A METHOD TO QUANTIFY SEVERAL TYROSINE KINASE INHIBITORS IN PLASMA BY MICELLAR LIQUID CHROMATOGRAPHY AND VALIDATION ACCORDING TO THE EUROPEAN MEDICINES AGENCY GUIDELINES

Iris Garrido-Cano[1], Aurelio García-García[1], Juan Peris-Vicente[1], Enrique Ochoa-Aranda[2], Josep Esteve-Romero[1]*

1. Departamento de Química Física i Analítica, ESTCE, Universitat Jaume I, 12071 Castelló, Spain

2. Biopatologia Molecular, Hospital Provincial, Castelló, Spain

** Corresponding author. E-mail: josep.esteve@uji.es - Tel.: 0034964728093*

This article was published by Talanta. The published version is available at:

https://doi.org/10.1016/j.talanta.2015.07.078

Abstract

A procedure based on micellar liquid chromatography has been developed to monitor five tyrosine kinase inhibitors in plasma, prescribed against several kinds of cancer: erlotinib, imatinib, sunitinib, sorafenib and lapatinib. The sample was diluted in a micellar solution and directly injected, thus clean-up steps were not required. The analytes were resolved without interferences in < 20 min using a C18 column and a mobile phase of 0.13 M SDS - 4 % 1-butanol, buffered at pH 3.5, running under isocratic mode at 1 mL/min. The detection was performed by UV-Visible absorbance, using a wavelength program to maximize the signal-to-noise ratio. The method was validated following the guideline of the European Medicines Agency in terms of: selectivity, calibration range (0.05 - 5 μg/mol), linearity ($r^2 > 0.990$), limit of detection (15 - 35 ng/mL), carry-over effect, accuracy (-10.4 to +11.0%), precision (< 9.2%), matrix effect, robustness (< 8.4%) and stability. The procedure is rapid, easy-to-handle, uses a low amount of toxic chemical provide reliable results. Finally, the method was successfully used to analyze the studied tyrosine kinase inhibitors in plasma from cancer patients.

Keywords: Direct injection; Micellar; Plasma; Tyrosine kinase inhibitors; Validation

1. Introduction

Tyrosine kinase receptors are proteins playing an important role in the transduction of the signals involved in growth and differentiation of cells. However, they are protooncogenes, because an increased activity provokes cell proliferation and stimulates formation of tumors [1]. Selective tyrosine kinase inhibitors (TKIs) have been developed to repress the activity of overexpressed tyrosine kinase membrane proteins by blocking its specific receptors or activity cores, in order to prevent cell growing and induce apoptosis. Besides, they are highly specific and may not affect normal cells. Therefore, these drugs have been prescribed for targeted therapy and hard improved progression free survival (PFS) and overall survival (OS) on the treatment of many cancer diseases [2]. Additionally, they notably increase the response rate.

Imatinib was the first approved TKI. It shows a strong activity against chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST) [3]. During the last years, other TKIs have been developed, as lapatinib, to treat solid tumors such as breast and lung cancer [4], erlotinib for lung and pancreatic cancer, among others [5], sorafenib for kidney, liver [6], and radioiodine-resistant thyroid (DTC) cancer [7], and sunitinib for renal cell carcinoma (RCC), imatinib-resistant GIST, and ovarian cancer (OC) [8,9] (The structures and characteristics of these drugs are described in Figure 1 and in Table 1 [9-13]). These TKIs are orally administered and must be taken during a long period, even lifelong [14]. TKI-based treatments have shown a high degree of success, they are generally well-tolerated and they cause weak adverse effects. Despite their excellent performances, some episodes of therapy failure, adverse effect or weak clinical effects have been reported [15].

Several studies have proven that predictive response to treatment is related to plasmatic concentration, rather than the ingested dose [16]. The therapeutic effects of the TKIs would be maximized if the plasmatic concentration remains in the therapeutic range, which is relatively narrow, throughout the whole treatment. Lower levels lead to treatment failure and stimulation of tumor resistance, whereas an excessive concentration can enhance the adverse effects. Both cases finally lead to an early interruption of the therapy [17]. However, it was observed that the same dosage of the studied TKIs produces different plasmatic concentrations, for different patients and even in the same patient at different stages of the treatment [18].

The plasmatic concentration of the TKIs is related to pharmacodynamics: bioavailability (absorption from the gastrointestinal system), metabolization (mainly in the liver through the enzyme cytochrome P450 3A4 pathway), and removal ways; and pharmacokinetics: drug-drug interactions, drug affinity constant and tumor cell resistance, besides to the ingested dose. Moreover, the TKIs are extensively bonded to plasma proteins, and less than 5 % are free to exert the pharmacological activity. Different studies have demonstrated that these factors depend on many characteristics (genetics, physiology, organ function, general health, diet, toxic habits, metabolism, adherence, time following the therapy and co-administration of other drugs), which

show high inter-individual differences, and even vary for the same patient over time. This explains the strong inter- and intrapatient variability of the plasmatic concentration of the TKIs [2,15,18,19].

Clinicians should personalize the dose, by prescribing to each patient the dose required to ensure his TKI-plasmatic concentration would remain in the therapeutic range, instead of setting a standard one, in order to improve the success of the therapy and limit the undesirable effects [16,18]. For this reason, the relationship between the ingested and plasmatic dose must be established for each patient at the beginning and along the therapy. This can be performed by means of therapeutic drug monitoring (TDM), which consists in quantification of drugs in plasma at several times after ingestion of drug formulation. TDM is useful for drugs showing narrow therapeutic range and highly variable biological activity, such as TKIs. It is also especially recommended for patients who develop an uncommon response. Plasmatic concentration at a specific moment would be also used to explain and predict the therapeutic and adverse effects, as well as to determine the adherence to the treatment [16-18]. Therefore, clinical laboratories are demanding practical and reliable analytical methods to quantify the most representative TKIs, such as erlotinib, imatinib, sunitinib, sorafenib and lapatinib in plasma.

Liquid chromatography coupled to mass spectrometry has been the technique of choice for the simultaneous analysis of these five TKIs in plasma for TDM purposes [20-23]. Likewise, ultra-high performance liquid chromatography (UPLC) has been coupled to MS for the simultaneous detection of these compounds [24]. However, mass spectrometry is an expensive instrumentation which requires a careful maintenance and a high throughput to be cost effective. Therefore, few clinical laboratories can afford it and external analyses at a high price are paid. Therefore, several aproaches based on HPLC-UV have been developed for the individual determination of imatinib [25], erlotinib [26], lapatinib [27], sorafenib [28] and sunitinib [29] in plasma. UPLC-UV has also been used to dectect imatinib [30] and erlotinib [31] in pharmaceutical formulations, and sunitinib in plasma [32]. However, at our knowledge, no method dealing with the simultaneous analysis of these five TKIs using UV-detection has been previously published. Although their different

perfomances, MS and UV detection provide similar results for TKI analysis in plasma [33,34]. These procedures use mobile phases containing a large amount of toxic and flammable organic solvent, that results in a risk for the laboratory staff and the environment. Another disadvantage of multi-TKI detection HPLC-methods is the need for mobile phases programmed as gradient, difficulting the analysis of a large amount of samples [20-24,30,32].

Analysis of plasma by HPLC has an important drawback due to the presence of a large amount of proteins and other macromolecules non-soluble in water, which must be removed prior injection to avoid their precipitation in the chromatographic system and the deterioration of the column. Therefore, tedious and time consuming clean up steps, which use large amounts of organic solvents, are required to separate TKIs from the matrix. The most popular is protein precipitation [20-23,25,27,28,34] although solid phase [24,29,32] and liquid/liquid [26,33] extraction have also been used. Besides, these sample pretreatment frequently lead to a variable recovery, and then an internal standard is usually added [20-24,26-28,33,34]. In order to automatize the analysis of TKIs in plasma, several authors have introduced an on-line extraction step using turbulent flow liquid chromatography, but it require specific instrumentation and a complex experimental assembly [21].

Micellar liquid chromatography (MLC), using sodium dodecyl sulfate (SDS) as the surfactant and a C18 column, has been proven as an interesting alternative to hydroorganic RP-HPLC to resolve drug mixtures in plasma [35]. Micelles interacts to proteins and other hydrophobic compounds, provoking their denaturation, solubilization and the release of bounded drugs. Therefore, direct injection of plasma samples, after a dilution with a micellar solution and filtration can be harmlessly directly injected, can be harmlessly performed, thus expediting the experimental protocol. Moreover, solubilized matrix substances are barely retained [36]. The presence of the micelle pseudophase and the modification of the stationary phase by the SDS monomer increase the number of interactions inside the column for the analytes, which complexes the retention mechanism. Besides, it only requires the addition of a small amount of short-chain alcohol (up to

15%) to improve the resolution [37]. This makes MLC a highly versatile technique and able to resolve mixtures of drugs wide range of hydrophobicity using an isocratic mode [38].

The aim of the work is to develop a reliable analytical procedure based on MLC to reliably determine erlotinib, imatinib, sunitinib, sorafenib and lapatinib in plasma. The method should be inexpensive, simple, environmentally friendly and enough sensitive to detect the usual concentrations of these drugs in plasma from cancer patients following a TKI-therapy. The method was validated following the guidelines of the European Medicines Agency (EMA) [39]. The developed method was applied to the study of plasma samples from cancer patients supplied by collaborating Hospitals.

2. Experimental

2.1 Standard and reagents

The standards of erlotinib hydrochloride (purity > 99.0%), imatinib methanesulfonate (> 99.0%), sunitinib free base (> 99.0%), sorafenib free base (> 99.0%) and lapatinib free base (> 99.0%) were supplied from LC laboratories (Woburn, MA, USA). SDS (> 99.0%), methanol, butanol and pentanol (HPLC grade) were purchased from Scharlab (Barcelona, Spain). Sodium dihydrogen phosphate monohydrate (> 98.0%) and HCl 37% come from Panreac (Barcelona, Spain). NaOH (> 99.0%) was supplied by Riedel-deHaën (Hanover, Germany). Ultrapure water was *in-lab* generated from deionized water using an ultrapure water generator device from Millipore S.A.S. (Molsheim, France). This ultrapure water was used to prepare all mobile phases.

2.2 Apparatus and instrumentation

A Metter-Toledo analytical balance (Greifensee, Switzerland) was used to weigh the analytes. The pH was measured with a Crison potentiometer (Barcelona) equipped with a combined Ag/AgCl/glass electrode. An ultrasonic bath was used to dissolve the standards (model Ultrasons-H, Selecta, Abrera, Spain). The mobile phases and the injected solutions were filtered through 0.45-μm nylon membranes.

The analyses were carried out in a chromatographic system HP1100 equipped with an isocratic pump, a degasser, an autosampler and a UV-visible diode array detector (DAD) supplied by Agilent Technologies (Palo Alto, CA, USA). Both instrumental control and the signal acquisition were performed using a personal computer connected by the chromatograph by means of the Agilent ChemStation (Rev. A.10.01) software. The obtained chromatograms were treated using the Michrom Software [40], to determine the chromatographic parameters: retention time (t_R), efficiency (N), asymmetry (B/A) and peak area (A). The capacity factor was calculated from t_R and the dead time (t_0). The meaning of these parameters can be found in [41].

2.3 Solutions and mobile phases

Stock solutions of each TKI (100 μg/mL of free drug) were prepared by weighing the appropriate amount of standard and dissolving it in methanol. Working solutions were made by successive dilution of these stock solutions with a micellar solution of 0.05 M SDS at pH 3.5. Combined solutions of the five TKIs were prepared in the same micellar solution.

Micellar solutions were prepared by weighing the appropriate amount of SDS and 0.01 M sodium dihydrogen phosphate monohydrate, and then solving them in ultrapure water. The pH was adjusted to the desired value by adding drops of HCl or NaOH solutions at 0.1 or 1.0 M. The adequate volume of alcohol was added, and the solution was filled up with ultrapure water, ultrasonicated and filtered.

181 *2.4 Chromatographic conditions*

182

183 The stationary phase was coated into a Kromasil C18 column (150 x 4.6 mm; 5 μm particle
184 size; 10 nm pore size). The mobile phase was a micellar solution of 0.13 M SDS - 4 %1-butanol
185 buffered at pH 3.5, running at isocratic mode. The following UV-Visible absorbance program
186 detection was run: 0.0 - 6.0 min, 345 nm; 6.0 - 9.2, 265 nm; 9.2 - 11.5, 370 nm; 11.5 - 20, 260 nm.
187 The injection volume and the flow rate were 20 μL and 1 mL/min, respectively. The aliquot vials
188 and the column remain at room temperature throughout the analysis.

189

190 *2.5 Chromatographic care*

191

192 A special care with the chromatographic system when dealing with micellar mobile phases
193 is required, to obtain a maximal performance and avoid column deterioration. Following these
194 instructions, the column has a lifespan of nearly 1000 injections.

195 A surfactant-free column must be previously saturated with the SDS monomers by pumping
196 a micellar solution for a minimum of 2 h at 1 mL/min. When a mobile phase is changed, the new
197 mobile phase must be pumped for 30 min at 1 mL/min to rinse the column and equilibrate the
198 stationary phase.

199 A micellar solution should never stay motionless in the chromatograpic system, to avoid the
200 precipitation of the salts (surfactant and buffer), which would seriously damage the components of
201 the chromatograph, especially needle, tubes and the column. If the operator has to work during
202 several days with the same mobile phase, it is possible to maintain the SDS solution overnight by
203 reducing the flow rate to a minimal value. Another possibility is to recycle the mobile phase.
204 Besides, both cases contribute to maintain a good equilibrium of the adsorbed SDS-monomers on
205 the stationary phase.

A cleaning procedure has to be implemented before switching off the pump. The SDS mobile phase must be replaced by 100 % pure water, which must be pumped at 1 mL/min during 1 h to eliminate the phosphate buffer. Later, the column should be rinsed with 100% methanol at 1 mL/min at least for 30 min to throw out the water and remove the adsorbed surfactant and strongly retained compounds from the stationary phase. Thereafter, the chromatograph power is ready to be turned off.

The removal process of the SDS is never complete, and the stationary phase remains modified. Therefore, it is not recommended to use this column in MLC with other surfactants or for hydroorganic-RP-HPLC.

2.6 Plasma extraction and sample preparation

Blood samples of cancer patients and healthy volunteers were provided by hospitals (after consent from the patients). The samples were collected with DB SST tubes (SBD Vacutainer Systems, Plymouth, UK), centrifuged at 4ºC at 3000 rpm for 10 min (3000 × *g*) to obtain the non-cellular fraction. The samples were filtered to remove the proteins, in order to quantify the free fraction of TKIs. Obtained plasma was frozen and kept at 20ºC in amber vials. Plasma samples were thawed at room temperature before the analysis.

Plasma samples were 1/5-diluted with a micellar solution of 0.05 M SDS buffered at pH 3.5, vigorously shaken, filtered and injected [42]. Samples used for the validation and QC samples were prepared as the same way. For spiked samples, the appropriate amount of the adequate working solution was added before the dilution. The diluted samples were not stored.

3. Results and discussion

3.1 Optimization of chromatographic conditions

232

233 The analysis for the optimization of the chromatographic conditions were performed using a
234 working solution containing 0.75 μg/mL of each TKI.

235

236 *3.1.1 Selection of the pH*

237

238 The studied drugs show acid/base activity, and then their structure and retention behavior
239 will depend on the pH of the mobile phase. The working range of the column is 1.5 - 9.5. However,
240 weakly alkaline solutions provoke a slow and continuous hydrolysis of the C18-alkyl bond,
241 resulting in a medium-term loss of performance of the column and a reduction of its lifespan.
242 Therefore, the study was restricted to acid and neutral pH. The first pKa values of the TKIs range
243 from 2.03 to 8.07 (Table 1), whereas the second pKa values were over 12. The pH was set at 3.5, far
244 from the first pKa of all the TKIs, to assure that the drugs would be quantitatively in one form.

245 The studied drugs are quite hydrophobic (log Po/w from 2.7 to 5.4, see Table 1) and then a
246 pure aqueous or propanol-hybrid mobile phase would be unable to elute them at an adequate
247 retention time using a C18 column. In order to obtain micellar mobile phases with increased elution
248 strength, the use of 1-butanol and 1-pentanol as organic modifier was envisaged.

249 A preliminary study with hybrid mobile phases containing SDS (0.05 - 0.15 M), and 1-
250 butanol or 1-pentanol (2-6 %) was performed. Five mobile phases were selected following a full
251 factorial design plus the center point: four by combining the minimal and maximal values of
252 concentration of SDS and alcohol, and one with the intermediate values. Therefore, the studied
253 mobile phases were aqueous solution buffered at pH 3.5 containing SDS/1-butanol or 1-pentanol
254 (M/% v/v): 0.05/2; 0.05/6; 0.10/4; 0.15/2 and 0.15/6.

255 The main chromatographic parameters (t_0; t_R, N and B/A) were taken for the five studied
256 TKIs in all the mobile phases. The chromatograph dead time (1.04 min) was calculated as the
257 average of the dead time determined for each analysis. The five drugs show a bending behavior

with the SDS micelles, that means their retention time increase at lower concentrations of SDS. As expected, mobile phases with 1-pentanol provide less retention times than 1-butanol, and higher values of alcohol increases the elution strength of the mobile phase, as usual in MLC. Therefore, 1-butanol was selected as organic modifier, in order to assure that the less retained compound would be eluted enough far from the protein band.

3.1.3 Optimization of SDS/1-butanol concentration

Use of an empirical strategy of testing mobile phases at several values of SDS/1-butanol to find the optimal mobile phase was judged too large, considering that five analytes should be resolved. Therefore, an interpretative strategy based on a chemometric approach was applied, in order to expedite the optimization process [37]. A mathematical model, able to predict the chromatographic parameters of medium-hydrophobic analytes from the composition of the mobile phase, was used. The Equation 1 is taken to model the retention factor:

$$k = \frac{K'_{AS}/(1+K_{AD\varphi})}{1+[M](K_{AM}(1+K_{MD\varphi})/(1+K_{AD\varphi}))} \quad (1)$$

where [M] and φ represent the concentration of SDS (M) and 1-butanol (%). The constants are partition coefficients and are described in further detail in [37]. K_{AD} and K_{AM} depend on the analyte and the surfactant, whereas K_{MD} and K_{AD} are different for each analyte, surfactant and alcohol. The simulation of the peak shape (efficiency and asymmetry) was carried out using the equation 2, which calculate the signal h(t) at each time of the chromatographic run because of the elution of the analyte:

$$h(t) = H \exp\left[-\frac{1}{2}\left(\frac{t - t_R}{s_0 + s_1(t - t_R)}\right)^2\right] \quad (2)$$

Value h(t) indicates the signal at each time (t) of the chromatographic run because of the elution of the analyte, which is considered as an asymmetrical normal function. H depends on the concentration and sensitivity, and t_R is the retention time. The constant s_0 is the central dispersion of the Gaussian curve, whereas s_1 quantifies the skewness. These parameters remain ideally invariant for each drug and mobile phase.

The experimental values of retention factor, N and B/A of the five TKIs measured from the analysis performed using the mobile phases described in section 3.1.2 were processed by the Michrom software [40] to fit the equations 1 and 2. Thus, the theoretical values of the retention time and peak shape of behaviour of erlotinib, imatinib, sunitinib, sorafenib and lapatinib can be estimated at any combination of SDS and 1-butanol inside the range 0.05 - 0.15 M, and 2 - 6%, respectively, by interpolation. Combining the information obtained for the five drugs at the same mobile phase, the theoretical values of resolution between each pair (r_{ij}), and the global resolution (Z) were calculated using the valley-peak and unnormalized product of all the r_{ij} criteria, respectively. Besides, Michrom software is able to draw simulated chromatograms, and then the analyst can easily visualize the variation of the chromatogram shape, and check the overlapping, when concentration of SDS or 1-butanol changes.

According to the chemometric model, the mobile phase providing the maximum resolution at the minimum analysis time was: 0.13 M SDS - 4 % 1-butanol - at pH 3.5 (R = 0.9990). The standard solution was analyzed under these conditions, and the studied TKIs were eluted without overlapping with adequate peak shape in < 20 min. Experimental chromatographic parameters were (t_R, N, B/A): erlotinib (4.7; 3422; 1.08), imatinib (8.1; 3687; 1.11), sunitinib (10.2; 2460; 1.15), sorafenib (12.6; 2965; 0.98) and lapatinib (17.3; 2474; 1.10). The error of the prediction of the retention times was < 5 %.

304

305 *3.1.4 Detection conditions*

306

307 A mixture of the drug was analyzed using the selected mobile phase and absorbance spectra
308 were measured between 200 nm and 800 nm. The maximal absorption wavelength were: erlotinib,
309 345 nm; imatinib, 265 nm; sunitinib, 370 nm; sorafenib, 260 nm and lapatinib, 260 nm. Therefore,
310 measured wavelength was modified through the chromatographic run to detect each TKI at its
311 corresponding maximal value.

312

313 *3.1.4 Advantages of the procedure*

314

315 The developed procedure shows several advantages over hydroorganic-RP-HPLC, because
316 of using micellar solutions.

317 Main feature of the procedure is the simplification of the sample preparation, exploiting the
318 possibility of direct injection allowed by MLC, eliminating need for clean-up steps. Sample is
319 quantitatively transferred to chromatographic system, without any step with variable recovery, and
320 then addition of an internal standard is not required. Besides, the optimized chromatographic
321 conditions allow the elution of five TKIs with a difference of three orders of hydrophobicity (2.7 -
322 5.4) in only < 20 min using an isocratic run. Therefore, the column does not require a stabilization
323 time between two injections, as in gradient. All these characteristics strongly reduce the global
324 analysis time and facilitate the successive analysis of a large number of samples.

325 Experimental procedure and the mobile phase use biodegradable salts. The mobile phase
326 contains only 4 % of toxic and flammable organic solvent, less than that normally used in
327 hydroorganic mobile phases (up to 100 %). Therefore, risk for the health of the laboratory staff due
328 to the handling of toxic chemicals and waste disposal are minimized. This fits the current trend in
329 analytical chemistry [43].

Using this analytical procedure, analyses can be performed at lower cost, because only basic chromatographic and laboratory instrumentation is used, and low amount of inexpensive reagents is required. Moreover, a large number of samples can be analyzed each day.

3.2 Method validation

The method was fully validated following the Guideline on bioanalytical method validation issued by the European Medicines Agency (EMA). Evaluated validation parameters were: selectivity, calibration curve, lower limit of quantification, precision, accuracy, carry-over, matrix effect, stability [39], limit of detection, and robustness [44]. Unless specified, validation was performed using drug-free plasma samples from healthy volunteers spiked with the studied TKIs.

3.2.1 Selectivity

Six blank plasma samples obtained from healthy volunteers (three males and three females) which do not take any drug, were analyzed by the developed procedure. In all cases, a broad band elutes from the dead time to nearly 3.0 min (Figure 2A). This band corresponds to the proteins and other endogenous compounds. These compounds strongly interact with the micelles of the mobile phase, and then are barely retained in the column. No other peaks were detected beyond 3.5 min. This result is similar to that obtained in other works also devoted to the analysis of plasma by MLC [35]. As the studied drugs show retention times > 3.5 min, there are no potential interfering compounds, and then the procedure is enough selective to unambiguously detect the analyte.

The same samples were spiked with 0.75 μg/mL of each analyte and analyzed. The TKIs were eluted without interference at the same retention time as indicated in section 3.1.3 (Figure 2B).

3.2.2 Calibration range and sensitivity

Seven plasma spiked samples containing increasing concentrations of the five studied TKIs, were analyzed by triplicate. Parameters of the calibration curve (slope and y-intercept) and degree of linearity (determination coefficient) were obtained using least-square linear regression (non-weighted). Five calibration curves were constructed in three different days (using different spiked samples) over a 1-month period, to consider influence of the between-run variability.

Upper limit of quantification was set at 5 μg/mL, whereas lower limit of quantification (LLOQ) was the lowest concentration which can be measured with enough accuracy and sensitivity (see section 3.2.3), 0.05 μg/mL. Limit of detection was calculated following the 3.3s criterion. Standard deviation of the blank was that of the y-intercept [44].

The results are shown in Table 2. The values for the determination coefficients ($r^2 > 0.990$) points to a good linearity in the considered range. Besides, the lower limit of quantification were below the lower limit of the therapeutic range. However For, the therapeutic range of sunitinib is too near its corresponding LLOQ, and then even slight subtherapeutic levels would provide unquantifiable peaks.

3.2.3 Accuracy and precision

These parameters were within-run determined at four concentrations (0.05, 0.15, 1.5 and 4 μg/mL) by six replicates, in the same day. Accuracy was the quotient of the average of the found concentrations minus the true value, divided by the true value, whereas the precision was taken as the relative standard deviation of the measured peak areas.

To calculate between-run values, the same experiments were performed three days over a 1-month period, by renewing the analyzed spiked samples. Accuracy was determined as difference of the average of average values of concentrations found each day and the true value, divided by the true value. Precision was the RSD of the average values of peak area taken each day.

Results can be seen in Table 3. Values of accuracy (-10.4 to +11.0%) and precision (RSD <9.2%) were inside maximum values accepted by the guide for both parameters (<20% for LLOQ and <15% for higher values). Therefore, the method provides reliable quantitative values for the studied TKIs with a low uncertainty.

3.2.4 Carry over effect

The carry over was evaluated by analyzing a plasma sample spiked at 5 μg/mL with the five analytes, and a blank plasma sample immediately after. No peak was observed in the second chromatogram near the retention time of the analytes. Thus, the carry-over was considered negligible at concentrations within the calibration range.

3.2.5 Matrix effects

Possible interference of the matrix compounds was evaluated by analysis of plasma samples spiked at same concentrations as for accuracy/precision, and standard working solutions (in micellar media) containing the same concentrations divided by five (to consider the dilution factor in the experimental procedure). All the analyses were performed by triplicate.

The peak area proved to be very similar, regardless the starting environment. Therefore, the matrix effect was not significant.

3.2.6 Robustness

In a realistic situation, the experimental parameters randomly oscillate in a short range, which introduce a new source of variance in the instrumental response. To verify the extent of this fact, the change in the retention time and peak area was studied when the chromatographic

parameters are at the minimum and maximum of their variation range, that can occur during the normal handling of the instrumentation: SDS concentration, 0.12-0.14 M; 1-butanol, 3.9-4.1%; pH, 3.4-3.6; flow-rate, 0.95-1.05 and wavelength absorbance ± 5 nm.

Influence of each parameter was separately studied by analyzing (n = 3) the standard solution using three mobile phases at: the optimal value, and the lower and upper values of the tested range, remaining the others constant. RSD of the three measurements was calculated for retention time and peak area. The results are shown in Table 4.

The method was considered quite robust, as low variations were observed for retention time (< 7.8%) and peak area (< 8.4 %), within the considered range.

3.2.7 Stability

Stability of the analytes in the plasma samples in two cases (freeze-and-thaw and processed) was studied at three concentrations (0.15, 1.5 and 4.0 μg/mL). Each measurement was performed by triplicate.

The freeze-and-thaw stability was determined during a two-weeks period, which is the usual storage time of the plasma samples in a Hospital. The plasma sample was spiked, analyzed, and stored in a fridge at -20ºC. After 12 h, the same plasma samples were thawed and reanalyzed. This cycle was repeated each day for two weeks. The peak areas remain nearly invariant the first ten days, and a diminishing of 10 % was observed between the 11^{th} and 15^{th} days.

Stability of the processed sample was studied at the experimental laboratory conditions. The plasma samples were analyzed after spiking. The remaining processed sample was stored at room temperature, and analyzed after 3; 6; 9 and 12 h. No significant decrease of the signal was observed for this period.

3.3 Analysis of real samples

The developed procedure was applied to the analysis of the free plasmatic fraction of TKIs in samples extracted from cancer patients, which are following a therapy based on one of the studied TKIs. Samples with concentrations over the ULOQ were diluted and reanalyzed. The results can be seen in Table 5. In all cases, the studied TKI can be quantified without interferences, and the QCs provide adequate values. Fig. 3 shows the chromatogram obtained from the analysis of a sample from the patient 3.

In the patient 20, sunitinib was detected, but the resulting concentration was under the LLOQ, and then show unacceptable uncertainty. For these reason, we only know that the value is between the LOD and the LLOQ.

4. Conclusions

Micellar liquid chromatography has been demonstrated as a useful technique for the analysis of erlotinib, imatinib, sunitinib, sorafenib and lapatinib in plasma. Main advantage of this procedure is the possibility of direct injection. Therefore, plasma samples were analyzed following an easy-to-handle, one-step, rapid and reproducible assay, instead of by long and tedious extractions. Hence, the main part of the experimental protocol is simplified, minimizing the probability of operator error and the use of reagents. The analytes were eluted without interference from the matrix in < 20 min using an isocratic mobile phase, resulting in a low global analysis time. These characteristics allow the analysis of a large number of samples per day. The method was validated in terms of selectivity, carry-over effect, linearity, sensitivity, matrix effect, precision, accuracy, robustness and stability with adequate results, which assesses reliability of quantitative data. The procedure is eco friendly and safe for the laboratory staff, according to the low amount of toxic chemicals handled and wasted. Moreover, it is relatively inexpensive, and then accessible to clinical laboratories with

459 limited economic power. Therefore, it can be useful by clinicians to monitor these drugs in plasma
460 of cancer patients.
461
462 **5. Acknowledgments**
463
464 This work was supported by the project P1-1B2012-36 (Universitat Jaume I).
465
466 **6. Conflict of interest disclosure**
467
468 The authors declare that they have no financial/commercial conflicts of interest.
469
470 **7. References**
471
472 [1] D.S. Krause, R.A. Van Etten, Tyrosine kinases as targets for cancer therapy, New Engl. J. Med.
473 353 (2005) 172–187, doi: 10.1056/NEJMra044389
474 [2] N.P. van Erp, H. Gelderblom, H.J. Guchelaar, Clinical pharmacokinetics of tyrosine kinase
475 inhibitors, Cancer Treat. Rev. 35 (2009) 692–706, doi: 10.1016/j.ctrv.2009.08.004
476 [3] R. Capdeville, E. Buchdunger, J. Zimmermann, A. Matter, Glivec (STI571, imatinib), a
477 rationally developed, targeted anticancer drug, Nat. Rev. Drug Discov. 1 (2002) 493–502,
478 doi:10.1038/nrd839
479 [4] A.J. Tevaarwerk, J.M. Kolesar, Lapatinib: a small-molecule inhibitor of epidermal growth factor
480 receptor and human epidermal growth factor receptor-2 tyrosine kinases used in the treatment of
481 breast cancer, Clin. Ther. 31 (2009) 2332–2348, doi:10.1016/j.clinthera.2009.11.029
482 [5] R. Rosell, E. Carcereny, R. Gervais, A. Vergnenegre, B. Massuti, E. Felip, R. Palmero, R.
483 Garcia-Gomez, C. Pallares, J.M. Sanchez, R. Porta, M. Cobo, P. Garrido, F. Longo, T. Moran, A.
484 Insa, F. De Marinis, R. Corre, I. Bover, A. Illiano, E. Dansin, J. de Castro, M. Milella, N. Reguart,

485 G. Altavilla, U. Jimenez, M. Provencio, M.A. Moreno, J. Terrasa, J. Muñoz-Langa, J. Valdivia, D.
486 Isla, M. Domine, O. Molinier, J. Mazieres, N. Baize, R. Garcia-Campelo, G. Robinet, D.
487 Rodriguez-Abreu, G. Lopez-Vivanco, V. Gebbia, L. Ferrera-Delgado, P. Bombaron, R. Bernabe, A.
488 Bearz, A. Artal, E. Cortesi, C. Rolfo, M. Sanchez-Ronco, A. Drozdowskyj, C. Queralt, I. de
489 Aguirre, J.L. Ramirez, J.J. Sanchez, M.A. Molina, M. Taron, L. Paz-Ares, Spanish Lung Cancer
490 Group in collaboration with Groupe Français de Pneumo-Cancérologie and Associazione Italiana
491 Oncologia Toracica. Erlotinib versus standard chemotherapy as first-line treatment for European
492 patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): A
493 multicentre, open-label, randomised phase 3 trial, Lancet Oncol. 13 (2012) 239–246, doi:
494 10.1016/S1470-2045(11)70393-X
495 [6] V. Di Marco, F. De Vita, J. Koskinas, D. Semela, P. Toniutto, C.Verslype, Sorafenib: From
496 literature to clinical practice, Ann. Oncol. 24(2) 2013 30-37, doi: 10.1093/annonc/mdt055
497 [7] O. Huillard, B. Blanchet, J.P. Durand, F. Goldwasser, Sorafenib for patients with differentiated
498 thyroid cancer, The Lancet 385 (2015) 227-228, doi: 10.1016/S0140-6736(15)60054-X
499 [8] U. Leone, R. Maggiore, M.V. Menada, P.L. Venturini, S. Ferrero, The potential of sunitinib as a
500 therapy in ovarian cancer, Expert. Opin. Investig. Drugs 22 (2013) 1671-1686, doi:
501 10.1517/13543784.2013.841138
502 [9] V. Law, C. Knox, Y. Djoumbou, T. Jewison, A.C. Guo, Y. Liu, A. Maciejewski, D. Arndt, M.
503 Wilson, V. Neveu, A. Tang, G. Gabriel, C. Ly, S. Adamjee, Z.T. Dame, B. Han, Y. Zhou, D.S.
504 Wishart, DrugBank 4.0: shedding new light on drug metabolism, Nucleic Acids Res. 42 (2014)
505 D1091-1097. Available at: http://www.drugbank.ca/ (Accessed: 15/07/2015)
506 [10] V.Gotta, T. Buclin, C. Csajka, N. Widmer, Systematic review of population pharmacokinetic
507 analyses of imatinib and relationships with treatment outcomes, Ther. Drug Monitor. 35 (2013)
508 150-167, doi: 10.1097/FTD.0b013e318284ef11
509 [11] M. Fukudo, Y. Ikemi, Y. Togashi, K. Masago, Y.H. Kim, T. Mio, T. Terada, S. Teramukai,
510 M. Mishima, K.I. Inui, T. Katsura, Population pharmacokinetics/pharmacodynamics of erlotinib

511 and pharmacogenomic analysis of plasma and cerebrospinal fluid drug concentrations in Japanese
512 patients with non-small cell lung cancer, Clin. Pharmacokinet. 52 (2013) 593-609, doi:
513 10.1007/s40262-013-0058-5
514 [12] K.T. Flaherty, C. Lathia, R.F. Frye, L. Schuchter, M. Redlinger, M. Rosen, P.J. O'Dwyer,
515 Interaction of sorafenib and cytochrome P450 isoenzymes in patients with advanced melanoma: a
516 phase I/II pharmacokinetic interaction study, Cancer Chemoth. Pharm. 68 (2001) 1111-1118, doi:
517 10.1007/s00280-011-1585-0, doi: 10.1007/s00280-011-1585-0
518 [13] M. Nagata, Y. Ishiwata, Y. Takahashi, H. Takahashi, K. Saito, Y. Fujii, K. Kihara, M.
519 Yasuhara, Pharmacokinetic-pharmacodynamic analysis of sunitinib-induced thrombocytopenia in
520 Japanese patients with renal cell carcinoma, Biol Pharm Bull. 38 (2015) 402-410, doi:
521 10.1248/bpb.b14-00636, doi: 10.1248/bpb.b14-00636
522 [14] G. Wilkes, Targeted cancer therapy. A Handbook for Nurses, Ed. Jones and Barlett
523 Publishing, Sudbury, MA, USA, 2011.
524 [15] W. Jeong, J.H. Doroshow, S. Kummar, United States Food and Drug Administration approved
525 oral kinase inhibitors for the treatment of malignancies, Curr. Probl. Cancer 37 (2013) 110–144,
526 doi: 10.1016/j.currproblcancer.2013.06.001
527 [16] H. Yu, N. Steeghs, C.M. Nijenhuis, J.H.M. Schellens, J.H. Beijnen, A.D.R. Huitema, Practical
528 Guidelines for Therapeutic Drug Monitoring of Anticancer Tyrosine Kinase Inhibitors: Focus on
529 the Pharmacokinetic Targets, Clin. Pharmacokinet. 53 (2014) 305-325, doi: 10.1007/s40262-014-
530 0137-2
531 [17] D.H. Josephs, D.S. Fisher, J. Spicer, R.J. Flanagan, Clinical pharmacokinetics of tyrosine
532 kinase inhibitors: Implications for therapeutic drug monitoring, Ther. Drug Monit. 35 (2013) 562-
533 587, doi: 10.1097/FTD.0b013e318292b931
534 [18] H.J. Klümpen, C.F. Samer, R.H.J. Mathijssen, J.H.M. Schellens, H. Gurney, Moving towards
535 dose individualization of tyrosine kinase inhibitors, Cancer Treat. Rev. 37 (2011) 251–260, doi:
536 10.1016/j.ctrv.2010.08.006

537 [19] R.W.F. van Leeuwen, T. van Gelder, R.H.J. Mathijssen, F.G.A. Jansman, Drug–drug
538 interactions with tyrosine-kinase inhibitors: a clinical perspective, Lancet Oncol. 15 (2014) e315–
539 e326, doi: 10.1016/S1470-2045(13)70579-5
540 [20] I. Andriamanana, I. Gana, B. Duretz, A. Hulin, Simultaneous analysis of anticancer agents
541 bortezomib, imatinib, nilotinib, dasatinib, erlotinib, lapatinib, sorafenib, sunitinib and vandetanib in
542 human plasma using LC/MS/MS, J. Chromatogr. B 926 (2013) 83–91, doi:
543 10.1016/j.jchromb.2013.01.037
544 [21] L. Couchman, M. Birch, R. Ireland, A. Corrigan, S. Wickramasinghe, D. Josephs, J. Spicer,
545 R. J. Flanagan, An automated method for the measurement of a range of tyrosine kinase inhibitors
546 in human plasma or serum using turbulent flow liquid chromatography–tandem mass spectrometry,
547 Anal. Bioanal. Chem. 403 (2012) 1685–1695, doi: 10.1007/s00216-012-5970-2
548 [22] L. Götze, A. Hegele, S.K. Metzelder, H. Renz, W.A. Nockher, Development and clinical
549 application of a LC-MS/MS method for simultaneous determination of various tyrosine kinase
550 inhibitors in human plasma, Clin. Chim. Acta 413 (2012) 143–149, doi:10.1016/j.cca.2011.09.012
551 [23] N. A. G. Lankheet, M. J. X. Hillebrand, H. Rosing, J. H. M. Schellens, J. H. Beijnena, A.D.R.
552 Huitema, Method development and validation for the quantification of dasatinib, erlotinib, gefitinib,
553 imatinib, lapatinib, nilotinib, sorafenib and sunitinib in human plasma by liquid chromatography
554 coupled with tandem mass spectrometry, Biomed. Chromatogr. 27 (2013) 466–476
555 [24] S. Bouchet, E. Chauzit, D. Ducint, N. Castaing, M. Canal-Raffin, N. Moore, K. Titier, M.
556 Molimard, Simultaneous determination of nine tyrosine kinase inhibitors by 96-well solid-phase
557 extraction and ultra performance LC/MS-MS, Clin. Chim. Acta 412 (2011) 1060–1067, doi:
558 10.1016/j.cca.2011.02.023
559 [25] B.A. Adeagbo, T.A. Olugbade, O.O. Bolaji, M.A. Durosinmi, Simple HPLC-UV Method for
560 Pharmacokinetic Studies of Imatinib in the Presence of Common Antimalaria Agents, J. Liq.
561 Chromatogr. R.T. 38 (2015) 1194-1198, doi: 10.1080/10826076.2015.1025143
562 [26] Y. Zheng, A. Thomas-Schoemann, L. Sakji, P. Boudou-Rouquette, N. Dupin, L. Mortier, M.

563 Vidal, F. Goldwasser, B. Blanchet, An HPLC-UV method for the simultaneous quantification of
564 vemurafenib and erlotinib in plasma from cancer patients, J. Chromatogr. B 928 (2013) 93-97,
565 doi:10.1016/j.jchromb.2013.03.017
566 [27] V. Escudero-Ortiz, J.J. Pérez-Ruixo, B. Valenzuela, Development and validation of a high-
567 performance liquid chromatography ultraviolet method for lapatinib quantification in human
568 plasma, Ther. Drug Monit. 35 (2013) 796-802, doi: 10.1097/FTD.0b013e3182959080
569 [28] V. Escudero-Ortiz, J.J. Pérez-Ruixo, B. Valenzuela, Development and validation of an HPLC-
570 UV method for sorafenib quantification in human plasma and application to patients with cancer in
571 routine clinical practice, Ther. Drug Monitor. 36 (2014) 317-325, doi:
572 10.1097/FTD.0000000000000027
573 [29] M. Helvenstein, S. Hambÿe, B. Blankert, Determination of three tyrosine kinase inhibitors and
574 one active metabolite by an identical and validated ultra-performance liquid chromatography-DAD
575 method in human plasma, Curr. Pharm. Anal. 10 (2014) 161-168, doi:
576 10.2174/1573412910666140619210406#sthash.V2qANJlh.dpuf
577 [30] A. Nageswari, K.V.S.R.K. Reddy, K. Mukkanti, Stability-indicating UPLC method for
578 determination of Imatinib Mesylate and their degradation products in active pharmaceutical
579 ingredient and pharmaceutical dosage forms, J. Pharmaceut. Biomed. Anal. 66 (2012) 109-115, doi:
580 10.1016/j.jpba.2012.03.022
581 [31] G. Naveen Kumar Reddy, V.V.S. Rajendra Prasad, P.K. Maharana, Development and
582 validation of a stability indicating uplc method for determination of erlotinib in pharmaceutical
583 formulations, Der Pharma Chem. 4 (2012) 2288-2297. Available at:
584 http://derpharmachemica.com/vol4-iss6/DPC-2012-4-6-2288-2297.pdf (Accessed: 15/07/2015)
585 [32] M. Helvenstein, S. Hambÿe, B. Blankert, Determination of three tyrosine kinase inhibitors and
586 one active metabolite by an identical and validated ultra-performance liquid chromatography-DAD
587 method in human plasma, Curr. Pharm. Anal. 10 (2014) 161-168, doi:
588 10.2174/157341291066614061921040 6

589 [33] M. Birch, P.E. Morgan, S. Handley, A. Ho, R. Ireland, R.J. Flanagan, Simple methodology for
590 the therapeutic drug monitoring of the tyrosine kinase inhibitors dasatinib and imatinib, Biomed.
591 Chromatogr. 27 (2013) 335-342, doi: 10.1002/bmc.2796
592 [34] M. Caterino, G.M. Casadei, R. Arvonio, S. De Francia, E. Pirro, F.M. Piccione, F. Pane, M.
593 Ruoppolo, Quantification of imatinib plasma levels in patients with chronic myeloid leukemia:
594 Comparison between HPLC-UV and LC-MS/MS, Int. J. Pept. Res. Ther. 19 (2013) 109-116, doi:
595 10.1007/s10989-012-9321-0
596 [35] P. Kawczak, T. Bączek, Recent theoretical and practical applications of micellar liquid
597 chromatography (MLC) in pharmaceutical and biomedical analysis, Cent. Eur. J. Chem. 10 (2012)
598 570-584, doi: 10.2478/s11532-012-0004-7
599 [36] J. Peris-Vicente, I. Casas-Breva, P. Roca-Genovés, J. Esteve-Romero, Application of micellar
600 liquid chromatography for the determination of antitumoral and antiretroviral drugs in plasma,
601 Bioanalysis 6 (2014) 1975–1988, doi: 10.4155/bio.14.154
602 [37] A. Berthod, M.C. García-Álvarez-Coque, Micellar liquid chromatography, in: J. Cazes (Ed.)
603 Chromatographic Science Series (Vol. 83), Ed. Marcel Dekker, New York, NY, USA, 2000
604 [38] J. Peris-Vicente, M. Villarreal-Traver, I. Casas-Breva, S. Carda-Broch, J. Esteve-Romero, Use
605 of micellar liquid chromatography to analyze darunavir, ritonavir, emtricitabine, and tenofovir in
606 plasma, J. Sep. Sci. 37 (2014) 2825–2832, doi: 10.1002/jssc.201400574
607 [39] Committee for Medicinal Products for Human Use, Guideline on bioanalytical method
608 validation, European Medicines Agency, London, UK, 2011. Available at:
609 http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109
610 686.pdf (Accessed: 29/04/2015)
611 [40] J. R., Torres-Lapasió, Michrom Software, Ed. Marcel Dekker, New York, NY, USA, 2000.
612 [41] W.J. Lough, I.W. Wainer (Eds.) High Performance Liquid Chromatography: Fundamental
613 Principles and Practice, Ed. Blackie Academic & Professional, Glasgow, UK, 1996

[42] J. Peris-Vicente, M. Villareal-Traver, I. Casas-Breva, S. Carda-Brocha, J. Esteve-Romero, A micellar liquid chromatography method for the quantification of abacavir, lamivudine and raltegravir in plasma, J. Pharm. Biomed. Anal. 98 (2014) 351–355, doi: 10.1016/j.jpba.2014.06.009

[43] A. Gałuszka, Z. Migaszewski, J. Namiesnik, The 12 principles of green analytical chemistry and the SIGNIFICANCE mnemonic of green analytical practices, TRAC Trend. Anal. Chem. 50 (2013) 78–84, doi: 10.1016/j.trac.2013.04.010

[44] B. Magnusson, U. Örnemark (Ed.), EURACHEM Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics (2nd ed.), EURACHEM, Leoben, Austria, 2014.

Available at: https://www.eurachem.org/images/stories/Guides/pdf/MV_guide_2nd_ed_EN.pdf (Accessed: 15/07/2015).

640 **Table 1**. Main characteristics of the studied TKIs

Compound	Trade name	Producer	pKa [9]	Log Po/w [9]	Therapeutic range
Imatinib	Gleevec/ Glivec	Novartis (Basilea, Switzerland)	8.27/12.45	3.0	0.56 - 1.39 μg/mL [10]
Lapatinib	Tykerb/ Tyverb	GlaxoSmithKline (London, UK)	7.2/15.99	5.4	not found
Erlotinib	Tarceva	Genentech, (Northbrook, IL, USA)	4.59/16.14	2.7	0.85-1.68 μg/mL [11]
Sorafenib	Nexavar	Bayer, (Leverkusen, Germany)	2.03/11.55	3.8	5.4–10.0 μg/mL [12]
Sunitinib	Sutent,	Pfizer, (New York, NY, USA)	9.04/11.46	5.2	0.050-0.100 μg/mL [13]

641

642

643

644

645 **Table 2.** Calibration parameters for the studied TKIs (concentrations in μg/mL).

TKI	Slope	y-intercept	r^2	LOD	LLOQ
Erlotinib	35.6 ± 0.84	-0.15 ± 0.41	0.998	0.035	0.05
Imatinib	50.8 ± 1.0	-0.13 ± 0.33	0.997	0.019	0.05
Sunitinib	29.9 ± 0.5	- 0.25 ± 0.27	0.990	0.027	0.05
Sorafenib	71.7 ± 2.2	- 0.02 ± 0.54	0.996	0.023	0.05
Lapatinib	63.2 ± 1.8	0.05 ± 0.32	0.998	0.015	0.05

646 n = 5

647

648

649

650 **Table 3.** Intra- and inter-day accuracy and precision of the method.

TKI	Concentration (μg/mL)	Within-run[a] Accuracy (%)	Within-run[a] Precision (RSD, %)	Between-run[b] Accuracy (%)	Between-run[b] Precision (RSD, %)
Elotinib	0.05	-8.5	6.3	- 9.0	8.1
	0.15	+5.2	4.7	+ 7.5	3.0
	1.5	+2.1	2.2	+ 3.1	1.3
	4	-0.5	2.3	+5.0	2.9
Imatinib	0.05	+7.5	6.9	+ 11.0	7.8
	0.15	-4.2	4.1	- 7.4	4.0
	1.5	+2.1	4.4	+5.2	3.9
	4	+3.4	2.9	+4.0	5.4
Sunitinib	0.05	-4.8	8.5	-8.7	5.4
	0.15	+4.5	2.0	+2.0	2.0
	1.5	-1.5	4.5	-3.0	3.8
	4	+3.1	2.1	+1.5	4.1
Sorafenib	0.05	-10.4	6.3	-6.1	8.3
	0.15	+7.5	2.1	+4.5	5.4
	1.5	+4.1	5.1	+3.1	3.5
	4	+5.2	1.5	+2.0	4.7
Lapatinib	0.05	+ 7.4	9.2	+4.7	7.2
	0.15	- 4.2	3.0	-3.5	1.5
	1.5	+3.8	2.1	+2.0	4.6
	4	+ 2.0	4.4	+1.7	3.8

651 [a]n=6; [b]n = 5

652

653

654

655 **Table 4.** Evaluation of the robustness of the MLC-method.

TKI	Chromatographic parameter	Retention time (R.S.D.; %)	Peak area (R.S.D; %)
Erlotinib	SDS concentration	4.4	3.2
	1-butanol	5.3	2.6
	pH	4.1	1.8
	Flow rate	6.5	3.5
	Wavelength	1.0	7.5
Imatinib	SDS Concentration	6.7	4.1
	1-butanol	4.0	2.8
	pH	5.1	3.5
	Flow rate pH	4.5	2.1
	Wavelength	1.6	5.3
Sunitinib	SDS Concentration	4.7	3.0
	1-butanol	5.1	1.9
	pH	4.3	3.1
	Flow rate pH	5.4	2.6
	Wavelength	2.0	6.5
Sorafenib	SDS Concentration	3.8	2.6
	1-butanol	4.7	1.3
	pH	3.1	2.6
	Flow rate (mL/min)	7.8	1.8
	Wavelength	1.8	8.4
Lapatinib	SDS Concentration	7.6	1.8
	1-butanol	3.9	1.6
	pH	4.9	2.5
	Flow rate	4.5	3.5
	Wavelength	1.9	7.2

656 n = 3

657

658

659

660

661

662 **Table 5**. Concentrations (µg/ mL) of the studied TKIs in plasma samples from cancer patients.

Taken TKI	Patient	Concentration
Imatinib	1	1.74
	2	2.04
	3	0.53
	4	3.24
Erlotinib	5	1.61
	6	0.84
	7	2.4
	8	1.21
Lapatinib	9	1.01
	10	0.41
	11	0.78
	12	1.90
Sorafenib	13	6.25
	14	4.35
	15	2.85
	16	3.86
Sunitinib	17	0.055
	18	0.061
	19	0.089
	20	Between LOD and LLOQ

663

664

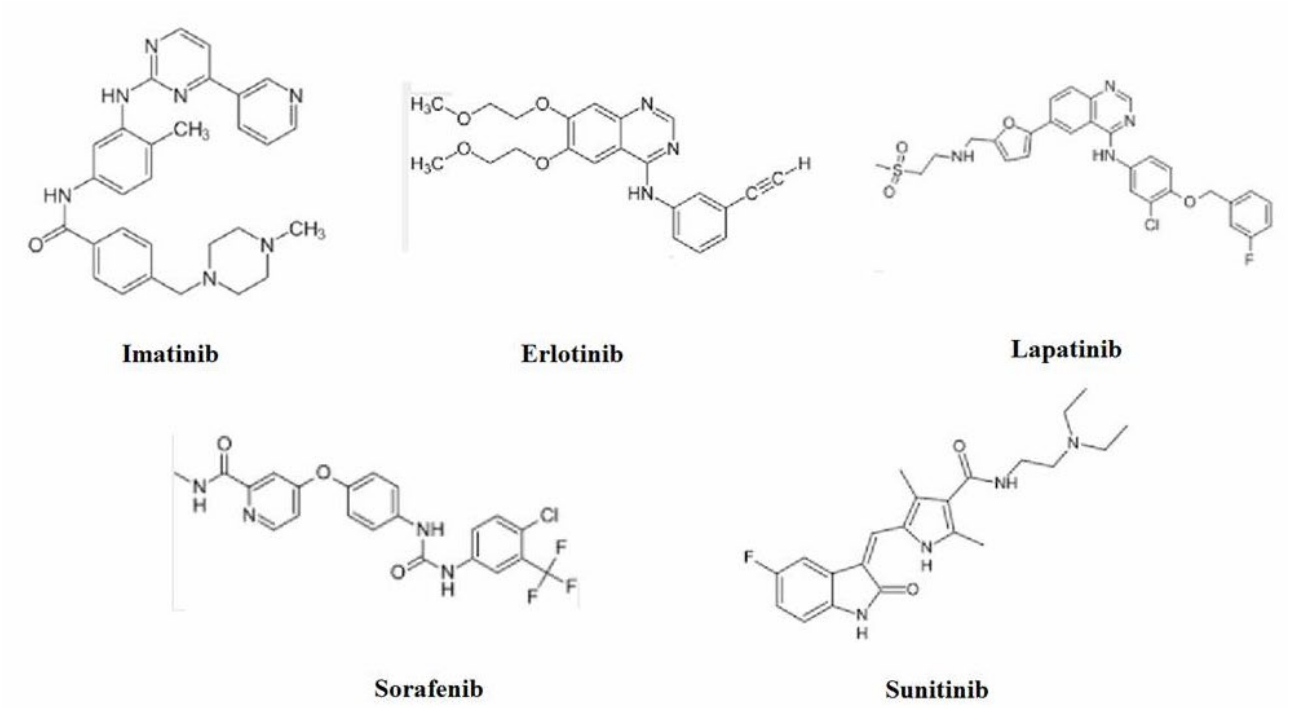


Figure 1. Structure of the studied tyrosine kinase inhibitors.

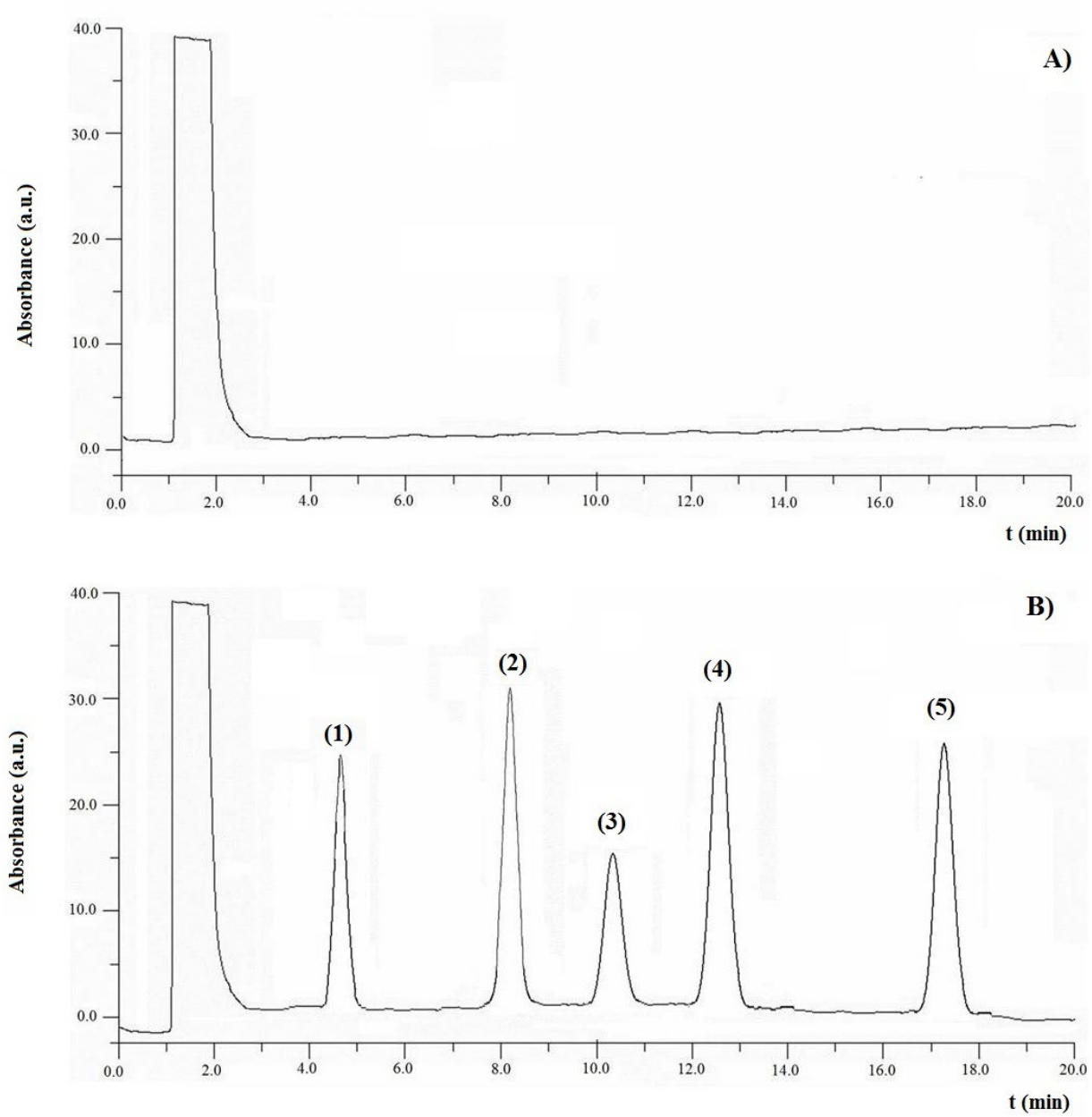
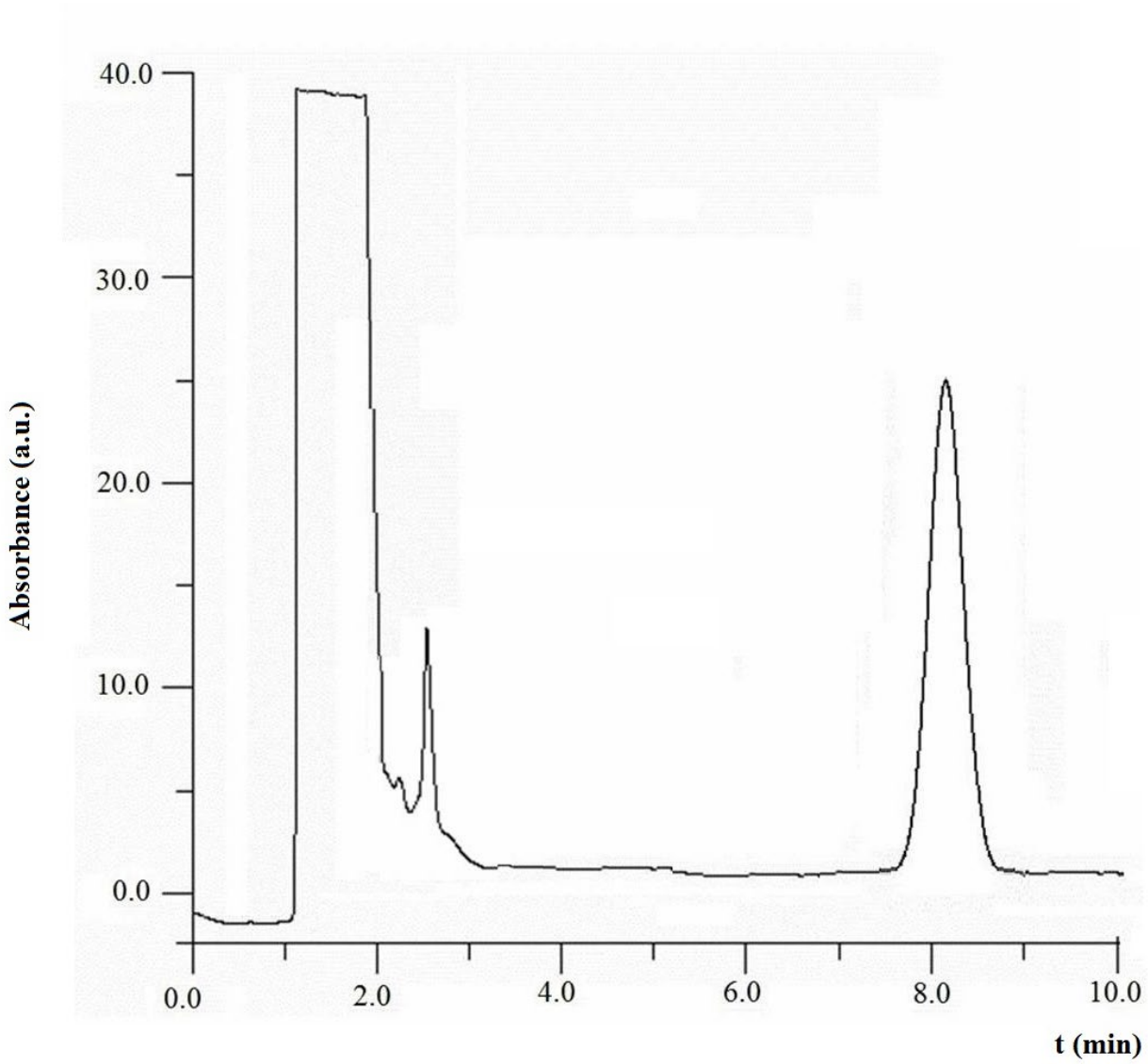


Figure 2. Chromatogram obtained by the analysis of plasma samples spiked with 0.75 μg/mL of 1) erlotinib, 2) imatinib, 3) sunitinib, 4) sorafenib and 5) lapatinib.



690

691 **Figure 3.** Chromatogram obtained from the analysis of sample 3, containing 0.53 μg/mL of

692 imatinib.