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Original Article

Method Development Combined with *in silico* Therapeutic and Toxicology Studies on Palbociclib and its Degradation Products to Assist in Discovery of New Molecule

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ABSTRACT

Background: Palbociclib is anti-cancer drug which interacts with cyclin-dependent kinase. There are no methods repoRTed for the characterization of palbociclib together with degradation products (DPs). The main aim of the study is to develop method combined with *in silico* therapeutic and toxicology studies on palbociclib and its DPs which assist in discovery of new molecule. **Methods:** A new analytical method was developed for the identification, characterization of palbociclib and DPs formed when subjected to forced degradation, using symmetry C_{18} column, 150mm x 4.6mm, 3.5µm in isocratic mode with acetonitrile: formic acid (0.1%) (50:50). *In silico* toxicity for drugs and DPs were predicted using the Swiss ADME web tool and StarDrop Derek Nexus software for all DPs. **Results:** Validation: LOD and LOQ were 0.01 and 0.1 µg/ml. which shows accuracy data at three different concentrations of 50, 100, and 150 % in triplicate analysis. The % RSD for intra-day and intermediate precision was 0.47% and 0.33%, respectively, indicating the method was sufficiently precise. Mass spectrums (LC-MS/MS) of drug with *m/z* 447(RT= 4.075 min), DPI with $[M+H]^+$ at *m/z* 503(RT= 1.328 min), DPII with $[M+H]^+$at *m/z* 540 (RT= 2.371 min), DPIII with *m/z* 46 (RT= 3.741 min) are reported. In docking studies, PS2 showed a higher docking score -12.289. **Conclusion:** The drug was found to degrade under forced degradation and DPs are characterized. All the DPs were found to be toxic except the major fragment ions (PS2), showed higher binding affinity than drug without toxicity. This is the lead molecule for further drug discovery.

Key words: Forced degradation, LC-MS/MS, Swiss ADME web tool and StarDrop Derek Nexus software, Analytical Method development, Anticancer drug.

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INTRODUCTION

According to International Council on Harmonisation guidelines,[1] stability testing of pharmaceutical drugs under different stress conditions should be performed because they undergo physic-chemical changes or degradation during the synthesis or upon storage produce process impurities and degradation products (DPs), which ends up in toxicity or side effects. Liquid Chromatography (LC) combined with Tandem mass spectrometry (MS/MS) are available for the identification and characterization of process impurities and DPs. Evaluating activity through molecular docking and toxicity[2-11] of DPs with the assistance of Computational tools helps in proposing the structures for the DPs, which normally explained by appropriate mechanisms. The molecular docking studies are useful for determining the binding capacity of drug molecules and their DPs towards the corresponding protein. Supported extensive literature search, LC-MS-MS[12-19] and LC techniques[20] were reported for therapeutic drug monitoring investigations in humans, animals and Pharmaceutical formulations. Hence, no method reported thus far for the characterization of Palbociclib together with DPs. During this study, we developed a method and validated by High-performance liquid chromatography coupled with electrospray ionization-quadrupole-time of flight-mass spectrometry (LC-ESI-QTOF-MS) method, SwissADME web tool was used for ADME, licensed StarDrop Derek Nexus used for the prediction of toxicological studies and Schrodinger software for Molecular Docking.

MATERIALS AND METHODS

Chemicals and reagents

Palbociclib standard was procured from Pharma Train Lab (Hyderabad, India). Acetonitrile and formic acid were purchased from Finar Chemicals (Ahmedabad, India). High performance liquid chromatography (HPLC) grade water. Analytical grade reagents of sodium hydroxide, hydrochloric acid, 30%hydrogen peroxide, and sodium bisulphate.

Instrumentation

Liquid chromatography instrument comprised Waters alliance model e2695, a Waters (2998) Photodiode array Detector, a Waters (2700) Auto sample injector, Solvent degasser, Quaternary pump, Temperature-controlled compartment. A thermal degradation study was administered employing a hot air oven.

LC conditions

Chromatographic separation was obtained with isocratic elution using mobile phase 50:50v/v Acetonitrile and formic acid (0.1%) pumped from a solvent reservoir with a flow rate of 1.0 mL/min to the analytical column of the Symmetry C_{18} column (150x4.6mm i.e., particle size 3.5µm) using column back pressure of 1570-1620 at the detector maximum wavelength of 220 nm. Detector performance was evaluated to assess the height peak area and other device suitability parameters using Empower-2 software. The injection volume was used at 0.1mL and

therefore the ambient column temperature was maintained throughout the analysis.

Forced degradation study

A standard solution of 50µg/mL was prepared by weighing 5mg of drug, transferring into 50 ml volumetric flask and diluted with mobile phase (as diluent). The chemical stability and pathway of degradation of any drug substance were determined by performing the stress degradation study in several stress environments as per ICH guidelines. The drug was exposed to hydrolytic degradation (acidic, neutral, and basic), photolytic, oxidative, reduction, and thermal degradation conditions specified in Table 1. Hydrolytic degradation studies were allotted using 1N HCl (acidic) and 1N NaOH (alkaline) and HPLC grade water (neutral) at 70°C for twenty-four hours. Oxidative degradation study was performed by using oxide (30%) at temperature for twenty-four hours and for performing the photolytic degradation, the sample was exposed to sunlight for twenty-four hours. The drug was exposed to 105°C for 3 hr in very hot air oven for thermal degradation study. The purity study of drug substances and all degradation products formed were checked using a PDA detector.

MS conditions

All the DPs generated during study were characterized by using Agilent 1200 array consisting of Q-TOF mass spectrometer. All the experiments were conducted under the positive mode of electron spray ionization (ESI). All the acquisition of statistics was achieved with the use of the software program MassHunter V 5.0. The parameters and conditions maintained for the optimized study explained in Table 2.

In silico Toxicity study

In silico ADME and toxicity predictions were performed using the Swiss ADME tool and StarDrop Derek Nexus (Optibrium). The basis of prediction software is the relationship of physicochemical properties such as log P and solubility with those of pharmacokinetics of the compound. The tools calculated physicochemical properties and predicted the pharmacological and toxic properties of the degradation products.

Table 1: Forced Degradation studies.

Type of Degradation	Stress Conditions	Degradation (%)	Peak purity	
			Purity Threshold	Purity Angle
Acidic hydrolysis	1N HCl at 70°C for 24 hr	23.1	0.125	1.063
Basic hydrolysis	1N NaOH at 70°C for 24 hr	21.1	0.129	1.034
Oxidation	30% H_2O_2 for 24 hr	23.7	0.168	1.069
Reduction	10% sodium bisulfate	22.6	0.125	1.044
Thermal	105°C for 3 hr	-	0.147	1.064
Neutral Hydrolysis	H_2O at 70°C	-	0.164	1.065
Photolytic	Sunlight for 24 hr	-	0.158	1.069

Table 2: Chromatographic conditions for optimized method.

LC Conditions		
Stationary phase	**:**	**Symmetry C_{18}, 150mm x 4.6mm, 3.5µm.**
Mobile Phase	:	0.1 % ORThophosphoric acid: acetonitrile
Elution mode	:	Isocratic A: B = 50:50 % v/v
Flow rate	:	1.0 ml/min,
Sample volume	:	10µl using Rheodyne 7725i injector
Oven Temperature	:	Ambient
MS Conditions		
Interface	:	ESI
Operation mode	:	MRM
Polarity	:	Positive
Capillary voltage	:	4 KV
Fragmentor voltage	:	170 V
Skimmer voltage	:	65 V
Nebulizer Gas flow	:	40 psig
Drying gas	:	10 L/min
Gasoline temperature	:	325°C
Detection	:	*m/z* : 0-800
Data station	:	MassHunter V 5.0

Molecular Docking

Preparation and grid generation of protein 5L2I

The protein preparation wizard in the maestro task was selected for protein preparation and energy minimization. The X-ray co-crystal structure of human CDK6 and Palbociclib (PDB code: 5L2I)[21] was imported into the maestro workspace, it is initially pre-processed all default parameters like assign bond orders, add hydrogens, create zero-order bonds t metals, create disulphide bonds, convert selenomethionines to methionine's and generate het states using Epik pH 7.0±2.0. Chain A of cyclin-dependent kinase 6 with 307 sequence length and co-crystal ligand LQQ (palbociclib) were selected in the review and modify tab. Water molecules were also deleted, as they were not making any interactions with both ligand and protein. Finally, the protein is optimized by using the OPLS3e force field[22] by keeping all default settings. The active site or binding site of the co-crystal ligand in the protein was selected for docking of compounds by generating a grid surrounding it. It was done by selecting any atom of the co-crystal palbociclib in the workspace and keeping all default settings, this enables a grid box in protein with X, Y, and Z coordinates 13.89, 28.25, 9.72 and the X, Y, Z grid dimensions 15, 15, 15 Å.

The Ramachandran (RP) plot, PROCHECK,[23] ERRAT[24] analysis, VERIFY3D[25] results were commonly used to indicate the quality of a protein model.

Ligand preparation

ChemDraw Ultra Version 8.0.3 (Cambridge Soft MA, USA) used to draw the structures of molecules. OpenBabelGUI version 2.4.1 was used to convert ChemDraw binary format .cdx files to .sdf format. The glide Ligprep tool was utilized to prepare ligands for docking studies, all the ligands in .sdf format were imported and subjected to energy minimization by using OPLS3e force field along with all default settings in Ligprep like ionization (possible states at target pH 7.0±2.0 using Epik), desalt, retain specified chirality's.

Glide ligand docking standard precision was selected for the docking studies of the ligands. The ligand docking findings were ranked from lowest to highest binding energy based on their interactions with the amino acid residues in the protein's binding pocket.

Validation

The developed method was validated as per ICH guidelines parameters.

RESULTS

Different run conditions, columns, and mobile phases were tried for optimizing chromatographic conditions. Different mobile phases like formic acid (in varying percentages from 0.05 to 0.5%), Ammonium acetate, and Ammonium format were used for optimizing the conditions. Formic acid at (0.1%) and Acetonitrile: (50:50v/v) using Symmetry C_{18} (150 X4.6mm, 3.5µm) showed good peak shape and short retention time with good resolution with no peak tailing. Among different flow rates, 1.0mL/min showed no peak tailing with a short retention time to permit high sample throughput analysis. The RT of Palbociclib is about 3.965 min.

Method of validation

Good linearity was achieved at concentrations of 0-20 µg/ml ($R2 = 0.999$). Limit of detection (LOD) and limit of quantification LOQ were 0.01 and 0.1 µg/ml. (Table 3) shows accuracy data at three different concentrations of 50, 100, and 150 % in triplicate analysis. The recoveries were found within the range of 99.9% and 100.3%. The % RSD for intra-day and intermediate precision was 0.47 percent and 0.33 percent, respectively, indicating that the method was sufficiently precise. Robustness was tested by varying the flow rate and percent of the Organic phase at three distinct concentrations. Each sample was injected three times (n=3), and the mean and %RSD values were calculated using the peak areas obtained. The %RSD was <1.

Stress decomposition behaviour

All DPs formed are resolved with drug substance indicating the method is specific. The purity of drug and DPs was less than the purity threshold. There was no interference of any other peak of DP, which ensures the selectivity of the method. Three DPs named as DPI, DP-II and DP-III were formed in hydrolytic (acidic and alkaline), reduction, oxidative stress conditions respectively. The chromatograms obtained for different stress conditions along with control are depicted in (Figure 1) In (Figure 2) all the mass spectrums of the drug and its degradations products are depicted.

MS/MS Palbociclib

Drug with m/z 447 (R*t*= 4.075 min) displayed the product ions at *m/z* 305, *m/z* 242, m/z 211, *m/z* 176. The high abundant product ion observed at *m/z* 305 due to the loss of $C_5H_{10,}$ C_2H_4O, H_2O, and other fragment ions of *m/z* 242 due to the loss of $C_4H_{11}N$ from the ion, *m/z* 211 due to the loss of C_2H_7N and *m/z* 176 due to the loss of C_3H_8.

MS/MS DPI

DPI with $[M+H]^+$ at *m/z* 503 (RT= 1.328 min), was formed under hydrolytic stress conditions. The DPI showed distinctive product ions at *m/z* 433, 417, 333,347, 323, 253, and 153. The product ions of *m/z* 433 (loss of C_5H_{10} from the parent ion at *m/z* 503), *m/z* 417 (loss of $C_4H_{10}N_2$ from the parent ion at *m/z* 503), *m/z* 333 (loss of $C_7H_{14}O$ from the fragment ion at *m/z* 433), *m/z* 347 (loss of C_5H_{10} from the ion at *m/z* 417), *m/z* 323 (loss of $C_5H_6N_2$ from the ion at *m/z* 417), *m/z* 253 (loss of C_5H_{10} from the ion at *m/z* 323), *m/z* 153 (loss of $C_7H_{14}O$ from the ion at *m/z* 253).

MS/MS DPII

DPII with $[M+H]^+$at m/z 540 (RT= 2.371 min), was formed under hydrolytic stress conditions. The DPII showed distinctive product ions at m/z 470, 439, 369,352, 283, 258, and 188. The product ions of *m/z* 470 (loss of C_5H_{10} from the parent ion at *m/z* 540), m/z 439 (loss of $C_5H_{10}O_2$ from the parent ion at m/z 540), m/z 369 (loss of C_5H_{10} from the fragment ion at *m/z* 438), m/z 352 (loss of $C_4H_{10}N_2$from the ion at *m/z*

Table 3: Summary of Validation.

S.no	Parameter			HPLC Results		Acceptance Criteria
1	System suitability	Tailing factor		1.07		NMT-2
		%RSD	0.27	NMT-2%		
2		Specificity	No interference of blank and placebo			-
3	Precision	Method precision	%RSD= 0.47			NMT-2%
		System precision	%RSD= 0.33	NMT-2%		
4		Accuracy	100.4-100.9%			97-103%
5		Linearity	r^2=0.9991			NLT-0.9999
6.		LOD	0.01			
7.		LOQ	0.1			
9.	Robustness	Change in flow rate	Tailing factor	1.29	1.28	1.29	NMT-2
		Plate Count					
		%RSD	3784	3723	3784	NLT-2000
			0.77	0.73	0.17	NMT-2%
		Change in the organic phase	Tailing factor	1.33	1.29	1.47	NMT-2
		Plate Count					
		%RSD	3322	3323	4741	NLT-2000
			0.25	0.24	0.15	NMT-2%

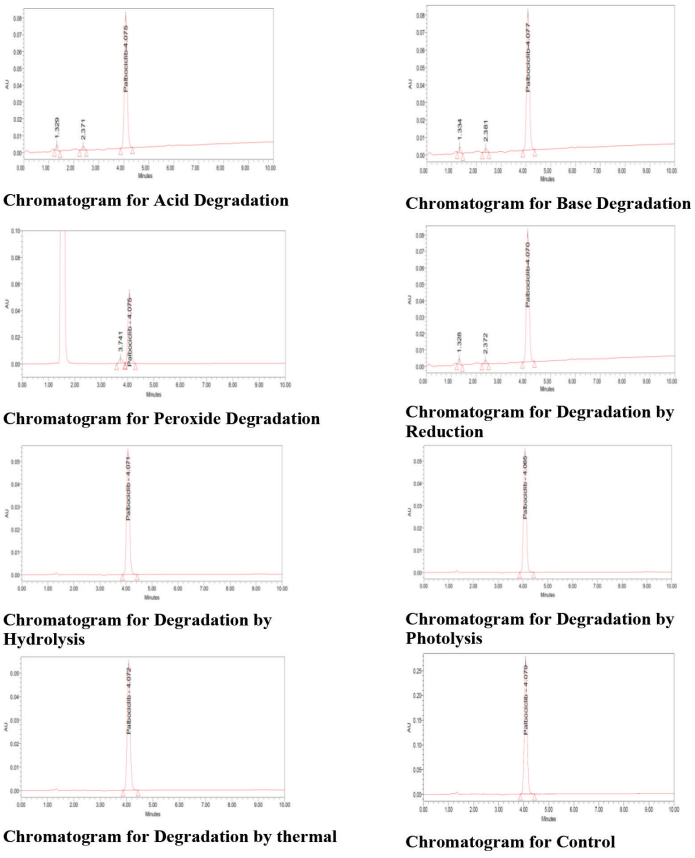


Figure 1: Representative Chromatograms for forced degradation conditions.

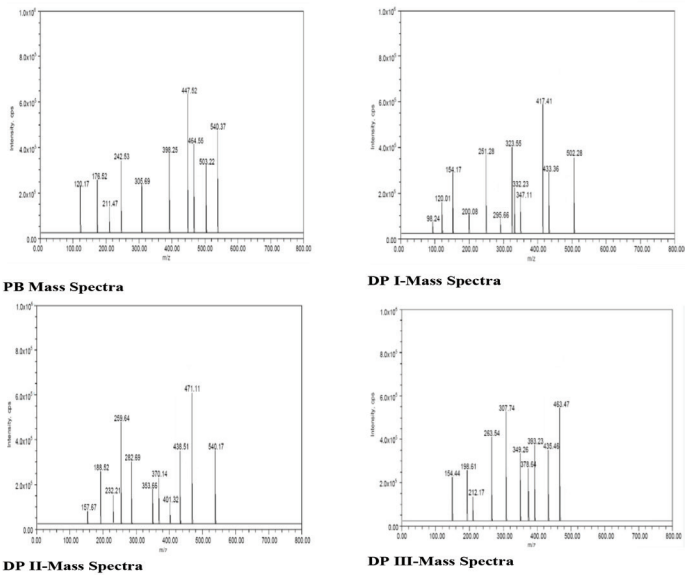


Figure 2: Representative MS/MS Spectra for Drug and Degradation products.

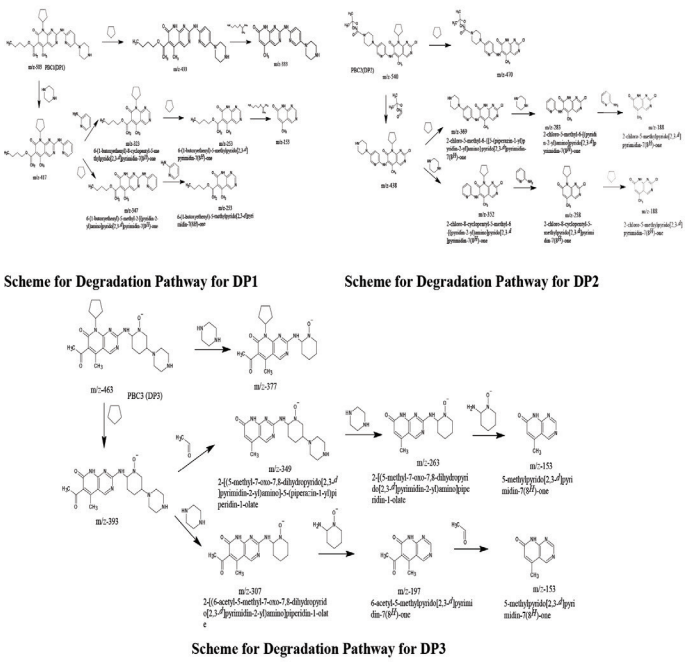


Figure 3: Schemes for proposed degradation pathway.

438), *m/z* 283 (loss of $C_4H_{10}N_2$ from the ion at *m/z* 369), *m/z* 258 (loss of $C_5H_6N_2$ from the ion at *m/z* 352), *m/z* 188 (loss of $C_5H_6N_2$ from the ion at *m/z* 283).

MS/MS DPIII

DPIII with *m/z* 463 (R*t*= 3.741 min) was formed under oxidative stress conditions. The product ions of *m/z* 393 (loss of C_5H_{10} from the parent ion at *m/z* 463), *m/z* 377 (loss of $C_4H_{10}N_2$ from the parent ion at *m/z* 463), *m/z* 349 (loss of C_2H_4O from the fragment ion at *m/z* 393), *m/z* 307 (loss of $C_4H_{10}N_2$from the ion at *m/z* 393), *m/z* 263 (loss of $C_4H_{10}N_2$ from the ion at *m/z* 349), *m/z* 197 (loss of $C_5H_{11}N_2O$ from the ion at *m/z* 307), *m/z* 153 (loss of $C_5H_{11}N_2O$ from the ion at *m/z* 263). All the schemes for proposed fragmentation pathways for degradation products depicted in (Figure 3).

Using Lipinski, the *in silico* techniques predicted drug breakdown products (Table 4). The GI absorption for drugs and their degradation products is high and may cause adverse effects by entering the bloodstream through the gastrointestinal tract. The capability to penetrate the blood-brain barrier (BBB) for drugs and their degradation products has significantly higher scores for this phenomenon. All the DPs show less toxicity because of the low log kp value than drug. The toxicity screening for the drug and DPs were performed and the results were elaborated in (Table 5). In the present research, all three degradation products were found to be toxic. Hence fragment ions obtained from degradation products which are non-toxic were screened for molecular docking. Total seven fragment ions are nontoxic which are named as PS1, PS2, PS3, PS4, PS5, PS6, and PS7. The docking results (Table 6, Figure 4), reveals that the co-crystal ligand Palbociclib showed hydrogen bond interactions with the amino acid residues VAL 101 and ASP 163 with a docking score of -11.571. Most of the all compounds PS1 to PS7 exhibited an identical type of interactions in the binding pocket, among all compounds, PS2 showed a higher docking score -12.289 than the standard Palbociclib without toxicity.

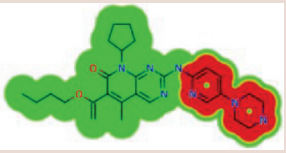
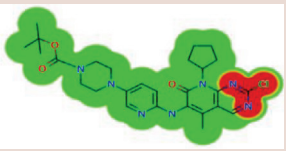
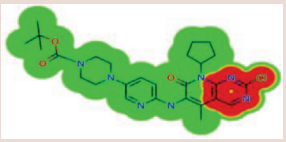
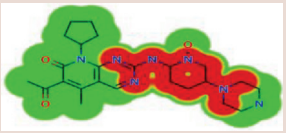
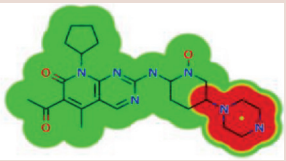
DISCUSSION

The mobile phase formic acid at (0.1%): acetonitrile (50:50v/v) ratio was used in the HPLC procedure with a Symmetry C_{18} (150 X4.6mm, 3.5m) column with 220 nm PDA detection under optimum chromatographic conditions. The optimal flow rate was determined to

Table 4: ADME studies by using the SWISS tool.

Drug	BBB Permeation	GI Absorption	CYP Inhibition					Log Kp	Lipinski Violation
			1A2	2C19	2C9	2D6	3A4		
Palbociclib	High	High	No	No	No	Yes	No	-8.33	0
DP1	High	High	No	Yes	Yes	Yes	Yes	-6.7	1
DP2	High	High	No	Yes	Yes	Yes	Yes	-6.73	1
DP3	High	High	No	No	Yes	Yes	Yes	-7.74	0

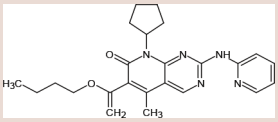
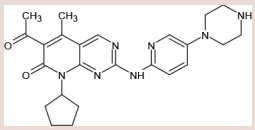
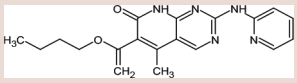
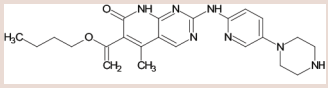
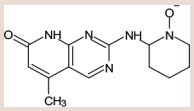
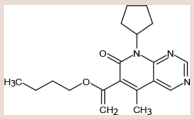
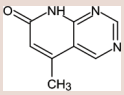
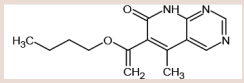
Table 5: Toxicological profile of the compounds using Star Drop Nexus.

Drug/ Degradation product	m/z	Toxicity	Structure	Group responsible for toxicity
Palbociclib	447.52	Phospholipidosis		1-(pyridin-3-yl)piperazine
DP1	503	Phospholipidosis		1-(pyridin-3-yl)piperazine
DP2	540	Hepatotoxicity		carbamimidyl chloride
		Respiratory sensitization		2-chloropyrimidine
DP3	463	HERG channel inhibition		2-carbamimidamido-5-(dimethylamino) piperidin-1-olate
		Skin sensitization		1-methyl piperazine

be 1mL/min. Palbociclib was isolated with improved sensitivity and excellent peak shape in 3.965 min. The linearity analysis for the HPLC technique yielded a regression coefficient value of 0.999, showing that the method is linear. The percentage of RSD for the precision analysis utilising the HPLC procedure was less than2, indicating that the HPLC methods developed were accurate. The average percentage recovery results for the HPLC technique of 100.4-100.9 percent demonstrated the accuracy of the methods produced because the values were within the acceptability limit. According to the findings of the LOD and LOQ tests, method established for palbociclib were shown to be sensitive and easily measurable. The robustness analysis results reveal that there are no significant changes in the outcome of slight deliberate adjustments in the mobile phase ratio and mobile phase flow rate, indicating that the approach method is resilient.

The drug found to be more vulnerable to acid, alkali, reduction, and peroxide degradation conditions, degrading by 23.1%, 21.1%, 22.6%, and

Table 6: Docking score and interactions of compounds with 5L2I protein using Schrodinger.

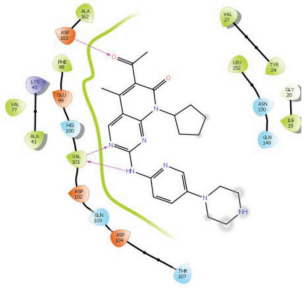
Code	Major fragment ions m/z	Chemical structure	Chemical formula	Docking score	Hydrogen bond interactions
PS2	417		$C_{24}H_{29}N_5O_2$	-12.289	VAL 101
Palbociclib	447		$C_{24}H_{29}N_7O_2$	-11.571	VAL 101, ASP 163
PS3	333		$C_{19}H_{21}N_5O_2$	-10.746	VAL 101
PS1	433		$C_{23}H_{29}N_7O_2$	-10.151	VAL 101, ASP 163
PS1	263		$C_{13}H_{16}N_5O_2$	-9.843	VAL 101
PS5	323		$C_{19}H_{25}N_3O_2$	-9.428	VAL 101, ASP 163
PS7	153		$C_8H_7N_3O$	-7.937	GLU 99, VAL 101
PS4	253		$C_{14}H_{17}N_3O_2$	-7.152	VAL 101

23.7% respectively. Palbociclib was found to be stable when subjected to neutral hydrolysis, photolysis, and heat deterioration.

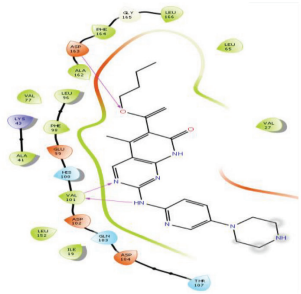
The DP-I and DP-II were created under acidic, basic, and reduction conditions, respectively, while the DP-III was formed under oxidative circumstances. All of the DPs were resolved using LC-MS/MS analysis. Palbociclib and DPs degradation pathways and mechanisms have been identified. Palbociclib and DPs degradation pathways and mechanisms have been identified. The Swiss ADME tool was used to determine the *in silico* Pharmacokinetics. The medication and its degradation products are absorbed in the GI tract and penetrate the blood-brain barrier. This is an orally accessible medicine, and it must have a high GI absorption rate in order to have a high bioavailability. It is not necessary to cross the BBB because it is primarily used to treat breast cancer. The toxicity of the medication and its degradants was predicted using a Star Drop Derek nexus model, and the results led to some interesting conclusions. The drug and DP1 were predicted to exhibit minor phospholipidosis toxicity due to the presence of the 1-(pyridin-3-yl) piperazine group. DPII causes hepatotoxicity and respiratory sensitization because to the presence of carbamimidyl chloride and 2-chloropyrimidine groups. DPIII inhibits HERG channels, causes skin sensitization, and the hazardous groups are 2-carbamimid amido-5-(dimethyl amino) piperidin-1-olate and 1-methyl piperazine. Glide (Schrodinger) molecular docking was used to determine binding affinities, and PS2 was identified as a fragment ion with a binding affinity greater than palbociclib. The binding affinities for the remaining fragment ions were discovered to be lower. Thus, one fragment ion was discovered to exhibit an intriguing binding affinity for the 5L2I protein, indicating that they may be bioactive in *in-vivo* experiments. As a result, PS2 has a good probability of being an effective anticancer agent. These experiments yielded several intriguing results, which may be useful in bridging pharmaceutical drug analysis and drug discovery.

CONCLUSION

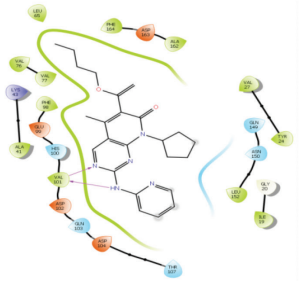
The study's goal is not only to investigate the forced degradation products produced under stress conditions utilising LC/MS-ESI-QTOF analysis, but also to perform a comparative *in silico* toxicological evaluation. In this study, degradation products were studied using *in silico* techniques and the developed method was precise, linear, accurate, highly sensitive, and dependable, with validation findings that were within the limitations. The developed method was compatible with hyphenated techniques of analysis. Palbociclib degradation routes were investigated under various



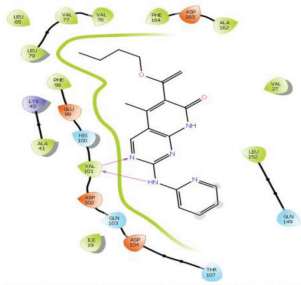
VAL 101, ASP 163 Hydrogen bond interactions of Palbociclib



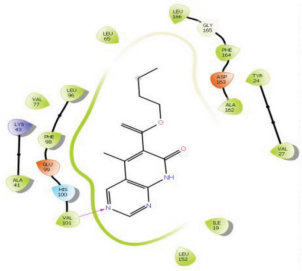
VAL 101, ASP 163 Hydrogen bond interactions of PS1



VAL 101 Hydrogen bond interactions of PS2

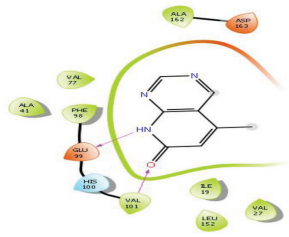


VAL 101 Hydrogen bond interactions of PS3

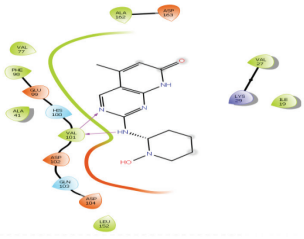


VAL 101 Hydrogen bond interactions of PS4

VAL 101, ASP 163 Hydrogen bond interactions of PS5



GLU 99, VAL 101 Hydrogen bond interactions with PS6



VAL 101 Hydrogen bond interactions with PS7

Figure 4: 2D interactions of compounds with 5L2I protein.

forced degradation stress conditions in accordance with ICH criteria. Palbociclib was used to forcefully degrade certain stressful conditions. It is a quick and sensitive HPLC approach capable of isolating Palbociclib and its three degradation products down to the nanogram level and there is a scope for the discovery of new molecules during anticancer therapy.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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