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AN UPLC METHOD FOR THE DETERMINATION OF SORAFENIB IN HUMAN PLASMA BY FLUORIMETRIC DETECTION WITH PRE-COLUMN DERIVATIZATION AND APPLICATION TO A PHARMACOKINETIC STUDY

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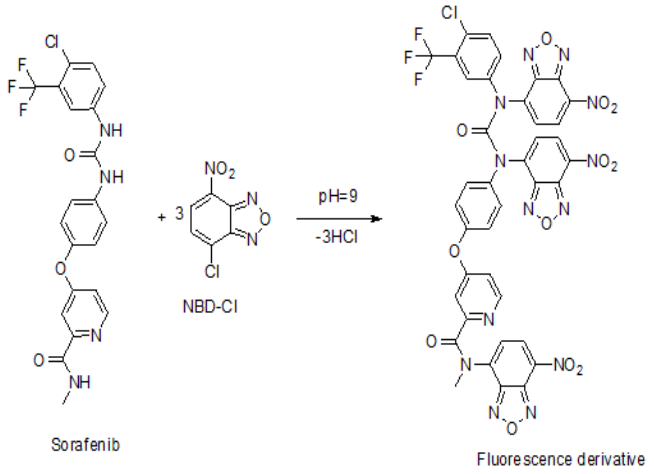
This research presents a new, sensitive and selective UPLC method with fluorometric detection for the determination of sorafenib in human plasma and application of the method to a pharmacokinetic study. Sorafenib was precolumn derivatized with 7-chloro-4-nitrobenzofurazan (NBD-Cl) and the separation of the fluorescent derivative was performed with a C18 column (50 mm x 2.1 mm, 1.7 µm) at 40ºC using a mobile phase composed of acetonitrile - 0.1% trifluoroacetic acid in water (60:40, v/v) by isocratic elution with flow rate of 0.5 mL min^{-1}. The injection volume was 7 µL. The method depends on the measurement of the derivative using fluorescence detection (λex = 398 nm, λem = 425 nm). The retention time of sorafenib was 3.10 ± 0.02 min. The novel method was validated in accordance with ICH criteria by studying on the parameters such as specificity, linearity, precision, accuracy and robustness. The method was determined to be linear in a concentration range of 0.25-10 µg mL^{-1} with the correlation coefficient of 0.9995. Limit of detection and quantitation were found to be 0.075 and 0.25 µg mL^{-1}, respectively. Intraday and interday RSD values were less than 5.48%. The plasma concentration–time profile and pharmacokinetic parameters such as AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, t_{max}, $t_{1/2}$ were measured according to the assays. The proposed method is feasible to investigate the bioequivalence and bioavailability and routine analysis of the drug in plasma.



INTRODUCTION

Sorafenib (SRB) is a multikinase inhibitor which is effective in the treatment of advanced renal and hepato-cellular carcinoma.[1] SRB is administrated orally and provides inhibition of many cellular targets (VEGFR-2, PDGFR, c-KIT, FLT-3, CRAF, wild-type BRAF or BRAFV600E).[2] Currently reported researches emphasize about the effects of SRB in different cancer types such as ovarian and brain cancers.[3-5] There are significant side effects that are reported for instance hand-foot reaction, fatigue and hypertension.[6]

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The chemical name of SRB is, 4-[4-[[4-chloro-3-(trifluoromethyl)phenyl]carbamoylamino] phenoxy]-*N*-methylpyridine-2-carboxamide; 4-methylbenzenesulfonic acid. However it is not a fluorogenic molecule, the carboxamide groups in its structure provides the molecule suitable for fluorogenic derivatization reactions.

There is not any fluorimetric method for determination of SRB in the literature. Additionally, there are not sufficient number of methods, that can provide quantitation of SRB in human plasma in order to use in pharmacokinetic studies. There are some LC-tandem mass spectrometric methods for the determination of SRB in human and rat plasma.[7-9] Also, some HPLC-UV methods exist in the literature, two of them,[10,11] are the assays in human serum, one of them provides determination in patients serum samples,[12] and one of them is for rat plasma.[13] In a UPLC-MS/MS method SRB determined with some same group of drugs; lenvatinib and apatinib in human plasma.[14] And by using a LC–MS/MS method for therapeutic drug monitoring of sorafenib, regorafenib and their active metabolites in patients with hepatocellular carcinoma was performed.[15] Another method provide determination of the analyte in its pharmaceutical preparations by RP-HPLC with UV detection.[16] Due to the continuing researches about drug interactions, side effects and therapeutic functions of SRB in different cancer types, the determination of SRB in human plasma in low amounts is a critical point. In this study, by the derivatization process it was aimed to gain sensitive and simple analysis that will be able to use in pharmacokinetic investigations.

The pre-column derivatization process conducted with a fluorogenic reagent; 7-chloro-4-nitrobenzofurazan (NBD-Cl), which is frequently preffered because of its basic reaction procedure, fast reactivity and high sensitivity. It was used as a fluorogenic agent for determination of amines, amino acids, thiol and sulphyryl groups.[17,18] The reagent has also been used for the determination of compounds which containes carbamate,[19] upon hydrolysis in alkali medium.

According to the chromatograhic conditions and calibration, validation studies were performed in terms of ICH criteria. The analysis for pharmacokinetic research were carried out, by the approval of ethic commitee, with the plasma samples of the healthy volunteer after administration of SRB.

RESULTS AND DISCUSSION

Derivatization

Reaction conditions of SRB with NBD-Cl were investigated and optimized for the efficiency of the derivative. Each parameter has been changed separately while the other parameters were constant. The optimum reaction time, temperature, pH, buffer type, volume proportions of organic and aqueous solutions, mole ratio of NBD-Cl/SRB and and the amount of HCl solution for acidification so as to stop the derivatization reaction were determined. Figure 1 indicates the derivatization reaction.

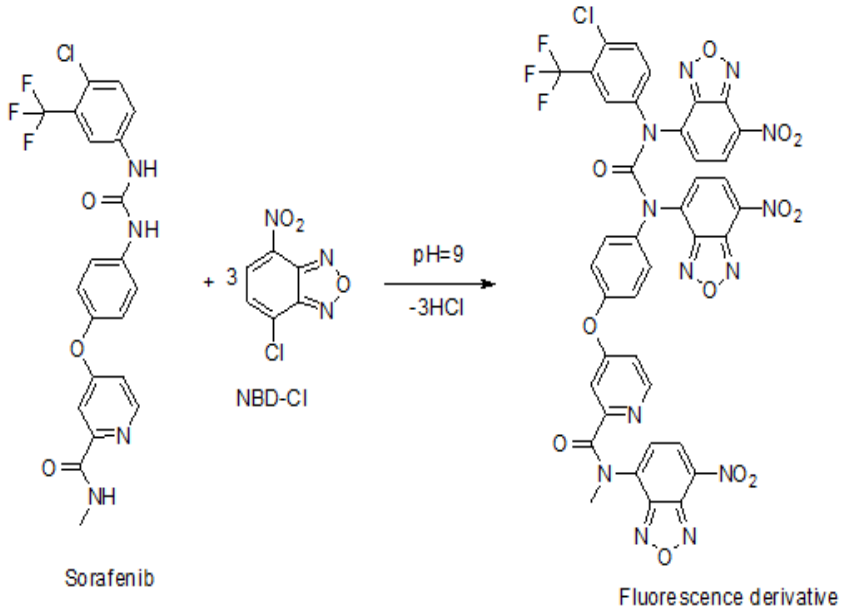

Fig. 1 – Derivatization reaction between SRB and NBD-Cl.

Effect of pH

NBD-Cl was used as an agent that provides fluoregenic features for the molecules including amines, amino acids, thiol, sulphyryl, carbamate and carboxamide groups in alkali medium. The pH range that is suitable for the occurence of the reaction is between 7-11 and mainly phosphate buffer is preffered.[17-19] In this method maximum absorbance was obtained at pH 8.5.

Effect of time and temperature

To determine the optimum temperature and time needed for the reaction, different temperatures and durations were trialed for the derivatization reaction. Occurence of the fluorophore heating at 80ºC for 5 min in a thermostated water bath was enough for derivatization.

Effect of NBD-Cl concentration

The effect of NBD-Cl concentration on the derivatization reaction was investigated. It was found that 0.025 mmol (500 µL of 0.5% (w/v)) NBD-Cl solution was sufficient to obtain maximum intensity.

Effect of acetonitrile to water ratio in derivatization medium

Different volumes of acetonitrile and water, were trialed where the concentrations of drug, buffer and NBD-Cl solutions were kept constant. The maximum peak area was observed by using a ratio of acetonitrile to water as 1:3.

Stoichiometry of the reaction

Stoichiometry of the reaction the molar ratio of NBD-Cl to SRB in the reaction mixture was studied according to Job's method of continuous variation.[20] SRB and NBD-Cl solutions were utilized to with a 1:3 ratio. According to peak areas, it is correct to say that all of the reagent was consumed, and there was no shortfall or excess of the reagent in this stoichiometric ratio. Upon testing derivatization reactions, all solutions were injected in to HPLC system and peak areas were measured to find the optimal conditions. Derivatives, prepared under the above mentioned conditions, remained stable for at least 24 h.

Effect of HCl Concentration for Acidification

To remove the excess of NBD-Cl, NBD-OH was produced by acidification of the medium, for this purpose 0.2 mL of 1.0 N HCl was sufficient.

Chromatographic Separation Procedure

A good separation of the derivatives and endogenous compounds of plasma was obtained using an isocratic elution system and HPLC-FL as described above. Representative chromatograms of the blank plasma, plasma samples spiked with SRB (25 µg mL^{-1}) and plasma samples of the volunteer that administered Nexavar® tablets containing 400 mg SRB at t_{max} are shown in Figure 2 a, b, c respectively. No interference was detected with the plasma constituents. The retention time of SRB is about 3.10 ± 0.02 min. Table 1 shows the chromatographic system suitability parameters that indicates the quality of the separation process.

Validation of the method

Validation of the method was carried out according to the following guidelines given by the International Conference on Harmonization (ICH).[21]

Calibration and sensitivity

The linearity of the method was evaluated by a calibration curve in the range 0.25–10.0 µg mL^{-1} of the drug (n = 6). Calibration curves were prepared by the analysis of 1 mL plasma samples spiked with various volumes of each working standard SRB solution. The samples were then submitted to the process of extraction, pre-column derivatization, chromatographic separation, and fluorometric detection described above. Calibration curves were obtained using linear least-squares regression analysis by plotting of peak areas of the derivative, versus the corresponding SRB concentrations. The equation of the calibration curve (n = 6) obtained from five points was: $y = 1835x + 3278$ (correlation coefficient = 0.9995) where y represents peak area of derivative and x represents the concentration of SRB.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined using the formula: LOD or LOQ= kSDa/b, where k=3 for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope. The parameters for the analytical performance of the proposed method are summarized in Table 2.

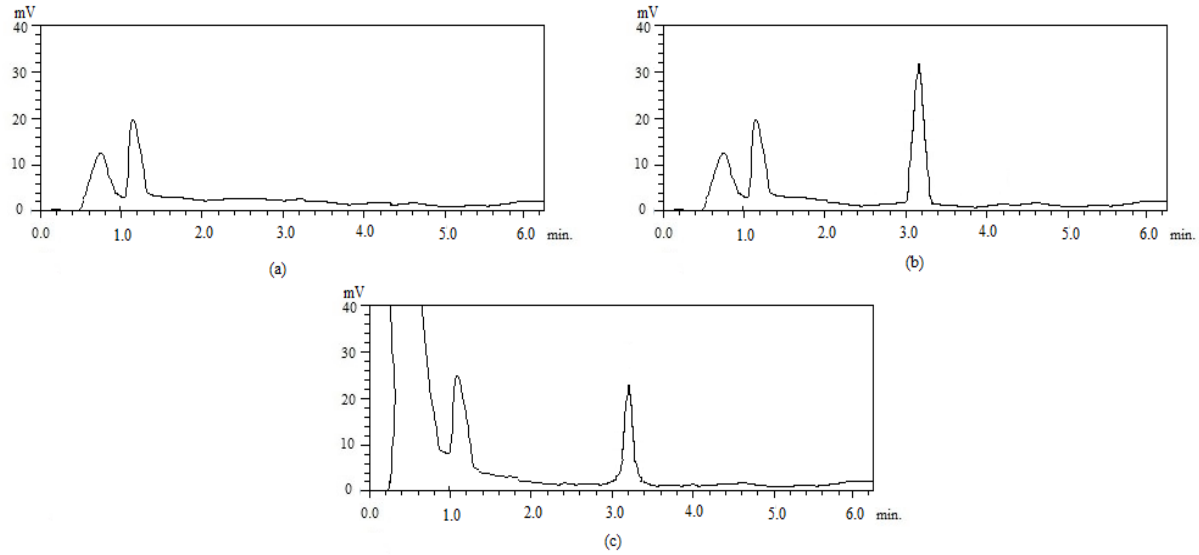

Fig. 2 – Chromatograms obtained from an extract of (a) blank plasma (b) plasma spiked with 20 µg mL^{-1} SRB, (c) plasma samples of the volunteer that administered Nexavar® tablets containing 400 mg SRB at t_{max}.

Table 1

Chromatographic system suitability parameters

capacity factor*	**resolution***	**HETP***	**tailing factor***	**asymmetry factor***
8.5	2.6	0.15	1.4	0.8

*mean values of the parameters of all the points of calibration

Table 2

Analytical parameters of the method

Parameters	**Method**
Concentration range [a] (µg mL^{-1})	0.25-10
Regression equation[b]	
Intercept ± SD	3278 ± 24.31
Slope ± SD	1835 ± 12.68
Correlation coefficient (r^2)	0.9995
LOD (µg mL^{-1})	0.075
LOQ (µg mL^{-1})	0.25

[a] Average of six determinatons
[b] $y=xC$ +b where C is the concentration in µg mL^{-1} and y is the peak area

Accuracy, precision and recovery

Accuracy and precision were assessed by determination of QC samples at three concentration levels. QC samples at three different concentrations (0.25, 5.0 and 10 µg mL^{-1}) that can be classified as low, medium and high concentration (n = 3) in plasma and aqueous sample were prepared. The accuracy was expressed by recovery values and RME and the precision by RSD. The absolute recovery of SRB from plasma samples was examined by extraction and derivatization of SRB spiked plasma samples

and comparison with peak areas obtained after derivatization of the same amounts of aqueous unextracted SRB solutions. The mean absolute recovery of SRB were of 94.20%. The mean relative recovery was calculated as 92.35% by the comparison of the amounts that is added on to spiked and measured by the calibration curve. Three replicates of samples at each concentration were assayed on the same day for intraday and on three different days for interday precision and accuracy. The RDS values of both intraday and interday assays were all less than 5.48%. According to all these results summarized in Table 3 good precision and accuracy were observed.

Robustness

Robustness was assessed by determination of the QC samples at three concentration levels as described at validation section above (n=3). The parameters, that are changed to measure the robustness of the method, are flow-rate, column oven temperature, acetonitrile and aqueous phase contents of the mobile phase. The column temperature was changed from 40ºC to 35ºC and 45ºC. The mobile phase proportions were changed from 60:40 (acetonitrile–acidic solution) to 65:35 and 55:45 and the flow rate was changed from 0.5 to 0.3 and 0.6 mL min^{-1}. These changes had no significant effect on peak area and resolution. According to standard conditions resolution of derivatives peak is 3.10 ± 0.02, in the trials for robustness resolution was observed between 3.0 to 3.5. Low RSD values indicates the robustness of the method Table 4.

Stability

The stability of working standard SRB solutions were tested at several storage conditions at QC levels and as three replicates. The trialed storage conditions are keeping at dark and at room temperature for 24 h, keeping in autosampler conditions for 24 h and keeping in refrigerated at 4ºC for 1 month. Recovery values for the trialed conditions are as follows; 98.1%, 97.9%, 96.5% respectively. The highest RSD % for all these experiments was 4.43%. It is possible to mention under all tested conditions SRB were found to be stable.

Table 3

Accuracy and precision of the method

Existant concentration (μg mL^{-1})	Added concentration (μg mL^{-1})	Found concentration (μg mL^{-1}) (Mean±SD[1])	Recovery (%)	RSD of recovery	RSD of intraday variation	RSD of interday variation
1	0.25	0.24 ± 0.01	94.37	1.24	3.23	4.11
	5	4.60 ± 0.52	91.16	3.21	3.55	5.16
	10	9.15 ± 0.87	91.52	3.35	4.27	5.48
Mean relative recovery = 92.35						

For each concentration n = 3

Table 4

Robustness of the method

Condition	Value	Recovery %	RSD %
Flow rate (mL min^{-1})	0.3	95.32	1.25
	0.6	93.21	2.33
Mobile phase composition (methanol:aqueous phase)	65:35	101.23	2.24
	55:45	94.12	1.68
Column temperature	35	95.42	3.56
	45	91.17	4.22

n=3 for all QC sample levels

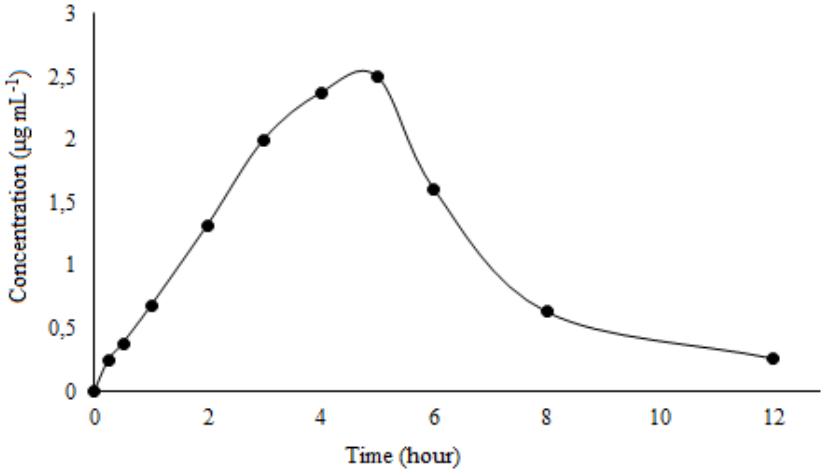

Fig. 3 – Pharmacokinetic curve of SRB after administration of 400 mg dose orall.

Table 5

Pharmacokinetic parameters of SRB

Parameter	Found value
T_{max} [a](h)	5
C_{max} [b](µg mL^{-1})	2.5
$t_{1/2}$[c](h)	30
AUC^{d}_{0-12} (µg h mL^{-1})	21.3
$AUC^{d}_{0-\infty}$ (µg h mL^{-1})	29.6

[a]Time to maximum concentration
[b]Maximum concentration,
[c]Elimination half life,
[d]Area under the concentration-time curve

Application of the Method to Pharmacokinetic Analysis

The presented method was applied to the determination of SRB in plasma for the prototype pharmacokinetic study. A healthy 42 year-old male volunteer was administered a two oral dose of SRB (2x200 mg). Approximately, 5 mL venous blood samples were collected prior to dosage and 0, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8 and 12 h, afterwards on the administration. For the following days, blood samples were collected once a day for 5 days. The blood samples were processed to plasma as described above. Figure 2c shows a chromatogram of the plasma sample obtained 5 h after the oral dose of 400 mg SRB from the volunteer. The samples were stored at –20ºC until analysis. Pharmacokinetic parameters were calculated by using the analysis carried out by the proposed method, which are given in Table 5. Area under the plasma concentration–time curves (AUC_{0-12}, $AUC_{0-\infty}$) were calculated using the TOPFIT 2.0 pharmacokinetic and pharmacodynamic data analysis system.[22] A plasma concentration–time curve of SRB after an oral administration of a two oral dose of 200 mg of drug is shown in Fig. 3. The results are compatible with a previous pharmacokinetic research by single dose 400 mg application.[23] In the cited study t_{max} was between 2.1-8 h, we measured as 5 h. C_{max} was found 1.93 µg mL^{-1} in our study it was found 2.5 µg mL^{-1}.

EXPERIMENTAL

Chemicals and Reagents

SRB was obtained from Shanghai Yingxuan Pharmaceutical Science & Technology (China), Nexavar® tablets containing 274 mg SRB tosylate equivalent to 200 mg SRB were purchased from local drug store. Acetonitrile, trifuoroacetic acide (HPLC grade), hexane and isoamyl alcohol (analytical grade) were supplied from Merck (Darmstadt, Germany). NBD-Cl was supplied from Sigma (MO, USA). Water was purified by Human (Japan) ultrapure water purification system.

Solutions

A stock solution of SRB tosylate (equivalent to 0.1 mg mL^{-1} SRB) was prepared and diluted with water to give standard solutions of from 0.25 to 10 µg mL^{-1}.

Phosphate buffer was prepared by 2.0209 g of sodium phosphate dibasic and 0.3394 g of sodium phosphate monobasic solution in 50 mL water. The pH level was adjusted to 8.5 with 0.1 M hydrochloric acid solution, and the

volume was made up to 100 mL with water. NBD-Cl solution was freshly prepared in methanol at 5 mg mL^{-1}. The other solutions were stored at 4ºC and were stable at least for 2 weeks.

Instrumentation

Fluorescence spectra and measurements were taken on a Shimadzu spectrofluorimeter Model RF-1501 equipped with xenon lamp and 1-cm quartz cells. Excitation and emission wavelengths were set at 398 nm and 425 nm. pH measurements were conducted with WTW pH 526 digital pH Meter.

The UPLC analyses were carried out on a Shimadzu (Japan) LC 20 liquid chromatograph which includes a binary LC-20AT pump, SIL AT-HT autosampler part, a SPD-20A HT fluorimetric detector, which was set at 398 nm for excitation and 425 for emission nm and CTO 10 AC column oven.

Different mobile phase, column types and size combinations were trialed with different flow rates and column temperatures in order to get the most efficient chromatographic separation. Chromatographic separation was achieved isocratically with Waters® C18 (50 mm x 2.1 mm, 1.7 µm) analytical column at 40ºC using a mobile phase composed of acetonitrile - 0.1% trifluoroacetic acid in water (60:40, v/v) by isocratic elution with flow rate of 0.5 mL min^{-1}. The injection volume was 7 µL.

Optimization Studies for Derivatization Procedure

The different experimental parameters affecting the development of the reaction product were trialed and optimal conditions were determined. Some parameters were changed individually while others were kept constant. These were: pH, reagent concentration, temperature and heating period, organic-aqueous solution ratio in the reaction medium.

Sample Preparation and General Procedure

Plasma samples were collected from a 42 years old healthy male volunteer (informed consent form was obtained according to ethical commitee approval) into polyethylene storage packs. The plasma samples were stored at −20°C. To extract the drug from the plasma samples, 1 mL plasma was alkalinized with 100 µL 0.1 M NaOH, and the solution was then extracted into 5 mL of hexane:isoamyl alcohol (4:1 v/v) mixture. The contents were mixed with vortex mixer at moderate speed for 5 min and centrifuged at 4500 × g for 2 min. After completing the extraction process, the aqueous layer was discarded. The organic layer was evaporated to dryness under a stream of nitrogen at 40ºC. To the residue, 1 mL water, 500 µL pH 8.5 phospate buffer and 500 µL of 5 mg mL^{-1} NBD-Cl solution were added, the system was heated at 80°C for 5 min. In order to stop the reaction, the tubes were cooled in an ice batch and then mixture was acidifed using 0.2 mL of 1 N HCl solutions. 7 µL of this solution including the derivatized sample was injected into the HPLC system.

CONCLUSIONS

SRB is a popular drug substance because of its therapeutic effect on advanced renal and hepatocellular cancer. Also, there are current studies that shows the effects of SRB for different cancer types. Due to the fact that this is a relatively new drug, it is a requirement to study on drug-drug and drug-food interactions and the side effects. For these purposes, sensitive and simple analytical methods are needed to carry out different types of clinical studies and measure the amount of SRB in human plasma. This presented study provides simple, selective and fast assay for SRB in human plasma and it is sensitive enough to investigate the pharmacokinetics of the drug. The chemical structure of SRB provided a dertivatization reaction for fluorimetric detection. After this pre-column derivatization a novel UPLC method developed and validated. This is the first fluorimetric detectin of SRB in the literature. A prototype pharmacokinetic study was carried out to calculate the parameters that indicates the pharmacokinetic features and bioavailability of the drug in oral administration. This new method can be used in the near future clinical researches and routine analysis of the drug.

Compliance with ethical standards

Ethical approval All procedures performed in 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. Bezmialem Vakıf University approved by the Clinical Trials Ethic Commitee (No: 24/23).

Informed consent Informed consent was obtained from all individual participants included in the study.

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