–MS/MS. The average analyte concentration in the extract was 97.63 ± 3.86%, which indicates an almost complete extraction of DKP with ethyl acetate. As human plasma is 90–92% water, its replacement with a corresponding volume of twice-distilled water is legitimate.

The analytical range of the procedure was estimated in the range of analyte concentrations from 0.01 to 8.50 μg/mL. The calibration curves were plotted as the ratio of the DKP peak area to the IBP peak area on the concentration of the calibration sample. The curves were then processed as linear using regression analysis (a quadratic function with a weighting factor of $1/x^2$). Deviations of back-calculated concentrations for at least 75% of the calibration samples should not exceed ±15% of their nominal values (except for the case of LLQ samples when ±20% deviations are acceptable). The curves should also be characterized by the coefficient of determination ($R^2 \geq 0.9900$).

The accuracy and precision of the procedure were assessed by analyzing quality control samples at the levels of LLQ and the low, medium, and high concentrations (six samples for each level). To assess interday accuracy, samples with different concentrations of DKP in plasma were analyzed, prepared within three different cycles; intraday accuracy was assessed within one cycle in the investigated calibration range.

The accuracy of the back-calculated concentrations for the QC samples should not go beyond the range of 85–115% (except for the LLQ samples, for which these values may be in the range of 80–120%). At least 67% of quality control samples and at least 50% of calibration samples should follow this criterion. The value of the variation coefficient of concentrations for the QC samples within one cycle and between the cycles should not exceed 15%; and for the LLQ samples, it should not exceed 20%. For each concentration of the QC samples, the mean values of the accuracy of all acceptable cycles were calculated.

The lower limit of quantification was determined by the value of the signal-to-noise ratio for the DKP peak at the LLQ level by comparing the average analyte response in the six treated calibration samples with the lowest concentration and the average noise level at zero level around the DKP retention time in six matrix blank samples. The LLQ value should correspond to a signal-to-noise ratio ≥10.0 and enable the determination of the standard analyte sample concentration with a variation coefficient ≤20%. Moreover, four of the six values of the LLQ should satisfy an accuracy of 100 ± 20% of the nominal value.

The selectivity of the method was assessed by comparing the chromatograms of six blank samples of intact plasma from different volunteers with samples containing an analyte in the LLQ concentration level and an IS. The response of the detector at the retention time of the analyte and IS should be ≤20% of the average response of the former and ≤5% of the average response of the latter for the found LLQ in 90% of the biological matrices under study.

The stability of DKP in plasma was assessed in an analytical cycle using six QC samples for high and low concentration levels.

To study the stability of the dexketoprofen trometamol substance on freezing and thawing, freshly prepared QC samples were placed in a refrigerator at −32°C for 12 h and then thawed at room temperature. After the complete thawing, the samples were again subjected to a repeated freeze–thaw cycle. Then, their sample preparation and analysis were carried out following the methods described in this work.

When evaluating the short-term stability of the analyte in the matrix, the QC samples that had undergone sample preparation were left in closed vials made of colorless glass at room temperature directly in the autosampler for 1 day (samples for checking stability). To determine stability during long-term storage, quality QC samples were placed in a refrigerator at −32°C for 2 months (samples for checking stability). After a lapse of time, their sample preparation and chromatography were carried out with similar freshly prepared QC samples (reference samples).

The short-term stability in a 50% acetonitrile solution at room temperature was evaluated using a similar principle.

The matrix effect was evaluated using freshly prepared QC samples with low and high concentrations in six samples of intact plasma from different volunteers. Sample preparation of QC samples was carried out as described above with the difference that the calculated amounts of analyte and IS were added into the samples immediately before analysis after the sample preparation stage. In the calculations, we used the values of the matrix factor, normalized to the IS, by the equations

$$\mathrm{MF} = S_\mathrm{m}/S_\mathrm{a},\ \ \mathrm{NMF} = \mathrm{MF_a}/\mathrm{MF_{IS}},$$

where S_m is the peak area of the analyte or IS in the presence of a biological matrix, S_a is the peak area of the analyte or IS in its absence, $\mathrm{MF_a}$ is the matrix factor (MF) of the analyte, $\mathrm{MF_{IS}}$ is the matrix factor of the IS, and NMF is the matrix factor normalized by the IS.

The variation coefficients of the matrix factor should not exceed 15%.

The evaluation of the matrix effect is an essential step in the development and validation of bioanalytical methods, because in some cases, a strong influence of the matrix on the analyte signal can be observed. The resulting ionic suppression often complicates the analysis of biosamples by HPLC–MS/MS, leading to underestimation of the analyte content being determined.

The carryover of the previous sample was assessed by analyzing blank samples after the chromatography of the QC samples with high concentrations (ULQ). The carryover to a blank sample after processing a high concentration standard should not exceed 20% of the LLQ value.

The effect of sample dilution on the accuracy of analysis was determined by the preliminary preparation of a DKP solution with the concentration twice as high as the high QC value (15.00 μg/mL), 150 μL of which was then diluted with 150 μL of intact plasma. Then, sample preparation and analysis were carried out according to the procedure described above.

RESULTS AND DISCUSSION

Selection of an internal standard. Because of the absence of isotopically labeled standards, a certified substance of ibuprofen was used as an internal standard. This substance is close in structure and chemical properties to dexketoprofen and is not an endogenous compound, which prevents significant distortion of the results. The possibility of using venlafaxine as an IS has also been studied. However, we abandoned this option because of the low reproducibility of the data obtained.

Selection of the sample preparation procedure. The extraction of DKP and IBP from the plasma was carried out by LLE with ethyl acetate with the addition of 0.6 M H_2SO_4. We also studied LLE versions with the simultaneous salting-out of plasma proteins with a 3 M $(NH_4)_2SO_4$ solution and with a mixture of a 3 M $(NH_4)_2SO_4$ solution with 0.6 M H_2SO_4. Satisfactory results were obtained only with the addition of H_2SO_4.

Taking into account the high degree of binding of the analyte and the IS with plasma proteins (99.2% for DKP [6]) and the fact that both substances contain a carboxyl group capable of ionization in their structures (Scheme 1), we assumed that the role of sulfuric acid in the sample preparation was reduced to the rupture of these bonds (the protein component of the plasma underwent denaturation, which contributed to the more complete precipitation of protein molecules and, thereby, to a decrease in the degree of their binding with the analyte and the IS) and the conversion of substances into a completely nonionized form (without the addition of acid, the target components can also be in solution in a partially ionized state). These facts should contribute to an increase in the recovery of DKP and IBP in the extraction.

Using a concentrated ammonium sulfate solution as a salting-out agent, on the one hand, helps to decrease the degree of binding of the target components with the plasma proteins because of the deposi-

tion of the latters, and on the other hand, slightly acidifies the solution because of possible hydrolysis. However, a more pronounced signal of the analyte, comparable to the signal in the case of using a mixture of $(NH_4)_2SO_4$ with H_2SO_4, was observed upon the addition of H_2SO_4, which indicates a significant role of the acidity factor in the extraction process.

Selection of eluent and optimization of HPLC–MS/MS conditions. The CH_3CN–HCOOH (0.1%) and CH_3CN–$HCOONH_4$ (5 and 10 mM) systems with an addition of HCOOH and in its absence were considered as the mobile phase. In developing the procedure, some preliminary tests were also carried out using several Phenomenex, Macherey–Nagel, and EcoNova C_{18} (octadecyl) reverse-phase chromatographic columns of equal sizes (50 × 2 mm) but differing in the size of adsorbent grain (3, 4, and 5 μm) and pores (80 and 100 Å). The highest separation efficiency, higher sensitivity with good peak shapes, and the optimal analysis time with the achievement of the desired selectivity at the minimum noise level were observed using a Phenomenex Luna $C_{18(2)}$ analytical column (50 × 2 mm, 3 μm, 100 Å) with a mixture of CH_3CN (eluent B) and 0.1% HCOOH (eluent A) in a ratio of 60 : 40 (v/v) in an isocratic mode. The following chromatographic conditions were selected: injected sample volume was 2 μL, eluent flow rate was 0.30 mL/min, column temperature was 40°C, and total analysis time was 3.5 min. The mean retention times of DKP and IBP were 1.62 ± 0.02 and 2.43 ± 0.03 min, respectively (Figs. 1a and 1b).

The use of a CH_3CN–HCOOH mixture (0.1%) in a volume ratio of 80 : 20 also led to optimal elution parameters; the retention times of DKP and IBP under these conditions were 1.43 ± 0.04 and 1.61 ± 0.03 min, respectively. The specified isocratic elution mode suggested a total analysis time of 3.0 min. However, at the stage of chromatography of the extracts obtained from volonteers plasma samples of volunteers, the analyte and its metabolite were coeluted at the specified MRM transitions (m/z 255.2 → 105.2, 255.2 → 208.8). In this regard, we selected the ratio of B and A eluents equal to 60 : 40 (v/v), which made it possible to separate the peaks of the metabolite (retention time 1.25 ± 0.03 min) and of the target component. Elution using the above mixture of substances in a volume ratio of 50 : 50 at a flow rate of 0.25 mL/min led to an increase in the total analysis time to 5.0 min (the retention times of DKP and IBP were 2.54 ± 0.04 and 4.27 ± 0.03 min, respectively). When a formate buffer solution with different buffer capacities was used as eluent A, no fundamental differences were observed in comparison with 0.1% HCOOH. In the case of $HCOONH_4$ solutions of different concentrations, the chromatographic peak of the analyte had a pronounced "tail" and gave a less intense signal.

To ensure the reproducibility of the results and minimize the cross-contamination of samples, the autosampler needle was washed before and after the injection of a sample with a CH_3CN–H_2O mixture (1 : 1, v/v). The determination of DKP was carried out by the method of internal standardization according to the ratio of chromatographic peak areas of the analyte and the IS.

Table 1. Conditions of mass spectrometric detection

Parameter	Value	
	DKP	IBP
Entrance potential, V	10	
Declustering potential, V	41	
Collision cell entrance potential, V	16	17
Collision cell exit potential, V	4	
Collision energy, V	17	13
Ionspray voltage, V	4000	
Electrospray source temperature, °C	450	
Flow rate of collision gas (N_2)	Medium	
Nebulizing gas (air), psi	50	
Curtain gas (N_2), psi	25	
Auxiliary gas (air), psi	25	

For identification, we used mass spectrometry in the MRM mode (positive ions were recorded) based on the following m/z values: 255.2 → 105.2 (main transition, DKP), 255.2 → 208.8 (confirmatory transition, DKP), and 206.8 → 161.0 (IBP). The mass spectra of the compounds (Figs. 2a and 2b) showed that the analyte had several characteristic transitions. This increased the reliability of its determination. The peak at m/z 255.2 corresponded to the protonated molecule $[M-H]^+$. The most intense fragment ions at m/z 208.8 and 105.2 formed by the loss of H_2O and CO by the precursor ion and in the cleavage of the C–C bond between the carbon atoms of the benzene ring and the carbonyl group with the formation of a positively charged benzoyl ion, respectively. For the IS, the parent ion was at m/z 206.8, and the fragment ion at m/z 161.0 was the result of the elimination of the H_2O and CO molecules. Both target compounds contained a –COOH group in their structure, which made detection in the negative ionization mode possible.

As the substances to be determined were polar, it was advisable to use electrospray ionization. Table 1 summarizes the conditions for the MS/MS detection (for DKP, data on the main transition are presented), preoptimized to ensure maximum sensitivity of the device by injecting analytes into the source chamber using syringe injection.

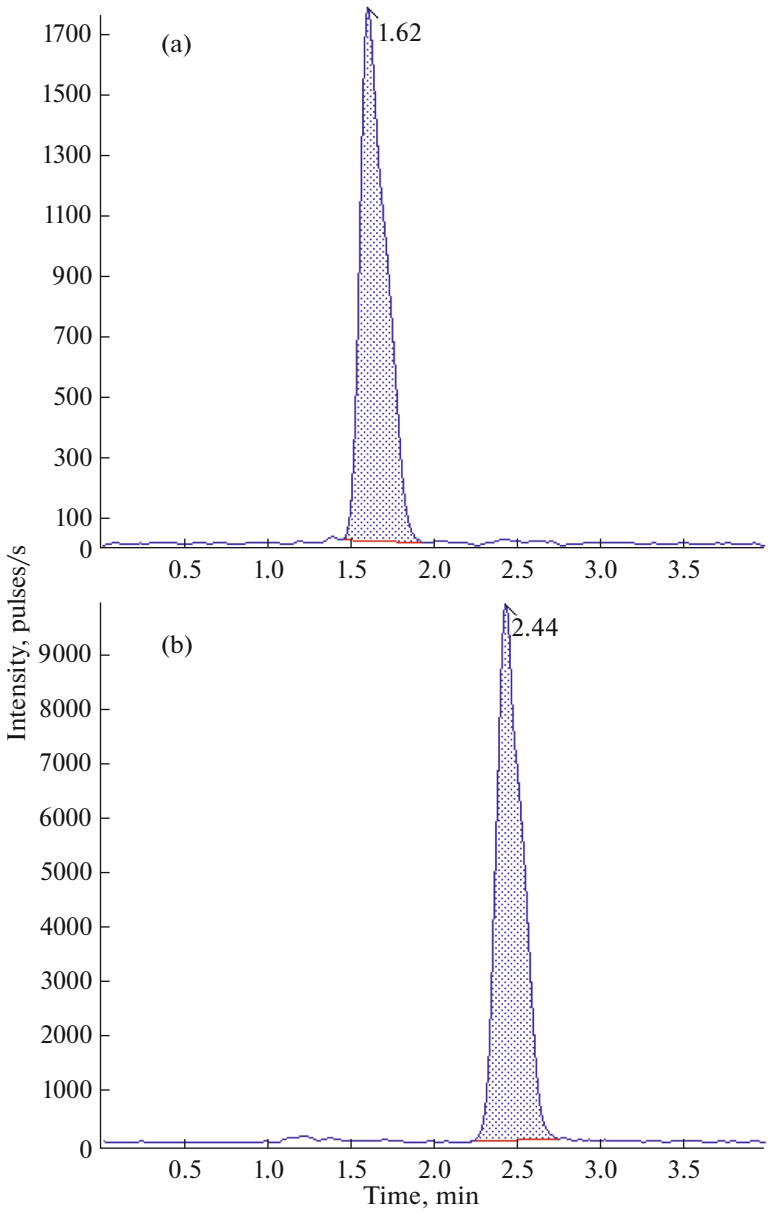


Fig. 1. Representative chromatograms of a standard samples mixture of (a) dexketoprofen (c = 0.25 μg/mL, transition m/z 255.2 → 105.2) and (b) ibuprofen (c = 37.5 μg/mL) extracted from plasma.

Validation of the procedure. According to the requirements of the EMA [19] and the State Pharmacopoeia of the Russian Federation XIV [20], the main parameters of the bioanalytical procedure, confirming the effectiveness and reliability of the results, include selectivity, limit of detection (LOD) and lower limit of quantification, linearity, accuracy, precision, stability of the analyte in the biological matrix and in the solution under the conditions of its storage and sample preparation, carryover of the previous sample, recovery of the analyte from the matrix, and magnitude of the matrix effect, which affects the degree of ionization and the response of the analyte. We have also added a parameter to this list that characterizes the effect of sample dilution to accurately determine the concentration of a substance exceeding the range of the calibration curve. The experimental data were processed according to the results of three consecutive analytical cycles, consisting of a blank sample (a processed sample of an intact matrix containing no analyte or IS), a zero sample (a processed intact matrix containing an IS), ten calibration samples, three QC samples, and samples corresponding to concentrations at the LLQ and ULQ levels. All of these samples passed the sample preparation stage as a single series. Each analytical cycle was accompanied by a calibra-

tion curve in the range of working concentrations to confirm the calibration coefficient and the performance characteristics of the procedure. The latter, in turn, must meet the acceptance criteria (possess the required accuracy and precision) [19, 20], which confirm the suitability of the developed method for the determination of DKP in human plasma.

Linearity. The back-calculated concentrations of the calibration samples corresponded to the above criteria in the studied concentration range. The percentage of samples that met the specified requirements was 96.67%. The calculated concentrations of the calibration standards for acceptable analytical cycles are given in Table 2. The R^2 value was 0.9974 (averaged value for three analytical cycles). As an example, we present the parameters of the calibration equation for the first analytical cycle: $y = 1.0177 \times 10^{-8}x^2 + 6.8158 \times 10^{-4}x - 4.0033 \times 10^{-4}$ ($R^2 = 0.9961$), where y is the peak area ratio of the analyte to the IS, and x is analyte concentration.

Accuracy and precision. According to the results of analysis, the intra- and interday accuracy are within 96.66–100.00% and 94.97–97.92% of the nominal concentrations, respectively; the variation coefficient for intra- and interday analysis is in the range of 4.93–9.25% and 4.24–9.57%, respectively. The data obtained satisfy the above acceptance criteria: the accuracy values at the levels of the LLQ and the low, medium, and high concentration ranges do not go beyond the range of 100 ± 20% and 100 ± 15%, respectively; the variation coefficients within one cycle and between them do not exceed ±15% for QC samples and ±20% for the LLQ samples. Table 3 shows experimental data on evaluating the intraday accuracy and precision of one of the analytical cycles.

Limit of detection and lower limit of quantitation. The results show that the average signal-to-noise ratio was 12.4. The variation coefficient and the accuracy for the LLQ within one cycle were 9.25 and 97.52%, respectively. In this case, the LOD coincides with the LLQ (0.01 μg/mL) (Table 4). Representative chromatograms of a blank sample and a sample containing an analyte with a concentration at the LLQ level are presented in Figs. 3a and 3b, respectively.

Selectivity. We have not revealed an effect of endogenous plasma components on the peak areas of DKP and IBP. The response of the detector at the retention time of the analyte and the IS was ≤20% of the average response of the former and ≤5% of the average response of the latter for the found LLQ in 90% of the biological matrices under study.

Stability. According to the results of the tests performed, DKP was recognized as stable both in plasma and in a water–acetonitrile solution in the studied time intervals. After three freezing at –32°C–thawing cycles, the change in the values of the accuracy of the determination of samples concentrations for testing the stability of relatively freshly prepared QC samples

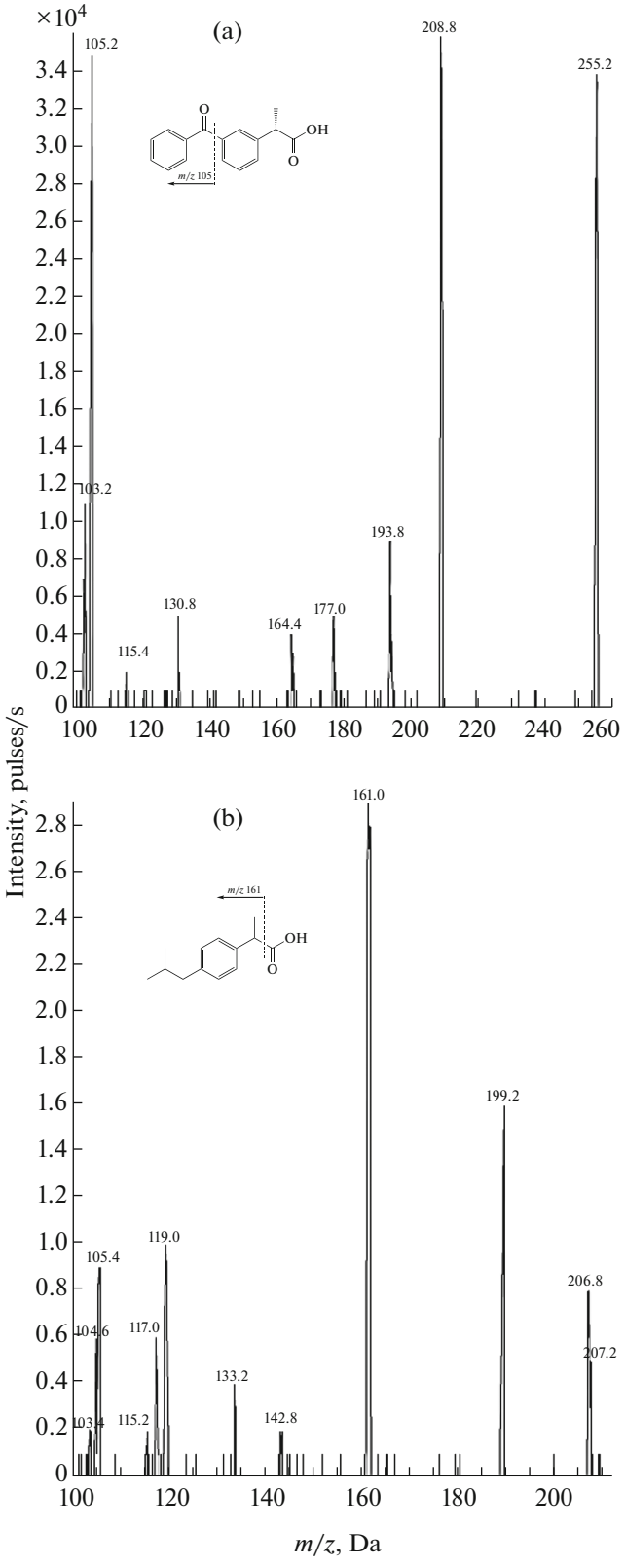


Fig. 2. Mass spectra of (a) dexketoprofen and (b) ibuprofen with the corresponding fragmentation schemes.

Table 2. Results of the calibration cycles evaluation for the determination of dexketoprofen trometamol in human plasma

No. of calibration level	$c \times 10^3$, μg/mL	No. of analytical cycle					
		1		2		3	
		$c \times 10^3$, μg/mL	accuracy, %	$c \times 10^3$, μg/mL	accuracy, %	$c \times 10^3$, μg/mL	accuracy, %
1	10.00	9.43	94.32	10.71	107.07	10.06	100.60
2	25.00	26.55	106.18	21.74	86.94	25.36	101.43
3	50.00	59.44	118.89*	45.27	90.54	47.55	95.11
4	100.00	97.46	97.46	97.86	97.86	98.88	98.88
5	250.00	236.73	94.69	252.00	100.80	240.57	96.23
6	500.00	488.30	97.66	526.59	105.32	506.04	101.21
7	1000.00	980.03	98.00	1100.97	110.10	1027.63	102.76
8	2500.00	2220.82	88.83	2632.01	105.28	2642.03	105.68
9	5000.00	5093.70	101.87	4863.76	97.28	4991.95	99.84
10	8500.00	8682.20	102.14	8396.33	98.78	8352.36	98.26

* The value does not meet the acceptance criteria.

with low and high concentrations was 8.70 and 3.29%, respectively. In evaluating short-term stability in plasma and solution, the change in the accuracy of determining the concentrations at the indicated levels was 4.35 and 0.80%, and 0.82 and 3.12%, respectively In the case of assessing long-term stability; the change was 1.53 and 2.50%.

Matrix effect. The variation coefficient of the matrix factor for the QC samples with low and high concentrations was 9.30 and 5.45%, respectively, which meets the prescribed requirements. The obtained values indicate an insignificant effect of the studied biological matrix on the analyte response.

Table 3. Results of the intraday accuracy and precision evaluation in the determination of dexketoprofen trometamol in human plasma

Sample no.	LLQ, $c = 0.01$ μg/mL		Low QC sample, $c = 0.03$ μg/mL		Medium QC sample, $c = 0.75$ μg/mL		High QC sample, $c = 7.50$ μg/mL	
	$c \times 10^3$, μg/mL	accuracy, %	$c \times 10^3$, μg/mL	accuracy, %	$c \times 10^3$, μg/mL	accuracy, %	$c \times 10^3$, μg/mL	accuracy, %
1	9.30	92.97	32.82	109.38	727.08	96.94	7715.09	102.87
2	8.70	86.97	26.34	87.78	675.67	90.09	6674.30	88.99
3	10.17	101.75	27.39	91.30	737.17	98.29	7080.64	94.41
4	10.75	107.49	31.60	105.32	747.40	99.65	7581.87	101.09
5	8.70	87.04	31.20	103.98	673.99	89.87	7352.51	98.03
6	10.89	108.87	30.66	102.21	788.29	105.10	7616.88	101.56
Mean	9.75	97.52	30.00	100.00	724.93	96.66	7336.88	97.83
SD*	0.90	–	2.33	–	40.20	–	362.03	–
CV**, %	9.25	–	7.77	–	5.55	–	4.93	–

* SD, standard deviation. ** CV, coefficient of variation.

Table 4. Results of the lower limit of quantification evaluation of dexketoprofen trometamol in human plasma (one cycle; nominal concentration 0.01 μg/mL)

Sample no.	$c \times 10^3$, μg/mL	Accuracy, %	Signal-to-noise ratio
1	9.30	92.97	11.7
2	8.70	86.97	13.0
3	10.17	101.75	10.0
4	10.75	107.49	13.9
5	8.70	87.04	14.1
6	10.89	108.87	11.7
Mean	9.75	97.52	12.4
SD*	0.90	–	–
CV**, %	9.25	–	–

* SD, standard deviation. ** CV, coefficient of variation.

Carryover. The results indicate the absence of the sample carryover, since the analysis of blank samples after the QC samples with high concentrations revealed no chromatographic peak with the DKP retention time.

The effect of dilution. The dilution of the samples by half did not significantly affect the parameters of the accuracy and precision of the proposed procedure. The average accuracy was 88.06%, and the change was 11.26%.

Table 5 summarizes the values of validation parameters of the developed procedure.

CONCLUSIONS

Thus, in this work, we proposed and validated an HPLC–MS/MS procedure for the determination of dexketoprofen trometamol in human plasma in the concentration range 0.01–8.50 μg/mL using ibupro-

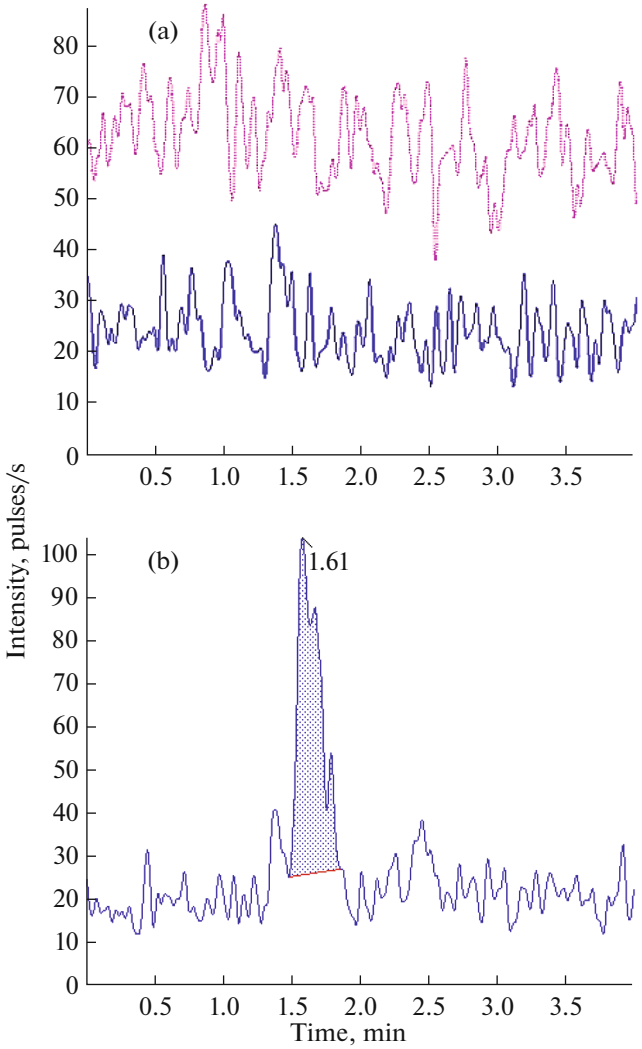


Fig. 3. Representative chromatograms of (a) a blank sample and (b) a standard dexketoprofen sample at the lower limit of quantitation (c = 0.01 μg/mL, transition m/z 255.2 → 105.2).

Table 5. Validation parameters of the developed procedure for the determination of dexketoprofen trometamol in human plasma

Parameter	Parameter value
Selectivity	≤20% of the LLQ response, ≤5% of the IS response
LOD	0.01 μg/mL
LLQ	0.01 μg/mL at a signal-to-noise ratio of 12.4
Linear range	0.01–8.50 μg/mL
Intra-day accuracy	96.66–100.00% of nominal values
Intra-day variation coefficients	4.93–9.25%
Inter-day accuracy	94.97–97.92% of nominal values
Inter-day variation coefficients	4.24–9.57%
Stability in the matrix during freezing at −32°C and thawing	Average change in accuracy 8.70 and 3.29%
Long-term stability in the matrix at −32°C for 2 months	Average change in accuracy 1.53 and 2.50%
Short-term stability in the matrix at room temperature	Average change in accuracy 4.35 and 0.80%
Short-term stability in the solution at room temperature	Average change in accuracy 0.82 and 3.12%
Carryover	≤20% of the LLQ value
Matrix effect	Variation coefficients of the matrix factor 9.30 and 5.45%
Diluting samples by half	Average change in accuracy 11.26%

fen as an internal standard. The MRM mode was selected for the determination of target compounds, which ensured sufficient selectivity in working with complex biological matrices. The developed procedure, which meets the requirements of the EMA [19] and the State Pharmacopoeia of the Russian Federation XIV [20], meets the criteria of accuracy, precision, rapidity, reliability, sensitivity, and also demonstrates acceptable performance. The optimized sample preparation conditions in comparison with those reported in the literature can significantly shorten the total analysis time. It is permissible to dilute samples with concentrations exceeding the analytical range of the developed procedure. In the experiments, special attention was paid to the evaluation of the matrix effect and the absence of the cross-contamination of samples to avoid erroneous results, which was controlled by analyzing a blank sample after the injection of the quality control sample with the maximum concentration. This procedure was successfully tested on real plasma samples from healthy volunteers (men and women) aged 20 to 45 years with a single intake of a generic drug at a dose of 25 mg on an empty stomach as part of clinical trials conducted according to the laws of the Russian Federation and ethical requirements.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement of compliance with standards of research involving humans as subjects. All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants involved in the study.

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