



Short communication

LC–MS/MS assay for the quantification of testosterone, dihydrotestosterone, androstenedione, cortisol and prednisone in plasma from castrated prostate cancer patients treated with abiraterone acetate or enzalutamide

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ABSTRACT

Prostate cancer is the most common malignancy among men in the Western world. Treatment of this patient population, e.g. by (chemical) castration, is primarily focused on depletion of tumor-stimulating androgens, with testosterone being the major androgenic hormone. After initial therapy, prostate cancer may progress to metastatic castration-resistant prostate cancer. Anti-hormonal drugs abiraterone acetate and enzalutamide are commonly used to treat patients with this disease as both drugs reduce tumor growth and increase time to tumor progression. To evaluate the pharmacodynamic effects of anti-hormonal drugs in this patient population, we developed an LC–MS/MS method for the quantification of testosterone, dihydrotestosterone, androstenedione, cortisol and prednisone in human plasma. The validated assay ranges from 10–10,000 pg/mL for testosterone and androstenedione, 100–10,000 pg/mL for dihydrotestosterone, 50–5000 pg/mL for cortisol and 500–50,000 pg/mL for prednisone. Intra-assay and inter-assay variabilities were within $\pm 15\%$ of the nominal concentrations for quality control (QC) samples at low, medium and high concentrations and within $\pm 20\%$ at the lower limit of quantification (LLOQ), respectively. The applicability of the method was demonstrated in plasma from patients with metastatic castration-resistant prostate cancer using either abiraterone acetate or enzalutamide.

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1. Introduction

Prostate cancer is the most common malignancy in men in the Western world [1–3]. Treatment of this patient population is primarily focused on depletion of tumor-stimulating androgens,

with testosterone being the major androgenic hormone [4]. Testosterone is produced from cholesterol in the testes and the adrenal cortex [5]. Furthermore, tumor cells may develop the ability to produce testosterone to autonomously stimulate growth [6]. Fig. 1 represents the simplified biosynthesis of testosterone from cholesterol via cytochrome P450 17 (CYP17) and 3 β -hydroxysteroid dehydrogenase (3 β HSD) [5]. A similar pathway is depicted for cortisol, which is also produced from cholesterol by CYP17 and 3 β HSD. Testosterone is further metabolized to the active metabolite dihydrotestosterone by 5 α -reductase [5].

Androgen-deprivation therapy is the basis for prostate cancer treatment. After initial therapy, prostate cancer may progress to metastatic castration-resistant prostate cancer (mCRPC) [7]. In this phase of the disease, abiraterone acetate and enzalutamide can be prescribed to prolong time to tumor progression. Abiraterone acetate, co-administered with prednisone, inhibits the enzyme CYP17, which is pivotal in the production of testosterone (Fig. 1)

Abbreviations: 3 β HSD, 3 β -hydroxysteroid dehydrogenase; AR, androgen receptor; CCS-FBS, charcoal-stripped fetal bovine serum; CYP17, cytochrome P450; EMA, European Medicines Agency; FDA, United States Food and Drug Administration; IS, internal standard; LC–MS/MS, liquid chromatography–mass spectrometry; LLOQ, lower limit of quantification; mCRPC, metastatic castration-resistant prostate cancer; MRM, multiple reaction monitoring; TBME, tert-butyl-methylether; ULOQ, upper limit of quantification; QC, quality control; WIS, working solution internal standard.

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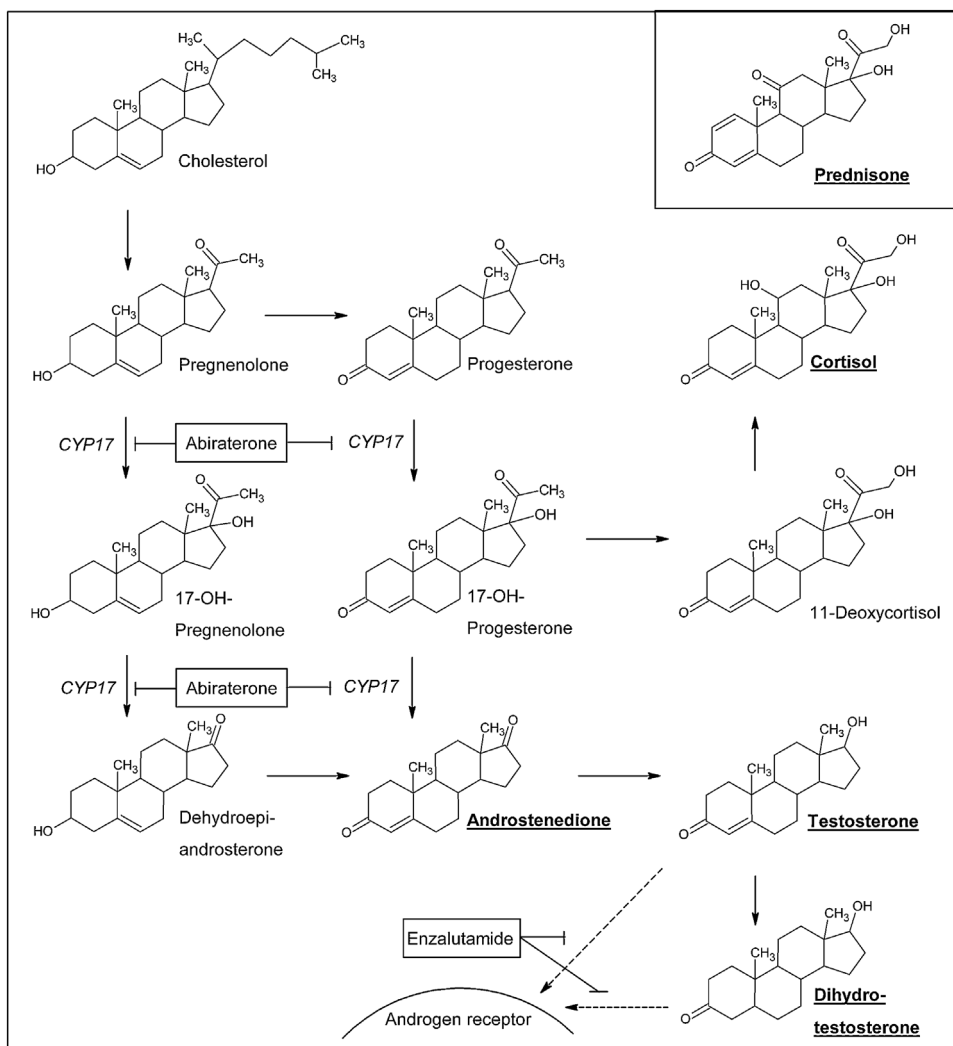


Fig. 1. Biosynthesis of testosterone and cortisol from cholesterol. Prednisone is not included in this pathway, but the chemical structure is visible in the top of the figure. Hormones in bold and underlined are included in this assay. Abiraterone inhibits CYP17 and enzalutamide inhibits androgen receptor activation by testosterone and dihydrotestosterone. Abbreviation: CYP = cytochrome P450 17.

[7], while enzalutamide inhibits the androgen receptor (AR) by competitive binding and thereby antagonizes AR activation [8].

Baseline testosterone and androstenedione serum levels in castrated patients prior to abiraterone acetate therapy are generally 0.1–309 pg/mL and 0.1–185 pg/mL, respectively [9]. Although enzalutamide itself does not decrease androgen levels, it is co-administered with a gonadoreline agonist (i.e. gosereline) to suppress testosterone concentrations below the castration limit of 500 pg/mL [10]. Circulating androgen concentrations could be used as biomarkers for efficacy of anti-androgen therapy. However, routine assays often lack sensitivity to measure below the testosterone castration level of 500 pg/mL [11]. Our institute has previously shown that these low testosterone concentrations can be quantified in human serum using liquid chromatography-mass spectrometry (LC–MS/MS) [12]. Previously published assays providing the possibility to quantify testosterone among other androgens at low concentrations do not focus on measuring these analytes in plasma from prostate cancer patients using androgen-deprivation therapy [13–15]. Besides androgen concentrations, plasma anti-androgenic drug concentrations are relevant to investigate whether the exposure is adequate to suppress androgenic effects. Recently, we published the bioanalytical validation of an LC–MS/MS method to measure plasma concentrations of anti-hormonal drugs for treatment of prostate- and breast cancer, among which abiraterone

and enzalutamide [16]. The assay that we present here consists of the same analytical system but with a different sample preparation and including four hormones to evaluate treatment effects. To our knowledge this is the first assay that combines quantification of androgens, cortisol and prednisone with the quantification of anti-hormonal drugs. Furthermore, this sensitive method is fast and easy to implement with the possibility to determine hormones and prednisone in human plasma instead of human serum, as is predominantly described in literature. In conclusion, we present now the development, validation and clinical application of this sensitive and high-throughput LC–MS/MS assay for measuring testosterone, dihydrotestosterone, androstenedione, cortisol and prednisone in plasma from castrated prostate cancer patients using abiraterone acetate or enzalutamide.

2. Experimental

2.1. Chemicals

Testosterone, androstenedione, cortisol, $^2\text{H}_4$ -testosterone, $^2\text{H}_7$ -androstenedione and $^2\text{H}_4$ -cortisol were purchased from Alsachim (Illkirch Graffenstaden, France). Dihydrotestosterone and $^2\text{H}_3$ -Dihydrotestosterone were from Sigma Aldrich (Zwijndrecht, the Netherlands). Prednisone and $^2\text{H}_6$ -Prednisone were purchased

Table 1

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

Analyte	Stock (mg/mL)	Calibration standards (pg/mL)	Quality control samples (pg/mL)
Androstenedione	1.00 (Methanol)	10; 20; 50; 100; 1000; 2500; 8,000; 10,000	10; 30; 100; 7,500
Cortisol	1.00 (Acetonitrile)	50; 100; 250; 500; 1000; 2500; 4000; 5000	50; 150; 500; 3,750
Dihydro-testosterone	1.00 (Methanol)	100; 200; 500; 1000; 2000; 5000; 8,000; 10,000	100; 300; 1000; 7,500
Prednisone	1.00 (DMSO)	500; 1000; 2500; 5000; 10,000; 25,000; 40,000; 50,000	500; 1500; 5000; 37,500
Testosterone	1.00 (DMSO)	10; 20; 50; 100; 1000; 2500; 8,000; 10,000	10; 30; 100; 7,500

Abbreviations: DMSO = Dimethylsulfoxide.

Table 2

Above: General mass spectrometric parameters. Below: Analyte specific mass spectrometric parameters for testosterone, dihydrotestosterone (DHT), androstenedione, cortisol, prednisone and the internal standards. The most sensitive transition was used for quantitation (quantifier, quan) and the second one was used for confirmation (qualifier, qual).

Run duration	7 min				
Ionspray voltage	5500 V				
Nebulizer gas	60 au				
Turbo gas / heater gas	40 au				
Curtain gas	25 au				
Collision gas	11 au				
Temperature	550 °C				
Dwell time	30 ms				
	MRM (Da)	Collision energy (V)	Collision exit potential (V)	Declustering potential (V)	Retention time (min)
Testosterone quan	289.1 → 97.1	27	12	27	2.8
Testosterone qual	289.1 → 109.1	27	12	27	2.8
Testosterone IS	293.1 → 98.1	27	12	27	2.8
DHT quan	291.3 → 225.3	21	10	86	4.6
DHT qual	291.3 → 159.1	21	10	86	4.6
DHT IS	294.2 → 258.3	21	10	86	4.6
Androstenedione quan	287.3 → 97.1	25	10	121	3.0
Androstenedione qual	287.3 → 109.1	25	10	121	3.0
Androstenedione IS	294.2 → 100.1	25	10	121	3.0
Cortisol quan	363.0 → 121.0	29	19	91	1.2
Cortisol qual	363.0 → 81.1	29	19	91	1.2
Cortisol IS	367.3 → 121.0	29	19	91	1.2
Prednisone quan	359.3 → 79.0	57	10	81	1.0
Prednisone IS	367.2 → 79.0	57	10	81	1.0

MRM = multiple reaction monitoring, IS = internal standard.

from Toronto Research Chemistry (Toronto, Canada). Acetonitrile, methanol, water and formic acid 99% (all ULC/MS-grade) were from Biosolve Ltd (Valkenswaard, The Netherlands). Water (distilled) used for sample preparation came from B. Braun Medical (Melsungen, Germany) and tert-butyl-methylether originated from Merck (Amsterdam, the Netherlands).

2.2. Charcoal-stripped fetal bovine serum

Testosterone, androstenedione, dihydrotestosterone and cortisol are present in human plasma. Therefore, we were unable to use control human plasma for the preparation of calibration standards and quality control (QC) samples. Charcoal-stripping human plasma reduced hormone levels, however, plasma concentrations of testosterone, androstenedione, dihydrotestosterone and cortisol were still elevated and too high for preferred assay sensitivity. Ultimately, we choose to use charcoal-stripped fetal bovine serum (CCS-FBS) as matrix for calibration standards and quality control samples, as this matrix did not contain interfering hormones. The matrix was produced by stripping FBS in triplicate with charcoal at the Division of Molecular Pathology of the Netherlands Cancer Institute (Amsterdam, The Netherlands).

2.3. Stock solutions and working solutions

Separate stock solutions were made for the preparation of calibration standards and QC samples according to Table 1. Working solutions were prepared in acetonitrile at concentrations of 0.4, 0.8, 2, 4, 40, 100, 320 and 400 ng/mL for testosterone and

androstenedione, at concentrations of 4, 8, 20, 40, 80, 200, 320 and 400 ng/mL for dihydrotestosterone, at concentrations of 2, 4, 10, 20, 40, 100, 160 and 200 ng/mL for cortisol and at concentrations of 20, 40, 100, 200, 400, 1000, 1600 and 2000 ng/mL for prednisone. Stock solutions for the internal standards (IS) were produced at a concentration of 1 mg/mL in DMSO for $^2\text{H}_4$ -testosterone and $^2\text{H}_6$ -prednisone, in methanol for $^2\text{H}_7$ -androstenedione and in acetonitrile for $^2\text{H}_4$ -cortisol. $^2\text{H}_3$ -dihydrotestosterone was purchased as a 0.1 mg/mL stock solution in acetonitrile. A mixture of IS stock solutions was prepared and diluted with water to obtain a working solution IS that was used for sample pretreatment. This working solution IS (WIS) contained 5 ng/mL $^2\text{H}_4$ -testosterone and $^2\text{H}_7$ -androstenedione, 0.5 ng/mL $^2\text{H}_3$ -dihydrotestosterone, 2.5 ng/mL $^2\text{H}_4$ -cortisol and 25 ng/mL $^2\text{H}_6$ -prednisone. Stock- and working solutions were stored at -20°C .

2.4. Calibration standards, quality control samples

Calibration samples were prepared freshly prior to each validation run, by spiking 25 μL working solution to 975 μL charcoal-stripped FBS. QC samples were prepared in batches and stored at -20°C . Calibration standards and QC samples were prepared in charcoal-stripped FBS according to Table 1.

2.5. Sample preparation

All samples were thawed prior to processing and 250 μL was aliquoted in 2 mL containers. Each sample was spiked with 20 μL

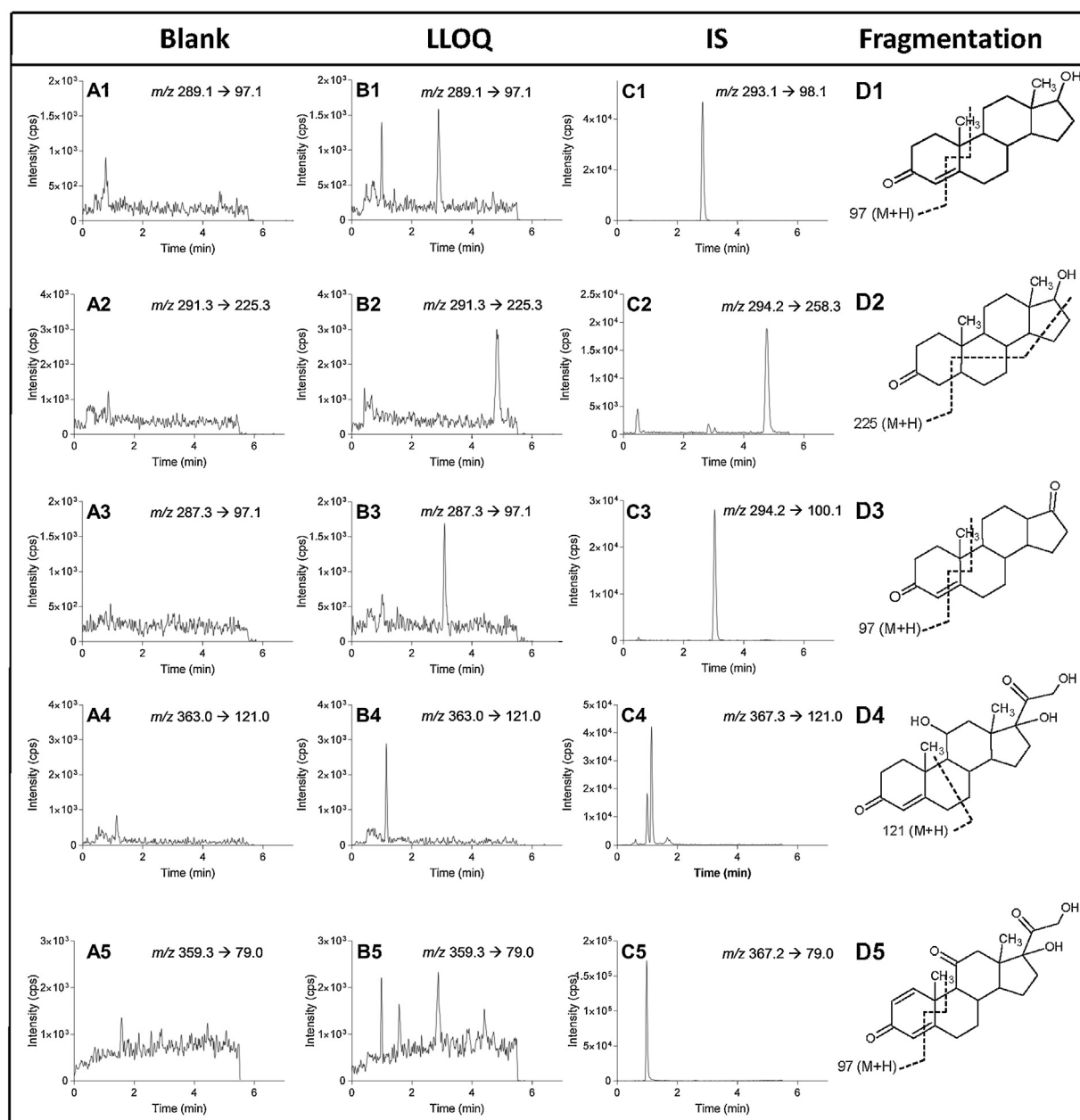


Fig. 2. Representative LC–MS/MS chromatograms of a blank sample (A-series) lower limit of quantification (LLOQ; B-series) and the internal standard (C-series), including proposed fragmentation patterns (D-series), for testosterone (1, $t = 2.8$ min), dihydrotestosterone (2, $t = 4.8$ min), androstenedione (3, $t = 3.0$ min), cortisol (4, $t = 1.2$ min) and prednisone (5, $t = 1.0$ min).

WIS, except for double blank calibration samples, and 1.5 mL tert-butyl-methylether (TBME). Samples were vortex-mixed for 10 s, shaken for 5 min at 1250 rpm and centrifuged for 3 min at 23,000 \times g. The aqueous layer was frozen in a bath of ethanol and dry ice and the organic layer was transferred into a clean 2 mL Eppendorf container. The samples were dried under a gentle stream of nitrogen at 40 °C. The residue was reconstituted in 50 μ L water-methanol (1:1 v/v), vortex-mixed for 10 s and centrifuged for 3 min at 23,000 \times g. The final extracts were transferred to autosampler vials with glass insert.

2.6. LC–MS equipment and conditions

The LC–MS system was similar to the previously published method for quantification of anti-hormonal drugs in the treatment of prostate- and breast cancer [16]. Table 2 depicts general and

analyte specific mass spectrometric parameters and the chemical structures of the analytes are shown in Fig. 1. The most sensitive transition was used for quantitation (quantifier, quan), while less abundant ions were monitored for confirmation (qualifier, qual). The ratio of quantifier/qualifier peak areas in unknown samples were compared to the average ratio of the calibration standards. The proposed fragmentation patterns of the quantifier transitions are given in Fig. 2.

2.7. Validation procedures

A full validation of the assay was performed based on the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation [17,18]. The following aspects were established during the validation: calibration model, accuracy, precision, carry-over,

Table 3

Assay performance data for testosterone, dihydrotestosterone, androstenedione, cortisol and prednisone.

Analyte	Nominal conc. (pg/mL)	Intra-assay (n = 15)		Inter-assay (n = 15)	
		Bias (%)	C.V. (%)	Bias (%)	C.V. (%)
Testosterone	10	−8.7 to 11.6	4.4 to 11.6	3.1	8.9
	30	−7.2 to 5.7	3.5 to 9.2	−1.4	6.0
	100	−4.1 to 5.6	3.2 to 3.6	−0.9	5.5
	7500	−1.4 to 2.6	0.5 to 8.1	0.4	*
Dihydrotestosterone	100	−6.6 to 1.1	10.1 to 12.5	−2.1	*
	300	2.1 to 7.2	1.7 to 6.9	4.2	0.6
	1000	−5.7 to −0.7	2.7 to 7.2	−2.5	2.0
	7500	−3.3 to −0.4	2.7 to 2.9	−1.9	0.8
Androstenedione	10	−4.9 to −1.4	6.6 to 13.3	−3.7	*
	30	−3.6 to 1.1	4.3 to 8.8	2.0	5.4
	100	−2.0 to 5.9	3.8 to 5.7	2.7	3.5
	7500	0.3 to 4.7	1.7 to 4.5	2.0	1.8
Cortisol	50	−9.2 to −1.0	8.6 to 13.0	−4.5	*
	150	−6.8 to −0.4	8.0 to 9.7	−3.3	*
	500	−7.0 to 0.3	3.4 to 5.0	−2.4	3.6
	3750	−4.1 to 0.4	1.6 to 5.4	−1.9	1.6
Prednisone	500	−10.5 to 11.5	6.3 to 10.9	0.0	10.4
	1500	−11.2 to 9.3	3.5 to 7.7	−1.1	10.1
	5000	−5.5 to 1.4	4.7 to 7.3	−2.9	2.7
	37500	−12.7 to 0.6	1.3 to 5.5	−7.1	7.2

*No significant additional variation was found due to the performance of the assay in different batches. Abbreviations: conc. = concentration, C.V. = coefficient of variation.

selectivity, matrix effect, dilution integrity, suitability of CCS-FBS as blank matrix, cross-validation and stability. Clinical application

This assay was developed to quantify of testosterone, androstenedione, dihydrotestosterone, cortisol and prednisone in samples from patients using abiraterone acetate or enzalutamide. Surplus plasma was collected from samples obtained during treatment with abiraterone acetate or enzalutamide at the Antoni van Leeuwenhoek – The Netherlands Cancer Institute as part of routine clinical care. These samples had been collected in K₂EDTA tubes and stored at −20°C until further processing as described in this report. This study was in accordance with the code of conduct for responsible use of human tissue and medical research [19].

3. Results and discussion

3.1. Development

3.1.1. Sample preparation

Starting method development, charcoal-stripped human plasma was used as a biomatrix for calibration standards and quality control samples. Endogenous testosterone, dihydrotestosterone, androstenedione, and cortisol levels, however, were too high to use as a matrix blank. Therefore, we decided to use charcoal-stripped fetal bovine serum to use as matrix to spike calibration standards and quality control samples as this matrix did not contain interfering hormones. Protein precipitation and liquid-liquid extraction were tested as a method for sample preparation. Protein precipitation showed poor sensitivity due to high background noise. Therefore, sample pretreatment was further developed with liquid-liquid extraction. Different extraction solvents were tried: TBME, ethyl acetate and a combination of both solvents in a 80:20 and 20:80 ratio, respectively. Furthermore, extraction ratios of 1:4, 1:9 and 1:14 (sample:extraction solvent) were tested to optimize sample preparation. The extraction recovery using TBME and ethylacetate was comparable, however, TBME was chosen for further development because this organic solvent has a lower boiling point and therefore the evaporation time was shortened. Extraction was optimal with a ratio of 1:14 (sample:extraction solvent), as the recovery did not improve by further increasing the volume of TBME.

3.1.2. Mass spectrometry and chromatography

We developed an analytical system for the quantification of anti-hormonal drugs and for quantification of testosterone, dihydrotestosterone, androstenedione, cortisol and prednisone. Developing such an assay is challenging because a highly selective chromatographic method is needed to separate structural analogues, such as testosterone, dihydrotestosterone and androstenedione. The composition of mobile phase B was pivotal for obtaining this high selectivity and could only be achieved by a combination of methanol-acetonitrile (50:50 v/v). Furthermore, high sensitivity was necessary for quantification of these hormones in plasma from patients treated with androgen-deprivation therapy. During assay development, the noise was high (>1000 cps) for the transitions of testosterone, dihydrotestosterone and androstenedione, which made it difficult to achieve this. However, by using ULC-quality solvents, the noise could be reduced to 200 cps thereby substantially improving assay sensitivity.

3.2. Validation procedures

3.2.1. Calibration model

Eight non-zero calibration standards were freshly prepared in duplicate prior to each run and were analyzed in three separate runs. All the calibration data were fitted linearly using a weighting factor of $1/x^2$, where x is the analyte concentration. For every calibration curve the calibration concentrations were back-calculated from the response ratios. Deviations of the nominal concentrations were within $\pm 15\%$ and within $\pm 20\%$ for the LLOQ. The assay was linear for the validated concentration ranges of 10–10,000 pg/mL for testosterone and androstenedione, 100–10,000 pg/mL for dihydrotestosterone, 50–5000 pg/mL for cortisol and 500–50,000 pg/mL for prednisone.

3.2.2. Accuracy and precision

Intra- and inter-assay accuracies and precisions of the method were assessed by analyzing five replicate QC samples in three consecutive runs at LLOQ, low, mid and high concentration levels. Accuracy and precision were calculated as described previously [20]. Assay performance data of all analytes are presented in Table 3. Inter-assay accuracy, intra-assay accuracy and the precision were $\leq 15\%$ for low, mid and high concentrations and $\leq 20\%$ for the LLOQ

concentrations. Therefore, accuracy and precision were within the acceptance criteria.

3.2.3. Carry-over

Carry-over was investigated by injecting two double blank samples subsequently after an upper limit of quantification (ULOQ) sample in three independent runs. There were no peaks observed in the first and second blank processed sample, which means that there was no carry-over for testosterone, dihydrotestosterone, androstenedione, cortisol, prednisone and the internal standards.

3.2.4. Specificity and selectivity

Six individual batches of charcoal-stripped FBS 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 transition chosen for the analytes and internal standards. Samples were processed according to the described procedures and analyzed. MRM chromatograms of the six batches of charcoal-stripped FBS contained no co-eluting peaks >20% of the area at the LLOQ level of the analytes and no co-eluting peaks >5% of the area of both internal standards. The accuracies at LLOQ level were within $\pm 20\%$ of the nominal concentration for at least 4 out of 6 batches charcoal-stripped FBS.

Cross-analyte interferences was assessed by preparing samples containing only one of the analytes at ULOQ concentration in control charcoal-stripped FBS. An interference was found for testosterone in the sample spiked with androstenedione. To further investigate this interference, an androstenedione sample at ULOQ concentration was prepared in methanol-water (50:50 v/v) and measured. This sample showed a similar cross-analyte interference for testosterone. Based on this information, we concluded that testosterone is not formed during sample preparation, but present in the reference standard of androstenedione. We quantified the amount of testosterone in the androstenedione reference standard to be 0.33% (w/w). Since the concentration ranges of both analytes are equal (10–10,000 pg/mL) and individual analyte concentrations per calibration standard were the same, we concluded that a bias of 0.33% was acceptable for this bioanalytical assay. For dihydrotestosterone, androstenedione, cortisol and prednisone, cross-interference of co-eluting peaks in separately spiked samples were $\leq 20\%$ of the QC LLOQ samples and thus within the required limits. For the internal standards, the interference was $\leq 5\%$ and thus also within the acceptance criteria [17,18].

To investigate the selectivity of the method, structurally related hormones and anti-hormonal drugs were injected onto the system. The method was selective with regard to 17-hydroxy-progesterone, estradiol, epitestosterone, dehydroepiandrosteron (DHEA), progesterone, abiraterone, $\Delta(4)$ -abiraterone (D4A) and exemestane. Estradiol, progesterone, abiraterone, $\Delta(4)$ -abiraterone (D4A) and exemestane showed no peak in the mass windows of analytes of interest. Furthermore, 17-Hydroxy-progesterone, epitestosterone and DHEA were baseline separated from testosterone, androstenedione and DHT, and therefore will not interfere with analyte quantification.

3.2.5. Matrix factor

The matrix effect was determined in six different batches of charcoal-stripped FBS at low and high concentration levels in singular. Peak areas of QC samples spiked after liquid-liquid extraction were compared to peak areas of QC samples of equivalent concentrations in methanol-water (50:50, v/v). Additionally, the IS-normalized matrix factor was calculated by dividing the matrix factor of the analyte by the matrix factor of the IS. The normalized matrix factor was between 0.957 and 1.09 for testosterone, between 0.915 and 1.04 for dihydrotestosterone, between 1.00

and 1.40 for androstenedione, between 0.912 and 1.29 for cortisol and between 0.474 and 0.707 for prednisone. The CVs for the IS-normalized matrix factor were $\leq 15\%$ at the tested concentrations for each compound. Prednisone showed a lower normalized matrix factor than the other compounds, which was caused by the presence of a small endogenous peak at the transition and retention time of the internal standard. Overall, the results indicate that the stable-isotopically labelled internal standards are most effective in minimizing the influence of matrix effects as the CV for IS-normalized matrix factor was $\leq 15\%$ for all analytes.

3.2.6. Dilution integrity cortisol

Dilution integrity was established for cortisol only, as plasma concentrations of the other hormones in patient samples were not expected to exceed the ULOQ. Five replicate charcoal-stripped FBS samples spiked with a cortisol concentration above the ULOQ (100 ng/mL) were diluted 100-fold with charcoal-stripped FBS. The concentrations of 100-fold diluted samples were within $\pm 15\%$ of the nominal concentration. Intra-assay bias and intra-assay variability were 11.0% and 3.9%, respectively. These results show that samples with cortisol concentrations > ULOQ can be diluted with charcoal-stripped FBS up to 100-fold to obtain plasma concentrations within the validated range.

3.2.7. Suitability charcoal-stripped FBS as blank matrix

Testosterone, androstenedione, dihydrotestosterone and cortisol are endogenous compounds and thus CCS-FBS was used as a matrix for calibration standards and QC samples. However, our method was applied to quantify testosterone, dihydrotestosterone, androstenedione, cortisol and prednisone in K₂EDTA plasma and, therefore, the suitability of CCS-FBS as a surrogate matrix was determined by spiking K₂EDTA plasma from patients using abiraterone acetate with unmeasurable testosterone, androstenedione, dihydrotestosterone and cortisol concentrations. Three batches of this 'hormone-free' plasma were used for the preparation of QC mid samples (n=3). Accuracy and precision were $\leq 15\%$ for all analytes. From these data it can be concluded that CCS-FBS is a suitable surrogate matrix for the quantification of these analytes.

3.2.8. Cross validation

The assay was cross validated for testosterone, androstenedione and cortisol with an LC-MS/MS method standardized against National Institute of Standards and Technology (NIST) reference material SRM 971 [12]. QC samples at three concentration levels and 17 patient samples were measured on both systems to check whether both methods were able to generate the same results. Accuracy of the QC samples was within $\pm 15\%$ and the bias between concentrations measured in patient samples was within $\pm 20\%$ of the mean for at least 67% of the repeats.

3.2.9. Stability

Stability experiments were performed under various conditions at low and high concentrations in triplicate. Analytes were considered stable in the stock solution when 95%–105% of the original concentration was recovered. Stability experiments demonstrate adequate stability in biomatrix of all analytes after 7 days at room temperature and 4 °C, both in the dark and exposed to light. Prednisone was unstable in final extract and dry extract after 7 days at nominally 4 °C, however, 2-day stability of prednisone in final extract and dry extract (4 °C) was established. Other analytes were stable in final extract and dry extract for at least 7 days at nominally 4 °C. Furthermore, analytes were stable undergoing 4 freeze/thaw cycles. Long-term stability assessment in charcoal-stripped FBS was demonstrated up to five months and is still ongoing. Stock solutions were stable for at least 124 days at -20 °C and working solutions were stable for at least 62 days at -20 °C.

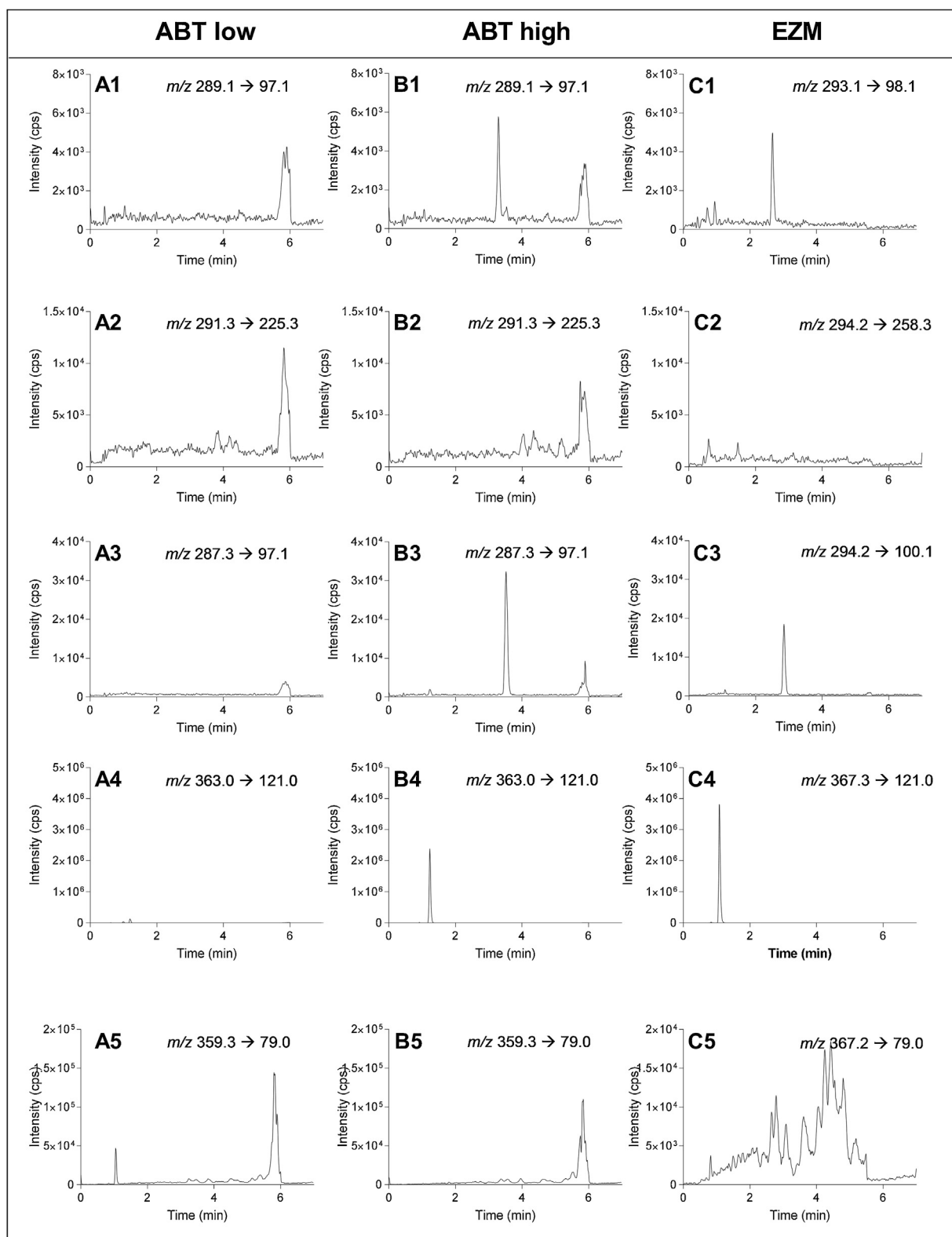


Fig. 3. Representative LC–MS/MS chromatograms of plasma from a patient using abiraterone acetate with adequate testosterone suppression (ABT low), a patient using abiraterone with insufficient testosterone suppression (ABT high), and a patient using enzalutamide (EZM) for testosterone (1, $t = 2.8$ min), dihydrotestosterone (2, $t = 4.8$ min), androstenedione (3, $t = 3.5$ min), cortisol (4, $t = 1.2$ min) and prednisone (5, $t = 1.0$ min).

4. Clinical application

The validated assay was used to quantify testosterone, dihydrotestosterone, androstenedione, cortisol and prednisone in

K₂EDTA plasma from prostate cancer patients with androgen deprivation therapy. Mean plasma concentrations in 20 patients using abiraterone acetate (1000 mg QD) were <10 pg/mL for testosterone, <10 pg/mL for androstenedione, <10 pg/mL for dihydrotestos-

terone, 9.6 ng/mL for cortisol and 9.8 ng/mL for prednisone, and mean plasma concentrations in 20 patients using enzalutamide (160 mg QD) were 97 pg/mL for testosterone, 289 pg/mL for androstenedione and <10 pg/mL for dihydrotestosterone. Testosterone and androstenedione could be determined in only two samples from patients using abiraterone acetate, indicating adequate androgen deprivation far below the castration limit of 0.50 ng/mL. Cortisol and prednisone were only quantified in plasma from patients using abiraterone acetate. Cortisol levels were lower in patients with adequate androgen suppression and a measurable prednisone concentration (3.4 vs 16 ng/mL, both $n = 10$). Fig. 3 shows representative chromatograms of plasma obtained from three separate patients: one patient using abiraterone acetate with adequate androgen deprivation, one patient using abiraterone acetate with measurable hormone levels and one patient using enzalutamide. Testosterone levels were <10 pg/mL, 92 pg/mL and 67 pg/mL, respectively. Androstenedione showed a similar trend, with plasma concentrations of <10 pg/mL, 576 pg/mL and 182 pg/mL, respectively. These results are in line with our expectations based on the mechanism of actions of both drugs. Abiraterone acetate inhibits the production of testosterone, androstenedione, dihydrotestosterone and cortisol via CYP17, while enzalutamide inhibits androgen receptor activation and does not directly inhibit androgen production. Thus, lower plasma concentrations of androgens and cortisol are expected in patients using abiraterone acetate compared to patients using enzalutamide. Furthermore, patients with measurable testosterone and androstenedione levels showed higher cortisol levels. Anti-androgen drugs, such as abiraterone acetate and enzalutamide, can be measured with the same analytical conditions for therapeutic drug monitoring, providing information on both the exposure to anti-androgen drugs and their effects on testosterone, dihydrotestosterone, androstenedione and cortisol.

5. Conclusion

The development and validation of a combined assay for the quantification of testosterone, dihydrotestosterone, androstenedione, cortisol and prednisone in samples from drug-treated castrated prostate cancer patients is described. The validated range was 10–10,000 pg/mL for testosterone and androstenedione, 100–10,000 pg/mL for dihydrotestosterone, 50–5000 pg/mL for cortisol and 500–50,000 pg/mL for prednisone. We are the first to describe a method to monitor plasma drug levels as well as hormones affected by the treatment. The method has been successfully implemented to support clinical pharmacology studies in castrated prostate cancer patients.

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References

- [1] C. Fitzmaurice, C. Allen, R.M. Barber, L. Barregard, Z.A. Bhutta, H. Brenner, et al., Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 32 cancer groups, 1990 to 2015: a Systematic Analysis for the Global Burden of Disease Study Global Burden, *JAMA Oncol.* 3 (4) (2017) 524–548.
- [2] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2016, *CA Cancer J. Clin.* 66 (1) (2016) 7–30.
- [3] American Cancer Society, Global Cancer Facts & Figures 3rd Edition, Available from: Internet]. American Cancer Society, 2015 <http://www.ncbi.nlm.nih.gov/pubmed/22019360>.
- [4] N. Sharifi, J.L. Gulley, W.L. Dahut, Androgen deprivation therapy for prostate cancer, *JAMA.* 294 (2) (2005) 238–244.
- [5] F.F.G. Rommerts, in: E. Nieschlag, H.M. Behre (Eds.), Testosterone: an Overview of Biosynthesis, Transport, Metabolism and Action BT - Testosterone: Action · Deficiency · Substitution, Springer Berlin Heidelberg, Berlin, Heidelberg, 1990, pp. 1–22, http://dx.doi.org/10.1007/978-3-662-00814-0_1, Available from:.
- [6] C. Cai, S.P. Balk, Intratumoral androgen biosynthesis in prostate cancer pathogenesis and response to therapy, *Endocr. Relat. Cancer* 18 (5) (2011) R175–82.
- [7] U.S. Food, Drug Administration (FDA), Clinical Pharmacology and Biopharmaceutics Review: Zytiga (abiraterone acetate) [Internet] [cited 2019 Jan 10] Available from: 2010, pp. 1–86 https://www.accessdata.fda.gov/drugsatfda_docs/nda/2011/202379orig1s000clinpharmr.pdf.
- [8] U.S. Food, Drug Administration, Clinical Pharmacology and Biopharmaceutics Review: Xtandi (Enzalutamide) [Internet] [cited 2019 Jan 10] Available from: Silver Spring, MD), 2012, pp. 1–75 https://www.accessdata.fda.gov/drugsatfda_docs/nda/2012/203415Orig1s000ClinPharmR.pdf.
- [9] C.J. Ryan, A. Molina, J. Li, T. Kheoh, E.J. Small, C.M. Haqq, et al., Serum androgens as prognostic biomarkers in castration-resistant prostate cancer: results from an analysis of a randomized phase III trial, *J. Clin. Oncol.* 31 (22) (2013) 2791–2798.
- [10] U.S. Food, Drug administration, in: Prescribing information: Xtandi (enzalutamide) [Internet], Silver Spring, MD), 2012, pp. 1–16 [cited 2018 Jun 28] Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2012/203415lbl.pdf.
- [11] J. Taieb, B. Mathian, F. Millot, M.-C. Patricot, E. Mathieu, N. Queyrel, et al., Testosterone measured by 10 immunoassays and by isotope-dilution gas chromatography-mass spectrometry in sera from 116 men, women, and children, *Clin. Chem.* 49 (8) (2003) 1381–1395.
- [12] L.J. van Winden, O. van Tellingen, H.H. van Rossum, Serum testosterone by liquid chromatography tandem mass spectrometry for routine clinical diagnostics, *Methods Mol. Biol.* 1730 (2018) 93–102.
- [13] M.R. Hakkinen, T. Heinosaalo, N. Saarinen, T. Linnanen, R. Voutilainen, T. Lakka, et al., Analysis by LC-MS/MS of endogenous steroids from human serum, plasma, endometrium and endometrial tissue, *J. Pharm. Biomed. Anal.* 152 (2018) 165–172.
- [14] R.M. Buttler, F. Martens, M.T. Ackermans, A.S. Davison, A.E. van Herwaarden, L. Kortz, et al., Comparison of eight routine unpublished LC-MS/MS methods for the simultaneous measurement of testosterone and androstenedione in serum, *Clin. Chim. Acta* 454 (2016) 112–118.
- [15] D.R. Taylor, L. Ghataore, L. Couchman, R.P. Vincent, B. Whitelaw, D. Lewis, et al., A 13-Steroid serum panel based on LC-MS/MS: use in detection of adrenocortical carcinoma, *Clin. Chem.* 63 (12) (2017) 1836–1846.
- [16] M. van Nuland, N. Venekamp, N. de Vries, K.A.M. de Jong, H. Rosing, J.H. Beijnen, Development and validation of an UPLC-MS/MS method for the therapeutic drug monitoring of oral anti-hormonal drugs in oncology, *J Chromatogr B [Internet]* (2019) 26–34, Available from: <http://www.sciencedirect.com/science/article/pii/S1570023218314260>.
- [17] U.S. Food, Drug administration (FDA), in: FDA Guidance for Industry: Bioanalytical Method Validation [Internet], Silver Spring, Maryland: US Food and Drug Administration, 2018 [cited 2018 Jun 25]. Available from: <https://www.fda.gov/downloads/drugs/guidances/ucm070107.pdf>.
- [18] European Medicines Agency (EMA), Guideline on Bioanalytical Method Validation. Committee for Medicinal Products for Human Use and European Medicines Agency. [Internet]. London [cited 2018 Jun 25]. Available from: 2011 http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf.
- [19] Federation of Dutch Medical Scientific Societies, Human Tissue and Medical Research: Code of Conduct for Responsible Use [Internet]. Available from: 2011, pp. 21 https://www.federa.org/sites/default/files/digital/version.first_part.code.of.conduct.in.uk.2011.12092012.pdf.
- [20] M. Herbrink, N. de Vries, H. Rosing, A.D.R. Huitema, B. Nuijen, J.H.M. Schellens, et al., Development and validation of a liquid chromatography-tandem mass spectrometry analytical method for the therapeutic drug monitoring of eight novel anticancer drugs, *Biomed. Chromatogr.* 32 (4) (2018).