

Liquid Chromatography Tandem Mass Spectrometry Determination of Bicalutamide in Human Plasma and Application to a Bioequivalence Study

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Abstract

This study aimed to develop a highly sensitive, rapid method to evaluate the pharmacokinetic bioequivalence of two formulations of the anticancer drug, bicalutamide. According to US and Korean regulatory requirements for a bioequivalence test, we developed an achiral, bioanalytical method to determine bicalutamide levels in human plasma. The method included liquid chromatography tandem mass spectrometry in negative mode and was validated with nilutamide as an internal standard. Quantitation was performed for the transition of 429.2 → 255.0 (m/z) for bicalutamide and 316.2 → 273.2 (m/z) for nilutamide. The lower limit of quantitation was 10 ng mL⁻¹ with a 50 μL plasma sample. This sensitivity was about 8 times higher than current methods in pharmaceuticals and 10 times higher than methods for human plasma samples. The concentrations of seven working standards showed linearity between 10 and 2000 ng mL⁻¹ ($r^2 \geq 0.9993$). Chromatographic separation was achieved within 4 min, compared to the 10 min of current methods. We used a Luna C18 column (100 mm x 2 mm, 5 μm) with distilled water/acetonitrile (30/70, v/v) as an isocratic mobile phase with a flow rate of 0.3 mL min⁻¹. The average extraction recoveries of 3 quality control concentrations were 94.43% for bicalutamide and 99.28% for nilutamide. The coefficient of variation was ≤15% for intra- and inter-batch assays. Thus, this method satisfied the US and Korean validation requirements. When applied to a pharmacokinetic bioequivalence study in 33 healthy Korean male volunteers, this method showed high sensitivity, selectivity, and accuracy.

Keywords: Validation; LC-MS-MS; Pharmacokinetic bioequivalence study; Bicalutamide

Introduction

Bicalutamide, N-[4-cyano-3(trifluoromethyl) phenyl]-3-[(4-fluorophenyl sulfonyl)]-2-hydroxy-2-methyl propanamide, is an oral, nonsteroidal, anti-androgen agent used for treating prostate cancer. Bicalutamide treatment prevents the activation of the androgen receptor, which inhibits the growth of prostate cancer cells in patients with early, non-metastatic, or advanced prostate cancer [1,2]. Bicalutamide is a racemate that shows anti-androgenic activity in the R-enantiomer form, but little or no anti-androgenic activity in the S-enantiomer form. Several early publications described methods for identifying bicalutamide enantiomers in pharmaceuticals with liquid chromatography with ultraviolet detection (LC-UV) [3-5]. In addition, a previous exploratory study described bicalutamide levels in human plasma with liquid chromatography and tandem mass spectrometry (LC-MS-MS) [6]. The present study was conducted to develop a method for evaluating the pharmacokinetic bioequivalence of two formulations of bicalutamide in clinical trials. Our methods were designed to meet US and Korean regulatory requirements for a bioequivalence test [7,8]. The guidance for bioequivalence studies recommends measuring the racemate with an achiral assay. We developed an achiral HPLC-MS-MS method for determining bicalutamide in human plasma. This sensitive, rapid, high throughput method requires only a simple plasma sample preparation. With a small plasma sample (50 μL), we quantitated the bicalutamide concentration in the range of 10-2000 ng mL⁻¹. The lower limit of quantitation (10 ng mL⁻¹) was about 8 times more sensitive than the previously published methods in pharmaceuticals and 10 times more sensitive than the methods in human plasma samples [3,6]. With our method, the total run time was reduced to 4 min from 10 min in a previous study [3]. In a bioequivalence study, the current method was suitable for comparing the pharmacokinetic profiles of two formulations with high sensitivity, selectivity, and accuracy.

Experimental Methods

Chemicals and reagents

Bicalutamide and nilutamide were obtained from Sandoz Korea Ltd. (Seoul, Korea) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Nilutamide was used as an internal standard (IS) for this assay. Acetonitrile (HPLC grade) was purchased from Fisher Scientific, Korea Ltd.

Chromatographic and mass spectrometry conditions

High performance liquid chromatography (HPLC) was performed on an Agilent (Santa Clara, CA, USA) 1100 series HPLC with a Luna C18 column (100 x 2 mm, 5 μm, Phenomenex, USA). The column temperature was set at 25°C. The mobile phase was distilled water and acetonitrile at a ratio of 30:70, applied at a flow rate of 0.3 mL min⁻¹. Sample analyses were performed on a tandem quadrupole mass spectrometer (MS/MS) API 4000 (Applied Biosystems/MDS Sciex, Toronto, Canada), operated in negative electrospray ionization mode. The MS/MS detection was conducted by multiple reaction monitoring at a transition of 429.2 → 255.0 (m/z) for bicalutamide and 316.2 → 273.2 (m/z) for the IS (Figure 1). For both bicalutamide and the IS, the source-dependent parameters were as follows: Gas 1 (nebulizer gas): 40

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psig; Gas 2 (heater gas flow): 50 psig; ion spray voltage: 4500 V; turbo heater temperature: 400°C; interface heater: On; entrance potential: 10 V; collision activation dissociation gas: 6 psig; and curtain gas, nitrogen: 10 psig. The optimized values for analyte-dependent parameters were set as follows: declustering potential, collision energy, and cell exit potential were set to: 75, 22, and 9 V, respectively, for bicalutamide and 90, 30, and 9 V, respectively, for the IS. Quadrupoles 1 and 3 were maintained at unit mass resolution and the dwell time was set at 400 ms.

Standard and quality control (qc) sample preparation

Bicalutamide and the IS stock solutions were prepared in methanol to a final concentration of 1 mg mL⁻¹. The appropriate dilutions with acetonitrile were performed to produce 7 working standards of 100, 50, 25, 5, 2.5, 1, and 0.5 µg mL⁻¹ of bicalutamide and one 50 ng mL⁻¹ sample of IS. Calibration standards ranging from 10 to 2000 ng mL⁻¹ were prepared by spiking 20 µL of the working standards into 1 mL of blank plasma. The QC samples were prepared with a different bicalutamide working solution and diluted in blank plasma. The QC bicalutamide concentrations included 10 ng mL⁻¹ for the lower limit of quantitation (LLOQ), 20 ng mL⁻¹ for a low quality control (LoQC), 200 ng mL⁻¹ for a Middle quality control (MeQC), 1600 ng mL⁻¹ for a High quality control (HiQC), and 4000 ng mL⁻¹ for a Di quality control (DiQC). All diluted samples were stored at -70°C.

Sample preparation

Frozen human plasma was thawed at room temperature. A plasma sample (50 µL) was placed in a 1.5 mL polypropylene tube, and 950 µL of IS (50 ng mL⁻¹) or one of the bicalutamide solutions was added. After vortexing for 30 s and centrifuging at 14,000 rpm for 5 min, 50 µL of supernatant was transferred to another polypropylene tube, and 950 µL

of 70% acetonitrile (mobile phase) was added. After vortexing for 30 s, 4 µL of each sample was injected into the LC-MS/MS system.

Bioanalytical Method Validation

Validation was performed according to the FDA guidelines for Bioanalytical Methods Validation for Human Studies [7,8].

Linearity and sensitivity

To test for acceptable linearity, 7 calibration standards were prepared, and more than 5 standards had to be within ±15% of the mean calculated concentrations. Calibration curves were calculated with weighted (1/x) linear regression analyses and fitted with regression coefficients of >0.99. To test for sensitivity, the LLOQ of 10 ng mL⁻¹ was allowed a deviation of ±20%.

Accuracy and precision

Intra-batch precision and accuracy were evaluated with 6 replicates of QC samples at each concentration in a single assay on 5 different days. Intra- and inter-batch precisions were expressed as coefficients of variation (CV%). For validation, the CV had to be within ±15%, except for the LLOQ, in which the deviation could not exceed ±20%. The accuracy was calculated as the percent deviation from the nominal concentration, and more than 5 replicates had to be within ±20%.

Extraction recovery

In order to evaluate the extraction recovery, we compared QC samples before and after extraction in 6 replicate samples. Before and after extraction, 3 different bicalutamide concentrations (20, 200, 1600 ng mL⁻¹) were spiked into blank plasma. Results were determined by comparing the analyte peak areas.

Selectivity and specificity

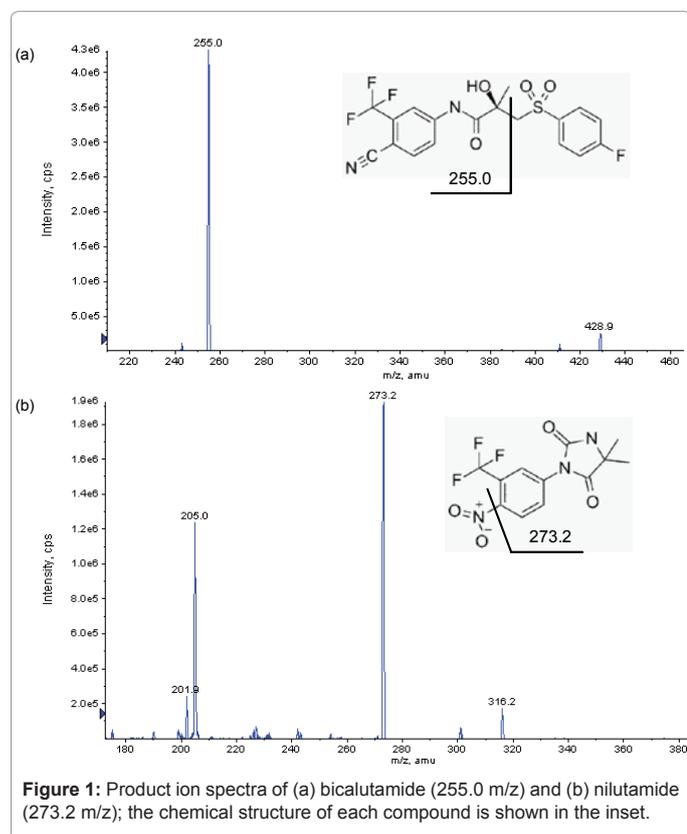
To determine whether there was any interference from endogenous plasma constituents, we analyzed 6 blank plasma samples and LLOQ samples made with the same blank plasma. Interference was considered acceptable only when the peak area of the interference did not exceed 20% (S/N ratio=5) of the peak area of the LLOQ.

Stability

The stability of the stock solution was validated after placing the bicalutamide stock solution at room temperature for 41 h. The concentration was compared with that of a freshly prepared stock solution and the stability was expressed as the percent difference; for validation, the difference could not exceed ±10%. The freeze/thaw stability was determined by comparing a back-calculated concentration of QCs after 3 freeze/thaw cycles to QCs after 1 freeze/thaw cycle. Extraction stability was also tested because it required time to inject the sample into the HPLC with the autosampler. To test extraction stability, each of 6 extracted samples from LLOQ to DiQC were stored at 4°C in the autosampler for 25 h. These were compared to freshly extracted QC samples, and the concentration was expressed as a percentage of the freshly extracted QC concentration. To validate whether the analyte was stable in the plasma, we performed short-term stability by storing LoQC and HiQC samples at room temperature for 24 h and comparing them to freshly prepared QCs.

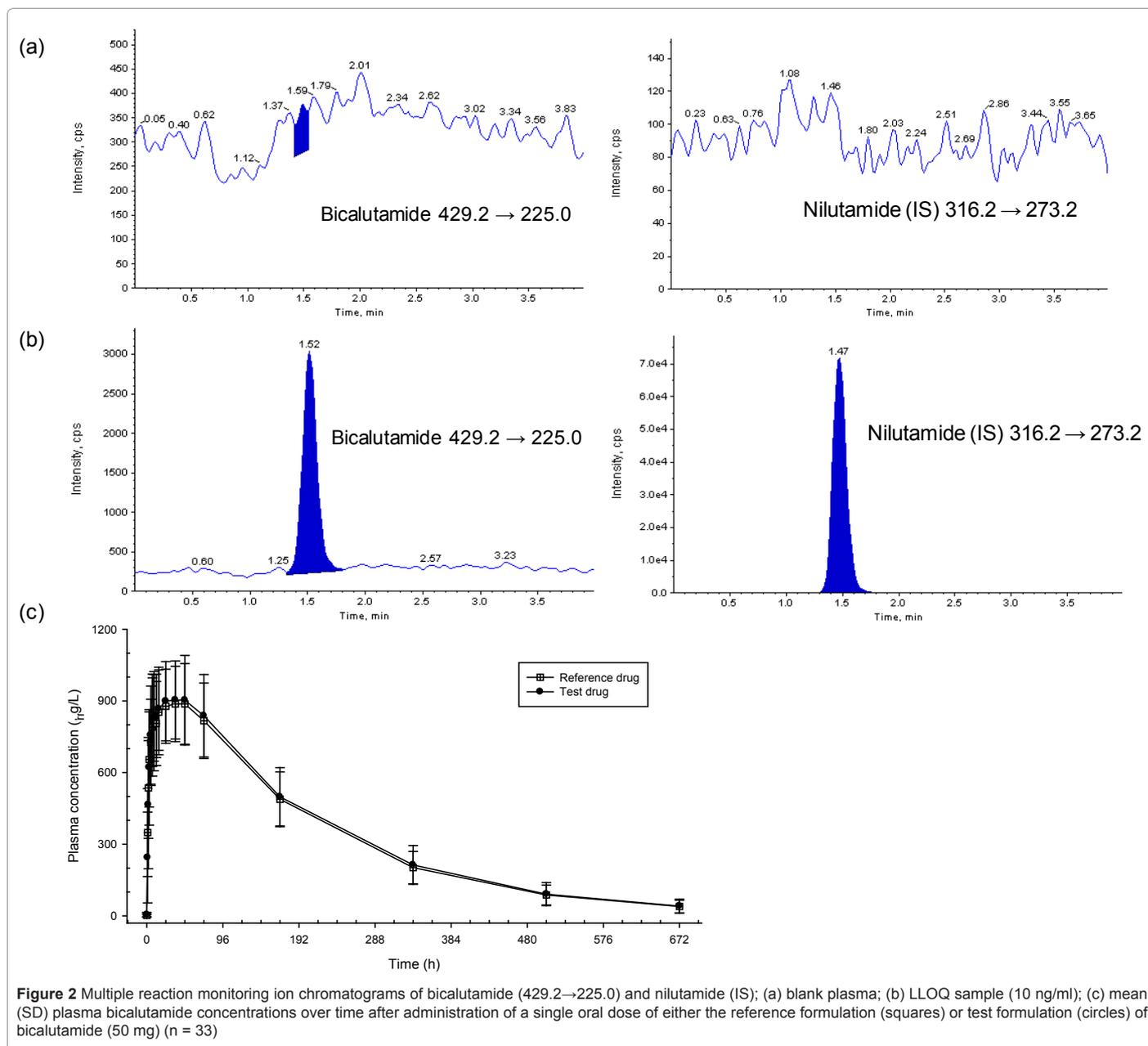
Application to a bioequivalence study

The validated analytical assay was applied to a pharmacokinetic bioequivalence study. A two-period, crossover design study was conducted with 34 healthy Korean male volunteers. The mean (SD)



age, height, and weight of subjects were 25.8 (4.1) years, 173.6 (5.7) cm, and 68.9 (7.8) kg, respectively. The clinical protocol was reviewed and approved by the institutional review board of Seoul National University Hospital (SNUH) and the Korean Food and Drug Administration. All subjects provided informed consent to participate in the study. In the

first period, subjects randomly received a single dose of bicalutamide in either the test formulation (30 mg tablet, Cadexo, Sandoz Korea Ltd., Seoul, Korea) or the reference formulation (Casodex, AstraZeneca UK Ltd, Macclesfield, United Kingdom). The first period was followed by an interval of x days before the second period began. In the second



QC	Concentration added (ng mL ⁻¹)	Intra-batch (n = 6)				Inter-batch (n = 6)			
		Mean (ng mL ⁻¹)	SD (±)	Precision (%CV)	Accuracy (%)	Mean (ng mL ⁻¹)	SD (±)	Precision (%CV)	Accuracy (%)
LLoQ	10	10.36	0.25	2.39	103.60	10.24	0.44	6.48	102.40
LoQC	20	19.72	0.98	4.98	98.59	19.87	0.97	3.60	99.37
MeQC	200	200.40	7.47	3.73	100.20	196.70	3.26	3.15	98.36
HiQC	1600	1657.00	89.54	5.41	103.60	1627.00	45.15	2.29	101.70
DiQC	4000	3918.00	58.37	1.49	97.95				

*DiQC was analyzed by 10-fold dilution

Table 1: Intra- and inter-day precision and accuracy for bicalutamide in human plasma.

Sample concentration (ng mL ⁻¹)	Mean concentration (ng mL ⁻¹) ± SD (%) (n=6)	Precision (%CV)	Accuracy (%)
Extract stability (4°C for 25 h)			
10	11.29 ± 0.98	8.69	112.93
20	21.09 ± 2.03	9.62	105.43
200	195.42 ± 3.16	1.62	97.71
1600	1650.33 ± 104.41	6.33	103.15
4000	3878.67 ± 68.54	1.77	96.97
Freeze/thaw stability ^a			
20	21.25 ± 2.10	9.86	106.23
1600	1547 ± 12	0.79	96.7
Short-term stability (room temperature for 24 h)			
20	18.34 ± 0.44	2.42	91.71
1600	1685 ± 13.52	0.8	105.33

^aFreeze thaw stability after third cycle

Table 2: Stability data for bicalutamide in human plasma.

period, subjects received a single dose of the alternate bicalutamide formulation (that not received in the first period). Serial blood samples were obtained before dosing and at 1, 2, 3, 5, 7, 9, 12, 15, 24, 36, 48, 72, 168, 336, 504, and 672 h after each administration. Pharmacokinetic analyses were performed with the noncompartmental method of Win-Nolin, version 5.2.1 (Pharsight Corporation, Mountain View, California). Maximum plasma concentration (C_{max}) and the time point of maximum plasma concentration were obtained directly by individual plasma concentration-time profiles. The terminal elimination constant (λ_z) was estimated with linear regression, and the elimination half-life ($t_{1/2}$) was calculated as $\ln 2/\lambda_z$. For AUC_{0-672h} and $AUC_{0-\infty}$, we used the linear-up/log-down trapezoidal method.

Results and Discussion

Method development

Determinations of bicalutamide and the IS were optimized with HPLC-MS/MS. The mass spectrometer was tuned in negative ionization mode, with deprotonated ions [M-H]⁻. For each selected multiple reaction monitoring transition of bicalutamide (m/z 429.2 → 255.0) and IS (m/z 316.2 → 273.2), retention times were 1.42 min and 1.47 min, respectively. The total run time was 4 min (Figure 2a and Figure 2b).

Validation of the Analytical Method

Linearity and sensitivity

The 7-point calibration curve was reproducible and linear over the range of 10-2000 ng mL⁻¹. The calibration curve showed good linearity for bicalutamide. The regression equation was $y = 0.154x + 0.00535$, and the correlation coefficient (r) was over 0.9993. The LLOQ was found to be 10 ng mL⁻¹, which gave a signal to noise ratio of 5 ($S/N \geq 5$).

Accuracy and precision

For the intra-batch assay, the precision ranged from 1.49% to 5.41%, and the accuracy ranged from 97.95% to 103.6%. Inter-batch precision and accuracy ranged from 2.29% to 6.48% and 98.36% to 102.4%, respectively (Table 1). These data indicated that the method had sufficient accuracy and precision for determining bicalutamide concentrations.

Extraction recovery

The pre-extraction, spiked QC samples were compared to post-extraction samples. For each of the 3 concentrations tested, the extraction recoveries were 98.91%, 89.88%, and 94.50%. The mean

extraction recovery was $94.43 \pm 4.52\%$. The recovery of nilutamide (IS) was $99.28\% \pm 4.30\%$.

Selectivity and specificity

We tested 6 blank plasma samples from different subjects. All 6 blank plasma samples had endogenous peaks at the retention time of bicalutamide. However, the peak area was within 9.781% of the peak area of the LLOQ (10 ng mL⁻¹) (Figure 2a and Figure 2b). Thus, the interference peak in blank plasma was not considered to be specific to bicalutamide.

Stability

The stock solution stored for 41 h at room temperature showed a -9.51% difference in concentration compared to that of freshly prepared stock solution. To be considered stable, the permissible percent difference from freshly prepared solution was $\pm 15\%$ for extract stability, freeze/thaw stability, and short-term stability. We found that the extract stability of QC samples stored at 4°C for 25 h was within the permissible range ($\pm 15\%$). The freeze/thaw stability did not show any relevant difference between QC samples that underwent 1 or 3 freeze/thaw cycles. The short-term stability of bicalutamide was stable at room temperature for 24 h (Table 2).

Application

The assay was applied in a clinical trial to determine the plasma concentration after an oral administration of bicalutamide in 33 healthy Korean male volunteers. The mean (SD) plasma concentration change over time is shown in Figure 2c. Concentrations were measured after administration of a single oral dose of one of two formulations of bicalutamide 50-mg tablets. The geometric mean ratio of the test formulation to the reference formulation showed a 90% CI within 0.97 to 1.06 for C_{max} and 0.98 to 1.07 for AUC_{0-672h} . The results indicated that there were no significant differences between the reference and test formulations [2].

Conclusion

Although an HPLC assay for bicalutamide enantiomers in pure and pharmaceutical dosage forms was published previously, we developed a highly sensitive, rapid, accurate method that was successfully applied to a bioequivalence study. Our method was based on LC-ESI-MS/MS analysis of a simple plasma sample preparation. The validation showed that this assay had a high sensitivity with a LLOQ at 10 ng mL⁻¹ and only required a small volume of plasma (50 μ L). This represented an advantage over the published method, which showed a 0.1 μ g mL⁻¹ of

LLOQ for each of the bicalutamide enantiomers [6]. In addition, our method required a total run time of 4 min, which was faster than the published 10 min required for other assays. Our method also showed good linearity in the dynamic range of 10–2000 ng mL⁻¹. These results provided validation for the method. This method was successfully applied in a human clinical study to compare the pharmacokinetic profiles of two formulations of bicalutamide in 33 healthy Korean male volunteers.

Acknowledgments

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