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Research Article

Analytical method development and validation for the evaluation of related substances in Apalutamide by RP-HPLC

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Abstract

Apalutamide is an anti-cancer agent used for the management of prostate cancer. A new stability indicating RP-HPLC method (Gradient mode) has been developed for the estimation of Apalutamide and its related substances using Waters Alliance system (Model no. 2996 and 2695) with Inertsil ODS-3 (250 × 4.6 mm, 5µm) column (PDA detector) was used for the present study. A mixture of Ammonium phosphate buffer solution and Acetonitrile (30: 70, v/v) was used as the mobile phase for the chromatographic study (Flow rate: 1.0 mL/min; Detection wavelength: 243 nm). Stress degradation studies were performed and the method was validated as per ICH guidelines.

Keywords: Apalutamide, RP-HPLC, Related substances, Impurities, Stability indicating, Validation, ICH guidelines.

INTRODUCTION

Apalutamide (Figure 1) is a selective competitive androgen receptor inhibitor[1]. It is chemically 4- [7- [6-cyano-5 -(tri fluoro methyl) pyridin-3-yl] -8-oxo -6-sulfanylidene-5, 7-diaza spiro [3. 4] octan-5-yl] -2-fluoro-N-methylbenzamide ($C_{21}H_{15}F_4N_5O_2S$) with molecular weight 477.435 g/mole. It is an anti-cancer agent used for the treatment of prostate cancer[2].

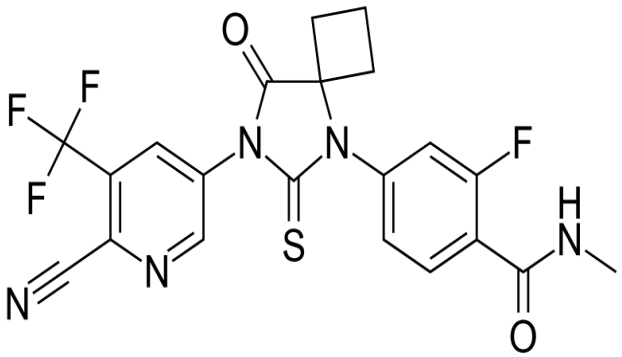
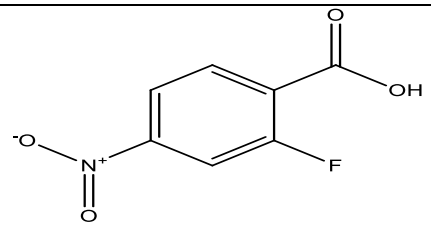


Figure 1: Chemical structure of Apalutamide

Bandaru *et al.,* have developed a HPLC method[3] for Apalutamide and its related substances (4 Impurities) on gradient mode using Luna Omega Polar C 18 column with flow rate 1.0 ml/min with detection wavelength 225 nm and the total run time was 85 min. Mobile phase consisting of a mixture of 0.01 M disodium phosphate dihydrate buffer (pH 4.20 ± 0.05) and acetonitrile (73:27, v/v) was used as mobile phase A and a mixture of water and acetonitrile (30:70, v/v) as mobile phase B.

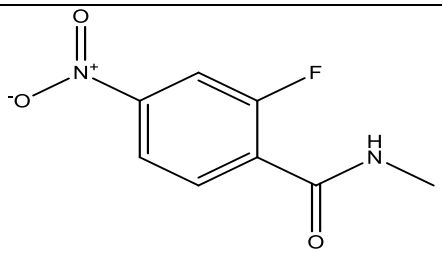
Lakka *et al.,* have developed a stability indicating HPLC and a LC-MS/MS method[4] for the separation of impurities and study of the degradation pathways of Apalutamide on gradient mode using Design of Experiments. Atlantis dC_{18} column with mobile phase consisting of a mixture 10 mM KH_2PO_4 (pH 3.5): acetonitrile was used with detection wavelength 270 nm

Sai Uday Kiran and Sandhya developed a LC-MS/MS method[5] for the estimation of Apalutamide in human plasma in presence of an internal standard, Canagliflozin. Agilent/1200 LC-MS/MS with ESI and Agilent/6460 triple-quadrupole was employed for the study. Inertsil C18 analytical column was used on an isocratic mode using a mobile phase mixture of 0.1% formic acid and acetonitrile (20:80, v/v). Linearity was observed over the concentration range 0.3-12 µg/ml. In the present study the authors have proposed a new stability indicating validated RP-HPLC method for the determination of Apalutamide and its related substances (4 Impurities) (IMP) and the method was validated as per ICH guidelines[6-8]. The chemical structures and other details of these 4 Impurities (IMP) were shown in Figure 2.



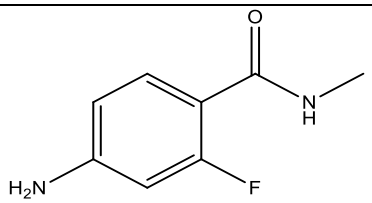
Apalutamide Impurity-1 (CAS: 403-24-7) (IMP-1) ($C_7H_4FNO_4$)

2-Fluoro-4-nitrobenzoic acid (Mo. Wt. 185.1 g/mol)



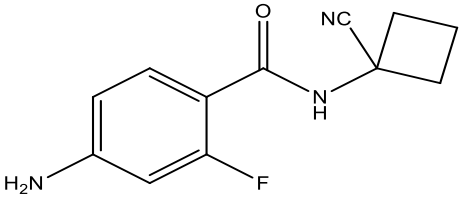
Apalutamide Impurity-2 (CAS: 915087-24-0) (IMP-2) ($C_8H_7FN_2O_3$)

2-Fluoro-4-(hydroxyamino)-N-methylbenzamide (Mo. Wt. 198.2 g/mol)



Apalutamide Impurity-3 (IMP-3) ($C_8H_9FN_2O$)

4-amino-2-fluoro-N-methylbenzamide (Mo. Wt. 168.17 g/mol)



Apalutamide Impurity-4 (IMP-4) ($C_{12}H_{12}FN_3O$)

4-amino-N-(1-cyanocyclobutyl)-2-fluorobenzamide (Mo. Wt. 233.124 g/mol)

Figure 2: Chemical structures of related substances (Impurities) of Apalutamide

MATERIALS AND METHODS

Apalutamide and other related substances IMP-1, IMP-2, IMP-3 and IMP-4 were of AR grade. Waters Alliance HPLC system with PDA/UV detector (Model No. 2996 and 2695), Digital Ultra Sonicator (Labman) (Model No. LMUC-3), analytical balance (Sartorius) (Model No. BT224S) and pH Meter (Polmon) (Model No. LP139SA) were used for the study. Ammonium phosphate (SRL), Ortho phosphoric Acid (Avra) HPLC grade Acetonitrile (Merck), hydrochloric acid, sodium hydroxide and hydrogen peroxide (30% w/v) were used. Milli-Q water and Millipore 0.45 µm membrane filters were used for the entire study.

Procedure

Preparation of mobile phase

1.32 grams of Ammonium phosphate was weighed and transferred into a 1000 mL volumetric flask, dissolved in Milli-Q water and pH was adjusted to 7.0 with Ammonia solution and made up to the volume with the diluent (Acetonitrile). A mixture of Ammonium phosphate buffer solution and Acetonitrile is used as the mobile phase on gradient mode.

Preparation of standard stock solutions

3.0 mg of each of IMP-1, IMP-2, IMP-3, IMP-4 and 2.0 mg of Apalutamide standard were weighed and transferred into a 100 mL volumetric flask, dissolved and made up to the volume with diluent (Acetonitrile) and further dilutions were made as per the requirement.

Preparation of test solution

10.0 mg of each of IMP-1, IMP-2, IMP-3, IMP-4 and Apalutamide samples were weighed, transferred into a 10 mL volumetric flask and diluted with diluent.

Method validation

Optimized chromatographic conditions

A mixture of Ammonium phosphate buffer solution and Acetonitrile (30: 70, v/v) was used as the mobile phase on gradient mode for the chromatographic study (Flow rate: 1.0

mL/min; UV detection: 243 nm; Injection volume: 20 µl). Waters Alliance HPLC system with PDA/UV detector (Model No. 2996 and 2695) was used for the chromatographic study and the run time was 40 min.

Linearity

Drug solutions containing Apalutamide and its impurities (5-40 µg/mL) were prepared n from their stock solutions and injected into the HPLC system (n=3) and the corresponding chromatograms were recorded. The peak area of Apalutamide and its impurities (IMP-1, IMP-2, IMP-3 IMP-4) were noted and calibration curves were drawn by plotting the concentration of Apalutamide and its impurities (IMP-1, IMP-2, IMP-3 IMP-4) solutions on the x-axis and the corresponding mean peak area on the y-axis.

Precision and Accuracy studies

Method precision was evaluated by test sample spiked with Apalutamide IMP-I, IMP-2, IMP-3 and IMP-4 at specification level with respect to the test sample concentration by injecting six different test preparations.

The accuracy of the method was proved by checking the recovery of known impurities. The test solution was spiked with the known impurities at LOQ, 50%, 100% and 200% level and the % RSD was calculated.

Assay of Apalutamide tablets

Apalutamide is available as tablets (Label claim: 60 mg) with brand names APATIDE, APNAT, APALUCIDE, PRYOR, ERLEADA etc from different pharmaceutical companies. 20 tablets of two different companies were accurately weighed, powdered and powder equivalent to 25 mg of Apalutamide was carefully transferred to two separate 25 ml volumetric flasks and Apalutamide was extracted with HPLC grade acetonitrile, sonicated and filtered. These solutions were diluted further with the mobile phase and 20 µL of each of these formulation solutions was injected in to the system (n=3) and the average peak area was calculated from the respective chromatograms and thereby the amount of Apalutamide was calculated from the calibration curve.

Stress degradation studies

Stress degradation studies of Apalutamide solution was carried out to confirm whether the known impurities are the degradation products or not.

For acidic degradation, the sample was treated with 2N hydrochloric acid at 80°C for 4 hours and then neutralized with 2N sodium hydroxide. For alkali degradation, the sample was treated with 2N NaOH at 80°C for 8 hours and then neutralized with 2N hydrochloric acid. For oxidative degradation, the sample was treated with 0.001N $KMnO_4$ on bench top for 30 min.

After degradation treatments the samples were cooled to room temperature, diluted with the diluent and injected for chromatographic analysis.

RESULTS AND DISCUSSION

The authors have developed a new validated stability indicating RP-HPLC method for the quantification of Apalutamide and its related substances on gradient mode using Waters Alliance system (Model no. 2996 and 2695) with Inertsil ODS-3 (250 × 4.6 mm, 5µm) column (PDA detector) (Column temperature 35ºC) and a mixture of Ammonium phosphate buffer solution and Acetonitrile (UV detection 271 nm) was chosen for the present study. The injection volume was 20 µl and the run time was 40 min. The analytical methods developed earlier for the estimation of Apalutamide summarised in Table 1 and the gradient program was given in Table 2.

Table 1: Review of literature

Method	Mobile phase (v/v)	Column	λ (nm)	Comment	Ref
HPLC	0.01 M disodium phosphate dihydrate buffer (pH 4.20 ± 0.05): Acetonitrile	Luna Omega Polar C 18	225	Related substances (Gradient mode)	3
HPLC LC-MS/MS	10 mM KH_2PO_4 (pH 3.5): Acetonitrile	Atlantis dC_{18}	270	Design of experiments (Gradient mode)	4
LC–ESI-MS/MS	0.1% Formic acid: Acetonitrile (20:80)	Inertsil C18	--	Human plasma Canagliflozin as internal standard (Isocratic mode)	5
RP-HPLC	Ammonium phosphate buffer (pH 7.0): Acetonitrile (30: 70)	Inertsil ODS-3	243	4 Impurities (Gradient mode)	Present method

Table 2: Gradient program

Time (minutes)	Acetonitrile (%)	Buffer (%)
0.01	80	20
9	75	25
20	30	70
25	20	80
30	80	20
35	80	20

Method validation[6-8]

Linearity, Precision and Accuracy

Linearity was conducted by preparing the five levels of linearity solutions for Apalutamide and for known impurities. The LOD and LOQ results were shown in Table 3 (Figure 2). The retention times of Apalutamide IMP-I, IMP-2, IMP-3 and IMP-4 were observed as 22.69, 3.48, 5.68, 16.34 and 16.83 min.

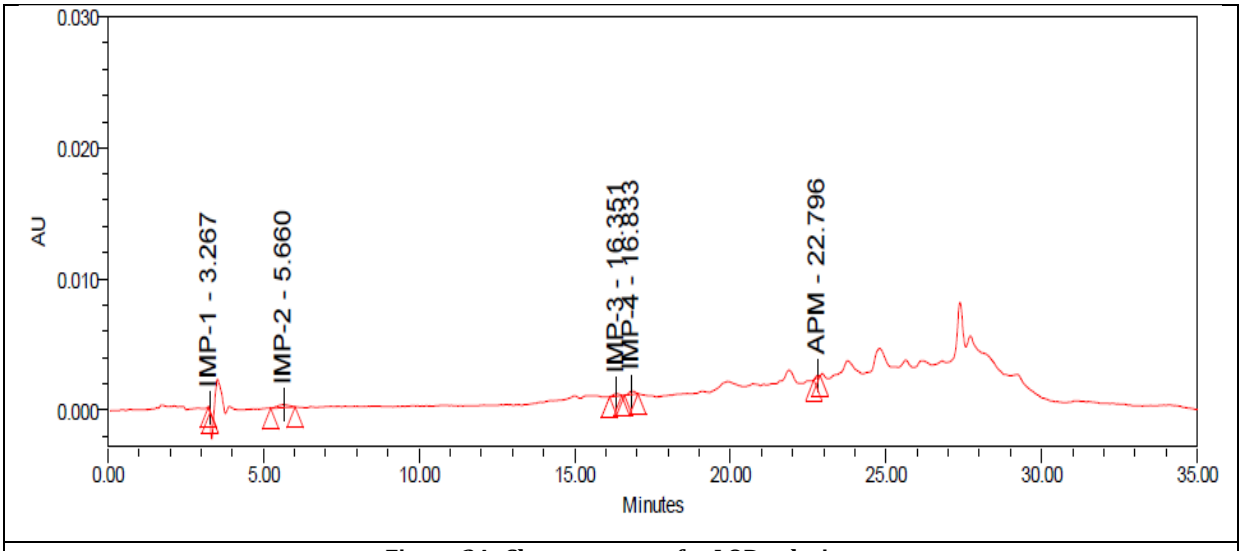


Figure 2A: Chromatogram for LOD solution

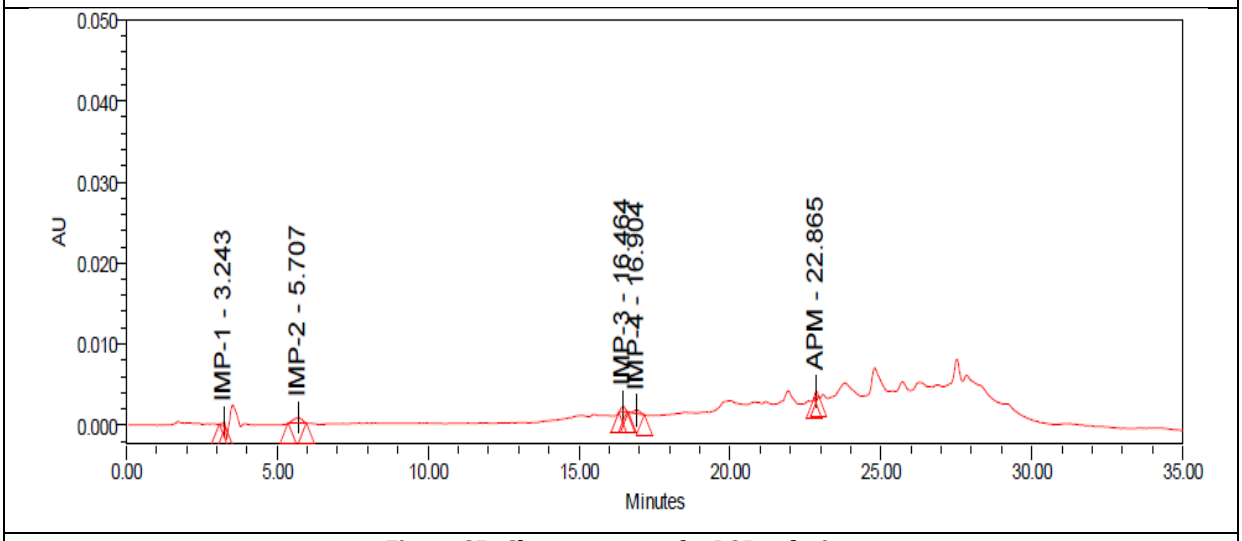


Figure 2B: Chromatogram for LOD solution

Table 3: Results of LOD & LOQ

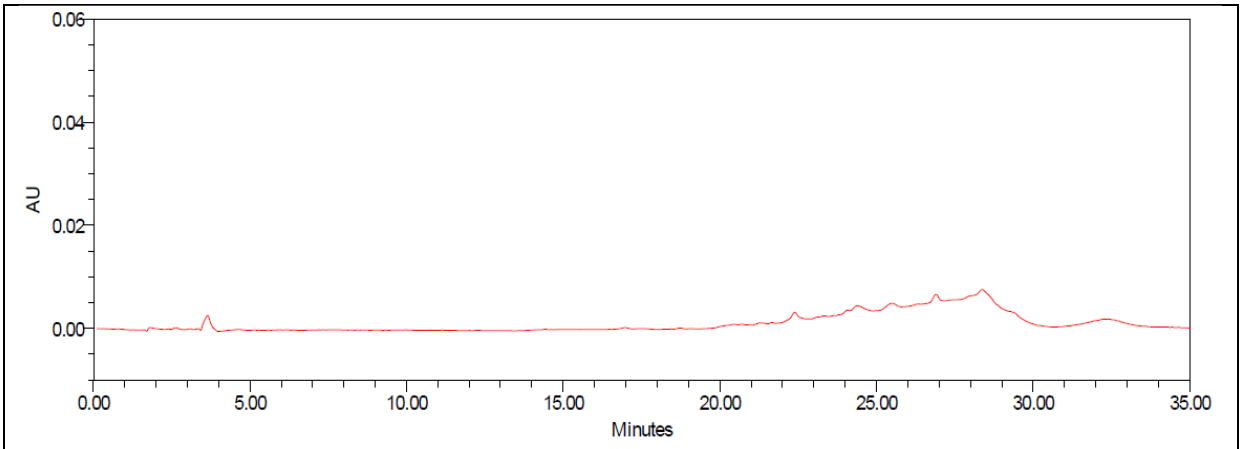
Injection	Limit of quantification				
	Peak area				
	IMP-1	**IMP-2**	**IMP-3**	**IMP-4**	**Apalutamide**
1	2985	13396	6332	6421	3956
2	2634	15407	7234	6530	4793
3	2856	13956	6395	6352	4025
4	2901	13263	7020	6329	4562
5	2790	13156	7325	6989	4489
6	2923	14434	6452	6524	4562
Mean	2848	13935	6793	6421	4398
%RSD	**3.96**	**5.68**	**6.06**	**3.40**	**6.90**
Limit of detection					
Injection	**Peak area**				
	IMP-1	**IMP-2**	**IMP-3**	**IMP-4**	**Apalutamide**
1	391	5713	2565	3227	1123
2	379	5625	2495	3312	1209
	S/N Ratio				
LOQ	14.2	15.3	15.0	10.0	15.9
LOD	4.4	4.2	3.7	4.4	3.0

The representative chromatograms of Apalutamide and its four impurities were shown in Figure 3. A calibration graph was drawn by taking the concentration of Apalutamide IMP-I, IMP-2, IMP-3 and IMP-4 on the x axis and the corresponding peak area on the y axis (Figure 4).

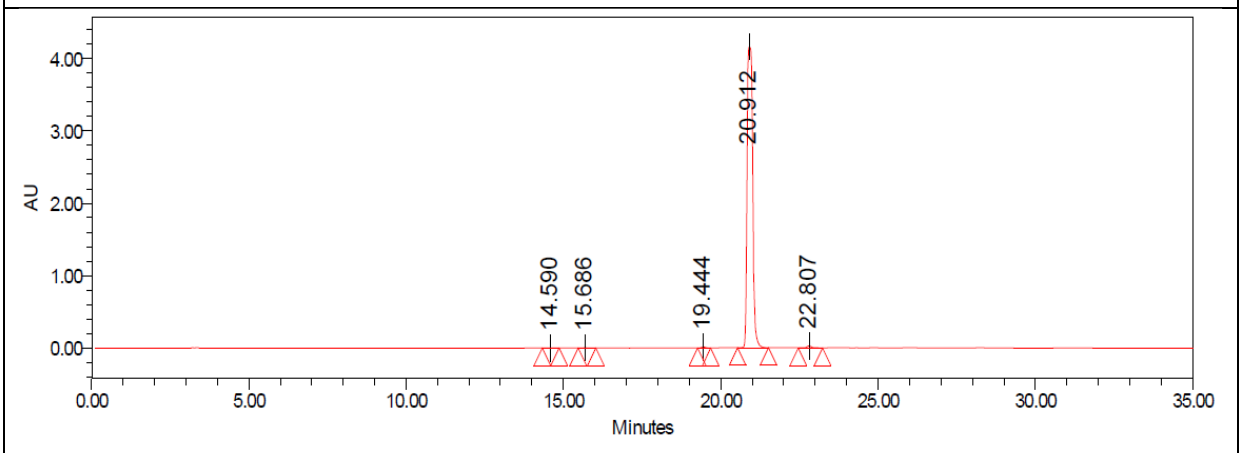
Table 4: Linearity of Apalutamide and impurities

Conc. (µg/ml)	Apalutamide	IMP-1	IMP-2	IMP-3	IMP-4
	Mean peak area				
5	17781	13249	50088	44793	18933
10	36453	47474	101464	85426	39794
20	74932	93859	204377	167120	80599
30	113295	143835	290240	260196	131188
40	150509	193274	408962	341451	165925
Rt (min)	22.69	3.48	5.68	16.34	16.83

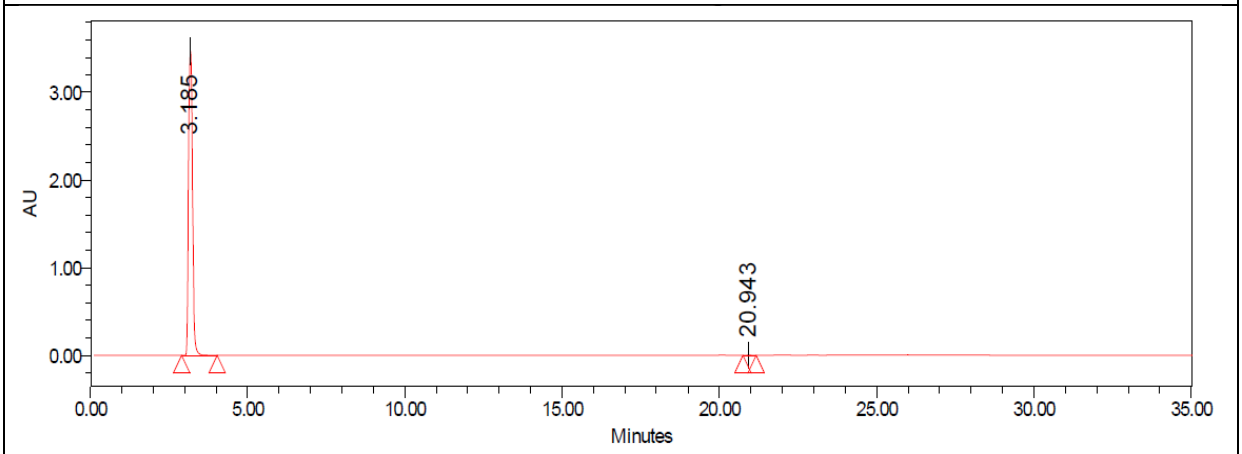
*Mean of three replicates



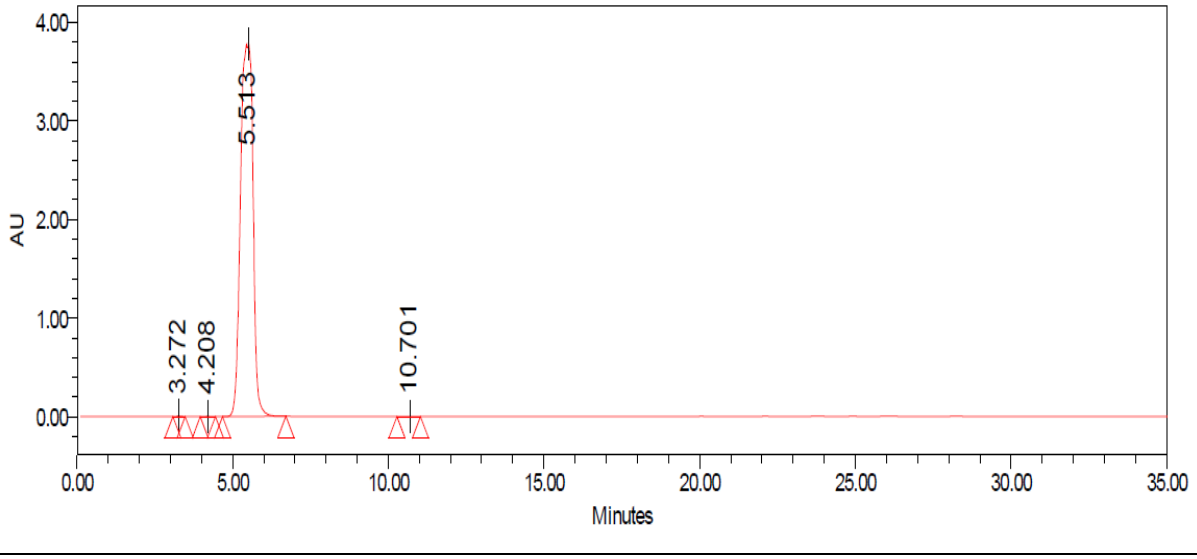
Blank solution



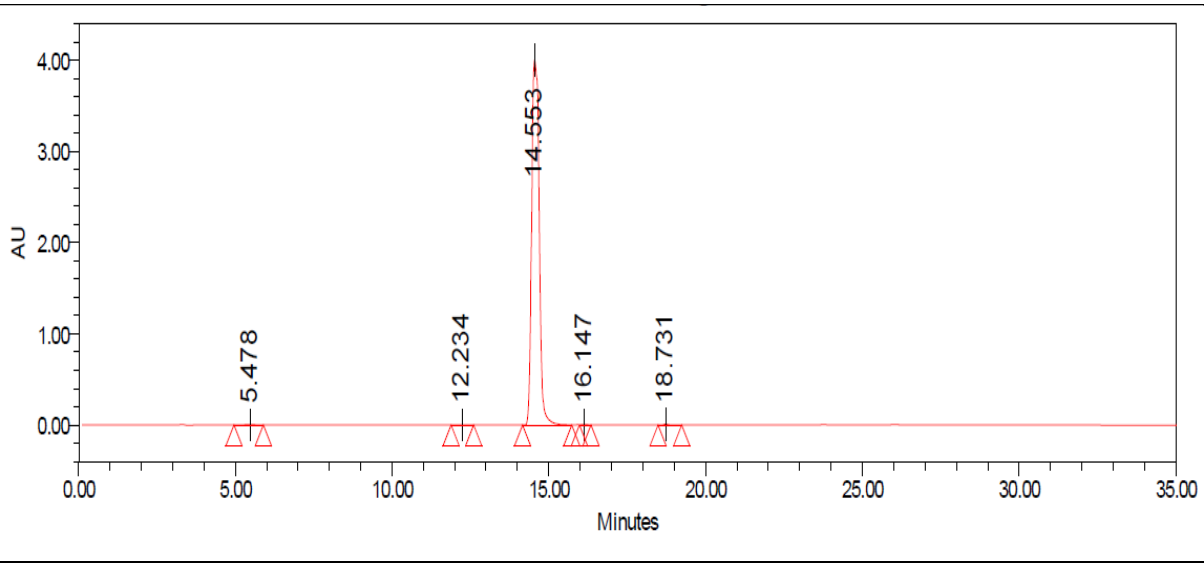
Apalutamide (Rt: 20.912 min)



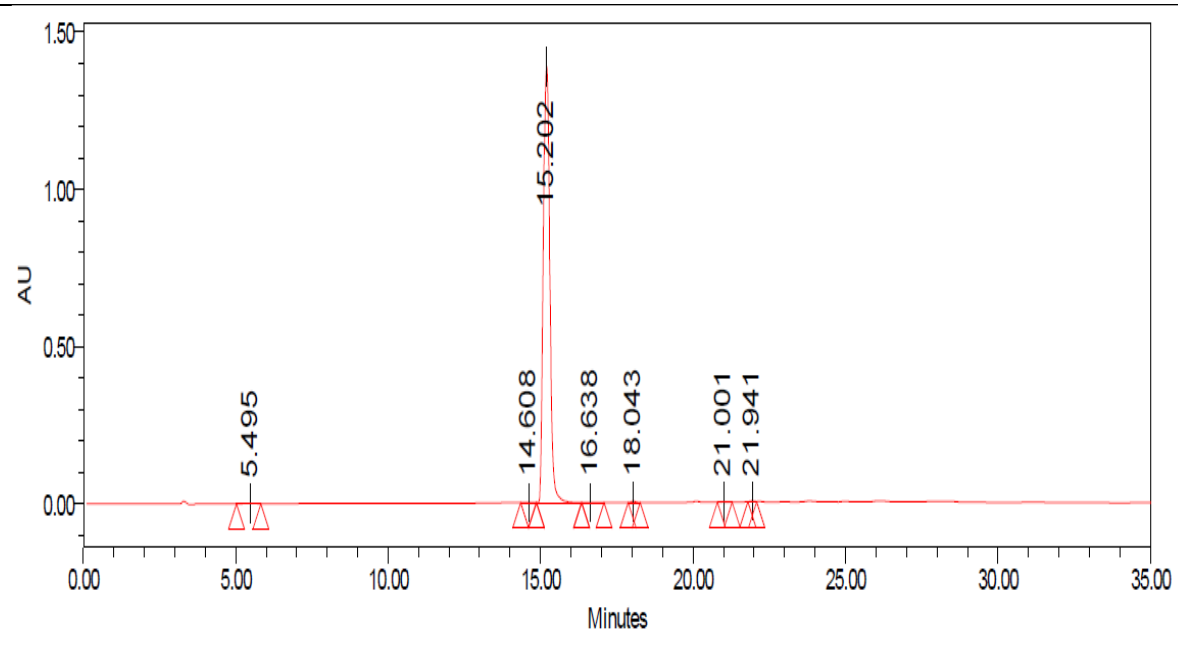
Impurity 1 (Rt: 3.185 min)



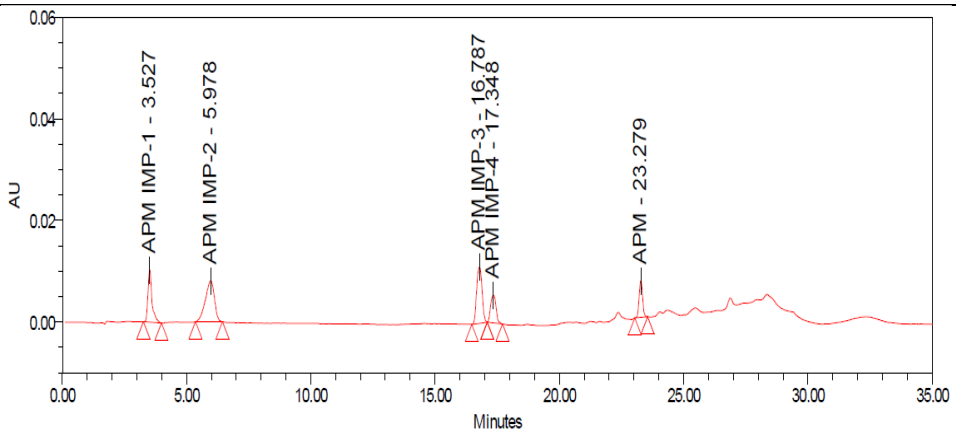
Impurity 2 (Rt: 5.513 min)



Impurity 3 (Rt: 14.553 min)

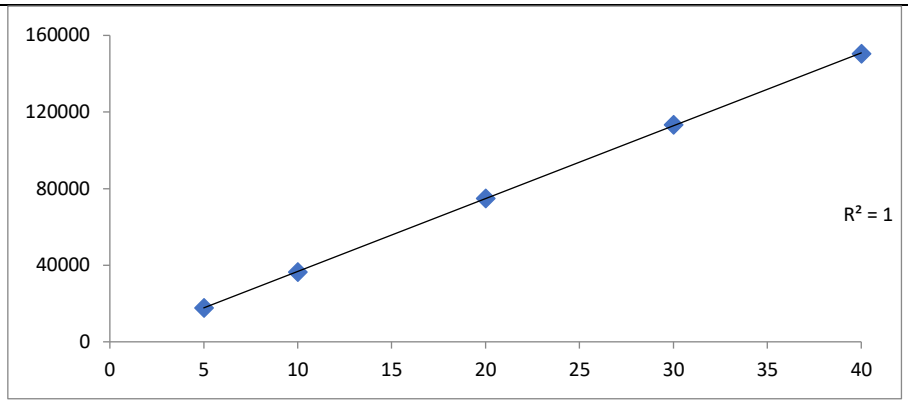


Impurity 4 (Rt: 15.202 min)

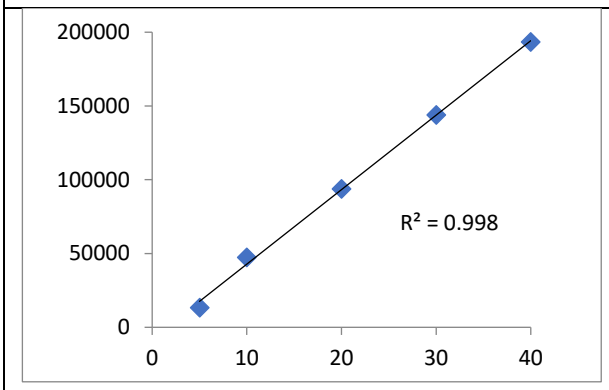


Standard solution

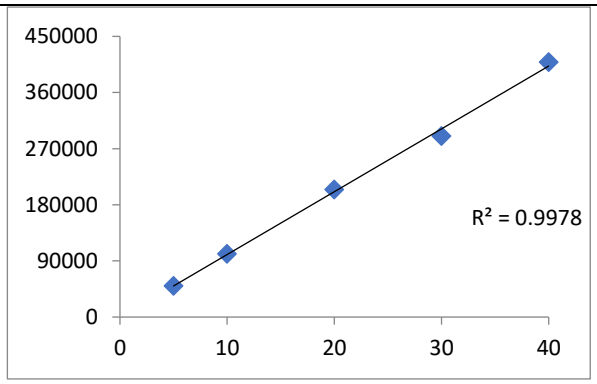
Figure 3: Representative chromatograms of Apalutamide and its impurities



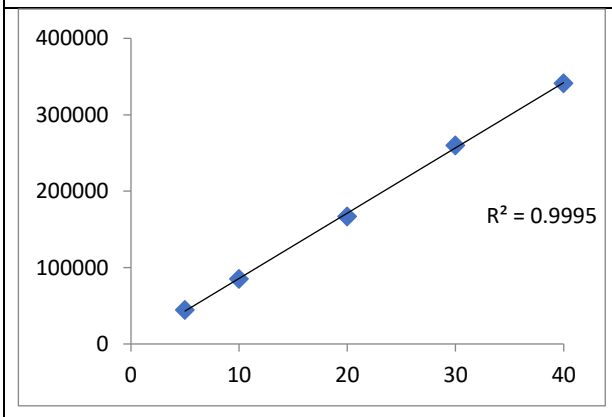
Apalutamide



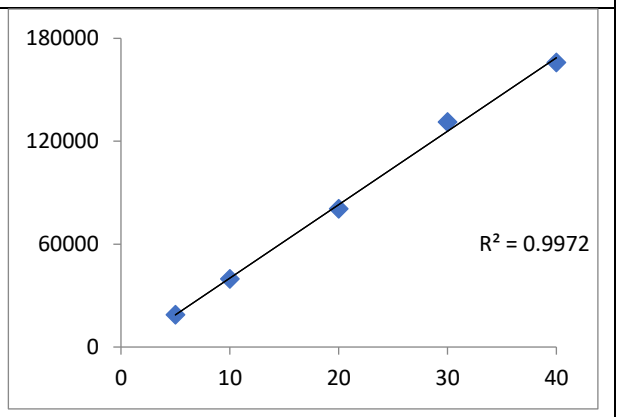
IMP-1



IMP-2



IMP-3



IMP-4

Figure 4: Calibration curves of Apalutamide and impurities (IMP)

The precision results of the impurities were shown in Table 5. The accuracy of the method was proved by checking the recovery of known impurities. Test solution was spiked with known impurities at LOQ, 50%, 100% and 200% level and the obtained results were summarized in Table 6-9.

Table 5: Precision study

S. No.	IMP-1 (%)		IMP-2 (%)		IMP-3 (%)		IMP-4 (%)	
	Analyst 1	Analyst 2	Analyst 1	Analyst 2	Analyst 1	Analyst 2	Analyst 1	Analyst 2
1	0.152	0.151	0.150	0.151	0.152	0.153	0.154	0.151
2	0.154	0.153	0.151	0.153	0.153	0.151	0.150	0.153
3	0.150	0.154	0.153	0.154	0.150	0.153	0.152	0.152
4	0.153	0.150	0.150	0.154	0.150	0.150	0.152	0.150
5	0.152	0.153	0.153	0.150	0.152	0.150	0.153	0.154
6	0.150	0.151	0.151	0.153	0.150	0.152	0.151	0.152
Mean	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150
%RSD	**0.96**	**0.93**	**0.82**	**0.98**	**0.80**	**0.83**	**0.85**	**0.85**

Table 6: Accuracy study of Impurity-1

	IMP-1 % Recovery			
	% Recovery at LOQ Level	% Recovery at 50 % Level	% Recovery at 100 % Level	% Recovery at 200 % Level
	95.5	99.9	99.5	98.9
	96.1	100.2	100.2	100
	91.2	98.2	98.9	98.9
	93.9	99.3	99.6	99.6
	94.6	100.5	99.9	100.9
	92.1	99.4	99.9	99.4
Mean %Recovery	93.90	99.6	99.67	99.6
Mean %RSD	**1.87**	**0.75**	**0.41**	**0.69**

Table 7: Accuracy study of Impurity-2

	IMP-2 % Recovery			
	% Recovery at LOQ Level	% Recovery at 50 % Level	% Recovery at 100 % Level	% Recovery at 200 % Level
	97.6	99.7	100.5	99.5
	95.2	100.6	98.5	98.6
	99.1	98.0	99.3	99.9
	98.5	99.5	98.9	100.3
	98.5	100.8	100.1	99.6
	100.0	99.5	98.7	100.9
Mean %Recovery	98.2	99.7	99.3	99.8
Mean %RSD	**1.53**	**1.10**	**0.74**	**0.71**

Table 8: Accuracy study of Impurity-3

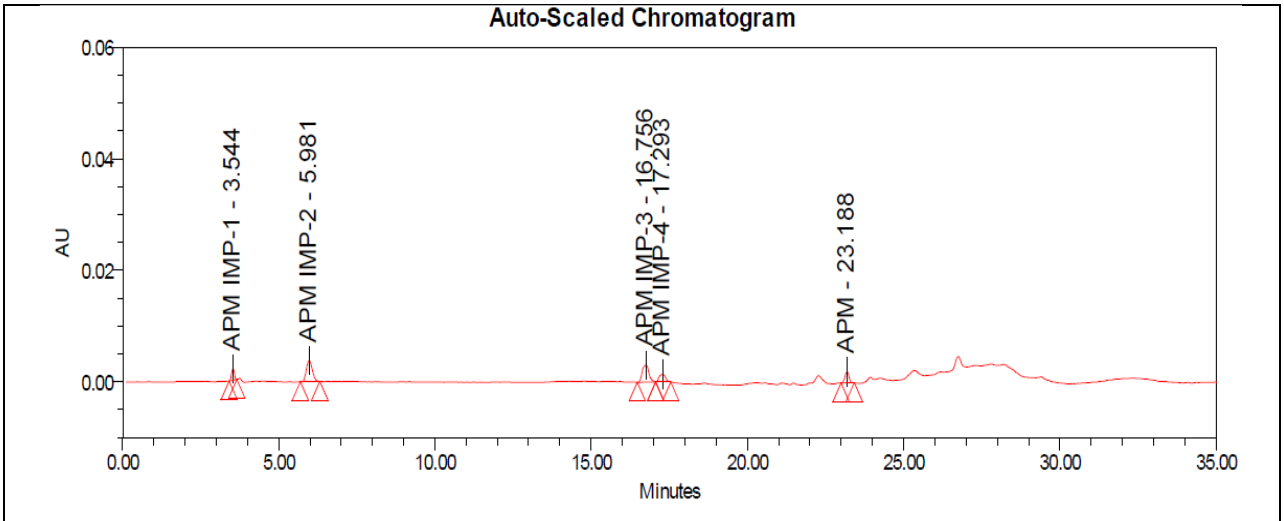
	IMP-3 % Recovery			
	% Recovery at LOQ Level	% Recovery at 50 % Level	% Recovery at 100 % Level	% Recovery at 200 % Level
	95.1	98.1	99.3	100.5
	96.8	99.6	98.2	100.9
	97.0	99.0	100.2	99.1
	97.8	98.8	99.8	98.2
	98.0	100.9	100.8	99.8
	96.8	100.9	99.8	100.9
Mean %Recovery	96.9	99.6	99.7	99.9
Mean %RSD	**0.97**	**1.05**	**0.81**	**0.99**

Table 9: Accuracy study of Impurity-4

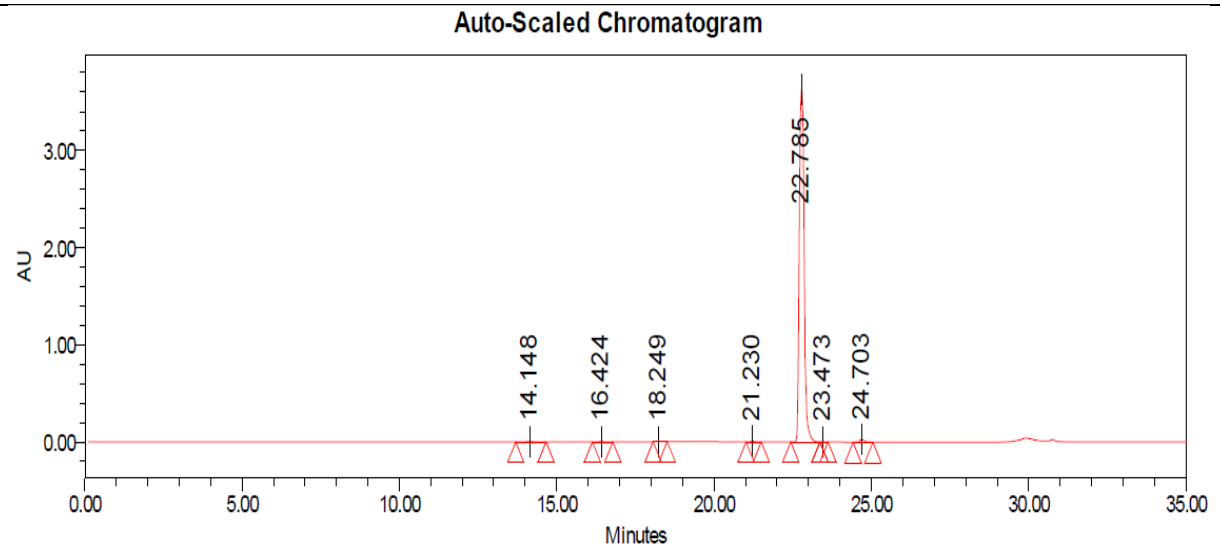
	IMP-4 % Recovery			
	% Recovery at LOQ Level	% Recovery at 50 % Level	% Recovery at 100 % Level	% Recovery at 200 % Level
	99.3	100.2	99.7	100.5
	98.2	99.8	98.6	100.6
	99.9	98.5	98.5	99.9
	100.5	98.6	98.2	98.9
	99.5	99.6	99.9	100.9
	98.6	98.6	99.9	98.7
Mean % Recovery	99.3	99.2	99.1	99.9
Mean % RSD	**0.77**	**0.68**	**0.72**	**0.85**

Stress degradation studies

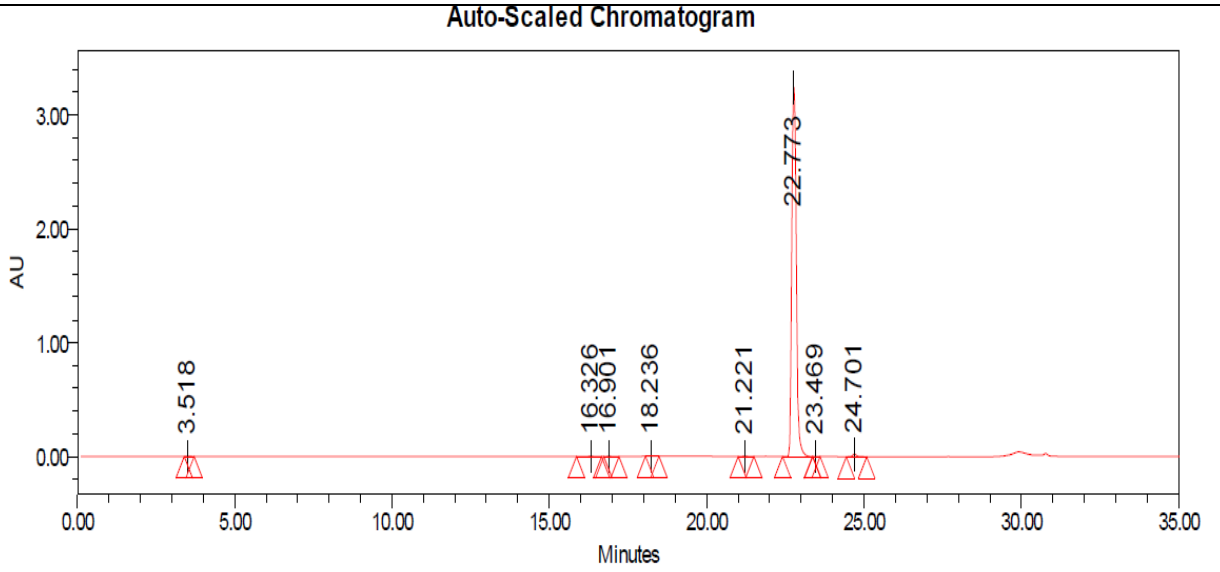
The pure drug Apalutamide API was eluted at 22.69 ± 0.1 mins with theoretical plates (>2000 and tailing factor <1.5 which are within the acceptable criteria. The % degradation was found to be less than 2% and the system suitability parameters were within the acceptable criteria. During the degradation studies of Apalutamide such as acidic, alkaline and oxidative degradation there is no interference of the drug peak indicating that the proposed method is highly specific and the resolution is more than 2.0 and the representative chromatograms were shown in Figure 5 and the results were shown in Table 10.



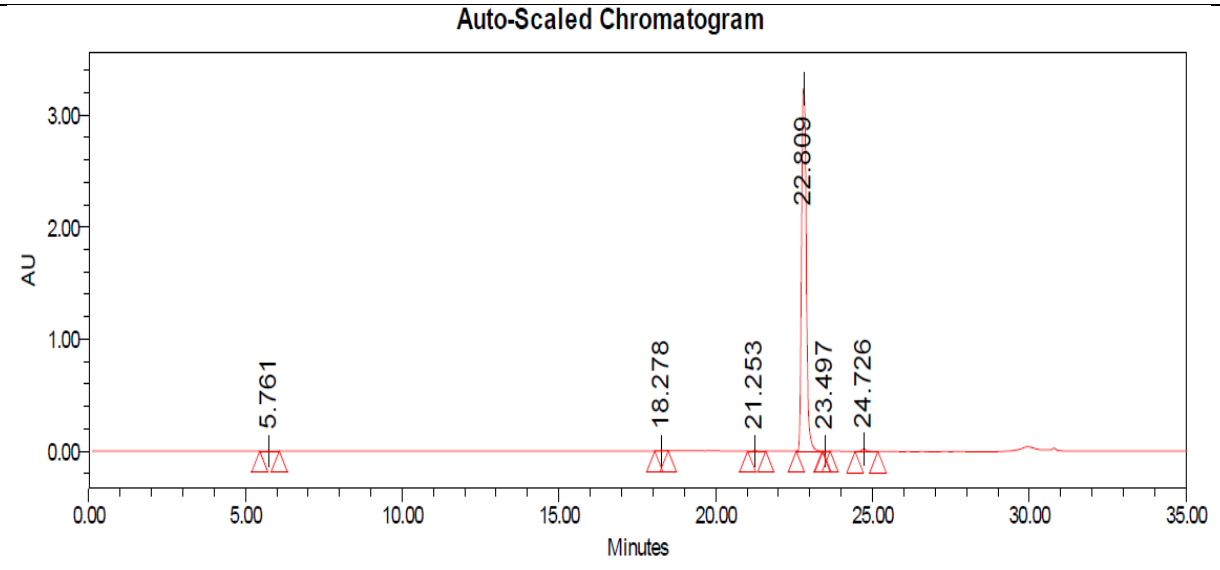
Chromatograms of Apalutamide & Impurities (Linearity level-1)



Alkaline degradation of Apalutamide & Impurities



Oxidative degradation of Apalutamide & Impurities



Acidic degradation of Apalutamide & Impurities

Figure 5: Chromatograms of Apalutamide & Impurities during stress degradation studies

Table 10: Stress degradation studies (ND: Not detected; NA: Not applicable)

Degradation condition	IMP-1 (%)	IMP-2 (%)	IMP-3 (%)	IMP-4 (%)	Total impurities (% w/w)	% Net degradation
Unstressed sample	ND	ND	ND	ND	1.07	NA
Acidic degradation 2N HCl /80°C for 4 hrs	ND	0.06	ND	ND	0.97	0.97
Alkaline degradation 2N NaOH /80°C for 8 hrs	ND	ND	0.12	ND	1.29	1.29
Oxidative degradation 10% H_2O_2 degradation at 80°C for 1hr	0.13	ND	ND	ND	1.27	1.27

CONCLUSION

A simple, rapid, accurate and precise RP-HPLC method for the analysis of Apalutamide and its related substances has been developed and validated as per ICH guidelines. It is concluded that the Apalutamide related substances and preservative content method is validated and suitable for its intended purpose and it is suitable for the testing of in-vitro, related substances and preservative content samples of Apalutamide suspension 100 mg/mL and tablets during routine quality control and stability testing.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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