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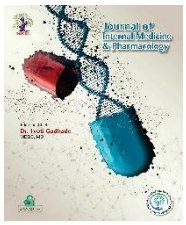

Research Article

Development and Validation of an RP-HPLC Assay for the Quantitative Analysis of Oseltamivir Phosphate in Bulk and Formulated Capsules

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ARTICLEINFO

ABSTRACT

Oseltamivir phosphate (OP), an inhibitor of the neuraminidase enzyme, is essential for treating Influenza A, B, and COVID-19. This study focuses on the synthesis of OP and the development and validation of an RP-HPLC method for determining its concentration. Through a Michael addition reaction with pyrimidine as a catalyst, OP was successfully synthesised with an 85 percent practical yield. Using a C18 Agilent Zorbax Bonus-RP column (240 mm x 4.6 mm, 5 m) at an oven temperature of 30°C and a mobile phase consisting of 0.1 percent trifluoroacetic acid (TFA) in water and acetonitrile (ACN) (65:35 v/v), the drug and capsule formulation were separated chromatographically. At a flow rate of 1 mL/min, 10 L was injected into the RP-HPLC apparatus, and peaks were identified at 218 nm. The retention time (RT) of the developed method was 2.53 minutes, with a linear calibration curve (R2 = 0.9999).This technique of synthesis is straightforward and effective, with the catalyst producing great results in practise. In addition, the RP-HPLC method for OP measurement was accurate, precise, and cost-effective. Consequently, RP-HPLC can be utilised for routine quantitative evaluations of pharmaceuticals and formulations. This research contributes significantly to the field of pharmaceutical analysis and quality control.

Keywords: Oseltamivir phosphate, RP-HPLC, Neuraminidase Inhibitor, Analytical validation

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1. Introduction

Large-scale attempts have been made to discover medications that can prevent or treat COVID-19 since the global breakout of the pandemic(Zendehdel, 2022). Numerous national and international research organisations have been striving to discover and grant access to medicines for SARS-CoV-2 variants and other pandemic viruses. The documented data showed that antiretroviral drugs were efficacious as the first line of treatment for COVID-19 patients [2]. Several medications, including Remdesivir, Hydroxychloroquine, and OP, are currently approved for the treatment of COVID-19(Indari, 2021). The antiviral drug neuraminidase inhibitor OP is used to treat COVID-19 and influenza A and B [4]. It is chemically Ethyl (3R, 4R, 5S)-4-acetylamino-5-amino-3-pentane-3-cyclohexene-1-carboxylate phosphate, as shown in Figure.1(Sharma,2010). Its active metabolite specifically suppresses the viral surface enzyme neuraminidase, prohibiting the virus from infecting cells. It is readily absorbed from the gastrointestinal tract after oral administration and is extensively converted by predominantly hepatic esterases to the active metabolite oseltamivir carboxylate. Following absorption, it is more than 90 % eliminated through conversion to oseltamivir carboxylate and subsequent elimination entirely through renal excretion(Fujiwara.2020). The previously reported data revealed several synthesis reactions such as aldol reactions and Horner-Wadsworth-Emmons olefination synthesis by employing different catalysts such as pyridine, purine and nitric acid(Li Sagandira and Watts, 2020; Trajkovic,2011). In terms of chromatographic techniques such as high-performance liquid chromatography, ultra-performance liquid chromatography and UV spectroscopy are utilized for individual estimation of OP and in combination with other drugs(da Ruos, 2022; Gungor, 2021; Upmanyu and Porwal, 2019). Recent data revealed that OP synthesis required a lot of time, chemicals and had a low practical yield. Similarly, chromatographic development methods include maximum RT for individuals and simultaneous estimation of OP. Hence, attempts have been made to synthesise OP by altering the catalyst and estimation of OP in the present investigation, and its capsule formulation was accomplished by the RP-HPLC chromatographic method. The OP was synthesized by Michael addition reaction using pyrimidine catalyst, which showed maximum % practical yield. Additionally, the analytical method was developed and validated in accordance with ICH Q2 (R1) guidelines. The detailed materials utilized in the present work and methodology are explored in a subsequent section.

2. Materials and Methods

2.1 Materials

Aadhaar life science Pvt. Ltd. Solapur, India, provided the OP as a gift sample. Analytical HPLC grade, acetonitrile, and trifluoroacetic acid were purchased from Merck Specialities Pvt. Ltd. Mumbai, India. The ultra-pure water of HPLC grade was procured from Merck Specialities Pvt. Ltd. Mumbai, India.

2.2. Chromatographic conditions

The RP-HPLC system deployed for the method development and validation comprised 1260 Infinity II with a G1311B pump, G1322A degasser, and G4212B Diode Array Detector (DAD) detector.

Analyses and separations have been performed using an Agilent zorbaz bonus -RP (240 mm x 4.6 mm, 5 μm) column at an oven temperature of 30°C. A 0.1% TFA Water: ACN (65:35 v/v) was employed as the mobile phase. The flow rate was adjusted to 1.0 mL/min with an injection volume of 10 μL. The absorbance was measured at 218 nm using a DAD detector.

2.3 Methods

2.3.1 Synthesis of OP

OP was synthesized by Michael addition reaction by using pyrimidine as a catalyst. The details procedure is outlined below. Tetriarybutyl-3-nitroacrylate and 2-pentane-3-oxyacetaldehyde were combined for a 6-hour reaction at 23 °C in the presence of chloroacetic acid and octadecytrimethoxysilane (OTMS). The combination was continually mixed for up to 4 hours while being added to the mixture of ethyl-2- (diethoxyphosphoryl acrylate) and cesium carbonate. Following gradual evaporation of the combination, ethanol was added to the solution mentioned above. P-tolylthiolate was added to the reaction mixture and allowed to stand at 15°C for 3 h. The purported reaction synthesised the ethyl -5 amino 4-methoxy carbonylamino 3-pentene 3-oxycyclohexene 1-carboxylate intermediate. It was filtered, dried, and treated with Triofloroacetic acid DMF at 0°C for 5 hrs in a toluene reagent. The organic solution was separated and reacted with trimethylsilyl azide and pyridine for 20 minutes. This was washed with acetic anhydride and acetic acid and then kept for reflux for upto 48 hr. Thus, the final intermediate was treated with zinc dust, trimethylsilyl chloride, and ethanol for 2 hrs at 70°C and ammonia was added at 0°C and subjected to this reflux for 10 minutes. Finally, potassium carbonate was added, and the reaction was kept for 9 h at room temperature. In the end, the product was added to ice cold water and then precipitated. It was filtered and washed with ethanol, and dried. Then the crude precipitate was subjected to further evaluations(Santos,2022).

2.3.2 Characterization of synthesised molecule

1.^{1}H NMR and ^{13}C NMR analysis

Nuclear Magnetic Resonance (NMR) spectrometry was performed on (Bruker Spectrometer), ^{1}H NMR was recorded at 400MHz, and ^{13}C NMR was recorded on Spect 5mm-PABBO BB (400MHz zgpg 30) in $CDCL_{3,}$ respectively. The sample was dissolved in a solvent, placed into NMR tube to make sample depth around 3.5 to 4 cm-1 and analysed by an NMR spectrometer. Chemical shifts were reported as δ units (ppm) with tetramethylsilane (TMS) as internal standard and coupling constants (J) in Hz. The 1H-NMR and 13C-NMR data were integrated using Topspin software(Ogasawara, 2017).

2.3.3 Preliminary analysis of OP

The melting point and solubility OP were determined in preliminary analysis. The melting point of OP was determined by the capillary method. The OP was placed within glass capillaries, with a flame used to close one end. The drug-containing capillary was immersed with an appropriate aperture in liquid paraffin inside Thiele's tube and heated with a silicon oil heater with a heating rate of 1°C/min. The melting point was monitored by analysing the temperature at which it began to melt. A 5 mL of the tube was laden with excess OP. Solubility was determined in water and organic solvents such as acetonitrile and methanol. Afterwards, the test tubes were covered with aluminium foil, and the amount of drug solubilized was observed (Upmanyu,2019).

2.3.4 Analytical method development

Preparation of stock solutions (Kousar, 2017)

1. Preparation of OP Standard Stock Solution (SSS-I)

A stock solution of OP was prepared in a 100 mL volumetric flask by dissolving an accurately weighed 7.5 mg of OP in 20 mL of ACN: Water (50:50, v/v) which was used as a diluent and sonicated for 10 minutes. Afterwards, the final volume comprised 100 mL of the diluent mixture(Sasikala, 2020).

2. Preparation of OP Working Standard (WS)

Working solutions were prepared from the corresponding SSS-1. In a 10 mL volumetric flask, a 1 mL SSS pipette was out and diluted with diluent up to 10 mL to obtain the concentration of working solution of (OP =75 µg/mL) (Kousar,2017).

3. Preparation of OP Capsule Stock Solution (CSS)

Five capsules of OP were weighed, powder equivalent to 7.5 mg of OP was transferred in a 100 mL volumetric flask, and a small amount of diluent was added. The solution was sonicated for 15 minutes, and the final volume was made with the same to obtain the solution of OP (750 µg/mL).

4. Preparation of OP capsule sample

A capsule sample solution was prepared from the corresponding CSS. In a 10 mL volumetric flask, 1 mL CSS pipette out and diluted with diluent up to 10 mL to obtain the concentration of capsule sample of (OP =75 µg/mL).

5. Selection of analytical wavelength

To investigate the appropriate wavelength for determining OP in the Water: ACN (50:50) was scanned on a UV-visible spectrophotometer (UV 1700, Shimadzu, Japan) in a 200-400 nm range.

6. Preparation and selection of mobile phase

The mobile phase was prepared and selected by mixing various concentrations of 0.1% TFA water: ACN (60:40,70:30, 65:35 v/v). To select a suitable one, all mobile phases were run at a flow rate of 1mL/min at different wavelengths. The selected mobile phase was filtered through 0.45µm and degassed before use.

2.3.5 RP-HPLC method validation

The optimized method for the determination of OP has been validated as per the ICH Q2 (R1) guidelines for assessing specificity, system suitability, linearity, accuracy, precision, the limit of detection (LOD), and the limit of quantitation (LOQ). The precise method for validation parameters is discussed in a subsequent section(Bano, 2015).

1. Specificity

Specificity of the proposed RP-HPLC method was performed by comparing blank, working standard and drug product solutions to justify the specificity of the target analyte.

2. System suitability

A system suitability study was done to confirm that the RP-HPLC system is working correctly and can provide accurate and precise results. System suitability parameters concerning tailing factor, repeatability, number of theoretical plates, and resolution of OP peaks were assessed by injecting 10 µL of OP solutions in five replicates.

3. Linearity

The linearity study was conducted by analyzing OP samples at different concentration ranges of (60-90 µg/mL) using the proposed RP-HPLC method. The calibration plots were generated by plotting the peak area of OP against the concentrations with least-square linear regression analysis.

4. Accuracy

The spiking OP -SSS samples established the proposed method accuracy at three levels 80, 100, and 120%. From SSS-I and CSSS-I 0.8, 1,1.2 mL solution pipette out and injected to RP-HPLC system and chromatogram were recorded under the

chromatographic conditions after a stable baseline. Duplicate determination of these three levels has been documented to obtain the % Recovery and % Relative standard deviation (% RSD).

5. Precision

The precision of an analytical method was studied by performing a repeatability study on five working standard solution samples of OP (75 µg/mL). A 10µl solution was injected into the RP-HPLC system, and chromatograms were recorded under the same chromatographic conditions after a stable baseline. The procedure was repeated five times, and the % RSD was calculated.

6. Limit of Detection and Limit of Quantitation

LOD and LOQ for OP were calculated using the formula from the linear regression equation based on a standard deviation of the intercept and the slope.

$LOD = 3.3 \times \sigma / S$ and $LOQ = 10 \times \sigma / S$

Where σ: the standard deviation of the response, S: the slope of the calibration curve.

3. RESULTS AND DISCUSSION

3.1 Synthesis of OP

The OP was synthesized successfully by using pyrimidine as a catalyst. The % practical yield of the synthesized OP was found to be 85%. The maximum practical yield showed that pyrimidine was the most suitable catalyst for OP synthesis. The schematic representation of OP synthesis is shown in Figure.2

3.1.1 Characterization of synthesised molecule

1.^{1}H NMR and ^{13}C NMR analysis

The ^{1}H-MMR spectrum of OP displayed δ 8.16-8.14 (d, 1H), 6.65 (br s, 1H), 4.28-4.10 (m, 4H), 4.08 (q, 1H), 3.65-3.63 (q, 1H), 3.38-3.35 (br s, 1H), 2.68 (dd, 1H), 2.51-3.49 (m, 1H), 1.87 (br s, 3H), 1.45-1.37 (m, 4H), 1.24-1.21 (t, 3H), and 0.86-0.77 (m, 6H) ppm. C13 NMR values shows δ 170.73, 165.25, 138.63, 127.46, 81.18, 74.53, 60.54, 53.00, 48.31, 40.12-38.87 (J = 125 Hz), 29.16, 25.58-25.06 (J = 52 Hz), 23.23, 14.05, and 9.36-8.83(J = 53 Hz) ppm. The obtained spectrum of NMR is indicated in Figure.3 and 4.

3.2 Preliminary analysis of OP

The melting point of OP was successfully ascertained by the glass capillary method. The melting point was found to be 190°C, in the range of its standard values of 190-206°C. The obtained value indicated that OP was in pure form. In the solubility study, it was observed that OP was soluble in water, acetonitrile and methanol; hence these solvents can be used for further studies.

3.3 Analytical method development

The presented RP-HPLC method was developed to provide a reliable, simple, and economical HPLC method.

3.3.1 Selection of analytical wavelength

The wavelength of OP was determined in the range between 200-400 nm in Water: ACN (50:50 v/v) by UV spectroscopy. It showed the appropriate intensity at 218 nm; hence this wavelength was selected for further studies, as demonstrated in Figure.5.

3.3.2 Preparation and selection of mobile phase

In varying proportions, solvents such as acetonitrile and methanol were employed for better resolution of the chromatogram. The mobile phase ratio of 0.1%TFA: ACN (70:30 v/v) at the wavelength of 227 nm and RT was found to be 2.80 minutes. Again, the mobile phase ratio changed to 0.1% TFA water: ACN (65:35 v/v) at a wavelength of 218 nm and RT was found to be 2.53 minutes, as shown in Figure.6. The obtained peaks of OP using 0.1% TFA: ACN (65:35 v/v) mobile phase at 218 nm exhibited less RT, better separation and resolution. Hence ACN: 0.1% TFA water (40:60, v/v) was used for further analysis.

3.4 RP-HPLC method validation

1. Specificity

The specificity study of OP and its formulation showed no significant interference in chromatograms due to diluent, mobile phase, and excipients. The WS and drug product (DP) RT was 2.53 and 2.52 minutes. The representative chromatograms are shown in Figure 7, and the specificity results are summarized in Table.1.

2. System suitability

The system suitability study demonstrates the proficiency of the column. It was analyzed by estimating peak area, asymmetry factor, and % RSD, which must be under 2 %. The OP solution system suitability study was found to be 2.53. The outcomes of system suitability are summarized in Table 2.

3. Linearity

The linearity was calculated by linear regression analysis, and it's observed within the range (60-90 µg/mL) for OP. The correlation coefficient, slope and intercept in the calibration curve were observed at 0.9998, respectively. The peak area of each concentration is given in the Table. 3 and the chromatogram of linearity shown in Figure.8

4. Accuracy

The accuracy of the proposed method established at three different concentrations levels of 80, 100, and 120 % is represented in Table.4. The data indicates that the % RSD for OP was found to be 0.08, 0.13, 0.06 % exhibiting that the method has acceptable accuracy within 2%. High accuracy results from the proposed method demonstrated that it can be used for analysis. The chromatograms of the accuracy study are shown in Figure.9.

5. Precision

The repeatability for five sample preparations was conducted successfully. The %RSD of five samples of OP was found to be 0.25%. The obtained value was within 2%, confirming the developed method's excellent precision. The summary and chromatogram of the precision study are shown in Table.5 and Figure.10.

6. Limit of Detection and Limit of Quantitation

The detection and quantification limits were calculated based on the response and slope standard deviation. The LOD and LOQ were found to be 2.12 µg/mL and 6.42 µg/mL for OP, indicating the method's sensitivity.

4. Conclusion

The established Michel addition method using pyrimidine catalyst produced maximum yield, so a large amount of OP was also synthesized using this catalyst. In addition, the RP-HPLC chromatographic method for estimating OP from bulk and formulated dosage forms was a sensitive, linear, accurate, selective, precise and reliable analytical method. The RT for OP was 2.53 minutes only; hence so many samples can be analyzed in a short period. As a result, this technique can be well-suited for routine quantitative investigation of the pharmaceutical dosage form.

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for providing facilities to perform the work.

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

Author statement

All individuals who fit the criteria for authorship have been credited, and they attest to having made substantial contributions to the conception, research, analysis, writing, and review of the text.

Abbreviations

OP: Oseltamivir Phosphate

RP- HPLC: -Reverse-Phase High-Performance Liquid Chromatography

RT: Retention Time

DAD: Diode Array Detector

OTMS: Octadecytrimethoxysilane

NMR: Nuclear Magnetic Resonance

TMS: Tetramethysilane

CAN: Acetonitrile

TFA: Trifluoroacetic Acid

CSS: Capsule Stock Solution

SSS: Standard Stock Solution

WS: Working Standard

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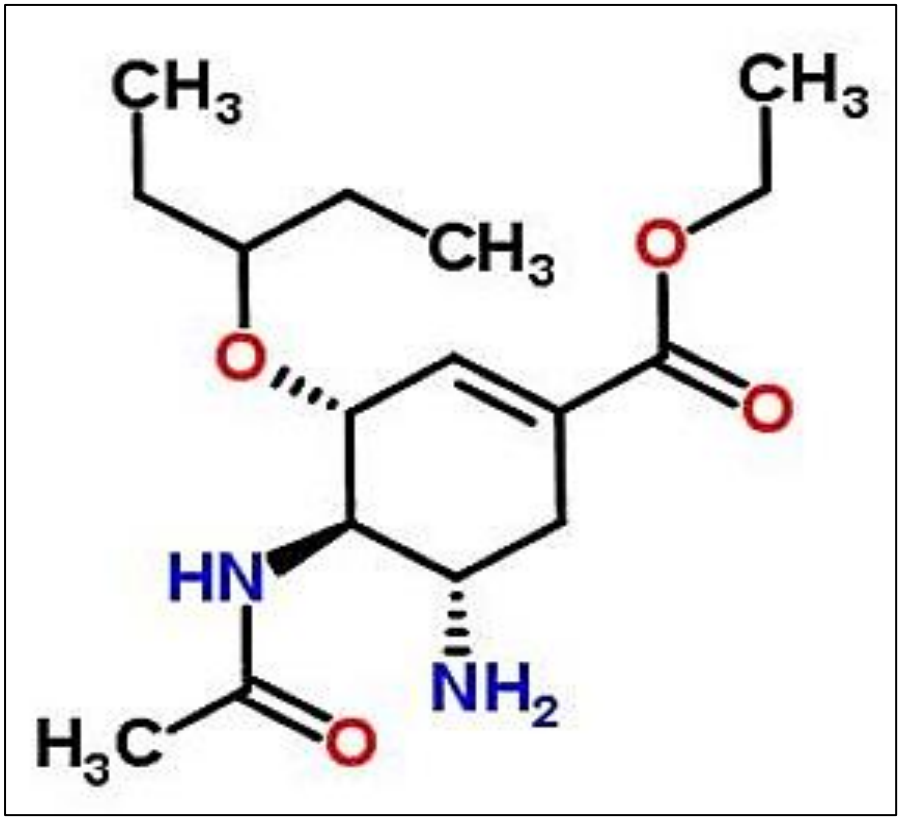

Fig 1: Chemical structure of OP.

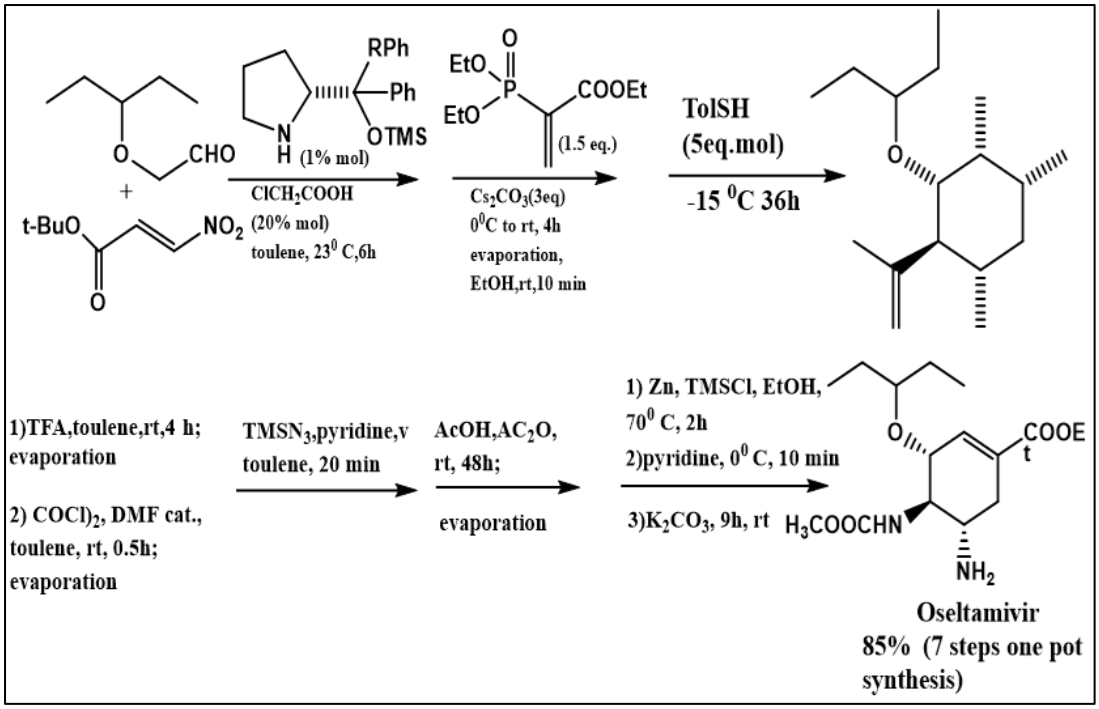

Fig 2: Schematic representation of OP synthesis.

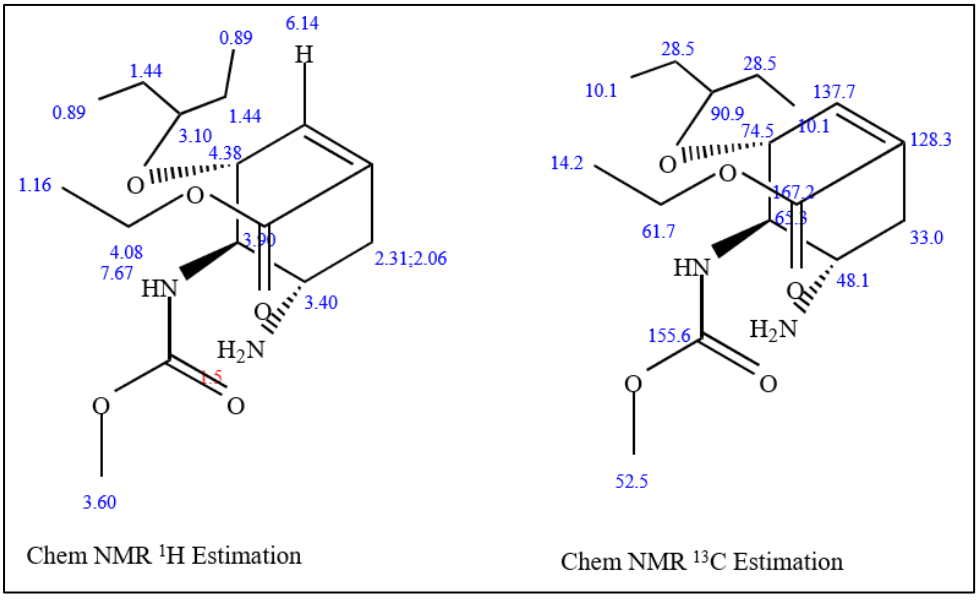

Fig. 3: Estimation of OP by NMR.

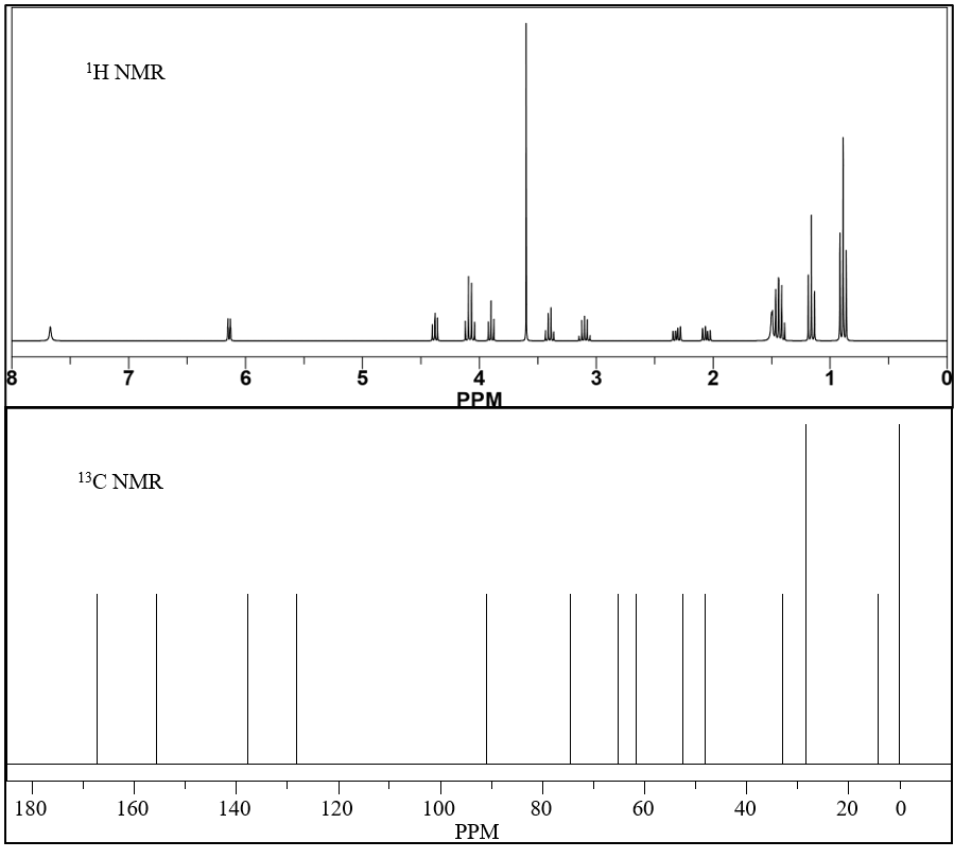

Fig. 4: ^{1}H NMR and 13C NMR spectrum of the OP.

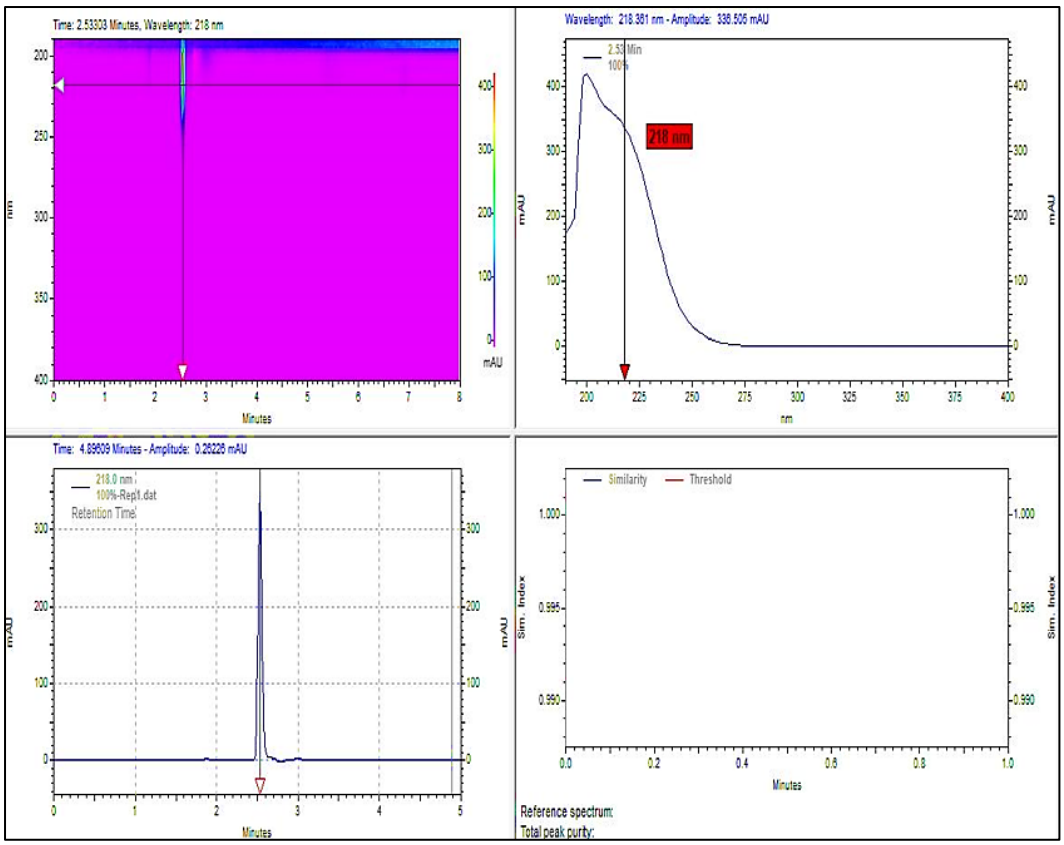

Fig..5: UV spectrums of OP between 200 to 400 nm.

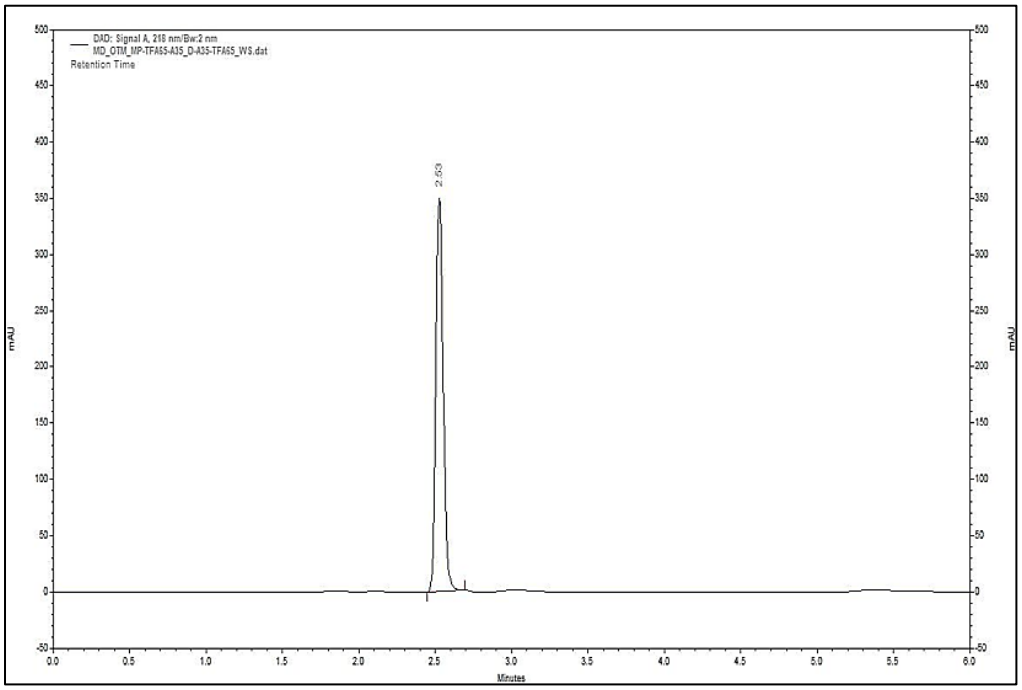

Fig. 6: RP-HPLC method development chromatogram of OP.

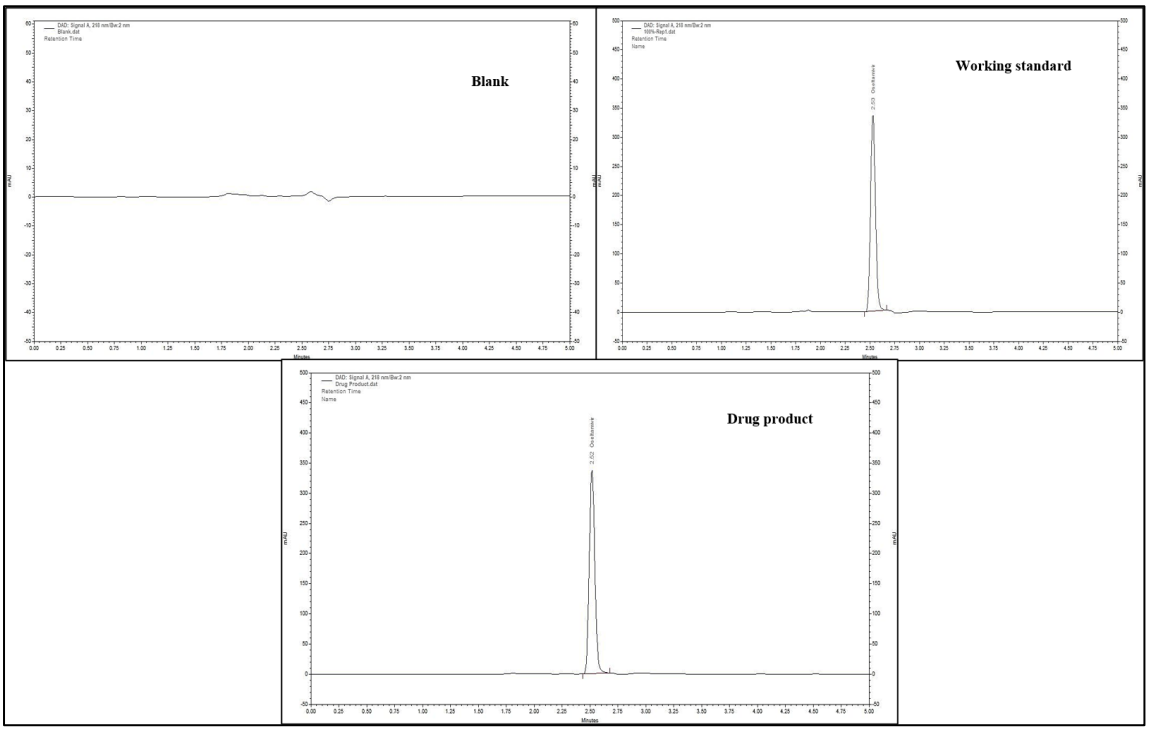

Fig. 7: Chromatograms for specificity study.

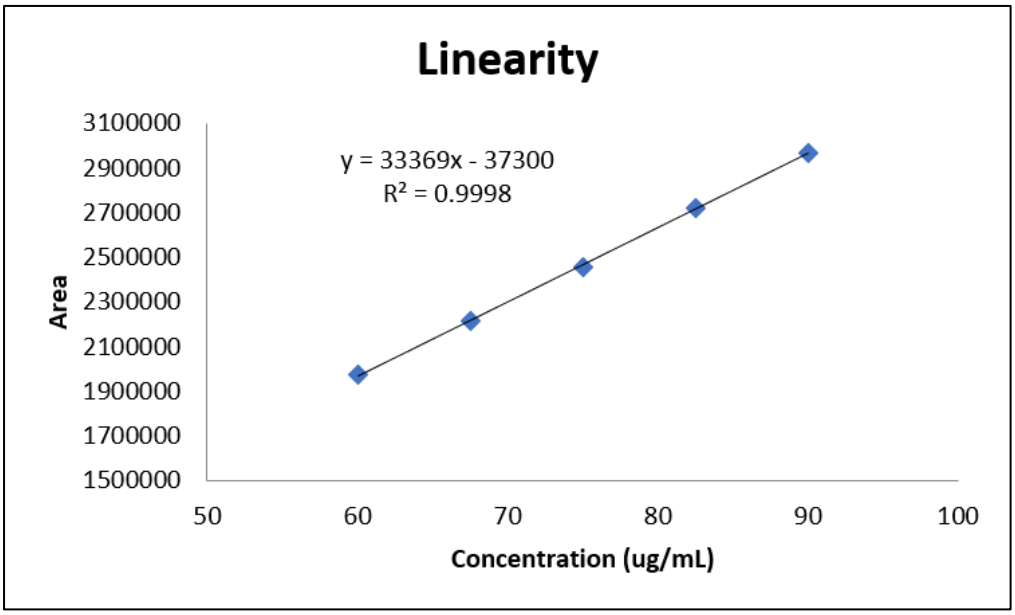

Fig. 8 Calibration curve of linearity study.

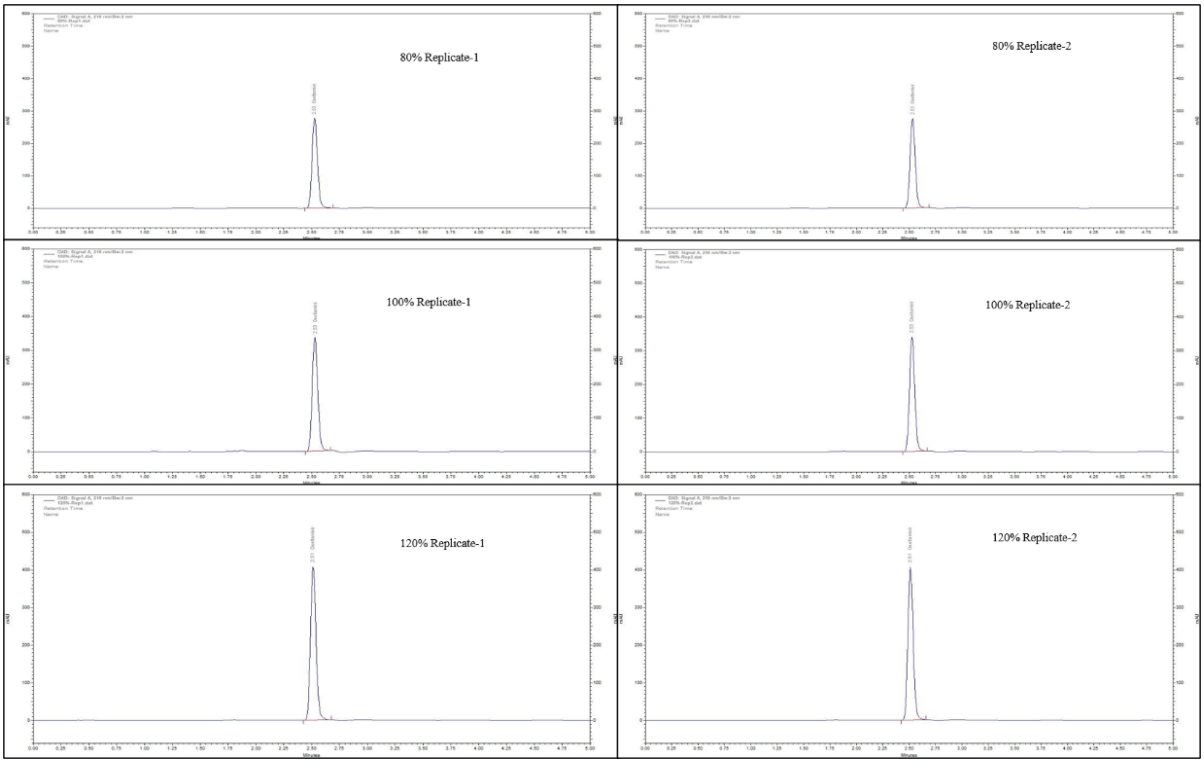

Fig. 9: Chromatograms of accuracy study.

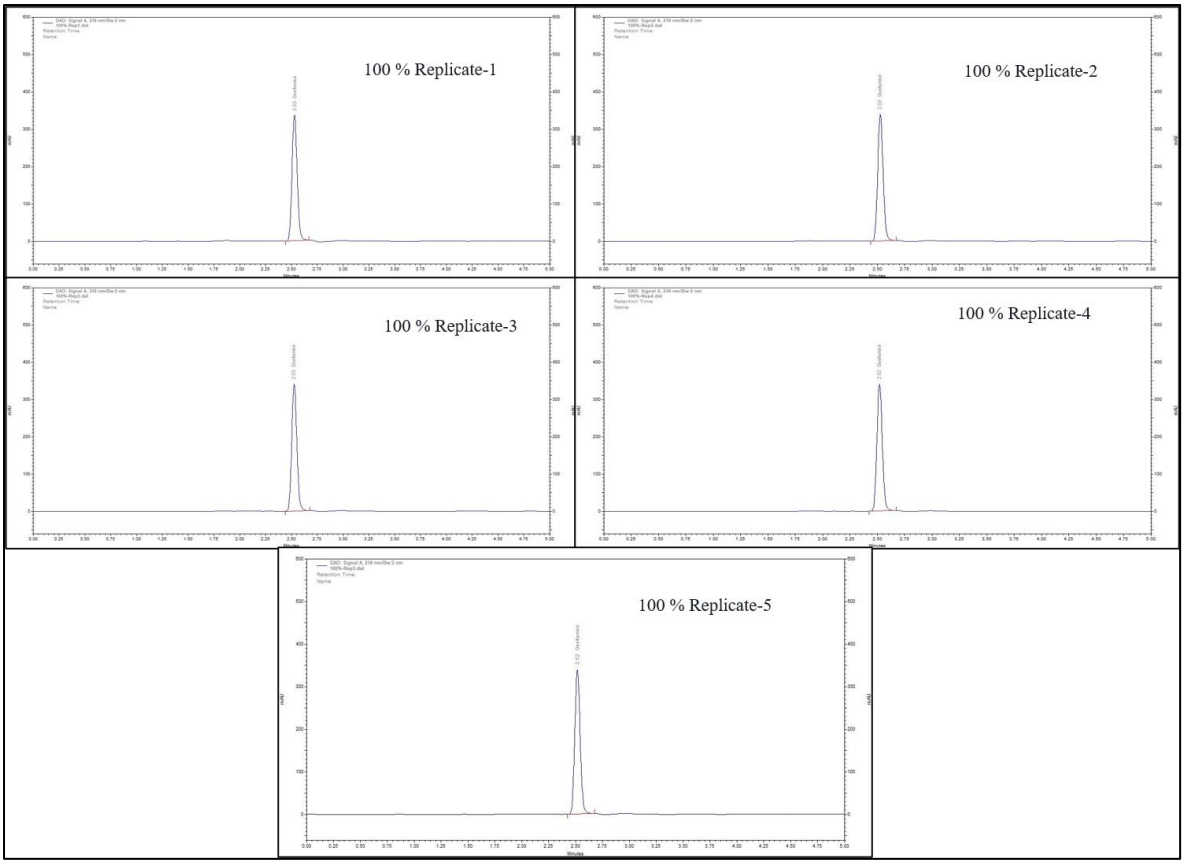

Fig. 10: Chromatograms of precision study.

Table.1: Summary of specificity study.

	RT	Area	% Assay
WS	2.53 minutes	2457163	-
DP	2.52 minutes	2471563	100.28

Table.2: Summary of system suitability study.

Sample ID	RT	Asymmetry	TP
100% Rep 1	2.53	1.05	12058
100% Rep 2	2.53	1.13	11939
100% Rep 3	2.53	1.12	11937
100% Rep 4	2.52	1.10	11822
100% Rep 5	2.52	1.05	11945

AVERAGE	2.53		
STDEV	0.005477		
% RSD	0.22		

Table.3: Response of OP at various linearity levels.

% Conc.	Concentration (ug/ml)	Area
80	60	1970776
90	67.5	2211335
100	75	2457163
110	82.5	2719305
120	90	2968110

Table.4: Summary of accuracy study.

Sample ID	Rep-licates	Spiked Conc. (ug/ml)	Area	Amount Recovered (ug/ml)	% Recovery	AVG	STDEV	% RSD
80%	Rep 1	59.82	1970776	59.88	100.09	100.04	0.076638	0.08
	Rep 2	59.82	1968642	59.81	99.98			
100%	Rep 1	74.78	2457163	74.65	99.84	99.74	0.130925	0.13
	Rep 2	74.78	2452606	74.51	99.65			
120%	Rep 1	89.73	2968110	90.18	100.50	100.45	0.062776	0.06
	Rep 2	89.73	2965488	90.10	100.41			

Table.5: Summary of precision study.

Sample ID	Area
100% Rep 1	2457163
100% Rep 2	2452606
100% Rep 3	2464268
100% Rep 4	2463547
100% Rep 5	2468300
AVERAGE	2461177
STDEV	6234.632
% RSD	0.25