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Stability Indicating RP-HPLC Method Development and Validation for the Estimation of Atezolizumab in Bulk and Its Injectable Dosage Form

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: A new simple reversed phase high performance liquid chromatographic method was developed for the estimation of Atezolizumab in bulk and its injectable formulation.
Study Design: Quantitative and qualitative estimation and degradation studies of AZM by using Rp-HPLC.
Place and Duration of Study: The work was performed at Santhiram College of Pharmacy and duration of study within 3 months.
Methodology: The method was developed and validated and better results were obtained by using Polaris C18-A (150 mm—4.6 mm i.d., 5µm particle size column by using mobile phase 0.1% TFA in acetonitrile: water 90:10 v/v. The mobile phase flow rate was fixed 0.75 mL/min.
Results: The mean retention time was 2.2 min. Correlation coefficient calibration curve within the detection range 75-225 µg/mL and R^2 value was found to be 0.999. The Detection limit and Quantification limit were performed for AZM and found to be 15.24-50.80 µg/mL. The assay was perfomed and the percentage purity of the AZM was found to be 100.27%. Forced degradation

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studies were performed with acid, base, water, H_2O_2 and UV-light.
Conclusion: The preliminary results indicated that the developed method was found to be a simple specific, precise, accurate and robust for the resolve of AZM in the bulk and marketed injectable formulations.

Keywords: Atezolizumab; HPLC.

1. INTRODUCTION

Atezolizumab (TECENTRIQ, Genentech, Inc.) is an Fc-engineered, humanized, monoclonal antibody that binds to programmed death-ligand 1 (PD-L1) and inhibits its interactions with the PD-1 and B7 receptors [1]. This releases the PD-L1/PD-1mediated inhibition of the immune response, including reactivation of the anti-tumor immune response. Programmed death ligand 1 (PD-L1) is an immune-checkpoint protein expressed on tumour cells and tumour-infiltrating immune cells that downregulates antitumoural T-cell function through binding to programmed death 1 (PD-1) and B7.1 (also known as CD80) receptors [2,3]. The engineered, humanised IgG1 monoclonal anti-PD-L1 antibody atezolizumab blocks PD-L1–PD-1 and PD-L1–B7.1 interactions, resulting in restoration of antitumour T-cell activity and enhanced T-cell priming [4,5,6]. Outcomes are poor for patients with previously treated, advanced or metastatic non-small-cell lung cancer (NSCLC); systemic chemotherapy (eg, docetaxel) provides only modest benefits [7] .Recently a phase-II clinical trail was conducted to evaluate an anti-PDLI monoclonal antibody atezoizumab for the treatment of Mucb after failure of PBCT or platinum-ineligible patients [8,14]. There are 117 ongoing clinical trails of atezolizumab currently. Given its efficacy in NSCLC and urothelial carcinoma; atezolizumab helds much potential in the future of cancer therapies [9,10]. The complexity of cancer chemotherapy requires pharmacists be familiar with the complicated regimens and highly toxic agents used [11,12]. A Phase III clinical trial of atezolizumab monotherapy for first-line, PD-L1–positive patients with NSCLC is underway; however, the potential of atezolizumab monotherapy as first-line treatment for NSCLC in a biomarker-unselected patient population remains to be investigated [13]. Atezolizumab holds much potential in the future of cancer therapeutics [15].

2. EXPERIMENTAL

2.1 Reagents

All the analytical grade chemicals and reagents were selected. Water was redistilled and filtered with a membrane filter. Acetonitrile – HPLC grade (Merck, India), 0.1%TFA and HPLC grade Water were used to prepare mobile phase. Pharmaceuical grade standard drug Atezolizumab was gifted by Natco Pharma Ltd, Hyderabad, India. The injectable formulation contains 1200 mg of atezolizumab purchased from local market of kurnool.

2.2 Chromatographic Conditions

The HPLC system consisted of a LC Waters (Waters, Milford, MA, USA) using a Polaris C_{18}-A (150 mm×4.6 mm i.d.,5 µm particle size; Varian, USA), a quaternary gradient system (600 Controller), in line degasser (Waters, model AF). The system was equipped with a photodiode array detector (Water, 2998 model) and auto sampler (Waters, model 717 plus). Data was processed using Empower Pro software (Waters, Milford, MA, USA). The Isocratic mobile phase consisting of a mixture of 0.1% TFA in acetonitrile and Water in the ratio of 90:10 v/v was used throughout the analysis. The flow rate of mobile phase was pumped at a rate of 0.75 mL/min. UV detection wavelength for AZM was 290 nm. Column temperature was kept at ambient and injection volume was 10µL. 90:10 v/v ratio of acetonitrile: water was used as diluent.

2.3 Solution Preparation

2.3.1 Standard stock solution preparation

100 mg of AZM was weighed accurately and transferred to 100 ml volumetric flask. Dissolved and diluted with acetonitrile: water to get a concentration of 1000 µg/ml.

2.3.2 Working standard Solution

Accurately measured 1.5 mL of AZM standard stock solution and transferred into a 10 mL volumetric flask, mixed well and diluted to final volume with diluent, so as get the final concentration of 150 µg/mL of AZM.

2.3.3 Sample solution preparation (Assay)

The average volume of injection was determined. A portion of volume equivalent to 1.66 mL of AZM was taken and transferred to a 100 mL volumetric flask. The volume was made with diluent.

2.3.4 Working sample solution

1.5 mL of AZM sample stock solution was accurately measured and transferred to a 10 mL volumetric flask, mixed well and diluted to final volume with diluents, so as get the final concentration of 150 µg/mL of AZM. The solution was injected in to HPLC system.

2.4 Method Validation

The developed Rp-HPLC method was validated in provisions of precision, accuracy, linearity and robustness according to ICH guidelines. System suitability tests were performed prior to method validation. System and method precision was determined using six independent test solutions. The method was evaluated for accuracy for the recovery of the standards from excipients. Dosage form mixture at the level of 50,100 & 150% were extracted and mean % recovery determined using developed HPLC method. The test solutions were prepared in the concentration range of 75– 225 µg/mL of AZM was estimated by injecting a series of dilute solutions with known concentration for the study of linearity curve. The robustness of the method was performed by change final experimental conditions were knowingly altered and the results were examined. The flow rate was varied by ± 0.2 mL/min the percentage of organic modifier was varied by ± 3%. Column temperature was varied by ± 5ºC. The drug was subjected to various stress conditions like acid (0.1 N HCl, refluxed for 1 H at 80ºC), base (0.1 N NaOH refluxed for 4H at 80ºC), peroxide (3% H_2O_2 Stored at room temperature for 2H), water and light (near UV ≥200 for 10 days) stability studies were conducted on the AZM.

3. RESULTS AND DISCUSSION

3.1 Method Development and Optimization of Chromatographic Conditions

During the optimization of the method, different columns (Inertsil C8, 250 mm×4.6 mm, 5 µm; Zorbax C18 250 mm×4.6 mm, 5 µm; Symmetry C18 250 mm×4.6 mm, 5 µm) and The chromatographic conditions Polaris C_{18}-A (150 mm×4.6 mm i.d.,5 µm particle size; Varian, USA), two organic solvents (methanol and acetonitrile) were tested. The method was also optimized by using different buffers like phosphate, acetates for mobile phase preparation. After a series of screening experiments, it was concluded that acetonitrile: water gave well peak shapes than their phosphates and methanol system of mobile phase, With acetonitrile as solvent both the peaks shows more theoretical plates and less retention time compared to methanol. The chromatographic separation was achieved on a Waters C18, 150 mm×4.6 mm, 5 µm column, by using a mixture of acetonitrile: water in the ratio of 90:10 v/v, as mobile phase. Temperature was maintained at ambient to facilitate mass exchange with the corresponding decrease of peak broadening and increase in sensibility. The flow rate was kept 0.75 mL/min to achieve adequate retention time AZM was 2.2 min and shown in Fig. 1 chromatogram. Table 1 shows the optimized chromatographic conditions.

3.2 Method Validation

When a method has been optimized it must be validated before routine analysis. By following ICH guidelines for analytical validation, Q2 (R1), the validation parameters were studied.

3.3 System Suitability

To ensure that all critical parameters were met as per the requirements system suitability tests were performed on all the days. As shown in the chromatogram 1, the analyte was eluted by forming symmetrical peak. That shown in the Fig. 1. Table 2 shows the System Suitability results.

3.4 Linearity

The calibration curves were constructed with five standard solutions were prepared over a concentration range of 75-225 µg/mL for AZM. The results, summarized in table , show a good-correlation between analytes peak area and concentration with $r > 0.999$ ($n = 5$). Linearity curve was shown in Fig. 3.

3.5 Precesion

The assay was studied with respect to repeatability and intermediate precesion. The repeatability of the system was examined by

injecting analyte solution with 6 replicate injections. The RSD values varied from 0.33-0.43% showed that the inter-day precesion of the method was satisfactory. Table no 4 shows the results of precesion.

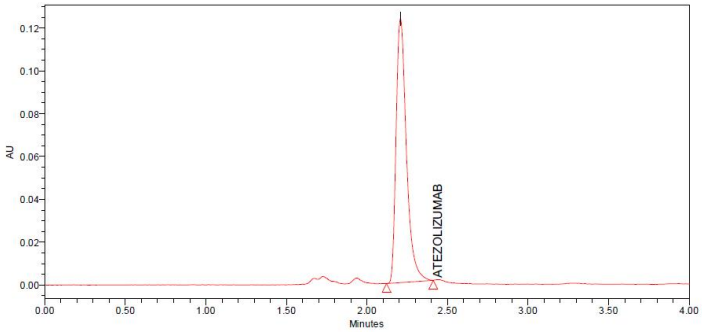


Fig. 1. Chromatograph of Atezolizuab

Table 1. Optimized HPLC conditions for simultaneous estimation Atezolizumab

S. No	Parameter	Description/Value
1.	Stationary Phase	Polaris C_{18}-A (150 mm×4.6 mm i.d., 5 µm particle size; Varian, USA)
2	Mobile Phase	0.1 % TFA in acetonitrile : water (90:10 v/v)
3	Flow rate	0.75 mL/min
4	Detection Wavelength (Isosbestic Point)	290 nm
5	Detector	Photo diode array
6	Injection	Autosampler -Waters, model 717 plus
7	Injection volume	10 µl
8	Column Temperature	Ambient
9	Run time	4 mins
10	Diluent	Acetonitrile: water (90:10)
11	Rt's	2.207 min

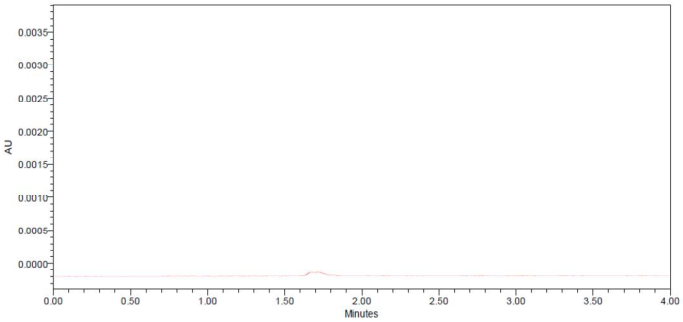


Fig. 2. Chromatogram of blank

Table 2. System suitability results

S. No	Parameters	Results	Limits
		Atezolizumab	
1	RSD of peak area	0.53	<2 n ≥ 6
2	Retention time	2.207	-
3	RSD of retention time	0.56	<2 n ≥ 5
4	USP plate count	13196	>2000
5	USP tailing factor	1.06	T<2
6	USP resolution	-	R >2

Table 3. Linearity results of AZM

S. no	Linearity leval	Concentratin (µg/mL)	Peak area
1	50	75	8977063
2	75	112.5	13465594
3	100	150	17954126
4	125	187.5	22442657
5	150	225	26731189
Slope			11862
R^2			0.999
Intercept			12000

3.6 Accuracy

To govern the accuracy results of the proposed method, recovery studies has been performed, known amount of pure drug sample solution at three different concentration levels ie, 50%, 100%, 150% was calculated. Accuracy was calculated as percentage recovery. The accuracy results were tabulated as Table 5.

3.7 Limit of detection and Limit of quantification (LOD & LOQ)

Estimation of limit of detection and limit of quantification considered as the signal- to- noice ratios 3:1 and 10:1 respectively. The LOD & LOQ was determined for AZM 15.24 µg/mL & 50.80 µg/mL respectively. The figure numbers 4&5 were chromatograms of LOD & LOQ.

3.8 Robustness

The robustness of the method was unaffected when small, deliberate changes like, flow rate change, mobile phase composition, column temperature were performed at 100% test concentration. The method was found to be robust for the said conditions. Results were tabulated in Table 7.

Table 4. Results of method precision

S. no	Peak area	% Assay
1	18045826	100.66
2	17952417	100.14
3	18036257	100.61
4	18045217	100.66
5	17915247	99.93
6	17936524	99.65
Average	17988581.33	100.27
STDEV	60254.91	0.43
% RSD	0.33	0.43

Table 5. Results of accuracy

% Concentration (Specified level)	Area	% recovery	% Mean recovery
% 50	8952983	99.84	99.84
% 100	18102356	100.49	100.49
% 150	26832147	99.61	99.61

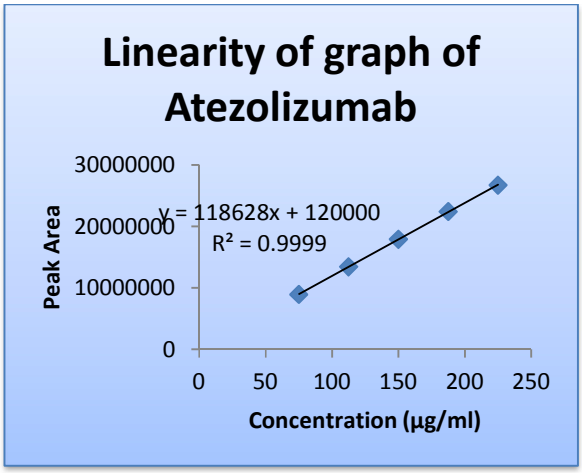


Fig. 3. Linearity graph of atezolizumab

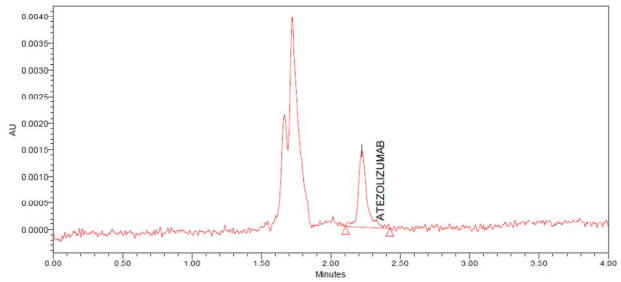


Fig. 4. Chromatogram of LOD

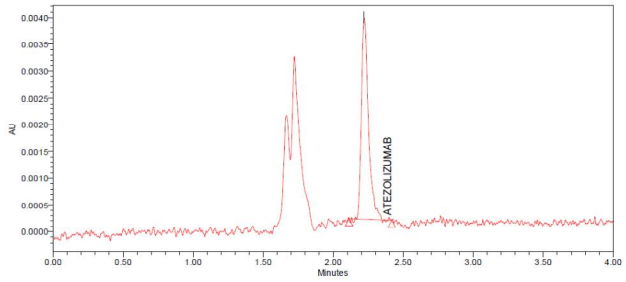
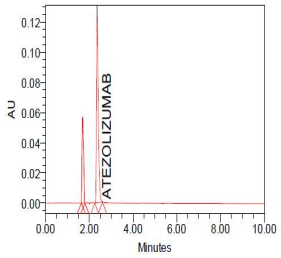
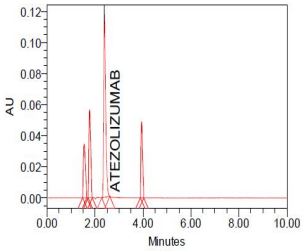


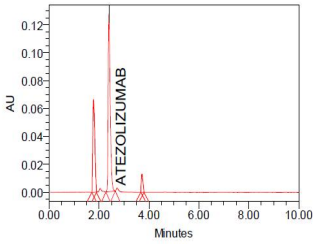
Fig. 5. Chromatogram of LOQ



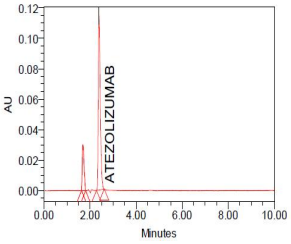
a. Acid degradtion chromatogram



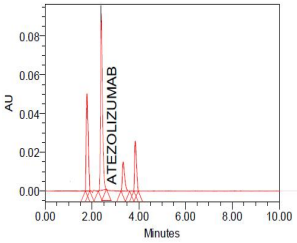
b. Base degradation chromatogram



c. Peroxide degradation chromatogram



d. Hydrolytic degradation chromatogram



e. UV-Light degradation chromatogram

Fig. 6-a-e. Degradation chromatograms

3.9 Analysis of Injectable Formulation

The developed method was applied for the analysis of atezolizumab in injectable dosage forms, the result was found to be 100.27%. The results tabulated as Table 8.

3.10 Forced Degradation Studies

There was no interference of blank and degradants, the developed HPLC method proves the capability stability indicating method for the analysis of AZM. Purity angle was less than the purity threshold and hence the proposed method was the specific and revealed its stability-indicating power. The results were tabulated as 9, Fig. (6 a-e) shows chromatograms of different stress conditions.

Table 6. Results of LOD & LOQ

Slope	11862
SD	60254.91
LOD	15.24 (µg/mL)
LOQ	50.80 (µg/mL)

Table 7. Results of robustness

S. no	Parameters	Condition	Atezolizumab		% Assay
			RT	Peak area	
1	Flow rate	0.55mL/min	2.537	18023698	100.53
2		0.75 mL/min	2.207	17988581	100.34
3		0.95mL/min	1.946	18102356	100.98
4	Temperature	65°C	1.947	17947851	100.12
5		70°C	2.207	17988581	100.34
6		75°C	1.955	18045217	100.66
7	Mobile phase	T:M 87:13 v/v	2.208	18007294	100.45
8		T:M 90:10 v/v	2.207	17988581	100.34
9		T:M 93:7 v/v	2.207	17998299	100.40

Table 8. Results of robustness

S. no	Volume of drug added (mL)	Peak area	% Assay
1	20	18045826	100.66
2	20	17952417	100.14
3	20	18036257	100.61
4	20	18045217	100.66
5	20	17915247	99.93
6	20	17936524	99.65
	Average	17988581.33	100.27
	STDEV	60254.91	0.43
	% RSD	0.33	0.43

Table 9. Results of Forced degradation studies

S. No	Condition	Atezolizumab		
		Peak Area	% Assay	% Degradation
1	Acid (0.1 N HCl at 80º for 24 H)	16448063	91.75	8.25
2	Base (0.1 N NaOH refluxed for 4H at 80ºC	16649491	92.87	7.13
3	Peroxide (3% H2O2 Stored at room temperature for 2 H)	16450918	91.77	8.23
4	Water	16583906	92.51	7.49
5	Light (near UV ≥200 for 10 days)	16152346	90.10	9.9

4. CONCLUSION

A simple, specific, précised and accurate isocratic Rp-HPLC method was developed for the estimation of Atezolizumab in the injectable formulation. Several stress conditions were applied for forced degradation studies. The proposed method was successfully separated atezolizumab and its degradants. The proposed method was specific, accurate and stability- indicating capability. The developed method can be used for routine analysis of atezolizumab in marketed formulations.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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