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A validated liquid chromatographic–tandem mass spectroscopy method for the quantification of abiraterone acetate and abiraterone in human plasma

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Abstract

A sensitive and selective LC–MS/MS method has been developed and validated for the quantification of abiraterone acetate and its metabolite, abiraterone (an androgen biosynthesis inhibitor) in human plasma. Analytes were extracted by SPE with cation mixed-mode polymer cartridges. Chromatography was performed on a Luna C5 5 μm, 50 mm × 2.1 mm i.d. column, using a mobile phase of 2% propan-2-ol in acetonitrile and 10 mM ammonium acetate. The assay was linear from 5 to 500 nM ($r^2 = 0.998$). The intra- and inter-day coefficients of variation were <13.9% for both analytes. This method will be applied to a clinical trial investigating the pharmacokinetics of abiraterone acetate and abiraterone in patients with prostate cancer.

Keywords: Abiraterone; Liquid chromatography–tandem mass spectrometry; Prostate cancer; Validation

1. Introduction

Prostate cancer is the second most common malignancy in western societies with hormonal or androgen deprivation therapy regarded as a mainstay treatment. In general, androgen deprivation induces remission in 80–90% of men with advanced disease, resulting in a median progression-free survival of 12–33 months, followed usually by the development of an androgen-independent phenotype [1,2].

As a high proportion of prostate tumours are dependent on androgens for growth, many agents have been developed to suppress either testicular or adrenal steroidogenesis. Luteinizing hormone (LH)-releasing hormone (LHRH) antagonists target testicular androgen synthesis by directly inhibiting the LHRH receptor. Preliminary clinical trial data indicates that these agents are effective in lowering serum testosterone [3], but do not affect the adrenal supply of androgens and their precursors. In a significant proportion of prostate cancer patients, studies have shown that extra testicular sources of testosterone represent an important alternative source of androgen stimulation, which supports tumour growth [1,4]. An alternative approach has thus been the complete inhibition of both testicular and adrenal androgen production. The cytochrome P450 enzyme, 17α-hydroxylase-$C_{17,20}$-lyase has become a promising therapeutic target, as it is key in the androgen biosynthetic pathway present in both the testes and adrenals [5]. The anti-fungal agent ketoconazole, an early inhibitor of this enzyme, has been shown to interfere with both testicular and adrenal steroid production [6–8]. However, ketoconazole is relatively unselective, inhibiting both cholesterol side chain cleavage and 11β-hydroxylation [8]. It is desirable in prostate cancer that a potential inhibitor should only inhibit the 17α-hydroxylase-$C_{17,20}$-lyase enzyme and not other cytochrome P450-dependent enzymes.

Abiraterone (17-(3-pyridyl-)androsta-5,16-dien-3β-ol) (Fig. 1) was developed as a selective mechanism-based steroidal inhibitor, following observations that nonsteroidal 3-pyridyl esters have improved selectivity for the inhibition of 17α-hydroxylase-$C_{17,20}$-lyase [9]. It is a potent inhibitor of the enzyme with a Ki_{app} of <1 nM [10]. Abiraterone acetate is a prodrug and 3β-*O*-acetate form of abiraterone (Fig. 1), and is de-acetylated to abiraterone *in vivo* [10,11].

The potential of abiraterone acetate to suppress testosterone levels, by the specific inhibition of the 17α-hydroxylase-$C_{17,20}$-lyase enzyme, in castrate and noncastrate males with prostate

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cancer, has been shown in clinical studies [12]. We present here a specific and selective validated liquid chromatography tandem mass spectrometry (LC–MS/MS) method for the quantification of abiraterone acetate and abiraterone in human plasma. This method will support an open label phase I/II study to evaluate the efficacy of abiraterone acetate in castrate males with hormone refractory prostrate cancer.

2. Experimental

2.1. Chemicals and reagents

Abiraterone acetate and abiraterone (Fig. 1) were provided by Cougar Biotechnology Inc. (Los Angeles, California, USA) with a purity of >99.7% and >99.2%, respectively. Dimethyl sulfoxide (DMSO), ammonium acetate and ammonia solution were obtained from Fisher Chemicals (Loughborough, Leicestershire, UK). Hydrochloric acid and propan-2-ol were obtained from VWR International Ltd. (Poole, Dorset, UK). HPLC grade acetonitrile and methanol were obtained from Laserchrom Chemicals Ltd. (Rochester, Kent, UK). Formic acid was obtained from Sigma (Poole, Dorset, UK). Drug-free (blank) human plasma was obtained from the Blood Transfusion Service (anticoagulant EDTA; Tooting, London, UK). Distilled water was generated using a Maxima-HPLC (18.2 MΩ cm) (Elga Ltd., High Wycombe, UK).

2.2. Mass spectrometry instrumentation

An Alliance Waters 2795 separations module liquid chromatography system (Waters Ltd., Hertford, UK), coupled to a Micromass Quattro Ultima mass spectrometer (Micromass UK Ltd., Manchester, UK), was used. Micromass MassLynx software (version 4.0 SP4) controlled the LC–MS/MS system.

2.3. Chromatography

The chromatographic separation of analytes was performed on a Luna C5 (5 μm, 50 mm × 2.1 mm i.d.) analytical column protected by a guard column (C5, 4.0 mm × 2.0 mm i.d.) (Phenomenex, Macclesfield, Chesire, UK) at ambient temperature. The mobile phase consisted of 2% propan-2-ol in acetonitrile and 10 mM ammonium acetate, with a linear gradient of 35% organic, increasing to 100% organic over 4 min, remaining at 100% organic for 4 min and then linearly decreasing to 35% over 0.5 min. The mobile phase was delivered at a flow rate of 0.2 mL min^{-1}. The injection volume for each sample was 10 μL with a total run time of 10.5 min.

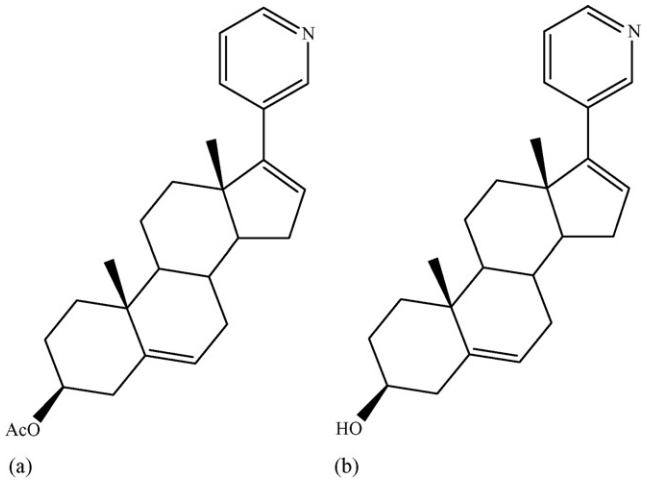

Fig. 1. Chemical structures of (a) abiraterone acetate and (b) abiraterone.

2.4. Mass spectrometry

Abiraterone acetate and abiraterone were ionised using electrospray ionisation in positive mode (ESI^+). Final conditions of capillary voltage (−3.2 kV), cone voltage (110 V), source temperature (100 °C), desolvation temperature (200 °C), cone gas flow (59 L h^{-1}) and desolvation gas flow (581 L h^{-1}) were optimal for the detection of parent and product ions. Standard solutions of analytes (10 μM) were infused into the mass spectrometer at a constant flow rate of 20 μL min^{-1}, using a Harvard Model 22 syringe pump (Harvard Apparatus Ltd., Kent, UK), for the optimisation of MS conditions.

2.5. Data acquisition and quantification

Analytes were detected using multiple reaction monitoring (MRM). The characteristic ion dissociation transitions were *m/z* 392.33 → 332.20 and 350.00 → 155.90 with collision energies of −30 and −40 eV, for abiraterone acetate and abiraterone, respectively. The MS response of each calibration standard was plotted against the prepared concentration and subjected to a least square regression weighting ($1/x^2$), to produce a calibration curve.

2.6. Stock solutions

Separate calibration and quality control stock solutions of abiraterone acetate and abiraterone were prepared in DMSO at a concentration of 1 mM. A mixed intermediate calibration and quality control stock solution (10 μM) was prepared by a 100-fold dilution of the individual stocks. Dilutions of the mixed intermediate stocks were made with DMSO, as appropriate, to give spiking solutions with concentrations of 5000, 3500, 2000, 1000, 500, 100 and 50 nM. Quality control (QC) spiking solutions were prepared at high (QCH: 4000 nM), medium (QCM: 2500 nM) and low (QCL: 150 nM) concentrations, as well as at the lower limit of quantification (QC LLOQ: 50 nM). All solutions were stored at −20 °C.

2.7. Preparation of calibration and QC samples

A mixed calibration curve with concentrations of 500, 350, 200, 100, 50, 10 and 5 nM, was prepared by adding 10 μL of the appropriate spiking solution to 100 μL of blank human plasma. QC samples were prepared by the addition of QCH, QCM, QCL and QC LLOQ spiking solutions (10 μL) to blank human plasma (100 μL) to give final concentrations of 400, 250, 15 and 5 nM.

2.8. Sample extraction

Plasma samples (100 μL) were acidified with 100 μL of 2% v/v formic acid in water and vortex mixed. Samples were centrifuged at 2000 × *g* for 10 min at 4 °C. Solid phase extraction was performed using an Aspec XL4 sample processor (Anachem, Luton, UK). Strata X-C 33 μM cation mixed-mode polymer cartridges (Phenomenex, Macclesfield, Chesire, UK) were conditioned with 1 mL methanol and 1 mL water. Sample (200 μL) was loaded at a rate of 6.0 mL min^{-1} followed by washing with 600 μL of 0.1 M hydrochloric acid and 600 μL methanol. Sample was eluted with 600 μL of 5% ammonium hydroxide in acetonitrile–methanol (80:20 v/v) at a rate of 6.0 mL min^{-1}. Eluant was evaporated to dryness for 45 min, on heat setting 1, in a speed vacuum sample concentrator (Model RC 10–22, Jouan, France) and reconstituted into 100 μL of acetonitrile–water (80:20 v/v).

2.9. Method validation

The method was validated according to the guidelines set by the US Food and Drug Administration (FDA) for bioanalytical method validation [13]. Validation consisted of three precision batches, each batch comprising of a human plasma standard curve, control human plasma and replicate ($n = 6$) QCH, QCM, QCL and QC LLOQ samples, in order to generate accuracy and precision data and assess the following parameters.

2.9.1. Specificity

Six independent batches of blank human plasma were assessed for the presence of chromatographic interferences, which could affect assay performance.

2.9.2. Linearity of calibration curves

The effect of weighting on the slope, intercept and coefficient of regression was evaluated.

2.9.3. Accuracy

The inter-batch accuracy of the method was expressed as relative error (%RE), which compares the mean observed QC concentrations with the theoretical concentrations ($n = 18$), and is calculated with the following equation:

$$\mathrm{RE}(\%) = \frac{(\text{mean observed conc} - \text{prepared conc})}{\text{prepared conc}} \times 100$$

2.9.4. Precision

Intra-batch and inter-batch precision of the assay were measured as coefficient of variations (CV), calculated from replicate ($n = 6$) samples at the LLOQ and QCL, QCM and QCH in assay batches 1–3.

2.9.5. Sensitivity

Sensitivity was determined by the assessment of the accuracy and precision at the lower limit of quantification, based on replicate ($n = 6$) analysis of the LLOQ (5 nM) in assay batches 1–3.

2.9.6. Stability

The stability of abiraterone acetate and abiraterone in human plasma was assessed at room temperature for 4 h, 2 weeks and 1 month at −20 °C and after three freeze–thaw cycles at −20 °C. Replicate ($n = 3$) sets of QCL and QCH were analysed. Stability of the stock solutions was assessed after storage at −20 °C for 4 months.

2.9.7. Recovery

To calculate extraction efficiency, abiraterone acetate and abiraterone were extracted from plasma or spiked into a blank plasma extract at QCL, QCM and QCH. The results were expressed as recovery (%) = (area of extracted analyte/unextracted analyte) × 100.

To determine the influence of endogenous material extracted from plasma on the analytical method, analytes were spiked into a blank plasma extract at QCL, QCM and QCH, and were compared to a standard of the same concentration.

2.9.8. Dilution

As abiraterone acetate and abiraterone concentrations in patient samples are likely to exceed the upper quantitative limit of the calibration curve, the accuracy (%RE) and precision (%CV) of diluting control samples into the calibration range, was assessed. Dilution factors of 5, 10 and 20 were applied.

3. Results

3.1. Method development

Preliminary tuning experiments were performed using positive electrospray ionisation (ESI) mode. Analytes (10 μM) were infused into the mass spectrometer at a rate of 20 μL min^{-1} with a mobile phase of either 0.1% v/v formic acid in water–methanol (50:50 v/v) or 2% propan-2-ol in acetonitrile–10 mM ammonium acetate (50:50 v/v). The latter mobile phase resulted in the greatest sensitivity in parent ion detection and was therefore the mobile phase of choice. Analytes were detected using multiple reaction monitoring. Transitions from the specific parent ion to product ion fragment were optimised (described in Sections 2.4 and 2.5). Chromatography was performed using a gradient mobile phase at a flow rate of 0.2 mL min^{-1} at ambient temperatures. The purge solvent was 2% propan-2-ol in acetonitrile and 10 mM ammonium acetate (35:65 v/v) and the needle/seal wash was methanol. A Luna C5 (5 μm, 50 mm × 2.1 mm i.d., Phenomenex, Chesire, UK) was chosen as the analytical column. The reduced retentive property of the Luna column proved optimal for the linearity of calibration curves, in comparison with other columns tested: C18 Discovery (Supelco, Sigma–Aldrich, Dorset, UK), Synergi Polar-RP and Synergi Max-RP (Phenomenex, Cheshire, UK). The injection volume was 10 μL and the run time 10.5 min. Under these LC–MS/MS conditions abiraterone acetate eluted at 7.22 min and abiraterone at 5.74 min. No interferences were present in blank human plasma (Fig. 2). A representa-

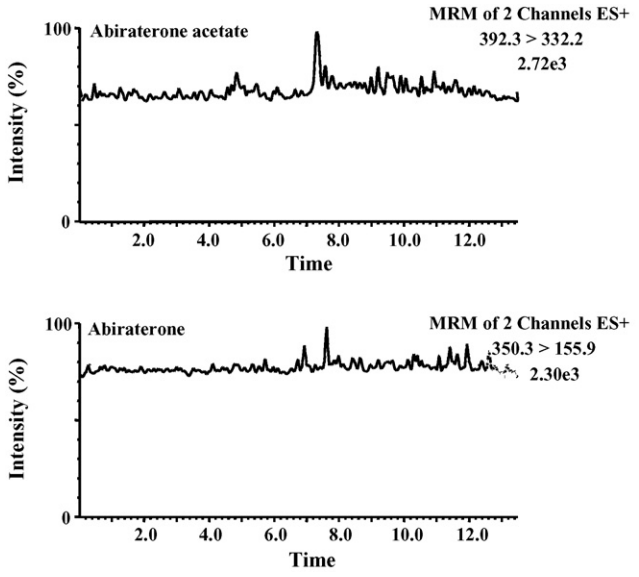

Fig. 2. Representative chromatogram of extracted blank human plasma.

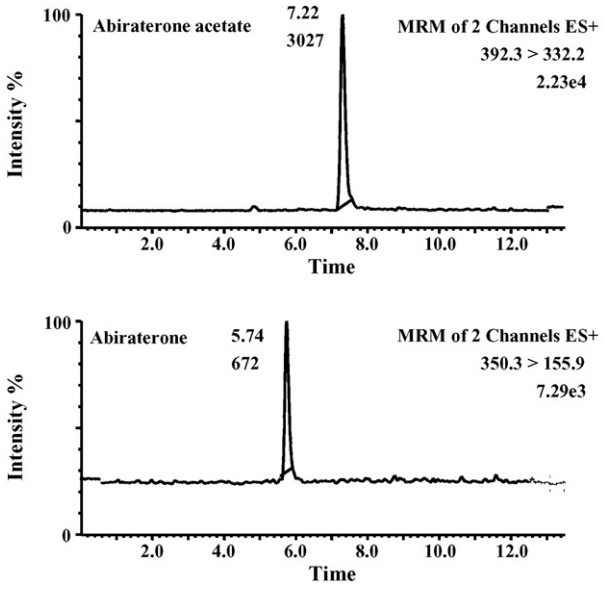

Fig. 3. Representative chromatogram of extracted LLOQ (5 nM) calibration standard.

tive chromatogram of analytes at 5 nM (LLOQ) is shown in Fig. 3.

Various techniques were investigated for the extraction of abiraterone acetate and abiraterone from human plasma. Protein precipitation with various organic solvents resulted in low sensitivity. Liquid–liquid extraction (LLE) improved sensitivity and was performed using ethyl acetate, dichloromethane and mixtures such as hexane-propan-2-ol (98:2 v/v) and hexane–butanol (98:2 v/v). LLE with the latter solvent mixture provided the most sensitivity, however the reproducibility of the assay was poor. Solid phase extraction was finally selected as it provided adequate sensitivity, assay reproducibility and cleaner extracts.

3.2. Specificity

No endogenous interferences from six independent batches of human plasma were observed, which if present could affect assay performance. Minimal carry-over (<5% of the lower limit of quantification) was observed for abiraterone acetate in acetonitrile washes, whilst no carry-over was seen for abiraterone. No cross talk was observed between compounds.

Table 1
Intra and inter-batch quality control samples for abiraterone acetate and abiraterone

QC	Abiraterone acetate				Abiraterone			
	LLOQ 5 nM	QCL 15 nM	QCM 250 nM	QCH 400 nM	LLOQ 5 nM	QCL 15 nM	QCM 250 nM	QCH 400 nM
Intra-batch								
Mean	4.7	14.0	222.1	350.2	4.6	14.5	226.6	355.1
SD	0.3	0.8	23.3	12.7	0.3	1.2	14.7	25.6
CV (%)	7.0	5.5	10.5	3.6	6.8	8.6	6.5	7.2
RE (%)	−5.7	−6.9	−11.2	−12.5	−8.3	−3.7	−9.4	−11.2
Mean	4.7	14.5	226.3	357.4	4.7	15.7	260.8	405.3
SD	0.3	0.6	5.1	13.3	0.5	1.5	16.3	31.8
CV (%)	6.2	4.3	2.3	3.7	9.8	9.6	6.2	7.9
RE (%)	−6.0	−3.3	−9.5	−10.7	−5.7	4.7	4.3	1.3
Mean	4.4	14.7	245.9	376.5	5.6	16.1	259.7	386.3
SD	0.6	0.8	14.1	24.9	0.8	1.4	16.4	26.2
CV (%)	13.9	5.6	5.7	6.6	13.8	8.6	6.3	6.8
RE (%)	−12.3	−2.2	−1.6	−5.9	11.0	7.4	3.9	−3.4
Inter-batch								
Mean	4.6	14.4	231.4	361.3	5.0	15.4	249.0	382.2
SD	0.4	0.8	18.4	20.3	0.7	1.5	22.1	33.9
CV (%)	9.5	5.3	8.0	5.6	13.7	9.7	8.9	8.9
RE (%)	−8.0	−4.1	−7.4	−9.7	−1.0	2.8	−0.4	−4.4

3.3. Human plasma standard curve

The calibration curve was linear over the range 5–500 nM, with a mean correlation coefficient determination of 0.998 ($n = 3$) (data not shown) for both analytes. Weighted least squares regression ($1/x^2$) was used.

3.4. Accuracy

For both analytes the mean intra-batch %RE was below 12.5% over all QC levels (Table 1). For abiraterone acetate the mean inter-batch %RE ranged from −4.1 to −9.7%, whilst for abiraterone %RE ranged from −4.4 to 2.8% (Table 1).

3.5. Intra-batch precision

The intra-batch precision of QC samples ($n = 6$) from assay batches 1–3, expressed as %CV, ranged from 6.2 to 13.9% at 5 nM (LLOQ), 4.3 to 5.6% at 15 nM, 2.3 to 10.5% at 250 nM and 3.6 to 6.6% at 400 nM for abiraterone acetate. For abiraterone, %CV ranged from 6.8 to 13.8%, 8.6 to 9.6%, 6.2 to 6.5% and 6.8 to 7.9% at 5, 15, 250 and 400 nM, respectively (Table 1).

3.6. Inter-batch precision

The inter-batch precision of QC samples ($n = 18$) from assay batches 1–3, expressed as %CV, were 9.5% at 5 nM, 5.3% at 15 nM, 8.0% at 250 nM and 5.6% at 400 nM for abiraterone acetate. For abiraterone, %CV was 13.7%, 9.7%, 8.9% and 8.9% at 5, 15, 250 and 400 nM, respectively (Table 1).

3.7. Sensitivity

At the LLOQ (5 nM) the intra-batch precision, expressed as %CV, ranged from 6.2 to 13.9% for abiraterone acetate and 6.8 to 13.8% for abiraterone. Inter-batch precision was 9.5% for abiraterone acetate, with a %RE of −8.0% ($n = 18$). For abiraterone inter-batch, precision was 13.7% with a %RE of −1.0% ($n = 18$) (Table 1).

3.8. Stability

3.8.1. Stability in human plasma

Abiraterone acetate and abiraterone were stable in human plasma at room temperature for 4 h and after storage for 2 weeks and 1 month at −20 °C. Analytes were also stable after three freeze–thaw cycles at −20 °C (Table 2).

Table 2
Stability of abiraterone acetate and abiraterone in human plasma

Treatment	Abiraterone acetate		Abiraterone	
	QCL 15 nM	QCH 400 nM	QCL 15 nM	QCH 400 nM
(a) Room temperature 4 h				
Mean	17.0	440.2	16.5	426.9
CV (%)	5.3	3.4	8.2	3.3
RE (%)	13.6	10.1	10.0	6.7
(b) 2 weeks stability (−20 °C)				
Mean	17.4	379.6	15.3	358.1
CV (%)	12.2	6.0	7.4	5.7
RE (%)	16.2	−5.1	2.2	−10.5
(c) 1 month stability (−20 °C)				
Mean	15.8	410.0	16.7	441.4
CV (%)	14.3	0.9	15.0	2.5
RE (%)	5.1	2.5	11.6	10.3
(d) Freeze–thaw stability				
Mean	15.5	374.3	14.6	364.0
CV (%)	3.9	12.2	13.8	4.8
RE (%)	3.3	−6.4	−2.9	−9.0

3.8.2. Stability in stock solutions

Abiraterone acetate and abiraterone were stable in standard stock solutions for 4 months at −20 °C (data not shown).

3.9. Recovery

Recoveries ($n = 6$) of abiraterone acetate from human plasma by solid phase extraction were 73%, 74% and 80% at QCL, QCM and QCH concentrations, respectively. Similar recoveries of 77%, 74% and 71% at QCL, QCM and QCH levels were obtained for abiraterone. No matrix effects were observed for abiraterone acetate over all concentrations. For abiraterone, a negligible mean difference of 2% was observed.

3.10. Dilution

The observed concentrations for dilution controls were in good agreement with the prepared concentrations. The relative errors (%RE) were 3.1%, 1.0% and 3.7% after dilution factors of 5, 10 and 20, respectively, for abiraterone acetate.

Table 3
Precision and accuracy summary of abiraterone acetate and abiraterone dilution quality control samples

Dilution QC Conc. (nM)	Dilution factor	Mean assayed Conc. (nM)	CV (%)	RE (%)
Abiraterone acetate				
300	20	15.6	10.2	3.7
2500	10	252.4	7.3	1.0
2000	5	412.3	7.0	3.1
Abiraterone				
300	20	14.8	8.6	−1.2
2500	10	227.3	7.4	−9.1
2000	5	380.4	7.1	−4.9

For abiraterone relative errors were −4.9%, −9.1% and −1.2% (Table 3).

4. Discussion

A liquid chromatography–tandem mass spectrometry (LC–MS/MS) method is described in this paper, for the quantification of abiraterone acetate and abiraterone in human plasma. In prior clinical studies, analytes were extracted from plasma using a one step liquid–liquid extraction with hexane–butanol (98:2 v/v) and detected by mass spectrometry using selected ion monitoring (SIM) [12]. In order to improve on the previous method various techniques were investigated for the extraction of analytes from plasma, such as liquid–liquid extraction with different organic solvents and protein precipitation. Solid phase extraction (SPE) proved to be the optimal method as it provided assay reproducibility and accuracy, clean sample extracts, reduced sample volumes and eliminated the use of undesirable solvents such as hexane. In addition the SPE method was automated using an Aspec XL4 sample processor, which significantly decreased the time required for sample preparation. Future work may include a cross-validation using a 96-well plate format, in order to achieve even higher sample preparation throughput.

Mass spectrometric detection of abiraterone acetate and abiraterone has also been improved. Previous methods used SIM [12] while our method employs tandem mass spectrometry, which confers increased selectivity. The dynamic range of abiraterone acetate and abiraterone has been extended and the assay is linear from 5–500 nM. Samples with analyte concentrations up to 20 times above the limit of this calibration range may be accurately and precisely diluted into range.

Numerous commercially available compounds, both steroidal and nonsteroidal, were investigated as potential internal standards for the analytical method. However, a suitable synthetic compound having sufficiently similar physicochemical properties to abiraterone acetate and abiraterone was not identified. Furthermore the robustness of the described analytical method, as demonstrated through its validation, showed that an internal standard was not necessary.

In conclusion, this assay is accurate, sensitive, and reproducible and meets the requirements set by the FDA for bioanalytical method validation [13]. The assay will support a forthcoming clinical trial investigating the pharmacokinetics of abiraterone acetate and abiraterone in patients with prostate cancer who have failed hormone therapy.

Acknowledgments

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