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LC-MS/MS BIOANALYTICAL METHO DEVELOPMENT AND VALIDATION OF ZANAMIVIR IN HUMAN PLASMA USING REMDESIVIR AS INTERNAL STANDARD

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ABSTRACT

Zanamivir is in a class of medications called neuraminidase inhibitors. It works by stopping the growth and spread of the flu virus in your body. Zanamivir helps shorten the time you have flu symptoms such as nasal congestion, sore throat, cough, muscle aches, tiredness, weakness, headache, fever, and chills. To establish a simple, sensitive and completely validated HPLC-MS-MS approach for the analysis of Zanamivir in Human plasma samples. The method was developed after protein precipitation by 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. The approach developed showed fine calibration curve in the quantity range of 4-80 ng/ml with correlation coefficient (r2) of ≥ 0.9994 and The intra-day and inter-day accuracy was ranged from 98.50% to 102.88%. The intra-day and inter-day precision was ranged from 0.81 to 1.98 % according to FDA guidelines**.**

Keywords: Zanamivir, Protein Precipitation, Remdesivir, FDA guidelines

INTRODUCTION

Zanamivir has been indicated to provide activity against influenza viruses [1-3]. Each year, influenza viruses continue to cause major health problems and economic loss worldwide [3,4]. Especially, A(H1N1) and H5N1 influenza viruses lead to an unacceptable number of deaths and serious concerns about global flu pandemics [1,5-7]. Influenza viruses carry two surface glycoproteins, a hemagglutinin and a neuraminidase, which are involved in the production processes for new virions [8]. Hemagglutinin binds to the cell surface by recognizing the cellular sialic acid receptor [9] Neuraminidase cleaves the terminal sialic acid residues and releases progeny virus from the surface of infected cell [10]. The World Health Organization (WHO) recommends two neuraminidase inhibitors, oseltamivir (Tamiflu®) and Zanamivir, for the treatment of A(H1N1) and H5N1 flu. Zanamivir has been reported to have activity against oseltamivir-resistant H1N1 and the H5N1 influenza viruses [7,11,12]. Unlike oseltamivir, Zanamivir cannot be orally administered due to its poor oral bioavailability (~2%) [8,13,14]. A study revealed that a median of 10 to 20% of an inhaled administration of Zanamivir was systemically absorbed

leading to low serum concentration [14]. Therefore, Zanamivir has been launched to the market only in a dry powder form for inhalation [8,13].

MATERIALS AND METHODS

Standards and reagents

The reference standards of Zanamivir and Remdesivir standards was obtained from Zydus Life Sciences LTD, Ahmedabad, India. HPLC grade acetonitrile and Methanol were got from Merck chemical division (Mumbai, India). Purified HPLC mark water was obtained by Milli-Q (Milli Q system, USA) water purification system

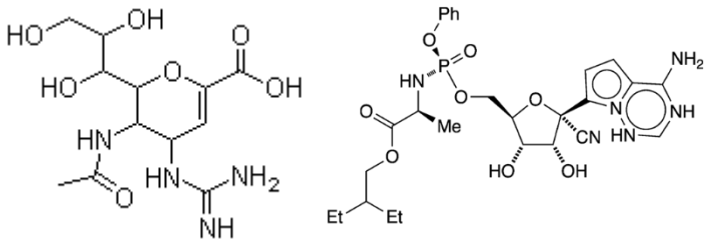

Fig.1: Chemical structures of A) Zanamivir B) Remdesivir

Instrumentation

Waters, Alliance e2695 model HPLC (Waters Corporation, Milford, USA) equipped with an auto sampler, column oven and degasser were operated for the analysis. The HPLC system was coupled to SCIEX QTRAP 5500 mass spectrometer (SCIEX, Canada) provided with electrospray ionization interface. To interpret chromatographic data, the software Analyst 1.6 was used. Waters X-bridge C8, 150mm x 2.1mm, 3.5µm analytical column was used for separation and analysis.

Buffer Preparation

6.3 g of Ammonium formate is dissolved in 1 Lt of HPLC grade water. Filtered through 0.45µ filter paper.

Mobile phase preparation:

Mix Methanol and buffer in 30: 70 v/v and filtered through 0.45 µ filter paper.

Zanamivir Standard Stock solution preparation

Weigh 8mg of Zanamivir working standard and transferred into a 10ml volumetric flask diluted to volume with diluent. Further diluted 0.2ml to 10ml with diluent. From this solution take 0.1 ml and transferred into 10 ml flask. This is the Zanamivir stock solution.

Remdesivir (IS) Standard Stock solution preparation

Weigh 6mg of Remdesivir working standard and transferred into a 10ml volumetric flask diluted to volume with diluent. Further diluted 0.2ml to 10ml with diluent. From this solution take 0.1 ml and transferred into 10 ml flask. This is the Remdesivir stock solution.

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Preparation of Standard Solution (40 ng/ml of Zanamivir)

Transferred 500µl of standard stock solution into 2ml centrifuged tube. To this add 200µl of plasma, 500µl of internal standard, 300µl of Methanol and 500µl of diluent. Centrifuge it to 20 min. Filter the supernatant liquid and transfer into HPLC vial.

Zanamivir Sample Stock solution preparation

Take 40 mg of Zanamivir sample and transferred into a 10ml volumetric flask diluted to volume with diluent. Further diluted 0.2ml to 10ml with diluent. From this solution take 0.2 ml and transferred into 10 ml flask. This is the Zanamivir sample stock solution.

HPLC conditions

Mobile phase was a mixture of MeOH: Ammonium Formate 30:70 v/v. Isocratic elution was done at 1.0 ml/min flow stream. The temperature of column and sample were maintained at ambient temperature. The volume of sample injection was 10 µL.

Mass spectrometer conditions

The mass spectrometer was managed in positive ion electrospray ionization interface mode. Multiple reaction monitoring (MRM)mode has been applied to quantify Zanamivir and Remdesivir. Mass parameters such as source temperature, IS, heater gas, nebulizer gas, curtain gas, and CAD gas (purged all gas channels with ultra-high pure nitrogen gas), EP, DP, CE, FP and CXP were optimized (table 1 &2). The ion transitions observed were m/z 333.31→93.42 for Zanamivir and m/z 603.5855 → 240.5395 for Remdesivir (internal standard).

Table 1: Optimized Chromatographic conditions

Parameter	Description
Column	Waters X-bridge C8, 150mm x 2.1mm, 3.5µm
Mobile phase	MeOH: Ammonium Formate 30:70 v/v
Flow rate	1.0 ml/min
Injection volume	10µL
Retention time	Zanamivir 2.600 min
Run time	6 min
Rinsing volume	100µl
Rinsing speed	High Speed
Needle stroke	Full stroke
Detector	PDA
Auto sampler temperature	Ambient
Column oven temperature	Ambient

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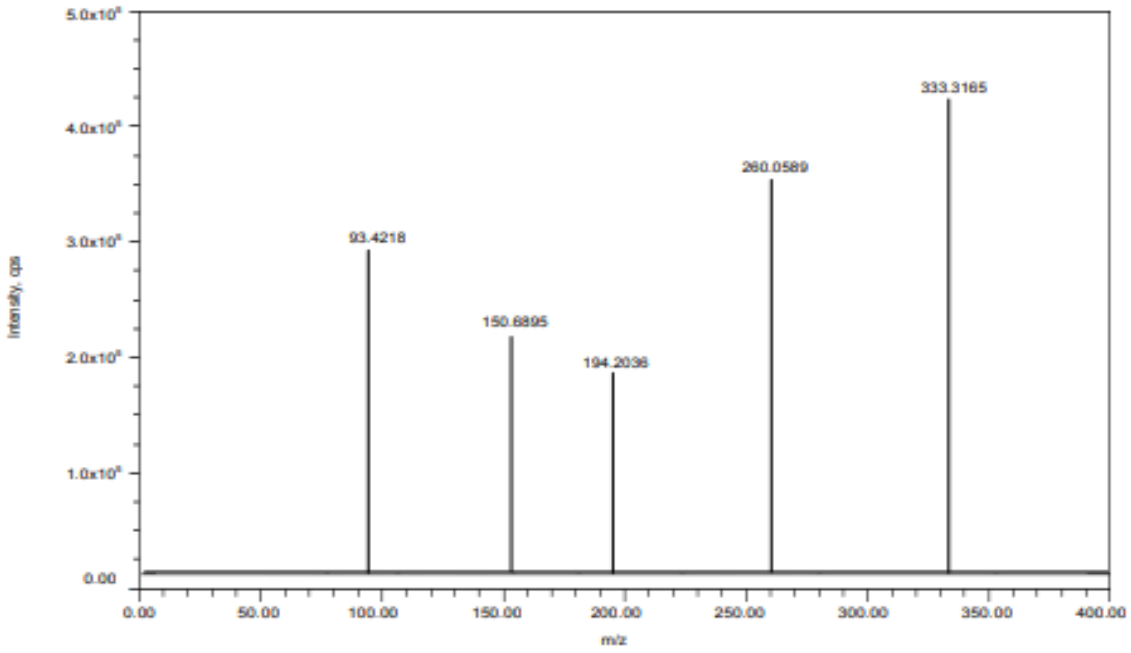
Purge time	5 min

Table 2: Source/Gas parameters

Parameter	Values
Spray voltage	5000V
Vaporizer temperature	500
Sheath gas pressure	60 psi
Auxiliary gas pressure	27 units (~8 L/min)
Capillary temperature	270 °C
Capillary offset	-500V
Polarity	Positive
Mode	ESI +

Fig.2: First and third (Q1 a



ndQ3) mass scans of Zanamivir

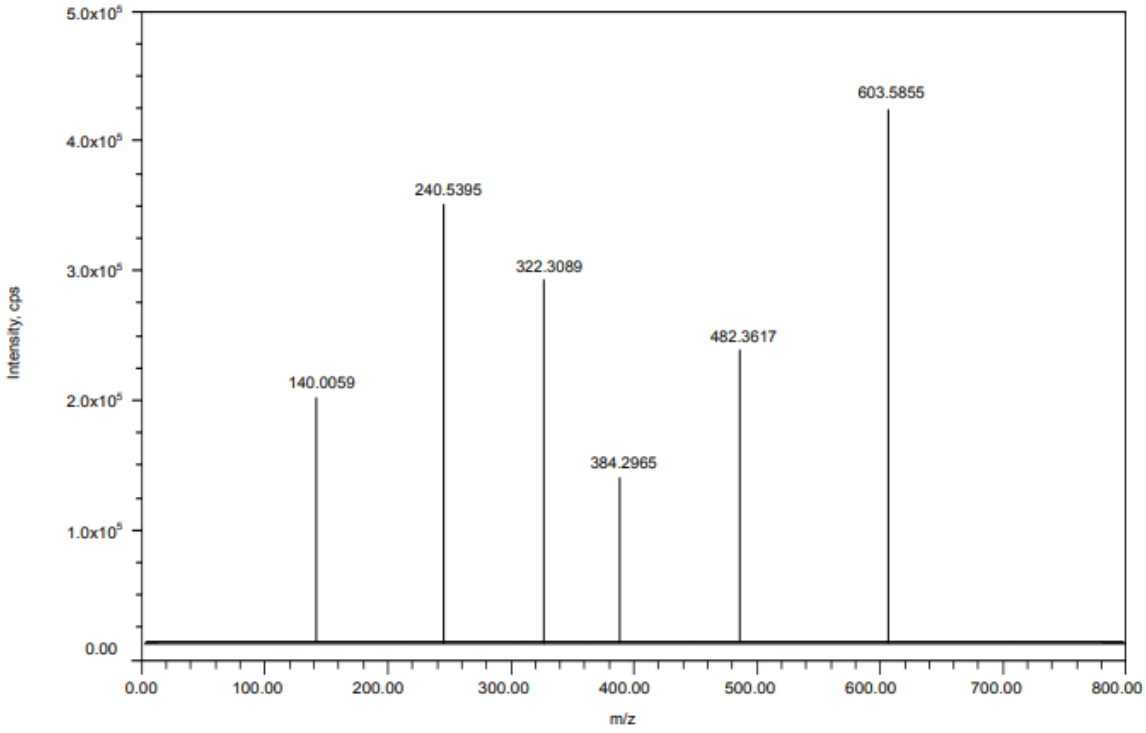


Fig.3: First and third (Q1 andQ3) mass scans of Remdesivir

Calibration standard solutions

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) were made in human plasma. To every calibration standard solution, 50µl of internal standard solution with concentration of Remdesivir was added. All the solutions were stored at-80 °C and prior to analysis they are brought to ambient temperature**.**

Quality control samples

Samples of quality control were made as explained above in the similar way at concentrations corresponding to 4 ng/ml (LLOQ), 20 ng/ml (LQC), 40 ng/ml (MQC) and 60 ng/ml (HQC). All the solutions were stored at-80 °C and prior to analysis they are brought to ambient temperature

Preparation of sample for analysis

Label the Centrifuged and treated plasma samples accordingly to their time intervals. To about 200 µL of plasma add 500 µL of diluent and mix well. Further add 300 µL of Methanol to precipitate all the proteins and mix in vortex cyclo mixture. Centrifuge at 4000 RPM for 15 – 20 min. collect the supernatant solution in HPLC vial and inject into the chromatograph. The supernatant solution was collected in a HPLC vial and 10 µL of prepared sample was injected to the HPLC-MS-MS system.

Validation of method [14-20]

Following the FDA bio-analytical method validation principles, the approach was validated to reveal the system appropriateness, auto sampler carryover, sensitivity, specificity, matrix consequence, linearity, precision, accuracy, extraction recovery, stability and ruggedness.

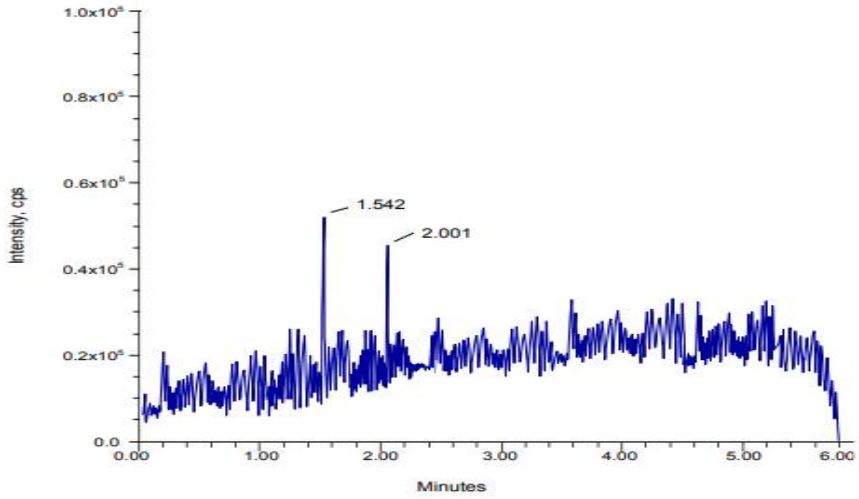


Fig.4 : Chromatogram of Plasma blank

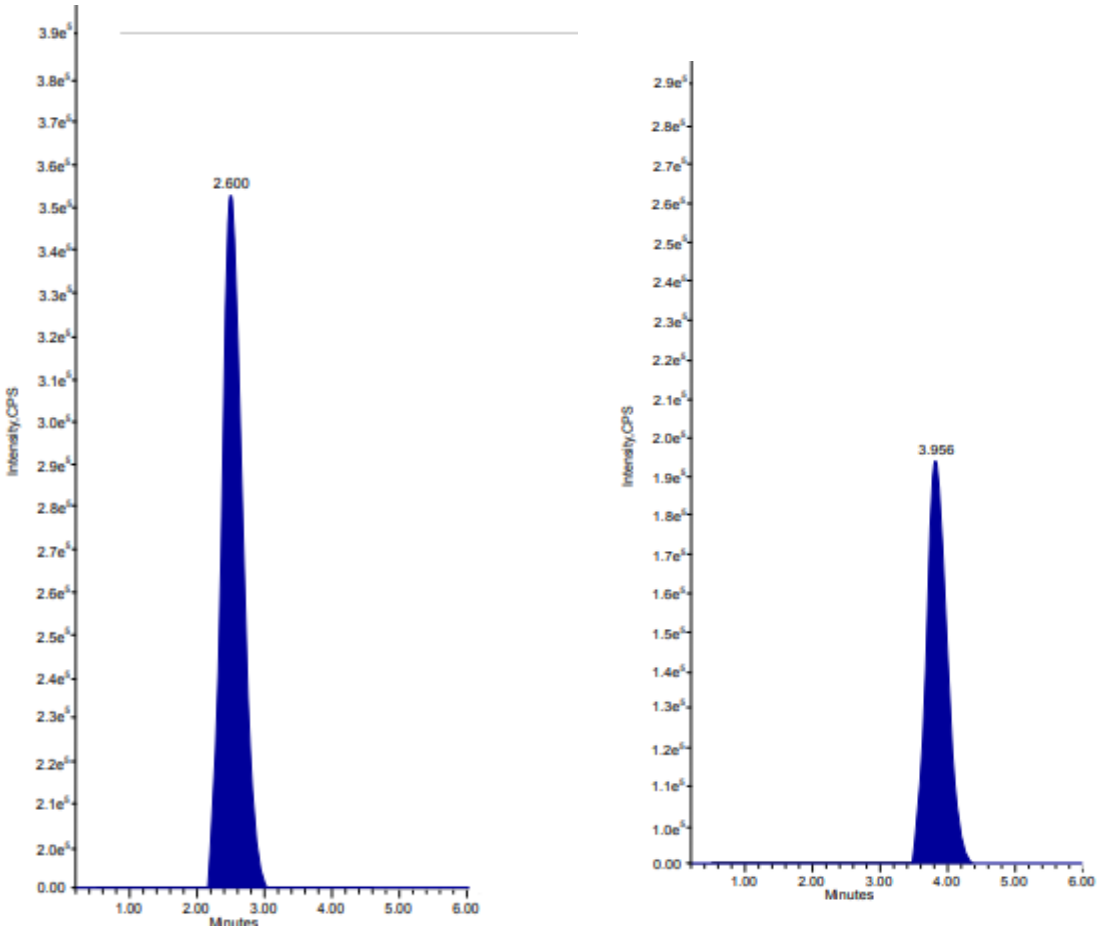


Fig.4: Chromatogram of Analyte and internal standard

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System suitability

System suitability was done to ensure that the HPLC-MS-MS system performs well by producing accurate and precise results. For this, MQC sample was injected in five replicates. The percent coefficient of variation (CV) was computed for the retention time and area response of Zanamivir and Remdesivir

Auto-sampler carryover

The auto-sample carryover was evaluated by injecting blank human plasma sample after ULOQ and HQC samples. The carryover of Zanamivir and Remdesivir should be lower than 20% mean peak area of Zanamivir in LLOQ sample. The carryover of internal standard should be lower than 1% of mean peak area**.**

Screening of biological matrix (specificity)

This test was done to indicate that the blank endogenous plasma components were not chromatographically interacted with Zanamivir and internal standard Remdesivir. Interference from endogenous human plasma components was assessed through evaluating 6 individual blank human plasma samples and Zanamivir and Remdesivir LOQ sample.

Sensitivity

LLOQ was the limit to quantify the molecule accurately and precisely. Signal to noise ratio should be at least 10. The LLOQ value was evaluated by analyzing LLOQ level samples in six replicates.

Matrix effect

The matrix consequence of human plasma on the simultaneous analysis of Zanamivir and Remdesivir was evaluated through comparison of peak areas of Zanamivir and Remdesivir in extracted blank plasma with that of obtained from Zanamivir and Remdesivir standard solutions. The matrix effect was studied at levels of LQC and HQC in 3 replicates.

Linearity

The linearity of Zanamivir was evaluated in the series of 4 ng/ml–80 ng/ml concentrations, respectively. Calibration curve of Zanamivir was plotted by plotting peak area ratios (analyte peak area/internal standard peak area) against the different concentrations of analytes. The linearity was checked by linear regression analysis using 1/x as weight

Table 3: Linearity results of Zanamivir

Conc. (ng/ml)	Zanamivir Peak Response	IS 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

Precision and accuracy

We ran six replicates in a single set with samples from the HQC, MQC, LQC and LLOQ concentration levels to measure intra-day precision and accuracy. By analyzing HQC, MQC, LQC and LLOQ concentration samples on 3 separate batches, the inter-day precision and accuracy were tested. The Precision and accuracy are reported as percent CV and percent recovery, respectively.

Recovery of internal standards and analytes

Zanamivir recovery was assessed by comparing peak areas of extracted LQC, MQC and HQC samples with spiked LQC, MQC and HQC samples after extraction. Remdesivir recovery was analyzed by equating peak areas of the extracted samples to spiked samples after extraction.

Ruggedness

Ruggedness was conducted by repeating the analysis of HQC, MQC, LQC and LLOQ samples in two different columns by two different analysts using the same bio-analytical procedure. The percent CV of recovery of Zanamivir and Remdesivir were determined.

Stability studies

The stability of Zanamivir in human plasma was evaluated by analyzing HQC, and LQC samples under different storage conditions like room temperature stability, freeze thaw at-80 °C, auto-sampler at 2-8 °C, 24 h and long term stability (30 d)-80 °C.

RESULTS AND DISCUSSION

Method establishing

The chromatography and mass spectrometer conditions were optimized to yield sensitive and efficient detection and quantification of Zanamivir. Remdesivir recovery was chosen as internal standard. Remdesivir is chromatographically equivalent (fig. 1) and they will prone to the same matrix effect. Both analytes were chemically extract from the matrix. Therefore, the accuracy of the method will be enhanced and matrix effects are avoided. In order to optimize electrospray ionization interface conditions for Zanamivir and, Remdesivir, triple quadrupole mass scan was done in positive detection mode. Good response was attained in positive mode of ionization. The ion transitions observed for quantification were m/z 333.31→93.42 for Zanamivir and m/z 603.5855 → 240.5395 for Remdesivir Fig. 2 shows the mass spectra of Zanamivir and 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)

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System suitability

The percentage CV values were less than 2.0% for retention times of Zanamivir and Remdesivir. Also, the percentage CV for area ratio was less than 2%. Hence, the system passed the system suitability test

Auto sample carryover

Peak area response of Zanamivir and Remdesivir was not observed in the blank Human plasma sample after successive injections of HQC and LLOQ at the retention times of Zanamivir and Remdesivir. Therefore, this method does not exhibit auto sample carryover.

Sensitivity

The LLOQ value for Zanamivir was 4 ng/ml. The accuracy and precision (%CV) determined at LLOQ quantity level and found to be within the approved limits.

Matrix effect

The percent CV of matrix factor for at MQC level of Zanamivir was found to be 0.83. The percent CV value indicated that there was no significant effect of the matrix on the bio-analytical methodology for simultaneous evaluation of Zanamivir.

Linearity

The Zanamivir and Remdesivir calibration curves were linear through the concentration series of 4 to 80 ng/ml and assayed in five replicates on five different days, which was depicted in fig. 4. The calibration curve had a coefficient correlation (r2) was>0.9994. The results were depicted in table 3.

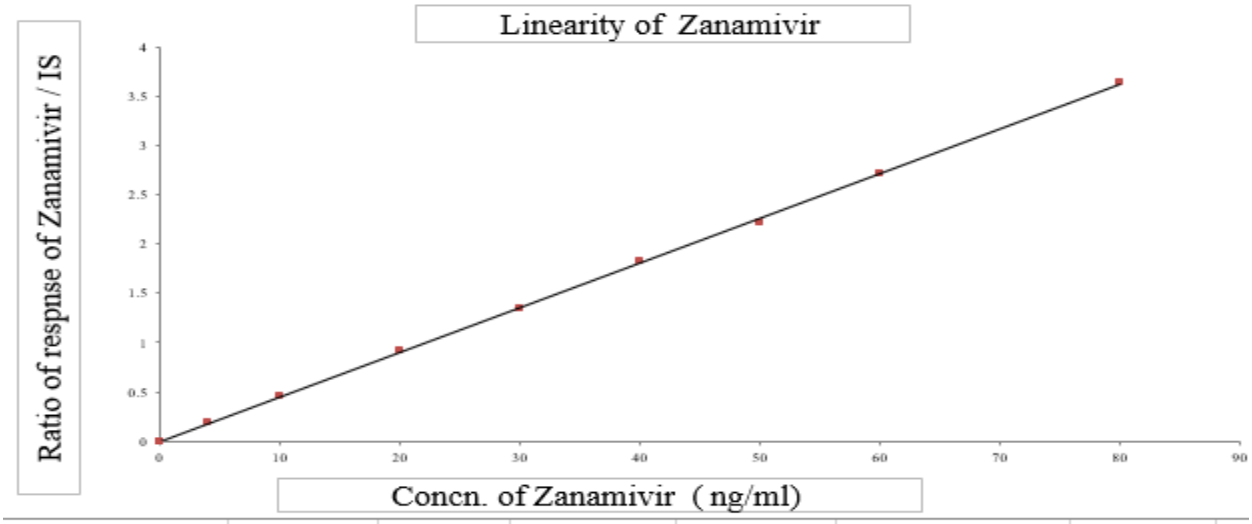

Fig 4: Calibration curve of Zanamivir

Precision and accuracy

The intra-day and inter-day accuracy was ranged from 98.50% to 102.88%. The intra-day and inter-day precision was ranged from 0.81 to 1.98 % (table 4). The accuracy and precision results met the acceptable bio-analytical criteria

Sample	Intraday	Interday

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	% Accuracy	**% RSD**	**% Accuracy**	**% RSD**
LQC	98.21	1.52	99.21	1.98
MQC	100.25	0.98	101.35	0.99
HQC	102.88	0.81	98.50	0.72

Recovery of internal standard and analyte

The extraction recoveries and percent CV for Zanamivir (92.15% to 99.43%) and Zanamivir and Remdesivir (96.43 to 105.08%) at LQC, MQC and HQC levels. The percent CV ranged from 0.27% to 1.75% for Zanamivir. The results demonstrated that the bio-analytical method had good extraction efficiency. This also showed that the recovery was not dependent on concentration

Stability studies

The results of Zanamivir stability tests were summarized in table 5. The findings showed that Zanamivir was durable in rat plasma under various storage conditions studied, including stored at Freeze-thaw at-80 °C, Bench top (normal room temperature) for 48 h, Auto-sampler at 2-8 °C for 24 h and Long term at–80 °C for 30 d

Table 5: Stability Results 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

In this investigation, a simple and sensitive HPLC–MS-MS method was established and validated to quantify Zanamivir in the sample plasma of human was five folds higher sensitive and more significant to other previously published methods. The method involved simple single step precipitation method using acetonitrile for sample preparation. The results showed satisfactory recovery as well as a lack of major matrix effects. The validation results are well within the criteria of acceptance.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not –Applicable

FUNDING

No funding and self-financed.

CONFLICT OF INTERESTS

No conflicts of interest present in this research

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