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Validation of an LC-MS/MS method for simultaneous quantification of abiraterone, enzalutamide and darolutamide in human plasma

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ABSTRACT

Currently, several oral androgen receptor signalling inhibitors are available for the treatment of advanced prostate cancer. Quantification of plasma concentrations of these drugs is highly relevant for various purposes, such as Therapeutic Drug Monitoring (TDM) in oncology. Here, we report a liquid chromatography/tandem mass spectrometric (LC–MS/MS) method for the simultaneous quantification of abiraterone, enzalutamide, and darolutamide. The validation was performed according to the requirements of the U.S. Food and Drug Administration and European Medicine Agency. We also demonstrate the clinical applicability of the quantification of enzalutamide and darolutamide in patients with metastatic castration-resistant prostate cancer.

1. Introduction

Androgen receptor signalling inhibitors (ARSi) form a mainstay in the treatment of hormone sensitive prostate cancer (HSPC) and castration resistant prostate cancer (CRPC) and are applied in both the non-metastatic and the metastatic setting [1]. In addition to androgen deprivation therapy (ADT) by chemical or surgical castration, ARSIs have proven to provide an increased survival benefit [2,3]. During the last decade, a new class of oral second generation ARSi, including abiraterone acetate, enzalutamide and darolutamide have been approved for clinical use in prostate cancer. Abiraterone acetate is a prodrug of abiraterone, which is a selective inhibitor of Cytochrome P450 (CYP) 17A1. It thereby hampers the conversion of testosterone precursors. Abiraterone acetate in combination with prednisone has been approved for metastatic HSPC and CRPC [4–6]. Enzalutamide is a selective blocker of the androgen receptor (AR) and has been approved for non-metastatic and metastatic CRPC and for metastatic HSPC [7–10]. Darolutamide is the most recently introduced AR antagonist and is currently approved for use in non-metastatic HSPC and for metastatic HSPC in combination with docetaxel and continued ADT [11,12].

In the era of precision medicine, personalized dosing strategies are preferred over fixed-dosing. For a wide range of therapeutics an exposure–response relationship has been established and large interindividual variability in exposure demands a tailored approach [13]. For abiraterone, a therapeutic threshold has recently been established. Based on a pharmacometric model, a minimum observed concentration threshold value of 8.4 ng/mL was found to be most predictive for PSA response. In patients with trough levels below the threshold, progression-free survival was 7.4 months compared to 12.2 months in patients with trough levels above the threshold [14]. With current dosing strategies (i.e. fixed dosing of 1,000 mg QD) 35–42% of patients have trough levels below the threshold value, stressing the need for Therapeutic Drug Monitoring (TDM) of this drug [14,15]. On the other hand, a recent study found that an exposure–response relationship could only be established in post-chemotherapy patients [16]. For enzalutamide, no clear relationship with survival could be established [17,18]. For darolutamide, only limited investigation of an exposure–response relationship in nmCRPC has been conducted yet, also lacking validation. No relationships were identified [19]. However, measurement of plasma concentrations of such novel agents in particular is crucial for investigational purposes, such as studies on drug-drug interactions or combination treatments.

In order to determine plasma concentrations of these compounds, a liquid chromatography/tandem mass spectrometric (LC–MS/MS) assay has to be established. Here, we describe the development and full validation according to U.S. Food & Drug Administration (FDA) and European Medicine Agency (EMA) guidelines of an LC–MS/MS method for the simultaneous quantification of the ARSi abiraterone, enzalutamide

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and darolutamide [20,21].

2. Material and methods

2.1. Chemicals

All chemicals used were of analytical grade or higher. Darolutamide (purity 98%) was obtained from BioConnect (Huissen, The Netherlands). Enzalutamide (purity 97%), abiraterone (purity 99%) and the Internal Standard abiraterone-d4 were purchased at Toronto Research Chemicals (Toronto, ON, Canada). The high purity (ULC/MS quality) solvents methanol, acetonitrile (ACN) and water were obtained from Biosolve (Valkenswaard, The Netherlands). Dimethyl sulfoxide (DMSO) and 2-propanol were purchased from Merck (Darmstadt, Germany) and formic acid (FA, purity 98%) was purchased at J.T. Baker (Deventer, The Netherlands). Blank human sodium citrated plasma was supplied by Sanquin Bloedbank Zuidwest (Rotterdam, The Netherlands) and potassium EDTA plasma was supplied by BioIVT (West Sussex, United Kingdom).

2.2. Preparation of stock solutions, calibration standards and quality control samples

Stock solutions of darolutamide, enzalutamide and abiraterone were prepared in DMSO at a concentration of 1 mg/mL. For darolutamide, enzalutamide and abiraterone, three independent stock solutions were made with a maximum concentration deviation of 5%. One stock solution of each compound was used to create a working stock solution containing 500 μg/mL darolutamide and enzalutamide and 10.0 μg/mL abiraterone. A second stock solution of each compound was used to prepare quality control samples. Stock solutions were stored at T < −70 °C. Two calibration curves were made for each validation run in which the working stock solution was diluted stepwise in ACN/DMSO (1:1, v/v) with final concentrations of 100, 75.0, 50.0, 25.0, 10.0, 2.50 and 1.00 μg/mL for darolutamide and enzalutamide and 2.00, 1.50, 1.00, 0.500, 0.200, 0.0500 and 0.0200 μg/mL for abiraterone. Each prepared standard solution was diluted in human plasma resulting in calibration lines with concentration levels of 50.0, 125, 500, 1,250, 2,500, 3,750, 4,500 and 5,000 ng/mL for darolutamide and enzalutamide and 1.00, 2.50, 10.0, 25.0, 50.0, 75.0, 90.0 and 100 ng/mL for abiraterone. The Lower Limit of Quantitation (LOQ) was established at 50.0 ng/mL for darolutamide and enzalutamide and at 1.00 ng/mL for abiraterone. Establishment of the LOQ was performed by analysing pools of quality control samples at the concentration of the LOQ on three independent days and analysing ten human plasma samples from different donors, spiked with darolutamide, enzalutamide and abiraterone at a concentration of the LOQ. Four pools of quality control samples were prepared in human plasma at concentrations of 150 ng/mL (QC-Low), 2000 ng/mL (QC-Middle), 4000 ng/mL (QC-High) and 20,000 ng/mL (QC-Diluted) for darolutamide and enzalutamide and 3.00 ng/mL (QC-Low), 40.0 ng/mL (QC-Middle), 80.0 ng/ml (QC-High) and 800 ng/mL (QC-Diluted) for abiraterone. All QC-samples were divided in cryo-vials and stored at T < −70 °C, while calibration lines were always prepared freshly on the day of analysis.

2.3. Sample preparation

An aliquot of 200 μL of the internal standard solution which consists of deuterated abiraterone (abiraterone-d4) at a concentration of 100 ng/mL in acetonitrile, was mixed with 25 μL of plasma sample. After vigorous mixing for 5 s, the samples were centrifuged for 10 min at a speed of 14,000*g*, resulting in a clear supernatant. An aliquot of 50 μL of this clear supernatant was transferred to a 96-well plate after which an aliquot of 150 μL of ACN/water/FA (70:30:0.1, v/v/v) was added. After mixing on a rocked platform for 5 min, the 96-well plate was placed in a temperature controlled sample organizer 10 °C. Sample analysis was done by injecting an aliquot of 2 μL onto the UPLC column.

2.4. Equipment

A Waters Acquity FTN-1 Sample Manager and a H-class Waters Quaternary Solvent Manager coupled to a Waters Xevo TQ-XS Mass Spectrometer (Waters Chromatography B.V., Etten-Leur, The Netherlands) were used for validation. Masslynx 4.2 SCN1007 package software (Waters Chromatography B.V.) was used for LC-MS/MS system control, while QuanLynx (Waters Chromatography B.V.) was used for data processing.

2.4.1. Chromatographic conditions

Separation of analytes was achieved using an Atlantis® dC18; 4.6 × 50 mm, 3.0 μm column (Waters Chromatography B.V), which was kept at 45 °C. The eluent consisted of water/FA (100:0.2, v/v; eluent A) and ACN/FA (100:0.2, v/v; eluent B), which were eluted at a speed of 0.400 mL/minute. A linear gradient from T = 0 to T = 0.5 min was used to increase eluent B from 30% to 50%, after which a further increase from 50% to 95% was created from T = 0.5 to T = 1.5 min. This was kept at steady state for 1 min and subsequently from T = 2.5 to 2.6 min brought back to the initial situation with a regeneration time of 1.4 min. The overall run-time was 4 min. The injection needle was washed before injection with a strong wash solvent, composed of ACN/methanol/water/2-propanol/FA (25:25:25:25:0.1, v/v/v/v/v).

2.4.2. Mass spectrometry

Quantitation of analytes and internal standard was achieved by MS/MS detection in positive ion mode. Mass transitions of *m/z* were optimized by direct infusion of the respective analytes in ACN/water/FA (40:60:0.1, v/v/v) and optimal MS settings were manually adjusted. The desolvation gas was set at 1200 L/h and the cone gas at 150 L/h (nitrogen). The ionspray voltage was kept at 3.80 kV and the cone voltage at 40 V for darolutamide, abiraterone and aberiterone-d4 and at 45 V for enzalutamide, with a source temperature of T = 150 °C and desolvation temperature of T = 600 °C. Multiple reaction monitoring (MRM) mode was applied for the quantitation with the following parameters: *m/z* 350 > 156, collision energy set at 50 V, *m/z* 465 > 209, collision energy set at 27 V, *m/z* 399 > 178, collision energy set at 20 V and *m/z* 354 > 160, collision energy set at 50 V for darolutamide, enzalutamide, abiraterone and aberiterone-d4, respectively. Primary to secondary ion ratios of darolutamide and enzalutamide were used to show the quality of the observed peaks. Dwell times were automatically set at 52 ms to perform at least 15 scans per peak. The column effluent was passed through the mass spectrometer and monitored between 2 and 4 min after start of the MS method, while 0 to 2 min and 4 to 5 min were sent to waste.

2.4.3. Quantitation

Calibration curves were linear in the range of 50.0–5000 ng/mL for darolutamide and enzalutamide and 1.00–100 ng/mL for abiraterone in human plasma using abiraterone-d4 as internal standard. The calibration curves were generated using peak area ratios of analytes to the internal standard versus the known concentrations with a linear regression equation of $1/\text{concentration}^2$.

2.5. Method validation

Potential presence of background contamination ions was determined by analysing blank human potassium EDTA plasma samples obtained from ten plasma donors. Validation parameters were determined by analysing pools of QC-samples in quintuplicate per validation run, spread over three independent days. Calculation of accuracy (ACC), within-run precision (WRP) and between-run precisions (BRP) was performed by using one-way analysis of variance, using the run as variable. Stability of darolutamide, enzalutamide and abiraterone in human plasma was investigated by storage of quality control samples at room

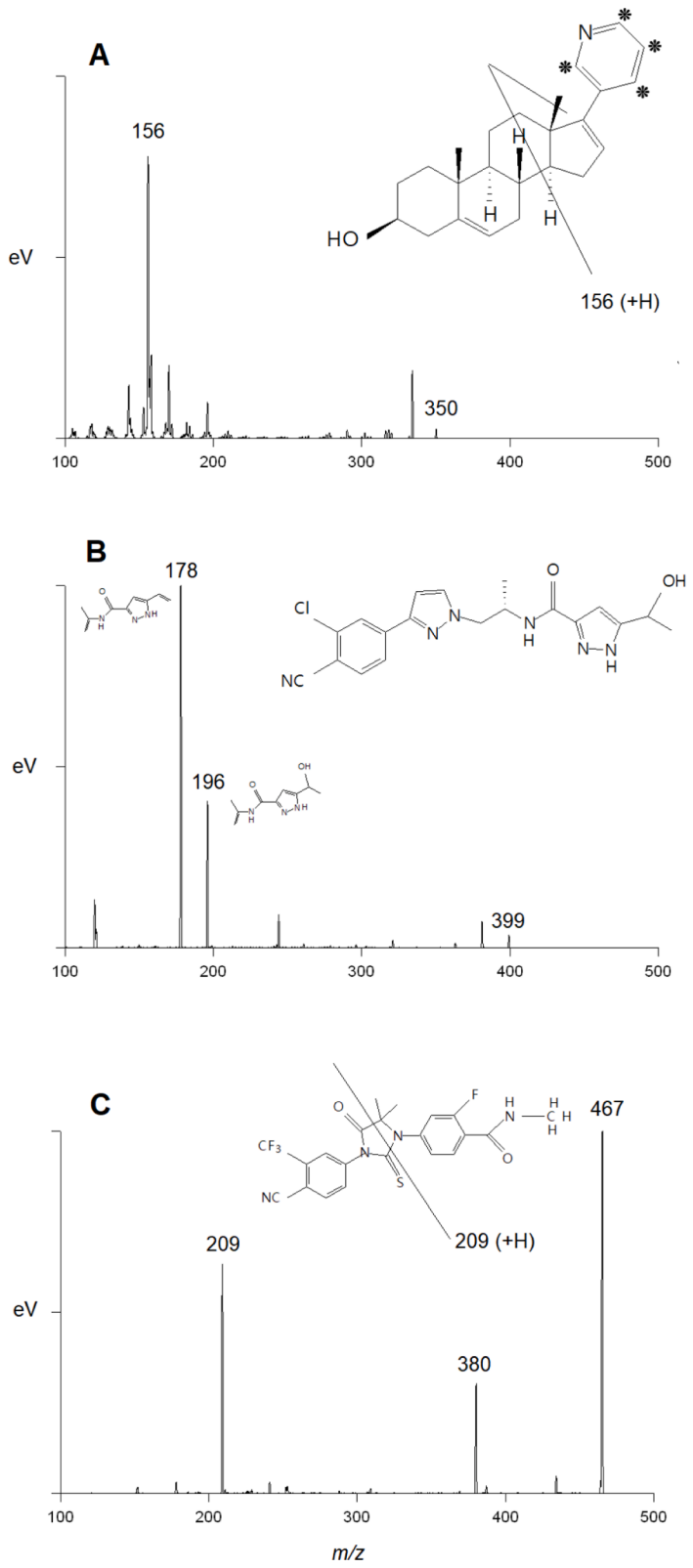

Fig. 1. Mass spectrum and chemical structure of abiraterone (A), darolutamide (B) and enzalutamide (C). The asterisks represent the deuterium atoms in the stably labeled internal standard abiraterone-d4.

temperature for a 3-day period and by subjecting quality control samples to three freeze–thaw cycles. Samples were thawed at room temperature and refrozen again at −80 °C. Stability of processed samples in the thermostatted autosampler was tested by repeated injections of processed samples. Evaluation of the matrix effect and recovery was tested at the concentrations of QC-Low and QC-High, as described previously [19].

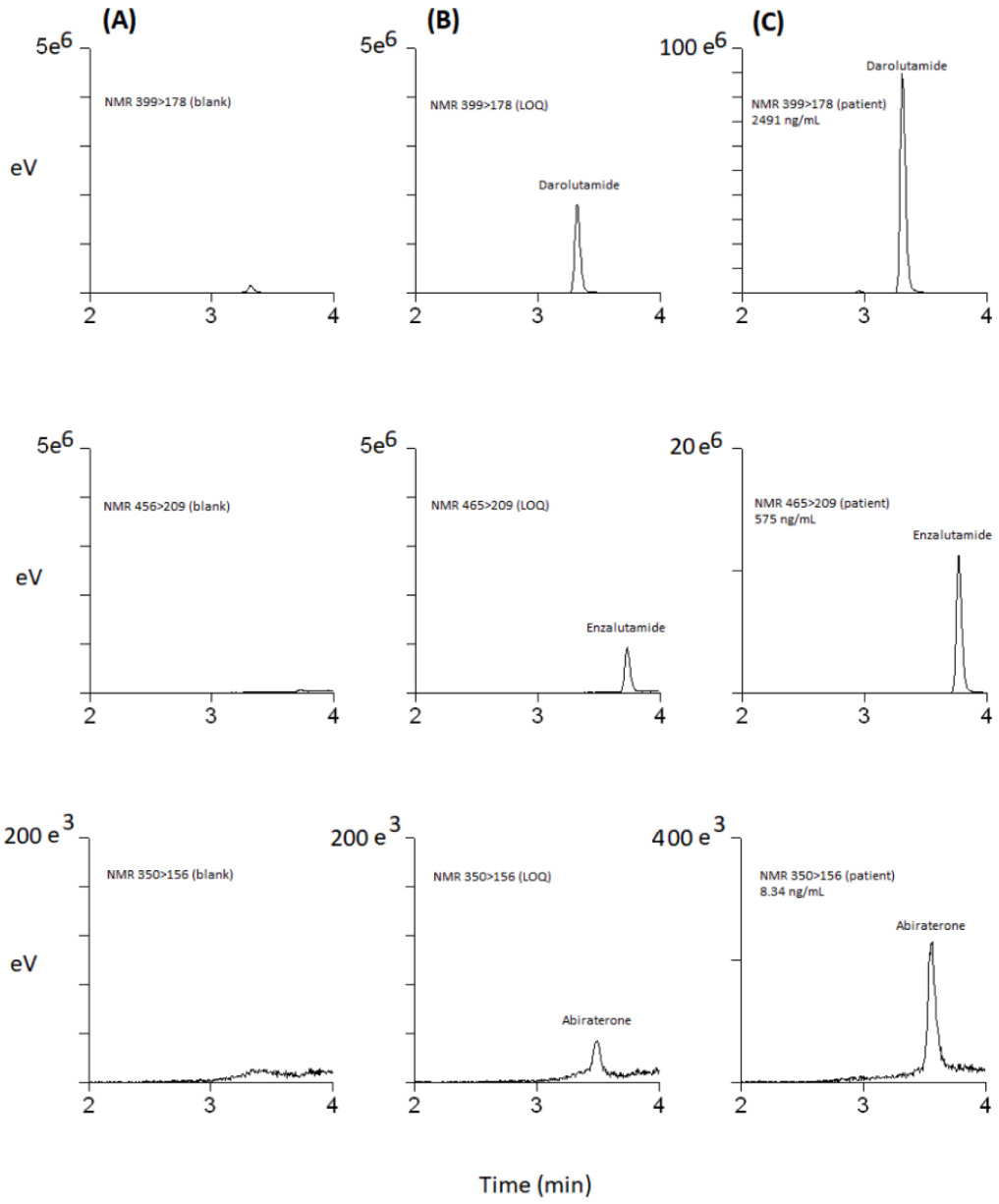

Fig. 2. Chromatograms of a blank human plasma sample (A), LOQ sample (B) and a patient sample (C) respectively for darolutamide, enzalutamide and abiraterone.

2.6. Stability of abiraterone

During the initial method validation, abiraterone was found to be highly unstable in fresh human plasma. Therefore, additional tests regarding the stability of abiraterone in a patient sample, after routine clinical blood processing (obtaining blood from a patient, shipping and storage at T < 7 °C for 3 days) were performed.

2.7. Application of method to clinical samples

To demonstrate the applicability of the validated bioanalytical method, blood samples were collected in the context of TDM for abiraterone and for two clinical studies in which enzalutamide and darolutamide, respectively, were administered orally in combination with cabazitaxel chemotherapy in metastatic CRPC patients [22,23]. Enzalutamide was dosed once daily at 140 mg, while darolutamide was dosed twice daily at a dose of 600 mg, according to the labels of the drugs. Samples were collected in EDTA tubes and processed within 15 min for plasma isolation, which was stored at T < −70 °C before analysis as described.

3. Results

3.1. LC-MS/MS conditions

Optimization of product ions was performed by direct infusion of abiraterone, darolutamide and enzalutamide into the mass spectrometer. Optimal sensitivity was achieved in the positive ion mode (electrospray positive). Product ion spectra (Fig. 1) showed multiple product ions suitable for MRM analysis with a main product ion of *m/z* 156 for abiraterone, *m/z* 178 and 196 for darolutamide, *m/z* 209 and 380 for enzalutamide and *m/z* 160 for the internal standard abiraterone d4. Abiraterone and the internal standard yielded only one product ion. Product ion *m/z* 178 and *m/z* 209 for darolutamide and enzalutamide respectively were selected as the MRM ion for quantitation, while the primary to secondary ion ratios of darolutamide and enzalutamide were used to show the quality of the observed peaks. Best performance was achieved by eluting at a flow-rate of 0.400 mL/min and a linear gradient

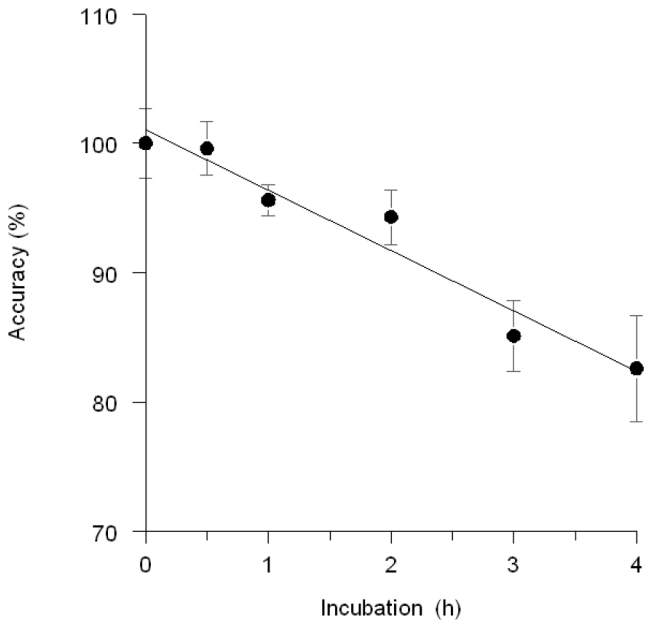

Fig. 3. Stability of abiraterone in human plasma at ambient temperature.

Table 1
Stability of darolutamide, enzalutamide and abiraterone in human plasma.

Condition	(% to concentration at the initial time point)		
	QC-Low	QC-High	QC-Diluted
Darolutamide			
Ambient temp (3-days)	109	89.6	123
3 freeze/thaw cycles	104	95.9	129
Processed sample (T = 10 °C, 20 h)	99.7	99.8	101
Enzalutamide			
Ambient temp (3-days)	103	101	101
3 freeze/thaw cycles	101	90.3	109
Processed sample (T = 10 °C, 20 h)	109	107	110
Abiraterone			
Ambient temp (3-days)	ND[1]	18.8	20.2
3 freeze/thaw cycles	91.3	93.6	93.9
Processed sample (T = 10 °C, 20 h)	97.8	97.7	101

[1] : Not Detectable.

with water and ACN containing 0.2% FA. A short analysis time of 4 min was maintained, with abiraterone and the internal standard eluting at 3.6 min, darolutamide at 3.2 min and enzalutamide at 3.7 min (Fig. 2).

3.2. Stability

The reason for setting up the current method for abiraterone was TDM [24]. Therefore, the stability of abiraterone was investigated in a patient sample during routine clinical blood processing. Abiraterone showed to be unstable during blood processing and during storage of a whole blood sample at ambient temperature or at T < 7 °C, with a loss of 40%. Furthermore, abiraterone was highly unstable in human plasma, with a 17% decrease in 4 h at ambient temperature (Fig. 3) and a >80% decrease after 3 days at ambient temperature (Table 1). Therefore patient samples should be processed within 30 min and plasma should be stored at T < −70 °C immediately. In contrast, darolutamide and enzalutamide showed to be stable in human plasma when stored for 3 days at ambient temperature. Abiraterone, enzalutamide, and darolutamide showed to be stable during three freeze–thaw cycles, where samples were thawed at room temperature and refrozen again shortly after thawing. Autosampler stability was tested for 20 h and abiraterone, enzalutamide, and darolutamide showed to be stable as processed samples in the chilled (T = 10 °C) autosampler for at least 20 h (Table 1).

Table 2
Within-run precision (WRP), Between-run precision (BRP) and the average accuracy (ACC) of the LOQ and QC-samples.

Concentration (ng/mL)	GM	WRP (%)	BRP (%)	ACC (%)	n
Darolutamide					
50.0 (LOQ)	46.9	2.39	1.89	93.8	5
150	146	1.70	1.93	97.3	5
2000	1883	1.44	0.75	94.2	5
4000	3810	1.64	0.97	95.3	5
20,000	18,997	2.60	[#]	95.0	5
Enzalutamide					
50.0 (LOQ)	44.4	2.17	0.41	88.8	5
150	137	2.17	[#]	91.3	5
2000	1,798	1.40	0.68	89.9	5
4000	3,606	0.63	1.67	90.2	5
20,000	18,143	2.47	1.33	90.7	5
Abiraterone					
1.00 (LOQ)	0.945	10.8	6.78	94.5	5
3.00	3.15	6.00	3.48	105.0	5
40.0	44.1	3.03	1.28	110.3	5
80.0	85.3	1.73	[#]	106.6	5
800	857	2.52	3.67	107.1	5

[#] : No additional variation observed by performing the assay in different runs.

3.3. Assay performance

The results of the method were linear in the concentration range of 50.0–5000 ng/mL with weighted linear regression of 1/concentration^2 in human plasma for darolutamide and enzalutamide, and 1.00–100 ng/mL with weighted linear regression of 1/concentration^2 for abiraterone. The LOQ was validated at 50.0 ng/mL in human plasma for darolutamide and enzalutamide and 1.00 ng/mL for abiraterone. For darolutamide and enzalutamide, all of the ten independently spiked plasma samples fell within the acceptable range of accuracy of 80–120%, while for abiraterone nine of the ten independently spiked plasma samples fell within the acceptable range of accuracy of 80–120%. The within-run and between-run precisions and the accuracies, including those at the level of the LOQ, are summarized in Table 2. All fell within the accepted ranges as specified by the Food and Drug Administration [18]. QC-Diluted was validated after a 20-times dilution in human plasma before processing. Response for darolutamide, enzalutamide and abiraterone in blank processed plasma sample after the highest concentration of the calibration line was <20% of the response observed for the LOQ. Therefore no carry-over was observed. No matrix effect was observed while the recovery was close to 100% for all compounds, determined at the concentration of QC-Low and QC-High in six different lots of human plasma.

3.4. Clinical application

A typical chromatogram of a patient treated with abiraterone is displayed in Fig. 2**C**. Enzalutamide concentrations were successfully determined in 14 metastatic CRPC patients, participating in a pharmacokinetic study on the drug-drug interaction between cabazitaxel and enzalutamide [22]. After oral administration of enzalutamide, twelve blood samples were drawn in 24 h (i.e. before the subsequent dosage) for determination of the Area Under the Curve from 0 to 24 h ($AUC_{0\text{-}24h}$), the maximum observed concentration (C_{max}) and the minimum observed concentration (C_{min}). The geometric mean (%CV) C_{max} of enzalutamide in 14 patients was 14,082 ng/mL (18%) (Fig. 4**A**) and the geometric mean C_{min} was 10,719 ng/mL (19%) (Fig. 4**B**). Darolutamide

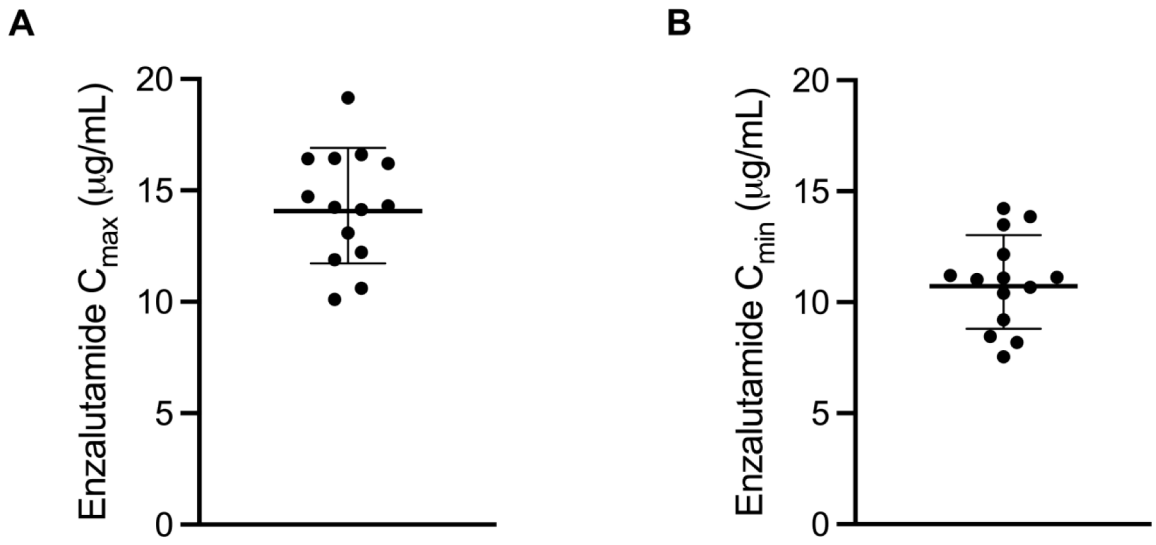

Fig. 4. Enzalutamide C_{max} (A) and C_{min} (B) (µg/mL) in 14 patients on steady state enzalutamide. Geometric mean and SD are shown.

concentrations were successfully determined in 18 metastatic CRPC patients. The geometric mean C_{max} and C_{min} of darolutamide in 18 patients was 4,791 ng/mL (39%) and 1883 ng/mL (79%), respectively. Additional pharmacokinetic parameters of darolutamide in these patients are described elsewhere [23].

4. Discussion

A sensitive, selective, accurate, and precise LC-MS/MS method was developed and validated for the analysis of abiraterone, enzalutamide, and darolutamide in human plasma. The performance of the assay was successful for all three compounds, using only one internal standard, abiraterone d4. The assay meets all of the current requirements of bioanalytical method validation.

In contradiction to previously described analytical methods for abiraterone quantification, we observed abiraterone to be highly unstable at ambient temperature [25–28]. Only one study reported abiraterone to be unstable in patient samples, as compared to spiked plasma samples [27]. We observed abiraterone even to be significantly unstable in QC samples at ambient temperature. Therefore, patient samples should be processed within 30 min and plasma should be stored at T < −70 °C immediately. By contrast, multiple freeze–thaw cycles did not seem to impact abiraterone concentrations in human plasma significantly. This is in line with our results that abiraterone concentrations decrease at a rate of 4% per hour. Methods that use differently prepared samples, such as dried plasma spots, may also be useful for quantification of abiraterone in patient samples [29]. However, the described method makes use of more advanced MS systems compared to the assay described here and could therefore complicate implementation in most laboratories. Furthermore, compared to a method making use of paper spray ionization, our data showed a higher accuracy [30]. Similar to previously published assays on enzalutamide and darolutamide, we report a robust assay for their determination and a proper stability of these analytes under various circumstances [27,28,31].

Overall, the method described here showed to be useful for TDM as well as for clinical pharmacokinetic studies with enzalutamide or darolutamide. Furthermore, we demonstrated the applicability of the determination of enzalutamide and darolutamide concentrations in two pharmacokinetic studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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