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Keywords:

Ibrutinib ; dihydrodiol-ibrutinib ; LC-MS/MS ; cerebrospinal fluid ; B-cell malignancies

Highlights:

- LC-MS/MS method was validated to quantify ibrutinib and PCI-45227 in CSF
- The biological CSF samples were prepared by a simple and rapid dilution
- The lowest quantifiable ibrutinib concentration in patient's CSF sample is 0.5ng/mL
- Accuracy profiles were established using the total error approach
- Methods were successfully applied to real human CSF samples

Abstract:

Ibrutinib is an orally administered first-in-class irreversible Bruton's tyrosine kinase (BTK) covalent inhibitor for the treatment of patients with B-cell malignancies. Several isolated clinical observations reported its efficacy in central nervous system dissemination. Herein, we described the development and validation of an ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) procedure for the quantification of ibrutinib and its active metabolite PCI-45227 in cerebrospinal fluid (CSF). This is the first complete validated method for quantification of ibrutinib and PCI-45227 in CSF. The compounds were eluted on a Waters BEH C18 column (50.0 x 2.1 mm ; 1.7 µm) using a gradient elution with a mobile phase composed of ammonium formate buffer 5 mM pH 3.2 and acetonitrile + 0.1% formic acid with a flow rate of 400 $\mu L.min^{-1}$. Two deuterated internal standards were used to obtain the most accurate quantification. The CSF samples were prepared by a simple and rapid dilution. The method was validated by testing the selectivity, response function, intra-day and inter-day precisions, trueness, limits of detection (LOD) and lower limits of quantification (LLOQ). The validation results proved that the methods were suitable to quantify ibrutinib and PCI-45227 in real biological CSF samples from 0.50 (ibrutinib) or 1.00 (PCI-45227) to 30.00 $ng.mL^{-1}$. Furthermore, the developed method was adapted to allow the quantification of both compounds in plasma and the results were compared to those reported in literature. The plasmatic samples were treated by protein precipitation and the method was validated to quantify ibrutinib and PCI-45227 in real biological plasmatic samples from 5.00 to 491 $ng.mL^{-1}$. Lastly, for both matrices, accuracy profiles were plotted from the trueness and precision results using a 20% α-risk (β=80%) and the tolerance intervals were comprised within the acceptance limits fixed at ±25% for the LLOQ and ±15% for the other concentrations. Finally, these methods were successfully applied to quantify ibrutinib and PCI-45227 in real human CSF and plasma samples.

1. Introduction

Tyrosine kinase inhibitors (TKI) is a recent pharmaceutical class which has recently undergone an extraordinary development. Several TKI are currently approved at diagnosis of neoplasms or at relapse as monotherapy or combined with chemotherapy or radiotherapy. These inhibitors block abnormally activated signaling pathways essential for cancer cell proliferation thus leading to apoptosis. Ibrutinib is an orally administered first-in-class irreversible Bruton's tyrosine kinase (BTK) covalent inhibitor. BTK is a signaling molecule of the B-cell receptor (BCR) [1] and is needed for abnormal B-lymphocytes to multiply and survive. By blocking BTK, ibrutinib helps move abnormal B-lymphocytes out of their nourishing environments in the lymph nodes, bone marrow and other organs [2]. Ibrutinib has been approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) since 2013 or 2014 for the treatment of various B-cell malignancies [3, 4] in relapsed/refractory mantle cell lymphoma (MCL) [5], previously untreated with 17p deletion or relapsed/refractory chronic lymphocytic leukemia (CLL) [6,7], relapsed/refractory or unsuitable for immunotherapy Waldenström macroglobulinemia (WM) [8], relapsed/refractory marginal zone lymphoma (MZL) [9] who require systemic therapy.

Central nervous system (CNS) dissemination, except in primary cerebral nervous system lymphoma (PCNSL), is uncommon in B-cell malignancies [10]. It is more frequent at relapse rather than at diagnosis and it is one of the more severe complications because of lack of effective and validated treatment. Efficacy of systemic drugs is limited because of impermeable brain blood-barrier (BBB) [11] and is a cogent area of under-researched application due to CNS dissemination being a common exclusion criterion in clinical studies of ibrutinib. However, many case reports evidenced that ibrutinib crosses the BBB because a complete remission was obtained in various B-cell malignancies [12-17].

The diffusion across the BBB could probably rely on an active influx transport across the BBB or a simple diffusion limited by the high plasma protein binding of ibrutinib. Simultaneous quantification of ibrutinib and its active metabolite, the dihydrodiol-ibrutinib (PCI-45227), in plasma and in CSF would help to establish a pharmacokinetic model in patients with CNS dissemination and could give more information about effects of ibrutinib and its active metabolite on tumoral cells in CSF. Analytical methods for the quantification of ibrutinib and/or PCI-45227 in rat, mouse or human plasma using various extraction procedures (solid-phase extraction, liquid-liquid extraction, protein precipitation) are largely described in literature [18-22]. However, to the best of our knowledge, no method has been specifically developed and validated for quantification of both ibrutinib and PCI-45227 in CSF. Some papers reported quantification of ibrutinib [12, 13, 23, 24] and PCI-45227 [15] in both plasma and CSF but very limited information about the analytical method was provided and the

same method was used for both matrices. Because plasma and CSF are different complex biological matrices and lower concentration are expected in CSF (ibrutinib crosses the BBB with a 1-7% CSF/plasma ratio [12, 13, 15]), a specific method has to be validated for the quantification of ibrutinib and PCI-45227 in CSF.

Authors described here the development and the validation of a sensitive, selective and rapid UHPLC-MS/MS method for the quantification of ibrutinib and PCI-45227 in human CSF. Since simultaneous quantification in CSF and plasma would be envisaged in a further clinical study, authors have also validated another method in plasma and the results obtained for each matrix are displayed in the paper. Attention was given to the sample preparation specific for each matrix. The method was validated according to the bioanalytical method validation guidelines of French Society of Pharmaceutical Science and Technology (SFSTP) [25, 26] and using the accuracy profile approach. Lastly, the applicability of the method to real human CSF samples was studied.

2. Materials and methods

2.1 Chemicals

Ibrutinib (>99.9%, MW: 440.5), dihydrodiol-ibrutinib or PCI-45227 (99.1%, MW: 474.5), [$^2H_5$]-ibrutinib (99.5%, MW: 445.5) and [$^2H_5$]-dihydrodiol-ibrutinib or [$^2H_5$]-PCI-45227 (99.5%, MW: 479.5) were purchased from Alsachim (Strasbourg, France). Methanol and acetonitrile (LC-MS grade) were obtained from Carlo Erba (Val-de-Reuil, France) and formic acid (LC-MS grade) from VWR Chemicals (Fontenay-sous-Bois, France). Ammonium formate (97%) was supplied by Sigma-Aldrich (Saint-Quentin-Fallavier, France). Ultra-pure water was obtained from a Milli-Q purification system (Millipore, Merck, Darmstadt, Germany). Human heparinized plasma was purchased from the Etablissement Français du Sang (EFS) (Rungis, France). One lot of normal human CSF was purchased from Biological Specialty (Colmar, PA, USA). Biological samples of plasma and CSF provided from patients treated in the department of hematology of Lille hospital. The CSF samples provided from 3 patients (A, B and C) and the plasmatic samples provided from 3 other patients (D, E and F). Signed informed consent was obtained from each patient in accordance with the declaration of Helsinki. Biological samples were stored at -20°C for one month maximum before analyzing. The stability of ibrutinib and PCI-45227 in human plasma at -20°C for 6 months was observed in accordance to de Vries et al. [21].

2.2 Instrumentation

The UHPLC system consisted of an Accela pump and a variable loop Accela autosampler (Thermo Fisher Scientific, Waltham, MA, USA). Separations were achieved on a BEH C_{18} column (50 x 2.1 mm;

1.7 µm) connected to a BEH C_{18} Vanguard pre-column (5 x 2.1 mm; 1.7 µm) (Waters, Guyancourt, France) with a mobile phase composed of ammonium formate buffer 5mM pH 3.2 (solvent A) and acetonitrile + 0.1% formic acid (solvent B) using the following gradient elution: 0-3.5 min, linear increase to 5% solvent A and 95% solvent B; 3.5-4.5 min, 5% solvent A and 95% solvent B; 4.5-6.0 min, linear decrease to 95% solvent A and 5% solvent B; 6.0-8.0 min, 95% solvent A and 5% solvent B for re-equilibration of the column. The flow rate was 400 µL.min^{-1} and the oven temperature was 30°C. Ten µL of the samples were injected via the loop injection system and the flush syringe was washed with a strong acidic solution (50% acetonitrile and 50% formic acid, 0.35 M) between injections. The temperature of the autosampler was set at 10°C. The UHPLC system was coupled to a Vantage triple quadripole mass spectrometer controlled by the Xcalibur® software (Thermo Fisher Scientific, Waltham, MA, USA). All experiments were acquired using positive electrospray ionization (ESI+). MS-MS parameters were optimized via the direct infusion of each analyte (5 µg.mL^{-1} in methanol) applying a flow rate of 400 µL.min^{-1} of the mobile phase (95% solvent A and 5% solvent B). Optimal parameters were as follows: capillary voltage, 3 kV; capillary temperature, 324°C; desolvation temperature, 371°C; sheath gas, 40 PSI; auxiliary gas, 55 PSI; sweep gas flow (N_2), 60 L.h^{-1}. Argon was used as the collision gas at a pressure of 1.0 mTor. Optimized S-lens were 140 V for ibrutinib and its internal standard and 120 V for PCI-45227 and its internal standard. Experiments were performed using the multiple reaction monitoring (MRM). Two transitions for each compound were chosen, the first transition was used for quantification and the second one for confirmation. Table 1 summarizes the MRM acquisitions parameters. The eluent from the first two minutes of the run was diverted to the waste (divert valve mode) to protect the mass spectrometer from the early eluting matrix components.

2.3 Solutions

Stock solutions of ibrutinib, PCI-45227, [$^2H_5$]-ibrutinib and [$^2H_5$]-PCI-45227 were individually prepared at 1.00 g.L^{-1} in methanol, and stored at -20°C.

2.3.1 Cerebrospinal fluid

2.3.1.1 Standard solutions

Calibration standards (CS) and validation standards (VS) were prepared by serial dilutions from separated stock solutions on three successive days. Samples were prepared by adding a constant CSF volume chosen according to the preparation of the biological samples. A solution containing both internal standards at 8.00 ng.mL^{-1} was prepared in methanol. A solution containing both ibrutinib and PCI-45227 was prepared at 6.00 ng.mL^{-1} in methanol. Two hundred µL of each CS and VS solutions

were prepared by adding 20 µL of blank CSF, 50 µL of the internal standards solution at 8.00 ng.mL^{-1}, a variable volume of the solution containing both analytes at 6.00 ng.mL^{-1} and a variable volume of methanol to reach 200 µL. Finally, the concentration of both internal standards was 2 ng.mL^{-1} in all solutions. Eight calibration standards were prepared with concentrations of ibrutinib and PCI-45227 at 0.05, 0.10, 0.20, 0.60, 1.20, 1.80, 2.40 or 3.00 ng.mL^{-1}. The validation standards were prepared at four concentration levels: 0.05 (low VS_1), 0.10 (low VS_2), 1.20 (medium VS) and 3.00 (high VS) ng.mL^{-1}. All these solutions were prepared each day (3 days) with four replicates per day for each VS (n=12).

2.3.1.2 Sample preparation

The method consisted in a simple dilution of the CSF matrix. For the CS and VS samples, the solutions (200 µL which contained 20 µL of blank CSF) were evaporated under nitrogen flow at 45°C and the residues were reconstituted in 60 µL of a 70:30 – acetonitrile:ammonium formate buffer mixture. Thus, the initial salts concentration in the CSF was diluted with a dilution factor equal to 3. Then, 10 µL were immediately injected in the chromatographic system. Thus, analyzed concentrations of ibrutinib and PCI-45227 in the CS samples were 0.17, 0.33, 0.67, 2.00, 4.00, 6.00, 8.00 and 10.00 ng.mL^{-1} and analyzed concentrations of ibrutinib and PCI-45227 in the VS samples were 0.17, 0.33, 4.00 and 10.00 ng.mL^{-1}. The concentration of both internal standards was 6.67 ng.mL^{-1} in all samples.

For the biological CSF samples, in order to strictly respect the protocol used for the standard samples, 50 µL of the internal standard solutions at 8.00 ng.mL^{-1} and 130 µL of methanol were added to 20 µL of the biological sample and prepared solutions were evaporated under nitrogen flow at 45°C. Residues were reconstituted in 60 µL of a 70:30 (v/v) – acetonitrile: ammonium formate buffer mixture. Then, 10 µL were immediately injected in the chromatographic system.

2.3.2 Plasma

2.3.2.1 Standard solutions

Calibration standards (CS) and validation standards (VS) samples were prepared by serial dilutions from separated stock solutions on three successive days. All solutions were prepared by dilution in blank plasma. A solution containing both internal standards at 80.0 ng.mL^{-1} was prepared in acetonitrile by dilution of both methanolic stock solutions at 1.00 g.L^{-1}. This solution was stored at -20°C before use. A solution containing both ibrutinib and PCI-45227 in plasma at 5 000 ng.mL^{-1} was used to prepare seven calibration standards. Final concentrations of ibrutinib and PCI-45227 were: 5.0, 50.0, 125.0, 259.5, 345.3, 429.6 and 491.0 ng.mL^{-1}. The VS were prepared at three concentration levels: 5.0 (low VS), 259.5 (medium VS) and 491.0 (high VS) ng.mL^{-1}. All these solutions were

prepared each day (3 days) with four replicates per day for each VS (n=12). Internal standards were added during the following sample preparation step.

2.3.2.2 Sample preparation

Samples were pretreated by protein precipitation. Sixty µL of each earlier prepared CS and VS solutions were mixed with 180 µL of the solution containing the internal standards at 80.0 $ng.mL^{-1}$ and the solutions were vortexed for 20 s. After centrifugation at 13 000 g for 10 minutes at 4°C, supernatants (195 µL) were removed and evaporated under nitrogen flow at 45°C. Residues were reconstituted in 120 µL of a 70:30 (v/v) – acetonitrile: ammonium formate buffer mixture. Then, 10 µL were immediately injected in the chromatographic system. Thus, analyzed concentrations of ibrutinib and PCI-45227 in the CS samples were 2.0, 20.3, 50.8, 105.4, 140.3, 174.5 and 199.5 $ng.mL^{-1}$ and analyzed concentrations of ibrutinib and PCI-45227 in the VS were 2.0, 105.4 and 199.5 $ng.mL^{-1}$. The concentration of both internal standards was 97.5 $ng.mL^{-1}$ in all samples.

For the biological plasmatic samples, in order to strictly respect the protocol used for the standard samples, 60 µL of the biological sample were mixed with 180 µL of a frozen solution containing the internal standards at 80.0 $ng.mL^{-1}$ and the solutions were vortexed for 20 s. After centrifugation at 13 000 g for 10 minutes at 4°C, supernatants (195 µL) were removed and evaporated under nitrogen flow at 45°C. Residues were reconstituted in 120 µL of a 70:30 (v/v) – acetonitrile: ammonium formate buffer mixture. Then, 10 µL were immediately injected in the chromatographic system.

2.4 Validation procedure

The whole method validation procedure was based on SFSTP requirements [25-26]. Selectivity, response function, precision (intra-day and inter-day precision), trueness, limits of detection (LOD) and lower limit of quantification (LLOQ) were studied. The validation was carried out on three consecutive days. Each day, a series of eight (for CSF) or seven (for plasma) calibrations of standards, four (for CSF) or three (for plasma) VS (four replicates each day), a single blank and a double blank were prepared and analyzed. The acceptance criteria for precision and trueness were fixed in accordance with the requirements of the SFSTP which recommends relative standard deviation (RSD) and relative bias under 15% for the VS samples (20% for the LLOQ). Lastly, accuracy profiles were established from the trueness and precision results. Accuracy profiles were plotted using a 20% α-risk (β=80%). Acceptance limits were fixed at ±25% for the LLOQ and ±15% for the other concentrations.

3. Results and discussion

3.1 Method development

3.1.1 MS/MS detection

To optimize the detection parameters, each compound was infused and ionized by positive ESI. The predominantly signals corresponding to the $[M+H]^+$ precursor ions were observed at m/z 441.3 (ibrutinib), 446.3 ($[^2H_5]$-ibrutinib), 475.5 (PCI-45227) and 480.5 ($[^2H_5]$-PCI-45227). After optimization of the MS/MS parameters (collision energy in particular), the most abundant product ions were 138.0 (ibrutinib), 138.0 ($[^2H_5]$-ibrutinib), 304.3 (PCI-45227) and 309.3 ($[^2H_5]$-PCI-45227), and were chosen as quantifier ions. Results were in accordance with the fragmentations described by previous reports [19, 20]. Fragmentation patterns of ibrutinib and PCI-45227 are displayed in figure 1.

3.1.2 Chromatography

The chromatographic conditions were optimized to obtain a sufficient resolution between ibrutinib and PCI-45227 in a short analysis time using a C_{18} stationary phase. The mobile phase composition was selected considering the results displayed in various papers dealing with the analysis of ibrutinib [18, 20]. Acetonitrile was preferred to methanol due to the highest sensitivity, best peak shape and resolution. Aqueous mobile phase was composed of a 5mM ammonium formate buffer with pH adjusted to 3.2. Formic acid 0.1% was added to the mobile phase in order to increase the protonation of analytes and then to enhance the response of analytes [18, 20]. After optimization of the gradient program, retention times of ibrutinib and PCI-45227 were 3.1 and 2.7 minutes, respectively, and the resolution was 3.2. The total run-time was 8 minutes allowing a slight return to the initial mobile phase composition and column equilibration. The optimal chromatogram obtained is displayed in figure 2.

3.1.3 Matrix effect

Preliminary study of the matrix effect was performed for CSF and plasma at two concentration levels (medium and high-VS concentrations) for ibrutinib and PCI-45227. Matrix effects were calculated from the ratio of the peak area ratio in presence of matrix to the peak area ratio in the absence of matrix (neat solvents: methanol for CSF and acetonitrile for plasma). For CSF, large matrix effects were pointed out since they were between -3% and -37%. For plasma, they were between -7% and +18%. These results were sufficient to impose the preparation of all standards samples (CS and VS) using blank matrices (CSF or plasma) and not the neat solvents.

3.1.4 Sample preparation

Taking into account the own characteristics of the CSF and plasma matrices, sample preparations peculiar to each matrix were developed.

The CSF is nearly protein-free compared with plasma but contains high salt concentrations which can result in ionic suppression in LC-MS/MS analysis. Since the expected concentrations in CSF biological samples were largely lower than those expected in plasma, particular attention must be paid to this problem and the sample preparation had to reduce salt concentrations. Protein precipitation was not adapted. In literature, when a sample preparation method is reported for this specific matrix, liquid-liquid extraction (LLE) is commonly described [27-31]. The preparation of CSF samples using dilution is reported in few studies with dilution factors between 4 and 10 [31-33]. The few studies that report ibrutinib quantification in CSF referred to the method described for plasma *i.e*. protein precipitation [19] or no information is given [15]. Authors choose to use dilution since it avoids the low recovery usually reported using LLE and reduces the preparation time. A dilution factor equal to 3 resulted from a compromise between the necessary dilution of salts and the undesired diminution of the analyte concentrations and it fulfilled our goal of being able to quantify ibrutinib and PCI-45227 in biological samples until 0.50 or 1.00ng.mL^{-1}. For the plasma, the removal of proteins prior to injection is necessary to preserve the integrity of the chromatographic system. Numerous studies have reported methods such as solid phase extraction, liquid-liquid extraction or protein precipitation for quantification of ibrutinib in plasma [18-22]. Here, precipitation was chosen since this methodology is simple, fast and provides relatively high recovery.

3.2 Method validation

3.2.1 Selectivity

Method selectivity was studied by analyzing blank CSF and plasma samples (n = 4). Single blanks (spiked with the internal standards) and double blanks (unspiked) were prepared as described in the experimental sections 2.3.1 and 2.3.2. The selectivity of the method was demonstrated by comparing the chromatograms obtained for the blank samples with those for the corresponding standard spiked samples. Two MRM transitions were monitored for each analyte and no interfering peaks were detected at the retention times of the analytes and internal standards. The results suggested that no endogenous component from the CSF and plasma matrix interfered with the measurement of the analytes demonstrating the selectivity of the method.

3.2.2 Response function

Calibration curves were based on the peak area ratio of the analyte to its internal standard. The regression model showing the best results for precision and trueness (for the VS samples) was chosen. For both analytes and both matrices, the best calibration model was obtained for the weighted 1/x quadratic regression. Standard curves exhibited excellent determination coefficient (r^2>0.999) in the range 0.17 ng.mL^{-1} – 10.00 ng.mL^{-1} for analyzed CSF samples and in the range 2.00 – 199.50 ng.mL^{-1} for analyzed plasma samples

3.2.3 Precision and trueness

Precision (intra-day and inter-day precision) and trueness were determined from 4 replicates at 4 (for CSF) or 3 (for plasma) VS concentrations over 3 days (n=12 for each concentration). Imprecisions were expressed as RSD of the ratio of the standard deviation on the theoretical value at each concentration level. Trueness was evaluated by comparing the nominal and back-calculated concentrations and was expressed as % relative bias. Validation parameters are displayed in Tables 2 and 3 for the quantification in CSF and in plasma, respectively. In CSF, the intra-day precision (RSD) ranged from 1.6% to 8.6% for ibrutinib and from 3.4% to 33.3% for PCI-45227. The inter-day precision (RSD) ranged from 2.1% to 19.7% for ibrutinib and from 5.0 % to 40.1% for PCI-45227. The trueness (relative bias) ranged from -14.0% to +4.6% for ibrutinib and from -5.8% to +16.0% for PCI-45227. Thus, for ibrutinib, all validation parameters on precision and trueness were under 20% at the low VS_1 and under 15% for other VS. For PCI-45227, the validation parameters obtained for the low VS_1 exceeded the allowed limits of 20% but were acceptable for the low VS_2, medium and high VS. In plasma, the intra-day precision ranged from 2.0% to 10.6% for ibrutinib and from 2.7% to 8.8% for PCI-45227. The inter-day precision ranged from 2.6% to 10.6% for ibrutinib and from 4.4% to 8.8% for PCI-45227. The trueness ranged from +0.3% to +6.2% for ibrutinib and from -2.8% to +0.2% for PCI-45227. Thus, these validation parameters on precision and trueness obtained in plasma were acceptable. It is worth mentioning that values obtained for the medium and high VS concentrations are, in all cases, lower than those obtained for the low VS concentrations.

3.2.4 Limits of detection and quantification

The limit of detection (LOD) was defined by the concentration for which the signal-to-noise ratio was 3. For ibrutinib and PCI-45227, LOD were estimated to be 0.01 and 0.03 ng.mL^{-1}, respectively. The lower limits of quantification (LLOQ), defined as the lowest concentration of analyte which can be quantified reliably, were set at the lowest VS concentrations with acceptable precision and trueness (RSD and relative bias < 20%). For quantification in CSF, the low VS concentrations were 0.17 ng.mL^{-1} (low VS_1) for ibrutinib and 0.33 ng.mL^{-1} (low VS_2) for PCI-45227. For quantification in plasma, LLOQ of 2.00 ng.mL^{-1} were defined for ibrutinib and PCI-45227. Thus, taking into account the preparation of

the biological samples, the developed method is suitable to quantify ibrutinib and PCI-45227 from 0.50 (ibrutinib) or 1.00 ng.mL^{-1} PCI-45227) in CSF and from 5.0 ng.mL^{-1} in plasma. Indeed, in CSF, for ibrutinib, the LLOQ was 0.17 ng.mL^{-1} in the analyzed sample which corresponds to 0.50 ng.mL^{-1} in the biological sample; for PCI-45227, the LLOQ was 0.33 ng.mL^{-1} in the analyzed sample which corresponds to 1.00 ng.mL^{-1} in the biological sample. In plasma, the LLOQ were 2.00 ng.mL^{-1} for both analytes in the analyzed sample which corresponds to 5.00 ng.mL^{-1} in the biological sample.

3.2.5 Accuracy profiles

The accuracy was based on the total error approach taking into account the precision and trueness of the method. Accuracy profiles are displayed in figure 3. Whatever the analyte (ibrutinib or PCI-45227), the tolerance intervals were comprised within the acceptance limits at the various VS concentrations in the two matrices. Therefore, for CSF, the method was validated on the whole range between 0.17 (ibrutinib) or 0.33 (PCI-45227) and 10.00 ng.mL^{-1}. Taking into account the preparation of the biological CSF samples, the developed method is suitable to quantify ibrutinb from 0.50 to 30 ng.mL^{-1} and PCI-45227 from 1.00 to 30 ng.mL^{-1} in the collected CSF samples. For plasma, the method was validated on the whole range between 2.00 and 199.50 ng.mL^{-1}. Taking into account the preparation of the biological plasmatic samples, the developed method is suitable to quantify both compounds from 5.0 to 491.0 ng.mL^{-1} in the collected plasmatic samples.

3.3 Applicability to biological samples

The suitability of the developed method was studied for the quantification of ibrutinib and PCI-45227 in CSF and plasma samples obtained from patients with various B-cell malignancies and treated with 420 mg per day of ibrutinib. Samples were taken more than 4 hours after administration. Three samples were analyzed for each matrix. Figure 4 presents the chromatograms obtained for double blank, blank spiked at the LLOQ and patients for Ibrutinib and PCI-45227 in both matrices. The concentrations of ibrutinib and PCI-45227 determined in the CSF and plasmatic patients' samples are displayed in Table 4. CSF concentrations ranged between 0.59 and 2.95 ng.mL^{-1} for ibrutinib, and 1.45 and 9.73 ng.mL^{-1} for PCI-45227. Plasma concentrations ranged between 6.61 and 19.44 ng.mL^{-1} for ibrutinib, and 12.00 and 47.89 ng.mL^{-1} for PCI-45227. These results were consistent with the few previously published data for quantification of ibrutinib and PCI-45227 reported in CSF and in plasma with a 1-7% CSF/plasma ratio [12, 13]. Moreover, these results show for the first time that PCI-45227 is found in CSF. Interestingly, as described in literature for plasmatic concentrations of both analytes [34, 35], concentrations are higher for PCI-45227 than for ibrutinib in CSF.

4. Conclusion

We developed a performant UHPLC-MS/MS method for the simultaneous quantification of ibrutinib and PCI-45227, its active metabolite, in CSF and in plasma. Attention was given to the sample preparation which needs to be developed for each matrix: dilution for CSF and protein precipitation for plasma. The method was validated according to the current SFSTP requirements and was successfully applied to quantify ibrutinib and PCI-45227 in human CSF and plasma samples from patients. To the best of our knowledge, this is the first study reporting validated quantification of ibrutinib and PCI-45227 in CSF.

Ibrutinib is a relatively recent drug and additional therapeutic indications are yet in current development. Several isolated clinical observations reported its efficacy in CNS dissemination of various B-cell malignancies, which were excluded in the large cohort studies. This combined method of quantification in CSF and in plasma could support pharmacokinetic studies of ibrutinib and PCI-45227 in the future. A best knowledge of diffusion and metabolism into the CNS could help to predict efficacy of ibrutinib in the CNS dissemination, a rare but serious complication of B-cell malignancies. Simultaneous quantification of ibrutinib and its metabolite PCI-45227 in CSF and in plasma would help establishing a pharmacokinetic model in patients with CNS dissemination and could give more information about effects of ibrutinib and its active metabolite on tumoral cells in CSF.

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Figure captions

Figure 1. Fragmentation patterns of ibrutinib (A) and PCI-45227 (B) with a collision energy of 26 V and 31 V, respectively.

Figure 2. Chromatographic separation of ibrutinib and PCI-45227 : total ion chromatogram (A), extracted ion chromatograms : 441.1→137.9 (B), 475.1→ 304.1. (C) (concentration of analytes: 199.5 $ng.mL^{-1}$ in blank plasma) RT : retention time in minute.

Figure 3. Accuracy profiles of ibrutinib and PCI-45227 in CSF (A) and (B) and in plasma (C) and (D). Accuracy profiles were plotted using a 20% α-risk (β=80%). Acceptance limits were fixed at ± 25% for the LLOQ and ± 15% for the other QC.

Figure 4 : Chromatograms obtained for double blank, blank spiked at the LLOQ and patient for Ibrutinib and PCI-45227 in both matrices. * : artefact at 2 minutes corresponds to the switch of the divert valve

541 Figure 1

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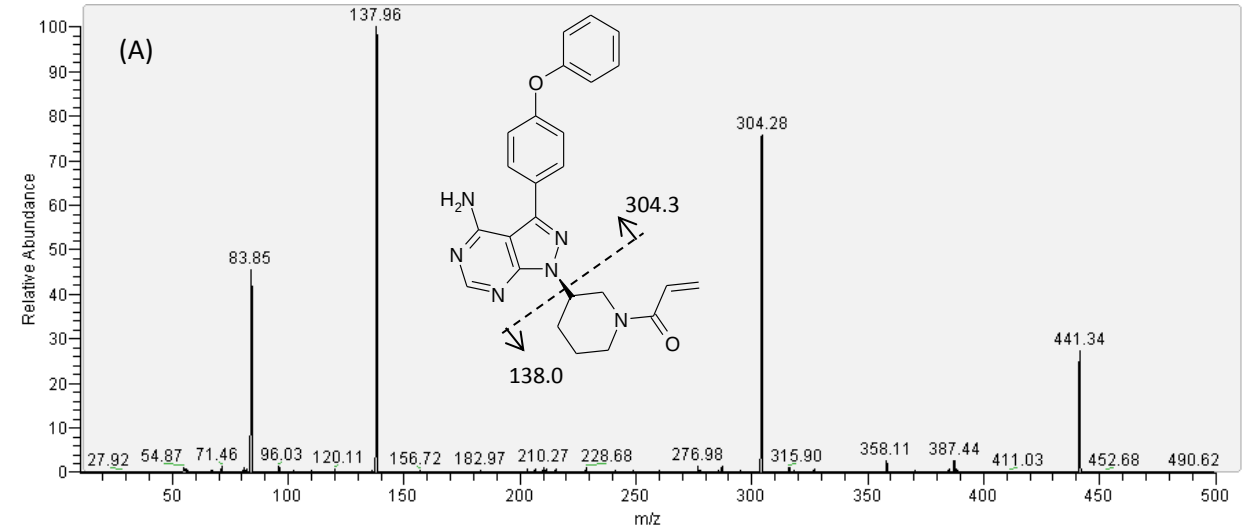


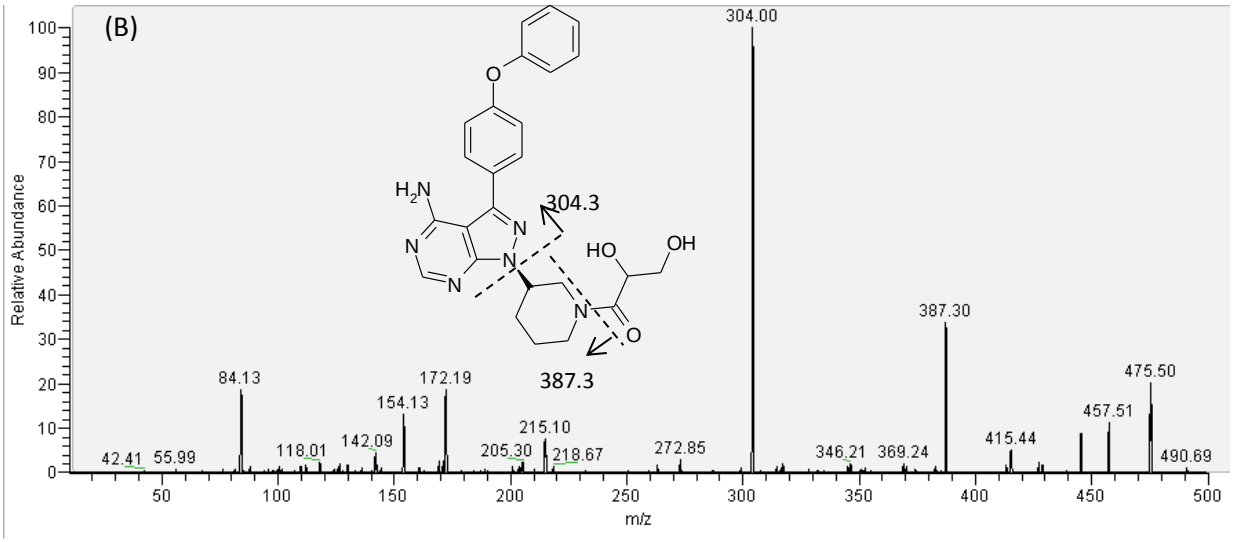
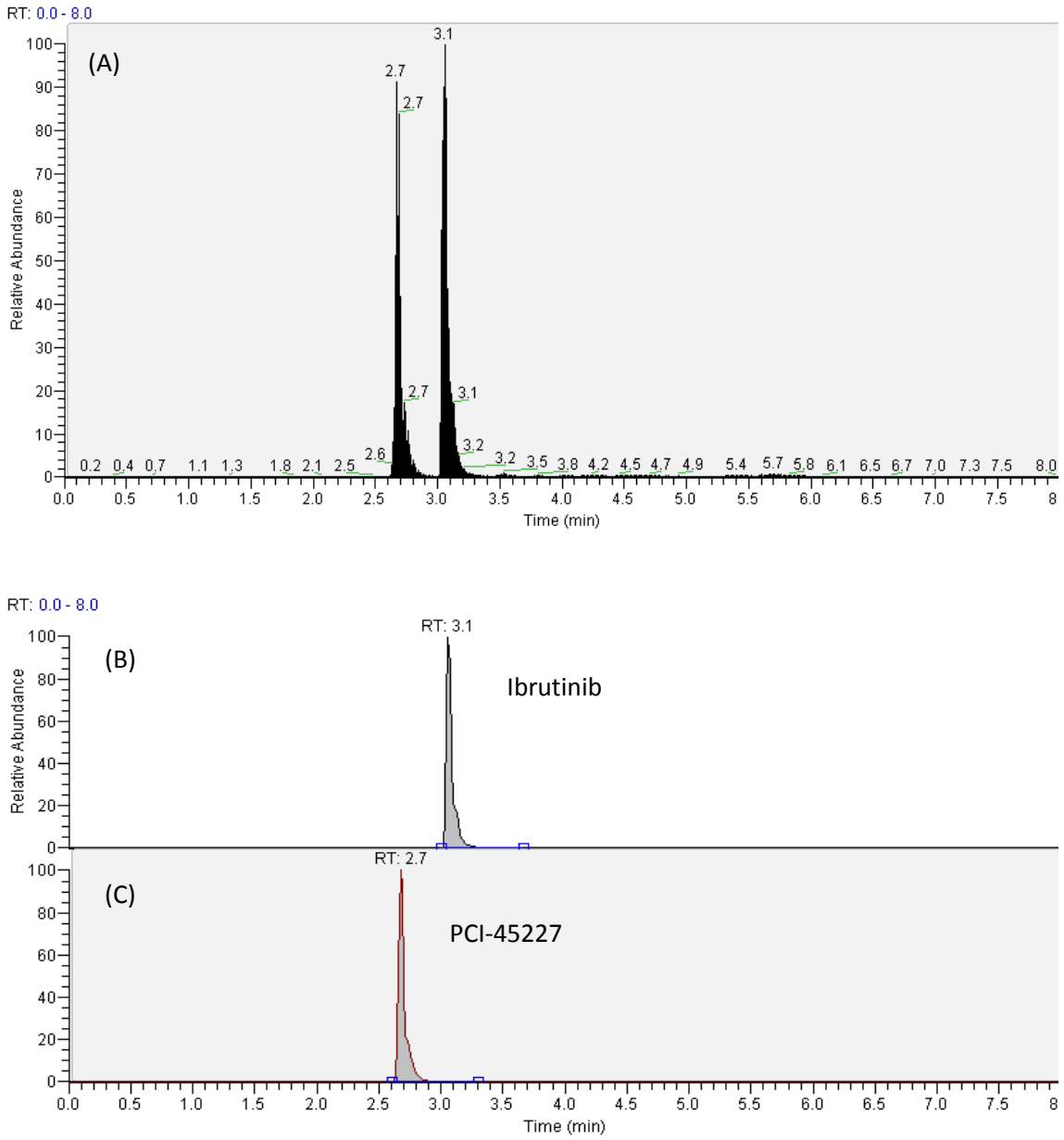


Figure 2


RT: 0.0 - 8.0
(A)
Relative Abundance
Time (min)
RT: 0.0 - 8.0
(B)
RT: 3.1
Ibrutinib
(C)
RT: 2.7
PCI-45227
Relative Abundance
Time (min)

559 Figure 3

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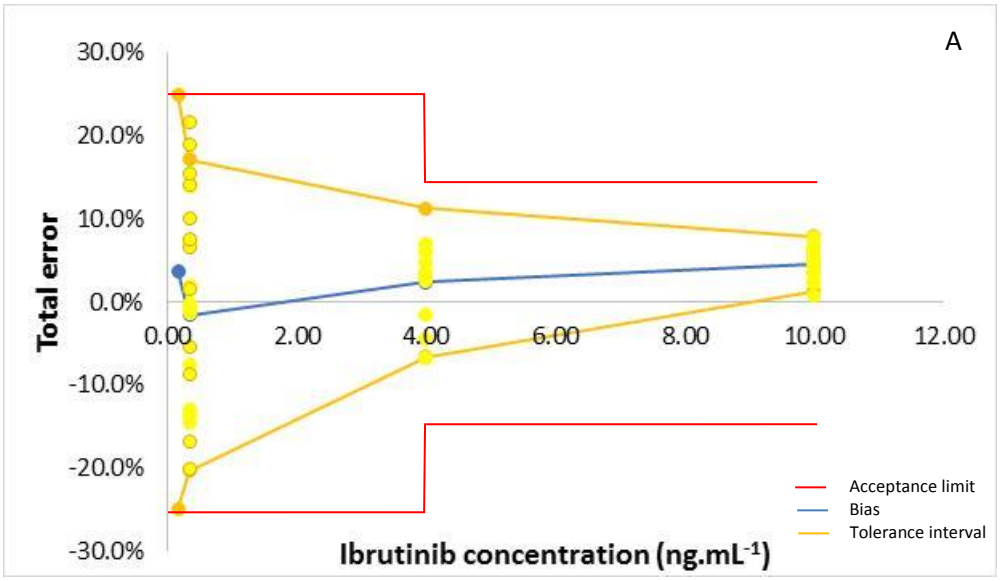

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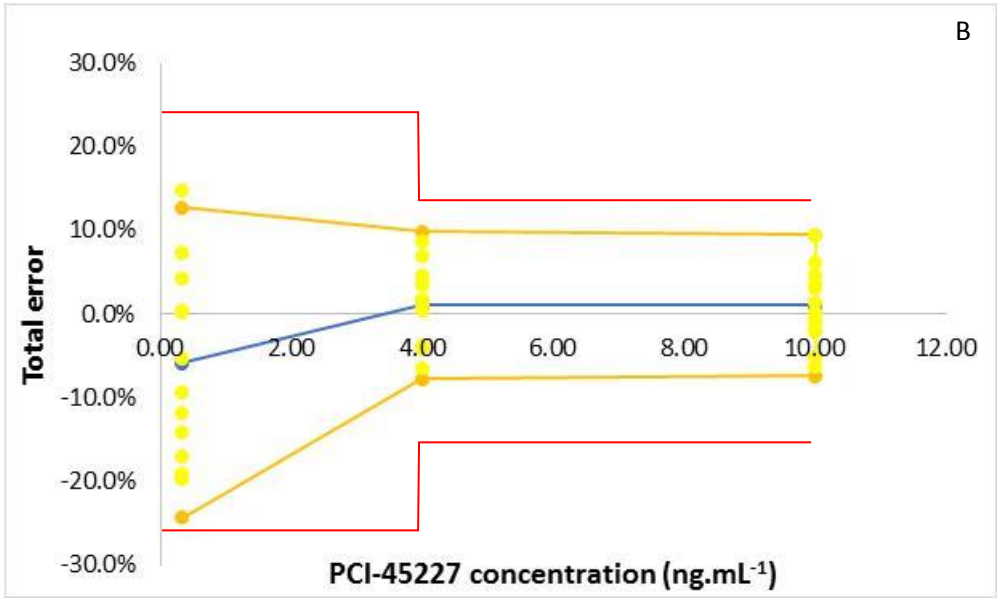

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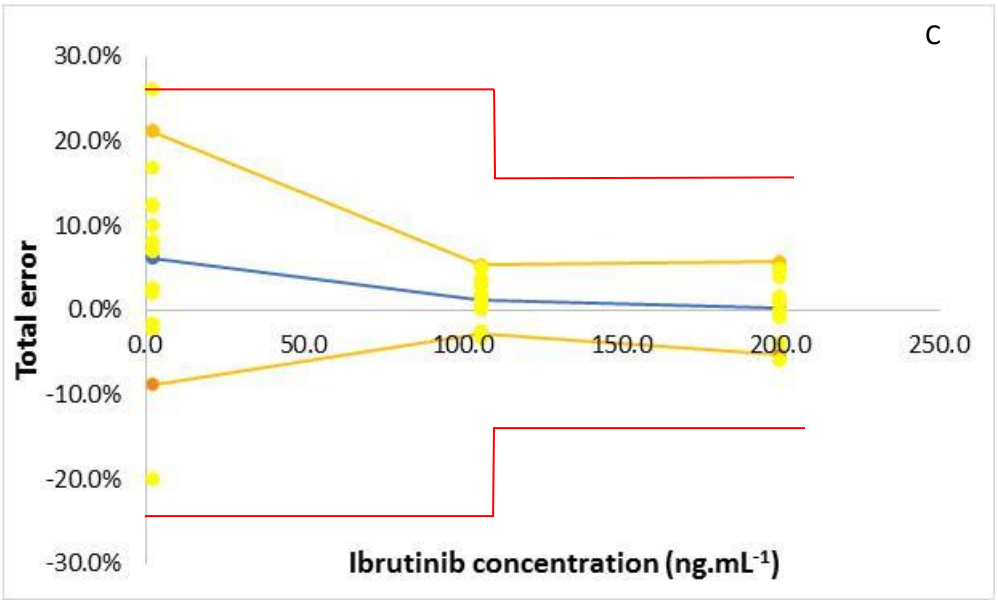

C
30.0%
20.0%
10.0%
0.0%
-10.0%
-20.0%
-30.0%
Total error
0.0
50.0
100.0
150.0
200.0
250.0
Ibrutinib concentration (ng.mL-1)

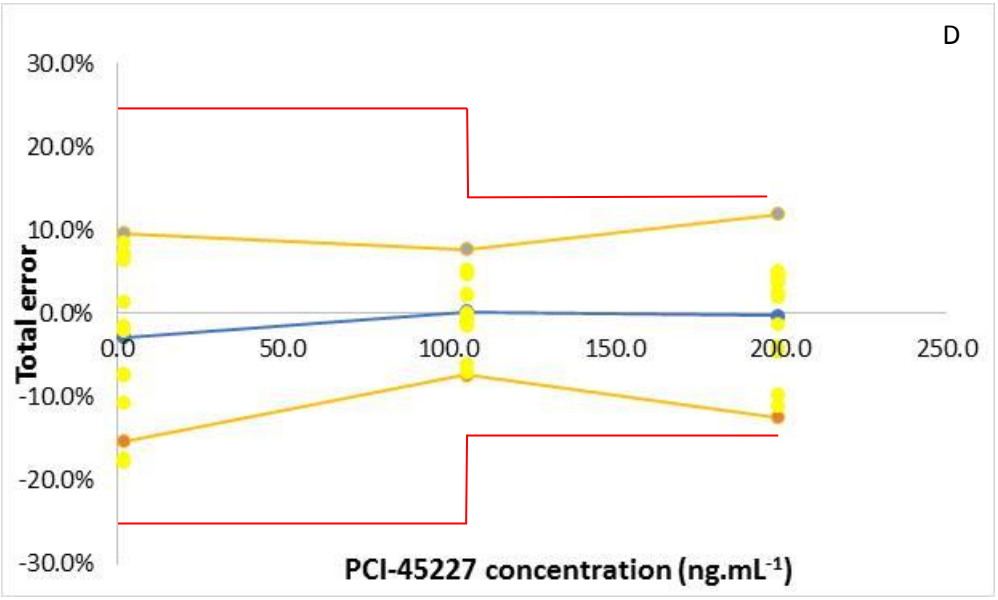
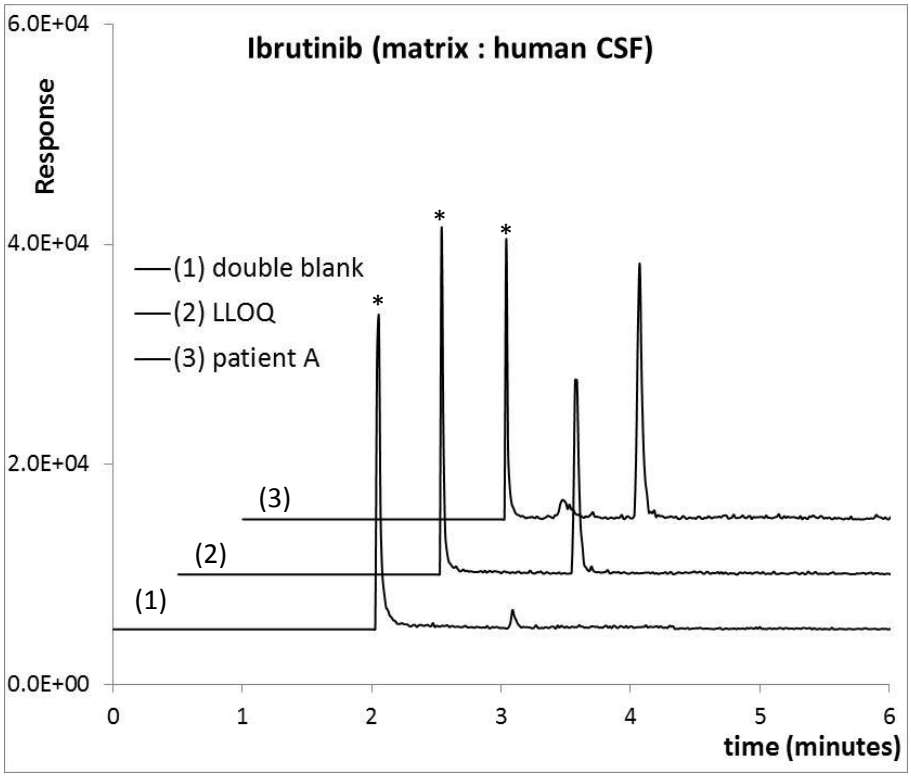
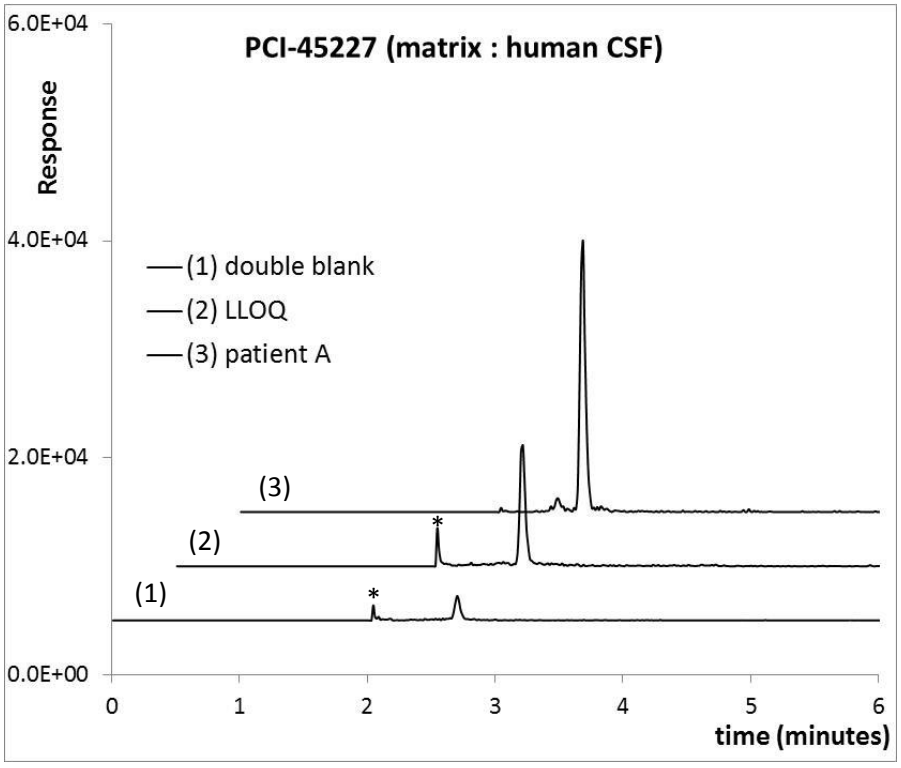
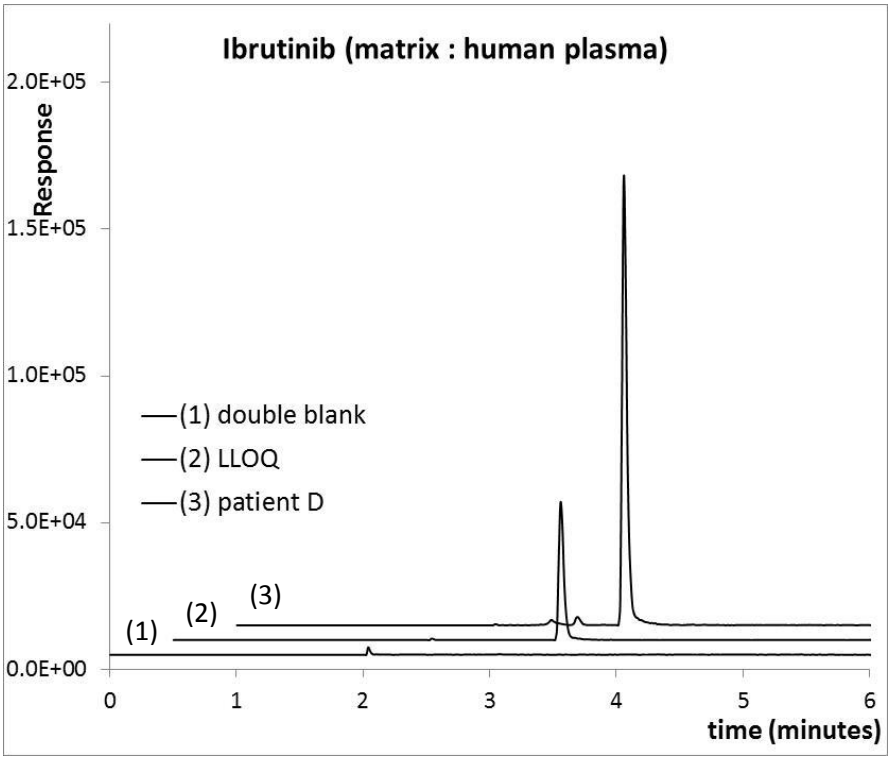
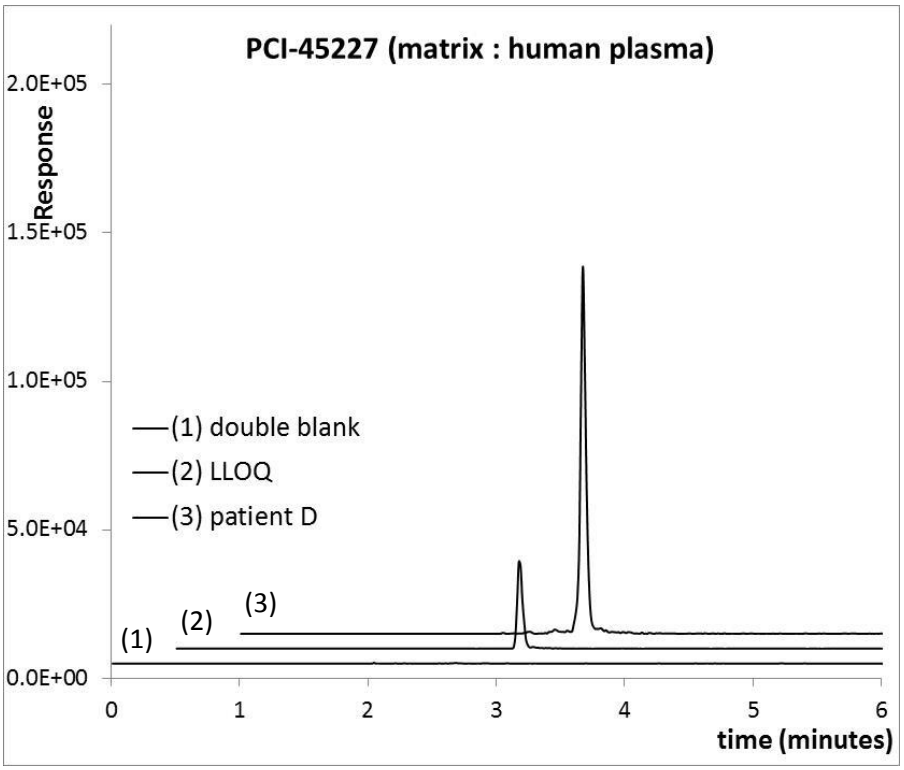

D
30.0%
20.0%
10.0%
0.0%
-10.0%
-20.0%
-30.0%
Total error
0.0
50.0
100.0
150.0
200.0
250.0
PCI-45227 concentration (ng.mL-1)

Figure 4


Ibrutinib (matrix : human CSF)
Response
6.0E+04
4.0E+04
2.0E+04
0.0E+00
(1) double blank
(2) LLOQ
(3) patient A
(1)
(2)
(3)
*
0
1
2
3
4
5
6
time (minutes)


PCI-45227 (matrix : human CSF)
Response
6.0E+04
4.0E+04
2.0E+04
0.0E+00
(1) double blank
(2) LLOQ
(3) patient A
(1)
(2)
(3)
*
0
1
2
3
4
5
6
time (minutes)


Ibrutinib (matrix : human plasma)
Response
2.0E+05
1.5E+05
1.0E+05
5.0E+04
0.0E+00
(1) double blank
(2) LLOQ
(3) patient D
(1)
(2)
(3)
0
1
2
3
4
5
6
time (minutes)


PCI-45227 (matrix : human plasma)
Response
2.0E+05
1.5E+05
1.0E+05
5.0E+04
0.0E+00
(1) double blank
(2) LLOQ
(3) patient D
(1)
(2)
(3)
0
1
2
3
4
5
6
time (minutes)

630 Table 1. Optimized MRM parameters for the determination of ibrutinib and PCI-45227

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Compound	Precursor ion mass (m/z)	Quantification ion		Qualification ion	
		mass (m/z)	$E_{collision}$ (V)	mass (m/z)	$E_{collision}$ (V)
ibrutinib	441.3	138.0	26	304.3	29
[$^2H_5$]-ibrutinib	446.3	138.0	28	309.3	26
PCI-45227	475.5	304.3	31	387.3	24
[$^2H_5$]-PCI-45227	480.5	309.3	32	392.3	23

632 $E_{collision}$: collision energy

633 Table 2. Intra-day precision, inter-day precision and relative bias data of the method for quantification of ibrutinib and PCI- 45227 in CSF at the four VS
634 concentrations (low VS_1, low VS_2, medium VS and high VS ; concentrations expressed are the concentration in the analyzed solutions)

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	Low VS_1 : 0.17 $ng.mL^{-1}$			Low VS_2 : 0.33 $ng.mL^{-1}$			Medium VS : 4.00 $ng.mL^{-1}$			High VS : 10 $ng.mL^{-1}$		
	Intra-day precision (% RSD)	Inter-day precision (% RSD)	Relative bias (%)	Intra-day precision (% RSD)	Inter-day precision (% RSD)	Relative bias (%)	Intra-day precision (% RSD)	Inter-day precision (% RSD)	Relative bias (%)	Intra-day precision (% RSD)	Inter-day precision (% RSD)	Relative bias (%)
ibrutinib	8.6	19.7	-14.0	4.7	10.1	-1.7	2.6	4.8	+2.3	1.6	2.1	+4.6
PCI-45227	33.3	40.1	16.0	9.4	11.9	-5.8	3.4	5.2	+1.1	3.4	5.0	+1.1

Table 3. Intra-day precision, inter-day precision and relative bias data of the method for quantification of ibrutinib and PCI- 45227 in plasma at the three VS concentrations (low VS, medium VS and high VS ; concentrations expressed are the concentration in the analyzed solutions))

	Low VS : 2.0 ng.mL^{-1}			Medium VS: 105.4 ng.mL^{-1}			High VS : 199.5 ng.mL^{-1}		
	Intra-day precision (% RSD)	Inter-day precision (% RSD)	Relative bias (%)	Intra-day precision (% RSD)	Inter-day precision (% RSD)	Relative bias (%)	Intra-day precision (% RSD)	Inter-day precision (% RSD)	Relative bias (%)
ibrutinib	10.6	10.6	+6.2	2.0	2.6	+1.3	2.1	3.2	+0.3
PCI-45227	8.8	8.8	-2.8	2.8	4.4	+0.2	2.7	6.6	-0.2

644 Table 4. Concentrations of ibrutinib and PCI-45227 in human CSF and plasmatic samples

645

	[ibrutinib] $ng.mL^{-1}$	[PCI-45227] $ng.mL^{-1}$
CSF samples		
Patient A	0.59	1.45
Patient B	0.85	5.06
Patient C	2.95	9.73
Plasmatic samples		
Patient D	19.44	20.90
Patient E	7.27	47.89
Patient F	6.61	12.00

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