International Journal of
Research in Chemistry and Environment
Available online at: www.ijrce.org

ISSN 2248-9649

Research Paper

Development and Validation of Stability Indicating RP-HPLC Method for the Determination of Oseltamivir Phosphate in Oseltamivir Phosphate Capsules

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(Received 27th September 2014, Accepted 17th December 2014)

Abstract: *A simple, precise, accurate and stability-indicating HPLC method was developed and Validated for the determination of Oseltamivir Phosphate, which is used to treat Influenza in Pharmaceutical Dosage forms. The separation was achieved on YMC Pro C8, (150 x 4.6 mm) 5 µm column and potassium dihydrogen phosphate (pH 3.2±0.05) as a buffer, Buffer: Methanol: Acetonitrile in the ratio 60:20:20 (v/v) as the Mobile Phase and Water, Methanol and acetonitrile 625:40:135 as a diluent. Chromatography was performed with sample injection volume of 20 µL, Flow rate 1.5mL/min and Column Temperature 45^{0}C as Instrumental parameters and the analyte was detected at 207nm. The method has been validated for Specificity, Linearity, Accuracy, Precision, Solution Stability and Robustness. The Oseltamivir phosphate was subjected to stress conditions of hydrolysis (acid, base), oxidation (5% H_2O_2), thermal, humidity and photolytic degradations. The degradation was observed for Oseltamivir phosphate in Acidic, Alkaline and Oxidative Conditions, while, it is found stable to remaining stress condition. The calibration curve was found to be linear in the range of 76.6-229.7 µg/mL. The precision of the method was determined by inter-day and intraday variation studies having RSD values less than 1.0% showing high precision of the method. The validated RP-HPLC method was successfully applied to the quantitative determination of Oseltamivir Phosphate in capsule dosage form.*

Keywords: Oseltamivir Phosphate (OSP), RP-HPLC, Validation and Stability Indicating.

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Introduction

Oseltamivir Phosphate[1,2] is an antiviral licensed to prevent or slow the spread of influenza A and influenza B (flu) virus between cells in the body by stopping the virus from chemically cutting ties with its host cell. The drug is taken orally in capsules or as a suspension. Oseltamivir is a prodrug, a (relatively) inactive chemical, which is converted into its active form by metabolic process after it is taken into the body. It was the first orally active neuraminidase inhibitor commercially developed. It was developed by C.U. Kim, W. Lew, and X. Chen of US-based Gilead Sciences, and is marketed by Genentech. Oseltamivir Phosphate is chemically described as ethyl (3R,4R,5S)-5-amino-4-acetamido-3-(pentan-3-yloxy)-cyclohex-1-ene-1-carboxylate (Figure 1).

An extensive literature survey revealed that few bio analytical LC-MS/MS methods are used for the determination of Oseltamivir Phosphate in human plasma[3]. The reported HPLC methods[4-11] were not capable to separate the placebo peaks and Oseltamivir phosphate impurities from Oseltamivir Phosphate. The literature survey also revealed that there was no stability-indicating RP-HPLC method for the determination of Oseltamivir Phosphate, a shorter version of method was developed for estimation of Oseltamivir Phosphate in Oseltamivir Phosphate capsules.

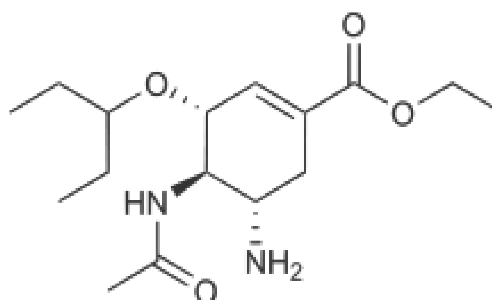

Figure 1: Oseltamivir Phosphate chemical structure

Material and Methods

Chemicals and Reagents

Oseltamivir Phosphate drug substance and impurities generously sponsored by Vendor, India. Commercially available Oseltamivir Phosphate capsules

were procured from the local market. All the chemicals and reagents sodium hydroxide, hydrochloric acid, potassium dihydrogen phosphate, hydrogen peroxide (30 %) and Orthophosphoric acid (88%) were used of Analytical grade. HPLC grade Methanol, Acetonitrile (Merck) was used. Milli-Q water was used in mobile phase and diluents preparation.

Instruments

A Chromatographic system , used for method development and method validation was Waters-Alliance HPLC equipped with separation module consisting of Binary gradient pump, Auto Sampler, thermostatted column compartment, Photo diode array detector (Model 2996), Computer with windows based Empower 2 Method validation manager software. The output signal was monitored and processed using Empower 2 software. Photo stability studies were performed in a photo stability chamber, from Thermolab (India).

Method development and optimization for an RP-HPLC method

Aim of this study was to develop the chromatographic system which was capable to separate Oseltamivir Phosphate from its process come degradation impurities and placebo with reduced run time. pKa of Oseltamivir Phosphate was found to be at about 7.7 and it was in basic nature. Drug product is official in united state pharmacopoeia and there was a limited analytical literature was available stability indicating assay method on this drug product. Since Oseltamivir Phosphate was in basic nature, it was proposed to keep the mobile phase pH on acidic side to reduce the silanol effect from the column. Trails were initiated using 10 mM (milli molar) monobasic phosphate buffer at different acidic pH (range pH 2.5 to 4.5) as mobile phase-A and Acetonitrile and methanol (50:50) as mobile phase-B. Considering nature of impurities present in drug compound, it is preferred to choose more aqueous buffer for initial elution purpose. Initially different programs at a flow rate of 1.0 mL per minutes were proposed for optimum separation of all the impurities. Different column chemistries were tried during initial trail purpose. However YMC Pro C8, (150 x 4.6 mm,) with 5.0 µm particle size column has shown better specificity. The same column was used for entire method development work.

Spectral data for main analyte has shown wavelength maxima at about 207nm, and hence the same wavelength has chosen for quantification purpose. The percentage of organic ratio in mobile phase was played a key role in separation of placebo and degradation impurities which may arise due to Oseltamivir Phosphate. After several logical trials with different flow rates, change in pH of buffer in Mobile phase, chromatographic condition was established with separation of degradants impurities and Placebo.

Finalized chromatographic conditions

Finalized column for chromatography was YMC Pro C8, (150 x 4.6 mm,) with 5.0 µm particle size. The mobile phase containing 20 mM of potassium dihydrogen phosphate in water (6.8 g of potassium dihydrogen phosphate in 1000 mL of water), adjusted the pH to 3.2 ± 0.05 with dilute ortho phosphoric acid. And mix methanol and acetonitrile in the ratio 600:200: 200 v/v and filtered through 0.45µ membrane filter. The flow rate of the mobile phase was 1.5 mL/min. The column temperature was maintained at 45°C and the detection wavelength was fixed at 207 nm. The injection volume was 20µL. Water, Methanol and Acetonitrile in the ratio 625:40:135 as a diluent. In the finalized chromatographic conditions, typical retention time of main analyte is about 6.5 min.

Preparation of Solutions

Preparation of Standard Solution

Accurately weigh and transfer about 75 mg of Oseltamivir (base) standard into a 50 mL volumetric flask. And add 150 mL of diluent and sonicate to dissolve and dilute with the same diluent to volume, and mix well. Further 5 mL of the above standard stock solution was taken in 50 mL volumetric flask and made up to mark with diluent to get a concentration of 150µg/mL.

Preparation of sample solution

Accurately weigh transfer 10 intact capsules into a 500 mL volumetric flask. Add 350 mL of purified water swirl and sonicate for 30 minutes with intermediate shaking to disperse the capsules completely. And add 20 mL of Methanol and sonicate for 5 minutes with intermediate shaking than add 20 mL of Acetonitrile and sonicate for 5 minutes with intermediate shaking and dilute to volume with purified water and mix well. Centrifuge a portion of the solution with lid at 5000 RPM about 10 minutes. Pipette out 5 mL of above clear centrifuged solution into a 50 mL volumetric flask, dilute to volume with diluent and mix well (Concentration 150µg/mL).

Analytical method validation

Oseltamivir Phosphate capsules are available in different strengths such as 30mg, 45mg and 75mg per capsules. However 75mg strength was considered for entire validation experimentation. The developed method was validated for Specificity, Forced degradation studies, Precision, Linearity, Accuracy, Solution Stability and Robustness as per ICH recommendation[12].

Results and Discussion

HPLC Method Development

The HPLC procedure was optimized with a view to develop a stability indicating assay method. Pure drug along with its available degraded products were injected and run in different solvent systems. Initially methanol and water and acetonitrile and water in different ratios were tried. It was found that acetonitrile and water system gives good results than methanol and water as the drug was more soluble in acetonitrile than methanol. Acetonitrile: water in the

ratio of 80:20 was not able to give good peak symmetry with acceptable retention time. An attempt to improve peak symmetry was made by adding phosphate buffer to the mobile phase. The presence of phosphate buffer in mobile phase resulted in excellent overall chromatography with appropriate peak symmetry and complete base line resolution. Finally the mobile phase consisting of Phosphate Buffer: Methanol: Acetonitrile in the ratio 60:20:20 (v/v) as the Mobile phase with pH 3.2±0.05 adjusted with diluted ortho phosphoric acid was selected for validation purpose and stability studies. The method was validated with respect to parameters including linearity, recovery, precision, robustness and System Suitability.

Analytical Method Validation

Specificity

Specificity-Blank and Placebo interference

To establish the interference of placebo, study was conducted. Assay was performed on placebo in duplicate equivalent to concentration of test preparation as per proposed method. Blank and Placebo chromatograms solutions showed no peaks at the retention time of Oseltamivir Phosphate peak. This indicates that the excipients used in the formulation do not interfere in estimation of Oseltamivir Phosphate in Oseltamivir Phosphate peak capsules. The chromatogram of blank, placebo, and standard using the proposed method is shown in Figure 2, Figure 3 and Figure 4.

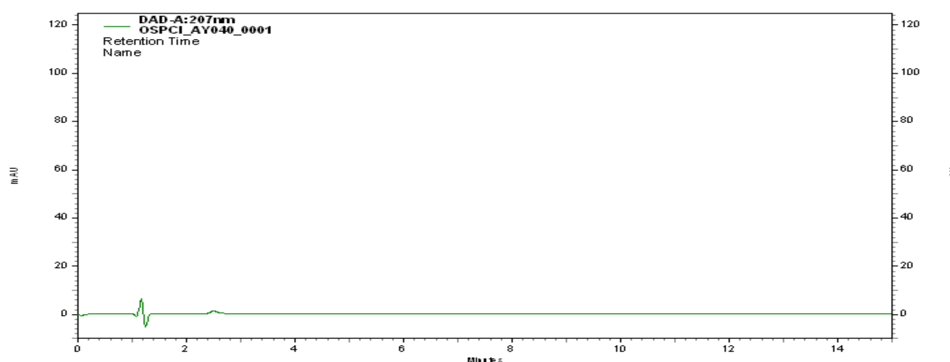

Figure 2: Chromatogram of Diluent

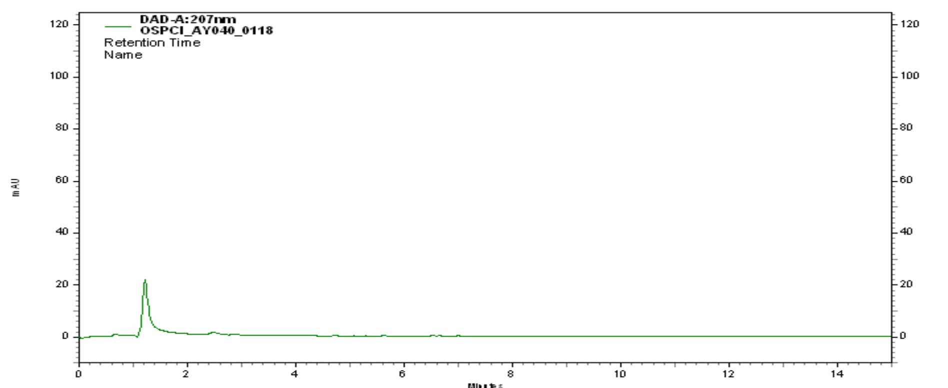

Figure 3: Chromatogram of placebo

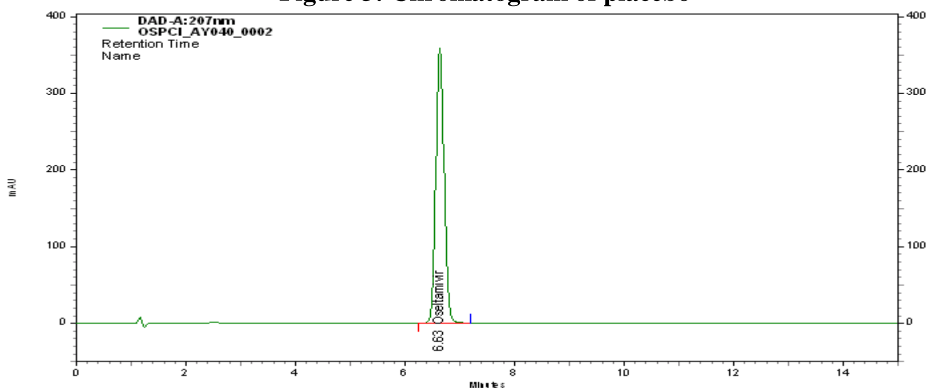

Figure 4: Chromatogram of Standard

Specificity-Known Impurity Interference

Sample Solution spiked with known related substances of Oseltamivir at 1% level (Spiked Sample) was prepared and injected into HPLC and also solutions of known related Substances were individually injected for the identification of retention time. From the retention times of individual known Related Substances that all the known Related Substances, eluted within assay run time of 15 minutes, and well separated from Oseltamivir Phosphate peak. The chromatogram of Impurity A, Impurity C and Spiked Sample are shown in Figure 5, Figure 6 and Figure 7.

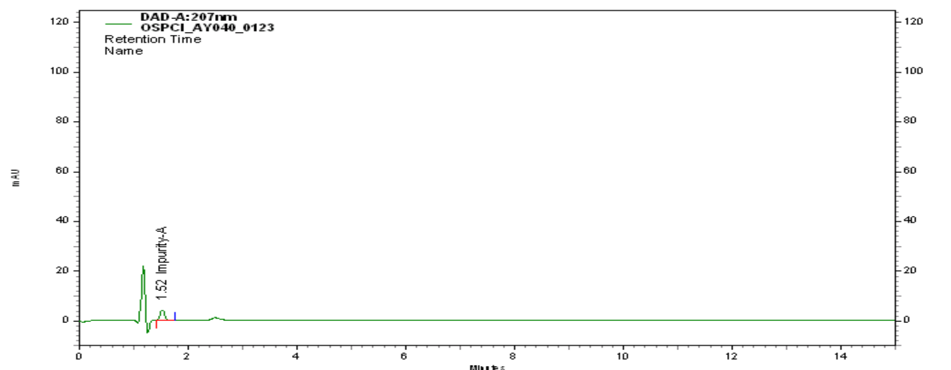

Figure 5: Chromatogram of Impurity-A

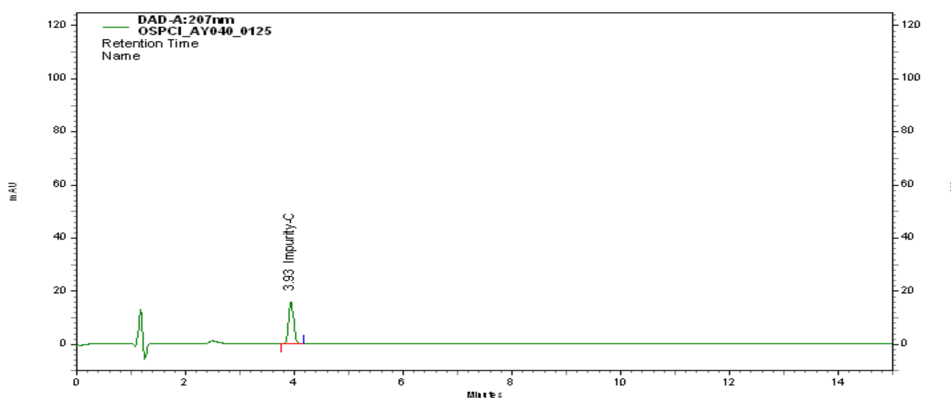

Figure 6: Chromatogram of Impurity-C

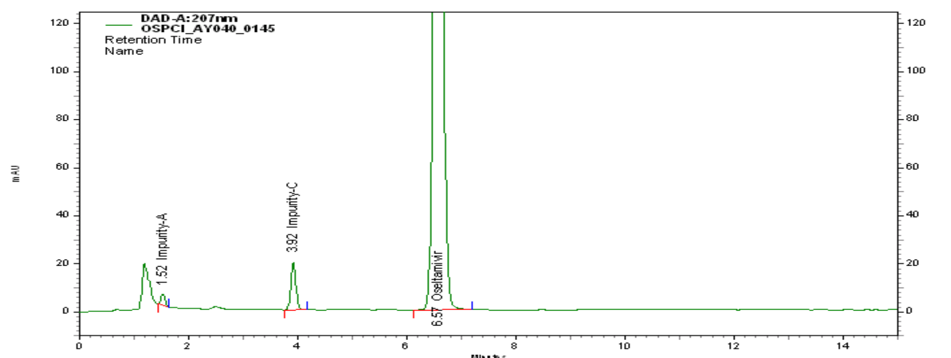

Figure 7: Chromatogram of Spiked Sample

Specificity-Forced Degradation Studies

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. The stress conditions used for degradation study are Acid hydrolysis (0.1M HCl / 70°C / 40 min), Base hydrolysis (0.1M NaOH / 25°C / 11 min), Oxidation (5% H_2O_2 / 50°C / 40 min), Thermal (105°C / 5 hours), Humidity (90%RH / 25°C / 120 Hours) and Photolytic (white fluorescent 1.2 million lux hours UV 200 watt hr/m^2 for 120 hours). The results of various stress conditions employed to degrade Oseltamivir Phosphate capsules indicates that the drug product is susceptible to degradation under Acidic, Alkaline and Oxidative Conditions, while, it is found stable to remaining stress condition employed. The percent degradation shown by Oseltamivir Phosphate at each stress condition given in Table 1. The chromatograms were extracted for Peak purity and demonstrated as in Fig. 8a, 8b, 8c, 8d, 8e, 8f and 8g.

Table 1: Summary of forced degradation study

Stress condition	Time	% Degradation
		Oseltamivir Phosphate
Acid hydrolysis (0.1M HCl / 70°C)	40 minutes	17.18
Base hydrolysis (0.1M NaOH / 25°C)	11 minutes	16.68
Oxidation stress sample (5% H_2O_2 / 50°C)	40 minutes	14.6
Thermal stress sample (105°C)	5 hours	0.68
Humidity stress sample (90%RH / 25°C)	120 hours	Nil
Photolytic stress sample (White Flouoscent Light 1.2 Millon Lux Hrs Uv 200 Watts/M^2)	120 hours	1.48

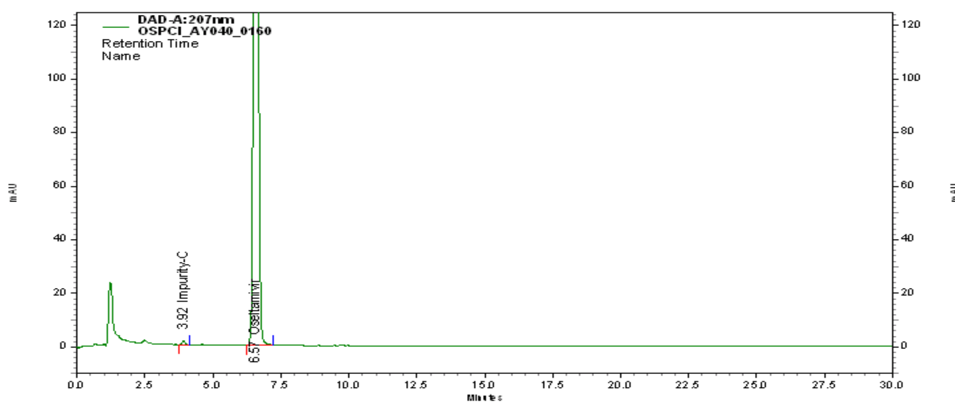

Figure 8a: Chromatogram of Control Sample

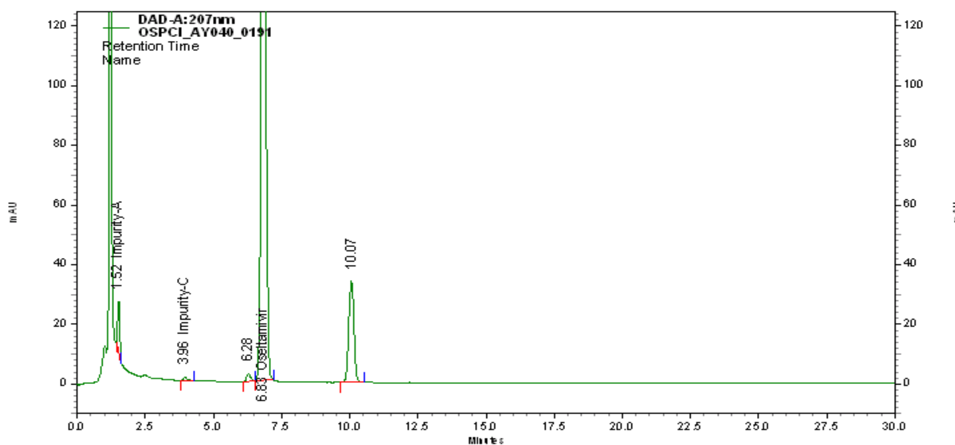

Figure 8b: Chromatogram of Acid degradation sample

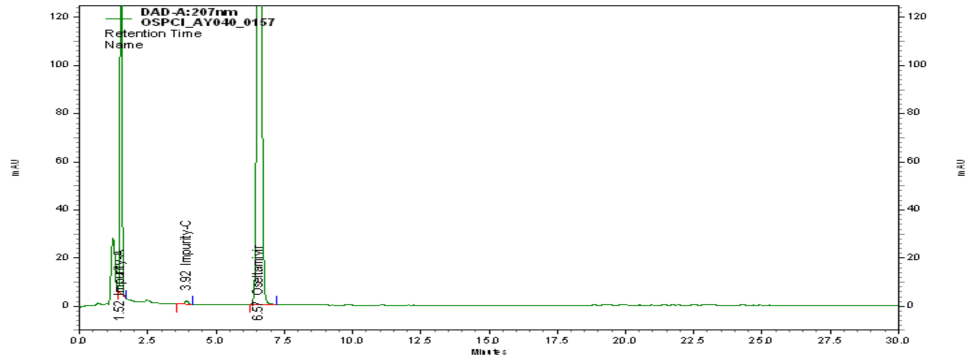

Figure 8c: Chromatogram of Base degradation sample

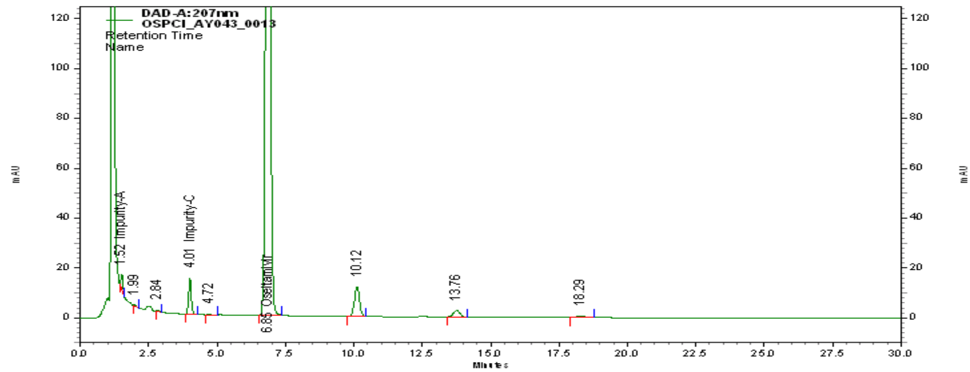

Figure 8d: Chromatogram of Peroxide degradation sample

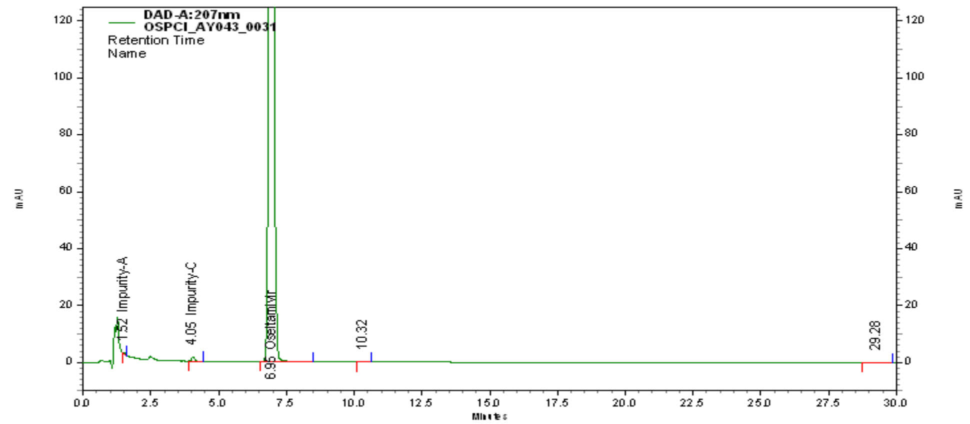

Figure 8e: Chromatogram of Photolytic degradation sample

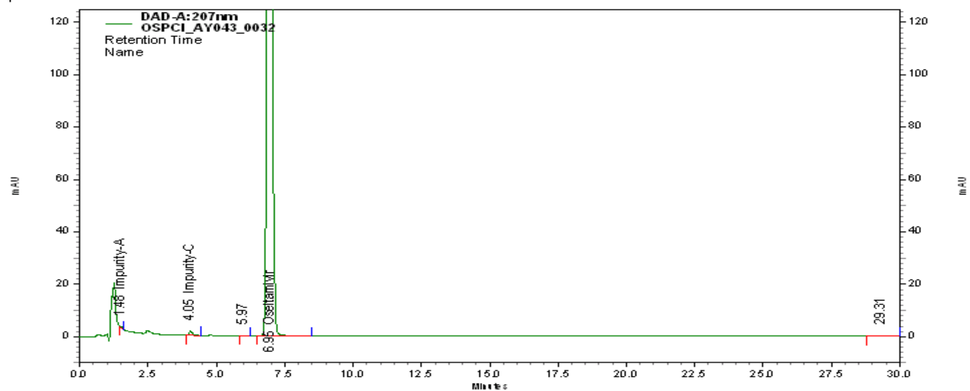

Figure 8f: Chromatogram of Humidity degradation sample

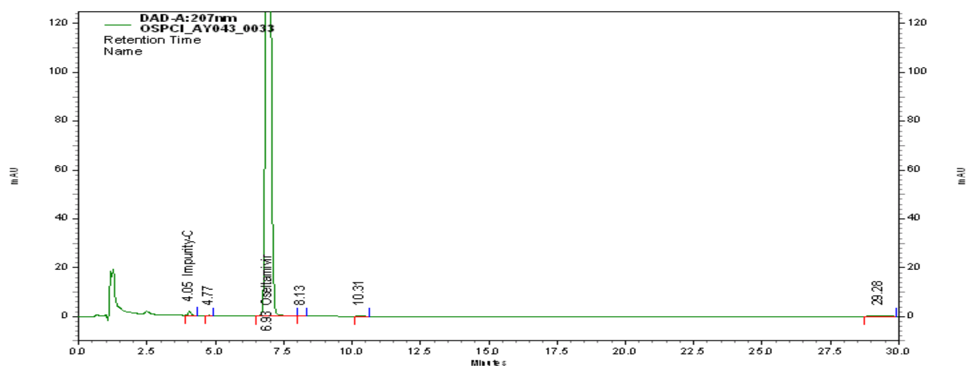

Figure 8g: Chromatogram of Thermal degradation sample

Linearity

Linearity was studied by plotting a graph of concentration versus response and determining the correlation coefficient, slope and Y-intercept. A series of solutions were prepared using Oseltamivir Phosphate standard at concentration levels from 50% to 150% of test concentration and each solution was injected into HPLC. Linearity results obtained are presented in Table 2. The linearity was found to be linear with a correlation coefficient of 0.999. Linearity graph of Oseltamivir Phosphate is shown in Figure 9.

Table 2: Linearity Study for Oseltamivir phosphate

Level	Oseltamivir phosphate	
	Concentration (μg/mL)	Response
50% Linearity Solution	76.6	1921539
60% Linearity Solution	91.9	2315650
80% Linearity Solution	122.5	3048712
100% Linearity Solution	153.1	3879911
125% Linearity Solution	191.4	4819392
150% Linearity Solution	229.7	5819684
Slope	25437	
Intercept	-33835	
% Y-Intercept	-0.9	
Correlation Coefficient	0.9999	

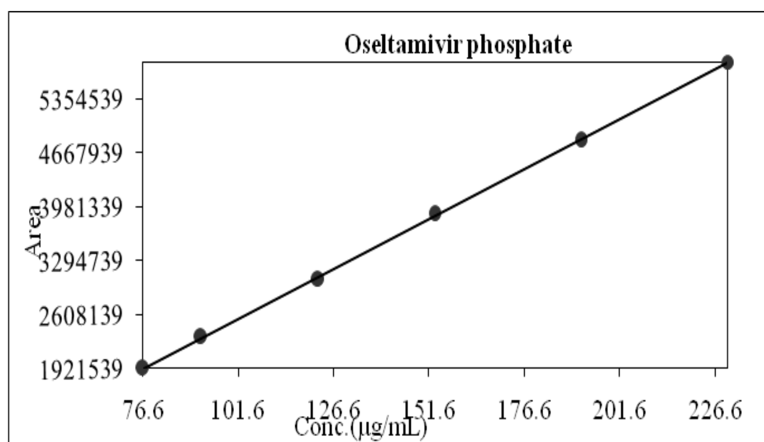

Figure 9: Linearity Graph of Oseltamivir phosphate

Method Precision and Intermediate Precision

The Method precision of test method was established by conducting assay in six samples of Oseltamivir phosphate capsules. The average % assays of Oseltamivir phosphate in capsules were found to be 99.0 and the %RSD found to be 0.8. Intermediate precision was carried out by analyzing the samples by a different analyst on another instrument. The results were given in Table 3.

Table 3: Method Precision and Intermediate Precision Results for Oseltamivir phosphate

Sample No.	% Assay in Method precision	% Assay in Intermediate precision
1	99.1	98.6
2	99.9	98.2
3	98.4	99.0
4	98.3	98.0
5	98.9	98.9
6	99.3	98.3
Average(n=6)	**99.0**	**98.5**
%RSD (n=6)	**0.6**	**0.4**
%RSD(n=12)	**0.6**	

Accuracy: A known amount of Oseltamivir phosphate drug is added to the placebo at 50%, 100% and 150% of the analyte concentration. Sample solutions were prepared in triplicate for each spike level and assay was determined as per proposed method. The observed recovery results were found in the range between 98 to 102%, demonstrating that the method is accurate within the desired range. The results were given in Table 4.

Table 4: Accuracy data of Oseltamivir phosphate

S. No.	% Level	Amount added(mg)	Amount recovered (mg)	% Recovery	%Mean recovery	%RSD
1.	50	374.52	377.92	100.9	100.6	0.3
2.		375.22	375.82	100.2		
3.		375.06	377.20	100.6		
1.	100	749.64	751.78	100.3	100.3	0.1
2.		749.65	752.69	100.4		
3.		750.00	751.90	100.3		
1.	150	1124.87	1135.03	100.9	100.9	0.1
2.		1124.62	1135.84	101.0		
3.		1124.81	1134.95	100.9		

Solution Stability

Standard and Sample Solutions were prepared as per proposed method and analyzed initially and at different time intervals by keeping the solutions at Room Temperature (~ 25°C) for 48 hours. % Difference between the assays obtained for Oseltamivir phosphate at initial and different time interval should not be more than 2.0. From the results it can be concluded that the Standard and Sample Solutions are stable upto 48 hours at room temperature (~ 25°C). The results were given in Table 5a and Table 5b.

Table 5a: Bench top Solution Stability for Standard

Time (hrs)	Similarity Factor
24	1.00
48	1.01

Table 5b: Bench top solution stability for sample

Time (hrs)	% Assay		% Difference	
	Sample-1	Sample-2	Sample-1	Sample-2
Initial	99.0	99.9	N/A	N/A
24	98.4	99.8	0.6	0.1
48	100.3	100.7	1.3	0.8

Robustness

The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. The conditions studied were flow rate (altered by ±0.1 mL min−1), wavelength (altered by ±5 nm), variation in mobile phase composition (±0.2% absolute), Column Oven temperature (±5°C) and pH of buffer in mobile phase (altered by ± 0.2), standard solution was prepared and injected into HPLC system. The system suitability parameters were evaluated. In all the cases, the %RSD obtained was less than 1. From the above study the proposed method was found to be robust. The results were given in Table 6.

Table 6: Results for Robustness

Parameter	Variation	Oseltamivir phosphate		
		USP Plate Count	USP Tailing	%RSD
STP	-	5205	1.1	0.4
Flow Rate	-10%	4642	1.1	0.8
	+10%	5602	1.3	0.6
Wavelength	-5 nm	5002	1.1	0.7
	+5 nm	5725	1.3	0.4
Organic in Mobile Phase	-2% absolute	5525	1.2	0.6
	+2% absolute	8526	1.3	0.5
Column Oven Temperature	-5°C	4325	1.2	0.7
	+5°C	4212	1.1	0.6
pH of Buffer	-0.2 units	7215	1.3	0.3
	+0.2 units	5105	1.1	0.9

Conclusion

The HPLC method developed is accurate, precise, reproducible, specific and stability indicating. There is allowable variation in flow rate, temperature, pH, and mobile phase composition which indicate that method is robust enough. The low RSD value for percent assay of test preparation revealed that the proposed method is rugged. This study shows that the drug is very sensitive to degradation with acid, alkali and oxidation degradation condition, and stable in case of other stress conditions. This stability indicating method was developed, which separates all degradation products formed under variety of conditions. The method was found to be simple, specific, Precise and Robust and can be applied for the routine and stability analysis for commercially available formulation.

Acknowledgement

The author wish to for supporting this work and also thankful to entire team of Vinayaka Missions University and Konde Abbulu nova collage of pharmaceutical education & research for their encouragement and support during the work.

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