**Quantitative Stability Indicating Bio-analytical Method Development and Validation of Apalutamide - Apalutamide D3 By Using Ultra Performance Liquid Chromatography in Human Plasma**

**D. China Babu\*1, G. Sai Sri Harsha1, Goli Venkateswarlu1, M. Nem Kumar Jain1, Alagusundaram1, SK. Aleesha2**

**1School of Pharmacy, ITM University Gwalior, Madhya Pradesh – 474001, India**

**2Narayana Pharmacy College, Nellore, Madhya Pradesh- 524002, India**

**\*Corresponding Author:**

**Mr. D. China Babu**

Asst. Professor,

Dept. of Pharmaceutical Analysis,

ITM University, Gwalior,

Madhya Pradesh, India.

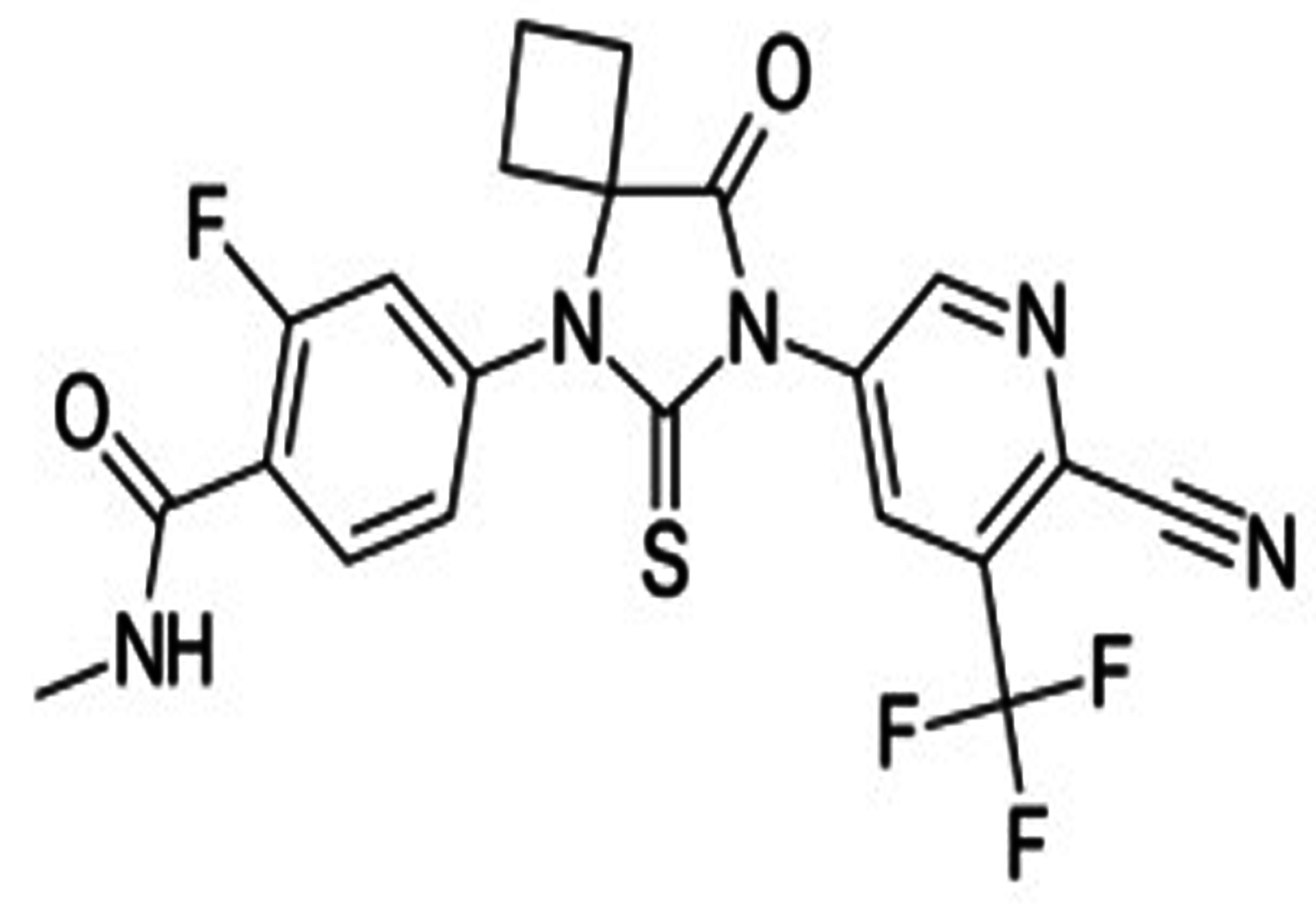
**ABSTRACT**

A simple, convenient, specific,precise and highly conventional stability indicating ultra performance liquid chromatographic‑ diode array method was developed for the quantification of Apalutamide in human plasma. The phenomenex Luna (100x4.6x5µ) column was used for apalutamide separation and mobile phase was composed with 5 mM ammonium fumarate and acetonitrile in the ratio of 15:85 v/v and buffer pH 3.5 was adjusted with glacial acetic acid and detected at 345 nm. The Apalutamide‑D3 used as internal standard and K2‑EDTA used as coagulant.The liquid‑liquid extraction process used for extraction of drug from human plasma with tert butyl methyl ether. The retention times of Apalutamide and Apalutamide D3 (ISTD) was 1.48 min & 1.97 min respectively. The assay of the method was validated in human plasma in the concentration range from 307.26-200013.87 pg/ml with the accuracy and precision ranging from 3.86 to 4.87. Recovery studies were found to be 103.79%, 90.93% & 96.83% for HQC, MQC and LQC respectively. The stability of the drug was evaluated in human plasma with different conditions of auto-sampler, freeze-thaw, bench top, short term and long term stability studies were performed. The method was proved as highly sensitive and selective for the quantification of Apalutamide and determined at picogram level. There was no matrix effect observed and proved as a stability indicating method.

**Keywords:**Apalutamide, Acetonitrile, Di Potassium ethylene diamino tetra acetic acid, High Quality control, Medium Quality control.

**INTRODUCTION**

Apalutamide (Figure 1) is an anti-androgen. The IUPAC name of the drug is 4-{7-[6-cyano-5-(trifluoromethyl) pyridine-3-yl]-8-oxo-6 sulfanylidine 5,7diazaspiro [3.4] octan-5-yl}-2-fluoro-N-methylbenzamide (Fig.1). It shows the antagonistic effect on androgenic receptors. The drug is under the class of non‑steroidal anti‑androgen.. It is second generation androgen receptor antagonist [Anjaneyulu Reddy R., *et al*.2019]. It is developed to inhibit androgen receptor mediate prostate cancer cell proliferation [Vadim Koshkin S *et al*., 2018].  Apalutamide evaluated in high-risk patients for its activity and safety of non-metastatic castration-resistance prostate cancer to identify it by conducting multicentre phase-2 trials on nm-CRPC patients with a high risk of advancement [Smith MR *et al.,* 2016, Zhou, Z., Hu.X. 2018].The efficacy of the apalutamide was evaluated in men with non-metastatic castration-resistance (nmcr) prostate cancer in the development of metastasis of high risk patients. They were conducted a double‑blind, placebo controlled, phase‑3 trial of nmcr prostate cancer and a prostate‑specific antigen doubling time of 10 months or less in men patients  [Smith MR *et al*., 2018, Small EJ *et al*., 2018]. The patients were taken apalutamide (240 mg/day) or placebo. The androgen deprivation therapy continued for patients.The prime end point was metastasis free survival [Sandler HM *et al*.,2016, Rathkopf DE *et al*.,2017].       The efficacy of apalutamide was evaluated with abiraterone acetate and prednisone in patients before or after treatment with progressive metastatic castration resistant prostate cancer  [Suresh P Sulochana *et al*., 2018, Dellis A *et al*., 2018]. The chemotherpy exposure shows  more effective on CRPC [Ranjan RK *et al*.,2018, Khan Z.G *et al*.,2016]. Few analytical  methods were reported on its related and core molecule of Apalutamide [Sandhya Rani J, *et  al*.,2018, Anjaneyulu Reddy R., *et al*. 2019, Ashok Zukkala *et al*. 2019, Chinababu D *et al* 2021, Sai Uday Kiran, G., Sandhya P. 2020].



**Figure1: Structure of Apalutamide**

**MATERIALS AND METHODS**

**Materials & reagents**

The apalutamide drug and internal standard of apalutamide D3 were procured from Ajanta Pharma LTD, Mumbai, India. The water used for analysis was prepared from milli-Q water purified system purchased from Millipore, Mumbai, India. The HPLC grade acetonitrile was purchased from Merk, Mumbai, India. Analytical grade of ammonium fumarate, K2-EDTA and tert-butyl methyl ether and glacial acetic acid purchased from SD fine chem, Mumbai, India. The plasma sample was purchased from Santhiram Medical College, Nandyal, AP, India.

**Instrumentation**

The liquid chromatographic system was Shimadzu UPLC‑2010 CHT (Shimadzu, Corporation,Kyoto,Japan) consisting of a quaternary pump, column heater, solvent degasser. The column used for separation was Phenomenex Luna (100 x 4.6mm x 5µ) Waters Corporation, Milford, USA. The column temperature was maintained at ambient and flow rate of the mobile phase was maintained at 1mL/min. The analyte was detected at 345 nm by using a photodiode array detector. The auto sampler temperature was maintained at 15℃ and pressure of the system was maintained at 6000 psi.

**Methodology**

**Statistical analysis**

The developed method in UPLC was validated to ensure the stability of the analytical method and consistency of the results. The statistical analysis was performed with one way variance analysis treatments.

**Preparation of standard solution**

Apalutamide (1mg/ml) and internal standard Apalutamide D3 standard solutions were prepared in 10 ml separate volumetric flask in the mobile phase. Apalutamide D3 internal standard, 0.5 µg/ml solution was prepared by diluting its stock solution with ammonium fumarate: acetonitrile (15:85 v/v). The plasma spiked working standard solutions prepared for Apalutamide in the concentration ranging from 200013.9 pg/ml (STD1) to 307.3 pg/ml (STD 10).

**Quality control samples**

Quality control samples of the APA were prepared for the qualitative evaluation of calibration curve. Lower limit of quantification (LLOQ), low quality control (LQC), Middle quality control 1 & 2 (MQC 1 & 2) and high quality control (HQC) has been prepared in drug free plasma and solutions were stored at 4℃.

**Extraction of APA from plasma**

The plasma samples were stored in a freezer at -70℃ and thaw at room temperature before processing. A 200 µl of plasma was transferred to the Ria vials, then 50µl of IS working standard solution 0.5 µg/ml was spiked and vortexed for 10 sec with100µl of 2% formic acid, tert-butyl methyl ether 2.5 ml was added and vortexed again for 10 min. After centrifugation at 3000 RPM for 10 min and transferred the organic layer in to new ria vials and evaporated until dry under a gentle stream of nitrogen gas at 45℃. The residue was reconstituted with 150 µl of the mobile phase and 10 µl aliquots were injected into UPLC system.

**Preparation of buffer**

Ammonium fumarate was prepared 1M solution from that collected 5 mL and transferred into 1000 mL volumetric flask. The volume was made up to mark with water and obtained 5 mM ammonium fumarate.

**Mobile phase**

Buffer and acetonitrile was taken in the ratio of 15:85 v/v used as mobile phase.

**RESULTS & DISCUSSION**

**Bio-analytical method validation**

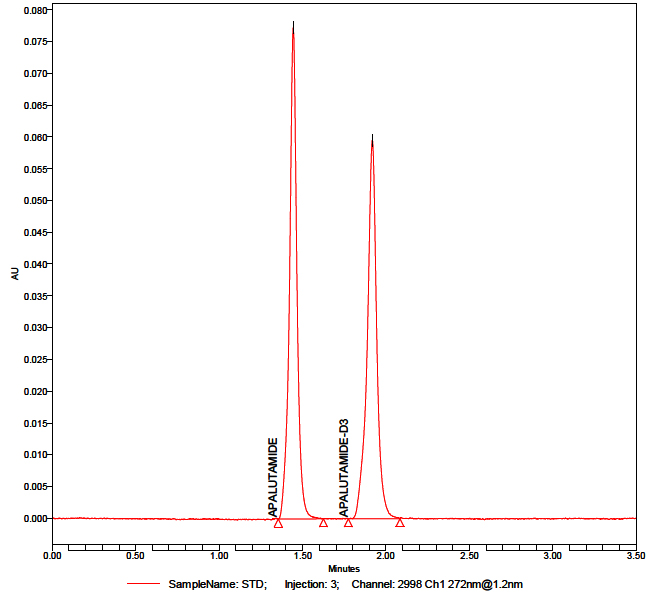
The validation was performed based on guidance for industry: Bio‑ analytical method validation from the US-FDA [US FDA Bio-analytical validation guidelines 2001, Smith G,2012, Zakkula A *et al*.,2019].

**Optimised parameters**

The separation was obtained with 5 mM ammonium fumarate: acetonitrile in the ratio of 15:85 v/v, buffer pH 3.5 was adjusted with glacial acetic acid and other optimized parameters discussed in Table 1. The standard chromatogram was shown in Figure 2.

**Table 1:** **Optimised parameters of the bio-analytical method**

|  |  |  |
| --- | --- | --- |
| **S.No** | **Parameters** | **Conditions** |
| 1 | Column | Phenomenex Luna (100x4.6x5µ) |
| 2 | Mobile phase | 5mM Ammonium fumarate: acetonitrile (15:85 v/v) and pH 3.5 |
| 3 | Column temperature | Ambient |
| 4 | Biological Matrix | Human plasma |
| 6 | Anti-coagulant | K2-EDTA |
| 7 | Flow rate | 1 mL/min |
| 8 | Wavelength | 345 nm |
| 9 | Run time | 3.5 min |
| 10 | Injection volume | 5 µL |
| 11 | Retention time | Apalutamide 1.48 min  Apalutamide D3 1.97min |



**Figure 2: Standard chromatogram of Apalutamide**

**System Suitability**

System suitability of current method was checked by injecting six replicate injections using an aqueous standard mixture equivalent to the MQC concentration of the calibration curve. The validation of the method on each day was started with system suitability as a first experiment.

**Specificity/ Selectivity**

The method specificity was established by viewed the standard blanks of different lots of commercially available human plasma. A different lot of plasma was screened for the specificity of the experiment. Out of ten, seven batches were of intended anticoagulant plasma, one of haemolytic plasma, one of lipidemic plasma and one lot containing heparin as an anticoagulant. The significant interferences were not observed in investigated human plasma lots at the retention times of drug and ISTD (Figure 1). In standard blank samples at the retention time the peak area of the drug was ≤ 20.00% of the peak area of the drug in the extracted LLOQ sample; for ISTD it was considered as ≤ 5.00%. The calibration curve standards and quality controls were prepared from blank matrix of pooled plasma lots.

**Linearity and Quality controls**

The linearity of the method was assessed by ten point standard curve. The weighted least square regression analysis 1/x2was used for the study of linearity from standard plots associated with ten point standard curve. All the three calibration curves analysed during the course of validation were found to be linear from the standard concentration ranging from 200013.87‑ 307.26 pg/mL and regression coefficient value was attained 0.999. A good linear relationship was shown between the peak area ratios of APA/ISTD. 

**Recovery studies**

The analyte was recovered from the plasma samples was studied at different levels of quality controls of LQC,MQC-2 and HQC. The % recovery values of LQC, MQC-2 & HQC were found 96.83%, 93.90% and 88.65% respectively for apalutamide.

**Matrix effect**

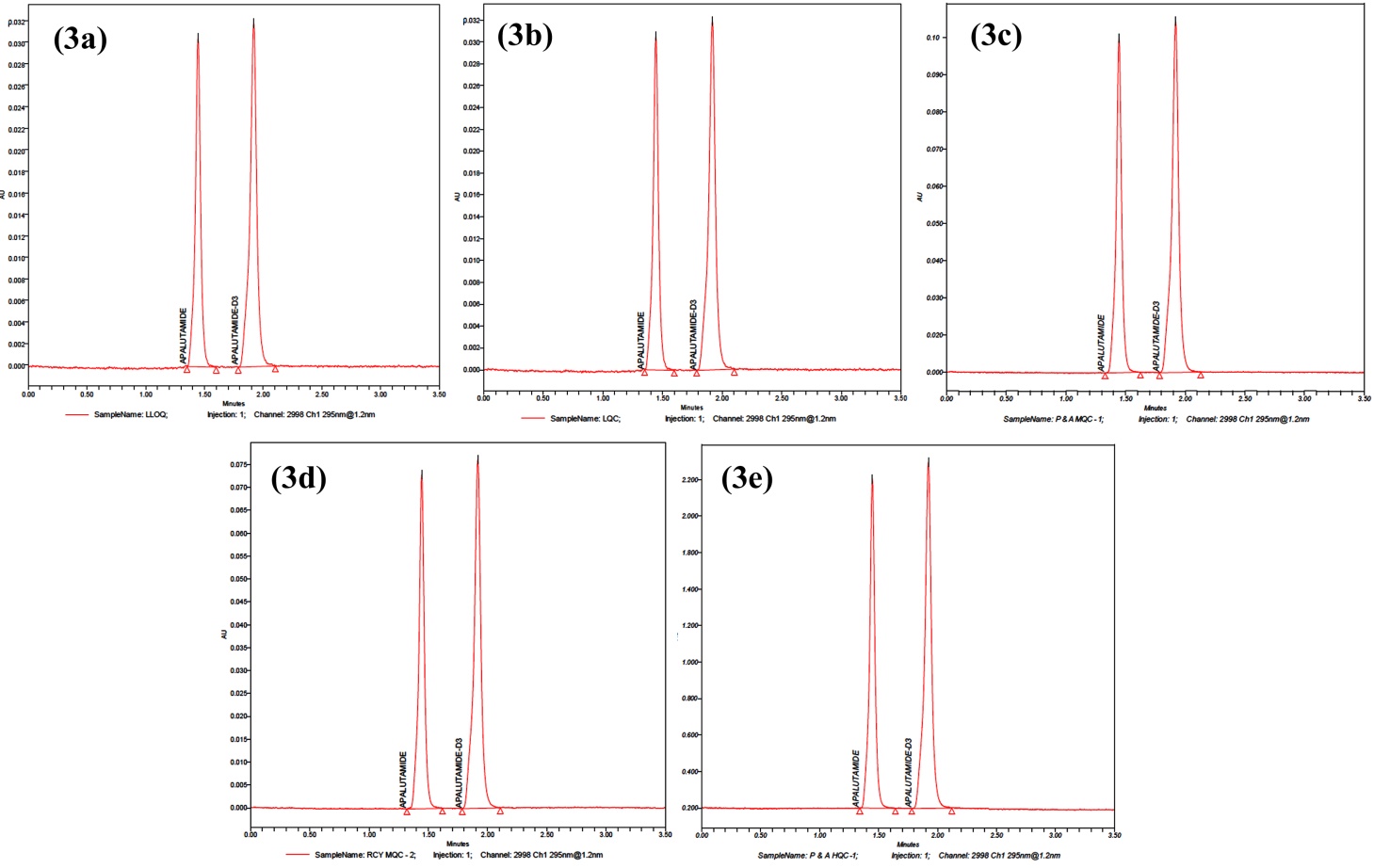
The matrix effect of UPLC method was determined (Table 2) by using six different lots of chromatographically screened human plasma, with each lot of plasma, sample concentrations equivalent to LQC and HQC of apalutamide solution was prepared and injected triplicate in each other. The mean percentage values were found to be 102.59% & 102.01% for HQC & LQC respectively.

**Table 2: Results of Matrix effect proposed UPLC-DAD method**

|  |  |  |  |
| --- | --- | --- | --- |
| **S. No.** | **QC** | **HQC (167199.079)** | **LQC (912.907)** |
| **Nominal Concentration (pg/mL)** | **142119.217– 192278.94\*** | **730.325 – 1095.48\*** |
| **Calculated Concentration (pg/mL)** | **Calculated Concentration (pg/mL)** |
| Mean | | 171543.57 | 931.31 |
| SD | | 7981.61 | 62.20 |
| % CV | | 4.65 | 6.67 |
| % Mean Accuracy | | 85.73 | 91.19 |

**Accuracy and Precision**

The precision was studied by % CV at different concentration levels corresponding to LLOQ, LQC, MQC1, MQC2 and HQC (Fig.3A- 3E) during the process of validation. The assay was assessed through accuracy by the ratio of the calculated mean values of the quality control samples to their respective nominal values expressed as a percentage. The Within batch and between the batch accuracy and precision was determined and % accuracy values were obtained 90.66%,95,05%,97.56%,98.15% & 93.16% for HQC,MQC-1,MQC-2,LQC & LLOQ. The accepted limits of % accuracy for all QC samples except LLOQ were 85%-115% and 80%-120% for LLOQ. The results were shown in Table 3.



**Figure (3a‑3e):**Representative accuracy & precision chromatograms of LLOQ, LQC, MQC-I, MQC-II & HQC

**Table3: Data of Accuracy and Precision for proposed UPLC-DAD method**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Nominal Concentration (pg/mL) | **HQC**  171316.60 | **MQC1**  102789.96 | **MQC2**  51394.98 | **LQC**  868.57 | **LLOQ QC**  312.687 |
| 14561911 – 197014.09\* | 87371.47 – 118208.45\* | 43685.733 – 59104.2282\* | 590.631 – 1042.29\* | 250.1496 – 375.224\* |
| Mean | 177297.45 | 110956.96 | 55544.30 | 799.87 | 290.83 |
| SD | 17753.36 | 5449.29 | 2157.49 | 34.64 | 33.95 |
| % CV | 10.01 | 4.91 | 3.8842 | 4.33 | 11.67 |
| % Mean Accuracy  Accuracy | 90.66 | 95.05 | 97.56 | 98.15 | 93.16 |

**\*** The percentage deviation ± 15 % from 100% of nominal concentration for all QC samples except for LLOQ (percentage deviation ± 20 %.

**Stability studies**

Stability studies were performed to determine stability of apalutamide and its internal standard in human plasma during sample preparation and sample analysis at different stress conditions.The bench top stability was determined for the spiked QC samples for a period of 6 hours at room temperature. Short term stability was studied for QC spiked samples for a period of 21 hours 40 min for analyte and 21hours 30 min for the ISTD. Long term stock solution and working standard solution stability of the analyte and ISTD were determined by using a standard equivalent to HQC & LQC concentration after a storage period of 6 days at 5± 3℃. The freeze-thaw stability of spiked QC samples was determined after third freeze thaw cycle stored at -28℃ ± 5℃. Auto sampler stability of QC was determined for a period of 54 hours 6 minutes by storing them in auto sampler maintained at temperature 5℃ ± 3℃. For all stability studies the concentrations of apalutamide and apalutamide D3 compared with nominal values**.**The results were tabulated in Table 4**.**

**Table 4: Results of stability studies of proposed UPLC-DAD method**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Stability** | **QC Level** | **Mean Measured concentrations (pg/ml); (n=6)** | | **% Change** | **%CV** | **% Mean stability** |
| **Comparison sample** | **Stability sample** |
| Auto sampler | HQC | 176633.75±  25310.24 | 184439.23±  8586.55 | 4.42 | 4.66 | 107.65 |
| LQC | 809.72±  38.75 | 757.78±  46.58 | -6.41 | 6.14 | 87.24 |
| Bench top | HQC | 170142.07±  23801.80 | 194207.65 ± 4718.00 | 14.14 | 2.71 | 106.11 |
| LQC | 834.96±  37.45 | 780.14±  42.56 | -6.56 | 5.45 | 93.43 |
| Freeze-Thaw | HQC | 185805.57±  5921.49 | 177829.18 ±  4342.64 | -4.29 | 2.44 | 95.70 |
| LQC | 772.37  ±  37.96 | 774.91  ±  131.30 | 0.32 | 16.94 | 100.32 |
| Short term | HQC | 166454.76  ±  11787.23 | 194363.99  ±  4718.00 | 16.76 | 2.91 | 108.74 |
| LQC | 875.30  ±  22.53 | 853.15  ±  94.42 | -2.53 | 2.12 | 106.62 |
| Long term | HQC | 173236.57  ±  21081.57 | 187812.47  ±  14960.47 | 8.41 | 1.69 | 98.65 |
| LQC | 923.78  ±  92.30 | 901.63  ±  57.75 | -2.39 | 3.35 | 109.47 |

**CONCLUSION**

**Bio-analytical method of Apalutamide by using UPLC**

Apalutamide used for treatment of urinary bladder cancer. The proposed method was performed in human plasma and no analytical method was reported on UPLC and few LC/MS works were reported on this drug.

The bio-analytical method was developed with suitable solvent system, column, nitrogen evaporator, UPLC system, mobile phases, vacuum pump and sonicator. The mobile phase was 5mM ammonium fumarate and acetonitrile in the ratio of 15:85 v/v and pH was  adjusted to 3.5 with glacial acetic acid used as mobile phase and also for preparation of sample solutions. The λmax was obtained at 345 nm, at this wavelength total analysis was done. The flow rate of mobile phase was maintained at 1 ml/min.The retention times were found to be 1.48 min for APA and 1.97 min for ISTD. The system suitability was performed at each day (6 days) %CV values of APA were found to be 0.58 -1.67 and 0.29‑1.68 for ISTD. Linearity concentrations were found to be 200013.87‑307.26 pg/mL and R2 value was found to be 0.999. The recovery of the sample from the matrix studied at HQC, MQC‑2 and LQC level. The % mean recoveries of HQC, MQC-2 and LQC were found to be 88.65%, 93.90%& 96.83% respectively. The precision & accuracy of the method have been conducted on APA at the level of HQC, MQC-1, MQC-2, LQC and LLOQC. The matrix effect was studied at HQC and LQC level and results were found to be 102.59 & 102.01 for HQC & LQC respectively. The % mean accuracy results were found to be 90.66, 90.05, 97.56, 98.15 & 93.16% were found to be HQC, MQC-1, MQC-2, LQC and LLOQC respectively. The acceptance criterion is % mean accuracy for all QC samples except of LLOQ QC should be in the range of 85-115% for LLOQ QC is 80-120%.Differnt stability studies were performed like freeze thaw, bench top, auto sampler, short term and Long term stability studies at HQC and LQC levels. The % mean stability of HQC and LQC obtained as 95.70% & 100.32%, to the bench top % mean stability was found to be 106.11% & 93.43% for HQC and LQC respectively. The auto sampler % mean stability was observed 104.41% & 93.58 for HQC & LQC. The %mean stability was obtained for HQC and LQC 108.74% & 106.62% in short term stability and 98.65% & 109.47% for long term stability. The validation parameters of the bio analytical method passed FDA guidelines.

**ACKNOWLEDGEMENT**

We heart fully thank to the ITM University ,Gwalior, Madhya Pradesh, India  and Ajanta Pharma Ltd for providing Apalutamide API and ISTD.

**Funding Support**

The authors declared that they have no funding support for this study.

**Conflict of Interest**

The authors are not declared conflict of interest. The authors are only responsible for content and writing of article.

**REFERENCES**

Anjaneyulu Reddy, R., *et al*.2019.A validated stability indicating RP‑HPLC method development for anticancer drug Enzalutamide in bulk and pharmaceuticals. *Int jour of pharm sci & drug res*,85-90.

Ashok Zukkala, *et al*. 2019. RP-HPLC-UV method for simultaneous Quantification of second generation non-steroidal androgens along with their active metabolites in mice plasma: Application to a pharmacokinetic study. *Drug Res*, 69: 537-544.

Chinababu, D., Madhusudhana Chetty, C., Mastanamma, SK. 2021. Forced indicating UPLC-DAD method development and validation for estimation of Apalutamide in bulk and in pharmaceutical dosage form. *Indian Drugs*, 58(09): 73-75.

Dellis, A., Papatsoris, A.G.2018. Apalutamide: The established and emerging roles in the treatments of advanced prostate cancer. *Expert Opinion on Investigational Drugs*,27(6) :553-559.

Khan, Z.G., *et al*.2016. Validated RP‑HPLC method for Determination of Enzalutamide in Bulk drug and pharmaceutical Dosage form. *Indian Drugs*, 53(11): 46-50.

Rathkopf, DE., *et al*.2017. Safety and antitumour activity of apalutamide (ARN-509) in metastatic castration-resistant prostate cancer with and without prior abiraterone acetate and prednisone. *Clin Cancer Res*, 23: 3544-3551.

Ranjan, RK.,Chandra, A.2018. Apalutamide : a better option for the treatment on non‑metastatic castration resistant prostatic carcinoma. *Int.J.Basic.Clin.Pharmacol*,7(9) :1853-1856.

Sandler, HM., *et al*.2016. ATLAS:a randomized, double blind, placebo-controlled, phase 3 trial of apalutamide (ARN-509) in patients with high-risk localized or locally advanced prostate cancer receiving primary radiation therapy. *J.Clin Oncol*,34: 5087.

Sandhya Rani,J., Devanna,N.2018.Method development and validation of Enzalutamide pure drug substance by using liquid chromatographic technique. *Jour of chem pharm sci*,1: 5-9.

Small, EJ., *et al*.2018. MP52 20 patient reported outcomes (PROs) in SPARTAN, a phase 3, double-blind, randomised study of apalutamide (APA) plus androgen deprivation therapy (ADT) Vs placebo plus ADT in men with non-metastatic castration-resistant prostate cancer (nm-CRPC). *Journal of Urology*, 99: 703-704.

Smith, MR., *et al*.2016. Phase 2 study of the safety and anti-tumour activity of apalutamide (ARN-509), a potent androgen receptor antagonist, in the high-risk non-metastatic castration-resistant prostate cancer cohort. *Eur Urol*, 70: 936-970.

Smith, MR., *et al*.2018.Apalutamide treatment and metastatic‑ free survival in prostate cancer. *N. Engl. J. med*, 378: 1408-1418.

Smith, G. 2012. European medicines agency guideline on bio-analytical method  validation: what more is there to say?, *Bioanalysis*, 4(8): 865-868.

Suresh P Sulochana., *et al*.2018. Validation of an LC‑MS/MS method for simultaneous quantitation of enzalutamide, N ‑desmethylenzulatamide, apalutamide, darolutamide and ORM-15341 in mice plasma and its application to a mice pharmacokinetic study. *J. Phar and Biomed Anal*,156: 170-180.

US Department of Health and Human Services (2001) Guidance for Industry, Bioanalytical Method Validation, Food and Drug Administration Centre for Drug Evaluation and Research (CDER),Centre for Veterinary Medicine (CVM),BP. Available at: <https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf>

Sai Uday Kiran, G., Sandhya P. 2020. Method development and validation for the analysis of Apalutamide in human plasma by LC-MS/MS. *International Journal of Current Research and Review,* 14(4): 74-79.

Vadim Koshkin, S.,Eric Small,J.2018. Apalutamide in the treatment of castrate-resistant prostate cancer: evidence from clinical trials. *Ther adv in urol*, 10(12) :445-454.

Zakkula, A., *et al*.2019.RP‑HPLC UV method for simultaneous quantification of second generation non‑steroidal antiandrogens along with their active metabolites in rat plasma: Application to a pharmacokinetic study. *Drug Res*, 6(10): 537-544.

Zhou, Z., Hu.X. 2018. PCN-153-Cost effectiveness analysis of apalutamide for treatment in non-metastasis castration-resistant prostate cancer. *Value in Health*, 21(3): S40-S41.