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1 Development and validation of an UHPLC-MS/MS method for simultaneous quantification of
2 ibrutinib and its dihydrodiol-metabolite in human cerebrospinal fluid.

3 D. Beauvais^{1,2}, J-F. Goossens¹, E. Boyle², B. Allal³, T. Lafont³, E. Chatelut³, C. Herbaux², F.
4 Morschhauser^{1,2}, S. Genay^{1,4}, P. Odou^{1,4}, C. Danel^{1*}

5

6 ¹Univ. Lille, EA 7365 – GRITA – Groupe de Recherche sur les formes Injectables et les Technologies
7 Associées, F-59000, Lille, France.

8 ²CHU Lille, Department of hematology, F-59000, Lille, France. ³Institut Claudius-Regaud, IUCT-
9 Oncopole, CRCT, Université de Toulouse, Inserm, Toulouse, France.

10 ⁴CHU Lille, Institut de Pharmacie, F-59000, Lille, France.

11

12 Corresponding author :

13 Dr Cécile Danel

14 Faculté de pharmacie de Lille – EA 7365 – GRITA

15 3 rue du Pr Laguesse – 59006 Lille – France

16 Cecile.danel@univ-lille2.fr - +33 3 62 28 30 26

17 Keywords:

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

19 Highlights:

- 20
- 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
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25

26 Abstract:

27 Ibrutinib is an orally administered first-in-class irreversible Bruton's tyrosine kinase (BTK) covalent
28 inhibitor for the treatment of patients with B-cell malignancies. Several isolated clinical observations
29 reported its efficacy in central nervous system dissemination. Herein, we described the development
30 and validation of an ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-
31 MS/MS) procedure for the quantification of ibrutinib and its active metabolite PCI-45227 in
32 cerebrospinal fluid (CSF). This is the first complete validated method for quantification of ibrutinib
33 and PCI-45227 in CSF. The compounds were eluted on a Waters BEH C18 column (50.0 x 2.1 mm ; 1.7
34 μm) using a gradient elution with a mobile phase composed of ammonium formate buffer 5 mM pH
35 3.2 and acetonitrile + 0.1% formic acid with a flow rate of $400 \mu\text{L}\cdot\text{min}^{-1}$. Two deuterated internal
36 standards were used to obtain the most accurate quantification. The CSF samples were prepared by
37 a simple and rapid dilution. The method was validated by testing the selectivity, response function,
38 intra-day and inter-day precisions, trueness, limits of detection (LOD) and lower limits of
39 quantification (LLOQ). The validation results proved that the methods were suitable to quantify
40 ibrutinib and PCI-45227 in real biological CSF samples from 0.50 (ibrutinib) or 1.00 (PCI-45227) to
41 $30.00 \text{ ng}\cdot\text{mL}^{-1}$. Furthermore, the developed method was adapted to allow the quantification of both
42 compounds in plasma and the results were compared to those reported in literature. The plasmatic
43 samples were treated by protein precipitation and the method was validated to quantify ibrutinib
44 and PCI-45227 in real biological plasmatic samples from 5.00 to $491 \text{ ng}\cdot\text{mL}^{-1}$. Lastly, for both matrices,
45 accuracy profiles were plotted from the trueness and precision results using a 20% α -risk ($\beta=80\%$)
46 and the tolerance intervals were comprised within the acceptance limits fixed at $\pm 25\%$ for the LLOQ
47 and $\pm 15\%$ for the other concentrations. Finally, these methods were successfully applied to quantify
48 ibrutinib and PCI-45227 in real human CSF and plasma samples.

49

50 1. Introduction

51 Tyrosine kinase inhibitors (TKI) is a recent pharmaceutical class which has recently undergone an
52 extraordinary development. Several TKI are currently approved at diagnosis of neoplasms or at
53 relapse as monotherapy or combined with chemotherapy or radiotherapy. These inhibitors block
54 abnormally activated signaling pathways essential for cancer cell proliferation thus leading to
55 apoptosis. Ibrutinib is an orally administered first-in-class irreversible Bruton's tyrosine kinase (BTK)
56 covalent inhibitor. BTK is a signaling molecule of the B-cell receptor (BCR) [1] and is needed for
57 abnormal B-lymphocytes to multiply and survive. By blocking BTK, ibrutinib helps move abnormal B-
58 lymphocytes out of their nourishing environments in the lymph nodes, bone marrow and other
59 organs [2]. Ibrutinib has been approved by the Food and Drug Administration (FDA) and the
60 European Medicines Agency (EMA) since 2013 or 2014 for the treatment of various B-cell
61 malignancies [3, 4] in relapsed/refractory mantle cell lymphoma (MCL) [5], previously untreated with
62 17p deletion or relapsed/refractory chronic lymphocytic leukemia (CLL) [6,7], relapsed/refractory or
63 unsuitable for immunotherapy Waldenström macroglobulinemia (WM) [8], relapsed/refractory
64 marginal zone lymphoma (MZL) [9] who require systemic therapy.

65 Central nervous system (CNS) dissemination, except in primary cerebral nervous system lymphoma
66 (PCNSL), is uncommon in B-cell malignancies [10]. It is more frequent at relapse rather than at
67 diagnosis and it is one of the more severe complications because of lack of effective and validated
68 treatment. Efficacy of systemic drugs is limited because of impermeable brain blood-barrier (BBB)
69 [11] and is a cogent area of under-researched application due to CNS dissemination being a common
70 exclusion criterion in clinical studies of ibrutinib. However, many case reports evidenced that
71 ibrutinib crosses the BBB because a complete remission was obtained in various B-cell malignancies
72 [12-17].

73 The diffusion across the BBB could probably rely on an active influx transport across the BBB or a
74 simple diffusion limited by the high plasma protein binding of ibrutinib. Simultaneous quantification
75 of ibrutinib and its active metabolite, the dihydrodiol-ibrutinib (PCI-45227), in plasma and in CSF
76 would help to establish a pharmacokinetic model in patients with CNS dissemination and could give
77 more information about effects of ibrutinib and its active metabolite on tumoral cells in CSF.
78 Analytical methods for the quantification of ibrutinib and/or PCI-45227 in rat, mouse or human
79 plasma using various extraction procedures (solid-phase extraction, liquid-liquid extraction, protein
80 precipitation) are largely described in literature [18-22]. However, to the best of our knowledge, no
81 method has been specifically developed and validated for quantification of both ibrutinib and PCI-
82 45227 in CSF. Some papers reported quantification of ibrutinib [12, 13, 23, 24] and PCI-45227 [15] in
83 both plasma and CSF but very limited information about the analytical method was provided and the

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

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

96 2. Materials and methods

97 2.1 Chemicals

98 Ibrutinib (>99.9%, MW: 440.5), dihydrodiol-ibrutinib or PCI-45227 (99.1%, MW: 474.5), [²H₅]-ibrutinib
99 (99.5%, MW: 445.5) and [²H₅]-dihydrodiol-ibrutinib or [²H₅]-PCI-45227 (99.5%, MW: 479.5) were
100 purchased from Alsachim (Strasbourg, France). Methanol and acetonitrile (LC-MS grade) were
101 obtained from Carlo Erba (Val-de-Reuil, France) and formic acid (LC-MS grade) from VWR Chemicals
102 (Fontenay-sous-Bois, France). Ammonium formate (97%) was supplied by Sigma-Aldrich (Saint-
103 Quentin-Fallavier, France). Ultra-pure water was obtained from a Milli-Q purification system
104 (Millipore, Merck, Darmstadt, Germany). Human heparinized plasma was purchased from the
105 Etablissement Français du Sang (EFS) (Rungis, France). One lot of normal human CSF was purchased
106 from Biological Specialty (Colmar, PA, USA). Biological samples of plasma and CSF provided from
107 patients treated in the department of hematology of Lille hospital. The CSF samples provided from 3
108 patients (A, B and C) and the plasmatic samples provided from 3 other patients (D, E and F). Signed
109 informed consent was obtained from each patient in accordance with the declaration of Helsinki.
110 Biological samples were stored at -20°C for one month maximum before analyzing. The stability of
111 ibrutinib and PCI-45227 in human plasma at -20°C for 6 months was observed in accordance to de
112 Vries et al. [21].

113 2.2 Instrumentation

114 The UHPLC system consisted of an Accela pump and a variable loop Accela autosampler (Thermo
115 Fisher Scientific, Waltham, MA, USA). Separations were achieved on a BEH C₁₈ column (50 x 2.1 mm;

116 1.7 μm) connected to a BEH C_{18} Vanguard pre-column (5 x 2.1 mm; 1.7 μm) (Waters, Guyancourt,
117 France) with a mobile phase composed of ammonium formate buffer 5mM pH 3.2 (solvent A) and
118 acetonitrile + 0.1% formic acid (solvent B) using the following gradient elution: 0-3.5 min, linear
119 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
120 min, linear decrease to 95% solvent A and 5% solvent B; 6.0-8.0 min, 95% solvent A and 5% solvent B
121 for re-equilibration of the column. The flow rate was 400 $\mu\text{L}\cdot\text{min}^{-1}$ and the oven temperature was
122 30°C. Ten μL of the samples were injected via the loop injection system and the flush syringe was
123 washed with a strong acidic solution (50% acetonitrile and 50% formic acid, 0.35 M) between
124 injections. The temperature of the autosampler was set at 10°C. The UHPLC system was coupled to a
125 Vantage triple quadrupole mass spectrometer controlled by the Xcalibur® software (Thermo Fisher
126 Scientific, Waltham, MA, USA). All experiments were acquired using positive electrospray ionization
127 (ESI+). MS-MS parameters were optimized via the direct infusion of each analyte (5 $\mu\text{g}\cdot\text{mL}^{-1}$ in
128 methanol) applying a flow rate of 400 $\mu\text{L}\cdot\text{min}^{-1}$ of the mobile phase (95% solvent A and 5% solvent B).
129 Optimal parameters were as follows: capillary voltage, 3 kV; capillary temperature, 324°C;
130 desolvation temperature, 371°C; sheath gas, 40 PSI; auxiliary gas, 55 PSI; sweep gas flow (N_2), 60 $\text{L}\cdot\text{h}^{-1}$.
131 Argon was used as the collision gas at a pressure of 1.0 mTor. Optimized S-lens were 140 V for
132 ibrutinib and its internal standard and 120 V for PCI-45227 and its internal standard. Experiments
133 were performed using the multiple reaction monitoring (MRM). Two transitions for each compound
134 were chosen, the first transition was used for quantification and the second one for confirmation.
135 Table 1 summarizes the MRM acquisitions parameters. The eluent from the first two minutes of the
136 run was diverted to the waste (divert valve mode) to protect the mass spectrometer from the early
137 eluting matrix components.

138 2.3 Solutions

139 Stock solutions of ibrutinib, PCI-45227, [$^2\text{H}_5$]-ibrutinib and [$^2\text{H}_5$]-PCI-45227 were individually prepared
140 at 1.00 $\text{g}\cdot\text{L}^{-1}$ in methanol, and stored at -20°C.

141 2.3.1 Cerebrospinal fluid

142 2.3.1.1 Standard solutions

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

148 were prepared by adding 20 μL of blank CSF, 50 μL of the internal standards solution at 8.00 $\text{ng}\cdot\text{mL}^{-1}$,
149 a variable volume of the solution containing both analytes at 6.00 $\text{ng}\cdot\text{mL}^{-1}$ and a variable volume of
150 methanol to reach 200 μL . Finally, the concentration of both internal standards was 2 $\text{ng}\cdot\text{mL}^{-1}$ in all
151 solutions. Eight calibration standards were prepared with concentrations of ibrutinib and PCI-45227
152 at 0.05, 0.10, 0.20, 0.60, 1.20, 1.80, 2.40 or 3.00 $\text{ng}\cdot\text{mL}^{-1}$. The validation standards were prepared at
153 four concentration levels: 0.05 (low VS₁), 0.10 (low VS₂), 1.20 (medium VS) and 3.00 (high VS) $\text{ng}\cdot\text{mL}^{-1}$.
154 All these solutions were prepared each day (3 days) with four replicates per day for each VS (n=12).

155 2.3.1.2 Sample preparation

156 The method consisted in a simple dilution of the CSF matrix. For the CS and VS samples, the solutions
157 (200 μL which contained 20 μL of blank CSF) were evaporated under nitrogen flow at 45°C and the
158 residues were reconstituted in 60 μL of a 70:30 – acetonitrile:ammonium formate buffer mixture.
159 Thus, the initial salts concentration in the CSF was diluted with a dilution factor equal to 3. Then, 10
160 μL were immediately injected in the chromatographic system. Thus, analyzed concentrations of
161 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
162 $\text{ng}\cdot\text{mL}^{-1}$ and analyzed concentrations of ibrutinib and PCI-45227 in the VS samples were 0.17, 0.33,
163 4.00 and 10.00 $\text{ng}\cdot\text{mL}^{-1}$. The concentration of both internal standards was 6.67 $\text{ng}\cdot\text{mL}^{-1}$ in all samples.

164 For the biological CSF samples, in order to strictly respect the protocol used for the standard
165 samples, 50 μL of the internal standard solutions at 8.00 $\text{ng}\cdot\text{mL}^{-1}$ and 130 μL of methanol were added
166 to 20 μL of the biological sample and prepared solutions were evaporated under nitrogen flow at
167 45°C. Residues were reconstituted in 60 μL of a 70:30 (v/v) – acetonitrile: ammonium formate buffer
168 mixture. Then, 10 μL were immediately injected in the chromatographic system.

169 2.3.2 Plasma

170 2.3.2.1 Standard solutions

171 Calibration standards (CS) and validation standards (VS) samples were prepared by serial dilutions
172 from separated stock solutions on three successive days. All solutions were prepared by dilution in
173 blank plasma. A solution containing both internal standards at 80.0 $\text{ng}\cdot\text{mL}^{-1}$ was prepared in
174 acetonitrile by dilution of both methanolic stock solutions at 1.00 $\text{g}\cdot\text{L}^{-1}$. This solution was stored at -
175 20°C before use. A solution containing both ibrutinib and PCI-45227 in plasma at 5 000 $\text{ng}\cdot\text{mL}^{-1}$ was
176 used to prepare seven calibration standards. Final concentrations of ibrutinib and PCI-45227 were:
177 5.0, 50.0, 125.0, 259.5, 345.3, 429.6 and 491.0 $\text{ng}\cdot\text{mL}^{-1}$. The VS were prepared at three concentration
178 levels: 5.0 (low VS), 259.5 (medium VS) and 491.0 (high VS) $\text{ng}\cdot\text{mL}^{-1}$. All these solutions were

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

181 2.3.2.2 Sample preparation

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

191 For the biological plasmatic samples, in order to strictly respect the protocol used for the standard
192 samples, 60 μL of the biological sample were mixed with 180 μL of a frozen solution containing the
193 internal standards at $80.0 \text{ ng}\cdot\text{mL}^{-1}$ and the solutions were vortexed for 20 s. After centrifugation at
194 13 000 g for 10 minutes at 4°C , supernatants (195 μL) were removed and evaporated under nitrogen
195 flow at 45°C . Residues were reconstituted in 120 μL of a 70:30 (v/v) – acetonitrile: ammonium
196 formate buffer mixture. Then, 10 μL were immediately injected in the chromatographic system.

197 2.4 Validation procedure

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

208

209 3. Results and discussion

210 3.1 Method development

211 3.1.1 MS/MS detection

212 To optimize the detection parameters, each compound was infused and ionized by positive ESI. The
213 predominantly signals corresponding to the $[M+H]^+$ precursor ions were observed at m/z 441.3
214 (ibrutinib), 446.3 ($[^2H_5]$ -ibrutinib), 475.5 (PCI-45227) and 480.5 ($[^2H_5]$ -PCI-45227). After optimization
215 of the MS/MS parameters (collision energy in particular), the most abundant product ions were
216 138.0 (ibrutinib), 138.0 ($[^2H_5]$ -ibrutinib), 304.3 (PCI-45227) and 309.3 ($[^2H_5]$ -PCI-45227), and were
217 chosen as quantifier ions. Results were in accordance with the fragmentations described by previous
218 reports [19, 20]. Fragmentation patterns of ibrutinib and PCI-45227 are displayed in figure 1.

219 3.1.2 Chromatography

220 The chromatographic conditions were optimized to obtain a sufficient resolution between ibrutinib
221 and PCI-45227 in a short analysis time using a C_{18} stationary phase. The mobile phase composition
222 was selected considering the results displayed in various papers dealing with the analysis of ibrutinib
223 [18, 20]. Acetonitrile was preferred to methanol due to the highest sensitivity, best peak shape and
224 resolution. Aqueous mobile phase was composed of a 5mM ammonium formate buffer with pH
225 adjusted to 3.2. Formic acid 0.1% was added to the mobile phase in order to increase the
226 protonation of analytes and then to enhance the response of analytes [18, 20]. After optimization of
227 the gradient program, retention times of ibrutinib and PCI-45227 were 3.1 and 2.7 minutes,
228 respectively, and the resolution was 3.2. The total run-time was 8 minutes allowing a slight return to
229 the initial mobile phase composition and column equilibration. The optimal chromatogram obtained
230 is displayed in figure 2.

231 3.1.3 Matrix effect

232 Preliminary study of the matrix effect was performed for CSF and plasma at two concentration levels
233 (medium and high-VS concentrations) for ibrutinib and PCI-45227. Matrix effects were calculated
234 from the ratio of the peak area ratio in presence of matrix to the peak area ratio in the absence of
235 matrix (neat solvents: methanol for CSF and acetonitrile for plasma). For CSF, large matrix effects
236 were pointed out since they were between -3% and -37%. For plasma, they were between -7% and
237 +18%. These results were sufficient to impose the preparation of all standards samples (CS and VS)
238 using blank matrices (CSF or plasma) and not the neat solvents.

239 3.1.4 Sample preparation

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

242 The CSF is nearly protein-free compared with plasma but contains high salt concentrations which can
243 result in ionic suppression in LC-MS/MS analysis. Since the expected concentrations in CSF biological
244 samples were largely lower than those expected in plasma, particular attention must be paid to this
245 problem and the sample preparation had to reduce salt concentrations. Protein precipitation was not
246 adapted. In literature, when a sample preparation method is reported for this specific matrix, liquid-
247 liquid extraction (LLE) is commonly described [27-31]. The preparation of CSF samples using dilution
248 is reported in few studies with dilution factors between 4 and 10 [31-33]. The few studies that report
249 ibrutinib quantification in CSF referred to the method described for plasma *i.e.* protein precipitation
250 [19] or no information is given [15]. Authors choose to use dilution since it avoids the low recovery
251 usually reported using LLE and reduces the preparation time. A dilution factor equal to 3 resulted
252 from a compromise between the necessary dilution of salts and the undesired diminution of the
253 analyte concentrations and it fulfilled our goal of being able to quantify ibrutinib and PCI-45227 in
254 biological samples until 0.50 or 1.00ng.mL⁻¹. For the plasma, the removal of proteins prior to injection
255 is necessary to preserve the integrity of the chromatographic system. Numerous studies have
256 reported methods such as solid phase extraction, liquid-liquid extraction or protein precipitation for
257 quantification of ibrutinib in plasma [18-22]. Here, precipitation was chosen since this methodology
258 is simple, fast and provides relatively high recovery.

259

260 3.2 Method validation

261 3.2.1 Selectivity

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

270 3.2.2 Response function

271 Calibration curves were based on the peak area ratio of the analyte to its internal standard. The
272 regression model showing the best results for precision and trueness (for the VS samples) was
273 chosen. For both analytes and both matrices, the best calibration model was obtained for the
274 weighted 1/x quadratic regression. Standard curves exhibited excellent determination coefficient
275 ($r^2 > 0.999$) in the range $0.17 \text{ ng.mL}^{-1} - 10.00 \text{ ng.mL}^{-1}$ for analyzed CSF samples and in the range $2.00 -$
276 $199.50 \text{ ng.mL}^{-1}$ for analyzed plasma samples

277 3.2.3 Precision and trueness

278 Precision (intra-day and inter-day precision) and trueness were determined from 4 replicates at 4 (for
279 CSF) or 3 (for plasma) VS concentrations over 3 days ($n=12$ for each concentration). Imprecisions
280 were expressed as RSD of the ratio of the standard deviation on the theoretical value at each
281 concentration level. Trueness was evaluated by comparing the nominal and back-calculated
282 concentrations and was expressed as % relative bias. Validation parameters are displayed in Tables 2
283 and 3 for the quantification in CSF and in plasma, respectively. In CSF, the intra-day precision (RSD)
284 ranged from 1.6% to 8.6% for ibrutinib and from 3.4% to 33.3% for PCI-45227. The inter-day
285 precision (RSD) ranged from 2.1% to 19.7% for ibrutinib and from 5.0 % to 40.1% for PCI-45227. The
286 trueness (relative bias) ranged from -14.0% to +4.6% for ibrutinib and from -5.8% to +16.0% for PCI-
287 45227. Thus, for ibrutinib, all validation parameters on precision and trueness were under 20% at the
288 low VS₁ and under 15% for other VS. For PCI-45227, the validation parameters obtained for the low
289 VS₁ exceeded the allowed limits of 20% but were acceptable for the low VS₂, medium and high VS. In
290 plasma, the intra-day precision ranged from 2.0% to 10.6% for ibrutinib and from 2.7% to 8.8% for
291 PCI-45227. The inter-day precision ranged from 2.6% to 10.6% for ibrutinib and from 4.4% to 8.8%
292 for PCI-45227. The trueness ranged from +0.3% to +6.2% for ibrutinib and from -2.8% to +0.2% for
293 PCI-45227. Thus, these validation parameters on precision and trueness obtained in plasma were
294 acceptable. It is worth mentioning that values obtained for the medium and high VS concentrations
295 are, in all cases, lower than those obtained for the low VS concentrations.

296 3.2.4 Limits of detection and quantification

297 The limit of detection (LOD) was defined by the concentration for which the signal-to-noise ratio was
298 3. For ibrutinib and PCI-45227, LOD were estimated to be 0.01 and 0.03 ng.mL^{-1} , respectively. The
299 lower limits of quantification (LLOQ), defined as the lowest concentration of analyte which can be
300 quantified reliably, were set at the lowest VS concentrations with acceptable precision and trueness
301 (RSD and relative bias < 20%). For quantification in CSF, the low VS concentrations were 0.17 ng.mL^{-1}
302 (low VS₁) for ibrutinib and 0.33 ng.mL^{-1} (low VS₂) for PCI-45227. For quantification in plasma, LLOQ of
303 2.00 ng.mL^{-1} were defined for ibrutinib and PCI-45227. Thus, taking into account the preparation of

304 the biological samples, the developed method is suitable to quantify ibrutinib and PCI-45227 from
305 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
306 ibrutinib, the LLOQ was 0.17 ng.mL⁻¹ in the analyzed sample which corresponds to 0.50 ng.mL⁻¹ in the
307 biological sample; for PCI-45227, the LLOQ was 0.33 ng.mL⁻¹ in the analyzed sample which
308 corresponds to 1.00 ng.mL⁻¹ in the biological sample. In plasma, the LLOQ were 2.00 ng.mL⁻¹ for both
309 analytes in the analyzed sample which corresponds to 5.00 ng.mL⁻¹ in the biological sample.

310

311 3.2.5 Accuracy profiles

312 The accuracy was based on the total error approach taking into account the precision and trueness of
313 the method. Accuracy profiles are displayed in figure 3. Whatever the analyte (ibrutinib or PCI-
314 45227), the tolerance intervals were comprised within the acceptance limits at the various VS
315 concentrations in the two matrices. Therefore, for CSF, the method was validated on the whole range
316 between 0.17 (ibrutinib) or 0.33 (PCI-45227) and 10.00 ng.mL⁻¹. Taking into account the preparation
317 of the biological CSF samples, the developed method is suitable to quantify ibrutinib from 0.50 to 30
318 ng.mL⁻¹ and PCI-45227 from 1.00 to 30 ng.mL⁻¹ in the collected CSF samples. For plasma, the method
319 was validated on the whole range between 2.00 and 199.50 ng.mL⁻¹. Taking into account the
320 preparation of the biological plasmatic samples, the developed method is suitable to quantify both
321 compounds from 5.0 to 491.0 ng.mL⁻¹ in the collected plasmatic samples.

322 3.3 Applicability to biological samples

323 The suitability of the developed method was studied for the quantification of ibrutinib and PCI-45227
324 in CSF and plasma samples obtained from patients with various B-cell malignancies and treated with
325 420 mg per day of ibrutinib. Samples were taken more than 4 hours after administration. Three
326 samples were analyzed for each matrix. Figure 4 presents the chromatograms obtained for double
327 blank, blank spiked at the LLOQ and patients for ibrutinib and PCI-45227 in both matrices. The
328 concentrations of ibrutinib and PCI-45227 determined in the CSF and plasmatic patients' samples are
329 displayed in Table 4. CSF concentrations ranged between 0.59 and 2.95 ng.mL⁻¹ for ibrutinib, and 1.45
330 and 9.73 ng.mL⁻¹ for PCI-45227. Plasma concentrations ranged between 6.61 and 19.44 ng.mL⁻¹ for
331 ibrutinib, and 12.00 and 47.89 ng.mL⁻¹ for PCI-45227. These results were consistent with the few
332 previously published data for quantification of ibrutinib and PCI-45227 reported in CSF and in plasma
333 with a 1-7% CSF/plasma ratio [12, 13]. Moreover, these results show for the first time that PCI-45227
334 is found in CSF. Interestingly, as described in literature for plasmatic concentrations of both analytes
335 [34, 35], concentrations are higher for PCI-45227 than for ibrutinib in CSF.

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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

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526 Figure 1. Fragmentation patterns of ibrutinib (A) and PCI-45227 (B) with a collision energy of 26 V and
527 31 V, respectively.

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

532

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

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

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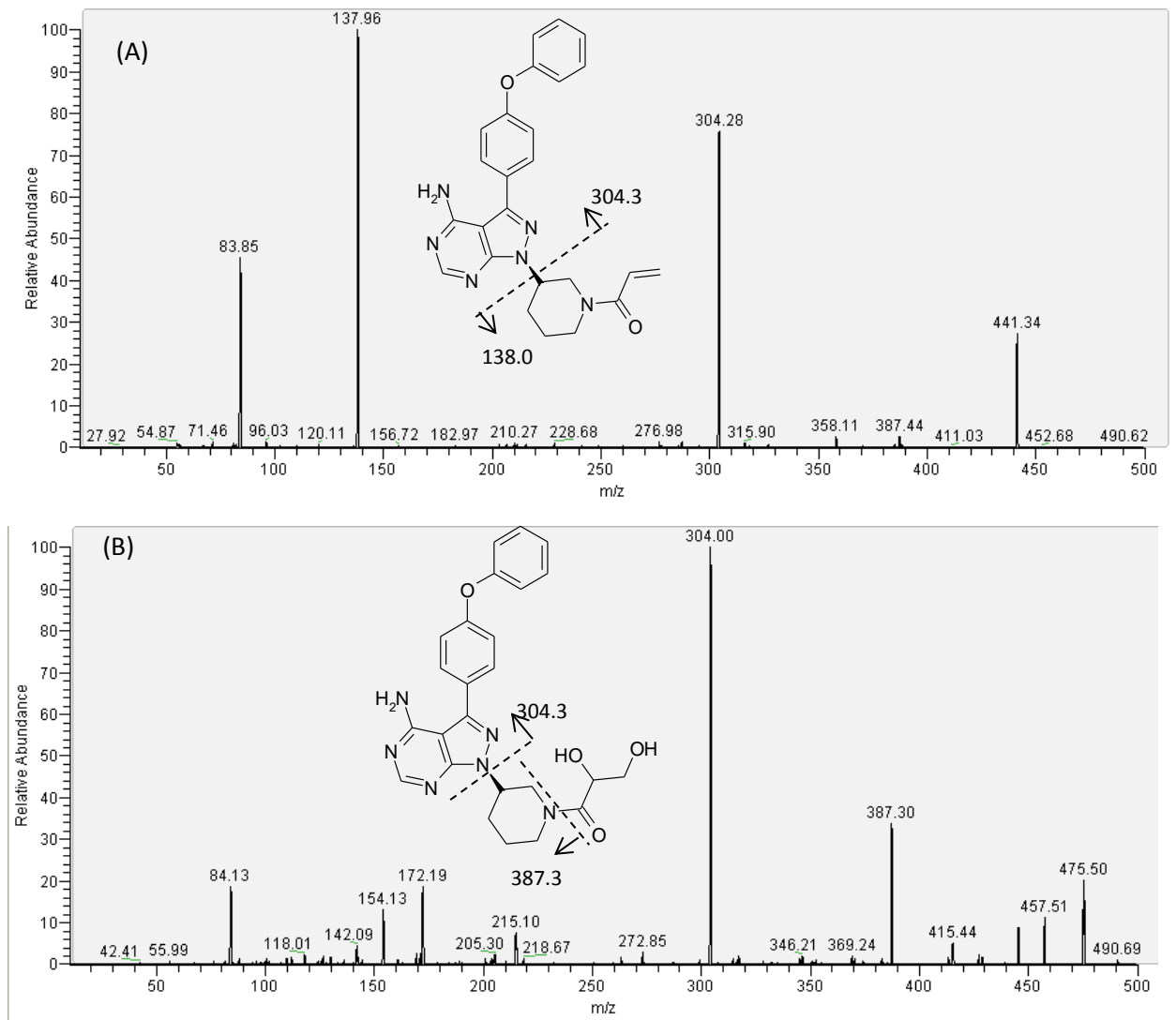
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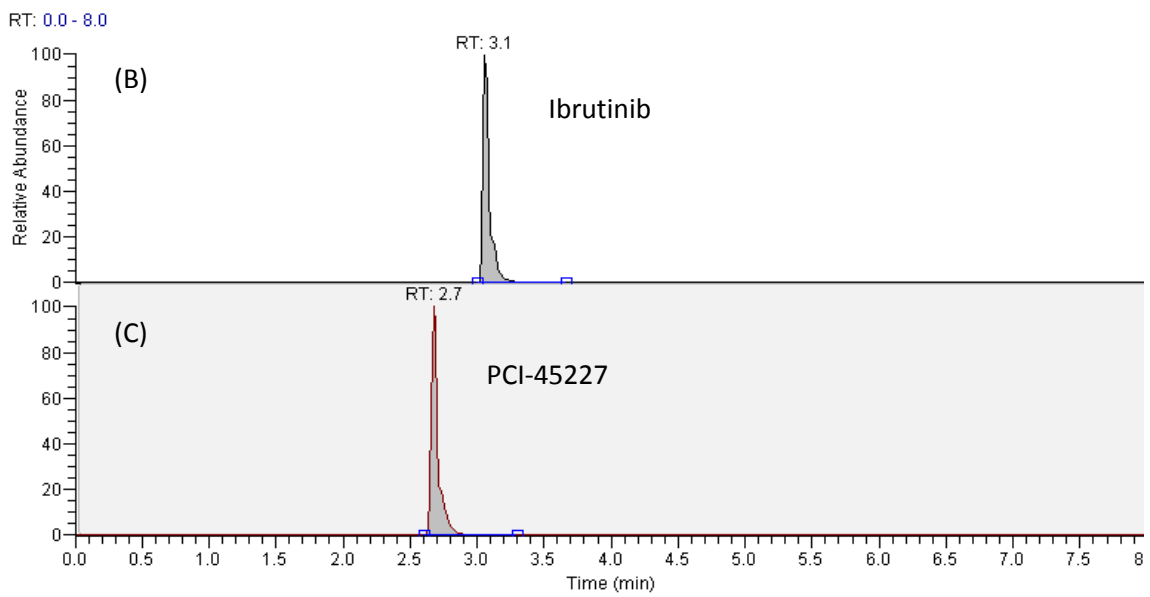
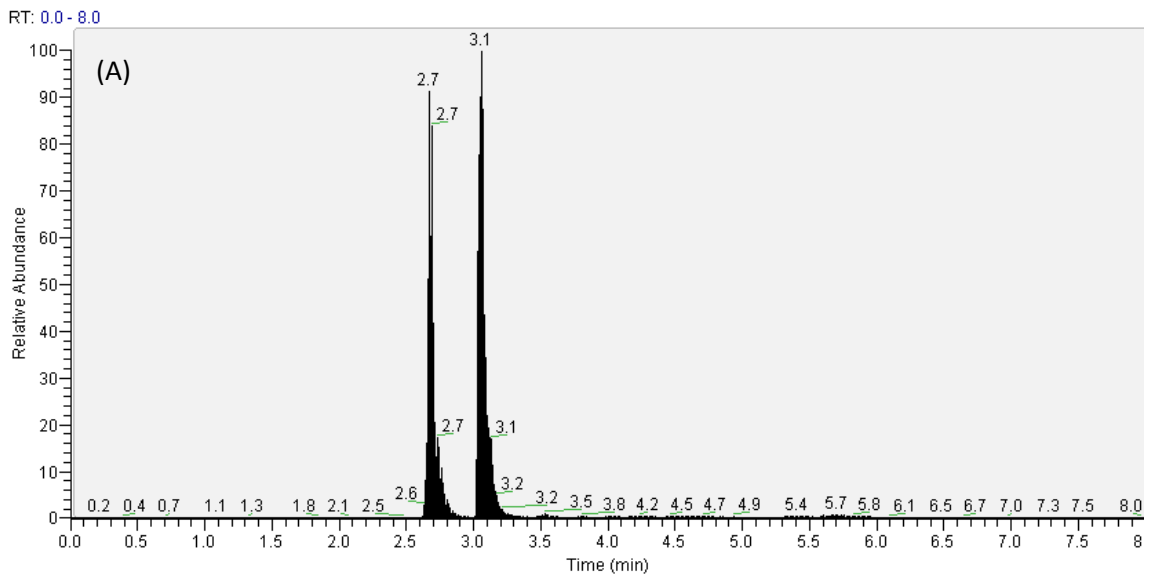
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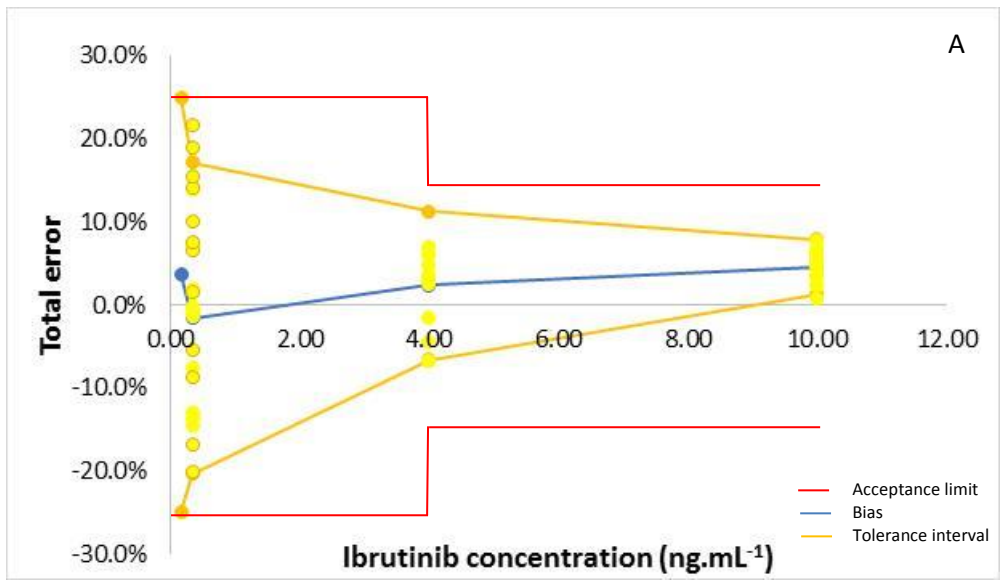
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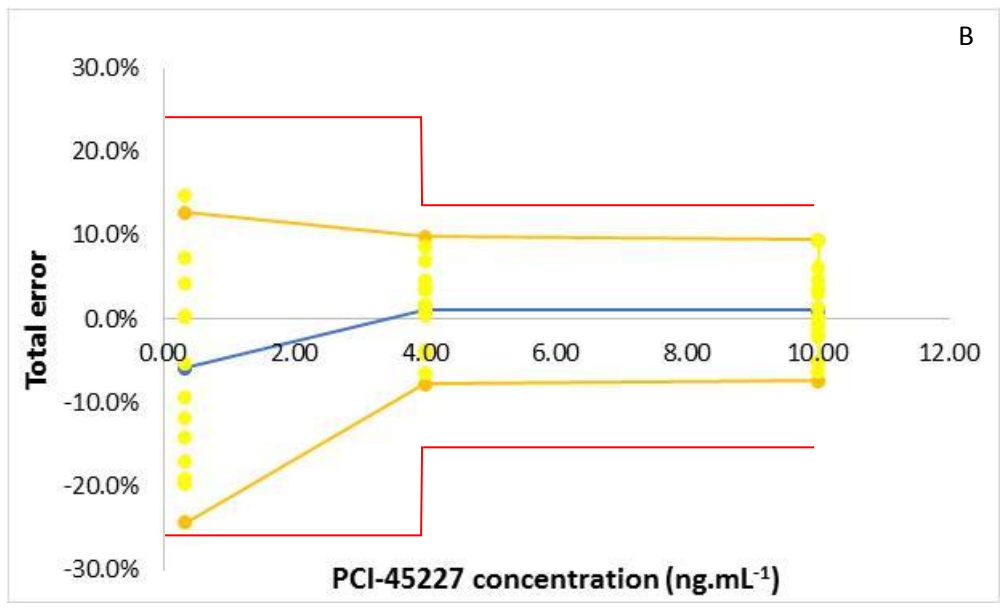
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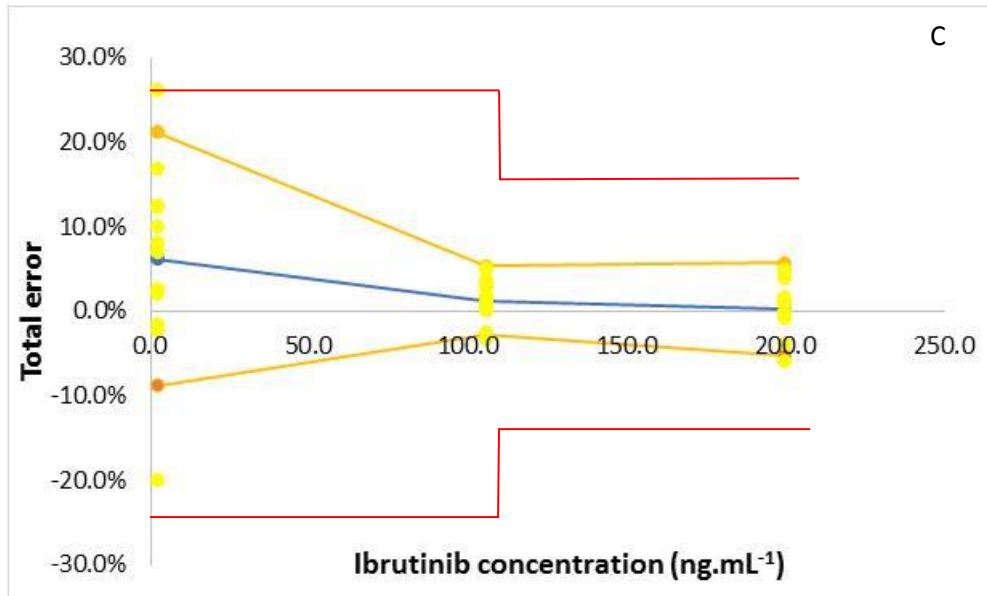
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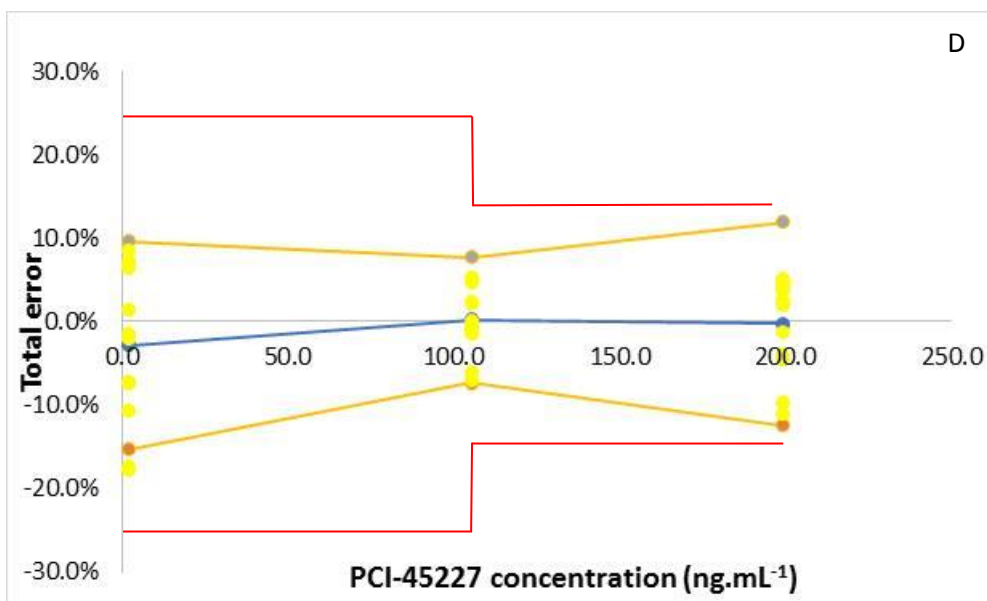
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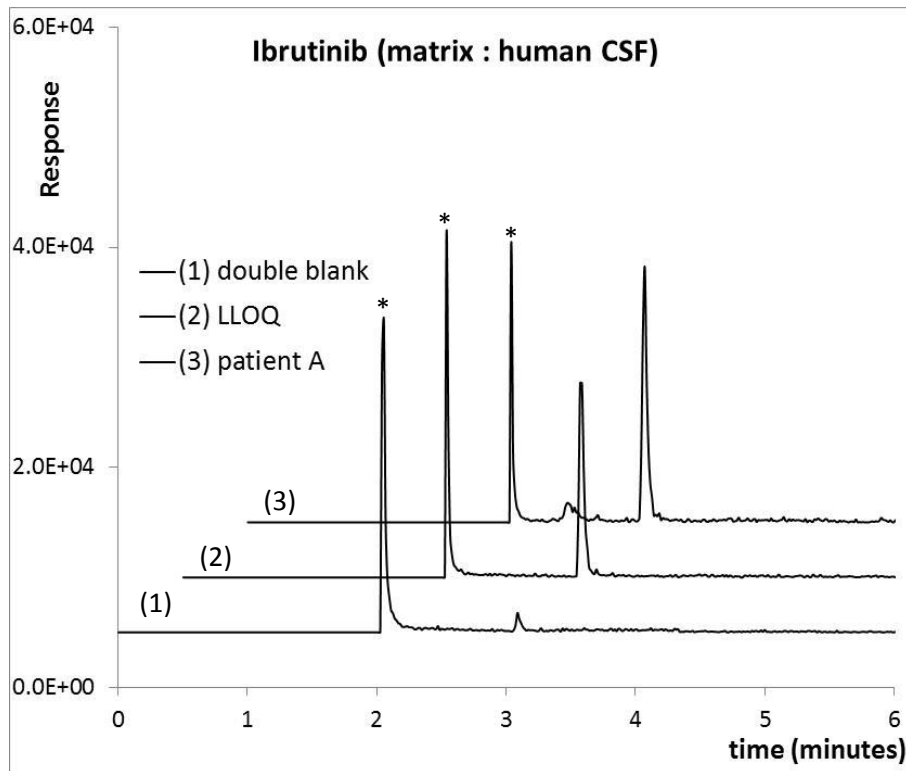
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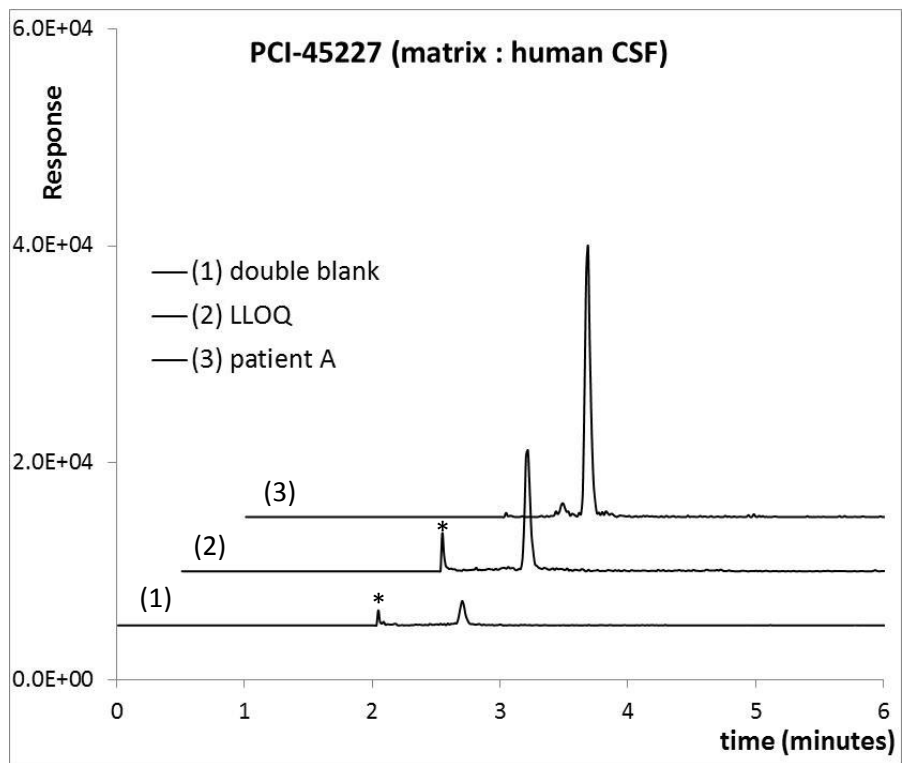
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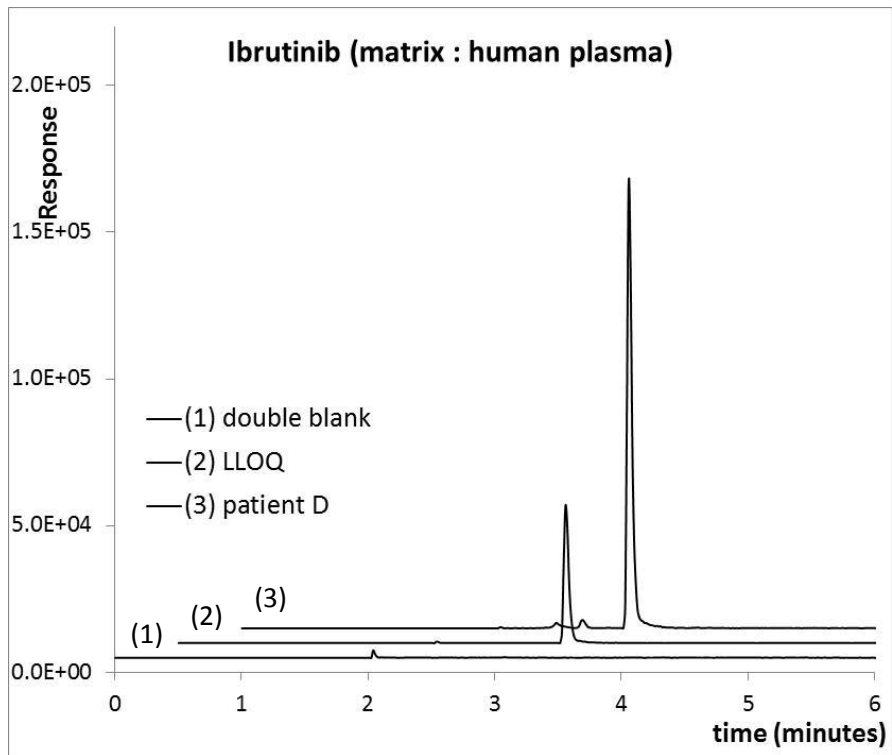
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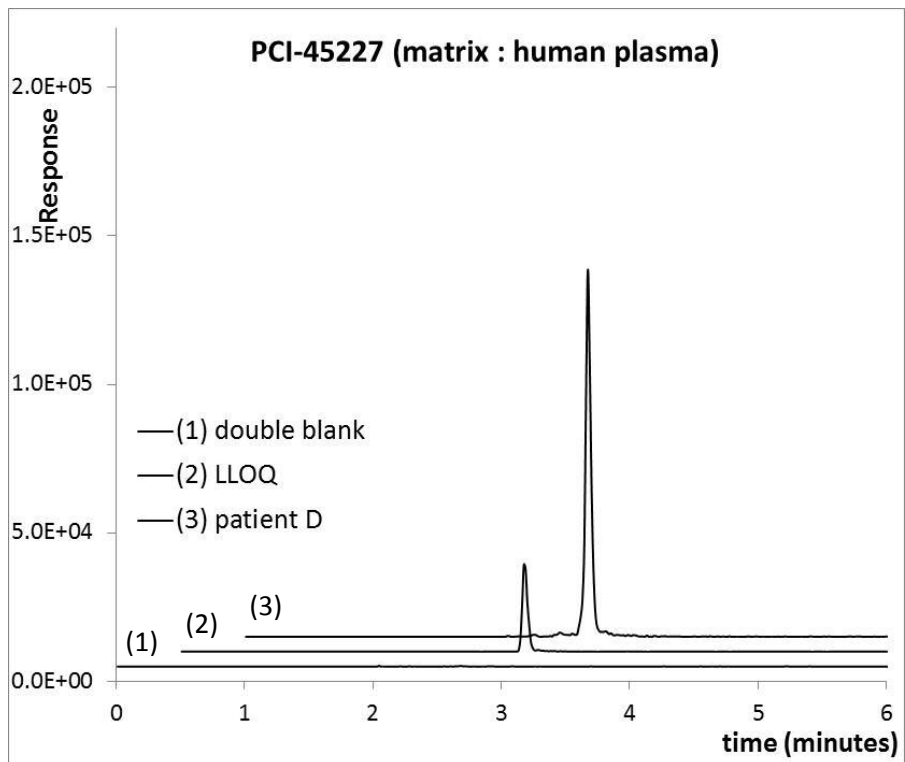
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630 Table 1. Optimized MRM parameters for the determination of ibrutinib and PCI-45227

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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
[² H ₅]-ibrutinib	446.3	138.0	28	309.3	26
PCI-45227	475.5	304.3	31	387.3	24
[² H 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 VS₁, low VS₂, 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

636 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
637 concentrations (low VS, medium VS and high VS ; concentrations expressed are the concentration in the analyzed solutions))

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	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

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

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	[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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