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Short communication

Quantification of ribociclib in dried blood spots by LC–MS/MS: Method development and clinical validation

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ABSTRACT

A reliable, specific, selective and robust liquid chromatography-tandem mass spectrometry (LC–MS/MS) method was developed for the determination of ribociclib in both dried blood spot (DBS) samples and potassium EDTA plasma. DBS samples were obtained simultaneously with a plasma sample in advanced breast cancer patients treated with ribociclib. A 6 mm disk from the central part of the dried blood spot sample was punched, followed by extraction of ribociclib using liquid-liquid extraction spiked with ribociclib-d6 as internal standard. Concentrations of ribociclib in DBS samples were correlated with corresponding plasma concentrations. From the blood sample also hematocrit was determined. The method was validated for selectivity, sensitivity, precision, lower limit of detection, linearity, stability and accuracy according to the food and drug administration (FDA) guideline. The within- and between-run precisions were ≤10.6 and ≤1.07 %, respectively; while the average accuracy ranged from 100 to 103 %. The influence of hematocrit on validation parameters was tested in the range of 0.20 – 0.40 L/L. No influence of hematocrit on validation parameters was observed. Regression analysis and a Bland-Altman plot indicated correlation between the results obtained from DBS and plasma samples. A strong correlation (R^2 >0.97) between DBS samples and plasma concentration from 17 breast cancer patients was found. A number of 12 out of 17 processed DBS samples (71 %) fell inside the acceptable range of 20 % difference of simultaneously obtained plasma samples. The lower limit of quantification in DBS is 10.0 ng/mL and linearity was demonstrated up to 1000 ng/mL. In conclusion, the newly developed assay met the required standard for validation. The methods were used to study ribociclib disposition in patients with advanced breast cancer.

1. Introduction

Worldwide, hormone sensitive breast cancer is the most common cancer diagnosis among women. In the US and Europe, about 6% of women are diagnosed with *de novo* metastatic breast cancer. [1] Endocrine therapy is an effective, well-tolerated treatment option for metastatic and advanced breast cancer, however almost all patients develop resistance. [2] In recent years, a novel treatment option, *i.e.* the class of oral cyclin dependent kinase (CDK) 4/6 inhibitors, has been introduced for patients with advanced or metastatic hormone sensitive breast cancer. [3] Clinical trials demonstrated that CDK4/6 inhibitors in combination with endocrine therapy lead to a statistically significant improvement in progression-free survival. [4]

There are currently three CDK4/6 inhibitors – palbociclib, ribociclib and abemaciclib – approved by the Food and Drug Administration (FDA) for hormone sensitive metastatic or advanced breast cancer in combination with endocrine therapy. [3] Furthermore, clinical trials are ongoing to potentially extend the indication area to other solid tumour types (NCT02933736; NCT04000529; NCT03673124; NCT02555189).

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In oncology, many pharmacokinetic targets for therapeutic drug monitoring (TDM) are being identified to individualise the dosage of oral anticancer agents. [5] A randomized phase III trial is ongoing to identify the pharmacokinetic targets and exposure-response relationship for the three CDK4/6 inhibitors. [6] For TDM, preferably a trough concentration is measured for interpretation of the systemic drug concentration. [7] This is difficult to achieve since conventional blood sampling often takes place immediately before a hospital visit at a random time point. For that reason and to offer a more patient friendly sampling technique, dried blood spot (DBS) sampling has been developed. With this technique a patient collects a drop of blood from his/her finger (<50 μL) on a small blood filter card at home and sends it by regular mail to the laboratory for analysis. Advantages of the DBS method are a better stability because of less enzymatic degradation compared to plasma; easier to sample a trough concentration by self-sampling and patients with phlebitis are no longer excluded from frequent blood sampling. [8–10]

Currently, some assays for ribociclib are available in human plasma and brain tumour tissue. [11–13] However, a DBS method for ribociclib has not yet been developed. Considering the advantages of a DBS method for patients and the possibility of applicability in pharmacokinetic research, the availability of a DBS method is desirable. The collection of a DBS sample is a more patient friendly method and is therefore useful for therapeutic drug monitoring in the near future. Therefore, the aim of this study was to develop and validate a DBS method for advanced breast cancer patients treated with ribociclib.

2. Materials and methods

2.1. Chemicals and materials

Ribociclib ($C_{23}H_{30}N_8O$) was obtained from TRC (Toronto, ON, Canada) and ribociclib-d6 was obtained from Clearsynth (Mississauga, ON, Canada). Acetonitrile, methanol and water were purchased from Biosolve (Valkenswaard, The Netherlands). Ammonium formate was obtained from Honeywell GmbH (Seelze, Germany). Dimethyl sulphoxide (DMSO) was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Formic acid was obtained from J.T. Baker (Deventer, The Netherlands). 2-propanol was obtained from Merck GmbH (Darmstadt, Germany). Nitrogen was purchased from Linde gas (Schiedam, the Netherlands). The Whatman 903™ protein saver cards were supplied by GE Healthcare companies (Cardiff, UK). Sterile safety 1.8 mm lancets were obtained from Vitrex Medical A/S (Herlev, Denmark). A regular puncher (Fiskars, Helsinki, Finland) was used for punching the DBS disks out of the spotting card. Blank human plasma was supplied by Biological Specialty Corporation (Colmar, PA, USA). In all experiments ribociclib-free human whole blood (Dutch blood donation center Sanquin, Rotterdam, The Netherlands) was used.

2.2. Preparation of stock solution, calibrators and QC samples

Ribociclib working stock solution (100,000 ng/mL) was prepared in DMSO and stored at T <−70 °C. Calibration curve working solutions were prepared in acetonitrile/DMSO (1:1, *v/v*). The internal standard ribociclib-d6 (100 ng/mL) was prepared in methanol (DBS) or acetonitrile (plasma) and stored in a fridge T = 3–7 °C. Calibration curve standards were diluted in human ribociclib-free EDTA whole blood (DBS) or lithium heparinized plasma (plasma) to construct the calibration curves yielding amounts of: 10.0, 25.0, 100, 250, 500, 750, 900 and 1000 ng/mL. The stock solution was diluted in organic solvent followed by preparation of individual levels from corresponding organic solvent dilutions. Four levels of quality controls (QCs) were prepared in EDTA blood (DBS) or lithium heparinized plasma (plasma), including the lower limit of quantification (LLOQ) (10.0 ng/mL), low-level (30.0 ng/mL), middle-level (400 ng/mL) and high-level (800 ng/mL). Calibration curves prepared in plasma were freshly prepared on the day of analysis, while pools of QC samples prepared in plasma were aliquotted and stored at T<−70 °C until analyses. 50 μL calibration curve standards and pools of QC-samples prepared in EDTA blood were spotted onto Whatman 903™ Protein Saver Cards in the center of the spot and dried for 24 h at ambient temperature. Hereafter they were stored in a sealed back at ambient temperature, protected from light, until analyses.

2.3. Study set-up and sample collection

A cross-sectional observational study was set up to determine ribociclib concentrations in both DBS and plasma samples. In this study, patients were eligible to enroll when they use ribociclib on a dose according to the prescription of the physician. On a random hospital visit at day 7–21 of a ribociclib cycle a DBS sample was obtained simultaneously with the peripheral venous sample. After an instruction from a nurse, a fingertip sample was performed by the patient. The blood sample was spotted onto a Whatman 903™ Protein Saver Card. The spotted cards were stored (protected from light in a sealed bag) at room temperature until analyses. From the peripheral venous blood sample used to generate plasma also hematocrit was determined by a DxH 500 hematology analyzer (Beckman Coulter Nederland B.V., Woerden, the Netherlands). Our study protocol was approved by the ethics committee of the Erasmus Medical Center (MEC 19−0467) and registered in the Netherlands Trial Register (www.trialregister.nl; number NL8197). Prior to collection of both plasma and DBS samples all patients provided written informed consent.

2.4. Plasma sample preparation and extraction

Ribociclib in human plasma was quantified by a validated LC–MS/MS method in our laboratory (unpublished data; Erasmus MC Cancer Institute, the Netherlands). An amount of 25 μL plasma was mixed with 100 μL of internal standard (200 ng/mL ribociclib-d6 in acetonitrile). After vortex mixing for 5 s and centrifugation for 10 min at 12,000*g*, an amount of 50 μL of the clear supernatant was transferred to a 96-well plate and mixed with 100 μL of 5 mM ammonium formate/water/formic acid (100:0.1, v/v) from which 10 μL was injected into the LC–MS/MS system.

2.5. DBS sample preparation and extraction

Using a manual disk puncher in the center of the spot, a 6 mm punch of the DBS sample was transferred to a 2-mL safe lock vial containing 200 μL internal standard working solution. After mixing on a vortex for 10 s, samples were treated with ultrasound for 20 min at T = 40 ± 5 °C. An aliquot of 50 μL supernatant was transferred into a 350-μL 96-well plate and 100 μL of 5 mM ammonium formate/water/formic acid (100:0.1 v/v) was added where after the plate was shaken for 5 min on a rocked platform. Aliquots of 10 μL were injected onto the UPLC column.

2.6. Apparatus and chromatographic system

The LC–MS/MS system (Waters Chromatography B.V. Etten-Leur, the Netherlands) consisted of a UPLC sample Manager (Waters Acquity), coupled to a Waters TQ mass spectrometer. Separation was achieved using a 2.1 mm x 50 mm, 1.8 μm UPLC column (Waters, Acquity UPCL® HSS T3). Data was processed with MassLynx software V4.1 SCN627 and concentrations were calculated by using an integrator (QuanLynx software). The mobile

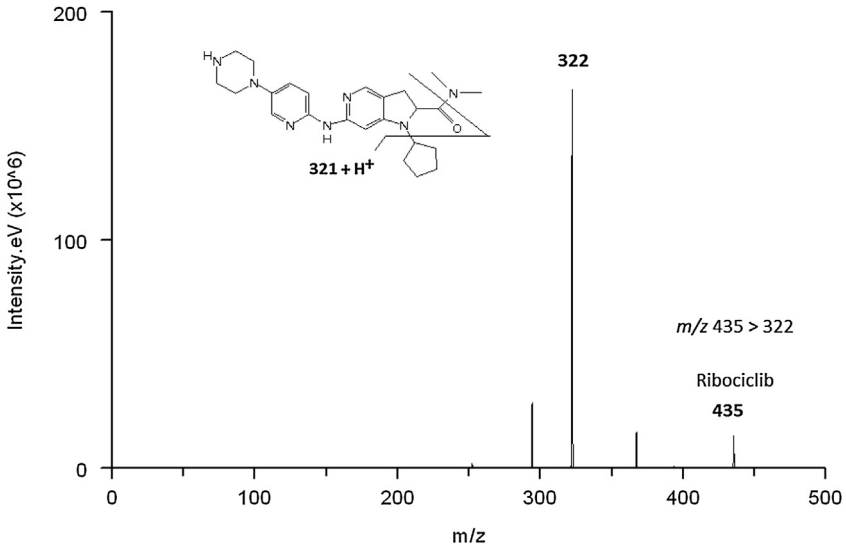

Fig. 1. Mass spectrum and chemical structure of ribociclib.

phase A consisted of 5 mM ammonium formate/water/formic acid (*0.5:99.5:0.1 v/v/v*) and 5 mM ammonium formate/methanol/formic acid (*0.5:99.5:0.1 v/v/v*) for mobile phase B. A linear gradient setting was used with 90–50% mobile phase A (*i.e.* 10–50 % mobile phase B) from 0 to 1 min, 50–0% from 1 to 2 min, 0–45 % from 2 to 2.5 min and held for 0.5 min and back to 0% mobile phase A from 3 to 4 min and held for 0.5 min and back to 45 % at 5.5 min. In 1 min, mobile phase was returned to initial situation and re-equilibrate for 0.5 min. To prevent a carry-over effect the second wash-step was incorporated into the method. The overall run-time was 7 min. The temperature of the column was set at T = 40 °C with a flow rate of 0.400 mL/min.

2.7. Mass spectrometry

Quantification was conducted with the positive multiple reaction monitoring (MRM+) mode, with use of argon gas collision induced dissociation, which resulted in the subsequent *m/z* ion transitions (*435 > 322*) for ribociclib and (*442 > 322*) for the internal standard. Primary to secondary ion ratios, *435 > 322 / 435 > 367* for ribociclib and *442 > 322 / 442 > 373* for the internal standard were used to show the observed peaks confirm identity. In Fig. 1 a typical mass spectrum for ribociclib is shown which was obtained with a cone voltage and collision energy of 50 V and 35 V, respectively. The following internal parameters of the device were used: capillary voltage 3.50 kV; source temperature 120 °C; desolvation temperature 350 °C; cone gas (nitrogen) 25 L/h; desolvation gas (nitrogen) 800 L/h and a collision cell pirani pressure of ~$5.5e^{-3}$ mbar (measure for organic gas flow). Calibration curves were constructed by linear-regression analysis in a range of 10.0–1,000 ng/mL. Weighted Linear regression ($1/concentration^2$) was performed in the range of 10–1000 ng/mL with peak area ratio (Analyte/IS) as dependent variable.

2.8. Method validation

The analytical method validation was performed at a standardized blood hematocrit value (0.40 L/L, *i.e.* 40 %). Selectivity, accuracy (ACC), within-run precision (WRP), between-run precision (BRP), extraction recovery, matrix effect, carry-over and stability for both DBS and plasma samples were assessed. The method validation was based on the recommendations and criteria for bioanalytical method validation of the Food and Drug Administration (FDA) and the draft of ICH M10 Only matrix effect and recovery of DBS samples were tested on five lots of individual donors instead of the recommended six lots.

2.8.1. Extraction recovery and matrix effect

Extraction recovery was determined by comparing the MS/MS response of ribociclib at QC-Low and QC-High in six different lots of human potassium EDTA plasma before extraction *versus* extracts of six different lots of blank human potassium EDTA plasma after extraction. The influence of matrix components on the ribociclib ionization was evaluated by comparing the MS/MS response of ribociclib at QC-Low and QC-High spiked concentrations to the MS/MS responses of ribociclib spiked in triplicate into extracts of six human potassium EDTA plasma samples. The matrix effect for DBS samples was assessed by spiking five different lots of blank human whole blood (of individual donors) with ribociclib at concentrations of 30.0 ng/mL (QC-Low) and 800 ng/mL (QC-High). Amounts of 50 µL of blood was applied on the Whatman 903™ protein saver card and dried for 24 h at room temperature. Hereafter, the samples were further prepared according to section 2.5. Matrix and recovery were determined as described earlier in literature. [14]

2.8.2. Stability

The stability of ribociclib in plasma during three freeze-thaw cycles was tested in triplicate at the concentrations of QC-Low, QC-High and QC-Diluted. The stability in human potassium EDTA plasma of ribociclib at ambient temperature was tested in triplicate at the concentrations of QC-Low and QC-High. The stability of ribociclib in DBS samples were tested in triplicate at concentrations of 30.0 ng/mL (QC-low) and 800 ng/mL (QC-high) ribociclib. The DBS samples were stored – protected from light – in a controlled cabin at 20 ± 5 °C, in a fridge at T = 3–7 °C (in a sealed bag of 2 g including silica gel desiccant) and at room temperature for at least 5 months.

2.8.3. Hematocrit effect

In general, a point of attention in dried blood spot methods is the inter-individual variability in hematocrit concentration; as this can greatly influence the spreadability and rheological properties of the blood spot on the filter paper. This is especially relevant for compounds with large differences between plasma and whole blood concentrations. [15–17] In addition, hematocrit levels can vary greatly in certain (oncological) diseases. Therefore, a potential hematocrit effect should be considered during the clinical validation of a DBS method. [18] To investigate the potential influence of

Table 1
Calculations of the grand mean, accuracy and within-run and between-run precisions of the LLOQ and QC samples[a].

Sample	Spiked (ng/mL)	GM (ng/mL)	ACC (%)	WRP (%)	BRP (%)	n[b]
Plasma						
LLOQ	10	10	100	8.6	3.58	15 of 15
QC Low	30	29.5	98.3	3.64	1.25	15 of 15
QC Middle	400	395	98.8	3.94	2.04	15 of 15
QC High	800	779	97.4	2.21	0.904	15 of 15
QC Diluted	8000	7628	95.4	3.51	2.64	15 of 15
DBS						
LLOQ	10	10.3	103	10.6	1.07	14 of 15
QC Low	30	30.6	102	6.52	#	15 of 15
QC Middle	400	400	100	4.1	#	15 of 15
QC High	800	810	101	4.52	#	15 of 15

Abbreviations: GM, grand mean; ACC, average accuracy; WRP, within-run precision; BRP, between-run precision; DBS, dried blood spot. Symbol: #: no additional variation observed by performing the assay in different runs. [a] n=5 in 3 separate runs. [b] Number of individual samples falling within acceptable range of accuracy of 85–115 % (80–120 % at LLOQ).

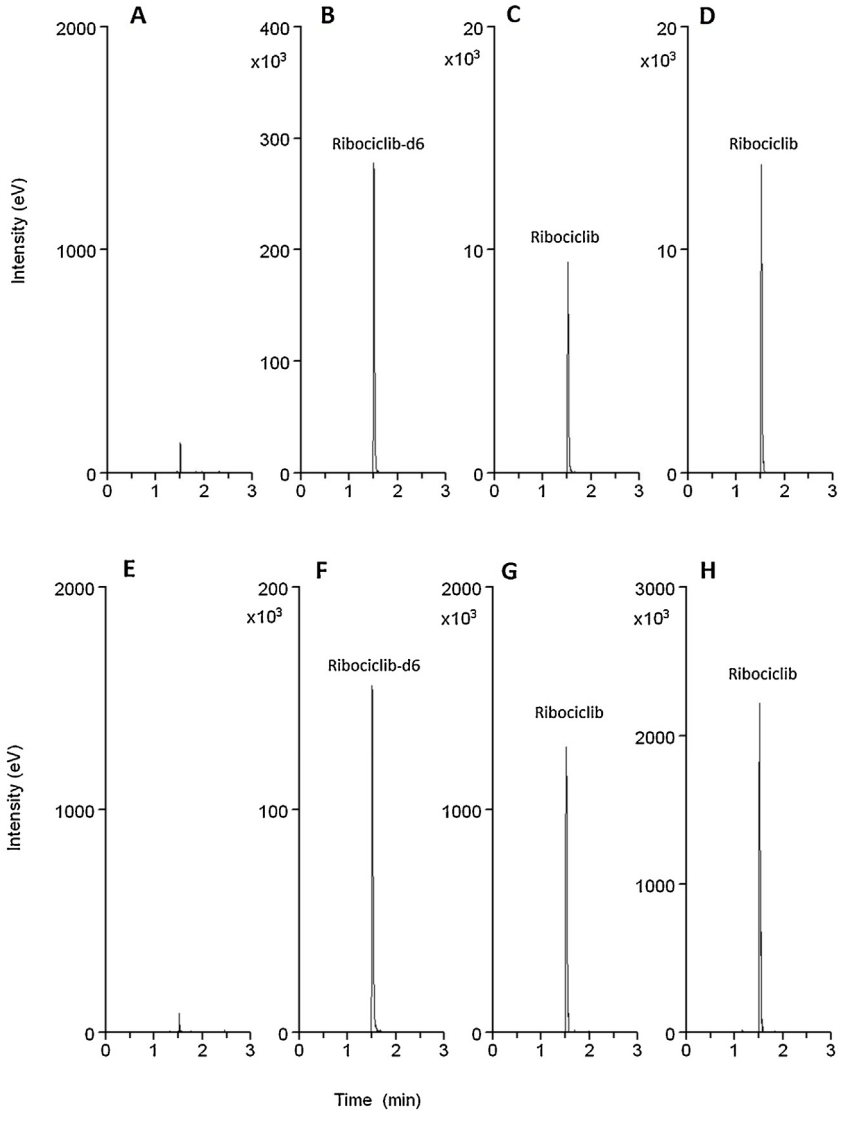

Fig. 2. Representative chromatograms of (A) double blank processed plasma sample, (B) blank processed plasma sample with Internal Standard, (C) spiked plasma sample containing 10.0 ng/mL ribociclib (LLOQ), (D) plasma sample collected prior to the administration of a fixed oral dose of 600 mg ribociclib once daily, containing 15.1 ng/mL ribociclib, (E) blank processed DBS sample, (F) blank processed DBS sample with Internal Standard, (G) DBS sample spiked with 10.0 ng/mL ribociclib (LLOQ) and (H) DBS sample collected simultaneously with a regular plasma sample (See D) containing 15.0 ng/mL ribociclib.

Table 2
Stability of ribociclib in DBS samples.

Condition	% to control Concentration	
	Low (RSD%)	High (RSD%)
Plasma ribociclib (n=3)		
5 days Ambient temperature[1]	91 (1.0)	97 (6.0)
3 freeze-thaw -cycles[1]	108 (12)	99 (2.7)
DBS ribociclib (n=3)		
Controlled cabin, T = 20 °C, RH 25% (5 months)[1]	106 (7.7)	102 (2.9)
Fridge, T= 3–7 °C (5 months)[1]	107 (9.8)	94 (3.7)
Ambient Temperature (5 months)[1]	106 (7.8)	103 (8.2)

[1] *versus* value non-treated.

hematocrit on the concentration measurement of ribociclib, DBS samples were prepared in whole blood with different relevant hematocrit concentrations (0.20, 0.35 and 0.40 L/L) and spiked at concentrations of 30.0 ng/mL (accuracy 94–104 % and RSD 2.7–8.3%, respectively) and 800 ng/mL (accuracy 90–108 % and RSD 2.2–4.8 %, respectively) ribociclib; with an acceptance criterion %RSD ≤ 15 %.

2.9. Data analysis

To evaluate the correlation between ribociclib concentrations in different biological matrices a correlation coefficient was calculated (Microsoft Excel 2016). The correlation coefficient measures the strength and direction of a relationship between two variables. Bland-Altman analyses were performed to evaluate the correlation between the ribociclib concentrations in both matrices. [19] At least 67 % of samples should have a prediction error of <20 %, in accordance with to the criteria for validation of the European guideline on bioanalytical method validation for industry. [17]

3. Results and discussion

3.1. LC–MS/MS conditions

For the quantification of ribociclib MRM transition, cone voltage and collision energy were optimized by direct infusion. The mass spectrum of ribociclib is displayed in Fig. 1. The lower limit of quantification (LLOQ) was 10.0 ng/mL and calibration curves were linear with concentrations in the range of 10.0 – 1000 ng/mL (weighting factor of 1/concentration^2), as shown by the mean correlation coefficient of 0.97 (n = 17; Y-intercept = 0.066; mean RSD 94.3 %). Ribociclib was also validated for quantification in human potassium EDTA plasma on our laboratory in the range of 10.0 – 1000 ng/mL (Table 1). In Fig. 2, representative chromatograms are shown which were obtained after processing of a blank DBS sample and DBS spiked with Internal Standard and 10.0 ng/mL (LLOQ) ribociclib. Also a DBS sample collected from a representative breast cancer patient – containing 15.0 ng/mL ribociclib – was included.

3.2. General method validation

The method fulfilled the acceptance criteria of the bioanalytical method validation FDA and ICH Q2 (R1) guidelines regarding accuracy and precision of the calibrators and QCs. The mean accuracy ranged from 100 to 103 %. For ribociclib, the within- and between-run precisions at four tested concentrations were ≤10.6 % and ≤1.07 % including the LLOQ. The absolute deviations from the nominal value of all analyzed calibrators met the acceptance criteria of ≤15 % for nominal concentrations (≤20 % for the LLOQ). The mean accuracy, within- and between-run precision – assayed in quintuplicate on three occasions with the calibrators in duplicate – for both plasma and DBS samples are depicted in Table 1. No interferences from endogenous compounds in human potassium EDTA whole blood and carry-over effects were observed. Also no major matrix effect (mean 126 ± 5.42 (QC-Low) and 112 ± 2.12 (QC-High), respectively) was observed and the mean extraction efficiency was 72.0 ± 7.29 (QC-Low) an 64.8 ± 2.73 (QC-High), respectively. Ribociclib DBS samples were proven to be stable for at least 5 months when stored in a controlled cabin (T = 20 °C; relative air humidity (RH) 25 %), at ambient temperature (T = 20 °C) protected from light or in a fridge (T = 3–7 °C); with mean percentages to control of 104 %, 101 % and 105 %, respectively (Table 2). The validation parameters were not influenced by hematocrit in a relevant range for patients with cancer of 0.20–0.40 L/L. The bias from nominal value of hematocrit on spot volume was maximal 5% and 10 % for QC-Low and QC-High, respectively.

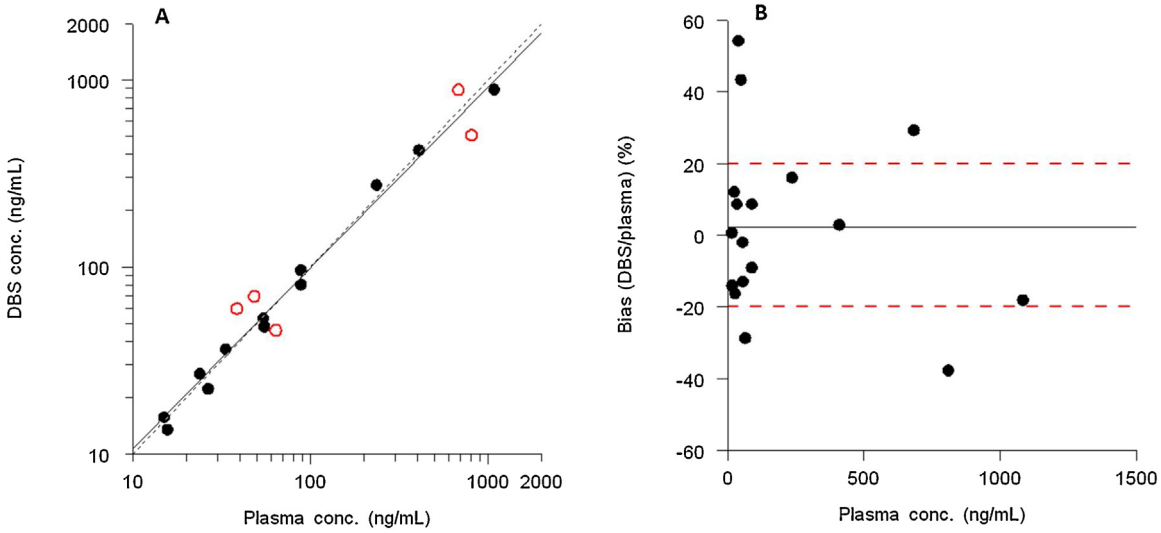

Fig. 3. (A) Correlation (R^2 >0.97) between ribociclib concentration in dried blood spot (DBS) and plasma samples from 17 breast cancer patients treated with ribociclib. Linear regression line (black lines) and the identity line (dashed line) are provided, while open dots represent DBS sample with a %DEV > 20 % compared with corresponding plasma sample. (B) Bland-Altman plot for total plasma and DBS. The dotted lines indicate the limits of agreement, and the solid line the mean ratio bias of 0.996.

3.3. Clinical application

Clinical validation has been performed by analyzing DBS samples and corresponding plasma samples. Our analysis showed that in 17/19 (89.5 %) of the patients plasma as well DBS samples could be determined. In one patient, the concentration in the DBS sample was above the highest calibration curve standard; and another patient was excluded due to an error in sampling of the DBS sample. In our clinical validation trial both DBS and plasma samples of ribociclib concentrations could be determined in the range of 10.0 – 1000 ng/mL (Fig. 2). A strong correlation – with a coefficient of determination of R^2 >0.97 – between ribociclib concentration in DBS and plasma concentration from 17 advanced breast cancer patients is shown in Fig. 3. Bland-Altman analysis showed that a number of 12 out of 17 processed DBS samples (71 %) fall inside the acceptable range of 20 % difference of simultaneously obtained plasma samples (acceptance criterion ≥67 %). Ribociclib concentrations of DBS and plasma samples were similar, as the bland-Altman plot showed a mean ratio of nearly one (mean ratio 0.996). A limitation of our study is the relatively small study population with 17 evaluable patients. [17] However, in our analysis no influence of hematocrit was found, therefore the expected variation in DBS sampling is considerably smaller than in other DBS studies with a clear hematocrit effect. The method meets the FDA requirements on clinical validation of this sampling approach. [20] Therefore, our method is suitable for clinical studies investigating the pharmacokinetic profile of ribociclib in an outpatient setting.

4. Conclusion

In conclusion, the assay was successfully applied to quantify dried blood spot and plasma samples of breast cancer patients treated with ribociclib. In order to investigate the disposition of ribociclib a reliable, reproducible, selective and sensitive dried blood spot method was developed. The dried blood spot sampling methods had been validated for whole-blood in both DBS and plasma samples over a range of 10.0 – 1000 ng/mL. No influence of hematocrit (range 0.20 – 0.40 L/L) on validation parameters was observed. Therefore, both plasma and DBS method are suitable for a pharmacokinetic study to determine ribociclib concentrations.

Funding

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Ethical approval

The study was approved by the Local Ethics Committee (Erasmus MC, Rotterdam; MEC 19-0467) and was registered in the Dutch Trial Registry (www.trialregister.nl; number NL8197).

Author contribution

LB, ML, RM, PdB and SK developed the concept and design of this method and clinical validation. LB, TR, CvT, WH, JH, MB recruited patients for the blood sampling. LB led the writing of the manuscript. ML and PdB were responsible for the analysis on the laboratory. All authors contributed to data interpretation and preparation of the manuscript for publication and they approved the final version.

Declaration of Competing Interest

This work was supported through an unrestricted grant by Novartis, the Netherlands. Stijn L.W. Koolen has received this research grant from Novartis. C. Louwrens Braal, Mei H. Lam, Tineke Rienks, Claudia van Tilborg, Wendy Heuts, Joan B. Heijns, Monique E.M.M. Bos, Ron H.J. Mathijssen and Peter de Bruijn declare they have no conflicts of interest that might be relevant to the contents of this manuscript.

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