https://doi.org/10.31925/farmacia.2023.5.17 **ORIGINAL ARTICLE**

QUANTITATIVE ANALYSIS OF SORAFENIB AND NILOTINIB IN HUMAN PLASMA BY SPE-LC-MS

MONICA DENISA ELENA POPESCU [1], COSTEL-VALENTIN MANDA [1]*, DANIELA MARIA CALUCICĂ [1], AMELIA MARIA GĂMAN [2], JOHNY NEAMȚU [1], ANDREI BIȚĂ [1], OLIVIA GAROFIȚA MATEESCU [2], SIMONA-DANIELA NEAMȚU [1], ȘTEFAN RĂILEANU [3], LILIANA STANCA [2], OCTAVIAN CROITORU [1]

[1]*Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova, Petru Rareș Street, 200638, Craiova, Romania*
[2]*Faculty of Medicine, University of Medicine and Pharmacy of Craiova, Petru Rareș Street, 200638, Craiova, Romania*
[3]*"Filantropia" Clinic Oncology Municipal Hospital, 1 Filantropiei Street, 200143 Craiova, Romania*

**corresponding author: valimanda052@gmail.com*

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Abstract

Sorafenib and nilotinib are two tyrosine kinase inhibitors (TKIs) used in the treatment of cancer. Plasmatic levels of the drugs show an important variability, so determining plasma concentration of the drugs, benefits in cancer treatment can be improved. Most papers published so far in the literature use protein precipitation followed by liquid chromatography - tandem mass-spectrometry (LC-MS/MS) as separation and detection method. With this work, we propose an alternative method for the analysis of both TKIs in human plasma. Solid phase extraction (SPE) involving Oasis PRiME HLB® cartridges was our choice for plasma "clean-up" procedure. Extraction recoveries were at least 85%. Chromatography was performed by an ultra-high-performance liquid chromatographic system (UHPLC), using a C18 (4.6 x 50 mm) column and a mobile phase consisting of ammonium acetate/acetic acid-acetonitrile gradient elution. Detector was a simple mass spectrometer (MS) in Single Ion Recording (SIR) mode. Intra- and inter-day precision data for both TKIs were 3.8 - 7.6% and 4.5 - 8.8% for sorafenib and nilotinib, respectively. Sorafenib and nilotinib calibration curves were linear between 500 and 20000 ng/mL and 5 and 5000 ng/mL respectively, with correlation coefficients higher than 0.998. Analytes were determined in a 15 min run-time. The validated LC-MS method was applied in real human plasma routine analysis. This method may improve dose adjustment of the drugs in patients involved in cancer therapy.

Rezumat

Sorafenib și nilotinib sunt doi inhibitori ai tirozin kinazei utilizați în tratamentul cancerului. Nivelurile lor plasmatice prezintă o variabilitate importantă, astfel încât determinarea concentrațiilor lor, poate ajuta în tratamentul cancerului. Majoritatea articolelor din literatură utilizează precipitarea proteinelor ca metodă de pregătire a probelor și cromatografia de lichide și spectrometria de masă în tandem (LC-MS/MS) ca metodă de separare și detecție. Propunem o metodă alternativă pentru analiza ambelor medicamente în plasma umană. Extracția pe fază solidă (SPE) cu cartușe Oasis PRiME HLB® a fost alegerea noastră pentru procedura de „curățare" a plasmei. Recuperările la extracție au fost de cel puțin 85%. Separarea a fost efectuată pe un cromatograf de lichide cu o coloană C18 (4,6 x 50 mm) și o fază mobilă cu gradient de eluție constând din acetat de amoniu/acid acetic-acetonitril. Detectorul a fost un spectrometru de masă (MS) în modul Single Ion Recording (SIR). Datele pentru precizia intra- și inter-zi pentru ambele TKI au fost 3,8 - 7,6% și respectiv, 4,5 - 8,8%. Curbele de calibrare pentru sorafenib și nilotinib au fost liniare între 500 și 20000 ng/mL și respectiv 5 și 5000 ng/mL, cu coeficienți de corelare mai mari de 0,998. Analiții au fost determinați într-un timp de 15 min. Metoda LC-MS a fost aplicată în analiza pe probe reale de plasmă. Această metodă poate îmbunătăți ajustarea dozei de medicamente la pacienții implicați în terapia anti-cancer.

Keywords: sorafenib, nilotinib, human plasma, SPE-LC-MS

Introduction

Tyrosine kinase inhibitors (TKIs) represent a large class of drugs used for therapy of diverse types of malignant pathologies [1, 9]. The first drug, commercialized in 2001 was imatinib (Gleevec®) approved by Food and Drug Administration (FDA) for the treatment of Chronic Myeloid Leukemia (CML). Since then, clinical use of all TKIs has grown significantly, especially in recent years [13]. Several TKIs are currently used in the diagnosis of neoplasms or tumour relapses, both in monotherapy and in combination with radiotherapy/ chemotherapy. The mechanism consists in blocking the abnormal signalling pathways that are essential in the development of malignant cell proliferation. TKIs possess different selectivity profiles which depend on the type of tyrosine kinase involved, therefore these molecules are involved in the therapy of a large number of malignancies [4]. In recent years, many new drugs have been created, with target on different kinase proteins, used against numerous cancers.

In 2020, a comprehensive review regarding analytical methods for determination of lymphocytic anti-leukaemia drugs, including sorafenib and nilotinib has been published by Suresh *et al*. [19]. Bioanalysis of these compounds is gaining more and more interest. Many examples are found in the literature [4, 11, 14, 25]. TKIs are mostly employed in cancer treatment and introduction in clinics revolutionized therapy by transforming potential deadly diseases into chronic malignancies. The tyrosine kinase inhibitor sorafenib was originally approved for the treatment of renal cell carcinoma, but currently, it is the only licensed drug approved by FDA for the therapy of hepatocellular carcinoma. Development of more promising treatment strategies is highly desirable for patients whose life expectancy is less than one year. Regorafenib is another example of treatment which exhibited survival benefits in hepatocellular carcinoma patients progressing on sorafenib treatment [16]. A novel therapy approach, using a combination of selective cyclin-dependent kinases (CDK4/6) inhibitor palbociclib with sorafenib reported to impair the tumour development and to significantly increase survival percentage in many preclinical studies of hepatocellular carcinoma [16]. The results of the study stated further clinical combination between palbociclib and sorafenib, in the treatment of patients affected with this carcinoma.

Since mass spectrometry as detection method has become more affordable, even small laboratories have begun to use these detectors for a more accurate chromatographic peak assignment and for their increased sensitivity in analysing low plasma levels of drugs (often ng/mL). Therefore, information on therapeutic drug monitoring (TDM) has become much more accessible [3, 20].

Bioanalysis of TKIs requires an appropriate pretreatment of blood or plasma samples. Like many drugs, TKIs possess a narrow therapeutic index, because the ratio between the minimum toxic and minimum effective plasma levels is typically less than 2 [22]. Therefore, extraction and analysis of these drugs from human plasma are important and crucial for medical research and patient care. TDM is essential to be performed for all narrow therapeutic index drugs. The narrow therapeutic index is added to the variability in pharmacokinetics regarding major mechanisms involved in determining drug exposure due to factors like age, gender, genetics, diet, different diseases or poly-pharmacy. TDM implies monitoring of drug levels followed by subsequent dose adjustment to achieve a plasma-drug concentration within an optimised therapeutic range. Therefore, accurate determination of plasma concentration is mandatory.

Interfering plasmatic compounds like salts, phospho-lipids, proteins and exogenous substances may alter chromatographic distribution process of the analytes and/or the electron ionization in MS [26]. Therefore, a sample pre-treatment method which provides at the same time a good "sample clean-up" and an increased extraction recovery is mandatory in resolving most of these problems.

By now in the literature, authors usually used protein precipitation [6, 8, 10, 13, 16, 22, 24] and liquid-liquid extraction (LLE) [23] to extract these compounds from biological fluids. To our knowledge, only a few papers presented so far used solid phase extraction (SPE) in TKIs sample pre-treatment [4, 11, 14]. The inconvenience in protein precipitation is represented by the lack of compatibility of the supernatant with the electrospray ionization process in the MS source due to large amounts of co-extracted endogenous compounds. LLE usually generates clean samples, but requires large amounts of solvents, a tedious working procedure, usually with multiple extraction steps.

SPE is an effective technique, involving low solvent consumption and low sample volumes which provides an even better "sample clean-up" than both protein precipitation and LLE. The extraction ratio, in some cases, is similar to SPE, but the main problem in simple protein precipitation and LLE is that a major quantity of phospholipids and plasma proteins are co-extracted in the upper organic layer. Koller *et al*. [11] used a SPE technique with a "Mixed-mode Cation exchange sorbent for bases". After applying this type of SPE sorbent (hydrophilic-lipophilic with mixed mode component) for amphiphilic molecules, they stated to remove more than 91% of early eluting phospholipids compared to protein precipitation and LLE techniques. The draw-back of this technique is the cartridge conditioning step with methanol and water.

Most authors employed tandem mass spectrometry for TKIs analysis in rat or human plasma [2, 4, 10-14, 17, 18, 22, 24, 27]. MS/MS detection methods are very sensitive and accurate, but also imply expensive instruments that, in most cases, are not affordable to small laboratories.

Our team already published two papers regarding some TKIs (ibrutinib and ruxolitinib) analysis in human plasma [5, 15]. The purpose of this work was to investigate the extraction process of sorafenib and nilotinib from human plasma, by analysing various conditions and types of cartridge in SPE. The aim was to optimize, but also simplify SPE working procedure, avoiding the problems that may occur in ESI-MS electrospray interface. All parameters for MS were assessed to obtain the best MS signals for both compounds. Full validation was performed and the method was applied on real patients' plasma samples.

Materials and Methods

Chemicals and reagents

Sorafenib and nilotinib were provided by Selleck Chemicals GmbH (Germany). Pentoxifylline (IS) was purchased from Merck. Ammonium acetate, acetic acid and HPLC solvents (acetonitrile, water, methanol)

were all MS grade and provided by Merck. Oasis PRiME HLB® cartridges were purchased from Waters (USA). Structures of the analytes, sorafenib, nilotinib and IS are presented in Figure 1.

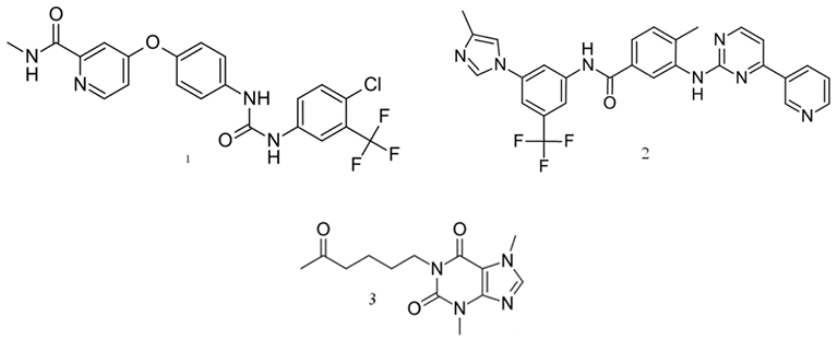

Figure 1.
Chemical structures of sorafenib (1), nilotinib (2) and IS (3)

Chromatographic system and MS detector parameters
The LC-MS instrument utilized for separation and detection was a Waters (Milford, Massachusetts, USA) Arc System (720006861EN) coupled with a Waters Acquity QDa mass detector, respectively. The chromatographic column CORTECS C18 (4.6 × 50 mm, 2.7 µm) was also purchased from Waters. The gradient elution was performed by using two solvents: solvent A (ammonium acetate/acetic acid 10 mM aqueous solution) and solvent B (acetonitrile). The following gradient was employed for the proper separation of the analytes: initial gradient, 75% (A) and 25% (B); 0 - 10 min, gradually increasing eluent B to 40%; 10 - 15 min, gradually increasing eluent B to 70%; 5 min post-run time are necessary between consecutive runs to equilibrate the column to the initial gradient. The chosen flow rate for the mobile phase was 0.5 mL/min. The separation of analytes was performed after the column was equilibrated at 40°C temperature. Injection volume was 5 µL. The temperature in the autosampler during the entire analysis was 20°C.

Separated analytes were detected using a QDa mass detector equipped with an electrospray ionization (ESI) source. The capillary voltage was set to 800 V and the cone voltage was maintained at 25 V. Mass spectra were all obtained in the positive ion mode, within the mass ranges m/z 100 - 500 for sorafenib and m/z 100 - 600 for nilotinib. Quantitative determination was achieved in SIR mode for sorafenib, nilotinib and pentoxifylline at m/z 465, 530 and 279, respectively. Equipment parameters, acquisitions and resulting data were controlled and processed using EmPower3 software.

Standard stock and working solutions
All stock solutions of sorafenib, nilotinib and IS were prepared in methanol at a concentration of 1 mg/mL. Then, separate working solutions of sorafenib (5, 10, 20, 50 and 200 µg/mL) and nilotinib (0.05, 0.5, 5, 10 and 50 µg/mL) were prepared by further dilution of these solutions with methanol, to further obtain target concentrations of the calibration curve. Two IS working solutions were prepared separately at 200 and 50 µg/mL concentration and used for sorafenib and nilotinib further analysis, according to the extraction procedure presented below. Standard solutions were kept at -20ºC and renewed every 30 days.

SPE procedure
Extraction procedure was performed separately for each analyte. A volume of 10 µL analyte working solution was evaporated to dryness in nitrogen stream at ambient temperature in a 0.5 mL conical glass vial. A volume of 100 µL blank human plasma was added. Then, 10 µL IS working solution (200 µg/mL for sorafenib analysis and 50 µg/mL for nilotinib analysis) were added and the sample was transferred to the Oasis PRiME HLB cartridge applying a positive pressure. Interfering compounds were eliminated in the washing step with 500 µL aqueous solution containing 5% methanol, then analytes were recovered in the elution step with 500 µL methanol. The obtained methanolic solution was evaporated in nitrogen stream to dryness and the residue was dissolved in 50 µL aliquot of initial mobile phase, consisting of aceto-nitrile:ammonium acetate/acetic acid 10 mM aqueous solution, 25:75 (v/v). The resulted solution was transferred in the autosampler vial and a 5 µL aliquot was automatically injected into the chromatographic system.

Validation data
All data was assessed according to International Council for Harmonisation guidelines (ICH guideline M10 on bioanalytical method validation and study sample analysis–step 5).

Criteria for selectivity should demonstrate that no significant response attributable to interfering components is observed at the retention times of the analyte or the IS in the blank samples. Responses which correspond to interfering components should not be more than 20% of the analyte response at the LLOQ and not more than 5% of the IS response in the LLOQ sample.

The calibration curve reveals the relation between the analyte concentration and the response of the LC-MS system to the analyte. Linear regression equation was employed. Calibration standards, prepared by spiking plasma with a known quantity of analyte, span the calibration range and comprise the calibration curve. Calibration standards are prepared in the same biological matrix as the real samples (human plasma). Calibration curves concentrations in plasma, obtained after extraction procedure were 500, 1000, 2000, 5000 and 20000 ng/mL for sorafenib and 5, 50, 500, 1000 and 5000 ng/mL for nilotinib. Determination of intra-day and inter-day accuracy (percentage ratio between mean found concentration and spiked concentration) and precision (coefficient of variance – CV%) of the analytes for all calibration levels was also performed (n = 5). The accuracy at each concentration level should be within 85 - 115% of the nominal concentration, except at the LLOQ, where it should be within 80 - 120%. The precision (%CV) of the concentrations determined at each level should not exceed 15%, except at the LLOQ, where it should not exceed 20%. Lower limit of quantitation (LLOQ) is accepted as limit of quantitation (LOQ) if the lowest standard has a signal to noise (S/N) ratio at least 10:1 and precision is no more than 10%.

Extraction recovery represents the percentage ratio between the peak area of the extracted compound and the peak area resulted after direct injection of the same amount of drug dissolved in the initial mobile phase.

Long term stability, freeze-thaw stability and room temperature stability of the analytes were also established in human plasma with potassium EDTA. Long term stability for 30 days at -20ºC and freeze-thaw stability after three freeze-thaw cycles were investigated (n = 5). Room temperature stability (20ºC) was determined for 12 hours (n = 5) to detect any degradation process of the analytes during SPE extraction procedure or actual analysis. The mean data obtained at each concentration level should be within ± 15% of the nominal concentration.

Carry-over effect

Carry-over assessment is a mandatory procedure when MS detection is employed. MS is a very sensitive detection method, so alteration of the measured concentration due to residual analytes that remain in the analytical instrument can occur. We evaluated carry-over by chromatography of a blank sample after previous injection of a calibration standard containing the analyte at the maximum concentration of the calibration curve (20000 and 5000 ng/mL for sorafenib and nilotinib, respectively). Acceptance criteria state that peak areas for the analytes in the blank samples should not be larger than 10% reported to LLOQ sample peak area (500 and 5 ng/mL for sorafenib and nilotinib, respectively). To avoid carry-over, the needle injection was automatically washed ten times with 10 µL mobile phase (initial gradient) between consecutive injections (using the option in Empower 3 instrument software). Furthermore, after injection of samples with an expected high concentration, before the next study sample, an injection of a blank sample is performed.

Method application to plasma samples

The method was applied for qualitative and quantitative determination of both drugs on real plasma samples. Sorafenib plasma levels were investigated in 7 patients with hepatocellular carcinoma, which received a daily dose of 800 mg sorafenib (Nexavar®), 400 mg twice a day. All samples were collected on day 10, two hours after administration of the morning dose. A volume of two mL of blood were collected into a vacutainer with potassium EDTA, followed by a 5 min centrifugation at 10000 rpm. When plasma samples were not immediately processed, they were put at -20ºC until analysis.

In a similar manner, nilotinib samples were collected from 5 chronic patients with chronic granulocytic leukaemia, treated with 400 mg nilotinib (Tasigna®), twice a day. On day 10 samples were collected, also two hours after the morning dose and then processed or kept at -20ºC as previously described.

Before analysis, frozen samples were thawed at ambient temperature, then extracted as in the SPE extraction procedure. Patients have given informed consent regarding collecting plasma samples.

Results and Discussion

Validation data

Typical chromatograms in SIR mode for the two analytes, with retention times of 14.48 for sorafenib and 13.33 min for nilotinib, are presented in Figure 2. Both peaks were confirmed by spiking the final extracts with standard working solutions. Regarding selectivity, at specified m/z values (465 and 530), no other peaks are observed on the chromatogram.

Calibration curves for both compounds reported to the IS are presented in Figure 3. On the ordinate axis, peak area ratios of the analyte/IS are represented; on the abscissa, plasma levels for sorafenib and nilotinib are represented. Correlation coefficients demonstrate the calibration curves are linear in the selected concentration range.

The extraction recovery was, for both compounds and for all concentrations, more than 85%. LLOQs were 500 and 5 ng/mL for sorafenib and nilotinib, respectively. Table I reveals stability tests results for sorafenib and nilotinib. Long term stability, freeze-thaw and room temperature stability were larger than 95%.

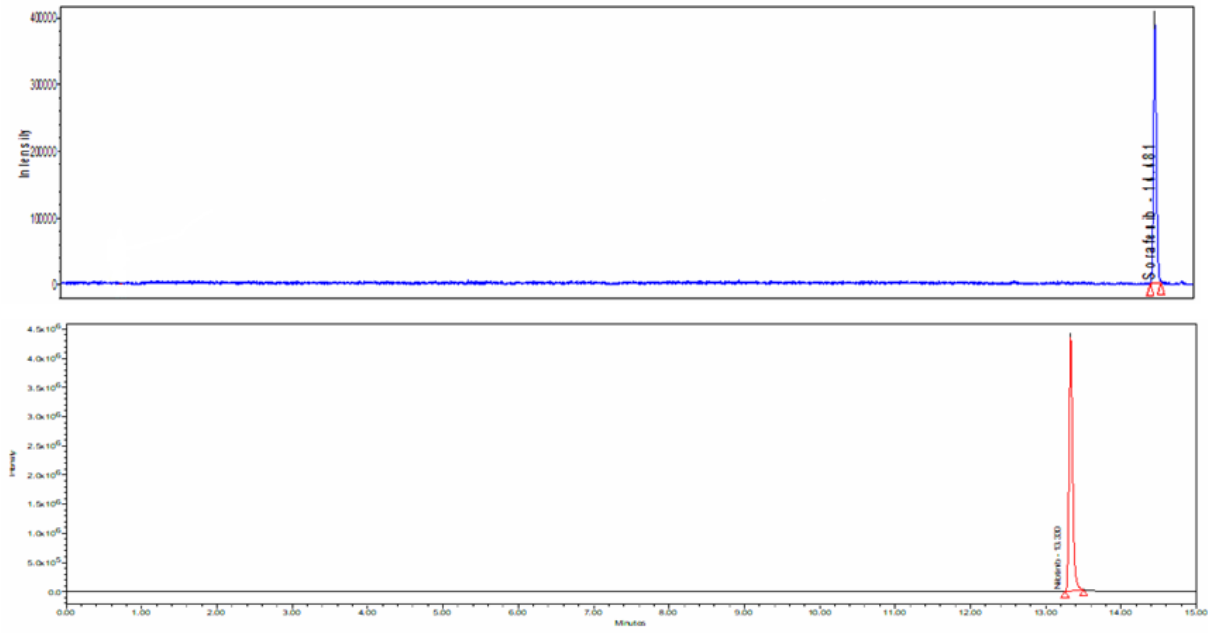

Figure 2.
Chromatograms (in SIR mode) for sorafenib (up) and nilotinib (down), with retention times of 14.48 and 13.33 min, respectively

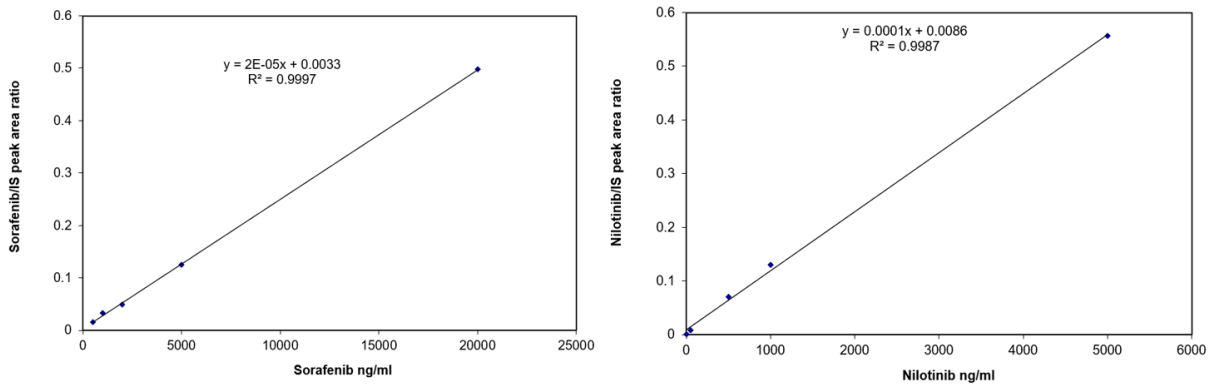

Figure 3.
Calibration curves, linear regression equations and correlation coefficients for sorafenib and nilotinib

Table I
Stability data for sorafenib and nilotinib (n = 5)

Target concentration sorafenib/nilotinib (ng/mL)	Mean stability (%) for sorafenib/nilotinib		
	Long term stability	Freeze-thaw stability	Room temperature stability
500/5	95.9/96.2	95.0/94.7	95.6/94.9
1000/50	96.7/96.0	97.3/97.0	95.6/95.3
2000/500	97.5/97.8	98.1/97.5	97.2/96.9
5000/1000	97.7/97.5	97.3/98.0	98.0/98.3
20000/5000	98.1/98.2	98.1/98.5	98.4/98.6

Samples were not processed and analysed immediately after collection. Therefore, first it is necessary to determine long term stability of the analytes in the matrix at -20ºC for a period of 30 days. Real plasma samples should not be kept at this temperature for a longer time before analysis. On the other hand, freeze-thaw stability must be investigated to establish possible degradation during the thaw process. Stability of the analyte in processed samples at 20ºC for 12 hours is necessary for evaluating both sample preparation process and time until completion of analysis (inside the autosampler/instrument).

The calibration curve data and stability tests demonstrate that the acceptance criteria are met for all concentration range on the calibration curve. Values for long term stability, freeze-thaw stability and room temperature stability show the reliability of the method.

Intra- and inter-day precision data were between 3.8 and 8.8%. Results for precision and accuracy are represented in Table II.

Table II

Precision and accuracy data (n = 5)

Analyte	Target concentration (ng/mL)	Mean found level (ng/ml)		CV %		Accuracy %	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Sorafenib	500	520.0 ± 37.4	540.2 ± 41.0	7.2	7.6	104.0	108.0
	1000	1019.8 ± 68.3	1029.3 ± 65.8	6.7	6.4	102.0	102.9
	2000	1958.2 ± 109.6	1960.3 ± 113.6	5.6	5.8	97.9	98.0
	5000	5066.1 ± 212.7	5074.7 ± 243.5	4.2	4.8	101.3	101.5
	20000	19945.6 ± 758	19717.9 ± 788.7	3.8	4.0	99.7	98.6
Nilotinib	5	4.7 ± 0.4	5.2 ± 0.4	8.6	8.8	95.1	104.7
	50	49.0 ± 3.8	48.6 ± 38.3	7.8	7.9	98.1	97.2
	500	512.1 ± 35.3	515.5 ± 36.0	6.9	7.0	102.4	103.1
	1000	988.1 ± 45.5	991.0 ± 51.5	4.6	5.2	98.8	99.1
	5000	5016.0 ± 225.7	5055.3 ± 242.6	4.5	4.8	100.3	101.1

Calibration standards are intended to mimic study samples and should be prepared by spiking matrix with a known quantity of analyte, storing them under the conditions anticipated for study samples and analysing them to assess the validity of the analytical method. Intra-day and inter-day accuracy and precision should be evaluated by analysing at least 5 replicates at each QC concentration level in each analytical run. Precision and accuracy data demonstrate the performance of the method. As one can see in Table II, values for both drugs are better than the acceptance criteria.

Plasma concentrations in real patients' plasma

Plasmatic levels of sorafenib and nilotinib in patients were obtained by calculus, using the linear regression equations. Sorafenib concentration levels for the investigated patients were 5980, 19347, 12342, 8857, 7160, 10289 and 15960 ng/mL with 11419 ± 4828 ng/mL (mean ± SD). Calculated values for nilotinib plasma levels were 1245, 1136, 1293, 1320 and 1170 ng/mL with 1232 ± 78 ng/mL (mean ± SD). Obtained values are in accordance with those presented so far in other studies [7, 21].

A detailed pharmacokinetic and dose-finding study was conducted by Fucile *et al.* [7] for the measurement of sorafenib plasma concentration by high-performance liquid chromatography in 12 patients with advanced hepatocellular carcinoma. With a similar administered dose, plasma levels were between 3.73 and 19.63 μg/mL, showing a large interindividual variability. This is explained by complex pharmacokinetics of sorafenib, starting with a variable absorption depending on the dosage form (tablet/oral solution), food intake or high-fat meals. Plasma protein binding is about 99.5%, with a half-life variable between 25 - 48 h. Sorafenib is metabolized by cytochrome P450 iso-enzyme 3A4, as well as glucuronidation. Sorafenib and metabolites are mainly eliminated in faeces. Regarding toxicity, skin toxicities like rash/desquamation, skin reaction, alopecia and pruritus, diarrhoea and hypertension were the most frequent adverse reactions showing symptoms of mild or moderate grade. All these data make TDM necessary for sorafenib dose adjustment.

Clinical pharmacokinetics of nilotinib were investigated in a review by Tian *et al*. [21]. Nilotinib is rapidly absorbed, with a peak plasma concentration approximately 3 hours after dose administration. Regarding maximum plasma concentration, according to the dose of 400 mg nilotinib twice daily, the mean value for 17 patients was 1595 ng/mL. The coefficient of variation of 47% also makes TDM necessary for treatment adjustment to each patient.

SPE procedure assessment

An important step in validation of an analytical method is the assessment of sample preparation process. The vast majority of papers use simple protein precipitation as sample pre-treatment. This approach generates a supernatant which, followed or not by a filtration step, is directly injected into the LC column. The shortcoming of this method is the incompatibility of the resulting supernatant with MS detection technique. LLE is tedious and pollutes the environment with large amounts of solvents.

SPE was previously applied as sample pre-treatment method in the analysis of sorafenib and nilotinib in human plasma [4, 11, 14]. They used Oasis PRiME (Mixed-mode Cation exchange sorbent for bases) MCX cartridges which imply an activation/pre-conditioning step with methanol and water. Within our approach, after performing proper investigations, we obtained the best results by SPE using Oasis HLB PRiME cartridges. The cartridges contain a water-wettable polymer capable of hydrophilic interactions with polar compounds. The advantage is that the procedure use a very simple working protocol, without a conditioning step. The lack of conditioning and equilibration steps reduces solvent consumption and also time analysis. The "three step clean-up" protocol was employed. The first step implies that the sample (110 μL, including the 10 μL IS solution) is loaded to the cartridge and adsorbed on sorbent surface. The second step is

represented by washing step with 500 µL solution 5% methanol. In the elution step (500 µL pure methanol), the analytes and the IS were recovered in a percentage larger than 85% for all analytes and all investigated concentration levels. The use of acetonitrile in elution step is not recommended in this case, because of low solubility of analytes in this solvent. Proper sample extracts were obtained by eliminating most interfering plasma matrix compounds, in particular phospholipids and proteins.

After SPE, concentration of the eluted solution was necessary to increase sensitivity of the method. The solvent (methanol) was evaporated in nitrogen stream and the remaining residue was redissolved in a 50 µL volume of mobile phase, so a 10-fold increase in analytes concentration level was achieved.

HPLC parameters were also investigated. Reverse phase CORTECS C18 chromatographic column was used for separation of the analytes. The use of formic acid 0.1% aqueous solution-acetonitrile as mobile phase generated important peak tailing, especially for nilotinib. Ammonium acetate/acetic acid-acetonitrile gradient elution provided improved peak shape and acceptable retention times. Mobile phase flow rate was adjusted to 0.5 mL/min and best resolution was obtained for constant column temperature of 40ºC.

Conditions for MS detection were optimized. Positive ionization mode was employed. Voltage for capillary and cone were assessed to obtain optimum signal for both analytes and IS. The values were 0.8 kV and 25 V for capillary and cone voltage, respectively.

Stability tests data (> 95%) revealed that no degradation of the analytes occurred during stock or sample preparation. Calibration curves were linear (correlation coefficient larger than 0.998). Precision (expressed as CV) was lower than 10% (8.8% for nilotinib at LLOQ). Data regarding accuracy was 95 - 110% to the nominal (spiked) concentrations, as presented in Table II.

Moreover, the validated method was applied for determination of real plasma drug levels. Blood samples were collected in day ten from starting the treatment for the drugs to reach the "steady state" concentration levels. Collection of sorafenib and nilotinib samples was performed at two hours post morning dose, according to the literature [13].

Real plasma concentrations were within the calibration curves ranges for both analytes. All results regarding sorafenib and nilotinib plasma levels show a large interindividual variability, as presented so far in the literature [7, 21]. Variability is determined by complex factors, starting with differences in absorption and metabolism, dependent on type of CYP isoenzymes, food in-take and/or co-administered drugs (enzymatic inhibition or induction) and adherence to the treatment [12].

Sorafenib and nilotinib concentration levels in human plasma determined for the 12 patients were 11419 ± 4828 ng/mL (mean ± SD) and 1232 ± 78 ng/mL (mean ± SD), respectively. Results reveal large inter-individual variability regarding the two TKIs pharmaco-kinetics, especially for sorafenib, making TDM necessary in individual dose adjustment.

Conclusions

A simple SPE-LC-MS method for precise and accurate determination of sorafenib and nilotinib in human plasma was developed. A small volume of 100 µL human plasma is necessary within this approach. Sample preparation (simple SPE technique, without cartridge equilibration or conditioning) produced clean extracts, providing the necessary LLOQs for accurate determination of the two TKIs. Application of the method in establishing plasmatic levels in patients undergoing treatment with sorafenib and nilotinib was performed. Both validation data and the results of real plasma levels presented above qualify this method for investigation of drug concentrations in clinical therapy.

Conflict of interest

The authors declare no conflict of interest.

References

1. Arora A, Scholar EM, Role of tyrosine kinase inhibitors in cancer therapy. *J Pharmacol Exp Ther.*, 2005; 315: 971-979.
2. Beauvais D, Goossens JF, Boyle E, Allal B, Lafont T, Chatelut E, Herbaux C, Morchhauser F, Genay S, Odou P, Danel C, Development and validation of an UHPLC-MS/MS method for simultaneous quantification of ibrutinib and its dihydrodiol-metabolite in human cerebrospinal fluid. *J Chromatogr B.*, 2018; 1093-1094: 158-166.
3. Birch M, Morgan PE, Handley S, Ho A, Ireland R, Flanagan RJ, Simple methodology for the therapeutic drug monitoring of the tyrosine kinase inhibitors dasatinib and imatinib. *Biomed Chromatogr.*, 2013; 27(3): 335-342.
4. Bouchet S, Chauzit E, Ducint D, Castaing N, Canal-Raffin M, Moore N, Titier K, Molimard M, Simultaneous determination of nine tyrosine kinase inhibitors by 96-well solid-phase extraction and ultra-performance LC/MS-MS. *Clin Chim Acta*, 2011; 412(11-12): 1060-1067.
5. Croitoru DM, Manda CV, Boldeanu MV, Rotaru I, Neamțu SD, Neamțu J, Croitoru O, New approach in determining ibrutinib in human plasma by HPLC-DAD and application of the method in a preliminary pharmacokinetic study. *Farmacia*, 2020; 68(4): 640-645.
6. De Francia S, D'Avolio A, De Martino F, Pirro E, Baietto L, Siccardi M, Simiele M, Racca S, Saglio G, Di Carlo F, Di Perri G, New HPLC–MS method for the simultaneous quantification of the antileukemia drugs imatinib, dasatinib, and nilotinib in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci.*, 2009; 877(18-19): 1721-1726.

7. Fucile C, Marenco S, Bazzica M, Zuccoli ML, Lantieri F, Robbiano L, Marini V, Di Gion P, Pieri G, Stura P, Martelli A, Savarino V, Mattioli F, Picciotto A, Measurement of sorafenib plasma concentration by high-performance liquid chromatography in patients with advanced hepatocellular carcinoma: is it useful the application in clinical practice? A pilot study. *Med Oncol.*, 2015; 32(335): 1-7.
8. Gheorghe GS, Ciobanu A, Hodorogea AS, Gheorghe ACD, Scăunașu RV, Nanea IT, Stoian MI, Iliesiu AM. Cardiotoxicity of anticancer therapies: focus on the role of the cardio-oncological team. A practical review. *Farmacia*, 2021; 69(6): 1053-1059.
9. Hartmann JT, Haap M, Kopp HG, Lipp HP, Tyrosine kinase inhibitors – a review on pharmacology, metabolism and side effects. *Curr Drug Metab.*, 2009; 10: 470-481.
10. Huynh HH, Pressiat C, Sauvageon H, Madelaine I, Maslanka P, Lebbe C, Thieblemont C, Goldwirt L, Mourah S, Development and validation of a simultaneous quantification method of 14 tyrosine kinase inhibitors in human plasma using LC-MS/MS. *Ther Drug Monit.*, 2017; 39(1): 43-54.
11. Koller D, Vaitsekhovich V, Mba C, Steegmann JL, Zubiaur P, Abd-Santos F, Wojnicz A, Effective quantification of 11 tyrosine kinase inhibitors and caffeine in human plasma by validated LC-MS/MS method with potent phospholipids clean-up procedure. Application to therapeutic drug monitoring. *Talanta*, 2020; 208: 120450.
12. Liu J, Liu H, Zeng Q, The effect of naringenin on the pharmacokinetics of ibrutinib in rat: A drug–drug interaction study. *Biomed Chromatogr.*, 2019; 33(5): e4507.
13. Llopis B, Robidou P, Tissot N, Pinna B, Gougis P, Cohen Aubart F, Campedel L, Abbar B, Roos Weil D, Uzunov M, Glidorov J, Salem JE, Funck-Brentano C, Zahr N, Development and clinical validation of a simple and fast UPLC-ESI-MS/MS method for simultaneous quantification of nine kinase inhibitors and two antiandrogen drugs in human plasma: Interest for their therapeutic drug monitoring. *J Pharm Biomed Anal.*, 2021; 197: 113968.
14. Merienne C, Rousset M, Ducint D, Castaing N, Titier K, Molimard M, Bouchet S, High throughput routine determination of 17 tyrosine kinase inhibitors by LC–MS/MS. *J Pharm Biomed Anal.*, 2018; 150: 112-120.
15. Neamțu SD, Manda CV, Boldeanu MV, Rotaru I, Neamțu J, Biță A, Croitoru O, Calucică DM, Extraction process assessment and LC-MS analysis of two tyrosine kinase inhibitors in human plasma. *Farmacia*, 2022; 70(5): 855-860.
16. Paul D, Chandrakala P, Surendran S, Bitla P, Satheeshkumar N, Pharmacokinetic interaction study of novel combination of palbociclib and sorafenib for hepatocellular carcinoma in SD rats. *J Chr B.*, 2019; 1108: 25-31.
17. Rood JJM, van Hoppe S, Schinkel AH, Schellens JHM, Beijnen JH, Sparidans RW, Liquid chromatography–tandem mass spectrometric assay for the simultaneous determination of the irreversible BTK inhibitor ibrutinib and its dihydrodiol-metabolite in plasma and its application in mouse pharmacokinetic studies. *J Pharm Biomed Anal.*, 2016; 118: 123-131.
18. Sun S, Cheng D, Kong S, Li H, Li T, Yu Q, Wang L, A rapid and sensitive method for quantification of ibrutinib in rat plasma by UPLC–ESI–MS/MS: validation and application to pharmacokinetic studies of a novel ibrutinib nanocrystalline. *Biomed Chromatogr.*, 2020; 34(1): e4703.
19. Suresh PS, Trivedi RK, Srinivas NR, Mullangi R, A review of bioanalytical methods for chronic lymphocytic leukemia drugs and metabolites in biological matrices. *Biomed Chromatogr.*, 2020; 34(1): e4742.
20. Teo YL, Ho HK, Chan A, Metabolism-related pharmacokinetic drug-drug interactions with tyrosine kinase inhibitors: current understanding, challenges and recommendations. *Br J Clin Pharmacol.*, 2015; 79(2): 241-253.
21. Tian X, Zhang H, Heimbach T, He H, Buchbinder A, Aghoghovbia M, Hourcade-Potelleret F, Clinical pharmacokinetic and pharmacodynamic overview of nilotinib, a selective tyrosine kinase inhibitor. *J Clin Pharmacol.*, 2018; 00(0): 1-8.
22. Van Dyk M, Miners JO, Kichenadasse G, McKinnon RA, Rowland A, A novel approach for the simultaneous quantification of 18 small molecule kinase inhibitors in human plasma: A platform for optimised KI dosing. *J Chromatogr B.*, 2016; 1033-1034: 17-26.
23. Van Eeckhaut A, Lanckmans K, Sarre S, Smolders I, Michotte Y, Validation of bioanalytical LC–MS/MS assays: evaluation of matrix effects. *J Chromatogr B Analyt Technol Biomed Life Sci.*, 2009; 877(23): 2198-2207.
24. Veeraraghavan S, Thappali S, Viswanadha S, Chennupati S, Nalla S, Golla M, Vakkalanka S, Rangasamy M, Simultaneous quantification of ruxolitinib and nilotinib in rat plasma by LC–MS/MS: Application to a pharmacokinetic study. *J Pharm Biomed Anal.*, 2014; 94: 125-131.
25. Veeraraghavan S, Viswanadha S, Thappali S, Govindarajulu B, Vakkalanka S, Rangasamy M, Simultaneous quantification of lenalidomide, ibrutinib and its active metabolite PCI-45227 in rat plasma by LC–MS/MS: Application to a pharmacokinetic study. *J Pharm Biomed Anal.*, 2015; 107: 151-158.
26. Xia YQ, Jemal M, Phospholipids in liquid chromatography/mass spectrometry bioanalysis: comparison of three tandem mass spectrometric techniques for monitoring plasma phospholipids, the effect of mobile phase composition on phospholipids elution and the association of phospholipids with matrix effects. *Rapid Commun Mass Spectrom.*, 2009; 23(14): 2125–2138.
27. Yang J, Yu H, Tang Q, Simultaneous determination of evobrutinib and its metabolite evobrutinib-diol in dog plasma by liquid chromatography combined with electrospray ionization tandem mass spectrometry. *Biomed Chromatogr.*, 2019; 33(9): e4575.