Development and validation of an UPLC-MS/MS method for the therapeutic drug monitoring of oral anti-hormonal drugs in oncology

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ABSTRACT

A liquid chromatography-mass spectrometry assay was developed and validated for simultaneous quantification of anti-hormonal compounds abiraterone, anastrozole, bicalutamide, Δ(4)-abiraterone (D4A), *N*-desmethyl enzalutamide, enzalutamide, Z-endoxifen, exemestane and letrozole for the purpose of therapeutic drug monitoring (TDM). Plasma samples were prepared with protein precipitation. Analyses were performed with a triple quadrupole mass spectrometer operating in the positive and negative ion-mode. The validated assay ranges from 2 to 200 ng/mL for abiraterone, 0.2–20 ng/mL for D4A, 10–200 ng/mL for anastrozole and letrozole, 1–20 ng/mL for Z-endoxifen, 1.88–37.5 ng/mL for exemestane and 1500–30,000 ng/mL for enzalutamide, *N*-desmethyl enzalutamide and bicalutamide. Due to low sensitivity for exemestane, the final extract of exemestane patient samples should be concentrated prior to injection and a larger sample volume should be prepared for exemestane patient samples and QC samples to obtain adequate sensitivity. Furthermore, we observed a batch-dependent stability for abiraterone in plasma at room temperature and therefore samples should be shipped on ice. This newly validated method has been successfully applied for routine TDM of anti-hormonal drugs in cancer patients.

1. Introduction

Breast cancer and prostate cancer are the most common malignancies in women and men in the Western world [1]. As these cancer types are highly dependent on growth-stimulating hormones, anti-hormonal therapy is a first-line treatment strategy. Anti-hormonal drugs are generally administered orally or subcutaneously. Oral drugs for treatment of breast cancer include tamoxifen, anastrozole, letrozole and exemestane and oral drugs for prostate cancer therapy include bicalutamide, abiraterone acetate and enzalutamide. The group of oral anticancer drugs is rapidly expanding [2–5], however, most oral anti-hormonal agents have been on the market for a longer period of time.

Although many patients benefit from anti-hormonal therapy in terms of progression-free survival, treatment outcome is variable. This may be attributed to variability in drug levels and exposure. For some anti-hormonal drugs, such as tamoxifen and abiraterone acetate, a clear exposure-response relationship has been described [6–8]. This relationship is the basis for therapeutic drug monitoring (TDM); individualized drug dosing by monitoring drug concentrations in patient blood, plasma or serum. In current practice, oral anti-hormonal drugs are administered at fixed doses, which could lead to suboptimal exposure or high blood concentrations and adverse-events. Recommendations for pharmacokinetic TDM are based on clinical studies and guidelines and proposed targets for anti-hormonal drugs for the treatment of breast cancer and prostate cancer can be found in literature [8]. Ultimately, implementation of individualized dosing with TDM may be an important tool to improve treatment outcome and efficacy in breast cancer and prostate cancer patients.

To facilitate TDM, there is a need for bioanalytical assays to quantify drugs of interest. Liquid chromatography-mass spectrometry (LC-MS/MS) is a useful and often applied analytical technique for determining drug concentrations. When developing an analytical method

Table 1
Concentrations of analytes in stock solution, calibration standards and quality control samples.

Analyte	Stock (mg/mL)	Calibration standards (ng/mL)	Quality control samples (ng/mL)
Abiraterone	1.00 (DMSO)	2; 10; 100; 200	2; 100; 200
Anastrozole	1.00 (methanol)	10; 20; 100; 200	10; 100; 200
Bicalutamide	3.00 (DMSO)	1500; 3000; 15,000; 30,000	1500; 15,000; 30,000
D4A	0.05 (DMSO)	0.2; 1; 10; 20	0.2; 10; 20
N-Desmethyl enzalutamide	3.00 (DMSO)	1500; 3000; 15,000; 30,000	1500; 15,000; 30,000
Z-Endoxifen	0.05 (methanol)	1; 2; 10; 20	1; 10; 20
Enzalutamide	3.00 (acetonitrile)	1500; 3000; 15,000; 30,000	1500; 15,000; 30,000
Exemestane	1.00 (DMSO)	62.5; 125; 625; 1250	1,88; 18,8; 37.5[a]
Letrozole	1.00 (DMSO)	10; 20; 100; 200	10; 100; 200

Abbreviations: D4A = Δ(4)-abiraterone, DMSO = Dimethylsulfoxide.
[a] Exemestane QC samples were concentrated prior to analysis.

Table 2
Above: General mass spectrometric parameters. Below: Analyte specific mass spectrometric parameters for abiraterone, anastrozole, bicalutamide, Δ(4)-abiraterone (D4A), *N*-desmethyl enzalutamide, endoxifen, enzalutamide, exemestane and letrozole. Bicalutamide is measured in negative ion mode, while the other analytes are measured in positive ion mode.

	Positive mode	Negative mode
Run duration	6 min	6 min
Ionspray voltage	5500 V	−4500 V
Nebulizer gas	40 au	30 au
Turbo gas/heater gas	60 au	40 au
Curtain gas	25 au	40 au
Collision gas	9 au	10 au
Temperature	450 °C	450 °C
Dwell time	20 ms	20 ms

	MRM (Da)	Collision energy (V)	Collision exit potential (V)	Declustering potential (V)	Retention time (min)
Abiraterone	350.1 ➜ 156.1	63	10	186	2.3
Anastrozole	294.3 ➜ 225.2	29	10	86	0.78
Bicalutamide	429.6 ➜ 255.2	−20	−19	−35	1.7
D4A	348.3 ➜ 156.1	57	6	111	2.2
N-Desmethyl enzalutamide	453.0 ➜ 197.1	37	18	131	2.0
Z-Endoxifen	374.3 ➜ 58.1	25	14	31	2.4
Enzalutamide	467.0 ➜ 211.1	61	18	171	2.4
Exemestane	297.1 ➜ 121.1	37	8	81	2.1
Letrozole	286.2 ➜ 217.1	17	8	56	0.76

for TDM, it is important to choose a clinically relevant calibration range. This quantitation range should be built around the proposed target concentration, covering the majority of samples as seen in the clinic. Our lab has experience developing and validating methods for TDM of anticancer agents [9–11]. Previously published LC-MS/MS assays for quantification of abiraterone [11–16], anastrozole [17], bicalutamide [13,18–20], Z-endoxifen [21–24], enzalutamide [9,11,13,25–28], exemestane [29,30] and letrozole [31,32] are limited to measuring one to four analytes. Furthermore, there are no articles reporting steady-state concentrations of anastrozole, letrozole and exemestane in humans for TDM purpose, and no articles describing Z-endoxifen analysis in plasma. To our knowledge this is the first bioanalytical assay for simultaneous quantification of six anti-hormonal drugs in oncology, including the active metabolites Z-endoxifen, *N*-desmethyl enzalutamide and Δ(4)-abiraterone (D4A), which enables concurrent quantification of these analytes to efficiently determine plasma concentrations for TDM purpose. Although methods have been developed for the combined analysis of anti-hormonal drugs for either prostate cancer or breast cancer, the development of an assay for both types of anti-hormonal drugs is complicated due to different chemical drug properties. Furthermore, the development of such an assay is challenging because target concentration ranges span from 0.2–30,000 ng/mL, compounds show a variety in MS response and a highly selective chromatographic method is needed to separate isomers of abiraterone and Z-endoxifen.

2. Materials and methods

2.1. Chemicals

Abiraterone, bicalutamide, enzalutamide, *N*-desmethyl enzalutamide, $^2H_4$-abiraterone, $^2H_4$-bicalutamide, $^2H_6$-enzalutamide and $^2H_6$-*N*-desmethyl enzalutamide were purchased from Alsachim (Illkirch Graffenstaden, France). Anastrozole, Z/E-endoxifen, exemestane, letrozole, $^2H_{12}$-anastrozole, $^2H_5$-Z/E-endoxifen, $^2H_3$-exemestane and $^2H_4$-letrozole were purchased from Toronto Research Chemistry (Toronto, Canada). 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. [33]. Acetonitrile, methanol, water and formic acid 99%, used to prepare mobile phase, were obtained from Biosolve Ltd. (Valkenswaard, The Netherlands). Water (distilled) used for sample preparation came from B. Braun Medical (Melsungen, Germany). Dimethyl sulfoxide (DMSO, seccosolv grade) was obtained from Merck (Darmstadt, Germany) and K_2EDTA plasma from Bioreclamations LLC (Hicksville, NY, USA).

2.2. Stock solutions and working solutions

Stock solutions containing abiraterone, anastrozole, bicalutamide, D4A, *N*-desmethyl enzalutamide enzalutamide, Z-endoxifen, exemestane or letrozole were stored in amber-colored containers. Separate stock solutions were prepared for calibration standards and
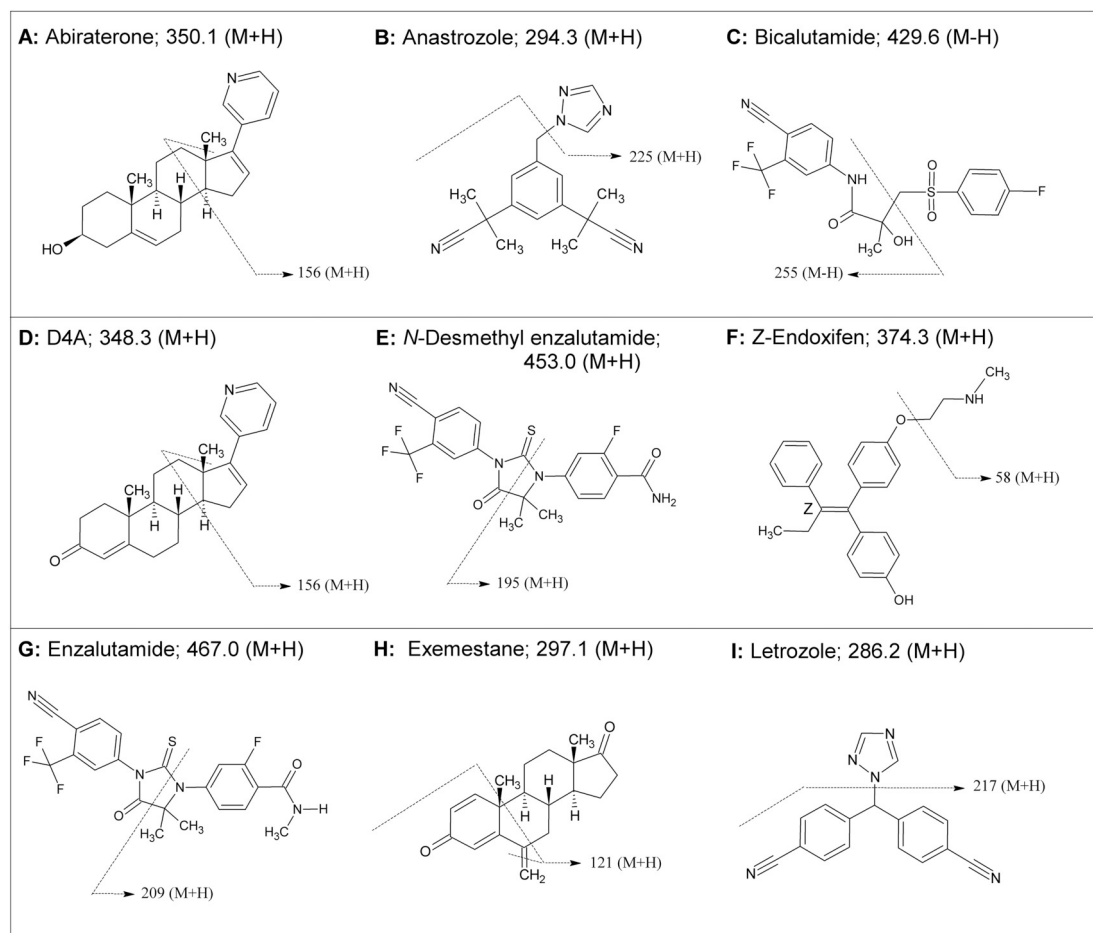
Fig. 1. Proposed fragmentation patters of abiraterone (A), anastrozole (B), bicalutamide (C), Δ(4)-abiraterone (D4A;D), *N*-desmethyl enzalutamide (E), enzalutamide (F), endoxifen (G), exemestane (H) and letrozole (I). The mass of both the parent ion and the product ion are given for each analyte.

quality control samples, according to Table 1. Stock solutions of the internal standards (IS) were prepared as 1 mg/mL concentration in the same solvent as the corresponding analyte. For D4A, no internal standard was available and therefore $^2H_4$-abiraterone was used as an internal standard for quantification. A mixture of IS stock solutions (IS working solution) was prepared in acetonitrile at concentrations of 125 ng/mL for $^2H_5$-Z-endoxifen, 250 ng/mL for $^2H_4$-abiraterone, $^2H_{12}$-anastrozole, $^2H_4$-bicalutamide and $^2H_4$-letrozole and 5000 ng/mL for $^2H_6$-enzalutamide and $^2H_6$-*N*-desmethyl enzalutamide.

Working solutions were prepared in control human K_2EDTA plasma to spike the calibration and quality control samples. Working solutions for spiking calibration standards were prepared at concentrations of 10, 20, 100, 200, 2000 ng/mL for Z-endoxifen, at 2, 10, 100, 200, 2000 ng/mL for D4A, 20, 100, 1000, 2000, 20,000 ng/mL for abiraterone, 100, 200, 1000, 2000, 20,000 ng/mL for anastrozole and letrozole, at 625, 1250, 6250, 12,500 ng/mL for exemestane and at 15,000, 30,000, 150,000, 300,000 ng/mL for bicalutamide, enzalutamide and *N*-desmethyl enzalutamide. Working solutions for spiking quality control (QC) samples were prepared at concentrations of 10, 20, 100, 200, 2000 ng/mL for Z-endoxifen, at 2, 100, 200, 2000 ng/mL for D4A, 20, 1000, 2000, 20,000 ng/mL for abiraterone, 100, 1000, 2000, 20,000 ng/mL for anastrozole and letrozole and at 15,000, 150,000, 300,000 ng/mL for bicalutamide, enzalutamide and *N*-desmethyl enzalutamide. Separate working solutions were prepared to spike exemestane quality control samples at 125, 1250, 25,000 ng/mL. All stock- and working solutions were stored at −20 °C.

### 2.3. Calibration standards, quality control samples

Calibration standards and QC samples were prepared by spiking 100 μL working solution to 900 μL K_2EDTA plasma. Independent working solutions were used for the preparation of calibration standards and QC samples. Combined QC samples were prepared for abiraterone, anastrozole, bicalutamide, D4A, *N*-desmethyl enzalutamide, enzalutamide and letrozole, while separate QC samples were prepared for exemestane. Final concentrations of calibration standards and quality control samples are depicted in Table 1. Calibration standards and QC samples were stored at −20 °C.

2.4. Sample preparation

2.4.1. Sample preparation of calibration standards and combined QC samples

This paragraph describes the sample preparation of calibration samples containing abiraterone, anastrozole, bicalutamide, D4A, *N*-desmethyl enzalutamide, enzalutamide, exemestane and letrozole and of QC samples containing abiraterone, anastrozole, bicalutamide, D4A, *N*-desmethyl enzalutamide, enzalutamide and letrozole. Directly after sample collection in the clinic, whole blood samples were centrifuged for 10 min at 2000 ×g at 4 °C and plasma was stored at −20 °C. To each 50 μL of plasma, a volume of 20 μL of IS working solution was added, except for double blank calibration samples. Proteins were precipitated to extract the analytes from the biomatrix with 100 μL of acetonitrile. Samples were then vortex-mixed for 10 s and centrifuged for 5 min at 23,000 ×g. The supernatant was transferred to amber-colored
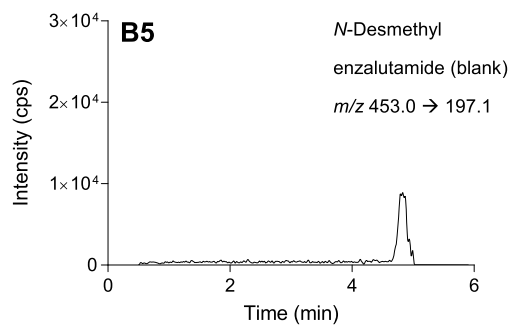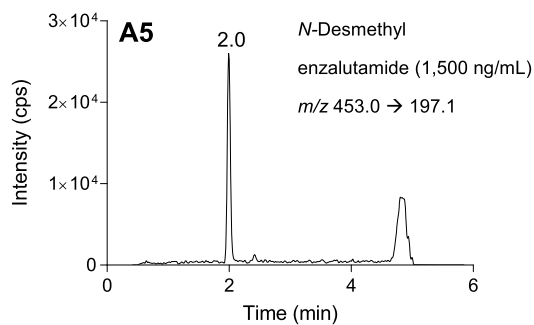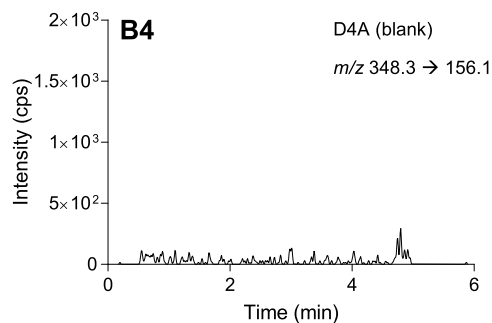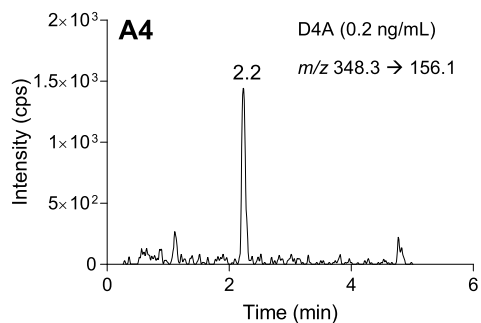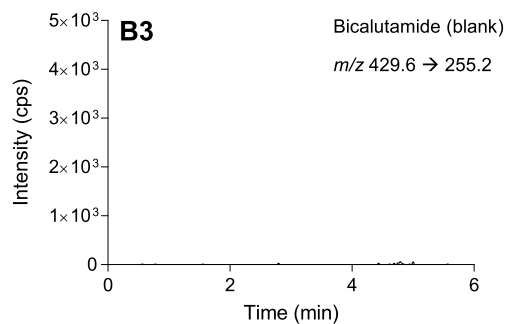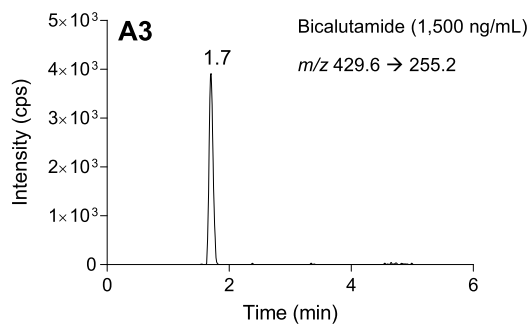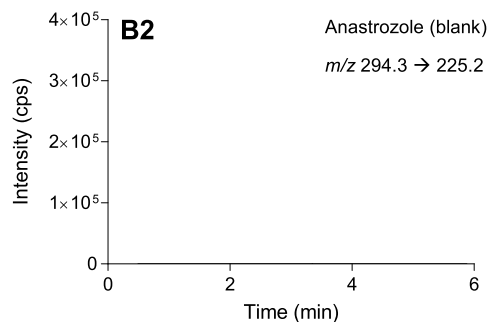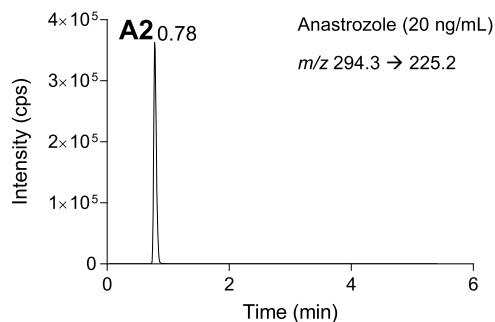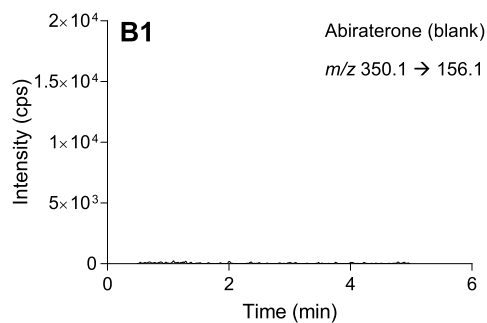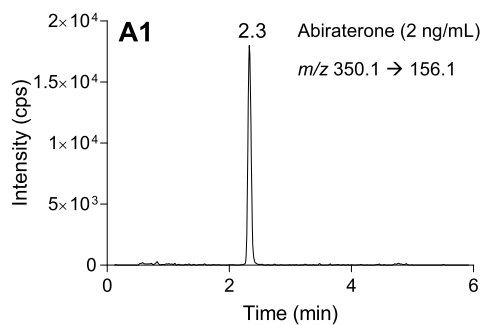
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Fig. 2. Representative LC-MS/MS chromatograms of the lower limit of quantification (A-series) and a blank sample (B-series): abiraterone (1), anastrozole (2), bicalutamide (3), D4A (4), *N*-desmethyl enzalutamide (5), enzalutamide (6), Z-endoxifen (7), exemestane (8) and letrozole (9). The isomer E-endoxifen (7) elutes at 2.1 min.

Representative LC-MS/MS chromatograms of the lower limit of quantification (A-series) and a blank sample (B-series): abiraterone (1), anastrozole (2), bicalutamide (3), D4A (4), *N*-desmethyl enzalutamide (5), enzalutamide (6), Z-endoxifen (7), exemestane (8) and letrozole (9). The isomer E-endoxifen (7) elutes at 2.1 min.
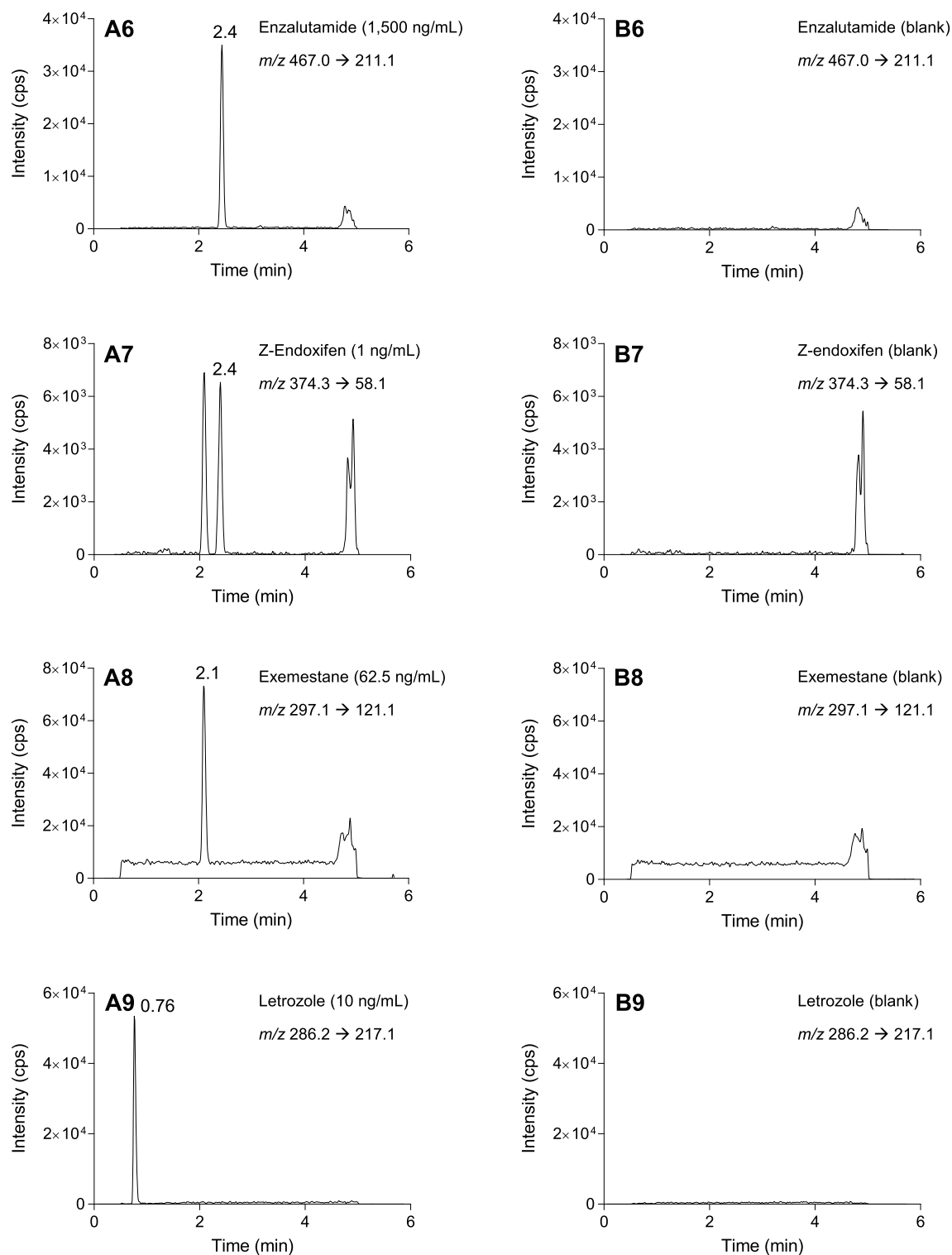
Fig. 2. (*continued*)
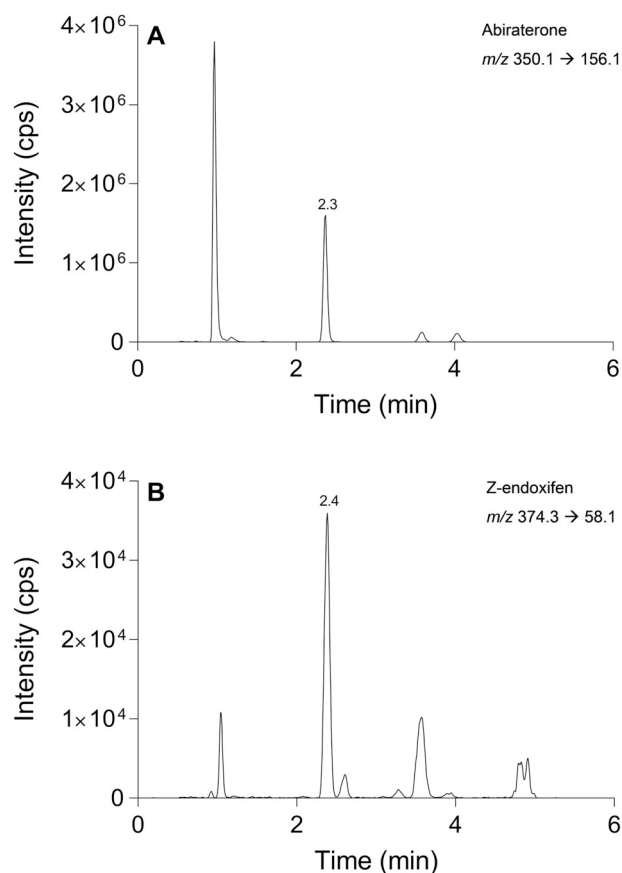
Fig. 3. Representative LC-MS/MS chromatograms of abiraterone (A: 2.3 min) and Z-endoxifen (B: 2.4 min), showing the isomer patterns as seen in patient samples from a patient using abiraterone acetate and tamoxifen, respectively.

autosampler vials with insert.

2.4.2. Sample preparation of exemestane patient samples and QC samples

Directly after sample collection in the clinic, whole blood samples were centrifuged for 10 min at 2000 $\times g$ at 4 °C and plasma was stored at −20 °C. For exemestane patient- and QC samples, 500 µL of plasma was aliquoted and a volume of 20 µL of IS working solution was added to each sample. Proteins were precipitated using 1000 µL of acetonitrile. Samples were vortex-mixed for 10 s and centrifuged for 5 min at 23,000 $\times g$. The supernatant was transferred to 2 mL containers and the samples were dried under a gentle stream of nitrogen at 40 °C. The residue was reconstituted in 50 µL water-methanol (1:1 v/v), vortex-mixed for 10 s and centrifuged for 5 min at 23,000 $\times g$. The supernatant was transferred to amber-colored autosampler vials with insert. To correct for the difference in sample preparation of exemestane calibration samples and QC samples, a dilution factor of 0.03 and an internal standard concentration of 3.4 was used for quantification of patient samples and quality control samples.

2.5. Analytical equipment and conditions

Analytes were separated chromatographically using a Shimadzu LC system with a binary pump, a degasser, an autosampler and a valco valve (Nexera 2 series, Shimadzu corporation, Kyoto, Japan). The temperature of the autosampler was kept at 4 °C and the column oven at 50 °C. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of acetonitrile-methanol (50:50, v/v). Gradient elution was applied at a flow rate of 0.6 mL/min through an Acquity BEH C_{18} column (100 Å, 2.1 × 15 mm, 1.8 µm) with an additional Acquity BEH C_{18} Vanguard pre-column (100 Å, 2.1 × 5 mm, 1.8 µm)

(Waters, Milford, MA, USA). The following gradient was applied: 45% B (0.0–4.0 min), 100% B (4.0–5.0 min), 45% B (5.0–6.0 min). The divert valve directed the flow to the mass spectrometer between 0.5 and 5 min and the remainder to the waste container.

A triple quadrupole mass spectrometer 6500 (Sciex, Framingham, MA, USA) with a turbo ion spray (TIS) interface operating in the positive and negative mode was used as a detector. Bicalutamide was determined in negative ion mode to obtain adequate assay sensitivity, while all the other compounds were measured in positive ion mode. For quantification, multiple reaction monitoring (MRM) chromatograms were acquired and processed using Analyst® 1.6.2 software (AB Sciex). General and analyte specific mass spectrometric parameters are listed in Table 2 and the structures and the proposed fragmentation patterns of the analytes are depicted in Fig. 1.

2.6. Validation procedures

The assay was validated for calibration model, accuracy and precision, LLOQ, sensitivity and selectivity, dilution integrity, carry-over and stability. Adjustments were made to typical validation practices to fit TDM purposes; four instead of six to eight calibrators were investigated, QC concentrations were prepared at three levels (LLOQ, medium, and high concentrations) and no matrix effects were evaluated. A reduced number of calibration standards increases the turnaround of the assay. We choose not to evaluate matrix effects as poor reproducibility due to the use of different matrices will also be reflected in the sensitivity experiments and because we use isotopically labeled internal standards to correct for matrix related effects. Accuracy and precision were calculated as described previously [9].

2.7. Clinical application

This assay was developed to support pharmacokinetic monitoring of abiraterone, anastrozole bicalutamide, D4A, *N*-desmethyl enzalutamide, Z-endoxifen, enzalutamide, exemestane and letrozole. As part of routine clinical care, K_2EDTA blood samples (4 mL) were collected from patients who were treated with one of these drugs at the Antoni van Leeuwenhoek – The Netherlands Cancer Institute. Plasma samples were collected and processed as described in this report.

3. Results and discussion

3.1. Development

3.1.1. Sample preparation

Previous validation procedures showed that abiraterone is not stable in acetonitrile [34], therefore, working solutions were prepared in K_2EDTA plasma. Protein precipitation was chosen as high-throughput method for sample preparation with a sample:acetonitrile ratio of 50:100 (v/v). With this composition of the final extract, no further dilution was necessary prior to injection. However, with this simple sample preparation we were unable to quantify exemestane in patient samples due to low sensitivity. Therefore, we developed a method for the quantification of exemestane in patient samples and QC samples, using a 10-fold larger volume of plasma (500 µL instead of 50 µL). The final extracts of these samples were evaporated to dryness and reconstituted in 50 µL of reconstitution solvent. To preserve a fast turn-around, we prepared combined calibration standards containing all analytes including exemestane at a higher concentration range (62.5 to 1250 ng/mL) these calibration standards were prepared with simple protein precipitation, without the need for concentrating the final extract. During development and validation it was shown that we could easily correct for the difference in sample preparation of exemestane calibration samples and QC samples by applying a dilution factor in the processing software.

Table 3
Assay performance data for abiraterone, anastrozole, bicalutamide, Δ(4)-abiraterone (D4A), *N*-desmethyl enzalutamide, endoxifen, enzalutamide, exemestane and letrozole.a

Analyte	Nominal conc. (ng/mL)	Intra-assay (n = 15)		Inter-assay (n = 15)	
		Bias (%)	Precision (%)	Bias (%)	Precision (%)
Abiraterone	2	4.9–5.8	1.8–2.5	5.1	a
	100	−1.1-10	2.7–5.5	2.9	5.7
	200	2.1–7.6	1.4–4.5	4.9	2.2
Anastrozole	10	−5.7–4.2	1.2–1.8	−5.0	0.5
	100	−14.4–7.0	1.9–5.0	−10.0	4.1
	200	−10.5–6.1	1.5–4.0	−8.4	2.0
Bicalutamide	1500	−10.0–7.2	1.7–3.1	−8.3	1.2
	15,000	−7.3–0.8	1.2–2.4	−4.8	3.6
	30,000	−7.3–1.1	1.4–3.2	−3.8	3.1
D4A	0.2	−16.6-7.5	2.5–12.5	−4.7	12.2
	10	−13.4–5.9	1.7–4.2	−10.6	4.3
	20	−11.1–8.9	2.2–2.8	−10.2	0.7
N-Desmethyl enzalutamide	1500	−6.1-2.1	3.4–5.9	0.0	5.0
	15,000	−8.9–5.5	2.3–5.2	−7.6	1.2
	30,000	−9.3–2.9	4.7–5.8	−5.5	2.7
Z-Endoxifen	1	2.7–16.2	2.9–6.6	8.2	6.2
	10	2.2–14.8	3.1–5.1	6.7	6.3
	20	2.6–13.8	0.7–3.2	7.0	5.5
Enzalutamide	1500	−5.9–2.1	1.6–4.7	−4.0	1.1
	15,000	−12.4-0.0	1.2–3.7	−6.6	6.6
	30,000	−3.5-0.7	0.7–3.4	−3.3	3.9
Exemestane	1.88	−19.0-5.1	0.8–3.1	−6.8	12.9
	18.8	−0.9-2.9	0.8–3.2	1.1	1.6
	37.5	−4.4-2.1	1.2–3.0	−0.4	3.4
Letrozole	10	−12.2–9.8	2.0–2.8	−10.9	0.7
	100	−10.0–6.0	1.3–4.4	−7.4	2.0
	200	−13.4–10.1	2.3–3.9	−11.2	1.6

a No significant additional variation was found due to the performance of the assay in different batches. Abbreviations: D4A = Δ(4)-abiraterone, conc. = concentration, C.V. = coefficient of variation.
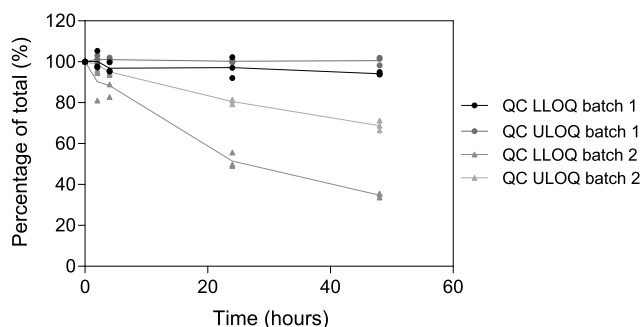
Fig. 4. Short-term stability of abiraterone at lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) concentration in 2-year old K_2EDTA plasma (batch 1) and fresh K_2EDTA plasma (batch 2).

3.1.2. Mass spectrometry and chromatography

The analytical setup was developed for simultaneous quantification of anti-hormonal drugs to monitor drug exposure. Chromatographic separation was pivotal and challenging for Z-endoxifen and abiraterone, as both analytes show extensive metabolism, including the formation of isomers. Therefore, baseline separation of these isomers was required. This was achieved by using an ultra-pressure liquid-chromatography (UPLC) column. Orbitrap MS (Thermo Fischer) spectra were obtained of the abiraterone metabolites to determine the accurate mass. These spectra confirm that both metabolites and abiraterone have the same accurate mass (349.24 g/mol) and are therefore considered isomers. Representative chromatograms of QC LLOQ and blank samples are presented in Fig. 2 for each analyte. Furthermore, Fig. 3 depicts the MRM chromatograms of Z-endoxifen and abiraterone of a patient sample, showing that the chromatographic system is capable of separating the isomers of these drugs. Calibration ranges were chosen so that

analyte concentrations in patient samples were within this range. Reported C_{trough} concentrations of enzalutamide and *N*-desmethyl enzalutamide are 11.4 mg/L and 13.0 mg/L, respectively [35]. A calibration range around these high plasma concentrations, however, caused saturation of the MS detector resulting in non-linearity of the calibration model. To overcome this, the MRM channel was adjusted (+2) to monitor *m/z* values of naturally occurring isotopes of both parent and product ions [36]. With this modification, enzalutamide and *N*-desmethyl enzalutamide could both be measured in a clinically relevant concentration range without the need for sample dilution.

3.2. Calibration model

Four non-zero calibration standards were prepared and analyzed in three separate runs. Linearity of the calibration model was determined by plotting the peak area ratio of the analyte/IS against the corresponding concentration (x) of the calibration standard. The reciprocal of the squared concentrations ($1/x^2$) was used as a weighting factor for all analytes. For each calibration curve the calibration concentrations were back-calculated from the response ratios. The deviations of the nominal concentrations should be within ± 15%. At the LLOQ level a deviation of ± 20% was permitted. All calibration curves (n = 3) of all analytes met these criteria. The assay was linear for the validated concentration ranges of 2–200 ng/mL voor abiraterone, 0.2–20 ng/mL for D4A, 10–200 ng/mL for anastrozole and letrozole, 1–20 ng/mL for Z-endoxifen, 62.5–1250 ng/mL for exemestane and 1500–30,000 ng/mL for enzalutamide, *N*-desmethyl enzalutamide and bicalutamide.

3.2.1. Accuracy and precision

Intra- and inter-assay bias and precisions of the method were determined by analyzing five replicate QC samples in three consecutive runs at LLOQ, mid and upper limit of quantification (ULOQ)

Table 4

Plasma concentrations of the analytes in patient samples of patients treated with these drugs (n = 10). Z-Endoxifen was measured in plasma from patients using tamoxifen and abiraterone and Δ(4)-abiraterone (D4A) were determined in plasma from patients using abiraterone acetate. Abbreviation: o.d. = once daily.

Analyte	Recommended dose (mg)	Mean plasma concentrations (ng/mL)	Range (ng/mL)	Validated range (ng/mL)
Abiraterone	1000 o.d.	43.0	3.31–136	2–200
Anastrozole	1 o.d.	38.9	19.6–64.6	10–200
Bicalutamide	50 o.d.	16,714	7270–31,200	1500–30,000
D4A	–[a]	4.67	0.382–9.45	0.2–20
N-Desmethyl enzalutamide	–[a]	11,069	9000–14,400	1500–30,000
Endoxifen	–[a]	9.73	2.38–17.1	1–20
Enzalutamide	160 o.d.	11,946	8320–17,800	1500–30,000
Exemestane	25 o.d.	12.4	0.62[b]–48.9	1.88–37.5
Letrozole	2.5 o.d.	107	37.9–356[c]	10–200

[a] D4A, N-desmethyl enzalutamide and endoxifen are active metabolites of abiraterone, enzalutamide and tamoxifen, respectively. The recommended dose of these drugs are 1000 mg o.d. for abiraterone, 160 mg o.d. for enzalutamide and 20 mg o.d. for tamoxifen.

[b] Two exemestane samples were below the lower limit of quantification (LLOQ).

[c] One letrozole samples was above the upper limit of quantification (ULOQ).

concentration levels. The intra- and inter-assay biases and precisions should be within ± 20% and ≤20%, respectively, for the LLOQ concentration and within ± 15% and ≤15%, respectively, for other concentrations. Table 3 summarizes the intra- and inter-assay biases and precisions of the assay. All values were within the acceptance criteria.

3.2.2. Carry-over

Carry-over was investigated by injecting two double blank samples subsequently after an ULOQ sample in three independent runs. The peak area in the blank processed samples should be ≤20% of the peak area in the LLOQ sample and ≤5% of the internal standard area. There were no peaks observed in the first blank processed sample for any analyte, which means that there was no carry-over.

3.2.3. Specificity and selectivity

Six individual batches of K_2EDTA plasma were used to assess the specificity and selectivity of the method. A double blank sample and a sample spiked at the LLOQ were processed of each batch. The samples were prepared to determine whether endogenous compounds interfere at the mass transitions chosen for the analytes and internal standards. Samples were processed and analyzed according to the described procedures. Interferences co-eluting with the analytes or internal standards in the blanks were all ≤20% of the peak area of the analytes at LLOQ or ≤5% of the internal standard areas. Deviations of the nominal concentrations were within ± 20% for at least 4 out of 6 batches for all analytes. Selectivity was therefore considered acceptable.

3.2.4. Stability

Stability of the analytes was tested under various conditions. All stability experiments were performed in triplicate. The analytes were considered stable in the plasma or processed sample when 85%–115% of the initial concentration was recovered. Furthermore, analytes were considered stable in the stock solution when 95%–105% of the original concentration was recovered.

All analytes were stable at − 20 °C in plasma for at least 21 weeks. Short-term stability in plasma was determined after five days at room temperature (20–25 °C) and at 4 °C in dark and exposed to light. Analytes were stable under these short-term storage conditions, except for abiraterone, which was unstable at room temperature in both light and dark. Additional stability experiments showed that abiraterone was stable for only 4 h in plasma at room temperature. However, when the experiment was repeated in a two-year old batch of plasma, abiraterone was proven stable at room temperature up to 48 h. Fig. 4 shows the stability of abiraterone, given as the recovery (%) of the original concentration up to 48 h in two different batches of plasma. The underlying mechanism for this batch-dependent stability remains to be elucidated but could possibly be caused by enzymes, which are active in fresh plasma and less active in older plasma.

The effect of three freeze (− 20 °C)/thaw cycles on the stability of each compound was investigated after thawing samples to room temperature with a minimum interval of 12 h on three separate occasions and comparison with freshly prepared calibration samples. All analytes were stable for three freeze/thaw cycles. Five-day stability was proven for all analytes in final extract at 4 °C. Furthermore, exemestane was stable in dried extract at 4 °C for at least five days. Stability in stock solution was demonstrated at 124 days at − 20 °C.

3.3. Clinical application

This analytical assay was used to determine plasma concentrations of abiraterone, anastrozole bicalutamide, D4A, N-desmethyl enzalutamide, Z-endoxifen, enzalutamide, exemestane and letrozole in samples from patients using these drugs. The chromatograms of abiraterone and Z-endoxifen show additional peaks with identical transitions, belonging to isomeric metabolites. The presence of these isomeric metabolites has been previously described [12,14,34,37]. Applicability of the assay was shown in samples from patients treated with these drugs and the results are listed in Table 4. Ten patients were included for each drug and one sample was drawn from each patient. All values were within the validated range, except for two exemestane samples being below the LLOQ and one letrozole sample being above the ULOQ. The quantitation ranges of previously published methods (exemestane 0.2–0.4 ng/mL, [29,30]; letrozole 6–430 ng/mL [32]) might be sufficient to determine exemestane and letrozole concentrations within the validated range. However, our method was developed for the purpose of therapeutic drug monitoring and therefore the quantitation range was chosen to measure the majority of samples from the clinic. These results demonstrate the applicability of this method for quantification of the selected oral anti-hormonal drugs and three active metabolites for therapeutic drug monitoring.

4. Conclusion

The development and validation of a combined assay for the quantification of abiraterone, anastrozole bicalutamide, D4A, N-desmethyl enzalutamide, Z-endoxifen, enzalutamide, exemestane and letrozole in plasma is described. The validated assay ranges from 2 to 200 ng/mL voor abiraterone, 0.2–20 ng/mL for D4A, 10–200 ng/mL for anastrozole and letrozole, 1–20 ng/mL for Z-endoxifen, 1.88–37.5 ng/mL for exemestane and 1500–30,000 ng/mL for enzalutamide, N-desmethyl enzalutamide and bicalutamide. Exemestane patient samples and QC samples should be concentrated to increase the sensitivity of the assay, and enzalutamide and N-desmethyl enzalutamide should be monitored at + 2 m/z values to prevent detector saturation and therefore the need for sample dilution. Furthermore, the chromatographic method of this assay is highly selective and capable of separating isomers of abiraterone and Z-endoxifen Due to instability of abiraterone in

plasma at room temperature, abiraterone patient samples should be shipped on dry ice. In conclusion, the presented assay is considered suitable to support therapeutic drug monitoring of oral anti-hormonal drugs in clinical daily oncology practice.

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