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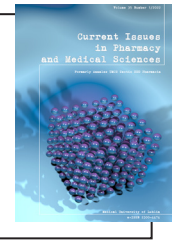

Development and validation of a new bioanalytical method for quantification of CDK4/6 inhibitor in Spiked Human Plasma by HPLC-UV

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ABSTRACT

A sensitive and accurate high performance liquid chromatography method utilizing ultraviolet/visible light detection (HPLC-UV) for the quantification of Ribociclib in Spiked Human Plasma by HPLC-UV was developed and validated. Ribociclib (RCB) and the internal standard (IS), Trifluridine, were first extracted from plasma samples by a simple Protein Precipitation extraction using Acetonitrile. Plasma concentration of RCB and internal standard were then analyzed by applying reversed phase chromatography using Orochem orosil C18 (4.6 mm × 250 mm, 5 µ) and elution with a isocratic mobile phase consisting of 10 mM phosphate buffer – Acetonitrile (60:40, v/v) adjusted to pH 3.0 at a flow rate of 1.0 ml/min. Detection of RCB and the IS was subsequently done at a wavelength of 260 nm. The limit of quantification was 10 ng/ml. The calibration curve was linear (R2>0.998) over the concentration range of 10-1000 ng/ml. For human plasma, the accuracy and precision were within ±15% and ≤15%, respectively, for all concentrations, except for the lower limit of quantification, where they were within ≤20%.

INTRODUCTION

Ribociclib (RCB) or 7-Cyclopentyl-N,N-dimethyl-2-{[5-(1-piperazinyl)-2-pyridinyl]amino}-7H-pyrrolo[2,3-d] pyrimidine-6-carboxamide is a pyrimidine derivative with a chemical structure unrelated to other anticancer drugs (Figure 1) It is a white, odorless, and slightly bitter crystalline lipophilic powder, with a logP of 0.88, low solubility in water (40 mg l-1) and limited solubility in 0.1 N hydrochloric acid (63 mg l-1) and simulated intestinal fluid (59 mg l-1) [1].

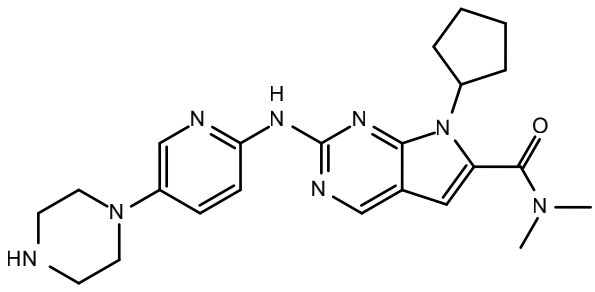

Figure 1. Chemical Structure of Ribociclib

In metastatic breast cancer patients with hormone receptor (HR)-positive and human epidermal growth factor receptor 2 (HER2)-negative tumours, endocrine therapy with or without targeted therapy, such as cyclin-dependent kinase 4/6 inhibitors or mTOR (mammalian target of rapamycin) inhibitors, effectively slows the progression of the disease [2]. The US Food and Drug Administration (USFDA) approved the selective CDK4/6 inhibitor ribociclib (RCB) in 2017. It is applied in conjunction with aromatase inhibitors to treat breast tumours that express the oestrogen receptor but lack the human epidermal growth factor receptor 2 [1,3-5]. Clinical trials showed that the use of CDK4/6 inhibitors in conjunction with endocrine treatment improves progression-free survival by a quantitatively considerable increase [6]. For small-molecule inhibitors of CDK4 and CDK6, ribociclib is highly selective and orally accessible, and ribociclib has shown therapeutic potential for a number of cancer types in preclinical and clinical studies with tolerable side effects.

Ribociclib was specifically approved (USDA, 2017) 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. It is used in combined with an aromatase

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inhibitor (such as letrozole) [7-10]. To personalize the dosage of oral anticancer agents, numerous pharmacokinetic targets for therapeutic drug monitoring (TDM) are being discovered via Anticancer Research development [11]. To interpret the systemic drug concentration for Theraputic drug Monitoring, a peak concentration is usually assessed [12].

Several high-performance liquid chromatographic methods coupled to mass spectrometry detection (HPLC–MS and HPLC–MS/MS) [13-16] and ultraviolet detection (HPLC–UV) [17] or for the simultaneous determination of RCB and some of their metabolites have been reported in the literature during the last 5 years. However, the methods reported involved the use of costly sample preparation methods such as solid phase extraction, and relied on less commonly available internal standards. Hence the purpose of the present work was to develop a new simple, economical Protein Precipitation RP-HPLC-UV method for the estimation of RCB from spiked human plasma.

EXPERIMENTAL

Chemicals and reagents

Authentic standards of RCB obtained from Intas Pharmaceuticals, Ahmadabad as a gift sample and Trifluridine (TRI) as an Internal Standard (IS) were purchased from (Sigma Aldrich, USA). HPLC grade acetonitrile, Analytical Grade Sodium acetate, glacial acetic and orthophsphoric acid were purchased from SD fine chemicals, Mumbai, India. HPLC grade water and Millipore membrane filter (0.22 mm, Millipore) were used throughout the experiments.

Stock solutions and calibrators

Stock solutions of RCB were first prepared in Water: Acetonitrile (50:50) diluent at the concentration of 10000 µg/ml, with two different stock solutions obtained for each compound – one for the calibration curve and the other for QCs. To create working solutions for the construction of the calibration curve (from A to H), the stock solutions of RCB were mixed together and diluted with diluent to achieve final RCB concentrations of 10, 20, 50, 100, 250, 500, 800 and 1000 ng/ml. The stock solutions for QCs (H-high, M-medium, L-low) were subsequently mixed together and diluted with Water: Acetonitrile (50:50) to obtain the final RCB concentrations of 50, 500, 800 ng/ml. Stock solutions of IS were prepared in water for Trifluridine at the concentrations of 50 ng/ml. The three working solutions were mixed together and diluted with Water:Acetonitrile (50:50). This solution was directly used to precipitate plasma proteins during sample treatment.

Biological sample

For the preparation of calibrators and QC's, pooled drug free human plasma was purchased from Dr. Vasantrao Pawar Medical College, Hospital & Research, Nashik, Maharashtra. Drug-free matrices were used for validation studies.

Sample preparation

The plasma samples were aliquoted into polypropylene centrifuge eppendorf tubes of 1.5 ml and stored at -20°C in a deep freeze until analysis, and on the day of analysis, the plasma was defrosted through centrifugation by REMI centrifuge. In the procedure, an aliquot of 975 µL of plasma samples was pipetted by using micropipette and transferred into labeled disposable polypropylene eppendorf tubes having capacity of 1.5 ml, and 25 µL of IS solution was added. Subsequently, 500 µL of acetonitrile was added after the tubes had been vortex-mixed for 30 seconds in order to precipitate the proteins. The tubes were then vortex-mixed for a further 60 s and centrifuged at 7000 rpm for 10 min at 4°C (Figure 2).

Protein precipitation procedure

After adding 25 µL of working solutions to 975 µL of blank human plasma, the mixture was vortexed, and then it was centrifuged for 15 minutes at a speed of 3000 revolutions per minute. The supernatant were transferred to a vial, and 10 µl was injected into the chromatograph for bioanalytical analysis. The final concentrations thus obtained were, 10, 20, 50, 100, 250, 500, 800 and 1000 ng/ml for RCB for the calibration curve; the same procedure was repeated for QC preparation.

HPLC-UV apparatus and conditions

The chromatographic system consisted of an Alliance e2695 Separation Module equipped with an online degasser and an automatic injector, as well as a 2998 photodiode array detector. This was set at 260 nm for the detection of RCB and IS. Separations were performed on an Orochem orosil C18 (4.6 mm × 250 mm, 5 µ) column maintained at 25°C. The mobile phase consisted of 10 mM Phosphate buffer at pH 3.0 (solvent A) and Acetonitrile (solvent B) in a (60:40, v/v) ratio delivered at a flow rate of 1 ml/min. The total run time of chromatographic separation was 5 min. Data were collected and processed using Empower 3 software for an HPLC system (Waters, Milan, Italy).

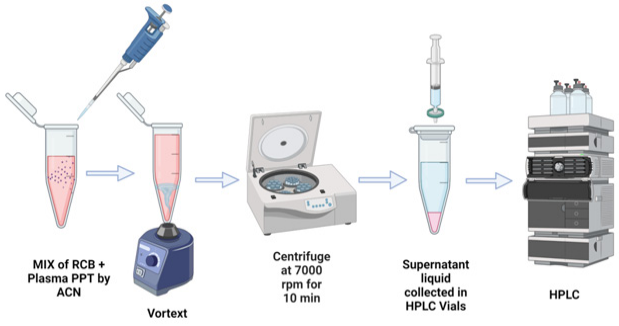

Figure 2. Sample extraction of RCB by protein precipitation

Chromatograph

The proposed HPLC-UV method was fully validated by using the specified acceptance criteria given in Bioanalytical Method Validation Guidance for Industry in May 2018 [18].

Lowest Limit Of Quantitation (LLOQ) & upper limit of quantitation (ULOQ)

The calibration range's precision and accuracy can be assessed separately or as a part of the LLOQ and ULOQ evaluation. The LLOQ is the least amount of an analyte for which a quantitative measurement may be made with

a reasonable degree of accuracy and precision. In contrast, the maximum amount of an analyte in a sample that can be precisely and accurately quantified is known as the ULOQ. The lower limit of quantification (LLOQ) was set as the first calibration curve point, while ULOQ was considered as the last point of the calibration curve. Practically, LLOQ=10% C_{max} and ULOQ=2 × C_{max}. For the reported analysis, concentration of LLOQ was 10 ng/ml and for ULOQ was 1000 ng/ml (Figure 3).

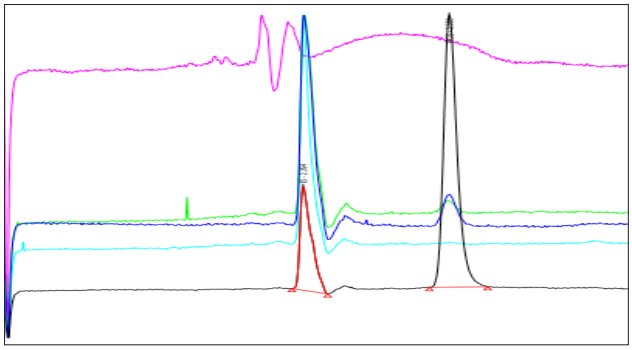

Figure 3. Overlay spectra of Aqueous blank, Plasma blank, LLOQ (10ng/ml), ULOQ (1000 ng/ml)

Specificity and selectivity

A peak purity test using a diode array detector was used to show the methods specificity. Herein, the diode array spectrum of RCB standard and IS (TRI) were recorded and compared. The retention time, Peak Area, % Peak Area, Peak Height, USP Plate Count, USP resolution and USP tailing were calculated by using Empower 3 software for the peaks of RCB & IS. The results obtain are shown in Figure 4 & details given in Table 1.

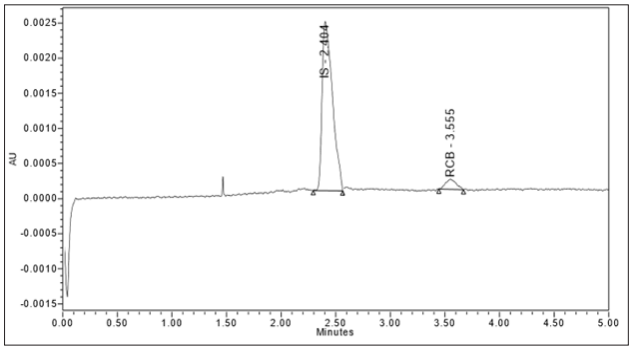

Figure 4. System suitability parameters of RCB at LLOQ (10 ng/ml)

Table 1. Details of System suitability parameters of RCB (10 ng/ml)

Peak Name	RT	Area	%Area	Height	USP Plate Count	USP Resolution	USP Tailing
IS	2.404	15779	94.29	2409	3060.0	---	1.8
RCB	3.555	956	5.71	141	5335.1	6.9	1.1

Calibration curve, precision and accuracy

Eight point Calibration curves were constructed by applying linear least-squares regression (1/×2 weighting regression) by taking ratios of Peak area and IS area peak (RCB area/IS area). The calibration curve of the x-axis demonstrated the concentration of RCB in ng/ml and the y-axis is the peak area ratio of RCB and IS. The method is linear if the coefficient of regression (r2) calculated as mean of 8 curves is ≥0.99 [19]. Eight concentrations of plasma samples served as the basis for the calibration curves used to estimate the levels of RCB in unknown samples. In each level, these samples were freshly prepared along with a plasma sample. Each calibration curve was obtained using 8 calibration levels; the ranges are listed in Table 1. Intraday and interday precision were determined by assaying 6 spiked plasma samples at 4 different RCB concentrations (LLOQ & 3QCs). Interday and intraday precisions were expressed as the relative standard deviation (RSD) at each QC concentration, including LLOQ.

Recovery from plasma using the extraction procedures was assessed by comparing the peak area ratio obtained from multiple analyses of spiked plasma samples (QCs) with IS excluding LLOQ with the peak height ratio from direct injections of the same amount of all analytes and IS. The Accuracy was calculated as the percent recovery from the nominal concentration at extracted and unextracted levels, and the accuracy of the method were assessed by calculating Mean, Standard deviation and % relative standard deviation (three replicates at each concentration of QC's. If recovery was above 80%, the assay was approved.

The results obtained for Intra-day and Inter-day precision were derived by calculating % RSD at each QC level, including LLOQ (Six replicates at each concentration). The result was considered acceptable if %RSD at each concentration was < ±5% of nominal concentrations (except ±20%) at LLOQ for both Intra-and inter-day precision, and the deviation of the mean from the true value was within < ±15% (excluding LLOQ) [19].

Stability

Drug stability in plasma samples was studied as per the Bioanalytical Method Validation Guidance [19]. During sample handling and collection, stability tests assessed the stability of RCB. These stability studies were performed as specified in guidelines and includes Short term (bench top) 8 hr at room temperature. Autosampler at 10°C for 24 hr was evaluated up to 24 h after extraction. The freeze-thaw stability was evaluated using 3 level QC samples determined after three FTS Cycles (freeze-thaw) of freezing at -60°C for 24 h and then thawing completely at room temperature. QC samples that were kept at -60°C for 28 days were used to assess the long-term stability.

RESULTS AND DISCUSSION

Optimization of HPLC-UV conditions

A new bioanalytical HPLC-UV method was established for RCB in our work. Various mobile phase systems (water (25): Acetonitrile (75), Phosphate Buffer pH – 5.0 (50): Acetonitrile (50), Phosphate Buffer pH – 3.0 (50): Acetonitrile(50) and Buffer pH – 3.0 (60): Acetonitrile (40)) were prepared and used to provide an appropriate chromatographic separation, but the proposed mobile phase comprising of Buffer and Acetonitrile in the ratio 60:40 gave a better resolution and sensitivity. The detection was carried out using a PDA detector at 260 nm using an Orochem orosil C18 (4.6 mm × 250 mm, 5 µ) column, and a 1 ml/min flow

Table 2. Retention time, UV wavelength, calibration levels and QC concentrations

Analyte	Retention time (min)	Wavelengths (nm)	Calibration Standards (ng/ml)								QC (ng/ml)		
			A	B	C	D	E	F	G	H	LQC	MQC	HQC
RCB	3.53	260	10	20	50	100	250	500	800	1000	50	500	800

rate yielded the best results for RCB in terms of retention durations and the theoretical plates, out of the three flow rates that were examined which included 0.9 ml/min and 0.8 ml/min. The best retention time was found to be 5.0 min for RCB. The USP tailing for RCB was found to be 1.1, demonstrating the symmetrical nature of the peak. For RCB, the number of theoretical plates was around 5335, confirming the column's effective operation. The details are shown in Table 2. Comparing this method to the HPLC-Tandem method published for RCB, these parameters show how specific the method is. Due to the easy sample extraction, reduced solvent consumption, quick analysis time, and economic impact, this method is safer, more environmentally beneficial and practical.

Calibration curve, model selection and verifications

A linear calibration range was selected based on weighting factor calculations. We found that 1/×2 was useable for calculations of equation of line which include slope, intercept and regression coefficient. The equation was applied to calculate the recovery, accuracy, precision, and stability using this calibration model, and validation over six days was sufficient to support usage. After model selection, the chosen calibration range was 10-1000 ng/ml. Its overlay, along with regression analysis are shown in Figures 5 and 6. Chromatograms were obtained from utilizing an Alliance e2695. Separation Module Data were collected and processed using Empower 3 software for HPLC systems (Waters, Milan, Italy).

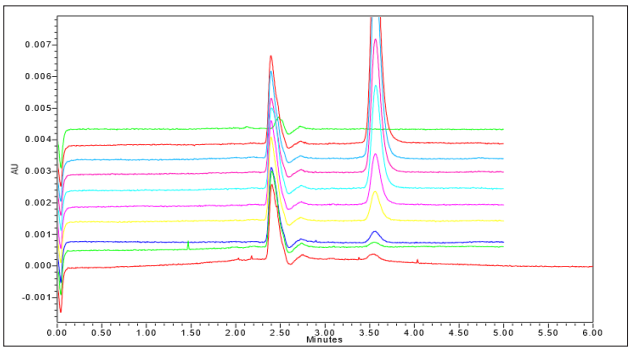

Figure 5. Overlay spectra of CC standards

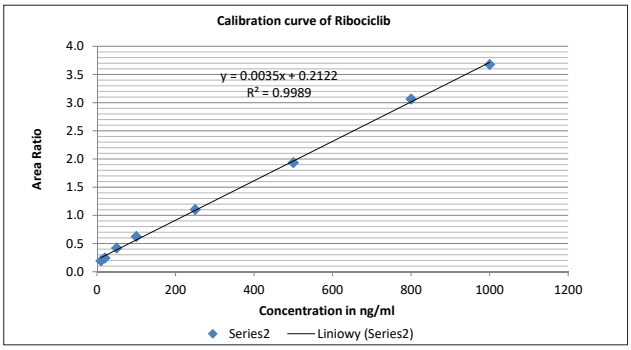

Figure 6. Eight point calibration curve of RCB

Accuracy and precision

In replicates over three days, spiked plasma samples were examined for inter- and intra-day precision and accuracy. Table 3 lists the overall % RSD at the three QC's concentrations, including lower limit of quantification (LLOQ-10, LQC-50, MQC-500, HQC-800 ng/ml) for the spiked human plasma. As per FDA guidance, the inter- and intra-day accuracy and precision are all within the acceptable limits of ±20% at LLOQ and ±15% at all other concentrations. In the Spiked human plasma, this shows that the process is Precise and accurate.

Table 3. Intra and inter-day precision and accuracy of RCB calibration standards in human plasma

		Nominal Conc (ng/ml)	Average Accuracy (%)	Mean	% RSD	% RE
RCB	Inter	LLOQ	98.65	9.87	3.99	1.98
		50	101.40	50.70	5.16	1.40
		500	96.64	483.19	2.80	3.36
		800	98.35	786.79	3.59	1.65
	Intra	LLOQ	98.67	9.87	4.19	1.67
		50	101.14	50.57	4.97	1.14
		500	96.65	483.26	3.05	3.34
		800	98.54	788	3.78	1.46

Recovery

Recoveries of sample in extracted and unextracted matrices at concentration ranging from 50 ng/ml to 800 ng/ml are represented in Table 4. The extraction recoveries for RCB in the Spiked human plasma ranged between 91.8-98.9%, with % RSD between 3.2-6.8%.

Table 4. Recovery of RCB in human plasma.

	Nominal Conc (ng/ml)	Mean Recovery	% RSD
RCB	50	96.48	0.38
	500	98.36	0.90
	800	97.04	0.94

Stability

The % RSD observed after one to three freeze-thaw (FTS) cycles were within 15% at LQC and HQC levels. Such deviations are representative of the effect of FTS cycles on stability of RCB and do represent inherent inter-/intra batch variations. The overlay in Figure 4 suggests that human plasma can handle such FTS cycles. However, repeated FTS cycles appear to have caused the % error to rise, adding to the unpredictability in estimating RCB concentrations in Low and High QC samples.

The stability results of RCB in spiked human plasma at Benchtop (8Hr at RT), auto-sampler (10°C for 48h), Freeze thaw stability (-20°C_3 Cycles) and Long term (-20°C for 28 days) are presented in Table 5. The % relative standard deviation observed in all the cases were below ±15% of the actual value. The experimental results signify good stability of RCB in plasma extracts at different storage and handling conditions.

Table 5. Stability of RCB in human plasma under operating conditions

	Nominal Conc (ng/ml)	Benchtop Stability (8hr at RT)	Autosampler Stability (10°C for 48h)	Freeze thaw stability -20°C 3 Cycles	Long term -20°C for 28 days
RCB	50	98.79±0.68	96.10±1.21	93.69±1.43	90.50±1.46
	800	98.03±.0.69	97.16±1.01	95.43±0.92	88.92±0.67

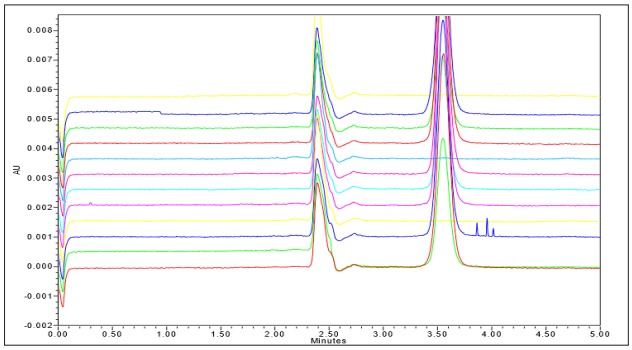

Figure 7. Overlay of 3 Freeze-Thaw stability studies

CONCLUSIONS

Chromatographic separation of RCB and IS from spiked human plasma was achieved by isocratic elution, and all the recoveries were within the acceptance limits of ±20% at LLOQ and ±15% at all QC's concentrations. Validation of human plasma was accurate and precise, as the standard deviation and the % relative standard deviation were within the acceptance limits of ±20% at LLOQ and ±15% at all QC levels. There were no significant changes in the predicted concentrations after FTS1, FTS2 and FTS3 freeze-thaw cycles – indicating that the analysis of frozen plasma samples is acceptable as per guidelines.

According to our knowledge, this study is the first to introduce and validate a simple, accurate and precise HPLC-UV method for RCB quantification in spiked human plasma, as compared to the standard HPLC-MS-MS technique used to analyze RCB. Sample preparation involved one-step protein precipitation with acetonitrile. The method was sensitive at a minimal LLOQ of 10 ng/ml RCB in Human plasma, and the eight point linear calibration curve was established at a RCB concentration range of 10-1000 ng/ml in plasma. This approach is more practical, effective, and affordable than the current technique. The proposed method can be used for routine bioanalysis of ribociclib in spiked plasma to support bioavailability bioequivalence studies.

ABBREVIATIONS

HR – Hormone receptor
USFDA – US Food and Drug Administration
RCB – Ribociclib
TDM – Therapeutic drug monitoring
HPLC-UV – High pressure liquid chromatography with UV detector
HPLC-MS – High pressure liquid chromatography with Mass detector
HPLC-MS/MS – High pressure liquid chromatography with MS/MS detector
TRI – Trifluridine
IS – Internal Standards
QCs – Quality Control samples
C_{max} – Maximum Plasma Concentration
LLOQ – Lower limit of quantification
ULOQ – Upper limit of quantification

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CONFLICT OF INTEREST

The authors do not have any conflict of interest.

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