# Development and Validation of an LC-MS/MS Method for the Simultaneous Quantification of Abiraterone, Enzalutamide, and Their Major Metabolites in Human Plasma

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**Background:** Abiraterone acetate and enzalutamide are 2 novel drugs for the treatment of metastatic castration-resistant prostate cancer. The metabolism of these drugs is extensive. Major metabolites are *N*-desmethyl enzalutamide, enzalutamide carboxylic acid, abiraterone N-oxide sulfate, and abiraterone sulfate; of which *N*-desmethyl enzalutamide is reported to possess antiandrogen capacities. A liquid chromatography-tandem mass spectrometry method for simultaneous quantification of abiraterone, enzalutamide, and the main metabolites has been developed and validated to support therapeutic drug monitoring.

**Methods:** Human plasma samples of patients treated with abiraterone or enzalutamide were harvested at the clinic and stored at −20°C. Proteins were precipitated by acetonitrile, and the final extract was injected on a Kinetex C18 column and separated with gradient elution. Analytes were detected by liquid chromatography-mass spectrometry (Triple Quad 6500).

**Results:** The method was validated over various linear ranges: 1–100 ng/mL for abiraterone, 5–500 ng/mL for enzalutamide and enzalutamide carboxylic acid, 10–1000 ng/mL for *N*-desmethyl enzalutamide, 30–3000 ng/mL for abiraterone N-oxide sulfate, and 100–10,000 ng/mL for abiraterone sulfate. Intra-assay and interassay variabilities were within ±15% of the nominal concentrations for quality control samples at medium and high concentrations and within ±20% at the lower limit of quantification, respectively.

**Conclusions:** The described method for simultaneous determination of abiraterone and enzalutamide was validated successfully and provides a useful tool for therapeutic drug monitoring in patients treated with these agents.

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The authors declare no conflict of interest.

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**Key Words:** abiraterone, enzalutamide, metabolites, *N*-desmethyl enzalutamide, LC-MS/MS, therapeutic drug monitoring

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## INTRODUCTION

Prostate cancer is a highly prevalent malignancy and accounts for approximately 20% of new oncological diagnoses in men.[1,2] Abiraterone acetate and enzalutamide are both oral antiandrogen drugs approved for treatment of metastatic castration-resistant prostate cancer. Abiraterone acetate was granted market access in 2011, and enzalutamide became available in 2012.[3,4] Both drugs inhibit tumor growth effects of androgens. Abiraterone acetate prevents the production of testosterone by inhibition of 17α-hydroxylase/C17,20-lyase (CYP17), whereas enzalutamide functions as an androgen receptor signaling inhibitor.[5,6] In human intestinal fluid, abiraterone acetate is rapidly deacetylated into the active compound abiraterone, which is measured in this bioanalytical method. Hepatic metabolism of abiraterone and enzalutamide is extensive, and the major metabolites *N*-desmethyl enzalutamide, enzalutamide carboxylic acid, abiraterone sulfate, and abiraterone N-oxide sulfate are produced in substantial quantities.[7,8] *N*-desmethyl enzalutamide is known to have clinically relevant antiandrogen capacities similar to enzalutamide.[4]

Pharmacokinetic monitoring of oral anticancer therapies has increased enormously over the past years.[9] Interpatient variability in exposure, known to be 41%–141% for abiraterone[10] and up to 31% for enzalutamide,[11] could lead to undesirable toxicities or subtherapeutic treatment. Therefore, plasma level measurement of abiraterone and enzalutamide could be beneficial in therapy optimization. It is currently unknown what target concentrations should be pursued, and this is now assessed in pharmacokinetic studies. Previously published liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays for the determination of abiraterone,[12–14] enzalutamide,[15] and both metabolites of enzalutamide[16] are limited to one antiandrogen drug in the assay and report no human clinical data. This assay enables concurrent quantification of these analytes to efficiently determine plasma concentrations of patients receiving abiraterone or enzalutamide. Quantification of the major

**TABLE 1.** General Mass Spectrometric Parameters

| | |
|---|---|
| Run duration | 13.5 min |
| Ion spray voltage | 5500 V |
| Nebulizer gas | 40 au |
| Turbo gas/heater gas | 40 au |
| Curtain gas | 20 au |
| Collision gas | 8 au |
| Temperature | 350°C |
| Dwell time | 50 ms |

metabolites gives insight into the metabolism of both drugs and provides information on hepatic clearance. We now report the development and validation of the simultaneous analysis of abiraterone, enzalutamide, and their major metabolites *N*-desmethyl enzalutamide, enzalutamide carboxylic acid, abiraterone sulfate, and abiraterone N-oxide sulfate in human plasma with LC-MS/MS. In addition, the clinical application of this assay was demonstrated.

## MATERIALS AND METHODS

### Chemicals

Abiraterone, enzalutamide, *N*-desmethyl enzalutamide, and the internal standards (ISs) $^2H_4$-abiraterone, $^2H_6$-enzalutamide, and $^2H_6$-*N*-desmethyl enzalutamide were purchased from Alsachim (Illkirch, France). Enzalutamide carboxylic acid was manufactured by TLC Pharmaceutical Standards Ltd (Ontario, Canada), and abiraterone sulfate sodium salt and abiraterone N-oxide sulfate sodium salt were obtained from Toronto Research Chemicals (Toronto, Canada). Acetonitrile, methanol (both Supra-Gradient grade), water, and formic acid (both LC-MS grade) were from Biosolve Ltd (Valkenswaard, the Netherlands). Dimethyl sulfoxide [(DMSO) seccosolv grade] was obtained from Merck (Darmstadt, Germany) and $K_2$EDTA plasma from the Medical Center Slotervaart (Amsterdam, the Netherlands) and Bioreclamation LLC (Hicksville, NY).

### Stock Solutions

Stock solutions, working solutions, calibrators, and all quality control (QC) samples were prepared in amber-colored 1.5-mL tubes. Separate stock solutions in DMSO were prepared for calibrators and QC samples. These contained 1 mg/mL of one of abiraterone, enzalutamide, or *N*-desmethyl enzalutamide, and 0.5 mg/mL of enzalutamide carboxylic acid, abiraterone sulfate, or abiraterone N-oxide sulfate. Stock solutions of 1 mg/mL in methanol were produced for the ISs $^2H_4$-abiraterone, $^2H_6$-enzalutamide, and $^2H_6$-*N*-desmethyl enzalutamide.

### Calibrators, QC Samples

The stock solutions were diluted with plasma to obtain working solutions. A combined working solution of abiraterone, enzalutamide, *N*-desmethyl enzalutamide, enzalutamide carboxylic acid, and abiraterone N-oxide sulfate was prepared. Since an interfering impurity of abiraterone was present in the reference standard of abiraterone sulfate, a separate working solution was prepared for this compound. A combined IS working solution was prepared in methanol at final concentrations of 25 and 100 ng/mL for $^2H_4$-abiraterone and both $^2H_6$-enzalutamide and $^2H_6$-*N*-desmethyl enzalutamide, respectively. Stock solutions and working solutions were stored at −20°C.

Both calibrators and QC samples were freshly prepared before each validation run by adding 80 μL of each working solution to 320 μL of blank human plasma. Concentrations of the calibrators were 1, 5, 50, and 100 ng/mL for abiraterone; 5, 25, 250, and 500 ng/mL for enzalutamide and enzalutamide carboxylic acid; 10, 50, 500, and 1000 ng/mL for *N*-desmethyl enzalutamide, and 30, 150, 1,500, and 3000 ng/mL for abiraterone N-oxide sulfate. A separate set of calibrators was produced for abiraterone sulfate containing 100, 500, 5,000, and 10,000 ng/mL. Concentrations of the lower limit of quantification (LLOQ) QC, QC mid, and QC high samples were 5, 40, and 100 ng/mL for abiraterone, respectively; 5, 200, and 500 ng/mL for enzalutamide and enzalutamide carboxylic acid, respectively; 10, 400, and 1000 ng/mL for *N*-desmethyl enzalutamide, respectively; 30, 1200, and 3000 ng/mL for abiraterone N-oxide sulfate, respectively; and 100, 4000, and 10,000 ng/mL, respectively, for the separately spiked abiraterone sulfate. Both calibrators and QC samples were processed in aliquots of 50 μL.

### Sample Preparation

Whole venous blood was obtained from treated patients and centrifuged for 5 minutes at 4°C at 1800*g*.

**TABLE 2.** Analyte Specific Mass Spectrometric Parameters for Abiraterone, Enzalutamide, and Both Major Metabolites

| | Parent Mass, m/z | Product Mass, m/z | Collision Energy, V | Collision Exit Potential, V | Declustering Potential, V | Retention Time, min |
|---|---|---|---|---|---|---|
| Enzalutamide | 465.0 | 209.1 | 37.0 | 18.0 | 131.0 | 4.77 |
| *N*-desmethyl enzalutamide | 451.1 | 195.2 | 37.0 | 18.0 | 186.0 | 4.64 |
| Enzalutamide carboxylic acid | 452.1 | 196.1 | 37.0 | 18.0 | 131.0 | 4.95 |
| Abiraterone | 350.1 | 156.1 | 63.0 | 10.0 | 186.0 | 7.10 |
| Abiraterone N-oxide sulfate | 446.0 | 348.3 | 63.0 | 10.0 | 186.0 | 5.10 |
| Abiraterone sulfate | 430.0 | 332.1 | 63.0 | 10.0 | 186.0 | 5.44 |
| $^2H_6$-enzalutamide | 471.2 | 215.1 | 37.0 | 18.0 | 131.0 | 4.76 |
| $^2H_6$-*N*-desmethyl enzalutamide | 457.1 | 201.2 | 37.0 | 18.0 | 131.0 | 4.63 |
| $^2H_4$-abiraterone | 354.1 | 160.1 | 63.0 | 10.0 | 186.0 | 6.95 |

The supernatant (plasma fraction) was isolated and stored at −20°C until analysis. Samples were thawed before processing, and a 50-μL aliquot was transferred to amber-colored 1.5-mL containers. A volume of 15 μL IS working solution was added to each sample. Proteins were precipitated with 150 μL of acetonitrile, after which the samples were vortexed, shaken (10 minutes at 11,300*g*), and centrifuged (10 minutes at 20°C at 23,100*g*). A volume of 100 μL of the supernatant was transferred to 1.5-mL tubes and diluted with 100-μL water (LC-MS grade). The final extracts were transferred to amber-colored glass autosampler vials.

## Analytical Equipment and Conditions

The analytical system was composed of a liquid chromatography Nexera 2 series (Shimadzu Corporation, Kyoto, Japan) coupled to a triple quadrupole mass spectrometer 6500 (Sciex, Framingham, MA) with a turbo ion spray interface. The liquid chromatography was equipped with a Nexera 2 series binary pump, a degasser, an autosampler, and a valco valve (Shimadzu Corporation, Kyoto, Japan).

Chromatographic separation was acquired using a Kinetex C18 column (15 × 2.1 mm, particle size 2.6 μm; Phenomenex, Torrance, CA). The mobile phase A consisted of formic acid–water (0.1:100, vol/vol), and mobile phase B was composed of formic acid–methanol (0.1:100, vol/vol). Analytes were eluted under isocratic conditions with 70% mobile phase B after a 2-minute hold of 30% mobile phase B at a flow rate of 0.3 mL/min. To elute hydrophobic compounds from the column, a 3-minute rinsing step with a 100% mobile phase B was applied before conditions were stabilized to initial settings. During the first 2.5 minutes and the last 4.5 minutes, the eluate was directed to waste.

The mass spectrometer operated in the positive ion mode and was configured in multiple reaction monitoring mode at unique transitions for each analyte and IS. Analyst software version 1.6.2 (Sciex) was used for system control and data analysis. A summary of general and specific mass spectrometric settings is provided in Tables 1 and 2. $^{2}H_4$-Abiraterone was used as IS for abiraterone, $^{2}H_6$-*N*-desmethyl enzalutamide for *N*-desmethyl enzalutamide, and $^{2}H_6$-enzalutamide for enzalutamide, enzalutamide carboxylic acid, abiraterone N-oxide sulfate, and abiraterone sulfate. The structures and the proposed fragmentation patterns of the analytes are depicted in Figure 1.

## Validation Procedures

Validation of the assay was completed based on the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation.[17,18] These guidelines apply for clinical and preclinical pharmacology and toxicology studies. For this therapeutic drug monitoring assay, all aspects of the validation were investigated; however, 4 instead of 6–8 calibrators were investigated, and QC concentrations were prepared at 3 levels: LLOQ, medium, and high concentrations.

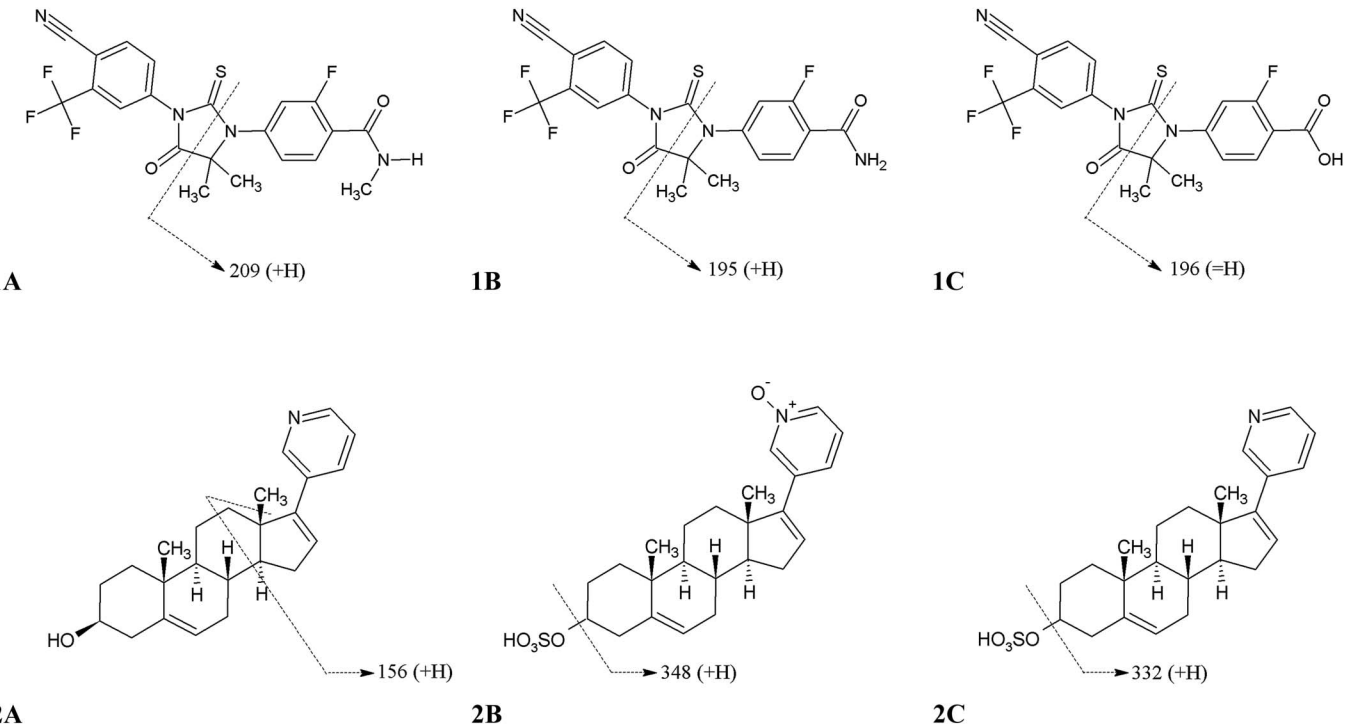


**FIGURE 1.** Molecular structures and proposed fragmentation of enzalutamide (1A), *N*-desmethyl enzalutamide (1B), enzalutamide carboxylic acid (1C), abiraterone (2A), abiraterone N-oxide sulfate (2B), and abiraterone sulfate (2C). Precursor ions m/z (+H) are 465.0, 451.1, 452.1, 350.1, 446.0, and 430.0, respectively.

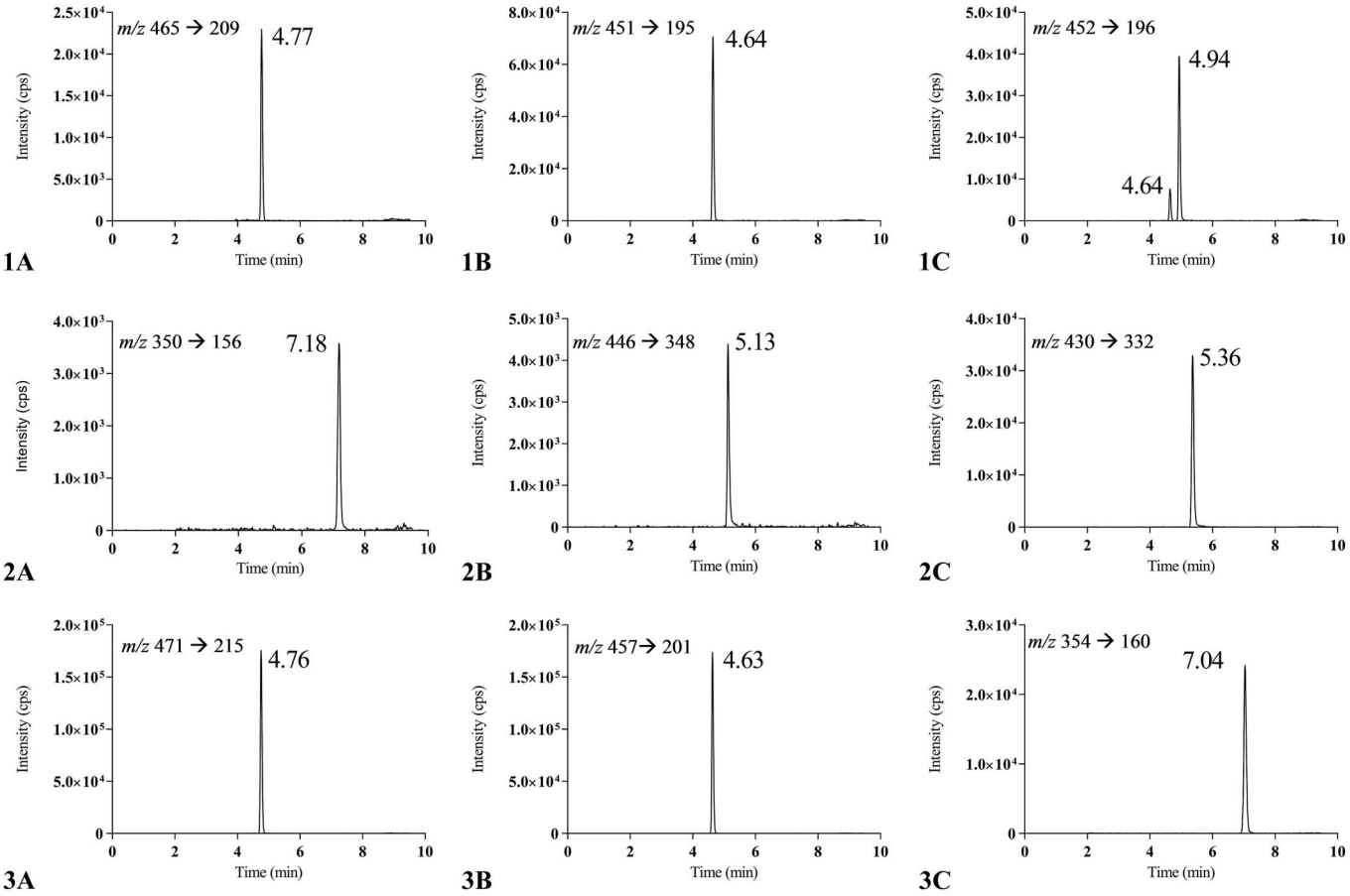


**FIGURE 2.** Representative LC-MS/MS chromatograms of enzalutamide (1A), *N*-desmethyl enzalutamide (1B), enzalutamide carboxylic acid (the additional peak belongs to enzalutamide carboxylic acid, the first peak corresponds with *N*-desmethyl enzalutamide) (1C), abiraterone (2A), abiraterone N-oxide sulfate (2B), abiraterone sulfate (2C), and the ISs $^{2}H_{6}$-enzalutamide (3A), $^{2}H_{6}$-*N*-desmethyl enzalutamide (3B), and $^{2}H_{4}$-abiraterone (3C).

Calibration model, accuracy and precision, carryover, selectivity (endogenous, cross analyte/IS interferences), matrix effect, recovery, dilution integrity, and stability were established during the validation.

## Calibration Model

Calibration linearity was determined by plotting the peak area ratio of the analyte/IS against the corresponding concentration (x) of the calibrator. Linear regression analysis was performed with $1/x^2$ weighting. Deviations from the mean calculated concentrations should be within ±15% (±20% for LLOQ calibrators) of nominal concentrations in at least 75% of nonzero calibrators.

## Accuracy and Precision

Five replicated QC samples were analyzed in 3 consecutive runs at LLOQ, medium, and high concentrations. Accuracy was expressed as the relative error (%), and precision was calculated as the relative standard deviation (%). Intra-assay variability (%) was determined from mean measured concentrations per run, and the interassay bias (%) was calculated from the overall mean measured concentrations. A one-way analysis of variance was used to calculate interrun variation. The acceptance criteria for both parameters were ±15% for QC mid and QC high and ±20% for QC LLOQ.

## Carryover

Carryover was determined by injecting 2 double blank samples after a calibrator with the highest concentration [upper limit of quantification (ULOQ)]. The peak area in the blank sample should not be higher than 20% of the peak area in the LLOQ.

## Selectivity

Six separate batches of blank human plasma were spiked at the LLOQ level with one of abiraterone, enzalutamide, *N*-desmethyl enzalutamide, enzalutamide carboxylic acid, and abiraterone N-oxide sulfate or abiraterone sulfate. An accuracy of within 80%–120% of the nominal concentration was strived for in at least 5 of 6 samples. Endogenous selectivity was assessed by analysis of 6 separate batches of blank human plasma. Interfering

**TABLE 3.** Assay Performance Data for Abiraterone, Enzalutamide, and Both Major Metabolites in Human Plasma Tested at LLOQ, Medium, and High Concentrations

| | | Intra-Assay (n = 15) | | Interassay (n = 15) | |
|---|---|---|---|---|---|
| **Analyte** | **Nominal Concentration, ng/mL** | **Bias (%)** | **C.V. (%)** | **Bias (%)** | **C.V. (%)** |
| Enzalutamide | 94.4 | 5.7 | 4.2 | 5.1 | 0.4 |
| | 189 | −4.3 | 5.6 | −0.1 | 3.3 |
| | 494 | 6.4 | 4.2 | 3.7 | 1.9 |
| *N*-desmethyl enzalutamide | 10 | 4.7 | 3.6 | 2.9 | 0.9 |
| | 400 | −10.4 | 3.2 | −6.3 | 3.9 |
| | 1000 | −5.1 | 2.6 | −4.8 | —* |
| Enzalutamide carboxylic acid | 5 | 7.4 | 9.0 | −4.0 | 5.6 |
| | 200 | −7.2 | 4.3 | −7.2 | 2.6 |
| | 500 | −9.1 | 3.8 | 2.0 | 5.9 |
| Abiraterone | 0.998 | 7.0 | 4.7 | 1.8 | 5.1 |
| | 40 | −6.8 | 2.2 | −4.6 | 1.9 |
| | 99.8 | −8.3 | 3.7 | −4.1 | 3.7 |
| Abiraterone N-oxide sulfate | 30 | 2.6 | 10.0 | 1.6 | —* |
| | 1200 | 12.7 | 7.4 | 8.2 | 4.0 |
| | 3000 | 13.5 | 4.4 | 4.7 | 8.0 |
| Abiraterone sulfate | 100 | −15.2 | 5.0 | −11.2 | 6.8 |
| | 4000 | −13.9 | 3.9 | −9.2 | 5.0 |
| | 10,000 | −13.3 | 4.0 | −12.5 | —* |

*No significant additional variation was found because of the performance of the assay in different batches.
C.V., coefficient of variation.

peak areas at the analyte retention time should not exceed 20% of the peak area at LLOQ level (n = 6). The cross-analyte and IS interference assay was performed with single samples that were separately spiked with one analyte or IS at ULOQ concentration. To ensure that compounds do not interfere with the quantification of the analyte, the cross-analyte or IS interference should be ≤20% of the peak area in LLOQ samples and ≤5% for the IS.

### Matrix Effect and Recovery

The matrix effect was investigated with 6 different batches of blank human plasma at QC LLOQ and QC high concentrations. Peak areas of QC samples spiked after protein precipitation were compared with peak areas of QC samples of equivalent concentrations in acetonitrile–water (50:50, vol/vol). In addition, the IS-normalized matrix factor was calculated by dividing the matrix factor of the analyte by the matrix factor of the IS.

Determination of the recovery was also performed at QC LLOQ and QC high (n = 6) concentrations. The recovery was calculated by dividing the peak area of processed QC samples by the peak area of blank plasma extract spiked with reference compound at equal concentrations. A total recovery of >70% and coefficient of variation of below 15% were required.

### Dilution Integrity

Five replicate plasma samples with a concentration above the ULOQ were diluted 200-fold with control human plasma. A volume of 30-μL sample was diluted with 570 μL of control human plasma. Subsequently, 30 μL of this solution was added to 270 μL of control human plasma. An accuracy of within −15% and +15% of the nominal concentration was acceptable.

### Stability

Short-term stability experiments were performed in plasma after storage at room temperature (20–25°C) and at −20°C. Further stability assessments were done in the final extracts at 4°C. The effect of 3 freeze (−20°C)/thaw cycles on the stability of each compound was investigated after thawing samples to room temperature for at least 12 hours on 3 separate occasions and comparison with freshly prepared samples. Long-term stability in plasma was determined after 1 month of storage at −20°C. Stock stability was described as the recovery percentage after 3 months storage at −20°C. All stability experiments were performed in triplicate at QC LLOQ and QC high levels. Analytes were considered stable under specific conditions when 85%–115% of the initial concentration at QC high levels and when 80%–120% of the initial concentration at QC LLOQ were recovered.

## Clinical Application

This assay was developed to facilitate pharmacokinetic monitoring of abiraterone and enzalutamide in patients with metastatic castration-resistant prostate cancer at the Antoni van Leeuwenhoek/Netherlands Cancer Institute. Whole blood was collected at the clinic as routine standard of care at steady-state situation, which is reached after at least 1 month of treatment with enzalutamide (half-life of 5.8 days[4]) and 1 week of treatment with abiraterone (half-life of 16.3 hours[10]). Samples were stored at −20°C. Further processing is

**TABLE 4.** Stability of Abiraterone, Enzalutamide, and Their Major Metabolites in Several Matrixes

| Matrix | Conditions | Compound | Nominal Concentration, ng/mL | Dev (%) | C.V. (%) |
|---|---|---|---|---|---|
| DMSO | −20°C, 3 mo | Enzalutamide | 1,000,000 | −1.2 | 3.9 |
| | | *N*-desmethyl enzalutamide | 1,000,000 | −3.7 | 6.2 |
| | | Enzalutamide carboxylic acid | 500,000 | −7.0 | 3.7 |
| | | Abiraterone | 1,000,000 | −4.8 | 3.5 |
| | | Abiraterone N-oxide sulfate | 500,000 | −8.8 | 2.4 |
| | | Abiraterone sulfate | 500,000 | 0.8 | 0.5 |
| Plasma | RT, 5 d | Enzalutamide | 4.94 | 0.29 | 2.5 |
| | | | 494 | −1.0 | 1.2 |
| | | *N*-desmethyl enzalutamide | 10 | −1.5 | 1.1 |
| | | | 1000 | −2.9 | 1.7 |
| | | Enzalutamide carboxylic acid | 5 | 0.84 | 2.6 |
| | | | 500 | −0.073 | 2.6 |
| | | Abiraterone | 0.998 | −2.9 | 0.99 |
| | | | 99.8 | −2.3 | 3.2 |
| | | Abiraterone N-oxide sulfate | 30 | 1.1 | 3.1 |
| | | | 3000 | −0.78 | 2.4 |
| | | Abiraterone sulfate | 100 | 2.7 | −2.3 |
| | | | 10,000 | −3.5 | −3.3 |
| Plasma | −20°C/RT, Freeze/thaw, 3 cycles | Enzalutamide | 4.94 | 0.29 | 2.5 |
| | | | 494 | −0.72 | 2.1 |
| | | *N*-desmethyl enzalutamide | 10 | −1.5 | 2.9 |
| | | | 1000 | 1.1 | −2.6 |
| | | Enzalutamide carboxylic acid | 5 | 0.44 | 3.3 |
| | | | 500 | 3.1 | 2.7 |
| | | Abiraterone | 0.998 | −3.1 | 1.3 |
| | | | 99.8 | 0.20 | 0.79 |
| | | Abiraterone N-oxide sulfate | 30 | 1.1 | 3.1 |
| | | | 3000 | 2.0 | 2.4 |
| | | Abiraterone sulfate | 100 | 0.95 | −3.9 |
| | | | 10,000 | 2.5 | −1.2 |
| Plasma | −20°C, 1 mo | Enzalutamide | 4.94 | 1.1 | 2.3 |
| | | | 494 | 0.26 | 1.5 |
| | | | 2470 | −0.97 | 2.7 |
| | | *N*-desmethyl enzalutamide | 10 | 0.83 | 1.9 |
| | | | 1000 | 0.56 | 1.5 |
| | | | 5000 | −1.4 | 4.8 |
| | | Enzalutamide carboxylic acid | 5 | 4.1 | 1.0 |
| | | | 500 | 0.45 | 5.2 |
| | | | 2500 | 2.0 | 2.8 |
| | | Abiraterone | 0.998 | 4.7 | 1.7 |
| | | | 99.8 | 5.3 | 2.2 |
| | | | 499 | 3.8 | 2.3 |
| | | Abiraterone N-oxide sulfate | 30 | −2.4 | 4.9 |
| | | | 3000 | 3.3 | 2.6 |
| | | | 15,000 | 3.1 | 3.2 |
| | | Abiraterone sulfate | 100 | −5.4 | 3.6 |
| | | | 10,000 | −2.7 | 1.0 |
| | | | 50,000 | −0.59 | 2.6 |
| Final extract | 2–8°C, 5 d | Enzalutamide | 4.94 | 1.9 | 4.0 |
| | | | 494 | −1.0 | 1.2 |
| | | *N*-desmethyl enzalutamide | 10 | −1.0 | 4.7 |
| | | | 1000 | −3.0 | 2.4 |
| | | Enzalutamide carboxylic acid | 5 | −0.64 | 3.8 |
| | | | 500 | 2.8 | 0.93 |

**TABLE 4.** (*Continued*) Stability of Abiraterone, Enzalutamide, and Their Major Metabolites in Several Matrixes

| Matrix | Conditions | Compound | Nominal Concentration, ng/mL | Dev (%) | C.V. (%) |
|---|---|---|---|---|---|
| | | Abiraterone | 0.998 | −0.22 | 5.6 |
| | | | 99.8 | −0.12 | 2.0 |
| | | Abiraterone N-oxide sulfate | 30 | 5.4 | 6.1 |
| | | | 3000 | 0.47 | 0.64 |
| | | Abiraterone sulfate | 100 | 9.9 | −4.4 |
| | | | 10,000 | 9.2 | −3.6 |

All experiments are performed in triplicate at LLOQ and high concentrations.
C.V., coefficient of variation; Dev, deviation; RT, room temperature.

performed as previously described in this report (Section *2.4 Sample preparation*).

## RESULTS AND DISCUSSION

## Development

### Sample Preparation

Several sample pretreatment methods have been tested. Protein precipitation was initially performed with methanol instead of acetonitrile to obtain a final extract similar to the composition of the mobile phase. This resulted, however, in large peak area variation (>50%) of the IS. Sample pretreatment with acetonitrile improved this variability to a nominal <5% per batch.

### Mass Spectrometry and Chromatography

During the development, carryover was observed for abiraterone, enzalutamide, and abiraterone N-oxide. Accordingly, a 3-minute rinsing step with 100% methanol was incorporated after each injection. Furthermore, an impurity of abiraterone was present in the abiraterone sulfate reference standard. As the abiraterone sulfate was expected to be 100-fold more abundant, the cross-analyte interference would exceed 20%. Therefore, separate calibrators and QC samples were produced for both abiraterone sulfate and a combination of abiraterone, enzalutamide, *N*-desmethyl enzalutamide, enzalutamide carboxylic acid, and abiraterone N-oxide sulfate. Representative chromatograms of QC LLOQ are presented in Figure 2 for each analyte and IS. Baseline separation was necessary for the metabolites *N*-desmethyl enzalutamide and enzalutamide carboxylic acid because *N*-desmethyl enzalutamide interferes at the transitions of enzalutamide carboxylic acid.

Three ISs were incorporated in the analysis. $^{2}H_{6}$-Enzalutamide was used to normalize concentrations of the compounds without a deuterated IS (enzalutamide carboxylic acid, abiraterone N-oxide sulfate, and abiraterone sulfate). Although $^{2}H_{6}$-Enzalutamide is not a structural analog of the abiraterone sulfated metabolites, this IS was able to compensate for variations in responses, resulting in acceptable accuracy and precision values.

After receiving clinical samples, it became clear that plasma concentrations of enzalutamide, *N*-desmethyl enzalutamide, enzalutamide carboxylic acid, abiraterone N-oxide sulfate, and abiraterone sulfate exceeded the ULOQ and therefore needed 10- to 100-fold dilution before analysis. Changing the calibration range was not possible because of nonlinearity at higher concentrations and increased carryover. It could be suggested, if available, to transfer this method to a less sensitive instrument with adjusted calibration ranges.

## Validation Procedures

### Calibration Model

Consistent with the requirement, at least 75% of the calibrators were within ±15% (±20% for the LLOQ) of the nominal concentrations. Calibration ranges of 1–100 ng/mL for abiraterone, 5–500 ng/mL for enzalutamide and

**TABLE 5.** Mean Steady-State Plasma Concentrations and the Range in 10 Patients Receiving Either Abiraterone (1000 mg/d) or Enzalutamide (160 mg/d)

| | Time After Dosing | | | |
|---|---|---|---|---|
| | 0–12 h (n = 5) | | 12–24 h (n = 5) | |
| Compound | Mean Concentration, ng/mL | Range, ng/mL | Mean Concentration, ng/mL | Range, ng/mL |
| Enzalutamide | 13,640 | 11,900–17,600 | 11,436 | 9620–13,100 |
| *N*-desmethyl enzalutamide | 10,737 | 6990–13,500 | 9120 | 5050–14,100 |
| Enzalutamide carboxylic acid | 4375 | 4160–4590 | 5560 | 2880–12,300 |
| Abiraterone | 37.4 | 12.7–121 | 19.6 | 7.70–30.7 |
| Abiraterone N-oxide sulfate | 7341 | 833–17,200 | 3234 | 1270–5270 |
| Abiraterone sulfate | 10,050 | 7000–14,000 | 11,936 | 7540–22,400 |

enzalutamide carboxylic acid, 10–1000 ng/mL for *N*-desmethyl enzalutamide, 30–3000 ng/mL for abiraterone N-oxide sulfate, and 100–10,000 ng/mL for abiraterone sulfate fulfilled the criteria.

### Accuracy and Precision

Assay performance data are presented in Table 3. Inter-assay accuracy, intra-assay accuracy, and the precision were within the acceptance criteria at LLOQ, medium, and high concentrations.

### Specificity and Selectivity

Mean measured QC LLOQ concentrations in 6 different batches of plasma were all within ±20% of the nominal concentrations for all tested analytes. In the double blank samples, no peaks were observed, with areas exceeding 20% of the analyte peak areas measured in QC LLOQ samples in the different batches of plasma. Cross-interference of coeluting peaks in separately spiked samples was <20% of the QC LLOQ samples and thus within the required limits. Hence, this method was proven to be selective.

### Dilution Integrity

The concentrations of 200-fold diluted samples were within ±15% of the nominal concentration in 5 replicates. Intra-assay bias and intra-assay variability were between −5.9% and 2.8% for abiraterone, 5.1% and 4.0% for enzalutamide, −4.1% and 14% for *N*-desmethyl enzalutamide, −7.7% and 5.4% for enzalutamide carboxylic acid, 2.4% and 14% for abiraterone N-oxide sulfate, and 1.6% and 3.5% for abiraterone sulfate. These results show that samples with concentrations >ULOQ can be diluted up to 200-fold to obtain plasma concentrations within the validated range.

### Carryover

Criteria were fulfilled in 3 separate runs, with a maximum carryover of 12% for abiraterone N-oxide sulfate. No carryover was observed for the ISs.

### Matrix Factor and Recovery

The coefficient of variation for the IS-normalized matrix factor was below 15% at the tested concentrations for each compound. Furthermore, the total recovery was >70% for all analytes, and the variation did not exceed 15%. These results show that the IS effectively minimizes the influence of matrix effects, and that protein precipitation with acetonitrile is an adequate sample pretreatment.

### Stability

Stability results of the tests are shown in Table 4. Abiraterone N-oxide and enzalutamide carboxylic acid are unstable in DMSO after 3 months storage at −20°C. To minimize degradation of these metabolites, working solutions were produced in plasma with freshly prepared abiraterone N-oxide and enzalutamide carboxylic acid stock solutions. All other experiments demonstrated adequate stability because results were within the acceptance criteria. Long-term stability assessment in plasma was measured up to 1 month and is still ongoing.

## CLINICAL APPLICATION

To establish applicability of the validated assay, steady-state plasma of patients receiving a regular dose of either abiraterone acetate or enzalutamide was analyzed. The mean measured plasma concentrations are presented in Table 5, and representative chromatograms are depicted in Figure 3. A second peak with identical transitions to abiraterone is observed in the augmented image presenting plasma from

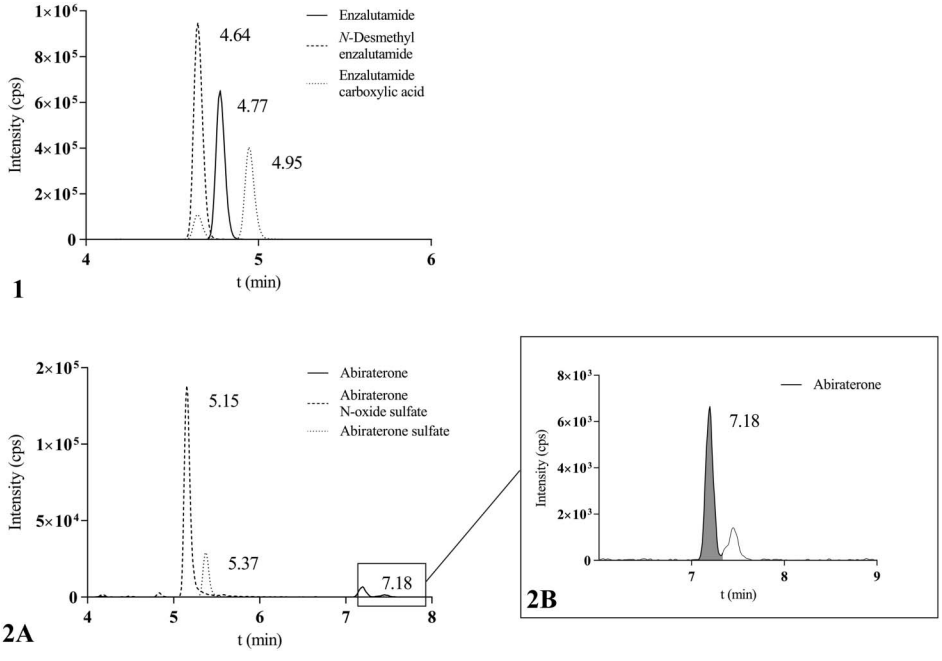


**FIGURE 3.** LC-MS/MS chromatograms of 100-fold diluted steady-state plasma from a patient using enzalutamide (1, from 4 to 6 minutes) and of 10-fold diluted steady-state plasma from a patient using abiraterone (2A, from 4 to 8 minutes). A limited part of the chromatogram is shown to provide a better image of the peaks, and the abiraterone chromatogram is enlarged to show the additional peak detected at the abiraterone transition (2B, from 6 to 9 minutes).

a patient using abiraterone acetate. The peak area of this additional peak differs per patient, and suggests that it belongs to an isomeric metabolite that is produced in varying quantities in vivo. Measurements were within the validated ranges after 100-fold dilution of enzalutamide samples and 10-fold dilution of abiraterone samples. One abiraterone concentration was below the LLOQ after 10-fold dilution and was therefore reanalyzed undiluted. The large range of the abiraterone concentrations can be explained by the sampling time after dosing and by reported variability in exposure and $C_{max}$ that is caused by intake with food or by decreased hepatic function.[3] These results also demonstrate the potential relevance of this assay for pharmacokinetic monitoring of abiraterone, enzalutamide, and their metabolites.

## CONCLUSIONS

An LC-MS/MS method was developed and validated for the simultaneous analysis of abiraterone, enzalutamide, *N*-desmethyl enzalutamide, enzalutamide carboxylic acid, abiraterone N-oxide sulfate, and abiraterone sulfate. Because of low stability of abiraterone N-oxide sulfate and enzalutamide carboxylic acid in DMSO, working solutions were produced in plasma with freshly prepared stock solutions. Concentration ranges of 1–100 ng/mL for abiraterone, 5–500 ng/mL for enzalutamide and enzalutamide carboxylic acid, 10–1000 ng/mL for *N*-desmethyl enzalutamide, 30–3000 ng/mL for abiraterone N-oxide sulfate, and 100–10,000 ng/mL for abiraterone sulfate were chosen to measure compound concentrations in steady-state plasma of patients. A combined assay for these analytes provides an efficient approach to measure plasma concentrations. This assay has now been successfully implemented to facilitate therapeutic drug monitoring of abiraterone, enzalutamide, and both major metabolites.

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