Short communication

Analysis of abiraterone stress degradation behavior using liquid chromatography coupled to ultraviolet detection and electrospray ionization mass spectrometry

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A B S T R A C T

A validated stability-indicating LC–UV–ESI-MS analytical method was established to analyze abiraterone (ABR) and its potential degradation products (DPs) and was performed according to ICH guidelines. Trace amounts of DPs that might be released under different environmental conditions were determined. Stress conditions, including the effect of heat, acid–base hydrolysis, oxidation and UV-light were investigated. ABR was found to be sensitive to UV light and oxidation. Five potential mono-oxygenated ABR products were generated upon exposure to UV-irradiation. Di-, tri-, and tetra-oxygenated ABR were detected and progressively increased in quantity upon longer exposure to UV light. The ESI-MS response factors (RF) of potential DPs and ABR were not comparable. The ESI-MS response factor of each single potential degradation product was derived from the LC–UV analysis of concentrated solutions of pure and degraded ABR. The ESI-MS limit of detection (LOD) and limit of quantification (LOQ) of ABR were 30 and 80 pg/μL, respectively. The intra-day RSD was 0.20%, and the inter-day RSD was 0.30%.

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1. Introduction

Abiraterone, (3β)-17-(pyridin-3-yl)androsta-5,16-dien-3-ol, was developed as a selective mechanism-based steroidal inhibitor based on observations that nonsteroidal 3-pyridyl esters have improved selectivity for the inhibition of 17α-hydroxylase-C17, 20-lyase [1–3]. Abiraterone (ABR) is a drug used in cases of prostate cancer that do not respond to androgen deprivation or treatment with anti-androgens. This drug was formulated as the produrg abiraterone acetate and was marketed under the trade name Zytiga® in tablet form [4,5]. Two different LC/MS methods have been reported for the analysis of ABR in humans [6] and in human and rat plasma [7]. The limits of detection and quantification were different due to the different LC–MS conditions and methods used. No reports could be found that describe the stability of ABR. For this reason, it was essential to develop a stability indicating assay method to monitor ABR and its potential degradation products. In this work, ABR was exposed to different stress conditions and then analyzed using HPLC–UV–ESI-MS. Normally, the ICH guidelines are not applicable to the calculation of unknown degradation products (DPs) using the ESI-MS response factor of a principal drug because of the wide variation in the protonation state of the compounds upon nebulization at an ESI source and the subsequent different positive MS responses [8]. A review article describing recent advances in the analysis of organic impurities of active pharmaceutical ingredients and formulations has been published [9]. This review showed many applications using LC–IT-MS. The developed method describes a stepwise procedure to realize two major points, including the stability-indicating assay of ABR using two detectors and the compliance with ICH guidelines concerning the calculation of the amounts of DPs in new drug substances [10–12].

2. Experimental

2.1. Chemicals

Abiraterone was purchased from SelleckChem in Houston, TX, USA. The ABR purity determined by the manufacturer using HPLC–UV analysis is 99.2–99.7% (batch S112301, analysis date 5/24/2010). All other materials were of analytical grade. All solvents were of HPLC grade. Solvents were saturated with pure nitrogen gas (99.9999%) before use by purging the cooled solvents with gas at a flow rate of 5 mL/min for 10 min.

2.2. Preparation of calibration solutions

Stock solutions containing 1 mg/mL of ABR were prepared by dissolving 10 mg of ABR in 0.5 mL of chloroform and were diluted to 10 mL with acetonitrile. Serial dilutions spanning the range...
10–200 ng/µL were prepared in acetonitrile and used to construct a UV calibration curve at 235 nm. Additional diluted standard ABR solutions spanning the range 80–2000 pg/µL were prepared and used to construct an ion trap-MS (IT-MS) calibration curve while applying the positive multiple reaction monitoring (MRM) mode.

2.3. Liquid chromatography–UV and mass spectrometry

The HPLC system consisted of an Agilent 1200 system, a solvent delivery module, a quaternary pump, an autosampler, a diode-array detector (DAD), and a column compartment (Agilent Technology, Germany). The column effluent was connected to an Agilent 6320 Ion Trap HPLC–ESI-MS. The column heater was set to 25 ± 2 °C. The control of the HPLC system and data processing were performed using ChemStation (Rev. B.01.03 SR2(204)) and 6300 Series Trap Control version 6.2 Build No. 62.24 (Bruker Daltonik Gmbh). The analytes were separated using an Agilent Zorbax Extend-C18 column (80 Å, 150 mm length × 4.6 mm, i.d., 5 µm) an Agilent-Zorbax Extend-C18 pre-column (Agilent Technologies, Palo Alto, CA, USA). The mobile phase was prepared by mixing 700 mL of 0.1 % formic acid in water with 300 mL acetonitrile and was pumped at a flow rate of 0.5 mL/min.

The MS conditions were optimized for maximum sensitivity by direct infusion of the ABR solution (5 ng/µL prepared in mobile phase) into the ESI-MS system at a flow rate of 10 µL/h, and all ion optics were optimized. General MS adjustments were set as follows: capillary voltage, 3500 V; nebulizer, 36 psi; drying gas, 12 L/min; desolvation temperature, 350 °C; ion charge control (ICC) smart target, 150,000; and max accumulation time, 150 ms. The UV detector was set at 235 nm.

Two MS modes were used, including the positive Auto-MS² scan mode and the MRM mode. The Auto-MS² mode was used to help with the identification of the chemical structures of the DPs, whereas the MRM mode was applied for the quantitative analysis of potential degradation products. Up to ten molecular positive ions could be retrieved by the MS machine simultaneously. The minor identified or unidentified DPs that were released due to severe stress conditions were not determined. A sample injection volume of 2 µL of concentrated degraded sample (500 ng/µL) was injected for qualitative analysis using positive Auto-MS² mode, whereas 5 µL of diluted sample was injected for the assay using positive MRM mode (selected m/z ± 0.3; 350.2, 366.2, 380.2, and 382.2). Other IT-MS software capabilities were used to determine the [M+H]+, the average MS² or to search for the origin of certain fragmentations.

2.4. Forced degradation

2.4.1. Effect of UV-irradiation

The effect of light was studied on powder and solution forms of ABR using a UVP-cabinet containing three types of lamps (each 15 watt: white light, 254 nm, and 365 nm lamps, UVP-Chromato-Vue, C-70G, Upland, CA, USA). The distance between the UV-lamp and the sample was 5 cm. The control sample was prepared and wrapped in aluminum foil. A sample film of ABR powder with a thickness of approximately 0.1 mm was placed in a flat glass dish and subjected to UV irradiation. A mass of 20 mg of ABR was mixed with 10 mL of water and sonicated for 1 min. This turbid solution was exposed to UV irradiation. A suitable concentration of this solution (claimed, 100 ng/µL and 1000 pg/µL) was prepared in acetonitrile after an exposure time of 0.25, 0.5, 2, and 12 h. A volume of 5 µL was injected for HPLC analysis and was detected simultaneously using UV at 235 nm and MS.

2.4.2. Effect of oxidation

A 0.5 mL aliquot of a sample solution of ABR was prepared in acetonitrile: water (1:1, v/v) at a concentration of 100 ng/µL and was mixed with 0.5 mL of 10% (v/v) hydrogen peroxide and left to stand for 15 min before injection. A volume of 5 µL was injected for HPLC–UV–ESI-MS analysis.

2.4.3. Effect of acid and base hydrolysis

In a 1-mL reaction vial, a 0.5 mL aliquot of a standard ABR solution (100 ng/µL prepared in acetonitrile:water, 1:1, v/v) and 0.5 mL of 0.1 M HCl or 1 M NaOH were added. Both samples were analyzed using HPLC–UV–ESI-MS immediately, after 1 h, and after heating at 80 °C for 15 min.

2.4.4. Effect of heat

Bulk powder and solution samples of ABR (1 mg/mL) were subjected to heat. The powder sample was left to stand at 80 °C in a hot air oven for 6 h, and a solution of 50 ng/µL was prepared in acetonitrile: water (1:1, v/v) and left to stand in the hot air oven at 90 °C for 60 min. The heated powder solution was dissolved in acetonitrile, and a solution with a concentration of 50 ng/µL was prepared in water; a volume of 5 µL was analyzed using HPLC–UV–ESI-MS.

3. Results and discussion

The development of a stability-indicating assay method for ABR was necessary because there are no reports describing the degradation products that might arise from exposure to different environmental conditions. The analytes were monitored simultaneously using UV and IT-MS detectors. The UV detector function was used to estimate the concentration of ABR and generated potential unknown degradation products (DPs) at relatively high concentration levels. Some of the released DPs were detected using UV techniques at a higher claimed ABR concentration (1 mg/mL). The ESI-MS detector was sensitive enough to trace low levels of DPs (30 pg/µL). The IT-MS system used is characterized by its capability to extract an ion chromatogram of MS¹ or MS² from a maximum of three overlapped data points. This capability enables the selective separation and identification of MS² target ions after data retrieval.

3.1. Optimization of chromatographic and IT-MS conditions

Five separate degraded ABR sample solutions were prepared as a result of the effect of heat, oxidation, acid hydrolysis, base hydrolysis, and light degradation. These samples were analyzed using LC–UV–ESI-MS by applying several interchangeable chromatographic and IT-MS conditions until the best resolution and ESI-MS detection were achieved. Finally, to obtain the best overall conditions, the mobile phase was optimized by examining the effect of the column type, the mobile flow rate, the percentage of formic acid, the percentage of acetonitrile and the settings of the IT-MS ion optics. The optimal chromatographic and ESI-MS conditions were achieved as described in the experimental section.

3.2. Method validation

ESI-MS was performed in positive ion mode, and the ion at m/z 350 corresponds to the protonated molecule of ABR. A linear relationship exists between the peak area and the concentration of ABR over a range of 10–200 ng/µL using a UV detector and over a range of 50–2000 pg/µL using an ESI-MS detector (EIC of m/z 350.2 ± 0.3). The coefficient of determination of both detection methods was 0.9998. The UV and MS slopes were 2.702 ± 0.33 and 68,573 ± 1.94, respectively. Both calibration curves were repeated six times using seven calibration levels. The limits of detection (LOD) of the UV and MS methods were 4 ng/µL and 30 pg/µL, respectively, whereas the
limits of quantification (LOQ) were 10 ng/µL and 80 pg/µL, respectively. The relative standard deviation of the LOD and LOQ values were not more than 1.2%. The peak purity index of ABR monitored using the UV detector was 100 ± 1%. The concentrations of the unknown peaks eluted at 3.0–6.0 were considered for LC–UV calculation at low levels because peak overlaps were observed at higher concentrations.

3.3. Accuracy (UV detection)

An analysis of (LC–UV) placebo solutions spiked with known amounts of ABR indicated the accuracy of the method (claimed, 10, 30, 50, 100, and 200 ng/µL; found, 10 ± 0.02, 30 ± 0.04, 50 ± 0.06, 100 ± 0.01, and 200 ± 0.11 ng/µL, respectively). A standard ABR solution (100 ng/µL) that was spiked with degraded sample covered the range of 50, 75 and 100%. The average percent error was calculated and is shown in Table 1. Because the results obtained were within the acceptable range of 100 ± 5%, the method was deemed to be accurate.

3.4. Selectivity, precision and system suitability

The precision of the method was estimated as a function of the RSD values of the determined amount of ABR (within the calibration range) using UV and ESI-MS detection methods. The RSD values of six repetitive injections did not exceed 1.30%. The RSD value of the characteristic two-potential DPs DEG-F and DEG-G were found to be 0.5 and 1.3%, respectively. The LC–UV chromatographic performance parameters, including the RSD of the retention time (tR, min), the selectivity coefficient (k'), the USP resolution (Rk), the peak asymmetry (As) and the USP theoretical plates (N) were within the acceptable range for ABR and the major degradation product eluted at 22.3 min (k'1: 17.5; k'2: 21.1; As: 1.0; and N: 45,390, n = 6).

The selectivity was evaluated from the UV and MS chromatograms. The peak corresponding to the standard ABR was UV-scanned at the apex, upslope, and downslope and normalized by estimating the matching percentage with the corresponding peak separated from the UV-degraded sample. The peak matching index was 100 ± 1%. Furthermore, the ESI-MS recorded [M+H]+ chromatograms of the same sample and the standard solution showed no peak overlapping. These data confirm the suitability and selectivity of the method.

3.5. Sample solution stability

Three UV-degraded samples (50 ng/µL) were kept under nitrogen gas at 4°C in brown autosampler vials until re-injection and monitoring at 235 nm. The samples were further diluted to obtain a suitable concentration for LC–ESI-MS monitoring (100 and 1000 pg/µL). All standard and degraded samples were analyzed three times within the valid claimed total concentration range. The RSD values of the found amounts were not more than 0.25% of the zero time samples. The precision was measured daily using a fresh daily standard ABR solution with a concentration of 50 ng/µL (RSD, did not exceed 0.3%). The stock and standard solutions of ABR were freshly prepared, stored at room temperature, wrapped with aluminum foil, and used within 12 h. After that time, some minor DPs were detected using ESI-MS.

3.6. Robustness

To evaluate the method robustness, one chromatographic parameter was changed while the other parameters remained unchanged. A standard solution (claimed, 50 ng/µL) of photo-decomposed ABR powder was injected three times after each change. The chromatographic performance parameters, including tR, k', R, As, and N, were calculated using UV detection and were compared with those of the system suitability. The method robustness was tested after changing the mobile phase composed of 30% acetonitrile and 70% (0.1 ± 0.01%) formic acid, the column temperature (25 ± 5°C), and the mobile phase flow rate (0.5 ± 0.01 mL/min). The LC–UV results revealed that the method was robust for these small changes in the mobile phase composition and the column temperature. However, the sensitivity to the ESI-MS detector was decreased when using a mobile phase containing less than 0.06% formic acid (v/v).

3.7. Quantitative monitoring with ESI-IT-MS detector

As stated by the ICH [9–11] guidelines, the amount of unknown degradation products can be calculated as if they are the principal drug. This statement is conventionally suitable for methods utilizing the UV detector. This assumption is based on the fact that DPs can retain some chromophore of the parent compound. In our case, mono- and multi-oxygenated ABR can possess comparable absorbivity values. However, using the ESI-MS detection method, completely different responses were observed due to differences in the ionization or protonation of the compounds to produce enough ions and the subsequent production of measurable IT-MS signals. The UV detection method was found to be unsuitable for the detection of trace amounts of related substances due to its very low sensitivity, as shown by the response factor (RF) of ABR. However, the ESI-MS (positive MRM mode) responses were approximately 200-fold more sensitive than those of the UV detection based on the limit of detection. For this reason, we conclude that a simple statistical procedure is required to determine the MS RF of ABR and each single m/z ion peak. The amount of ABR could be calculated from the UV or LC–MS response considering the linearity, range and other validation parameters as defined by the ICH guidelines [10,11]. Unknown degradation products can be calculated from the calibration curve of the principal drug. For this reason, we calculated the concentration of each unknown DP using a standard ABR solution measured using UV–UV (three samples exposed to UV light for 1, 2, and 6 h). These UV-degraded ABR samples were analyzed using HPLC–UV at 235 nm, and the concentration of each single-potential degradation product was calculated. Each peak detected by UV was simultaneously detected by ESI-MS to determine the retention time and corresponding ion mass (m/z). The same UV-degraded samples (using a 200-fold diluted solution) were again analyzed using LC–ESI-IT-MS in MRM positive mode. The ESI-MS responses were then calculated based on the concentration that was pre-defined from the LC–UV data. The RSD values of MS-RF did not exceed 1.0%. The ESI-MS response factors of all the detected degradation products are listed in Table 1 as a function of peak area per concentration (ng/µL). The ESI-MS detection method showed the advantage of the identification of eluted peaks based on the retention time and positive exact mass.

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Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>tR (min)</th>
<th>Max. m/z</th>
<th>MS RF (RSD)</th>
<th>% Error*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEG-A</td>
<td>5.2</td>
<td>380.1</td>
<td>18383(1.0)</td>
<td>0.8</td>
</tr>
<tr>
<td>DEG-B</td>
<td>5.7</td>
<td>382.1</td>
<td>37912(1.1)</td>
<td>1.2</td>
</tr>
<tr>
<td>DEG-C</td>
<td>6.7</td>
<td>366.1</td>
<td>91812(1.1)</td>
<td>1.2</td>
</tr>
<tr>
<td>DEG-D</td>
<td>7.6</td>
<td>366.1</td>
<td>46702(1.0)</td>
<td>1.6</td>
</tr>
<tr>
<td>DEG-E</td>
<td>8.1</td>
<td>366.1</td>
<td>46702(0.7)</td>
<td>0.9</td>
</tr>
<tr>
<td>DEG-F</td>
<td>22.3</td>
<td>377.290</td>
<td>376,005(1.1)</td>
<td>1.3</td>
</tr>
<tr>
<td>DEG-G</td>
<td>25.1</td>
<td>366.1</td>
<td>77,169(0.2)</td>
<td>0.4</td>
</tr>
<tr>
<td>ABR</td>
<td>43.7</td>
<td>350.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Equal to (spiked amount – found amount/spiked amount).
3.8. Forced degradation of ABR

Abiraterone that was subjected to heat in dry powder form or solution form showed a relatively low percentage (w/w) of the degradation product eluted at 5.2 min (DEG-A; 0.4%) and the highest percentage (w/w) of DEG-F (22.3 min; 0.8%). The acidic solution of ABR (0.1 M HCl) showed three types of degradation products, including a considerable amount of DEG-A (1.2%) and a low amount of DEG-F (0.1%). Table 2 shows the recovered amount of ABR and DPs under different stress conditions. The ABR powder sample exposed to UV light resulted in many degradation products (see Table 2). The percentage amounts (w/w) of the UV-released DP were increased as the exposure time was increased.

Exposure to UV light (irradiated for 2 h), oxidation (in 5% H₂O₂) and exposure to an acidic solution (15 min, 0.1 M HCl) were the most critical stress conditions applied. Photolytic and oxidative conditions resulted in multi-oxygenated ABR DPs and detectable amounts of DEG-F at 23.3 min. Exposure of ABR to UV light (for 2 h) or exposure to a 5% (v/v) H₂O₂ solution (for 15 min at room temperature) resulted in the same degradation products with approximately the same percentage range of 10–20% (w/w). Fig. 1 shows the overlaid chromatograms of the signals monitored using UV and ESI-MS detectors. The relative signal intensities of the peaks monitored by UV were not proportional to the corresponding signals monitored by MS. The UV response depends on the absorptivity of the compound, whereas the ESI-MS response depends on the capability of nebulized compounds to form protonated molecules. The potential DPs that eluted at 22.3 and 25.1 min (DEG-F and DEG-G) were detected by UV monitoring at a high concentration (5 ng/µL) and were sensitively detected by HPLC–ESI-MS monitoring (30 ng/µL). This method enables the detection of a low amount of these two particular compounds in the ABR bulk material stored at room temperature for approximately 6 months. The pure ABR material was analyzed using this method at a concentration of 50 ng/µL. The percentages of unchanged ABR were determined to be 99.7%, with 0.2% of DEG-F and 0.05% of DEG-G.

3.9. Identification of DPs

The fragmentation pathway of ABR is proposed in Figs. 2 and 3A. ABR is preferably cleaved to lose one methyl group to form a base peak at m/z 335 [M−CH₃+H]+, and the loss of another methyl group leads to an ion at m/z 320 [M−2CH₃+H]+ (50%). A loss of water forms an ion with m/z 302 [M−2CH₃−H₂O+H]+ (42%). The dotted lines in Fig. 3 were drawn to show the characteristic MS² fragments that contain no oxygen (Fig. 3A) or one oxygen atom within an epoxide (Fig. 3B) or N-hydroxyl group (Fig. 3C).

A characteristic MS² fragment of 366.2 (Fig. 3B) gave rise to peaks at m/z 120 and m/z 134, whereas the MS² spectrum of the m/z 368.2 ion (Fig. 3C) showed fragments at m/z 122 and 136. This difference in the [M+H]+ is due to the conversion of the epoxide to an oxirane group. The MS² spectrum of 335.3 (Fig. 3B) showed the fragment ion at m/z 302, which is consistent with the presence of a 3-carbon chain in the molecule.

![Fig. 1. Representative overlaid LC–UV spectra at 235 nm (100 µg/mL) and HPLC–ESI-IT-MS chromatograms at m/z 50–500 (5 µg/mL) of the UV-irradiated ABR powder. (A) Full chromatogram; (B) magnified UV and extracted ion chromatograms at 4–11 min.](image)

![Fig. 2. Proposed MS fragmentation of abiraterone (the relative abundances of the fragment ions are given in brackets).](image)
compound to the N-hydroxyl form. The MS² spectrum of the N–OH form showed the highest percentage abundance of the m/z 350 ion due to the preferable loss of one molecule of water.

The fragmentation pattern of the compound shown in Fig. 3D (m/z 366.2 at 3.5–3.6 min) and Fig. 3E (m/z 366.2 at 3.9–4.0 min) was similar to that of ABR (Fig. 3A). Both Fig. 3D and Fig. 3E show the m/z 348 ion that resulted from the loss of a water molecule and the m/z 334 ion (100%) that is due to the loss of methanol [365–CH₃OH+H]. The m/z 318 fragment corresponds to the radical ion [365–CH₃OH–CH₃]⁺, and the m/z 300 fragment corresponds to the radical ion [318–H₂O]⁺. These data confirm oxidation at one methyl group of ABR. We were unable to confirm on which methyl group the oxidation occurred. However, the m/z 128 and 195 fragments might also be due to oxidation at the peripheral methyl group. The MS² peak at 3.9 min (m/z 366.2) (Fig. 3E) could be an isomeric form of the compound shown in Fig. 3D because of the similarity of the MS² fragments; however, the percentage abundance of the fragment at m/z 348 is equal to 77%. Additionally, the compound shown in Fig. 3F (m/z 364.2) that eluted at 4.6–4.7 min showed m/z 349 (100%) as a major fragment corresponding to [M–CH₃+H]⁺, m/z 334 (29%) due to [M–2CH₃+H]⁺, and m/z 316 (8.8%) due to [M–2CH₃–H₂O+H]⁺.

Multi-oxygenated ABR DPs, including m/z 382 [ABR+2 oxygen+H]⁺, 398 [ABR+3 oxygen+H]⁺, and 414 [ABR+4 oxygen+H]⁺, were detected by IT-MS. The MS² profile of these DPs was characterized by molecular ion fragments of [M of DPs–CH₃ (one or two)+H]⁺. The chemical structure of these multi-oxygenated products could not be proposed. Minor DPs, including m/z 368 and 364, were not determined because they were released only upon severe stress testing.

4. Conclusion

A validated, sensitive and selective stability-indicating LC–UV–ESI-IT-MS analytical method was developed for the analysis of ABR. ABR was found to be sensitive to UV light, oxidation and acidic media. Multi-oxygenated products were detected. Five potential mono-oxygenated ABR products were detected and analyzed in the picogram range, and their chemical structures were proposed. The ESI-MS response factor of each potential degradation product was calculated from the information obtained during the LC–UV analysis procedure. The determination of the degradation products using LC–MS alone is not in compliance with the ICH guidelines concerning the stability study of active pharmaceutical ingredients because of the wide differences in the response factors of each unknown peak from the parent drug. The simultaneous detection with UV and MS detectors leads to three major pieces of information, including quantitative results,
high sensitivity, and information about the chemical structures of the degradation compounds. The proposed procedure is suitable for the quantification of trace amounts of potential degradation products due to the photolytic effect.

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