Degradation Behavior of Oseltamivir Phosphate under Various Stress Conditions using Stability-indicating HPLC Method

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Abstract

Degradation of the active pharmaceutical ingredient (API) and excipients is a major concern for the drug industry. Fast and reliable analytical method can help in quick investigation and possible corrective action(s). The present work deals with the development of a simple, accurate, precise and robust liquid-chromatographic method and subsequent validation of the method for assessing degradation behavior of oseltamivir phosphate (OP). A stability-indicating isocratic reverse-phase high-performance liquid chromatography method is presented to detect the presence of related impurities and degradation products. Efficient chromatographic separation could be achieved on Inertsil® ODS-2 column (250 mm x 4.6 mm, 5 µ) with buffer (pH 2.5): MeOH (55:45, v/v) in isocratic mode with 1% orthophosphoric acid at 1.0 mL/min flow rate and the eluent monitored at 215 nm. The method validation was performed as per ICH guidelines and found to be linear with regression coefficient 0.999. The proposed method was further used to investigate the degradation kinetics of OP under various stress conditions employed. The drug was less stable under acidic condition. The method was consistent with recoveries for OP (99.8-101.2%) and for its known impurities (97.2-101.3 %).

Keywords: Oseltamivir phosphate; degradation kinetics; validation; HPLC; stability-indicating

Introduction

Oseltamivir phosphate (OP) is an antiviral drug aimed at treating influenza [1]. After metabolism by hepatic esterases, it yields oseltamivir carboxylate (OC), the active form of drug. Dose for adults is 75 mg twice a day for five days (treatment) and 75 mg once a day for at least seven days (prophylaxis) as per World Health Organization (WHO) guidelines [2].

Literature is full of analytical methods for the determination of OP in branded and generic dosage forms and in biological fluids [3-12]. The only stability-indicating assay method validation of OP was described by Narasimhan et. al., (2008), without a mention of known and unknown impurities. The potential impurities must be investigated before product release into the market with the help of a suitable analytical method [13].

The present study is aimed at developing and validating a simple liquid-chromatographic (LC) method for the determination of OP and its known and unknown impurities along with the potential degradation product(s) in capsule dosage form, and validating the method in accordance with the International Conference on Harmonization (ICH) guidance document [14]. The method was specifically aimed at precisely resolving the degradation product(s) from the known process impurities.

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Experimental

Chemicals

Qualified standards of OP and its related impurities were received as a gift (Torrent Research Centre, Ahmadabad, India). The chemicals and solvents were purchased from Ranbaxy Fine Chemicals Limited (Delhi, India) and were used as such unless otherwise specified.

Chromatographic conditions

For the method development, Agilent 1100 HPLC Value System was used. The system included quaternary pump, diode-array detector or variable wavelength detector, autosampler and vacuum degasser. The data were processed by ChemStation® software.

The method was developed and optimized by small deliberate variations in different parameters such as column, buffer and relative amount of organic phase. Combinations of acetonitrile (ACN) and MeOH in varying ratios were used under isocratic condition with deliberate changes in flow rate of 1.0-1.5 mL/min on C_8 and C_{18} column as stationary phase. Measurements made with an injection volume of 20 µL and UV detection at 220 nm, showed reasonably good response. A combination of buffer and organic phase (1:1, v/v) was used as sample diluent.

Preparation of solutions

Standard and resolution solutions

Standard stock solutions of OP (28 µg/mL), impurity-I and impurity-II (56 µg/mL) were prepared by dissolving appropriate amounts in the diluent. Standard of OP (2.8 µg/mL) was prepared by dissolving appropriate amount in diluent.

Sample solution

Dry powder equivalent to 140 mg of OP was transferred to a 100 mL volumetric flask to yield a concentration of 1.4 mg/mL. The solution was filtered through 0.45 µ Nylon-66 membrane filter and used for the analysis.

System suitability parameters

System suitability parameters such as peak retention factor, tailing factor, column plate number, resolution between Impurity-I and Impurity-II, resolution between Impurity-II and OP and %relative standard deviation (RSD) of theoretical area obtained from two diluted standard solutions of OP (in triplicate), were evaluated.

Filter compatibility studies

Diluted standard, sample solution and impurity stock solution were injected in the HPLC system. The difference between concentration of impurities and OP standard preparation in filtered and unfiltered sample solution was calculated using Whatman® filter paper No. 42 and 0.45 µ nylon filter.

Analytical method validation

Specificity and mass balance study

In the specificity study, the developed LC method, OP standard preparation, placebo and its two known impurities were checked for separation and resolution. In addition, the samples were subjected to various levels of degradation conditions as described by Singh et. al. (2000) [19].

Linearity

The linearity of OP and its related substances were determined over the range of limit of quantitation (LOQ) to 300% of impurity specification limit. Mixed standard solutions at LOQ, 50%, 80%, 100%, 120%, 150%, 200% and 300% of

specification limit concentration, were prepared by spiking from stock solutions. The specification limit for related substances was 0.3% sample concentration (1.4 mg/mL). Placebo stock solution was prepared and required volume was added to each preparation. The calibration curve was plotted using the peak area of impurities and OP versus its corresponding concentration. Linearity test was performed for three consecutive days in the same concentration range for related substance method. Percentage RSD value of the slope and Y-intercept of the calibration curve was calculated. Response factors (RF) were calculated for all impurities and purity was adjusted accordingly.

Precision

System and method precision of the standard and sample were checked by injecting six individual diluted standard solution preparations and homogenous sample of OP capsules, respectively. Percentage RSD peak area and percentage concentration for each known impurity and all unknown impurities (≥0.05%) was calculated for system precision and method precision respectively. Intermediate precision (ruggedness) of the method was also evaluated by different analysts, on different days with different instruments in the same laboratory. Limit of detection (LOD) and LOQ were estimated by signal-to-noise (S/N) ratio of 3:1 and 10:1, respectively.

Accuracy

Four concentration levels, i.e., 50, 100, 150 and 300% of sample concentration were used for this study. Accuracy of the related substance method in solutions comprising the drug–matrix used in capsule formulation were determined by standard addition and recovery experiments.

Robustness

Robustness of the method was evaluated by analysis of samples with deliberate small changes in the method with respect to pH (±0.2 unit), Organic phase ratio (± 2%), mobile phase flow rate (±0.2 mL/min) and in the column oven temperature (±5 ºC). The resolution between Impurity-I and Impurity-II, Impurity-II and OP, tailing factor of OP peak and %RSD of theoretical area obtained from two diluted standard solution preparations of OP (in triplicate), were studied with each change.

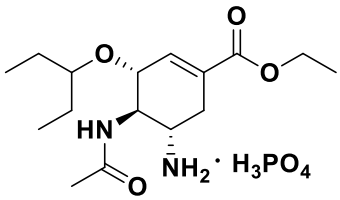
Results and Discussion

Development of chromatographic method

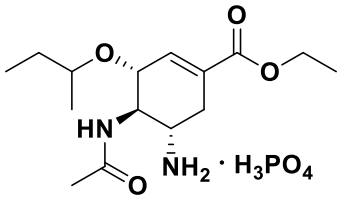
Of the columns tested, C_8 column gave sufficient separation of all the compounds under consideration. Yet, C_{18} column was preferred due to peak shape and especially, resolution between placebo and the degradation products. Preliminary mobile phase was 50 mM potassium phosphate buffer : ACN : MeOH (50:25:25) in the isocratic mode. The product showed degradation under acidic conditions. The peak of impurity-I at 0.31 RRT was not meeting the acceptance criteria for peak purity, which indicated that the peak was not a single peak. The buffer phase was changed to 1% orthophosphoric acid in water (pH 2.5) and the flow rate adjusted to 1.0 mL/min, which resulted in proper resolution of the peak. Optimization of the mobile phase was performed by increasing the concentration of buffer and MeOH. The method resulted in three individual peaks (0.27, 0.32 and 0.34 RRT). The final method parameters are listed in Table 1.

System suitability parameters

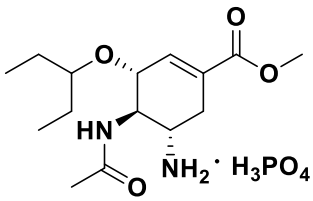
A representative chromatogram (Figure 2) corresponds to the chromatographic separation of these substances. The capacity factor (k') of the first peak was 1.12 and 5.69 for the last peak. The impurity-I, impurity-II and OP resulted in higher number of theoretical plates (acceptance limit >2000) in order of elution as 5860, 7637 and 8587, respectively. The results obtained for the tailing factor were - 1.097 for impurity-I, 1.129 for the impurity-II, and finally 1.198 for OP. The resolution of impurity-I with respect to impurity-II and resolution of impurity-II with respect to OP were 7.1 and 8.0, respectively (acceptance limit: NLT 1.5). The ratio of the peak area of duplicate injection of standard was 0.995, where the limits were 0.95 to 1.05. The %RSD of peak area was 1.6, and within limit of not more than 2%. The tailing factor was also within the established limits. Hence, it was concluded that this method could be applied to routine analysis without any problem.



Oseltamivir phosphate (Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-pentan-3-yloxycyclo-hexene-1-carboxylate phosphate)



Impurity-A (Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(sec-butoxy)cyclohex-1-ene-1-carboxy-late phosphate)



Impurity-B (Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(sec-butoxy)cyclohex-1-ene-1-carboxy-late phosphate)

Figure 1. Molecular structures of oseltamivir phosphate and its known impurities

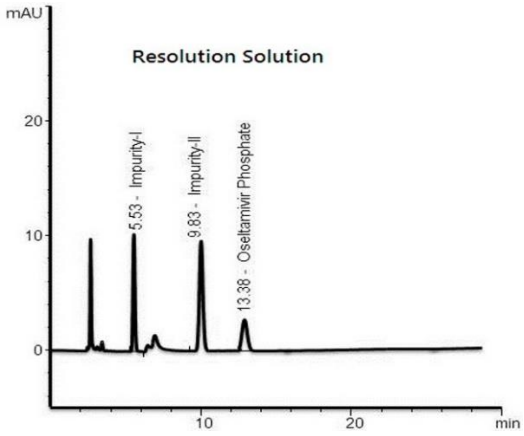


Figure 2. Representative chromatogram of OP and its known impurities

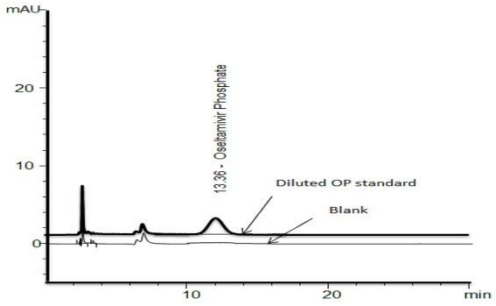


Figure 3. Overlaid chromatogram of diluted solution of OP with blank

Compatibility of filter

The filter compatibility was checked by comparing %peak area of centrifuged sample (unfiltered), Whatman filter paper No. 42 and 0.45 µ nylon filter. The %peak area of the filtered sample was within ±5.0% from the unfiltered sample (centrifuged sample). From the data (Table 2), it was clearly evident that there were no interactions between the sample and the filter.

Specificity

There were no peaks for excipients within the established retention time of drug and impurity as seen from Figure 3 (overlaid chromatograms of sample and blank). There were no peaks for excipients and related substance along with drug, which indicated that the developed method was selective for OP. Degradation behavior of the sample was noted by the method as part of method specificity and the results are summarized in Table 3.

Degradation under acidic conditions. The acidic hydrolysis resulted in 74% degradation of OP with 1.0 N HCl for 30 min at 80 °C. More than eleven degradation products were seen (Figure 4a). Major degradation products (12.22 and 7.71%) were observed at 0.34 and 0.91 RRT, respectively. OP was reduced to about 90.14% with nine degradation products under milder acidic conditions (0.1 N HCl for 30 min at 80 °C).

Alkaline degradation. Alkaline hydrolysis resulted in 85.2% degradation with 0.1 N NaOH solution for 10 min at 80 °C. Six degradants were detected at the RRTs of 0.27, 0.36, 0.55, 0.81, 0.91 and 1.18 as depicted in Figure 4b. Also, OP was reduced to about 95.65% with five degradation products in milder basic conditions (0.1 N NaOH kept at RT) as shown in Figure 4b.

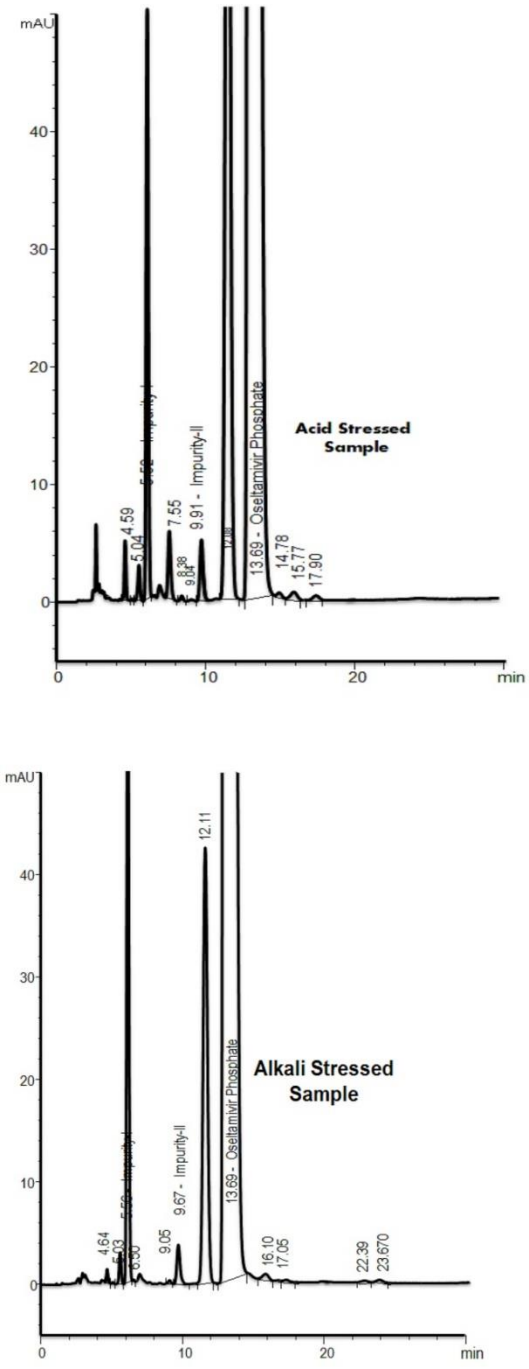


Figure 4. Chromatograms of (a) acid-stressed sample; (b) alkali stressed sample

Oxidative degradation. This resulted in 96.96% degradation of OP, when treated with 3% v/v H_2O_2 and heating for 2 hrs at 80 °C in water-bath. The major degradation product (1.5%) were observed at 0.91 RRT as shown in Figure 5. Under milder condition, the degradation was negligible to about 0.59%.

Photolytic degradation. The photolytic degradation of OP was recorded to about 1.1% under the standard conditions mentioned. This suggested that the drug was stable under light exposure. Representative chromatograms for UV stressed and sunlight stressed samples are shown in Figures 6a and 6b, respectively.

Table 1. Optimized method parameters

Parameter	Conditions
Column	C_{18}, ODS (Inertsil® ODS-2, 250 mm×4.6 mm i.d., 5 µm particle size) column
Mobile Phase	Buffer:MeOH (55:45)
Column temperature	25 °C
Injection Volume	20 µL
Column oven temperature	25 °C
Detection Wavelength	220 nm

Table 2. %Concentration difference (CD) of filtered sample solution with unfiltered (centrifuged) sample

	Centrifuged (Unfiltered)	0.45 µ filter		Whatman filter paper	
		%CD	%RSD	%CD	%RSD
OP	99.321	99.228	0.99%	99.153	1.8%
Impurity-I	0.092	0.094	-2.17 %	0.096	-4.35 %
Impurity-II	0.202	0.201	0.50 %	0.200	1.0 %
Single Unknown	0.164	0.160	2.44 %	0.161	1.54 %
Total Impurities	0.665	0.664	0.15 %	0.648	5.11 %

Table 3. Forced degradation study at various conditions of OP in the presence of known impurities

Conditions	OP (By area % remaining)	IMP-I (By area % remaining)	IMP-II (By area % remaining)	No. of degradation products	Major degradation product (By area % (RRT))	Mass balance (in %)
As such	99.402	0.096	0.196	-	-	-
Acid degradation 0.1 N HCl (30 min at 80 °C)	90.314	0.096	0.249	9	7.129 (0.91)	99.95

Acid degradation 1N HCl (30 min at 80 °C)	73.743	0.071	0.673	13	12.022 (0.34)	99.99
Base degradation 0.1 N NaOH (RT)	95.653	0.095	0.194	5	3.825 (0.36)	99.91
Base degradation 0.1 N NaOH (10 min at 80 °C)	85.269	0.093	0.189	6	9.897 (0.91)	99.98
Peroxide degradation (3% H_2O_2; 5 min at 80 °C)	96.96	0.095	0.199	7	1.495 (0.91)	100.00
Neutral degradation	99.577	0.097	0.204	2	0.149 (0.36)	100.00
Thermal stress	98.148	0.092	0.415	4	1.136 (0.91)	100.00
UV-light exposed	99.484	0.415	0.184	4	0.117 (0.74)	100.00

Table 4. Range of linearity OP and related substance

Compound	Linearity range (µg/ml)	R^2	Slope	Intercept	Std error	t-Stat	p-value	RRF	Precision at LOQ level (% RSD)	Precision at higher level (% RSD)
OP	LOQ-4.162	0.999	23.87	0.42	0.777	-0.546	0.60	-	4.52 %	1.05 %
Impurity-I	LOQ - 12.633	1.000	25.22	-0.08	0.469	-0.172	0.87	1.05	1.51 %	0.65%
Impurity-II	LOQ - 12.008	1.000	24.16	-0.29	0.298	-0.980	0.35	1.01	4.15 %	0.5 %

Table 5. LOD and LOQ results for OP and related substances

Compound	LOD				LOQ			
	C[a] (µg/ml)	**% sample C[a]**	**S/N ratio**	**% RSD**	**C[a] (µg/ml)**	**% sample C[a]**	**S/N ratio**	**% RSD**
OP	0.090	0.007	3.84	9.56	0.281	0.02	12.74	4.52
Impurity-I	0.087	0.006	3.91	10.99	0.292	0.02	11.98	1.51
Impurity-II	0.088	0.006	4.09	11.73	0.294	0.02	13.01	4.15

[a]C: Concentration

Table 6. Ruggedness of OP, known impurities and unknown impurities in term of % CD

Compound	Method precision (Day-1)		Method precision (Day-2)	
	%CD	% RSD	%CD	% RSD
OP	99.107	0.91	99.038	0.53
Impurity-I	0.096	0.43	0.095	0.26
Impurity-II	0.191	0.56	0.193	0.66
unknown Impurity-1	0.052	3.77	0.050	1.30
unknown Impurity- 2	0.089	4.59	0.087	4.21
unknown Impurity-3	0.054	4.56	0.052	4.05
Total Impurities	0.545	2.50	0.546	4.15

Table 7. % Recovery of OP and its related substances at various level

	OP		Impurity-I		Impurity-II	
Level	**%CD**	**%RSD**	**%CD**	**%RSD**	**%CD**	**%RSD**
50	98.9	0.6%	97.4	0.9 %	97.6	2.0 %
100	99.3	0.6 %	99.3	1.6 %	101.8	2.2%
150	100.3	0.9 %	97.3	0.8 %	99.2	2.1 %
HL	99.0	1.1 %	99.0	1.3 %	99.2	0.9 %

Table 8. Stability of OP, known impurities and single unknown maximum impurity in analytical solution at various temperatures

Time (hrs)	OP		Impurity-I		Impurity-II		Single maxima		Total impurities	
	25 °C	**10 °C**	**25 °C**	**10 °C**	**25 °C**	**10 °C**	**25 °C**	**10 °C**	**25 °C**	**10 °C**
Initial Area	66.53234		29.50558		57.99929		35.1432		156.300	
2	0.0	0.9	-0.6	-0.1	1.8	0.2	5.4	7.8	5.0	4.8
4	0.3	0.3	-0.7	-	0.3	-	4.2	-	3.6	-
6	0.4	0.4	-0.8	-0.1	0.9	-0.2	6.2	12.2	5.1	9.2
8	0.7	0.7	-1.3	-	1.2	-	4.6	-	4.9	-
12	1.0	1.0	-1.2	-0.7	2.0	0.5	4.7	13.6	3.7	-3.1
18	1.5	1.5	-1.8	-0.8	3.8	0.5	7.8	10.6	11.2	0.5
24	1.9	1.9	-0.7	-0.6	3.2	0.6	10.8	11.5	12.6	1.6
30	2.4	2.4	-0.4	-1.2	3.6	0.6	12.0	19.7	15.7	0.5

Table 9. Effect of various deliberated changes on the system suitability parameters

System suitability conditions		Resolution b/w Impurity-I and Impurity-II	Resolution b/w Impurity-II and OP	Tailing factor of Standard	%RSD of Replicate Standard Injection
Change in flow	1.2 ml	12.6	13.9	1.04	0.50
	0.8 ml	14.4	15.8	1.03	0.44
Change in column	35°C	13.4	14.8	1.04	0.42
Temperature	25°C	13.6	14.7	1.06	0.45
Change in pH	pH 2.7	12.4	13.8	1.07	0.99
units	pH 2.3	12.7	14.1	1.05	0.43
Change in	- 2.0 %	14.0	15.4	1.02	0.47
Organic phase	+ 2.0 %	11.2	13.3	1.05	0.59

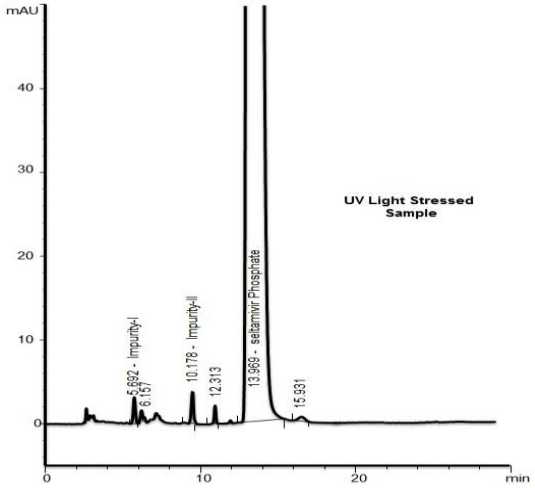


Figure 5. Representative chromatogram of sample subjected to oxidative stress

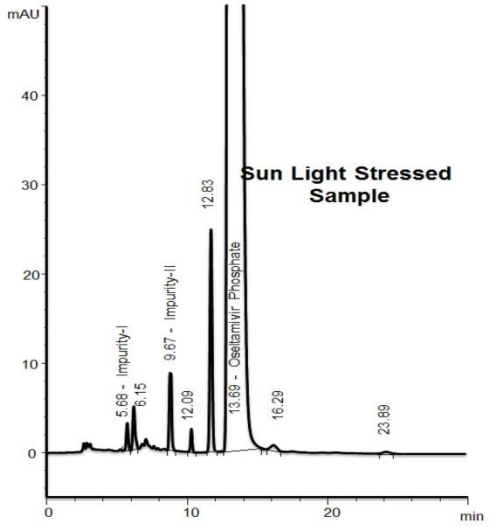


Figure 6. Chromatogram of photolytic stressed (a) UV light stressed and (b) sun light stressed sample

Degradation by heat. Dry-heat degradation was observed to be negligible (<1.5%) when exposed to at 80 °C for 8 hrs. Four degradation products were observed with only one degradation product >1% (by area) at 0.91 RRT.

Linearity. Linearity parameters of the calibra-tion curves for OP and the related compounds as (Table 4) showed r^2 0.995 which suggested that the response was linear between LOQ to highest level.

LOD and LOQ. The sensitivity of the method can be measured by LOD and LOQ, by means of the S/N ratio. The acceptance criterion for %RSD of the peak area of in the case of LOD and LOQ were 33.33 and 10.00%, respectively (Table 6). The results suggested that the method could be used to quantity very small quantities of impurities generated.

Precision and repeatability. % RSD values for system precision of the retention time and peak area response for OP were 0.17 and 0.87, respectively (Table 6). Method precision exhibited %RSD of 1.9 and 1.4, for repeatability and intermediate precision, respectively. This indicated that the method was precise and reproducible.

Accuracy. As shown in Table 7, the percent recoveries of OP and its two related were consistent (98.9–100.3% with % RSD ranging from 0.6-1.1%). However, the range for related substances was from 97.4 to 101.8 with % RSD ranging from 0.9 to 2.2%. These values of recoveries proved that the method was accurate in determining the drug and the related substances.

Stability in analytical solution. The standard and sample solutions were stable for 24 hrs. Percentage area changed was < 3.0 for OP while the %area changed <5% for individual impurities and total impurity (Table 8). This suggests the sample can be used upto 24 hrs.

Robustness. Small deliberate changes in the method with respect to certain parameters mentioned in Table 9 had no detrimental effect on the method performance. The data suggested that the flow rate to be maintained within the established limits otherwise could lead to poor resolution.

Conclusions

The developed analytical HPLC method for oseltamivir phosphate was validated and found accurate, precise, robust and specific. The method was extrapolated to determine the related substances; therefore, the developed method could be considered as stability-indicating method for determination of osetamivir in any dosage form.

Conflict of Interest

The authors declare no conflict of interest.

Disclaimer

The views, thoughts and opinions expressed in this research article belong solely to the authors, and not necessarily to the author's employer, organization, committee or other group or individual.

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