**Determination of Abiraterone and Its Metabolites in Human Serum by LC-ESI-TOF/MS Using Solid-phase Extraction**

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We developed and validated a liquid chromatography-electrospray ionization-time of flight/mass spectrometry method for the determination of abiraterone (Abi) and its metabolites (Δ^2-Abi, 3-keto-5α-Abi, 3α-OH-5α-Abi and 3β-OH-5α-Abi) in human serum using Abi-d as the internal standard. As a pretreatment procedure of serum samples, solid-phase extraction based on a silica-gel cartridge was used. The relative recovery of Abi and its metabolites was over the ranges of 84.5 – 109.2% at a concentration of 6.0 ng mL⁻¹ for Abi and 0.6 ng mL⁻¹ for its metabolites. The method was free from matrix effects. The calibration curve of Abi was linear over the range of 2.0 – 400 ng mL⁻¹ and those of its metabolites over the ranges 0.2 – 40 ng mL⁻¹. The results of the intra- and inter-day accuracy and precision data were within the FDA acceptance criteria. The optimized method was applied for the determination of Abi and its metabolites in human serum after oral administration of Abi acetate.

**Keywords** Liquid chromatography, mass spectrometry, solid-phase extraction, abiraterone, prostate cancer

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**Introduction**

Prostate cancer is the most common malignant tumor in men; its progression depends on continued androgen receptor activity. Either medical or surgical castration, which is androgen deprivation therapy (ADT), has been used for treating patients with this kind of disease. However, one of the problems is that the cancer becomes castration-resistant prostate cancer (CRPC) after the initial response to ADT. Several novel effective drugs have been developed for CRPC. One of them is abiraterone (Abi), marketed as Abi acetate.

Abi was metabolized by 3β-hydroxysteroid dehydrogenase (3β-HSD) to Δ^2-Abi (D4A), which is more potent than Abi (Fig. 1). D4A was further metabolized by steroid 5α-reductase (5α-reductase) to 3-keto-5α-Abi, which promoted prostate cancer progression. Furthermore, 3-keto-5α-Abi was metabolized to 3α-OH-5α-Abi and 3β-OH-5α-Abi, which were reversibly converted to 3-keto-5α-Abi by 3β-HSD and 3α-hydroxysteroid dehydrogenase (3α-HSD), respectively. In addition, D4A was similarly metabolized by steroid 5β-reductase (5β-reductase) to 3-keto-5β-Abi, which is further metabolized to 3α-OH-5β-Abi and 3β-OH-5β-Abi. Recently, clinical trials of a combination use of Abi and dutasteride, which is a 5α-reductase inhibitor, resulted in a decrease of 3-keto-5α-Abi concentrations together with an increase of D4A concentrations. This co-administration is promising for the treatment of CRPC because the concentrations of D4A, which is more potent than Abi, increase, while the concentrations of 3-keto-5α-Abi, which promotes prostate cancer progression, decrease.

The determination of Abi and/or its metabolite(s) (mainly D4A) in human plasma or serum has been performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and LC-high resolution MS. Dried plasma spots in combination of LC-MS/MS were used for the monitoring of Abi and D4A from patients, aimed therapeutic drug monitoring. A paper spray ionization-MS/MS method was developed for measuring Abi in patients’ plasma. Sharifi and his group reported on the simultaneous determinations of Abi, D4A, the three 5α-reduced metabolites (3-keto-5α-Abi, 3α-OH-5α-Abi and 3β-OH-5α-Abi) and three 5β-reduced metabolites (3-keto-5β-Abi, 3α-OH-5β-Abi and 3β-OH-5β-Abi). Pretreatment methods used so far for the determination of Abi and its metabolites in plasma or serum samples have included liquid-liquid extraction, protein precipitation and solid-phase extraction (SPE). For liquid-liquid extraction of Abi and its metabolites methyl tert-butyl ether was used. For protein precipitation, acetone was mainly used; and 10 vol% tetrahydrofuran in acetone was also used. For the SPE, the mixed-mode (cation-exchange and reversed-phase) or reversed-phase mode cartridges were used for extracting Abi and its metabolites, including glucuronide derivatives.

In this study, we developed for a new SPE method for Abi, D4A and the three 5α-reduced metabolites in human serum using silica cartridges for attaining good recovery and reducing matrix effects. Furthermore, a sensitive and selective LC-electrospray ionization-time of flight/MS (LC-ESI-TOF/MS) method has been developed for the determination of Abi and its metabolites in human serum after the administration of Abi acetate.
Experimental

Reagents and chemicals
Abi was purchased from Tokyo Chemical Industry (Tokyo, Japan). D4A was purchased from KNC Laboratories (Kobe, Japan). 3-Keto-5α-Abi, 3α-OH-5α-Abi and 3β-OH-5α-Abi were synthesized according to a method reported previously. A ZORBAX Eclipse Plus C18 column (3.5 μm particle size, 150 × 2.1 mm i.d.) was purchased from Agilent Technologies (Santa Clara, CA, USA). Cosmosil 5C18-MS-II and Cosmosil 5C18-AR-II columns (5 μm particle size, 150 × 2.0 mm i.d.) were purchased from Nacalai Tesque (Kyoto, Japan). Strata SI-1 (100 mg, 1 mL) and Strata-X (60 mg, 3 mL) cartridges were purchased from Phenomenex (Torrance, CA, USA). Water, methanol and acetonitrile of LC-MS grade were obtained from FUJIFILM Wako Pure Chemical (Osaka, Japan). Other reagents and solvents were of analytical-reagent grade and were used without further purification. The structures of Abi, D4A, 5α-reduced metabolites (3-keto-5α-Abi, 3α-OH-5α-Abi and 3β-OH-5α-Abi) and 5β-reduced metabolites (3-keto-5β-Abi, 3α-OH-5β-Abi and 3β-OH-5β-Abi) are illustrated in Fig. 1.

Preparation of standard and quality control samples
Stock solutions (1 mg mL⁻¹) of Abi and its metabolites were prepared by dissolving the respective compounds in 100 vol% methanol. The solutions were further diluted with 50 vol% methanol in water, and then mixed to prepare the working standard solutions at concentration of 5.0 μg mL⁻¹ for Abi and 0.50 μg mL⁻¹ for each metabolite. The stock solution (1 mg mL⁻¹) of the internal standard (IS) was prepared by dissolving Abi-d₄ in 100 vol% methanol, and further diluting it with 50 vol% methanol in water to a final concentration of 250 ng mL⁻¹. Freshly prepared working standard and IS solutions were used to prepare the serum calibrators and serum quality control (QC) samples. To 45 μL of human serum, 5 μL of working standard solutions and 10 μL of IS solutions were added. The concentrations of serum calibrators were 2.0, 6.0, 20, 50, 200, 320, and 400 ng mL⁻¹ for Abi and 0.20, 0.60, 2.0, 5.0, 20, 32, and 40 ng mL⁻¹ for each metabolite. QC samples were prepared at three levels (low-level QC (LQC), mid-level QC (MQC) and high-level QC (HQC), respectively): 6.0, 200, and 320 ng mL⁻¹ for Abi and 0.60, 20, and 32 ng mL⁻¹ for each metabolite. Stock and working standard solutions were stored at −20°C, and the calibrators and QC samples were freshly prepared through the validation.
LC-ESI-TOF/MS conditions

LC separation was performed using a Nexera SFC/SFE-HPLC system (Shimadzu, Kyoto, Japan). A ZORBAX Eclipse Plus C18 column, Cossmol 3C5-MR-II and Cossmol 3C8-AR-II columns, whose temperatures were maintained at 40°C, and were used at a flow rate of 200 μL min⁻¹. The mobile phase consisted of (A) H₂O and (B) 60 vol% methanol in acetonitrile. A linear-gradient elution and re-generation program were optimized as follows: 0.0 - 5.0 min, 70 vol% B; 5.0 - 18.0 min, 70 - 80 vol% B; 18.0 - 18.5 min, 80 - 98 vol% B; 18.5 - 23.0 min, 98 vol% B; 23.0 - 23.5 min, 98 - 70 vol% B; 23.5 - 30.0 min, 70 vol% B. The injection volume was 5 - 10 μL and the autosampler temperature was set at 4°C.

MS was performed using an impact II TOF/MS system (Bruker Daltonics, Bremen, Germany) equipped with an ESI source. MS was operated in the positive ion mode using the following operation parameters: source voltage, 500 V; capillary voltage, 4500 V; nebulizer pressure, 8.0 L min⁻¹; dry gas temperature, 200°C; collision induced dissociation energy, 0.0 eV; quadrupole ion energy, 5.0 eV; quadrupole mass, 100 m/z; collision energy, 10.0 eV; transfer time, 30 - 50 μs; pre pulse storage, 5 μs. The mass range was m/z 50 to 700. Nitrogen was used for the nebulizer, drying and collision gas. The concentrations of Abi and its metabolites were measured using the exact mass value of the protonated molecule ([M+H]⁺) of each compound.

Method validation

Linearity. The seven-point calibration curves for Abi and its metabolites, respectively, were constructed from 2.0 to 400 and 0.20 to 40 ng mL⁻¹ in human serum samples. The ratio of the analyte peak area to the IS peak area was plotted against the nominal concentration. The correlation was fitted by linear regression by a weighting factor of 1/x.

Accuracy and precision. The accuracy and precision data were evaluated using the lower limit of quantitation (LLOQ) and three QC samples. The intra-day accuracy and precision were determined by the analysis of five replicates; the inter-day accuracy and precision were determined by the analysis of three consecutive days.

Matrix effects and recovery. Matrix effects and recovery were evaluated using the ratio of the analyte peak area to the IS peak area in spiked serum samples before and after extraction, and standard samples according to the method, as reported previously.⁷

Results and Discussion

Pretreatment conditions

To separate Abi and its metabolites, we checked three columns: C18 column, Cosmosil 5C 18-MR-II and Cosmosil 5C 18-AR-II (Shimadzu, Kyoto, Japan) were administered before breakfast every day. Informed consent from all patients was obtained for the research subjects. Human serum was obtained at 2 – 4 h after a 4-week administration of Abi acetate. To 45 μL of human serum, 5 μL of 50 vol% methanol in water and 10 μL of an IS solution were added and treated using a Strata SI-1 cartridge, as described in a section of sample pretreatment.

Patients samples

To patients with CRPC at Yamaguchi University Hospital (Ube, Japan) 1000 mg of Abi acetate (Janssen Japan, Tokyo, Japan) were administered before breakfast every day. Informed consent from all patients was obtained for the research subjects. Human serum was obtained at 2 – 4 h after a 4-week administration of Abi acetate. To 45 μL of human serum, 5 μL of 50 vol% methanol in water and 10 μL of an IS solution were added and treated using a Strata SI-1 cartridge, as described in a section of sample pretreatment.

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Matrix effects and recovery. Matrix effects and recovery were evaluated using the ratio of the analyte peak area to the IS peak area in spiked serum samples before and after extraction, and standard samples according to the method, as reported previously.⁷

Selection of column and mobile phase

To separate Abi and its metabolites, we checked three columns:
Table 2  Comparison of the recovery of Abi and its metabolites by protein precipitation and SPE procedures

| Protein precipitation \( (n = 5) \) | Strata SI-1 \( (n = 10) \) | Strata-X \( (n = 8) \) |
|-------------------------------------|-----------------|-----------------|
| LQC \( \text{LQCa} \) | LQC \( \text{LQC} \) | LQC \( \text{LQC} \) |
| Mean, % | RSD, % | Mean, % | RSD, % | Mean, % | RSD, % |
| Abi     | 101.4 | 5.47 | 84.5 | 2.72 | 90.6 | 1.00 |
| D4A     | 33.0 | 5.68 | 109.2 | 3.91 | 68.7 | 3.02 |
| 3-Keto-5α-Abi | 607.0 | 136.7 | 100.8 | 5.84 | 88.2 | 16.1 |
| 3α-OH-5α-Abi | 376.7 | 112.1 | 96.4 | 3.74 | 78.6 | 4.01 |
| 3β-OH-5α-Abi | 607.0 | 61.6 | 90.8 | 5.29 | 88.9 | 4.33 |

Table 3  Recovery of Abi and its metabolites by a Strata SI-1 cartridge

| MQC\(^a (n = 9) \) | HQC\(^b (n = 10) \) |
|-------------------|-------------------|
| Abi | Mean, % | RSD, % | Mean, % | RSD, % |
| D4A | 95.8 | 1.75 | 98.5 | 1.37 |
| 3-Keto-5α-Abi | 126.5 | 3.42 | 134.5 | 4.37 |
| 3α-OH-5α-Abi | 100.6 | 3.55 | 107.7 | 2.68 |
| 3β-OH-5α-Abi | 112.7 | 3.32 | 119.2 | 6.13 |
| 3β-OH-5α-Abi | 101.1 | 4.14 | 103.7 | 4.58 |

a. Concentrations of Abi and its metabolites, respectively, are 20 and 2.0 ng mL\(^{-1}\).

b. Concentrations of Abi and its metabolites, respectively, are 32 and 3.2 ng mL\(^{-1}\).

Fig. 2  EICs of Abi and its metabolites in the standard solution (A) and human serum (B) after the administration of Abi acetate on an Eclips Plus C18 column.  A: Concentrations of Abi and its metabolites were 5.0 and 0.5 ng mL\(^{-1}\), respectively.  B: Concentrations of Abi, D4A, 3-Keto-5α-Abi, 3β-OH-5α-Abi and 3α-OH-5α-Abi, respectively, were estimated to be 20.5, 2.54, 4.41, <0.20 and 0.66 ng mL\(^{-1}\).  EICs were recorded at (a) \( m/z \) 348.2322, (b) \( m/z \) 350.2478, (c) \( m/z \) 352.2635 and (d) \( m/z \) 354.2729.
Eclipse Plus C18, Cosmosil 5C18-MR-II and Cosmosil 5C18-AR-II columns. The first column was used to separate Abi, D4A, 5α-reduced metabolites (3-keto-5α-Abi, 3α-OH-5α-Abi and 3β-OH-5α-Abi) and 5β-reduced metabolites (3-keto-5β-Abi, 3α-OH-5β-Abi and 3β-OH-5β-Abi).\textsuperscript{6,7} Since we do not have the standard of 5β-reduced metabolites, their peak assignments were done using human serum samples after an oral administration of Abi acetate, with taking into account elution order of Abi and its metabolites on an Eclipse Plus C18 column reported previously.\textsuperscript{6,7} Figures 2A and 2B, respectively, show extracted ion chromatograms (EICs) of the standard sample and serum sample 4 weeks after administrating Abi acetate. Traces a, b, c and d, respectively, were monitored for D4A at \(m/z\) 348.2322, for Abi, 3-keto-5α-Abi and 3-keto-5β-Abi at \(m/z\) 350.2478 and for 3α-OH-5α-Abi, 3β-OH-5α-Abi, 3α-OH-5β-Abi and 3β-OH-5β-Abi at \(m/z\) 352.2635, and for Abi-d\(_4\) at \(m/z\) 354.2729. As shown in Fig. 2B, seven peaks, except for 3α-OH-5α-Abi, which was below LLOQ, were observed on EICs, and all peak assignments were performed taking into account the elution order of Abi and its metabolites on an Eclipse Plus C18 column. It is interesting that two unknown peaks were observed on traces a and b of Fig. 2B. Further studies are ongoing concerning the structure elucidation of unknown peaks in our laboratory.

However, 3-keto-5α-Abi and 3-keto-5β-Abi were not completely separated on an Eclipse Plus C18 column (see trace b of Fig. 2B). We tried to use another column, a Cosmosil 5C18-AR-II column. As shown in trace b on Fig. 3B, 3-keto-5α-Abi and 3-keto-5β-Abi seem to be completely overlapped on a Cosmosil 5C18-MR-II column, while the unknown compound observed on the Eclipse Plus C18 column could elute before 3-keto-5α-Abi and 3-keto-5β-Abi. Furthermore, 3β-OH-5α-Abi seems to be overlapped with 3β-OH-5β-Abi as shown in trace c of Fig. 3B taking account into the peak intensity of trace c of Fig. 2B and the fact that 3β-OH-5α-Abi is below LLOQ. Furthermore, though we tested a Cosmosil 5C18-AR-II column, the separations of four OH metabolites on trace c were incomplete (data not shown). These results suggest that an Eclipse Plus C18 column is the best column for the separation of Abi and its seven metabolites.
The slop, intercept and coefficient of the determination (recovery were evaluated as described above. Furthermore, the method validation study. In addition of formic acid in the mobile phases was omitted in this metabolites, but it decreased their sensitivity. Therefore, the formic acid did not affect the separation of Abi and its metabolites. However, the inclusion of water and 60 vol% methanol in acetonitrile, including 0.1 vol% formic acid as an isocratic mode. The optimized method was applied to the determination of Abi and its metabolites in human serum after the oral administration of Abi acetate. The concentrations of Abi, D4A, 3-keto-5α-Abi, 3α-OH-5α-Abi and 3β-OH-5α-Abi, respectively, were 34.0 ± 38.1, 2.30 ± 0.85, 4.56 ± 1.38, 1.43 ± 1.26 and 1.44 ± 1.81 ng mL⁻¹ (n = 5).

Conclusions
An LC-ESI-TOF/MS method was established for the determination of Abi and its metabolites (D4A, 3-keto-5α-Abi, 3α-OH-5α-Abi and 3β-OH-5α-Abi) in human serum using Abi-d₃ as the internal standard. For pretreatment procedures of serum samples, protein precipitation and SPE procedures using the normal- and reversed-phase cartridges were examined. The Strata SI-1 cartridge, which is based on the normal phase mode, gave the highest recovery and the lowest matrix effects among the sample pretreatment procedures tested. The relative recovery of Abi and its metabolites were over the ranges of 84.5 – 109.2% at a concentration of 6.0 ng mL⁻¹ for Abi and 0.6 ng mL⁻¹ for its metabolites. This method was free from matrix effects. The calibration curve of Abi was linear over the range of 2.0 – 400 ng mL⁻¹ and those of its metabolites over the ranges 0.2 – 40 ng mL⁻¹. The results of intra- and inter-day accuracy and precision data were within the FDA acceptance criteria. The optimized method was successfully applied for the determination of Abi and its metabolites in human serum after the oral administration of Abi acetate.

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| Analyte     | Slope   | Intercept | R²   |
|-------------|---------|-----------|------|
| Abi         | 2.33 × 10⁻² | −1.55 × 10⁻² | 0.9966 |
| D4A         | 2.33 × 10⁻² | 2.54 × 10⁻³  | 0.9988 |
| 3-Keto-5α-Abi | 1.61 × 10⁻² | 3.96 × 10⁻³  | 0.9944 |
| 3α-OH-5α-Abi | 2.33 × 10⁻² | −6.60 × 10⁻⁴  | 0.9890 |
| 3β-OH-5α-Abi | 2.33 × 10⁻² | 1.18 × 10⁻³  | 0.9938 |

| Analyte     | Added/ ng mL⁻¹ | Intra-day (n = 5) | Inter-day (n = 15) |
|-------------|----------------|------------------|-------------------|
|             | Accuracy, Precision, % |                   |                   |
| Abi         | 2.0 | 98  | 5.9  | 101  | 5.5  |
|             | 6.0 | 92  | 0.7  | 93   | 2.4  |
|             | 200 | 106 | 0.6  | 105  | 1.0  |
|             | 320 | 115 | 0.6  | 110  | 3.1  |
| D4A         | 0.20 | 92  | 4.7  | 103  | 12   |
|             | 0.60 | 115 | 12   | 99   | 14   |
|             | 20  | 102 | 2.8  | 105  | 3.7  |
|             | 32  | 103 | 3.1  | 95   | 5.5  |
| 3-Keto-5α-Abi | 0.20 | 109 | 19   | 115  | 17   |
|             | 0.60 | 100 | 2.1  | 92   | 9.1  |
|             | 20  | 102 | 2.1  | 105  | 6.2  |
|             | 32  | 103 | 1.0  | 96   | 5.2  |
| 3α-OH-5α-Abi | 0.20 | 109 | 2.6  | 96   | 18   |
|             | 0.60 | 103 | 2.3  | 94   | 9.6  |
|             | 20  | 110 | 3.9  | 110  | 5.2  |
|             | 32  | 103 | 3.3  | 99   | 3.8  |
| 3β-OH-5α-Abi | 0.20 | 110 | 4.5  | 99   | 18   |
|             | 0.60 | 105 | 3.4  | 104  | 7.1  |
|             | 20  | 92  | 2.3  | 98   | 4.5  |
|             | 32  | 105 | 3.2  | 102  | 3.4  |
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