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A stability indicating LC method for oseltamivir phosphate

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ABSTRACT

An Isocratic reverse phase liquid chromatographic (RP-LC) assay method was developed for the quantitative determination of Oseltamivir Phosphate in bulk drug and in pharmaceutical dosage form, used to treat Antiviral (Influenza). The developed method is also applicable for the related substances determination .The chromatographic separation was achieved on Oyster-RP18e, 250mm×4.6 mm, 5µm column. The LC method employs Solution A as mobile phase. The Solution A contains a mixture of phosphate buffer pH 3.0: Methanol : Acetonitrile (60:25:15, v/v). The flow rate was 1.0 ml $min^{-1}$and the detection wavelength was 207 nm. In the developed HPLC method the resolution between Oseltamivir Phosphate and its potential impurities, namely Imp-1, Imp-2 and Imp-3 was found to be greater than 2.0. The drug was subjected to stress conditions of hydrolysis, Acid , Alkaline ,oxidation, photolysis and thermal degradation. Considerable degradation was found to occur in Thermal, Photolysis,alkaline medium, Acid medium, and Oxidative stress conditions. Major degradation product formed during Photolysis was found to be Imp-1, Acid hydrolysis , Base and oxidation hydrolysis was found to be un specified impurities. The stress samples were assayed against a qualified reference standard and the mass balance was found close to 99.6%. The developed RP-LC method was validated with respect to linearity, accuracy, precision and robustness.

KEYWORDS

Column liquid chromatography;
Oseltamivir phosphate;
Forced degradation;
Validation;
Stability indicating.

INTRODUCTION

Oseltamivir phosphate ethyl (3R,4R,5S)-4-(acetyl amino)-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate is a orally active inhibitor of influenza virus neuraminidase figure 1. It is a new class of specific anti-influenza agents, the neuraminidase inhibitors, has demonstrated potent inhibition of both influenza A and B viruses. These inhibitors are potent and selective inhibitor of influenza A and B virus neuraminidases. Oseltamivir is approved for treatment of uncomplicated acute illness caused by influenza A or B virus in persons greater than or equal to twelve years of age and who have been symptomatic for no more than two days

Few Chromatographic methods have been appeared in the literature. for the determination of Oseltamivir phosphate., in human plasma and in pharmaceutical dosage form[1-3]. So far, to our present knowledge there is no stability indicating LC method for the related substance determination and quantitative estimation of Oseltamivir phosphate. This paper describes the assay and related substances method validation for accurate quantification of Oseltamivir phosphate and all 3 impurities in bulk samples and in phar

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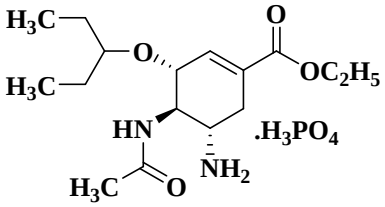
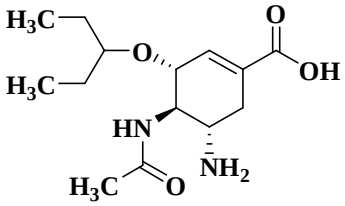
Oseltamivir Phosphate



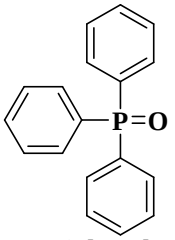
(3R,4R,5S)-4-acetylamino-5-amino-3(1-ethyl proopoxy)-1-cyclohexene-1-carboxylic acid, ethyl ester, phosphate

Imp-1



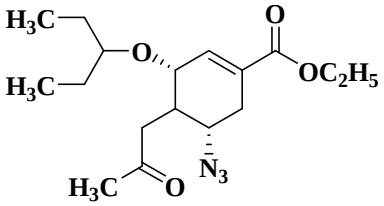
(3R, 4R, 5S)-4-(Acetylamino)-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid

Imp-2



Triphenyl Phosphine oxide

Imp-3



Ethyl (3R, 4R, 5S)-4-(Acetyl amino)-5-azido-3-(1-ethylpropoxy) -1-cyclohexene-1-carboxylate

Figure 1: Chemical structures and labels of oseltamivir phosphate and its impurities

maceutical dosage forms also (TABLE 7).

EXPERIMENTAL

Chemicals

Samples of Oseltamivir Phosphate its related impurities and 75 mg Oseltamivir Phosphate tablets are manufactured at Hetero labs Limited India.. HPLC grade acetonitrile was purchased from Merck, Darmstadt, Germany. Analytical reagent grade Potassium dihydrogen phosphate monohydrate and Ortho phosphoric acid were purchased from Merck, Darmstadt, Germany. High pure water was prepared by using Millipore Milliq plus water purification system.

Equipment

The LC System, used for method development, forced degradation studies and method validation was Shimadzu Prominence photo diode array detector. The output signal was monitored and processed using LC Solutions software on Pentium computer (Digital equipment Co).

Chromatographic conditions

The chromatographic column used was Oyster-RP18e, (250×4.6) mm with 5 μm particles. The mobile phase contains a mixture of pH 3.0 Phosphate Buffer and Methanol and Acetonitrile in the ration of 60:25:15 (v/v) Buffer consist of 0.05mM Potassium dihydrogen Orthophosphate, pH adjusted to 3.0±0.05 using Orthophosphoric acid.

The solvent mixture contains a mixture of 62 ml of water, 45 ml of Methanol and 135 ml of Acetonitrile. Filter and degas the solvent mixture.

The flow rate of the mobile phase was 1.0 ml min^{-1}. The column temperature was maintained at 50°C and the detection was monitored at a wavelength of 207 nm. The injection volume was 20 μl. Solvent mixture was used as diluent.

Preparation of solutions

Preparation of standard solutions

Weigh accurately about 3.0 mg of each Impurity-1, Impurity-2, Impurity-3, and 2.0 mg of Oseltamivir Phosphate working standard into a 100 ml volumetric flask. Dissolve and dilute to the mark with mobile phase. Take 1.0 ml of above solution into a 20 ml volumetric flask. Dilute to the mark with solvent mixture.

Preparation of sample solution

Twenty capsules were weighed and the content transferred into a clean and dry mortar, grinded well. Then equivalent to 100 mg of drug was transferred to 100 ml volumetric flask, 70 ml of diluent added and kept on rotatory shaker for 10 min to disperse the material completely and sonicated for 10 min and diluted to 100 ml (500 μg ml^{-1}). The resulting solution was centrifuged at 3,000 rpm for 5 min. Supernant solution was taken 10 ml and diluted to 100ml with diluent (100 μg ml^{-1}). This was filtered using 0.45 μ nylon 66-membrane filter.

Specificity

Specificity is the ability of the method to measure

the analyte response in the presence of its potential impurities[4]. Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used.

The specificity of the developed LC method for Oseltamivir Phosphate was determined in the presence of its impurities namely Imp-1, Imp-2, Imp-3 and degradation products. Forced degradation studies were also performed on Oseltamivir Phosphate to provide an indication of the stability indicating property and specificity of the proposed method. The stress conditions employed for degradation study includes light (carried out as per ICH Q1B), UV light 254 nm, heat (105°C), acid hydrolysis (0.5M HCl), base hydrolysis (0.5M NaOH), water hydrolysis and oxidation (30% H_2O_2). For heat, UV light and light studies, study period was 48 hrs whereas for acid, base, water hydrolysis and oxidation, it was heat the solution 80°C for 2 hours. Peak purity of stressed samples of Oseltamivir Phosphate was checked by using PDA. The purity angle is within the purity threshold limit obtained in all stressed samples demonstrates the analyte peak homogeneity. All stressed samples of Oseltamivir Phosphate [heat (80°C), acid hydrolysis (0.5M HCl), base hydrolysis (0.5M NaOH), water hydrolysis and oxidation (30% H_2O_2).] were studied for extended run time of 90 min to check the late eluting degradants

Assay studies were carried out for stress samples against qualified reference standard and the mass balance (% assay+% of impurities+% of degradation products) was calculated. Assay was also calculated for bulk samples and drug product by spiking all 3 impurities (Imp-1, Imp-2 and Imp-3) at the specification level.

Method validation

Precision

The precision of the related substance method was checked by injecting six individual preparations of (100% sample) Oseltamivir Phosphate spiked with 0.15% each Imp-1, Imp-2, and Imp-3. The %RSD of area for each Imp-1, Imp-2, and Imp-3 was calculated.

The intermediate precision of the method was also evaluated using different analyst and a different instrument in the same laboratory.

Assay method precision was evaluated by carrying out six independent assays of test sample of Oseltamivir Phosphate against qualified reference standard. The %RSD of six assay values obtained was calculated.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for Imp-1, Imp-2, and Imp-3 were estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration[6]. The precision study was also carried out at the LOQ level by injecting six individual preparations of Imp-1, Imp-2, Imp-3 and calculated the %RSD for the areas of each impurity.

Linearity

Linearity test solutions for assay method were prepared from stock solution at seven concentration levels from LOQ to 150% of assay analyte concentration (LOQ, 50%, 80%, 100%,120% and 150%). The peak area versus concentration data was performed by least-squares linear regression analysis.

Linearity test solutions for related substance method were prepared by diluting the impurity stock solution to the required concentrations. The solutions were prepared at five concentration levels from LOQ to 150% (LOQ, 0.075%, 0.120%, 0.15%, 0.18% and 0.225%. The correlation coefficient, slope and Y-intercept of the calibration curve was reported.

Accuracy

The accuracy of the assay method was evaluated in triplicate at four concentration levels, i.e. LOQ, 50%, 100% and 150% in bulk sample and drug product. The percentages of recoveries were calculated.

The study was carried out in triplicate at 0.075%, 0.15% and 0.225% of the analyte concentration. The percentage of recoveries for Imp-1, Imp-2, Imp-3 were calculated.

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately changed and the resolution between Oseltamivir Phosphate, Imp-1, Imp-2, Imp-3 was evaluated. The flow rate of the mobile phase was 1.0 mL min^{-1}. To study the

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TABLE 1: System suitability report

Compd.	USP resolution (R_S)	USP tailing	USP theoritical plates (N)
Imp-1	--	1.04	13,892
Oseltamivir phosphate	27.271	0.88	16,410
Imp-2	40.293	0.95	17,564
Imp-3	2.063	0.96	21,786

TABLE 2: Summary of forced degradation results

Stress condition	Time	% Assay of active substance	Mass balance (%assay+% impurities+% degradation products)	Remarks (major degradant)
Acid hydrolysis (0.5M HCl)	2 hr	91.1	99.9	Impurity-1
Base hydrolysis (0.5M NaOH)	2 hr	94.3	99.9	Impurity-1
Oxidation (30%H_20_2)	2 hr	98.8	99.7	Impurity-1
Water hydrolysis	2 hr	99.3	99.7	Unknown impurities
Thermal (105°C)	48 hrs	99.2	99.8	Impurity-1
UV light	254 nm	99.3	99.7	Impurity-1
Light (photolytic degradation)	1200 KLUX	99.4	99.8	Impurity-1

TABLE 3: Results of accuracy study for drug substance

Added (%) (n=3)	Recovered (µg)	% Recovery
50	50.1	100.2
100	100.6	100.6
150	150	99.5

n=3, Number of determinations

effect of flow rate on the resolution, 0.2 units changed it from 0.8 to 1.2 mL min^{-1}. The effect of pH on solution of impurities was studied by varying ±0.1 pH units (at 2.9 and 3.1 buffer pH). In the all above varied conditions, the components of the mobile phase were held constant as stated in section 2.3.

Solution stability and mobile phase stability

The solution stability of oseltamivir phosphate in the assay method was carried out by leaving both the test solutions of sample and reference standard in tightly capped volumetric flasks at room temperature for 24 h. The same sample solutions were assayed for 6 h interval up to the study period. The mobile phase stability was also carried out by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions for 24 h interval up to 7 days. Mobile phase prepared was kept constant during the study period. The % RSD of assay of Oseltamivir Phosphate was calculated for the study period during mobile phase and solution stability experiments.

The solution stability of Oseltamivir Phosphate and its impurities in the related substance method was carried out by leaving spiked sample solution in tightly capped volumetric flask at room temperature for 48 h. Content of Imp-1, Imp-2, Imp-3 were determined for every 12 h interval up to the study period.

Mobile phase stability was also carried out for 7 days by injecting the freshly prepared sample solutions for every 24 h interval. Content of Imp-1, Imp-2, Imp-3 were checked in the test solutions. Mobile phase prepared was kept constant during the study period.

RESULTS AND DISCUSSION

The chromatographic separation was achieved on a Oyster-RP18e, (250×4.6) mm with 5 µm particles. The mobile phase contains a mixture of pH 3.0 Phosphate Buffer and Methanol and Acetonitrile in the ration of 60:25:15 (υ/υ) Buffer consist of 0.05mM Potassium dihydrogen Orthophosphate, pH adjusted to 3.0±0.05 using Orthophosphoric acid.

The flow rate of the mobile phase was 1.0 mL min^{-1}. The column temperature was maintained at 50°C and the detection was monitored at a wavelength of 207 nm. The injection volume was 20µL. Solvent mixture was used as diluent. There was no interference of blank with Impurities (Imp-1, Imp-2, and Imp-3) and Oseltamivir.The interference of excipients (Dicalcium phosphate,Lactose, micro crystalline cellulose) was also checked by injecting sample solutions of excipients. There was no interference of excipients with Impurities (Imp-1, Imp-2 and Imp-3) and Oseltamivir peak. In the optimized conditions Oseltamivir, Imp-1, Imp-2, and Imp-3, were well separated with a resolution of greater than 2 and the typical retention times of Imp-1, Oseltamivir, Imp-2, and Imp-3 were about 5.1, 13.8, 72.3 and 77.7 min respectively. The system suitability results are given in TABLE 1. and the developed LC method was found to be specific for Oseltamivir and its three impurities namely Imp-1, Imp-2, and Imp-3 (TABLE 2).

Analysis was performed for different batches of bulk drug samples (n=3) and for pharmaceutical dosage forms (n=3). Results were given in TABLE 7. Stability study results as per ICH Q1A (R2) for Oseltamivir[4]

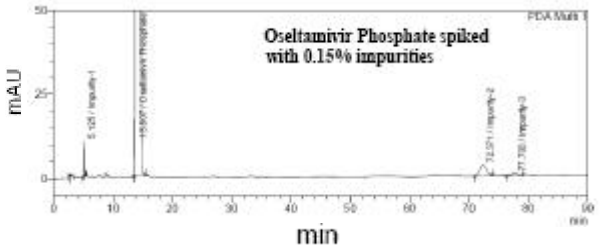


Figure 2: Typical chromatogram of oseltamivir phosphate spiked with impurities at 0.15% level

were given in TABLE 8 and TABLE 9.

Method validation

Precision

The %RSD of assay of Oseltamivir Phosphate during assay method precision study was within 1.0% and the %RSD of area of Imp-1, Imp-2, Imp-3 in related substance method precision study were within 5%. The %RSD. of assay results obtained in intermediate precision study was within 1.0% and the %RSD of content of Imp-1, Imp-2, Imp-3 were well within 10.0%, confirming good precision of the method(TABLE 6).

Limit of detection and limit of quantification

The limit of detection of Imp-1, Imp-2, and Imp-3 were 0.002, 0.012, and 0.004% (of analyte concentration, i.e.1000μg mL^{-1}) respectively for 20 μL injection volume. The limit of quantification of Imp-1, Imp-2, and Imp-3 were 0.02, 0.05, and 0.02% (of analyte concentration, i.e. 1000 μg ml^{-1}) respectively for 20L injection volume. The precision at LOQ concentration for Imp-1, Imp-2, Imp-3 were below 5%

Linearity

Linear calibration plot for assay method was obtained over the calibration ranges tested, i.e. 50%-150% specification level and the correlation coefficient obtained was greater than 0.999. The results show that an excellent correlation existed between the peak area and concentration of the analyte. The slope and Y-intercept of the calibration curve were 59 and -18 respectively.

Linear calibration plot for related substance method was obtained over the calibration ranges tested, i.e. LOQ to 0.225% for Imp-1, Imp-2, Imp-3. The correlation coefficient obtained was greater than 0.998. Linearity was checked for related substance method over the same concentration range. The percentage of R.S.D. values of the slope and Y-intercept of each component is within the limit. The results show that an excellent

TABLE 4: Results of accuracy study for drug product

Added (μg) (n=3)	Recovered (μg)	% Recovery
50	49.7	99.4
100	100.2	100.2
150	148.2	98.8

n=3, Number of determinations

TABLE 5: Results of robustness study

S. no.	Parameter	Variation	Resolution between Imp-2 and Imp-3
1	Flow rate (±20% of the set flow)	(a) At 0.8ml/min	2.08
		(b) At 1.2ml/min	2.12
2	pH (± 0.1 unit of set pH)	(a) At 2.9	2.21
		(b) At 3.1	2.16
3	Temperature (±5^{0}C of set temperature)	(a) At 45°C	2.12
		(b) At 55°C	2.04

TABLE 6: Results of intermediate precision

S. no.	Parameter	Variation	%RSD for assay	%RSD for related substances	Resolution between Imp-2 and Imp-3
1	Different system	(a)Shimadzu	0.12%	< 5.0%	2.12
		(b)Agilent 1100 series VWD system	0.18%	< 5.0%	2.05
2	Different column	(a)B.No:11050170	0.24%	< 5.0%	2.24
		(b)B.No:11050176	0.26%	< 5.0%	2.13
3	Different analyst	(a) Analyst-1	0.12%	< 5.0%	2.12
		(b) Analyst-2	0.23%	< 5.0%	2.08

TABLE 7: Batch analysis

Batch no.	Imp-1	Imp-2	Imp-3	Purity by HPLC	Assay by HPLC
Drug substance DS001	0.02	ND	ND	99.68%	99.9%
DS002	0.02	ND	ND	99.71%	99.9%
DS003	0.02	ND	ND	99.71%	100.0%

Where ND =Not detected

TABLE 8: Accelerated stability data (storage conditions: 40°±2°C / 75 ±5% RH)

Bulk drug b.no:DS 001	Temperature 40 ±2°C Relative Humidity 75±5%					
Duration	Imp -1	Imp -2	Imp -3	Total impurities	Assay by HPLC	Remarks
Initial	0.03	ND	ND	0.35%	99.8%	No significant change observed
1st month	0.03	ND	ND	0.37%	99.9%	No significant change observed
2nd Month	0.03	ND	ND	0.36%	99.9%	No significant change observed
3rd Month	0.02	ND	ND	0.32%	99.9%	No significant change observed

Where ND=Not Detected

correlation existed between the peak area and concentration of Imp-1, Imp-2, Imp-3.

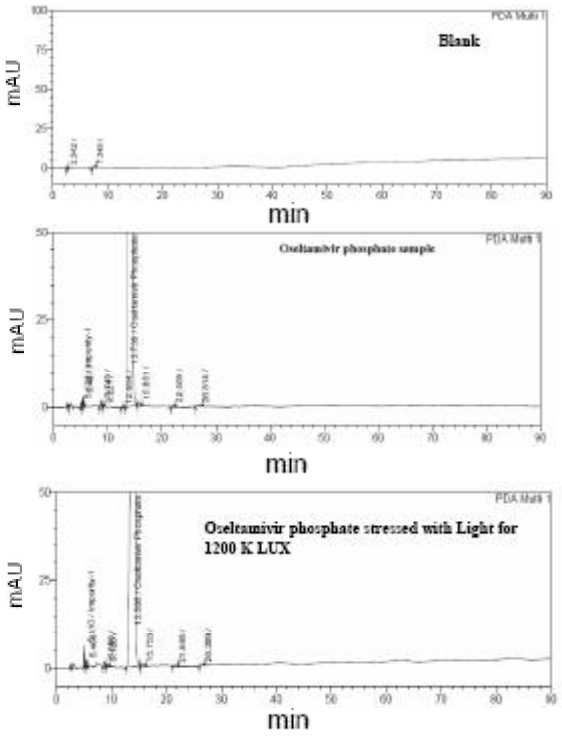
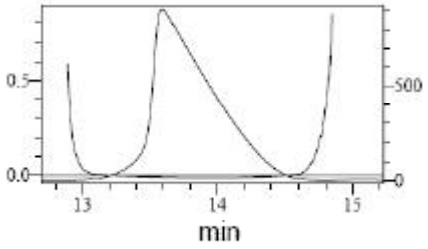


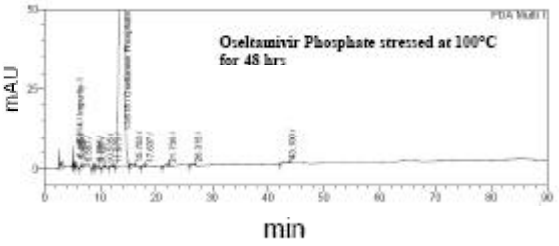
Figure 3: Typical chromatogram of oseltamivir phosphate sample and stressed oseltamivir phosphate samples

Peak purity results for Photo degradation :

ID# : 2
Retention Time : 13.602
Compound Name : Oseltamivir Phosphate

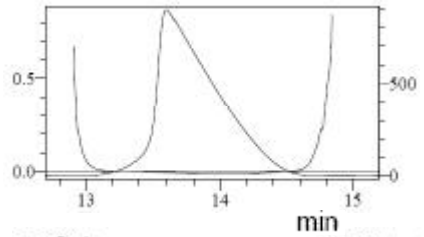


Impurity :Not Detected
Peak purity index : 1.000000
Single point threshold : 0.999997
Minimum peak purity index : 3

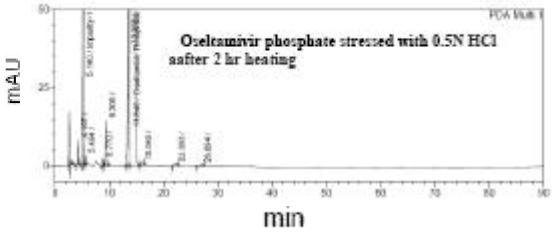


peak purity results for thermal degradation :

ID# : 2
Retention Time : 13.615
Compound Name : Oseltamivir Phosphate

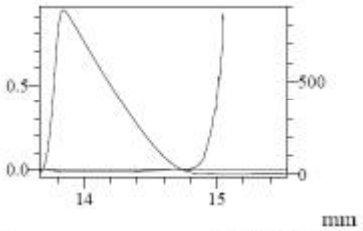


Impurity :Not Detected
Peak purity index : 1.000000
Single point threshold : 0.999996
Minimum peak purity index : 3

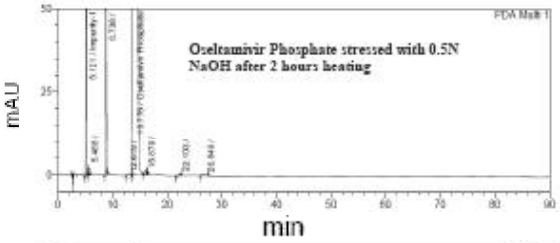


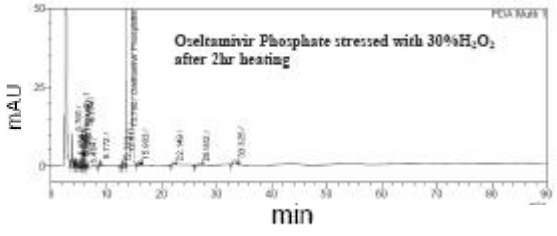
Peak purity results for acid degration :

ID# : 2
Retention Time : 13.849
Compound Name : Oseltamivir phosphate



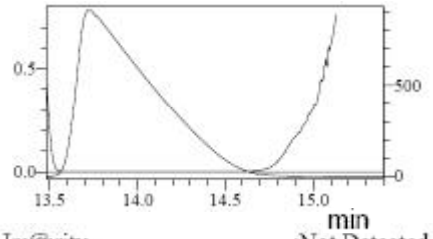
Impurity :Not Detected
Peak purity index : 1.000000
Single point threshold : 0.999996
Minimum peak purity index : 3



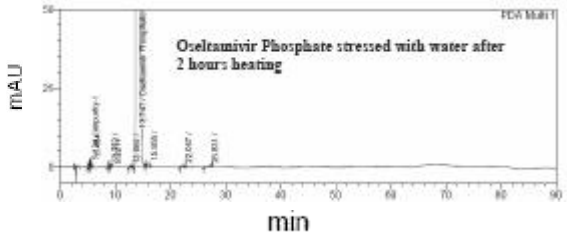


peak purity results for peroxide degradation

ID# : 2
Retention Time : 13.729
Compound Name : Oseltamivir Phosphate

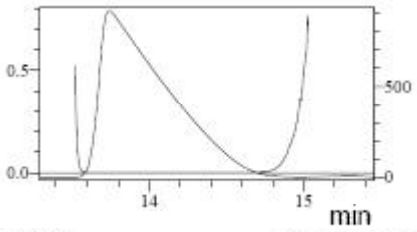


Impurity :Not Detected
Peak purity index : 1.000000
Single point threshold : 0.999996
Minimum peak purity index : 3



Peak purity results for water degradation :

ID# : 2
Retention Time : 13.747
Compound Name : Oseltamivir Phosphate



Impurity :Not Detected
Peak purity index : 1.000000
Single point threshold : 0.999996
Minimum peak purity index : 3

Accuracy

The percentage recovery of Oseltamivir Phosphate in bulk drug samples ranged from 98.0 to 102.0 (TABLE 3). The percentage recovery of Imp-1, Imp-2, and Imp-3 in bulk drugs samples ranged from 85 to 115. HPLC chromatogram of spiked sample at 0.15% level of all 3 impurities in Oseltamivir Phosphate bulk drug sample are shown in figure 2.

Robustness

In all the deliberate varied chromatographic conditions carried out as per Section 2.6.6 (flow rate and pH), the resolution between closely eluting impurities, namely Imp-2 and Imp-3 was greater than 2.0, illustrating the robustness of the method (TABLE 4).

Solution stability and mobile phase stability

The %RSD of assay of Oseltamivir Phosphate during solution stability and mobile phase stability experiments was within the limit. No significant changes were observed in the content of Imp-1, Imp-2, Imp-3, during solution stability and mobile phase experiments when performed using related substances method. The solution stability and mobile phase stability experiments data confirms that sample solutions and mobile phase used Assay Solution 24 hrs , Assay mobile phase 7 Days and Related substances Solution stable at 48 hrs and Mobile phase 7 days.

Results of forced degradation studies

Degradation was not observed in Oseltamivir Phosphate stressed samples that were subjected to Thermal , light, UV light and heat study. The degradation of drug substance was observed under acid, water hydrolysis, base hydrolysis, and oxidative conditions (Figure 3). Oseltamivir Phosphate is highly sensitive towards base and it was degraded into Imp-1 during base hydrolysis (in 0.5M NaOH after 2 hrs heating at 80^0C). The drug substance Oseltamivir Phosphate was degraded into Impurity-1 during acid hydrolysis, (in 0.5M HCl after 2 hrs heating at 80^0C). The drug substance Oseltamivir Phosphate it was degraded into Imp-1 during in water hydrolysis ((in Water after 2 hrs heating at 80^0C) and The drug substance Oseltamivir Phosphate was degraded into Impurity-1 during Oxidation,(in 30% H2O2 after 2 hrs heating at 80^0C). Peak purity test results derived from PDA detector, confirmed that the Oseltamivir Phosphate peak is homogeneous and pure in all the analyzed stress samples. Peak purity results for degraded peaks from PDA detector confirm that all-degradant peaks are homogeneous and pure in all analyzed stress samples. No degradants were observed after 30 min in the extended runtime of 90 min for all the Oseltamivir Phosphate samples [heat (105^0C), UV light, photolysis, acid hydrolysis (0.5M HCl), base hydrolysis (0.5 M NaOH), water hydrolysis and oxidation (30% H_2O_2).]

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TABLE 9: Long stability data (storage conditions: 25°±2°C/60± 5% RH)

Bulk drug b.no: DS001	Temperature 25 ±2°C relative humidity 60± 5%					
Duration	Imp-1	Imp-2	Imp-3	Total impurities	Assay byHPLC	Remarks
Initial	0.03	ND	ND	0.34%	99.9%	No significant change observed
1st Month	0.03	ND	ND	0.38 %	99.8%	No significant change observed
2nd Month	0.03	ND	ND	0.40%	99.8%	No significant change observed
3rd Month	0.03	ND	ND	0.40%	99.8%	No significant change observed

Where ND=Not Detected

The mass balance of stressed samples was close to 99.8%(TABLE 2). The assay of Oseltamivir Phosphate is unaffected in the presence ofImp-1, Imp-2, Imp-3 and its degradation products confirm the stability indicating power of the developed method(TABLE 6).

CONCLUSIONS

The isocratic RP-LC method developed for quantitative and related substance determination of Oseltamivir Phosphate in both bulk drug and pharmaceutical dosage form is precise, accurate and specific. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicating and can be used for the routine analysis of production samples and also to check the stability of samples of Oseltamivir Phosphate.

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