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

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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. The 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 a 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 the 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 the picogram level. There was no matrix effect observed and proved as a stability-indicating method.

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 (Figure 1). It shows the antagonistic effect on androgenic receptors. The drug is under the class of non-steroidal anti-androgen. It is a second-generation androgen receptor antagonist (Reddy and R, 2019; Rani and Devanna, 2018). It is developed to inhibit androgen receptor mediate prostate cancer cell...
proliferation (Koshkin and Small, 2018). Apalutamide evaluated in high-risk patients for its activity and safety of non-metastatic castration-resistance prostate cancer to identify it by conducting multi-centre phase-2 trials on nm-CRPC patients with a high risk of advancement (Smith et al., 2016). 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, 2018; Small, 2018; Saad, 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 et al., 2016; Rathkopf 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 (Sulochana et al., 2018; Dellis and Papatsoris, 2018). The chemotherapy exposure shows more effective on CRPC (Ranjan and Chandra, 2018; Khan et al., 2016).

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 (100x4.6mmx5 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°C, 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 the 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 the calibration curve. The lower limit of quantification (LLOQ), low-quality control (LQC), Middle-quality control 1 & 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                                                                |
| 5    | Anti-coagulant      | K2-EDTA                                                                     |
| 6    | Flow rate           | 1 mL/min                                                                    |
| 7    | Wavelength          | 345 nm                                                                      |
| 8    | Run time            | 3.5 min                                                                     |
| 9    | Injection volume    | 5 µL                                                                        |
| 10   | Retention time      | Apalutamide 1.48 min                                                        |
|      |                     | Apalutamide D3 1.97 min                                                     |

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

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

Table 3: Data of Accuracy and Precision for proposed UPLC-DAD method

| Nominal Concentration (pg/mL) | HQC | MQC1 | MQC2 | LQC | LLOQ QC |
|-------------------------------|-----|------|------|-----|---------|
|                               | 171316.60 | 102789.96 | 51394.98 | 868.57 | 312.687 |
| Mean                          | 14561911 – 1497014.09* | 87371.47 – 118208.45* | 43685.73 – 59104.2282* | 590.631 – 1042.29* | 250.1496 – 375.224* |
| SD                            | 177297.45 | 110956.96 | 55544.30 | 799.87 | 290.83 |
| % CV                          | 17753.36 | 5449.29 | 2157.49 | 34.64 | 33.95 |
| % Mean                        | 10.01 | 4.91 | 3.8842 | 4.33 | 11.67 |
| % Mean                        | 90.66 | 95.05 | 97.56 | 98.15 | 93.16 |

(MQC 1 & 2) and high-quality control (HQC) has been prepared in drug-free plasma and solutions were stored at 4°C.

**Extraction of APA from plasma**

The plasma samples were stored in a freezer at -70°C 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 into new ria vials and evaporated until dry under a gentle stream of nitrogen gas at 45°C. The residue was reconstituted with 150 µl of the mobile phase, and 10 µl aliquots were injected into a 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.
## 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 of sample Stability sample       |          |     |                  |
| Autosampler        | 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            |
| Benchtop           | 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           |

### Mobile phase

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

### RESULTS AND DISCUSSION

#### Bio-analytical method validation

(US Department of Health and Human Services, 2001) The validation was performed based on guidance for industry: Bioanalytical method validation from the US-FDA (Smith, 2012; Zakkula et al., 2019).

#### Optimized 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.

#### 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
Linearity and Quality controls

The linearity of the method was assessed by a ten-point standard curve. The weighted least square regression analysis $1/x^2$ was used for the study of linearity from standard plots associated with a 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 2000 to 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.

Accuracy and Precision

The precision was studied by % CV at different concentration levels corresponding to LLOQ, LQC, MQC-1, MQC-2 and HQC (Figure 3 A-Figure 3 E) 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, MQC & 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.

\[
\text{% Mean accuracy} = \frac{\text{Mean concentration QCs}}{\text{Nominal concentration}} \times 100
\]

* 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 the 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 sam-
amples 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 the analyte and 21 hours 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°C. The freeze-thaw stability of spiked QC samples was determined after the third freeze-thaw cycle stored at -28°C ± 5°C. Auto-sampler stability of QC was determined for a period of 54 hours 6 minutes by storing them in autosampler maintained at temperature 5°C ± 3°C. For all stability studies, the concentrations of apalutamide and apalutamide D3 compared with nominal values. The results were tabulated in Table 4.

\[ \% \text{Change} = \frac{\text{Mean stability sample} - \text{Mean comparison}}{\text{Mean comparison}} \times 100 \]

**CONCLUSION**

Stability indicating UPLC-DAD bio-analytical method was developed and validated for quantification of apalutamide, and the method was shown high sensitive and specificity. In addition, the method successfully applied to separate the active pharmaceutical ingredient from the degradation product. The plasma sample of apalutamide was determined at the picogram level without matrix effect was observed. The new method is specifically suitable for routine bio-analytical and bioequivalence studies of apalutamide in pharmaceutical industries.

**Conflict of Interest**

The authors declare that they have no conflict of interest for this study.

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