RESEARCH ARTICLE

Development and validation of a bioanalytical method using LC-MS/MS to quantify Zanamivir in human plasma

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ABSTRACT

This study presents the development and validation of a highly sensitive and accurate LC-MS/MS method for the quantification of Zanamivir in human plasma. Utilizing a mobile phase of MeOH and Ammonium Formate (30:70 v/v) and a protein precipitation extraction technique, the method achieves excellent specificity and reproducibility. The calibration curve demonstrated linearity from 4-80 ng/ml with a correlation coefficient (r^2) ≥ 0.9994. The method adheres to FDA guidelines, exhibiting high precision and accuracy with intra-day and inter-day variations of 0.81-1.98%. The developed method, requiring a minimal sample volume and a short run time, ensures robustness and stability across various analytical conditions, making it an ideal tool for Zanamivir plasma concentration studies.

Keywords: Zanamivir, Human plasma, Mobile phase, Ammonium formate, Protein precipitation.

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INTRODUCTION

There is evidence that Zanamivir can inhibit the growth of influenza viruses.[1] Viruses that cause influenza still endanger countless lives and drain global economies every year. Worldwide flu pandemics are a significant cause for concern, and the A(H1N1) and H5N1 influenza viruses, particularly, cause intolerable fatalities.[2,3] Influenza viruses carry two surface glycoproteins crucial in forming new virions: hemagglutinin and neuraminidase. Hemagglutinin attaches to cells using the sialic acid receptor on their surface.[4,5] The enzyme known as neuraminidase is responsible for releasing the progeny virus from infected cells by cleaving the terminal sialic acid residues. Oseltamivir (Tamiflu®) and Zanamivir are two neuraminidase inhibitors that the World Health Organization suggests for treating A(H1N1) and H5N1 flu. According to reports, the oseltamivir-resistant H1N1 and H5N1 influenza viruses have been susceptible to Zanamivir.[6,7] Because of its low oral bioavailability (~2%), Zanamivir cannot be used orally as oseltamivir. Researchers found that only 10-20% of Zanamivir breathed reached the bloodstream, resulting in a deficient serum concentration. This is why the only commercially available form of Zanamivir is a dry powder for inhalation.[8]

MATERIALS AND METHODS

Chemicals and Reagents

Zydus Life Sciences LTD of Ahmedabad, India, provided the Remdesivir (Figure 1) and Zanamivir (Figure 1) reference standards. The Merck chemical division in Mumbai, India, supplied the HPLC-grade acetonitrile and methanol. The Milli-Q water purification system (Milli Q system, USA) was used to obtain purified HPLC-marked water.

Instrumentation

For the analysis, a Waters Alliance e2695 type HPLC (Waters Corporation, Milford, USA), which included a degasser, column oven, and Autosampler, was used. A mass spectrometer from SCIEX (Canada), SCIEX QTRAP 5500, was connected to the HPLC system using an electrospray ionization interface. Analyst 1.6 was employed to interpret chromatographic results.

Preparation of the Zanamivir and Internal Standard Stock Solution

Put 8mg of Zanamivir working standard in a 10-volumetric flask with diluent diluent-diluted 0.2ml to 10 mL. Transfer 0.1 ml of this solution to a 10 ml flask. Transfer 6mg Remdesivir working standard to a 10-volumetric flask diluted with diluent

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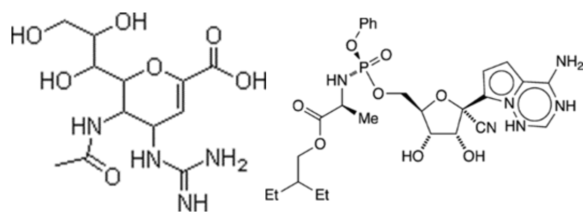

Figure 1: Chemical structures of zanamivir and remdesivir

diluent-diluted 0.2 to 10 mL. Transfer 0.1 ml of this solution to a 10 ml flask.

HPLC and Mass Spectrometer Conditions

MeOH/Ammonium Formate 30:70 v/v was the mobile phase. Isocratic elution occurred at 1.0 ml/min. Column and sample temperatures were ambient. The sample injection volume was ten μL. The mass spectrometer operated in positive ion electrospray ionisation interface mode. Multiple reaction monitoring (MRM) quantifies Zanamivir and Remdesivir. Optimized mass parameters include source temperature, IS, heater gas, nebulizer gas, curtain gas, CAD gas (purged all gas channels with ultra-high pure nitrogen gas), EP, DP, CE, FP, and CXP.

Calibration Curve and Quality Control Standards

Human plasma was used to create spiked calibration standard solutions of Zanamivir (4.00, 10.00, 20.00, 30.00, 40.00, 50.00, 60.00, and 80.00 ng/ml). 50μl of an internal standard solution containing Remdesivir was added to each calibration standard solution. Before being analyzed, all solutions were allowed to come to room temperature from their initial 80 °C storage. Quality control samples were prepared in the same manner as previously described, with concentrations equal to 4 ng/ml (LLOQ), 20 ng/ml (LQC), 40 ng/ml (MQC), and 60 ng/ml (HQC). Before being analyzed, all solutions were cooled to room temperature from their initial 80 °C storage temperature.

Validation of Method

Following the FDA bio-analytical method validation principles, the approach was validated to reveal the system appropriateness, Autosampler carryover, Sensitivity, specificity, matrix consequence, linearity, precision, accuracy, extraction recovery, and stability.[9-15]

System Suitability and Sensitivity

System appropriateness was carried out to guarantee that the HPLC-MS-MS system operates well and delivers precise and accurate results. For this, the MQC sample was injected into five duplicates. For Zanamivir and Remdesivir, the percent coefficient of variation (CV) was calculated for the retention time and area response.

The limit of precise and accurate molecular quantification was known as LLOQ. A minimum of 10 should be the signal-to-noise ratio. Six duplicates of LLOQ level samples were analysed to determine the LLOQ value.

Matrix Effect

By comparing the peak regions of Remdesivir and Zanamivir in extracted blank plasma with those from Remdesivir and Zanamivir standard solutions, the matrix effect of human plasma on the simultaneous analysis of Remdesivir and Zanamivir was assessed. Three replicates of the matrix effect were examined at the LQC and HQC levels**.**

Linearity

Zanamivir linearity was examined at doses ranging from 4 ng/ml to 80 ng/ml. The calibration curve for Zanamivir was generated by graphing peak area ratios (analyte peak area/internal standard peak area) versus analyte concentrations. Linear regression analysis, with 1/x as the weight, was used to verify linearity.

Precision and Accuracy

To assess intra-day precision and accuracy, we performed six repetitions in a single set using samples from the HQC, MQC, LQC, and LLOQ concentration levels. Next, we examined HQC, MQC, LQC, and LLOQ concentration samples in three different batches to evaluate inter-day precision and accuracy. Percentage CV and percent recovery express precision and accuracy, respectively.

Recovery

The recovery of Zanamivir was evaluated by comparing the peak areas of extracted low, medium, and high-quality control samples with spiked samples of the same quality after extraction. The analysis of Remdesivir recovery involved comparing the peak areas of the extracted samples to those of the spiked samples after extraction.

Stability Studies

The stability of Zanamivir in human plasma was assessed by examining HQC and LQC samples under various storage circumstances, such as room temperature stability, freeze-thaw at -80 °C, auto-sampler at 2- 8 °C, 24 hours, and long-term stability (30 d) at -80 °C.

RESULTS AND DISCUSSION

Method Establishing

The mass spectrometer and chromatography conditions were adjusted to produce accurate and efficient quantification and detection of Zanamivir. The internal benchmark that was selected was Remdesivir recovery. The two analytes were extracted from the matrix chemically. Thus, matrix effects are prevented, improving the method's accuracy. A triple quadrupole mass scan conducted in positive detection mode achieved an electrospray ionization interface condition optimization for Remdesivir and Zanamivir. A good reaction was obtained while ionizing in the positive mode. The ion transitions detected for quantification were m/z 603.5855 → 240.5395 for Remdesivir and m/z 333.31→93.42 for Zanamivir. The mass spectra of Remdesivir and Zanamivir are shown in Figure 2 and 3.

To achieve good peak shape and mass spectrometer response for Zanamivir and Remdesivir. Various chromatographic conditions were optimized. Finally, Zanamivir and Remdesivir were chosen for separation and analysis. The Mobile phase was a mixture of MeOH: Ammonium Formate 30:70 v/v. was

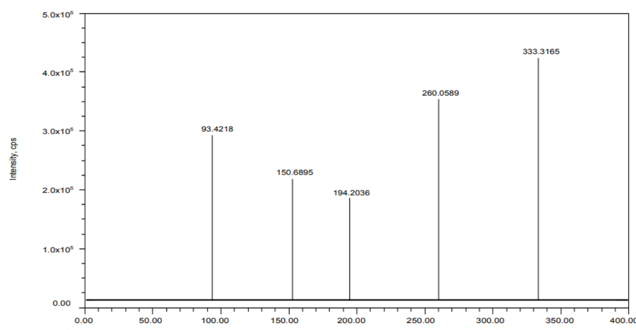

Figure 2: Mass spectrum of zanamivir

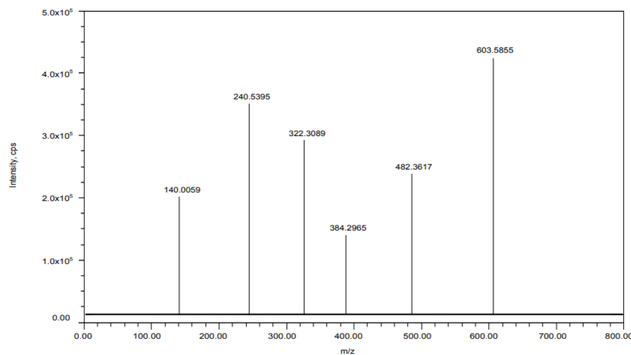

Figure 3: Mass spectrum of remdesivir

selected and directed at an isocratic flow stream of 1.0 ml/min with 10µl of sample injection volume. The retention times of Zanamivir and Remdesivir were 2.600 min and 3.956 min, correspondingly, with 6 min run time (Figure 4)

Specificity and Selectivity

At their ISTD, six different randomly blank human plasma specimens with no conflicting findings between Zanamivir and Remdesivir (IS) were examined. Their quantification chromatogram's lowest limit should be dependable, clear, and repeatable (Figures 5 and 6).

System Suitability and Sensitivity

It is a critical parameter for determining the chromatography's sSensitivity which is essential for increasing the system's authenticity and firmness and reducing its level of uncertainty, as illustrated in the following Table 1.

The system suitability parameter predicts the accuracy and efficacy of the system's chromatographic state under a variety of analytical conditions. The chromatogram can be analyzed by conducting six replications of MQC-1 through MQC-6. Results are presented in Table 2. It was determined to be compatible with the system.

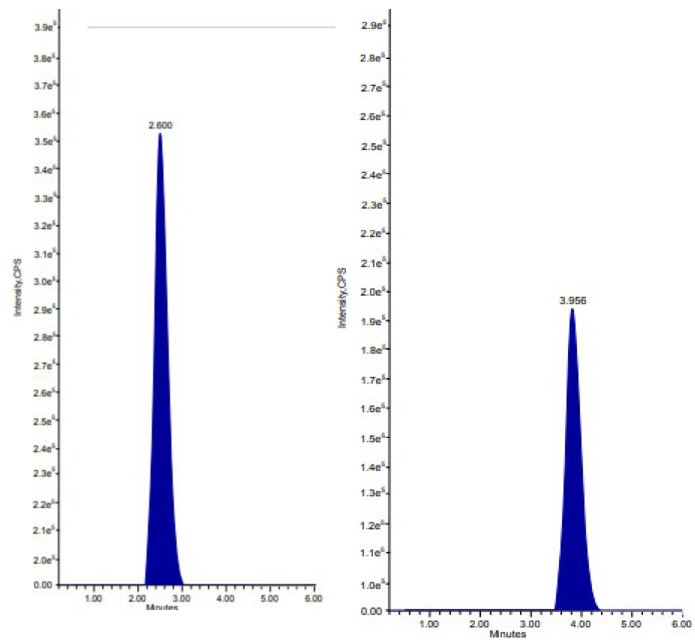

Figure 4: Chromatogram of analyte and internal standard

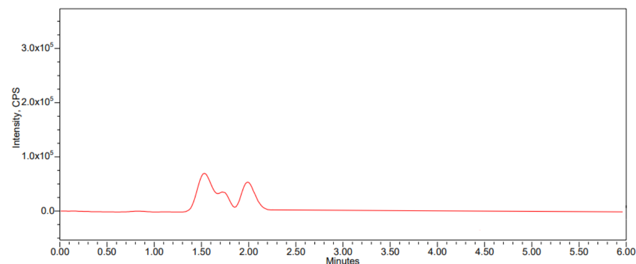

Figure 5: Blank chromatogram

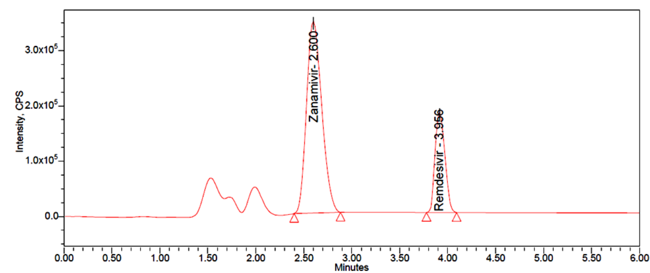

Figure 6: LLOQ Chromatogram Zanamivir and Remdesivir (IS) in Human Plasma

Table 1: System Suitability Results of Zanamivir

Sample name	*Analyte response*	*Analyte RT (min)*	*ISTD Response*	*ISTD RT (min)*	*Response Ratio*
Average	3.535×10^5	2.626	1.909×10^5	3.948	1.851
SD	0.027	0.002	0.006	0.003	0.013
%CV	0.76	0.076	0.31	0.075	0.70

Table 2: Zanamivir sensitivity results

Number of repetitions	*LLOQ*
	Zanamivir strength in ng/mL
	4.00
	Analyte Peak Area
Average	0.353 x10^5
SD	0.005
%CV	1.41
% Average	99.34

Table 3: Linearity results of zanamivir

Final conc. in ng/mL	*Drug response*	*ISTD response*	*Area response ratio*
4.00	0.356	1.925	0.185
10.00	0.875	1.942	0.451
20.00	1.754	1.922	0.913
30.00	2.637	1.955	1.349
40.00	3.554	1.939	1.833
50.00	4.251	1.926	2.207
60.00	5.253	1.938	2.711
80.00	7.105	1.949	3.645
Slope	0.0449		
Intercept	0.00490		
R^2 value	0.99941		

Table 4: Precision and accuracy results of zanamivir

Injections	*HQC*	*MQC*	*LQC*	*LLQC*
	Nominal concentration (ng/mL)			
	60.0	*40.0*	*20.0*	*4.0*
	Analyte peak area			
Mean	5.259 x 10^5	3.535 x 10^5	1.754 x 10^5	0.353 x 10^5
SD	0.029	0.027	0.026	0.005
% CV	0.55	0.76	1.48	1.41
% Mean Accuracy	100.12	99.48	99.99	99.34

Linearity

The standard curves were linear over the 3–60 ng/mL Zanamivir concentration range. Mean correlation coefficient: 0.999. The peak area of the analyte to that of IS was the ratio used in sample quantification. Plotted against plasma concentrations were peak area ratios(Figure 7). The results are shown in Table 3

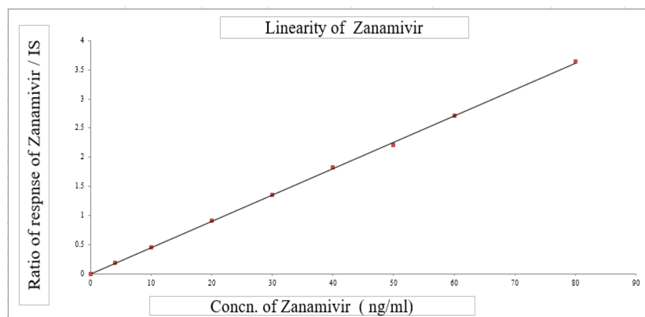

Figure 7: Calibration plot for concentration *v/s* area ratio of zanamivir

Recision and Accuracy

The intra-assay precision and accuracy were estimated by analyzing six replicates containing Zanamivir at four different QC levels. The inter-assay precision was determined by analyzing the four levels of QC samples on four different runs. The results are shown in Table 4

Recovery of Analyte

Three separate concentrations low, medium, and high qualitative and quantitative medication and IS recovery evaluations were conducted. Comparative reactions of a sample to those of a neat, normal solution help to define its recovery. The extraction efficiency depends on the analyte used; thus, the results match those of analytical techniques. The results are shown in Table 5.

Matrix Effect

It was discovered that the percent CV of the matrix factor for Zanamivir at the MQC level was 0.83. The percentage CV value showed that the matrix had no discernible impact on the bioanalytical approach used for the concurrent assessment of Zanamivir.

Stability Studies

Table 6 summarises the Zanamivir stability test findings. Under several storage settings examined, including kept at Freeze-thaw at-80 °C, Benchtop (normal room temperature) for 48 h, Auto-sampler at 2-8 °C for 24 h, and Long term at 80°C for 30 d, the results revealed that Zanamivir was durable in human plasma.

Table 5: Recovery results of zanamivir

Injections	*Extracted LQC*	*Un extracted LQC*	*Extracted MQC*	*Un extracted MQC*	*Extracted HQC*	*Un extracted HQC*
SD	0.026	0.026	0.027	0.040	0.029	0.024
%CV	1.48	1.47	0.76	1.12	0.55	0.45
%Mean Recovery	99.99	100.62	99.48	100.13	100.12	100.36
Overall Recovery	100.11					

Table 6: Stability studies of zanamivir

Statistical value	*Zanamivir*	
	LQC	*HQC*
Bench Top Stability		
% Recovery	100.25	98.23
%RSD	0.89	1.25
Freeze thaw stability		
% Recovery	98.25	101.89
% RSD	0.15	0.82
Autosampler stability		
% Recovery	103.52	104.23
% RSD	1.82	0.59

CONCLUSION

The validated LC-MS/MS method developed for the bioanalysis of Zanamivir in human plasma provides a reliable and efficient means of quantification essential for clinical pharmacokinetic studies. The technique demonstrates high specificity, sensitivity and reproducibility, meeting all FDA bioanalytical validation requirements. Stability tests confirm that Zanamivir remains stable under various storage conditions, ensuring the integrity of samples during analysis. This method's efficiency, combined with its rigorous validation, supports its application in both clinical settings and pharmacokinetic research, contributing valuable insights into the effective use of Zanamivir as an antiviral agent.

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