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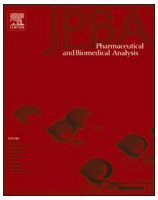

The first RP-UHPLC method for simultaneous quantification of abiraterone acetate, its four degradants, and six specified process impurities and correct identification of all analytes based on molecular weight

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ABSTRACT

This article describes the first simple, fast, time-saving, and cost-effective UHPLC method that was developed and validated for simultaneous quantification of abiraterone acetate, its four degradation products, and six specified process impurities in bulk and tablet form. Moreover, when coupled with a mass spectrometer detector, the proposed method provides additional advantages for confirmation of peak and correct identification based on molecular weight. The eleven peaks were separated on a Water Acquity BEH C18, (150 mm length, 2.1 mm internal diameter, 1.7 μm particle size) column maintained at a 50.0 °C temperature. Using 0.05% formic acid in 10 mM ammonium formate, acetonitrile, and methanol as mobile phases in gradient elution at a flow rate of 0.40 mL/min. provides excellent separation at 260 nm. The linearity curves of all analytes showed promising results with a correlation coefficient of 0.999 with a lower limit of detection and quantification. A forced degradation study on solid abiraterone acetate proved its specificity with improvements and significance. This proposed method provides improved separation with a lower flow rate, which offers faster analysis, reduces wastage and cost, and specifies the greener advantages compared to reported methods. The outcome of the specificity, linearity, precision, and trueness as per ICH guidelines proved that the proposed method is fast, time-saving, and cost-effective for the intended purpose.

1. Introduction

Worldwide, prostate cancer is the second-most common cancer and is the fifth-leading cause of cancer-related death in men [1]. As per the Global Cancer Statistics 2020 report, the number of new cases of prostate cancer in 2020 was 1.41 million, which was approximately 7.3%, and the number of new deaths was 375,304 (3.8%) [2]. The androgens (male sex hormones) found in men are testosterone, dihydrotestosterone, and androstenedione, which are required for normal growth and function of the prostate gland that helps make semen in the male reproductive system and also necessary for prostate cancer to grow. Hence, hormone therapy (androgen suppression therapy) plays an important role in the treatment of prostate cancer since it uses hormone medicines to reduce the body's natural synthesis of androgen [3].

The drug abiraterone acetate, with multiple (six) chiral centers, is on the World Health Organization's list of essential medicines used in the treatment of prostate cancer. It is a prodrug for the active ingredient named abiraterone, a potent, selective, irreversible inhibitor of the 17α-hydroxylase and C17,20-lyase enzymatic activities of cytochrome P450 (CYP17A1) produced by the testes, adrenal glands, and prostate cancer cells. The hormone-sensitive prostate cancer cells shrink when CYP17A1 is inhibited by abiraterone because it prevents the tissues from converting cholesterol into testosterone and other androgens. In other words, abiraterone acetate is an anti-androgen medication that reduces testosterone levels and deprives prostate cancer cells of the fuel they require, thus slowing the growth of prostate cancer tumors. It is under development for the treatment of breast cancer and ovarian cancer. In the years 2011–2012, abiraterone acetate was first approved by the Food and Drug Administration and followed by the European Medicines Agency, Medicines and Healthcare Products Regulatory Agency, and Therapeutic Goods Administration for this indication [4–7].

To check the efficacy, quality, purity, and safety of pharmaceutical

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drug substances and drug products, analysts need a simple, fast, accurate, linear, and precise analytical method. While selecting such an analytical method, the user evaluates each method in detail and prefers the one that covers all impurities mentioned in different pharmacopeias. The USP monograph for 'abiraterone acetate' and 'abiraterone acetate tablets' provides a list of key specified impurities and degradation products (7-ketoabiraterone acetate, abiraterone, α-epoxy abiraterone acetate, ß-epoxy abiraterone acetate, 3-deoxy-3-acetyl abiraterone-3-ene, abiraterone ethyl ether, abiraterone isopropyl ether, anhydro abiraterone, 3-deoxy 3-chloroabiraterone, and *O*-chlorobutylabiraterone) of abiraterone acetate [8]. The molecular structure of abiraterone acetate, its specified impurities, and degradation products defined in the United States Pharmacopeia (USP) monograph are presented in Fig. 1. These impurities and degradation products in drug substances and drug products might occur or increase because of synthesis or manufacturing processes, degradation (hydrolysis, oxidation, and thermal), or other factors (storage conditions, containers, excipients, or contamination). Regulatory agencies are now closely monitoring impurity trending in drug substances and in various drug products with information on the source or pathway of such impurities [9].

Based on the findings of the literature review, abiraterone acetate was determined in pharmaceutical (bulk, tablets, etc.) and biological samples (plasma/serum/urine, etc.) using various techniques. The techniques include spectrofluorimetric [10], spectrophotometry [11], reverse-phase high-performance liquid chromatography (RP-HPLC) with different detection [8,12–19], and liquid chromatography coupled with mass spectrometry (as LC-MS or LC-MS/MS) [20–27]. Some of these methods are chromatography-based techniques that have been reported for determining abiraterone, abiraterone acetate, their metabolites, and degradation products in bulk, tablet formulations, serum, and plasma. A thorough literature review uncovers that these reported methods mainly focus on a maximum three of specific impurities mentioned in the USP monograph. The presence of degradation products such as 7-ketoabiraterone acetate, abiraterone, α-epoxy abiraterone acetate, ß-epoxy abiraterone acetate, as well as specified impurities abiraterone ethyl ether, and abiraterone isopropyl ether, proves the quality, purity, and safety of drug substances and drug products of abiraterone acetate [8]. None of these degradation products and impurities were covered in the reported methods. As per our knowledge, the compendial HPLC method described in the USP monograph is so far the only method for assay and purity analysis with its own limitations on speed, sensitivity, and separation. The literature review also revealed that there was no published and reported single UHPLC method available to estimate assay, specified impurities (six), and degradation products (four) mentioned in the USP monograph of abiraterone acetate from bulk and tablet formulation. Based on these facts, we have been motivated to develop a new UHPLC method that is mass spectrometry compatible and should be able to separate and quantify all six specified impurities, four degradation products, and abiraterone acetate from each other with improved speed, separation, and sensitivity. Section 1 and Tables S1 to S3 in the supplementary information contain further details regarding the introduction section, the additional review literature, a comparison of the reported methods, and the compendial HPLC method with the proposed UHPLC method.

This article describes the development and optimization of the first selective, rapid, timesaving, and cost-effective UHPLC method for the simultaneous determination of abiraterone acetate, its six specified impurities, and four degradation products in the bulk and tablet form. Finally, the developed UHPLC method has been validated as per ICH guidelines in terms of specificity, precision, trueness, linearity, detection limit, quantification limit, solution stability, forced degradation, and robustness studies and has demonstrated the improved capabilities and significance of the proposed methods [28,29]. Additionally, when the mass spectrometer detector with electrospray ionization is coupled with the proposed UHPLC method, it is useful for accurate identification and confirmation of all analytes based on observed molecular weight.

2. Materials and methods

2.1. Chemicals, reagents, materials, and standards

Acetonitrile and methanol (LC/MS grade of Thermo Fisher Scientific and HPLC grade of Merck), ammonium formate (BioUltra, Sigma-Aldrich), formic acid (Merck), and Milli-Q water were used to prepare

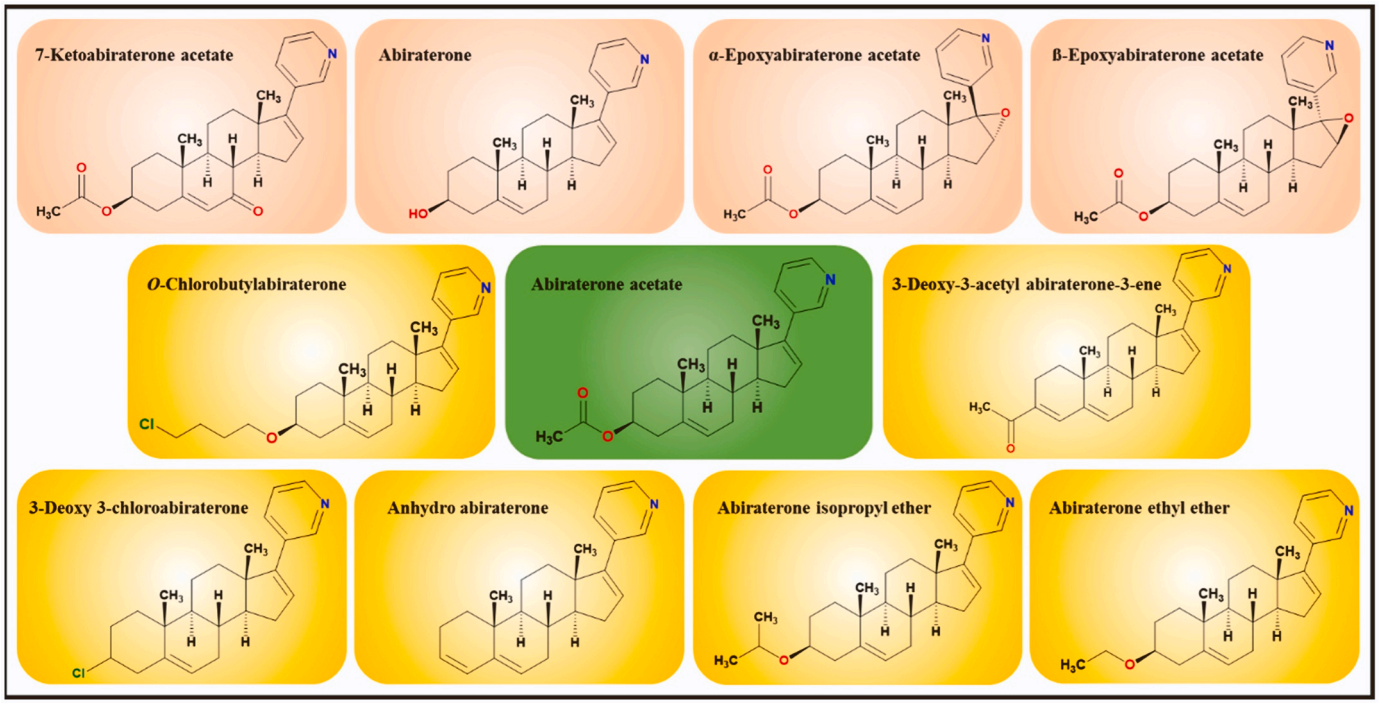

Fig. 1. The molecular structure of abiraterone acetate, its four degradation products, and six specified process impurities.

mobile phases, and solvents. The prepared mobile phase-A was filtered through Millipore 0.20 μm, White Nylon, GNWP 47 mm (Merck). The in-house standards of all specified process impurities, degradation products, and abiraterone acetate were used (details are listed in Table S4). The placebo of tablet formulations was of standard grade, and samples of abiraterone acetate active pharmaceutical ingredients (APIs) and tablets (250 mg and 500 mg) were used wherever applicable.

2.2. Instrument and software

The experiments were performed on a Waters Acquity ultra-high-performance liquid chromatography (UHPLC) H-class system consisting of a quaternary solvent delivery pump, an autosampler, a column manager, a photodiode array (PDA) detector, and a quadrupole dalton (QDa) mass spectrometer (MS) detector with an electrospray ionization source (Waters Corporation). Instrument control, chromatographic data acquisition, and monitoring were performed using Empower 3 software.

2.3. Preparation of solutions

2.3.1. Preparation of standard solution

Weighed approximately 25 mg of abiraterone acetate reference standard and transferred it into an amber-colored 25 mL volumetric flask. The content was dissolved and diluted to volume with dilution solvent to obtain a 1.0 mg/mL concentration of abiraterone acetate.

2.3.2. Preparation of impurity stock solutions

Individually and separately, weighed approximately 5 mg of each impurity and degradation product into an amber-colored 50 mL volumetric flask. The impurity was dissolved and diluted to volume with 100% acetonitrile to obtain a stock solution with a concentration of 0.10 mg/mL.

2.3.3. Preparation of spiked standard solution/system suitability test (SST) solution

Weighed approximately 50 mg of abiraterone acetate reference standard and transferred it into an amber-colored 50 mL volumetric flask. Then 1.0 mL of the stock solution of each impurity was added. The solution was further diluted to volume with dilution solvent. The resulting spiked standard solution contained each impurity at a 0.20% level.

2.3.4. Preparation of API sample solution

Weighed approximately 25 mg of abiraterone acetate API sample and transferred it into an amber-colored 25 mL volumetric flask. The content was dissolved and diluted to volume with dilution solvent to obtain a 1.0 mg/mL concentration of abiraterone acetate.

2.3.5. Preparation of tablet sample solution

We accurately weighed 20 Tablets of abiraterone acetate tablet (500 mg) sample. Determined the mean tablet weight and ground the tablets to a fine powder. Immediately weighed accurately an amount of homogenized powder equivalent to 1/5 of the mean tablet weight and transferred it into an amber-colored 100 mL volumetric flask. Added approximately 60 mL of dilution solvent and shaken mechanically for 45 min. The content was diluted to volume with dilution solvent to obtain a 1.0 mg/mL concentration of abiraterone acetate.

2.4. Chromatographic conditions for UHPLC-UV and UHPLC-PDA-ESI-MS

All chromatographic analysis was performed using a Waters, Acquity UHPLC, H-CLASS system with a PDA detector, and a quadrupole Dalton (QDa) mass detector with an electrospray ionization source. The chromatographic conditions consisted of the Acquity BEH C18, 150 mm length x 2.1 mm i.d., 1.7 μm particle size, P/N 186002353 column (Waters Corporation, USA) maintained at a 50 °C column temperature. Mobile phase-A (0.05% formic acid in an aqueous 10 mM ammonium formate), mobile phase-B (acetonitrile), and mobile phase-C (methanol) were pumped at 0.40 mL/min in gradient program mode. The gradient conditions were like % of mobile phases A-B-C; at the time in min.: composition: 94–4–2, at 0 min, composition: 50–33–17, at 10.0 min, composition: 15–55–30, at 40.0 min, composition: 94–4–2, at 41 min, and finally composition: 94–4–2, at 45 min for equilibration. The autosampler was maintained at 20 °C and the injection volume was 4.0 μL. For the chromatogram, the PDA spectra need to be extracted at a 260 nm detection wavelength. For the preparation of dilution solvent, mobile phase A and mobile phase B were mixed in a ratio of 20/80, v/v. With the use of the mentioned chromatographic parameters of the proposed UHPLC method, the mass spectrometer parameters were as follows for analysis with UHPLC-PDA-ESI-MS. The *m/z* was extracted in positive ESI modes at 550 °C as the probe temperature, with a capillary voltage for a positive mode of 1.2 kV and a negative mode of 0.8 kV. The MS scan in positive and negative modes was set to scan the *m/z* from 100.00 to 1000.00. The sampling rate was 10 points/second, and the gain value was 1.0, with 10 V as the cone voltage.

2.5. Analytical method development

In the method development, a number of diverse aqueous buffers were evaluated with methanol, acetonitrile, and ethanol as organic phases on selected UHPLC columns. Additionally, the parameters such as the selection of suitable detection wavelength, injection volume, and concentration of 100% solution were studied using optimized mobile phases and columns. The parameters such as peak separation, peak responses, peak symmetry factor, signal-to-noise ratios, and theoretical plates for all peaks were examined and assessed in the optimization of different parameters. The spiked standard solutions and sample solutions of the thermally stressed API were used in method development activities for the optimization of chromatographic conditions and mass spectrometer parameters.

2.6. Method robustness

The robustness of the method (ability to remain unaffected and be confirmed to be valid even when executed with a small modification in defined parameters) was studied to ensure that the results were not impacted by the variation in chromatographic parameters. The robustness of the developed UHPLC method was investigated and proven with the minimum and maximum influence of six different parameters indicated in the proposed chromatographic conditions. The robustness of the chromatographic conditions was tested by varying the following parameters: column temperature (± 5.0 °C), mobile phase flow rate (± 0.05 mL/min), slope gradient (± 1.0 min), the concentration of 0.05% formic acid in mobile phase A (± 0.05 mL), the concentration of buffer of 10 mM ammonium formate in mobile phase A (± 20%), change in detection wavelength (± 5 nm), and finally change in column lot to lot, different lot of chemicals and reagents.

2.7. Forced degradation study

Forced degradation studies need to be performed to prove the ability of proposed UHPLC methods to simultaneously separate, detect, and quantify the assay of API with various drug-related degradants and impurities. It is also essential to demonstrate the specificity and stability-indicating nature of methods. So, based on the outcome, we can assure the formation of degradation products and their degradation pattern (in solid form and solution form) of the APIs and drug products that could form during the storage, manufacturing, and packaging of drug products (degradant). In these forced degradation studies, different conditions were investigated, such as i) acid (HCl) hydrolysis, ii) base (NaOH) hydrolysis, iii) neutral (water) hydrolysis, iv) thermal degradation at

80 °C, v) oxidation (H_2O_2) degradation, and v) photolytic (ICH Light) degradation. These stressing conditions were projected to harvest all potential degradants and separate them from all the eleven desired peaks in APIs and drug products of abiraterone acetate.

2.8. Analytical method validation

In order to achieve the targeted outcome and to prove that the developed UHPLC method is suitable for its intended purpose, method validation was planned and executed as per ICH guidelines. The validation parameters such as system repeatability, analysis repeatability, intermediate precision, trueness, linearity, specificity, range, solution stability, filtration study, relative response factor, system suitability test, detection limit, and quantification limit were tested according to the ICH Q2(R1) [28,29].

2.9. Proposed methods applicability

To prove the applicability of the proposed methods and to confirm their intended purpose on real samples, which will be helpful to glance over all the possibilities and their uses, the assessment of real samples was conducted by using the proposed UHPLC and UHPLC-PDA-ESI-MS methods. In the market, the abiraterone acetate molecule was available in various pharmaceutical dosage forms, including tablets and drug substances. The two different batches of API and tablet forms of abiraterone acetate were selected and analyzed by using the proposed methods for simultaneous quantification (by UHPLC), correct identification, and confirmation of results (by UHPLC-PDA-ESI-MS). To get true values of selected real samples of abiraterone acetate (API and tablets) for result comparison purposes, we analyzed all the selected samples by the reported method.

3. Results and discussion

3.1. Analytical method development

The main goal of this research work was to develop an improved UHPLC technique to separate and quantify all specified process impurities (six), degradation products (four), and the main peak (abiraterone acetate) from the peak of used solvents, formulation excipients (placebo), and all other unknown degradation products. Furthermore, the developed approach should be rapid, timesaving, cost-effective, and simple enough to be used easily in the quality control lab of a pharmaceutical company. The method development started with the target criteria of separating all eleven analytes with a maximum resolution between two consecutive peaks and an overall run time of less than 50 min. Numerous UHPLC parameters and conditions were tuned to achieve the targeted goal with adequate peak separation, sharp peak shape, stable baseline with little matrix influence, and higher peak responses.

3.1.1. Criteria for analytical method development evaluation

In method development activities, spiked standard solutions and thermally stressed sample solutions were used to optimize chromatographic conditions and mass spectrometer parameters. The parameters such as peak separation (resolution), peak responses (peak area), peak symmetry factor (tailing), peak height (peak sharpness), and theoretical plates (USP Plate count) for all peaks were examined and assessed in the optimization of different parameters. Based on the outcome of stage-1 of method development (For details refer to Section 3.1.1 in supplementary information with Tables S5 and S6), the minimum evaluation criteria for these parameters were set as the resolution in any two peaks should be more than 1.2; the peak area for DL of all analytes should be more than 1000; tailing factors for all analytes should be between 0.8 and 2.5; every peak observed in any solution should be sharp, and theoretical plates for all desired peaks should be more than 100000.

3.1.2. Selection of mobile phases (aqueous buffer and organic modifier)

The number of published and reported works revealed that the pKa value for abiraterone acetate is 5.19 and for abiraterone is 4.8, and hence, based on this data, we have concluded that the mobile phase with an acidic pH (2–4 pH) would be helpful for separation. Further pharmacopeial HPLC methods for abiraterone acetate showed the use of acetonitrile and ethanol as organic modifiers. Based on this, we selected the diverse aqueous buffers in mobile phase A with acetonitrile and methanol as organic modifiers, which are compatible with the mass spectrometer detector. The different types of aqueous buffers with and without the addition of acidic modifiers for pH 3.0–7.0 were tested as mobile phase A. The aqueous buffers for acidic pH include 0.1% acetic acid, 0.1% formic acid, and diverse ratios of acetic acid (0.05%, 0.1%, and 0.2%) in ammonium acetate (10 and 20 mM), and formic acid (0.05%, 0.1%, and 0.2%) in ammonium formate (10 and 20 mM). The aqueous buffers with neutral pH include ammonium acetate (10 and 20 mM) and ammonium formate (10 and 20 mM) with the use of diluted ammonia solution. These selected buffers in conjugation with acetonitrile as mobile phase B was investigated with and without mobile phase C (methanol). These mobile phases were pumped at a constant flow rate (0.40 mL/min) in gradient elution mode along with acetonitrile as diluents on the octadecylsilane (C18) stationary phase column individually and separately. Most of all selected aqueous buffers were helpful with acetonitrile and methanol for the separation of all desired peaks, but only acidic acetate and formate buffers were able to provide maximum separation in desired peaks. Hence, 0.05% acetic acid in 10 mM ammonium acetate and 0.05% formic acid in 10 mM ammonium formate were shortlisted for further evaluation. The high concentration of formic acid and a high concentration of ammonium formate in mobile phase A increases the resolution between pairs of impurities peaks 1 and 2 as well as in impurities peaks 9 and 10 with additionally increased run time. The buffers with acidic pH (less than 4.0) were more helpful in the separation of the first two peaks than the buffers with neutral pH (approx. 7.0). Additionally, the buffers with pH approx. 7.0 were impacting on separation and elution of peak 3 and peak 4 and their interference with peak 5. The use of buffers with neutral pH (approx. 7.0) and lower column temperature ($\leq$35 °C) was quite helpful and able to provide higher separation. However, it cost a total run time of more than 65 min. The use of formate or acetate salts in an acidic mobile phase was responsible for the improvement in the sharpness of the peak of abiraterone acetate. The use of methanol or ethanol in mobile phases plays an important role in the separation of the peak of anhydro abiraterone and 3-deoxy 3-chloroabiraterone. The representative chromatograms and overall outcome generated for the system suitability test (SST) solution during the optimization of selected buffers are presented in the supplementary information (Figs. S1 to S6).

3.1.3. Selection of column and column temperature

Most of the published and reported work on abiraterone acetate combined with impurities showed their separation on the octadecylsilane (C18) stationary phase column. The C18 column, with a 150 mm length, a smaller internal diameter, and a small particle size, confirms the higher performance with respect to separation. In stage-1 of analytical method development, investigated the different octadecylsilane (C18) columns (mentioned in Table S5) with various buffers, and organic solvents. Based on the evaluation concluded that analytical columns with higher lengths (150 mm) and carbon load (17–18%) are more helpful and promising for separation with sharp peak shape and peak symmetry when compared with the columns with lower carbon load and shorter lengths. Hence, two separate C18 columns (mentioned in Table S7) were chosen for further evaluation, and these two columns were identified as Waters, Aquity BEH C18 (BEH C18), and Waters Acquity UPLC BEH Shield RP18 (RP Shield 18) with the same dimensions (150 mm × 2.1 mm×1.7 μm). During the further evaluation of both selected columns, we also observed that the column temperature stimulates the separation between all impurities. The column of RP

Shield 18 offers the best separation in the range of 35–45 °C, while column BEH C18 provides the same separation in the range of 45–60 °C. Out of two investigated columns, the most desirable results were achieved by using a BEH C18 column with optimized three mobile phases at 50 °C, where the optimized gradient could differentiate all specified impurities, degradation products, related substances, and abiraterone acetate compared to the results on the RP Shield 18 column. We also investigated the old and new columns of both column details and concluded that after multiple injections and use, the RP Shield 18 column showed increased column pressure and could not work above 45 °C column temperature. However, the BEH C18 column shows quite a stable backpressure. The representative chromatogram and overall outcome generated for the SST solution during optimization of RP Shield 18 at maximum temperature (45 °C) are presented in the supplementary information (Fig. S7).

3.1.4. Selection of detection wavelength, injection volume, and concentration of 100% solution

Basically, observed peak areas are directly proportional to the injection volume and concentration of analytes in the solution. However, in UHPLC technique analysis, we observed that injection volumes of more than 5 µL also influence the peak sharpness, tailing factor, peak shape, and peak height. Hence, the responses of all analytes with diverse injection volumes were investigated in solutions containing different concentrations of analytes. The different injection volumes in the range of 1.0–5.0 µL of spiked standard solution were injected, and the 100% standard solution concentration was evaluated as 1.0 mg/mL based on the outcome of responses at DL for α and ß-epoxy abiraterone acetate, the signal to noise ratio of DL peaks, and the outcome regarding peak area, peak height, tailing factor, and theoretical plates for all desired analyte peaks. The generated chromatographic data in the form of recorded PDA spectra (190–400 nm) of all injected solutions was extracted and checked at different wavelengths to finalize a single detection wavelength for all eleven analytes in search of an interference-free baseline and higher peak response. Based on peak area responses, the detection wavelength of 254 nm was suitable for the quantification of nine analytes, whereas the detection wavelength of 260 nm was excellent for the quantification of two degradants (α and ß-epoxy abiraterone acetate). It is important to monitor these two degradants (α and ß-epoxy abiraterone acetate) in drug substances and drug products to prove their safety and purity. Hence, considering the higher responses of degradants α and ß-epoxy abiraterone acetate than the 254 nm wavelength, we finalized 260 nm as the detection wavelength. Additionally, it was finalized based on the stable baseline on the positive scale and had no major impact on the other nine analytes' responses. The representative chromatograms and overall outcome generated for the SST solution during optimization of suitable detection wavelength, injection volume, and concentration of 100% solution are presented in the supplementary information (Figs. S8 to S10 and Table S8).

All optimized UHPLC chromatographic parameters and mass spectrometer parameters are summarized in the supplementary information (Table S9). The representative chromatogram of the spiked API sample solution observed by the UHPLC-PDA-ESI-MS method is represented in Fig. 2.

3.2. Method robustness

Robustness is the capability to repeat the analytical outcome and procedure in separate and diverse laboratories or working conditions without major variations in the obtained results. The outcome of the method robustness study proved that the minor variation in chromatographic condition parameters was not impacting the analytical results. The robustness of the developed method was demonstrated using six different parameters, including a. column temperature (± 5.0 °C), b.

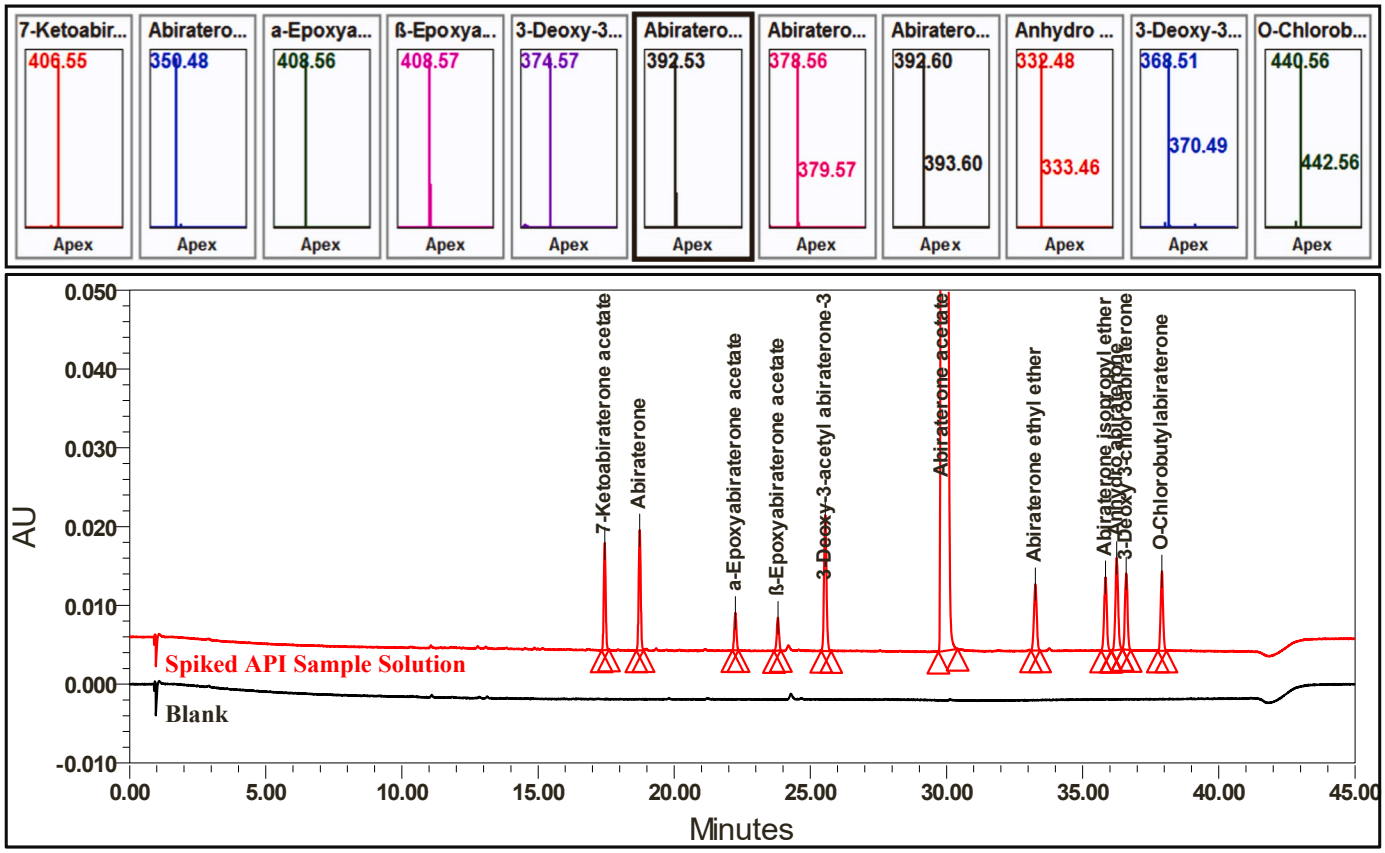

Fig. 2. : Overlay chromatogram of blank and spiked abiraterone acetate API sample solution generated by proposed UHPLC-PDA-ESI-MS method.

mobile phase flow rate (± 0.05 mL/min), c. slope gradient (± 1.0 min), d. the concentration of 0.05% formic acid in mobile phase A (± 0.05 mL), e. the concentration of buffer of 10 mM ammonium formate in mobile phase A (± 20%), f. column lot to lot, different lot of chemicals and reagents, and finally g. change in wavelength. The robustness study evaluations of the developed UHPLC method were carried out simultaneously on two separate UHPLC instruments: one UHPLC with PDA and a second UHPLC with a TUV detector. The UHPLC with PDA detector was a new instrument (2021) and recently calibrated, while the UHPLC with TUV detector was an old instrument (2016) with one month due for yearly calibration. One new column of Acquity BEH C18, 150 mm length x 2.1 mm i.d., 1.7 μm particle size, P/N 186002353, and a second old and used column with the same description were used in the robustness study. The sets of injections such as blanks, DL solution, QL solution, spiked standard solution-1 (six replicates), and spiked standard solution-2 were injected in the evaluation of each robustness parameter.

3.2.1. Impact of variations in column temperature

The variation in column temperature demonstrates its influence on the observed chromatographic outcome when compared to the outcome of nominal conditions. The peak retention times for all analytes decreased slightly as column temperature increased by 5 °C. Due to the higher temperature (55 °C), the faster exchange of all analytes between the mobile phase and the stationary phase happened, and early elution of all analytes was seen in the respective chromatograms. The outcome of chromatographic data with a lower column temperature of 5 °C was shown to be influenced inversely by the back pressure because increased back pressure was observed with the decreased column temperature. The viscosity of mobile phases was changed due to a change in column temperature, which impacted the amount of pressure and elution time of all analytes. As the column temperature increased, the viscosity of mobile phases decreased, and the column backpressure also decreased. However, when the column temperature decreased, the viscosity of mobile phases increased, and the column backpressure also increased. The total chromatographic outcome of variation in column temperature illustrated some of the effects on column back pressure, peak retention times, and a negligible effect on total system suitability requirements. However, it also showed that there was no impact on the expected results when the temperature was changed to 5 °C. The overlay chromatogram and critical chromatographic outcome of the SST solution demonstrating the impact of column temperature are presented in the supplementary information (Fig. S11 and Table S10).

3.2.2. Impact of variations in flow rate

The mobile phase flow rate demonstrates the influence on the chromatographic outcome. Column back pressure was influenced linearly by the changed flow rate of mobile phases; it was increased with increased flow rate and vice versa. The lower flow rate (0.35 mL/min.) shows higher peak responses with an increased retention time of all analytes and a moderate impact on system suitability requirements, especially the decreased resolution between peak 9 and peak 10 from 2.0 to 1.5 when compared to the outcome of the nominal condition. However, compared to the outcome of the nominal conditions, the higher flow rate (0.45 mL/min.) shows decreased peak responses with the reduced retention time of all analytes and a positive impact on system suitability requirements, such as increased resolution between peak 9 and peak 10 from 2.0 to 2.4. Based on the total outcome of both changes in flow rate, it was demonstrated that even with these slight changes, the proposed method was capable of providing accurate and precise results. The overlay chromatogram and critical chromatographic outcome of the SST solution demonstrating the impact of flow rate are presented in the supplementary information (Fig. S12 and Table S11).

3.2.3. Impact of variations in gradient slop

The proposed method's first gradient slop is 10.0 min, so it was investigated at 9.0 and 11.0 min to demonstrate the influence of a slight change (1.0 min) in it on the chromatographic outcome. Due to slightly increased organic phases in the lower gradient slop of 9.0 min, it shows slightly early elution of all analytes with negligible impact on system suitability requirements when compared to the nominal conditions. However, in the case of higher gradient slop, as at 11.0 min, it shows slightly increased retention time and a positive impact on system suitability requirements, such as increased resolution between all peaks. After a comparison of all the outcomes obtained with these slight changes in slop, it was revealed that, even with slight shifting in the retention time of all analytes, these changed gradient slop in the proposed method were able to deliver the expected results with trueness and precision. The overlay chromatogram and critical chromatographic outcome of the SST solution demonstrating the impact of gradient slop are presented in the supplementary information (Fig. S13 and Table S12).

3.2.4. Impact of variations in concentration of formic acid in mobile phase-A

The chromatographic outcome can be influenced by the pH of mobile phases through different parameters such as sensitivity, peak shape, retention time, and resolution, which purely depend on the molecule being investigated. The pH of a proposed mobile phase-A (0.05% formic acid in an aqueous 10 mM ammonium formate solution) was 3.3. We confirmed that the pH of a buffer solution containing 0.04% formic acid in an aqueous 10 mM solution was 3.5, which was higher than nominal conditions, while we achieved a pH value of 3.0 with the buffer solution of 0.10% formic acid in an aqueous 10 mM ammonium formate. The observed outcome concluded that the decrease in the pH value (from 3.3 to 3.0) induced the eluent strength of the mobile phase-A and increased the resolution between the critical pair of peaks 9 and 10. On the other hand, the increase in the pH value (from 3.3 to 3.5) reduced the eluent strength of the mobile phase-A (MP-A) and decreased the resolution between the critical pair of peaks 9 and 10. The slight impact of pH variations was seen on the retention times of all analytes' peaks when compared to the nominal conditions. The overlay chromatogram and critical chromatographic outcome of the SST solution demonstrating the impact of the concentration of formic acid in MP-A are presented in the supplementary information (Fig. S14 and Table S13).

3.2.5. Impact of variations in concentration of ammonium formate in mobile phase-A

The concentration of ammonium formate in the proposed mobile phase-A was 10 mM, and hence the effects of increasing and decreasing the concentrations of ammonium formate by 20% (2 mM) in mobile phase-A were studied. After comparison with the outcome by nominal conditions, it was confirmed that even with a 20% change in the concentration of buffer, there was no major impact on the pH of mobile phase-A, peak shape, or all system suitability requirements. Slightly early elution of all analytes and a very slightly decreased peak area of some analytes were observed in the decreased concentrations of ammonium formate (8 mM) in mobile phase-A. While in the case of mobile phase-A with 12 mM ammonium formate, a slightly late elution of all analytes was observed. The overlay chromatogram and critical chromatographic outcome of the SST solution demonstrating the impact of the concentration of ammonium formate in MP-A are presented in the supplementary information (Fig. S15 and Table S14).

3.2.6. Impact of variations in detection wavelength

The total chromatographic data outcomes obtained with different wavelengths showed very good precision of the retention times of all analytes. However, the % RSD for peak areas of all analytes is less precise. Based on observed peak area responses, the detection wavelength of 255 nm showed a higher peak area for the five analytes, while the wavelength of 260 nm showed the best response for the two important degradants (α and ß-epoxy abiraterone acetate). The

detection wavelength of 265 nm was showing decreased peak areas for all analytes and, hence, due to that, showed a slight impact on the system suitability outcome and especially on the area % and S/N ratio. However, the outcome observed with a change in wavelength shows that all peaks can be easily detected and can be quantified correctly with the use of relative response factors at respective wavelengths. The overlay chromatogram and critical chromatographic outcome of the SST solution demonstrating the impact of detection wavelength are presented in the supplementary information (Fig. S16 and Table S15).

3.2.7. Impact of different lot numbers of columns, chemicals, and reagents on separate UHPLC instruments

The chromatographic data obtained by using a different lot of columns, chemicals, and reagents on two separate UHPLCs (Waters) revealed that these changes had no significant effect on the results. However, it had a moderate impact on the peak elution time of all analytes and the peak shape of abiraterone acetate in spiked standard solution on the old UHPLC, most likely due to the multiple times used. Both columns have the same stationary phase (octadecyl silane, C18), with the same dimensions and the same manufacturer (Waters). The only changes in the lot number, age, and use of columns resulted in the main peak broadening and the increased retention time for all analytes. During analysis with an old column (multiple times used) on new and aged UHPLC, we observed quite a higher backpressure than with a new column.

Based on reported research work, we understood that different vendors, such as Agilent (1290 Infinity I and II), Shimadzu (Nexera X3), and Thermo Fisher Scientific (Vanquish), are also the leading providers of UHPLC instruments like Waters Corporation. As per our knowledge, if this proposed UHPLC method is used for its intended purpose on UHPLC from different vendors, then there could be the possibility of a slight impact on the expected outcomes. However, based on the investigational outcome (impact of variations in flow rate, column temperature, and gradient slope), we also believe that the analysis of samples on UHPLC with different vendors would give accurate and precise results. The reason for the impact of the UHPLC instrument from different vendors can be because of slightly different configurations such as the varied internal diameter of the tubing, dwell volume, extra-column volume, and optical path length in the flow cell of the detector. The dwell volume (the volume between the mixer of the pump and the start end of the column) for UHPLC instruments from these vendors is reported in the range of 150–225 μL while the extra-column volume is reported in the range of 10–15 μL. The extra-column volume is the area where peak dispersion can take place and includes such areas as an injector, tubing, pre-heating of mobile phases, post-column cooling, needle volume, sample loop volume, and the flow cell volume in the detector. The variation in UHPLC instrument volumes (dwell volume and extra-column volume) had the possibility of having a slight impact on the elution time of analytes. However, the application of the correction of these instrument volumes can minimize such an impact. If such a correction of volume is not applied, then slight alterations in system suitability parameters may be encountered for the proposed method. The variation in optical path length in the flow cell of the UV/PDA detector of UHPLC instruments can impact sensitivity. Increased optical path length in the flow cell can enhance sensitivity, while decreased path length can reduce sensitivity, and accordingly, it impacts LOD and LOQ values. The effect of the diversity in the working of the pump with a lower internal diameter of the connector or tubing (pressure due to viscosity, mixing of mobile phases, and reproducibility in flow rate) can create periodic pressure pulses and have a slight impact on the baseline noise and drift of the detector [30]. The overlay chromatogram and critical chromatographic outcome of the SST solution demonstrating the impact of different lot numbers of columns, chemicals, and reagents on separate UHPLC are presented in the supplementary information (Fig. S17 and Table S16).

3.3. Forced degradation study

3.3.1. Stressing of abiraterone acetate under various conditions

The hydrolysis of abiraterone acetate is one of the most important and widest degradation ranges, which covers acidic, neutral, and basic conditions. In this hydrolysis degradation process, a weighed known amount of abiraterone acetate drug substance was stressed with 2 mL of 1 M HCl (acidic), Milli-Q water (neutral), and 1 M NaOH (basic) at ambient temperature for 2, 5, and 60 days to yield breaking into degradation products. A weighed amount of abiraterone acetate was also stressed for 12 h at 60 °C in 2 mL of 1 M HCl and 1 M NaOH. Because the rate of the degradant's formation increases with temperature, the API of abiraterone acetate was exposed in an oven at 80 °C for two days to study the thermal stress conditions. In thermal degradation, different reactions happen, like decarboxylation, dehydration, and polymerization. A solution of 3.0% H_2O_2 (hydrogen peroxide) was used to perform oxidative degradation. In oxidative stressing, the weighted amount of abiraterone acetate was stressed for 2, 5, and 60 days with 2 mL of 3.0% H_2O_2.

To study the hydrolysis degradation process in the drug product, a weighed known amount of powdered sample of 500 mg abiraterone acetate tablets and 100% tablet placebo was stressed with 2 mL of 0.1 M HCl (acidic), Milli-Q water (neutral), and 0.25 M NaOH (basic) at 60 °C for 2 and 5 days. A powdered sample of 500 mg abiraterone acetate tablets and 100% tablet placebo was exposed in an oven at 80 °C for two days to study the thermal stress conditions in the drug product. A solution of 3.0% H_2O_2 (hydrogen peroxide) was used to perform oxidative degradation and for that a weighed known amount of powdered sample of 500 mg abiraterone acetate tablets and 100% tablet placebo was stressed for 2 and 5 days with 2 mL of 3.0% H_2O_2.

The photolytic (ICH Light) degradation study was performed by exposing the solid API of abiraterone acetate, a powdered sample of 500 mg abiraterone acetate tablets, and a 100% tablet placebo in a separate Petri dish to ICH light. This ICH light is able to provide an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet (UV) energy of not less than 200-watt hours per square meter in the photostability chamber. After the appropriate stressing condition was finished, the solution of the 2-day hydrolysis (acid and base) stressing was appropriately neutralized by using 2 mL of 1 M NaOH and HCl. To examine and confirm the subsequent degradations, the remaining hydrolysis (acid and base) stressed sample solutions were not neutralized. To achieve a final concentration of 1.0 mg/mL, all stressed solutions were prepared. Then all prepared solutions were subjected to analysis with proposed methods to establish the number of degradation products formed.

3.3.2. Unstressed conditions

To ensure that the degradants produced were caused solely by the stressing condition and not by the representative API sample used for stressing, we prepared an unstressed API sample solution and injected it at 0, 2, 5, and 60 days. The unstressed sample solution was prepared in an amber-colored volumetric flask and stored in a closed cabinet at room temperature (22 °C). In all chromatograms of unstressed sample solution injected at 0, 2, 5, and 60 days, we observed traces of three degradants and an unknown impurity at RRT_1.13, and all are below the detection limit. The representative chromatogram of the unstressed solution of abiraterone acetate API and unstressed solution of powdered abiraterone acetate 500 mg Tablets are presented in the supplementary information (Figs. S18 and S19).

3.3.3. HCl (acidic) stressed conditions

The chromatograms of all the acid-hydrolyzed samples with 1 M HCl showed the hydrolysis of abiraterone acetate with the formation of a degradant named abiraterone by losing the acetate group. Within two days of stressing with 1 M HCl at room temperature, abiraterone acetate was degraded and formed into 2% of abiraterone, while it was observed

up to 12% in a sample stressed at 60 °C for 12 h. The outcome of un-neutralized sample solutions of HCl stressing conditions at 5 days (injected solution after 60 days) and 60 days at room temperature revealed the approximately complete degradation of abiraterone acetate (assay: 0.7%) and the formation of abiraterone as the main degradant (up to 99%) with minor degradants (0.5%). The formation of anhydro abiraterone (up to 0.2%) and traces of 3-Deoxy-3-Chloroabiraterone (up to 0.1%) as a degradant were discovered in all chromatograms of un-neutralized HCl stressing sample solutions at room temperature for 5 days (solution injected after 60 days), 60 days, and stressed at 60 °C for 12 h. Based on the total outcome observed in all chromatograms of un-neutralized HCl stressed sample solutions, we concluded that abiraterone was generated as a degradant due to the hydrolysis of abiraterone acetate. Further, due to the loss of water molecules from the generated degradant abiraterone, it formed the specified impurity anhydro abiraterone. Because all solutions were un-neutralized and contained a chloride environment, the generated anhydro abiraterone began to form another specified impurity known as 3-Deoxy-3-chloroabiraterone. These two specified impurities (anhydro abiraterone and 3-Deoxy-3-chloroabiraterone) were not observed in HCl stressed for two days and neutralized sample solution, not even traces, and injected after 60 days. In neutralized 2-days HCl stressed sample solutions, no secondary degradation was observed, whereas the formation of anhydro abiraterone and 3-Deoxy-3-chloroabiraterone impurities reveals a slight secondary degradation in un-neutralized HCl stressed sample solutions. The representative chromatograms of the solution of abiraterone acetate API and powdered abiraterone acetate 500 mg Tablets stressed with HCl for 12 h at 60 °C, for 60 days at ambient temperature, for 5 days at ambient temperature (un-neutralized solution injected after 60 days) and for 2 days at ambient temperature (neutralized solution injected after 60 days) are presented in the supplementary information (Figs. S20 to S24).

3.3.4. Water (neutral) stressed conditions

The chromatograms of stressed samples with neutral (Milli-Q water) conditions showed that there was no degradation of abiraterone acetate when stressed at room temperature for 60 days. The observed chromatogram was exactly matched to the unstressed sample solution with assay values. Indeed, we also observed traces of two degradants and unknown impurities in the sample solution stressed with neutral conditions for 60 days, as we identified in the unstressed sample solution. The chromatogram of the stressed abiraterone acetate API with neutral (Milli-Q water) conditions at ambient temperature (22 °C) for 60 days is presented in the supplementary information (Fig. S25).

3.3.5. NaOH (basic) stressed conditions

As observed in acid hydrolysis, the chromatograms of all the 1 M NaOH hydrolyzed samples also showed the hydrolysis of abiraterone acetate and the formation of an abiraterone by losing the acetate group. Abiraterone acetate was degraded and formed 1.7% of abiraterone (within 2 days at RT) and 9.7% of abiraterone (within 5 days at RT), while when the same solutions were injected after 60 days, it was observed to be up to 25% and 67%, respectively. The chromatograms of un-neutralized sample solutions stressed at 60 °C for 12 h and at room temperature for 60 days also showed the formation of abiraterone as a single and main degradant up to 7% and 32%, respectively. There was no secondary degradation observed in any NaOH stressed sample solutions and only one degradant abiraterone formation in neutralized and un-neutralized stressed sample solutions. The chromatograms of the solution of abiraterone acetate API and powdered abiraterone acetate 500 mg Tablet stressed with NaOH for 12 h at 60 °C, for 60 days at ambient temperature, for 5 days at ambient temperature (un-neutralized solution injected after 60 days) and for 2 days at ambient temperature (neutralized solution injected after 60 days) are presented in the supplementary information (Figs. S26 to S30).

3.3.6. Thermally (heat) stressed conditions

The chromatograms of thermally stressed samples presented the total 2% degradation of abiraterone acetate when stressed at 80 °C in an oven for 2 days. Out of a total of 2% degradants, α-epoxy abiraterone acetate was observed up to 0.5%, ß-epoxy abiraterone acetate was observed up to 1.1%, and 0.5% of other degradants. The color and description of an unstressed sample of abiraterone acetate were a solid white powder, but after 2 days of stressing at 80 °C in an oven, it turned into a pale-yellow solid powder. As thermal stressing was performed in the open petri dish, it was also exposed to the environmental oxygen and, due to that α-epoxy abiraterone acetate and ß-epoxy abiraterone acetate were observed. The chromatograms of the thermally stressed abiraterone acetate API and powdered abiraterone acetate 500 mg Tablet at 80 °C for 2 days are presented in the supplementary information (Figs. S31 and S32).

3.3.7. Hydrogen peroxide, H_2O_2 (oxidative) stressed conditions

The molecular structure of abiraterone acetate shows the two double bonds at carbon C5 and C16, which possibly get the active site to form the degradation due to their nature as tertiary carbon. Based on the chromatographic outcome of stressed samples with 3.0% H_2O_2 at ambient temperature for 60 days, it was confirmed the formation of 7-Ketoabiraterone acetate, α-epoxy abiraterone acetate, and ß-epoxy abiraterone acetate as degradants due to oxidation of abiraterone acetate, and maximum degradation was approximately 3%. The maximum degradation was observed at approximately 0.2% in the stressed samples with 3.0% H_2O_2 at ambient temperature for 2 days and 5 days. However, when the same solutions of 2 days and 5 days stressed with 3.0% H_2O_2 were injected after 60 days, then their chromatograms showed the major degradant observed at RRT_0.66, up to 38% and 5% respectively with 7-Ketoabiraterone acetate, α-epoxy abiraterone acetate, and ß-epoxy abiraterone acetate. Based on the comparison of results of stressing for 60 days and injection of a 2-day stressed solution after 60 days, it was concluded that abiraterone acetate was degraded rapidly in solution form and formed the major degradant observed at RRT_0.66 with molecular weight 408.8 as $[M+H]^+$. The molecular ion for abiraterone acetate appeared at *m/z* 392.6 in all stressed solutions, while the major degradant generated due to solution form was identified at RRT_0.66 and the same degradant appeared at *m/z* 408.6 in mass spectral data. The molecular weight difference between both was 16 *m/z*, and it revealed that there was an addition of one oxygen to the abiraterone acetate molecule and that this degradant was somehow different in molecular structure compared to α-epoxy abiraterone acetate and ß-epoxy abiraterone acetate. The representative chromatograms of the solution of abiraterone acetate API and powdered abiraterone acetate 500 mg Tablets stressed with 3.0% H_2O_2 at ambient temperature (22 °C) for 60 days, for 2 days and solution injected after 60 days, and stressed for 5 days and solution injected after 60 days are presented in the supplementary information (Figs. S33 to S36).

3.3.8. Photolytic (ICH Light) stressed conditions

The chromatograms of photolytic (ICH light) stressed samples showed the minor degradation of abiraterone acetate when stressed for 8 h of ICH light cycles. The two degradants named α-epoxy abiraterone acetate and ß-epoxy abiraterone acetate were slightly increased. As photolytic (ICH light) stressing was performed in the open petri dish, it was also exposed to the environmental oxygen and, due to that α-epoxy abiraterone acetate and ß-epoxy abiraterone acetate were generated. The representative chromatograms of the photolytic (ICH light) stressed abiraterone acetate API and powdered abiraterone acetate 500 mg Tablet are presented in the supplementary information (Figs. S37 to S38).

3.3.9. Total outcome of forced degradation study

The generated chromatograms show that the concentrations of abiraterone acetate in the solutions stressed with acidic (HCl), basic

(NaOH), oxidative (3.0% H_2O_2), thermal (at 80 °C), and photolytic (ICH light) conditions were lower as compared to the concentration in the unstressed abiraterone acetate solution. However, the concentration in the stressed solution with neutral (water) stress conditions remained unaffected as compared to the added concentrations. Abiraterone acetate was found to be stable under neutral conditions at room temperature for 60 days. Significant degradation was observed under acidic and basic hydrolysis, and due to ester hydrolysis resulting in the formation of major degradant abiraterone. Partial degradation was observed in oxidative (3.0% H_2O_2), thermal conditions, and when exposed to ICH light. When compared to stressing in solid form (without dissolving), almost complete degradation of abiraterone acetate was formed only when it was stressed in solution form or once abiraterone was dissolved. The molecular weight (m/z) of each separate peak was checked and confirmed as a degradant and specific known process impurity.

In the case of forced degradation of the drug product, it is observed that the abiraterone acetate 500 mg tablets are stable under neutral conditions. The drug product is prone to minor degradation under thermal conditions, oxidative conditions, and when exposed to ICH light. The drug product is unstable under the acidic and alkaline conditions. The primary degradation process for abiraterone acetate in drug products is ester hydrolysis resulting in the formation of abiraterone. However, under thermal conditions, oxidative conditions, and exposure to ICH light conditions, it undergoes oxidation and form mainly α-epoxy abiraterone acetate and its isomer ß-epoxy abiraterone acetate. Under all stressed conditions, unidentified degradation products have also been observed. The values of the purity threshold were found to be greater than the values of the purity angle for major degradants, impurities, and abiraterone acetate peak, which reproduced the purity of the peak and recognized the capability, significance, and specificity of the proposed method. All known specified degradation products and impurities, such as abiraterone, anhydro abiraterone, 3-Deoxy 3-chloroabiraterone, 7-Ketoabiraterone acetate, α-epoxy abiraterone acetate, and ß-epoxy abiraterone acetate, were confirmed as degradants using PDA data and mass spectral data. Based on the total outcome of forced degradation studies, the proposed UHPLC method is suitable for its intended use as a stability-indicating method because it separates, detects, and accurately measures the process impurities, degradation products, and assay contents without any interference from process impurities, degradation products, excipients, or any other potential impurities. The foremost outcome of forced degradation studies (hydrolysis and oxidation) on the solid form of abiraterone acetate in reaction representation forms is presented in Fig. 3. The stressed samples were evaluated against the average peak area of a known amount of standard and each content was calculated and summarized as the mass balance (% assay + % impurities + % of degradation impurities) and was found to be close to 100%. The detailed outcomes in tabular form are presented in the supplementary information (Tables S17 and S18).

3.4. Analytical method validation

The foremost goal of our research study was to develop the enhanced UHPLC method for the intended purpose of comprehensive quality assessment. Analytical method validation was carried out according to the ICH Q2(R1) guidelines, to confirm that the proposed UHPLC method with developed chromatographic parameters is appropriate for the execution of the analysis. The validation parameters such as specificity, precision, trueness, linearity, quantification limit (QL), detection limit (DL), and stability of solutions were checked and confirmed in the method validation. The total representative outcomes of the method validation of the UHPLC method (for quantification) and the UHPLC-ESI-MS method (for confirmation) are presented in Table 1.

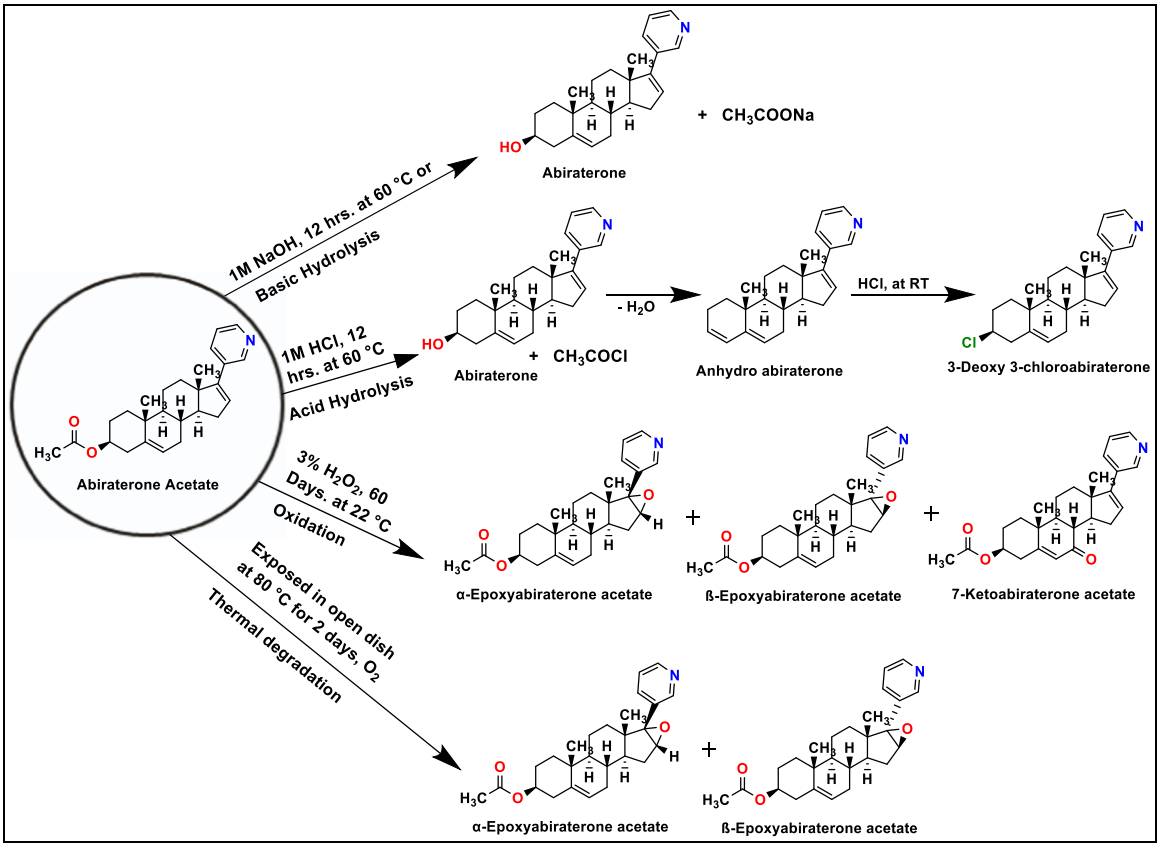

Fig. 3. : Representation of forced degradation studies on the solid form of abiraterone acetate (hydrolysis and oxidation).

Table 1
Outcome of method validation of UHPLC method (for quantification) and UHPLC-PDA-ESI-MS method (for confirmation).

Parameter	Degradant				Process Impurity	API	Specified Process Impurity				
	7-Keto abiraterone acetate	Abiraterone	α-Epoxy abiraterone acetate	ß-Epoxy abiraterone acetate	3-Deoxy-3-acetyl abiraterone-3-ene	Abiraterone Acetate	Abiraterone ethyl ether	Abiraterone isopropyl ether	Anhydro abiraterone	3-Deoxy 3-chloroabiraterone	*O*-Chlorobutyl-abiraterone
Peak Number	1	2	3	4	5	6	7	8	9	10	11
Retention time (min.)	17.5	18.8	22.3	23.9	25.7	30.0	33.4	36.0	36.4	36.7	38.0
Relative Retention Time	0.58	0.63	0.74	0.80	0.86	1.00	1.11	1.20	1.21	1.22	1.27
DL (μg/mL)	0.30	0.30	0.30	0.30	0.30	0.10	0.30	0.30	0.30	0.30	0.30
Lower QL (μg/mL)	0.50	0.50	0.50	0.50	0.50	0.30	0.50	0.50	0.50	0.50	0.50
Upper QL (μg/mL)	20.0	20.0	20.0	20.0	20.0	1300.0	20.0	20.0	20.0	20.0	20.0
S/N in DL Solution	26.4	27.2	6.2	7.2	29.5	7.3	15.1	16.8	20.7	17.2	18.4
S/N in QL Solution	50.2	52.2	13.6	13.2	55.4	23.8	28.9	32.1	39.2	31.9	35.7
Regression equation	y = 13520x - 5.9316	y = 14613x + 244.68	y = 4651.6x + 275.79	y = 4364.8x + 317.04	y = 19996x + 284.44	y = 13610x + 8193.2	y = 10614x + 229.65	y = 12073x + 291.41	y = 15101x + 1121.1	y = 11578x + 388.33	y = 11829x + 475.66
Coefficient of determination (R^2)	0.9999	0.9993	0.9999	0.9999	0.9999	1.0000	0.9999	0.9999	0.9999	0.9997	0.9999
% RSD for Response factor	5.8	2.9	7.3	7.6	5.4	3.0	5.4	3.6	5.0	0.7	1.2
Linearity range (μg/mL)	0.50–20.0	0.50–20.0	0.50–20.0	0.50–20.0	0.50–20.0	0.30–1300	0.50–20.0	0.50–20.0	0.50–20.0	0.50–20.0	0.50–20.0
Stability of solutions (at RT)	7 days	7 days	7 days	7 days	7 days	7 days	7 days	7 days	7 days	7 days	7 days
Filter for Sample filtration[a]	0.2 μm	0.2 μm	0.2 μm	0.2 μm	0.2 μm	0.2 μm	0.2 μm	0.2 μm	0.2 μm	0.2 μm	0.2 μm
Relative Response Factor	0.993	1.074	0.342	0.321	1.469	1.000	0.780	0.887	1.110	0.851	0.869
Purity Angle	17.392	19.714	21.576	18.140	23.269	0.217	29.032	23.811	18.019	41.880	27.968
Purity Threshold	18.871	22.314	24.712	20.074	27.925	0.260	35.924	28.265	20.272	62.010	34.934
Molecular formula	$C_{26}H_{31}NO_3$	$C_{24}H_{31}NO$	$C_{26}H_{33}NO_3$	$C_{26}H_{33}NO_3$	$C_{26}H_{31}NO$	$C_{26}H_{33}NO_2$	$C_{26}H_{35}NO$	$C_{27}H_{37}NO$	$C_{24}H_{29}N$	$C_{24}H_{30}ClN$	$C_{28}H_{38}ClNO$
USP Resolution	NA	9.9	25.0	9.6	10.3	20.5	15.6	13.2	2.2	2.0	7.7
Maximum USP Tailing	1.0	1.1	1.1	1.2	1.0	2.2	1.0	1.1	1.1	1.0	1.1
Minimum USP Plate Count	320350	251479	375351	305362	387293	233340	488226	634241	510557	662460	679451
Molecular Weight (g/mol)	405.5380	349.5180	407.5540	407.5540	373.5400	391.5550	377.5720	391.5990	331.5030	367.9610	440.0680
Exact mass [M] (g/mol)	405.2304	349.2406	407.2460	407.2460	373.2406	391.2511	377.2719	391.2875	331.2300	367.2067	439.2642
Molecular ion [M ± H]	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$	$[M+H]^+$
m/z value in Standard	406.5	350.5	408.6	408.6	374.5	392.6	332.5	392.6	332.5	368.5	440.6
m/z value in Sample	406.5	350.5	408.6	408.6	374.5	392.6	332.5	392.6	332.5	368.5	440.6

S/N: Signal-to-noise ratio, NA: Not Applicable, [a]: Pall, Acrodisc CR 25 mm syringe filter with 0.20 μm PTFE membrane, part No. 4225 T filter, RT: Room temperature

3.4.1. Specificity

The results of the specificity test showed that this suggested UHPLC method can reliably and specifically separate, identify, and measure with accuracy each targeted analyte in the presence of unknown impurities and degradants in the sample matrix. The specificity was proved by analyzing the solutions containing blank, placebo solutions of the tablet, system suitability test solution, standard solution, individual impurity solution of all specified process impurities and degradation products, spiked sample solution, and unspiked sample solution of API and tablet formulation. The peak purity was checked and calculated by the UHPLC-PDA scan using "Empower" software. The purity angle values were observed to be lower than the purity threshold values for abiraterone acetate. By visual observation of all the chromatograms, it was confirmed that there was no coelution of peaks with each other and that the peak of the abiraterone acetate was pure. The peak purity was also confirmed with the help of data obtained from the mass spectra. Sometimes, in the chromatogram of a blank matrix, small blank peaks were observed when analytical grade solvent was utilized instead of LC-MS grade solvent. However, the magnitude of these blank peaks was very negligible, and they were separated from the mentioned peak in that region. There were no placebo peaks found. The overlay chromatogram of the blank, tablet placebo, individual impurity solution, and tablet spiked sample solution is shown in the supplementary information (Fig. S39).

3.4.2. Trueness

The trueness of the method for abiraterone acetate at lower concentrations was determined at seven levels in the range of 0.03–5.0%w/w of sample concentrations in triplicate preparations. At higher concentrations, the trueness for abiraterone acetate was calculated at seven levels in the range of 25–150%w/w of sample concentrations in triplicate preparations. The trueness for all six specified process impurities and four degradation products was determined at seven concentration levels in the range of 0.05–2.0%w/w of sample concentrations in triplicate preparations. The recoveries for the abiraterone acetate at a lower level and for all impurities and degradation products were found to be in the accepted range of 89.8–113.0%. However, for abiraterone acetate, the assay was within 99.8%− 101.2%. The determined relative response factor was considered for the calculation of the trueness of all impurities and degradation products. The results of the recoveries confirm that the method is accurate. The trueness results for all four degradation products and six process impurities (Tables S19 and S20), for abiraterone acetate with tablet placebo (Table S21), and for abiraterone acetate without placebo (Table S22) are provided in the supplementary information.

3.4.3. Precision

The precision of the proposed method was expressed as system repeatability, analysis repeatability, and intermediate precision. The system repeatability parameter was checked and confirmed in every sample set injected in method validation, by injecting six replicates of 100% standard solution of the abiraterone acetate with pre-defined acceptance criteria for a percent RSD of $\leq$ 0.73%. The parameter analysis repeatability was assessed as a percentage of RSD at all seven-concentration levels injected and determined as an individual recovery in the trueness parameter. The percent relative standard deviation was found to be below 0.6% for the assay range of abiraterone acetate and that for all specified process impurities, degradation products, and abiraterone acetate at a lower range was within 6.3%. The third parameter, intermediate precision, was confirmed by analyzing two separate sets of spiked samples on different instruments using different columns on different days with separate solution preparations. The selected samples of abiraterone acetate API did not contain all impurities or degradation products above the reporting threshold limit (0.05%), and samples of abiraterone acetate tablets contained only three degradation products above 0.05%. Hence, all impurities and degradation products were spiked into the separate six sample solutions of abiraterone acetate API and tablet samples at the 0.3% level and 0.5% level, respectively. For both analysts, the RSD values obtained for the six replicates of spiked sample solution yielded for each impurity and degradation product were less than 6.8%, while the RSD for abiraterone acetate assay values was less than 0.8%. The total results of system repeatability, analysis repeatability, and intermediate precision confirm the precision of the method. The representative outcome of the system repeatability case study, in which six replicates of the same standard solution were injected on three different UHPLCs by using different lot numbers of columns, is presented in the supplementary information (Table S23).

3.4.4. Detection limit (DL) and quantitation limit (QL)

The detection limit was demonstrated by three injections of a solution containing abiraterone acetate at a DL level of 0.1 μg/mL (0.01%) and all ten impurities (process impurities + degradation products) at a DL level of 0.3 μg/mL (0.03%). The signal-to-noise ratio (USP s/n) obtained in the solution of the detection limit for all eleven analytes was in the range of 6.2–29.5. The quantitation limit was demonstrated by injecting three injections of a solution containing abiraterone acetate at a QL level of 0.3 μg/mL (0.03%) and all ten impurities (process impurities + degradation products) at a QL level of 0.5 μg/mL (0.05%). The signal-to-noise ratio (USP s/n) obtained in the solution of the quantitation limit for all eleven analytes was in the range of 13.2–55.4. The overlay chromatogram of blank, detection limit (DL), and quantification limit (QL) solutions for all eleven analytes are presented in the supplementary information (Fig. S40) and the individual value of signal-to-noise ratio is in Table 1.

3.4.5. Linearity

For abiraterone acetate (API), the linearity of the proposed UHPLC method was tested in three replicates from QL to 130.0% of the concentration of abiraterone acetate in the sample solution. While for all ten impurities (process impurities + degradation products), the linearity was tested in three replicates from QL to 2.0% of the concentration sample solution. The three separate stock standard solutions of all ten impurities were prepared with a concentration of approximately 0.1 mg/mL and were further diluted to obtain seven solutions of different concentrations in the range of 0.5–20 μg/mL (0.05–2.0%). Three separate stock standard solutions (1.0 mg/mL) were prepared for a series of lower concentration abiraterone acetate solutions, which were further diluted to yield six solutions with concentrations ranging from 0.30 to 50 μg/mL (0.03–5.0%). At a higher concentration of abiraterone acetate, seven levels of solutions were prepared in the range of 250–1300 μg/mL (25–130%) in triplicate preparations. A correlation coefficient of $\geq$ 0.999 was observed for abiraterone acetate, six process impurities, and four degradation products, confirming that the proposed UHPLC method is linear in the concentration range tested. The percent RSD of response factors was less than 4.5% for all ten impurities, and it was less than 2.2% for abiraterone acetate (for the range of 0.30–1300 μg/mL) and less than 0.2% for abiraterone acetate in the assay range (250–1300 μg/mL). The linearity graphs for all validated eleven analytes are presented in the supplementary information (Fig. S41), and the observed regression equation with values of the correlation coefficient is presented in Table 1.

3.4.6. Range

The range for abiraterone acetate was defined as the verified interval between a lower concentration of 0.03% (0.30 μg/mL) and a higher concentration of 130% (1300 μg/mL). While the range for all six process impurities and four degradation products was between 0.05% (0.50 μg/mL) and 2.0% (20 μg/mL).

3.4.7. Solution stability

The solution stability was checked and confirmed on the solutions of

unspiked and spiked standards solutions (0.3 and 1000 μg/mL), and sample solutions of API and tablets (1000 μg/mL) and was found to be stable at ambient temperature for seven days.

3.4.8. Filtration study

The filtration study was conducted on the unspiked and spiked sample solutions of the tablet formulation. Based on the generated chromatogram and outcome, it was determined that there were no interfering contaminants extracted from the filter and that there was no significant adsorption of any analyte under investigation onto the filter bed when compared to the same unfiltered (centrifuged) sample solution.

3.4.9. Relative response factor (RRF)

The relative response factors were calculated for all six specified process impurities and four degradation products by using the respective slope values observed in linearity tests with the linearity curves in the prescribed range, as well as the slope value for abiraterone acetate in the assay range. The obtained RRF values are listed in Table 1.

3.4.10. System suitability test

The system suitability test is nothing but an assurance of performance qualification of UHPLC, column, mobile phases, software, and the analytical method that is tested. Additionally, it is the reconfirmation of validation parameters of an analytical method where we check the specificity (resolution), trueness (% recovery), precision (% RSD), DL (signal-to-noise ratio), QL (% recovery), robustness (5% RT shift window), and carry over (interference-free blank). Meeting the acceptance criteria of system suitability indicates that the used LC instrument system, column, mobile phases, and generated chromatographic conditions are suitable for the measurement of the respective samples. The system suitability parameters were examined, confirmed, and verified during sample analysis and ensured that all chromatographic conditions were suitable prior to sample injection, intermittently after 12 injections of the sample, and after the last injection of the sample. The parameters listed in the supplementary information (Table S24) were assured throughout all of the method validation sample sets, and each time, they fulfilled the specified acceptance criteria.

Based on validation results it is concluded that the proposed UHPLC method is selective, accurate, precise, linear, and robust for the measurement of abiraterone acetate, its four degradants, and six specified impurities in drug substances and drug products. Additionally, the proposed methodology offers improvements in sensitivity, speed, and separation with respect to previously published and reported methods. The stability of all analytes in the solution was checked and confirmed that all analytes were stable for at least seven days in the solution if stored in the light-protecting flask at ambient temperature.

3.5. Proposed methods applicability

To demonstrate the applicability, usefulness, and effectiveness, and to prove the integrity, rationality, and reliability of the proposed analytical methods, we analyzed the unspiked and spiked sample solutions of the drug substance and drug products (250 mg and 500 mg tablets) of abiraterone acetate. Simultaneous quantification of all eleven desired analytes was estimated by using the UHPLC method and additionally with the UHPLC-PDA-ESI-MS method for correct identification and confirmation of results. The generated chromatographic data with the PDA detector and mass spectrometer detector of all unspiked API sample solutions revealed the absence of all six specified process impurities and the presence of traces of four degradation products and two unknown impurities. However, the total amount of all detected peaks in both API batches was less than DL (0.03%), and hence total impurities were less than 0.05%. While in the case of unspiked tablet sample solutions, the generated data revealed the absence of all six specified process impurities and the presence of all four degradation products. In both batches of tablets, the observed amount of degradant 7-Ketoabiraterone acetate was 0.05–0.1%, abiraterone was 0.03–0.05%, α-epoxy abiraterone acetate was 0.3%, ß-epoxy abiraterone acetate was 0.2%, all unknown impurities were less than 0.04%, and hence total impurities were up to 0.5–0.7%. The simultaneous quantification of the assay of abiraterone acetate and degradation products was calculated against the average peak area of a known amount of abiraterone acetate standard solution, and the generated results showed that all results were well within the acceptance criteria mentioned in the USP monographs. All batches of API and abiraterone acetate tablets were evaluated using the reported compendial HPLC method in order to confirm and compare the results obtained by the proposed methods. The observed results of both batches of API and abiraterone acetate tablets through the proposed methods and the reported HPLC method are summarized in the supplementary information (Table S25) and chromatograms (Figs. S42 to S49).

The increased sensitivity and detection efficiency with the significance of the proposed methods were confirmed based on the observed results and comparison of both (the reported method and the proposed methods), which led to the conclusion that there were no significant differences in the results. All of the peaks shown in the sample chromatograms were clean, and there was no coelution, as confirmed by the precise identification and confirmation of all chromatographic information generated by the PDA detector against the observed mass spectrometer data. The reliability of the observed molecular weight from mass spectral data in all samples was confirmed by comparing the observed molecular weights of all eleven analytes with the observed m/z values of individual standard solutions and with expected m/z values taken from ChemDraw Software for ESI-MS. In mass spectral data, the molecular ions of all eleven analytes were identified as $[M+H]^+$ in the positive mode. The generated MS data (listed in Table S26) explained that simultaneous quantifications of all analytes are possible against the standard solution of each analyte. The comprehensive outcome confirmed that the proposed method was successfully developed, validated, and applied for qualitative and quantitative analysis of drug substances and drug products of abiraterone acetate. The mass spectra for all four degradation products, abiraterone acetate, and six specified process impurities are shown in Figs. 4 and 5. The representative online UV absorption spectra recorded by the PDA detector and data taken from ChemDraw Professional for all eleven analytes are shown in the supplementary information (Figs. S50 to S53).

4. Conclusions

This research article presented the development of a new reverse-phase ultra-high performance liquid chromatographic method compatible with mass spectrometers for qualitative and quantitative analysis. Compared with the previously reported methodologies, this proposed work provides the first specific method for the separation and simultaneous quantification of abiraterone acetate, its six specified process-related impurities, and four degradation products with UHPLC. In conjugation with a mass spectrometer, the proposed method additionally offers correct identification and confirmation of results based on molecular weight.

Finally, the developed UHPLC method was validated in terms of specificity, precision, trueness, linearity, DL, QL, and solution stability. Based on validation results, it was proven to be rapid, timesaving, cost-effective, linear, robust, and appropriate for its intended purpose. The detailed robustness study revealed that although slight chromatographic condition variations have a slight influence on the elution time of analytes, they are still able to achieve their intended purpose. The forced degradation studies explained the degradation of abiraterone acetate and the formation of abiraterone (due to hydrolysis), the formation of 7-Ketoabiraterone acetate, α-epoxy abiraterone acetate, ß-epoxy abiraterone acetate (due to oxidation), and the formation of anhydro

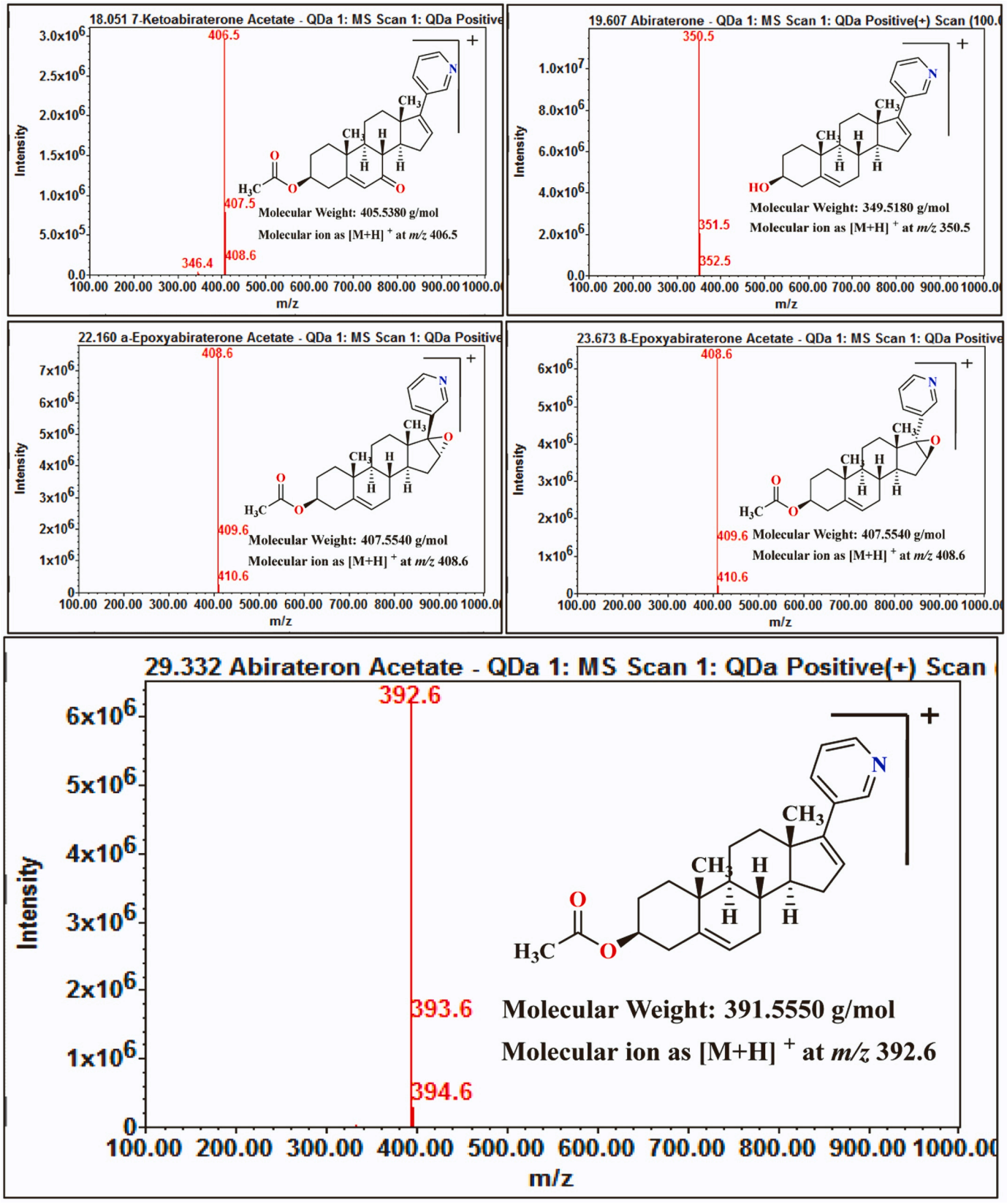

Fig. 4. : Mass spectrum of all four degradation products and abiraterone acetate.

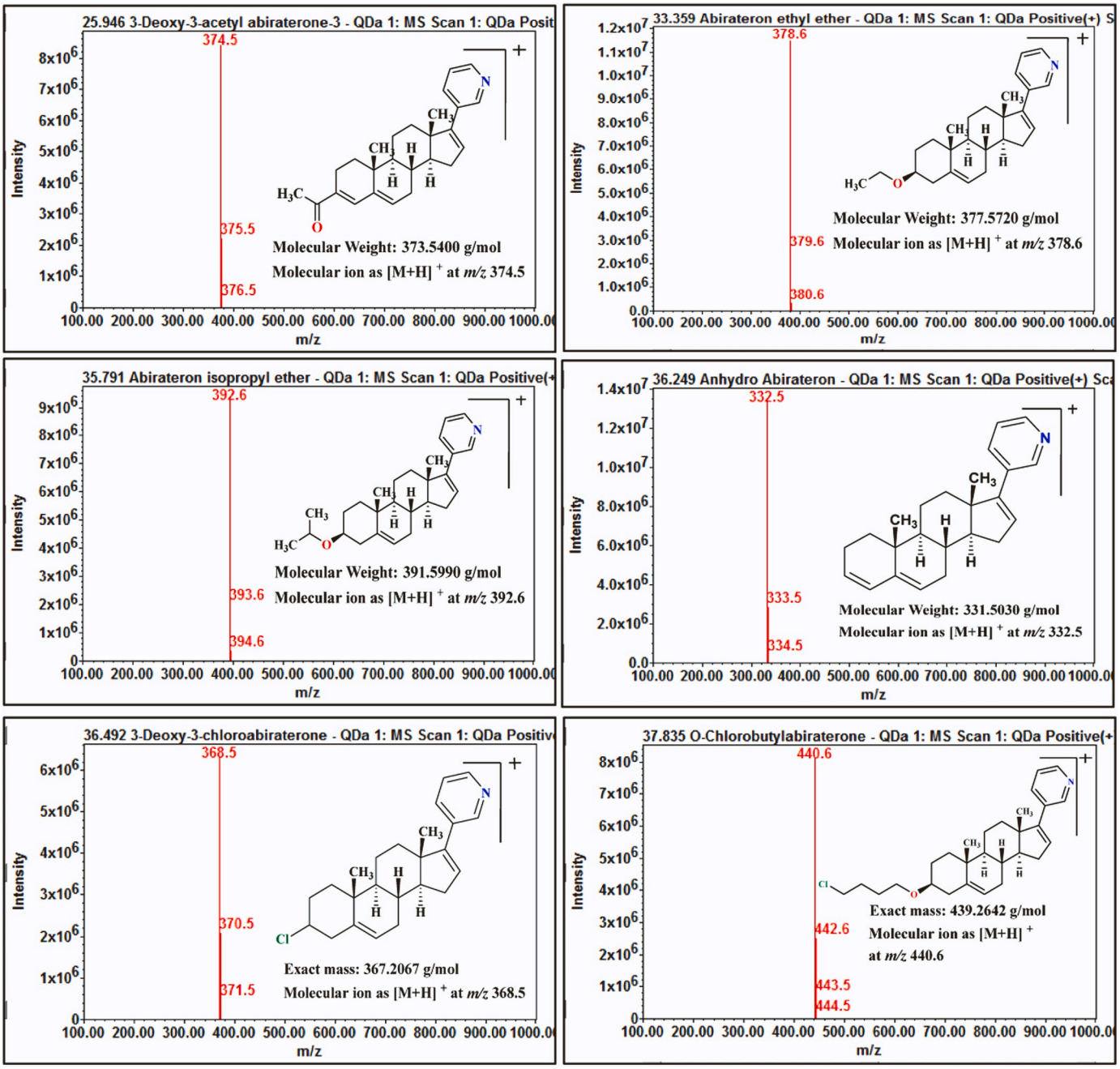

Fig. 5. : Mass spectrum of all six specified process impurities of abiraterone acetate.

abiraterone and 3-Deoxy-3-chloroabiraterone (due to temperature, HCl medium, and degradation of abiraterone).

We are confident that these proposed methods will be very useful as the first effective UHPLC methods for comprehensive quality assessment of abiraterone acetate for the manufacturers and suppliers of abiraterone acetate drug substances and drug products. The proposed methods will be of significant benefit to the pharmaceutical industry for routine quality control analysis, stability studies of pharmaceutical products, developmental studies, investigational studies, pharmacokinetics, and pharmacological mechanisms.

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CRediT authorship contribution statement

Deepak Krishna Mhaske: Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing. **Arjun Shankar Kumbhar:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Conflict of interest statement

The authors declare that they have no conflict of financial interests or personal relationships that could have influenced the findings of this study.

Data Availability

All related data is included in the manuscript and supplementary information. Additional data are available from the authors upon request.

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Submission declaration and verification

Submission of an article implies that the work described in this article has not been published previously anywhere and is not under consideration for publication elsewhere. This publication is approved by all authors, and it will not be published elsewhere in the same form.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115568.

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