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1 **Liquid chromatography-tandem mass spectrometric assay for the quantification of CDK4/6**
2 **inhibitors in human plasma in a clinical context of drug-drug interaction.**

3

4 Authors:

5 Fanny Leenhardt^{1,2,3}, Matthieu Gracia^{1,3}, Catherine Perrin⁴, Claudia Muracciole-Bich⁴,
6 Bénédicte Marion⁴, Celine Roques⁴, Marie Alexandre⁵, Nelly Firmin^{3,5}, Stephane Poudroux⁵,
7 Litaty Mbatchi^{1,3,6}, Celine Gongora³, William Jacot^{3,5}, Alexandre Evrard^{1,3,6}.

8

- 9 1- Laboratoire de Pharmacocinétique, Université de Montpellier, Faculté de Pharmacie,
10 France
- 11 2- Service Pharmacie, Institut du Cancer de Montpellier, Université de Montpellier, 208 rue
12 des Apothicaires, 34298, Montpellier, France.
- 13 3- Institut de Recherche en Cancérologie de Montpellier (IRCM), INSERM U1194, Université
14 de Montpellier, Montpellier, France.
- 15 4- Institut des Biomolécules Max Mousseron (IBMM), UMR 5247-CNRS-UM-ENSCM,
16 Montpellier, France.
- 17 5- Département d'oncologie médicale, Institut du Cancer de Montpellier, Université de
18 Montpellier, 208 rue des Apothicaires, 34298, Montpellier, France
- 19 6- Laboratoire de Biochimie et Biologie moléculaire, Centre Hospitalier Universitaire Nîmes,
20 France

21

22 Corresponding author: Fanny Leenhardt, fanny.leenhardt@icm.unicancer.fr, Institut du
23 cancer de Montpellier, 208 avenue des Apothicaires, Montpellier, France. Phone number :
24 00 336 83 44 04 06

25

26

27 Credit authorship contribution statement:

28 Fanny Leenhardt : Conceptualization, Methodology, Investigation, Formal analysis, Writing -
29 Original Draft, Supervision, Funding acquisition

30 Matthieu Gracia : Methodology, Investigation, Formal analysis, Writing - Original Draft

31 Catherine Perrin : Methodology, Validation, Formal analysis, Writing - Original Draft,
32 Supervision Claudia Muracciole-Bich : Methodology, Resources, Writing - Original Draft
33 Bénédicte Marion : Methodology, Investigation, Resources
34 Celine Roques : Methodology, Investigation, Resources
35 Marie Alexandre : Investigation, Writing - Original Draft
36 Nelly Firmin : Investigation, Writing - Original Draft
37 Stephane Pouderoux : Investigation
38 Litaty Mbatchi : Writing - Original Draft
39 Celine Gongora : Writing - Original Draft, Project administration
40 William Jacot : Conceptualization, Investigation, Writing - Original Draft, Supervision, Project
41 administration, Funding acquisition
42 Alexandre Evrard : Conceptualization, Methodology, Writing - Original Draft, Supervision,
43 Project administration, Funding acquisition

44

45

46 **Abstract**

47

48 The CDK4/6 inhibitors palbociclib and ribociclib are kinase inhibitors used in association with
49 hormonal therapy for the management of patients with metastatic breast cancer. Like most
50 kinase inhibitors, therapeutic drug monitoring may be used for personalize their dosage. To
51 this aim, we developed and validated a sensitive and specific HPLC-MS/MS method for
52 palbociclib and ribociclib quantification in blood samples. We then quantified exposure to
53 palbociclib (plasma trough concentration; C_{trough}) in a real-life cohort of patients with locally
54 invasive or metastatic breast cancer (n=18) at day 15 of the first cycle of palbociclib
55 treatment to characterize palbociclib concentration at steady state (Clinicaltrials.gov
56 identifier NCT04025541). The geometric mean (\pm standard deviation [min-max]) of
57 palbociclib plasma C_{trough} was 88.58 ng/ml (\pm 26.4 [46.5 ng/mL – 133 ng/mL]) at day 15.
58 Some covariates, such as drug-drug interactions, could explain the concentration variations

59 observed in our Caucasian cohort. These first results in real-life settings obtained with our
60 HPLC-MS/MS method give important information on palbociclib monitoring and
61 pharmacokinetic variability.

62

63 Keywords: CDK4/6 inhibitor; HPLC-MS/MS; metastatic breast cancer; therapeutic drug
64 monitoring; drug-drug interactions

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68

69 **1. Introduction**

70 The cyclin-dependent kinase 4/6 (CDK4/6) inhibitors palbociclib and ribociclib in combination
71 with endocrine therapy are gradually becoming the first-line treatment for patients with
72 locally advanced or metastatic hormone receptor-positive (HR+) /HER2-negative breast
73 cancer. However, these combination therapies show specific adverse events, such as severe
74 neutropenia (grade 3-4 for about 50% of patients), anemia, asthenia and liver toxicity [1].
75 Nevertheless, their safety profile is globally good, and the febrile neutropenia incidence is
76 about 1% in the pivotal clinical trials. Moreover, they have shown a clear benefit in terms of
77 progression-free survival compared with endocrine therapy alone [1]. As palbociclib and
78 ribociclib require only a single, fixed oral dose per day, their administration is easy to
79 manage but requires good patient's compliance for optimal drug exposure and therapeutic
80 effect. However, neutropenia often leads to treatment interruption/delay or dosage
81 modification, possibly linked to the lack of baseline dose adaptation in function, for instance,
82 of age or body weight. In addition, targeted oral anti-cancer treatments, including CDK4/6
83 inhibitors, can display intra- and inter-individual pharmacokinetic variability that can
84 influence their efficacy and tolerance. Drug-drug interactions (DDI), food intake and genetic
85 polymorphisms in drug metabolizing enzymes are among the many factors that can
86 influence drug-exposure variability. For instance, the observed variability concerning the
87 Area Under the plasma concentration Curve (AUC) and the steady state trough
88 concentration (C_{trough}) of tyrosine kinase inhibitors are about 32% for imatinib, 34% for
89 sunitinib, 24% for pazopanib, and 28% for vemurafenib [2]. For drugs with a linear
90 pharmacokinetics, efficacy and tolerance are mostly related to plasma exposure. Therefore,
91 therapeutic drug monitoring (TDM) is a useful tool to monitor this parameter. Practical

92 recommendations on TDM use are based on pharmacokinetic data, availability of analytical
93 techniques, and clinical trials that used TDM for dosage adjustments [3]. TDM data and
94 recommendations are available for some oral targeted therapies used in solid tumors, such
95 as sunitinib in renal carcinoma and imatinib in gastrointestinal stromal tumor and leukemia
96 chronic myeloid [4]. Conversely, very few evidence-based data are available on TDM
97 usefulness for oral targeted therapies used in breast cancer, such as everolimus and
98 lapatinib. Some methods have been validated for the plasma quantification of CDK4/6
99 inhibitors, but they are less used in real-life settings [5–7]. Therefore, we developed a
100 specific high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)
101 method suitable for TDM of palbociclib and ribociclib in real-life settings. Here, we report the
102 method validation and also preliminary results obtained in 18 patients with breast cancer at
103 day 15 of the first cycle of palbociclib treatment.

104 **2. Material, patients and methods**

105 2.1. Chemicals

106 Palbociclib, ribociclib, palbociclib $^2\text{H}_8$ and ribociclib $^2\text{H}_6$ were obtained from Alsachim (Illkirch,
107 France). HPLC-grade acetonitrile (ACN) and methanol were purchased from Carlo Erba
108 Reagents (Val De Reuil, France). Formic acid (FA) (98% pure) was obtained from PanReac
109 AppliChem ITW Companies (Darmstadt, Germany). Dimethylsulfoxide (DMSO) was from
110 Carlo Erba Reagents (Val De Reuil, France). Ultrapure water (H_2O) was produced with a Milli-
111 Q® Simplicity apparatus (Millipore Corp., Burlington, MA, USA).

112 2.2. Control plasma and blood sample collection

113 Control plasma samples (blank samples) were obtained from Nîmes University Hospital
114 Center (UHC) and were stored at -20°C. Patient blood samples were collected in EDTA tubes
115 at the Montpellier Regional Institute of Cancer (ICM, Montpellier, France) and at Nîmes UHC,
116 centrifuged and stored at -80°C till analysis. Patient blood samples were from patients with
117 metastatic breast cancer treated with an aromatase inhibitor (letrozole or anastrozole) and
118 CDK4/6 inhibitor (palbociclib or ribociclib) and enrolled in a multicenter prospective clinical
119 trial (ALCINA 2, NCT04025541) initiated at ICM in 2018 to assess the clinical usefulness of
120 various cancer biomarkers. Patients were included after signature of the informed consent.
121 Cohort 1 (palbociclib) recruitment is already ongoing for patients received 125mg oral
122 palbociclib as per therapeutic indication, once per day for 3 weeks followed by one week off.
123 Gold standard of TDM for oral therapy in cancer is to assess the plasma concentration at the
124 predose (C_{trough}) at the steady-state (one to two weeks after treatment start depending on
125 elimination half-lives). Blood samples were collected at day 15 (steady-state reached) of the
126 first and second treatment cycle before drug administration to estimate plasma exposure
127 (C_{trough}). Cohort 2 (ribociclib) is active but not recruiting yet. Palbociclib plasma concentration
128 at the first treatment cycle and at the predose was analyzed for the first twenty patient of
129 cohort 1 (n=18; two patients excluded because blood samples were not collected at C_{trough}).
130 Patients were classified according to their risk of DDI that might lead to inhibition of CYP3A4
131 and/or P-glycoprotein. Database search (e.g. DDI predictor®, Drugs.com®, Pubmed®) allowed
132 the identification of the following drugs that could cause DDI: fluconazole, ivabradine,
133 atorvastatin, amlodipine, losartan and nifedipine [8–12].

134 2.3. Stock and working solutions

135 Individual stock solutions (1 mg/mL) of palbociclib, ribociclib and internal standards (IS;
136 palbociclib $^2\text{H}_8$ and ribociclib $^2\text{H}_6$) were prepared in DMSO and stored at -20°C . Mixed
137 palbociclib, ribociclib and IS working solutions were then prepared in ACN/ H_2O (50:50, v/v)
138 with 0.1% (v/v) FA by mixing the appropriate volumes of analyte and IS stock solutions. The
139 palbociclib and ribociclib working solution concentrations were 78.12, 156.25, 312.5, 625,
140 1250, and 2500 ng/mL. They were used for the preparation of calibration standards and
141 quality control (QC) samples. The final IS concentrations were 2500 ng/mL for all working
142 solutions. Independent stock solutions were used for the preparation of the calibration and
143 quality control standards. We also have performed quality control sample testing in two
144 laboratories to ensure reproducibility of the method (data not shown).

145 2.4. Calibration standards, quality control, and patient plasma samples

146 To compute the calibration curve over a specific concentration range, calibration standards
147 were prepared by spiking 200 μL of blank plasma with 10 μL of working solution that
148 contained known concentrations (3.9, 7.8, 15.6, 31.5, 62.5, and 125 ng/mL) of palbociclib
149 and ribociclib, respectively. For each calibration standard, the final IS concentration was 125
150 ng/mL. To determine the lower limit of quantification (LLOQ), medium, and upper limit of
151 quantification (ULOQ), palbociclib and ribociclib QC samples were prepared at the
152 concentrations of 3.9, 15.6, 50, and 100 ng/mL. All solutions were prepared according to the
153 recommendations for bioanalytical method validation [13,14]. Plasma sample (100 μl) was
154 mixed with 100 μL of blank plasma and 10 μL of the final IS (palbociclib $^2\text{H}_8$) working solution
155 before sample extraction to avoid concentration measurements out of the limit of
156 calibration range.

157 2.5. Sample extraction procedure

158 Sample (calibration standards, QC, patient plasma samples) extraction was performed by
159 Solid Phase Extraction (SPE) with Oasis Hydrophilic-Lipophilic Balance (HLB) columns (1cc;
160 30gr) (Waters®) on a vacuum support. 100 µL of methanol/H₂O (50:50, v/v) and 1mL of FA
161 28% (v/v) were added to each sample (final volume: 1.310 mL). SPE columns were first
162 conditioned with 2 mL of methanol and 2 mL of H₂O. Then, samples were loaded on the SPE
163 column followed by two rinses (1 mL) with water. Samples were eluted with 1 mL of
164 methanol. Eluted samples were dried and concentrated under a nitrogen stream using a
165 TurboVap® device (Air Liquid, France) coupled with a 37°C water bath. Dried extracts were
166 reconstituted with 200 µL ACN/H₂O (50:50, v/v) and 0.1% (v/v) FA and analyzed (10 µL) by
167 HPLC-MS/MS. We also compared SPE to liquid-liquid extraction in order to use the most
168 cost-effective and reliable extraction technique. SPE has proven to be the technique with the
169 highest repeatability and reproducibility, as well as the best performance (data not shown).

170

171 2.6. HPLC-MS-MS equipment

172 An Agilent 1100 HPLC instrument linked to a triple quadrupole mass spectrometer (MS/MS)
173 (API3000, PE Sciex) with a turbo ion spray interface was used for all analyses.
174 Chromatographic separations were carried out on a Waters Symmetry® C18 column (4.6 µm
175 x 75mm; 3.5 µm). Data were treated with the Analyst 1.5.2 software.

176

177 2.7. HPLC-MS/MS conditions

178 The column and autosampler temperatures were maintained at 25 °C and 4°C, respectively.
179 Eluent A was 0.1% (v/v) of FA in water and eluent B was ACN with 0.1% (v/v) of FA. Complete
180 separation of palbociclib, ribociclib and IS was obtained using a carefully optimized 22min
181 stepwise gradient with a flow rate of 0.5mL/min: 100% A (0-2 min), 0-50% B (2-8min), 50% B

182 (8-11 min, end of analytical run), 50-100% B (11-14min), 100% B (14-17 min), 100-0% B (17-
183 19 min), and 100% A (19-22 min). The ion source temperature was set to 450°C and the ion
184 spray voltage at 5000 V. The nebulizer, curtain and collision gas pressures were 8, 8, and 4
185 psi, respectively. MS/MS transitions and optimal potential settings were determined for
186 each analyte/IS and are listed in Figure 1.

187

188 2.8. Method validation

189 The HPLC-MS/MS method was validated according to the FDA and EMA recommendations
190 [13,14].

191

192 2.9. Statistical analysis

193 The Wilcoxon rank-sum test was used to compare the distribution of quantitative variables
194 (palbociclib plasma exposure and risk of DDI).

195

196 3. Results

197 3.1 Method development

198 Precursor and product ions were obtained for the investigated analytes and IS with collision
199 energy values of 39 V and 45 V for palbociclib and its IS and for ribociclib and its IS,
200 respectively (Figure 1). Precursor ions (Q1, m/z) for palbociclib and its IS was 448 and 456
201 respectively, and ribociclib and its IS was 435 and 441. Product ions (Q3, m/z) obtained were
202 379.9, 388, 322 and 322 for palbociclib, its IS, ribociclib and its IS respectively.

203 After HPLC gradient optimization, the method specificity was evaluated by replicating
204 analyses using the multiple reaction monitoring (MRM) mode. This mode allows the specific
205 and selective detection, identification and quantification of the components of interest in a
206 mixture, based on monitoring the precursor-fragment ion pair. Figure 1 shows the

207 chromatogram and retention times obtained for the ribociclib and palbociclib working
208 solutions at 125 ng/mL and their IS. Elution step was maintained up to 22 minutes in order
209 to limit the risk of sample carry-over.

210

211 3.2 Method validation

212 3.2.1 Selectivity

213 The selectivity of the method (i.e. the ability to differentiate between palbociclib, ribociclib
214 and IS) for endogenous plasma matrix components was evaluated in four different batches
215 of blank plasma. Selectivity was good with no interference observed between analytes and
216 plasma components (Figure 1). Indeed, the signal of the blank matrix was <2% of that
217 obtained for palbociclib and ribociclib at the LLOQ (and <0.2% for the IS).

218

219 3.2.2 Calibration curve

220 Linearity was assessed using calibration standards at increasing concentrations: 3.9 (LLOQ),
221 7.8, 15.6, 31.2, 62.5, and 129 (ULOQ) ng/mL of palbociclib and ribociclib, respectively. Five
222 calibration curves were analyzed on different days for each analyte (i.e. palbociclib and
223 ribociclib). All correlation coefficients were >0.998 for palbociclib and ribociclib. All back-
224 calculated concentrations were within 15% of the nominal concentrations ($\pm 20\%$ for the
225 LLOQ). For both analytes, the LLOQ signals were >5 times greater than the signal of the blank
226 sample.

227

228 3.2.3 Accuracy and precision

229 Within-run and between-run accuracy and precision were studied using calibration
230 standards with the following concentration levels: 3.9 ng/mL (LLOQ), 15.6 ng/mL (about 3
231 times the LLOQ), 50 ng/mL (about 40% of the calibration curve), and 100 ng/mL (about 78%

232 of the calibration curve). Accuracy was expressed as the difference (%) between the mean
233 measured concentration and the nominal concentration (bias). Precision was expressed as
234 the coefficient of variation (CV) (%). ANOVA was used to assess the between-run precision.
235 Within-run and between-run accuracy and precision were within the acceptance criteria: \leq
236 15% for low, medium and high QC and $\leq 20\%$ for the LLOQ of the QC (Table 1). Accuracy and
237 precision measurements were analyzed by pooling repeated results using statistical methods
238 (ANOVA). The standard deviation and the common coefficient correlation were checked
239 (Table 1).

240

241 3.2.4. Carry-over

242 Blank samples were injected after analysis of high-concentration QC samples to evaluate the
243 carry-over. For palbociclib, interference was $<5\%$ of the peak area observed at the LLOQ. For
244 ribociclib, interference was $<2\%$ of the peak area observed at the LLOQ. For both IS,
245 interference was $<0.5\%$ of the IS peak area. These results are fully compliant with the
246 required limits: $\leq 20\%$ of peak areas at the LLOQ for the analytes and $\leq 5\%$ of IS area for IS.

247

248 3.2.5 Matrix effect

249 The matrix effect was evaluated at the LLOQ and ULOQ. For each analyte, the normalized
250 matrix effect was estimated by dividing the analyte matrix factor (i.e. analyte peak area in
251 plasma divided by the analyte peak area in the water/ACN mixture) by the IS matrix factor
252 (i.e. IS peak area in plasma divided by IS peak area in the water/ACN mixture). For palbociclib
253 and ribociclib, the normalized matrix effect varied between 0.83 and 1.14. The overall CV of
254 the normalized matrix effect was $<15\%$.

255

256 3.2.6 Stability

257 Plasma samples were stable at ambient temperature for at least 6 hours with observed
258 variations <5% compared with freshly prepared samples for both palbociclib and ribociclib.
259 Samples stored at -20°C were stable for at least one month with <15% of difference with the
260 nominal value.

261

262

263 3.3 Clinical application

264 The plasma concentration of palbociclib was assessed using our HPLC-MS/MS assay in the
265 first 18 patients treated with palbociclib and aromatase inhibitor in the framework of the
266 ALCINA 2 trial (see Table 2 for their description). The C_{trough} (geometric mean \pm standard
267 deviation [min-max]) was 88.58 ng/mL \pm 26.4 [46.5 ng/mL – 133 ng/mL] at day 15 of the first
268 cycle of palbociclib treatment, with a CV(%) of 29.8 (Figure 2).

269 No correlation between plasma concentration and body weight or area was found. DDI-
270 linked pharmacokinetic variabilities, such as drug absorption or metabolism, can modulate
271 palbociclib plasma exposure. Besides palbociclib and aromatase inhibitor treatment, the 18
272 patients were taking other drugs (mean number: 3.22 per day; min-max: 0-7). Therefore,
273 they were divided in two groups based on the presence (n=7) or not (n=11) of potential DDI
274 (CYP3A4 and P-glycoprotein inhibitors listed in 2.2). Palbociclib C_{trough} at day 15 was
275 significantly different in patients with and without potential DDI ($p<0.01$) (Figure 2).

276

277

278 4. Discussion and conclusion

279

280 Here, we described a specific, accurate and sensitive HPLC-MS/MS method that we
281 developed and validated for the simultaneous estimation of ribociclib and palbociclib

282 exposure in patient plasma samples. Most efficient extraction and interlaboratory control
283 have allowed, between quality criteria, a useful and effectiveness method in TDM clinical
284 use. In this first study, the method was also used to monitor palbociclib exposure in a real-
285 life cohort of 18 patients with locally advanced metastatic breast cancer treated with
286 palbociclib and an aromatase inhibitor. Like for most kinase inhibitors used in oncology, the
287 relationship between pharmacokinetics and pharmacodynamics of CDK4/6 inhibitors
288 assessed by TDM could help to improve the treatment efficacy and reduce toxicities.
289 However, with the exception of the data from the PALOMA trials or from small cohorts,
290 clinicians do not have much information on palbociclib plasma exposure [7]. Our preliminary
291 analysis in 18 patients showed a mean C_{trough} of 88.58 ng/mL, similar to what reported in the
292 PALOMA trials. In the PALOMA 1 trial, the geometric mean C_{trough} was 88.5 ng/mL (n=6) [15].
293 In the PALOMA 2 trial, the mean C_{trough} of palbociclib (%CV) at the steady state was 61 ng/mL
294 (42%), with a mean C_{max} at 116 ng/mL (28%) [16]. In the PALOMA 2 subgroup, ethnicity
295 influenced plasma exposure. Specifically, palbociclib C_{trough} was higher in Japanese patients
296 (n=27) than in non-Asian patients (n=142) (95.4 ng/mL versus 61.7 ng/mL) [17]. Our cohort
297 included only Caucasian patients treated with palbociclib (125mg per day; full dose). DDI
298 mediated by drug metabolizing enzymes and transporters are a major source of
299 pharmacokinetic variability. In our subgroup with potential DDI, interactions between
300 palbociclib and CYP3A4 or P-glycoprotein inhibitors could explain the palbociclib
301 concentration variability (74.15 ng/mL vs 111.26 ng/mL with DDI). Additional studies are
302 needed to characterize palbociclib pharmacokinetic variabilities that could explain plasma
303 concentration variations between patients, and their clinical impact. These preliminary data
304 must to be confirmed in both cohorts of the ALCINA 2 study (once enrollment will be

305 completed) in order to evaluate the interest of individualizing CDK4-6 inhibitor dosage
306 according to pharmacokinetic and pharmacogenetic data integrated in a decision algorithm.

307

308

309 Declaration of competing interest:

310 No competing interests to declare for all authors/provided financial support for the conduct
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371 Figure :

372 Figure. 1. Chemical structures of the analytes (palbociclib and ribociclib) and their IS
373 (palbociclib $^2\text{H}_8$ and ribociclib $^2\text{H}_6$) with their retention time (RT) and peak intensity. The
374 table specifies the precursor (Q1) and fragment (Q3) ions selected for each compound of
375 interest. The HPLC-MS/MS conditions are described in section 2.6.

376 Figure 2: Palbociclib plasma exposure at day 15 (n=18) in the whole cohort (n=18) and in the
377 two subgroup with (n=7) and without (n=11) potential DDI. Black crosses represent the
378 subpopulation arithmetic mean values and open circles represent individual patient values.