Bioanalytical HPLC Methodfor the Determination of Apalutamide from Human Plasma

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Abstract

An accurate, uncomplicated, and repeatable high-performance liquid chromatography (HPLC) technique was developed and validated for quantifying Apalutamide in spiked human plasma, with Budesonide serving as the internal standard. The experiment was executed using an HPLC system equipped with a UV detector. The chromatographic separation was successfully carried out using an Agilent Eclipse XDB C8 Column (150 × 4.6 mm) and a mobile phase was run at a flow rate of 1.0 ml/min. Detection was performed at 245 nm. A protein precipitation technique was applied for pre-treating plasma samples before injecting them into the HPLC system.The mobile phase consists of a mixture of Acetonitrile: 0.1M Phosphate Buffer (pH-4.60) in a ratio of 60:40 respectively.The process includes sample pre-treatment, chromatographic separation and UV detection. The retention time for Apalutamide was determined to be 4.5 minutes with a margin of error of ±0.02. This method demonstrated linearity within the 2–10 µg/ml concentration range. Validation was conducted following the guidelines outlined in ICH M10 for parameters such as selectivity, specificity, linearity, accuracy, precision, stability, carry-over, recovery, and matrix effect.Precision and accuracy within the specified range were observed for both intra- and inter-day measurements. The recovery studies yielded results ranging from 90% to 100%. The relative standard deviation (RSD) fell within the range of 0% to 2%. The stability of Apalutamide in plasma was assessed for bench-top stability, freeze-thaw stability, and long-term stability, with results meeting the acceptable limits outlined by ICH.The suggested HPLC-UV technique is precise and rapid for the determination of Apalutamide in human plasma and can be suitable for pharmacokinetic investigations and therapeutic drug monitoring. This bioanalytical approach holds importance in assessing the bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic aspects of Apalutamide.

Keywords:Apalutamide, HPLC-UV, bioanalytical method, validation, human plasma

INTRODUCTION

It is essential to validate bioanalytical methods in order to accurately measure drugs and their metabolites in biological fluids.This procedure is crucial for evaluating and understanding data obtained from bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic studies, as well as for sample preparation, storage, transportation, handling, and sample collection.[1]It is crucial to validate analytical methods in order to guarantee the accuracy of quantitative measurements of substances in various biological samples like blood, plasma, serum, or urine.It is crucial to validate bioanalytical methods in order to ensure accurate and reliable results for drug dosage and patient safety monitoring.[2]Apalutamide is indicated for the management of prostate cancer, prostatic neoplasms, castration-resistant non-metastatic prostate cancer, and hepatic impairment. The maximum concentration (C_{max}) of Apalutamide is 6.0 µg/ml.[3]The medication is available for sale in tablet form.The IUPAC name of the Apalutamide is 4-[7-[6- cyano-5-(trifluoromethyl) pyridin-3-yl]-8- oxo-6-sulfanylidene-5,7-diazaspiro[3.4] octan-5-yl]-2-fluoro-N-methylbenzamide and its chemical formula is $C_{21}H_{15}F_4N_5O_2S$. Its molecular mass is 477.435 g/mol.This is a non-steroidal anti-androgen antagonist that binds to the ligand bonding of the androgen receptor and prevents the nuclear translocation of the androgen receptor.The structure of Apalutamide is depicted in Figure 1.

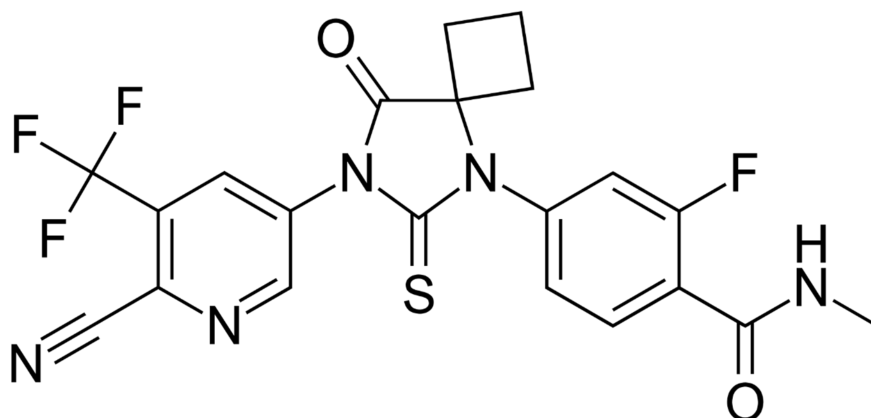

Fig. 1. Structure of Apalutamide

Upon conducting an extensive review of the literature, we identified several bioanalytical techniques that have been reported for Apalutamide analysis in mouse plasma[4] and human plasma[5] by LC-MS/MS. Based on our current understanding, there is potential for the creation of a simple HPLC bioanalytical technique for Apalutamide in human plasma. The primary objective of this study is to establish a basic bioanalytical chromatographic method. The utilization of an internal standard (IS) is essential as it helps to correct for different analytical errors in the method, ultimately enhancing the accuracy of the bioanalytical method. The approach adopted in this research is distinguished by its simplicity, reliability, and cost-efficiency.

METHODS

Instrumentation:

A bioanalytical technique for Apalutamide was conducted using a High Performance Liquid Chromatographic System with an HPLC pump (model PU 2080 Plus) and Plus 2075 Ultra Violet (UV) detector. The analysis utilized an Agilent Eclipse XDB C8 (4.6 x 150mm, 5µm) column and detection was achieved with Borwin Chromatographic Software (version 1.5).The study utilized various instruments, such as a UV-Visible spectrophotometer (JASCO, Model V730), an electronic balance (Shimadzu, Model ATX224R), a sonicator (PRAMA, Model SM15 US), a centrifuge (make-REMI), and a cyclomixer (make-REMI).

Chemicals and reagents:

Chemicals and reagents, including Acetonitrile(HPLC grade), Potassium Dihydrogen Orthophosphate Anhydrous were purchased from Loba chemie Pvt. Ltd. Mumbai India. HPLC grade water was collected with the conductivity below 0.05 μscm^{-1} using Extrapure Lab Link Water Purifier System. The pooled human plasma was obtained from Sasoon blood bank.

Selection of analytical wavelength:

Additional dilutions were made from the original stock solution using acetonitrile, followed by scanning within the wavelength range of 200–400 nm.

Selection of Internal Standard (I.S.):

An internal standard (I.S.) is a compound that is included in samples to ensure the accuracy and precision of sample pre-treatment as well as to test the reliability of the bioanalytical method. The API reference standards in the laboratory were chosen based on their proximity to a wavelength of 245 nm, which is the wavelength of Apalutamide. For example, Didanosine (250 nm), Semaglutide (239 nm), Vandetanib (249 nm), and Budesonide (246 nm) were tested for recovery studies. Budesonide was ultimately selected as the internal standard because the peaks of both the drug and the I.S. were clearly separated and both were successfully recovered from spiked plasma samples.

Preparation of stock solution:

The Apalutamide and Budesonide (I.S.) stock solution was individually prepared by accurately transferring 10 mg of the drug into a 10 ml volumetric flask and filling it up with acetonitrile to achieve a concentration of 1000 µg/ml. The working solution for Apalutamide was then created by diluting the appropriate stock solution to achieve a final concentration ranging from 2–10 µg/ml, while the working solution for Budesonide was prepared to achieve a final concentration of 10 µg/ml.

Preparation of Spiked plasma sample:

The Apalutamide clinical Cmax was reported to be 6µg/ml,to the selection of a linearity range of 2–10 µg/ml. A spiked plasma sample was created by adding 0.1 ml from each of the stock solutions (100, 150, 250, 300, 400, and 500 µg/ml) to 4.8 ml of plasma, along with 0.1 ml of Budesonide (500 µg/ml) as an internal standard, individually. After vortexing the content for 10 minutes, a 1:1 ratio of vortexed solution and precipitating agent (Acetonitrile) was taken. Following another round of vortexing, the solution was centrifuged for 10 minutes and filtered through a 0.22 µ membrane filter. This process was repeated for blank plasma (only plasma), zero plasma (plasma spiked with an internal standard), and spiked plasma (plasma spiked with an internal standard

and Apalutamide). Protein precipitation was chosen as the preferred method for sample pretreatment due to its simplicity and efficiency in extracting the drug from the matrix.

Mobile Phase Preparation:

HPLC-Grade acetonitrile was combined with a phosphate buffer (0.1 M, pH = 4.6) prepared in HPLC-Grade water (60:40 v/v). The resulting solution underwent additional filtration using a 0.45 µm membrane filter and was then subjected to sonication for a duration of 10 minutes.

Chromatographic Conditions:

After conducting multiple experiments, we have identified the most effective mobile phase. This particular mobile phase, which is composed of Acetonitrile: 0.1 M Phosphate Buffer in a 60:40 v/v ratio, has demonstrated superior performance in terms of enhanced resolution and well-defined peaks. The Agilent Eclipse XDB C8 chromatographic column was employed in this investigation, operating at a flow rate of 1 ml/min. Figure 2 illustrates the typical chromatogram of Apalutamide.

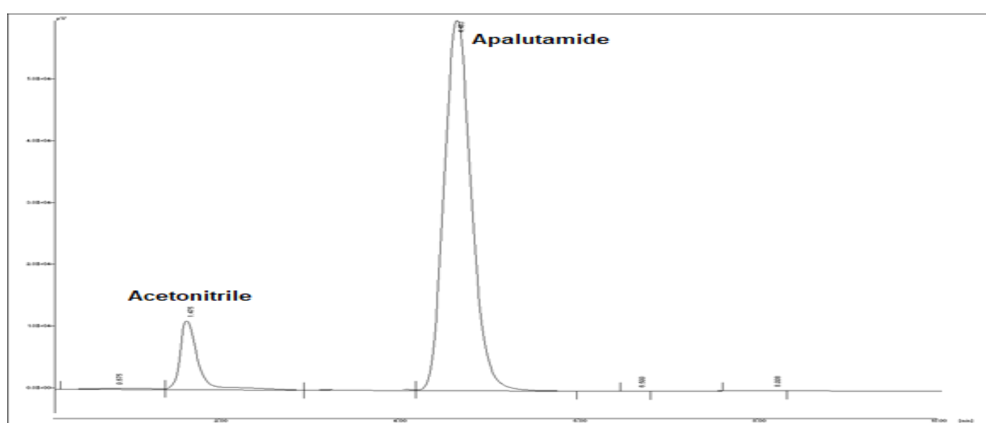

Fig. 2. Chromatogram of Apalutamide (10 µg/ml) RT- 4.5 ±0.02 min

Preparation of Quality Control Samples:

In the process of method validation, it is necessary to prepare quality control samples for accuracy and precision testing at a minimum of four concentration levels across the calibration curve range. These levels include the Lowest Limit of Quantification (LLOQ), a level within three times the LLOQ (low QC), a level around 30–50% of the calibration curve range (medium QC), and a level at least 75% of the upper limit of quantification (high QC). The standard curve's lowest concentration that can be measured with acceptable accuracy and precision is known as LLOQ.

BIO-ANALYTICAL METHOD VALIDATION

There are multiple guidelines for Bioanalytical method validation. We have adhered to the ICH M10 guidelines.[6-8]

Selectivity:

The bioanalytical method's selectivity refers to its capacity to accurately measure and distinguish the active drug in a sample, even when there are other substances that could potentially interfere, such as those found in the pooled plasma matrix.

Matrix Effect:

The phenomenon known as the matrix effect refers to a change in the analyte's response due to interfering substances present in the sample matrix, which are often not identified. It is crucial to evaluate the matrix effect when comparing results from various sources or batches during method validation. The analysis was conducted using a pooled plasma sample.

Calibration Curve:

The relationship between the established concentration and response was assessed by conducting a regression analysis on the calibration curve, which was created using a six-point (2, 3, 5, 6, 8, and 10 µg/ml) standard calibration curve. The calibration curve was plotted with the ratio of the peak area of the drug to the internal standard (I.S.), referred to as the response factor, on the Y-axis and concentration on the X-axis.

Accuracy:

The accuracy of all QC samples was determined by calculating the mean percentage. The response factors were used in the regression equation to determine the concentration of the sample. The deviation of the average from the theoretical value was used to estimate accuracy, expressed as a percentage (%). This can be shown by spiking the sample matrix with a known concentration of analyte. The percentage deviation should be less than 15% for all four concentrations (LLOQ, LQC, MQC, and HQC).

Precision:

The proximity of the single measured value of the drug component in all portions of the plasma of equal volume was evaluated by injecting six duplicates at LLOQ, LQC, MQC, and HQC concentrations. The accuracy of the technique carried out on the HPLC system was assessed by calculating the % CV of the repeated injections.

Carry Over:

Carryover refers to the influence of the prior injection on the subsequent injection, resulting in a change in the measured concentration of the analyte due to residual analyte from the previous sample. This phenomenon was identified by introducing blank samples following the injection of a high-quality control sample with a concentration of 8 µg/ml.

Stability Studies:

The stability procedure must assess the stability of the analyte while the sample is being collected and handled under long-term (frozen at the intended storage temperature) and short-term (room temperature) storage conditions.

Recovery:

The recovery experiments involved analyzing the chromatographic response of samples extracted at LQC, MQC, and HQC in comparison to the standard, each in three replicates as per US FDA guidelines.[9] It is not necessary for the recovery to reach 100%, but it should demonstrate consistent and reproducible levels of the analyte.

RESULTS

Detection Wavelength:

Apalutamide demonstrated significant absorbance at 245 nm, leading to the selection of this wavelength for detection, as shown in Figure 3.

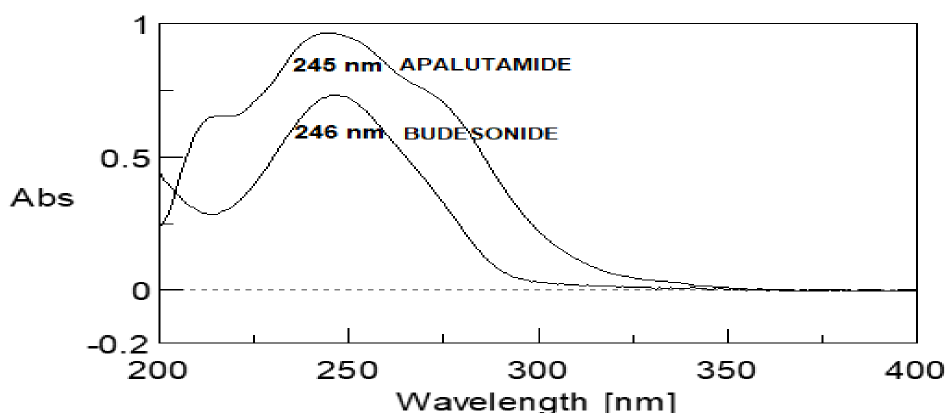

Fig. 3. UV-Spectral overlay of Apalutamide and Budesonide (20 µg/ml)

Selectivity:

The method's selectivity can be illustrated by examining blank plasma, zero plasma, and spiked plasma, as depicted in figures 4, 5, and 6, respectively. It was confirmed that there is no interference of more than 5% at the retention time of the internal standard and no more than 20% of the analyte.

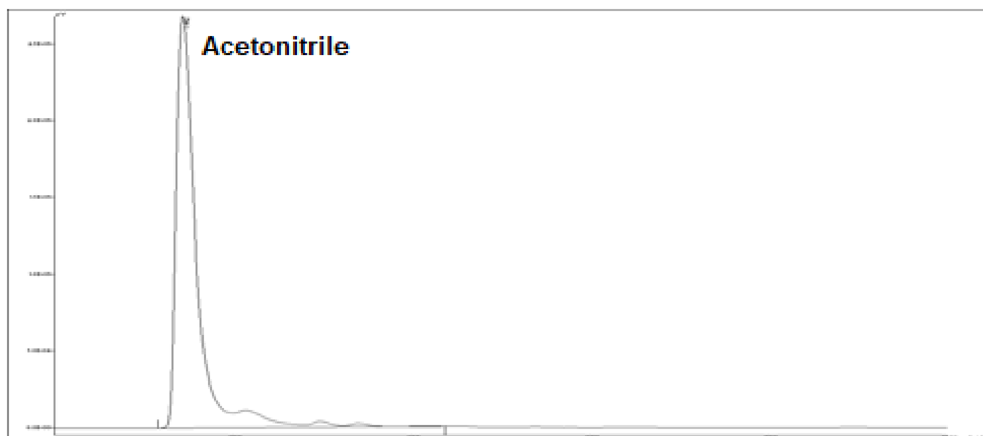

Fig. 4. Chromatogram of blank human plasma

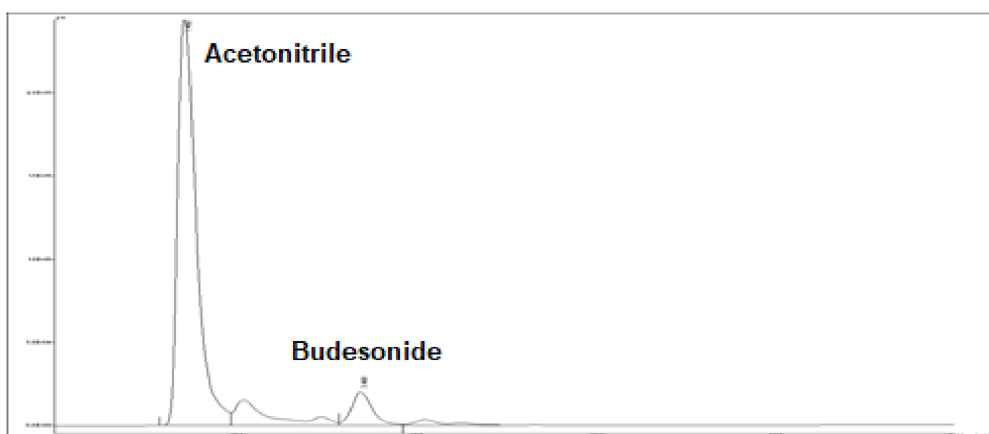

Fig. 5. Chromatogram of Zero Plasma.Budesonide (I.S.) Rt-3.5 min.

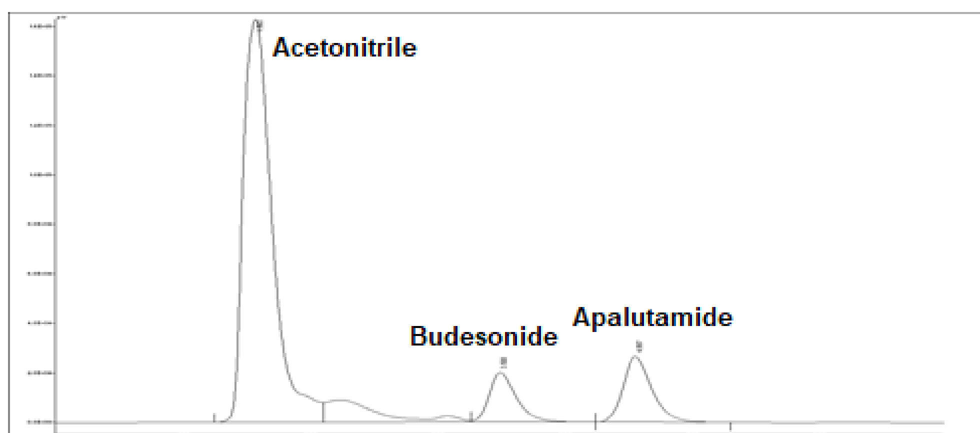

Fig. 6. Chromatogram of Spiked Plasma.
Budesonide (I.S.) Rt-3.5 min, Apalutamide Rt-4.5 min

Linearity:

Linearity within the concentration range of 2–10 µg/ml was assessed. Peak areas were recorded following the examination of six replicates of quality control samples. A regression analysis was conducted on a standard calibration curve to assess the relationship between known concentrations and response factors, utilizing six points (2, 3, 5, 6, 8, and 10 µg/mL). The linearity range of the solutions, along with their corresponding area response ratios are presented in Table 1. An R2 value of 0.9835 was determined.

Table 1. Linearity of Apalutamide

Replicate No.	Concentration (µg/ml)					
	2	3	5	6	8	10
	Response Factor					
1	0.3540	0.4769	0.6342	0.6993	0.8696	1.0237
2	0.3586	0.4660	0.6289	0.7058	0.8606	1.0312
3	0.3585	0.4751	0.6380	0.6893	0.8512	1.0478
4	0.3587	0.4693	0.6310	0.6869	0.8598	1.0341
5	0.3593	0.4773	0.6299	0.7101	0.8479	1.0621
Mean	0.3578	0.4729	0.6324	0.6982	0.8578	1.0397
SD	0.0021	0.0050	0.0037	0.0100	0.0085	0.0152
%CV	0.6030	1.0613	0.5866	1.4453	0.9971	1.4640

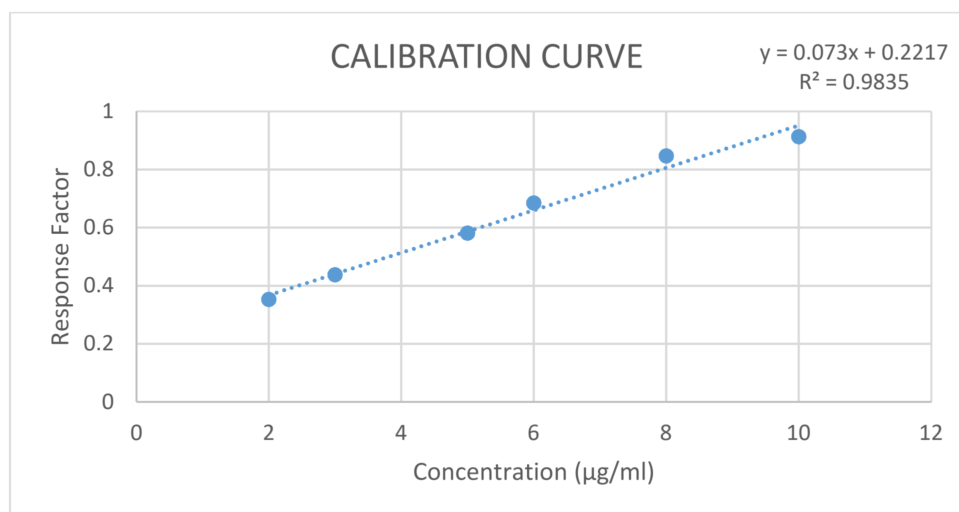

Fig.7. Calibration curve of Apalutamide

Accuracy:

Accuracy was assessed by employing at least 5 replicates of 4 different concentrations, namely LLOQ, LQC, MQC, and HQC. The accuracy was determined and presented as both a percentage of recovery and a percentage of the coefficient of variation (% CV). The accuracy was considered acceptable if it fell within the range of 85–115%. The accuracy study findings are displayed in Table 2.

Table 2. Results of accuracy studies

Replicates	Calculated concentration			
	LLOQ (2 µg/ml)	LQC (3 µg/ml)	MQC (6 µg/ml)	HQC (8 µg/ml)
1	1.8221	3.5064	6.5532	8.8862
2	1.8862	3.3571	6.6415	8.7617
3	1.8846	3.4820	6.4153	8.5889
4	1.8876	3.4021	6.3830	8.6330
5	1.8956	3.5120	6.7012	9.0531
Mean	1.8751	3.4519	6.5388	8.7840
SD	0.0298	0.0688	0.1384	0.1898
%CV	1.5942	1.9947	2.1170	2.1615
%Mean	93.75%	115.06%	108.98%	109.80%

Acceptance Criteria: The accuracy at each concentration level should be within ±15% of the nominal concentration, except at the LLOQ, where it should be within ±20%.

Precision:

The precision was determined by injecting six duplicates at LLOQ, LQC, MQC, and HQC concentrations. Intra-day precision was assessed by calculating the % CV of the response from the repeated injections performed on the same day. Conversely, inter-day precision was determined by comparing the measured values of the samples injected on three separate days. The findings are detailed in Tables 3 and 4.

Table 3. Intraday precision studies

Concentration Level	LLOQ (2 µg/ml)	LQC (3 µg/ml)	MQC (6 µg/ml)	HQC (8 µg/ml)
Mean Concentration	1.8735	3.4467	6.5420	8.7791
Standard Deviation	0.0271	0.0628	0.1240	0.1703
%CV	1.4485	1.8234	1.8963	1.940

Acceptance Criteria: The precision (%CV) of the concentrations determined at each level should not exceed 15%, except at the LLOQ, where it should not exceed 20%.

Table 4. Interday precision studies

Concentration Level	Mean Concentration		
	Day 1	Day 2	Day 3
LLOQ(2µg/ml)	1.8735	1.8521	1.8921
LQC(3µg/ml)	3.4467	3.5227	3.3189
MQC(6µg/ml)	6.5420	6.3952	6.6298
HQC(8µg/ml)	8.7791	8.5974	8.6287
Acceptance Criteria: The precision (%CV) of the concentrations determined at each level should not exceed 15%, except at the LLOQ, where it should not exceed 20%.			

Recovery:

The chromatographic response of the samples post-extraction at LLOQ, LQC, MQC, and HQC was compared with standard samples in three replicates for the purpose of conducting recovery studies. The extraction recoveries for the four QC levels (lowest, lower, medium, and high) for Apalutamide were 94.58%, 91.96%, 92.77%, and 93.07%, respectively. For Budesonide, the recoveries were 95.98%, 95.76%, 96.61%, and 95.58%, respectively. The results obtained have been presented in Table 5.

Table 5. Results of Recovery Studies

Concentration level	Area of Apalutamide		Recovery	Area of Budesonide (I.S)		Recovery
	Standard	Spiked plasma	%Mean Recovery	Standard	Spiked plasma	%Mean Recovery
LLOQ (2µg/ml)	114086.21	107902.74	94.58	313503.59	300900.75	95.98
LQC (3µg/ml)	182432.41	167783.00	91.96	312447.61	299206.12	95.76
MQC (6µg/ml)	256206.25	237702.12	92.77	319632.00	308813.31	96.61
HQC (8µg/ml)	313214.70	291533.00	93.07	337986.21	323073.72	95.58
Overall	Overall % Mean Recovery		93.09	Overall % Mean Recovery		95.98

Carry Over:

Upon injection of ULOQ samples, analysis of blank plasma samples showed no peaks at the retention time of either analytes or internal standard (I.S.), suggesting that the method utilized does not exhibit any carryover effect.

Stability Studies:

Apalutamide stability was assessed at two concentration levels, specifically at LQC and HQC. The mean of three samples was calculated for each sample under testing. Subsequently, the samples were processed, stored, and analyzed. The findings have been compiled in Table 6.

1. **Freeze and thaw stability:**
   The freeze-thaw stability of quality control samples spiked with analytes was evaluated following three freeze-thaw cycles at -20 °C and then compared to freshly spiked quality control samples to evaluate stability. The mean percentage stability for high-quality control (HQC) samples at 8 µg/ml and low-quality control (LQC) samples at 3 µg/ml was determined to be 92.84% and 100.00%, respectively.

2. **Long-Term Stability:**
   The long-term stability of the LQC and HQC over an extended period was evaluated following storage at -20 °C for 7 days in comparison to a freshly prepared stock solution tested for stability. The average stability percentages for HQC (8 µg/ml) and LQC (3 µg/ml) were determined to be 100.86% and 103.73%, respectively.

3. **Short-Term Stability:**
   The stability of spiked quality control samples over a 4-hour period at room temperature was assessed and compared to freshly spiked samples to determine their short-term temperature stability. The percent mean stability for HQC (8 µg/ml) and LQC (3 µg/ml) were found to be 94.45% and 95.63%, respectively.

4. **Stock solution stability:**
   The stability of the stock solution of the drug and I.S. was evaluated over a period of 2 hours at room temperature and then compared to the freshly prepared stock solution to assess stability. The mean stability of Apalutamide at HQC (8 µg/ml) and LQC (3 µg/ml) levels was determined to be 101.32% and 98.12%, respectively.

Table 6. Results of stability study

Stability	Conc.(µg/ml)	Mean Stability (%)
Freeze thaw stability (three cycles)	LQC	100.00
	HQC	92.84
Short term stability (for 4h at R.T.)	LQC	95.63
	HQC	94.45
Long term stability (for 7 days at -20 °C)	LQC	103.73
	HQC	100.86
Stock solution stability (for 2h)	LQC	98.12
	HQC	101.32
Acceptance Criteria: The % mean stability for drug and I.S. should be within range 85-115%.		

DISCUSSION

A literature review indicates that there are two bioanalytical methods employed to determine Apalutamide in human plasma and mice plasma using a complex technique such as LC-MS/MS. Thus, a simple, quicker and more efficient bioanalytical technique has been developed and validated. Protein precipitation was utilized for the extraction process in human plasma and the later response factor of Apalutamide was subsequently calculated.The validation parameters were determined to comply with all the acceptance criteria set by ICH. The research findings indicate that Apalutamide can be efficiently extracted from human plasma, with Budesonide serving as an internal standard to guarantee extraction efficiency. No interfering peaks were observed between the working standard and the internal standard.The retention time is optimized to ensure well-resolved peaks in the method. The stability of Apalutamide in human plasma depends on various factors, such as storage conditions, the chemical properties of the drug, the matrix, and the storage system. It was observed that the drug remained stable in human plasma.

CONCLUSION

We have effectively created and thoroughly verified a high-performance liquid chromatography with ultraviolet detection (HPLC-UV) technique for measuring Apalutamide in biological samples. Our approach enables precise quantification of Apalutamide across a broad concentration range, with a minimum quantification limit (LLOQ) of 2 µg/ml. The validation outcomes, encompassing precision and accuracy within the same day and across different days, showcase the method's reliability and consistency.
The HPLC-UV method that has been developed provides numerous benefits, such as simplicity, cost-effectiveness, and speed, which makes it an appealing option for regular analysis in pharmaceutical laboratories and clinical environments. The effective validation of this method creates possibilities for pharmacokinetic research, bioequivalence evaluations, and therapeutic monitoring of Apalutamide in various biological samples. It is recommended to conduct additional research and utilize this method in various applications to fully exploit its potential in drug development and clinical use.

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