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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS DETERMINATION OF AVELUMAB AND AXITINIB

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Abstract

The aim of this work was to propose an antibacterial mouthwash formulation based on aqueous extracts of the leaves of Prosopis africana (Fabaceae) for maintaining oral hygiene. Three types of mouthwashes were formulated, incorporating the liquid extract, freeze-dried extract, and oven-dried liquid extract of Prosopis africana as the active ingredient. Quality control of the formulations involved the evaluation of macroscopic and organoleptic characteristics, pH, phytochemical composition, and in vitro antimicrobial activity. The mouth washes were liquid in consistency, clear in appearance, homogeneously green in color, with a menthol odor and a sweet taste. The pH values ranged from 5.05 ± 0.03 for the mouthwash formulated with the liquid extract, 5.24 ± 0.1 for the mouthwash formulated with the oven-dried extract, and 5.18 ± 0.01 for the mouthwash formulated with the freeze-dried extract. The formulations containing the liquid extract showed higher levels of phytochemical content. All formulated mouthwashes exhibited broad-spectrum antimicrobial activity against the tested oral pathogens.

Keywords: Oral pathogens, Prosopis Africana extracts, mouthwashes, Photochemical, Antimicrobial activity.

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Introduction

Avelumab is also known as Bavencio. It is a fully human monoclonal antibody used to treat Merkel cell carcinoma, urothelial carcinoma, and renal cell carcinoma [1-3]. Fatigue, diarrhea, nausea, musculoskeletal pain, infusion-related reactions, reduced appetite, rash, and limb swelling are all known side effects of Avelumab. It is a monoclonal antibody that targets the protein programmed death-ligand 1 (PDL1) [4]. In January 2017, the European Medicines Agency (EMA) designated it as an orphan drug for the treatment of gastric cancer [5].

Axitinib (AG013736; trade name Inlyta), manufactured by Pfizer, is a small molecule and acts as tyrosine kinase inhibitor [6]. It has been shown to prevent breast cancer growth in animal models and showed partial responses in clinical trials with renal cell carcinoma (RCC) [7] and many other tumor forms [8]. The US Food and Drug Administration approved it for RCC after it showed a small improvement in progression-free survival, though there have been reports of fatal side effects. The most common side effects are hypertension, nausea, diarrhea, fatigue, dysphonia, reduced appetite, weight loss, hand-foot syndrome, asthenia, vomiting, and constipation, which affect more than 20% of patients [9]. The objective of this study was to separate the pharma ingredients of Avelumab and Axitinib by using RP-HPLC. Till date there are some UV, HPLC, and LCMS methods reported in the literature for Axitinib, but no methods are developed in the individual analysis of Avelumab. Hence, a method for the simultaneous quantification of Avelumab and Axitinib was developed in the present research. The developed RP-HPLC method was utilized for the estimation of the combined drugs in *in-vitro* method.

Materials and Method

Chemicals and Materials: HPLC grade acetonitrile (99.99% purity), Milli Q water, and Orthophosphoric acid were obtained from Rankem. Both Axitinib (99.99% purity) and Avelumab (99.99% purity) APIs were obtained as reference standards from Zydus Cadila, Ahmadabad.

Equipment: Axitinib, and Avelumab, were isolated using a Waters alliance e2695 model HPLC with a PDA (photodiode array) detector and the chromatographic program Empower 2.0 [10].

Chromatographic Conditions: Using a symmetry C18 (150x4.6mm, 3.5) column, chromatographic separation was performed in an isocratic mode at room temperature [10]. The mobile phase is an isocratic mixture of Acetonitrile and Ammonium formate pH-3.0 adjusted with OPA (20:80) with a flow rate of 1 ml/min with a detection wavelength of 228nm. The injection volume was 10 µl, with a 6- minute run time.

Preparation of Standard Solution: Working standards of 10 mg Axitinib and 4 mg Avelumab was correctly weighed. These standards were put in a 10 mL volumetric flask, filled with diluents, and sonicated for 10 minutes to dissolve the contents before being made up to the mark with the same diluents. Using the diluents, dilute 1 mL of the above solution to 10 mL.

Preparation of Sample Stock Solution: In a 10 ml volumetric flask, measure correctly the 17.2 mg equivalent weight of Axitinib and 0.2 mg equivalent weight of Avelumab sample. Add about 7 mL of diluents, sonicate for 30 minutes to fully dissolve the contents, and makeup up the mark with diluents. Using a 0.45 syringe filter, filter the solution.

Validation of the Proposed Method

The developed method for Axitinib and Avelumab was validated according to ICH guidelines of the parameters like Specificity, linearity, precision, accuracy, robustness, ruggedness, and forced degradation [11, 12].

i. System suitability

System suitability was studied under each validation parameter by injecting six replicates of the standard solution. The system suitability parameters were shown in Table 01.

Table 01: System suitability parameters for Axitinib & Avelumab

S.NO	PARAMETER	AXITINIB	AVELUMAB
1	Retention time	2.246	4.574
2	Plate count	9128	15437
3	Tailing factor	0.98	1.06
4	Resolution	----	10.23
5	%RSD	0.17	0.28

ii. Specificity

Placebo Interference: Specificity was the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. Typically, these include impurities, degrades, matrix, etc. It was observed that there is no interference at a retention time of Avelumab and Axitinib peaks with placebo peak and the results are summarized in Figure 1 & 2.

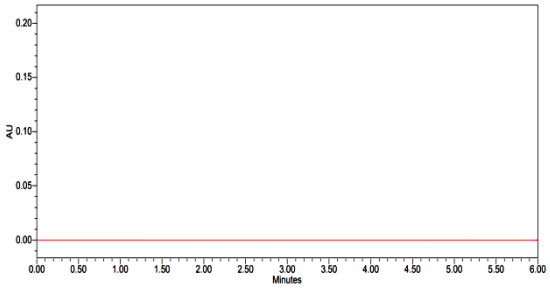


Fig 01: Blank Chromatogram

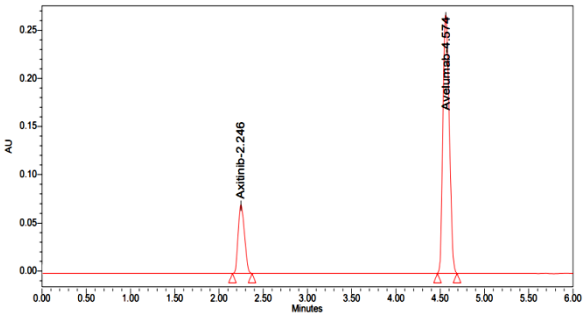


Fig 02: Optimized chromatogram

iii. Precision

Precision is the degree of repeatability of an analytical method under operation conditions. Precision is of three types.

a) System Precision: System precision is checked by using standard chemical substances to ensure that the analytical system is working properly. In this peak area and % drug of six determinations should be measured and % RSD should be measured.

b) Method Precision: The precision of an analytical technique is the degree of closeness of agreement between the series of measurements from samplings. The process of Method precision was performed by injecting six individual injections of Axitinib and Avelumab. In method precision, a homogeneous 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 measure the % RSD. The precision of the instrument was checked by repeatedly injecting (n=6) solutions of 10ppm of Axitinib, 40ppm of Avelumab.

c) Intermediate precision: Intermediate Precision of assay method was conducted on Axitinib and Avelumab using two different systems by different analysts using the different columns and analyzed under Day1 and Day2 similar conditions as per the test method. The system suitability parameters are evaluated and found to be within the limits. The system precision test for Axitinib and Avelumab demonstrated % RSD values of 0.17% and 0.28%, respectively, which are within the acceptable limit of less than 2%. These results confirm that the analytical method is precise and reliable for consistent measurements, and the system precision test is successfully passed. The results of system precision of Axitinib and Avelumab are summarized in Table 02.

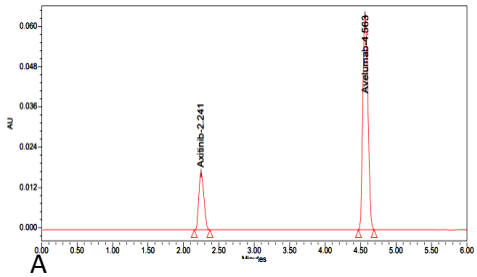
Table 02: System precision table of Axitinib & Avelumab

S.NO.	CONCENTRATION AXITINIB (µG/ML)	AREA OF AXITINIB	CONCENTRATION OF AVELUMAB (µG/ML)	AREA OF AVELUMAB
1.	10	731043	40	2948402
2.	10	733617	40	2929073
3.	10	730667	40	2935956
4.	10	731479	40	2929647
5.	10	733308	40	2931617
6.	10	732627	40	2944456
Mean	732124		2936525	
S.D	1232.180		8138.980	
%RSD	0.17		0.28	

iv. Linearity

Linearity was assessed for Axitinib and Avelumab across a concentration range of 25% to 150% of the standard working concentration. For Axitinib, the regression equation was determined to be y = 71875.83x + 3567.14 with a slope of 71875.83, an intercept of 3567.14, and an R^2 value of 0.99962. For Avelumab, the regression equation was y = 73091.23x + 5758.39 with a slope of 73091.23, an intercept of 5758.39, and an R^2 value of 0.99984. The calibration curves for both drugs demonstrated a strong linear relationship between concentration and peak area, with R^2 values exceeding the minimum requirement of 0.999.

The results of the linearity test indicate excellent linearity for Axitinib and Avelumab, as evidenced by R^2 values of 0.99962 and 0.99984, respectively. These values exceed the acceptance criterion of $R^2 \geq 0.999$, confirming that the analytical method is capable of accurately and consistently correlating concentration with peak area over the tested range. Therefore, the method is validated for linearity and is suitable for quantitative analysis of both drugs.



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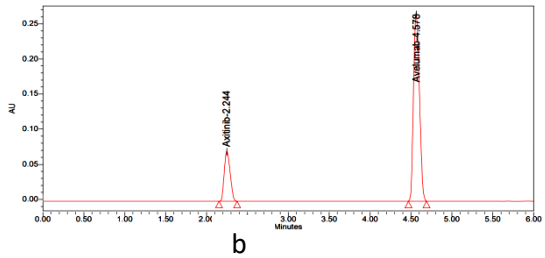
a



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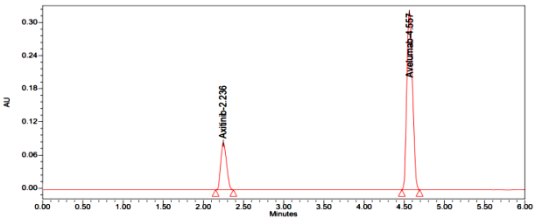

c

Fig 03: a. Chromatogram of Linearity-25%
b. Chromatogram of Linearity-100%
c. Chromatogram of Linearity-150%

v. Robustness

The results of Robustness for Axitinib and Avelumab are given in the table 03 & 04.

Table 03: Robustness results of Axitinib

PARAMETER	AXITINIB					
	CONDITION	RETENTION TIME(MIN)	PEAK AREA	TAILING	PLATE COUNT	%RSD
Flow rate Change (mL/min)	Less flow (0.9ml)	2.301	720846	0.93	9075	0.36
	Actual (1.0ml)	2.246	731043	0.98	9128	0.17
	More flow (1.1ml)	2.023	746116	1.03	9241	0.15
Organic Phase change	Less Org (18:82)	2.461	711565	0.90	9032	0.47
	Actual (20:80)	2.241	733617	0.94	9145	0.17
	More Org (22:78)	1.908	763443	0.97	9288	0.40

Table 04: Robustness results of Avelumab

PARAMETER	AVELUMAB						
	CONDITION	RETENTION TIME(MIN)	PEAK AREA	RESOLUTION	TAILING	PLATE COUNT	%RSD
Flow rate Change (mL/min)	Less flow (0.9 ml)	4.757	2791586	11.02	1.03	15391	0.81
	Actual	4.574	2948	10.23	1.06	1543	0.2

	(1.0 ml)		402			7	8
	More flow (1.1 ml)	4.303	30863900	9.96	1.09	15513	0.75
Organic Phase change	Less Org (18:82)	4.826	26095988	10.52	1.02	15329	0.55
	Actual (20:80)	4.577	29290733	10.28	1.07	15406	0.28
	More Org (22:78)	4.115	32367455	9.47	1.12	15572	0.21

vi. Accuracy

The accuracy of the method should demonstrate that the % Recovery for each concentration level (50%, 100%, and 150%) falls within the range of 98% to 102%. Additionally, the mean % Recovery at each level should also lie within this range, ensuring that the method is accurate and capable of quantifying the drugs accurately.

For Axitinib, the accuracy results showed recoveries at 50%, 100%, and 150% concentration levels. At 50%, the % Recovery ranged from 99.6% to 100.0%, with a mean recovery of 99.8%. At 100%, the % Recovery ranged from 99.8% to 100.6%, with a mean recovery of 100.1%. At 150%, the % Recovery ranged from 100.0% to 101.3%, with a mean recovery of 100.7%. All recoveries were within the acceptance criteria. For Avelumab, accuracy testing at the same concentration levels also met the criteria. At 50%, the % Recovery ranged from 99.5% to 101.0%, with a mean recovery of 100.2%. At 100%, the % Recovery ranged from 99.8% to 100.3%, with a mean recovery of 99.9%. At 150%, the % Recovery ranged from 98.7% to 99.7%, with a mean recovery of 99.2%. All values were within the acceptable range.

Accuracy results for Axitinib and Avelumab across 50%, 100%, and 150% concentration levels demonstrated recoveries within the acceptable range of 98% to 102%. The mean recoveries for Axitinib were 99.8%, 100.1%, and 100.7%, while for Avelumab, they were 100.2%, 99.9%, and 99.2%, respectively. These findings confirm that the analytical method is accurate and suitable for quantifying both drugs at varying concentration levels.

Table 05: Accuracy results of Axitinib

% CONCENTRATION(AT SPECIFICATION LEVEL)	AREA	AMOUNT ADDED (MG)	AMOUNT FOUND (MG)	% RECOVERY	MEAN RECOVERY
50%	365176	0.5	0.499	99.8	99.8
	364595	0.5	0.498	99.6	
	362441	0.5	0.500	100.0	
100%	736167	1.0	1.006	100.6	100.1
	730866	1.0	0.998	99.8	
	732064	1.0	1.000	100.0	
150%	1103501	1.5	1.510	100.7	100.7
	1109358	1.5	1.520	101.3	
	1101035	1.5	1.500	100.0	

Table 06: Accuracy results for Avelumab

% CONCENTRATION (AT SPECIFICATION LEVEL)	AREA	AMOUNT ADDED (MG)	AMOUNT FOUND (MG)	% RECOVERY	MEAN RECOVERY
50%	1459857	2.0	1.99	99.5	100.2
	1479278	2.0	2.02	101.0	
	1471133	2.0	2.00	100.0	
100%	2931441	4.0	3.99	99.8	99.9
	2945598	4.0	4.01	100.3	
	2926891	4.0	3.99	99.8	
150%	4375608	6.0	5.96	99.3	99.2
	4391934	6.0	5.98	99.7	
	4344588	6.0	5.92	98.7	

vii. LOD and LOQ

For Axitinib, the LOD was determined to be 0.15 µg/ml with an S/N ratio of 3, and the LOQ was determined to be 0.50 µg/ml with an S/N ratio of 10. For Avelumab, the LOD was determined to be 0.60 µg/ml with an S/N ratio of 3, and the LOQ was 2.00 µg/ml with an S/N ratio of 10. These results indicate that the method is sufficiently sensitive to detect and quantify both drugs at their respective low concentrations.

The LOD and LOQ values for Axitinib and Avelumab meet the acceptance criteria, with S/N ratios of 3:1 for LOD and 10:1 for LOQ. This demonstrates that the analytical method is highly sensitive and reliable for detecting and quantifying Axitinib and Avelumab at low concentrations.

Table 07: Sensitivity parameters (LOD & LOQ)

NAME OF DRUG	LOD(µG/ML)	S/N	LOQ(µG/ML)	S/N
Axitinib	0.15	3	0.50	10
Avelumab	0.60	3	2.00	10

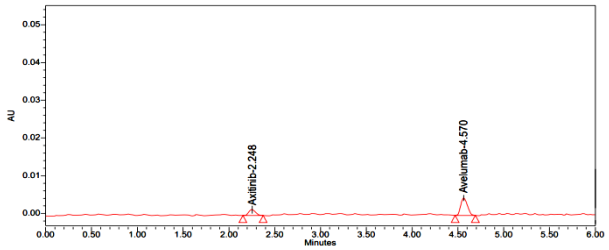

Fig 04: Chromatogram for LOD

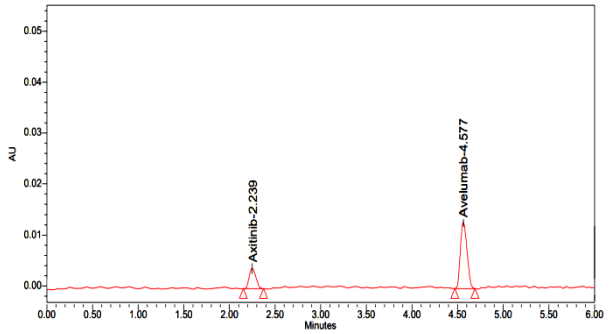

Fig 05: Chromatogram for LOQ

Degradation Studies

To determine the analytical method and assay for the study of stability indicating method in the formulation of axitinib and Avelumab studied under various stress conditions to conduct forced degradation studies. Forced degradation conditions such as acidic, basic, peroxide, hydrolysis, reduction, and thermal stress were studied at 1N concentration levels. The discovery of such conditions is shown in Table 08.

Table 08: Forced Degradation results for Axitinib and Avelumab

% DEGRADATION	AXITINIB					AVELUMAB				
	AREA	% ASSAY	% DEG	PURITY ANGLE	PURITY THRESHOLD	AREA	% ASSAY	% DEG	PURITY ANGLE	PURITY THRESHOLD
Control	732197	100	0	2.065	5.778	2933561	100	0	1.598	7.664
Acid	652853	89.2	10.8	2.046	5.725	2546565	86.8	13.2	1.532	7.628
Alkali	643054	87.8	12.2	2.023	5.731	2563561	87.3	12.7	1.545	7.649
Peroxide	637084	87.0	13.0	2.058	5.767	2508103	85.5	14.5	1.569	7.621
Reduction	705126	96.3	3.7	2.041	5.745	2618585	89.2	10.8	1.525	7.654
Photolytic	710487	97.1	2.9	2.037	5.723	2834659	96.6	3.4	1.537	7.683
Thermal	721354	98.5	1.5	2.059	5.721	2896508	98.7	1.3	1.554	7.652
Hydrolysis	718522	98.2	1.8	2.026	5.779	2880023	98.1	1.9	1.533	7.646

Conclusion

The HPLC approach that has been devised for the measurement of certain medications is quick, easy, precise, accurate, reliable, and cost-effective. The solvents and mobile phase are cheap, easy to make, dependable, sensitive, and quick to prepare. The sample recoveries were consistent with the promises made on the labels, which means that the formulation receivers did not interfere with the estimate. This means that the medications may be routinely tested in labs. It has been concluded that the suggested methods, which are both simple and brief, would be the most useful for analysis because the system validation parameters of the HPLC method have demonstrated satisfactory, accurate, and repeatable results (without recipient interference, of course). Results showed that the RP-HPLC stability indicating assay technique was free of interference with degradation products and placebos, and it was also easy to use, accurate, exact, and specific. You may use them for your regular Avelumab and Axitinib analyses.

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Conflicts of interest

The authors declare no conflicts of interest.

Inform Consent and Ethical Clearance

Not Applicable

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