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Analytical Method Development and Stability Studies for Estimation of Oseltamivir In Bulk and Capsules Using RP-HPLC

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Abstract : A simple and reproducible method of isocratic reverse phase liquid chromatography (RP-LC) was developed for the quantitative determination of oseltamivir phosphate in bulk drug and capsules, used to treat antiviral (influenza). The proposed RP-HPLC method uses X terra C_{18}, 4.6 mm, 150 mm 4.6 mm i.d. column (at room temperature), using 0.1% octa-sulfonic acid: acetonitrile 30: 70 v / v, effluent flow rate (1.0 ml / min) and UV detection at 237 nm for oseltamivir analysis. The method was validated according to the ICH guidelines in terms of specificity, linearity, precision and accuracy. The retention time for oseltamivir was 2.31 min. The recovery determinations allowed the calculation of a confidence interval from 99.79 to 101.30% with a relative standard deviation value of 0.5%. LOD and LOQ were estimated at 2.98 and 9.98 µg/mL respectively. The validated method was successfully applied to the determination of oseltamivir in dosage form in capsules (Tamiflu 75 mg, Roche). Oseltamivir was exposed to conditions of acid, basic, oxidative and thermal stress and the stressed samples were analyzed with the proposed method. The chromatographic peak purity results indicated the absence of elution peaks with the main oseltamivir peak, which demonstrated the specificity of the test method for estimating oseltamivir in the presence of degradation products. This method has advantages that include a short execution time, a simple and rapid sample preparation which makes this method used for routine oseltamivir analysis in quality control laboratories.
Key words : Oseltamivir phosphate, X terra, octa-sulfonic acid, acetonitrile, Tamiflu.

Introduction:

The ethyl ester of Oseltamivir phosphate (OSP), [(3R, 4R, 5S) -4-acetylamino-5-amino-3 (1-ethylpropoxy) -1-cyclohexene-1-carboxylic, phosphate] (Figure 1), is a prodrug of ethyl ester of the neuraminidase inhibitor which is extensively metabolized in the human liver by the active metabolite,

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oseltamivir carboxylate[1,2]. The hydrophobic group of Oseltamivir is responsible for its poor oral absorption; therefore, phosphate salt was developed which allows the oral administration of this medicine, recommended for both treatment and prophylaxis of influenza. The recommended doses for adults according to the World Health Organization (WHO) are 75 mg twice a day for 5 days (treatment) and 75 mg once a day for at least 7 days (prophylaxis).[3]

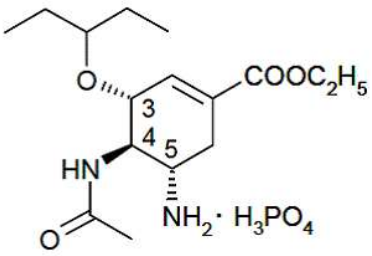

Figure 1: Chemical structure of Oseltamivir phosphate (OSP)

It was developed by Gilead Sciences and is currently marketed by Hoffmann-La Roche (Roche) under the trade name Tamiflu. Tamiflu® is the main avian flu drug. The manufacturer has allowed several companies to produce Tamiflu under license to meet the growing global demand for this drug.[4]

Hitherto there are few reported analytical methods for estimating oseltamivir based on different techniques, such as bioassay, capillary electrophoresis, cyclic voltammetry, fluorescent liquid chromatography, mass spectrometry and UV detection, among others[5 -7]. To date, there are some reports published on HPLC methods to measure oseltamivir[5, 8]. A sensitive HPLC mass spectrometry assay for oseltamivir carboxylate in plasma and urine has been described[9,10] and an HPLC assay for OSP in pharmaceutical preparations[11]. Joseph-Charles et al.[12] reported an HPLC method for the determination of OSP in dosage forms at 226 nm using a Zorbax CN column with a mobile phase consisting of methanol and formic acid 0.04 M pH 3.0 (50:50). Fukeet al.[13] describe the detection by HPLCUV of the oseltamivir carboxylate in biological materials, after extraction of the cation exchange in mixed mode. Furthermore, Bahrami et al.[14] presented a simple RP-HPLC method to determine carboxylated oxeltamivir in human serum using a C18 reverse phase column with a mobile phase containing 0.05 M phosphate buffer - acetonitrile, pH 3, 70:30 (V / V) and a wavelength detection of 215 nm, while Narasimhan et al.[15] developed a reverse phase HPLC method of gradient-grade elution indicative of the same detection wavelength with a Kromasil C18 column and a gradient that used a mobile phase consisted of triethylamine and acetonitrile. More recently, Aydogmuset al.[16] developed an RP-HPLC method for the determination of OSP in capsules and plasma, based on the reaction of the drug with 4-chloro-7-nitrobenzofurazan in the borate buffer, while Chabaia et al.[17] used the monolithic silica HPLC column to determine the OSP in the pharmaceutical preparation.

Therefore, our goal was to develop an economical and time-saving HPLC method with UV detection for oseltamivir determination, avoiding long sample preparation steps and using acetonitrile in a mobile phase composition, which retained adequate sensitivity for analysis routine in a pharmaceutical analytical laboratory.

To establish the stability indicating nature of the method, the forced degradation of oseltamivir was performed under various stress conditions (base, acid, oxidative and thermal) and the proposed method analyzed the samples subjected to stress. The proposed LC method was able to separate the drug from the degradation products generated during the forced degradation studies. The method developed was validated according to the ICH guidelines.[18]The linearity of response, precision, robustness and robustness of the described method was checked.

Experimental:

Chemicals and solvents:

The pure OSP drug was procured as a gift sample from Hetero Drugs, Hyderabad, India. The OSP tablets were purchased at the local Kurnool pharmacy. HPLC grade Acetonitrile and water were obtained from SD Fine Chem, Mumbai, India. All other chemicals used were of AR grade.

HPLC–PDA instrumentation and chromatographic conditions

The HPLC system was an LC Waters (Waters, Milford, MA, USA) consisting of quaternary gradient system (600 Controller), in line degasser (Waters, model AF), photodiode array detector (Water, 2998 model) and auto sampler (Waters, model 717 plus). Data was processed using Empower Pro software (Waters, Milford, MA, USA). Chromatographic separation assay was performed with a X-terra C-18 analytical column (150 mm × 4.6 mm inner diameter, 5 µm particle size, Waters, Dublin, Ireland) maintained at ambient temperature. The mobile phase consists of 0.1% Octasulphonic acid: Acetonitrile 30:70v/v. The mobile phase was pumped at a flow rate of 1.0 mL min^{-1}. The detection wavelength was 237 nm. Mobile phase was used as diluent for the preparation of working standards of OSP.

Preparation of Standard Solution:

10 mg of OSP was accurately weighed and transferred into 10 ml volumetric flask, added 50 ml of mobile phase and sonicated for 5 mins to dissolve and diluted to volume with the same. Further diluted 7 ml of the above solution to 10 ml with diluent, to get the final concentration of 700µg/mL. The Solution was filtered through 0.45 µm membrane filter prior to injection and 10 µL was injected into chromatograph.

Sample Preparation (Assay of pharmaceutical preparation):

Twenty capsules were weighed and average weight was determined. Then all the capsule contents were ground to fine powder. A quantity of powder equivalent to 10 mg of OSP was weighed and transferred to 10 ml volumetric flask. Added 5 ml of mobile phase and sonicated for 5 mins to dissolve and made up to mark with the same. The solution was filtered if necessary. 7 ml of a clear filtered solution was pipetted out in to a 10 ml volumetric flask and make up to the mark with mobile phase.

Method validation

The proposed method has been validated in accordance with the guidelines established by the International Harmonization Conference for the validation of analytical procedures.[18] The parameters used to validate the method of analysis were: system suitability, specificity, linearity, precision and precision, limit of detection (LOD), limit of quantification (LOQ), specificity and robustness.

System suitability

The system suitability test is an integral part of the validation of the liquid chromatography method performed to verify and assure the continuous performance of a chromatographic system. The repeatability of the system was estimated by 6 repeated injections of standard working solution at 100% of the test concentration (700 µg / ml of OSP). The suitability parameters of the system have been calculated according to the recommendation of ICH.[18]

Specificity

Specificity is the ability to unequivocally evaluate the analyte in the presence of components that can be assumed to be present (impurities, degrading, matrix, etc.)[18,19]. Specificity has been demonstrated by determining OSP in the presence of degradation products generated by forced decomposition. The stress conditions applied in the study were: basic hydrolysis (0.1 molNaOH, at 60 °C, for 5 hours), acid hydrolysis (1 molHCl, at 90 °C, for 3 hours) and oxidative degradation (H_2O_2 at 3%, at 90 °C, for 30 minutes) and thermal degradation (60 °C, for 24 minutes). The blank solutions have been treated in the same way.

Linearity

For the evaluation of linearity, the calibration curve was obtained at 6 concentration levels of standard OSP solutions (100–1600 µg / ml). The solutions (20 µl) were injected in triplicate in a chromatographic system with the chromatographic conditions previously provided. For linearity assessment, the peak area and concentrations were subjected to a least squares regression analysis to calculate the calibration equation and the coefficient of determination.

Precision

The precision of the analytical procedure (intra-assay precision) was investigated by analyzing six sample solutions obtained by multiple sampling of the same homogeneous sample under the prescribed conditions (at 100% of the test concentration of OSP (700 µg/mL)) on the same day, by the same analyst and using the same equipment. The intermediate precision of the analytical procedure was investigated by analyzing sample solutions on three consecutive days. The precision of the analytical procedure was expressed as the relative standard deviation of a series of measurements.

Limit of detection and limit of quantification

The detection limit (LOD) and the limit of quantification (LOQ) of the proposed method were determined by consecutively injecting low concentrations of the standard solutions (0.125-1.25 µg ml-1) using the proposed RP-HPLC method. LOD and LOQ were calculated according to ICH[18, 19] guidelines as follows:

LOD = 3.3 SD/*S* and

LOQ = 10 SD/*S*,

where SD is the standard deviation of the response (*y* intercept) and *S* is the slope of the calibration curve obtained.

Accuracy

To study the accuracy of the proposed analytical method, recovery tests were conducted. To discover whether excipients interfered with the analyte, equivalent amounts at 50, 100 and 150% of OSP were evaluated from capsule formulation and the resulting mixtures were analyzed by the proposed methods. The percent of recovery was calculated as follows:

Amount Added:

$$\frac{\mu g}{ml} Added = \frac{Sample\ Weight}{Sample\ Dilution} X \frac{Average\ Assay}{100} X \frac{Labelled\ Claim}{Average\ Weight} X\ 1000$$

Amount Found:

$$\frac{\mu g}{ml} Found = \frac{Sample\ Area}{Standard\ Mean\ Area} X \frac{STD\ Weight}{STD\ Dilution} X \frac{STD\ Potency}{100} X\ 1000$$

% Recovery:

$$\%\ Recovery = \frac{\frac{\mu g}{ml}\ Found}{\frac{\mu g}{ml}\ Added} X100$$

Robustness

The ability of the proposed method to remain unaffected by small (deliberate) variations in parameters was evaluated in order to determine method robustness. Changes were made to the following method parameters: flow rate (±0.1 ml min–1), organic solvent concentration (± 2%), wavelength of detection (± 5 nm), and temperature (± 3 °C).

Results & Discussion

Method Development

Various mobile phases have been studied in the development of an HPLC method for OSP analysis. These include: methanol - water, 50:50 (V/V), acetonitrile - water, 30:70 (V / V), methanol - orthophosphoric acid buffer (pH 4.5–6.5), 50:50 (V / V), Methanol buffer - phosphate (pH 3.0–6.5), 25:75 (V / V) and

acetonitrile - phosphoric acid buffer (pH 3.2-4.5) 60:40 v / v . The suitability of the mobile phase was decided based on the sensitivity of the assay, the suitability for stability studies, the ease of preparation and the use of readily available solvents. Therefore, the mobile phase consisting of 0.1% octa-sulfonic acid: acetonitrile 30: 70v / v, has been found to be optimal for isocratic determination of oseltamivir in pharmaceutical products. The wavelength was selected by scanning the standard OSP solution at more than 200-400 nm and the wavelength of 237 nm was chosen for the detection of oseltamivir phosphate.

Oseltamivir has been identified as a function of retention time compared to the oseltamivir standard. Furthermore, oseltamivir was identified by adding the standard to the sample prior to analysis, which resulted in an increase in the sample peak area that was proportional to the amount added. The mean oseltamivir retention time was approximately 2.31 minutes at a flow rate of 1.0 ml min-1. Oseltamivir was rapidly determined as a single sharp peak. The OSP retention time we reached in our study was shorter than previously reported. No interference was observed from other degradation products. Figure 2-4 shows the blank, standard and sample chromatograms.

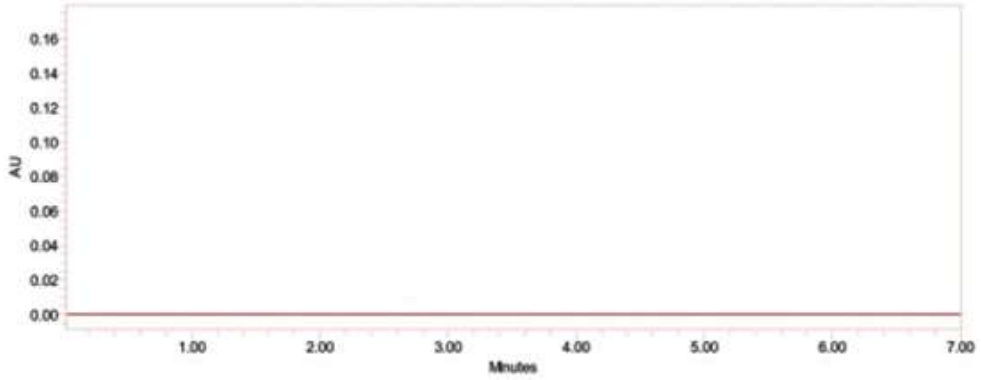

Figure 2: Blank chromatogram

Method Validation

System suitability

The results of system suitability test were found within the acceptable range[18] indicatingthat the system was suitable for the intended analysis (Table 1).

Table 1: Results of system suitability test

S. No	Parameters	Results	Acceptance Limits
1.	Retention time (Rt)*	2.306	--
2.	Number of theoretical plates (N)*	3100.2	More than 3000
3.	Tailing factor (T)*	1.65	Less than 2
4.	Similarity factor*	1.0	0.9-1.2
	* Number of injections: 6 replicates		

Specificity

In the specificity study, standard OSP solutions and the sample solution were injected and a single peak was obtained for OSP, indicating that there was no interference from the excipients used or from the mobile phase. Furthermore, a forced degradation study was conducted to demonstrate the specificity of the proposed method. This study also provides information on degradation pathways and degradation products that could be formed during storage. The result of the studies on forced degradation, with an approximate percentage of degradation and a relative retention time of the degradation products, is shown in Table 2. Figure 6 (a-d) shows the OSP chromatograms

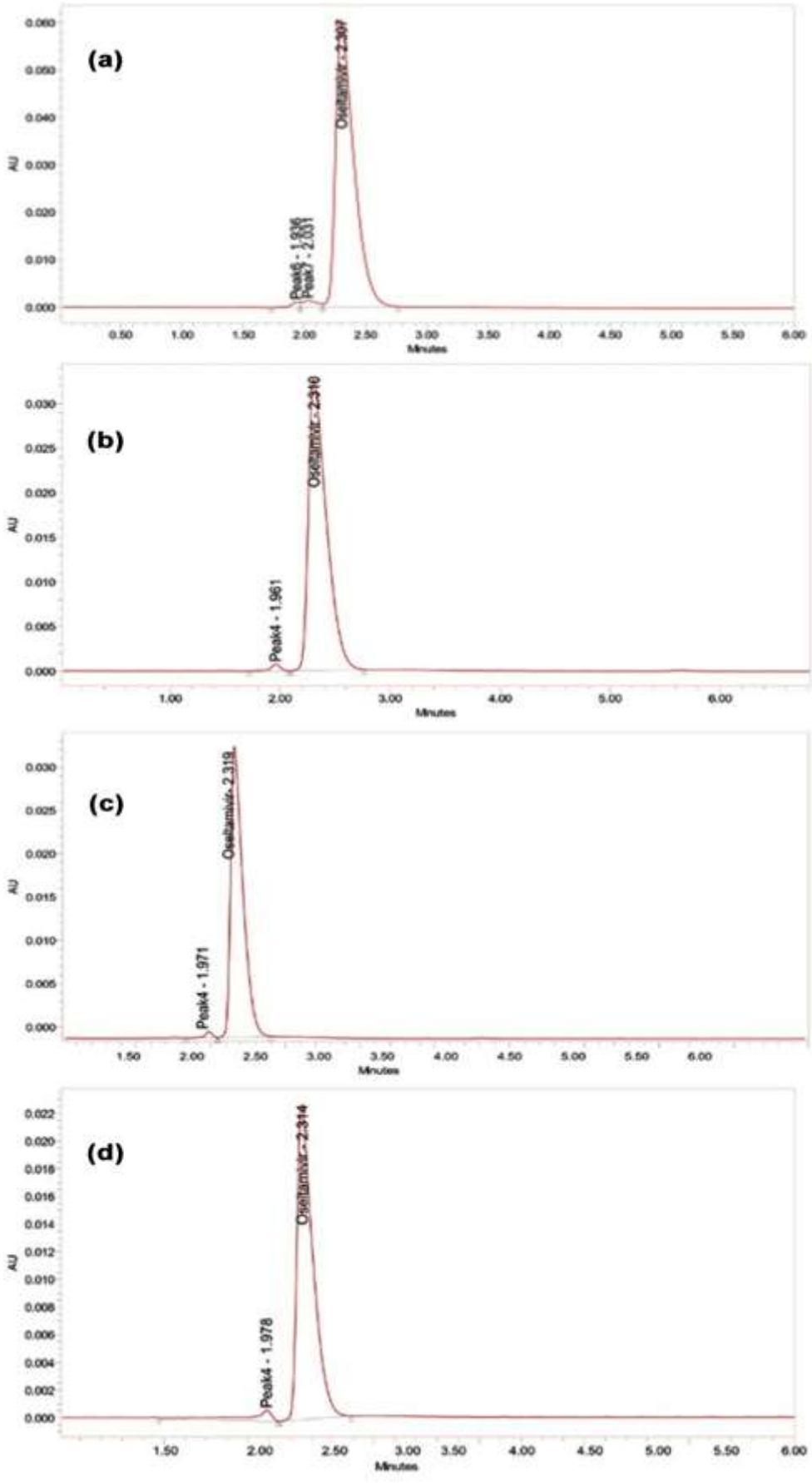

Figure 6: Chromatograms of acid degradation (a), basic degradation (b), Oxidative degradation (c), and thermal degradation of oseltamivir (d)

Table 2: Results of forced degradation study for Oseltamivir

Stress Degradation condition	Oseltamivir		
	% Assay	% Degraded	Relative RT (min) of degradation products
Acid	92.24	7.76	1.936, 2.031
Base	91.41	8.59	1.961
Peroxide	90.76	9.24	1.971
Thermal	95.64	4.36	1.978

HPLC chromatograms of acid, base, oxidative and thermal degradation of OSP show that OSP peak is well separated from all the degradation products formed during the different stress conditions. Thus specificity study ensures that the developed analytical method is able to separate and quantify OSP in presence of different degradation products.

Linearity

In the present study, linearity was studied in the concentration range 100-1600 µg/mL OSP and the following regression equation was found by plotting the peak area (y) expressed in mAU versus the OSP concentration (x) expressed in µg/mL: Y=855.4x+1E+06 (r^2 = 0.998). The determination coefficient (r^2) demonstrates the excellent relationship between the peak area andconcentration of OSP. The calibration curves of oseltamivir API and oseltamivir with placebo were linear. The excipients had no influence and there was no matrix effect observed. Figure 5 shows the linearity curve of OSP.

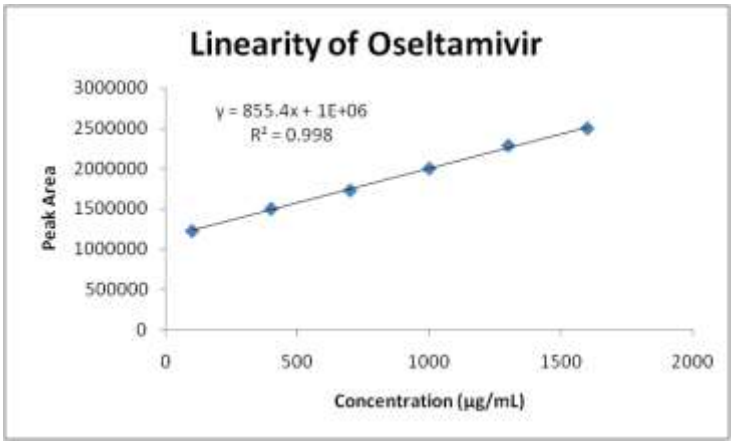

Figure 5: Linearity of OSP

Precision and accuracy

Precision was demonstrated by Interday and intraday variation studies. In the intraday andInterday studies the solutions were injected 6times and %RSD was calculated which was found to be less than 1%. Accuracy (R %) of the proposed methods was demonstrated by analyzing different concentrations covering the points in the calibration range. The average percentage recovery was found to be 100.32 and 99.18%. The precision and accuracy was shown in Table 3 and 4 respectively.

Table 3: Precision of the method

S. No	Interday		Intraday	
	Peak Area	% Assay	Peak Area	% Assay
1.	1707855	99.41	1690999	98.18
2.	1720536	100.15	1716574	99.66
3.	1712803	99.70	1721186	99.93

4.	1726599	100.51	1725670	100.19
5.	1716136	99.90	1733700	100.66
6.	1723573	100.33	1745975	101.37
Avg	1717917.00	100.00	1722350.67	100.00
SD	6997.08	0.41	18523.43	1.08
%RSD	0.41	0.41	1.08	1.08

Table 4: Accuracy at various levels of concentrations

Concentration	Peak area	Recovery	Mean recovery	Standard deviation	% RSD
80%	858843	101.33	101.30	4792.4	0.5
	853761	100.73			
	863340	101.86			
100%	1691682	99.8	99.79	8938.5	0.5
	1700606	100.32			
	1682729	99.27			
120%	2564856	100.87	100.86	13240.1	0.5
	2566204	100.92			
	2588433	100.80			

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were 2.98 µg/mL and 9.98 µg/mL respectively. The proposed HPLC method for OSP determination was demonstrated to be sensitive for performing the stability indicating assay and the assay evaluation for product release and stability studies and profiling of Tamiflu® capsules.

Robustness

Based on the obtained results the proposed HPLC analytical method was demonstrated to be robust (Table 5).

Table 5: Robustness of the method

S. No	Change in the parameter	%RSD (n=3)
1.	Flow rate (1.01 mL/min)	0.12
2.	Flow rate (0.90 mL/min)	0.18
3.	Organic solvent concentration (68)	1.25
4.	Organic solvent concentration (72)	1.05
5.	Wavelength of detection (232 nm)	1.08
6.	Wavelength of detection (242 nm)	0.95
7.	Temperature (22 °C)	1.26
8.	Temperature(28 °C)	1.40

Method Applications

The validated method was applied for the determination of OSP in commercially available Tamiflu® capsules. Figure 3-4 shows two typical HPLC chromatograms obtained later the test of the standard OSP reference solution and of the Tamiflu® capsule sampling solution, respectively. The results of the trial (n = 6) produced 100.00% (RSD = 0.14%) of the label declaration for oseltamivir in Tamiflu® 75 mg capsules, respectively (Table 3). The average retention time of oseltamivir was approximately 2,306 minutes. The test results indicate that the method is specific for the analysis of oseltamivir without interference from the excipients used to formulate and produce these capsules.

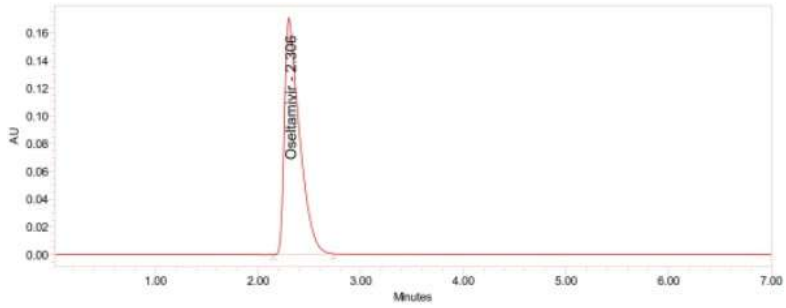

Figure 3: Standard chromatogram

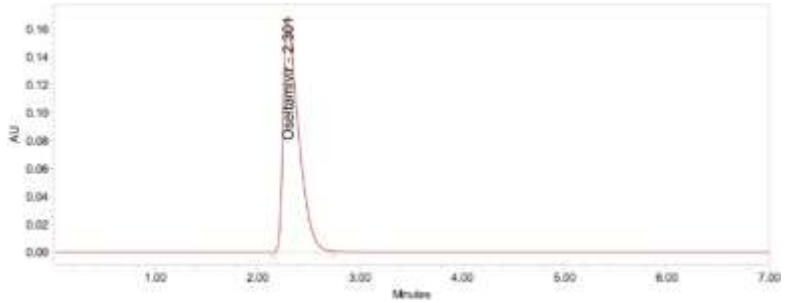

Figure 4: Sample chromatogram

Conclusion

In conclusion, a sensitive and selective stability indicating RP-HPLC method has been developed and validated for OSP analysis in API and capsules. Based on the peak purity results obtained from the analysis of force degraded samples using the described method, it can be concluded that the absence of a co-eluent peak together with the main oseltamivir peak indicated that the developed method it is specific for the estimation of oseltamivir in the presence of degradation products. Furthermore, the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility. Although no attempt has been made to identify degraded products, the proposed method can be used as a stability indicator method for oseltamivir dosage.

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