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BIO ANALYTICAL LC-MS METHOD DEVELOPMENT AND VALIDATION FOR RAVULIZUMAB IN RAT PLASMA USING ECULIZUMAB (INTERNAL STANDARD)

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Abstract:

The objective of the study was to develop and validate simple, selective, specific Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS) method for the determination of ravulizumab and eculizumab(Internal Standard) in rat plasma. The accuracy and precision data must fulfill the requirements for the quantification of analytes in biological matrices to produce data for bioavailability, bioequivalence, etc. The separation of the analyte was carried out on Waters, X-Bridge, C18, 5µm column having 4.6×50 mm internal diameter and the mobile phase containing acetonitrile and 0.1 % formic acid (90:10 v/v) at a flow rate of 0.6 mL/min. The retention times of ravulizumab and eculizumab(Internal Standard) were 2.886 min and 4.439 min simultaneously and the total run time was 7.0 min. Monitoring of the fragmentation of m / z 473.54 → 157.6 performed during MS/MS detection of ravulizumab and eculizumab(I.S.) on the mass spectrometer. The overall recovery of ravulizumab and eculizumabwas 92.5 % and 89.9 % respectively. The matrix effect of ravulizumab and eculizumabwas 5.51 and 1.33 % respectively. The method was validated over the concentration range of 10ng/mL to 5000ng/mL. Multiple Reaction Monitoring (MRM) mode was used as an operating mode in the mass spectrometer. Ion spray was kept in positive mode for the detection of Analyte and IS during the production of ions. The method was validated for linearity, accuracy, precision, specificity, selectivity, inter and intraday precision, LQC, HQC.

***Key Words**: Ravulizumab, EculizumabBioavailability, Validation, Accuracy, LC-MS/MS.*

1. Introduction

Ravulizumab is a Monoclonal antibody complement inhibitor**,** final complement inhibitor that binds to C5 and prevents it from being cleaved into C5a (proinflammatory anaphylatoxin) and C5b (terminal complement). PNH (paroxysmal nocturnal hemoglobinuria) is an extremely rare blood disorder characterized by hemolysis (the loss of red blood cells) caused by uncontrolled complement

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activation, an immune system feature. Having molecular formula $C_{6430}H_{9888}N_{1696}O_{2028}S_{48}$and molecular mass 144938.56 g $mol^{-1.}$

Eculizumab belongs to a group of medicines called monoclonal antibody.Eculizumab is a typical hemolytic uremic syndrome is a type of hemolytic uremic syndrome stops the breakage of protein that complements C5 into C5a and C5b, as well as the development of C5b-9, the final complement complex. Complement-mediated intravascular hemolysis, complement-mediated microangiopathy, and immune-mediated inflammation and CNS damage are all prevented by inhibiting this complex in paroxysmal nocturnal hemoglobunuria, atypical hemolytic uremic syndrome, and neuromyelitisoptica spectrum disorder. Molecular weight:148 Kg mol^{-1}

2.Material and methods:

2.1 Materials:

Biological Source:

Buffered blank plasma was procured from rat for the putting together plasma quality management samples and calibration requirements. For specificity check experiment different samples of plasma were procured form Vivo Bio Tech Ltd., Hyderabad.

2.2 Method Optimization:

The objective was to develop suitable method that might give require accuracy, precision and specificity for Ravulizumab and Eculizumab at LQC level in rat plasma. The trial associated with optimization of following parameters.

2.2.1 Protein Precipitation:

Using a micro pipette, move 200µl of plasma sample into an Eppendrof tube (system suitability, zero requirements, calibration level, quality control samples, and validation samples). Now add 300 µlitres of organic solvent and vortex for 10 minutes, then add 500 µlitres of Ravulizumab normal stock, 500 µlitres of IS stock, and 500 µlitres of diluent vortex for 10 minutes. After centrifuging for 20 minutes at 5000 RPM, the resulting solution is used for analysis.

2.2.2. Optimization Process

The sections that follow describe a functional approach for improving analytical efficiency that organises the method collection, growth, and optimization phase.

The focus of this study is to evolve an easy-to-use and high-efficiency analytical method for the estimation of assays for the drug component Ravulizumab.

2.2.2.1 Selection of Mobile phase: The mobile process assisted the chemical properties of ravulizumab. The high ratio of organic modifiers and buffers used will affect peak elution. In this situation, the buffer's solubility in the organic phase would have a major effect on the analytical system.

2.2.2.2 Selection of Column: Following the selection of the appropriate mobile phase, the LC column was chosen by trial and error. The bio analytical LCMS method differs from the traditional HPLC method for analysing raw drugs. The matrix used in bioanalysis poses a challenge to LCMS analysts because, in the vast majority of cases, the matrix compounds co-elute with the analyte. To differentiate the interfering peak from the analyte, different stationery stages were used, such as cyano, amino, and nitrile. All other chromatographic conditions were kept constant during the operation, with the exception of the column.

2.2.2.3 Optimization of the final mobile phase: Final tuning with mobile phase composition and buffer concentration was done based on the retention time of the Ravulizumab with the IS after selection of the internal norm. The final mobile step was chosen so that the Ravulizumab, as well as the IS, could be eluted with appropriate peak separation.

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2.2.2.4 Optimization of flow rate: Following the optimization of the mobile phase composition, different flow rates are measured to ensure proper RT, peak asymmetry, and resolution for the drug and the IS. The finalised flow rate is measured using the RT, proper peak asymmetry, and resolution.

3.Validation of the Procedure:

The advanced technique was verified to meet the industrial advice for bio analytical technique acceptance criteria.

3.1 Specificity and Selectivity

At the eluting times of Ravulizumab and the ISTD, there are no interfering peaks were observed in 6-separate blank rat plasma samples at random

3.2 The effect of the matrix: The plasma matrices component in comparison to ionisation of analytes was calculated by comparing the response of post-extracted plasma standard QC samples (n=6) with analyte response from neat samples at similar levels of concentration. The intended method for the matrix effect was tested utilising rat plasma that had been chromatographically screened

3.3 Linearity

Over the concentration ranges of 1-20 ng/ml Ravulizumab, the normal the curves were straight. The correlation coefficient was 0.999 on average. The samples were quantified using the ratio of analyte peak area to IS peak area. Plasma concentrations were plotted against peak area ratios.

3.4 LOD and LOQ

The calibration curve approach was used to calculate LOD and LOQ separately. The compound's LOD and LOQ were determined by using an established LCMS method that involved injecting progressively lower concentrations of standard solutions. The LOD concentrations for Ravulizumab was 0.013 ng/ml (s/n = 6). The LOQ concentrations for Ravulizumab was 0.043 ng/ml, with s/n value of 27.

3.5 Accuracy and precision

The precision and accuracy of the intra-assay were determined by analysing 6-replicates containing Ravulizumab at six different QC levels. The precision of inter-assay comparisons was determined by checking the four stages of QC samples on four different plays. Except for LLQC, where accuracy and precision should be within 85-115 percent of actual values and 15 percent relative standard deviation (RSD), data acceptability requirements include accuracy within 85-115 percent of real values and precision within 15 percent RSD

3.6 Recovery of analyte

At three different concentration levels, the drug and IS recovery were tested (low, medium, and high-quality control). To test recovery, replicate sample responses were compared to neat standard solution responses. Extraction efficiency is defined as the ratio of systematic reaction from the calculation of the sample matrix after the addition of analyte.

3.7 Ruggedness on precision accuracy: The %CV for Ravulizumab passed the Ruggedness on precision accuracy.

4.Stability

4.1 Bench Top Stability: It passed the Bench top stability.

Auto Sampler Stability: It passed the Auto Sampler Stability.

4.2Freeze Thaw: It passed the freeze thaw stability.

4.3Wet Extract: It passed the Wet Extract stability.

4.4 Dry Extract: It passed the Dry Extract stability

4.5 Short term Stability: It passed the short-term stability.

Long term Stability: The %CV and mean accuracy for Ravulizumab was found to be within the acceptable limit. Hence it passed the long-term stability.

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Results and discussion

Optimization of flow rate: Following the optimization of the mobile phase composition, different flow rates are measured to ensure proper RT, peak asymmetry, and resolution for the drug and the IS. The finalisedflow rate is measured using the RT, proper peak asymmetry, and resolution.

Table1: Optimized liquid chromatography and mass spectroscopic conditions

LC parameters		MS parameters	
UPLC	Waters Acquity	MS	Sciex QTRAP 5500
Isocratic step mobile	ACN: 0.1percent Formic acid in the ratio of 40:60	Ionization source	Drying gas: N_2 gas Drying flow: 5 ml per min Pressure: 55 psi
	Flow rate: 1 mL per min		Source temperature: 550°C
	Injection vol: 10µl		Capillary voltage: 5500V
X-bridge phenyl	150mm length	Collision gas	Nitrogen with high purity
	4.6 mm diameter	Mode	MRM[b]
	3.5 µm PS		

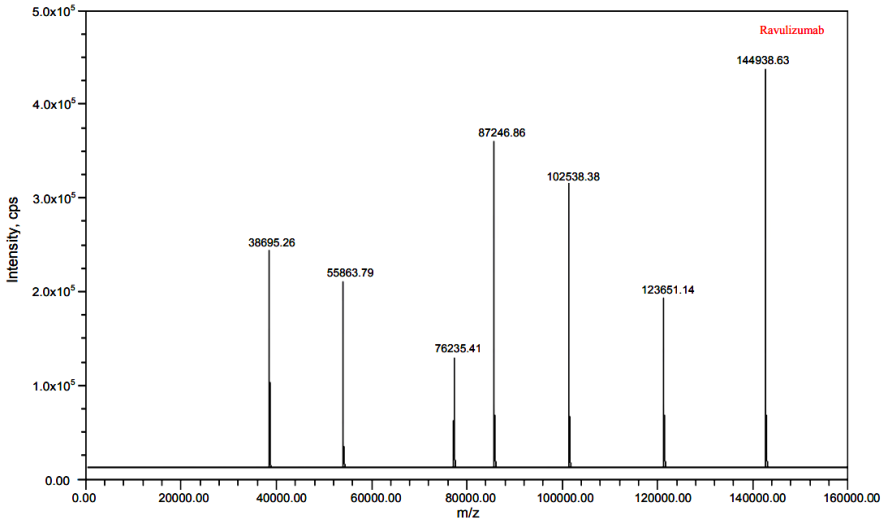

Fig.1: Ravulizumab MS Spectra

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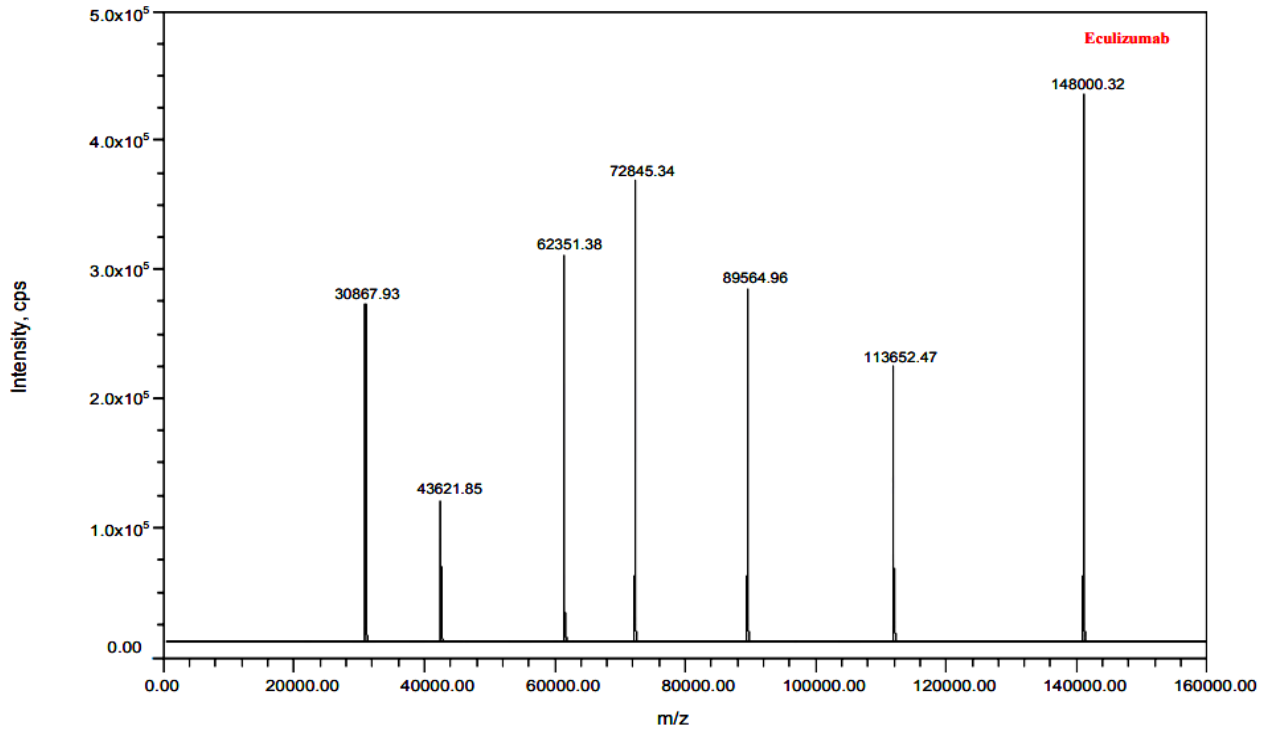

Fig.2: Eculizumab MS Spectra

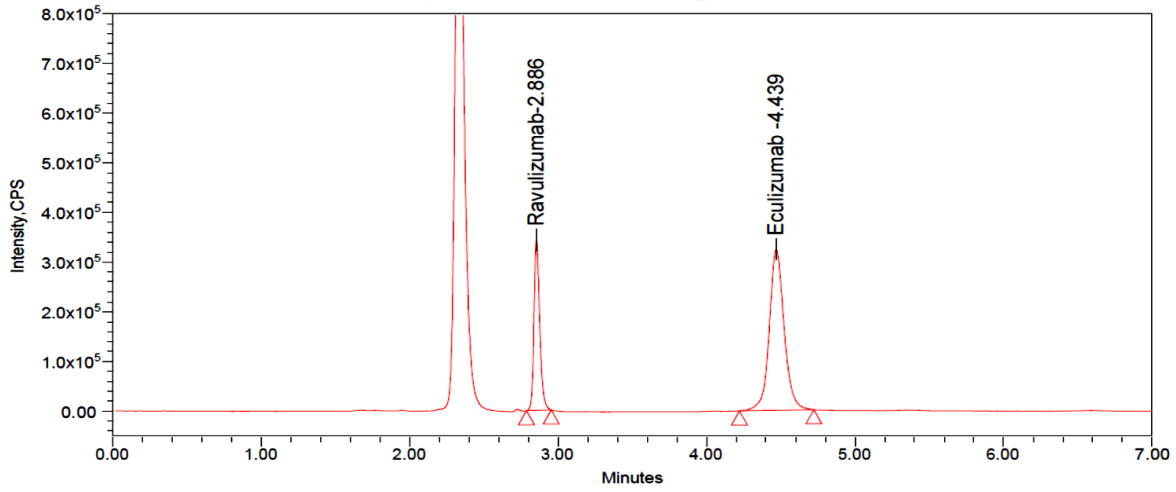

Fig.3: Chromatogram of standard

Validation of the Procedure:

The advanced technique was verified to meet the industrial advice for bio analytical technique acceptance criteria.

Specificity and Selectivity

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At the eluting times of Ravulizumab and the , there are no interfering peaks were observed in 6-separate blank rat plasma samples at random.

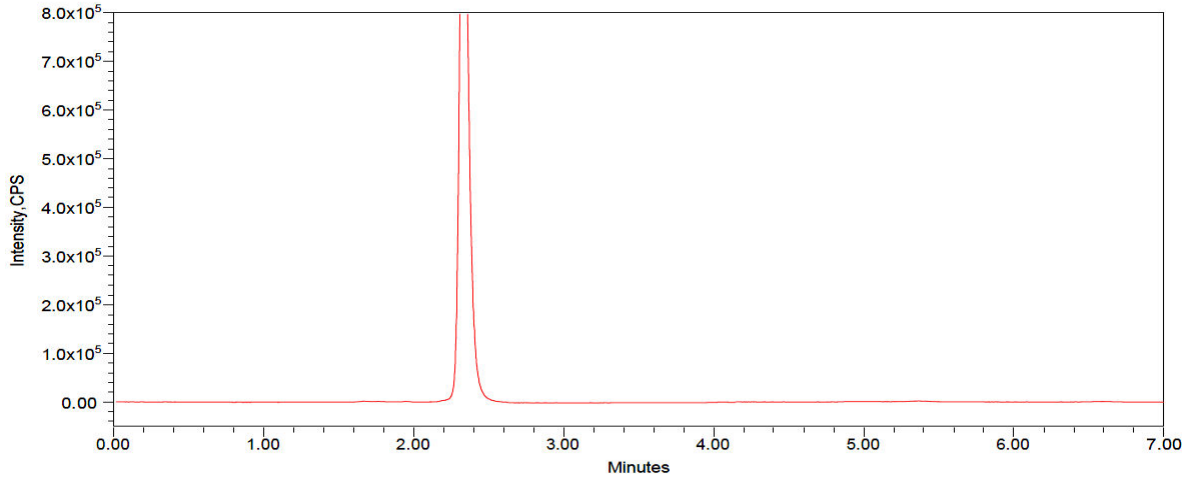

Fig.4: Chromatogram for Blank rat plasma

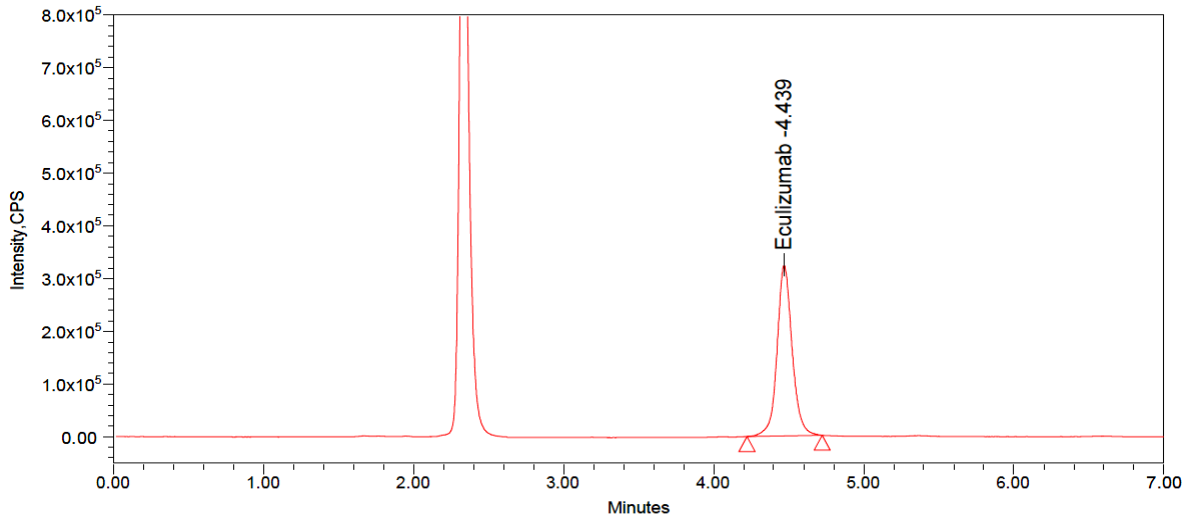

Fig. 5: Chromatogram for Blank-IS

Table 2: System precision outcomes of Ravulizumab

Samlple Name MQC (10ng/ml)	Analyte intensity (cps)	Analyte RT (min)	Eculizumab intensity (10ng/ml)	Eculizumab RT (min)	Area Ratio
MQC-1	$3.521x10^5$	2.881	$3.334x10^5$	4.436	0.9469
MQC-2	$3.584x10^5$	2.886	$3.364x10^5$	4.439	0.9375
MQC-3	$3.562x10^5$	2.889	$3.35x10^5$	4.442	0.9405
MQC-4	$3.581x10^5$	2.892	$3.350x10^5$	4.446	0.9355
MQC-5	$3.59x10^5$	2.896	$3.320x10^5$	4.446	0.9248
MQC-6	$3.53x10^5$	2.898	$3.334x10^5$	4.451	0.9445
Mean	$3.561x10^5$	2.89	$3.342x10^5$	4.443	0.9383
Std Dev	0.02943	0.00635	0.01565	0.00543	0.00785
%CV	0.83	0.22	0.47	0.12	0.84

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Table3: Sensitivity outcomes of Ravulizumab

	Ravulizumab
Recreate Number	LLOQ
	Nominal Concentration (ng/ml)
	1.325
	Analyte peak area
1	$0.352x10^5$
2	$0.345x10^5$
3	$0.355x10^5$
4	$0.358x10^5$
5	$0.353x10^5$
6	$0.359x10^5$
N	6
Mean	$0.354x10^5$
SD	0.00505
% CV	1.43
% Mean Accuracy	99.41%

Table 4: Matrix effect Results of Ravulizumab(HQC-15ng/ml, LQC-5ng/ml)

S.No.	Plasma Lot No.	HQC	LQC
		Nominal Concentration(ng/ml)	
		15.258	5.647
		Analyte peak area	
1.	Lot 1	$5.281x10^5$	$1.754x10^5$
		$5.297x10^5$	$1.795x10^5$
		$5.264x10^5$	$1.735x10^5$
2.	Lot 2	$5.281x10^5$	$1.725x10^5$
		$5.25x10^5$	$1.79x10^5$
		$5.24x10^5$	$1.768x10^5$
3.	Lot 3	$5.254x10^5$	$1.798x10^5$
		$5.297x10^5$	$1.726x10^5$
		$5.287x10^5$	$1.700x10^5$
4.	Lot 4	$5.281x10^5$	$1.711x10^5$
		$5.251x10^5$	$1.779x10^5$
		$5.284x10^5$	$1.769x10^5$
5.	Lot 5	$5.23x10^5$	$1.768x10^5$
		$5.222x10^5$	$1.779x10^5$
		$5.203x10^5$	$1.719x10^5$
6.	Lot 6	$5.287x10^5$	$1.735x10^5$
		$5.257x10^5$	$1.78x10^5$
		$5.291x10^5$	$1.728x10^5$
N		18	18
Mean		$5.264x10^5$	$1.753x10^5$

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Std Dev	0.02761	0.03112
%CV	0.52	1.77
% Mean Accuracy	98.55%	98.46%
Number of QCs that Failed	0	0

Linearity

Table 5: Stock solution Preparation

Standard	Drug taken	Acetonitrile added	Further diluted	Further diluted	Final stock concentration
Ravulizumab	5 mg	100 ml	0.8 ml/10 ml	0.1 ml/10 ml	40 ng/ml

Table 6: Ravulizumab working stock solution preparation for regular curve

Linearity	Plasma (µl)	ACN (µl)	Std Stock (µl)	IS (µl)	Diluted to ml (µl)	RavulizumabConc (ng/ml)	Ravulizumab response	Area res ratio
Linearity-10%	200	1250	50	500	2000	1.00	0.356	0.105
Linearity-25%	200	1175	125	500	2000	2.50	0.875	0.262
Linearity-50%	200	1050	250	500	2000	5.00	1.754	0.522
Linearity-75%	200	925	375	500	2000	7.50	2.537	0.755
Linearity-100%	200	800	500	500	2000	10.00	3.554	1.069
Linearity-125%	200	675	625	500	2000	12.50	4.251	1.276
Linearity-150%	200	550	750	500	2000	15.00	5.253	1.558
Linearity-200%	200	300	1000	500	3000	20.00	7.105	2.099
Slope						0.1038		
Intercept						0.00118		
r square						0.99917		

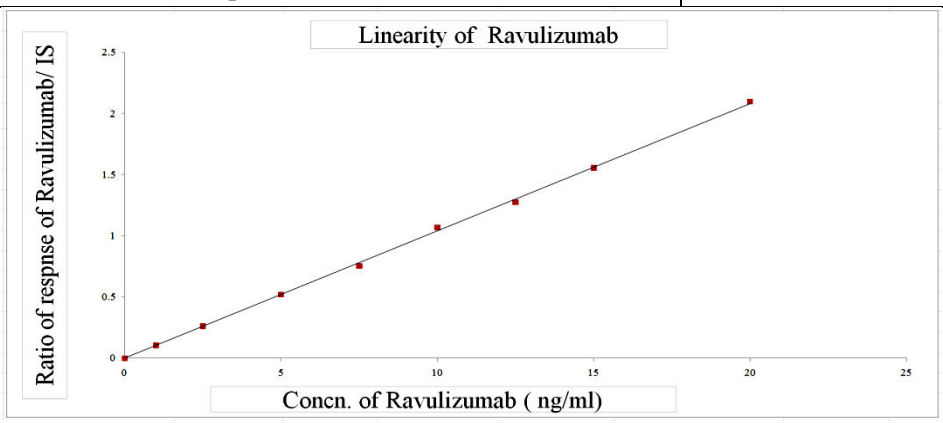

Fig.6: Calibration plot for concentration vs area ratio of Ravulizumab

LOD and LOQ

Table 7: LOD and LOQ data

Name	LOD		LOQ	
	Conc.(ng/ml)	s/n	Conc. (ng/ml)	s/n
Ravulizumab	0.013	6	0.043	27

Accuracy and precision

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Table 8: Precision and accuracy Results of Ravulizumab

Drug name	HQC	MQC	LQC	LLQC
	Nominal Concentration (ng/ml)			
	15.257	10.158	5.652	1.854
	Analyte peak area			
Ravulizumab	$5.280x10^5$	$3.561x10^5$	$1.740x10^5$	$0.369x10^5$
	$5.251x10^5$	$3.561x10^5$	$1.796x10^5$	$0.365x10^5$
	$5.280x10^5$	$3.587x10^5$	$1.784x10^5$	$0.389x10^5$
	$5.282x10^5$	$3.597x10^5$	$1.790x10^5$	$0.367x10^5$
	$5.231x10^5$	$3.520x10^5$	$1.778x10^5$	$0.334x10^5$
	$5.250x10^5$	$3.523x10^5$	$1.735x10^5$	$0.300x10^5$
N	6	6	6	6
Mean	$5.262x10^5$	$3.558x10^5$	$1.771x10^5$	$0.354x10^5$
SD	0.02132	0. 03178	0.02630	0.0318
% CV	0.41	0.89	1.49	8.98
% Mean Accuracy	98.51%	99.92%	99.47%	99.41%

Recovery of analyte

Table 9: Recovery of analyte of RavulizumabHQC (15 ng/ml)

Replicate Number	HQC (15 ng/ml)		
	Extracted Response	Un Extracted Response	Matrix Factor
1.	$5.259x10^5$	$5.338x10^5$	0.9852
2.	$5.213x10^5$	$5.387x10^5$	0.9677
3.	$5.250x10^5$	$5.346x10^5$	0.9820
4.	$5.294x10^5$	$5.391x10^5$	0.9820
5.	$5.251x10^5$	$5.386x10^5$	0.9749
6.	$5.290x10^5$	$5.383x10^5$	0.9827
N	6	6	6
Mean	$5.26x10^5$	$5.372x10^5$	0.9791
SD	0.02982	0.02339	0.00655
%CV	0.57	0.44	0.67
%Mean Recovery	98.47%	100.57%	-

Table 10: Recovery of analyte of RavulizumabMQC(10 ng/ml)

Rep No.	MQC(10 ng/ml)		
	Extracted Response	Un Extracted Response	Matrix Factor
1.	$3.592x10^5$	$3.686x10^5$	0.9745
2.	$3.598x10^5$	$3.634x10^5$	0.9901
3.	$3.507x10^5$	$3.668x10^5$	0.9561

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4.	$3.567x10^5$	$3.675x10^5$	0.9706
5.	$3.511x10^5$	$3.600x10^5$	0.9753
6.	$3.578x10^5$	$3.635x10^5$	0.9843
N	6	6	6
Mean	$3.559x10^5$	$3.65x10^5$	0.9752
SD	0.04011	0.03233	0.01176
%CV	1.13	0.89	1.21
%Mean Recovery	99.94%	102.5%	-

Table 11: Recovery of analyte of Ravulizumab LQC (5 ng/ml)

LQC (5 ng/ml)		
Extracted Response	Un Extracted Response	Matrix Factor
$1.767x10^5$	$1.800x10^5$	0.9817
$1.762x10^5$	$1.803x10^5$	0.9773
$1.706x10^5$	$1.806x10^5$	0.9446
$1.756x10^5$	$1.857x10^5$	0.9456
$1.749x10^5$	$1.838x10^5$	0.9516
$1.784x10^5$	$1.893x10^5$	0.9424
6	6	6
$1.754x10^5$	$1.833x10^5$	0.9572
0.02633	0.03720	0.0176
1.5	2.03	1.84
98.16%	105.14%	-

Ruggedness on precision accuracy: The %CV for Ravulizumab passed the Ruggedness on precision accuracy.

Table 12: Ruggedness on precision accuracy of Results of Ravulizumab

P& A ID	Acquisition Batch ID	HQC (15 ng/ml)	MQC (10ng/ml)	(Q)	LQC (5ng/ml)
		Nominal Concentration (ng/ml)			
		15.627	10.385		5.269
		Analyte peak area			
Different Column		$5.25x10^5$	$3.550x10^5$		$1.720x10^5$
		$5.23x10^5$	$3.560x10^5$		$1.786x10^5$
		$5.284x10^5$	$3.554x10^5$		$1.738x10^5$
		$5.281x10^5$	$3.521x10^5$		$1.795x10^5$
		$5.277x10^5$	$3.597x10^5$		$1.750x10^5$
		$5.264x10^5$	$3.582x10^5$		$1.795x10^5$
n		6	6		6
Mean		$5.264x10^5$	$3.561x10^5$		$1.764x10^5$
SD		0.02102	0.02649		0.03229

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% CV	0.4	0.74		1.83
% Mean Accuracy	98.55%	100.00%		99.07%

Stability

Bench Top Stability: It passed the Bench top stability.

Table 13: RavulizumabBenchtop Stability

Replicate No.	HQC	LQC	MQC
	Nominal Conc. (ng/ml)		
	15.216	5.652	10.582
	Peak region of the analyte		
1	$5.210x10^5$	$1.724x10^5$	$3.581x10^5$
2	$5.230x10^5$	$1.706x10^5$	$3.587x10^5$
3	$5.241x10^5$	$1.754x10^5$	$3.587x10^5$
4	$5.261x10^5$	$1.755x10^5$	$3.520x10^5$
5	$5.212x10^5$	$1.778x10^5$	$3.521x10^5$
6	$5.250x10^5$	$1.792x10^5$	$3.584x10^5$
n	6	6	6
Mean	$5.234x10^5$	$1.752x10^5$	$3.563x10^5$
SD	0.02054	0.03221	0.03325
%CV	0.39	1.84	0.93
% Mean Accuracy	97.99%	98.40%	100.06%

Auto Sampler Stability: It passed the Auto Sampler Stability.

Table 14: Auto Sampler Stability of Ravulizumab

Replicate No.	HQC	MQC	LQC
	Nominal Concentration (ng/ml)		
	15.547	10.329	5.565
	Analyte peak area		
1	$5.251x10^5$	$3.510x10^5$	$1.728x10^5$
2	$5.234x10^5$	$3.540x10^5$	$1.772x10^5$
3	$5.297x10^5$	$3.531x10^5$	$1.743x10^5$
4	$5.227x10^5$	$3.527x10^5$	$1.776x10^5$
5	$5.297x10^5$	$3.572x10^5$	$1.725x10^5$
6	$5.231x10^5$	$3.520x10^5$	$1.725x10^5$
7	$5.282x10^5$	$3.594x10^5$	$1.784x10^5$
8	$5.234x10^5$	$3.535x10^5$	$1.715x10^5$
9	$5.220x10^5$	$3.572x10^5$	$1.762x10^5$
10	$5.297x10^5$	$3.572x10^5$	$1.754x10^5$
11	$5.211x10^5$	$3.594x10^5$	$1.768x10^5$
12	$5.231x10^5$	$3.534x10^5$	$1.783x10^5$
13	$5.294x10^5$	$3.534x10^5$	$1.734x10^5$
14	$5.220x10^5$	$3.520x10^5$	$1.781x10^5$
15	$5.297x10^5$	$3.527x10^5$	$1.778x10^5$
16	$5.220x10^5$	$3.522x10^5$	$1.759x10^5$
17	$5.220x10^5$	$3.589x10^5$	$1.751x10^5$

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18	$5.273x10^5$	$3.594x10^5$	$1.708x10^5$
19	$5.284x10^5$	$3.597x10^5$	$1.791x10^5$
20	$5.223x10^5$	$3.520x10^5$	$1.718x10^5$
21	$5.250x10^5$	$3.591x10^5$	$1.744x10^5$
22	$5.240x10^5$	$3.520x10^5$	$1.738x10^5$
23	$5.274x10^5$	$3.513x10^5$	$1.783x10^5$
24	$5.204x10^5$	$3.567x10^5$	$1.747x10^5$
N	24	24	24
Mean	$5.250x10^5$	$3.552x10^5$	$1.753x10^5$
SD	0.03222	0.03083	0.02514
%CV	0.61	0.87	1.43
% Mean Accuracy	98.29%	99.75%	98.46%

Table 15: Freeze Thaw Stability of Ravulizumab

Replicate No's	HQC	LQC	MQC
	Nominal Conc. (ng/ml)		
	15.528	5.241	10.358
	Peak region of the analyte		
1	$5.230x10^5$	$1.754x10^5$	$3.549x10^5$
2	$5.272x10^5$	$1.786x10^5$	$3.564x10^5$
3	$5.243x10^5$	$1.784x10^5$	$3.534x10^5$
4	$5.230x10^5$	$1.767x10^5$	$3.594x10^5$
5	$5.271x10^5$	$1.778x10^5$	$3.597x10^5$
6	$5.282x10^5$	$1.721x10^5$	$3.528x10^5$
n	6	6	6
Mean	$5.255x10^5$	$1.765x10^5$	$3.561x10^5$
SD	0.02310	0.02463	0.02952
%CV	0.44	1.40	0.83
% Mean Accuracy	98.38%	99.13%	100.00%

Table 16: Ravulizumab Wet Extract Stability at 12 Hours

Replicate No.	HQC	LQC	MQC
	Nominal Conc. (ng/ml)		
	15.256	5.482	10.475
	Peak region of the analyte		
1	$5.291x10^5$	$1.743x10^5$	$3.534x10^5$
2	$5.231x10^5$	$1.786x10^5$	$3.569x10^5$
3	$5.227x10^5$	$1.765x10^5$	$3.594x10^5$
4	$5.267x10^5$	$1.794x10^5$	$3.534x10^5$
5	$5.220x10^5$	$1.758x10^5$	$3.530x10^5$
6	$5.267x10^5$	$1.784x10^5$	$3.594x10^5$
n	6	6	6
Mean	$5.251x10^5$	$1.772x10^5$	$3.559x10^5$
SD	0.02845	0.01954	0.03047
%CV	0.54	1.10	0.86

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% Mean Accuracy	98.31%	99.52%	99.94%

Table 17: Ravulizumab Wet Extract Stability at 18 Hours

Replicate No.	HQC	LQC	MQC
	Nominal Concentration(ng/ml)		
	15.228	5.605	10.741
	Peak region of the analyte		
1	$5.291x10^5$	$1.756x10^5$	$3.564x10^5$
2	$5.292x10^5$	$1.781x10^5$	$3.520x10^5$
3	$5.297x10^5$	$1.716x10^5$	$3.569x10^5$
4	$5.230x10^5$	$1.758x10^5$	$3.591x10^5$
5	$5.251x10^5$	$1.729x10^5$	$3.589x10^5$
6	$5.297x10^5$	$1.789x10^5$	$3.528x10^5$
n	6	6	6
Mean	$5.276 x10^5$	$1.755x10^5$	$3.56x10^5$
SD	0.02865	0.02842	0.03008
%CV	0.54	1.62	0.84
% Mean Accuracy	98.77%	98.57%	99.97%

Dry Extract: It passed the Dry Extract stability.

Table 18: Dry Extract Stability of Ravulizumab at 12 Hr

Replicate No.	HQC	LQC	MQC
	Nominal Conc.(ng/ml)		
	15.528	5.244	10.329
	Peak region of the analyte		
1	$5.297x10^5$	$1.720x10^5$	$3.534x10^5$
2	$5.271x10^5$	$1.787x10^5$	$3.579x10^5$
3	$5.230x10^5$	$1.700x10^5$	$3.520x10^5$
4	$5.287x10^5$	$1.765x10^5$	$3.524x10^5$
5	$5.251x10^5$	$1.708x10^5$	$3.514x10^5$
6	$5.230x10^5$	$1.769x10^5$	$3.594x10^5$
n	6	6	6
Mean	$5.261x10^5$	$1.742x10^5$	$3.544x10^5$
SD	0.02861	0.03657	0.03377
%CV	0.54	2.10	0.95
% Mean Accuracy	98.49%	97.84%	99.52%

Table 19: Dry Extract Stability of Ravulizumab at 18 Hrs

Replicate No.	HQC	LQC	MQC
	Nominal Conc.(ng/ml)		
	15.264	5.166	10.852
	Peak region of the analyte		
1	$5.261x10^5$	$1.785x10^5$	$3.567x10^5$
2	$5.261x10^5$	$1.734x10^5$	$3.594x10^5$
3	$5.234x10^5$	$1.735x10^5$	$3.587x10^5$

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4	$5.265x10^5$	$1.780x10^5$	$3.591x10^5$
5	$5.213x10^5$	$1.730x10^5$	$3.574x10^5$
6	$5.267x10^5$	$1.713x10^5$	$3.524x10^5$
n	6	6	6
Mean	$5.250x10^5$	$1.746x10^5$	$3.573x10^5$
SD	0.02182	0.02928	0.02607
%CV	0.42	1.68	0.73
% Mean Accuracy	98.29%	98.06%	100.34%

Short term Stability: It passed the Short term stability.

Table 20: Short term Stability of Ravulizumab

Replicate No.	HQC	LQC	MQC
	Nominal Conc.(ng/ml)		
	15.458	5.217	10.257
	Peak region of the analyte		
1	$5.184x10^5$	$1.702x10^5$	$3.425x10^5$
2	$5.186x10^5$	$1.709x10^5$	$3.426x10^5$
3	$5.183x10^5$	$1.703x10^5$	$3.421x10^5$
4	$5.184x10^5$	$1.708x10^5$	$3.425x10^5$
5	$5.188x10^5$	$1.702x10^5$	$3.423x10^5$
6	$5.185x10^5$	$1.703x10^5$	$3.428x10^5$
n	6	6	6
Mean	$5.185x10^5$	$1.705x10^5$	$3.425x10^5$
SD	0.00179	0.00315	0.00242
%CV	0.03	0.18	0.07
% Mean Accuracy	97.07%	95.76%	96.19%

Long term Stability: The %CV and mean accuracy for Ravulizumab was found to be within the acceptable limit. Hence it passed the Long term stability.

Table 21: Long term Stability of Ravulizumab at Day 1

Replicate No.	HQC	LQC	MQC
	Nominal Conc.(ng/ml)		
	15.265	5.274	10.538
	Peak region of the analyte		
1	$5.256x10^5$	$1.758x10^5$	$3.556x10^5$
2	$5.254x10^5$	$1.753x10^5$	$3.558x10^5$
3	$5.258x10^5$	$1.754x10^5$	$3.551x10^5$
4	$5.255x10^5$	$1.752x10^5$	$3.554x10^5$
5	$5.259x10^5$	$1.759x10^5$	$3.557x10^5$
6	$5.251x10^5$	$1.755x10^5$	$3.553x10^5$
n	6	6	6
Mean	$5.256x10^5$	$1.755x10^5$	$3.555x10^5$
SD	0.00288	0.00279	0.00264
%CV	0.05	0.16	0.07
% Mean Accuracy	98.40%	98.57%	99.83%

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Table 22: Long term Day-7 Stability of Ravulizumab

Replicate No.	HQC	LQC	MQC
	Nominal Conc.(ng/ml)		
	15.658	5.147	10.522
	Peak region of the analyte		
1	$5.174x10^5$	$1.698x10^5$	$3.415x10^5$
2	$5.173x10^5$	$1.692x10^5$	$3.412x10^5$
3	$5.179x10^5$	$1.693x10^5$	$3.427x10^5$
4	$5.174x10^5$	$1.695x10^5$	$3.422x10^5$
5	$5.172x10^5$	$1.699x10^5$	$3.429x10^5$
6	$5.175x10^5$	$1.694x10^5$	$3.426x10^5$
n	6	6	6
Mean	$5.175x10^5$	$1.695x10^5$	$3.422x10^5$
SD	0.00243	0.00279	0.00691
%CV	0.05	0.16	0.20
% Mean Accuracy	96.88%	95.20%	96.11%

Table 23: Long term Day-14 Stability of Ravulizumab

Replicate No.	HQC	LQC	MQC
	Nominal Conc.(ng/ml)		
	15.581	5.652	10.728
	Peak region of the analyte		
1	$5.024x10^5$	$1.629x10^5$	$3.341x10^5$
2	$5.029x10^5$	$1.626x10^5$	$3.346x10^5$
3	$5.022x10^5$	$1.621x10^5$	$3.349x10^5$
4	$5.021x10^5$	$1.624x10^5$	$3.345x10^5$
5	$5.027x10^5$	$1.629x10^5$	$3.344x10^5$
6	$5.023x10^5$	$1.631x10^5$	$3.346x10^5$
n	6	6	6
Mean	$5.024\ x10^5$	$1.627x10^5$	$3.345x10^5$
SD	0.00308	0.00372	0.00264
%CV	0.06	0.23	0.08
% Mean Accuracy	94.06%	91.38%	93.93%

Table 24: Long term Day-21 Stability of Ravulizumab

Replicate No.	HQC	LQC	MQC
	Nominal Conc.(ng/ml)		
	15.547	5.625	10.865
	Peak region of the analyte		
1	$4.907x10^5$	$1.576x10^5$	$3.198x10^5$
2	$4.905x10^5$	$1.574x10^5$	$3.196x10^5$
3	$4.906x10^5$	$1.578x10^5$	$3.194x10^5$
4	$4.902x10^5$	$1.575x10^5$	$3.190x10^5$
5	$4.901x10^5$	$1.579x10^5$	$3.197x10^5$

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6	$4.903x10^5$	$1.578x10^5$	$3.192x10^5$
n	6	6	6
Mean	$4.904x10^5$	$1.577x10^5$	$3.195x10^5$
SD	0.00237	0.00197	0.00308
%CV	0.05	0.12	0.10
% Mean Accuracy	91.81%	88.57%	89.72%

Table 25: Long term Day-28 Stability of Ravulizumab

Replicate No.	HQC	LQC	MQC
	Nominal Conc.(ng/ml)		
	15.521	5.628	10.745
	Peak region of the analyte		
1	$4.734x10^5$	$1.498x10^5$	$3.106x10^5$
2	$4.755x10^5$	$1.492x10^5$	$3.108x10^5$
3	$4.777x10^5$	$1.495x10^5$	$3.100x10^5$
4	$4.794x10^5$	$1.493x10^5$	$3.106x10^5$
5	$4.759x10^5$	$1.499x10^5$	$3.106x10^5$
6	$4.753x10^5$	$1.494x10^5$	$3.103x10^5$
n	6	6	6
Mean	$4.762\ x10^5$	$1.495x10^5$	$3.105x10^5$
SD	0.02084	0.00279	0.00286
%CV	0.44	0.19	0.09
% Mean Accuracy	89.15%	83.97%	87.19%

Discussion

By performing the trails, the optimized method was developed on LC-MS system the conditions were in LC Waters Acquity with mobile phase ACN: 0.1percent Formic acid in the ratio of 35:65 with Flow rate of 1 mL/min and Injection volume 10µl. column of XBridge BEH Phenyl Column, 130Å, 3.5 µm, 4.6 mm X 150. MS parameters MS Sciex QTRAP 5500 Drying gas: N2 gas Drying flow: 5 ml per min with Pressure: 55 psi Source temperature: 550°C Capillary voltage: 5500V Collision gas Nitrogen with high purity ModeMRMb.the method is specific and selective at the eluting times of Ravulizumab and the ISTD, there are no interfering peaks were observed. The period of retention % CV (RT) ought to be ≤ 2.00 %. The intensity ratio % The field ratio's CV should be ≤ 5.00 % At least 67 % (4 out of 6) of samples should be within 80.00-120.00 %. % The average accuracy should be between 80.00 and 120.00 percent. At the very least, 67 percent (2 out of 3) of samples should fall within this range 85-115 percent at each point. At least 80% a matrix tone should be satisfying the criterion for acceptance. Back measured concentration accuracy for LQC and HQC samples prepared from the intended method for the matrix effect was tested utilizing rat plasma that had been chromatographically screened Over the concentration ranges of 1-20 ng/ml Ravulizumab, the normal the curves were straight. The correlation coefficient was 0.999 on average. The samples were quantified using the ratio of analyte peak area to IS peak area. Plasma

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concentrations were plotted against peak area ratios. different biological matrix lots should be 85 to 115 percent. The calibration curve approach was used to calculate LOD and LOQ separately. The compound's LOD and LOQ were determined by using an established LCMS method that involved injecting progressively lower concentrations of standard solutions. The LOD concentrations for Ravulizumab was 0.013 ng/ml (s/n = 6). The LOQ concentrations for Ravulizumab was 0.043 ng/ml, with s/n value of 27. The precision and accuracy of the intra-assay were determined by analyzing 6-replicates containing Ravulizumab at six different QC levels. The precision of inter-assay comparisons was determined by checking the four stages of QC samples on four different plays. Except for LLQC, where accuracy and precision should be within 85-115 percent of actual values and 15 percent relative standard deviation (RSD), data acceptability requirements include accuracy within 85-115 percent of real values and precision within 15 percent RSD. At three different concentration levels, the drug and IS recovery were tested (low, medium, and high-quality control). To test recovery, replicate sample responses were compared to neat standard solution responses. Extraction efficiency is defined as the ratio of systematic reaction from the calculation of the sample matrix after the addition of analyte. At each QC stage and for Internal standard, the percent RSD of recovery should be less than 15%. The average mean recovery percent RSD for all QC levels should be less than 20%. Between 85.00-115.00 percent for at least 67 percent (8 out of 12) of 50 QC samples in total percent (3 out of 6) at each standard. LQC and HQC should have a percent mean accuracy of 85.00-115.00 percent. LQC and HQC samples should have a percent CV of less than 15%.

Conclusion:

The proposed method of LC-MS/MS has proved to be simple, sensitive, accurate, precise and reliable. The method is specific due to the selectivity of the mass spectrometry. So, the proposed study is proved to apply this method for the estimation of ravulizumab in rat plasma.

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