https://doi.org/10.31925/farmacia.2022.5.10 **ORIGINAL ARTICLE**

EXTRACTION PROCESS ASSESSMENT AND LC-MS ANALYSIS OF TWO TYROSINE KINASE INHIBITORS IN HUMAN PLASMA

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Abstract

The class of tyrosine kinase inhibitors (TKIs) is represented by a group of compounds which are currently used in the treatment of different types of cancer. As plasma concentration profiles of these drugs reveal a large interindividual variability, therapeutic drug monitoring (TDM) might be necessary to adjust the therapeutic protocol. With this purpose, we developed and validated a simple and accurate method for determining plasma concentrations for two TKIs. Solid phase extraction (SPE) using Oasis PRiME HLB® cartridges was used for plasma sample preparation procedure. The method provided clean extracts with a recovery extraction of more than 85%. Separation was achieved by high-performance liquid chromatography (HPLC), using a C18 (4.6 x 50 mm) column, with a gradient elution of ammonium formate-acetonitrile as mobile phase. Detection was performed by mass spectrometry (MS) in Single Ion Recording (SIR) mode. Intra-day and inter-day precision data for both analytes were 3.8 - 7.2% and 3.6 - 7.4%, respectively. Calibration curves were both linear between 2 and 400 ng/mL with a correlation coefficient higher than 0.998. The LC-MS method was used to quantify both TKIs in human plasma in routine analysis. The method was applied to TDM of ibrutinib and ruxolitinib and may help physicians for dose adjustment.

Rezumat

Clasa inhibitorilor de tirozin kinază (TKI) este reprezentată de un grup de compuși care sunt utilizați în prezent în tratamentul diferitelor tipuri de cancer. Deoarece profilurile concentrației plasmatice ale acestor medicamente dezvăluie o variabilitate interindividuală mare, monitorizarea terapeutică a medicamentelor (TDM) ar putea fi necesară pentru ajustarea protocolului terapeutic. În acest scop, am dezvoltat și validat o metodă simplă și precisă pentru determinarea concentrațiilor plasmatice pentru doi inhibitori de tirozin kinază. Extracția pe fază solidă (SPE) folosind cartușe Oasis PRiME HLB® a fost utilizată pentru procedura de pregătire a probelor de plasmă. Metoda a furnizat extracte curate cu o recuperare la extracție de peste 85%. Separarea a fost realizată prin cromatografie de lichide de înaltă performanță (HPLC), folosind o coloană C18 (4,6 x 50 mm), cu o eluție în gradient de formiat de amoniu-acetonitril ca fază mobilă. Detecția a fost efectuată prin spectrometrie de masă (MS) în modul *Single Ion Recording* (SIR). Datele de precizie „intra-zi" și „inter-zi" pentru ambii analiți au fost 3,8 - 7,2% și, respectiv, 3,6 - 7,4%. Curbele de calibrare au fost ambele liniare între 2 și 400 ng/mL, cu un coeficient de corelație mai mare de 0,998. Metoda LC-MS a fost utilizată pentru a cuantifica ambele TKI din plasma umană în analiza de rutină. Metoda a fost aplicată pentru monitorizarea terapeutică a ibrutinibului și ruxolitinibului și poate ajuta medicii pentru ajustarea dozei.

Keywords: TKIs, Human plasma, SPE, LC-MS

Introduction

Clinical use of tyrosine kinase inhibitors (TKIs) has grown significantly in recent years [9]. A full review of analytical methods regarding lymphocytic anti-leukaemia drugs including ibrutinib has already been published [13]. Bioanalysis of these compounds is gaining increasing interest; initially, only a few research organizations and some universities were involved in determining TKIs. The analytical methods were used rather for the development of the drug in pre-clinical and clinical studies, mandatory for implementation of these medicines on the drug market. Later, with the development of mass spectrometry, smaller laboratories have begun to use highly sensitive detectors (including triple quadrupole mass analysers), which are more accurate in determining chromatographic peak assignment and more sensitive in analysing low plasma concentrations (often ng/mL levels). So, information on therapeutic drug monitoring (TDM) has become much more accessible [2, 14].

TKIs are widely metabolized by CYP enzymes, especially by CYP3A4 [23]. These enzymes are subject to large interindividual variations due to

enzymatic inhibition or induction, but also due to different expression which undergoes genetic polymorphism [3, 15]. The metabolic profiles of these drugs could generate lower and ineffective plasmatic levels or, by contrary, higher drug levels, leading to side effects such as thrombocytopenia, neutropenia, anaemia or fibrillation. Therefore, individual dosing is necessary, which can only be achieved by TDM.

Determination of TKIs concentration levels require a proper pre-treatment of plasma samples. Interfering plasma compounds like phospholipids, proteins, salts and exogenous substances may alter the electron ionization in MS and/or the chromatographic separation of target TKIs [20]. Therefore, an appropriate sample pre-treatment method which provides at the same time a good sample-clean-up and a high extraction recovery in human plasma is required. So far in the literature, authors usually used simple protein precipitation [4-6] or liquid-liquid extraction (LLE) [17] to extract these compounds from biological fluids. The main problem in protein precipitation is the incompatibility of the resulted supernatant with ESI source in MS detection, because of large amounts of co-extracted endogenous compounds. LLE usually generates clean samples, but requires a tedious working procedure, usually with multiple extraction steps and uses, also, large amounts of solvents. Solid phase extraction (SPE) uses low solvent and sample volumes and, in most cases, an even better sample clean-up than LLE.

Some authors used UV detection for ibrutinib quantitation. They monitored the mobile phase at 260 nm wavelength [22]. The lack of sensitivity with this detection method provided a lower limit of quantitation (LLOQ) of 10 ng/mL, which in some cases (like fasting conditions as the authors admit) may not be low enough to determine plasma concentrations.

Tandem mass spectrometry was also employed in TKIs quantitation in rat or human plasma [1, 3, 6-12, 16, 18, 19, 21]. MS/MS detection methods are very sensitive and accurate, but also involve instruments that, in most cases, are not available to small laboratories or clinics.

The aim of this work was to investigate the extraction process of two TKIs, ibrutinib and ruxolitinib, by using a variety of solvents and solvent mixtures in LLE and SPE. The purpose was to optimize the cleaning procedure, therefore, to avoid the problems that may occur in ESI-MS electrospray interface. MS parameters were also assessed obtaining the best signal-to-noise ratio for both compounds. The method was fully validated and applied in the TDM of the two TKIs in human plasma.

Materials and Methods

Chemicals and Reagents

Ibrutinib and ruxolitinib were purchased from Biomedica Medizinprodukte (Romania). Pentoxifylline (IS) was provided by Merck. Ammonium formate and MS grade ultrapure LC solvents (water, acetonitrile and methanol) came from Merck. Oasis PRiME HLB® cartridges were purchased from Waters (USA).

LC-MS chromatographic system and analytical parameters

The separation was carried out on a Waters (Milford, Massachusetts, USA) Arc System coupled with a Waters QDa mass detector. The column used was a Waters CORTECS C18 (4.6 × 50 mm, 2.7 µm). Elution was carried out using solvent A (ammonium formate 10 mM aqueous solution) and solvent B (acetonitrile). The following gradient was used for the analytical separation of the compounds: 0 - 1.0 min, 75% (A) and 25% (B); 1 - 9 min, gradually increasing eluent B to 75%; 9 - 10 min, gradually decreasing eluent B to 25%. The flow rate of the mobile phase was set to 0.5 mL/min. The column was equilibrated at 40°C temperature. The injection volume was 5 µL. All samples were kept at 20°C in the autosampler during the entire analysis.

Eluted compounds were analysed using a QDa mass detector equipped with an electrospray ionization (ESI) source. Capillary voltage was maintained at 0.8 kV, cone voltage was kept at 25 V and the mass spectra were recorded in positive ion mode in the range m/z 100 – 400 for ruxolitinib and m/z 100 – 500 for ibrutinib. Quantification was performed in SIR mode for ruxolitinib, ibrutinib and pentoxifylline at m/z 307, 441 and 279, respectively. The equipment was controlled using the EmPower 3 software package.

Preparation of Stock and Working Standard Solutions

Stock solutions of ibrutinib, ruxolitinib and pentoxifylline at a concentration of 1 mg/mL were prepared in methanol. Ibrutinib and ruxolitinib mixed working solutions were prepared by diluting these solutions with methanol, to obtain final concentrations of 0.02, 0.1, 0.5, 2 and 4 µg/mL. Pentoxifylline working solution was prepared separately at 4 µg/mL concentration. All standard solutions were kept at -20°C and renewed every 30 days.

SPE procedure for standards and samples

10 µL mixed working solution was evaporated to dryness with nitrogen in a conical glass vial and 100 µL blank human plasma were added. Then, 10 µL IS working solution were added and the mixture was loaded on Oasis PRiME HLB cartridges by using a positive pressure. The washing step

consisted of 500 µL water with 5% methanol and analytes elution step was performed with 500 µL methanol. The elution solvent was evaporated to dryness and the residue was redissolved in 50 µL mixture consisting of acetonitrile: ammonium formate 10 mM, 25:75 (v/v) representing the initial gradient of the mobile phase. The solution was put in the autosampler and a volume of 5 µL was injected in the LC column.

Validation data

Validation was carried out according to International Council for Harmonisation (ICH) guidelines. Calibration curves concentrations obtained as described above for ibrutinib and ruxolitinib were 2, 10, 50, 200 and 400 ng/mL. Both intra-day and inter-day accuracy (percentage ratio between mean found concentration and spiked concentration) and precision (coefficient of variance - CV%) of the analytes for all calibration levels were determined (n = 5).

Extraction recovery was also investigated for all concentrations. It represents the percentage ratio between the peak area of the extracted compound and the peak area resulted after direct injection of the same amount of drug dissolved in the initial mobile phase.

LLOQ was established as the lower concentration of the analytes where the ratio between peak height and the baseline noise is at least 10:1 and precision is no higher than 10%.

Stability of the analytes was also investigated. Long term stability (30 days at -20°C) and freeze-thaw stability after three freeze-thaw cycles were established (n = 5). Stability at room temperature (20°C) was determined for 12 hours (n = 5) to detect the possible degradation of the analytes during sample preparation or in the autosampler, prior to injection.

Carry-over

Carry-over effect was tested by injecting a blank sample after the injection of standards containing both analytes at 400 ng/mL (maximum concentration of the calibration curve). The peak areas for the analytes in the blank sample should be less than 10% from the LLOQ peak area (2 ng/mL). The needle injection was automatically washed (ten times) with mobile phase (initial gradient) between consecutive injections.

Human plasma samples analysis

To evaluate application of the method to real plasma samples, ibrutinib plasmatic levels were investigated in 5 chronic patients with chronic leukaemia treated with 420 mg ibrutinib daily. Samples were collected on day 10, two hours after administration of the daily dose. Two mL of blood were collected into a vacutainer containing potassium EDTA, followed by centrifugation at 10,000 rpm for 5 minutes. Obtained plasma samples were kept at -20°C before analysis.

Similarly, ruxolitinib samples were obtained from 6 chronic patients with myelofibrosis, treated with 20 mg ruxolitinib twice a day. On day 10, samples were collected, one hour after the morning dose and processed as previously described.

Prior to analysis, all samples were thawed at room temperature and extracted as described in SPE procedure. All patients have given the informed consent regarding acquisition of plasma samples.

Results and Discussion

Validation data

The obtained chromatograms in SIR mode for the two analytes emphasized the retention times of 4.15 and 6.75 min for ruxolitinib and ibrutinib, respectively. Both peaks were identified by spiking the final extracts with standard working solutions. Regarding selectivity, at specified m/z values (307 and 441), no other peaks are observed on the chromatogram.

From the calibration curves for both compounds reported to the IS, thecorrelation coefficients demonstrate the curves are linear in the selected concentration range.

The extraction recovery was, for both compounds and for all concentrations, more than 85%. LLOQ was 2 ng/mL. Table I reveals the results for stability tests for ibrutinib and ruxolitinib. Long term stability, freeze-thaw and room temperature stability were higher than 95%.

Intra-day and inter-day precision were between 3.6 and 7.4%. All data for accuracy and precision are presented in Table II.

Table I

Stability tests for ibrutinib and ruxolitinib (n = 5)

Target concentration (ng/mL)	Mean stability (%) for ibrutinib/ruxolitinib		
	Long term stability	Freeze-thaw stability	Room temperature stability
2	96.1 / 96.4	95.2 / 94.8	95.9 / 95.1
10	96.7 / 96.1	97.9 / 97.1	95.7 / 95.5
50	97.9 / 97.2	98.0 / 97.7	97.5 / 96.4
200	97.2 / 97.4	98.2 / 98.1	98.3 / 98.0
400	98.1 / 98.2	98.3 / 98.5	98.5 / 98.7

Table II

Precision and accuracy data (n = 5)

Analyte	Target concentration (ng/mL)	Mean found level (ng/mL)		CV %		Accuracy %	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Ibrutinib	**2**	2.1 ± 0.1	2.2 ± 0.1	7.2	7.3	105.0	110.0
	10	10.2 ± 0.7	10.3 ± 0.6	6.8	6.5	102.0	103.0
	50	48.9 ± 2.8	49.0 ± 2.9	5.7	5.9	97.8	98.0
	200	202.5 ± 8.3	202.9 ± 9.5	4.1	4.7	101.2	101.4
	400	397.4 ± 15.1	395.2 ± 15.4	3.8	3.9	99.35	98.8
Ruxolitinib	**2**	1.9 ± 0.1	2.1 ± 0.1	7.2	7.4	95.0	105.0
	10	9.8 ± 0.6	9.7 ± 0.6	6.8	6.5	98.0	97.0
	50	51.3 ± 3.0	51.5 ± 3.1	5.9	6.0	102.6	103.0
	200	197.7 ± 8.3	198.1 ± 10.3	4.2	5.2	98.8	99.0
	400	402.7 ± 15.3	403.6 ± 14.5	3.8	3.6	100.6	100.9

Plasmatic levels of ibrutinib and ruxolitinib

Plasmatic levels of ibrutinib and ruxolitinib in patients TDM were obtained using the linear regression equations. Ibrutinib levels for the five patients were 6.2, 7.9, 11.5, 13.2 and 19.7 ng/mL with 11.7 ± 13.5 ng/mL (mean ± SD). Calculated values for ruxolitinib plasma levels were 12.4, 27.5, 45.9, 48.3, 74.3 and 95.8 ng/mL with 50.7 ± 83.4 ng/mL (mean ± SD). The values are in accordance with those presented so far by other authors.

Extraction Assessment

The first step in validation of an analytical method is the assessment of sample preparation process. Protein precipitation generates a supernatant usually incompatible with MS detection. LLE is tedious and pollutes the environment with large amounts of solvents. After performing proper investigations, we obtained the best results by SPE using Oasis HLB PRiME cartridges. The cartridges contain a water-wettable polymer capable of hydrophilic interactions with polar compounds. These cartridges use a very simple working protocol. The lack of conditioning and equilibration steps reduces solvent consumption and time analysis also. The "three step clean-up" protocol was employed. In the first step, the sample (110 µL, including the IS solution) is applied with a positive pressure to the cartridge and absorbed on sorbent surface, followed by washing step with 500 µL solution 5% methanol and elution step with 500 µL methanol. The use of acetonitrile in elution step is not recommended because of low solubility of TKIs in this solvent. Clean sample extracts were obtained by elimination of most interfering plasma matrix compounds, especially proteins and phospholipids.

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

HPLC parameters were also investigated. A reverse phase CORTECS C18 chromatographic column was used for separation. Formic acid 0.1% aqueous solution as mobile phase produced important peak tailing, especially for ruxolitinib. Ammonium formate 10 mM provided improved peak shape and acceptable retention times within 7 minutes. Mobile phase flow rate was adjusted to 0.5 mL/min and best resolution was obtained at 40°C column temperature.

MS conditions were optimized. Positive ionization mode was employed. Capillary voltage and cone voltage were assessed to obtain the best signal for both analytes. Optimal values were 0.8 kV and 25 V for capillary and cone voltage, respectively.

Stability tests data (> 95%) revealed no degradation of the analytes occurred during stock or sample preparation. Calibration curves were linear (correlation coefficient > 0.998). Precision (expressed as CV) was lower than 10% (7.4% for ruxolitinib at LLOQ). Accuracy data was 95 - 110% to the nominal (spiked) concentrations.

The method was applied for the analysis of real plasma drugs levels. All blood samples were collected in day ten from the beginning of the treatment for the drugs to get to their steady-state concentrations. Collection of ibrutinib and ruxolitinib blood samples was performed at two hours and one hour post dose respectively, representing the mean time (T_{max}) to reach the maximum concentration in plasma (C_{max}) according to literature [9].

All detected plasma concentrations were in the 2 - 400 ng/mL range for both analytes. In our study, as presented so far in the literature, ibrutinib and ruxolitinib plasma levels also presented a large interindividual variability. This is due to complex factors, starting with absorption and metabolism rates which depend on type of cytochrome P450 izo-enzymes, co-administration of food and other drugs (enzymatic inhibition or induction) or patient's adherence to the treatment [7].

Ibrutinib and ruxolitinib plasma concentration levels determined for 11 patients were 11.7 ± 13.5 ng/mL and 50.7 ± 83.4 ng/mL (mean ± SD). These

data demonstrate the large interindividual variability regarding TKIs pharmacokinetics, making TDM necessary in individual dose adjustment.

Conclusions

A reliable LC-MS method was developed and validated for precise and accurate quantification of two TKIs in plasma. The method required only 100 µL human plasma. The proper sample preparation (using a SPE technique without cartridge conditioning) produced clean extracts providing a high sensitivity (LLOQ 2 ng/mL). The method was applied in determining TKIs plasma concentrations to 11 patients undergoing treatment with ibrutinib and ruxolitinib. Validation data and results obtained on real plasma samples qualify this method to be used in analytes TDM in human plasma.

Conflict of interest

The authors declare no conflict of interest.

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