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QBD BASED HPLC METHOD DEVELOPMENT AND VALIDATION OF OSELTAMIVIR PHOSPHATE IN API AND PHARMACEUTICAL FORMULATION

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ABSTRACT

Background: Quality By Design is systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management. An essential element of the QbD is the understanding of dependent variables, various factor, and their interaction consequences with the aid of a desired set of test on the response to be analysed. A simple, rapid, selective, and reproducible Quality by Design based high performance liquid chromatography (HPLC) method has been developed and validated for the estimation of Oseltamivir Phosphate in API of Oseltamivir Phosphate tablet. **Results:** The chromatographic conditions were optimized with the help of Design expert software. The total analysis was carried out by using Waters XBridge C18 (250 mm x 4.6 mm, 5 µm particle size) column. A mobile phase composed of Methanol: Buffer (70: 30 v/v) at a flow rate of 1.0 ml/min was used for the separation. Detection was carried out at 207 nm. The linearity range used was 8-48 µg/ml and (Rt) was 3.184 min and temperature was adjusted at ambient. The correlation coefficient values were found to be 0.9993. precision studies showed % RSD values less than 2 % for all the selected concentrations. The percentage recovery of Oseltamivir Phosphate was found to be 99.95 – 100.23 additionally mentioned method found satisfactory plus robust at different level of mobile phase, change in pH and wavelength and flowrate. The assay results of Oseltamivir Phosphate were within the limits of 95-105 %. Validation parameters such as specificity, linearity, precision, accuracy, and robustness, limit of detection (LOD) and limit of quantitation (LOQ) were evaluated for the method according to the International Conference on Harmonization (ICH) Q2 R1 guidelines. **Conclusion:** The Central composite design method is used to determine the number of experiments to be evaluated for the optimization of the variables and responses. The experimental design describes the relationship between mobile phase and pH at different levels and response observed were retention time, theoretical plate and tailing factor with the help of design expert software program. The QbD approach in method development used for the better understanding of method variables at different levels. The developed method was successfully used for the quantitative analysis of commercially available dosage form.

KEYWORDS: QbD, HPLC, Oseltamivir Phosphate, Method Development, Validation.

BACKGROUND

Oseltamivir phosphate is an active metabolite (Oseltamivir carboxylate) which is a potent and selective inhibitor of the influenza virus neuraminidase enzymes, which are glycoproteins found in the translation site. Oseltamivir is used for the prevention and treatment of influenza A and B infections. Listed by the World Health Organization's Essential Medicines. The WHO supports its use in critical illnesses due to confirmed or suspected influenza infections in critically ill people hospitalized. The function of the enzyme viral neuraminidase is important for the entry of the virus into non-infected cells, in order to release the newly formed viral particles from the infected cells, as well as for the spread of the infectious virus in the body. The activity of oseltamivir reduces viral load and infection.

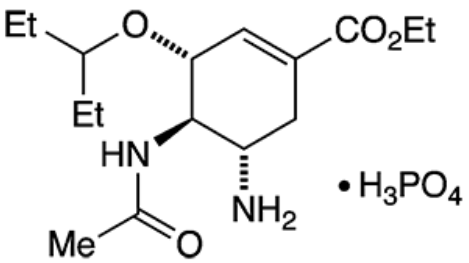

Figure 1 Structure of Oseltamivir Phosphate.

Methods

Instruments

Shimadzu LC-2030 HPLC, The Waters XBridge C_{18} (4.6× 250mm id. particle size 5µm) analytical column was used as a stationary phase, Chromatographic

data was acquired using LabSolutions software, pH Meter (Lab India), Balance (Mettler Toledo), Sonicator (Rolex).

Reagents and Materials

Oseltamivir Phosphate API, Tamiflu 100mg
Chemicals- Acetonitrile (HPLC Grade), Methanol (HPLC Grade), Potassium Dihydrogen Phosphate, Sodium Hydroxide, Distilled Water.

Preliminary Drug Analysis

a) Description
Oseltamivir is a white crystalline powder
b) Solubility
Oseltamivir Phosphate is soluble in organic solvents such as water, Methanol, Ethanol, Acetonitrile
c) UV analysis
UV analysis was performed by scanning the Oseltamivir solution at 200-400 nm.

Design of Experiments

Central Composite Factorial design

Factory design is able to change / add / remove any parameter at any time during our testing. The software provides a place to provide standard functionality simultaneously in one mobile phase. Two independent features have been selected.

Central Composite Factorial design facilitate only one mobile phase at a time

- ✓ Change pH Range: 6-8
- ✓ Change Mobile phase proportion Range: 60-80% (Consider Organic Phase)

Where all the above ranges are put in the Central Composite Factorial design. Provided 13 runs with different pH range and Mobile phase.

The same procedure was followed for each mobile phase. The column C-18 has two mobile phase. Total runs perform is 26. After the completion of all the testing software provides the best single value optimized for chromatographic conditions. Optimization means finding an alternative with the most cost effective or highest achievable performance under the given constraints, by maximizing desired factors and minimizing undesired ones. In comparison, maximization means trying to attain the highest or maximum result or outcome without regard to cost or expense.

A central composite design was used in method development to assess buffer value results, buffer pH and flow rate in the responses. In total 13 runs have been suggested by software. Research considerations are shown in Table No 1. The range of organic matter was 60 to 80% v / v, at a pH of bath 6 to 8 and a flow rate of 1 ml / min.

Table 1: Run suggested by software for each mobile phase.

Sr. No	Mobile Phase Composition (Organic Phase)	pH of Buffer
1	70.00	7.00
2	60.00	8.00
3	80.00	8.00
4	84.14	7.00
5	70.00	7.00
6	70.00	8.41
7	70.00	7.00
8	80.00	6.00
9	60.00	6.00
10	70.00	5.59
11	70.00	7.00
12	70.00	7.00
13	55.86	7.00

Preparation of mobile Phase

To 70 ml of Methanol add 30 ml of Phosphate buffer i.e. in 70:30 v / v proportions. The pH was adjusted at 6,7 and 8 with Trimethylamine and orthophosphoric acid. The solution was filtered through a 0.45µ membrane filter and placed in a sonicator bath for 10 minutes.

Following mobile phases selected

- ✓ Buffer: Acetonitrile
- ✓ Buffer: Methanol

Preparation of Oseltamivir Phosphate stock solutions

The stock solution was prepared by dissolving 10 mg of Oseltamivir Phosphate in water and Methanol as 50:50 v / v Part and then diluted with Water in a 10 ml volume flask to obtain a concentration of 1000 µg / ml. From the resulting solution 0.1 ml diluted into 10 ml of water to obtain a concentration of 10 µg / ml of Oseltamivir Phosphate and labelled standard Oseltamivir Phosphate.

Selection of wavelength

From the standard stock solution some dilutions were performed using water and was scanned at 200-400 nm

and the spectra was covered. It was noted that the drug shows a high absorption of 207 nm.

Optimization

Experimental screening design for chromatographic condition

Determination of the appropriate column and solvent system based on peak parameters.

- Methanol: Phosphate Buffer
- Acetonitrile: Phosphate Buffer

These two types of mobile phase were selected for screening study on the C18 columns at pH 6.0, 7.0 and 8.0. These mobile phases were tested with a variation of organic phase formation from 60 to 80% v / v and a flow rate of 1.0 mL / min.

Table 2: Chromatographic Condition For Methanol Mobile Phase.

Parameter	Optimized Condition
Mobile Phase	(Methanol: Phosphate Buffer 60-80%)
pH	6.0-8.0
Wavelength	207 nm
Injection volume	20 µL
Flow rate	1.0 ml/min
Temperature	Ambient (25° C)
Column	C18 column Waters XBridge (4.6× 250mm id. particle size 5µm),

Result of various trials having methanol organic phase with different pH concentration are shown in Table no: 3

Table 3: Trials response from mobile phase (Methanol: Buffer).

Sr.No	Mobile Phase Composition(Methanol: phosphate buffer)	pH of Buffer	Retention Time	Asymmetry	Theoretical Plates
1	70.00	7.00	3.18	0.932	5491
2	60.00	8.00	6.52	1.245	4098
3	80.00	8.00	1.68	1.349	5002
4	84.14	7.00	0.98	1.982	4651
5	70.00	7.00	3.18	1.11	9784
6	70.00	8.41	3.21	1.34	2187
7	70.00	7.00	3.19	1.21	9860
8	80.00	6.00	1.51	2.341	3021
9	60.00	6.00	6.59	1.10	10200
10	70.00	5.59	3.21	1.368	9254
11	70.00	7.00	3.18	1.22	10245
12	70.00	7.00	3.18	1.332	9854
13	55.86	7.00	9.31	1.478	4175

Table 4: Chromatographic Condition For ACN Mobile Phase.

Parameter	Optimized Condition
Mobile Phase	(Acetonitrile: Phosphate Buffer, 60-80%)
pH	6-8
Wavelength	207 nm
Injection volume	20 µL
Flow rate	1.0 ml/min
Temperature	Ambient (25° C)
Column	C18 column Waters XBridge (4.6× 250mm id. particle size 5µm),

Result of various trials having ACN organic phase with different pH concentration are shown in Table no: 5

Table 5: Trials results from mobile phase (Acetonitrile: Buffer)

Sr.No	Mobile Phase Composition (Organic Phase)	pH of Buffer	Retention Time	Asymmetry	Theoretical Plates
1	70.00	7.00	5.13	1.289	2468
2	60.00	8.00	7.25	1.241	3410
3	80.00	8.00	3.44	1.748	2422
4	84.14	7.00	1.60	2.072	4012
5	70.00	7.00	5.13	1.117	8484

6	70.00	8.41	5.97	1.924	2351
7	70.00	7.00	5.13	2.001	3458
8	80.00	6.00	3.56	2.347	7648
9	60.00	6.00	7.25	1.175	6241
10	70.00	5.59	5.89	1.746	8467
11	70.00	7.00	5.13	1.354	7141
12	70.00	7.00	5.13	1.302	6849
13	55.86	7.00	9.11	2.114	3524

This methodology is initially based on constructing a desirability function for each individual response. The scale of individual desirability function ranges between i= 0, for completely undesirable response and i =1, for fully desired response. Selection of the trial was based on maximum desirability value. Therefore, the first trial which was having desirability one (i=1) selected for method optimization.

Results from different HPLC trials are placed into design expert software and afterward software has optimized the following chromatographic conditions with respect to desirability value.

Table 6: Optimized trials suggested by software based on desirability value (Methanol: Phosphate buffer)

Sr. no.	Amount of Methanol	pH of buffer	Flow rate	Retention time	Tailing factor	Theoretical Plates	Desirability
1	68.00	7.0	1.0	3.18	0.932	9945.67	0.984
2	60.16	6.5	1.0	5.9	0.898	9458.16	0.892

Table 7: Optimized trials suggested by software based on desirability value (ACN: Phosphate buffer)

Sr. no.	Amount of ACN	pH of buffer	Flow rate	Retention time	Tailing factor	Theoretical Plates	Desirability
1	84.140	6.000	1.0	2.161	1.970	7400.80	0.873
2	83.814	6.000	1.0	2.235	1.964	7396.27	0.868

The outcomes found that the desirability value of methanol: phosphate buffer is higher than the ACN: phosphate buffer, subsequently higher estimated results were continuing further for the experiment.

Optimized chromatographic conditions

Mobile phase: Methanol: Phosphate Buffer (68.00: 32.00 v/v), pH of buffer: 7, Analytical column: C_{18} column Waters XBridge (4.6× 250mm id. particle size 5µm), UV detection: 207nm, Injection volume: 20 µL, Flow rate: 1.00 mL min^{-1}, Temperature: Ambient, Run time: 10 min.

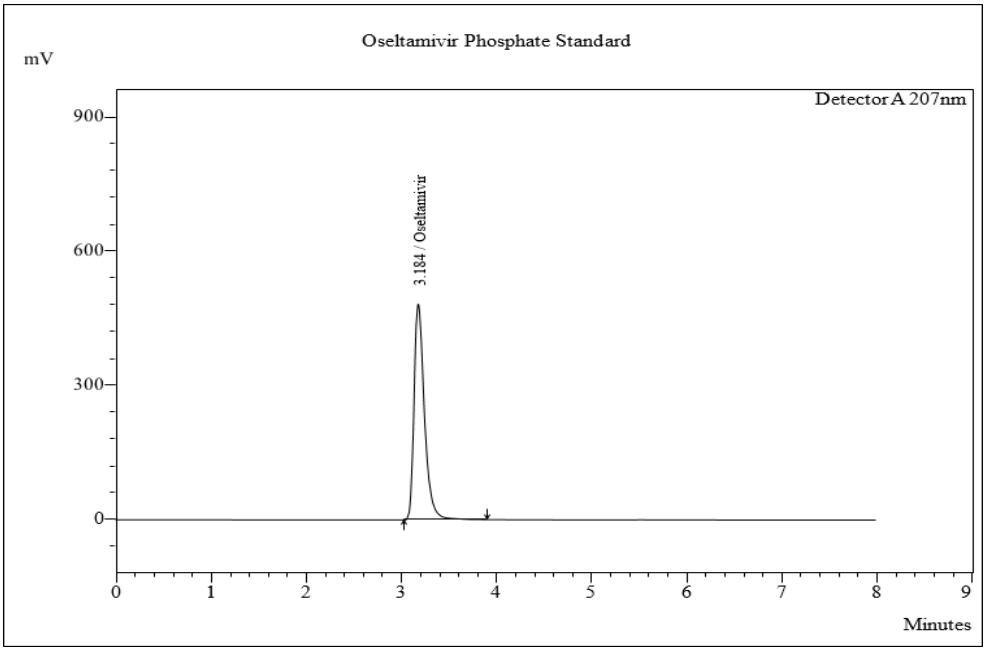

Figure 2 Typical Chromatogram of Optimized chromatographic conditions.

Effect of independent variables on retention time (X):
After applying experimental design, suggested Response Surface Linear Model was found to be significant with model F value of 53.43, p value less than 0.005 and R^2 value of 0.9129. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of % C.V. and adjusted R^2 were 8.93 and 0.8955 respectively. The model for response X (Retention time) is as follows:

The equation for response surface quadratic model is as follows
Retention Time= +4.8 -1.5* A+0.0 * B

Figure 2 shows a graphical representation of pH of buffer (B) and amount of Methanol (A), while flow rate (C) is maintained constant at its optimum of 1 mL min^{-1}. Change in pH of buffer showed slightly change in retention time (X), also increase in amount of Methanol showed decreases the retention time.

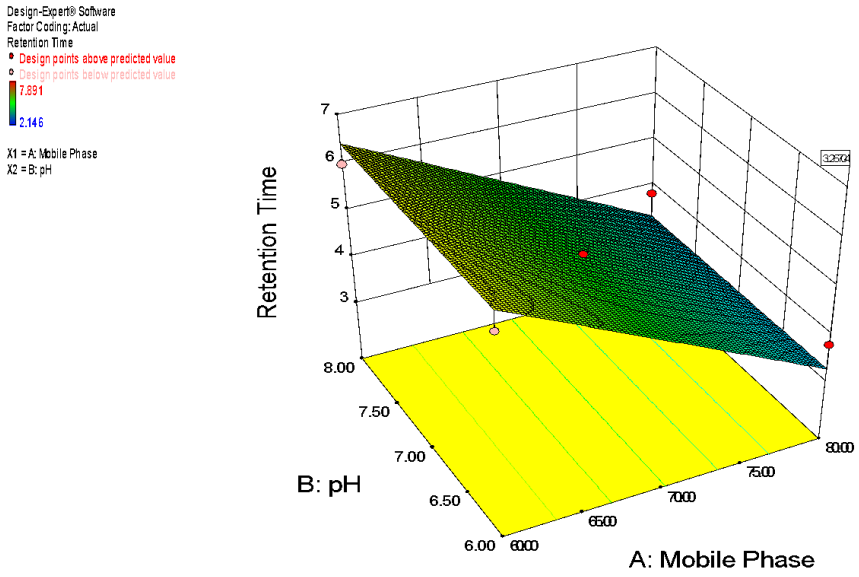

Figure 3 Three-dimensional plot for retention time as a function of pH of buffer and amount of buffer. Constant factor (flow rate- 1mL min-1)

Fit summary: Linear model was suggested by the software.
ANOVA: ANOVA of developed Full three level factorial model for retention time (Y_1).

Values of "Prob > F" (p- value) less than 0.0500 indicate model terms are significant.
In this case A and B are significant model terms.

Table 8: Significance of p value on model terms of retention time.

Model terms	p value	Effect of factor	Remarks
A	0.0001	19.59	Significant
B	0.0001	1.01E-003	Significant
Overall model	0.008	-	Significant

Effect of independent variables on tailing factor (Y):
After applying experimental design, suggested Response Surface Linear Model was found to be significant with model F value of 31.79, p value less than 0.005 and R^2 value of 0.8641. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of % C.V. and adjusted R^2 were 8.66 and 0.8369 respectively. The model for response.

Y (Tailing factor) is as follows:
Asymmetrical Factor = +1.54 +0.031* A +0.3 * B
Figure 3 shows a graphical representation of pH of buffer (B) and amount of Methanol (A), while flow rate (C) is maintained constant at its optimum of 1.0 mL min^{-1}. A decrease in pH of buffer decrease the tailing factor, it is synergistic effect on response (Y) while increase in amount of Methanol showed no drastic change in the asymmetry.

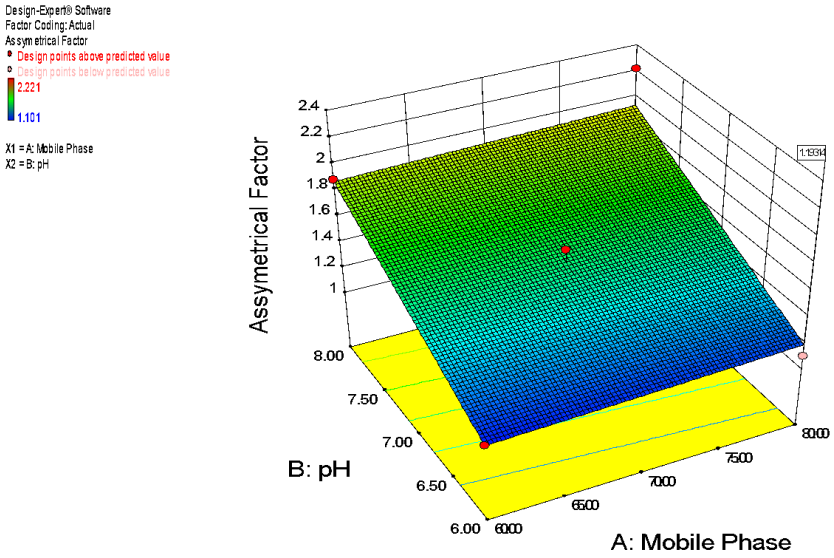

Figure 4 Three-dimensional plot for tailing factor as a function of pH of buffer and % v/v of buffer. Constant factor (flow rate- 1mL min-1)

Fit summary: Response Surface Linear Model was suggested by the software.

ANOVA: ANOVA of developed CCD model for tailing factor (Y_2).

Table 9: Significance of p value on model terms of tailing factor.

Model terms	p value	Effect of factor	Remarks
A	0.5260	7.632	Insignificant
B	0.0001	1.12	Significant
Overall model	0.0944	-	Insignificant

Effect of independent variables on theoretical plates (Z):

After applying experimental design, suggested Response Surface Linear Model was found to be significant with model F value of 95.61, p value less than 0.005 and R^2 value of 0.9503. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of % C.V. and adjusted R^2 were 5.55 and 0.9404 respectively. The model for response Z (theoretical plates) is as follows:

Theoretical Plates = +7554.46 +2049.96 * A +69.69 * B

Figure 4 shows a graphical representation of amount of Methanol (A) and pH of buffer (B), while flow rate (C) is maintained constant at its optimum value 1mL min^{-1}. A decrease in pH of buffer showed not a significant effect on number of theoretical plates (Z), while increase in amount of Methanol showed increases response.

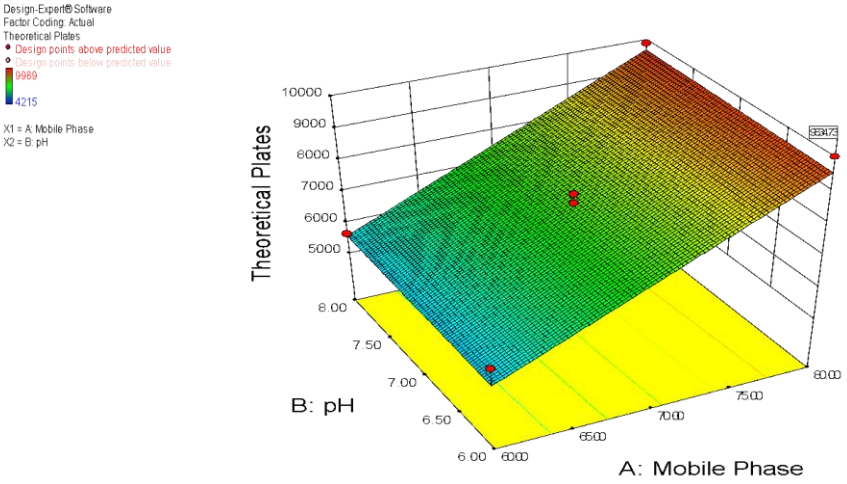

Figure 5 Three-dimensional plot for theoretical plates as a function of pH of buffer and % v/v of buffer. Constant factor (flow rate- 1 mL min-1)

Fit summary: Linear model was suggested by the software

ANOVA: ANOVA of developed CCD model for theoretical plates (Y_3).

Table 10: Significance of p value on model terms of theoretical plates.

Model terms	p value	Effect of factor	Remarks
A	0.0001	3.366	Significant
B	0.6468	-1872.01	Insignificant
Overall model	0.4482	-	Insignificant

RESULTS

Validation of method

An improved chromatographic method was validated using ICH guidelines. Verified validation parameters include linearity, limit of detection and quantification, precision, accuracy, robustness and repeatability according to ICH guidelines.

Linearity and range

The linearity of this method was determined from 8-48 µg / ml in Oseltamivir Phosphate at 207 nm. Oseltamivir Phosphate measurement was obtained with a linear concentration range of 8-48 µg / ml with regression equation with y = 157018x + 13333, a slope of 157018, a breakdown of 13333 and a correlation coefficient is 0.9. The linearity data shown in Table No. 11

Figure 5 shows the measurement curve of Oseltamivir Phosphate and Figure 6 represents the linear chromatographs obtained.

Table 11: Linearity Result of Oseltamivir Phosphate.

Sr. No	Concentration (µg/mL)	Peak Area
1	8	1254714
2	16	2509428
3	24	3714142
4	32	5018856
5	40	6273570
6	48	7528284
Slope		157017.82
Standard Error		22625.31

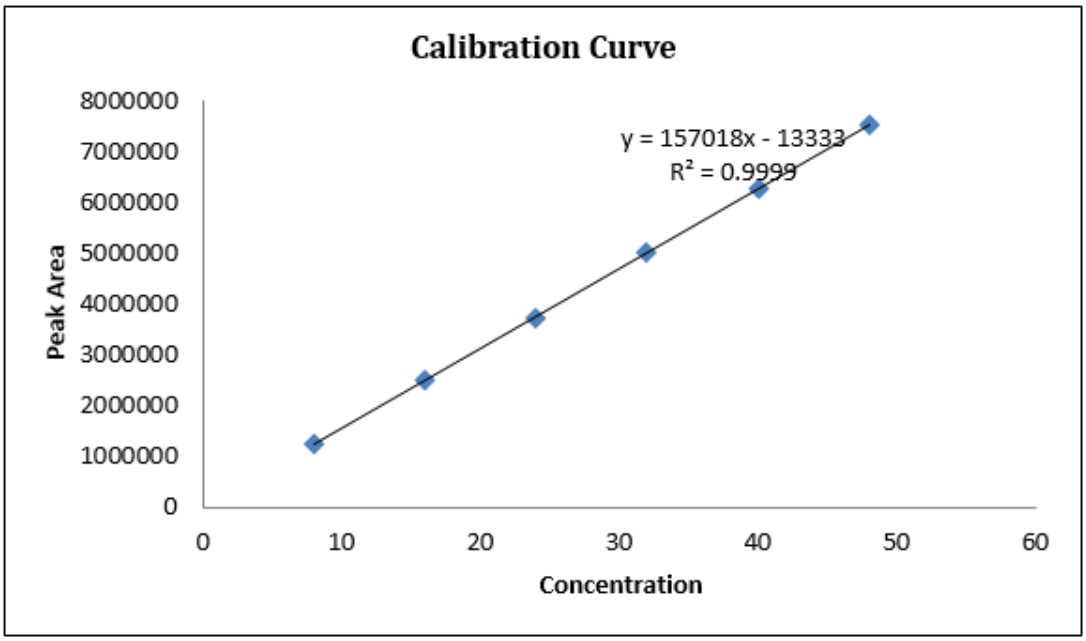

Figure 6 Calibration Curve of Oseltamivir Phosphate.

System suitability

System suitability testing is an important part of system development and is used to ensure adequate functioning of the chromatographic system. Retention time (Rt), number of theoretical plates (N) and tailing factor (T) were tested for six repeated injections of the drug at a concentration of 8 µg / ml. The results provided in Table No 12 were within the acceptable range.

Table 12: System Suitability Studies of Oseltamivir Phosphate.

Retention time (min)	Concentration (µg/mL)	3.18
Peak area	8	1254714
Theoretical plates		9854
Asymmetric Factor		0.984

Specificity

An empty Chromatogram was taken, the Chromatogram of Oseltamivir Phosphate showed a peak retention time of 3.184 min. The mobile phase designed for the method dissolved the drug very successfully. The retention time of the Oseltamivir Phosphate sample was 3.181 min. A wavelength of 207nm was selected for detection because; resulting in better sensitivity to the drug.

Table 13: Specificity of Oseltamivir Phosphate by HPLC Method.

Sample	Label Claim (mg)	Amount Found	Recovery	Retention Time
Tablet	75	74.5	99.4	3.181

Precision

Precision study was conducted to ensure the productivity of the proposed method. This study is divided into repeatability, intraday and interday.

The frequency of injection (System accuracy) was tested using six injections of standard Oseltamivir Phosphate solution and % RSD of replicate injections. Additionally, in order to demonstrate method accuracy (Method accuracy), six samples from the same batch of formulation were analyzed individually and the assay content of each sample was estimated. Average of six determinations was calculated with % RSD for duplicate resolutions. Both the precision of the system and the accuracy of the method have been subjected for interday and intraday variation as reported in Table No. 14

Table 14: Precision.

Sr. No	Concentration (µg/mL)	Intraday (Mean)	Interday (Mean)
1	32	5043332.5	5031695
2	32	5068357	5072605.333
3	32	4894970	5158018
4	32	5050337	4937648
5	32	5082092	5018558
6	32	5055543.25	4984279
Average		5032438.6	5033800.6
Standard Deviation		62750.91	69366.48
RSD%		1.2469	1.378

Accuracy

The accuracy of the Oseltamivir Phosphate analysis was determined at 80%, 100% and 120% concentrations of the standard solution and the results were expressed in terms of % recoveries. Standard deviations and % RSD are calculated. The results are presented in Table No. 15

Table 15: Accuracy of Oseltamivir Phosphate at 207 nm.

Sr. No	Concentration (µg/mL)	Peak area	Found Concentration (µg/mL)	% Recovery
1	25.6	4040688.8	25.59	99.95
2	32	5050861	31.99	99.97
3	38.4	5788145.75	38.49	100.23

Robustness

The robustness of method is not affected by small variations, are indicators of the Robustness of the method. The method is robust, if not affected by minor changes in operating conditions. To determine the robustness of this method, the experimental conditions were intentionally altered at three different levels and the retention time and chromatographic response were evaluated. One factor at a time was changed to study the result. Variations in wavelength, mobile phase, pH and flow rate of 1.0 ml / min (0.9 and 1.1 ml / min) had no significant effect on retention time and chromatographic response of 8 µg / ml solution, indicating that the method was robust. Results are shown in Table No. 16

Table 16: Robustness of Oseltamivir Phosphate.

Sr. No	Parameter		Response	Parameter	Response
Methanol : Water (V/V)			Retention Time (min)	Detection Wavelength (nm)	Peak Area
1	67	33	3.183	205	4960729
2	68	32	3.182	207	5019044
3	69	31	3.179	209	5052308
Average			3.181	Average	5010694
Standard Deviation			0.002	Standard Deviation	37850.36
RSD%			0.065	RSD%	0.755
Flow Rate (mL/min)			Retention Time (min)	pH of Buffer (mmol/L)	Peak Area
1	0.9		3.311	6.8	5047116
2	1		3.181	7	5019217
3	1.1		3.110	7.2	4943596
Average			3.200	Average	5003310
Standard Deviation			0.101	Standard Deviation	43733.13
RSD%			3.18	RSD%	0.874

LOD and LOQ

The LOD and LOQ of Oseltamivir phosphate based on slandered deviation of slop and intercept were found to be 1.08 µg / ml and 3.28 µg / ml respectively.

DISCUSSION

Improvement of the quality analysis method by designing the HPLC method for Oseltamivir phosphate dosage and pharmaceutical formulation has was developed. Both the Mobile phase and the pH were identified affecting the analytical method or profile of the target analytical product. Central composite design was applied for two factors with the use of Design expert software Version 8. In chromatographic separation, column selection variations, instrument configurations, injection volume were maintained during method while variables such as pH and mobile phase, column flow rate, temperature is assigned to the study of robustness. The quality by design has successfully improved the HPLC method of Oseltamivir phosphate.

CONCLUSION

For general analytical purposes, it is always necessary to develop methods that are able to analyse large numbers of samples in a short period of time with due accuracy and precision. Oseltamivir Phosphate is legal in Indian Pharmacopoeia.

Very few analytical methods that have appeared in Oseltamivir Phosphate literature include HPLC, HPTLC and UV-Visible spectrophotometric methods. In view of the above, some simple analysis methods have been developed to improve sensitivity, accuracy, precision and economy. In the current study the HPLC (Quality by Design) method of measuring Oseltamivir Phosphate in bulk and formulation drugs as per ICH guidelines. HPLC methods were validated as linearity results, accuracy, precision, specificity, system suitability and robustness pass the limit. The HPLC method is more sensitive, precise and accurate compared to the previously reported method. There was no disruption of excipient in the recovery study. A low value of % RSD, molar extinction coefficient suggested that developed methods are sensitive. The proposed high performance liquid chromatographic method of has also been tested on accuracy, precision and robustness and proved to be convenient and effective in controlling the quality of Oseltamivir Phosphate. The improved method was found to be simple and less expensive for the quality control of Oseltamivir Phosphate.

In addition, the use of low solvent leads to an inexpensive and environmentally friendly chromatographic process. Thus, the proposed method is fast, selective, requires a simple sample preparation process, and represents a good Oseltamivir Phosphate process.

List of Abbreviations

HPLC: High Pressure Liquid Chromatography
SD: Standard Deviation
nm: Nanometre
nm: Nanometre
UV: Ultraviolet
V: Volt
i.e: That is
cm: Centimetre
RSD: Relative Standard Deviation
ppm: Parts Per Million
USP: United State Pharmacopoeia
ACN: Acetonitrile
Me OH: Methanol
tR: Retention time
r2: Regression Coefficient
LOD: Limit of detection
LOQ: Limit of Quantification
ICH: International Conference on Harmonization
°C: Degree Celsius
No.: Number
Fig: Figure

US: United State
LC: Liquid Chromatography
Std: Standard
%: Percentage
etc: Extra
e.g.: For example
max: Maximum Wavelength

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