Assay of Bio Analytical Method for the Estimation of Avelumab and Axitinib Using UPLC and its Application to Pharmacokinetic Studies

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ABSTRACT

Aims: Bioanalytical methods for the estimation of Avelumab and Axitinib using UPLC have been validated and may be used in Pharmacokinetic investigations.
Place and Duration of Study: Department of Engineering Chemistry, AUCE (A), Visakhapatnam, Andhra Pradesh, between September 2021 to February 2022.
Methodology: Using a C18 column (50x2.1 mm, 1.7) and an organic mobile phase containing 0.1 percent formic acid and Acetonitrile in a 70:30 ratio, this article provides an overview of recent advances in bioanalytical UPLC procedures.
Results: Avelumab and axitnib had linear calibration curves in the 5-100 ng/ml and 5-100 ng/ml ranges, respectively. Avelumab's precision and accuracy trial results were from 100.18 to 103.94, whereas Axitinib's were 99.39 to 100.81, according to the findings. The LQC and HQC of Avelumab and axitinib were 99.9 and 99.1 percent, respectively, in the matrix effect. There was a 0.67 and a 0.59 percent CV for the two medicines at the LQC and HQC levels respectively. In pharmacokinetic investigations, a simple and effective approach was created and used to observe the analyte of interest in bodily fluids.
Conclusion: It shows that the system's appropriateness, precision, linearity, and accuracy are all in accordance with USFDA criteria and may be used successfully for pharmacokinetic research in rabbits.

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

1. INTRODUCTION (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

Avelumab, marketed as Bavencio, is a completely human monoclonal antibody [1, 2] used to treat Merkel cell carcinoma [3, 4], urothelial carcinoma [5], and renal cell carcinoma [6, 7]. Fatigue, musculoskeletal discomfort [8], diarrhoea [9], nausea, infusion-related responses, rash, reduced appetite, and swelling of the limbs (peripheral edoema [10]) are common adverse effects. Avelumab is a monoclonal antibody that targets the protein programmed death-ligand 1 (PD-L1) [11, 12]. The European Medicines Agency (EMA) has designated it as an orphan medication for the treatment of gastric cancer [13, 14]. Immune-mediated adverse events (pneumonitis [15], hepatitis [16], colitis [17], adrenal insufficiency, hypo- and hyperthyroidism [18], diabetes mellitus [19], and nephritis) and life-threatening infusion reactions are the most prevalent significant adverse reactions to avelumab. Patients who have severe or life-threatening infusion-related events should discontinue use of avelumab. Women who are pregnant or nursing should avoid using avelumab since it may damage a growing foetus or newborn infant. The structural formula of Avelumab was $C_{6374}H_{9898}N_{1694}O_{2010}S_{44}$.

Pfizer developed axitinib, marketed as Inlyta, as a small molecule tyrosine kinase inhibitor [20, 21]. It has been proven in animal (xenograft) models [23] to dramatically limit the development of breast cancer [22], as well as in human studies with renal cell carcinoma (RCC) and numerous other tumour types [24]. The most frequent adverse effects include diarrhoea, hypertension [25], tiredness, reduced appetite, nausea, dysphonia, hand-foot

syndrome [26], weight loss, vomiting, asthenia, and constipation, which occur in more than 20% of individuals. Co-administration with powerful CYP3A4/CYP3A5 [27] inhibitors should be avoided as it may impair axitinib plasma clearance. The structural formula of Axitinib was $C_{22}H_{18}N_4OS$.

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

When utilising a chromatography column of size C18 (50mmx2.1mm,1.7), the separation was performed in an isocratic mode at temperature using a C18 (50mmx2.1mm,1.7) column in a chromatographic condition of isocratic operation. In this experiment, formic acid (0.1 percent) and acetonitrile (70:30 v/v) were utilised as the mobile phase. As the greatest concentrations of Avelumab and Axitinib were observed at 220 nm, the injection volume was 5 l, and the eluent was measured at that wavelength. So, the wavelength of 220 nm was chosen.

2.4 Preparation of standard solutions

2.4.1 Preparation of standard stock solution

In a 100ml volumetric flask, add 5 mg of Avelumab working standards and 70 mg of diluents; sonicate for 10 minutes to dissolve the contents fully and bring the diluent volume to the mark. Add 4 ml to a 100 ml volumetric flask for further dilution. 1 ml of the aforementioned solution is placed into a 10 ml volumetric flask, and the diluent is added to bring the volume to the mark.

2.4.2 Preparation of internal standard stock solution

Daunorubicin, 5 mg, is dissolved in 10 minutes by ultrasonically dissolving it in 100 millilitres of diluent, which is then transferred to a volumetric flask for analysis. To get 100 mL of this solution, use a volumetric flask with a neck diameter of 100 mm. One millilitre of the aforementioned solution is transferred to a 10 millilitre volumetric flask and diluted with the diluent.

2.4.3 Preparation of standard solution

Preparation for a typical meal A 2 ml centrifuge tube was filled with 200 ml of plasma, 300 ml of acetonitrile, and 500 ml of standard stock solutions, IS, and diluents, then vortexed for 10 minutes. Centrifuged for 30 minutes at 5000 rpm on these samples. The solution was collected, filtered via a 0.45 nylon syringe filter, and then put into a vial before being injected into the machine.

2.5 Pharmacokinetic Study

All animals are fed and watered ad libitum overnight prior to research. The technique that was employed was a topical anaesthetic. These formulations were subjected to in-vitro testing for pharmacokinetics. Each of the rabbits was given the samples when they were

fasting from food and water. 0.5, 2, 4, 8, 12, 16, 20, 24, 28, 32, and 36 hours after oral administration of avelumab and axitinib, blood samples were taken from the rabbit marginal ear vein using a 25-gauge,5/8 inch needle by clipping it with a paper clip. The blood was drawn in an Eppendorf tube having an EDTA solution concentration of 10%. 5000 rpm for 30 minutes at 2-8°C was used to centrifuge the blood. The crystal-clear plasma supernatant was collected and kept at -30°C until it could be analysed. A new analytical approach was used to assess the plasma samples for drug content after liquid-liquid phase extraction. The animals were returned to the animal home for rehabilitation when the research was completed.

These characteristics were derived from plasma concentration data for avelumabs and axitinis that were administered orally. An AUC, Cmax, Tmax, the time at which the peak of the pharmacokinetic parameters Kel, t12, were determined using this information. The concentration-time curve was plotted against time, and data was collected using the trapezoidal rule approach. From the graph, we were able to determine Cmax and Tmax.

3. RESULTS AND DISCUSSION

A recovery study, re-injection repeatability and stability were tested using the method's selectivity, sensitiveness, linearity, accuracy and preciseness, matrix condition, and recovery.

3.1 Specificity

Avelumab and Axitinib studies using this approach was shown to be very specific. Figures 1 and 2 show the chromatograms of the blank and the standard. Analysis of the two samples showed no interference in their chromatograms.

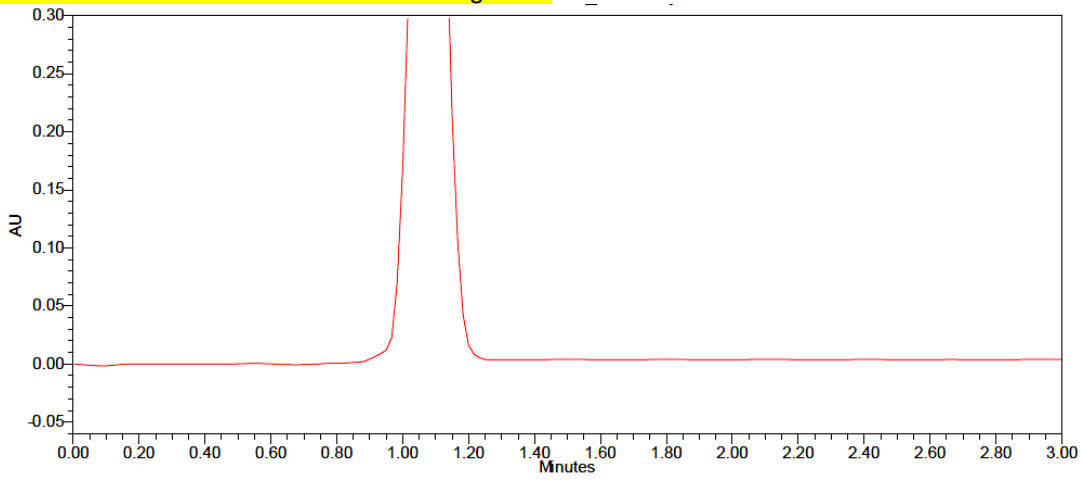


Fig. 1. Chromatogram of blank

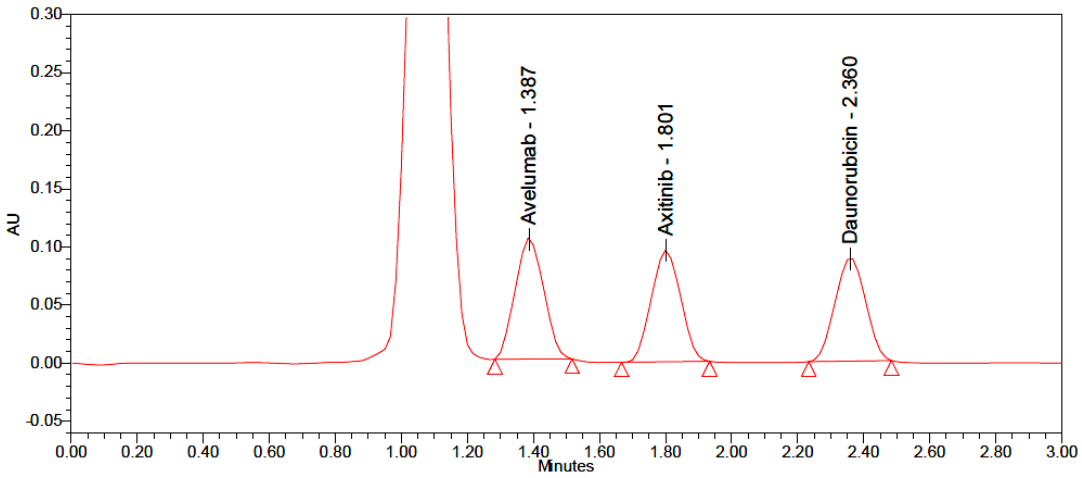


Fig. 2. Chromatogram of Standard

3.2 Matrix effect

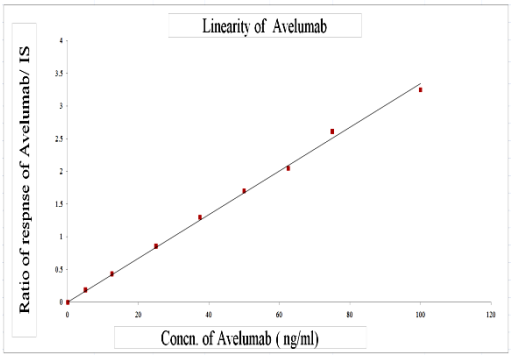
The percent RSD (Relative standard deviation) for ion suppression/enhancement within the signal was reported to be 1.0 percent for Avelumab and Axitinib in UPLC, indicating that the matrix influence on analyte ionisation is within an acceptable range of ionisation under these conditions. Avelumab had 99.9 percent LQC (Low Quality Control) and 99.1 percent HQC (High Quality Control) in the matrix effect, whereas axitinib had 99.3, 100.2 percent LQC and HQC, respectively. The percent CV of the two medicines was 0.67 and 0.59 at the LQC level, and 0.73 and 1.24 at the HQC level, respectively. It demonstrates that the matrix influence on the ionisation of the analyte is within the acceptable range of values.

3.3 Linearity

The peak area ratio of calibration standards was proportional to the concentration of the calibration standard solution. Avelumab has a concentration range of 5 - 100 ng/ml, whereas Axitinib has a concentration range of 5-100 ng/ml. In the accompanying table 1, the linearity findings of Avelumab and Axitinib are provided, as well as their calibration plots, which are displayed in picture 3. Calibration curves for Avelumab and Axitinib looked to be linear, and the coefficient of correlation was determined to be 0.999 for both drugs.

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	



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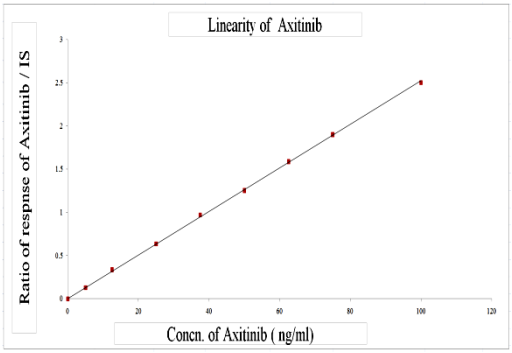

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Fig. 3. Calibration plots of (A) Avelumab and (B) Axitinib

3.4 Precision and Accuracy

The accuracy and precision of internal control samples were determined by combining the findings of all individual assays. Based on the information presented, it was clear that the technique used was exact and successful. Table 2, 3 shows the precision findings for avelumab and axitinib.

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 bio-analytical method's extraction effectiveness was proven by the recoveries of Avelumab and Axitinib at the LQC, MQC (Middle Quality Control), and HQC levels of the study. This further demonstrated that healing was not dependent on mental focus. At LQC, MQC, and HQC levels, Avelumab's recovery rates varied from 0.15-2.67 percent CV (Coefficient Variation) while Axitinib's recovery rates ranged from 0.36-2.47 percent CV (Coefficient Variation). A good extraction efficiency was established by the bio-analytical approach.

3.6 Ruggedness

In HQC, LQC, MQC, and LLQC (Lower Limit of Quality Control) samples, the percentage recoveries and percentage CV of Avelumab and Axitinib were within acceptable parameters. Results show that the approach has a long-term viability. For Avelumab, the percentage of recoveries varied from 96.45 to 104.72 percent; for Axitinib, it was 97.58 to 103.51. Avelumab's percent CV varied from 0.08 to 2.45, whereas Axitinib's ranged from 0.26 to 2.98. Results show that the approach has a high level of durability.

3.8 Stability

Diluents were added to Avelumab and Axitinib solutions for stability analysis and stored at 2-8oC in a refrigerator. There was a correlation between recently created stock solutions and previously prepared stock solutions prepared 24 hours earlier. For a total of twenty-four hours, the plasma on both the bench top and the auto sampler remained steady at 20oC. Axitinib and Avelumab were found to be stable at -30oC for up to 24 hours, based on the stability of the products in the future. Table 4 shows the overall stability data for avelumab and Axitinib.

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

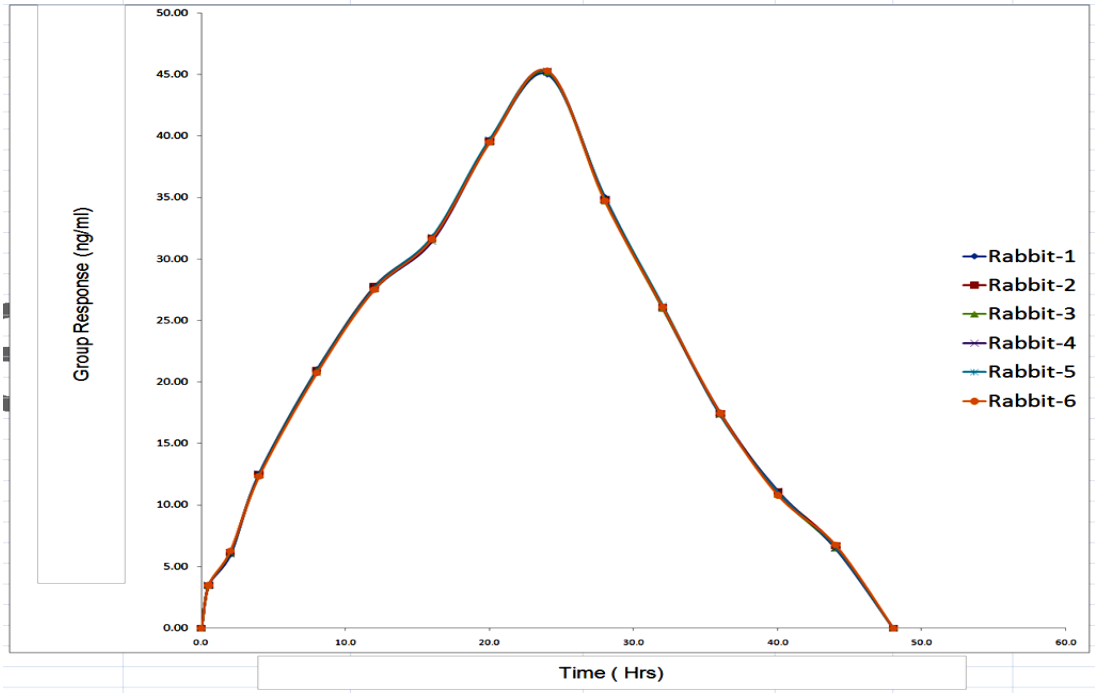
In both examples of experimental formulation, the graph showed a bell-shaped curve. The presence of Avelumab and Axitinib in the blood for 24 and 4 hours after oral treatment reflects the efficiency of the formulation's drug release.

Table 6 displays the results of the calculations of the pharmacokinetic parameters Cmax, Tmax, T1/2, Kel, AUC0-t, and AUC0-. Both Avelumab and Axitinib had a maximum plasma concentration (Cmax) of 45.203 ng/ml and 46.232 ng/ml. For Avelumab and Axitinib, the Tmax was determined to be 24 hours and four hours, respectively. ' Avelumab and Axitinib had t12 values of 44 and 20 hours, respectively, in table 6 and 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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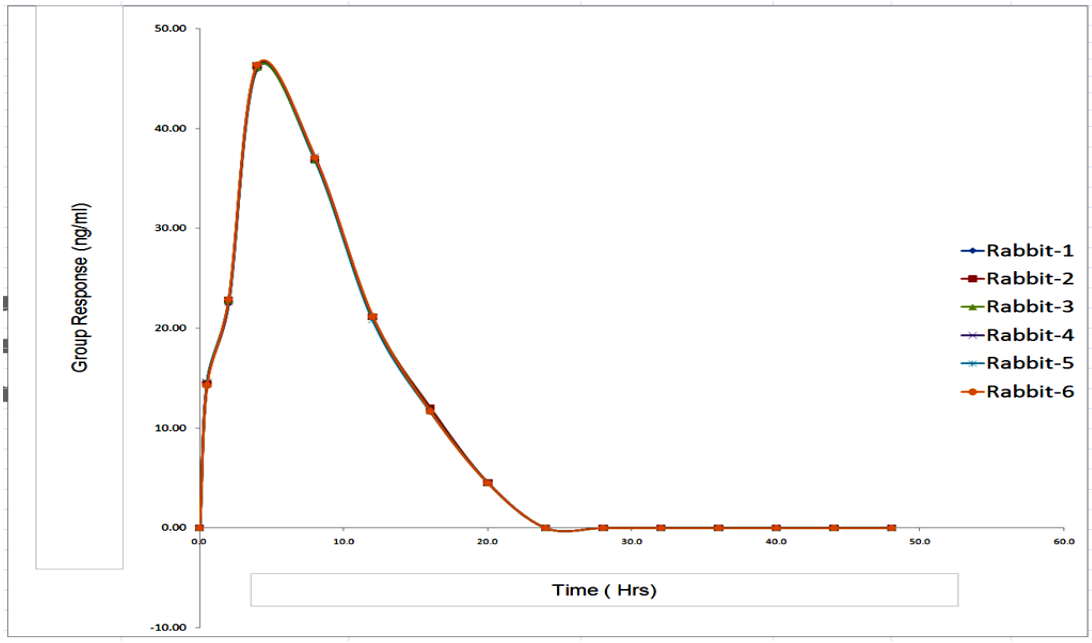

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Fig. 4. Recovery plots of (A) Avelumab and (B) Axitinib

4. CONCLUSION

For the first time, a more sensitive UPLC technique for the detection of Avelumab and Axitinib in rabbit plasma was developed and validated. Methods given here are robust, rapid and repeatable in the bio-analytical field. This approach has been shown to be safe and effective by the US Food and Drug Administration (FDA). For pharmacokinetic research and to observe the analyte in bodily fluids, an effective and simple approach has been devised.

ETHICAL APPROVAL

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

ACKNOWLEDGEMENTS

I thankful to my guide for encouragement and supporting to finish this research work.

COMPETING INTERESTS

No financial conflicts of interest were identified.

AUTHORS' CONTRIBUTIONS

Dr G. Himabindu designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. S. Prasanthi managed the analyses of the study, managed the literature searches. All authors read and approved the final manuscript.

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