Journal of Applied Pharmaceutical Research
Volume 12 Issue 4, Year of Publication 2024, Page 99 – 113
DOI: https://doi.org/10.69857/joapr.v12i4.607

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Research Article

JOURNAL OF APPLIED PHARMACEUTICAL RESEARCH | JOAPR
www.japtronline.com ***ISSN: 2348 – 0335***

BIOANALYTICAL METHOD FOR THE SIMULTANEOUS ESTIMATION OF ATOLTIVIMAB, MAFTIVIMAB AND ODESIVIMAB IN RAT PLASMA BY LCMS/MS AND ITS APPLICATION TO A PHARMACOKINETIC STUDY

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Article Information
Received: 16th May 2024
Revised: 28th June 2024
Accepted: 12th July 2024
Published: 31st August 2024

Keywords
HPLC-MS/MS, Atoltivimab, Maftivimab, Odesivimab, validation, rat plasma.

ABSTRACT
Background: A quick, accurate, reproducible, and straightforward liquid chromatography-tandem mass spectrometry(LC-MS/MS) system employing Atoltavimab, Maftivimab, and Odesivimab as an internal standard for Zanamivir quantification was achieved. Zanamivir is a neuraminidase inhibitor that effectively treats influenza caused by influenza A and B viruses. **Methodology:** Whenever we use the Kinetex C-18 column, all HPLC parameters and conditions are obeyed, so we use this column. Separation was performed on a Kinetex C18 column (100 mm x 4.6 mm, 2.6µm) using isocratic elution with a buffer containing 1mL of formic acid in 1Lit of water and a mobile step consisting of a 40:60 v/v mixture of two elements, buffer and acetonitrile, with a flow rate of 1mL/min at 30^0C temperature was used. **Results & Discussion:** We used different stationary phases in the optimization process, such as C18, C8, and CN-propyl. Using a kinetex C18 column with dimensions of (100 mm x 4.6 mm, 2.6 µm) connected to a PDA detector, we obtain strong peak shapes of Atoltivimab, Maftivimab, and Odesivimab from various trials. Flow rates in the mobile process were set to 1 mL/min. **Conclusion:** Atoltivimab, Maftivimab, and Odesivimab analysis was completed in 7 minutes over a good linear concentration range of 5ng/mL to 100ng/mL (r^2 = 0.999), 5ng/mL to 100ng/mL (r^2 = 0.999), and 5ng/mL to 100ng/mL (r^2 = 0.9998). The findings of the precision and recovery studies are within the appropriate range.

INTRODUCTION

Ebola virus disease (EVD) is a rare, severe, and life-threatening disease that affects both human beings and non-human primates (monkeys, gorillas, and chimpanzees) [1-4]. EVD is also called Ebola hemorrhagic fever (EHF). According to the World Health Organization (WHO), fruit bats belonging to the Pteropodidae family may serve as the natural host for the Ebola virus [5]. Ebola virus (EBOV) is a single-stranded RNA virus belonging to the Filoviridae family. Depending on the topographic studies, there are five sub-types of Ebolaviruses, including Zaire, Bundibugyo, Sudan, Reston, and Tai Forest. Initially, people who are suffering from EVD may experience symptoms like

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fever, headache, muscle pain, and chills. In a later stage, these patients may experience internal bleeding or coughing blood. The Ebola virus is mainly transmitted through direct contact with blood, body fluids (saliva, blood, urine, feces, sweat, breast milk, semen), and tissues of infected persons or wild animals. The mortality rate ranges from 25% to 90% depending on the type of ebolavirus strain. The Ebola virus replicates after penetrating the host cell membrane by binding with glycoprotein spikes and clathrin-mediated endocytosis. So, monoclonal antibodies protect against several viral diseases [6-8].

Inmazeb is the combination of three human IgG1 monoclonal antibodies (mAbs) that consist of atoltivimab (ATO), maftivimab (MAF), and odesivimab (ODE). These mAbs exert their effect against the glycoprotein (GP 1,2) of the Zaire ebolavirus (ZEBOV) [9-12]. The glycoprotein attaches to the cell receptor and fuses the viral and host cell membranes that allow the virus to enter the cell. So, the three antibodies in Inmazeb can bind to GP and block the attachment and entry of the virus [13-17]. GP is mainly responsible for the pathogenic differences between ebolaviruses [18-19]. ZE-BOV is a species of Ebola virus and is a causative agent of Ebola virus disease. These antibodies are administered to neutralize viral particles and also activate the immune effectors to destroy infected cells and viral particles. It was first approved by the US FDA in 2020 for treating infection caused by Zaire ebolavirus in adult and pediatric patients [20-23]. The recommended dosage of atoltivimab, maftivimab, and odesivimab is 50 mg/kg each. The mechanism of action of these antibodies includes antibody-dependent cell cytotoxicity, phagocyte stimulants, and virus internalization inhibitors [24-26]. The literature survey reported that few analytical methods have been developed for the quantitative analysis of Atoltivimab in combination with other medications [27-29].

MATERIALS AND METHOD

Chemicals and Materials

Zydus Cadila, Ahmadabad, provided Atoltivimab, Maftivimab, Odesivimab, and Zanamivir (Internal Standard) with purity levels of 99 percent. Merck (India) Ltd., Worli and Mumbai, India, provided acetonitrile (LCMS Grade, 99.99 purity), water (Milli Q), and formic acid (HPLC grade, 99.0 percent). All other reagents and components were of AR quality and readily available. Whenever these chemicals and materials are used, exact reproducibility will occur.

Instruments & Conditions

A Waters Alliance e-2695 version HPLC equipped with a column oven, autosampler, and degasser was used for analysis. The SCIEX QTRAP 5500 mass spectrometer, which has an electrospray ionization interface, was connected to the HPLC system. The results from the chromatogram were interpreted using SCIEX software. The multiple reaction monitoring mode was adopted to record the transformation of protonated precursors to final ions at m/z 145097.63, m/z 143947.82, m/z 146164.32 for Atoltivimab, Maftivimab, and Odesivimab, m/z 332.65 for Zanamivir (Internal standard) sample and IS, correspondingly. The source-dependent variables that were retained for the sample and IS were as follows: GS1: 50.00 psi, GS2: 50.00 psi, IS voltage: 5,500.00V, turbo heater temperature: 550.00°C, collision activation dissociation: 7.00psi, and curtain gas: 20.00psi. The compound-dependent factors such as de-cluttering potential were adjusted at 40.00V, and entrance potential, collision energy, and cell exit potential were 10.00V, 15.00V, and 7.00V, respectively [30-32]. The instrumentation specifications are detailed in Table 1.

Stock Preparedness, Calibration, and Quality Control Specimens

The diluting solvent (Buffer 0.1% FA 40:60 v/v mixture of two elements, Buffer and Acetonitrile) was used to create successive dilution series from stock solutions. Weighed each 5 mg of Atoltivimab, Maftivimab, and Odesivimab into a 100 ml volumetric flask, added approximately 70 ml of diluents, and sonicated for 15 minutes to dissolve. Then, using diluents, get it up to par. Take 1 mL of this solution and dilute it with diluents to make up 10 mL. Take 0.4 mL of this solution and dilute it to 10 mL. This is referred to as a stock solution. (Each Conc. 200ng/mL of Atoltivimab, Maftivimab, and Odesivimab).

A standard solution of 50 ng/mL Atoltivimab, 50 ng/mL maftivimab, 50 ng/mL Odesivimab, and 50 ng/mL Zanamivir (IS) was prepared by dissolving the drugs in Formic acid 0.1 percent ACN (40:60, v/v) at concentrations ranging from 5 to 100 ng/mL for each drug. Calibration and quality control specimens were made by diluting the working solutions previously described and mixing them with blank plasma. Eight calibration specimens had concentrations of 5, 12.5, 25.0, 37.5, 50.0, 62.5, 75.0, and 100.0 ng/mL, while QC specimens had concentrations of 5 ng/mL (LLOQ), 25 ng/mL (LQC), 50 ng/mL (MQC), and 75 ng/mL (MQC) (HQC). Both specimens were

stored at -20°C and then returned to room temperature for examination. Whenever the stock solution standard solution and sample preparation are prepared, all necessary precautions are followed, and errors are omitted.

Table 1: Optimized liquid chromatography and mass spectroscopic conditions

LC conditions		**MS conditions**		
HPLC	Waters Alliance e2695	MS	Sciex QTRAP 5500	
Isocratic step mobile	ACN: Formic acid 0.1% 60:40 v/v	Ionization source	Drying gas: N_2 gas; Drying flow rate: 5 ml/min; Pressure: 55 psi	
	Flow level: 1 ml/min		Source temperature: 550°C	
	Injection volume: 10 µL		Capillary voltage: 5500V	
kinetex C_{18}	100mm length	Collision cell gas	Nitrogen with high-purity	
	4.6 mm ID	Mode	MRM	
	2.6 µm PS			
Analyte	Atoltivimab	Atoltivimab MRM transitions	m/z-145097.63 m/z-38695.26;	CE^a – 15V
	Maftivimab	Maftivimab MRM transitins	m/z-143947.82 m/z-37542.16;	CE^a - 14V
	Odesivimab	Odesivimab MRM transitions	m/z-146164.3 m/z-30867.93;	CE^a – 15V
Internal standard	Zanamivir	Zanamivir MRM transitions	m/z-607.33 m/z-193.48;	CE^a – 14V

CE-Collision energy, MRM- Multi reaction monitoring transitions

Preparation of a solution for plasma samples

Aliquots of 200 microliters of rat plasma specimens were spiked with 500µL of internal ordinary (IS) and 500µL of standard stock working solution for sample preparation. Following that, 300 microlitres of acetonitrile and 500 microlitres of diluents were vortex mixed for 15 minutes, the samples were centrifuged at 5000 rpm for 15 minutes, and the supernatant handled solution was separated, collected, and filtered through a 0.45 nylon syringe filter into a vial before being injected into the HPLC system. Optimized trials plasma sample is partially dissolved in different solvents like (MeOH, 0.1Sulpuric acid, 0.1 Triethylamine, etc), but soluble in diluting solvent (Buffer 0.1% FA and 40:60 v/v mixture of two elements, Buffer and Acetonitrile) [33-34]

Animals parameters

Six healthy white albino rats (body weight 250-350 grams) were obtained from Biological E Limited in Hyderabad, India, for this research. Both animals were fasted overnight and given free access to water before the experiment. Atoltivimab, Maftivimab, and Odesivimab solid injection powders were evaluated for pharmacokinetics. Each medication was given orally to all rats at a 1.004 mg/kg dose. A 1.5mL blood sample was taken from the rat body at 0.5, 1, 2, 3, 4, 5, 6, and 7 hours and then centrifuged the plasma for 30 minutes at 5000 rpm. The supernatant solution was injected into a chromatographic column, and plasma specimens were held at 2-8 degrees Celsius until the study was completed.

Table 2: Mean average weight of rats

Group name	Average weight of rats
Rat-1	259.14±2.53
Rat-2	263.18±3.97
Rat-3	267.56±2.55

Ethical statement

This study was carried out in strict accordance with the recommendations and compliance of the committee to control and supervise experiments on animals(CPCSEA'S) norms. The Institute of Animal Ethics Committee accepted the animal research protocol (Reg.No:1074/PO/Re/S/05/CPCSEA). CPCSEA is a statutory body constituted by the Government of India that regulates experiments on animals as part of this research work. The Institute of Animal Ethics Committee approved the animal study protocol. Animals were housed in similar laboratory conditions with access to endives, carrots, and fresh corn (few amounts only), and the animals were kept at a temperature of 21°C–24°C, and humidity was 50%–55%. Before experimentation, all animals fasted overnight and had water adlibitum. Experiments are done without anesthesia and all such measures as necessary to ensure that animals are not subjected

to unnecessary pain or suffering before, during, or after the experiments on them.

Procedure for preparing mobile phase and buffer

1 ml of FA and 1000 ml of deionized water were combined, and the solution was passed through a 0.45-micron filter membrane. 40:60 v/v mixture of ACN and 0.1% FA was mixed, followed by filtration using 0.45 micron filter paper.

Validation of Bioanalytical Method

The technique was validated for sensitivity, selectivity, linearity, precision, matrix condition, accuracy, reinjection, reproducibility, recovery study, and stability.

Selectivity and sensitivity

Six diverse rat samples were examined, and interference at respective retention times (RTs), sensitivity, and selectivity were examined.

Matrix Effect

The comparability of height area ratios of six diverse drug-free samples for Atoltivimab, Maftivimab, and Odesivimab was assessed to estimate the matrix effect. Six diverse plasma batches were studied in repeated trials at LQC and HQC concentrations with an adequate precision below 15%.

Recovery

The extraction efficiencies of atoltivimab, maftivimab, and odesivimab were determined by looking at six repeats at each concentration of QC. The recovery degree was determined by comparing highlights of separate guidelines to non-extricated peak areas of standards.

Dilution Integrity

The integrity of the dilution must be explicable by injecting the matrix with the sample over the ULOQ levels and then reconstituting using a blank matrix. Spiking the matrix above the ULOQC with analyte concentration and diluting this test with a blank matrix should demonstrate dilution integrity.

Carryover

Carryover refers to the analyte recovered by the chromatographic column after reconstitution of this sample using a matrix with a sample concentration above the upper limit of quantification (ULOQ) and beyond.

Precision and Accuracy

An LLOQ, LQC, MQC, and HQC level investigation of IS samples was used to assess it. Replication analysis of quality control specimens (n=6) was used to assess it at the lower quantification limit (LLOQ), low-quality control (LQC), medium quality control (MQC), and high-quality control (HQC) levels. Except for LLOQ, where the CV should be less than 20%, the CV amount should be less than 15%.

Stability

Benchtop stability:

Atoltivimab, Maftivimab, and Odesivimab stability in rat plasma was assessed by exposing six replicates of three different concentrations (LQC, MQC, and HQC) for 18 h on a benchtop and injecting them into the system.

Short-term and long-term stability

Short-term and long-term stability was assessed for Atoltivimab, Maftivimab, and Odesivimab. Three different analyte concentrations were spiked into six duplicates of rat plasma for QC. LQC, MQC, and HQC samples were prepared and stored at (5±3)°C for 7 days, and short-term stability was assessed. LQC, MQC, and HQC samples were prepared and stored at −20±3 °C. These samples were injected from day 1 to 28 days for every seven days (as days 1,7, 14, 21, and 28), and long-term stability was assessed.

Freeze–thaw stability

The stability of Atoltivimab, Maftivimab, and Odesivimab was evaluated after freeze-thaw cycles, respectively. Each LQC, MQC, and HQC had six duplicates held at −20°C, thawed at 30°C, and then immediately refrozen at −20°C. After this cycle was done twice, the samples were removed for injection into the LC-MS.

Autosampler stability

LQC, MQC, and HQC samples of Atoltivimab, Maftivimab, and Odesivimab in plasma were injected at one-hour up to 24-h intervals. Mean accuracy (%) and CV (%) were calculated.

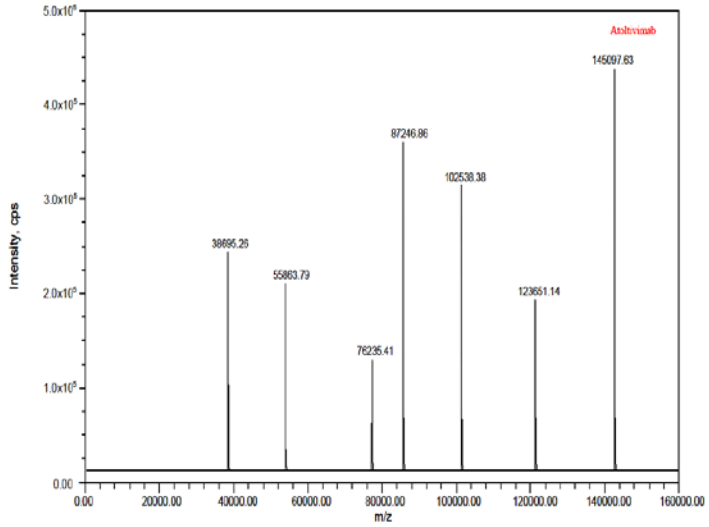
Dry extract and wet extract stability

Wet extract stability was evaluated by assessing the six LQC, MQC, and HQC sets after 12 h and 18 h that were stored at 2–8°C. The dry extract stability test used six sets of LQC, MQC, and HQC after 12 h and 18 h that were stored at 22°C.

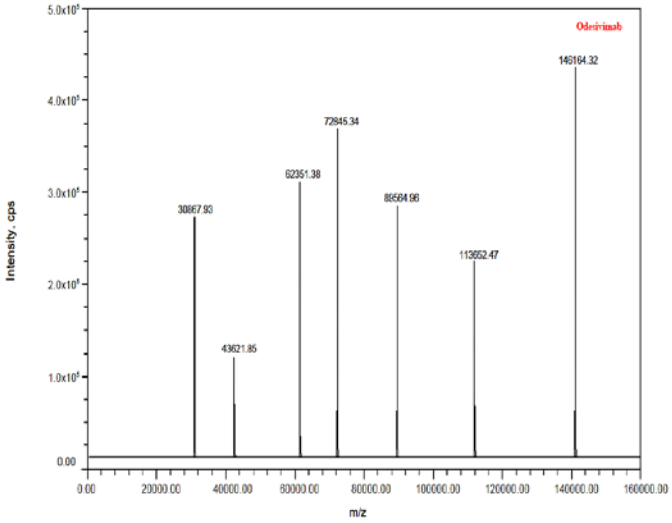
RESULTS

Bioanalytical Method development

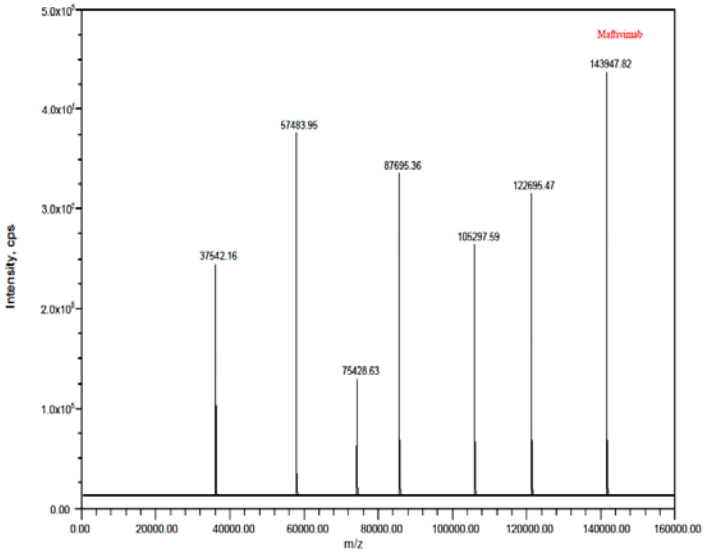
In this step, the ESI has the most intense reaction over the chemical ionization by atmospheric pressure (APCI) mode. The MRM mode has been used to quantify the ions of Atoltivimab, Maftivimab, Odesivimab, and Zanamivir. Atoltivimab, Maftivimab, and Odesivimab have a solid positive ion response mode compared to the ion-negative mode. The details of the mass spectrum are shown in the figures below.



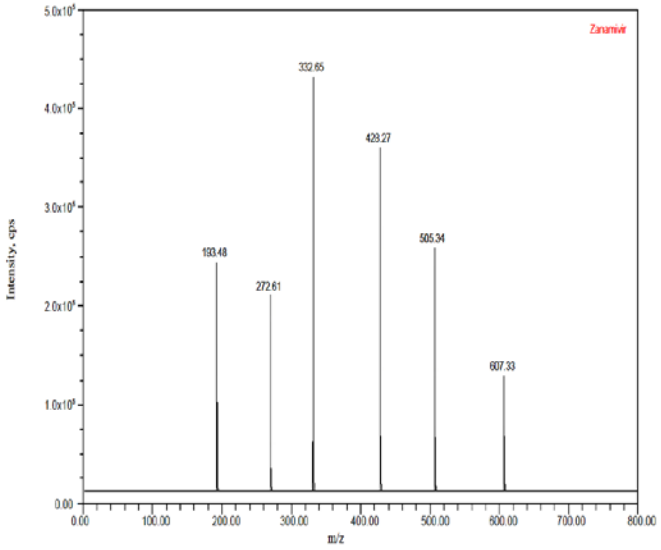
Mass spectra of Atoltivimab



Mass spectra of Odesivimab



Mass spectra of Maftivimab



Mass spectra of Zanamivir

Figure 1: Mass spectra of Atoltivimab, Maftivimab, Odesivimab and Zanamivir

To obtain the best chromatographic conditions, we evaluated different buffers with acetonitrile as the mobile phase in various ratios for isocratic and gradient modes. The mobile step composition was tweaked at each trial to improve resolution and achieve reasonable retention times. Finally, the mobile step was chosen to be 0.1 percent formic acid and ACN in isocratic mode at 40:60 v/v ratios because it provides the best response of the drugs. We used different stationary phases in the optimization process, such as C18, C8, and CN-propyl. Using a kinetex C18 column with dimensions of (100 mm x4.6 mm, 2.6 µm) connected to a PDA detector, we obtain strong peak shapes of Atoltivimab, Maftivimab, and Odesivimab from various trials. Flow rates in the mobile process were set to 1 mL/min. The retention times of the four drugs, Atoltivimab, Maftivimab, Odesivimab, and Zanamivir, were 2.456 minutes, 3.201 minutes, 4.131 minutes, and 5.710 minutes, respectively. Six replicate injections yield a percent CV in the allowable limit, indicating that the suggested technique is very specific. According to USFDA guidelines, the development method has been validated.

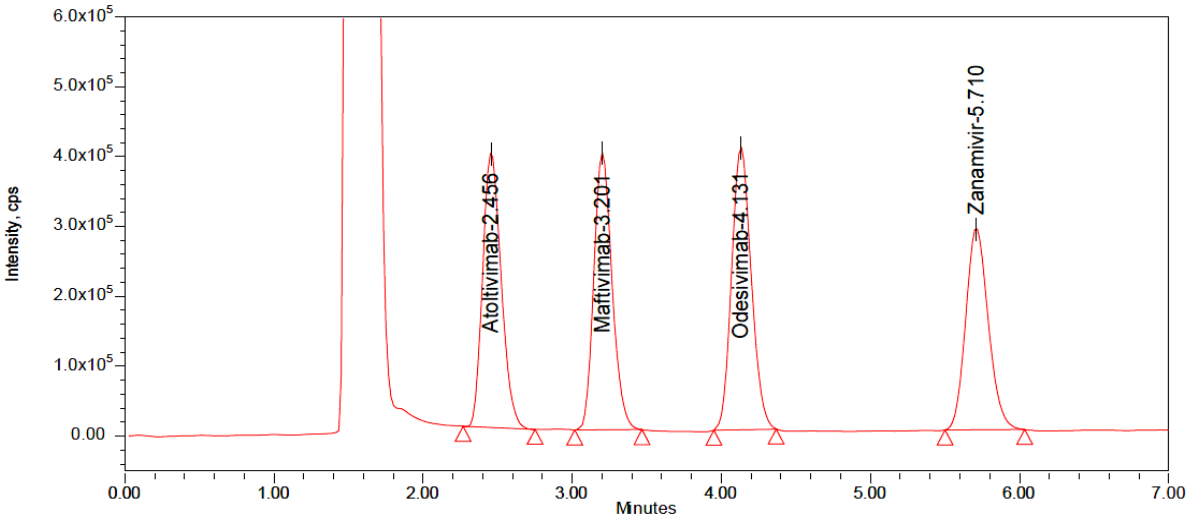


Figure 2: Chromatogram of Standard

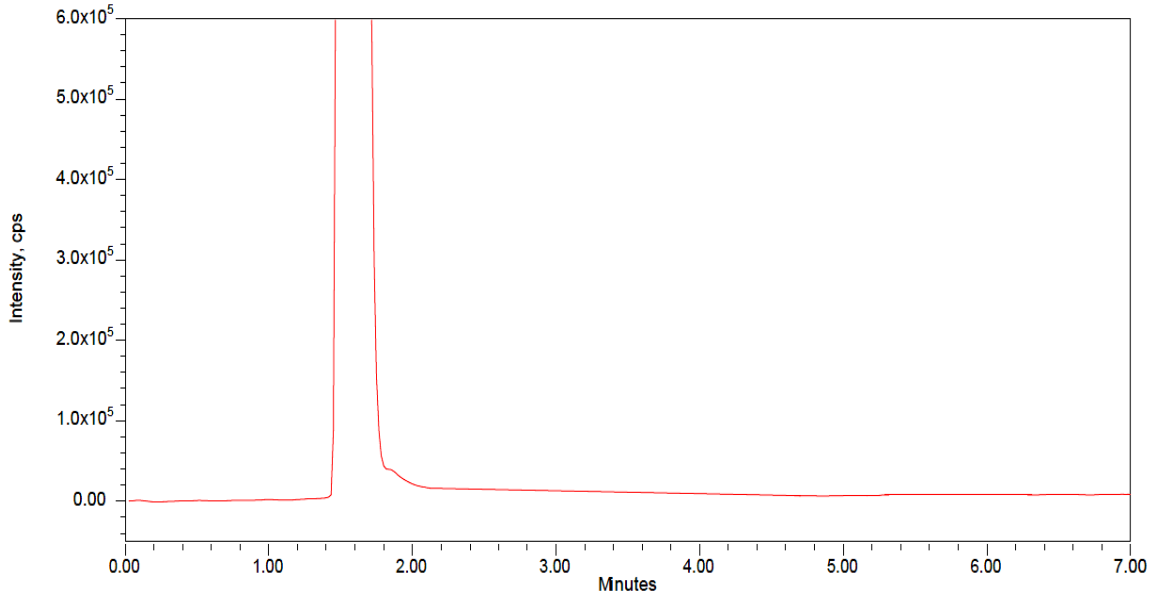


Figure 3: Chromatogram of blank

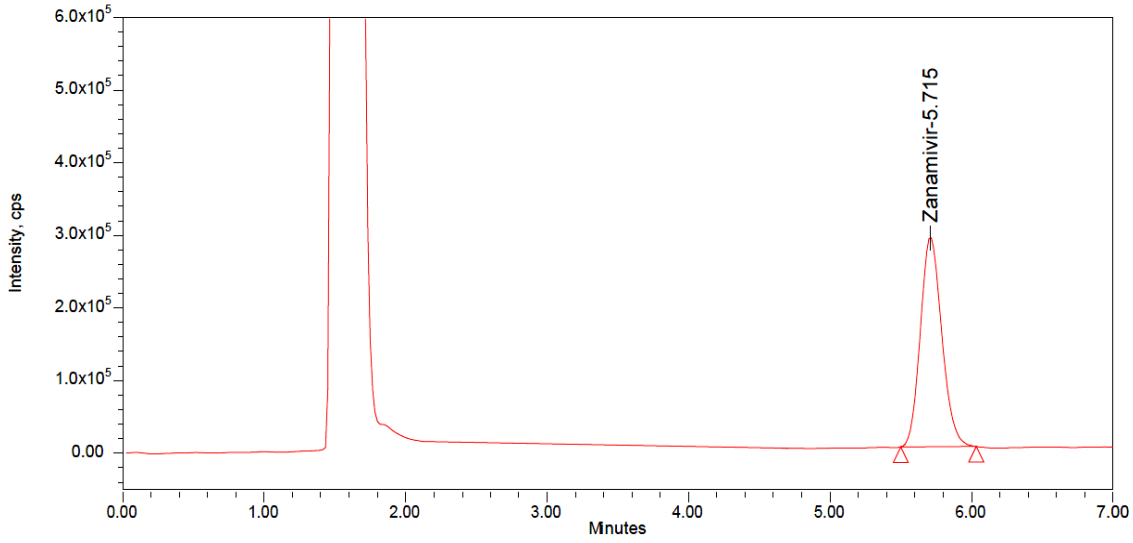


Figure 4: Chromatogram of blank plasma spiked with internal standard

Validation of Bio-analytical process

Specificity

The chromatographs of blank plasma samples, STD and IS, are depicted in Figures 2–4, respectively. There were no interfering peaks visible in the obtained chromatographs.

Matrix Effect

Atoltivimab, Maftivimab, and Odesivimab had matrix impact results of 98.27, 99.89 percent and 97.78, 99.23 percent and 98.21, 99.95 percent at LQC and HQC stages, respectively. At LQC and HQC levels of 1.89, 0.75, and 1.11, 0.96, and 0.64, 1.42, respectively, the drugs' percent CV was found to be 1.89, 0.75, and 1.11. The findings show that the matrix effect on analyte ionization and internal specifications was within reasonable limits.

Recovery

Atoltivimab, Maftivimab, and Odesivimab have 99.21-99.35,0.84 percent recovery at low and high concentration focal levels in rat plasma and 98.47,0.77-98.56,0.66 and 96.32,1.25-97.48,0.93 percent recovery at 25, 25, 25ng/mL 50, 50, 50ng/mL and 75, 75, 75ng/mL concentrations. Atoltivimab, Maftivimab, and Odesivimab have high extraction performance.

Linearity

The calibration curve was generated by analyzing eight plasma concentrations of Atoltivimab, Maftivimab, and Odesivimab. Samples were measured by comparing the peak area of Atoltivimab, Maftivimab, and Odesivimab to that of Zanamivir. The peak area ratios vs. plasma concentrations were plotted. Table 4 shows the linearity and association findings for Atoltivimab, Maftivimab, and Odesivimab.

Table 3: Results of matrix variability and Recovery (%) of Atoltivimab, Maftivimab and Odesivimab in plasma

Analyte	Matrix	Matrix factor bias (%)		% RSD	% Recovery			
		LQC	HQC		LQC	HQC	Mean	% RSD
Atoltivimab	Plasma	98.27	99.89	1.04	99.21	99.35	99.28	0.53
Maftivimab	Plasma	97.78	99.23	0.62	98.47	98.56	98.52	0.74
Odesivimab	Plasma	98.21	99.95	0.87	96.32	97.48	96.90	1.24

Table 4: Linearity results of Atoltivimab, Maftivimab, and Odesivimab

Linearity	Atoltivimab conc. (ng/mL)	Atoltivimab area response ratio	Maftivimab conc. (ng/mL)	Maftivimab area response ratio	Odesivimab conc. (ng/mL)	Odesivimab area response ratio
1	5.00	0.151	5.00	0.155	5.00	0.152
2	12.50	0.379	12.50	0.382	12.50	0.376
3	25.00	0.744	25.00	0.745	25.00	0.759
4	37.50	1.128	37.50	1.112	37.50	1.142
5	50.00	1.496	50.00	1.511	50.00	1.522
6	62.50	1.868	62.50	1.878	62.50	1.901
7	75.00	2.250	75.00	2.246	75.00	2.284
8	100.00	2.971	100.00	2.940	100.00	2.994
Slope	0.0299		0.0297		0.0303	
Intercept	0.00233		0.00669		0.00068	
CC	0.99997		0.99971		0.99986	

Table 5: Co-relation results of Atoltivimab, Maftivimab and Odesivimab

Validation parameter	Atoltivimab			Maftivimab			Odesivimab		
Quality control levels	Low	Medium	High	Low	Medium	High	Low	Medium	High
QC Conc. (ng or pg/ml)	25	50	75	25	50	75	25	50	75
Linearity range	5-100 ng/mL			5-100 ng/mL			5-100 ng/mL		
Correlation (r^2)	0.9999±0.022			0.9997±0.013			0.9998±0.007		

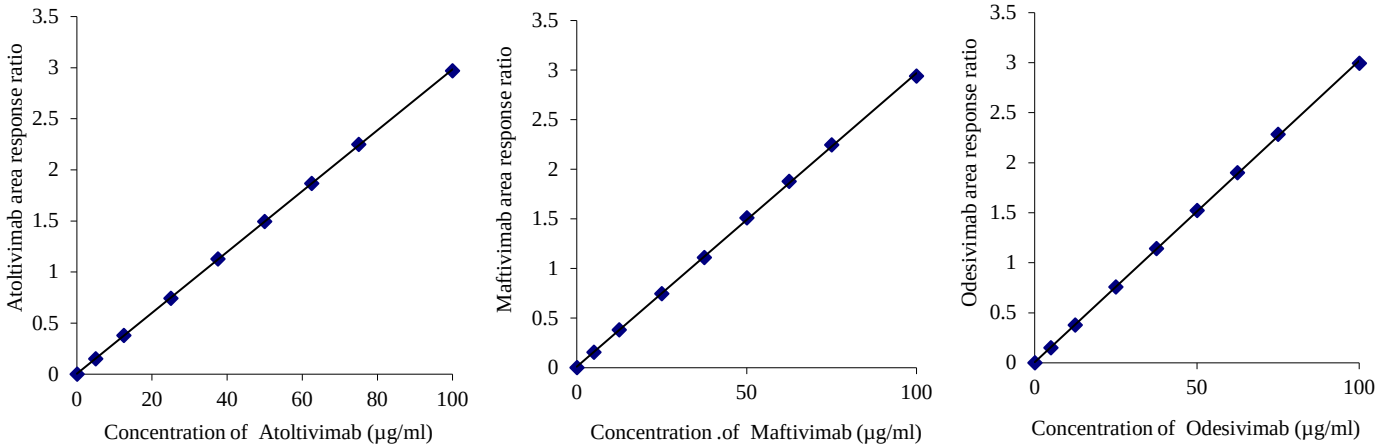


Figure 5: Calibration plots of Atoltivimab, Maftivimab and Odesivimab

Precision and accuracy

By adding together all of the discrete test outcomes from the discrete IS samples, the accuracy and precision were computed. It was clear from the information presented that the system was precise and efficient. Table 6 presents the Atoltivimab, Maftivimab, and Odesivimab precision and accuracy findings. They could monitor precision and exactness by pooling all test results from various QC specimens. For all quality control samples at different concentrations, the % CV of Atoltivimab, Maftivimab, and Odesivimab was less than 5%. The exactness and accuracy of the results were all within the quantification limit. The specifics of the findings are shown in Table 6.

Table 6: Precision and accuracy results of Atoltivimab, Maftivimab, and Odesivimab in rat plasma

Matrix	Sample	Atoltivimab			Maftivimab			Odesivimab		
		Accuracy bias (%)	Precision RSD (%)		Accuracy bias (%)	Precision RSD (%)		Accuracy bias (%)	Precision RSD (%)	
			Intraday	Interday		Intraday	Interday		Intraday	Interday
Plasma	LLOQC	-1.24	2.67	2.44	-1.68	0.52	1.85	-0.87	1.16	1.49
	LQC	0.58	1.26	0.87	0.62	0.48	0.67	0.54	0.71	0.52
	MQC	0.17	1.54	0.52	0.18	0.22	0.57	0.76	0.69	1.63
	HQC	0.06	0.59	0.39	0.27	1.93	0.94	0.12	0.54	0.88

Dilution integrity

Spiking the analyte matrix fixation over the ULOQC and diluting this specimen with a blank matrix can demonstrate dilution integrity. Dilution integrity was tested at 2ULOQC (100ng/mL for Atoltivimab, Maftivimab, and Odesivimab). For Atoltivimab, Maftivimab, and Odesivimab, six replication samples of 1:2 dilutions (50ng/mL) and 1:4 dilutions (25ng/mL) were used. The percent CV for the three components was within suitable ranges at 3.57, 1.49, and 2.18. Table 7 shows the specifics of the results.

Carryover

System error that may affect the measured value of the sample is called carryover. Sample carryover on an LC/MS system configured with Waters Alliance was evaluated using the following procedure. A system blank injection volume of 10µL for 0.1% Formic acid and Acetonitrile (40:60) was performed on a waters Z-spray triple quadruple mass detector by flow injection. From this procedure, we can say that it didn't influence the accuracy and precision of the proposed strategy. Sample carry-over results of Atoltivimab, Maftivimab, and Odesivimab were LLQC (4.21%), ULQC (0.22%), and LLQC (7.06%), ULQC (0.65%) within the permissible limit. Details of carryover results are shown in Table 8.

Reinjection Reproducibility

The reinjection was reproducible during real subject sample analysis to verify the system after complex product disabling because of instrumental disappointment. The shift in levels at LQC and HQC was below 2.0. Thus, during genuine subject specimen investigation, the group was re-infused on account of instrument failure, and samples were prepared and re-injected after 24 hours. It shows a change under 2% at LQC and HQC levels. Henceforth, during genuine specimen analysis, in the case of instrument failure, the batch can be re-injected after 24 hours.

Table 7: Results of dilution integrity

Analyte	ULOQC conc.	Calculated conc.	%CV
Atoltivimab	100 ng/mL	99.97 ng/mL	3.57
Maftivimab	100 ng/mL	99.86 ng/mL	1.49
Odesivimab	100 ng/mL	100.07 ng/mL	2.18

Table 8: Results of carryover

Concentration	% of carryover		
	Atoltivimab	Maftivimab	Odesivimab
Blank	0	0	0
LLOQC	6.23	5.41	5.72
ULOQC	2.48	1.63	0.49

Stability

Benchtop stability

Bench-top stability is the stability of an analyte in a matrix under sample handling conditions during sample processing. The Accuracy (%) of Atoltivimab, Maftivimab, and Odesivimab in three different samples, HQC, LQC, and MQC, was found to be 1.85, 2.35, 4.16, 4.06, 0.47, 1.25, 1.85, 0.67, and 1.04 respectively. The % mean recovery of Atoltivimab, Maftivimab, and Odesivimab in three different samples, HQC, LQC, and MQC, was found to be 98.52, 101.36, 100.75, 98.46, 100.34, 98.95, 100.95, 100.62, and 100.38 respectively. The results of benchtop stability are shown in Tables 9, 10 & 11.

Short-term and long-term stability

Long-term stability assesses the degradation of an analyte in the matrix relative to the starting material after periods of frozen storage. The results showed that Atoltivimab, Maftivimab, and Odesivimab QC low, medium, and high samples were stable in short-term and long-term stability. Short-term stability results & long-term stability results are summarized in Tables 9, 10, 11.

Autosampler stability

Autosampler stability is the stability of the analyte in the processed sample under the conditions in the autosampler. The accuracy, recovery, and RSD (%) of HQC, LQC, and MQC were found to be three different drugs results are shown in table 9,10 and 11, respectively.

Table 9: Stability results of Atoltivimab in plasma of rats under different storage conditions

Stability	Storage condition	Conc. level	Measured conc (ng/ml) (Mean±SD, n=6)	% RSD	% Recovery	Accuracy (% RE)
Benchtop stability	18 hours at room temperature	25	25.201±0.64	0.56	98.52	2.35
		50	50.264±0.49	0.48	101.36	4.16
		75	75.157±0.83	0.27	100.75	1.85
Auto sampler stsbility	24 hrs in autosampler at room temperature	25	25.049±0.41	0.39	100.64	1.72
		50	50.043±0.52	1.15	100.36	-0.64
		75	75.162±0.38	1.07	100.84	0.85
Long term stability	28 days at (-20±3)°C	25	24.968±0.95	2.32	98.36	2.58
		50	49.712±0.85	1.43	97.31	1.76
		75	75.241±0.34	0.85	100.23	0.55
Freeze thaw stability	24 hrs at (-28±5)°C, then exposed to three freeze and thawed cycles	25	25.192±1.07	1.26	101.62	4.57
		50	50.117±0.81	1.11	99.48	-0.64
		75	74.896±0.59	0.96	98.26	-0.74
Wet extract stability	18 hrs at 2-8°C	25	24.997±0.27	2.29	99.14	1.19
		50	50.121±0.42	0.49	100.24	2.63
		75	75.036±1.28	0.35	100.36	1.04
Dry extract stability	18 hrs at (-20±3)°C	25	25.069±3.14	2.48	100.48	0.87
		50	50.223±1.08	1.06	101.63	0.55
		75	75.163±2.96	0.87	100.94	-1.62
Short term stability	7 days at (5±3)°C	25	24.831±1.42	3.31	98.75	0.86
		50	50.169±2.85	0.48	99.93	0.72
		75	74.893±2.03	0.71	97.42	0.91

Table 10: Stability results of Maftivimab in rat plasma under different storage conditions

Stability	Storage condition	Conc. level	Quantified conc. (pg/ml) (Mean±SD, n=6)	% RSD	% Recovery	Accuracy (%)
Benchtop stability	18 hrs at room temperature	25	24.759±1.25	0.29	98.46	-0.47
		50	50.326±4.29	0.48	100.34	1.25
		75	74.625±0.49	0.27	98.95	4.06
Autosampler stability	24 hrs in autosampler at room temperature	25	25.623±0.68	1.06	99.98	1.25
		50	50.321±0.63	0.85	100.78	1.11
		75	75.053±1.58	0.49	100.31	0.46
Long term stability (Day 28)	28 days at (-20±3)°C	25	25.174±1.24	2.45	100.14	0.82
		50	49.639±0.63	1.32	97.63	0.43
		75	75.858±0.78	0.44	100.62	0.62
Freeze thaw stability	24 hrs at (28±5)°C then exposed to three freeze and thaw cycles	25	25.743±0.49	1.04	100.57	-1.65
		50	49.632±0.47	0.87	99.47	0.48
		75	75.632±0.33	1.63	101.43	0.37
Wet extract stability	18 hrs at 2-8°C	25	25.852±0.68	3.45	100.02	-2.46
		50	49.247±0.47	0.48	99.48	1.47
		75	75.675±0.89	0.92	100.29	0.63
Dry extract stability	18 hrs at (-20±3)°C	25	25.284±0.54	2.24	101.45	0.49
		50	50.339±1.62	1.65	101.62	2.41
		75	75.264±3.47	0.49	100.74	1.86
Short term stability	7 days at (5±3)°C	25	24.893±1.22	0.67	99.67	0.59
		50	50.124±1.54	0.51	100.46	0.82
		75	74.457±2.38	0.96	98.65	0.73

Freeze–thaw stability

Freeze–thaw stability refers to the stability of the analyte in the matrix upon freezing and thawing. The accuracy (%) of Atoltivimab, Maftivimab, and Odesivimab in three different samples, HQC, LQC, and MQC, was found to be 0.74, 4.57, 0.64, 0.37, 1.65, 0.48, 1.42, 2.59, and 2.48 respectively. The mean recovery (%) of Atoltivimab, Maftivimab, & Odesivimab in three different samples, HQC, LQC, and MQC, was found to be 98.26, 101.62, 99.48, 101.43, 100.57, 99.47, 98.43, 100.47, and 100.62 respectively. The results are tabulated in Tables 9, 10, and 11.

Dry extract and wet extract stability

Extract stability assesses the degradation of the processed sample relative to the starting material. The results of wet extract stability are tabulated in Tables 9,10 and 11. Similarly, the results of dry extract stability were summarized in Tables 9,10,11. From this, we observed a % change of Atoltivimab, Maftivimab, and Odesivimab were 1.14%, 0.96%, and 0.53%, respectively, which indicates that solutions are stable up to 24h. At room temperature, Atoltivimab, Maftivimab, & Odesivimab were stable in plasma for different conditions. It was evaluated that LQC, MQC, and HQC levels continued freezing and defrosting of plasma specimens spiked with Atoltivimab, Maftivimab, and Odesivimab, which didn't influence its stability. It was clear from long-term stability that Atoltivimab, Maftivimab, and Odesivimab were stable at a capability temperature of -30°C up to 24h. The overall stability results of Atoltivimab, Maftivimab, and Odesivimab are shown in Tables 9, 10, and 11.

Pharmacokinetic studies

Six distinct rats were given injections of Atoltivimab, Maftivimab, and Odesivimab samples at various intervals, including 2, 4, 6, 8, 12, and 24 days. Samples are then concocted following the test methodology, loaded into the chromatographic device, and the findings are noted and tabulated in Table 12. By incurred sample reanalysis (ISR), the

stability of the research samples was determined. Close to Cmax and the elimination phases in the pharmacokinetics, three samples from every subject were chosen for ISR. The percentage difference should not exceed 20%, and the samples were considered stable. Figures 6,7, and 8 represent the recovery graph for Atoltivimab, Maftivimab, and Odesivimab in rat plasma. Table 12 shows the pharmacokinetic parameters of Atoltivimab, Maftivimab, and Odesivimab.

Table 11: Stability results of Odesivimab in rat plasma under different storage conditions

Stability	Storage condition	Conc. level	Quantified conc. (pg/ml) (Mean±SD, n=6)	% RSD	% Recovery	Accuracy (%)
Benchtop stability	18 hrs at room temperature	25	25.505±0.64	0.57	100.95	0.67
		50	50.416±0.49	0.19	100.62	1.04
		75	75.527±0.83	0.32	100.38	1.85
Autosampler stability	24 hrs in autosampler at room temperature	25	25.638±0.41	0.49	100.74	2.96
		50	50.965±0.52	1.45	100.26	1.03
		75	75.857±0.38	1.08	100.26	0.95
Long term stability (Day 28)	28 days at (-20±3)°C	25	24.521±0.95	2.07	99.12	1.46
		50	49.471±0.85	1.49	98.16	1.74
		75	75.398±0.34	1.05	100.12	1.83
Freeze thaw stability	24 hrs at (28±5)°C then exposed to three freeze and thaw cycles	25	25.685±1.07	0.75	100.47	2.59
		50	50.622±0.38	0.83	100.62	2.48
		75	74.574±0.75	0.52	98.43	1.42
Wet extract stability	18 hrs at 2-8°C	25	24.889±0.69	0.24	99.14	1.38
		50	50.105±0.47	1.79	100.54	3.49
		75	75.968±2.20	0.58	100.52	0.89
Dry extract stability	18 hrs at (-20±3)°C	25	25.718±4.51	0.66	100.47	0.74
		50	50.365±1.63	0.71	100.25	1.45
		75	75.889±2.58	1.98	100.58	1.09
Short term stability	7 days at (5±3)°C	25	24.851±1.47	0.58	99.36	1.42
		50	50.637±0.49	0.43	100.42	0.83
		75	74.414±0.85	1.77	97.48	0.46

DISCUSSION

LC-MS/MS is a sensitive method for the quantification of monoclonal antibodies. Several elution conditions were tested for the chromatographic separation. In trial 1, a mobile phase composition of acetonitrile and triethylamine buffer in the ratio 60:40 was used. Peak splitting was observed, so further trial was carried out. In trial 2, a mobile phase ratio of acetonitrile and ammonium formate buffer (60:40) was used. The plate count was not within the limit. Hence, further trial was carried out. A mobile phase ratio of acetonitrile and ammonium formate buffer (50:50) was used in trial 3. Peak heights were not within the limit, so further trial was conducted. In trial 4, an isocratic elution with a buffer containing 1mL of formic acid in 1Lit of water and a mobile step consisting of a 40:60 v/v mixture of two elements, buffer and acetonitrile, with a flow rate of 1mL/min at 30^0C temperature was used. System suitability parameters were within the limit, so this method was validated. The developed method quantified Atoltivimab, Maftivimab, and Odesivimab in a biological matrix. The system was deemed suitable for usage if the area ratio's CV (%) was less than five and the retention time's CV (%) was less than 2. It thus passed the system suitability test. The calibration curve was deemed agreeable when % accuracy for all calibration curve standards ranged from 85.00 to 115.00%. The correlation coefficient (R^2) was 0.99 or better. The method was found to be linear. The response of any interfering peaks at the analyte retention time was to be ≤20.00% of Atoltivimab, Maftivimab, and Odesivimab at LLOQ and ≤ 5.00% of that in LLOQ in the case of Zanamivir. The method

was found to be specific and selective. Sensitivity acceptance criteria were 4 out of 6 samples, or at least 67.00%, and fell within the 80.00–120.00% range. The recommended range for mean accuracy (%) was 80.00–120.00%. The CV's (%) accuracy was to be 20.00%. The outcomes fell within the permitted range. The method was found to be sensitive. The standards for data acceptance included accuracy (%) within 85.00–115.00% of the actual values and precision within 15.00% relative standard deviation (RSD).

Table 12: Pharmacokinetic studies of Atoltivimab, Maftivimab, Odesivimab and Zanamivir

Pharmacokinetic parameters	Atoltivimab	Maftivimab	Odesivimab
AUC_{0-t} (ng h/ml)	187	179	189
C_{max} (ng/ml)	46.1	44.6	46.4
$AUC_{0-\infty}$(ng h/ml)	187	179	189
$T_{1/2}$ (h)	7	7	7
T_{max} (h)	3	3	3

These findings demonstrated that the accuracy and precision were reproducible and dependable for quantifying Atoltivimab, Maftivimab, and Odesivimab in rat plasma. If the analyte concentration detected in the double blank sample was less than 20.00% for Atoltivimab, Maftivimab, and Odesivimab, carryover was deemed significant. Hence, there was no carryover effect. CV (%) and mean accuracy in dilution integrity were within the limits for Atoltivimab, Maftivimab, and Odesivimab. The minimum acceptance standard required two out of three samples at every level to fall under the 85.00 to 115.00% range. The matrix lot was to be within the agreeable criteria in at least 80.00% (5 out of 6 cases). The results were within the tolerable range. Hence, the matrix effect was found to be negligible. For each QC level, the CV (%) of recovery was to be under 15.00%. For all QC levels, the mean recovery CV (%) was to be under 20.00% overall. All of the results fell within desirable limits. The overall mean recovery (%) and CV (%)were less than 20.00% for all QC levels. The range of the mean accuracy for low, medium, and high-quality control samples was between 85.00 and 115.00%.

The results were within tolerable limits, specifying that the extraction technique was effective. The limitations were all met in reinjection reproducibility. The method was found to be reproducible. The CV (%) of low and high-quality control samples was ≤ 15.00%. The CV (%) and mean accuracy were within the standard limits.

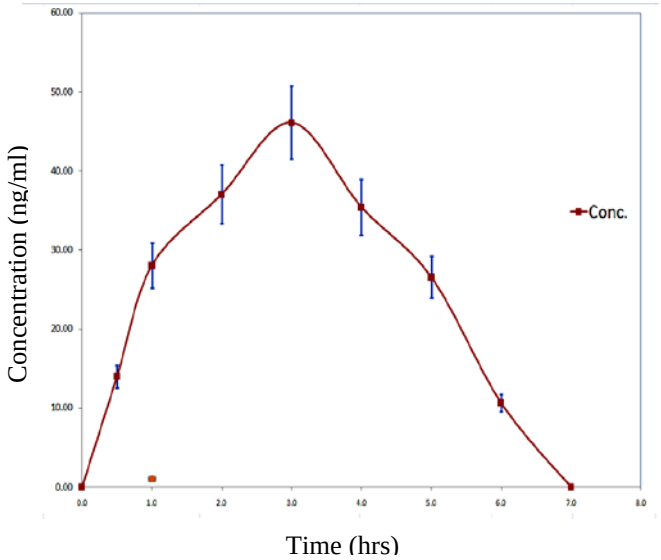


Figure 6: Recovery plot of Atoltivimab

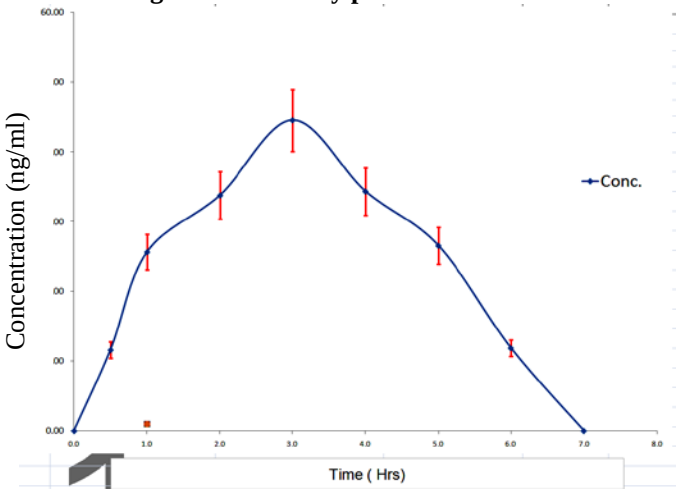


Figure 7: Recovery plot of Maftivimab

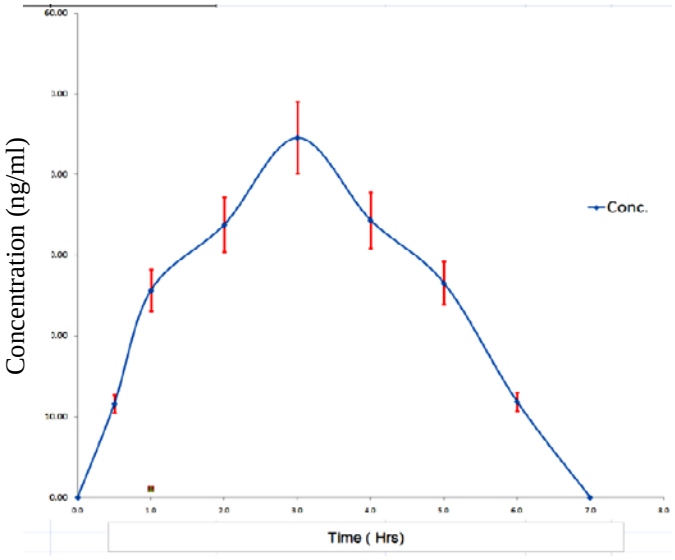


Figure 8: Recovery plot of Odesivimab

The CV (%) of low and high-quality control samples was ≤ 15.00%. The CV (%) and mean accuracy were within the standard limits. Any condition, period, or analyte concentration examined had less than 15.00% of CVs. All the stability results were within the tolerable range. The LQC and HQC samples' mean concentration accuracy range was between 85.00 and 115.00%. LQC and HQC samples were to have a CV (%) of less than 15.00%. The results showed that Atoltivimab, Maftivimab, and Odesivimab were stable in rat plasma. CV (%) and mean accuracy (%) were within the limits. Samples were deemed stable if the CV (%) for the low, medium, and high-quality control samples was less than 15.00%. It showed that the stability of the autosampler was determined to be within limits. Moreover, the mean accuracy and CV (%) were within limits. The CV (%) and mean accuracy (%) for Atoltivimab, Maftivimab, and Odesivimab passed the wet and dry extract stability. As a result, the approach was accurate in various conditions. By studying three QC samples of Atoltivimab, Maftivimab, and Odesivimab, the application of multiple storage conditions and the stability of the drug were evaluated. The findings were consistent throughout the studies conducted. These stability results indicate that Atoltivimab, Maftivimab, and Odesivimab were stable during bench top, freeze-thaw, autosampler, short-term, long-term, wet extract, and dry extract stability studies. Also, Atoltivimab, Maftivimab, and Odesivimab were stable during the storage and handling of samples in a rat plasma matrix. The study confirmed that the bioanalytical method was accurate and can be used to study pharmaceutical dosage forms. The validated technique was sensitive enough to accurately quantify the analyte in experimental rats' plasma samples. In rats, the pharmacokinetic findings illustrate less absorption and metabolism effects on Atoltivimab, Maftivimab, and Odesivimab. These findings will be helpful in further pharmacokinetic assessments [33].

CONCLUSION

These present studies performed experiments on Atoltivimab, Maftivimab, and Odesivimab. The instrument used in these experiments is LCMS/MS coupled with a PDA detector. This is the first time a new MS/HPLC technique has been successfully developed and validated for evaluating Atoltivimab, Maftivimab, and Odesivimab in 7 minutes of rat plasma. In this method, we used liquid-liquid extraction for sample preparation because of its increased sensitivity and column life compared to the protein precipitation method. The solid phase extraction method was avoided because of its high economic rate. After intravenous administration, Atoltivimab, Maftivimab, and Odesivimab were rapidly absorbed from the rat body and showed pharmacokinetic behavior. The described method followed USFDA guidelines and is fast, rugged, and reproducible. It can be applied successfully for pharmacokinetic studies and check the investigated analyte concentrations in body fluids with acceptable accurate results and good linear concentration range. These studies are warranted to validate our results shortly as a reference.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Shree Icon Pharmaceutical Laboratories, Vijayawada, India, and Sir Eswar College of Engineering, Kesanupalli Village, Narasaraopet, Guntur, India, for providing the necessary facilities to carry out this work.

FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

All authors made substantial contributions to conception and design, data acquisition, or data analysis and interpretation, took part in drafting the article or revising it critically for important intellectual content, agreed to submit it to the current journal, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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