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New method development and validation for the simultaneous estimation of Avelumab and Axitinib by using rp-HPLC

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ABSTRACT: Background: 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. 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 any other medicine. **Aim:** To develop a simple, rapid, cost-effective methodology for the determination of Avelumab and Axitinibl simultaneously by RP-HPLC. **Method:** A stability indicating reverse phase-HPLC method has been developed and validated for the simultaneous determination of Avelumab and Axitinib in pharmaceutical dosage form. The method was developed using a Waters Alliance-e2695 by using Hyperclone 5µ BDS C18 130A (150x4.6 mm, 5 µ) 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 observed to be about 4.771 min 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 topical dosage form consisting of Avelumab and Axitinib for routine analysis.

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Keywords: Avelumab, Axitinib, Stability Indicating, RP-HPLC, Validation.

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 143831.79 $g{\cdot}mol^{-1}$ with an empirical formula $C_{6374}H_{9898}N_{1694}O_{2010}S_{44}$ [1,2].

Axitinib belongs to a family of drugs known as kinase inhibitors. Axitinib is an anticancer drug (cancer medicine). It inhibits the development of cancer cells, causing them to be eliminated. Its molecular weight is 386.47 $g{\cdot}mol^{-1}$with an empirical formula $C_{22}H_{18}N_4OS$. It works by preventing an aberrant protein from signaling

cancer cells to proliferate. This aids in slowing or stopping the spread of cancer cells [3-5].

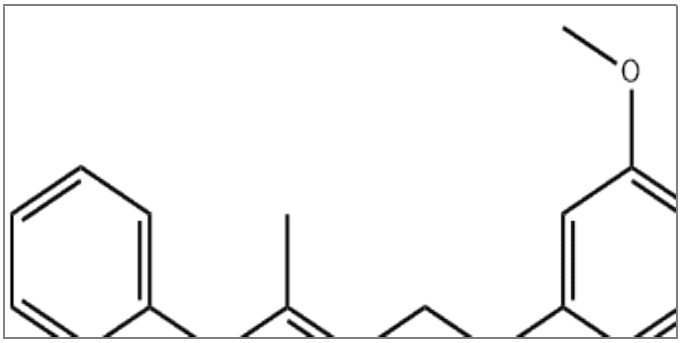

Fig 1. Molecular structure of Avelumab.

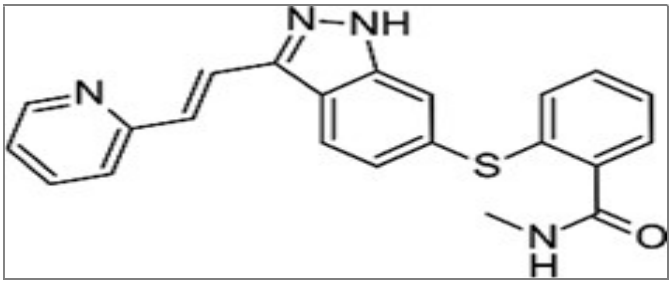

Fig 2. Molecular structure of Axitinib.

MATERIALS AND METHODS:

Chemicals and reagents:

Avelumab and Axitinib. Topical formulation containing Clotrimazole 1 % w/w and Betamethasone 0.05 % w/w was taken from the commercial batch manufactured at Oman Pharmaceutical Products Co L.L.C. HPLC grade Acetonitrile and Methanol was procured from LobaChemie and Merck Ltd. All other chemical reagents were of analytical grade.

Preparation of 0.1% TEA Buffer:

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

Preparation of Mobile Phase:

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 in the final chromatogram.

Preparation of standard solution:

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

Further 1 ml of the above stock solutions was pipetted into a 10 ml volumetric flask and diluted up to the mark with diluent (50 ppm of Axitinib, 200 ppm of Avelumab).

Sample Solution Preparation:

Accurately 834 mg of Axitinib was weighed and 1ml of Avelumab sample was measured, which were transferred into a 10 ml clean dry volumetric flask. The diluent was added to the solution and sonicated it up to 30 min to dissolve, and centrifuged for 30 min to dissolve it completely and the volume was made up to the mark with the same solvent. Then it was filtered through 0.45 µ Injection filter (Stock solution). Further 1 ml of the above stock solutions was pipetted into 10 ml volumetric flask and diluted up to the mark with diluents (50 ppm of Axitinib, 200 ppm of Avelumab).

Chromatographic study:

Avelumab and Axitinib in all solutions were determined by HPLC by using the chromatographic conditions as mentioned in Table 1.

Table 1. Chromatographic conditions for analytical study.

Parameters	Observation
Instrument used	Waters HPLC with auto sampler and PDA detector.
Injection volume	10 µL
Mobile Phase	Acetonitrile: 0.1 % TEA pH-2.5/OPA(40:60)
Column	Hyperclone5µBDSC18130A(150 ×4.6 mm, 5 µ)
Detection Wave Length	219 nm
Flow Rate	1 ml/min
Run time	8 min
Column Temperature	Ambient (40 ºC)
Mode of separation	Isocraticmode

The Chromatographic data were analysed 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.

Table 2. System suitability data of Axitinib and Avelumab.

Parameter	Axitinib	Avelumab
Retention time	3.122	4.773
Plate count	2227	3084
Tailing factor	1.27	1.19
Resolution	----	5.91
% RSD	0.14	0.25

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 3.

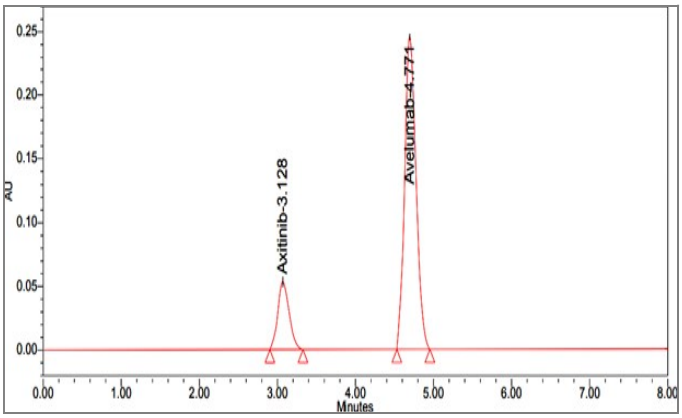

Fig 3. Optimized chromatogram.

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

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 coefficient of correlation, slope and intercept. In general, a value of correlation coefficient (r) > 0.999 is considered as the evidence of an acceptable fit for the data to the regression line. The results obtained are presented in the 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 4 and 5.

Table 3. Linearity data of Axitinib and Avelumab.

Sl. No.	Axitinib		Avelumab	
	Conc. (µg/ml)	Peak area	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	

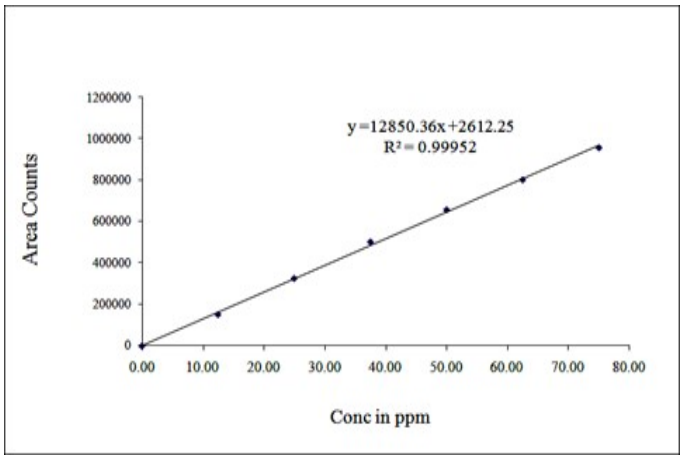

Fig 4. Calibration curve for Avelumab.

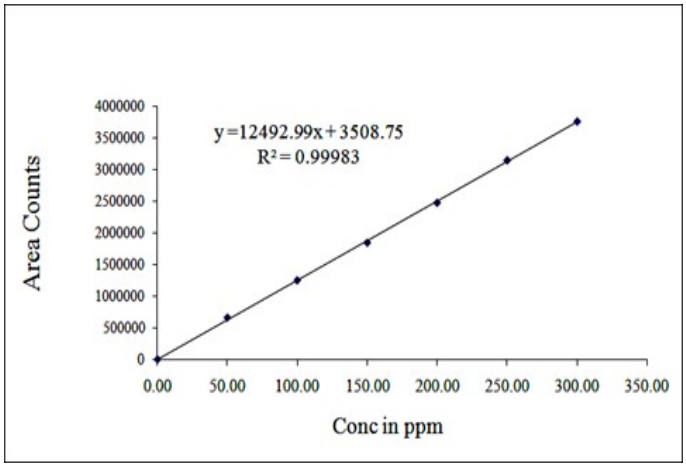

Fig 5. Calibration curve for Axitinib.

Table 4. Forced Degradation study summary of Axitinib and Avelumab.

Results: % Degradation results	Axitinib					Avelumab				
	Area	% Assay	% Deg	Purity Angle	Purity Threshold	Area	% Assay	% Deg	Purity Angle	Purity Threshold
Control	657196	100.0	0	1.538	10.826	2481247	100.0	0	4.185	15.236
Acid	585241	89.0	11.0	1.542	10.833	2192624	88.4	11.6	4.116	15.234
Alkali	582147	88.5	11.5	1.525	10.841	2175387	87.7	12.3	4.123	15.243
Peroxide	572478	87.1	12.9	1.515	10.886	2116837	85.3	14.7	4.152	15.284
Reduction	645530	98.2	1.8	1.548	10.842	2410021	97.1	2.9	4.145	15.245
Thermal	641478	97.6	2.4	1.543	10.847	2417487	97.4	2.6	4.149	15.241
Photolytic	650619	99.0	1.0	1.555	10.874	2391724	96.4	3.6	4.144	15.243
Hydrolysis	656204	99.8	0.2	1.538	10.856	2425894	97.8	2.2	4.135	15.259

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 Table 12 and 13.

System Precision:

Table 5. System precision table of Axitinib and Avelumab.

Sl. No.	Conc. of Axitinib (µg/ml)	Area of Axitinib	Conc. of 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	

Intermediate precision:

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

Sl. No.	Area for Axitinib		AreaforAvelumab	
	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

Repeatability:

Table 7. Method Precision for Axitinib and 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

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.

Table 8. Accuracy results of 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 9. 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	

Three levels of Accuracy samples were prepared by 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 10. Robustness results of Axitinib by RP-HPLC.

Parameters	Condition	Retention Time (min)	Peak area	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

Robustness:

Ruggedness of 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 11.

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

Parameter	Condition	Retention Time (min)	Peak area	Resolution	Tailing	Plate count
Flow rate Change (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 (1.1 ml)	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

Ruggedness:

***Limit of detection and Limit of quantification*:**

Table 12. Sensitivity parameters (LOD and 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:

Table 13. Assay of Axitinib and Avelumab.

Drug	Avg Sample area (n=5)	Std. Conc. (µg/ml)	Sample Conc. (µg/ml)	Label Amount (mg)	Std. purity	Amount Found (µg/ml)	% assay
Axitinib	659401	50	50	5	99.8	5.02	100.4
Avelumab	2481224	200	200	20	99.9	19.99	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 Axitinib and Avelumab.

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