DEVELOPMENT AND VALIDATION OF A MICELLAR LIQUID CHROMATOGRAPHIC METHOD TO DETERMINE THREE ANTITUMORALS IN PLASMA

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Abstract

Background: A micellar liquid chromatographic-method, to determine several anticancer drugs (pazopanib, dabrafenib and regorafenib) in plasma was developed and validated by the guidelines of the European Medicines Agency. *Results*: Plasma samples were directly injected, after a 1/5-dilution in a micellar solution. The drugs were resolved in < 18 min using a C18 column. The mobile phase was an aqueous solution of 0.12 M sodium dodecyl sulphate - 2 % 1-pentanol, buffered at pH 7. The detection was performed by absorbance at 260 nm. The main results of the validation were: limit of detection (0.1 - 1 mg/L), calibration range (0.2-2 to 80 mg/L), accuracy (-12.5 to +11.7%) and precision (<11.9%). *Conclusions*: The procedure was conducted by a minimum cost, effort, manipulation, time, and quantity of hazardous chemicals. The method was useful to determine the drugs at their respective target concentrations, and was found useful for clinical analysis.

Keywords: Cancer; Dabrafenib; Direct injection; Factorial design; Modeling; Optimization; Pazopanib; Regorafenib; Surface Response Methodology; Validation.

1. Introduction

Cancer is a group of diseases involving an uncontrolled cell proliferation. The development of oncogenic malignancies is caused by genetic modifications, which induces numerous abnormal biological processes. Tyrosine kinases are involved in the control of several biological processes and have been recognized as hot spots of oncogenic transformation. The tyrosine kinase inhibitors (TKIs) are a potent group of antineoplastic agents that specifically target tyrosine kinases that participate in the abnormal growth of tumor cells. TKIs have minimal side effects compared to cytotoxic chemotherapeutic agents and are often synergistic in combination with radiotherapy and/or chemotherapy [1-3]. Within this group of drugs that are getting satisfactory results, we found pazopanib (PAZO) [4], regorafenib (REGORA) [5] and dabrafenib (DABRA) [6]. The main chemical and pharmacological parameters can be seen in Table 1 [7-11], and the structures in the Figure 1.

Pazopanib (Votrient®) is angiogenesis agent and multi-TKI, which is able to block a large number of receptors: platelet-derived growth factor receptors (PDGFR)-α and -β, the vascular endothelial growth factor receptors (VEGFR)-1, VEGFR-2, VEGFR-3), cytokine receptor (Kit), fibroblast growth factor receptor (FGFR)-1 and -3, transmembrane glycoprotein receptor tyrosine kinase (cFms), interleukin-2 receptor-inducible T-cell kinase (Itk), and leukocyte-specific protein tyrosine kinase (Lck). Pazopanib is administered to patients suffering from advanced soft tissue sarcoma (STS), or advanced renal cell carcinoma (RCC), who have already been treated by other chemotherapeutic agents [7,12,13].

Regorafenib (Stivarga®, BAY 73-4506) is a multi-kinase inhibitor for RET, VEGFR-1, VEGFR-2, VEGFR-3, KIT, PDGFR-α, PDGFR-β, FGFR-1, FGFR-2, TIE-2, DDR2, Trk2A, Eph2A, RAF-1, BRAF, BRAFV600E, SAPK2, PTK5, and Ab. On September 27, 2012, the US Food and Drug Administration approved regorafenib for the treatment of patients with metastatic colorectal cancer (mCRC) [7,14,15]

Dabrafenib (TAFINLAR®) is a tyrosine kinase inhibitor prescribed as monotherapy for unresectable or metastatic melanoma with BRAF V600E or V600k mutation. At high concentrations, Dabrafenib is also able to inhibit wild-type BRAF, CRAF, SIK1, NEK11 and LIMK1 kinases [7,16].

Separation techniques, especially HPLC, are nowadays preferred for bioanalysis for clinical purposes [17]. Literature reveals a few chromatographic methods to determine pazopanib [18,19], regorafenib [20,21] and dabrafenib [22,23] in plasma. However, these methods require large and complicated sample preparations, with several extraction and purification steps because of the extremely complex chemical composition of plasma. Besides, they use specific instrumentation, a large amount of toxic solvents and the analysis results at a high price. In our knowledge, no HPLC method has been developed for the simultaneous analysis of these compounds. The availability of an easy, rapid and reliable HPLC method to determine the plasmatic concentration of these TKIs analytes at values over their Cthrough - Cmax (Table 1) would be very useful to monitor and control the therapeutic treatments [17].

Micellar liquid chromatography, using hybrid mobile phases of the anionic surfactant sodium dodecyl sulfate (SDS), is an alternative to the determination of drugs in biological fluids. Indeed, the micellar solutions have different solubilization sites: bulk water, polar; the surface of the micelle, electrostatic and polar; the core of the micelle, hydrophobic; and miscellaneous, at the palisade layer between these two zones. Micelles and monomers tend to bind proteins and other macromolecules competitively releasing bound drugs. Therefore, they are denatured and solubilized, and then, in the column, they are harmless eluted at the front of the chromatogram, instead of precipitating. This allows the direct injection of the sample. The environment in the column is strongly modified. The monomer settles on the C18 stationary phase, with the polar group facing outwards. This increases the hydrophobicity of the mobile phase and generates an outer anionic layer. The mobile phase contains free monomers, at the critical micellar concentration, the corresponding organic solvent, and the micelles. The solute can partition between three phases

(modified stationary phase, bulk mobile phase and micellar pseudophase). This high multiplicity of interactions and equilibria complexes the retention mechanism and improves the stability and versatility of the technique, enabling its modeling by chemometrics. Besides, the use of hybrid micellar mobile phases make a method more innocuous, non-flammable, biodegradable and inexpensive than those based on hydro organic ones [24,25].

The aim of the work was the development of an analytical method to quantify pazopanib, dabrafenib and regorafenib in plasma. The use of micellar liquid chromatography was explored. The method should be able to reliably quantify the drugs at the clinical concentrations, and be useful for routine analysis in a clinical laboratory. Especially, the sample preparation must be easy-to-handle, and the use of hazardous chemicals must be limited. In order to test the analytical performances, the procedure must be validated by the guidelines of the European Medicines Agency [26]. Finally, its reliability should be demonstrated by analyzing samples of plasma from cancer patients following a therapy based on these drugs, provided by a local Hospital.

2. Experimental

2.1 Standard and reagents

Powdered standards of pazopanib hydrochloride (purity >98.0%), dabrafenib mesylate (>98.0%) and regorafenib (>98.0%) were purchased from LGM Pharma (Nashville, TN, USA). Sodium dodecyl sulphate (>98.0%), 1-pentanol and dimethyl sulfoxide (DMSO) (HPLC grade) were supplied by Scharlab (Barcelona, Spain). Sodium dihydrogen phosphate monohydrate (>98.0%) and hydrochloric acid (37%) were bought to Panreac (Barcelona, Spain). Sodium hydroxide (>98%) comes from Riedel-deHaën (Hannover, Germany). Ultrapure water was generated in the laboratory from deioinized water, supplied by the University as tap water, using an

ultrapure water generator device Simplicity UV (Millipore S.A.S., Molsheim, France). This ultrapure water was used to prepare all solutions and mobile phases.

2.2 Preparation of solutions and mobile phases

Micellar solutions were prepared by weighting the appropriate quantity of SDS and $NaH_2PO_4.H_2O$ and solving in ultrapure water using an automatic stirrer. Afterwards, the pH was adjusted to the desired value by adding drops of HCl or NaOH solutions. Furthermore, the adequate volume of organic solvent was added and the flask was filled up with ultrapure water. Finally, the solutions were vigorously shaken, ultrasonicated for 15 min to ensure the total solubilization and filtered through a 0.45-µm Nylon membrane filters (Micron Separations, Westboro, MA, USA) situated on a Büchner funnel, with the aid of a vacuum pump. They were stored in amber bottles.

Individual stock solutions of the studied anti-tumour drugs (200 mg/L) were prepared in dimethyl sulfoxide. Working solutions (individual and mixtures) were prepared from stock solutions by successive dilution in a micellar solution of 0.05 M SDS buffered at 7 with 0.01 M phosphate salt.

2.3 Chromatographic conditions

The chromatographic system was a HP1100 (Agilent Technologies, Palo Alto, CA, USA) equipped with an isocratic pump, an autosampler, a degasser, a 20-µL loop, a Kromasil C18 column (150 x 4.6 mm; 5 µm particle size; 10 nm pore size) and a UV-Absorbance diode array detector (DAD). The software Agilent ChemStation (Rev. A.10.01) was used to control the system and to register the signal. The dead time (t_0), retention time (t_R) and peak area (A) were taken from each chromatogram using the same software.

The mobile phase was an aqueous solution of 0.12 M SDS – 2 % 1-pentanol, buffered at pH 7 with 0.01 M phosphate buffer, running at 1 mL/min under isocratic mode without controlling the temperature. The detection absorbance wavelength was set to 260 nm. The chromatographic vials and the column remained at room temperature throughout the whole analysis. The special care required with the chromatographic instrumentation when dealing with micellar mobile phases is described in [27].

2.4 Sample collection and preparation

Samples of plasma from cancer patients and healthy volunteers were provided by a local Hospital, after consent of patients and doctors at the time of blood collection. The blood was collected by the clinical staff using a DB SST tube (SBD Vacutainer Systems, Plymouth, UK), and centrifuged at +4ºC at 3000 rpm for 10 min (3000 x *g*), to obtain the non-cellular fraction. The samples were frozen and stored at -20ºC from collection to analysis. For confidentiality reasons, our laboratory did not receive any personal or clinical information about the patients. The samples were only used for this study and later destroyed. No additional analyses were carried out, and no information was shared with other institutions.

Patient and blank samples were processed as the same way. Before the analysis, the samples were thawed for 30 min at room temperature. Afterwards, they were 1/5 diluted using a micellar solution of 0.05 M SDS buffered at pH 7 with 0.01 M phosphate salt, vigorously shaken, filtered through a 0.45-µm Nylon membrane filter (Micron Separations) with the aid of a 3-mL syringe, and directly injected. For spiked samples, the appropriate amount of the standard solution of drugs was added before dilution. The processed samples were destroyed after analysis.

3. Results and discussion

3.1 Optimization of the chromatographic conditions

The anionic surfactant sodium dodecyl sulphate is considered as the most adequate for the analysis of drugs in biological fluids. Indeed, it shows a proven ability to denature and solubilize plasma proteins and other non-water soluble macromolecules, as well as other interesting practical advantages (purity, solubility, low viscosity of the solutions, and low Kraft point). Therefore, it was selected for this study. Therefore, a C18 column was chosen because SDS monomers are easily adsorbed on C18 hydrocarbon chain, with a saturation point of only 10 mM. Isocratic mode was chosen for its higher simplicity [24,28]. According to the hydrophobicity (log Po/w 3.6-5.5) and charge of the drugs (neutral), they would be strongly retained on the modified stationary phase, and then the addition of 1-pentanol was envisaged to increase the hydrophobicity of the mobile phase, thus resulting in a faster elution and improving the peak shape [24].

The following experimental parameters were optimized: mobile phase composition (pH, and concentration of SDS and 1-pentanol), and the detection conditions. The chromatographic analyses were carried out using a working standard solution containing 1 mg/L of each TKI.

3.1.1 Selection of the pH

The effect of the pH on the retention was evaluated, inside the working range of the column (1.5-9.5). However, the study was restrained to acidic (3 and 5) and neutral (7) pH, in order to limit the damage to the stationary phase. At pH 3, no peaks were observed, whereas at 5, strongly distorted peaks were observed. At pH 7, the peaks show a normal shape. Therefore, the optimal pH was set to this value.

3.1.2 Effect of SDS and 1-pentanol amount on the retention

The influence of SDS and 1-pentanol (qualitative and quantitative) on the retention factor of the three anti-tumour drugs was investigated to model the retention factor and the global resolution, using an interpretative strategy. The experimental design was a face centered composite design, taking as -1 and +1 the minimal and maximal concentrations recommended in MLC for both chemicals. Therefore, the analytes were injected in nine micellar mobile phases, containing the following concentrations of SDS (M)/1-pentanol (%): 0.05/2; 0.05/4; 0.05/6; 0.1/2; 0.01/4; 0.01/6; 0.15/2; 0.15/4 and 0.15/6. The experimental dead time was 1.00 min. For each one, the retention factor (k) of the TKIs was calculated.

In the nine mobile phases, pazopanib was the less retained, followed by dabrafenib and regorafenib. The three drugs showed a binding behavior with the micelles, as the retention time diminished at increasing concentrations of SDS. This is probably due to the hydrophobic interaction between the drug and the inner core of the micelle. Otherwise, the augmentation of the proportion of 1-pentanol results in an earlier elution of the three compounds, as expected in MLC [28].

3.1.3 Modeling of the retention factor

A chemometric model was proposed to model the retention factor, in order to diminish the time and effort required for the optimization. Eq. (1) has been proposed for moderately hydrophobic compounds, to predict the retention factor depending on the concentration of SDS and 1-pentanol (φ) in the hybrid micellar mobile phase, running under isocratic mode [29]:

$$1/k = c_0 + c_1[SDS] + c_2\varphi + c_{12}\,[SDS]\,\varphi$$

[SDS] and φ are in normalized unities (-1 to +1) from (0.05 - 0.15 M) and (2 - 6 %), respectively, in order to better compare the constants. The constant c_0 indicates the retention factor at average values of each chemical, while c_1, c_2 and c_{12}, quantify the effect of each factor and their

interaction. For each drug, the equation was adjusted using the values of retention factor obtained in the experimental design (5 freedom degrees), by curve-fitting non-linear least-square regression [30]. The accuracy of the mathematical model was proven experimentally by the values of the multiple regression coefficient (R^2), and results are showed for pazopanib, dabrafenib and regorafenib and respectively:

$1/k = (0.531\pm0.024)+(0.28\pm0.03)[SDS]+(0.13\pm0.03)\varphi+(0.14\pm0.04)[SDS]\varphi \quad R^2 = 0.96$

$1/k = (0.1556\pm0.0016)+(0.0401\pm0.0021)[SDS]+(0.0376\pm0.0021)\varphi+(0.004\pm0.003)[SDS]\varphi$

$R^2 = 996$

$1/k=(0.101\pm0.003)+(0.041\pm0.004)[SDS]+(0.043\pm0.004)\varphi+(0.013\pm0.005)[SDS]\varphi$

$R^2= 0.990$

Figure 1 shows the theoretical retention times of the three drugs at concentrations of SDS and 1-pentanol ranging from 0.05-0.15 M and 2-6 %, respectively. The order of retention times (regorafenib > dabrafenib > pazopanib) was maintained in the whole interval of surfactant and alcohol concentrations.

The effect of each factor and the interaction on the retention factor was inferred from the values of the constants. For the three drugs, the significance of the constants was < 7 %, and then all of them were included in the model. Besides, they have a positive value, that means an increasing of the concentrations of SDS and 1-pentanol effectively reduce the retention. These deductions are similar to those extracted from the unprocessed results of the experimental design. Besides, the effect of SDS is enhanced at higher amounts of 1-pentanol, and *vice versa*. This may be caused by the decrease of the CMC and the aggregation number, and then the augmentation of the total number of micelles, caused by the introduction of the organic modifier [25]. For pazopanib and dabrafenib, SDS has a higher influence than 1-pentanol, unlike for regorafenib. The interaction was the less relevant.

3.1.4 Optimization of the SDS/1-pentanol concentration

The optimal concentrations of SDS and 1-pentanol were established by surface response methodology, in order to maximize the global resolution (*Z*) and minimize the analysis time (retention time of the most retained drug).

The elementary resolutions at any combination of SDS/1-pentanol were calculated between two consecutive peaks as the modified selectivity, from the equations adjusted in 3.1.2. The paired peaks were (pazobanib: dabrafenib) and (dabrafenib: regorafenib), as the elution order was maintained:

$$\alpha'(\text{PAZO/DABRA}) = 1-k(\text{PAZO})/k(\text{DABRA})=1-(0.1556+0.0401\,[\text{SDS}]+0.0376\varphi+0.004[\text{SDS}]\varphi)/(0.531+0.28[\text{SDS}]+0.13\varphi+0.14[\text{SDS}]\varphi)$$

$$\alpha'(\text{DABRA/REGORA})\ 1-\ (0.101+0.041[\text{SDS}]+0.043\varphi+0.013[\text{SDS}]\varphi)/\ (0.1556+0.0401\,[\text{SDS}]+\ 0.0376\varphi+0.004[\text{SDS}]\varphi)$$

The global resolution was modelled as the minimal elementary resolution (Figure 1D). At proportions of 1-pentanol of 2 %, the maximal resolutions were reached, and remained acceptable (0.45-0.64) for the entire interval of SDS. The optimal SDS concentration was fixed to 0.12 M, where the global analysis time was acceptable (15.6 min) and the less retained compound was eluted enough far (3.2 min) to prevent overlapping with the front of the chromatogram when plasma samples are injected. Thereafter, a mixture of the three drugs was analyzed using a mobile phase of 0.12 M SDS - 2 % 1-pentanol buffered at pH 7 with 0.01 M phosphate salt. The peaks showed adequate shape, and the experimental retention times (min) were: PAZO, 3.4; DABRA, 8.8 and REGORA, 15.9. The error in the prediction of the retention times was < 6.5 %. The experimental modified selectivity was 0.47.

3.1.5 Detection conditions

A working solution containing the three drugs was analyzed under the optimized conditions, and the absorbance spectra was measured between 200 and 800 nm at their retention time. The three drugs showed a high absorptivity at 260 nm. Therefore, the detection was performed at this value, and no wavelength changes were required.

3.2 General comments about the procedure

The developed method shows several practical features, because of the specific properties of micellar solutions.

The foremost advantage of the method is the simplification and shortening of the sample preparation. Indeed, the ability of SDS-micellar solutions to solubilize the proteins has eradicated the needing of extraction or cleanup intermediate steps. Therefore, plasma can be quantitatively injected into the column, thus minimizing the sources of variance and limiting the loss of the analytes, and then an internal standard is not compulsory. The dilution decreases the sensitivity, but it is necessary to reduce the amount of proteins and other macromolecules in the chromatographic system.

The analysis has a scarcely harmful effect on the environment and the health of the laboratory staff. The pure micellar solution (used in the sample pretreatment) contains only innocuous and biodegradable chemicals, and the hybrid micellar solution (mobile phases) additionally includes a lower amount (<2%) of volatile, toxic and flammable organic solvent, less than usually required in RP-HPLC (up to 100 %). Besides, its volatility is reduced by the interaction with SDS. Therefore, the handling and waste of toxic chemicals are minimal.

The method is adapted to the analysis of a large number of samples per day, as the successive analysis of the samples is facilitated and the global analysis time is significantly reduced. A single operator can successively process many samples, due to the short duration of the sample treatment (2 - 3 min).

The use of this method makes the analyses of individual plasma samples relatively inexpensive. The cost and the amount of the consumed chemicals are relatively low, and only basic laboratory materials and instrumentation are used. Otherwise, the characteristics of the chromatographic conditions (pH of the mobile phase, sample dilution and isocratic mode) have been selected to enlarge the lifespan of the stationary phase.

3.3 Method validation

The developed procedure was *in-lab* fully validated by the Guideline on Bioanalytical method validation, published by the European Medicines Agency (EMA) [26]. The studied parameters were: selectivity, calibration curve, lower limit of quantification, within-run and between-run accuracy and precision, carry-over, dilution integrity, matrix effect and stability [26]. The detection limit [31] and the robustness [32] were as well investigated. The validation was carried out using blank plasma samples from healthy volunteers fortified with the studied TKIs, unless specified.

3.3.1 Selectivity

Blank samples of plasma were taken from six healthy individuals, which does not take any medication: three males and three females, and for each gender, at three different age frame: twenty, forty and fifty. Each sample was analyzed before and after (Figure 2A) spiking with 5 mg/L of each analyte. The retention times (min)/capacity factor were: PAZO, 3.4/2.4; DABRA, 8.8/7.8 and REGORA, 15.9/14.9. The selectivity values were: αDABRA/PAZO = 3.3 and αREGORA/DABRA= 1.9. The resolution parameters were: RsPAZO-DABRA, 5; RsREGORA-DABRA: 5.8. These values indicate the high quality of the chromatographic resolution.

In the chromatograms obtained by the analysis of blank plasma, only a high and broad front of the chromatogram (dead time to 2.5 min) and a quite stable baseline without peaks (>2.5 min) were observed. Indeed, as the matrix compounds are strongly associated with the micelles, they barely interact with the modified stationary phase, and are quickly eluted. In addition, the dilution has contributed to reduce the size of the protein band.

The spiked samples provide chromatograms with a similar protein band and baseline, and without other peaks, except those corresponding to the analytes. These ones were eluted as similar retention time (<2 %) and showed the same absorbance spectra, as those obtained by injecting a standard micellar solution (section 3.1.4, respectively). The less retained drug (pazopanib) was eluted after the front of the chromatogram and then no overlapping was observed. Therefore, the procedure was enough selective to unequivocally identify these three drugs in plasma.

3.3.2 Calibration range and sensitivity

For calibration purposes, samples of plasma were spiked at increasing concentrations of the three drugs (up to 80 mg/L) and analyzed by triplicate. For each drug, the peak area was related to the corresponding concentration by a first-grade equation, using the least-square linear regression method. The lowest limit of quantification was the smallest level which can be measured (using the calibration curve) with an accuracy and precision inside the requirements of the guide, while the highest limit of quantification was established at to the maximal concentrations usually reached in the clinical practice. Levels under LLOQ were removed from the definitive calibration curve. The limit of detection was determined by the 3s criterion [31]. The values of these calculated parameters can be seen in Table 2. A chromatogram obtained by the analysis of a plasma sample spiked at the LLOQ for each drug is shown in Figure 2B.

An adequate linearity ($r^2 > 0.9999$) was obtained along the studied range for the three drugs. Besides, the lower limits of quantification were below their target concentration.

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338 *3.3.3 Accuracy and precision*

339

340 These parameters were studied in matrix at four concentrations for the three drugs: LLOQ,
341 near 3 times the LLOQ, 20 and 60 mg/L. The within-run measurements were performed out by six
342 successive injections of a spiked sample. The accuracy was calculated as the average relative error
343 of the found concentration, whereas the precision was the relative standard deviation (RSD) of the
344 peak areas. The between-run values were determined to evaluate their consistency over time. The
345 within-run experiment was repeated in five days over a two month period, using renewed spiked
346 samples each time. The accuracy was determined as the average of the five within-run errors, while
347 the precision was the RSD between the five average peak area (each one calculated from the six
348 analysis). The results can be seen in Table 3.

349 The values of error (12.6 to 11.7 %) and variability (< 11.9 %), were inside the requirements
350 of the guide (20 % for LLOQ and 15 % for upper levels). These results were reached by the
351 minimization of the sources of variance and the quantitative injection of the sample. Therefore, the
352 analytical method provides reliable quantitative values for the three drugs throughout the studied
353 calibration range.

354

355 *3.3.4 Dilution integrity*

356

357 The within- and between-run accuracy and precision was determined at 50 mg/L of each
358 drug, using the protocol indicated in section 3.2.2, but introducing a 1/10 dilution in a solution of
359 0.05 M SDS buffered at pH 3.

360 The values of intraday accuracy and precision were determined as in section 3.3.3 and were
361 similar to those shown in Table 3, indicating that the introduction of the dilution step does not

significantly affects the quantitative information, and then can be a useful strategy to analyze samples contaminated at levels over the HLOQ.

3.3.5 Carry over effect

The carry over effect was evaluated, in order to evaluate the possibility of cross-contamination in an analytical run. A plasma sample spiked at 80 of each TKI, and a blank sample was processed and successively analyzed. No peak was observed at the window time of the analytes in the chromatogram obtained from the blank sample. Consequently, the transfer of drugs to the following analyzed sample can be considered insignificant, at concentrations under the HLOQ.

3.3.6 Matrix effects

The possible interference of endogenous compounds on the quantitative results was investigated at two levels for each drug: PAZO, 5 and 60 mg/L, DABRA, 2 and 60 mg/L and REGORA, 0.5 and 60 mg/L. A micellar standard solution, and a plasma spiked samples were 1/5-diluted and analyzed. For the three drugs, the peak areas were similar in both cases, indicating that the matrix effect can be neglected. Indeed, after the dilution, the micellar solution is predominant, and then the chemical environment of the drugs in quite similar, in spite of the original matrix. Besides, the micelles bind the most important matrix compounds (proteins and macromolecules), weakening their interaction with the drugs [24].

3.3.7 Stability

The stability of the drugs was evaluated at the same concentrations as in section 3.3.5 in working solutions, and in plasma matrix (freeze and thaw and long term freeze), at their usual

storage conditions. The possible degradation was considered by considering the diminishing of the peak area of each analyte in the chromatogram obtained by the analysis of the samples, and the appearance of other peaks, corresponding to decomposition products. A stored solution must be discarded if the concentration varies more than 15 % of the initial concentration.

The working solutions were kept at +4ºC in a fridge. Each day, during two months, the solutions were thawed, 1/5-diluted, analyzed and stored another time. No variation of the peak area was observed during the study, and then the working solutions can be used for this period without introducing a systematic error in the method.

Spiked plasma samples were kept at the usual storage conditions of biological fluids for clinical purposes (-20ºC in a freezer for 15 days). Two situations were considered:

- Freeze-and-thaw stability: A spiked sample for each matrix and concentration was stored. Each 12 h, the sample was thawed, an aliquot analyzed, and replaced in the freezer. No variation of the peak area was observed the considered period. Therefore, the same sample can be used for the clinical and analytical studies each day for a minimum of two weeks.
- Long term freeze stability: 15 samples (for each matrix and concentration) were spiked and kept. Each day, a sample (for each category) was thawed, analyzed and discarded. The concentration of the drugs was the same in all the measures, and this storage protocol can be applied for the quantification of these drugs in biological samples extracted from patients.

When many samples arrive at the laboratory and must be successively analyzed, a processed sample may remain a long time in the autosampler tray before injection. Therefore, the short term stability of the drugs in the processed matrix at room temperature must be evaluated. A sample (for each concentration and matrix) was processed, placed in the autosampler tray, and successively injected for one day. The time between two successive injections was the duration of a single chromatographic run (18 min), and then the sample was injected 80 times. No variation of the peak area was observed for the three drugs. Therefore, until 80 samples.replicate can be processed and analyzed in one day.

414

415 *3.3.8 Robustness*

416

417 The effect of small oscillations of the experimental factors on the chromatographic response
418 (retention and sensitivity) was evaluated. The studied minimal and maximal values were that we
419 consider than can easily be reached during the preparation of the mobile phase and the normal use
420 of the instrumentation: SDS concentration, 0.11-0.13 mM; 1-pentanol, 1.8-2.2 %, v/v; phosphate
421 salt, 0.008-0.012 M; pH, 6.8-7.2; flow-rate, 0.95-1.05 mL/min; injection volume 18-22 µL and
422 detection wavelength 255-265 nm.

423 A working standard solution containing 1 mg/L of the three anti-tumour drugs were
424 analyzed by stating each experimental parameter as indicated in the Youden approach [32], to
425 calculate the difference in retention time and peak area caused by the variation of each parameter.
426 The relative differences are shown in the Table 4.

427 For the three drugs, the detection wavelength and the injection volume make an appreciable
428 influence in peak area, because of the variation of the coefficient of extinction and quantity of
429 sample, respectively. Retention time was significantly affected by the variation of flow. Although
430 this parameter does not alter the retention, it has a direct influence on dead time. Besides, for
431 pazopanib and regorafenib, retention time was excessively affected by the considered variation of 1-
432 pentanol, probably by its role in the retention mechanism. Dabrafenib peak area was influenced by
433 SDS and pH. The effect of the other factors was insignificant.

434 The most influential parameters (detection wavelength, injection volume and flow-rate) are
435 adjusted by the instrumentation and remain constant, and then will not cause analytical troubles.
436 However, the operator must pay a special attention to the manipulation of 1-pentanol, SDS and
437 buffer when preparing the mobile phase. Considering this, the developed procedure is enough
438 robust to be unaffected by slight changes in the main experimental conditions, that can arise from
439 random errors. This proves the system suitability of future chromatographic analysis.

3.4 Analysis of real samples

The developed method was employed for the quantification of pazopanib, dabrafenib and regorafenib in plasma from cancer patients taking these medications by medical prescription (five for each one), provided by a local Hospital. These samples were frozen from collection to delivery. A blank sample and a blank plasma sample spiked at low QC (PAZO, 5 mg/L; DABRA, 2 mg/L; REGORA, 0.5 mg/L), medium QC 20 and high QC 60 mg/L were analyzed, in order to verify the keeping of the analytical quality of the measurements. All the samples were processed and analyzed in a single batch in the same analytical run (Table 5). The chromatogram obtained by the analysis of the biological matrices from patient 5 is shown in Figure 2C.

The blanks and the QCs samples provide adequate values (all under 15 % error), and the anti-tumour drugs were quantified without interferences. Therefore, the method can be applied in a realistic clinical analysis.

4. Conclusions

Micellar liquid chromatography has been proven as an interesting alternative to determine pazopanib, dabrafenib and regorafenib in plasma. The sample preparation was a simple dilution and direct injection, thus avoiding time consuming and cumbersome extraction steps. This contributes to shorten the global analysis time and to improve the reproducibility. The effect of the main components of the mobile phase (SDS and 1-pentanol) on the retention in a C18 column was quantified using an experimental design. Therefore, the retention time and the resolution were modeled, which expedited the optimization of the mobile phase from few assays. The drugs were eluted in < 18 min using a hybrid micellar mobile phase running under isocratic mode, without interferences from endogenous compounds. The method was validated by the guidelines of the

European Medicines Agency in terms of selectivity, linearity, calibration range, sensitivity, carry over effect, accuracy, precision, dilution integrity, matrix effect, stability and robustness. The results were in agreement with the requirements of the guideline, thus ensuring the reliability of the quantitative data at the concentration levels that can be found in clinical samples. The procedure also holds practical advantages, like being half-automated, short-time, safe for the laboratory staff, eco-friendly, inexpensive, available, able to analyze may samples per day, and applicable to routine analysis. The outstanding achievements of the procedure are possible because of the specific properties of micellar solutions. Therefore, it can be implemented in clinical laboratories to monitor these drugs in biological samples.

5. Future perspective

The current trend in Analytical Chemistry is the development of inexpensive, simple, automated and ecofriendly analytical procedures. Therefore, the implementation of these kinds of methods will increase in the future in routine analysis for clinical purposes. An interesting approach is the substitution of current analytical methods by other ones, based on direct injection, and using a lower quantity of toxic chemicals. Micellar liquid chromatography can play a major role in this process.

The application of the here-described method may be enlarged to determine other drugs for therapeutic drug monitoring, by varying the separation and the detection conditions. Besides, it can also be applied to other biological fluids (urine, cerebro-spinal, gastric, saliva, sweat, *etc*.), and further to solid tissues (feces, organs and muscles). In this case, a solid-liquid extraction would be necessary. The chromatographic conditions will be similar, as the micellar environment reduces the matrix effect.

A modification of micellar liquid chromatography, based on the use of pure mixed micellar mobile phases (using a biodegradable and safe nonionic surfactant), instead of hybrid ones (using

toxic, flammable and volatile organic solvent), has been recently proposed and has attracted a huge interest. This new technique can be applied to this method, in order to totally remove the use of hazardous chemicals and then totally fulfil the requirements of "green" chemistry.

6. Executive summary

Background

- The tyrosine kinase inhibitor shows a high variability in the clinical (therapeutic and adverse) effects depending on the patient and even at different stages of the treatment. Therefore, the application of therapeutic drug monitoring may be helpful to investigate the pharmacokinetics in specific cases to optimize the treatment.
- We explore the use of micellar liquid chromatography to develop a method to determine pazopanib, dabrafenib and regorafenib in human plasma.

Experimental

- The sample was diluted and directly injected in the chromatographic system.
- The retention time was measured using nine mobile phases containing different SDS/1-pentanol concentrations, which were selected using an interpretative strategy.
- The mobile phase was an aqueous solution of 0.12 M SDS - 2 % 1-pentanol, buffered at pH 7, and the stationary phase was in a C18 column.
- The method was validated by the guidelines of the EMA and tested by the analysis of samples from patient cancer taking the corresponding medication, and QC samples in the same run.

Results and Discussion

- Chemometrics has allowed to model the retention factor from the concentration of sodium dodecyl sulfate and 1-pentanol in the mobile phase.
- The proper values of SDS/1-pentanol concentration were selected by surface response methodology, to maximize the resolution and minimize the analysis time.

- The Tyrosine Kinase Inhibitors can be determined in a single run using isocratic mode, without interferences from the matrix.
- The plasma can be directly injected, after a simple dilution, despite its complexity. Therefore, the experimental is strongly simplified.
- Cumbersome, time-consuming and hard-to-manipulate liquid or solid extraction, re-dissolutions or solvent evaporation, centrifugation steps are not required.

Conclusions

- The method minimizes the cost, effort, and use of chemicals, laboratory material and hazardous compounds, and fits the requirement for "green chemistry".
- The method was suitable for the analysis of plasma samples containing the analytes near to their target concentrations.

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TABLES

Table 1. Physicochemical and pharmacological parameters of the studied drugs [7-11].

Drug	Pazopanib	Dabrafenib	Regorafenib
Developer	GlaxoSmithKline (London, UK)	GlaxoSmithKline (London, UK)	Bayer (Leverkusen, Germany)
Log Po/w	3.6	5.5	4.5
pKa (basic)	2.1/6.4	2.2/6.6	2.3
pKa (acidic)	10.2	-----	12
Charge at pH 7	0	0	0
Bioavailability	14-39 %	95 %	69-83 %
Usual treatment	800 mg once daily for 22 days	150 mg each 12 h, until disease progression or unacceptable toxicity	160 mg once daily for 21 days
Time to peak plasma	2 - 4 h	2 h	4 h
Cmax	58.1 mg/L	1.5 mg/L	2.5 mg/L
C trough	20.5 mg/L	0.03 mg/L	3.9 mg/L
Half life	35 h	8 h	28 h
Route of elimination (unchanged)	82.2 % feces 4 % urine	71 % feces	47 % feces 2 % urine

Table 2. Calibration curves and sensitivity of the method (concentrations in mg/L)

TKI	Slope	y-intercept	r^2	LOD	LLOQ-HLOQ
Pazopanib	12.14±0.11	-2±2	0.9990	1.0	2-80
Dabrafenib	68.8 ± 0.3	0.0±0.8	0.9990	0.2	0.5-80
Regorafenib	98.6±1.3	-1.1±1.5	0.997	0.1	0.2-80

n = 7

Table 3. Intra- and inter-day accuracy and precision for the studied drugs.

Drug	Concentration (mg/L)	Intra-day[a]		Inter-day[b]	
		Accuracy (ε, %)	Precision (RSD, %)	Accuracy (ε, %)	Precision (RSD, %)
Pazopanib	2	+4.2	11.9	+6.1	8.1
	5	-2.1	7.7	-4.1	6.9
	20	+3.8	0.3	1.7	4.1
	60	-2.2	0.4	-2.3	1.9
Dabrafenib	0.5	-1.9	6.2	-4.5	5.3
	2	-2.0	3.6	-2.6	4.7
	20	+2.2	0.3	+1.2	1.3
	60	-6.1	2.3	-2.2	4.0
Regorafenib	0.2	+11.7	8.3	+11.2	8.8
	0.5	-12.6	10.8	-12.5	5.3
	20	-8.5	2.7	-4.8	2.1
	60	+5.8	0.5	+6.8	0.7

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

Table 4. Robustness of the method. Difference between upper and lower level of each factor divided by the optimal value (%)

Drug	Instrumental response	λ	[SDS]	[1-pentanol]	pH	Flow	Injection Volume
Pazopanib	Retention time	+4.4	+8.7	-18.6	-8.5	-18.1	-1.8
	Peak area	+34.1	+7.1	+10.6	+1.4	-4.2	+12.2
Dabrafenib	Retention time	+1.4	+5.0	-6.8	-8.4	-15.8	-9.7
	Peak area	-57.0	-14.7	+3.4	+19.4	+6.4	+37.7
Regorafenib	Retention time	+3.0	-4.1	-10.1	-0.0	-10.4	-4.4
	Peak area	+26.5	+5.0	+23.1	+1.2	-1.7	+18.5

Table 5. Concentrations (mg/L) of the studied TKIs in plasma samples from cancer patients taking

this medication (blanks and QCs are included in the analytical run).

Patient	PAZO	DABRA	REGORA
Blank	Under LOD	Under LOD	Under LOD
1	23.4		
2	12.8		
3	48.4		
4	6.9		
5	25.7		
Low QC	1.8	5.3	0.54
6		2.1	
7		0.7	
8		Under LOQ	
9		Under LOQ	
10		1.6	
Medium QC	18.7	21.4	19.1
11			2.7
12		<0.2	4.1
13			3.2
14			4.5
15			2.9
High QC	64.8	57.4	63.1

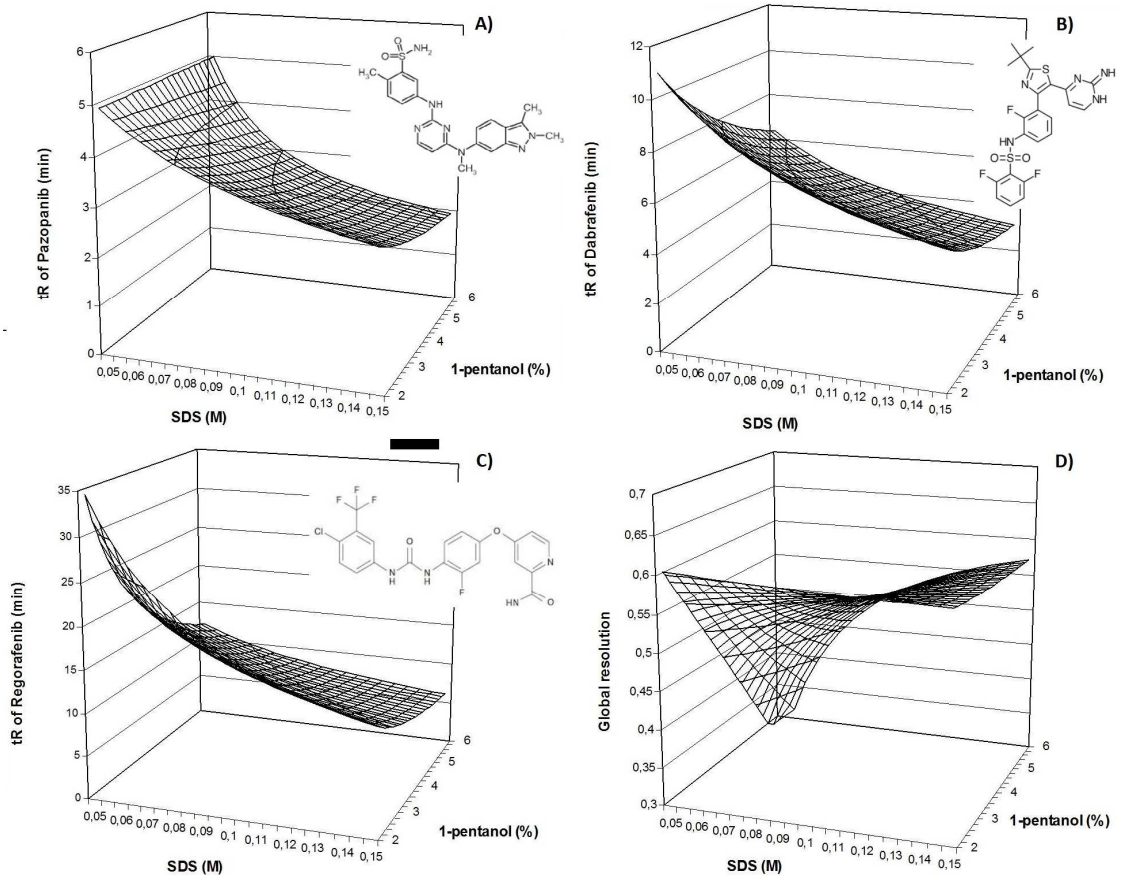

Figure 1. Predicted retention time of A) pazopanib, B) dabrafenib and C) regorafenib, and D) global resolution, at several SDS/1-pentanol concentration. The structure of each drug is also shown.

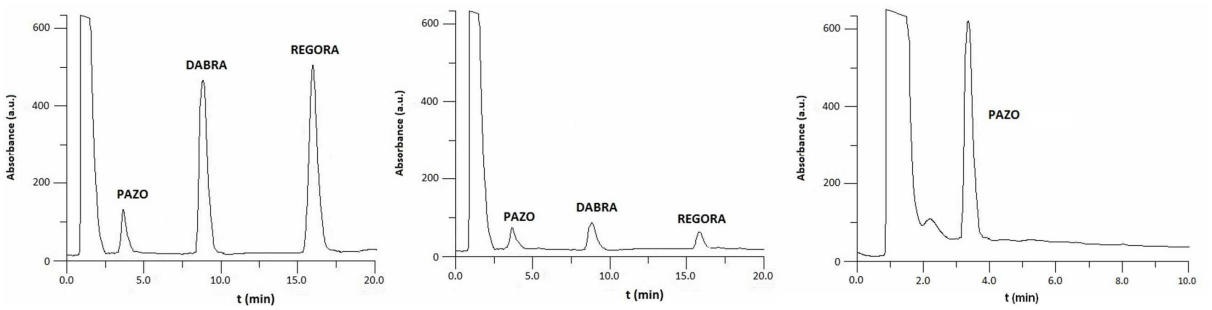

Figure 2. Chromatogram obtained by the analysis of plasma samples spiked with each drug at A) 5 mg/L and B) their corresponding LOQ, and C) from patient 5.

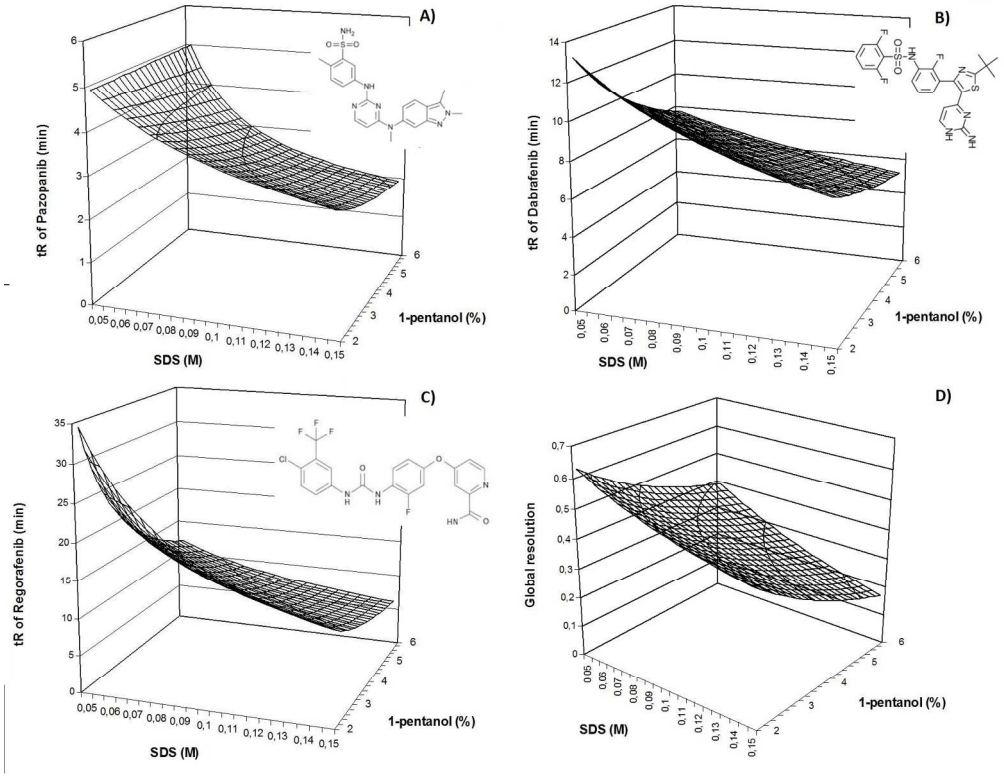

Figure 1. Predicted retention time of A) pazopanib, B) dabrafenib and C) regorafenib, and D) global resolution, at several SDS/1-pentanol concentration. The structure of each drug is also shown

336x261mm (150 x 150 DPI)

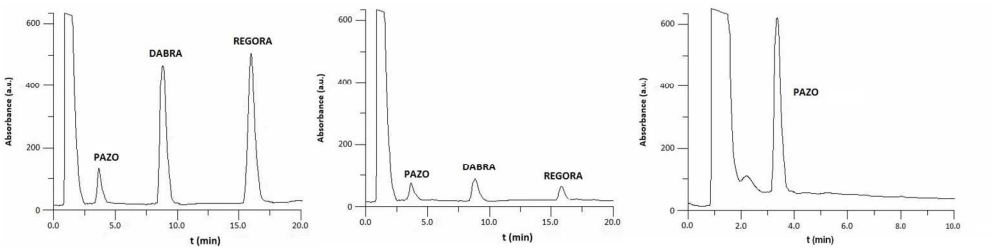

Figure 2. Chromatogram obtained by the analysis of plasma samples spiked with each drug at A) 5 mg/L and B) their corresponding LOQ, and C) from patient 5.

458x111mm (150 x 150 DPI)