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DETERMINATION OF PALBOCICLIB IN HUMAN PLASMA USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY - ULTRAVIOLET DETECTION

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ABSTRACT: A simple, sensitive, and high-performance liquid chromatography ultraviolet detection (HPLC-UV) method was developed and validated for the quantification of palbociclib in human plasma. Plasma samples were processed by solid phase extraction using an Oasis hydrophilic-lipophilic balance extraction cartridge (1 mL, 30 mg). Commercial imatinib was used as an internal standard. Palbociclib and imatinib (IS) in human plasma were analyzed using a mobile phase of acetonitrile and 0.1% Triethylamine TEA (pH 3.3; adjusted with 50% Ortho-phosphoric acid) (70:30, v/v), on a Agilent Zorbax C18 column (150 × 4.6 mm i.d., 5 µm) using isocratic elution at a flow rate of 1.0 mL/min with ultraviolet detection at 266 nm. The method was validated over a concentration range of 0.1-3.0 µg/mL ($r^2 \geq 0.998$) with coefficient of variation for intraday precision of 3.92%, 3.56% and 2.28% for 0.5, 1.5, and 2.5 µg/mL respectively. The lower limit of detection was 50 ng/mL. The extraction recovery rates for palbociclib ranged from 66.69% to 80.97%. The intra- and inter-day precision was below 5.0%, and the accuracy ranged from 89.40 - 112% over the linear range. No notable matrix effects were observed. The proposed RP-HPLC method was successfully applicable for clinical therapeutic drug monitoring programs and found suitable application in design pharmacokinetic studies.

INTRODUCTION: Palbociclib (PALBO) [6-Acetyl-8-cyclopentyl-5-methyl-2-{[5-(piperazin- 1-yl) pyrindin-2-l] amino} pyrido [2, 3-d]-pyrimidin-7(8H)-one] **Fig. 1** is a cyclin-dependent kinase (CDK) inhibitor with potential antineoplastic activity for the treatment of advance breast cancer [1]. It selectively inhibits cyclin-dependent kinase CDK4 and CDK6, thereby inhibiting retino-blastoma (Rb) protein phosphorylation early in the G1 phase leading to cell cycle arrest.

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It has been approved by the Food and Drug Administration (FDA), in combination with the aromatase inhibitor (AI) letrozole, for the treatment of postmenopausal women with estrogen receptor-positive (ER^+) human epidermal growth factor receptor (HER^{2-}) advanced breast cancer [2].

Palbociclib is soluble in diluted acidic aqueous solutions. It is well absorbed, maximum peak plasma concentrations were reached in 4 - 8 h after oral administration and its concentration increased with food. Mean bioavailability of palbociclib after a 125 mg dose is about 46%. Plasma protein binding in humans *in vitro* was about 85%. Studies have shown that drug is metabolized mainly by CYP3A and SULT2A1 with oxidation and sulfonation and that none of the metabolites contribute considerably to its pharmacological

activity. After a single oral dose palbociclib, recovery was evaluated over 15 days and found 1.75% in urine and about 74.1% [3]. Few LC-MS/MS analytical methods have been reported for the determination of palbociclib in human plasma [4, 5, 6] but without considering a validation procedure from the analytical point of view. Various high-performance liquid chromatographic (HPLC) based methods, with ultraviolet (UV) detection have been reported for the quantification of imatinib in plasma [7]. However, there are no analytical methods officially reported for determination of palbociclib in biological matrix. The development and validation of simple, sensitive, rapid and reliable method proposed herein for determining the concentration of palbociclib in plasma consists of one-step solid-phase extraction, followed by HPLC-UV.

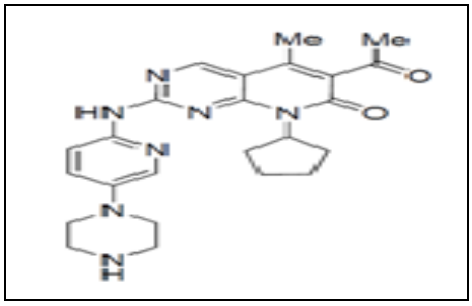

FIG. 1: CHEMICAL STRUCTURE OF PALBOCICLIB

Experimental Work:

Chemicals and Reagents: Palbociclib (purity 98.6%) and imatinib as internal standard (purity 99.0%) were obtained from Pharma Train Lab (Hyderabad, India). Solid phase extraction cartridge (Oasis HLB, 1 mL, 30 mg) were procured from Waters, Bangalore. All other reagents used were of analytical grade except acetonitrile, which was HPLC grade. The lyophilized human plasma was purchased from Sri Laxmi Sai Clinicals (Hyderabad, India). Milli-Q water was used throughout the study.

Equipment and Chromatographic Conditions: A Waters 2695 HPLC system with auto sampler, equipped with UV detector was used. The wavelength was set at 266 nm. The software used was Empower 2. The chromatographic analyses was carried out in the isocratic mode using Agilent Zorbax C18 reversed phase column (150 × 4.6 mm i.d, 5 µm) at ambient temperature. The mobile phase was acetonitrile and 0.1% Triethylamine (Tea, pH 3.3; adjusted with 50% ortho-phosphoric acid) (70:30, *v/v*), which was filtered through a Millipore vaccum filter system (0.45 µm) and degassed in an ultrasonic bath prior to use. The flow rate was 1.0 mL/min. The injection volume was 50 µL.

Preparation of Standard and Quality Control Samples: The standard stock solution of palbociclib (1.0 mg/mL) was prepared in diluent (acetonitrile: water, 50: 50 *v/v*). The standard working solution (10 µg/mL) was made by dilution from the stock solution. Two separate stock solutions were made for the preparation of calibration curve standards (CC) and quality control samples (QC). The working solutions required for plotting calibration curve were also prepared with diluent in a concentration range between 0.1 - 3.0 µg/mL. Similarly, QC samples at the lower limit (0.1 µg/mL), low (0.5 µg/mL), middle (1.5 µg/mL), and high (2.5 µg/mL) concentrations were prepared. Internal standard (IS) solution of imatinib (1.0 mg/mL) was prepared in diluent. From the stock solution, 1.0 mL of solution taken into a 100 mL volumetric flask and the volume was made up with diluent to produce 10 µg/mL solutions.

Sample Preparation: Plasma (300 µL) was spiked with 50 µL of palbociclib (1.5 µg/mL) and an internal standard (IS), except in blank plasma samples and vortexed. To this 0.3 mL of 0.1% ortho-phosphoric acid was added and vortexed. On the SPE unit using Oasis HLB (30 mg/mL) cartridges, preconditioned with 2 mL of methanol followed by 2 mL of Milli-Q water. Load the plasma sample and wash with 1 mL of Milli-Q water. Finally elute with 1 mL of methanol. Then evaporate to dryness under nitrogen atmosphere at 40 °C and the dried residue was reconstituted with 0.5 mL of mobile phase, vortexed thoroughly. Volume of 50 µL was injected into HPLC system.

Validation of the Bioanalytical Method: The method was validated by the determination of the following parameters: specificity, linearity, precision and accuracy, recovery, lower limit of detection and quantification (LLOQ) and stability studies according to the currently accepted US Food Drug Administration (FDA) bioanalytical method validation guidelines [8].

Selectivity: Selectivity is the ability of an analytical method to differentiate the analyte in the presence of other components in the sample. Before the preparation of the pooled calibration standards and QC samples, six lots of blank plasma were screened for matrix effects or interferences. The interferences from individual blank plasma in the LC-UV chromatograms at the retention times of the studied drug and internal standard was investigated to ensure the selectivity of the method.

Linearity: Calibration curves were made from blank sample (a plasma sample processed without IS) a zero sample (a plasma processed with IS) and six concentrations of palbociclib (0.1 - 3.0 µg/mL) including LLOQ were constructed by linear least squares regression by plotting peak area of the drug versus concentration. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ which was set at 20%.

Precision and Accuracy: The precision of the method based on intra-day variability was determined by replicate analysis of the calibration standards in the sample. The reproducibility was taken as the inter-day variability and was determined by replicate analysis of the calibration standards in different days with one replicate being analyzed each day. The percentage coefficient of variation values (% CV) were calculated from the ratios of the standard deviation (SD) to the mean. The accuracy of the analytical method describes the closeness of the mean test results obtained by the method to the true value of the analyte. The evaluation of precision was based on the criteria that the deviation of each concentration level should be within ± 15%, similarly for accuracy, the mean value should not deviate by ± 15% of the nominal concentration.

Recovery: Recovery from plasma was determined for QC samples (LQC, MQC and HQC) of drug by comparing the peak area of each analyte after extraction with the respective non-extracted standard solution at the same concentration. The percentage of the drug recovered from the plasma samples was determined by comparing the peak area ratio after extraction, with those of non-extraction sample containing same concentration of the drug as in plasma.

Stability: The concentration of the studied drug after each storage period was related to the initial concentration as zero cycle (samples that were prepared and processed immediately). The samples were considered as stable if the standard deviation (expressed as percentage bias) from the zero cycles was within ± 15%.

Bench Top Stability: The stability of the low and high unprocessed QC samples were maintained at a temperature of 25 ºC for 9 h and the samples were analyzed and the results were compared with that of zero cycle.

Long Term Stability: The stability of the low and high QC samples frozen at - 20 °C for 15 days was studied for long term stability. The samples were analyzed and results were compared with that of zero cycles.

Auto - Sampler Stability: The stability of the low and high QC samples stored in the auto-sampler tray for 24 h at 4 °C was studied for auto-sampler stability. The samples were analyzed and results were compared with that of zero cycle.

RESULT AND DISCUSSION: The developed HPLC method was optimized for the analysis of palbociclib in human plasma. In order to obtain proper chromatographic conditions, different columns like C_{18} of Phenomenex, YMC and Agilent Zorbax were used. The run time of analysis was higher when a longer reverse phase column (250 × 4.6 mm i.d,) was used. The resolution between the peaks decreased and peaks were not of acceptable shape when the experiment was performed using a shorter column (50 × 4.6 mm i.d,). However better resolution, less tailing and high theoretical plates were obtained with Agilent Zorbax column C_{18} (150 × 4.6 mm i.d., 5 µm). Methanol-water and acetonitrile-water in different proportions were tested as mobile phase. In addition, orthophosphoric acid, triethylamine (TEA) and acetate buffer in different proportions were tested instead of water for mobile phase. There is no interference was found between the drug and internal standard, from the extracted blank plasma [9]. The peak shape and symmetry were found to be good when the mobile phase composition of acetonitrile and 0.1% TEA (pH 3.3, orthophosphoric acid) (70:30 v/v) at a flow rate of

1 mL/min with UV detection at 266 nm. The retention time for extracted peaks of palbociclib and imatinib were 2.51 min and 3.22 min respectively.

Method Validation:

Selectivity: Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Six plasma samples were chromatographed to check for endogenous components which might interfere with palbociclib and imatinib (IS). Spiked plasma samples representing a low (0.5 µg/mL), medium (1.5 µg/mL) and high (2.5 µg/mL) palbociclib concentrations were analyzed to verify the selectivity of the method of analysis.

Both the peaks of palbociclib and imatinib did not interfere with any endogenous components. **Fig. 2** and **3** show a good resolution between the peaks of drug and internal standard.

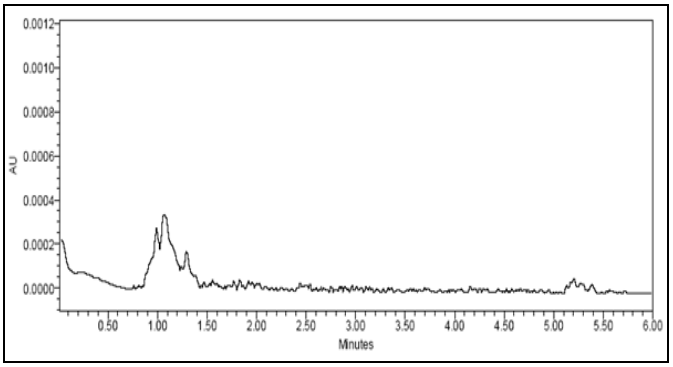

FIG. 2: CHROMATOGRAM OF BLANK PLASMA

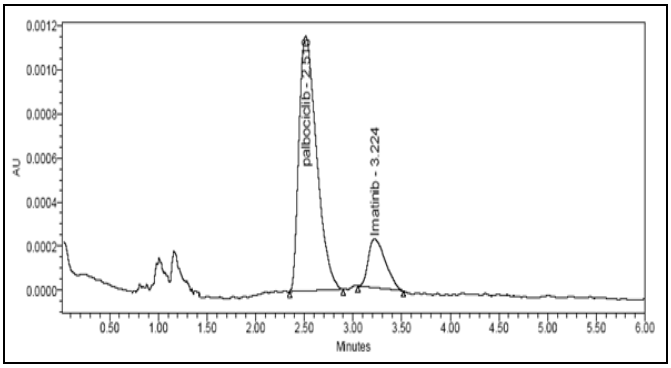

FIG. 3: CHROMATOGRAM OF PLASMA SPIKED SAMPLES WITH PALBOCICLIB AND INTERNAL STANDARD (IMATINIB)

Linearity: The linearity of the method was determined by a weighted ($1/X^2$ where X is concentration) least square regression analysis of the standard plots associated with the six point standard curve for palbociclib. The calibration curve was obtained by plotting chromatographic peak area versus concentration of palbociclib. The calibration line was linear in the range of 0.1 - 3.0 µg/mL of the drug **Table 1**, **Fig. 4**. A straight line fit made through the data points showed a constant proportionality with minimal data scattering with the correlation coefficient (r^2) of 0.997.

TABLE 1: RESULTS OF REGRESSION ANALYSIS OF THE LINEARITY DATA

S. no.	Parameters	Results
1	Linearity range (µg /mL)	0.1-3.0
2	Quantification limit (µg /mL)	0.1
3	Regression Equation	Y = 5.129x – 0.077
4	Slope	5.129
5	Intercept	0.077
6	Correlation co-efficient (r^2)	0.997

Lower Limit of Quantification (LLOQ) and Lower Limit of Detection (LLOD): The analyte concentration that produced a signal to noise ratio greater than 5 and 3 was considered as the LLOQ and LLOD respectively. The LLOQ and LLOD as per criteria were found to be 0.100 and 0.052 µg/mL when 50 µL of sample was injected.

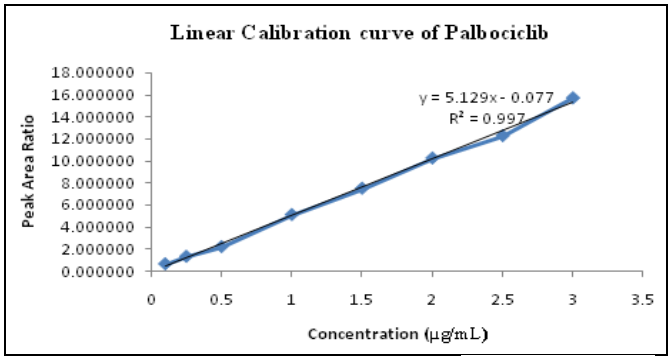

FIG. 4: CALIBERATION CURVE OF PALBOCICLIB

Precision and Accuracy: For accuracy, the mean value of three concentrations should be within 15% of the actual value and for the precision, the coefficient of variation (CV) should not exceed 15% at each concentration level. The intra-day and inter-day coefficient of variations was 5.0% or less for all the selected concentrations.

The percentage accuracy at all the studied concentrations was 89 to 115%. These data indicated a considerable degree of precision and accuracy of the method both during the analytical run and between different runs. The percentage accuracy showed that the method is remarkably accurate which ensures that reliable results are obtained. The results are summarized in **Table 2**.

TABLE 2: INTRA-DAY AND INTER-DAY ACCURACY AND PRECISION OF HPLC (n = 3)

Parameter	Concentration added (µg/mL)		
	0.5 (LQC)	1.5 (MQC)	2.5 (HQC)
Intra-day			
Mean	0.467	1.40	2.51
SD	0.01	0.05	0.05
% CV	3.92	3.56	2.28
% Accuracy	93.35	93.86	100.5
Inter-day			
Mean	0.49	1.52	2.58
SD	0.01	0.02	0.05
% CV	1.5	3.54	4.03
% Accuracy	99.33	101.73	103.50

Each mean value is the result of triplicate analysis

Recovery: The recovery was determined by comparing the aqueous solution and the spiked drug. The mean recovery of palbociclib from plasma spiked, in terms of LQC, MQC and HQC levels were respectively, 67.69%, 67.76%, and 80.94%. The overall recovery of palbociclib was 72.1 % **Table 3**.

TABLE 3: RECOVERY STUDIES OF PALBOCICLIB (n = 6)

Concentration (µg/mL)	% Recovery (mean ± SD)	% CV
0.5	67.69 ± 1.01	1.50
1.5	67.76 ± 2.40	3.54
2.5	80.94 ± 3.26	4.03

Stability: The stability of the studied drug in human plasma was assessed by analyzing six replicate QC samples at the low, medium and high concentration levels at room temperature over 9 h (Bench top stability). The measured concentrations of the drug in these QC samples kept at room temperature for 9 h were compared with the corresponding QC sample freshly prepared and analyzed immediately. The results in **Table 4** indicated that the studied drug was stable for at least 9 h in human plasma when stored at ambient temperature.

TABLE 4: STABILITY OF THE SAMPLES

Concentration (µg/mL)	Concentration found (µg/mL)	% CV	% RE
Bench top stability			
0.5	0.49 ± 0.02	4.85	-2
2.5	2.36 ± 0.06	2.63	-14
Long-term stability after 15 day at - 20 °C			
0.5	0.40 ± 0.04	9.56	-20
2.5	2.03 ± 0.04	2.15	-18.8
Auto sampler stability (24 h) at 4 °C			
0.5	0.53 ± 0.01	2.53	6
2.5	2.47 ± 0.05	1.98	-1.2

Also the studied drug showed the long-term stability in human plasma when stored at -20 °C for 15 days when compared with the freshly prepared QC samples. The studied drug was also stable, when stored at 4 °C for 48 h in the auto-sampler and compared with the freshly prepared sample. All the above stability studies indicated that the samples in various phases were within the acceptance limits.

CONCLUSION: The optimized HPLC-UV method is simple, accurate, precise and reproducible. The method is linear over a wide range and utilizes a mobile phase which can be easily prepared. Simple sample preparation procedure and a relatively short chromatographic run time make this method suitable for processing of multiple samples in a limited amount of time for pharmacokinetic studies. The method developed was complies with the validation criteria laid down by the US-FDA guidelines. Hence the developed method can be applied for pharmacokinetic studies and therapeutic drug monitoring in humans.

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CONFLICT OF INTEREST: The authors declared no conflict of interest.

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