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A validated HPTLC densitometric method for quantitative determination of Zanamivir in bulk and pharmaceutical formulation

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ABSTRACT

The main purpose of the present study was to develop and validate a high performance thin layer chromatographic (HPTLC) method for quantitative determination of an antiviral agent, zanamivir in pure drug and diskhaler powder formulation. Chromatography was performed on aluminum TLC plates pre-coated with silica gel $60F_{254}$, employing a mixture of chloroform:methanol:ammonia (9.5:3.2:0.2,*v:v:v*) as mobile phase. The TLC scanner was operated in the absorbance mode at a wavelength of 230 nm for evaluation of chromatograms. The system has given well resolved peak of zanamivir (R_f = 0.56). The linearity of the method was established in the range of 20-300 ng/spot; correlation coefficient (*r*) was 0.9995. The low values of limit of detection and limit of quantification (12.4 and 37.5 ng/spot, respectively) have demonstrated the sensitivity of the developed method. The reported method was precise in both intra-day as well as inter-day analysis; % RSD of peak area was found to be less than 2%, and has an accuracy within 100 ± 2%. The developed method has a potential to quantify zanamivir from its diskhaler formulation without any interference from other components. The applicability of the method was demonstrated by excellent recovery of analyte (99.8%) from diskhaler formulation. The current analytical method can be applied for routine analysis of zanamivir in pure form and pharmaceutical formulation in quality control laboratories.

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1. Introduction

Zanamivir, a sialic acid analogue, is an antiviral agent which is first in a category known as neuraminidase inhibitors [1,2]. Zanamivir is effective against influenza viruses, especially H1N1 and H5N1. The two surface glycoproteins carried by influenza viruses are hemagglutinin and neuraminidase. Hemagglutinin participates in the bonding of virus particles to the cell surface through sialic acid receptor [3], whereas, neuraminidase is an enzyme that cleaves the bond between cell proteins and sialic acid residue and hence, permitting the progeny viruses to release from the infected cell surface and infect the surrounding uninfected cells [1,4]. In addition to that, the enzyme also inhibits the aggregation of viral particles and probably reduces the inactivation of viruses by mucus in the respiratory tract [5]. Zanamivir acts by inhibiting the influenza neuraminidase enzyme and thus prevent the spread of virus to the neighboring uninfected cells within the respiratory tract. As neuraminidase is a critical viral enzyme, its inhibition also results in the reduction of multiplication of virus in the body. Zanamivir has been found to be effective against both influenza A and B viruses and used in the individuals with typical influenza symptoms; however, the treatment should be started within hours of appearance of the symptoms [1,6-8]. The peak viral titers has been reduced by a factor of approximately 100, when the treatment was started one day after the viral infection and also frequency of the febrile illness was decreased by 85 percent [1,2].

Overall, zanamivir has been proved to be a promising drug in decreasing the severity and duration of influenza in the individuals who begins the treatment after the appearance of the symptoms. Oseltamivir and zanamivir are the two neuraminidase inhibitors recommended by World Health Organization (WHO) for the treatment of H1N1 and H5N1 influenza, however, the latter has been found to be active against Oseltamivir-resistant viruses as well [9-11].

Chemically zanamivir is (6*R*)-5-acetamido-2,6-anhydro-4-carbamimidamido-3,4,5-trideoxy-6-[(1*R*,2*R*)-1,2,3-trihydroxy-propyl]-*L*-threo-hex-2-enonic acid (Figure 1).

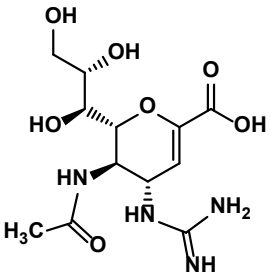

Figure 1. Chemical structure of zanamivir (ChemSpider ID: 54842).

The literature review reveals that various analytical methods based on UV-spectrophotometry [12], HPLC [1,13-16], and voltametry [17] have been developed for quantitative estimation of zanamivir in pure drug as well as in pharmaceutical preparations. Analytical methods based on HILIC tandem mass spectrometry have also been reported for the analysis of zanamivir in blood plasma samples [18,19]. Similarly, Lindberg *et al*. has reported a rapid solid-phase extraction liquid chromatography tandem mass spectrometric method for trace analysis of zanamivir [20]. However, insignificant attention has been paid towards the development of analytical method based on HPTLC for quantification of zanamivir in pure drug as well as in pharmaceutical formulations. Hence, the present study was an attempt to develop a simple and precise HPTLC densitometric method for estimation of zanamivir in bulk drug and diskhaler formulation and validate the new method as per The International Conference on Harmonisation (ICH) method validation guidelines [21]. The validation results were compared with the results obtained by the HPLC and HPTLC methods reported in the literature [22].

2. Experimental

2.1. Chemicals and apparatus

All the chemicals and reagents used in this study were of analytical grade. Zanamivir standard sample was provided as gift by GlaxoSmithKline Pharm Co (Cairo, Egypt). Chloroform, ammonia and methanol (HPLC grade) were procured from Sigma Aldrich, Steinheim, Germany and used as received. Zanamivir 5 mg diskhaler formulation (RELENZA®, Glaxo Smithkline) was procured from pharmacy store in Jazan, Saudi Arabia. The silica gel 60F_{254} pre-coated TLC plate with aluminum sheet support (20 cm × 10 cm, 0.2 mm thickness) was purchased from E. Merck, Darmstadt, Germany. The samples and standards were weighted using Metler Toledo analytical balance (Kern & Sohn GmbH, Germany) and the aliquots were taken using micropipettes (Eppendorfs, USA).

2.2. Instruments and chromatographic conditions

The chromatographic experiment was carried out using TLC plates consisting of silica gel 60F_{254} precoated on aluminum sheet with dimension of 20 cm × 10 cm × 0.2 mm. The sample application was performed by CAMAG Linomat V automatic sampler (CamagMuttenz, Switzerland), using Hamilton micro-syringe (100 µL). The developing chamber Camag glass twin trough (20 × 10 cm) was used. Densitometric scanning was carried out in CAMAG TLC Scanner 3 operating in the absorbance mode at 230 nm wavelength. The system was linked to WINCATS software (Camag Muttenz, Switzerland) for the evaluation of densitometry results. Deuterium lamp with 200-400 nm UV spectrum range was used as a radiation source and the experiment was commenced at laboratory temperature (25±2°C) and 40% relative humidity.

Accurately measured amount of sample and standard solutions (10 µL) were spotted on the TLC at a fixed application rate (0.1 µL/sec). The spots were applied 30 mm apart from each other at a distance of 10 mm from both sides and 20 mm from bottom edge of the TLC plate. Then the TLC plates were air dried for 5 min before transferring to the development chamber. Solvent mixture consisting of chloroform:methanol:ammonia at the ratio of 9.5:3.2:0.2 (*v*:*v*:*v*) was used as mobile phase. The TLC plates were developed using linear-ascending mode in the developing chamber that was pre-saturated with effluent mixture at room temperature for 20 min and the development distance was 8 cm. After that, the developed plates were dried with the help of a hot air dryer and subjected to densitometric scanning at a speed of 20mm/sec, the slit dimension 6.0 × 0.3 µm was employed. The TLC chromatograms were evaluated by WINCATS software.

2.3. Preparation of standard solution

A stock standard solution of zanamivir (100 µg/mL) was prepared by transferring accurately weighed amount (5 mg) of zanmivir to a volumetric flask (50 mL) and dissolving in methanol. The volume was completed to the mark with the same solvent. Aliquots (0.2, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL) of stock standard solution were taken to 10 mL volumetric flasks and the volumes were adjusted with methanol to prepare zanamivir working standard solutions of 2, 5, 10, 15, 20, 25 and 30 µg/mL concentrations respectively.

2.4. Preparation of sample solution

A weight of zanamivir diskhaler powder equivalent to 5 mg of zanamivir was transferred to a volumetric flask (50 mL). To the flask methanol (approximately 10 mL) was added and sonicated for 15 min. The volume was completed upto the mark with the same solvent. The resulting mixture was centrifuged at 3000 rpm for 5 min. Aliquots of supernatant were diluted to 10 mL in volumetric flasks with methanol to achieve solutions of desired concentrations. The solutions were applied on the TLC plates, developed in the developing chamber as per the above described chromatographic condition. The developed plates were scanned and the chromatograms were recorded.

2.5. Method validation

The developed analytical method was evaluated by performing validation in compliance with The International Conference on Harmonisation (ICH) guidelines [21]. The following parameters were evaluated:

2.5.1. Linearity

Linearity of the new method was assessed by analyzing standard solutions (n = 3) of zanamivir at seven concentration levels, in the range of 2 to 30 µg/mL. Accurately measured amount (10 µL) of different working standard solutions were applied on the TLC plate to achieve spots of 20, 50, 100, 150, 200, 250 and 300 ng/spot and the plates were developed by applying the above-described chromatographic condition. After the development, the plates were dried and densitometrically scanned at 230 nm. The area of the analyte peak at each concentration was recorded and the calibration graph of zanamivir was constructed by plotting average peak area against corresponding concentration of zanamivir.

Table 1. Validation parameters for determination of zanamivir by the developed TLC densitometric method.

Parameter	Zanamivir
System precision (% RSD, n = 6)	0.91
R_f value	0.56±0.02
Linearity range (µg/mL)	2-30
Slope (a)	18.6
Intercept (b)	170.1
Correlation coefficient (*r*)	0.9995

The linear least square regression analysis was performed to evaluate the linearity of the method and slope, intercept, correlation coefficient (*r*) were calculated.

2.5.2. Precision and accuracy

Instrumental precision was tested by applying standard solution at concentration of 100 ng/spot on the TLC plate into six replicates and scanned as per the chromatographic condition of the current method. The system precision was exhibited as %RSD of analyte peak area. The method precision and accuracy were evaluated by applying standard solution at three concentration levels (10, 15 and 30 µg/mL) on the TLC plate to achieve concentrations of 100, 150 and 300 ng/spot of zanamivir, respectively. The intra-day precision and accuracy was established by analyzing the samples at the same day, while the inter-day precision and accuracy was checked by conducting the experiment at three consecutive days by the proposed method. The analysis was carried out in three replicates and the precision results were represented as percent RSD of the peak area of the analyte. The accuracy results were represented as percentage recovery of zanamivir at each concentration level.

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

To assess the sensitivity of the newly developed method, LOD and LOQ were determined using the equations; LOD = 3.3 × SD/s and LOQ = 10×SD/s, respectively; where 'SD' is the standard deviation of the *y*-intercept regression line and 's' represents the slope of the calibration plot.

2.5.4. Robustness

In the present analysis, robustness of the method was assessed by applying the standard solution of zanamivir (100 ng/spot) on the TLC plate in six replicates. Small but deliberate changes in the chromatographic parameters of the proposed method such as mobile phase composition, wavelength, saturation time for developing chamber, time from spotting to development (5, 20 and 60 min) and time from development to scanning (5, 20 and 60 min) were introduced and the effects on recovery and R_f value of the analyte were examined. The TLC plates were developed using mobile phase of slightly varying proportions (±0.1 mL) of the components; chloroform: methanol: ammonia (9.6:3.2:0.2; 9.4:3.2:0.2; 9.5:3.1:0.2; 9.5:3.3:0.2; 9.5:3.2:0.1 and 9.5:3.2:0.3; *v*:*v*:*v*). The wavelength and the duration of chamber saturation were varied as ±5 nm (225 and 235 nm) and 20 min±25% (15 and 25 min), respectively.

2.5.5. Specificity

To examine the specificity of proposed HPTLC method, the sample (prepared from diskhaler formulation) and standard solutions were analyzed by using above described chromatographic parameters. The peak due to zanamivir from sample solution was ascertained by comparing the R_f value of the peak corresponding to standard solution. Moreover, the peak purity of the zanamivir peak from sample and standard solutions were tested.

3. Results and discussion

3.1. Method development

HPTLC is considered to be an effective tool for the estimation of drug substance as well drug products, including those from herbal origin because of easy method development, simplicity of the sample preparation, low cost and the possibility to analyze multiple analytes simultaneously within short time. In the current study, a new thin layer chromatographic method for the quantitative determination of an antiviral agent, zanamivir in bulk powder and diskhaler formulation was developed using HPTLC. The sample and standard solutions were applied in the form of spot. Twenty minutes was found to be optimum for the saturation of the developing chamber and the slit dimension was fixed to 6 × 0.30 µm. The wavelength was selected on the basis of the maximum absorbance with optimal sensitivity. To optimize the mobile phase that give sharp and well defined peak, different solvents with varying polarities and proportions were tried. Finally, the solvent system consisting of chloroform: methanol in the ratio of 9.5:3.2 was found to offer acceptable resolution with R_f value of 0.56, however the analyte peak with higher tailing was observed. Addition of small amount of ammonia solution to the above composition of mobile phase gave a compact spot, and finally, a mobile phase containing chloroform: methanol: ammonia (9.5:3.2:0.2, *v*:*v*:*v*) was considered to be optimum, as it has offered a good resolution and sharp and symmetrical peak of the analyte. Figure 2 showed the representative chromatograms of zanamivir from standard solution.

3.2. Method validation

The new HPTLC method was validated according to ICH guidelines [21]. The following parameters were evaluated:

3.2.1. Linearity

Linearity of the developed analytical method has been established by applying a series of dilution of standard solution (20-300 ng/spot). Calibration graph was plotted using analyte concentrations against the peak area recorded from the densitograms of zanamivir at each level. A good linear relationship was observed, the correlation coefficient (*r*) was calculated as 0.9995, which indicates the linearity of the proposed method. The slope and intercept were also determined and depicted in Table 1. The linearity chromatograms have been depicted in Figure 3.

3.2.2. Precision and accuracy

The standard solution was analyzed and the percent RSD of the peak area of six replicate applications was calculated to be 0.91% (Table 1). The observed percent RSD indicates the instrumental precision and suitability of the instrument to carry out the analysis.

Table 2. Intra- and inter-day precision and accuracy of zanamivir evaluated by the developed HPTLC method.

Parameter	Concentrations of analyte (ng/band)	% RSD of peak area*	Average percent recovery±SD *
Intra-day (n = 3)	100	0.96	99.6±1.33
	150	0.78	99.7±1.24
	300	0.67	99.9±1.19
Inter-day (n =3)	100	1.21	98.2±0.27
	150	1.03	98.5±0.30
	300	0.94	99.5±1.11

* Values for 3 replicate applications; %RSD = percent relative standard deviation.

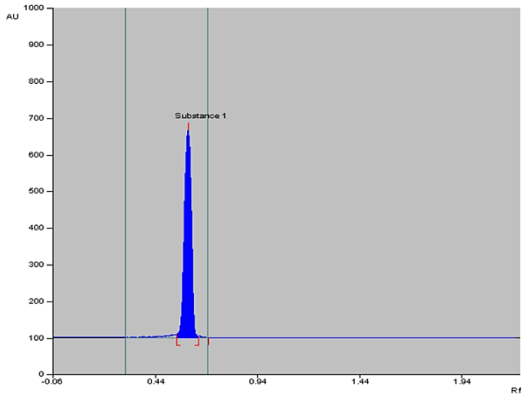

Figure 2. Typical densitogram of zanamivir spot of standard solution obtained using proposed HPTLC method.

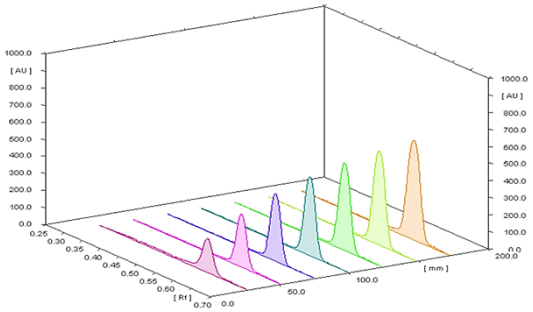

Figure 3. Linearity chromatograms of zanamivir obtained by the developed HPTLC method.

The intra-day and inter-day method precision and accuracy was evaluated by performing the analysis at three levels of concentrations in triplicate. The percent RSD of peak area for all the precision experiments were found to be <2%. The average percent recoveries of zanamirvir for intra-day analysis were 99.6, 99.7 and 99.9%, while the recoveries for inter-day analysis were calculated as 98.2, 98.5 and 99.5 at 100, 150 and 300 ng/band concentration levels, respectively. The precision and accuracy data have suggested that the developed method is precise, accurate, and reproducible. The precision and accuracy results have been summarized in Table 2.

3.2.3. Limit of detection and limit of quantification

The LOD and LOQ values for the analyte were calculated as 1.24 and 3.75 µg/mL, (12.4 and 37.5 ng/spot), respectively. On the basis of calculated LOD and LOQ values, the proposed method has been considered to be sensitive.

3.2.4. Robustness

The robustness was assessed by finding out the capability of the method to remain unaffected by small but deliberate changes in the chromatographic parameters such as composition of mobile phase (±0.1 mL), wavelength (±5 nm), time of saturation of developing chamber (±25%), time from sample application to development (5, 20 and 60 min) and time from development to densitometric scanning of the plates (5, 20 and 60 min). By these small variations, no marked change in percent recovery (within 100.0±2%) and R_f values (< 0.05 units) of zanamivir were observed, this means that the new method was unaffected by small change in the chromatographic conditions that could happen during routine analysis. Therefore, the present HPTLC method is considered to be robust. The robustness data has been represented in Table 3.

3.2.5. Specificity

The specificity of the developed HPTLC method was examined by peak purity test for zanamivir peak recorded from standard and sample solutions (extracted from diskhaler formulation). The closeness in the peak purity results proved the specificity of the method. Furthermore, the peak of zanamivir in standard and sample solutions were found to be resolved efficiently.

3.3. Analysis of Zanamivir diskhaler formulation

The proposed HPTLC method was successfully applied for the estimation of zanamivir in its commercially available diskhaler powder dosage form.

Table 3. Robustness data for determination of zanamivir by the proposed HPTLC method.

Variations	R_f value	% Recovery
Mobile phase (9.4:3.2:0.2, *v*:*v*:*v*)	0.57	100.0
Mobile phase (9.6:3.2:0.2, *v*:*v*:*v*)	0.56	99.9
Mobile phase (9.5:3.1:0.2, *v*:*v*:*v*)	0.57	100.1
Mobile phase (9.5:3.3:0.2, *v*:*v*:*v*)	0.54	100.1
Mobile phase (9.4:3.2:0.1, *v*:*v*:*v*)	0.56	99.9
Mobile phase (9.4:3.2:0.3, *v*:*v*:*v*)	0.56	99.8
Wavelength 225 nm	0.56	100.1
Wavelength 235 nm	0.55	100.0
Time of developing chamber saturation (±25%)	0.56	99.3
Time from sample application to development (5, 20 and 60 min)	0.54	99.5
Time from development to densitometric scanning (5, 20 and 60 min)	0.55	100.2
Without variation	0.56	100.1

* The values are the mean of three replicate applications.

Table 4. Recovery data of the developed HPTLC method (by standard addition method) for zanamivir diskhaler formulation.

Concentration level (%)	Initial amount (µg/mL)	Amount of zanamivir standard added (µg/mL)	Amount of zanamivir recovered (µg/mL)	Recovery (%) ± SD*	Average recovery (%)
50	10	5	14.96	99.7±0.21	99.8
100	10	10	19.98	99.9±0.11	
150	10	15	24.98	99.9±0.12	

* SD = Standard deviation; n = 3.

Table 5. Comparison of current HPTLC method with reported HPLC and HPTLC method for the determination of zanamivir in bulk and pharmaceutical formulation.

Parameter	This study	Reported HPTLC method [22]	Reported HPLC method [22]
Matrix	Bulk and diskhaler	Bulk and tablet	Bulk and capsule
Mobile phase	Chloroform:methanol:ammonia (9.5: 3.2:0.2, *v*:*v*:*v*)	Chloroform:methanol:acetic acid (4.5:0.5:0.3, *v*:*v*:*v*)	Methanol:0.02 M phosphate buffer (50:50, *v*:*v*), pH = 3.5
R_f	0.56	0.29	-
Linearity range	2-30 µg/mL (20-300 ng/band)	500-3000 ng/band	2-12 µg/mL
Correlation coefficient (r^2)	0.9995	0.9999±0.0001	-
System precision (n = 6)	0.91	-	1.45
Intra- and inner-day precision	%RSD of peak area (n = 3): 0.67-1.03	% RSD of recovery values (n = 3): 0.70-1.75	% RSD of recovery values (n = 3): 0.12-1.32
Accuracy (% recovery)	99.7-99.9	99.99-100.74	99.47-100.30
LOD	1.24 µg/mL (12.4 ng/band	36.4 ng/band	0.15 µg/mL
LOQ	3.75 µg/mL (37.5 ng/band)	110.3 ng/band	0.46 µg/mL
Robustness	Robust	Robust	Robust
Specificity	Specific	Specific	Specific

As discussed above, the method was proved to be specific through comparison of peak purity of the analyte from standard and formulation sample solutions. The applicability of the method was further demonstrated by recovery (accuracy) experiment using sample solution by standard addition method, where the sample solution was spiked with known amount of the zanamivir standard substance. The recovery experiment was performed at three concentration levels (50, 100 and 150% addition of zanamivir to target concentration of 100 ng/spot) to obtain final concentrations within the specified linearity range. For every concentration the recovery samples were prepared in triplicate. The application of sample spot, development, and scanning of TLC plates were carried out as described under chromatographic conditions. The peak area of zanamivir at each concentration level was recorded and the results were expressed as % recoveries of the analyte ±standard deviation (Table 4). The recovery values were found to be in good agreement with the label claim of the tested formulation. Furthermore, good recoveries indicate that there is no interference from inactive ingredients used in diskhaler preparation. The representative densitogram of zanamivir obtained from diskhaler formulation sample solution has been depicted in Figure 4.

3.4. Comparison with the reported HPLTC and HPLC methods

The currently developed HPTLC method was compared with already reported HPTLC and HPLC methods, which has been developed for the determination of zanamivir in bulk drug and tablet formulation [22]. A comparable overall performance was observed between these methods. Notably, the present HPTLC method was found to be more sensitive than the reported HPTLC method, while the later has exhibited greater performance in terms of linearity. The data comparison has been summarized in Table 5.

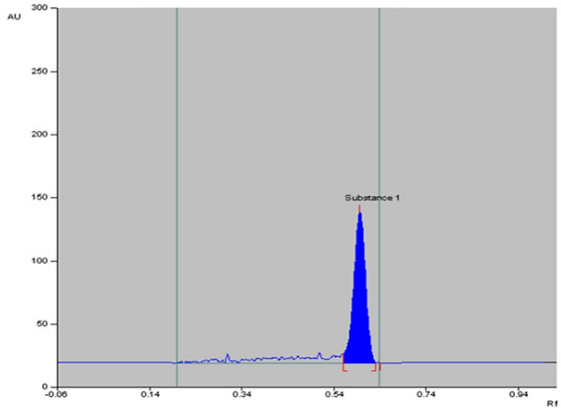

Figure 4. Typical densitogram of zanamivir in diskhaler sample obtained by analyzing with the developed method.

4. Conclusion

The HPTLC has been proved to be advantageous in many aspects over HPLC and hence it is sometime preferred over the later. HPTLC is considered to be more economical and environmental friendly, because it consumes lesser amount of solvents and offers fast analytical methods allowing simultaneous screening of large number of samples. In the present

work, a simple, accurate and robust HPTLC densitometric method was successfully developed for the quantification of zanamivir in pure form and diskhaler formulation. The proposed method was evaluated with respect to linearity, precision and accuracy, robustness, sensitivity and specificity and found to meet the ICH guideline acceptance criterion. The sensitivity of the method was proved by significantly lower LOD and LOQ values (12.4 and 37.5 ng/spot, respectively). The applicability of the method was established by the analysis of zanamivir diskhaler formulation procured from pharmacy store, the recovery results were found to be in good agreement with label claim. Overall, the satisfactory analytical performance of the proposed method reinforces its aptness for routine analysis of zanamivir in bulk drug and dosage forms in quality control laboratories.

Disclosure statement

Conflict of interests: The authors declare that they have no conflict of interest.
Author contributions: All authors contributed equally to this work.
Ethical approval: All ethical guidelines have been adhered.

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