Original Research Article

Bio-Analytical Method Development and Validation of Avelumab, Axitinib and its Application to Pharmacokinetic Studies In Rabbit Plasma By Using UPLC

ABSTRACT

Aims: New validated bio analytical method for the estimation of Avelumab and Axitinib using UPLC and its application to Pharmacokinetic studies.
Place and Duration of Study: Department of Engineering Chemistry, AUCE (A), Visakhapatnam, Andhra Pradesh, between September 2021 to February 2022.
Methodology: This article summarizes the recent progress on bioanalytical LC-MS/MS methods using C_{18} column (50x2.1 mm, 1.7µ) and organic mobile phase of 0.1% formic acid and Acetonitrile in 70:30 ratio.
Results: The calibration curve was linear in the range of 5-100 ng/ml for avelumab and 0.5-50 ng/ml axitnib. Accuracy, precision, recovery, matrix effect and stability results were found to be within the suitable limits. Simple and efficient method was developed and utilized in pharmacokinetic studies to see the investigated analyte in body fluids.
Conclusion: The application denotes all the parameters of system suitability, specificity, linearity and accuracy are in good agreement with USFDA guidelines and applied effectively for the investigation of pharmacokinetic studies in rabbit.

Keywords: Avelumab, Axitinib, Rabbit plasma, Validation, RP-UPLC.

1. INTRODUCTION

Avelumab, sold under the brand name Bavencio, is a fully human monoclonal antibody [1, 2] medication for the treatment of Merkel cell carcinoma [3, 4], urothelial carcinoma [5], and renal cell carcinoma [6, 7]. Common side effects include fatigue, musculoskeletal pain [8], diarrhea [9], nausea, infusion-related reactions, rash, decreased appetite and swelling of the limbs (peripheral edema) [10]. Avelumab targets the protein programmed death-ligand 1 (PD-L1) [11, 12]. It has received orphan drug designation by the European Medicines Agency (EMA) for the treatment of gastric cancer [13, 14]. The most common serious adverse reactions to avelumab are immune-mediated adverse reactions (pneumonitis [15], hepatitis [16], colitis [17], adrenal insufficiency, hypo- and hyperthyroidism [18], diabetes mellitus [19], and nephritis) and life-threatening infusion reactions. Patients who experience severe or life-threatening infusion-related reactions should stop using avelumab. Women who are pregnant or breastfeeding should not take avelumab because it may cause harm to a developing fetus or a newborn baby.

Axitinib, sold under the brand name Inlyta, is a small molecule tyrosine kinase inhibitor [20, 21] developed by Pfizer. It has been shown to significantly inhibit growth of breast cancer [22] in animal (xenograft) models [23] and has shown partial responses in clinical trials with renal cell carcinoma (RCC) and several other tumour types [24] Diarrhea, hypertension [25], fatigue, decreased appetite, nausea, dysphonia, hand-foot syndrome [26], weight decreased, vomiting, asthenia, and constipation are the most common side effects occurring

in more than 20% of patients. Co-administration with strong CYP3A4/CYP3A5 [27] inhibitors should be avoided where possible as they may reduce plasma clearance of axitinib.

To date, there have been no bio analytical UPLC methods for Avelumab and Axitinib estimation. Thus, the goal of the study is to predict Avelumab and Axitinib, which is a pharmaceutical component, using RP-UPLC.

2. MATERIAL AND METHODS

2.1 Chemicals and Reagents

Acetonitrile, HPLC-grade formic acid, water were purchased from Merck India Ltd, Mumbai, India. APIs of Avelumab and Axitinib standards were procured from Glenmark, Mumbai.

2.2 Equipment

Agilent1290 Infinity II LC System with quaternary pump, PDA detector with empower 2.0 software was used.

2.3 Chromatographic Conditions

To conduct chromatography using isocratic conditions, an C_{18} (50 mm x 2.1 mm, 1.7 µ) column was utilized at temperature using a Chromatographic conditions separation was administered in isocratic mode at temperature employing C_{18} (50 mm x 2.1 mm, 1.7 µ) column. Formic acid (0.1%) and acetonitrile (70:30 v/v) with a flow rate of 0.5 mL/min were used as a mobile phase in this experiment. Injection volume was 5 µl, and the eluent was found at 220 nm, as the maximum concentration of Avelumab and Axitinib were found at this wavelength. So, it was decided to use the wave length of 220 nm.

2.4 Preparation of standard solutions

2.4.1 Preparation of standard stock solution

Take 5mg each of Avelumab and Axitinib working standards were taken into a 100ml volumetric flask and 70ml of diluents and sonicate for 10minutes to dissolve the contents completely and make up to the mark with diluent. Further dilute by taking 4ml into 100ml volumetric flask. From the above solution 1ml of the solution is taken into the 10ml volumetric flask and make up to the mark with the diluent.

2.4.2 Preparation of internal standard stock solution

Take 5mg internal standard of Daunorubicin into a 100ml volumetric flask and make up to the mark with diluent and sonicate for ten minutes to dissolve the contents completely. From this solution take 4ml of solution into 100ml volumetric flask. From the above solution 1ml is taken into the 10ml volumetric flask and make up to the mark with the diluent.

2.4.3 Preparation of standard solution

For standard preparation 200µl of plasma was taken and 300µl of acetonitrile into a 2ml centrifuge tube and 500µl of standard stock solutions and 500µl of IS and 500 µl of diluents were added and vortexed for 10 min. These samples further subjected for centrifuge at 5000rpm for 30 min. Collect the solution and filter through 0.45µ nylon syringe filter and the clear solution was transferred into vial and injected into a system.

2.5 Pharmacokinetic Study

Before experimentation all animals are starved overnight and had water ad-libitum. Topical anesthetic procedure was used. Pharmacokinetic evaluation was performed for avelumab and axitinib formulations. The samples were administered to each rabbit under fasting conditions. After oral administration of avelumab and axitinib, blood samples were collected from rabbit marginal ear vein using a 25-guage,5/8 inch needle by clipping the marginal ear vein with a paper clip with volume of 0.5 ml to 1.0 ml at 0.5,2,4,8,12,16,20,24,28,32 and 36 hrs. The blood was collected in Eppendorf containing 10% EDTA solution. Blood was

centrifuged at 5000 rpm for 30 mins at 2-8°C temperature. The clear supernatant plasma were collected & stored at -30°C till its analysis. The plasma samples were treated for liquid-liquid phase extraction and analyzed for drug content with developed analytical method. After the study the animals were returned to animal house for rehabilitation.

The pharmacokinetic parameters for avelumab and axitinib oral administration were determined from plasma concentration data. Pharmacokinetic parameters like AUC, C_{max}, T_{max} the time at which C_{max} occurred, K_{el}, $t_{½}$, were calculated using the data. Data was measured by the trapezoidal rule method from time zero to infinity of concentration-time curve. C_{max} and T_{max} were obtained from the graph.

3. RESULTS AND DISCUSSION

The method was validated in selective, sensitive, linearity, accuracy and precise, matrix condition, recovery study, re-injection reproducibility and stability.

3.1 Specificity

The specificity of the method to research Avelumab and Axitinib simultaneously is proved. The chromatograms of blank and standard as shown in figure 1, 2. The chromatograms of blank rabbit plasma and standard having no interference peaks were observed.

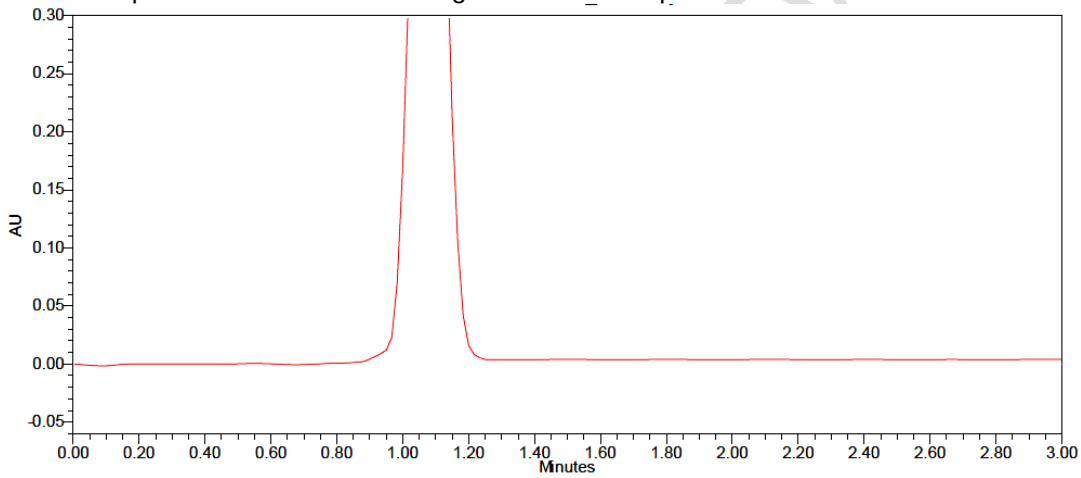


Fig. 1. Chromatogram of blank

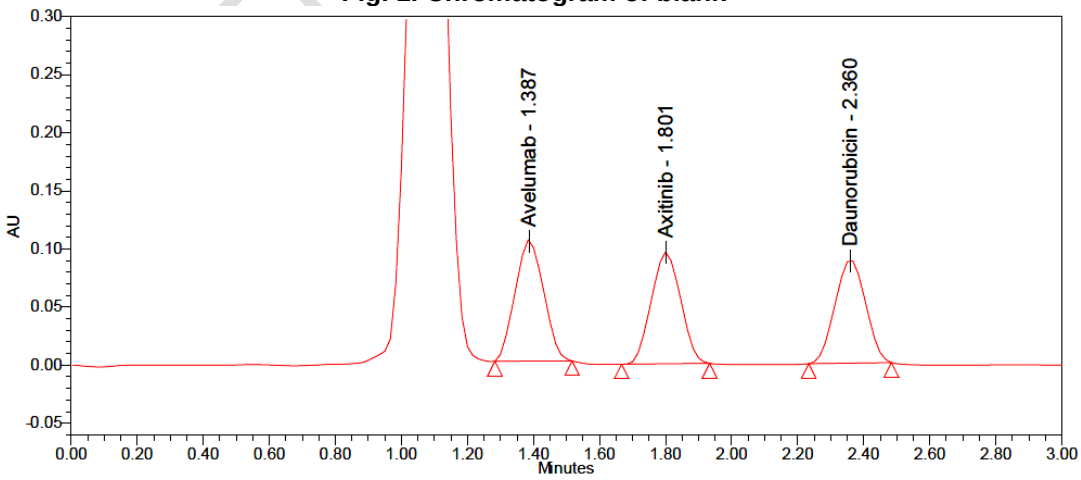


Fig. 2. Chromatogram of Standard

3.2 Matrix effect

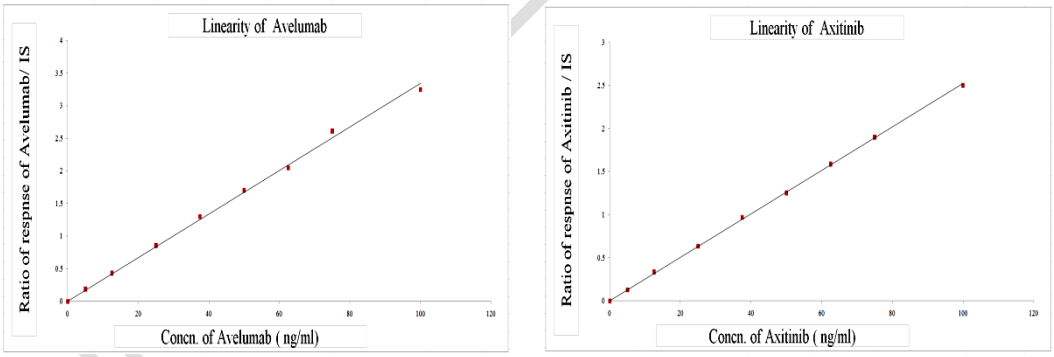
Percent RSD for within the signal, ion suppression/enhancement was observed as 1.0 % for Avelumab and Axitinib in UPLC, suggesting that under these circumstances the matrix effect on analyte ionization is within an acceptable range of ionization. In matrix effect LQC and HQC of Avelumab were 99.9 and 99.1 and axitinib were 99.3, 100.2%. %CV of the both drugs at LQC level were 0.67, 0.59 and HQC level is 0.73, 1.24 respectively. It indicates that the matrix effect on the ionization of the analyte is within the suitable limit.

3.3 Linearity

The peak area ratio of calibration standards was proportional to the concentration. The concentration range of Avelumab is 5 - 100 ng/ml and Axitinib is 5-100 ng/ml. Linearity results of Avelumab and Axitinib were shown in following table 1 and their calibration plots were shown in figure 3. The calibration curves were appeared linear and coefficient of correlation was found to be 0.999 for Avelumab and Axitinib.

Table 1: Results of Linearity

Linearity	Avelumab		Axitinib	
	Conc. (ng/ml)	Area response ratio	Conc. (ng/ml)	Area response ratio
Linearity-1	5.00	0.189	5.00	0.130
Linearity-2	12.50	0.435	12.50	0.337
Linearity-3	25.00	0.860	25.00	0.635
Linearity-4	37.50	1.303	37.50	0.972
Linearity-5	50.00	1.705	50.00	1.253
Linearity-6	62.50	2.052	62.50	1.587
Linearity-7	75.00	2.616	75.00	1.901
Linearity-8	100.00	3.252	100.00	2.502
Slope	0.0331		0.0254	
Intercept	0.02393		0.00452	
CC	0.99921		0.99976	



A B

Fig. 3. Calibration plots of (A) Avelumab and (B) Axitinib

3.4 Precision and Accuracy

By pooling all individual assay results of different internal control samples, the accuracy and precision were calculated. It was obvious, based on the data provided, that the strategy was precise and effective. The precision results of avelumab and axitinib was shown in Table 2, 3.

Table 2: Precision and Accuracy of Avelumab

QC Name	LLQC	LQC	MQC	HQC
Conc.(ng/ml)	5 ng/ml	25 ng/ml	50 ng/ml	75 ng/ml
QC sample -1	5.231	25.317	50.143	75.054

QC sample -2	5.124	25.415	50.351	75.428
QC sample -3	5.247	25.258	50.429	75.120
QC sample -4	5.119	25.216	50.448	75.315
QC sample -5	5.138	25.314	50.352	75.241
QC sample -6	5.342	25.247	50.217	75.349
Mean	5.200	25.295	50.323	75.251
SD	0.089	0.071	0.120	0.142
%CV	1.71	0.28	0.24	0.19
Accuracy	103.94	100.89	100.32	100.18

Table 3: Precision and Accuracy of Axitinib

QC Name	LLQC	LQC	MQC	HQC
Conc.(ng/ml)	5 ng/ml	25 ng/ml	50 ng/ml	75 ng/ml
QC sample -1	5.136	25.174	50.195	74.492
QC sample -2	5.071	25.863	49.963	74.449
QC sample -3	4.986	25.074	49.975	75.054
QC sample -4	4.951	24.615	49.955	74.315
QC sample -5	4.832	24.998	50.256	74.456
QC sample -6	5.267	25.441	49.914	74.487
Mean	5.041	25.194	50.043	74.542
SD	0.152	0.424	0.144	0.259
%CV	3.02	1.68	0.29	0.35
Accuracy	100.81	100.76	100.08	99.39

3.5 Recovery

The recoveries for Avelumab and Axitinib at LQC, MQC and HQC levels the results demonstrated that the bio-analytical method had good extraction efficiency. This also showed that the recovery wasn't hooked into concentration. The recoveries for Avelumab (95% - 105%) and Axitinib (95% - 105%) at LQC, MQC and HQC levels and % CV ranged from 0.15-2.67 for Avelumab and 0.36-2.47 for Axitinib. The results demonstrated that the bio-analytical method had good extraction efficiency.

3.6 Ruggedness

The percent recoveries and percent CV of Avelumab and Axitinib determined with two different analysts and on two different columns were within acceptable criteria in HQC, LQC, MQC and LLQC samples. The results proved method is ruggedness. The percent recoveries ranged from 96.45 – 104.72% for Avelumab and 97.58% -103.51% for Axitinib. The %CV values ranged from 0.08-2.45 for Avelumab and 0.26 – 2.98 for Axitinib. The results proved method is ruggedness.

3.8 Stability

Avelumab and Axitinib solutions were prepared with diluents for solution stability analysis and placed in a refrigerator at 2-8^{o}C. Fresh stock solutions were associated with stock solutions that were prepared 24 hours earlier. The plasma stability of the bench top and auto sampler was stable for twenty four hours, and 24 hours at 20^{o}C in the auto sampler. It became apparent from future stability that Avelumab and Axitinib were stable at a storage

temperature of -30^{o}C for up to 24 hours. The overall stability results of avelumab and Axitinib have been stated in the below table 4, 5.

Table 4: Stability results of Avelumab

Stability experiment spiked plasma		Spiked plasma conc.(n=6,ng/ml)	Conc.measured (n=6,ng/ml)	%CV
Bench top stability	LQC	25	25.241	1.24
	MQC	50	50.301	1.05
	HQC	75	75.224	0.74
Auto sampler stability	LQC	25	25.063	2.24
	MQC	50	50.135	0.56
	HQC	75	75.364	0.65
Long term(Day28) stability	LQC	25	25.118	1.68
	MQC	50	50.169	0.86
	HQC	75	75.214	0.54
Wet extract stability	LQC	25	25.039	0.85
	MQC	50	50.412	0.44
	HQC	75	75.512	0.31
Dry extract stability	LQC	25	25.331	1.7
	MQC	50	50.412	1.16
	HQC	75	75.324	1.41
Freeze thaw stability	LQC	25	25.269	0.86
	MQC	50	50.342	0.51
	HQC	75	75.148	0.23
Short term stability	LQC	25	25.339	2.21
	MQC	50	50.124	1.57
	HQC	75	75.312	1.12

Table 5: Stability results of Axitinib

Stability experiment spiked plasma		Spiked plasma conc.(n=6,ng/ml)	Conc.measured (n=6,ng/ml)	%CV
Bench top stability	LQC	25	25.114	1.54
	MQC	50	50.213	0.56
	HQC	75	75.316	0.74
Auto sampler stability	LQC	25	25.056	0.28
	MQC	50	50.225	0.54
	HQC	75	75.359	0.99
Long term (Day 28)stability	LQC	25	24.457	1.43
	MQC	50	49.395	0.52

	HQC	75	74.512	0.74
Wet extract stability	LQC	25	25.314	0.64
	MQC	50	50.226	1.83
	HQC	75	75.219	0.51
Dry extract stability	LQC	25	25.431	0.84
	MQC	50	50.162	0.73
	HQC	75	75.527	1.18
Freeze thaw stability	LQC	25	25.334	1.45
	MQC	50	50.246	0.58
	HQC	75	75.485	0.66
Short term stability	LQC	25	24.965	1.48
	MQC	50	49.966	0.52
	HQC	75	74.481	0.97

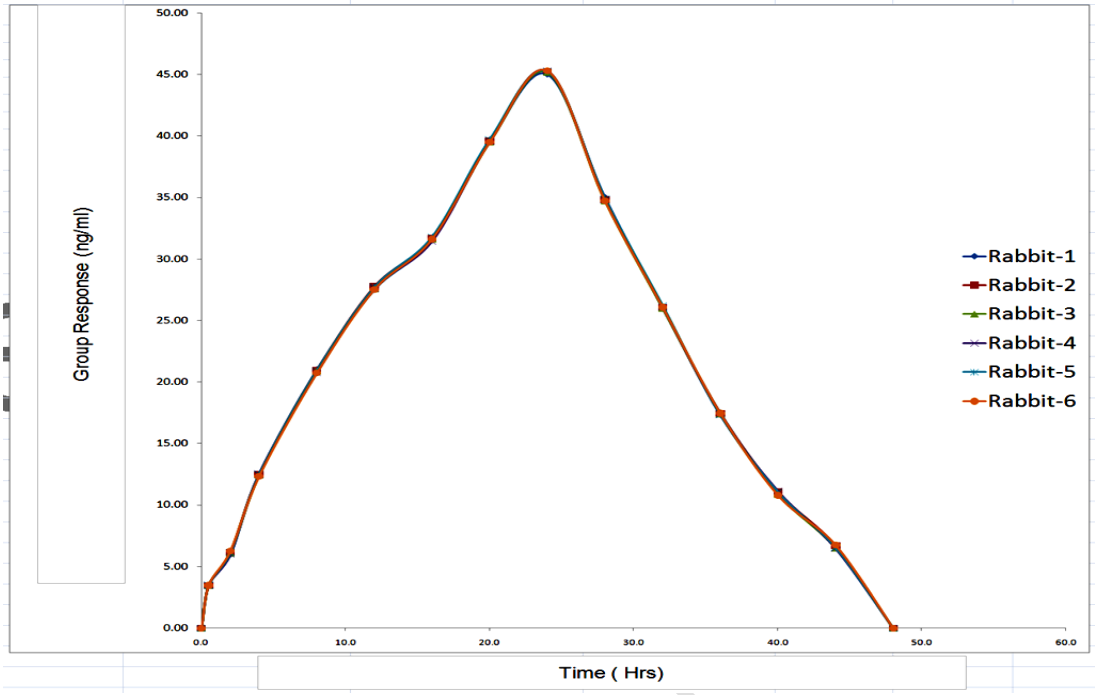
In Vivo Pharmacokinetic Evaluation

The plasma concentration time profiles of Avelumab and Axitinib in rabbit are shown in figure 5. The graph indicated bell shaped curve in both the cases of experimental formulation. Avelumab and Axitinib could be traced to be present in the blood for 24 h and 4 h after oral administration, which indicates the effectiveness of drug release from the formulation.

The pharmacokinetic parameters C_{max} , T_{max}, $T_{1/2}$, K_{el}, AUC_{0-t} , $AUC_{0-\infty}$, were calculated and the data is shown in Table 6. The C_{max} for Avelumab and Axitinib were found to be 45.203 ng/ml and 46.232 ng/ml respectively. The T_{max} for Avelumab and Axitinib were found to be 24h and 4h respectively. The t½ values were 44h and 20h respectively for Avelumab and Axitinib shown in table 6 and the recovery plots were shown in figure 4.

Table 6: Pharmacokinetic parameters of Avelumab and Axitinib

Pharmacokinetic parameters	Avelumab	Axitinib
AUC_{0-t}	688 ng-hr/ml	762 ng-hr/ml
C_{max}	45.203 ng/ml	46.232 ng/ml
$AUC_{0-\infty}$	688 ng-hr/ml	762 ng-hr/ml
t_{max}	24 hr	4hr
$T_{1/2}$	44 hr	20hr



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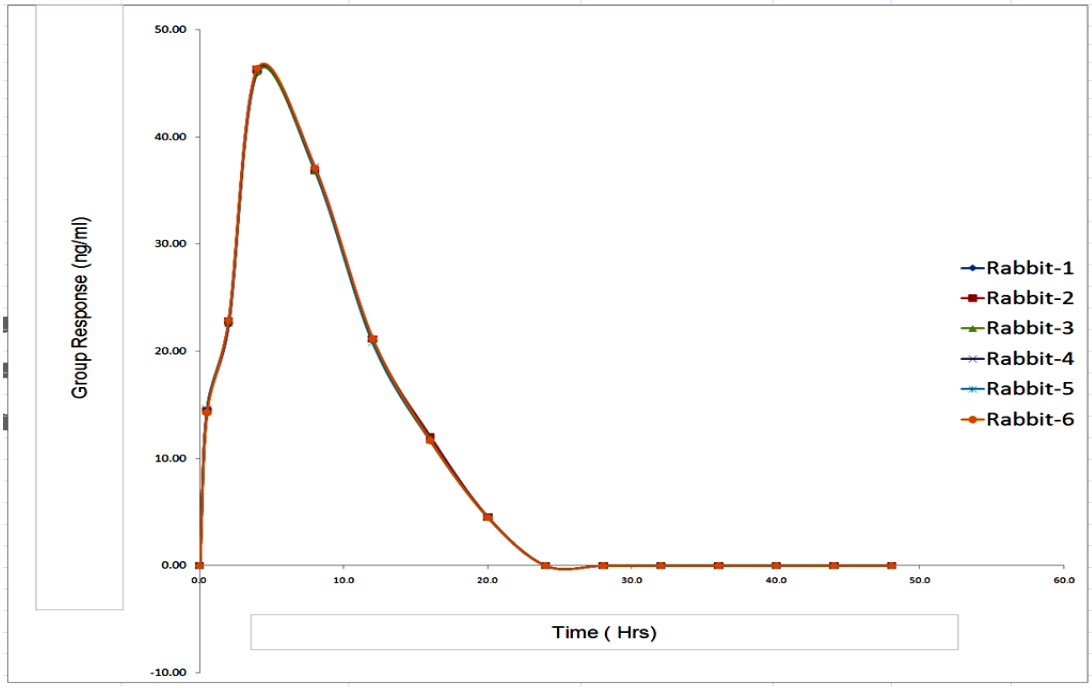

Fig. 4. Recovery plots of (A) Avelumab and (B) Axitinib

4. CONCLUSION

For the primary time higher sensitive UPLC method was developed and validated for the determination of Avelumab and Axitinib in rabbit plasma. Here the described method is rugged, fast, reproducible bio analytical method. This method was validated according to

USFDA guidelines. Simple and efficient method was developed and may be utilized in pharmacokinetic studies and to see the investigated analyte in body fluids.

CONSENT

This manuscript not published at any other journals.

ETHICAL APPROVAL

The protocol of animal study was approved by institute of animal ethics committee (reg.no:1074/po/re/s/05/cpcsea).

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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