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

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Project administration, Funding acquisition

Abstract

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

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

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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

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

2. Material, patients and methods

2.1. Chemicals

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

2.2. Control plasma and blood sample collection

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

2.3. Stock and working solutions

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

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

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

2.5. Sample extraction procedure

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

2.6. HPLC-MS-MS equipment

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

2.7. HPLC-MS/MS conditions

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

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

2.8. Method validation

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

2.9. Statistical analysis

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

3. Results

3.1 Method development

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

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

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

3.2 Method validation

3.2.1 Selectivity

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

3.2.2 Calibration curve

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

3.2.3 Accuracy and precision

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

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

3.2.4. Carry-over

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

3.2.5 Matrix effect

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

3.2.6 Stability

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

3.3 Clinical application

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

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

4. Discussion and conclusion

Here, we described a specific, accurate and sensitive HPLC-MS/MS method that we 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

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

Declaration of competing interest:

No competing interests to declare for all authors/provided financial support for the conduct of the research

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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.