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DEVELOPMENT AND VALIDATION OF A QUANTITATIVE DETERMINATION METHOD FOR FLUOROTHIAZINONE IN HUMAN BLOOD PLASMA

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An HPLC-MS/MS technique for quantitative determination of the new antibacterial drug fluorothiazinone in human blood plasma using an internal standard was developed and validated. The validation protocol proved the selectivity, accuracy, and precision of the method and the lack of significant carryover, matrix, and sample-dilution effects. The calibration curve was linear in the concentration range 0.1 – 300 ng/mL with a correlation coefficient $r^2 > 0.9990$. The lower limit of quantitation was 0.1 ng/mL. The studied analyte was shown to be stable during storage in a stock solution (for 6 h at room temperature) and in plasma samples (for 6 h at room temperature, after three freeze–thaw cycles, and for 30 d at −70°C) and in an autosampler after sample preparation (for 24 h at +10°C).

Keywords: quantitative determination, HPLC-MS/MS, fluorothiazinone, plasma.

The growth of antibiotic resistance in microorganisms is currently a global problem because the resistance of pathogens to antibiotics reduces the effectiveness of pharmacotherapy of infectious diseases [1 – 4]. Although the problem has long been solved by the constant introduction of new antibiotics, this strategy has slowed in the last decade [5]. Microorganisms can develop resistance to antimicrobial drugs because of the high mutability of the bacterial genome, which can adapt to environmental conditions [6]. The genes responsible for the adaptability are passed into the population and transferred between microorganisms [4, 7 – 9].

The bacteria *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* were included in the World Health Organization high-priority list of pathogenic microorganisms against which new effective drugs are needed [10].

The new drug fluorothiazinone or 4-(3-ethoxy-4-hydroxybenzyl)-5-oxo-5,6-dihydro-4*H*-[1,3,4]-thiadiazine-2-(2,4-difluorophenyl)carboxamide (Fig. 1) was developed at Gamaleya NRCEM and was characterized with antibacterial activity against Gram-negative bacteria [11 – 13]. Its main mechanism of action includes inhibition of the type III secretion system (TTSS), which is involved in excretion of exotoxins of Gram-negative bacteria [14 – 17]. Fluorothiazinone was active *in vitro* and *in vivo* against *A. baumannii*, among others, which characteristically lacks a TTSS, suggesting that an additional mechanism of action exists [12].

Quantitative analysis methods for fluorothiazinone in human blood plasma have not been reported in the literature because the active species has not previously been investigated and was not included in existing drug compositions. The development of a bioanalytical method enabling pharmacokinetic studies of fluorothiazinone is a timely endeavor.

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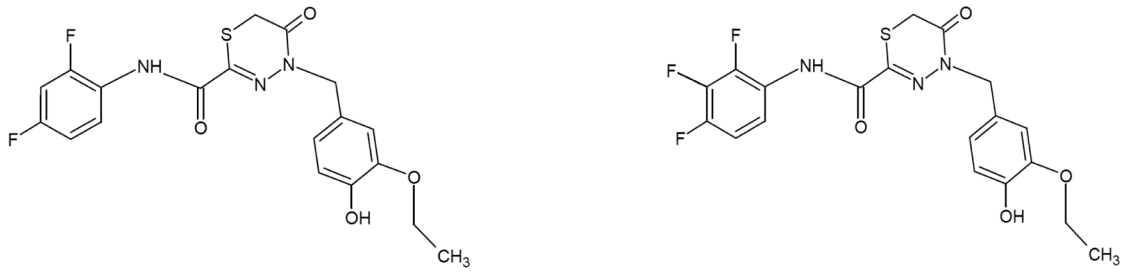

a) *b*)

Fig. 1. Structural formulas of fluorothiazinone (*a*) and internal standard (*b*).

The present work was aimed at development and validation of a chromatography–mass-spectrometry method for quantitative determination of fluorothiazinone in human blood plasma to determine the pharmacokinetic parameters.

The fluorothiazinone concentration in blood plasma was determined by high-performance liquid chromatography in combination with tandem mass spectrometry (HPLC-MS/MS), which is most common for conducting pharmacokinetic studies [18].

EXPERIMENTAL PART

Reference standard fluorothiazinone [4-(3-ethoxy-4-hydroxybenzyl)-5-oxo-5,6-dihydro-4*H*-[1,3,4]-thiadiazine-2-(2,4-difluorophenyl)carboxamide] of 99% purity was used. The internal standard was CL-130 [4-(3-ethoxy-4-hydroxybenzyl)-5-oxo-5, 6-dihydro-4*H*-[1,3,4]-thiadiazine-2-(2,3, 4-trifluorophenyl)carboxamide] of 99% purity (supplied by Gamaleya NRCEM, MH of Russia) [19]. Eluent was prepared using MeCN (Reidel-de Haen, Germany), formic acid (Sigma-Aldrich, Germany), and deionized H_2O (Milli-Q plus system, Millipore, France). Enzymatic hydrolysis of conjugates used β-glucuronidase from *E. coli* K12 (Roche Diagnostics GmbH, Germany) and phosphate buffer (pH 6.2, Sigma-Aldrich, Germany). Deionized H_2O was produced using a Millipore Direct Q-3 UV system (Merck KGaA, Germany). Blank human blood plasma samples were obtained from the Sechenov University biobank. The local ethics committee of the university approved the use of the plasma for research purposes (note from protocol No. 1755 of Nov. 25, 2022).

Preparation of calibration solutions and quality control (QC) samples

Stock solutions of fluorothiazinone and the internal standard with concentrations of 1 mg/mL were prepared in MeCN. Working solutions were prepared by successive dilution of the standard solutions in the appropriate volume of MeCN.

Calibration solutions were prepared by adding the appropriate volumes of working solutions to blank human blood plasma to produce the following concentrations: 0.1, 1, 5, 10, 50, 100, 200, and 300 ng/mL. QC samples with concentrations 0.1, 0.3, 150, and 250 ng/mL (LLOQQC, LQC, MQC, and HQC, respectively) were prepared analogously but using separately prepared independent working solutions. Samples of calibration solutions and QC solutions were prepared according to the general method for samples.

Sample preparation

Human blood plasma (100 μL) was treated with internal standard solution (10 μL) in MeCN (1000 ng/mL) and phosphate buffer (100 μL, 0.1 M, pH 6.2), incubated for 30 min at room temperature, and treated with MeCN (400 μL) to precipitate proteins. The tube was shaken on a shaker and centrifuged at 13,000 rpm for 5 min. The transparent supernatant (400 μL) was evaporated to dryness in a vacuum evaporator at 37°C. The dry solid was redissolved in MeCN (100 μL). The solution was transferred to a vial and analyzed by HPLC-MS/MS.

The concentration of glucuronic-conjugated metabolite was determined by HPLC-MS/MS analysis after enzymatic hydrolysis. For this, human blood plasma (100 μL) was treated with internal standard solution (10 μL) in MeCN (1000 ng/mL) and β-glucuronidase from *E. coli* K12 solution (100 μL) in phosphate buffer (0.1 M, pH 6.2). The tube was incubated at 37°C for 4 h. Then, proteins were precipitated analogously to samples without added glucuronidase.

Chromatographic and mass-spectrometric analytical conditions

The analysis used a Waters Acquity I-Class CM-A UPLC HPLC in combination with a Waters TQS tandem quadrupole mass spectrometric detector (Waters Corp., USA) and an Acquity UPLC BEH C18 analytical column (2.1 × 50 mm, 1.7 μm) (Waters Corp., USA). The temperature of samples in the autosampler was 10°C. An electrospray ionization source in positive-ion mode (ESI+) with a capillary voltage of 3500 V; drying gas temperature, 300°C; spray gas flow rate, 10 L/min; and drying (Aux) gas flow rate, 8 L/min were used. The multiple reaction monitor-

ing (MRM) method was used for the analysis. The transitions of fluorothiazinone and internal standard CL-130 were 422 → 151.1 → 123.1 and 440.2 → 151.1, respectively.

The chromatographic separation used an Acquity UPLC BEH C18 analytical column (2.1 × 50 mm, 1.7 μm; Waters Corp., USA) and column temperature 40°C. The mobile phase composition was 0.1% formic acid in H_2O (A) and 0.1% formic acid in MeCN (B). The gradient was 0 min (10% B), 3 min (45% B), 4.1 min (90% B), and 5.1 min (10% B). The total analysis time was 7 min; mobile phase flow rate, 0.4 mL/min. The volume of injected sample was 5 μL.

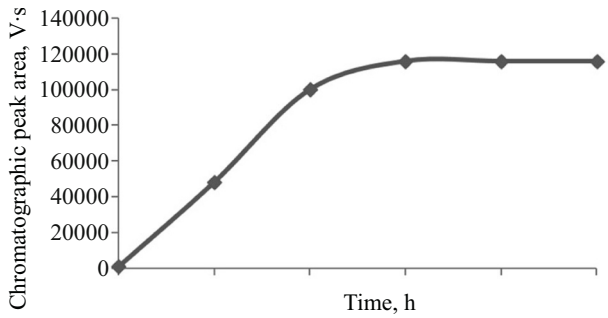

Fig. 2. Dependence of fluorothiazinone chromatographic peak area on incubation time.

RESULTS AND DISCUSSION

Fluorothiazinone is transformed by *in vivo* metabolism primarily into the glucuronic conjugate, which forms more hydrophilic and readily excreted molecules [20]. Several factors including the pH of the buffer solution, enzyme concentration, incubation time, and temperature affected the completeness of the enzymatic reaction using β-glucuronidase [23].

Figure 2 shows the dependence of fluorothiazinone chromatographic peak area on the duration of the enzymatic reaction. The reaction temperature and enzyme concentration were selected according to the documentation for β-glucuronidase from *E. coli* K12. The studies found that the optimal hydrolysis time was 4 h.

Figures 3 and 4 show the chromatogram and mass-spectral fragmentation of fluorothiazinone.

Validation protocol

The method was validated according to guidelines for validating bioanalytical methods [21 – 23]. The goal of the validation was to demonstrate that the method was suitable for determining the concentrations of the free and glucuronide forms of fluorothiazinone in human blood plasma. The suitability of the developed method was evaluated based on validation characteristics of selectivity, carry-

TABLE 1. Evaluation of Accuracy and Precision from Analytical Results for Quality Control Samples LLOQQC, LQC, MQC, and HQC

	LLOQQC (0.1 ng/mL)			LQC (0.3 ng/mL)			MQC (150 ng/mL)			HQC (250 ng/mL)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
C_{act}, ng/mL (n = 6)	0.10	0.09	0.10	0.28	0.28	0.28	158	164	162	217	235	230
	0.11	0.09	0.09	0.33	0.28	0.28	144	163	138	221	249	268
	0.10	0.09	0.10	0.33	0.28	0.29	168	166	155	218	259	227
	0.09	0.09	0.10	0.31	0.29	0.33	134	159	139	278	234	229
	0.10	0.10	0.09	0.32	0.32	0.28	168	149	149	240	235	232
	0.10	0.11	0.10	0.28	0.31	0.29	168	165	161	235	246	250
Mean, ng/mL	0.100	0.095	0.097	0.308	0.293	0.292	157	161	150	235	243	239
	0.097			0.298			156			239		
*SD*within-run, ng/mL	0.006	0.008	0.005	0.023	0.018	0.019	14	6	11	23	10	16
*SD*between-run, ng/mL	0.007			0.020			11			17		
*RSD*within-run, %	6.3	8.8	5.3	7.5	6.0	6.7	9.2	4.0	7.1	9.9	4.2	6.8
*RSD*between-run, %	6.9			6.9			7.2			7.0		
Accuracy, %	100.0	95.0	96.7	102.8	97.8	97.2	104.4	107.3	100.2	93.9	97.2	95.8
	97.2			99.3			104.0			95.6		

Note: C_{act}, experimentally calculated concentration (ng/mL); *Mean*, mean value; *SD*, mean-square deviation; *RSD*, relative standard deviation.

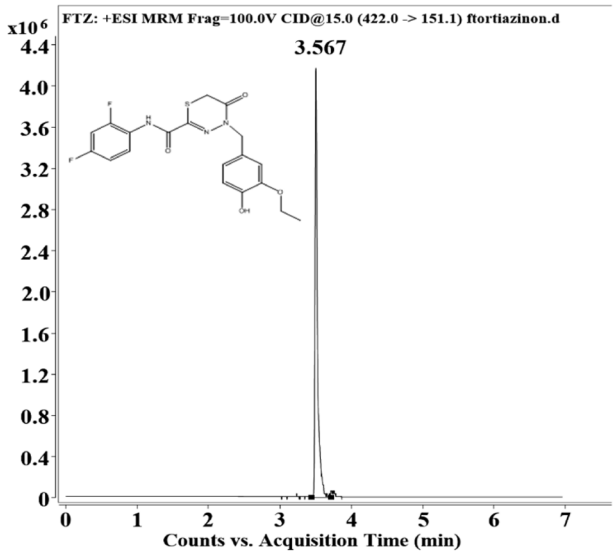

Fig. 3. Chromatogram of fluorothiazinone. Along the abscissa, time (min); along the ordinate, area under chromatographic peak (mAU).

over effect, LLOQ, linearity, accuracy (within-run and between-run), precision (within-run and between-run), lack of a dilution effect on samples, matrix effect, and stability of the studied analyte.

Selectivity

The selectivity of the developed method was evaluated by analyzing six blank plasma samples. The resulting chromatograms were compared with chromatograms of QC samples containing the studied analyte at a concentration equal to the lower limit of quantitation (LLOQ). An analysis of data obtained from the evaluation of the blank samples of the biological matrix (Fig. 5) did not reveal any interfering peaks with retention times corresponding to that of the studied analyte and internal standard (Fig. 6).

Carryover effect

The carryover effect was evaluated using successive injections of the calibration standard with a fluorothiazinone concentration of 300 ng/mL, an internal standard concentration of 100 ng/mL, and a blank sample of the biological matrix. The chromatogram of the intact blood plasma sample did not contain peaks corresponding to the retention times of the analyte and the internal standard.

Lower limit of quantitation

The LLOQ was evaluated by analyzing the calibration standard with a concentration of studied analyte of 0.1 ng/mL (Fig. 7). The signal at the LLOQ level was >5 times that of the blank sample of the biological matrix. An analysis of the data from the experiment evaluating the LLOQ found that the LLOQ of the studied analyte in the biological matrix was 0.1 ng/mL.

Linearity

The calibration curve was constructed using eight levels of calibrators distributed evenly in the range from 0.1 to 300 ng/mL (Fig. 8). The calibration curve was constructed by the internal standard method. A weighted linear regression was used to describe the dependence. The accuracy of the experimentally calculated calibrator concentrations was within ±20% for the lower point of the calibration curve and ±15% for other solutions. The determination coefficient R^2 was 0.99985.

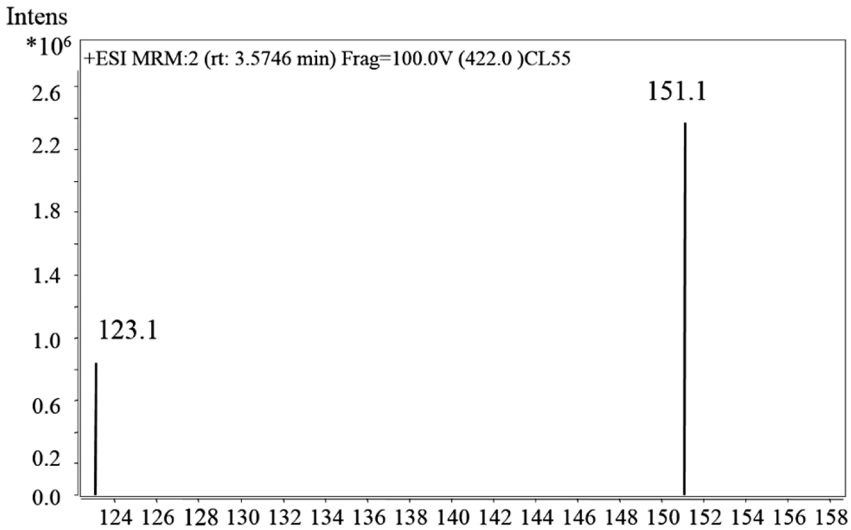

Fig. 4. Mass spectrum of fluorothiazinone fragments. Along the abscissa, molecular mass (Da); along the ordinate, area under chromatographic peak (mAU).

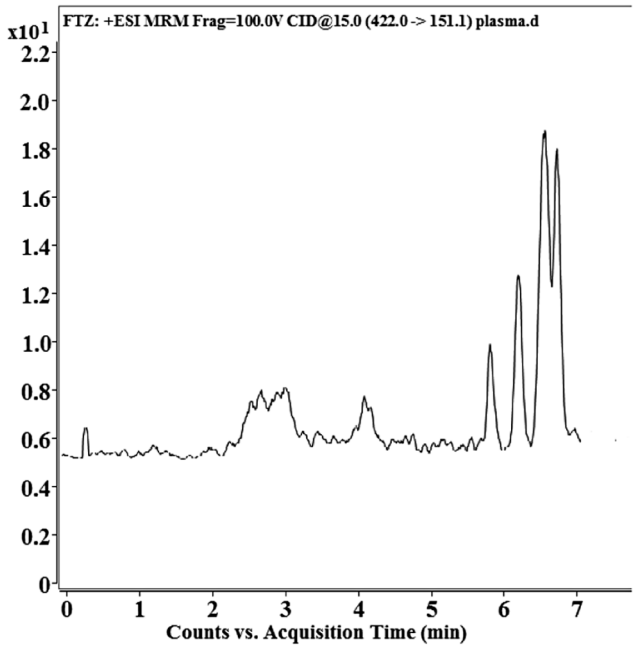

Fig. 5. Chromatogram of plasma not containing fluorothiazinone reference standard solution. Along the abscissa, time (min); along the ordinate, area under chromatographic peaks (mAU).

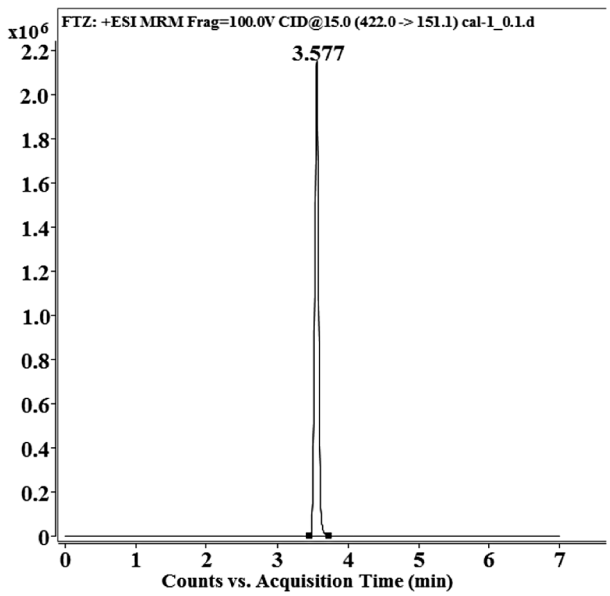

Fig. 7. Chromatogram of plasma containing fluorothiazinone at LLOQ level. Along the abscissa, time (min); along the ordinate, area under chromatographic peak (mAU).

Accuracy and precision

The accuracy and precision of the developed method were evaluated by analyzing QC samples at four concentration levels (0.1, 0.3, 150, and 250 ng/mL). For this, the appropriate standard solutions were added to blank plasma samples. The resulting solutions were analyzed during the first day to evaluate the within-run precision and during three days to evaluate the inter-run precision. The results showed that the experimental values met the acceptance criteria of ±20% for the LLOQQC and ±15% for other QC samples (Table 1).

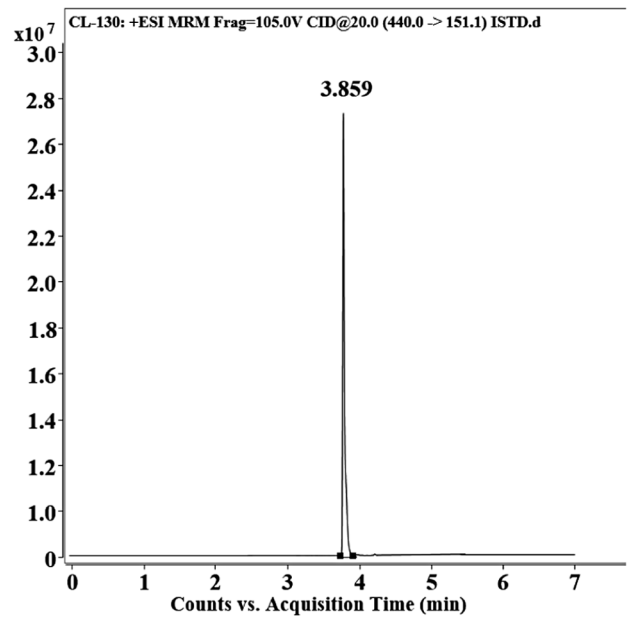

Fig. 6. Chromatogram of internal standard. Along the abscissa, time (min); along the ordinate, area under chromatographic peak (mAU).

Lack of dilution effect of samples

The effect of diluting the samples by two (F = 2) and five times (F = 5) was evaluated by adding fluorothiazinone stan-

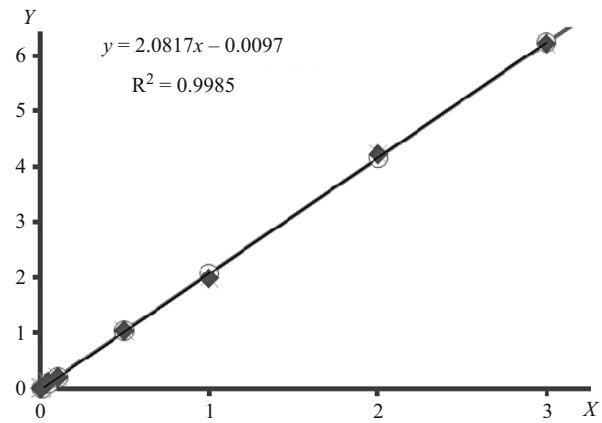

Fig. 8. Calibration curve of fluorothiazinone in the range 0.1 – 300 ng/mL. Along the abscissa, ratio of analyte concentration to internal standard concentration; along the ordinate, ratio of area of analyte chromatographic peak to area of internal standard peak.

TABLE 2. Evaluation of Sample Dilution Effect

HQC (250 ng/mL)	F = 2	F = 5
C_{act}, ng/mL (n = 6)	249	255
	251	257
	248	255
	251	254
	246	253
	245	253
Mean, ng/mL	248	255
SD, ng/mL	2.5	1.5
RSD, %	1.0	0.6
Accuracy, %	99.3	101.8

TABLE 3. Matrix Effect and Degree of Analyte and Internal Standard Extraction

Compound	Parameter	LQC, 0.3 ng/mL	HQC, 250 ng/mL
Fluorothiazinone	Matrix effect	0.94 (CV = 4.66 %)	0.97 (CV = 10.45 %)
	Extraction, %	93.1 (CV = 6.4 %)	91.9 (CV = 7.5 %)
Internal standard	Matrix effect	0.97 (CV = 3.12 %)	0.98 (CV = 8.32 %)
	Extraction, %	95.6 (CV = 3.8 %)	98.3 (CV = 5.9 %)

dard solution to intact plasma samples to produce concentrations equal to F × 250 ng/mL. The samples were diluted with blank plasma to produce the HQC concentration. The accuracy and precision of the quantitative determination of the analyte in samples diluted by 2 and 5 times were within the limits of the acceptance criteria and were % (Table 2). Thus, the degree of dilution of the samples did not affect the accuracy and precision of the analyte concentration determination.

Matrix effect and degree of extraction

The matrix effect was evaluated by comparing chromatographic peak areas of fluorothiazinone and LQC and HQC samples with working solutions at the same concentrations. The results led to the conclusion that the matrix effect and degree of extraction had insignificant effects on the results of the quantitative analysis because the possible effect was compensated by the presence of the internal standard (Table 3).

Stability of studied analyte

The stability of fluorothiazinone was evaluated in working solutions and biological samples with analyte contents at the LQC and HQC levels after storage at room temperature for 6 h, in biological samples after sample preparation, after 24 h in the autosampler at 10°C, in plasma samples with added fluorothiazinone working solutions at the LQC and HQC levels, after three freeze–thaw cycles, and after storage at −70°C for one month.

An analysis of the experimental stability evaluation data (Table 4) demonstrated that substantial degradation of fluorothiazinone in the samples was not observed. The analyte concentrations in the QC samples after storage under these conditions were within ±15% of their nominal values.

Thus, a quantitative determination method for fluorothiazinone in human blood plasma that enabled clinical trials of the drug was developed and validated during the research. The parameters and conditions of the HPLC-MS/MS method were optimized. The method had high sensitivity, accuracy, precision, and selectivity. The lack of substantial carryover, matrix, and dilution effects of the samples was established. Thus, the method could be used for quantitative determination of fluorothiazinone in human blood plasma to study the pharmacokinetic parameters.

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Conflict of interest

We declare no conflict of interest.

Contribution of authors

SNB reviewed the literature on the research topic and prepared the draft manuscript; MVS performed the experi-

TABLE 4. Evaluation of Accuracy and Precision Obtained from Analytical Results of Quality Control Samples LQC and HQC

Stability evaluation conditions/Concentration level	Storage of working solutions for 6 h at room temperature	Storage of biological samples for 6 h at room temperature	Storage of biological samples after preparation in autosampler for 24 h	Samples after three freeze–thaw cycles	Storage of samples in freezer at −70°C for 1 month
Accuracy of LQC samples, %	99.44	98.89	96.67	96.11	104.44
Accuracy of HQC samples, %	98.22	96.52	101.64	97.51	103.38

mental part of the work; NEM developed the method and processed the results; VMS reviewed the literature; SAA approved the research concept and the final manuscript; NLL analyzed, processed, and prepared the graphical material; SIL reviewed the literature on the research topic; AVS reviewed the literature on the research topic; CAZ processed the results; and NAZ approved the research concept and final manuscript.

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