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A Sensitive And Rapid LC-MS/MS Method For The Determination Of Zanamivir In Human Plasma: Method Development And Validation

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ABSTRACT

A sensitive and rapid LC-MS/MS method was developed and validated for the quantification of Zanamivir in human plasma using Remdesivir as an internal standard (IS). The method employed a Waters Alliance HPLC system coupled with a SCIEX QTRAP 5500 mass spectrometer using an electrospray ionization interface in positive ion mode. Chromatographic separation was achieved on an isocratic system with a mobile phase of MeOH and ammonium formate (30:70 v/v) at a 1.0 mL/min flow rate. The method demonstrated excellent linearity in the 4–80 ng/mL concentration range with a correlation coefficient (R^2) of 0.99941. The retention times of Zanamivir and Remdesivir were 2.600 min and 3.956 min, respectively, with a total run time of 6 minutes. Validation studies were performed by FDA guidelines, assessing system suitability, sensitivity, accuracy, precision, recovery, and stability. The % recovery for Zanamivir across all QC levels was above 99%, and the matrix effect was negligible with a %CV of 0.83. Stability studies showed Zanamivir remained stable under varied storage conditions, including freeze-thaw cycles, bench-top, and long-term storage at −80 °C. The developed method was precise, accurate, and robust, making it suitable for pharmacokinetic studies and therapeutic drug monitoring of Zanamivir.

Keywords : Zanamivir, LC-MS/MS, Remdesivir, Method Validation, Human Plasma, Quantification, Stability.

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% to 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 METHODOLOGY

Chemicals and reagents

Zydus Life Sciences LTD of Ahmedabad, India, provided the Remdesivir (Fig.1) and Zanamivir(Fig.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.

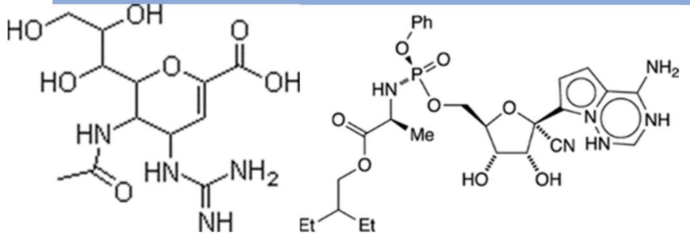

Fig.1: Chemical structures of Zanamivir and Remdesivir

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 ionisation 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 10ml. 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. Diluent-diluted 0.2ml to 10ml. 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. Optimised mass parameters include source temperature, IS, heater gas, nebuliser 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 analysed, 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 analysed, 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, 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 was used to verify linearity, with 1/x as the weight.

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 ionisation interface condition optimisation for Remdesivir and Zanamivir. A good reaction was obtained while ionising 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 Fig. 2 and 3.

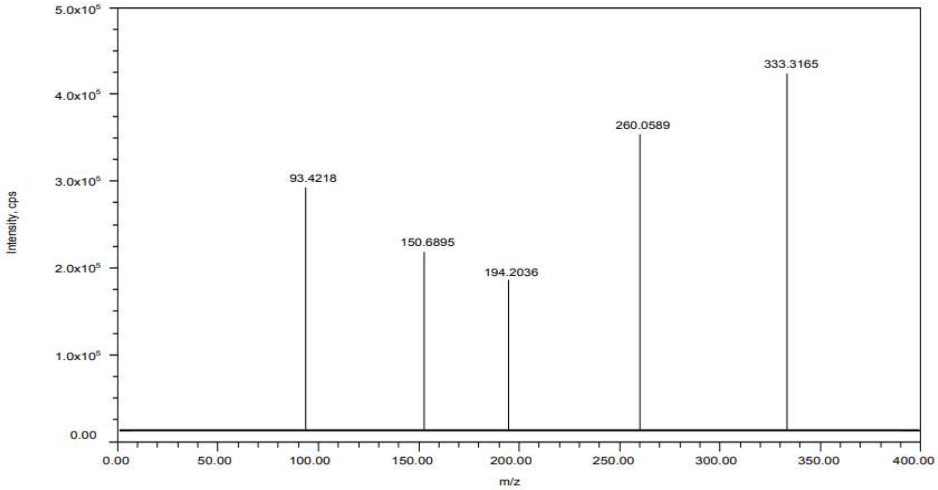

Fig.2: Mass spectrum of Zanamivir

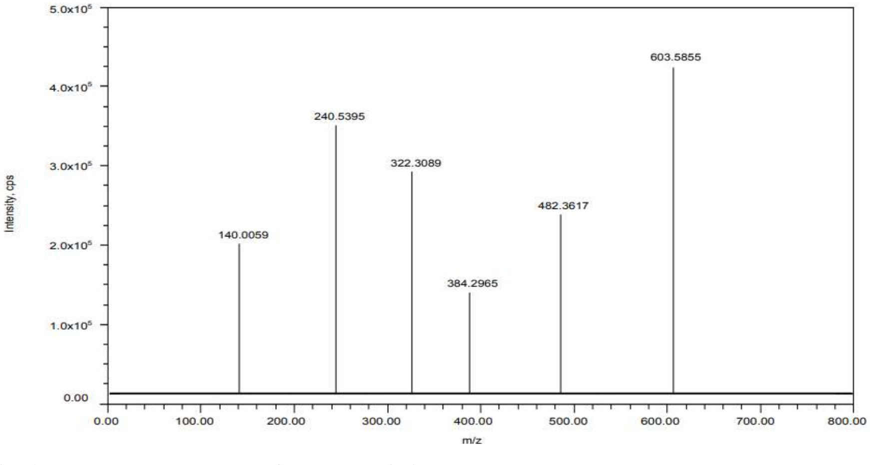

Fig.3: Mass spectrum of Remdesivir

To achieve good peak shape and mass spectrometer response for Zanamivir and Remdesivir. various chromatographic conditions were optimized. Finally, Zanamivir and Remdesivir was chosen for separation and analysis. The Mobile phase was a mixture of MeOH: Ammonium Formate 30:70 v/v. was 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 (Fig. 4)

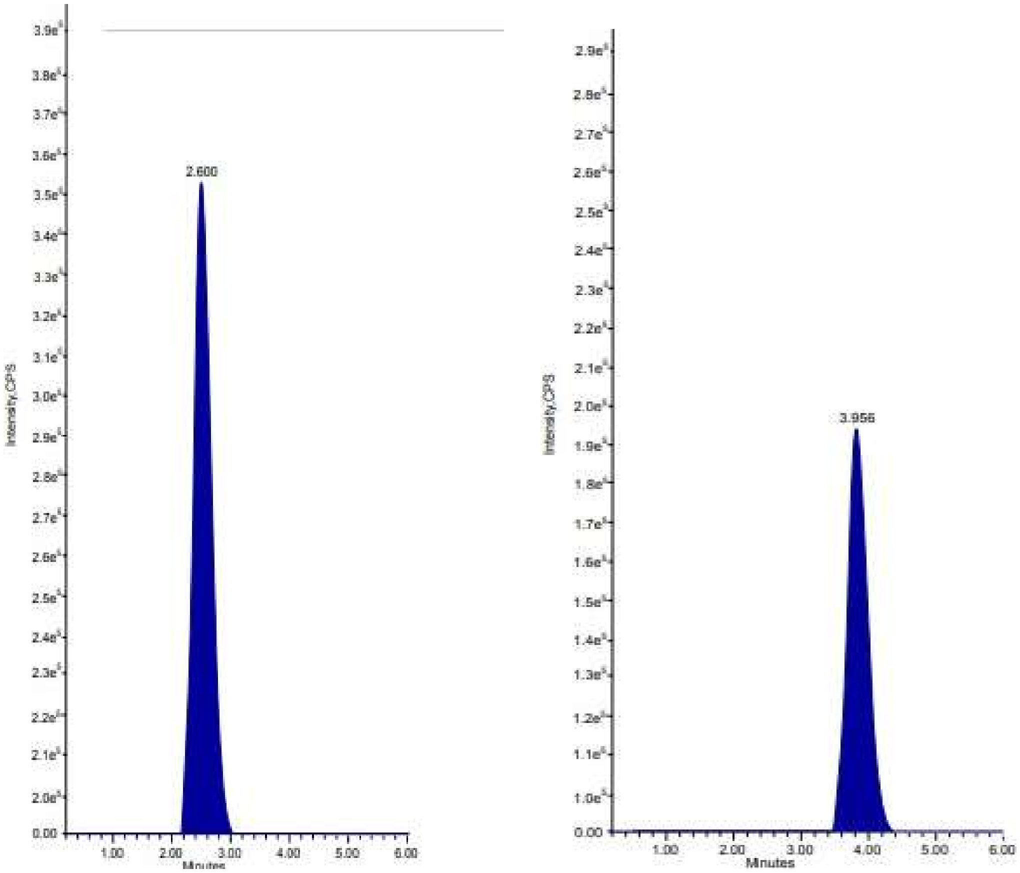

Fig.4: Chromatogram of Analyte and internal standard

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 (Figs. 5 and 6).

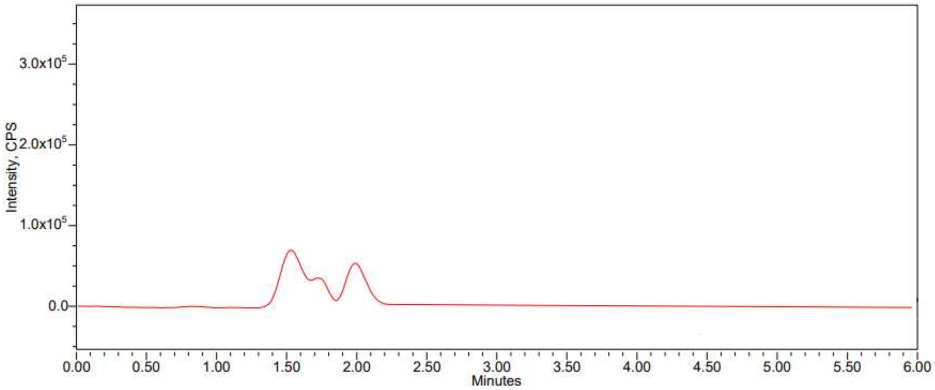

Fig :5 Blank chromatogram

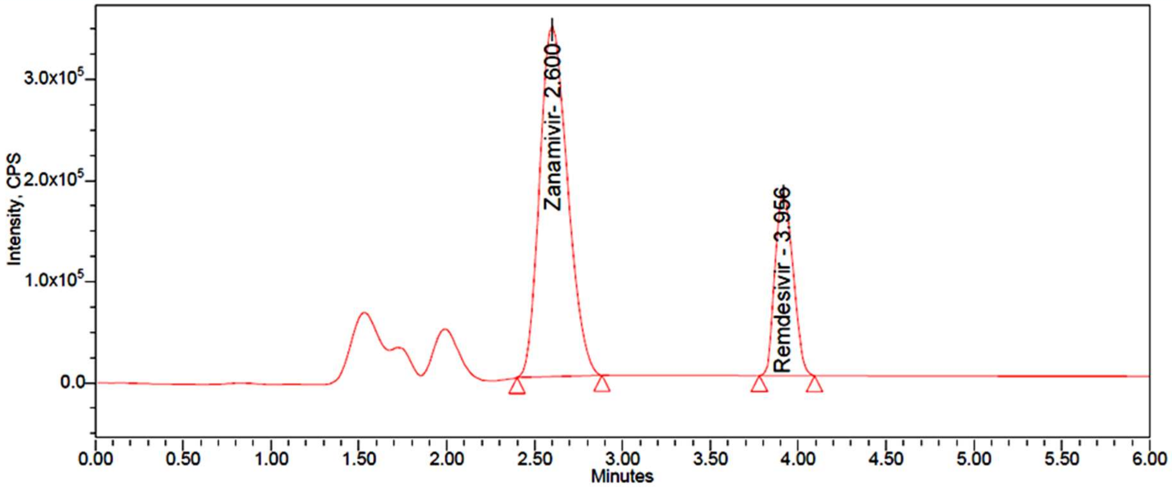

Fig.6: LLOQ Chromatogram Zanamivir and Remdesivir (IS) in Human Plasma

System suitability and Sensitivity

It is a critical parameter for determining the chromatography's sensitivity, 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 analysed 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.

Table 1: System suitability Results of Zanamivir

Sample Name	**Analyte Response**	**Analyte RT (min)**	**ISTD Response**	**ISTD RT (min)**	**Response Ratio**
Average	3.535 x10^5	2.626	1.909 x10^5	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

Linearity

The standard curves were linear over the 3.00–60.00 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(Fig7). The results are shown in Table 3

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		

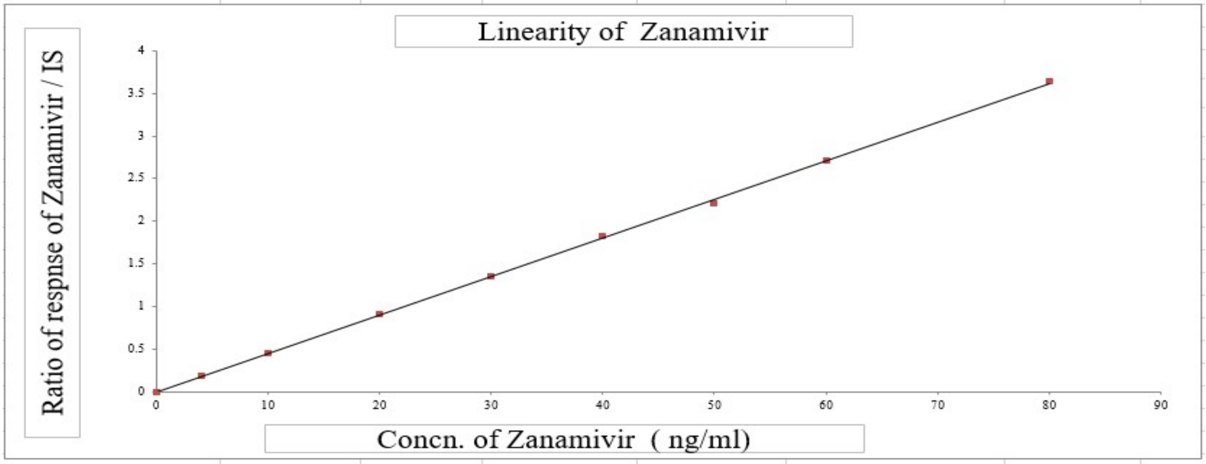

Fig.7: Calibration plot for concentration v/s Area ratio of Zanamivir

Precision and accuracy

The intra-assay precision and accuracy were estimated by analysing 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

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	5.259×10^5	3.535×10^5	1.754×10^5	0.353×10^5
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

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.

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					

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 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
Auto sampler stability		
% Recovery	103.52	104.23
% RSD	1.82	0.59

CONCLUSION

A sensitive, selective, and rapid LC-MS/MS method for the quantification of Zanamivir in human plasma was successfully developed and validated. The method exhibited excellent linearity, precision, and accuracy, with recoveries above 99%. The retention time of Zanamivir was short, allowing for a total run time of 6 minutes, ensuring high throughput for bioanalytical applications. Stability studies demonstrated the robustness of the method, with Zanamivir remaining stable under various storage and handling conditions. The negligible matrix effect and high system suitability confirmed the reliability of the method. This validated method can be applied to pharmacokinetic studies and therapeutic drug monitoring, contributing to the efficient evaluation of Zanamivir in clinical research and drug development.

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