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Short communication

An LC–MS/MS method for quantification of the active abiraterone metabolite Δ(4)-abiraterone (D4A) in human plasma

Merel van Nuland[a,f,*], Hilde Rosing[a], Jelle de Vries[b,c], Huib Ovaa[b,c], Jan H.M. Schellens[d,e,f], Jos H. Beijnen[a,e,f]

[a] Department of Pharmacy & Pharmacology, Antoni van Leeuwenhoek/The Netherlands Cancer Institute and MC Slotervaart, Amsterdam, The Netherlands
[b] Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands
[c] Department of Chemical Immunology, Leiden University Medical Center, Leiden, The Netherlands
[d] Division of Clinical Pharmacology, Department of Medical Oncology, Antoni van Leeuwenhoek/The Netherlands Cancer Institute, Amsterdam, The Netherlands
[e] Division of Pharmacoepidemiology and Clinical Pharmacology, Faculty of Science, Department of Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands
[f] Division of pharmacology, Antoni van Leeuwenhoek/The Netherlands Cancer Institute, Amsterdam, The Netherlands

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ABSTRACT

Δ(4)-Abiraterone (D4A) is a recently discovered active metabolite of the oral anti-androgen drug abiraterone acetate. For quantification of this metabolite in human plasma, a liquid chromatography-tandem mass spectrometry (LC–MS/MS) method was developed and validated. Human plasma samples of patients treated with abiraterone acetate were prepared by protein precipitation with acetonitrile. The method was validated over a linear range of 0.2–20 ng/mL. Intra-assay and inter-assay variabilities were within ± 15% of the nominal concentrations for quality control (QC) samples at medium and high concentrations and within ± 20% at the lower limit of quantification (LLOQ), respectively. The described method for quantification of D4A was validated successfully and implemented to support therapeutic drug monitoring in patients treated with abiraterone acetate.

1. Introduction

Abiraterone acetate is an oral drug for the treatment of metastatic castration-resistant prostate cancer [1]. Its anti-androgen capacities can be allocated to 17α-hydroxylase/C17,20-lyase (CYP17) inhibition [2]. CYP17 is responsible for the production of androgens, such as testosterone, which are natural ligands for the androgen receptor. Inhibition of this receptor reduces testosterone levels and prolongs the survival of prostate cancer patients [3].

Abiraterone undergoes extensive hepatic metabolism. The main circulating metabolites abiraterone sulfate and abiraterone N-oxide sulfate account for about 43% of exposure each and are inactive [1,4]. Li et al. recently discovered the active metabolite Δ(4)-Abiraterone (D4A), that is formed by conversion of abiraterone by the enzyme 3β-hydroxysteroid-dehydrogenase (3βHSD). D4A inhibits multiple steroidic enzymes and blocks androgen receptor signaling. This combined mechanism of action makes D4A even more active than abiraterone [5].

We recently published a liquid chromatography-mass spectrometry (LC–MS/MS) method for determination of abiraterone, enzalutamide and their major metabolites to support therapeutic drug monitoring (TDM) of these compounds [6]. D4A possesses relevant anti-androgen capacities that contribute to the efficacy of abiraterone treatment in prostate cancer and is therefore a relevant metabolite to be incorporated in the previously published assay. The aim of the presented study was to include D4A in the existing assay for quantification of abiraterone, enzalutamide and their major metabolites to obtain further insight into the metabolism of these drugs to optimize the treatment of prostate cancer patients.

2. Experiments

2.1. Chemicals

D4A was produced at the Chemical Immunology laboratory, Leiden University Medical Centre (LUMC, Leiden, the Netherlands) according to a previously published method by Li et al. [7]. Abiraterone and $^{2}H_{4}$-abiraterone were purchased from Alsachim (Illkirch, France). Acetonitrile, methanol (both Supra-Gradient grade), water, and formic acid

Abbreviations: D4A, Δ(4)-Abiraterone; CYP17, 17α-hydroxylase/C17,20-lyase; LC–MS/MS, liquid chromatography-mass spectrometry; IS, internal standard; QC, quality control; LLOQ, lower limit of quantification; DAD, diode array detection; TDM, therapeutic drug monitoring

* Corresponding author at: Department of Pharmacy & Pharmacology, Antoni van Leeuwenhoek/The Netherlands Cancer Institute and MC Slotervaart, Amsterdam, The Netherlands.
E-mail address: m.v.nuland@nki.nl (M. van Nuland).

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(both LC–MS grade) were from Biosolve Ltd. (Valkenswaard, The Netherlands). Dimethyl sulfoxide (DMSO, seccosolv grade) was obtained from Merck (Darmstadt, Germany) and K_2EDTA plasma from Bioreclamations LLC (Hicksville, NY, USA).

2.2. Calibration and quality control samples

Stock solutions of D4A and the internal standard (IS) $^2H_4$-abiraterone were prepared at a concentration of 1 mg/mL in DMSO and methanol, respectively.

Calibration standards and quality control (QC) samples were prepared from stock solutions in K_2EDTA plasma. The calibration standards were freshly produced before each validation run in a concentration range of 0.2–20 ng/mL. QC samples were produced in batches at concentrations of 0.2, 8 and 20 ng/mL. The IS working solution contained 25 ng/mL of $^2H_4$-abiraterone. Stock solutions, working solutions and QC samples were stored at −20 °C.

2.3. Sample preparation

Samples were collected in the clinic by venipuncture and centrifuged for 5 min at 4 °C at 1800 g. After centrifugation, plasma was isolated and stored at −20 °C until further analysis. Samples were thawed and vortex-mixed prior to processing, and a 50 µL aliquot was used for analysis. Fifteen microliters of IS working solution and, after mixing, 150 µL of acetonitrile were added to precipitate proteins. Samples were shaken for 10 min at 1250 rpm and centrifuged for 10 min at 20 °C at 23,100*g*. The supernatant was transferred to an autosampler vial.

2.4. Liquid chromatography-tandem mass spectrometry

The chromatographic separation was performed using a Nexera 2 series liquid chromatograph equipped with a Nexera 2 series binary pump, a degasser, an autosampler, and a valco valve (Shimadzu Corporation, Kyoto, Japan). The autosampler temperature was kept at 4 °C and the column oven at 45 °C. Analytes were separated using a Kinetex C18 column (15 × 2.1 mm ID, particle size 2.6 µm; Phenomenex, Torrance, CA, USA) with mobile phase A consisting of formic acid-water (0.1:100, v/v) and mobile phase B consisting of formic acid-methanol (0.1:100, v/v). The following gradient program was used to achieve separation: 30% B (0.0–2.0 min), 70% B (2.0–10 min), 30% B (10–13 min) at a flow rate of 0.3 mL/min. A triple quadrupole mass spectrometer API6500 (Sciex, Framingham, MA, USA) operating in positive mode was used for quantification of D4A. The instrument was equipped with a turbo ion spray (TIS) interface and was configured in multiple reaction monitoring (MRM) mode. Analyst software version 1.6.2 (Sciex) was used for system control and data analysis. Table 1 summarizes the general and specific mass spectrometric settings.

2.5. Identification and purity of the reference standard D4A

The identity and purity of the D4A reference standard were determined. Identification was performed by nuclear magnetic resonance (NMR) and LC–MS and the purity was determined by LC-UV (diode array detection, DAD) and LC–MS. For NMR, a solution of 7.5 mg/mL D4A was prepared in deuterated chloroform and this solution was further diluted to 0.26 mg/mL in formic acid-acetonitrile-water (0.1:1:100, v/v) for LC–MS and LC-UV analysis.

NMR was performed with a Bruker Avance 300 (75.00 MHz for 13C) using the residual solvent as internal standard. LC–MS and UV for identification were done with an LCT Premier equipped with an LC Allience 2795 and PDA1996 (Waters, Milford, MA, USA). Chromatographic separation was performed using an XBridge column (30 × 2.1 mm ID, particle size 10 µm, Waters), using mobile phase A consisting of formic acid-acetonitrile-water (0.1:1:100, v/v) and mobile phase B consisting of formic acid-water-acetonitrile (0.1:1:100, v/v). The following gradient was applied to the column with a 0.8 mL/min flow: 5% B (0.0–0.2 min), 5% → 95% B (0.2–3.2 min), 95% B (3.2–4.2 min), 95% → 5% B (4.2–4.4 min), 5% B (4.4–6.2 min).

Table 1
General and analyte specific mass spectrometric parameters.

General settings		
Run duration (min)	13.5	
Ion spray voltage (V)	5500	
Nebulizer gas (au)	40	
Turbo gas/heater gas (au)	40	
Curtain gas (au)	20	
Collision gas (au)	8	
Temperature °C	350	
Dwell time (msec)	50	
Analyte specific settings	D4A	$^2H_4$-abiraterone
Parent mass	348.3 *m/z*	354.1 *m/z*
Product mass	156.1 *m/z*	160.1 *m/z*
Collision energy	61 V	63 V
Collision exit potential	18 V	10 V
Declustering potential	171 V	186 V
Retention time	6.16 min	7.08 min

To establish the D4A purity we used an LC-20AD pump with a SIL-HTc autosampler (Shimadzu, Kyoto, Japan) coupled to a LTX-XL linear ion trap mass spectrometer (Thermo Electron, Waltham, MA, USA) in combination with diode array detection (DAD). Chromatographic conditions were as described for the LC–MS identification method However, the flow was reduced to 0.2 mL/min in order not to exceed the upper pressure limit. After chromatographic separation, a post column splitter directed 1/4th of the flow to the MS/MS and 3/4th to the DAD. The peaks in the UV chromatogram could therefore be directly correlated with the retention time of the peaks in the LC chromatogram. Peak identification of D4A and abiraterone were assessed with LC–MS/MS and purity was determined by LC-DAD 254 nm.

Additional peaks in the chromatograms beside the D4A and abiraterone peaks and not observed in the blanks, were assigned as unknown impurities. The total area of these unknown impurities was expressed as relative impurity compared to the peak area of D4A. Furthermore, the amount of abiraterone in the D4A reference standard was determined with a validated LC–MS/MS method [6].

2.6. Validation procedures

Validation of the assay was based on the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation [8,9]. All aspects of the validation were investigated. However, four instead of six to eight calibrators were included and three instead of four QC concentrations were prepared. These adaptations were made since the method will be used for routine TDM. Therefore, we focused on the development of a fast turn-around method, while still offering a bioanalytical validation approach.

2.7. Clinical application

The applicability of the assay for TDM was demonstrated with steady-state plasma samples of patients receiving abiraterone acetate, collected after at least one week after the start of the abiraterone treatment (half-life of 16.3 h [10]). Samples were collected for routine TDM at the Netherlands Cancer Institute according to the declaration of Helsinki.

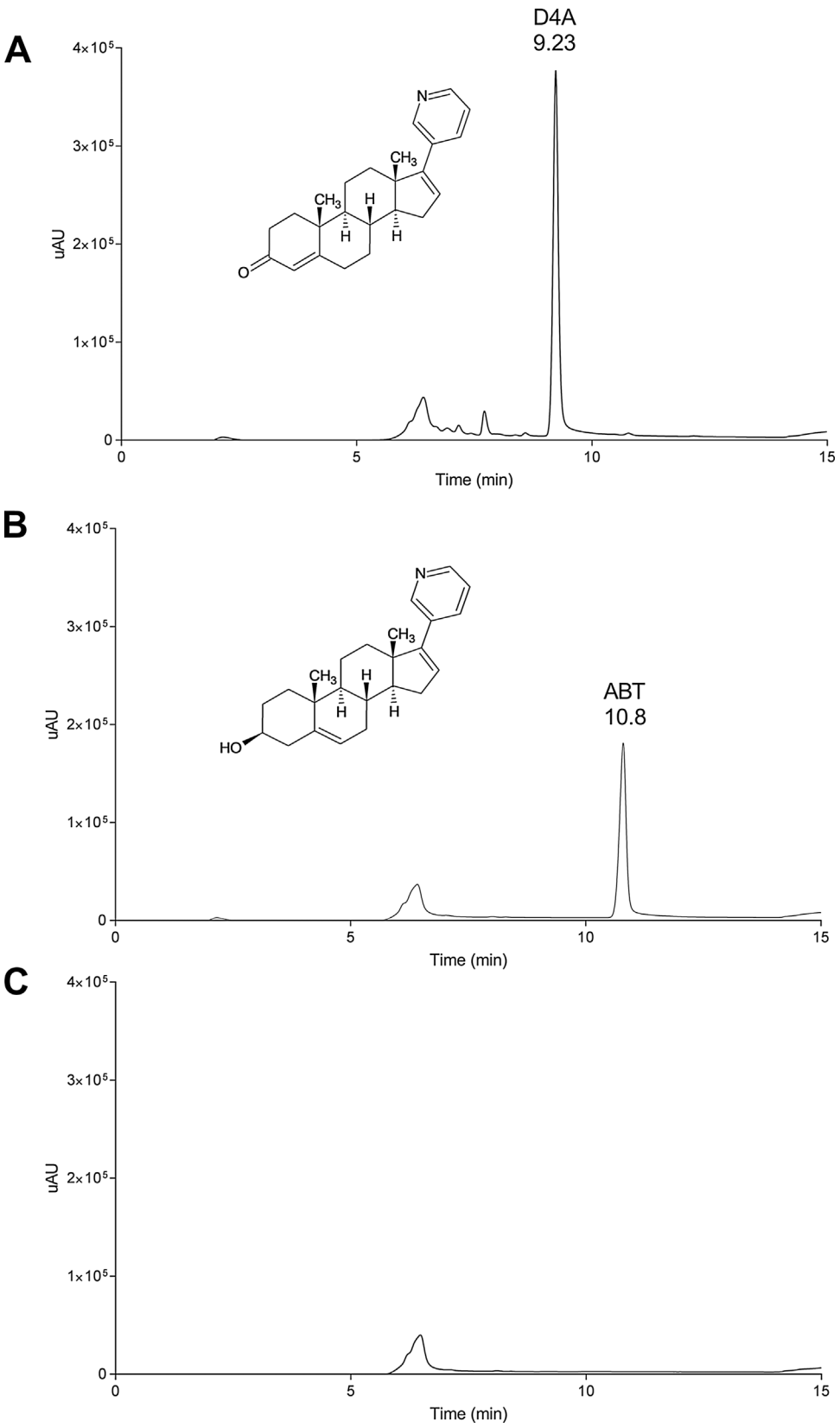

Fig. 1. Representative UV chromatograms of D4A (A), abiraterone (ABT) (B) and water (C) at λ = 254 nm.

3. Results and discussion

3.1. Identification and purity of the reference standard D4A

The identity of D4A was determined using NMR and LC–MS. The position and number of chemical shifts in the NMR spectrum were diagnostic of the structure of D4A, as presented in literature [7]. Furthermore, the MS spectrum clearly showed a response at *m/z* 348 corresponding to the protonated parent mass of D4A.

The percentage of unknown impurities was determined with LC-UV.

Fig. 1 shows the UV chromatograms of D4A, abiraterone and a blank sample. D4A and abiraterone elute at a retention time of 9.23 min and 10.8 min, respectively. Ten unknown impurities were visible in the D4A chromatogram and the total peak area of these impurities accounted for 8.30% of the D4A peak area. The percentage of abiraterone in the reference standard, determined with LC–MS/MS, was 0.938%. Taken together, the assigned purity of the reference standard was 90.8% (100%-8.30%-0.938%) and a correction factor of 0.908 was used to calculate the D4A concentration in the stock solutions that were used for the preparation of calibration standards and QC samples during the validation and routine application of the method.

3.2. Validation procedures

3.2.1. Calibration curve

Calibration standards were analyzed in duplicate in three separate analytical runs. Linear regression was used with a weighting factor of $1/x^2$ to fit the calibration data (peak area ratios versus the concentration of D4A). The calibration range of D4A consisted of four calibration standards with concentrations of 0.2, 1, 10 and 20 ng/mL. The calibration plots were consistent and the back-calculated D4A concentrations were within the requirements, as at least 75% of the calibration standards were within ± 15% (± 20% for the lower limit of quantification (LLOQ)) of the nominal concentrations.

3.2.2. Accuracy and precision

Five replicates of QC LLOQ (0.182 ng/mL), QC Mid (7.28 mg/mL) and QC High (18.2 g/mL) were analyzed in three consecutive runs. Accuracy was expressed as the relative error (% deviation) and one-way ANOVA was used to calculate the intra- and inter-assay variation. The acceptance criteria for accuracy were within ± 15% for QC mid and QC high and within ± 20% for QC LLOQ. Precisions should be ≤15% for QC mid and QC high and for QC LLOQ the criterion was set to ≤20%. As shown in Table 2, all parameters were within the acceptance criteria.

3.2.3. Specificity and selectivity

Six separate batches of blank human K_2EDTA plasma were spiked at the LLOQ level and were processed together with blank samples to assess whether endogenous constituents interfere with the assay. The accuracy of the LLOQ samples was within 80–120% of the nominal concentration in all batches of plasma and no interference was observed in the blanks at the retention time of the analyte with areas > 20% (or > 5% for the internal standards) of the LLOQ areas in all tested batches.

Cross-analyte interference was tested by spiking blank human plasma separately at the highest concentration of the calibration range (upper limit of quantification, ULOQ) with D4A, abiraterone, abiraterone sulfate, abiraterone N-oxide sulfate, enzalutamide, desmethyl-enzalutamide or enzalutamide carboxylic acid. Internal standard interference was tested by spiking blank samples separately at nominal concentrations of internal standard. 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.

The interference of D4A at the retention time of abiraterone was 49% and the interference of abiraterone sulfate at the retention time of D4A was 100%. These percentages exceeded the acceptance criteria of 20% and which can be explained by impurities in the reference standards of D4A and abiraterone sulfate. The impurity of abiraterone in the D4A reference standard will have no significant influence in the quantification of abiraterone, since the calibration range of D4A is 5-fold lower than the calibration range of abiraterone. Therefore, this interference was considered acceptable. However, the interference of abiraterone sulfate at the retention time of D4A was unacceptably high as the concentration range of abiraterone sulfate is 500-fold higher than the calibration range of D4A. Therefore, separate calibration standards should be prepared for abiraterone sulfate while combined calibration standards can be prepared for D4A, abiraterone, abiraterone N-oxide sulfate, enzalutamide, desmethyl-enzalutamide and enzalutamide carboxylic acid. The interference of other analytes and internal standards was ≤20% (≤5% for the IS) of the peak area in LLOQ samples and therefore within the acceptance criteria.

Table 2
Assay performance data for D4A in human plasma tested at LLOQ, mid-, and high concentrations.

Run	Nominal conc. (ng/mL)	Measured conc. (ng/mL)	Inaccuracy (% deviation)	Precision (%)	No. of replicates
1	0.182	0.183	0.8	10	5
2	0.182	0.170	−6.6	5.8	5
3	0.182	0.186	2.2	8.1	5
Inter-assay	0.182	0.180	−1.2	3.0	15
1	7.28	7.01	−3.8	5.2	5
2	7.28	6.74	−7.4	6.7	5
3	7.28	7.27	−0.1	2.2	5
Inter-assay	7.28	7.01	−3.8	3.1	15
1	18.2	18.8	3.5	5.3	5
2	18.2	19.4	6.6	5.3	5
3	18.2	19.4	6.8	4.9	5
Inter-assay	18.2	19.2	5.6	-[a]	15

[a] Inter-run precision could not be calculated because mean square between group was less then mean square within groups.

3.2.4. Dilution integrity

The concentrations of 10-fold diluted samples (30 µL sample in 270 µL control K_2EDTA plasma) were within the criteria of ± 15% for accuracy and ≤15% for precision in five replicates. From these data it can be concluded that samples exceeding the ULOQ can be diluted up to 10-fold to obtain plasma concentrations within the validated range.

3.2.5. Carry-over

Two blank samples were injected after the ULOQ to determine the carry-over. In three separate analytical runs, the peak areas in blank samples were ≤20% of the peak areas in the LLOQ and therefore considered acceptable.

3.2.6. Matrix effect

The matrix effect was investigated for six different batches of blank human K_2EDTA plasma at QC LLOQ and QC high concentration. The matrix factor (MF) was calculated by comparison of the D4A response in presence and in absence (acetonitrile-water (50:50, v/v)) of the biomatrix. The following formula was used to calculate the IS-normalized MF:

$$IS-normalized\ MF = \frac{MF\ of\ the\ analyte}{MF\ of\ the\ internal\ standard} \quad (1)$$

The IS-normalized MF ranged from 1.23 to 1.45. The CV for the IS-normalized matrix factor at LLOQ and high concentration was respectively 3.1% and 7.9% and fulfilled the criteria (≤15%).

3.2.7. Stability

Stability experiments were performed in triplicate at QC LLOQ and QC high levels. D4A was 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. In plasma, D4A was stable for at least 5 days at ambient temperature (20–25 °C), for at least a month at −20 °C and after 3 freeze/thaw cycles (4 °C/20–25 °C). Final extracts could be injected up to 3 days after sample preparation.

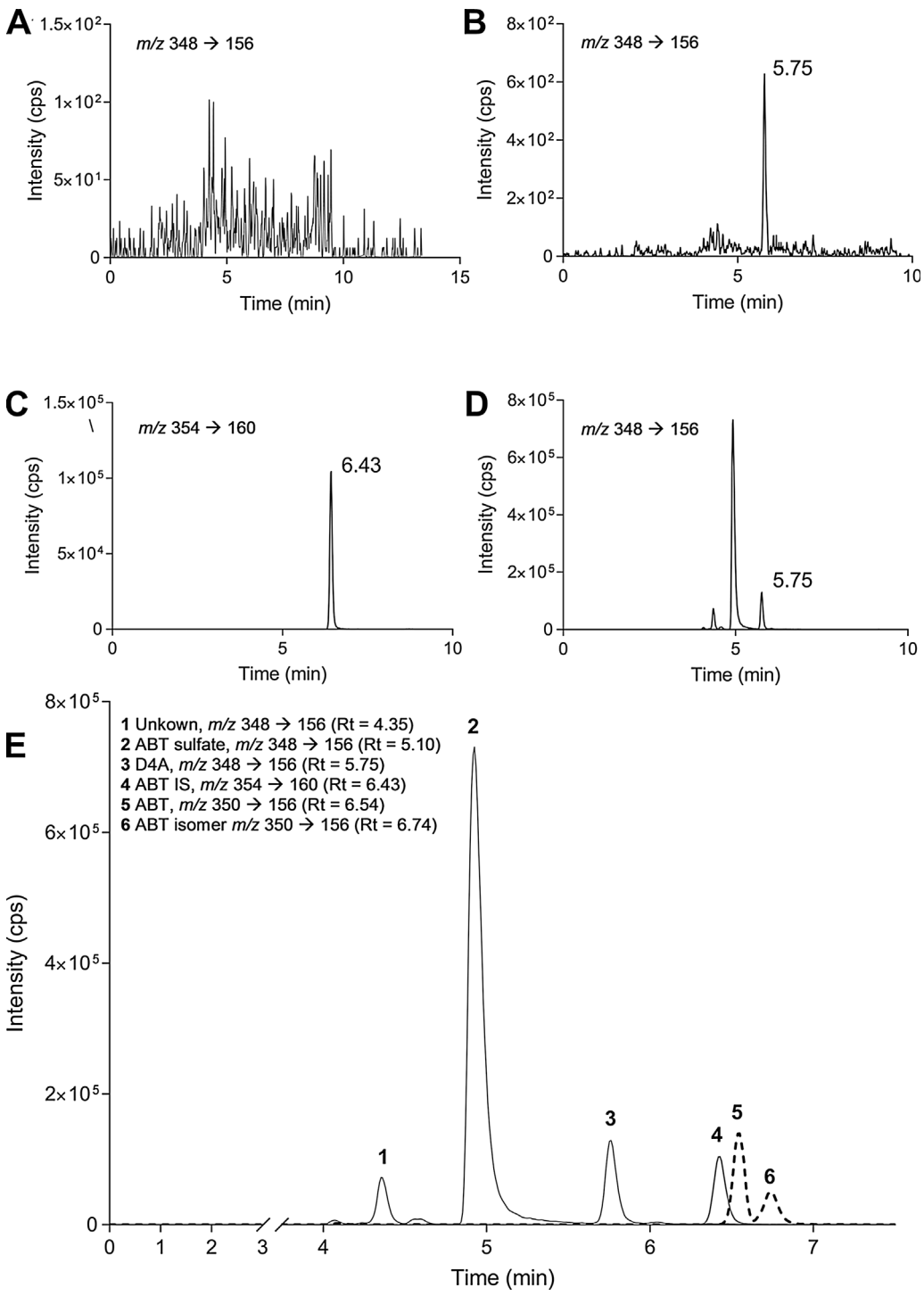

Fig. 2. Representative LC-MS/MS chromatograms of a blank sample (A), D4A LLOQ (B), $^{2}H_{4}$-abiraterone (C), D4A in steady-state plasma from a patient using abiraterone acetate (D) and a chromatogram showing abiraterone, D4A and $^{2}H_{4}$-abiraterone in a steady-state plasma sample collected from a patient using abiraterone acetate (E).

4. Clinical application

Steady-state plasma samples of 15 patients receiving abiraterone acetate were analyzed; all results were within the validated range. Representative selective ion chromatograms of a blank sample, spiked calibration standards and a patient sample at steady-state (abiraterone acetate 1000 mg daily dose) is depicted in Fig. 2. D4A elutes at 5.75 min and abiraterone at 6.54 min. Another peak at a retention time of 5.10 min belongs to the metabolite abiraterone sulfate and is observed in the transitions of abiraterone and D4A. Measured plasma concentrations of D4A and abiraterone are presented in Table 3 with a median concentration of 1.99 ng/mL (0.329-12.1 ng/mL) and 32.6 ng/mL (0.980–452 ng/mL), respectively. The median conversion ratio of abiraterone to D4A was 6.56%, which is comparable to the 5% conversion ratio as described in literature [11]. Interpatient variability of D4A plasma concentrations and conversion ratios demonstrate the additional relevance of monitoring D4A in plasma of patients treated with abiraterone acetate.

5. Conclusion

An LC–MS/MS method for the quantification of D4A was validated successfully over a concentration range of 0.2–20 ng/mL. A median D4A steady-state plasma concentration of 1.99 ng/mL and a 6.56%

Table 3
Steady-state plasma concentrations of D4A and abiraterone and the conversion ratio.

Patient number	D4A conc. (ng/mL)	ABT conc. (ng/mL)	Conversion ratio (%, D4A/ABT)
1	1.30	5.06	25.7
2	1.00	15.9	6.30
3	2.54	38.7	6.56
4	2.13	32.6	6.53
5	12.1	452	2.69
6	1.99	44.8	4.44
7	0.329	4.47	7.36
8	0.458	0.980	46.7
9	0.361	1.00	36.1
10	1.50	14.8	10.1
11	1.70	32.0	5.31
12	7.91	417	1.90
13	3.31	41.5	7.98
14	3.35	44.1	7.60
15	10.4	207	5.02
Median (range)	1.99 (0.329–12.1)	32.6 (0.980–452)	6.56 (1.90–46.7)

Abbreviations: conc = concentration, ABT = abiraterone.

conversion ratio of abiraterone to D4A were determined for 15 patients treated with abiraterone acetate. The active metabolite D4A has been successfully incorporated in the assay for quantification of abiraterone, enzalutamide and their major metabolites to support TDM.

Conflicts of interest

None.

Funding

None.

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