Published in final edited form as:

J Pharm Biomed Anal. 2019 March 20; 166: 197–204. doi:10.1016/j.jpba.2019.01.017.

Determination of total and unbound ribociclib in human plasma and brain tumor tissues using liquid chromatography coupled with tandem mass spectrometry

Xun Bao¹, Jianmei Wu¹, Nader Sanai², Jing Li^{1,*}

¹Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201

²Barrow Neurological Institute, St. Joseph's Hospital & Medical Center, Phoenix, AZ 85013

Abstract

Ribociclib is a selective, orally bioavailable inhibitor of cyclin-dependent kinase (CDK) 4/6, which has therapeutic potential for a variety of cancer types. This study was to develop and validate a liquid chromatography with tandem mass spectrometry (LC-MS/MS) method for determining total and unbound concentrations of ribociclib in human plasma and brain tumor tissue samples. Plasma and tumor homogenate samples were extracted using protein precipitation with acetonitrile. Unbound fraction in plasma or tumor homogenate was determined by equilibrium dialysis method. Chromatographic separation was achieved based on aqueous normal-phase chromatography mechanism on a Waters XBridge™ Amide column under isocratic elution with acetonitrile-ammonium formate (10 mM, pH 3) (75:25, v/v) at a flow rate of 0.8 mL/min. Ribociclib and the internal standard ([¹³C₆]ribociclib) were monitored at the mass transitions m/z , 435.3 > 367.2 and 441.3 > 373.2, respectively, using positive electrospray ionization mode. The lower limit of quantitation (LLOQ) was 0.5 nM of ribociclib in plasma. Linear calibration curve was established at the concentration range of 0.5 – 1000 nM in plasma. Intra- and inter-day precision and accuracy were within the generally accepted criteria for bioanalytical method. The developed method was successfully applied to determine the plasma pharmacokinetics and central nervous system penetration of ribociclib in patients with malignant primary brain cancer.

Keywords

ribociclib; liquid chromatography with tandem mass spectrometry (LC-MS/MS); aqueous normal-phase liquid chromatography; protein binding; blood-brain barrier; pharmacokinetics

*Requests for reprints addressed to: Jing Li, Ph.D., Karmanos Cancer Institute, 4100 John R Street, HWCRC, Room 523, Detroit, MI 48201, USA. Phone: 313-576-8258; lijn@karmanos.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Declarations of interest: none

1. Introduction

Abnormal cellular proliferation and dysregulation of cell-cycle control is associated with tumorigenesis of many cancers [1–4]. The cyclin D-cyclin-dependent kinases (CDK) 4/6 are key proteins in a cell cycle regulatory pathway, which also involves p16 (an endogenous suppressor of CDK4/6), cyclin D1 (the regulatory subunit of CDK4/6), and the retinoblastoma protein (pRB, a tumor suppressor protein) [5–7]. In response to mitogenic signaling, CDK4 and CDK6 form a complex with their regulatory subunit, cyclin D1. The active cyclin D1-CDK4/6 complex phosphorylates pRB, relieving its repression of E2 transcription factors (E2F), which subsequently activates the transcription of genes required for the G1–S transition and cell-cycle progression [5–7]. Malignant cells frequently acquire unrestricted CDK4/6 activity, leading to unhindered cell cycle stimulation and uncontrolled cell proliferation. Given the importance of CDK4/6 activity in cancer cells, CDK4/6 inhibitors have emerged as promising candidates for cancer treatment.

Ribociclib (LEE011) is a highly specific, orally bioavailable, small-molecule inhibitor of CDK4 and CDK6 [1, 8]. In preclinical and clinical studies, ribociclib demonstrated a manageable tolerability and therapeutic potential for a variety of cancer types [1]. Ribociclib was approved by the United States Food and Drug Administration in 2017 for use in combination with an aromatase inhibitor (e.g., letrozole) for the treatment of postmenopausal women with advanced or metastatic breast cancer that is hormone receptor (HR) positive and human epidermal growth factor receptor 2 (HER2) negative [1, 8]. The efficacy and safety of single-agent ribociclib in patients with neuroblastoma was evaluated as part of the phase I study in pediatric patients, where ribociclib demonstrated acceptable toxicity profile and promising antitumor activity [9]. Preclinical data suggest that ribociclib can cross the blood–brain barrier, supporting further clinical development for the treatment of central nervous system (CNS) tumors [10]. Currently, a Phase 0/II study is ongoing in our institution to evaluate the CNS penetration, pharmacodynamics, and antitumor activity of single-agent ribociclib in patients with primary malignant brain tumors (Trial Identifier number: , PHX-16-0116-80-12). Patients with recurrent high-grade glioma and meningioma were treated with daily oral ribociclib (900 mg) for 5 days prior to surgical resection of tumors. Plasma, tumor, and cerebrospinal fluid (CSF) samples were collected to assess the plasma and tumor pharmacokinetics and CNS penetration of ribociclib.

To accurately characterize the plasma and tumor pharmacokinetics of ribociclib, a specific, sensitive, and reliable bioanalytical method is critically needed. A reversed-phase liquid chromatography with tandem mass spectrometry (LC-MS/MS) method has been reported for determining ribociclib concentrations in mouse plasma and CSF samples [11]. The reported method, however, is limited by tedious sample preparation with solid-phase extraction method and poor sensitivity [lower limit of quantitation (LLOQ), 62.5 ng/mL (144 nM) in mouse plasma]. In this study, we developed and fully validated an aqueous normal-phase LC-MS/MS method for the determination of both total and unbound ribociclib in human plasma, brain tumor, and CSF samples. Compared to the published method, our method involved simple, one-step protein precipitation for sample preparation, while achieving significantly greater sensitivity (LLOQ, 0.5 nM in human plasma and tissue homogenate).

2. Experimental

2.1. Chemicals and reagents

Ribociclib succinate and the stable isotope-labeled internal standard, [$^{13}\text{C}_6$] ribociclib, were provided by Novartis Pharmaceuticals (Basel, Switzerland). All other chemicals and reagents were purchased from VWR International (Radnor, PA, USA) (LC-MS grade). Water was filtered and deionized with a US Filter PureLabPlus UV/UF system (Siemens, Detroit, MI, USA) and used throughout in all aqueous solutions. Drug-free (blank) human plasma from six different healthy donors and pooled plasma (with Na-EDTA anticoagulant) were purchased from Innovative Research Inc. (Novi, MI, USA).

2.2. Chromatographic and mass-spectrometric conditions

All LC-MS/MS analyses were performed on an AB SCIEX QTRAP 6500 LC-MS/MS system (Foster City, CA). It is equipped with a SHIMADZU Nexera ultra-high performance liquid chromatography (Kyoto, Japan) coupled with a hybrid triple quadrupole / linear ion trap mass spectrometer. The system operation and data acquisition were controlled by Analyst@1.6 software, and data processing and quantitation were performed using Multi Quant 3.0 software.

Chromatographic separation was optimized on Waters Xterra MS C8 column (2.1×50 mm, $3.5 \mu\text{m}$), Acquity UPLC BEH C18 column (2.1×50 mm, $1.7 \mu\text{m}$), and Waters XBridgeTM Amide column (4.6×100 mm, $3.5 \mu\text{m}$). The best chromatographic separation was achieved on a Waters XBridgeTM Amide column (4.6×100 mm, $3.5 \mu\text{m}$) under isocratic elution with acetonitrile-ammonium formate (10 mM, pH 3.0) (75:25, v/v) at the flow rate of 0.8 mL/min. Column oven temperature was maintained at 40°C. External and internal washes were implemented prior to and post the injection for both the auto-sampler syringe and injection port to eliminate potential carryover.

The column eluent was monitored using the multiple reaction monitoring mode (MRM) under electrospray positive ionization mode. MS parameters were optimized to obtain the most sensitive and specific MS transitions for ribociclib and [$^{13}\text{C}_6$] ribociclib by direct infusion of standard solutions (at 10 nM) into the ion source with a syringe pump. The Turbo ion-spray voltage was 4500 V and the source temperature was 500 °C. Collision gas was optimized at medium level with curtain gas, ion source gas 1 and ion source gas 2 delivered at 10, 30 and 30 psi, respectively. Decluttering potential, collision energy, collision cell exit potential, and entrance potential were optimized at 111, 30, 22, and 10 V for both ribociclib and [$^{13}\text{C}_6$] ribociclib. The dwell time was set for 50 ms. Ribociclib and [$^{13}\text{C}_6$] ribociclib were monitored at the most sensitive and specific MS transitions m/z , $435.3 > 367.2$ and $441.3 > 373.2$, respectively (Fig. 1).

2.3. Sample Preparation

2.3.1. Stock solutions, calibration standards, and quality control (QC)

samples—Ribociclib succinate stock solution (5 mM) were prepared in dimethyl sulfoxide (DMSO), and [$^{13}\text{C}_6$] ribociclib stock solution (100 μM) was prepared in acetonitrile. All stock solutions were stored in brown glass vials at -80 °C. Ribociclib working solution was

prepared freshly by serial dilutions of the stock solution with 50% acetonitrile on the day of analysis. For quantitation of ribociclib in plasma and brain tissue samples, calibration standards were prepared in blank human plasma at the concentrations of 0.5 – 1000 nM. For quantitation of ribociclib in CSF samples and post-dialysis phosphate buffer solution (PBS, pH 7.4), calibration standards were prepared in PBS. QC samples were prepared in blank human plasma at the concentrations of 0.5 (LLOQ), 1.5, 300, and 800 nM. All calibrators and QC samples were prepared fresh daily. For long-term and freeze-thaw stability, QC samples were prepared as a batch and stored at –80 °C.

2.3.2. Plasma and brain tumor specimens for total ribociclib concentrations

—Frozen plasma and tumor samples were thawed at room temperature. Patient plasma samples were diluted 2-fold with blank human plasma to ensure measured drug concentration to fall within the calibration curve range (0.5 – 1000 nM). Five volumes (500 µL) of ice-cold acetonitrile containing [¹³C₆] ribociclib (5 nM) was added into 100 µL plasma, followed by vortex-mixing (10 s) and centrifugation (9000 g, 4°C, 10 min). The supernatant was transferred to an autosampler vial and 5 µL was injected into the LC-MS/MS system.

Tissue homogenate of a brain tumor sample was prepared by adding 5 volumes of distilled water into the tissue sample, followed by homogenization in a Precellys® homogenizer (at 2000 g for two 10 s with 5 s pause). An aliquot of 20 µL of tissue homogenate was diluted 10-fold with blank human plasma, followed by protein precipitation with 5 volumes (500 µL) of ice-cold acetonitrile containing [¹³C₆] ribociclib (5 nM). Five µL supernatant was injected into the LC-MS/MS system.

2.3.3. Equilibrium dialysis for determination of unbound fraction in plasma and tumor tissue

—Ribociclib fraction unbound in plasma and brain tumor tissue homogenate was determined using equilibrium dialysis on a 96-Well Equilibrium DIALYZER™ with 5 KDa molecular weight cut-off regenerated cellulose membrane (Harvard Apparatus Holliston, MA), as described previously by us [12–14]. The time to equilibrium was optimized by dialysis of 2, 4, 6, 16, and 24 h at ribociclib concentrations of 0.1, 1 and 10 µM in human plasma. Post-dialysis plasma and PBS were transferred into Eppendorf tubes, and extracted with acetonitrile containing [¹³C₆] ribociclib (5 nM) as described above. The total drug recovery was assessed as the ratio of total drug concentration recovered in post-dialysis buffer and plasma (or homogenate) compartments to drug concentration in pre-dialysis plasma (or homogenate) sample. Fraction unbound in plasma or diluted tissue homogenate was estimated as ribociclib concentration ratio of post-dialysis PBS to post-dialysis plasma or homogenate. Fraction unbound in original brain tissue ($f_{u, hom}$) was calculated based on the fraction unbound in diluted tissue homogenate ($f_{u, hom}$) and dilution factor (D_f), using the below equation [15]:

$$f_{u, tissue} = \frac{1/D_f}{\left(\frac{1}{f_{u, hom}} - 1\right) + 1/D_f}$$

2.4. Method validation

The method was fully validated for the assessment of specificity, linearity, precision and accuracy, matrix effect and recovery, as well as short- and long-term stability, based on the US FDA Guidance on Bioanalytical Method Validation [16].

Selectivity was assessed by the inspection of potential endogenous interfering peaks in blank matrix (i.e., blank plasma from 6 donors or PBS). The interfering peak area should be less than 10% of the peak area of the analyte at the lower limit of quantitation (LLOQ) and less than 5% of the peak area of the internal standard.

Linearity was assessed at ribociclib concentrations of 0.5 to 1000 nM in human plasma. Calibration curves were built by fitting calibrator concentrations versus peak area ratios of ribociclib to the internal standard using least-squares non-linear regression analysis with the weighting scheme of $1/x^2$. The intra- and inter-day precision and accuracy were assessed for the calibrators (each in duplicate) and QCs (including LLOQ, low, medium, and high QCs, each in quintuplicate) on three days. The accuracy was assessed as the relative percentage of the determined concentration to nominal concentration. The intra- and inter-day precisions were estimated by one-way analysis of variance (ANOVA) using the JMP™ statistical discovery software version 5 (SAS Institute, Cary, NC, USA), as described previously by us [12, 14]. The analyte response at the LLOQ should have a signal to noise ratio of at least 10, and has an accuracy of 80-120% and precision of 20%. The analyte response at the lower limit of detection (LLOD) should have a signal to noise ratio of at least 5.

Matrix effect and recovery were assessed using plasma samples of 6 different donors, as described previously by us [12, 17]. In brief, three sets of QC samples were prepared. Set 1 QCs were prepared by spiking ribociclib (at 1.5, 300, or 800 nM) and [$^{13}\text{C}_6$] ribociclib (at 5 nM) into 100 μL human plasma. Set 1 samples were processed by adding 500 μL acetonitrile, followed by centrifugation (9000 g, 4°C, 10 min), and 5 μL of the supernatant was injected into the LC-MS/MS system. Set 2 and 3 QC samples were prepared by spiking the same amounts of ribociclib and [$^{13}\text{C}_6$] ribociclib as those in Set 1 samples into 600 μL mobile phase and blank matrix extract (i.e., post-precipitation supernatant of blank plasma), respectively. Set 2 and 3 samples were directly injected into the LC-MS/MS system. Matrix factor is estimated as ribociclib peak area ratio of the post-extraction (set 3) to neat solution (set 2). The recovery is calculated as ribociclib peak area ratio of plasma (set 1) to post-extraction matrix (set 3).

The short-term (bench-top) stability of ribociclib in 50% acetonitrile (at 100 and 1 μM) and in plasma (at 1.5 and 800 nM) were assessed at ambient temperature (25°C) for 1, 2, 3, 4, and 6 hours. The autosampler stability of ribociclib in post-extracted matrix was examined at 4°C for up to 12 hours. Freeze-thaw stability in plasma (at 1.5 and 800 nM) was evaluated for three freeze-thaw cycles with at least 1 day of storage at -80°C between each thawing. The long-term stability of ribociclib in plasma (at 1.5 and 800 nM) was investigated up to 7 months. All QCs were run in triplicate.

2.5. Applications

The developed method was used to assess the plasma and brain tumor pharmacokinetics of total and unbound ribociclib in patients with primary brain cancer in a clinical trial (Trial Identifier number: , PHX-16-0116-80-12). The protocol was approved by the Institutional Review Board of Barrow Neurological Institute, St. Joseph's Hospital & Medical Center (Phoenix, AZ). All the patients provided a written informed consent. Eligible patients were treated with oral ribociclib daily at the dose of 900 mg for 5 days prior to planned cranial tumor resection. Blood samples were collected from each patient at pre-dosing, 0.5, 1, 2, 4, 6, 8 and 24 h after the administration of the 4th dose of ribociclib. Plasma was separated from the whole blood by centrifugation (at 4°C, 1500 g for 10 mins), and plasma samples were stored at -80°C until analysis. Cranial tumor resection was performed after the administration of the 5th dose of ribociclib. Blood, tumor (including contrast enhancing and non-enhancing regions of the tumor), and CSF samples were collected intraoperatively at predefined time points following the administration of the 5th dose. Tumor samples were immediately rinsed with ice-cold PBS to remove residual blood, dried on tissue paper, and snap frozen in liquid nitrogen. All samples were stored at -80°C until analysis.

Plasma and brain tumor tissue samples were prepared, as described in Section 2.3.2. Ribociclib total concentrations in plasma and tumor tissue homogenate samples were determined using the validated LC-MS/MS method. Unbound fractions of ribociclib in plasma and brain tumor tissue were determined using the equilibrium dialysis method as described in Section 2.3.3. Unbound drug concentrations in plasma or tumor tissue were calculated by multiplying total drug concentrations and fraction unbound. The plasma pharmacokinetic parameters were estimated using the non-compartmental analysis.

3. Results and discussion

3.1. Method development

Ribociclib is a weak base with pK_a value of 8.87, and it has a predicted unionized species octanol/water partition coefficient (LogP) of 2.5. It is very soluble in DMSO and slightly soluble in water (with a solubility of 0.231 mg/mL). Given the hydrophobic nature of ribociclib (logP, 2.5), reversed-phase liquid chromatography methods were initially tested. However, under traditional reversed-phase chromatographic conditions using either Waters Xterra MS C8 column or Acquity UPLC BEH C18 column, ribociclib was poorly retained, as demonstrated by wide, unsymmetrical, and tailing peaks (Fig. 2A and 2B). By contrary, ribociclib was well retained on a Waters XBridge™ Amide column, as demonstrated by a sharp, symmetrical peak (Fig. 2C). Waters XBridge™ Amide column is based on a chemically stable, trifunctionally bonded amide stationary phase, which is originally designed to retain and separate extremely polar analytes using hydrophilic interaction liquid chromatography (HILIC) retention mechanism. Our previous study has demonstrated that the XBridge Amide column has a dual hydrophilic-hydrophobic retention property [12], rendering its ability to retain compounds with both polar function groups (e.g., -OH and -NH) and a significant hydrophobic component such as ribociclib. This dual hydrophilic-hydrophobic retention property has been illustrated for silica-hydride-based stationary phase, in which by varying the percentage of water in the mobile phase, reversed-phase

retention mode, normal-phase mode, or a combination of both can take place [18]. As shown in Fig. 3, when increasing acetonitrile concentration from 10% to 90% in the mobile phase, ribociclib exhibited a U-shape retention curve on the Waters XBridge Amide column, indicating the retention mechanism changed from reversed-phase to aqueous normal-phase chromatography. Under the isocratic elution with acetonitrile-ammonium formate (10 mM, pH 3.0) (75:25, v/v), which was used in the present study, aqueous normal-phase chromatography was the main retention mechanism for ribociclib.

3.2. Method validation

Full assay validation using pooled human plasma demonstrated the technical feasibility, reliability, and reproducibility of the developed method. The selectivity was shown by symmetrical resolution of the chromatographic peaks, with no significant interference from 6 different donors of plasma. Fig. 4 presents the extracted ion chromatograms of blank plasma, plasma spiked with ribociclib at the LLOQ, and a patient plasma sample collected at 7 h after the oral administration of ribociclib 900 mg.

Linear calibration curves were constructed at ribociclib concentration range of 0.5 – 1000 nM in human plasma, with a linear correlation coefficient (R^2) of > 0.99 in all analytical runs. The LLOQ was established at 0.5 nM of ribociclib in human plasma, at which the mean signal-to-noise ratio was 939 ± 175 ($n = 30$). For all calibrators, the average accuracy in terms of the percentage of back-calculated concentrations relative to nominal concentrations ranged from 90% to 110% and the intra- and inter-day precisions were within 6.7% (Table 1). For all QC samples (including LLOQ, low, medium and high QCs), the average accuracy ranged 94% - 106% and intra- and inter-day precisions were within 9.5% (Table 2).

The matrix effect of human plasma from 6 different donors was assessed for potential ionization suppression or enhancement on ribociclib and [$^{13}\text{C}_6$] ribociclib. The average matrix factor of ribociclib from 6 donors of plasma ranged from 0.98 to 1.22 across three QC concentrations, with the interindividual variability (i.e., coefficient of variation of 6 donors) $< 6.4\%$ (Table 3). The average matrix factor of [$^{13}\text{C}_6$] ribociclib (at 5 nM) from 6 different donors of plasma was 1.09. The relative (or internal standard-normalized) matrix factor of ribociclib ranged from 0.92 to 1.04 across three QC concentrations, with the interindividual variability $< 2.8\%$ (Table 3). Collectively, these data suggested that while human plasma had no apparent matrix effect on the ionization of ribociclib or [$^{13}\text{C}_6$] ribociclib, the use of isotope-labeled internal standard could effectively reduce the interindividual variability of different source of matrices.

The average recovery of ribociclib from 6 donors of plasma across three QC concentrations ranged from 94% to 104%, with the inter-individual variability within 3.4% (Table 3). The average recovery of [$^{13}\text{C}_6$] ribociclib from 6 donors of human plasma was 112% (Table 3). The relative (or internal standard-normalized) recovery of ribociclib was consistently ($\sim 99\%$) across all three QC concentrations, with the interindividual variability $< 2.6\%$ (Table 3).

Table 4 summarizes the short- and long-term stability data of ribociclib. Bench-top stability test showed that ribociclib was stable in both 50% acetonitrile (at 1 and 100 μM) and human plasma (at 1.5 and 800 nM) at ambient temperature ($\sim 25^\circ\text{C}$) for at least 6 hours.

Autosampler stability test suggested that ribociclib was stable in post-extracted samples at 4°C for at least 12 hours, allowing the assay to be performed continuously overnight for a large number of samples. Freeze-thaw stability test indicated no apparent degradation of ribociclib after three freeze-thaw cycles. Long-term stability test demonstrated that the ribociclib was stable in human plasma (at 1.5 and 800 nM) at -80°C for at least 7 months, suggesting clinical samples can be stored at -80°C for at least 7 months before the analysis.

3.3. Applications

Because the use of a stable isotope-labeled internal standard can adequately correct for variations in the matrix effect and recovery of the analyte across different matrices, the present method demonstrated a broad application for quantitation of ribociclib in patient plasma, brain tumor, and CSF samples. Ribociclib plasma pharmacokinetics and CNS penetration were evaluated in the first cohort of 5 patients who were treated with oral ribociclib daily (900 mg) for 5 days and cranial tumor resection was performed at 2-4 h after the administration of the last dose. Fig. 5 shows the total ribociclib plasma concentration – time profiles following the administration of 4th dose in 5 individual patients. Ribociclib reached the steady-state maximum plasma concentration (C_{max}) of 4906.8 ± 1261.6 nM at 3.2 ± 2.2 h and maintained the trough steady-state plasma concentration of 1495.9 ± 674.8 nM following daily oral dose. It exhibited an elimination half-life ($T_{1/2}$) of 16.0 ± 5.2 h and apparent oral clearance (CL/F) of 38.8 ± 17.0 L/h.

The fraction unbound of ribociclib in plasma or tissue homogenate was determined by equilibrium dialysis, with the optimized equilibrium time of 16 h (Fig. 6). Ribociclib was modestly bound to human plasma proteins, showing a mean fraction unbound of 28.9% in pooled healthy human plasma at clinically relevant concentrations (0.1 – 10 μM) and a mean fraction unbound of 12.7% (with coefficient variation of 17.6%) in 5 brain cancer patients. Ribociclib was highly bound to brain tumor tissues, which showed a mean fraction unbound in tumor of 4.6% (with coefficient variation of 23%) in 5 patients. The unbound drug concentrations in paired non-enhanced and contrast-enhanced tumor regions were 455.4 ± 235.3 and 2316.4 ± 1786.3 pmol/g (or nM), respectively, which significantly exceeded the *in vitro* IC_{50} for the inhibition of CDK4 (10 nM) and CDK6 (39 nM) in cell-free assay [8] , The CSF concentrations, which are generally considered as the surrogate of unbound drug concentration in normal brain, were 538.8 ± 165.7 nM. Collectively, these data indicated that ribociclib well penetrated across not only the disrupted blood-brain tumor barrier but also the largely intact blood-brain barrier, supporting further clinical development of this agent for the treatment of central nervous system tumors.

4. Conclusion

A simple, sensitive, and reliable LC-MS/MS method based on aqueous normal-phase chromatography was developed for the determination of ribociclib concentrations in patient plasma, brain tumor, and CSF samples. The method was fully validated with human plasma.

Sample preparation involved one-step protein precipitation with acetonitrile. The method was sensitive, with the LLOQ of 0.5 nM ribociclib in plasma and tissue homogenate. The linear calibration curve was established at ribociclib concentration range of 0.5 – 1000 nM in plasma. The validated method was successfully applied to assess the plasma pharmacokinetics and CNS penetration of ribociclib in patients with malignant brain cancer.

Acknowledgements

This study was supported in part by the United States National Institute of Health (NIH) Cancer Center Support Grant P30 CA022453 and the Ben & Catherine Ivy Foundation. We thank Novartis Pharmaceuticals (Basel, Switzerland) for providing the study drug (ribociclib), and we are indebted to the patients who participated in this study.

References:

- [1]. Edessa D, Sisay M, Recent advances of cyclin-dependent kinases as potential therapeutic targets in HR+/HER2– metastatic breast cancer: a focus on ribociclib, *Breast Cancer* (Dove Med Press) 9 (2017) 567–579. [PubMed: 29263697]
- [2]. Hamilton E, Infante JR, Targeting CDK4/6 in patients with cancer, *Cancer Treat Rev* 45 (2016) 129–38. [PubMed: 27017286]
- [3]. Iwata H, Clinical development of CDK4/6 inhibitor for breast cancer, *Breast Cancer* (2018).
- [4]. Klein ME, Kovatcheva M, Davis LE, Tap WD, Koff A, CDK4/6 Inhibitors: The Mechanism of Action May Not Be as Simple as Once Thought, *Cancer Cell* (2018).
- [5]. Otto T, Sicinski P, Cell cycle proteins as promising targets in cancer therapy, *Nat Rev Cancer* 17(2) (2017) 93–115. [PubMed: 28127048]
- [6]. Sherr CJ, Cancer cell cycles, *Science* 274(5293) (1996) 1672–7. [PubMed: 8939849]
- [7]. Suarez-Arroyo IJ, Loperena-Alvarez Y, Rosario-Acevedo R, Martinez-Montemayor MM, *Ganoderma spp.: A Promising Adjuvant Treatment for Breast Cancer*, *Medicines* (Basel) 4(1) (2017).
- [8]. Tripathy D, Bardia A, Sellers WR, Ribociclib (LEE011): Mechanism of Action and Clinical Impact of This Selective Cyclin-Dependent Kinase 4/6 Inhibitor in Various Solid Tumors, *Clin Cancer Res* 23(13) (2017) 3251–3262. [PubMed: 28351928]
- [9]. Geoerger B, Bourdeaut F, DuBois SG, Fischer M, Geller JI, Gottardo NG, Marabelle A, Pearson ADJ, Modak S, Cash T, Robinson GW, Motta M, Matano A, Bhansali SG, Dobson JR, Parasuraman S, Chi SN, A Phase I Study of the CDK4/6 Inhibitor Ribociclib (LEE011) in Pediatric Patients with Malignant Rhabdoid Tumors, Neuroblastoma, and Other Solid Tumors, *Clin Cancer Res* 23(10)(2017) 2433–2441. [PubMed: 28432176]
- [10]. Raub TJ, Wishart GN, Kulanthaivel P, Staton BA, Ajamie RT, Sawada GA, Gelbert LM, Shannon HE, Sanchez-Martinez C, De Dios A, Brain Exposure of Two Selective Dual CDK4 and CDK6 Inhibitors and the Antitumor Activity of CDK4 and CDK6 Inhibition in Combination with Temozolomide in an Intracranial Glioblastoma Xenograft, *Drug Metab Dispos* 43(9) (2015) 1360–71. [PubMed: 26149830]
- [11]. Kala A, Patel YT, Davis A, Stewart CF, Development and validation of LC-MS/MS methods for the measurement of ribociclib, a CDK4/6 inhibitor, in mouse plasma and Ringer's solution and its application to a cerebral microdialysis study, *J Chromatogr B Analyt Technol Biomed Life Sci* 1057 (2017) 110–117.
- [12]. Wu J, Sanai N, Bao X, LoRusso P, Li J, An aqueous normal-phase chromatography coupled with tandem mass spectrometry method for determining unbound brain-to-plasma concentration ratio of AZD1775, a Wee1 kinase inhibitor, in patients with glioblastoma, *J Chromatogr B Analyt Technol Biomed Life Sci* 1028 (2016) 25–32.
- [13]. Li J, Brahmer J, Messersmith W, Hidalgo M, Baker SD, Binding of gefitinib, an inhibitor of epidermal growth factor receptor-tyrosine kinase, to plasma proteins and blood cells: in vitro and in cancer patients, *Invest New Drugs* 24(4) (2006) 291–7. [PubMed: 16502356]

- [14]. Bao X, Wu J, Sanai N, Li J, A liquid chromatography with tandem mass spectrometry method for quantitating total and unbound ceritinib in patient plasma and brain tumor, *J Pharm Anal* 8(1) (2018) 20–26. [PubMed: 29568664]
- [15]. Wan H, Rehngren M, Giordanetto F, Bergstrom F, Tunek A, High-throughput screening of drug-brain tissue binding and in silico prediction for assessment of central nervous system drug delivery, *J Med Chem* 50(19) (2007) 4606–15. [PubMed: 17725338]
- [16]. FDA Guidance on Bioanalytical Method Validation. <https://www.fda.gov/downloads/drugs/guidances/ucm070107.Pdf>.
- [17]. Wiegand R, Wu J, Sha X, LoRusso P, Li J, Simultaneous determination of ABT-888, a poly (ADP-ribose) polymerase inhibitor, and its metabolite in human plasma by liquid chromatography/tandem mass spectrometry, *J Chromatogr B Analyt Technol Biomed Life Sci* 878(3-4) 333–9.
- [18]. Pesek JJ, Matyska MT, A new approach to bioanalysis: aqueous normal-phase chromatography with silica hydride stationary phases, *Bioanalysis* 4(7) (2012) 845–53. [PubMed: 22512801]

Highlights:

- A validated LC-MS/MS method based on aqueous normal-phase chromatography mechanism
- Determination of total and unbound concentrations of ribociclib in human plasma and brain tumors
- Evaluation of the central nervous system penetration of ribociclib

Fig. 1. Representative parent ion scan spectra (left) and product ion scan spectra (right) of (A) ribociclib and (B) the internal standard $^{13}\text{C}_6$ ribociclib. The mass transitions m/z 435.3 $\rightarrow$ 367.2 and 441.3 $\rightarrow$ 373.2 were selected for monitoring ribociclib and $^{13}\text{C}_6$ ribociclib, respectively.

Fig. 2.

Extracted ion chromatograms of ribociclib (200 nM in the mobile phase) monitored at m/z 435.3 $\rightarrow$ 367.2 using (A) Waters Xterra MS C8 column (2.1×50 mm, $3.5 \mu\text{m}$) under gradient elution with the mobile phase of acetonitrile (containing 0.1% formic acid) and 0.1% formic acid in water (at a flow rate of 0.3 mL/min); (B) Waters Acquity UPLC BEH C18 column (2.1×50 mm, $1.7 \mu\text{m}$) under gradient elution with the mobile phase of acetonitrile (containing 0.1% formic acid) and 0.1% formic acid in water (at a flow rate of 0.3 mL/min); and (C) Waters Xbridge Amide column (4.6×100 mm, $3.5 \mu\text{m}$) under isocratic elution with the mobile phase of acetonitrile-ammonium formate (10 mM, pH 3.0) (75:25, v/v) (at a flow rate of 0.8 mL/min).

Fig. 3. Retention behavior of ribociclib on an Waters XBridge Amide column (4.6×100 mm, $3.5 \mu\text{m}$) under isocratic elution with the mobile phase consisting of different ratios of acetonitrile and ammonium formate (10 mM, pH 3.0). As increasing acetonitrile ratio from 10% to 90% in the mobile phase, ribociclib exhibited a U-shape retention curve on the Waters XBridge Amide column, indicating the retention mechanism was changing from reversed-phase to aqueous normal-phase chromatography.

Fig. 4. Extracted ion chromatograms monitored at m/z 435.3 $\rightarrow$ 367.2 for ribociclib and 441.3 $\rightarrow$ 373.2 for $^{13}\text{C}_6$ ribociclib in (A and B) blank human plasma; (C and D) human plasma spiked with ribociclib at LLOQ (0.5 nM); and (E and F) a patient plasma sample collected at 7.0 h after oral administration of ribociclib (900 mg). All plasma samples were protein precipitated with 5 volumes of acetonitrile containing the internal standard $^{13}\text{C}_6$ ribociclib (5 nM).

Fig. 5. Observed plasma concentration – time profiles of total ribociclib in 5 individual patients with malignant primary brain cancer, following oral administration of the 5th daily dose of ribociclib (900 mg)

Fig. 6. Fraction unbound of ribociclib (at 0.1, 1, and 10 μM) in pooled human plasma, determined by the equilibrium dialysis method at the equilibrium time of 2, 4, 6, 16, and 24 h. The optimum equilibrium time was established at 16 h, at which the dialysis reached equilibrium. Data are shown as the mean of triplicate measurements.

Table 1

Intra- and inter-day precision and accuracy of ribociclib calibrator standards in human plasma^a

Nominal concentration (μM)	Determined concentration (μM)	Average accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
0.5 (LLOQ)	0.50 ± 0.00	100	4.0	2.0
1	0.90 ± 0.00	90	2.5	₋ ^b
2	1.85 ± 0.15	92	6.6	5.4
5	5.17 ± 0.14	103	3.0	₋ ^b
10	10.15 ± 0.55	102	6.7	₋ ^b
20	20.48 ± 0.63	102	1.1	3.2
50	54.93 ± 1.42	110	1.7	2.2
100	101.48 ± 4.21	102	2.5	3.7
200	198.50 ± 6.21	99	3.9	₋ ^b
500	492.62 ± 13.95	98	2.3	1.8
1000	993.18 ± 24.72	99	2.6	₋ ^b

^a Each calibrator standard was evaluated in duplicate on three different days.

^b No additional variation was observed as a result of performing assay in different days.

Table 2

Intra- and inter-day precision and accuracy of ribociclib quality control samples in human plasma^a

Nominal concentration (μM)	Determined concentration (μM)	Average accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
0.5 (LLOQ)	0.53 ± 0.05	106	8.1	4.2
1.5 (LQC)	1.41 ± 0.10	94	4.7	6.1
300 (MQC)	287.09 ± 10.51	96	3.0	2.4
800 (HQC)	753.01 ± 63.79	94	2.6	9.5

^a Each QC was performed in quintuplicate on three different days.

Table 3

Matrix effect and recovery of ribociclib and $^{13}\text{C}_6$ ribociclib from 6 different donors of human plasma

Analyte	Nominal concentration (μM) ^a	Absolute		Internal standard-normalized	
		Matrix factor ^b	Recovery (%) ^c	Matrix factor ^e	Recovery (%) ^f
ribociclib	1.5	1.11 (6.4)	98 (2.7)	0.94 (1.8)	98 (2.6)
	300	1.06 (6.0)	100 (2.3)	1.01 (2.8)	100 (2.2)
	800	1.05 (4.7)	99 (3.4)	0.99 (2.1)	99 (2.0)
$^{13}\text{C}_6$ ribociclib	5	1.09 (4.0)	112 (6.4)	NA	NA

^aThe nominal concentrations of the analyte spiked in 100 μL of plasma before precipitation with 5 volumes of acetonitrile (Set 1). The same amounts of the analyte as in Set 1 were spiked in 600 μL mobile phase and in plasma extract for Set 2 and 3, respectively.

^bAbsolute matrix factor is calculated as the ratio of the mean peak area of ribociclib spiked post precipitation (Set 3) to the mean peak area of the same amount of analyte spiked in the mobile phase (Set 2). Data are shown as the mean (%CV) from six donors of plasma extract.

^cAbsolute recovery is calculated as the ratio of the mean peak area of ribociclib spiked in plasma before precipitation (Set 1) to that spiked post extraction (Set 3). Data are shown as the mean (%CV) from six different donors of plasma.

^eInternal standard-normalized matrix effect is expressed as the ratio of the mean peak area ratio of ribociclib to the internal standard in post-extraction matrix (Set 3) to that in the mobile phase (Set 2). Data are shown as the mean (%CV) from six donors of plasma extract.

^fInternal standard-normalized recovery is calculated as the ratio of the mean peak area ratio of ribociclib to the internal standard in plasma (Set 1) to that in post-extraction matrix (Set 3). Data are shown as the mean (%CV) from six different donors of plasma.

Table 4

Assessment of stability of ribociclib

	Ribociclib ^c	
	1 µM	100 µM
Bench-top stability (in 50% acetonitrile) (25°C) (%) ^a		
1.0h	89	97
2.0h	94	98
4.0h	103	104
6.0h	108	107
Bench-top stability (in plasma) (25°C) (%) ^a	1.5 nM	800 nM
1.0h	98	97
2.0h	96	104
4.0h	109	102
6.0h	109	101
Auto-sampler stability (in post-extracted plasma samples) (4°C) (%) ^a	1.5 nM	800 nM
1.0h	100	99
4.0h	96	100
6.0h	100	98
8.0h	100	99
10.0h	98	100
12.0h	96	100
Freeze-thaw stability (in plasma) (−80°C) (%) ^b	1.5 nM	800 nM
Cycle 1	100	101
Cycle 2	92	93
Cycle 3	85	94
Long-term stability (in plasma) (−80°C) (%) ^b	1.5 nM	800 nM
1months	102	114
3month	92	105
7months	96	105

^aStability data is expressed as the mean percentage of the peak area determined at certain time relative to that at time zero.

^bStability data is expressed as the mean percentage of the analyte concentration determined at certain time point relative to the nominal concentration (%).

^cEach concentration at each time point was assessed in triplicate.