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Original Article

Development and Validation of the Stability-Indicating RP-HPLC Method to Estimate Olaparib in Graphene Quantum Dot Nanoformulation Using Analytical Quality by Design (AQbD) Principles

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ABSTRACT

Introduction: This study aimed to develop and validate a reliable Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) method for the accurate quantification of Olaparib in a Graphene quantum dot nanoformulation. The research employed the principles of Analytical Quality by Design (AQbD) to optimize and validate a stability-indicating RP-HPLC method. The primary objective was to optimize key chromatographic parameters, including retention time, theoretical plate count, and tailing factor, to ensure precise and robust analysis of Olaparib formulations. **Materials and Methods:** A central composite design was utilized to optimize the chromatographic conditions. The method was then validated rigorously to assess its performance characteristics, including accuracy, Linearity, and Limit of Detection (LOD), Limit of Quantification (LOQ), precision, and % RSD. **Results:** The optimized RP-HPLC method demonstrated excellent performance with a retention time of 4.6 min. The method exhibited high sensitivity with an LOD of 0.4996 µg/mL and LOQ of 1.5139 µg/mL. Precision was demonstrated by a %RSD of less than 2%. The evaluation of stability parameters confirmed the method's suitability for the accurate and precise quantification of Olaparib. **Conclusion:** This study successfully developed and validated a robust, cost-effective HPLC method for OLA using QbD principles. The method, optimized via CCD, accurately quantifies OLA in both pure and nanoformulation forms, demonstrating excellent stability-indicating properties and broad applicability in pharmaceutical research and development.

Keywords: Olaparib, Graphene Quantum dots, Central composite design, Quality by design, Force Degradation.

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INTRODUCTION

Cancer is defined as a malignant tumour with potentially infinite growth that spreads both locally through invasion and systemically through metastasis.[1] Cancer represents a significant global health challenge and is the second leading cause of death in the United States.[2] The Food and Drug Administration (FDA) has approved Olaparib (OLA) which is a selective and potent inhibitor of poly (ADP-ribose) Polymerase (PARP) enzymes, PARP1 and PARP2.[3,4] A novel class of anti-cancer therapies is represented by PARP inhibitors which function by taking advantage by dysfunctioning the DNA repair that causes cell death in cancer cells with BRCA gene (Breast Cancer) mutations. OLA is used to treat various BRCA-associated malignancies, such as prostate, pancreatic, ovarian, and breast cancers. In December 2014, OLA received initial approval from both the FDA and the EU, and subsequently, in April 2016 by Health Canada. Figure 1 illustrates the chemical structure of OLA.

However, OLA is also constrained by a number of factors, including toxicity, also it exhibits poor bioavailability (12-17%), a short biological half-life (6-12 hr), and limited stability within the body. Furthermore, it lacks the ability to effectively target and accumulate within tumor tissues.In addition to the previously mentioned restrictions, adverse reactions such exhaustion, nausea, vomiting, and anaemia significantly impede the clinical application of this treatment.[5] In terms of biopharmaceutical classification, OLA is classified as a Class IV medication, with poor aqueous solubility and low permeability leading in a low oral bioavailability.[6]

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Various analytical methods, such as UV spectroscopy, UPLC, and high-performance thin-layer chromatography HPTLC, are available for quantifying OLA, but High-Performance Liquid Chromatography (HPLC) is often preferred due to its specificity, broad applicability, and automation capabilities.

HPLC method was developed, drawing on different existing methods that employ varied mobile phases and conditions. For instance, Daumar P *et al.*, developed one method used a acetonitrile-water (50:50), achieving a retention time of 10 min.[7] Another method utilized a 0.1% orthophospheric acid in water and acetonitrile, resulting in a retention time of 15 min.[8] A third method combined 0.1% tri fluoroacetic buffer- acetonitrile (60:40), with a retention time of 5.14 min.[9]

Literature surveys have revealed that various analytical techniques, including spectrophotometry, HPLC, and HPTLC, are employed for the quantification of Olaparib in bulk drug substances. However, to the best of our knowledge, no validated methods using Quality by Design approach have been previously reported for the specific quantification of OLA within nanoformulations. This study aimed to develop a cost-effective and robust HPLC method for the accurate and precise determination of Olaparib in its nanoformulation.

The developed HPLC method, based on Quality by Design (QbD) principles using Central Composite Design (CCD) approach, employed Methanol: water (61.36:38.64) as the mobile phase, resulting in a notably lower retention time of 4.6 min. Unlike previous methods, which did not utilize the QbD principle, this method has higher aqueous phase concentration ratio which offers several advantages for OLA analysis, including the assessment of encapsulation efficiency and stability which makes it a valuable resource for future studies in pharmaceutical formulation development. The FDA strongly supports the QbD, advocating for its systematic, evidence-based, and risk-centered integration into developmental processes. Additionally, Standardization of QbD implementation is guided by the International Conference on Harmonization (ICH). In recent years, numerous articles have explored comprehensive strategies for incorporating QbD principles into analytical measurements, particularly in the development of HPLC methodologies. This study aims to establish a precisely validated RP-HPLC technique that adheres to QbD principles to evaluate various parameters of OLA.

MATERIALS AND METHODS

Chemicals and Materials

Olaparib was received as a gift sample from Cdymax (India) Pharma Private Limited. HPLC grade methanol, Milli-Q water other necessary chemicals were procured from from Hi-Media (Mumbai, India). In addition Olaparib nanoformulation were prepared by sacrifial template method.

Chromatography Instrument and Conditions

A Shimadzu HPLC instrument (LC-2010, Japan) adapted with pump (LC-20AD), degasser (DGU-20ASVP), auto-injector (SIL-20ACHT), PDA detector (SPD-M20A), CTO-10AS VP column oven and injecting value with 10 µL loop. The analytical data was interpreted using Lab Solutions software. For the chromatographic separation, a Phenomenex Luna C18 column (4.6×150 mm, 5-µm particle size) was utilised, thus ensuring precise and reliable chromatographic analyses. The mobile phase was thought up of different ratios of methanol and water. Elution was performed at a flow rate of 1.08 mL/min, at a column temperature of 30°C. Sample analysis was performed with an injection volume of 10 µL and a detection wavelength of 254 nm. The samples were filtered through a syringe filter (0.45 µ; Millex HV, Millipore, USA) prior to analysis.

Preparation Stock Solution

The initial stock solution of Olaparib was prepared by dissolving 1 mg of Olaparib in 1 mL of methanol at a concentration of 1 mg/mL. Furthur, samples were prepared by diluting the stock solution with the mobile phase to obtain concentrations ranging from 2 to 10 µg/mL. Prior to HPLC analysis, all solutions were stored securely sealed in volumetric flasks at a temperature of 4°C. Each working standard solution was subsequently injected individually, and chromatograms were generated for analysis.

Preliminary Method Development Studies

Due to its limited solubility in aqueous phases, Olaparib required the utilization of organic solvents such as methanol for dissolution. Methanol was initially selected as the ideal solvent due to its potential to fully solubilize Olaparib. Accordingly, methanol was selected as the mobile phase for chromatographic analysis. However, initial trials using acetonitrile-water (50:50), achieving a retention time of 10 min. Subsequently, acetonitrile was replaced with methanol, resulting in the observation of discernible peaks. To optimize separation and peak characteristics, we used a quality-by-design approach with design of experiment methodologies. This systematic approach ensures the methodical optimization of chromatographic conditions, leading to robust and reliable analytical results.

Developing an RP-HPLC Technique with the Use of CCD

This study utilized response surface methodology, a statistical technique that integrates detailed HPLC analysis with controlled factors (independent variables) and their corresponding measurements (dependent variables) as shown in Table 1. The response surface method used in this study involves a comprehensive HPLC method with both independent and dependent variables.[10-12] The optimization process for method development of Olaparib was executed utilizing a specialized design known as CCD in the software Design Expert® (Version

13, State-.Ease Inc., Minneapolis, USA). To attain the desired optimal conditions, two independent variables Solvent ratio and Flow rate and three dependent variables, namely Retention Time (RT), Tailing Factor (TF) and Theoretical Plate (TP), resulting in 13 experimental runs was evaluated as optimization response parameters. A polynomial equation was formulated according to the responses provided, and to determine the optimal levels of factors A and B for the desired response, ANOVA screening was conducted. The optimal model was rigorously validated to ensure its predictive accuracy.

Method Validation

Validation of the developed analytical method was conducted in accordance with ICH Q2 (R1) guidelines at the optimized conditions, which comprised a specific set of variables.[13-19]

System Suitability

To assess system suitability, six replicate injections of a 20 µg/mL standard solution were performed. The percent Relative Standard Deviation (% RSD) for the Critical Quality Attributes (CQAs) was calculated.

Linearity and range

Linearity was evaluated by analyzing olaparib solutions at concentrations ranging from 2 to 10 µg/mL in triplicate. A calibration curve was generated by plotting peak area versus concentration. The linearity of the method was confirmed over three consecutive days of analysis within the same concentration range.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ was conducted utilizing the standard deviation method and was calculated using a specific formula:

$$LOD=3.3\times\sigma/S \quad (1)$$

$$LOQ=10\times\sigma/S \quad (2)$$

Here, σ is the standard deviation of the response, and S stands for slope.

Precision

To assess precision, Olaparib samples were analyzed at three quantification levels: Higher (HQL), Intermediate (IQL), and Lower (LQL), corresponding to concentrations of 2, 6, and 10 µg/mL. Intra-day and inter-day precision were evaluated by performing three injections on three consecutive days %RSD and recovery were calculated to assess precision.

Accuracy

The results of recovery experiments are based on the sample's % mean recovery. This was conducted at three concentration levels (50%, 100%, and 150%) in triplicate to assess method accuracy for estimation of Olaparib. The samples were analysed, and the percentage recoveries were reported.

Robustness

The robustness of the method was evaluated by introducing small changes in the chromatographic conditions, such as altering the wavelength (252, 254 and 256 nm), flow rate (1.06, 1.08 and 1.10 mL/min).

Force degradation studies

Acid degradation study was conducted by treating 1 N HCl (1 mL) solution with OLA stock solution (1 mL).[20,21] The resulting solution was sealed in a volumetric flask and heated at 80°C for a duration of 2 hr. Further, the solution was neutralized with 1 N sodium hydroxide and diluted upto 10 mL using the mobile phase prior to HPLC analysis.

Thermal Degradation

Thermal degradation study was done by mixing 1 mL of OLA stock solution with 2 mL of methanol. The flasks were sealed and heated at 80°C for 2 hr. The solution was cooled at room temperature and then diluted to a final volume of 10 mL with the mobile phase. The resultant solution was filtered and was subjected for further HPLC chromatographic analysis.

Alkaline Degradation

OLA stock solution (1 mL) was treated with 1 N NaOH (1 mL) solutions in a base degradation study. The solutions were sealed and then heated at 80°C for 2 hr. The samples was neutralized with 1 N HCl (1 mL) and made the volume upto 10 mL using the mobile phase before the HPLC analysis.

Oxidative Degradation

Stock solution (1 mL) of OLA was mixed with 1 mL of 30% hydrogen peroxide. The mixture was heated at 80°C for 2 hr in a thermostatically controlled water bath. After cooling, the mixture was diluted with the mobile phase and injected into the HPLC.

Photo-Degradation Study

Photolytic study was carried out by placing 1 mL of OLA stock solution in a transparent volumetric flask and mobile phase was added to make up the final volume of 10 mL. The flask was sealed and placed in direct sunlight for 2 hr. After the desired time of heat exposure, the resultant solution was filtered and analysed by HPLC system.

Analysis of Developed Nanoformulation

Graphene Quantum dot gated copper sulfide nanoparticles modified with hyaluronic acid were synthesized using the facile sacrificial templating method at a mild temperature.[22-26] The preparation utilized PVP, Copper chloride, Sodium sulphide, Hyaluronic acid, NHS and EDC. For particle size analysis, 1 mg of nanoparticles was diluted with 10 mL of methanol and measured using a Malvern Zetasizer. The developed RP-HPLC method was employed for analysis. For analysis, a 1 mL aliquot of the prepared nanoformulation was diluted to 10 mL with the mobile phase, sonicated for 10 min, filtered through a 0.45 µm Nylon syringe filter, and injected into the HPLC system. Finally the chromatogram was obtained and the peak responses were analysed.

RESULTS

Method Validation

To ensure the suitability of the optimized HPLC method for its intended application, we conducted thorough validation procedures in accordance with the standards outlined in ICH Q2 (R1 and R2). The proposed HPLC method's validation parameters are listed in Table 2 and all of them adhere to the standard limits specified in the ICH guidelines. A linear relationship was established by creating a calibration curve that plotted the average peak area of the analyte against its concentrations ranging from 2 to 10 µg/mL. The linear regression equation obtained from the calibration curve was utilized to calculate the LOD and LOQ. Additionally, the % RSD for various parameters, including peak area, Retention Time (RT), Theoretical Plates (TP), and Tailing Factor (TF), was evaluated to confirm the validity of the method and were all below 2%, indicating the suitability of the method. The robustness of the analytical procedure was tested by making slight adjustments to the HPLC parameters, including wavelength and flow rate. The results demonstrated that the %RSD for both peak area and RT of the analyte remained well within the acceptable limit of ≤2 %. The precision assessments confirmed the method's high precision. Additionally, recovery studies at 50%, 100%, and 150% demonstrated excellent accuracy. These results collectively highlight the method's reliability and robustness.

Development of an RP-HPLC Method Employing CCD

To optimize the chromatographic conditions CCD was employed, using the volume of the organic phase (methanol: water) and flow rate as the independent variables. The dependent variables, including RT, TF, and TP were set as responses. The experimental design consisted of 13 runs, including five center points, to determine the superlative HPLC conditions, as shown in Table 3.

DISCUSSION

Experimental responses were tested against various kinetic models (quadratic, linear and second-order), with the quadratic model yielding the best fit (p<0.0001). The quadratic model also showed the highest R^2 value, approaching 1, confirming that all independent variables had both individual and combined effects on the dependent variables. To visualize these effects, 3D plots were generated for each response, illustrating the impact of two factors on a single response. The results showed that the response surface for Y1 (Retention time) of all batches varied from 3.97 min to 5.87 min, depending upon the concentration of solvent ratio and flow rate. The regression equation clarified as shown in Figure 2 that, with an increase in solvent ratio (X1) and Flow rate (X2) concentrations, the retention time decreases (Figure 2a). For Y2 (Tailing Factor) of all batches varied from 1.089 to 1.117, depending upon the concentration of solvent ratio and flow rate.

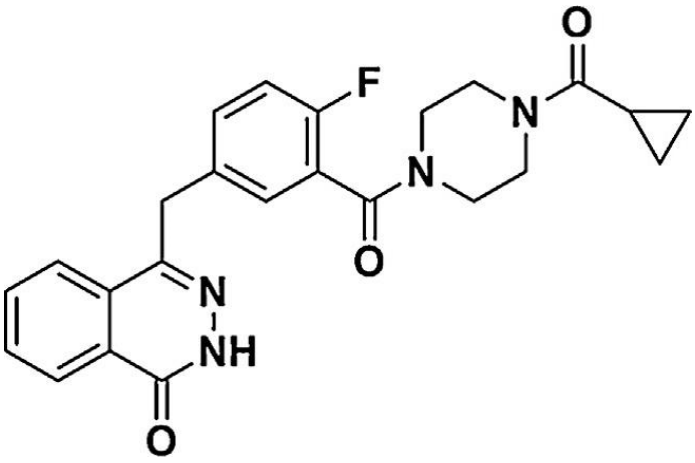

Figure 1: Chemical Structure of Olaparib.

Table 1: Optimization Parameters for HPLC method development.

Code	Variables	Levels		
Independent Variables		**Low (-1)**	**Medium (0)**	**High (+1)**
X1	Solvent ratio	60	62.5	65
X2	Flow rate	0.9	1	1.1
Dependent Variables				
Code	Variables	Constrains		
Y1	Retention Time (RT)	Minimum		
Y2	Tailing Factor (TF)	Minimum		
Y3	Theoretical Plate (TP)	Maximum		

The regression equation clarified that, with an increase in solvent ratio (X1) and Flow rate (X2) concentrations, the tailing factor increases (Figure 2b). For Y3 (Theoretical Plate) of all batches varied from 4077 to 4809, depending upon the concentration of solvent ratio and flow rate. The regression equation clarified that, with an increase in solvent ratio (X1) and Flow rate (X2) concentrations, the theoretical plate decreases (Figure 2c).

The experimental data was analyzed using ANOVA within the software to assess the significance of factors and their interactions on the response variables. Polynomial equations were developed to model the relationship between independent (factors) and response variables. Positive coefficients in these equations indicated a favorable effect on optimization, while negative coefficients suggested opposing interactions among the factors. Correlations between all equations are provided in Table 4.

These predictions closely matched experimental results, particularly in Run 13, with a low prediction error of 5%. Under these optimal conditions, excellent chromatographic of the drug was achieved as shown in Figure 3.

Table 2: **Outline of Validation parameters.**

Parameters	Result
Detection wavelength (nm)	254 257
Linear dynamic range (µg/mL)	2-10 µg/mL
Correlation coefficient	0.9983
Limit of Detection (LOD) (µg/mL)	0.499602
Limit of Quantification (LOQ) (µg/mL)	1.513946
% RSD	0.043
Accuracy of Standard Olaparib	
50% recovery	101.62
100% recovery	100.75
150% recovery	99.94
Accuracy of Nanoformulation	
50% recovery	100.78
100% recovery	99.95
150% recovery	100.12

Recovery Assay in prepared Nanoformulation

The proposed method was effectively applied for the determination of olaparib in prepared nanoformulation, with six replicate measurements conducted. Quantitative estimation revealed prominent peaks for olaparib in the presence of nanoformulation, underscoring superior performance in terms of recovery assay as shown in Figure 4 and Retention Time, Theoretical plates, Tailing factor, Peak area as shown in Table 5. This ensures the reliability of the obtained results. The particle size and zeta potential of nanoformulation was performed, and particle size was found to be 110.3 nm and zeta potential was found to be -18.36 respectively.

Force Degradation Studies

The results of the stress degradation study (acid, base, oxidative, thermal, and photolytic) conducted on the standard solution of OLA is represented in Table 5.

In the acid degradation study, olaparib was minimally affected, exhibiting degradation with the formation of one minor degradation peak while maintaining a significant peak height. In the thermal degradation study, olaparib showed a significant

Table 3: **Optimization of Olaparib HPLC method development using CCD.**

Runs	Factors		Responses		
	X1-Solvent ratio (%)	X2-Flow rate (min/mL)	Y1- Retention Time (min)	Y2- Tailing Factor	Y3-Theoretical Plate
1	65	0.9	4.82	1.106	4434
2	62.5	1	4.79	1.101	4439
3	60	1.1	4.83	1.103	4354
4	62.5	1	4.78	1.092	4452
5	65	1	4.36	1.105	4481
6	62.5	1.1	4.35	1.098	4152
7	62.5	0.9	5.3	1.095	4734
8	62.5	1	4.8	1.089	4236
9	60	1	5.31	1.102	4574
10	60	0.9	5.87	1.105	4809
11	62.5	1	4.79	1.092	4478
12	62.5	1	4.78	1.093	4478
13	65	1.1	3.97	1.117	4077

Table 4: Regression coefficient equation.

Sl. No.	Variables	Constant	A	B	AB	A^2	B^2
1	Retention time(min)	+4.79	-0.4767	-0.4733	+0.0475	+0.0472	+0.0372
2	Tailing factor	+1.09	+0.0030	+0.0020	+0.0033	+0.0107	+0.0037
3	Theoretical plate (N/m)	+4438.31	-124.17	-232.33	-	-	-

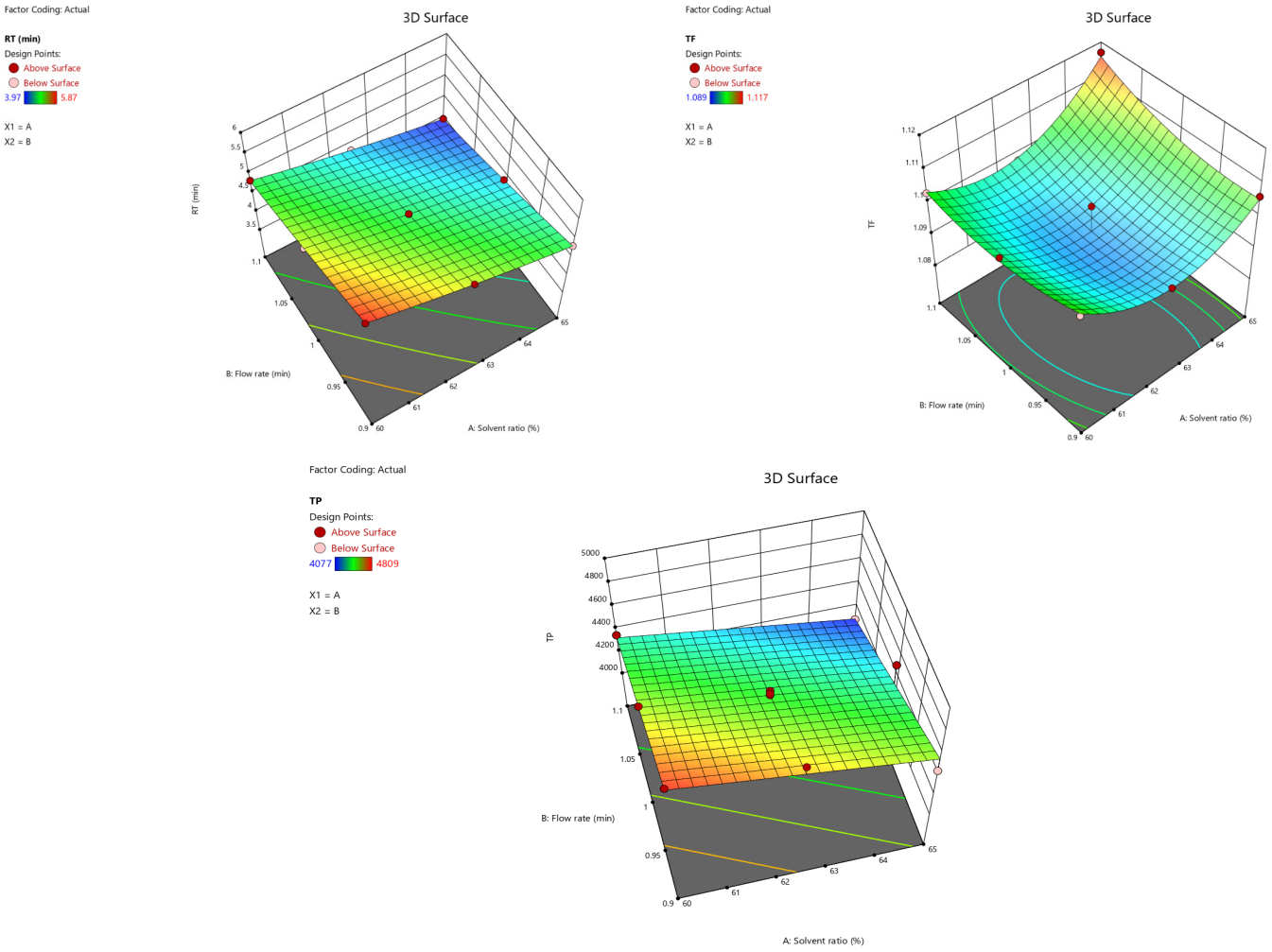

Figure 2: 3D-Response surface graphs of Olaparib (a) retention time (b) tailing factor (c) theoretical plate.

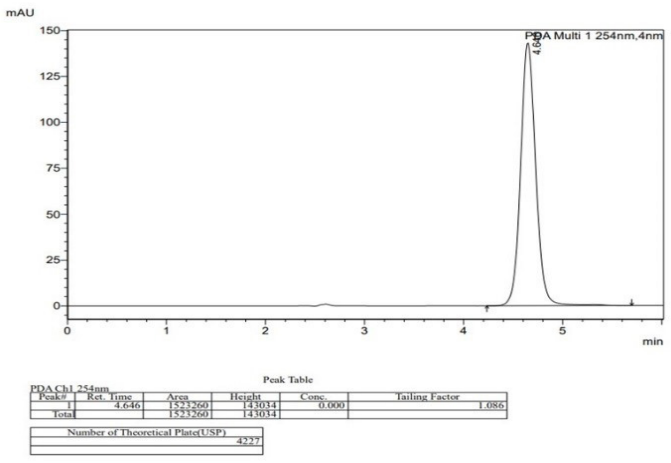

Figure 3: Chromatogram of Olaparib.

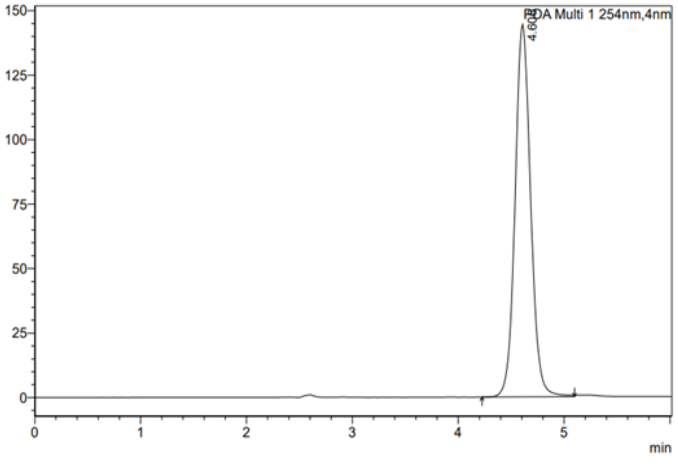

Figure 4: Chromatogram of Olaparib nanoformulation.

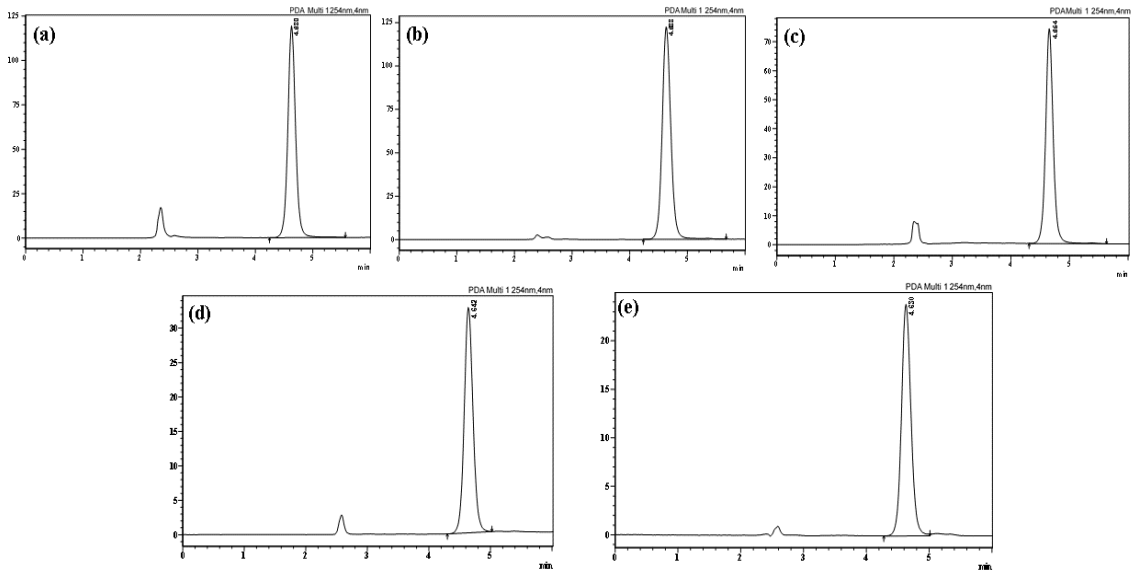

Figure 5: Force degradation study of Olaparib (a) Acid degradation (b) Thermal degradation (c) Base degradation (d) Oxidative degradation (e) Photolytic degradation.

Table 5: Chromatographic Condition of HPLC and force degradation study.

Parameters	Conditions	
HPLC system	Shimadzu HPLC system (LC-2010)	
Column	C-18 (Phenomenex Luna, 4.6 mm×150 mm, 5 µm)	
Wavelength (nm)	254	
Flow rate (mL/min)	1.08	
Injection volume (µL)	10	
Mobile phase ratio	Methanol: water (61.36:38.64)	
Column Temperature (°C)	30	
	Standard Olaparib	Nanoformulation
Retention Time	4.606	4.639
Theoretical plates	4401	4377
Tailing factor	1.079	1.078
Peak area	1478891	1504017
Force Degradation Study		
Exposed degradation conditions	Retention time (min)	% Degradation
1 N HCL, 60°C, 2 hr	4.630	7.05
Thermal, 60°C, 2 hr	4.633	10.19
1 N NaOH, 60°C, 2 hr	4.654	12.34
30% H2O2, 60°C, 2 hr	4.642	27.02
Photolytic sunlight, 2 hr	4.630	31.53

decrease in peak height. Similarly, the base degradation study revealed olaparib with the presence of small degraded peaks. The oxidative degradation study was characterized by a reduced peak height when exposed to oxidative stress and the presence of minor degraded peaks for olaparib. In the photolytic degradation study, olaparib demonstrated a significant reduction in peak height and the appearance of degradation peak. The peak area and retention time of olaparib remained unchanged throughout all degradation studies, indicating no significant degradation or interference. The purity of the olaparib peak was not significantly affected, confirming the method's robustness and reliability. Figure 5 shows the chromatographic data of blank and stressed standard solutions after 2-hr exposure.

CONCLUSION

A simple, rapid, cost-effective, and stability-indicating HPLC method for OLA has been successfully developed using a Quality by Design (QbD) approach. This technique reliably detects and measures OLA in both its pure and nanoformulation forms. The Central Composite Design (CCD) was instrumental in optimizing chromatographic conditions, revealing the significant influence of organic phase concentration and flow rate on response variables such as RT, TF, and TP. Optimal conditions were determined through numerical optimization within the defined analytical design space. Method validation encompassed parameters such as linearity, specificity, accuracy, precision, robustness, LOD, LOQ, and system suitability. Furthermore, to quantify total drug degradation, forced degradation studies were conducted under different stress conditions. This method is adaptable to various OLA formulations, highlighting the effectiveness of the QbD approach in HPLC method development and validation. The

validated method also shows strong potential and cost effective for broader analytical applications of OLA.

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ABBREVIATIONS

ANOVA: Analysis of variance; **CCD:** Central Composite Design; **OLA:** Olaparib; **ICH:** International Council for Harmonisation; **LOD:** Limit of Detection; **LOQ:** Lower Limit of Quantification; **QbD:** Quality by Design; **RP-HPLC:** Reversed-Phase High-Performance Liquid Chromatography; **%RSD:** Relative Standard Deviation; **RT:** Retention Time; **TFL:** Tailing Factor; **TP:** Theoretical Plates.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

This study developed and validated a reliable RP-HPLC method for quantifying Olaparib in a Graphene quantum dot nanoformulation using Analytical Quality by Design (AQbD) principles. A central composite design optimized key chromatographic parameters, including retention time, theoretical plate count, and tailing factor. The optimized method achieved a retention time of 4.6 min, high sensitivity with LOD and LOQ of 0.4996 µg/mL and 1.5139 µg/mL, respectively, and excellent precision with %RSD below 2%. The method's stability-indicating nature ensures accurate quantification of Olaparib in quantum dot nanoformulation. This robust and reliable RP-HPLC method provides a valuable tool for pharmaceutical research and development, demonstrating the effectiveness of AQbD in enhancing drug quantification.

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