Assay Method development and validation of Oseltamivir Phosphate API and its Pharmaceutical Formulation.

*Sachin A. Babar[1], Sudhakar L. Padwal[2]

[1,2]*Department of Chemistry, K.B.P. Mahavidyalaya Pandharpur, Punyashlok Ahilyadevi Holkar Solapur University, Pandharpur, India-413 304*

***Abstract*:** *A simple, rapid, economical, and highly sensitive Assay HPLC method was developed and fully validated for determination of Oseltamivir Phosphate in bulk and its pharmaceutical dosage form namely Tamiflu. Chromatographic separation was achieved on ZORBAX Eclipse XDB C8 EPS column (250 mm X 4.6 mm, 5 µm). Chromatographic separation was carried out at ambient temperature (25 °C +0.5 °C). All analytes were separated isocratically with a mobile phase consisting of acetonitrile and Acetate buffer pH 3.5 (75/25 v/v), at a flow rate 1.0 ml/min with injection volume 20 µL. The wavelength was monitored at 210 nm. The proposed method was validated according to ICH guidelines with total run time less than 6 min. The correlation coefficient (r2) was noted as 0.9998 which states that the method was good linear to the concentration versus peak area responses. The average percentage recovery were in the range of 95.0-105.0 %. A rapid, economical, simple and sensitive HPLC method was developed and validated for the determination of Oseltamivir Phosphate in capsule. The developed, validated method was successfully applied for the determination of Oseltamivir Phosphate in formulation.*

Keywords: **Oseltamivir Phosphate, Assay, RP-HPLC, Validation, ICH guidelines.**

1. Introduction

Oseltamivir Phosphate is chemically ethyl (3R, 4R, 5S)-5-amino-4-acetamido-3-(pentan-3-yloxy)-cyclohex-1-ene-1-carboxylate. Oseltamivir, sold under the brand name Tamiflu, is an antiviral medication used to treat and prevent influenza A and influenza B (flu). It is used first treatment on COVID 19. Oseltamivir phosphate is a pro-drug of the active metabolite (oseltamivir carboxylate) which is a potent and selective inhibitor of influenza virus neuraminidase enzymes, which are glycoproteins found on the version surface. Viral neuraminidase enzyme activity is important for viral entry into uninfected cells, for the release of recently formed virus particles from infected cells, and for the further spread of the infectious virus in the body. Oseltamivir activity reduces viral shedding and infectivity.

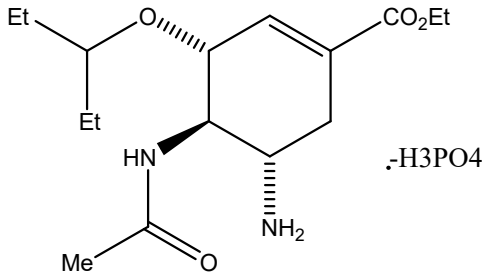

Figure 1. Structure of Oseltamivir Phosphate

2. Materials and Method

2.1 Materials:

Oseltamivir Phosphate (API) - White powder, use as an antiviral

Tamiflu- 75 mg drug contain each Capsule

Acetonitrile, Methanol, Acetate Buffer (HPLC grade) by Merck Life Science, Distilled Water.

Instruments:

Table 1. List of Instruments

Sr. No.	Name of Equipment's/ Instruments	Model / Specification	Manufacturer
1	HPLC	Series LC2030	Shimadzu
	Pump	PU2030	
	Sample Injection Port	Rheodyne Injector	
	UV/Vis Detector	UV 2075 plus	
	Software	LabSolutions	
2	pH Meter	101	Chemiline
3	Balance	AY-120	Shimadzu
4	Sonicator	UCB-40	Rolex

2.2 Method:

2.2.1 Optimized chromatographic conditions: HPLC system equipped with Shimadzu LC2030, ZORBAX Eclipse XDB C8 EPS column (250 mm X 4.6 mm, 5 µm). Chromatographic separation was carried out at ambient temperature (25 °C $\pm$0.5 °C). All analytes were separated isocratically with a mobile phase consisting of acetonitrile and Ammonium acetate buffer pH 3.5 (75/25, v/v), at a flow rate 1.0 ml/min with injection volume 20 µL. The wavelength was monitored at 210 nm.

2.2.2 Preparation of buffer solution for mobile phase: Weigh accurately about 3.4gm of ammonium acetate and transfer in to 250 ml of HPLC grade water then shake and sonicate to dissolve completely. Finally make the solution up to 500 ml with HPLC grade water.

2.2.3 Preparation of mobile phase: In 25 ml of Phosphate Buffer and 75 ml of HPLC grade Acetonitrile i.e. in 25:75 v/v proportions. The pH was adjusted to 3.5 with orthophosphoric acid. The solution was filtered through 0.45µ membrane filter and then sonicated in sonicator bath for 10 min.

2.2.4 Preparation of standard solution: Stock solution was prepared by dissolving 10 mg Oseltamivir Phosphate in water and then diluted with Water in 10 ml of volumetric flask to get concentration of 1000 µg/ml. From the resulting solution 1.0 ml was diluted to 10 ml with water to obtain concentration of 100 µg/ml, labelled as standard stock Oseltamivir Phosphate.

2.2.5 Preparation of sample solution: For Assay, 20 capsule of Oseltamivir Phosphate labelled as containing 75mg with excipients was weigh accurately, and made a fine powder (except body and cap). Take accurate weight of powder equivalent to 100 mg of Oseltamivir Phosphate transferred in to 100 ml volumetric flask and 50 ml of mobile phase was added, sonicated for 10 min. Cool it and make volume up to the mark with mobile phase. Filter sufficient amount of this solution through 0.45µm membrane syringe filter. The final solution was prepared by transferring 10 ml of this filtered solution in to 100 ml volumetric flask then make volume up to the mark by adding mobile phase to get 100µg/ml of Oseltamivir Phosphate. A figure 2.0 represents the typical chromatogram of the sample Oseltamivir Phosphate.

3. Result and Discussion

3.1 Linearity and range:

In above method linearity was determined by constructing the calibration curves. For this purpose different standard solution of Perindopril erbumine& Amlodipine besylate of different concentration level (10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml and 50 µg/ml) were used. Measurement of each concentration was carried out & the peak areas of the chromatograms were plotted against the concentrations to obtain the calibration curves & correlation coefficients. A table 2 represents the results were directly proportional to the concentration of analyte in the given sample. Fig 3a to 3e are shown linearity chromatogram respectively.

Table 2. Linearity Result of Oseltamivir Phosphate

Sr.No.	Concentration (µg/ml)	Peak Area
1	10	89765
2	20	165840
3	30	248782
4	40	322510
5	50	401288

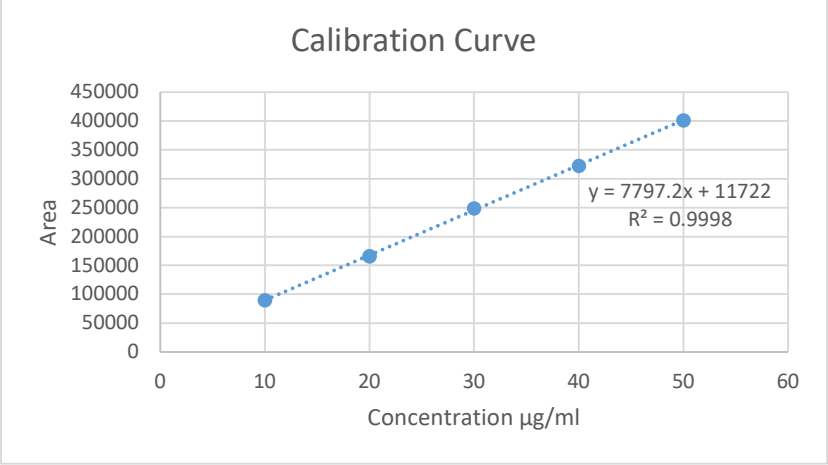

Figure 2. Calibration Curve of Oseltamivir Phosphate

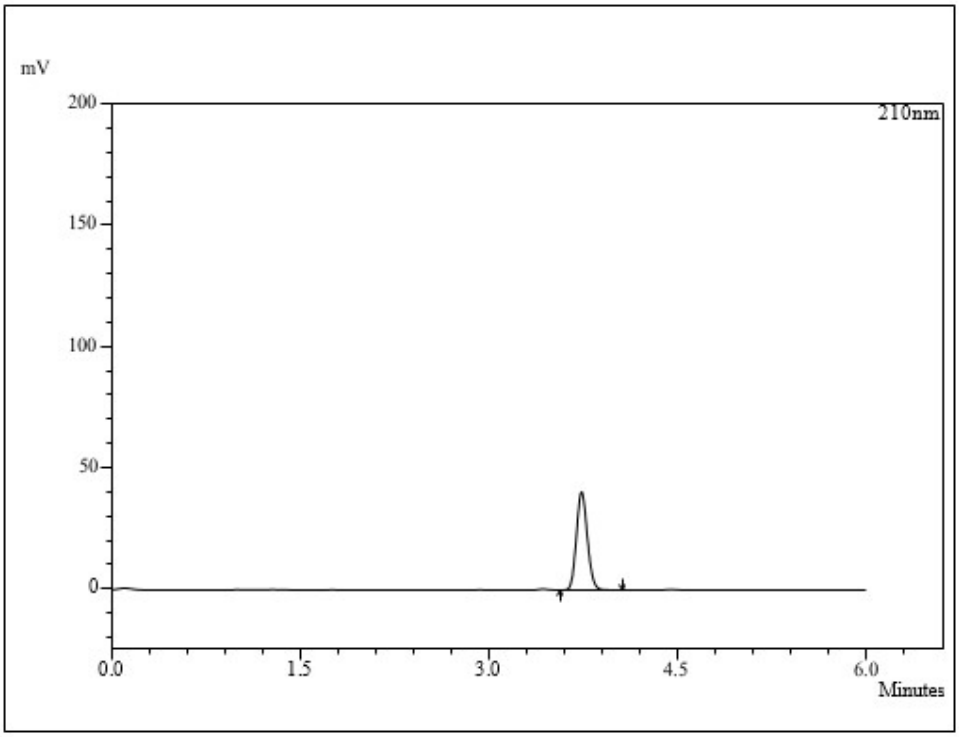

Figure 3a. Chromatogram for Oseltamivir Phosphate at 10ppm

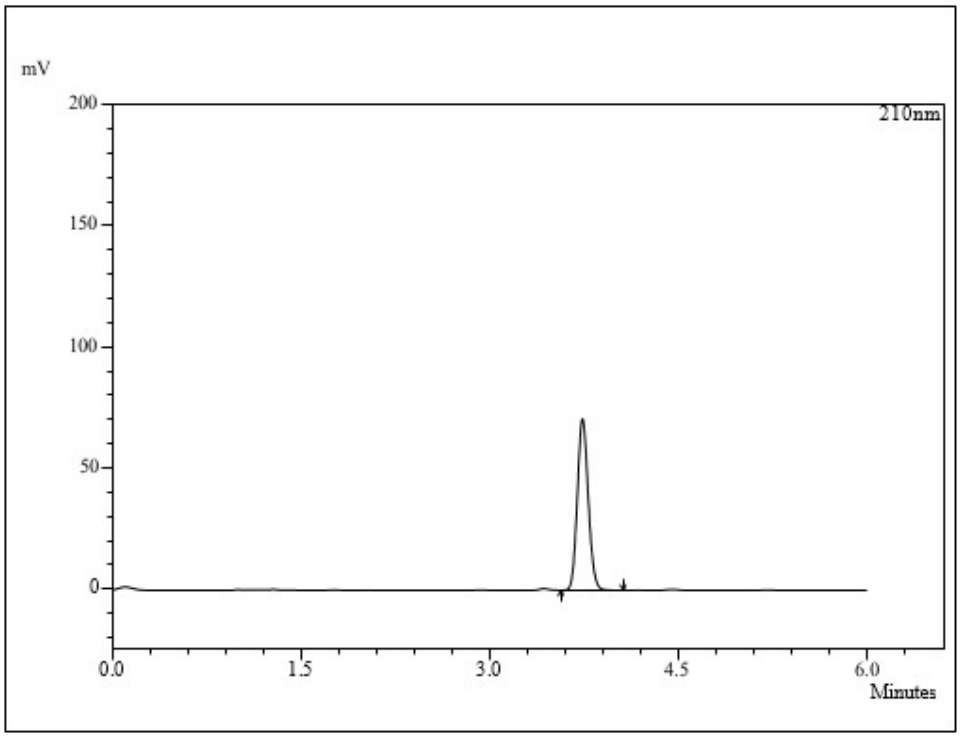

Figure 3b. Chromatogram for Oseltamivir Phosphate at 20ppm

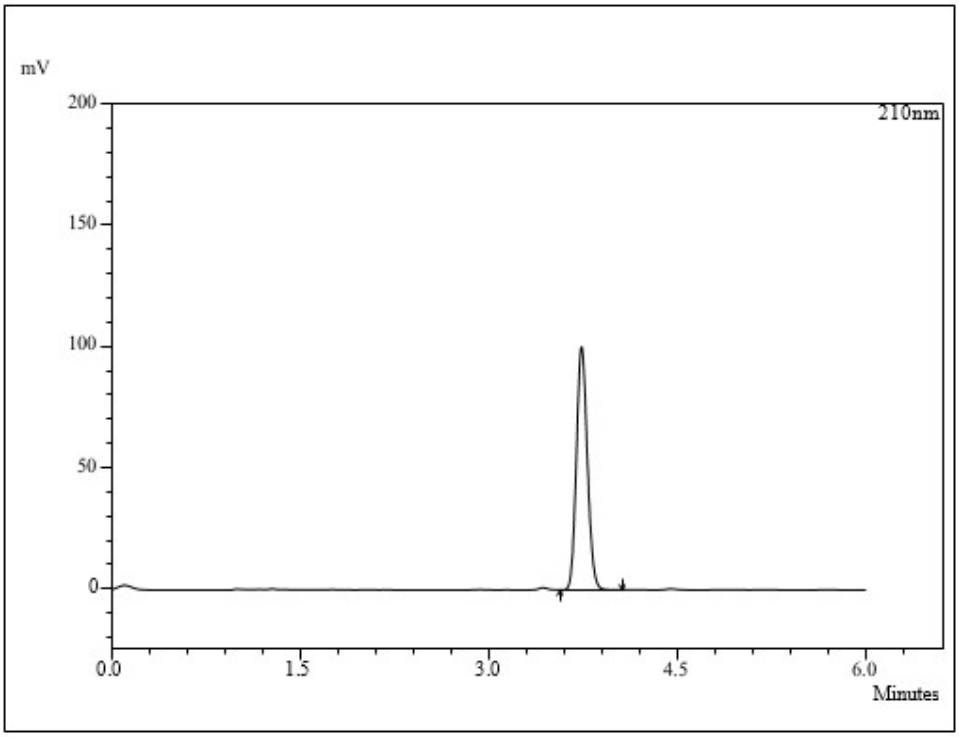

Figure 3c. Chromatogram for Oseltamivir Phosphate at 30ppm

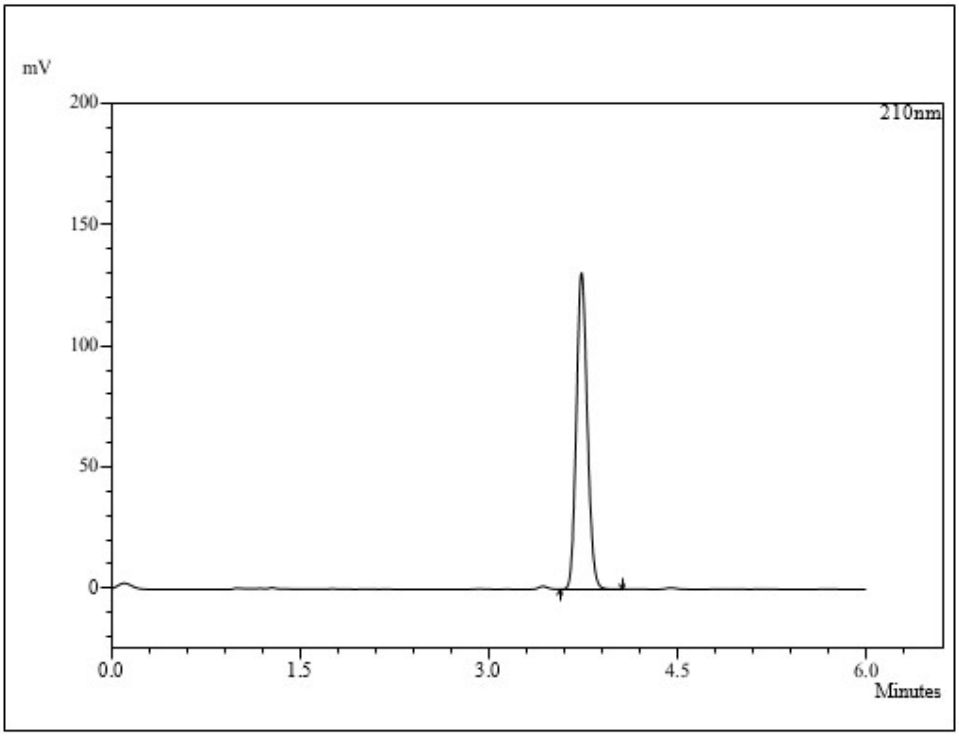

Figure 3d. Chromatogram for Oseltamivir Phosphate at 40ppm

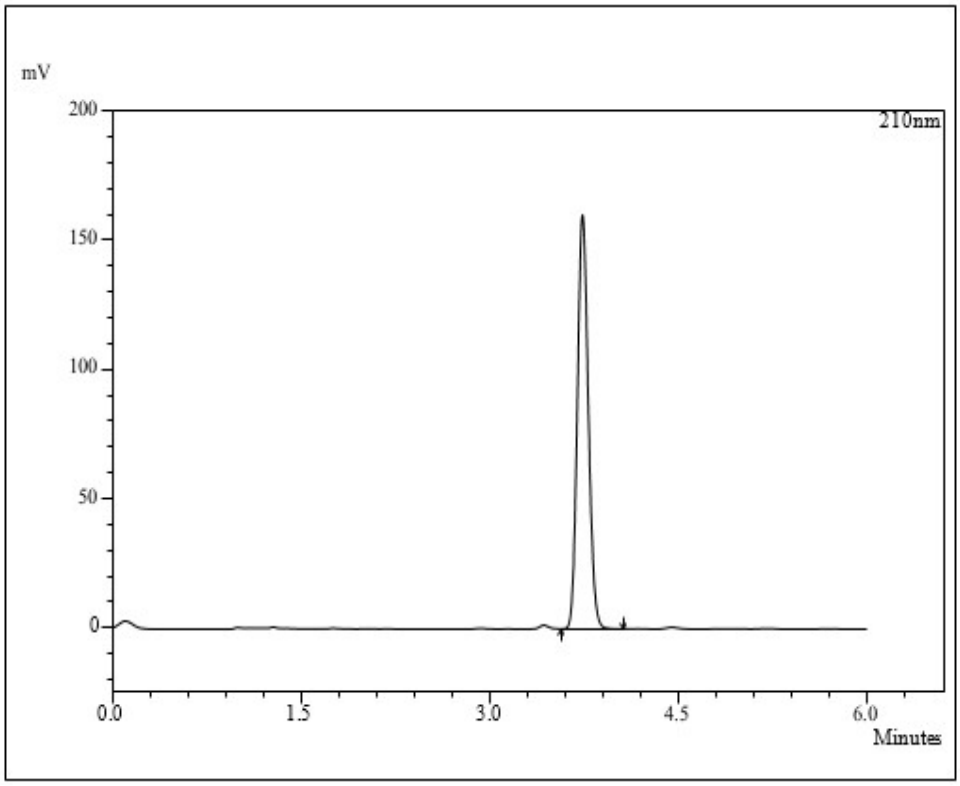

Figure 3e. Chromatogram for Oseltamivir Phosphate at 50ppm

Table 3. Characteristic parameters of Oseltamivir Phosphate for the proposed HPLC method.

Parameter	Result
	Oseltamivir Phosphate
Calibration range (µg/ml)	10-50
Detection wavelength (nm)	210
Solvent (Acetonitrile: Buffer)	25:75
Regression equation (y*)	y = 7797.2x + 11722
Slope (b)	7797.2
Intercept (a)	11722
Correlation coefficient(r2)	0.9998
Limit of Detection (µg/ml)	0.042
Limit of Quantitation (µg/ml)	0.153

3.2 System Suitability:

System-suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (Rt), number of theoretical plates (N) and tailing factor (T) were evaluated for six replicate injections of the drug at a concentration of 20 µg/ml. The results which are given in Table 4. Were within acceptable limits.

Table 4. System suitability studies of Oseltamivir Phosphate by HPLC method.

Sr. No.	Properties	Values
1.	Retention time	**3.8**
2.	Area	**165840**
3.	Asymmetry	**0.93**

3.3 Specificity:

Chromatogram of blank was taken as shown in Fig 4. Chromatogram of Oseltamivir Phosphate showed peak at a retention time of 3.868 min (fig 5). The mobile phase designed for the method resolved the drug very efficiently. The Retention time of Oseltamivir Phosphate sample was 3.849 min (fig 6). The wavelength 210 nm was selected for detection because; it resulted in better detection sensitivity for the drug. The peak for Oseltamivir Phosphate from the tablet formulation was Oseltamivir Phosphate. Specificity of Oseltamivir Phosphate represent in table 5.

Table 5. Specificity of Oseltamivir Phosphate by HPLC method

Concentration	API Area	Tablet Area
20	165887	162087
20	166423	158988
20	165025	158246
20	166352	161289
20	169909	162328
20	167852	157998
Mean	166908	160156
SD	1733.76	1969.74
RSD	1.04	1.23

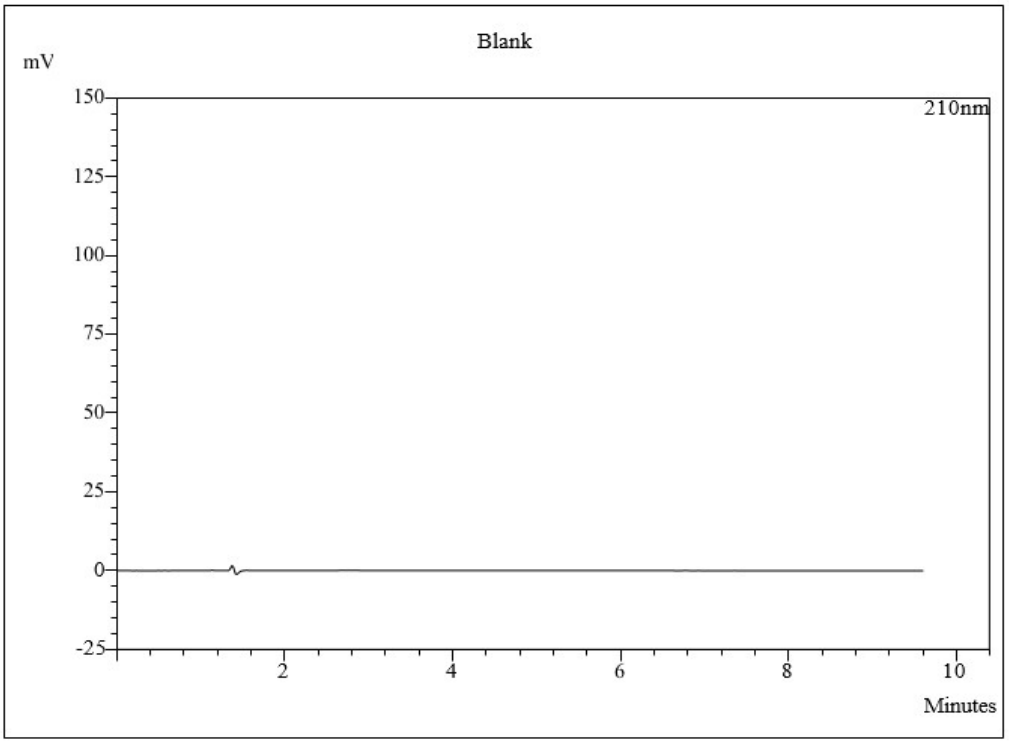

Figure 4. A typical chromatogram of Blank

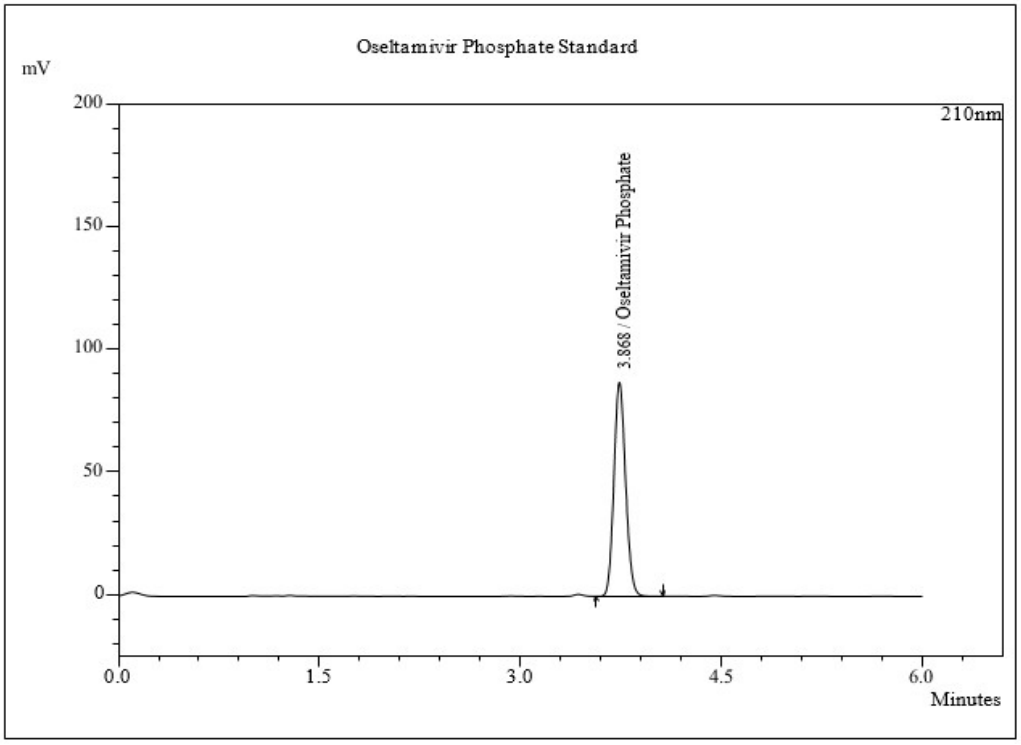

Figure 5. A typical chromatogram of Oseltamivir Phosphate Standard [Concentration 20ug/ml]

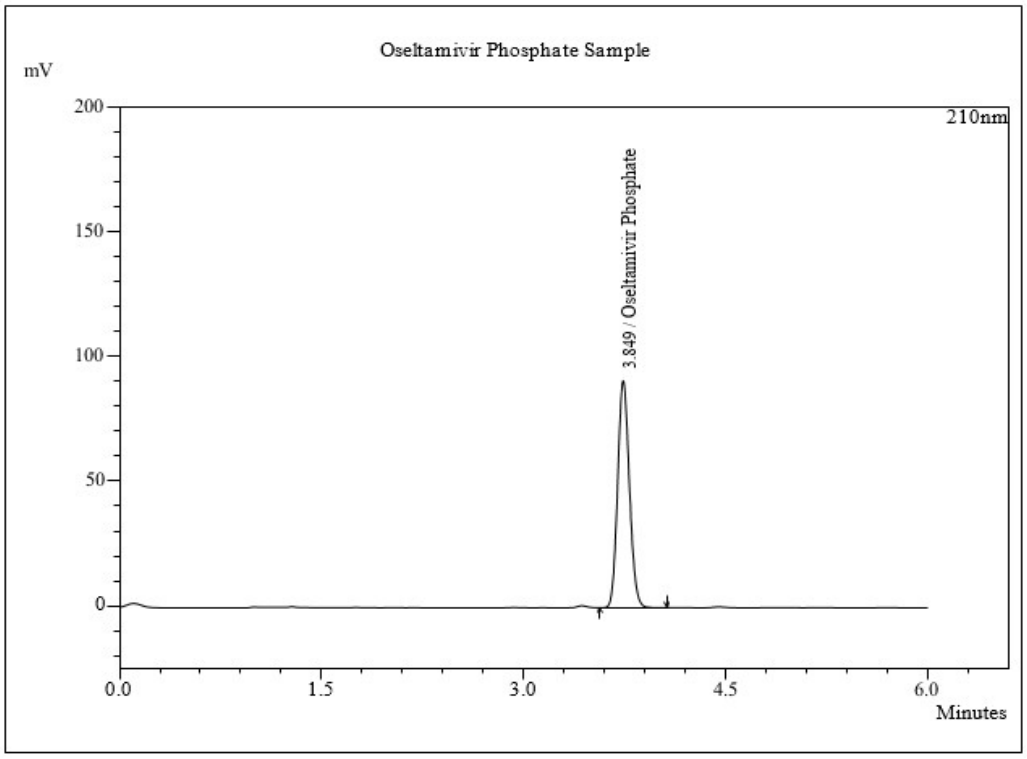

Figure 6. A typical chromatogram of Oseltamivir Phosphate Sample [Concentration 20ug/ml]

3.4 Precision:

Demonstration of precision was done under two categories. The injection repeatability (System Precision) was assessed by using six injections of the standard solution of Oseltamivir Phosphate and the % RSD of the replicate injections was calculated. In addition, to demonstrate the precision of method (Method Precision), six samples from the same batch of formulation were analysed individually and the assay content of each sample was estimated. The average for the six determinations was calculated along with the % RSD for the replicate determinations. Both the system precision and method precision were subjected for inter-day and intra-day variations as reported in Table No 6and 7 respectively.

Table 6. Intraday Precision of Oseltamivir Phosphate at 210

Concentration	Peak Area		
	0 Hrs	2 Hrs	3 Hrs
20	165887	165426	165234
20	166423	166015	166287
20	165025	165149	164814
20	166352	165648	165476
20	169909	169875	169457
20	167852	165868	165284
Mean	166908	166330	166092
SD	1733.76	1763.90	1718.31
RSD	1.04	1.06	1.03

Table 7. Interday Precision of Oseltamivir Phosphate at 210

Concentration	Peak Area				
	1 day	2 day	3 day	4 day	5 day
20	165887	165579	165782	165587	165484
20	166423	166984	166574	166478	166004
20	165025	165598	165188	165876	161478
20	166352	166359	166599	166684	163878
20	169909	169228	169108	168769	164876
20	167852	163938	163644	162874	166700
Mean	166908	166281	166149	166045	164737
SD	1733.76	1768.28	1815.05	1913.61	1865.21
RSD	1.04	1.06	1.09	1.15	1.13

3.5 Accuracy:

Recovery studies by the standard addition method were performed with a view to justify the accuracy of the proposed method. Previously analysed samples of Oseltamivir Phosphate (20 µg/ml) were spiked with 80, 100, and 120 % extra Oseltamivir Phosphate standard and the mixtures were analysed by the proposed method. Standard deviation of the % recovery and % RSD were calculated and reported in Table 8.

Table 8. Accuracy of Oseltamivir Phosphate

Sr. No.	Concentration	Peak Area	recovery%
1	40	322510	98.53
2	40	323014	99.07
3	40	323514	99.71
4	50	401289	100.08
5	50	401098	99.94
6	50	401116	100.07
7	60	481545	99.01
8	60	481496	98.94
9	60	481652	99.91

3.6 Robustness:

To evaluate the robustness of the developed RP-HPLC method, small deliberate variations in the Optimized method parameters were done. The effect of change in flow rate, wavelength, on the retention time and tailing factor were studied. The method was found to be unaffected by small changes in flow rate and changes in wavelength. The results are shown in Table 9 and 10.

Table 9. Robustness of Oseltamivir Phosphate at 210 nm & 215 nm

Conc. (µg/ml)	Wavelength	
	210 nm	215 nm
20	165887	157894
20	166423	158497
20	165025	160004
20	166352	159803
20	169909	155678
20	167852	155776
Mean	166908	157942
SD	1733.76	1889.06
RSD	1.04	1.20

Table 10. Robustness of Oseltamivir Phosphate at 1.0 ml/ min & 1.1 ml/ min

Conc. (µg/ml)	Flow rate	
	1.0 ml/ min	1.1 ml/ min
20	165887	156566
20	166423	154673
20	165025	156278
20	166352	153548
20	169909	155422
20	167852	152138
Mean	166908	154771
SD	1733.76	1694.40
RSD	1.04	1.09

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