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New method development and validation for the simultaneous estimation of Avelimab and Axitinib by using RP-HPLC

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ABSTRACT: Background: Avelumab injection is used to treat adults, children 12 years of age and older with Merkel cell Carcinoma (MCC, a kind of skin cancer) that has progressed to other regions of the body. Axitinib is used alone to treat advanced renal cell carcinoma (RCC, a kind of cancer that starts in the kidney cells) in persons who have not been effectively treated with other medicines. **Aim:** To develop a simple, rapid, cost-effective methodology for the determination of Avelumab and Axitinib simultaneously by RP-HPLC. **Method:** A stability indicating reverse phase HPLC method has been developed and validated for the simultaneous estimation of Avelumab and Axitinib in pharmaceutical dosage forms. The method was developed using a Waters Alliance-e2695 by using Hyperclone 5 µ BDS C18 130A (150×4.6 mm, 5 µm) column and the mobile phase containing Acetonitrile: 0.1% TEA PH-2.5/OPA in the ratio of 40:60 v/v. The flow rate was adjusted at 1.0 ml/min. The column oven was set at 40 °C and the detection wavelength was set at 219 nm. The retention time of Avelumab and Axitinib was found to be 4.771 and 3.128 min respectively. **Results:** The developed method was validated according to the ICH Q2 R1 guidelines and values of accuracy, precision and other statistical analysis were found to be in good accordance with the specified acceptance criteria. **Conclusion:** The proposed method was successfully applied to the oral dosage form consisting of Avelumab and Axitinib for routine analysis.

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Keywords: Avelumab, Axitinib, Stability Indicating, RP-HPLC, Validation, ICH Q2 R1 guidelines, Retention time.

INTRODUCTION:

Avelumab injection is used to treat adults and children 12 years of age and older with Merkel cell carcinoma (MCC; a kind of skin cancer) that has progressed to other regions of the body. Its molecular weight is 14381.79 g/mole with an empirical formula $C_{6374}H_{9898}N_{1694}O_{2010}S_{44}$. Axitinib belongs to a family of drugs known as kinase inhibitors. It is an anticancer drug (cancer medicine). It inhibits the development of cancer cells, causing them to be eliminated. Its molecular weight is 386.47 g/mole with an empirical formula $C_{22}H_{18}N_4OS$.

It works by preventing an aberrant protein from signalling cancer cells to proliferate. This aids in slowing or stopping the spread of cancer cells. The objective of the study is to develop a simple, rapid, and precise method for the simultaneous estimation of Avelumab and Axitinib by using RP-HPLC [1,2].

Chemicals and reagents:

Avelumab and Axitinib were obtained as reference standards from Zydus, Ahmedabad. HPLC-grade Acetonitrile and Methanol were procured from Loba Chemie and Merck Ltd. All other chemical reagents were of analytical grade.

0.1% TEA Buffer Preparation:

About 1 ml of Triethylamine was dissolved in 1 l of HPLC water and the pH is adjusted to 2.5 with OPA and filtered through 0.45 µ membrane filter paper.

Preparation of Mobile Phase:

The mobile phase was prepared by mixing Acetonitrile and 0.1 % TEA pH-2.5/OPA taken in the ratio 40:60. It was filtered through a 0.45 µ membrane filter to remove the impurities which may interfere with the final chromatogram.

Preparation of Standard Solution:

Accurately weighed and transferred 5 mg of Axitinib, and 20 mg of Avelumab working standard into a 10 ml clean dry volumetric flask Diluent was added and sonicated to dissolve it completely and the volume was made up to the mark with the same solvent (Stock solution).

Table 1. Chromatographic conditions for analytical study.

Parameters	Observation
Instrument used	Waters HPLC with autosampler and PDA detector.
Injection volume	10 µL
Mobile Phase	Acetonitrile: 0.1% TEA pH-2.5/OPA(40:60)
Column	Hyperclone 5µ BDS C18 130A (150×4.6 mm, 5 µ)
Detection Wavelength	219 nm
Flow Rate	1 ml/min
Runtime	8min
Column Temperature	Ambient (40 ºC)
Mode of separation	Isocratic mode

Further, 1 ml of the above stock solutions was pipetted into a 10 ml volumetric flask and diluted up to the mark with diluent. The strength of the solution was 50 ppm of Axitinib, and 200 ppm of Avelumab) [3,4].

Preparation of Sample Solution:

Accurately weighed and transferred 834 mg of Axitinib and 1ml of Avelumab sample into a 10 ml clean dry volumetric flask. Diluent was added and sonicated up to 30 min, and centrifuged for 30 min. Then it was dissolved completely, and the volume was made up to the mark with the same solvent. Then it was filtered through a 0.45 µm Injection filter (Stock solution). Further 1 ml stock solution was pipetted out into a 10 ml volumetric flask and diluted up to the mark with diluents. The strength of the solution was 50 ppm of Axitinib, 200 ppm of Avelumab) [3,4].

Table 2. System suitability data of Axitinib and Avelumab.

Sl. No.	Parameter	Axitinib	Avelumab
1	Retention time	3.122	4.773
2	Plate count	2227	3084
3	Tailing factor	1.27	1.19
4	Resolution	--	5.91
5	% RSD	0.14	0.25

Chromatographic study:

Avelumab and Axitinib in all solutions were determined by HPLC by using the chromatographic conditions as mentioned in Table 1. The Chromatographic data were analyzed and Specificity, Linearity and range, Robustness, precision, and accuracy were determined.

RESULTS AND DISCUSSION:

The developed method for determination of Avelumab and Axitinib were validated by using the following parameters.

System suitability:

For establishing the system suitability, the procedure described in the methodology was followed before starting the analysis. System suitability data has been presented in Table 2.

Tailing factor for the peaks due to Axitinib and Avelumab in Standard solution shouldnot be more than 2.0.

Theoretical plates for the Axitinib and Avelumab peaks in Standard solution should not beless than 2000.

Resolution for the Axitinib and Avelumab peaks in standard solution should not be lessthan 2 [5].

Specificity:

There were no interfering peaks at the retention time of Avelumab and Axitinib in the presence of excipients.

Further, to demonstrate the specificity of the method, the sample was subjected to acid, base, oxidation, thermal, and photolytic degradation. This was evaluated by using a Photo Diode Array detector (PDA). The chromatograms are presented in Fig 1 [6].

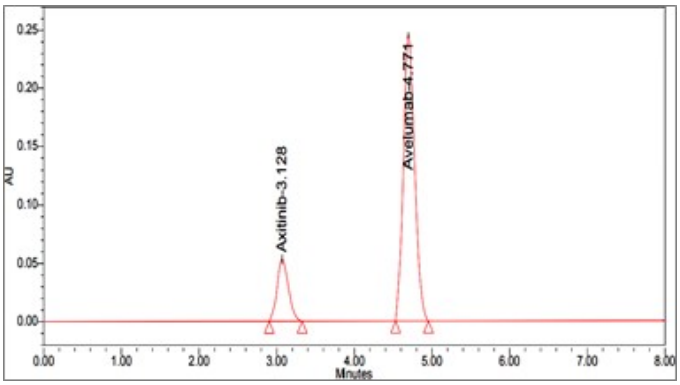

Fig 1. Optimized chromatogram.

Retention times of Axitinib and Avelumab were 3.128 min and 4.771 min respectively. We did not find any interfering peaks in blank and placebo at the retention times of these drugs in this method. So, this method was said to be specific.

Table 3. Linearity data of Axitinib and Avelumab.

Sl. No.	Axitinib		Avelumab	
	Conc. (µg/ml)	Peakarea	Conc. (µg/ml)	Peak area
1	12.50	151356	50.00	661818
2	25.00	325500	100.00	1248822
3	37.50	501265	150.00	1843374
4	50.00	656456	200.00	2475546
5	62.50	801404	250.00	3150828
6	75.00	955525	300.00	3761815
Regression equation	y = 12850.36x +2612.25		y =12492.99x + 3508.75	
Slope	12850.36		12492.99	
Intercept	2612.25		3508.75	
R^2	0.99952		0.99983	

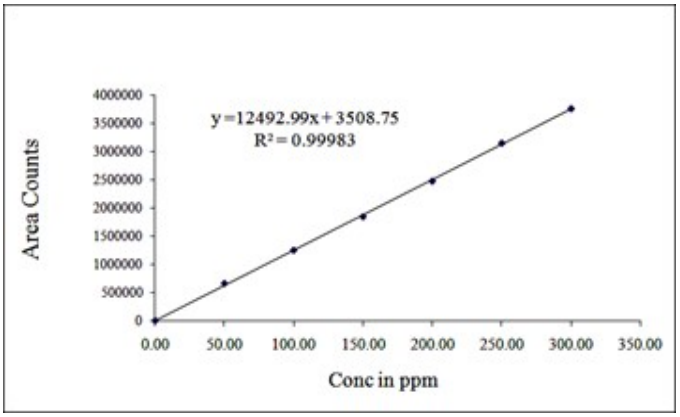

Fig 2. Calibration curve for Avelumab.

Linearity:

Standard solutions containing Avelumab and Axitinib were prepared. Linearity levels at five different concentrations of 50, 100, 150, 200, and 250 % for Avelumab and 12.50, 25.00, 37.50, 50.00, and 62.50 respectively. The average peak areas were plotted against concentration. Then, linearity was evaluated using the calibration curve to calculate the coefficient of correlation, slope, and intercept. In general, a correlation coefficient (r) > 0.999 is considered as evidence of an acceptable fit for the data to the regression line. The results obtained are presented in Table 3 which demonstrates that the current method was linear for the two analytes in the range specified above with a correlation coefficient better than 0.999. The plots have been represented in Fig 2 to 3 [7,8].

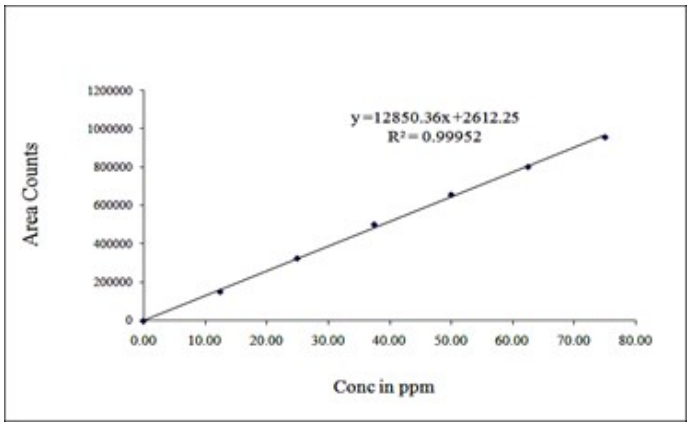

Fig 3. Calibration curve for Axitinib.

Table 5. System precision table of Axitinib & Avelumab.

Sl. No.	Conc. Axitinib (µg/ml)	Area of Axitinib	Conc. Avelumab (µg/ml)	Area of Avelumab
1.	50	659032	200	2488271
2.	50	656772	200	2479520
3.	50	656624	200	2471478
4.	50	657856	200	2484328
5.	50	656947	200	2483809
6.	50	657119	200	2486728
Mean	657392		2482356	
S.D	911.23		6106.97	
%RSD	0.14		0.25	

Precision:

For Assay, precision was determined by preparing the standard and sample as per the methodology. The sample was prepared in six replicates and injected into the chromatograph. The % Assay value of each preparation

was calculated and finally the % RSD of the six replicate preparations was deduced. The data obtained for six replicate standard injections and the six sample preparations have been presented in Tables 12 and 13 [8-11].

Table 6. Intermediate Precision (Day variation) for Axitinib and Avelumab.

Sl. No.	Area for Axitinib		Area for Avelumab	
	Day-1	Day-2	Day-1	Day-2
1	653125	655847	2437379	2470417
2	655871	656901	2475559	2476891
3	652154	652304	2446358	2485421
4	656245	657210	2462652	2461469
5	658719	651346	2486768	2452634
6	650652	654288	2483883	2455736
Average	654461	654649	2465433	2467095
Standard Deviation	2997.230	2433.293	20281.702	12748.638
%RSD	0.46	0.37	0.82	0.52

Table 7. Method Precision for Axitinib & Avelumab.

Sl. No.	Area for Axitinib	Area for Avelumab
1	651286	2465977
2	656871	2487574
3	652783	2457354
4	652874	2438856
5	657136	2479669
6	656784	2458983
Average	654622	2464736
Standard Deviation	2593.020	17340.554
%RSD	0.40	0.70

System Precision:

System precision is checked by using standard chemical substances to ensure that the analytical system is working properly. In this peak ar ea and % of the drug of six determinations is measured and % RSD should be calculated [12].

Intermediate precision:

In method precision, a homogenous sample of a single batch should be analyzed 6 times. This indicates whether a method is giving constant results for a single batch. In this analyze the sample six times and calculate the % RSD [13].

Repeatability:

The precision of the instrument was checked by repeatedly injecting (n=6) solutions of 50 ppm of Axitinib, and 200 ppm of Avelumab).

Accuracy:

For Assay, the accuracy of the proposed method had been demonstrated by the recovery study performed by the standard addition method at levels 50, 100, and 150 % of the target concentration. The data obtained had been presented in Table 8. was calculated and finally the % RSD of the three replicate preparations was deduced. Three levels of Accuracy samples were prepared by the standard addition method. Triplicate injections were given for each level of accuracy and mean % Recovery was obtained as 99.7 and 100.1 % for Axitinib and Avelumab respectively.

Table 9. The Accuracy results for Axitinib by RP-HPLC method.

% Conc. (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50 %	327848	2.5	2.49	99.6	99.7
100 %	658742	5.0	5.01	100.2	
150 %	979871	7.5	7.45	99.3	

Table 10. The Accuracy results for Avelumab by RP-HPLC method.

% Conc. (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50 %	1251822	10	10.09	100.9	100.1
100 %	2481471	20	19.99	100.0	
150 %	3698617	30	29.80	99.3	

Robustness:

As part of the Robustness, deliberate change in the Flow r ate, Mobile Phase composition,Temperature Variation wa s made to evaluate the impact on the method.

Ruggedness:

The ruggedness of the method was demonstrated by preparing the standard and sample as per the methodology by a different analyst on a different day, using a different column lot, and using a different HPLC system. The sample was prepared in six replicates and injected into the chromatograph. The % Assay value of each preparation was calculated and finally, the % RSD of the six replicate preparations was deduced. The data obtained for six replicate standard injections and the six sample preparations have been presented in Table 10 and Table 11 [14-16].

Table 10. Robustness results of Axitinib by RP-HPLC.

Parameter	Axitinib					
	Condition	Retentiontime (min)	Peak area	Resolution	Tailing	Plate count
Flow rate Change (ml/min)	Less flow (0.9 ml)	3.314	642135	-	1.25	2335
	Actual (1 ml)	3.128	659032	-	1.29	2216
	More flow (1.1 ml)	2.866	665241	-	1.31	2175
Organic Phase change	Less Org (36:64)	3.458	635586	-	1.22	2396
	Actual (40:60)	3.122	656772	-	1.27	2227
	More Org(44:56)	2.721	684029	-	1.28	2140

Table 11. Robustness results of Avelumab by RP-HPLC.

Parameter	Avelumab					
	Condition	Retentiontime (min)	Peak area	Resolution	Tailing	Plate count
Flow rateChange (ml/min)	Less flow (0.9 ml)	4.935	2318474	6.10	1.20	3124
	Actual (1 ml)	4.771	2488271	5.96	1.18	3092
	More flow (1.1ml)	4.432	2521639	5.84	1.13	2971
Organic Phase change	Less Org(36:64)	4.991	2169482	6.03	1.22	3185
	Actual(40:60)	4.773	2479520	5.91	1.19	3084
	More Org(44:56)	4.318	2684573	5.90	1.16	2942

Limit of detection and Limit of quantification:

The limit of detection (LOD) limit of quantification (LOQ) of the drug carry was calculated using the following equation as per International Conference Harmonization (ICH) guidelines [17].

$LOD = 3.3 \times \sigma / S$ …..(1)

$LOQ = 10 \times \sigma / S$ ……(2)

LOD for Axitinib, was found to be 0.15 µg/ml and LOQ for Axitinib, was found to be 0.5 µg/ml, LOD for Avelumab was found to be 0.6 µg/ml, and LOQ for Avelumab was found to be 2 µg/ml.

Table 12. Sensitivity parameters (LOD & LOQ) by RP-HPLC.

Name of drug	LOD (µg/ml)	s/n	LOQ (µg/ml)	s/n
Axitinib	0.15	3	0.50	10
Avelumab	0.6	3	2.0	10

Assay:

The assay and % purity were calculated. The observed value was compared with that of standard value without interference from the recipients used in the formulation.

Table No.13: Assay of Axitinib & Avelumab.

Brand	Axitinib	Avelumab
Avg. sample area (n=5)	659401	2481224
Std. Conc. (µg/ml)	50	200
Sample Conc. (µg/ml)	50	200
Label amount(mg)	5	20
Std. purity	99.8	99.9
Amountfound (µg/ml)	5.02	19.99
% assay	100.4	100.0

CONCLUSION:

This intended study concludes that the proposed method is economical, simple, sensitive, and reliable. Also, it is found to be accurate, precise, specific, stability indicating and rugged. Hence, it can be employed for the routine estimation of Axitinib and Avelumab.

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REFERENCES:

1. Smith K, Garman L, Wrammert J, Zheng NY, Capra JD, Ahmed R, *et al*. Rapid generation of fully human monoclonal antibodies specific to a vaccinating antigen. Nature Protocols, 2009; 4(3): 372-384.

2.Ho M, Feng M, Fisher RJ, Rader C, Pastan I. A novel high-affinity human monoclonal antibody to mesothelin. Int J Cancer, 2011; 128(9): 2020-2030.

3.Sunshine JC, Jahchan NS, Sage J, Choi J. Are there multiple cells of origin of Merkel cell carcinoma? Oncogene, 2018; 37(11): 1409-1416.

4.Vander HY, Nijhuis A, Smeyers-Verbeke J, Vandeginste BG, Massart DL. Guidance for robustness/ruggedness tests in method validation. J Pharm Biomed Anal, 2001; 24(5-6): 723-753.

5.Himabindu G, Prasanthi S. Assay of Bio analytical method for the estimation of Avelumab and Axitinib using UPLC and Its application to Pharmacokinetic Studies. J Pharm Res Int, 2022, 34(27A): 1-10.

6.Vander HY, Nijhuis A, Smeyers-Verbeke J, Vandeginste BG, Massart DL. Guidance for robustness/ruggedness tests in method validation. J Pharm Biomed Anal, 2001; 24(5-6): 723-753.

7.Vuehitha G, Kunduri LM, Kamireddy S, Chatla S,,*et al*. Development and validation of stability indicating RP-HPLC method for simultaneous estimation of Avelumab and Axitinib. J Cardiovasc Dis Res, 2021; 12(4): 1089-1099.

8.Sunshine JC, Jahchan NS, Sage J, Choi J. Are there multiple cells of origin of Merkel cell carcinoma? Oncogene, 2018; 37(11): 1409-1416.

9.Syn NL, Teng MW, Mok TS, Soo RA. De-novo and acquired resistance to immune checkpoint targeting. Lancet Oncol, 2017; 18(12): e731-e741.

10.Siu AL. Screening for breast cancer: US Preventive Services Task Force recommendation statement. Ann Intern Med, 2016; 164(4): 279-296.

11.Escudier B, Gore M. Axitinib for the management of metastatic renal cell carcinoma. Drugs R & D, 2011; 11: 113-126.

12.Simler R, Walsh G, Mattaliano R, Guziewicz N, Perez-Ramirez B. Maximizing data collection and analysis during preformulation of biotherapeutic proteins. BioProcess Int, 2008; 6: 38-45.

13.Rafi S, Rambabu K. Bio analytical Method development and validation of Avelumab, Axitinib and its application to Pharmacokinetic studies in rabbit plasma by using LC-MS/MS. Int J Appl Pharm, 2021; 13(5): 198-204.

14.Zielinski MR, Systrom DM, Rose NR. Fatigue, sleep, and autoimmune and related disorders. Front Immunol, 2019: 10: 1827.

15.Ho M, Feng M, Fisher RJ, Rader C, Pastan I. A novel high-affinity human monoclonal antibody to mesothelin. Int J Cancer, 2011; 128(9): 2020-2030.

16.Bornstein SR, Allolio B, Arlt W, Barthel A, Don-Wauchope A, Hammer GD, *et al*. Diagnosis and treatment of primary adrenal insufficiency: an endocrine society clinical practice guideline. J Clin Endocrinol Metab, 2016; 101(2): 364-389.

17.Gurusamy K, Vale CL, Pizzo E, Bhanot R, Davidson BR, Mould T, Mughal M, *et al*. Cytoreductive surgery (CRS) with hyperthermic intraoperative peritoneal chemotherapy (HIPEC) versus standard of care (SoC) in people with peritoneal metastases from colorectal, ovarian or gastric origin: protocol for a systematic review and individual participant data (IPD) meta-analyses of effectiveness and cost-effectiveness. BMJ open, 2020; 10(5): e039314.

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