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Simultaneous quantitation of abiraterone, enzalutamide, N-desmethyl enzalutamide, and bicalutamide in human plasma by LC-MS/MS

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Abstract

Inhibiting the androgen receptor (AR) pathway is an important clinical strategy in metastatic prostate cancer. Novel agents including abiraterone acetate and enzalutamide have been shown to prolong life in men with metastatic, castration-resistant prostate cancer (mCRPC). To evaluate the pharmacokinetics of AR-targeted agents, we developed and validated an LC-MS/MS assay for the quantitation of enzalutamide, N-desmethyl enzalutamide, abiraterone and bicalutamide in 0.05 mL human plasma. After protein precipitation, chromatographic separation was achieved with a Phenomenex Synergi Polar-RP column and a linear gradient of 0.1% formic acid in methanol and water. Detection with an ABI 4000Q mass spectrometer utilized electrospray ionization in positive multiple reaction monitoring mode. The assay was linear over the ranges of 1–1,000 ng/mL for abiraterone and bicalutamide and 100–30,000 ng/mL for N-desmethyl enzalutamide and enzalutamide and proved to be accurate (92.8–107.7%) and precise (largest was 15.3% CV at LLOQ for bicalutamide), and fulfilled FDA criteria for bioanalytical method validation. We demonstrated the suitability of this assay in plasma from patients who were administered enzalutamide 160 mg, abiraterone 1000 mg and bicalutamide 50 mg once a day as monotherapy or in combination. The LC-MS/MS assay that has been developed will be an essential tool that

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further defines the pharmacology of the combinations of androgen synthesis or AR-receptor targeted agents.

Graphical abstract

Keywords

bicalutamide; enzalutamide; abiraterone; tandem mass spectrometry; metabolites; assay; validation

1 Introduction

Prostate cancer is the second most common cancer in men, with an estimated 220,800 cases and 27,540 deaths during 2015 in the US [1]. Androgens are critical to prostate cancer growth, which is the underlying scientific rationale for the development of androgen receptor (AR)-targeted therapy in prostate cancer [2]. Androgen deprivation therapy (ADT) via GnRH agonist or antagonist suppression of gonadal androgens or orchiectomy is generally the initial treatment for men with metastatic prostate cancer [3]. Although the initial response rate to ADT is high, the duration of response is highly variable, and most

prostate cancer patients eventually experience disease progression despite treatment. Patients who have progressed while on ADT are said to have castration resistant disease (CRPC), during which time the androgen receptor continues to signal, and many patients may remain responsive to additional therapies directed against androgen receptor signaling [4].

Bicalutamide, enzalutamide and abiraterone acetate are the most frequently prescribed drugs targeting the androgen receptor (AR) pathway [5]. Bicalutamide binds to AR and competitively inhibits the interaction with testosterone and dihydrotestosterone and is often used as an initial anti-androgen in metastatic prostate cancer [6]. Enzalutamide, a second generation anti-androgen, is not only a competitive antagonist of androgen binding to AR, but also inhibits the nuclear translocation of AR and its interaction with DNA [4], additionally, N-desmethylenzalutamide has similar *in vitro* activity. Abiraterone, the active product of abiraterone acetate, is an inhibitor of cytochrome P450 C17, a critical enzyme in extragonadal and testicular androgen synthesis. Enzalutamide and abiraterone acetate have emerged to be important agents in patients who have failed initial ADT with bicalutamide [7, 8].

The combined use of enzalutamide and abiraterone acetate has shown promising results that may indicate more efficacious androgen signaling inhibition in men with castration resistant disease [9]. Combining anti androgen agents may result in additional suppression of AR signaling. However, combinations of these drugs could potentially have detrimental side effects. A phase 1 trial of the combination of enzalutamide and abiraterone acetate has suggested that pharmacokinetic interaction is not significant [10]. In addition, there is an ongoing phase 3 trial in which sparse pharmacokinetic sampling is being performed on both drugs (ClinicalTrials.gov Identifier: NCT01949337).

With the oral ADT drugs, it would probably be beneficial to minimize pharmacokinetic variability due to drug oral bioavailability, drug-drug interaction and noncompliance [11]. Therefore, a sensitive and selective method can be used to answer clinically relevant questions. Various analytical methods have been reported in the literature to detect each drug separately. However, no method has been reported to simultaneously determine bicalutamide, enzalutamide and abiraterone in plasma [12-14]. The aim of this study was to develop and validate a new method to simultaneously determine bicalutamide, enzalutamide, N-desmethylenzalutamide, and abiraterone in human plasma using LC-MS/MS, providing an assay that allows one-run analysis of the agents commonly used in prostate cancer anti-androgen therapy. This method is currently supporting the ongoing phase 3 trial of abiraterone combined with enzalutamide (ClinicalTrials.gov Identifier: NCT01949337).

2 Experimental

2.1 Chemicals and reagents

Abiraterone, bicalutamide, N-desmethyl enzalutamide, enzalutamide, [D₄]-abiraterone, [D₄]-bicalutamide, were purchased from Toronto Research Chemicals (Toronto, Canada). [D₆]-enzalutamide and [D₆]-N-desmethyl enzalutamide were purchased from Alsachim (Strasbourg, France). Chemical structures are shown in. Methanol and water (all HPLC grade) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Formic acid was

purchased from Sigma-Aldrich (St. Louis, MO, USA). Human EDTA K2 plasma was obtained from Valley Biomedical (Winchester, Virginia, USA). Control human plasma was obtained from Sera Care Life Sciences (Gaithersburg, MD). Nitrogen for evaporation of samples was purchased from Valley National Gases, Inc. (Pittsburgh, PA, USA). Nitrogen for mass spectrometrical applications was purified with a Parker Balston Nitrogen Generator (Parker Balston, Haverhill, MA, USA).

2.2 Chromatography

The LC system consisted of an Agilent (Palo Alto, CA, USA) 1200 SL autosampler and binary pump, a Phenomenex (Torrence, CA USA) Synergi Polar-RP (4 μ m, 100 $\times$ 2 mm) column, and a gradient mobile phase pumped at 0.3 mL/min. Mobile phase solvent A was 0.1% (v/v) formic acid in methanol, and mobile phase solvent B was 0.1 % (v/v) formic acid in water. The initial mobile phase composition of 65% solvent A increases linearly to 70% from 0 min to 4 min. Solvent A is then increased from 70% to 95% from 4 min to 6 min. Solvent A is held at 95% from 6 min to 8 min and at 8.1 min solvent A is decreased to 65%. These conditions were held until 15 min, followed by the next injection.

2.3 Mass spectrometry

Mass spectrometric detection was carried out using a ABI SCIEX (San Jose, CA, USA) 4000Q hybrid linear ion trap tandem mass spectrometer with electrospray ionization in positive multiple reaction monitoring (MRM) mode. The parameters were as follows: curtain gas 40, IS voltage 5000 V, probe temperature 500°C, GS1 40, GS2 40, declustering potential 60 V, a collision energy of 20 V, and an exit potential of 15 V. The declustering potential for bicalutamide and its internal standard was 60 V. The declustering potential for abiraterone, N-desmethyl enzalutamide, enzalutamide and their respective internal standards was 50 V. The collision energy for bicalutamide and its internal standard was 30 V. The collision energy for N-desmethyl enzalutamide, enzalutamide and their internal standards was 40 V and the collision energy for abiraterone and its internal standard was 60 V. MRM *m/z* transitions monitored were: 350.0>156.0 for abiraterone; 354.0>160.0 for [D₄]-abiraterone; 431.0>217.0 for bicalutamide; 435.0>221.0 for [D₄]-bicalutamide; 453.0>197.0 for N-desmethyl enzalutamide; 457.0>201.0 for [D₆]-N-desmethyl enzalutamide; 467.0>211.0 for enzalutamide; and 471.0>215.0 for [D₆]-enzalutamide. The LC system and mass spectrometer were controlled by Analyst software (version 1.5.2), and data were collected with the same software.

2.4 Preparation of calibration standards and quality control samples

Stock solutions of enzalutamide and N-desmethyl enzalutamide were prepared independently at 5 mg/mL in DMSO. Abiraterone and bicalutamide were prepared independently at 1 mg/mL in DMSO. A working stock mixture was prepared in methanol that had a final concentration of 1 mg/mL of enzalutamide and N-desmethyl enzalutamide and 0.1 mg/mL of abiraterone and bicalutamide. Each internal standard was prepared separately at a final concentration of 1 mg/mL in the same solvent as its respective analyte. An internal standard mixture was prepared in methanol that had a final concentration of 10 μ g/mL [D₆]-enzalutamide, 10 μ g/mL [D₆]-N-desmethyl enzalutamide, 10 μ g/mL [D₄]-abiraterone, and 5 μ g/mL [D₄]-bicalutamide. This solution was diluted 100-fold with

methanol to obtain the internal standard working solution. All analyte stocks were stored at -80 °C until use. On the day of assay, the working stock mixture was serially diluted (in steps of 10-fold, to generate 3 additional levels) in methanol to obtain the lower calibration working solutions. These calibration working solutions were diluted in human plasma to produce the following analyte concentrations for enzalutamide and N-desmethyl enzalutamide: 100, 300, 1000, 3,000, 10,000 and 30,000 ng/mL and for abiraterone and bicalutamide: 1, 3, 10, 30, 100, 300 and 1,000 ng/mL. LLOQ For each calibration series, zero and blank samples were also prepared from 50 µL of control plasma.

Quality control (QC) stock solutions were diluted in human plasma to produce the following QC samples: Lower Limit of Quantitation (LLOQ) 1 ng/mL, Low QC (QCL) 2 ng/mL; Middle QC (QCM) 50 ng/mL, and high QC (QCH) 800 ng/mL for abiraterone and bicalutamide and: LLOQ 100 ng/mL, QCL 200 ng/mL; QCM 8000 ng/mL, and QCH 24,000 ng/mL for enzalutamide and N-desmethyl enzalutamide.

2.5 Sample preparation

Ten µL of internal standard working solution was added to each microcentrifuge tube followed by 50 µL standard, QC, or sample plasma, while kept on ice. Next, 150 µL of methanol was added to each tube and the samples were vortexed for 1 min on a Vortex Genie-2 set at 10 (Model G-560 Scientific Industries, Bohemia, NY, USA) and then centrifuged at 13,000 × g for 10 min. The resulting supernatants were transferred to autosampler vials, followed by injection of 10 µL into the LC-MS/MS system.

2.6 Validation procedures

2.6.1 Calibration curve and lower limit of quantitation (LLOQ)—Decreasing concentrations of analytes were injected into the analytical system to determine the minimal concentration with a signal-to-noise ratio of at least 5:1. Calibration standards and blanks were prepared (see paragraph 2.4) and analyzed in triplicate to establish the calibration range with acceptable accuracy and precision. The analyte-to-internal standard ratio (response) was calculated for each sample by dividing the area of the analyte peak by the area of the internal standard peak. Standard curves were constructed individually by plotting the analyte-to-internal standard ratio *versus* the known concentrations in each sample. Standard curves were fitted by linear regression with weighting by $1/y^2$, followed by back-calculation of concentrations. The deviations of these back-calculated concentrations from the nominal concentrations were expressed as percentage of the nominal concentration.

2.6.2 Accuracy and precision—The accuracy and precision of the assay were determined by analyzing samples at the LLOQ, QCL, QCM, and QCH concentrations in 6 replicates each in 3 analytical runs, together with independently prepared, triplicate calibration curves. Accuracy was calculated at each test concentration as: (mean measured concentration / nominal concentration) × 100%.

Assay precision was calculated by ANOVA as previously described [15], by using SPSS 23 for Windows (SPSS Inc., Chicago, IL, USA). Back-calculated concentrations of calibration and QC samples were entered with the run number as factor. From the resulting mean

squares of the within runs and mean squares of the between runs, the intra-assay and inter-assay precisions were calculated.

2.6.3 Selectivity and specificity—To investigate whether endogenous matrix constituents interfered with the assay, six individual batches of control, drug-free human plasma were processed and analyzed according to the described procedure. Responses of analytes at the LLOQ concentrations were compared with the response of the blank samples. Cross-talk of abiraterone, bicalutamide, N-desmethyl enzalutamide, and enzalutamide in their mutual MRM channels was characterized.

2.6.4 Extraction recovery and matrix effect—We determined the extraction recoveries of all analytes from plasma by comparing the absolute response of an extract of control plasma to which these analytes had been added after protein precipitation, with the absolute response of an extract of plasma to which the same amounts had been added before protein precipitation. The matrix effect by plasma matrix components was defined as the change of the absolute response of an extract of control plasma to which analyte had been added after the protein precipitation relative to the absolute response of reconstitution solvent to which the same amount of each respective analytes had been added. Experiments were performed at two QC concentrations, in triplicate.

2.6.5 Stability—Long-term stability experiments were performed in plasma and in stock solution after storage at -80 °C. Stability of the stock solution was expressed as the percentage recovery of the stored solution (3 months) relative to a fresh solution. The stabilities of all compounds in plasma at -80 °C were determined by assaying samples before and after 4 months of storage. In addition, the stabilities of the compounds in stock solution at room temperature for 4 h were determined in triplicate. All stability testing in plasma was performed in triplicate at the LLOQ, QCL, QCM and QCH concentrations. The effect of 3 freeze/thaw cycles on analyte concentrations in plasma was evaluated by assaying samples after they had been frozen (-80 °C) and thawed on 3 separate days and comparing the results with those of freshly prepared samples. The stabilities of compounds in plasma during sample preparation were evaluated by assaying samples before and after 4 h at room temperature. To evaluate the stabilities in reconstituted samples in the autosampler, we re-injected QC samples and calibration curves approximately 72 h after the first injection and compared the concentrations derived from the second injection with those derived from the first injection. Stability during human whole blood sample collection and processing was evaluated by assessing 4 h bench stability at room temperature.

2.6.6 Dilutional integrity—To demonstrate dilutional integrity, the ability to dilute samples from above the upper limit of quantitation to within the validated concentration range, plasma samples containing all four compounds above the upper limit of quantitation were diluted to within the assay range. Plasma samples (N=3) with analyte concentrations of 50,000 ng/mL were diluted 10-fold and 100-fold (5,000 and 500 ng/mL) with control plasma and assayed.

2.7 Application of the assay

To document the potential applicability of the assay, we quantitated abiraterone, enzalutamide and N-desmethyl enzalutamide in a sample from a patient enrolled on the ongoing phase III trial of abiraterone and enzalutamide (NCT01949337).

3 Results and Discussion

3.1 Validation of the assay

3.1.1 Chromatography—The approximate retention times of each compound were as follows: abiraterone and [D₄]-abiraterone at 8.0 min, bicalutamide and [D₄]-bicalutamide at 4.5 min, N-desmethyl enzalutamide and [D₆]-N-desmethyl enzalutamide at 5.0 min, and enzalutamide and [D₆]-enzalutamide at 6.0 min.

The analytes at the retention time extremes of approximately 4.5 and 8.0 min had corresponding retention factors of 4.6 and 9.0, respectively, with a void time of 0.8 min. Representative chromatograms of each compound (at the LLOQ), and internal standards in plasma are displayed in Fig. 1.

3.1.2 Calibration curve and LLOQ—According to the FDA guidelines for bioanalytical method validation [16], the calibration curve adequately describes the concentration *versus* response relationship if the observed deviation and precision are 20% for the LLOQ and 15% for all other calibration concentrations. At least 4 of 6 calibration points should meet the above criteria [16].

The selected assay range of 1-1,000 ng/mL (abiraterone and bicalutamide) and 100-30,000 ng/mL (N-desmethyl enzalutamide and enzalutamide) fulfilled the FDA criteria for the LLOQ concentration and the calibration curve. Accuracies and precisions at the different concentrations were determined from triplicate calibration curves on 3 separate days and are reported in. At most concentrations, the mean square of the within runs was greater than the mean square of the between runs, indicating that there was no significant additional variability due to the performance of the assay in different runs [15]. Representative calibration curves and corresponding correlation and regression coefficients are shown in.

3.1.3 Accuracy and precision—FDA guidelines specify that the accuracies for all tested concentrations should be within $\pm 15\%$, and the precisions should not be $>15\%$ CV except for the LLOQ, in which case these parameters should not exceed 20% [16].

The accuracies and intra- and inter-assay precisions for the tested concentrations (LLOQ, QCL, QCM, QCH) were all within the defined acceptance criteria (Table 1).

3.1.4 Selectivity and specificity—According to FDA guidelines, the signal at the LLOQ must be at least 5 times the signal of any co-eluting peaks [16].

Chromatograms of six individual control plasma samples contained no co-eluting peaks $>20\%$ of the analyte areas at the LLOQ concentration (interference $<4.8\%$ for abiraterone,

<2.8% for bicalutamide, <7.0% for N-desmethyl enzalutamide, and <6.2 for enzalutamide) (Fig. 1).

3.1.5 Extraction recovery and matrix effect—As outlined in the FDA-guidelines, there is a requirement that recovery be consistent and precise [16]. A recovery of 70% with a variation of 15% is generally accepted [15, 16]. There is no specific requirement for matrix effect. Ultimately, the assay performance, as expressed in the precision and accuracy, is most relevant; however, a large and/or variable matrix effect may result in lack of assay robustness.

The recoveries of analytes ranged from 77.2 to 115.2 %, with CVs between 1.1 and 11.3%. Matrix effect ranged from -35.2 (i.e. ionization suppression) to 0.6% (i.e. ionization enhancement), with CVs between 4.4 and 13.4% (Table 2).

3.1.6 Stability—Stability in biological samples is acceptable when 85% of the analyte is recovered. The stabilities of abiraterone, bicalutamide, N-desmethyl enzalutamide and enzalutamide stock solutions at room temperature for 4 h were 94.9%-96.7% (Table 3). Stabilities in stock solutions for 3 months at -80 °C were 99.4-100.3%. The stabilities of the analytes after 3 freeze thaw cycles (-80°C to RT) were between 92.8 and 111.2%. Long-term stabilities of the analytes in plasma at -80 °C were adequate with recoveries between 91.6 and 107.5%. The absolute responses of plasma extracts of abiraterone at the QCL and QCH concentrations, when reconstituted and kept in the autosampler for 72 h, were 162.9% to 168.1% of the initial responses (CV 21.3-38.3%), while the response of abiraterone relative to the internal standard signal ranged from 112.0% to 112.8% (CV 3.2-7.8%). The absolute responses of plasma extracts of bicalutamide at the QCL and QCH concentrations, when reconstituted and kept in the autosampler for 72 h, were 116.2% to 141.6% of the initial responses (CV 40.7-41.6%), while the response of bicalutamide relative to the internal standard signal ranged from 100.5% to 101.4% (CV 7.2-7.9%). The absolute responses of plasma extracts of N-desmethyl enzalutamide at the QCL and QCH concentrations, when reconstituted and kept in the autosampler for 72 h, were 95.0% to 114.3% of the initial responses (CV 3.6-37.9%), while the response of N-desmethyl enzalutamide relative to the internal standard signal ranged from 97.1% to 100.4% (CV 2.6-6.6%). The absolute responses of plasma extracts of enzalutamide at the QCL and QCH concentrations, when reconstituted and kept in the autosampler for 72 h, were 124.0% to 139.6% of the initial responses (CV 1.6-36.5%), while the response of enzalutamide relative to the internal standard signal ranged from 97.5% to 109.0% (CV 2.4-3.5%). Importantly, the reinjection run passed the requirements of any run set by the FDA [16]. The whole blood stabilities of abiraterone, bicalutamide, N-desmethyl enzalutamide and enzalutamide at room temperature for 4 h were all acceptable (<15% difference).

3.1.7 Dilutional integrity—The samples diluted from 50 µg/mL to 5,000 ng/mL displayed the following accuracies for: N-desmethyl enzalutamide: 102.7%, with a CV of 2.9%; and enzalutamide: 101.2%, with a CV of 5.8%. The samples diluted from 50 µg/mL to 500 ng/mL displayed the following accuracies for: abiraterone: 104.0%, with a CV of 8.7%; and bicalutamide: 85.0%, with a CV of 6.3%.

3.2 Development

The method development of the assay included multiple variations in column type, extraction solvents, and HPLC gradients, and a strategy to quantitate analytes with vastly different concentration ranges without saturating the mass spectrometric detector signal.

3.2.1 Mass Spectrometry—Each analyte was scanned in both negative and positive ionization modes until the most sensitive mode was identified for parent drug and the respective metabolites. We determined that abiraterone, N-desmethyl enzalutamide, and enzalutamide were optimally sensitive in the positive ionization mode, while bicalutamide was most sensitive in negative ionization mode. We initially employed a MRM method that switched polarity between positive and negative mode, however, this led to high background signal for all analytes. The method was then optimized for detection of bicalutamide in positive mode and this proved to be sensitive enough to achieve a clinically useful LLOQ of 1 ng/mL for bicalutamide.

The method was optimized so that the expected analyte concentration from patient samples being analysed would fall within the standard curves for each analyte without the need for separate dilution and analysis of the plasma sample. The ranges were 1-1,000 ng/mL for abiraterone and bicalutamide and 100-30,000 ng/mL for N-desmethyl enzalutamide and enzalutamide. A major problem during this phase of development was the efficient ionization of enzalutamide and N-desmethyl enzalutamide when the $[M+H]^+$ ion was used as the parent mass, resulting in mass spectrometric detector saturation. To overcome this, we modified the MRM channels (+2) to monitor the m/z values of the respective $^{13}C_2$ -isotopologues for both the precursor and product ions of N-desmethyl enzalutamide and enzalutamide, avoiding signal saturation. Monitoring the $^{13}C_2$ -isotopologue amounts to a signal dilution by approximately a factor of 12. This approach has been described previously [17, 18].

3.2.2 Extraction and sample preparation—Because of the need to include four analytes with diverse physico-chemical properties in our assay, both protein precipitation (methanol and acetonitrile separately) and liquid-liquid (methyl *tert*-butyl ether) extraction methods were tested to extract the analytes from plasma. Methanol protein precipitation (1:3, v/v) was chosen as a suitably non-specific sample preparation. The methyl *tert*-butyl ether extracted plasma showed high sensitivity and saturated the mass spectrometer signal in higher concentrations. However, the “dilute and shoot” method with methanol avoided saturation while sensitivity of the assay was not compromised and was proven to be sufficient for the intended application (see below). A plasma sample volume of 50 μ L was applied to keep sample and solvent use low, resulting in a practical sample preparation, executable in microtubes.

3.2.3 Chromatography—We evaluated the following four columns: Acquity BEH C18 UPLC (50 $\times$ 2.1 mm, 1.7 μ m), Synergi Polar RP 80A (100 $\times$ 2.0 mm, 4 μ m), Phenomenex Synergi Hydro-RP 80A (100 $\times$ 2.0 mm, 5 μ m), and Develosil (150 $\times$ 2 mm). Acquity BEH and Develosil columns inadequately separated the analytes. The Phenomenex Synergi Hydro-RP adequately separated the compounds; however, the run time exceeded 10 minutes.

The Synergi Polar RP displayed the best combination of retention, peak shape and separation of analytes and was therefore selected.

3.3 Application of the assay

We applied the assay to a sample from a patient enrolled on the ongoing phase III trial of abiraterone acetate combined with enzalutamide. This patient had signed informed consent on an institutional review board approved protocol. Similarly, we obtained a sample from a patient being treated with bicalutamide consented on an institutional review board approved protocol. As seen in Fig. 2, the assay was capable of quantitating abiraterone, enzalutamide and N-desmethyl enzalutamide in these patients with exposures in line with previous reports for abiraterone (25.3 ng/mL), enzalutamide (18.6 µg/mL), N-desmethyl enzalutamide (17.5 µg/mL), and bicalutamide (11.3 µg/mL) [19-21]. While analyzing these patient samples, we observed additional peaks in the abiraterone MRM channel (3.5 min and 8.9 min) and the N-desmethyl enzalutamide channel, see Fig. 2. Exploring more shallow gradients revealed an additional very early peak in the abiraterone channel. Given the baseline separation of all these peaks from the analytes of interest, the quantitation of our analytes of interest was not compromised. Precursor scans were not able to postulate the identity of the peak in the N-desmethyl enzalutamide channel, but did suggest a glucuronide (very early peak observed with shallow gradient) and a sulfate metabolite (retention of 3.5 min in the validated assay) of abiraterone. Anyone quantitating abiraterone and enzalutamide should be aware of these drug-derived peaks with the potential to cross-talk, and that these peaks need to be chromatographically separated to ensure generation of unbiased data.

Because conjugate metabolites may hydrolyse during sample preparation to produce parent compound, thereby resulting in an overestimate of the analyte, we assessed the 4 h bench stability of actual patient plasma samples in triplicate in 5 individual patients. Any change in enzalutamide and N-desmethyl enzalutamide concentrations in patient samples was within 10%. Counter to our expectation, abiraterone concentrations appeared to decrease on average 38% with a range of 10-52% between individual patients (CV% of triplicates were within 6.5%). Concomitantly incubated QC plasma samples showed 9.1% loss of spiked abiraterone, confirming our previous result of acceptable (<15%) difference (see Table 3). This suggested that abiraterone in patient samples was somehow more susceptible to degradation than abiraterone in spiked QC samples. We excluded the possibility of a labile metabolite cross-talking into the abiraterone MRM channel, which would be peculiar to patient samples. This was done by slowing the gradient (no secondary peaks appeared in patient samples), and by injecting the following reference compounds to exclude cross-talk at the abiraterone retention time: abiraterone acetate, abiraterone acetate N-oxide, abiraterone N-oxide (data not shown).

Although we could not further elucidate the increased rate of degradation of abiraterone in patient samples relative to spiked QC samples (see Fig. 3 for illustration), it was clear that this phenomenon had the potential to bias our assay results if not properly controlled. Using patient samples, we established that during the maximum 30 min time window in which patient plasma samples would be unprocessed on the bench at room temperature, stability was acceptable, whereas at 60 min some samples started to display loss of abiraterone in

excess of 15%. Furthermore, keeping patient plasma samples on ice for 60 min resulted in virtually no change in abiraterone concentrations. These results provided us with confidence that clinical samples analysed thus far (processed within 30 min) had yielded reliable results, yet to improve the robustness of our assay, we proceeded to perform further sample processing on ice. Autosampler stability was also repeated with patient samples and confirmed 72 h stability (data not shown).

3.4 Incurred sample reanalysis and external validation

Upon completion of the validation, incurred sample reanalysis for abiraterone, enzalutamide and N-desmethyl enzalutamide was performed on samples from the ongoing clinical trial being supported with this assay (NCT01949337). Reanalysis of 50 samples yielded the following average difference: abiraterone 3.0%, enzalutamide 0.8%, and N-desmethyl enzalutamide 1.4%, with no sample exceeding 20% difference.

Duplicate sets of QCs were exchanged with the Department of Clinical Pharmacy, Radboud University Nijmegen Medical Centre, where separate quantitative abiraterone and enzalutamide assays are in use. For abiraterone, 6 out of 6 QCs analysed were within 7.5% difference, for enzalutamide, 5 of 6 QCs analysed were within 20% difference, and for N-desmethyl enzalutamide, 4 of 5 QCs analysed were within 3.4% difference, while the 6th QC experienced an assay failure (no peaks).

4 Conclusion

The objective of this study was to develop and validate an analytical method for the simultaneous quantitation of abiraterone, bicalutamide, enzalutamide and its metabolite N-desmethyl enzalutamide, in human plasma. We accomplished this using reversed phase chromatography for separation with triple quadrupole mass spectrometric MRM detection.

Previously reported assays, which have quantitated abiraterone, bicalutamide, or enzalutamide separately, were validated over the ranges of 1-2,000 ng/mL with a run time of 5-10 min. However, combining AR targeted agents is becoming more important in the clinical practice of treating prostate cancer. We plan to apply the assay to samples obtained from a phase 3 trial comparing enzalutamide in combination with or without abiraterone in patients with metastatic castration-resistant prostate cancer (NCT01949337). The assay was capable of simultaneously quantitating abiraterone, bicalutamide, enzalutamide and its active metabolite in samples and the LLOQs were 1 ng/mL, 1 ng/mL, 100 ng/mL and 100 ng/mL, respectively. Patient samples suggested metabolic products capable of cross-talking into analyte channels. Our assay was not affected by these compounds, and future assays developed for these analytes will need to take these observations in consideration during the assay development phase. This work also shows the care which should be taken in utilizing patient derived samples to confirm critical stability results for the analytes of interest obtained with spiked QC samples. Furthermore, though abiraterone responses did not increase upon incubation of our patient samples, the potential for conjugated metabolites to be hydrolyzed and yield parent abiraterone under conditions other than those used in our current assay should be considered in future quantitative assays. To our knowledge, this is the first assay published to date that can quantitate these androgen deprivation agents

simultaneously, and that is validated according to the FDA guidance for Bioanalytical Method Validation [16]. The analytical method presented herein will be a valuable tool in quantitating androgen deprivation agents in plasma during further clinical development of these agents when used as monotherapy or in combinations, as well as to document exposure variability in clinical practice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Bicalutamide, enzalutamide and abiraterone target the androgen receptor pathway
- A phase 3 trial of enzalutamide and abiraterone in combination is ongoing
- An LC-MS/MS assay quantitating all compounds in 0.05 mL plasma was developed and validated
- Isotopologue monitoring allowed enzalutamide signal dilution and direct sample analysis

Fig. 1.

Representative chromatograms of: A) abiraterone (m/z 350.0>156.0; 8.0 min) added to control plasma at the LLOQ concentration of 1 ng/mL (top trace with an offset of 300 counts) and control human plasma (bottom trace); B) bicalutamide (m/z 431.0>217.0; 4.1 min) added to control plasma at the LLOQ concentration of 1 ng/mL (top trace with an offset of 300 counts) and control human plasma (bottom trace); C) enzalutamide (m/z 467.0>211.0; 5.7 min) added to control plasma at the LLOQ concentration of 100 ng/mL (top trace with an offset of 500 counts) and control human plasma (bottom trace); D) N-desmethyl enzalutamide (m/z 453.0>197.0; 4.8 min) added to control plasma at the LLOQ concentration of 100 ng/mL (top trace with an offset of 300 counts) and control human plasma (bottom trace); E) [D₄]-abiraterone internal standard (m/z 354.0>160.0; 8.0 min) added to control plasma at a concentration of 20 ng/mL (top trace with an offset of 300

counts) and control human plasma (bottom trace); F) [D₄]-bicalutamide internal standard (m/z 435.0>221.0; 4.1 min) added to control plasma at a concentration of 10 ng/mL (top trace with an offset of 300 counts) and control human plasma (bottom trace); G) [D₆]-enzalutamide (m/z 471.0>215.0; 5.7 min) added to control plasma at a concentration of 20 ng/mL (top trace with an offset of 500 counts) and control human plasma (bottom trace); H) [D₆]-N-desmethyl enzalutamide (m/z 457.0>201.0; 4.8 min) added to control plasma at a concentration of 20 ng/mL (top trace with an offset of 300 counts) and control human plasma (bottom trace).

Fig. 2.

Treated patient plasma chromatograms of abiraterone (A), bicalutamide (B), enzalutamide (C), and N-desmethyl enzalutamide (D) with baseline separated analytes cross-talking into the respective MRM channels.

Fig. 3.
Stability of abiraterone over time in room temperature in spiked QC plasma samples (Δ) and in patients plasma samples after being dosed with abiraterone (○).

Table 1
Assay performance data for the quantitation of LLOQ, QCL, QCM and QCH for all analytes

Analyte	Concentration (ng/mL)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
Abiraterone	1 (LLOQ)	97.0	9.4	6.6
	2 (QCL)	101.5	6.3	5.3
	50 (QCM)	99.6	12.1	8.5
	800 (QCH)	103.9	6.4	3.2
Bicalutamide	1 (LLOQ)	104.0	15.3	11.5
	2 (QCL)	101.0	9.3	8.0
	50 (QCM)	104.4	14.2	9.6
	800 (QCH)	95.3	9.0	6.6
Enzalutamide	100 (LLOQ)	94.8	11.4	7.7
	200 (QCL)	94.1	7.2	5.2
	8000 (QCM)	104.1	4.4	3.1
	24000(QCH)	106.7	4.9	3.7
N-desmethyl enzalutamide	100 (LLOQ)	97.3	10.7	7.7
	200 (QCL)	92.3	6.9	5.4
	8000 (QCM)	103.5	5.8	3.5
	24000(QCH)	107.6	4.0	3.1

N=18; 6-fold results, each in 3 separate runs, for each concentration.

Table 2
Recoveries of analytes from human plasma and their respective matrix effects in human plasma, with coefficients of variation (CV)

Analyte	Concentration (ng/mL)	Recovery (%)	CV (%)	Matrix effect (%)	CV (%)
Abitraterone	2 (QCL)	115.2	2.5	-35.2	5.7
	50 (QCM)	83.0	2.8	-30.5	13.3
	800 (QCH)	87.6	1.1	-29.3	9.1
Bicalutamide	2 (QCL)	84.5	11.3	-11.9	13.4
	50 (QCL)	77.3	5.6	-14.8	7.8
	800 (QCM)	83.2	3.6	-11.7	7.1
Enzalutamide	200 (QCL)	77.2	9.6	-13.0	6.8
	8000 (QCL)	85.2	2.4	-9.2	6.4
	24000 (QCM)	105.7	6.2	0.6	7.0
N-desmethyl enzalutamide	200 (QCL)	91.9	9.7	-18.8	11.0
	8000 (QCL)	84.3	4.3	-11.3	7.0
	24000 (QCM)	106.7	4.2	-0.5	4.4

N=3, for each concentration.

Table 3
Stability of analytes under varying conditions

Storage condition	Concentration (ng/mL)	Stability (%)	CV (%)	Replicates
Abiraterone				
Stock solution 4 h				
Ambient temp.	1,000,000	96.3	1.9	3
Stock solution 3 months				
-80 °C	1,000,000	99.4	4.1	3
Plasma 4 h				
Ambient temp.	QCL	99.8	7.5	3
	QCH	102.3	3.3	3
Plasma 3 freeze-thaw cycles				
-80 °C	QCL	92.8	7.4	3
	QCH	99.6	5.4	3
Plasma 4 months				
-80 °C	QCL	103.2	5.7	3
	QCH	91.6	2.8	3
Bicalutamide				
Stock solution 4 h				
Ambient temp.	1,000,000	95.8	3.6	3
Stock solution 3 months				
-80 °C	1,000,000	99.4	7.5	3
Plasma 4 h				
Ambient temp.	QCL	114.7	12.2	3
	QCH	95.7	7.1	3
Plasma 3 freeze-thaw cycles				
-80 °C	QCL	111.2	16.4	3
	QCH	98.1	7.5	3
Plasma 4 months				
-80 °C	QCL	94.8	3.9	3

Storage condition	Concentration (ng/mL)	Stability (%)	CV (%)	Replicates
	QCH	104.6	3.7	3
Enzalutamide				
Stock solution 4 h				
Ambient temp.	1,000,000	96.7	3.0	3
Stock solution 3 months				
-80 °C	1,000,000	100.2	2.3	3
Plasma 4 h				
Ambient temp.	QCL	95.2	2.6	3
	QCH	103.3	4.2	3
Plasma 3 freeze-thaw cycles				
-80 °C	QCL	96.3	2.6	3
	QCH	101.2	3.6	3
Plasma 4 months				
-80 °C	QCL	107.5	8.7	3
	QCH	93.3	2.9	3
N-desmethyl enzalutamide				
Stock solution 4 h				
Ambient temp.	1,000,000	94.9	4.8	3
Stock solution 3 months				
-80 °C	1,000,000	100.3	1.6	3
Plasma 4 h				
Ambient temp.	QCL	105.2	7.3	3
	QCH	102.6	6.3	3
Plasma 3 freeze-thaw cycles				
-80 °C	QCL	100.2	5.5	3
	QCH	98.7	5.1	3
Plasma 4 months				
-80 °C	QCL	100.7	6.5	3
	QCH	96.3	3.5	3