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Development and Validation of Stability Indicating HPTLC Method for the Determination of Oseltamivir Phosphate in Bulk and Dosage Form

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ABSTRACT

The objective of the present work is to develop a simple, precise, accurate, validated stability indicating HPTLC method for the determination of Oseltamivir in bulk and tablet dosage form. The HPTLC method employed TLC aluminum plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of Toluene: methanol: ammonia (3.5:1.5:0.2v/v) and then scanned. The system was found to give the compact spot for Oseltamivir (Rf value of 0.45 ± 0.02). The linearity was found to be in the concentration range 800-4800 ng/spot. The reliability and analytical performance of the proposed methods, including linearity, range, precision, accuracy, detection and quantitation limits, were statistically validated. When Oseltamivir was subjected to different stress conditions; the proposed methods could effectively separate the drug from its degradation products and were thus considered as good stability-indicating procedures. It is concluded that this method can be applied for routine quality control of Oseltamivir in dosage forms as well as in bulk drug.

INTRODUCTION

Oseltamivir (OTV) is a white crystalline solid with the chemical name (3R,4R, 5S)-4-acetylamino-5-amino3(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid, ethyl ester, phosphate (1:1) **(Fig. 1)**. The chemical formula is $C_{16}H_{28}N_2O_4$ (free base). The molecular weight is 312.4 for oseltamivir free base and 410.4 for oseltamivir phosphate salt. [1, 2].Oseltamivir is a novel agent approved for the treatment and prevention of influenza infection and illnesses in adults and children.[3]. Its active metabolite selectively blocks the viral surface enzyme neuraminidase thereby preventing the release of virus particles from infected cells. It is active against influenza A and B virus and is the drug of choice for treatment of swine flu. It comes under the category of drugs called neuraminidase inhibitors [4]. The drug is taken orally in capsules or as a suspension. Oseltamivir is a prodrug, a (relatively) inactive chemical, which is converted into its active form by the metabolic process after it is taken into the body. It was the first orally active neuraminidase inhibitor commercially developed [5].

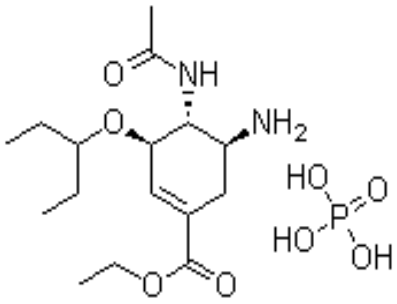

Fig.1 Chemical structure of Oseltamivir Phosphate

Literature survey revealed, few analytical methods, which include UV-spectrophotometric methods [6-11] liquid chromatographic methods [12-25], and colorimetric method [26] have been reported for estimation of OTV. However, to our knowledge, no information related to the stability-indicating UV and high-performance thin-layer chromatography (HPTLC) determination of OTV in pharmaceutical dosage forms has ever been mentioned in the literature. According to the stability test guidelines issued by ICH [27-29], in the present study, the stress induced stability studies were carried out for OTV to establish its stability characteristics. Hence, an attempt has been made to develop an accurate, specific and reproducible method for the determination of OTV in presence of degradation product for the content analysis during stability studies from the pharmaceutical dosage form.

2. MATERIALS AND METHODS

2.1 Materials and Reagents

A pharmaceutical grade of Oseltamivir kindly supplied as a gift sample by Cipla Ltd., Mumbai, India. All chemicals and reagents used were of HPLC of analytical grade and were purchased from Merck Chemical. India. Analytical grade sodium hydroxide, hydrochloric acid, and 30% hydrogen peroxide were used.

2.2 Instrumentation and chromatographic conditions

HPTLC was performed on 20 cm x 10 cm aluminum plates coated with silica gel aluminum plate 60F-254 (0.2 mm thickness E. Merck, Germany).OTV was spotted in the form of bands of width 6 mm with Camag microlitre syringe using Camag Linomat V (Switzerland). A constant application rate of 150 ml/sec was employed and space between two bands was 15 mm. The slit dimension was kept 6 mm x 0.45 mm micro, 20 mm/s scanning speed was employed. The mobile phase consisted of Toluene: methanol: ammonia (3.5:1.5:0.2*v/v*). The chromatogram was developed in twin trough glass chamber saturated with mobile phase using the linear ascending technique. The optimized chamber saturation time for mobile phase was 25 min at room temperature. The length of chromatogram run was approximately 80 mm. The system was found to give a compact spot for OTV (Rf value of 0.45 ± 0.02). Subsequent to the development, TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed using Camag TLC scanner III in the absorbance mode at 220 nm. The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum in the range of 200 - 400 nm.

2.3. Preparation of Standard solution and calibration graphs

Accurately weighed quantity of OTV (10 mg) was transferred to 10.0 ml volumetric flask. Then small amount methanol was added and ultrasonicated for 5 min and diluted up to the mark with methanol (Concentration: 1000 µg/ml). Different volume of stock solution 0.8,1.6,2.4,3.2,4.0,4.8 µl were spotted in six replicates on TLC plates to obtain concentration of 800,1600,2400,3200,4000,4800 ng/spot of OTV respectively. The plate was developed on previously described mobile phase. The peak areas were plotted against corresponding concentrations to obtain the calibration graphs.

2.4 Preparation of sample solution

Twenty capsules were accurately weighed and average weight per tablet was determined. Tablets were ground to fine powdered, and weighed tablet powder equivalent to 100mg of OTV was transferred to 100 mL volumetric flask. The powder was dissolved in 30 mL methanol by intermittent shaking and the volume was made up to the mark with methanol. The solution was then filtered through Whatman filter paper no.45. 1µLof above solution was spotted on TLC plate followed by development and scanning as described in Section 2.2.3.The analysis was repeated for six times. OTV gave sharp and well-defined peaks at Rf 0.45 when scanned at 220 nm.

2.5 Method validation

The method was validated for its linearity range, accuracy, precision, sensitivity, and specificity. Method validation is carried out as per ICH guidelines.

2.5.1 Precision

Intra- and inter day precisions of the methods were determined by performing replicate (n=3) analyses of standards and samples. This procedure was replicated on different days (n=3). Recovery studies by standard addition method were performed in view of justifying the accuracy of the proposed methods. Previously analyzed samples containing OTV was spiked with standard OTV, and the mixtures were analyzed in triplicate (n=3) by proposed methods. Precision was calculated from percentage relative standard deviation (RSD %) for repeated measurements, whereas accuracy was expressed as % of recovery.

2.5.2. Robustness and ruggedness of the method

Robustness was studied in six replicate at the concentration level of 1000 ng/spot. In this study, seven parameters (mobile phase composition, mobile phase volume, development distance, relative humidity, duration of saturation, time from spotting to chromatography and chromatography to spotting) were studied and the effects on the results were examined.

Two different analysts using the same experimental and environmental conditions studied the ruggedness of the proposed method. The spots 1000 ng/band of OTV was applied on TLC plates. This procedure was repeated in triplicates.

2.5.3. Limit of detection and limit of quantitation

The sensitivity of the proposed method was estimated in terms of the limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ were calculated by the use of the equation LOD = 3.3 X ASD/S and LOQ = 10 X ASD/S; where, 'ASD' is Average standard deviation of the peak height and area of the drugs, taken as a measure of noise, and 'S' is the slope of the corresponding calibration curve.

The different volume of stock solution in the range 1000 - 1500 ng/band was spotted on TLC plate. The procedure was repeated in triplicate.

2.5.4. Specificity

To confirm the specificity of the proposed method, OTV sample was spotted on TLC plate. The mobile phase designed for the method resolved OTV very efficiently**.** The peak purity of OTV was tested by correlating the spectra of OTV extracted from capsules and standard OTV at the peak start (S), peak apex (A) and at the peak end (E) positions. Correlation between these spectra indicated purity of OTV peak {correlation r (S, M) = 0.9994, r (M, E) = 0.9998}.

2.5.5. Recovery studies

Recovery experiments were performed at three different levels i.e. 80, 100 and 120 %. To the pre-analyzed sample solutions, a known amount of standard drug solution of OTV was added at three different levels.

2.6 Forced degradation studies

A stock solution containing 10 mg for OTV in 10 mL methanol was prepared. This solution was used for forced degradation to provide an indication of the stability-indicating property of the method.

2.6.1 Acid degradation

A methanolic solution of the drug (10 mg) was separately dissolved in 10 mL of 1M HCl and these solutions were kept for 8 h at room temperature in dark in order to exclude the possible degradative effect of light. The solutions (1 mL) were taken and neutralized and then diluted

up to 10 mL with methanol. The resultant solutions were applied on TLC plate in triplicate (10 µL each, i.e. 1000 ng/band). The plate was chromatographed as described above.

2.6.2 Base degradation

A methanolic solution of the drug (10 mg) was separately dissolved in 10 mL of 1 M NaOH solution. These solutions were kept for 8 h at room temperature in dark in order to exclude the possible degradative effect of light. The solutions (1 mL) were taken and neutralized and then diluted up to 10 mL with methanol. The resultant solutions were applied on TLC plate in triplicate (10 µL each, i.e. 1000 ng/band). The plate was chromatographed as described above.

2.6.3 Oxidative degradation

The drug (10 mg) was dissolved in 10 mL of the methanolic solution of hydrogen peroxide (30% v/v) and kept for 8 h at room temperature in the dark, to exclude the possible degradative effect of light. The solution (1 mL) was then diluted to 10 mL with methanol and treated as described for acid and base-induced degradation.

2.6.4 Photochemical degradation

The drug solution was left in sunlight for 8h. The resultant solution was treated as described for hydrogen peroxide-induced degradation.

2.6.5 Dry heat degradation

A solution of the treated powder was then prepared and 1000 ng/band was applied to a plate in triplicate. The plate was then chromatographed and treated as described above.

3. RESULTS AND DISCUSSION

3.1. Optimization of procedures

The TLC procedure was optimized with a view to developing a stability indicating assay method. Initially, Toluene was tried but OTV does not show any movement. Therefore, methanol was added in various proportions to toluene. Finally, toluene and methanol in the ratio of 3.5:1.5 *v/v* was selected as mobile phase. The developed spot was diffused. To the above mobile phase, 0.2 ml ammonia was added. It gave good resolution, sharp and

symmetrical peak with Rf value of 0.45 for OTV. Also, the spot for OTV was compact and not diffused. It was observed that pre-washing of TLC plates with methanol (followed by drying and activation) and pre-saturation of TLC chamber with mobile phase for 25 min ensure good reproducibility and peak shape of OTV.

3.2. Linearity

A series of dilutions and standard curves were prepared over a concentration range from 800-4800 ng/band of OTV from a stock solution. In UV method absorbance versus concentration, in HPLC and HPTLC peak area versus concentration data was performed by least square linear regression analysis, whereby slope, intercept, and the correlation coefficient was determined.

3.3. Precision

An Intra-day variation was assessed by analyzing three different concentrations 1600, 2400 and 3200 ng/band of OTV for three times within a day.

Inter-day variation was assessed using same concentration of drug (mentioned above) and analyzing it for three different days, over a period of the week. The results are as shown in **Table 1.**

Table 1. HPTLC Intra-day and Inter-day Precision Studies

Drug	Conc. [ng/band]	Intra- day Amount found [ng]		Inter- day Amount found [ng]	
		Mean ± SD [*n* = 3]	% RSD	Mean ± SD [*n* = 3]	% RSD
OTV	1000	996.72 ± 6.56	0.70	998.09 ± 37.56	0.75
	1500	1504.47 ± 21.32	1.61	1503.63 ± 42.37	1.02
	2500	2497.49 ± 19.92	0.89	2499.84 ± 24.57	1.68

3.4. Robustness and ruggedness of the method

The parameters such as mobile phase composition, the volume of the mobile phase, development distance, relative humidity, activation of plates, duration of saturation were

studied. The standard deviation of peak areas was calculated for each parameter. The low %R.S.D. 0.12-1.37 indicates the robustness of the method.

The ruggedness of the proposed method was evaluated by two different analysts. The results for OTV were found to be 99.81% and 99.63% respectively

3.5. LOD and LOQ

Detection limit and quantification limit was calculated by the method as described in Section **2.5.3.3.** The LOD and LOQ were found to be **36.40 ng and 119.38 ng**, respectively.

3.6. Recovery studies

To the analysed 2400 ng/band solutions, a known amount of standard drug solutions of OTV was over spotted at 80 %,100 % and 120 % levels i.e.1600, 2400 and 3200 ng/band. The chromatogram was developed and scanned. The result of % recovery was found to be 0.47-1.37 indicated accuracy of the method.

3.7 System Suitability

System suitability tests were also carried out to verify reproducibility. The system suitability was assessed in HPTLC by using six replicate analysis of drugs at concentration of 1000 ng/spot of OTV respectively.% RSD was found to be 1.22 which was less than 2 indicated the method is reproducible.

Summary of validation parameter is as shown in **Table 2.**

Table 2.Summary of Validation Parameter

Parameter	HPTLC
Linearity range	500-3000[ng /spot]
Regression equation [Y = mX + C]	Y = 1050X + 868.4
Correlation coefficient	0.997
Limit of detection	36.40ng
Limit of quantitation	119.38 ng
% Recovery [n = 3]	99.92 – 100.34
Ruggedness [%]	
Analyst I [n = 3]	99.81
Analyst II [n = 3]	99.63
Precision [% RSD]	
Repeatability [n = 6]	1.47
Inter-day [n = 3]	0.70-1.61
Intra-day [n = 3]	0.75-1.68
Robustness	Robust
Specificity	Specific

3.8 Stability- indicating property

The chromatogram of samples degraded with acid, base, hydrogen peroxide and light showed well-separated spots of pure OTV as well as some additional peaks at different Rf values. Degradation of OTV in acid, base, H_2O_2, dry heat and light heat are as shown in **Fig 2**.

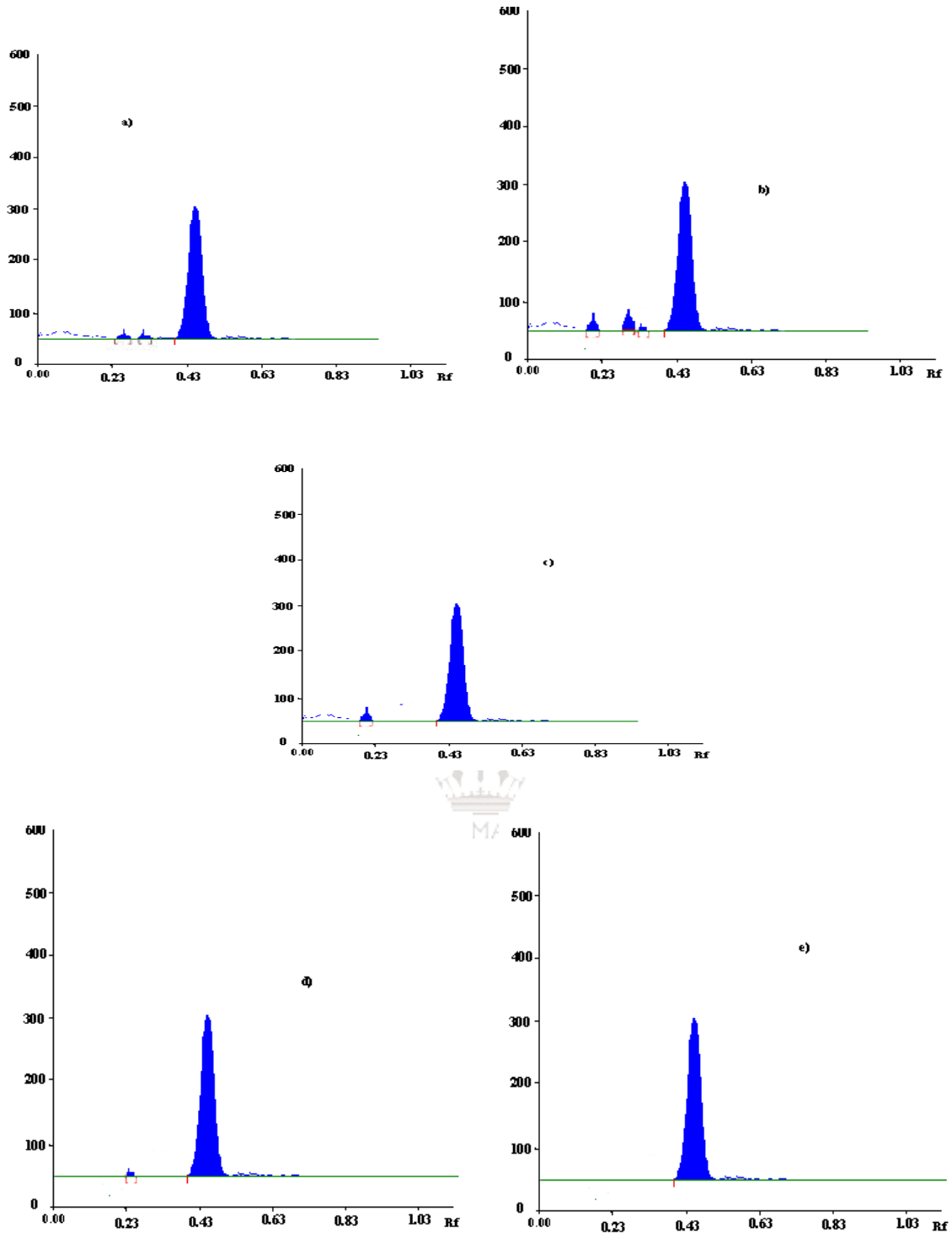

Fig. 2 Forced degradation of OTV by HPTLC: A) 1N HCl + OTV; B) 1N NaOH + OTV; C) 30% H_2O_2 + OTV; D) Dry heat OTV; E) Light heat OTV

The content of OTV remained, and percentage recovery was calculated and listed in **Table 3.**

Table 3. Forced Degradation study

Sample exposure condition	Number of degradation products [R_f values]	OTV remained [1000 ng/spot]	SD	Recovery [%]
1 M HCl, 8h, RT	2 (0.25,0.29)	821.9	9.68	82.19
1 M NaOH, 8h, RT	3 (0.21,0.33,0.35)	831.6	10.40	83.16
10 % H_2O_2, 8h, RT	1 (0.21)	871.1	5.48	87.11
Heat, 3h, 55^0C	1 (0.25)	884.1	4.98	88.41
Photo, 8 h	No degradation	998.5	3.21	99.85

[a]RT = Room Temperature

3.9 Analysis of the marketed formulation

Six replicate determinations were performed on the commercially available capsules. The spots of R_f 0.45 was observed in chromatograms obtained from drug samples extracted from tablets and recovery was found to be 99.12±0.65%. There was No interference was observed from the excipients commonly present in the tablets. It may, therefore, be inferred that degradation of OTV had not occurred in the marketed formulations analyzed by this method. The low RSD indicated that the method is suitable for routine estimation of OTV in pharmaceutical dosage forms.

4. CONCLUSION

The proposed HPTLC method provide simple, accurate and reproducible quantitative analysis for determination of OTV in Capsules. The method was validated as per ICH guidelines. As the method could effectively separate the drugs from their degradation products; therefore, it can be employed as a stability indicating study.

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Conflict of Interests

The authors declare that they have no conflict of interests.

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