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Development and Validation of a Novel LC–MS/MS Method for Simultaneous Determination of Abiraterone and its Seven Steroidal Metabolites in Human Serum: Innovation in Separation of Diastereoisomers Without Use of a Chiral Column

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Development and validation of a novel LC–MS/MS method for simultaneous determination of abiraterone and its seven steroidal metabolites in human serum: Innovation in separation of diastereoisomers without use of a chiral column

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1. Introduction

Prostate cancer (PCa) progression depends on continued androgen receptor activity [1,2]. Androgen receptor activation occurs through the binding of the potent androgens, testosterone and dihydrotestosterone (DHT), which has been shown to promote malignant cell growth [3]. Androgen deprivation therapy (ADT), either by medical or surgical castration, is the treatment for patients with advanced disease [4]. However, in most cases, although the cancer initially responds to ADT, after time, it becomes resistant, and castration-resistant prostate cancer (CRPC) develops, which is the cause of nearly all PCa deaths [5–7]. In 2016 in the United States, the number of new PCa cases is estimated to be 180,000, with about 26,000 PCa-related deaths, making prostate cancer the most frequently diagnosed cancer and the second-leading cause of cancer death in men [8].

Abiraterone [abi; 17-(3-pyridyl)-androsta-5,16-dien-3 β -ol] is a 17-heteroazole steroidal compound and a potent inhibitor of steroid 17 α -hydroxylase/17,20-lyase (CYP17A1), an enzyme required for androgen synthesis [9]. Abi's structure makes it a potent CYP17A1 inhibitor, and the double bond at C16 is necessary for

functionally irreversible inhibition of the enzyme [10–12]. Abi is administered as the prodrug, abiraterone acetate (AA), for the treatment of CRPC and prolongs survival for these patients [13–15]. In 2011, the United States Food and Drug Administration (FDA) approved AA in the post-chemotherapy setting, and in December 2012 it approved the use of AA for patients with chemotherapy-naïve CRPC [16]. Prednisone is administered with AA to block mineralocorticoid excess that occurs with the simultaneous inhibition of cortisol synthesis [17,18].

It has been reported that the main circulating Abi metabolites are abiraterone sulfate and N-oxide abiraterone sulfate [19]. We have recently shown that Abi is converted by 3 β -hydroxysteroid

dehydrogenase (3 β -HSD) to its Δ^4 , 3-keto congener (Δ^4 -abiraterone; D4A) [20] both *in vitro* and *in vivo*. The steroid A and B rings of D4A (Fig. 1) are identical to that of testosterone and androstenedione, which enables further metabolism of D4A [21]. Either the 5 α -reductase or 5 β -reductase enzymes could initiate the downstream metabolism of D4A. The 5 α -reduced metabolites are 3-keto-5 α -Abi, 3 α -OH-5 α -Abi and 3 β -OH-5 α -Abi, while the corresponding 5 β -reduced metabolites are 3-keto-5 β -Abi, 3 α -OH-5 β -Abi and 3 β -OH-5 β -Abi (Fig. 1).

The separation of the Abi metabolites and the parent Abi compound is expected to be difficult, given their structural similarities. Here, we describe and validate a novel method for

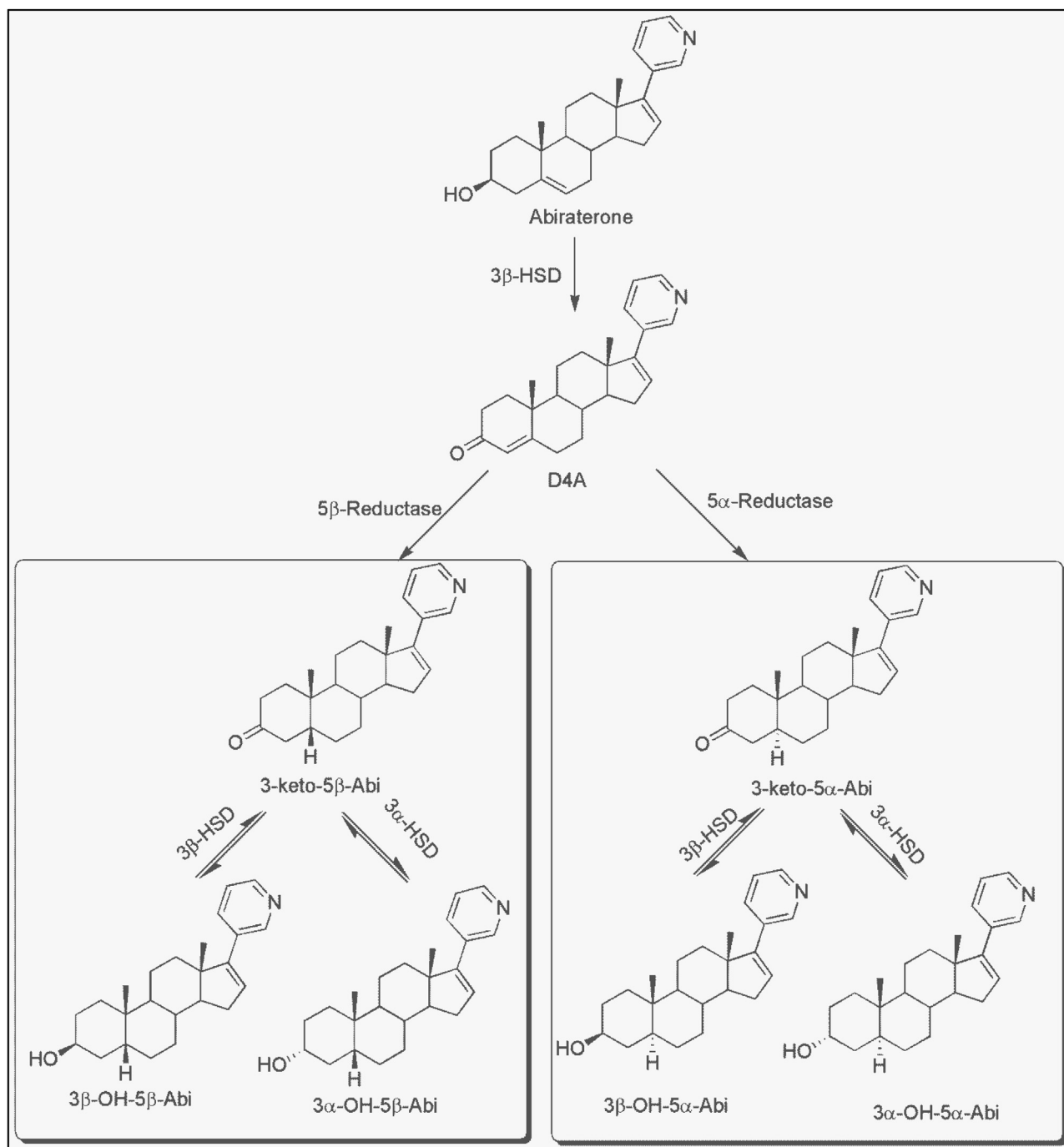


Fig. 1. Steroidogenic enzyme metabolism of abiraterone and structure of the resultant metabolites. 3 β -HSD: 3 β -hydroxysteroid dehydrogenase, 5 α -Reductase: steroid 5 α -reductase, 5 β -reductase: steroid 5 β -reductase, 3 α -HSD: 3 α -hydroxysteroid dehydrogenase.

the determination of Abi and its 7 metabolites (Fig. 1) in human serum. Separation of all diastereomeric metabolites was achieved using a reversed-phase chromatographic technique, not requiring a chiral column. We demonstrated that all seven steroidal Abi metabolites are present in serum samples from patients with CRPC undergoing treatment with AA [21].

2. Experimental

2.1. Materials

Abiraterone and its seven metabolites 3-keto- Δ^4 -Abi, 3-keto-5 α -Abi, 3 α -OH-5 α -Abi, 3 β -OH-5 α -Abi, 3-keto-5 β -Abi, 3 α -OH-5 β -Abi and 3 β -OH-5 β -Abi were synthesized as described previously [21]. The internal standard (abiraterone-d4) was purchased from Toronto Research Chemicals, (Toronto, Canada). Methanol, acetonitrile, water, and formic acid were LC-MS grade, methyl tertiary butyl ether (MTBE) was HPLC grade and all were from Fisher Scientific (Fair Lawn, NJ). Double charcoal-stripped human serum (Golden West Biological Inc., Temecula, CA) was used for all samples in the validation studies (controls, calibrators) which were processed by spiking with known concentrations of the analytes.

2.2. Preparation of standards and quality control (QC) samples

The stock solutions (1 mg/mL) of Abi and its metabolites were prepared by dissolving the synthesized powder in 100% methanol. These solutions were further diluted with methanol:H₂O, 1:1, and then mixed in one flask to prepare the working standard at concentration of 5.0 μ g/mL Abi and 0.25 μ g/mL for each metabolite. Freshly prepared working standard was used to prepare the serum calibrators and serum quality control samples, all of which contained appropriate concentrations of all the analytes. Quality control (QC) samples were prepared at three levels, based on the lower limit of quantitation (LLOQ) and the upper limit of quantitation (ULOQ): QC Low (3x LLOQ), QC Mid (half ULOQ), and QC High, which is 80% of ULOQ. The final concentrations of the calibrators and quality control in serum are listed in Table 1. The internal standard stock (2 mg/mL) was prepared by dissolving the stock powder in 100% methanol and further diluting it with methanol:H₂O, 1:1 to a final concentration of 1.25 μ g/mL. Stock and working standard solutions were stored at -20°C, and the calibrators with quality controls were freshly prepared through the validation.

2.3. Sample preparation

Each calibrator, quality control and patient serum sample was taken through the following sample preparation steps. The

analytes were extracted from human serum using a liquid-liquid extraction procedure. 100 μ L of serum with spiked analytes was placed in a glass tube, and 20 μ L of 1.25 μ g/mL internal standard working solution was added. The samples were vortexed for 30 s. After addition of 2 mL MTBE, the samples were vortexed for 1 min. The samples were then centrifuged for 5 min at 4000 rpm at 4°C. The organic layer was transferred to another tube and evaporated to dryness under nitrogen at 40°C. The dried extract was then reconstituted with 300 μ L 1:1 methanol:H₂O, and 200 μ L was transferred to an HPLC vial.

2.4. Instrumentation and data analysis

The LC-MS/MS system was an ultra-pressure liquid chromatography (UPLC) system (Shimadzu Corporation, Japan), consisting of a LC-30AD solvent delivery system, a DGU-20A5R vacuum degasser, a CTO-30A thermostated column oven, SIL-30AC autosampler, and a system CBM-20A controller, that was coupled with a Qtrap 5500 mass spectrometer (AB Sciex, Redwood City, CA). Data acquisition and processing were performed using Analyst software (version 1.6.2) from AB Sciex. The peak area ratio of the analyte over the internal standard was used for quantification purposes.

2.5. UPLC-MS/MS conditions

Separation of drug metabolites was achieved using a Zorbax Eclipse Plus C₁₈ column 150 mm x 2.1 mm, 3.5 μ m (Agilent, Santa Clara, CA) at 40°C with an isocratic mobile phase consisting of 35% A (0.1% formic acid in water) and 65% B (0.1% formic acid in methanol:acetonitrile, 60:40), at a flow rate of 0.2 mL/min. Sample injection volume was 10 μ L, and analytical run time was 13 min.

The mass spectrometer was operated in positive ion mode using an electrospray ionization (ESI) source. Tuning parameters were optimized for the Abi metabolites and internal standard by infusing a solution containing 200 ng/mL of each analyte. Nitrogen was used as the nebulizing (40 L/min) agent and drying gas (30 L/min). Ion spray voltage and nebulizer temperature were regulated at 2500 V and 500°C, respectively. All the analytes have the same declustering potential (120 V), collision energy (60 V), entrance potential (10 V) and collision exit potential (13 V). The analytes were quantified using multiple reaction monitoring (MRM) with the mass transitions for each compound as listed in Table 2.

2.6. Method validation

The method was validated following the US FDA guidance for bioanalytical method validation [22]. The validation parameters evaluated were linearity, accuracy and precision, selectivity, recovery, matrix effect, and stability.

Table 1
Calibrators and quality control samples preparation and final concentration.

Sample	Volume of Working Standard (μ L)	Final Volume (mL)	Final Concentration in Serum ng/mL	
			Abi	Metabolites
Cal.1	20	10	2	0.1
Cal. 2	40	10	4	0.2
Cal. 3	120	10	12	0.6
Cal. 4	300	5	60	3
Cal. 5	600	5	120	6
Cal. 6	1400	5	280	14
Cal. 7	2000	5	400	20
QC Low	60	10	6	0.3
QC Mid	1000	5	200	10
QC High	1600	5	320	16

Table 2
Mass transition parameters for abiraterone, its metabolites, and the internal standard.

Analyte	Q1 (m/z)	Q3 (m/z)
Abiraterone	350.5	156.1
D4A	348.3	156.1
3-keto-5 α -Abi	350.3	156.2
3 α -OH-5 α -Abi	352.4	156.2
3 β -OH-5 α -Abi	352.3	156.1
3-keto-5 β -Abi	350.4	156.1
3 α -OH-5 β -Abi	352.4	156.4
3 β -OH-5 β -Abi	352.1	156.1
Abiraterone-d4 (IS)	354.4	160.1

IS = internal standard.

2.6.1. Linearity

Six calibration curves were constructed in same serum matrix for each analyte to assess the linear range. The ratio of the analyte peak area over the internal standard peak area was plotted versus the nominal concentration. The correlation was then fit by linear regression, applying a weighting factor of $1/x$. To assess selectivity, blank and zero (blank spiked with internal standard) samples were prepared with each calibration curve. The calibration curve consisted of 7 non-zero points with single repeat for each point. Based on the FDA guidelines, the acceptable deviation from the nominal concentration is <20% for the LLOQ calibrator and <15% for the other calibrators. Validation of the calibration curve requires 67% of the calibrators, including LLOQ and ULOQ, being within these acceptance criteria.

2.6.2. Accuracy, precision, and LLOQ

In order to determine the accuracy and precision, QC samples (LLOQ, low, mid, and high) were run. Intra-day precision was determined with five replicates and inter-day precision was determined running one of each QC sample on 3 separate days. Accuracy was determined by how close the mean of the intra-day QC sample results were to the nominal value. The method is considered accurate if the measured concentration is within 85–115% (80–120% for LLOQ) of the expected value. The acceptable precision criterion is a coefficient of variation percent (CV%) of the QC sample analyte concentration being no more than 15% (LLOQ not more than 20%).

2.6.3. Selectivity

Single runs of six different serum batches were used to study the selectivity of the method. To insure that the samples were free of interference, LLOQ samples were prepared and compared to the corresponding blank samples. The requirement of LLOQ is to be at least 5 times the peak height (or peak area) of any peak detected in the blank samples at the same retention time of the analyte.

2.6.4. Recovery

Relative recovery was assessed by calculating the ratio of the analyte/internal standard peak area in spiked samples before extraction over the analyte/internal standard peak area in spiked samples after extraction. Three QC samples (low, mid, and high) were determined. Each of the three QC samples, including both the pre- and post-extraction samples were assayed in triplicate. The FDA criterion for acceptable recovery results need to be consistent, precise and reproducible, as assessed by the coefficient of variation for each QC sample type being no more than 15%. The CV% was calculated using this formula $CV\% = [(DX/X)^2 + (DY/Y)^2]^{1/2}$, where DX is the standard deviation for the pre-extraction samples, X is the mean area ratio of pre-extraction samples, DY is the standard deviation for the post-extraction samples, and Y is the mean area ratio of post-extraction samples.

2.6.5. Matrix effect

Matrix effect, calculated as a matrix factor percent (MF%), was assessed as a ratio of peak area ratio of spiked analyte/internal standard determined for QC low sample after extraction ($n = 18$) over the peak area ratio of the spiked analyte/internal standard in methanol: H_2O , 1:1 ($n = 3$). The QC sample was tested in triplicate on each of six different serum batches. CV% was calculated as mentioned in section 2.6.4.

2.6.6. Stability

Analyte stability was evaluated for analytes in both solution and serum. Diluted standard solution stability was evaluated after 6 h at room temperature and after 9 days storage at 4 °C for all eight analytes at LLOQ and ULOQ levels and for the internal standard

working solution. The stability was assessed in triplicate by comparing peak area of the old (6 h and 9 days) solutions with freshly prepared from stock stored at –20 °C.

In order to ensure that the processed samples were stable during the analytical run, post-preparative stability was studied at the three QC levels for 43 h at the auto sampler temperature of 4 °C by analyzing five samples per QC level. Analyte stability in serum was determined by preparing samples at two QC levels (low and high) and analyzing six serum samples containing a known concentration of each analyte as follows: bench top stability at room temperature after 21 h, after three freeze-thaw cycles (24 h at –80 °C with thaw unassisted at room temperature), and long-term stability at –80 °C after 28 weeks of storage. In order to evaluate the stability, the concentrations in these samples were compared to the nominal values by running a freshly prepared calibration curves. The accuracy should fall within the 85–115% acceptance criteria and RSD not to exceed 15%

3. Results and discussion

3.1. Method development

In the development of a comprehensive pharmacokinetic technique determining parent drug and metabolites, the challenge is to separate all of these compounds, while achieving the highest sensitivity without compromising linearity. Several mobile phases were evaluated including different organic modifiers (methanol, acetonitrile and methanol/acetonitrile mixtures) at various concentrations in water, as well mobile phases containing ammonium formate and/or formic acid without organic modifier. The Prodigy Phenomenex C_{18} analytical columns were also evaluated. Optimization of sample preparation according to recovery was also performed, varying volumes of MTBE, as well as evaluating acidic and neutral reconstitution solutions. The best method was then selected based on the separation of all metabolites as well as based on the analytical qualities of linearity and sensitivity.

3.2. Optimization of the mass analyzer and chromatographic conditions

Because these metabolites are steroidal compounds containing a pyridyl moiety, the mass analyzer was operated with electrospray ionization in the positive mode. The parameters were optimized to ensure that the highest sensitivity possible would be achieved. The settings of the mass spectrometric parameters used in the optimized method are listed in Section 2.5, and the analyte mass transitions are given in Table 2.

3.3. Optimization of the chromatographic conditions

Many of the metabolites are isomeric compounds, and thus it is not possible to distinguish between them based on their MRM transitions. Therefore, we separated the metabolites (as well as the parent compound) by chromatography. Optimization of isocratic chromatographic conditions was done investigating the effect of various mobile phase components at different concentrations. A mobile phase of 35% water mixed with 65% methanol/acetonitrile (60:40) (both containing 0.1% formic acid) resulted in the optimized chromatographic performance, separating the parent compound and metabolites, as shown in Fig. 2. It should be noted that the method was able to resolve diastereomeric metabolites with a C_{18} column, not requiring chiral columns for separation of these stereoisomers. The employment of a methanol/acetonitrile mixture was critical to achieve this separation, as methanol or acetonitrile as sole organic modifiers in the mobile phase did not resolve the chiral compounds.

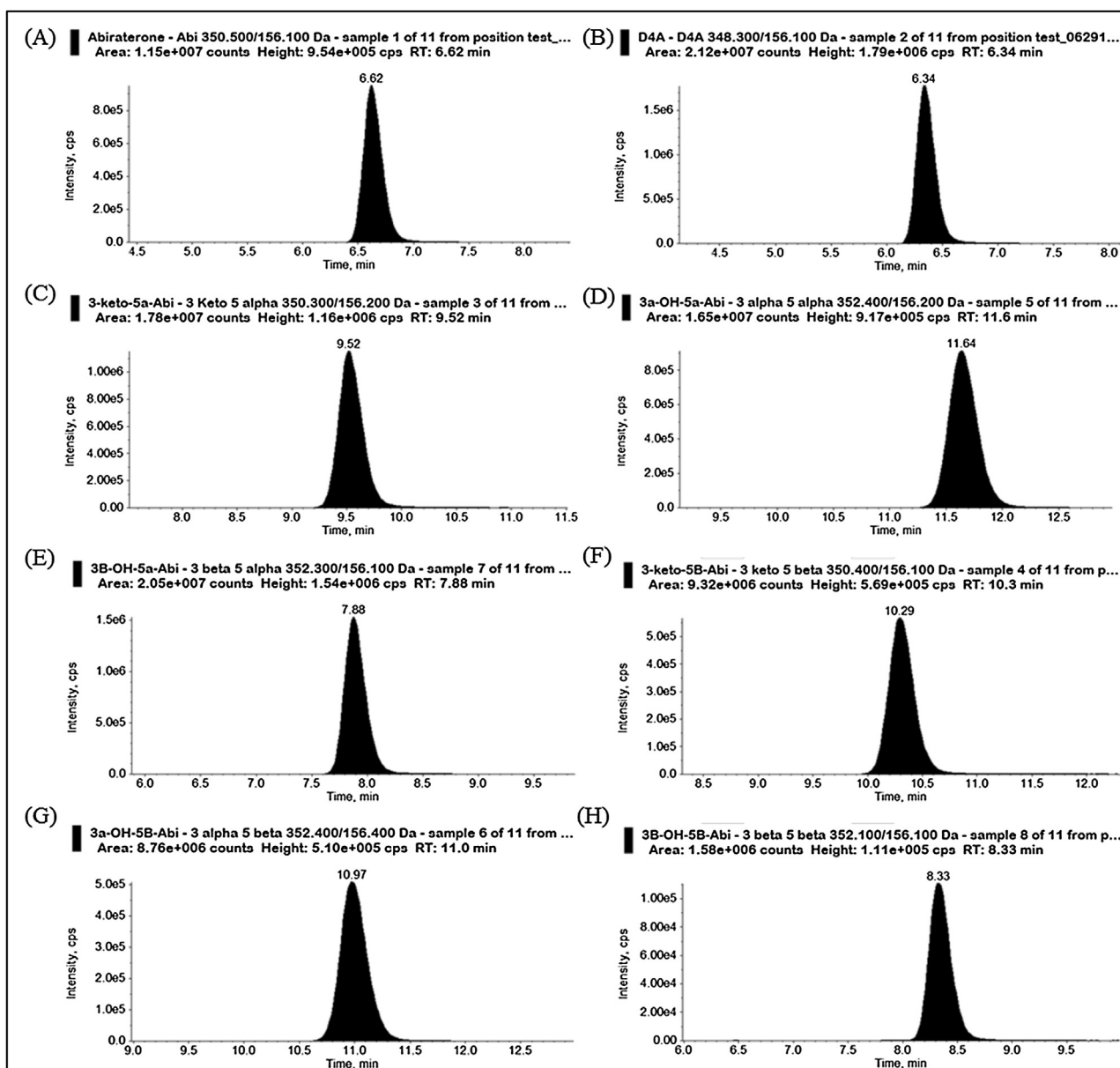


Fig. 2. Chromatogram for abiraterone and its metabolites. The results were obtained by injecting 10 μ L of 50 ng/mL standard solutions for A, Abi; B, D4A; C, 3-keto-5 α -Abi; D, 3 α -OH-5 α -Abi; E, 3 β -OH-5 α -Abi; F, 3-keto-5 β -Abi; G, 3 α -OH-5 β -Abi; and H, 3 β -OH-5 β -Abi.

3.4. Method validation

The method was validated according to FDA guidelines. As discussed below, all criteria of the guidelines were met for the present method. All the analytes were stable in solution and serum, meeting the stability criterion. The criteria for linearity, accuracy and precision fell within the acceptance criteria. The method gave excellent recovery without matrix effects or interference.

3.4.1. Linearity

Six sets of calibrators in the same serum matrices were used to generate calibration plots to evaluate the linearity for each analyte. Each calibration curve consisted of seven non-zero points. The plot of the response ratio (analyte peak area over internal standard peak area) versus the analyte concentration was linear, the R^2 values for all analytes were ≥ 0.9979 . The mean values for slope, intercept and R^2 values for each analyte are listed in Table 3.

3.4.2. Accuracy, precision, and LLOQ

The results for inter- and intra-day precision and accuracy determination are given in Table 4. Five replicates of each QC

Table 3

Mean values for slope, intercept and R^2 values obtained from six different calibration curves.

Analyte	Slope	Intercept	R^2
Abiraterone	1.80E-02	8.17E-03	0.9990
D4A	2.47E-02	3.00E-06	0.9996
3-keto-5 α -Abi	2.00E-02	1.04E-04	0.9995
3 α -OH-5 α -Abi	2.16E-02	-6.33E-05	0.9995
3 β -OH-5 α -Abi	2.56E-02	1.21E-04	0.9996
3-keto-5 β -Abi	1.15E-02	3.80E-05	0.9995
3 α -OH-5 β -Abi	1.26E-02	-3.67E-05	0.9995
3 β -OH-5 β -Abi	2.03E-03	5.00E-06	0.9991

Table 4

Intra- and inter-day accuracy and precision for abiraterone and its metabolites in LLOQ and QC samples.

Analyte	Intra-day (n = 5)			Inter-day (n = 15)		
	Concentration ^a Mean ± SD	Accuracy %	RSD %	Concentration ^a Mean ± SD	Accuracy %	RSD %
Abiraterone ^b	2.05 ± 0.06	102.7	2.89	n.a	n.a	n.a
	6.40 ± 0.17	106.8	2.65	6.21 ± 0.31	103.5	4.95
	203.8 ± 4.2	102.0	2.06	206.3 ± 5.9	103.3	2.84
	316 ± 12	98.7	3.86	317 ± 22	99.1	6.80
D4A ^c	0.111 ± 0.004	111.2	3.89	n.a	n.a	n.a
	0.307 ± 0.024	102.3	7.76	0.297 ± 0.020	98.9	6.68
	10.02 ± 0.19	100.2	1.86	9.74 ± 0.38	97.4	3.94
	16.4 ± 0.5	102.3	3.02	16.0 ± 1.0	100.1	6.35
3-keto-5α-Abi ^c	0.111 ± 0.004	111.0	3.43	n.a	n.a	n.a
	0.322 ± 0.005	107.4	1.56	0.301 ± 0.02	100.1	7.86
	10.32 ± 0.29	103.2	2.86	9.87 ± 0.5	98.7	5.03
	16.8 ± 0.8	104.9	4.68	16.5 ± 1.2	103.1	7.56
3α-OH-5α-Abi ^c	0.106 ± 0.010	106.2	9.23	n.a	n.a	n.a
	0.314 ± 0.020	104.6	5.96	0.304 ± 0.030	101.5	9.45
	9.91 ± 0.44	99.1	4.41	9.39 ± 0.59	93.9	6.33
	14.8 ± 0.7	92.6	4.97	15.1 ± 1.2	94.5	7.61
3β-OH-5α-Abi ^c	0.103 ± 0.003	103.0	2.63	n.a	n.a	n.a
	0.305 ± 0.010	101.5	4.21	0.301 ± 0.01	100.3	4.66
	9.92 ± 0.20	99.2	2.07	9.86 ± 0.39	98.6	3.99
	16.0 ± 0.7	99.9	4.63	16.1 ± 1.0	100.6	6.51
3-keto-5β-Abi ^c	0.106 ± 0.004	106.4	4.13	n.a	n.a	n.a
	0.316 ± 0.008	105.4	2.56	0.303 ± 0.03	101.0	8.77
	9.95 ± 0.27	99.5	2.70	9.56 ± 0.44	95.6	4.61
	15.1 ± 0.5	94.2	3.61	15.5 ± 1.2	96.5	7.80
3α-OH-5β-Abi ^c	0.108 ± 0.004	108.4	4.26	n.a	n.a	n.a
	0.308 ± 0.020	102.6	6.74	0.301 ± 0.02	100.4	7.55
	9.74 ± 0.29	97.4	2.98	9.37 ± 0.40	93.8	4.26
	15.0 ± 0.7	93.8	4.65	15.3 ± 1.1	95.8	7.46
3β-OH-5β-Abi ^c	0.085 ± 0.010	85.3	16.89	n.a	n.a	n.a
	0.275 ± 0.030	91.7	10.75	0.29 ± 0.03	97.6	12.18
	9.68 ± 0.09	96.8	0.99	9.76 ± 0.36	97.6	3.67
	14.9 ± 0.8	93.3	5.60	16.0 ± 1.5	99.7	9.66

^a Measured Concentration in ng/mL.^b Nominal Concentrations: (LLOQ = 2, QC Low = 6, Mid = 200, and High = 320 ng/mL).^c Nominal Concentrations: (LLOQ = 0.1, QC Low = 0.3, Mid = 10, and High = 16 ng/mL).

sample were prepared in three different days, to test the method accuracy and precision. The CV% of the 5 replicates determining intra-day precision and the CV% of the 15 replicates determining inter-day precision for the eight analytes ranges between 0.99–10.75% and 2.84–12.18%, respectively. The accuracy values for intra-day and inter-day fall between 91.7–107.4% and 93.8–103.5%, respectively. Five LLOQ samples were prepared in the first batch, and the intraday accuracy mean values were 85.3–111.2% and CV% 2.63–16.89%. All the results fall within the FDA acceptance criteria.

3.4.3. Selectivity

Six LLOQ samples, each prepared in different serum batches, were compared to the blank samples showed that the serum was free from interference. The MRM chromatograms for the six representative blank samples compared to the MRM chromatograms of LLOQ samples of Abi and its 7 metabolites are shown in Fig. 3. The analyte peak in the spiked LLOQ samples ranged from a factor 6.7–592.6 greater than the blank serum matrix with largest interference peak at the same retention time, meeting the LLOQ requirement being a factor greater than 5 for the technique to be judged free of interference.

3.4.4. Recovery

Three QC levels were used to study the relative recovery, which is determined by a percent ratio of the analyte/internal standard peak area of a spiked sample before extraction over the analyte/internal standard peak area of a spiked sample after extraction. The results should be consistent, precise, and reproducible. The recovery was calculated as the mean of three triplicates for each analyte at each QC level. Recovery results were good, ranging from 85.1–105.2% (Table 5). CV% given in Table 5 ranged from 1.09–13.47% for all QC samples, meeting the 15% CV% criterion for acceptable recovery.

3.4.5. Matrix effect

The matrix effect is quantified as a matrix factor percent (MF%) by calculating the ratio the peak area ratio of analyte/internal standard of a QC low sample (post-extraction addition of analyte) over the peak area ratio of analyte/internal standard in a methanol/water solution. Matrix effect results are given in Table 5, showing that the technique is essentially free of interference, with results ranging from 88.5–114.7% for QC low samples.

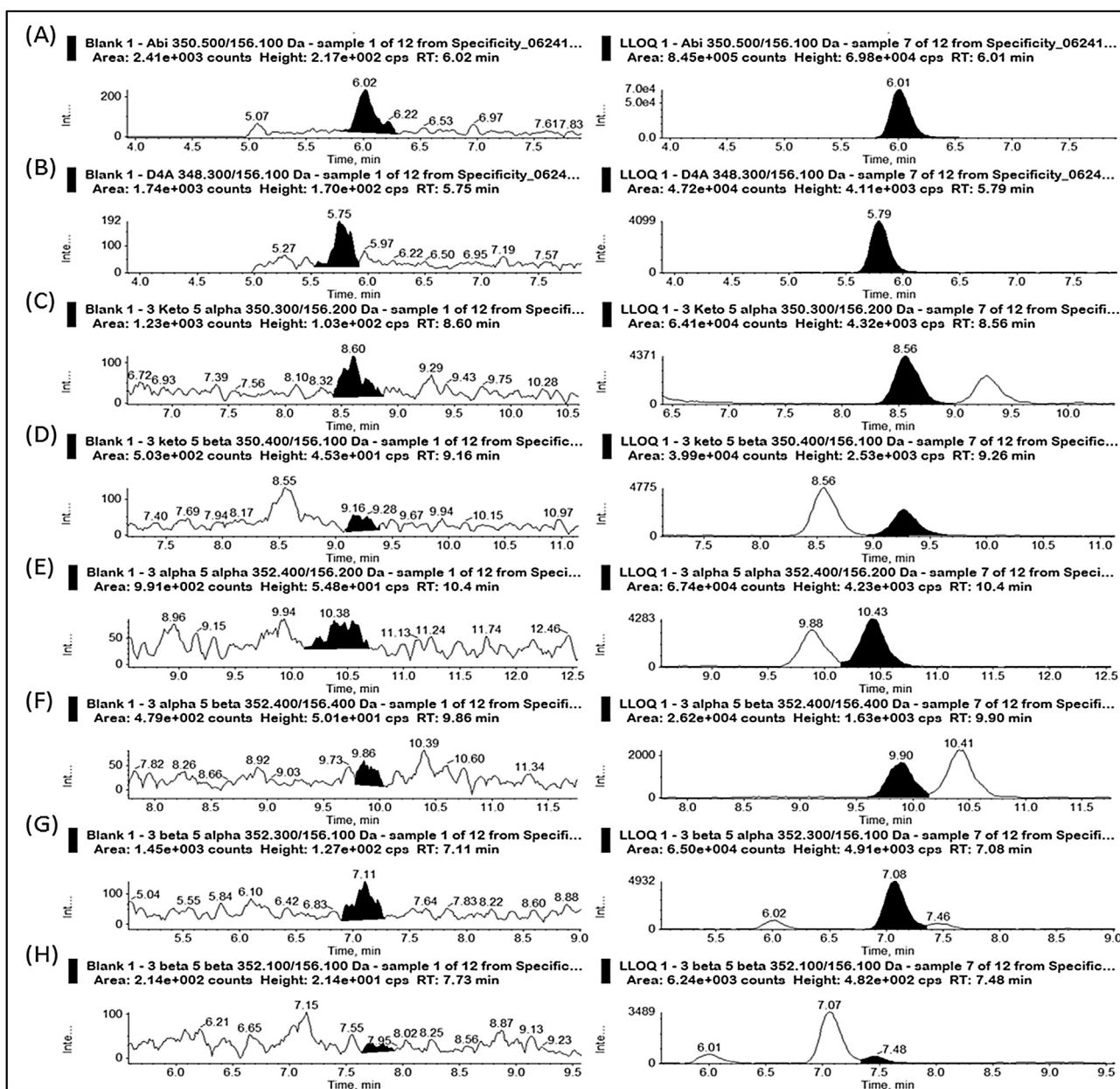


Fig. 3. Representative chromatogram of selectivity studies comparing blank samples (left chromatograms) matched to LLOQ (right chromatograms) for A, Abi; B, D4A; C, 3-keto-5 α -Abi; D, 3-keto-5 β -Abi; E, 3 α -OH-5 α -Abi; F, 3 α -OH-5 β -Abi; G, 3 β -OH-5 α -Abi; and H, 3 β -OH-5 β -Abi.

Table 5
Matrix effect and recovery for abiraterone and its metabolites.

	MF% (n = 18)		Recovery% (n = 3)					
	QC low		QC low		QC Mid		QC High	
	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)
Abi	114.7	5.04	95.7	6.54	91.7	1.49	93.4	4.65
D4A	88.5	5.99	105.2	4.78	99.0	3.39	94.6	7.81
3-keto-5 α -Abi	96.1	5.22	91.6	7.47	89.1	7.62	97.7	9.12
3 α -OH-5 α -Abi	92.0	5.05	91.2	7.48	85.1	4.51	95.1	5.61
3 β -OH-5 α -Abi	99.4	4.57	88.5	6.87	86.9	2.12	92.1	5.78
3-keto-5 β -Abi	93.2	8.31	91.0	13.47	87.1	6.33	96.3	6.39
3 α -OH-5 β -Abi	92.0	6.74	86.0	8.47	85.1	1.09	91.1	5.64
3 β -OH-5 β -Abi	100.0	11.13	96.6	11.50	86.3	4.28	91.4	8.16

RSD% – relative standard deviation percent which is the same as CV%.

Table 6

Stock stability for abiraterone, its metabolites and the internal standard.

	6 hours at RT (n = 3)		9 days at 4 °C (n = 3)	
	LLOQ (%)	ULOQ (%)	LLOQ (%)	ULOQ (%)
Abi	98.5	103.5	109.5	98.4
D4A	100.2	102.4	91.1	86.9
3-keto-5 α -Abi	112.1	103.7	101.6	88.4
3 α -OH-5 α -Abi	103.8	102.7	97.9	85.7
3 β -OH-5 α -Abi	105.2	102.1	101.7	89.3
3-keto-5 β -Abi	107.6	103.7	97.5	83.2
3 α -OH-5 β -Abi	104.1	103.3	105.0	85.4
3 β -OH-5 β -Abi	115.0	104.6	95.4	89.7
Internal standard	105.7		86.2	

RT, Room temperature.

3.4.6. Stability

Analytes were stable in the diluted solution stored at room temperature for 6 h 98.5–115.0% and at 4 °C for 9 days 83.2–109.5% as given in Table 6. Results of serum-based stability studies employing QC samples are given in Table 7. Post-preparative stability in which the QC samples were stored in the autosampler at 4 °C for 43 h were found to be acceptable 96.1–103.3%. Short-term (21 h) stability of all analytes in the prepared QC samples at each concentration were within the acceptance criteria 88.2–114.8% for the determined mean concentration values. Even though all analytes did show a decrease in concentration when subjected to three freeze-thaw cycles 86.3–96.7% and long term stability 86.7–95.6%, the results were less than 15%, which is within the criteria for accuracy.

4. Conclusion

We have developed and validated an LC–MS/MS MRM method for the determination and accurate quantification of Abi and its seven steroidal metabolites in human serum. To our knowledge, this is the first published report of a method to determine Abi metabolites that result from steroidogenic metabolism. The validated LC–MS/MS method resolved and quantitated all the metabolites despite the similarity in their structures, including resolving diastereomers, which precludes analysis of co-eluting isomers based solely on their MRM transitions. Reversed-phase chromatographic conditions were identified to accomplish the separation of all metabolites and their subsequent accurate quantification. This validated method can be applied to determine Abi and the aforementioned metabolites in human serum in clinical trials in which patients are treated with AA.

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Table 7

Stability of abiraterone and its metabolites after preparation and in serum.

		Post-Preparative 42 h			Short-term 21 h		Freeze- thaw 3 cycles		Long-term stability 28 weeks	
		QC Low	QC Mid	QC High	QC Low	QC High	QC Low	QC High	QC Low	QC High
Abi ¹	Concentration	5.9	203.4	314.6	5.3	293.8	5.4	284.9	5.7	290.7
	Accuracy%	97.8	101.7	98.3	88.2	91.8	89.5	89.0	95.5	90.8
	% RSD	3.26	0.75	3.04	6.03	1.97	11.38	9.45	0.82	2.28
D4A ²	Concentration	0.3	9.6	16.0	0.3	18.4	0.3	15.3	0.3	14.2
	Accuracy%	96.7	96.1	100.3	100.0	114.8	86.7	95.8	90.0	88.6
	% RSD	3.68	2.87	2.67	3.03	1.86	8.82	11.71	3.64	1.42
3-keto-5 α -Abi ²	Concentration	0.3	10.0	16.3	0.3	15.7	0.3	14.7	0.3	14.4
	Accuracy%	100.0	100.2	101.6	93.3	98.1	86.7	91.6	86.7	90.1
	% RSD	4.49	1.25	3.95	8.04	2.57	12.84	14.11	5.90	5.59
3 α -OH-5 α -Abi ²	Concentration	0.3	10.1	16.3	0.3	15.8	0.3	14.1	0.3	14.2
	Accuracy%	103.3	100.7	102.0	96.7	98.7	86.7	88.2	86.7	88.6
	% RSD	3.48	2.02	3.89	7.16	3.40	9.82	14.26	7.32	4.34
3 β -OH-5 α -Abi ²	Concentration	0.3	9.9	15.7	0.3	15.2	0.3	13.9	0.3	14.1
	Accuracy%	100.0	98.7	98.1	90.0	95.1	86.7	86.7	86.7	87.9
	% RSD	3.16	1.37	4.20	10.28	2.22	14.55	11.27	4.58	2.50
3-keto-5 β -Abi ²	Concentration	0.3	10.2	16.3	0.3	16.3	0.3	14.2	0.3	14.4
	Accuracy%	103.3	101.7	102.0	100.0	101.8	93.3	88.9	86.7	90.1
	% RSD	6.40	1.62	4.03	7.58	3.66	14.57	13.44	4.92	4.26
3 α -OH-5 β -Abi ²	Concentration	0.3	9.6	15.6	0.3	16.1	0.3	13.8	0.3	15.0
	Accuracy%	100.0	96.2	97.5	96.7	100.8	86.7	86.3	90.0	93.5
	% RSD	4.81	2.06	4.20	7.77	2.29	10.66	12.19	3.65	3.53
3 β -OH-5 β -Abi ²	Concentration	0.3	9.8	16.1	0.3	16.5	0.3	14.4	0.3	14.7
	Accuracy%	103.3	98.2	100.4	93.3	103.0	96.7	89.8	93.3	91.9
	% RSD	7.82	1.27	3.19	10.44	5.30	14.41	13.84	5.21	3.56

RSD% = relative standard deviation% is the same as CV%; ¹ Nominal Concentrations: (QC Low = 6, Mid = 200, and High = 320 ng/mL); ² Nominal Concentrations: (QC Low = 0.3, Mid = 10, and High = 16 ng/mL).

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