Comparative pharmacokinetic study of bicalutamide administration alone and in combination with vitamin D in rats

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ABSTRACT

Bicalutamide (BCL) has been approved for treatment of advanced prostate cancer (Pca), and vitamin D is inevitably used in combination with BCL in Pca patients for skeletal or anti-tumor strategies. Therefore, it is necessary to study the effect of vitamin D application on the pharmacokinetics of BCL.

We developed and validated a specific, sensitive and rapid UHPLC–MS/MS method to investigate the pharmacokinetic behaviours of BCL in rat plasma with and without the combined use of vitamin D. Plasma samples were extracted by protein precipitation with ether/dichloromethane (2:1 v/v), and the analytes were separated by a Kinetex Biphenyl 100Å column (2.1 × 100 mm, 2.6 μm) with a mobile phase composed of 0.5 mM ammonium acetate (PH 6.5) in water (A) and acetonitrile (B) in a ratio of A:B = 35:65 (v/v). Analysis of the ions was run in the multiple reactions monitoring (MRM) mode. The linear range of BCL was 5–2000 ng mL⁻¹. The intra- and inter-day precision were less than 14%, and the accuracy was in the range of 94.4–107.1%. The mean extraction recoveries, matrix effects and stabilities were acceptable for this method. The validated method was successfully applied to evaluate the pharmacokinetic behaviours of BCL in rat plasma. The results demonstrated that the pharmacokinetic property of BCL is significantly affected by combined use of vitamin D, which might help provide useful evidence for the clinical therapy and further pharmacokinetic study.

KEYWORDS

pharmacokinetics, bicalutamide, vitamin D, prostate cancer, UHPLC–MS/MS

1. INTRODUCTION

Androgen deprivation therapy (ADT), which inhibits androgen receptor (AR) signalling, has been the mainstay treatment for advanced prostate cancer (Pca) for decades [1–3]. Bicalutamide (BCL), known as an oral nonsteroidal anti-androgen drug that suppress the growth-simulating effect of 5α-dihydrotestosterone on the prostate tumor via competing for AR binding sites, thus effectively inducing the apoptosis of PCa cells and inhibiting the growth of adenocarcinoma [4]. BCL has been approved for treatment of locally advanced nonmetastatic Pca at a dose of 150 mg per day, and advanced Pca combined with luteinizing hormone releasing hormone (LHRH) analogue or surgical castration at a dose of 50mg with overall safety and good tolerability [5–7].

PCa is the most commonly diagnosed cancer in aging males, who are likely have a higher prevalence of osteoporosis. To make matters worse, long-term ADT accelerates bone loss and is associated with an increased incidence of osteoporosis and osteoporotic fracture [8–11]. So calcium and vitamin D supplementation are recommended to reduce skeleton related events (SREs) in Pca patients on long-term ADT in addition to bisphosphonates [12, 13].

Vitamin D3, generated endogenously or acquired from diet or supplements, is converted into 25-hydroxyvitamin D [25(OH)D] by the enzyme CYP2R1 in the liver, and subsequently
is further hydroxylated into 1α,25-dihydroxyvitamin D \([1,25(\text{OH})_2\text{D}]\) by the enzyme CYP27B1 in renal proximal tubules [14–16]. 25(\text{OH})\text{D} is the major circulating form of vitamin D and is regarded as a representative biomarker of estimating vitamin D status. 1,25(\text{OH})_2\text{D}, the active form of vitamin D, also called calcitriol, is a steroid hormone which binds to a specific high affinity receptor, the vitamin D receptor (VDR), regulating calcium and bone homeostasis and many extraskeletal processes.

VDR belongs to a class of nuclear transcription factors, and has been found in most human tissues and cells [14, 17–24], although at variable concentrations, and in a variety of cancer cells, including lung cancer cells [25], hepatocellular carcinoma (HCC) cells [26], colorectal cancer cells [27, 28], breast cancer cells [29], and Pca cells [30]. Accumulating data indicate that vitamin D deficiency is commonly diagnosed among patients with Pca [31, 32]. There is crosstalk between androgen and vitamin D signaling pathways [33]. 1,25(\text{OH})_2\text{D} could upregulate the expression of AR and other androgen responsive genes, and androgens were involved in the regulation of VDR [34, 35]. Data from pre-clinical studies showed that vitamin D has anti-tumor effects both directly via controlling proliferation, differentiation and apoptosis of tumor cells as well as indirectly through regulating immune cells in tumor microenvironment [36]. Lines of epidemiological studies, not all, suggest that there are no associations of 25(\text{OH})\text{D} with the risk of total prostate cancer, but a strong association of low circulating 25(\text{OH})\text{D} level with increased incidence of high-grade Pca and worse cancer, but a strong association of low circulating 25(\text{OH})\text{D} level with increased incidence of high-grade Pca and worse outcomes [34, 37–39]. Meta-analysis of randomized controlled trials (RCTs) suggested that vitamin D3 supplementation leads to a significantly lower total cancer mortality [40]. Some scholars expressed positive attitude toward vitamin D to be a novel and economical anticancer agent [41].

For the above reasons, vitamin D supplementation is inevitably used in combination with BCL in Pca patients for skeletal or anti-tumor strategies. It is necessary to get more information on the pharmacokinetic behaviours between the two drugs. However, few studies on vitamin D combination strategies have focused on pharmacokinetic interactions. Likewise, few LC-MS/MS methods have been reported to determine BCL in biological matrices of rodents [42]. It is crucial to determine whether the combination of the two drugs affects the pharmacokinetics of BCL, yet has never been reported. Our study aims to establish a selective, sensitive and fast method to investigate the pharmacokinetic behaviours of BCL in rat plasma with and without the combined use of vitamin D. The application of this method in pharmacokinetics study may help provide useful evidence for more efficient and safe therapies in clinical application and further pharmacokinetic study.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Bicalutamide tablets (50 mg) and Vitamin D Drops (Soft Capsules, 400 IU) were procured from AstraZeneca (UK) and DoubleWale (Qingdao, China), respectively. Standards of bicalutamide (purity >98%) and tolbutamide [ purity >98%, internal standard (IS) ] were purchased from Shanghai Yuanye Biotechnology Institute (Shanghai, China). Acetonitrile, methanol and ammonium acetate of HPLC-grade were obtained from Fisher Scientific (Nanjing, China). All other chemical reagents were of analytical grade. The Distilled water was purchased from Wahaha Co., Ltd (Hangzhou, China).

2.2. Animals

Male Sprague-Dawley rats (body weight 220–240 g) were purchased from Hebei Experimental Animal Research Center (Hebei, China), and were housed at an ambient temperature of 24–26°C and relative humidity of 40–60%, and kept under 12–12h light-dark cycle per day. After an acclimatization for 7 days, rats were fasted for 12 h prior to and 2 h post dosing with free access to water. Then blood samples were taken from the postorbital venous plexus and plasma samples were separated and stored at −80°C prior to use. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and under the approval and supervision of the Ethics Committee of Hebei General Hospital (No. 202192).

2.3. Apparatus and UHPLC–MS/MS conditions

The LC–MS/MS system consisted of a Shimadzu Nexera UHPLC LC-30A (Kyoto, Japan) equipped with an AB Sciex Q Trap 6,500 system (Massachusetts, USA). The analytes were separated using a Kinetex Biphenyl 100A column (2.1 × 100 mm, 2.6 μm, Phenomenex, USA) maintained at a temperature of 40°C. The mobile phase was composed of 0.5 mM ammonium acetate (pH 6.5) in water (A) and acetonitrile (B) in a ratio of A:B = 35:65 (v/v), which was filtered through a 0.45 μm microporous membrane and degassed ultrasonically for 5 min. The flow rate was 0.3 mL min⁻¹ with isotropic elution. Finally, 1 μL sample solution was injected into the UHPLC-MS/MS system via an autoinjector. Analysis of the ions was run in the multiple reactions monitoring (MRM) mode.

The MS condition and parameters were set as follows:

- Curtain gas: 20 psi, nebulizer gas: 35 psi, auxiliary gas: 45 psi and collision gas: 6 psi, with high-purity nitrogen respectively. Declustering potential: 80 and 55 V, respectively; collision energy: both 26 V; collision exit potential: 9 and 15 V, respectively; and entrance potential: both 10 V. For BCL, the precursor ion (Q1) was m/z 428.80 and the product ion (Q3) was m/z 254.70, respectively. For IS, the Q1 was m/z 269.00 and Q3 was m/z 169.60, respectively.

2.4. Preparation of stock solutions, calibration standards, and quality control (QC) solutions

Accurately weighed BCL and tolbutamide standards. The stock solution was prepared in methanol at a concentration of 1 mg mL⁻¹ both for BCL and tolbutamide (IS). The stock solutions were stored at 4°C for further analysis.
Then the stock solution of BCL were progressively diluted with methanol to prepare working solutions of BCL. The concentrations of QC solutions were 0.1, 1.5, and 15 μg mL⁻¹, respectively. The concentrations of calibration standards were 0.05, 0.25, 0.5, 1, 2, 5 and 20 μg mL⁻¹, respectively. The working solution of IS (20 μg mL⁻¹) was prepared in the same manner. All the working solutions were stored at 4 °C for further analysis.

2.5. Plasma sample preparation

Plasma samples were pre-treated as described below: Firstly, 20 μL of IS working solution and 20 μL of methanol were added to 200 μL of plasma sample, and the mixture was vortexed for 30 s. Then, 800 μL of extraction solvent, ether/dichloromethane (2:1 v/v), was added to an EP tube and vortexed for 3 min. After centrifugation at 4 °C and 13,000 rpm for 5 min, the supernatant was harvested into a flesh tube and dried with nitrogen flow at 45 °C. The residue was dissolved in 200 μL of methanol and filtered through a 0.45 μm microporous membrane, and 1 μL of the sample solution was then injected for analysis.

2.6. Method validation

The method was validated in accordance with the guidelines of bioanalytical method validation issued by the U.S. Food and Drug Administration (FDA, 2018) and related reference (U.S. 2001).

2.6.1. Specificity, linearity, and lowest limit of quantitation (LLOQ). Specificity: The specificity of the method was assessed by analyzing the chromatograms of blank rat plasma, standard solution of IS and BCL at the LLOQ, and blank rat plasma spiked with IS and BCL at the LLOQ.

The calibration curve (CC) and linearity: 20 μL of the calibration standards and 20 μL of IS working solution were added to 200 μL of rat blank plasma, and the following steps were carried out according to 2.5 items: the mixture was vortexed for 30 s. Then, 800 μL of extraction solvent, ether/dichloromethane (2:1 v/v), was added to an EP tube and vortexed for 3 min. After centrifugation at 4 °C and 13,000 rpm for 5 min, the supernatant was harvested into a flesh tube and dried with nitrogen flow at 45 °C. The residue was dissolved in 200 μL of methanol and filtered through a 0.45 μm microporous membrane, and 1 μL of the sample solution was then injected for analysis.

The final concentrations of calibration samples at plasma were 5, 25, 100, 200, 500 and 2,000 ng/mL, respectively. The final concentration of IS solution was 2,000 ng mL⁻¹. The linearity was evaluated on three validation days with two replicates at each level.

The LLOQ was defined as the lowest concentration on the calibration curve with a signal-to-noise ratio (S/N) of no less than 10, and with acceptance accuracy (RE, within 80–120%) and precision (RSD, less than 20%).

2.6.2. Accuracy and precision. QC samples at three final concentrations (10, 150, 1,500 ng mL⁻¹) were used to determine the intra- and inter-day precision and accuracy. 20 μL of the QC solutions and 20 μL of IS working solution were added into 200 μL of blank rat plasma, and the following steps were carried out according to 2.6.1 items.

Six replicates for each concentration were measured on the same day and on three consecutive days. The intra- and inter-day precision was expressed as relative standard deviation (RSD%) of the measured concentration, and the accuracy was expressed as relative error (RE%) of the measured mean value deviated from the nominal value.

2.6.3. Extraction recovery. The recovery was assessed by comparing the mean peak area of BCL or IS of six replicates in the QC samples with the reference samples spiked in neat solution to blank plasma extracts (n = 6).

2.6.4. Matrix effect. Matrix effects of BCL at three QC concentration levels (n = 6 different lots at each concentration) and IS (n = 6) were assessed by comparing the mean peak area of the BCL and IS from blank after extracting into blank plasma with the mean area of the BCL and IS from distilled water at equivalent concentration.

2.6.5. Stability. Three concentrations of QC samples (n = 3 of each concentration) were placed under different conditions including room temperature for 4 h, −80 ± 10 °C for 30 days, three freezing and thawing cycles from −80 °C to room temperature, and placed in the automatic sampler (4 °C) for 12 h to investigate the stability of short-term, long-term, freeze-thaw and post-preparation.

The stability data were obtained by comparing the measured concentration with the nominal concentration. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (RE, within 85–115%) and precision (RSD, less than 15%).

2.7. Pharmacokinetic study

Bicalutamide was prepared by dissolving one Bicalutamide tablet (50 mg) with 50 mL of sterilized distilled water to a concentration of 1 mg mL⁻¹. VD was prepared by diluting one Vitamin D3 capsule (400 IU) with 20 mL of sterilized soybean oil to a concentration of 20 IU/mL.

For the pharmacokinetic study, 12 male SD rats were randomly divided into two groups with 6 rats in each group, namely, bicalutamide group and combination group. Rats in the bicalutamide group were dosed with bicalutamide 4.5 mg kg⁻¹ by gavage, and rats in the combination group were dosed with bicalutamide 4.5 mg kg⁻¹ combined with vitamin D 72 IU/kg. Blood samples (about 300 μL) were collected prior to dosing (0 h) and at 1, 2, 4, 6, 8, 24, 48, 72, 96, 120, 144 and 168 h post-dosing, respectively. Then placed in heparinized microtubes and centrifuged at 4,000 rpm for 10 min at 4 °C to separate the plasma, and store at −80 °C until assayed.

2.8. Statistical analysis

DAS 2.0 software was used to calculate all the pharmacokinetic parameters, including area under the concentration-
time (AUC), the maximum observed concentration (C_{max}),
biological half-life (t_{1/2}), peak time (T_{max}), mean residence
time (MRT), oral clearance rate (Clz/F), and apparent vol-
ume of distribution (Vz/F) by non-compartmental model.
The data were plotted with Origin software (ver 7.5; Ori-
ginLab, USA). The major pharmacokinetic parameters were
listed in Table 3 and were statistically analysed by SPSS 19.0
software. All data were expressed as mean ± standard devi-
ation (SD). A Student’s t-test or nonparametric rank sum
test was used to assess the difference between two groups.

P < 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Specificity
Typical chromatograms are shown in Fig. 1. The total
chromatographic run time was 3.0 min. The retention time
of BCL and IS in plasma were 1.03 and 0.75 min, respec-
tively, with no interference was observed. The peaks were
well shaped and the baselines were stable, indicating a higher
degree of specificity of this method.

3.2. Calibration curve, linearity and LLOQ
Y is the peak area ratio of BCL to IS, and X is the concen-
tration of BCL. The standard curve of BCL was obtained by
linear regression using a 1/X^2 weighted least square linear
regression model. The calibration curves exhibited excellent
linearity over the concentration range of 5–2,000 ng mL^{-1},
and the LLOQ was 5 ng mL^{-1}. The typical regression
equation was Y = 5.424 \times 10^{-3} X + 5.925 \times 10^{-2} with a
correlation coefficient (R) of 0.9920. All the nonzero cali-
brators were within ±15% of the nominal concentrations.

3.3. Accuracy and precision
The accuracy and the inter- and intra-day precision results
of BCL in three concentrations (10, 150, and 1,500 ng mL^{-1})
are presented in Table 1. For each QC sample, the accuracy
(RE, %) was in the range of 94.4–107.1%, and the intra- and
inter-day precision values (RSD, %) were all less than 14%,
suggesting that the accuracy and precision of this method
were satisfactory.

3.4. Absolute recovery
The recoveries of QC samples at three different concen-
trations in rat plasma are presented in Table 1, ranged from
86.8 ± 3.5 to 97.8 ± 5.7. Meanwhile, the recovery of IS at
2000 ng mL^{-1} was 89.1 ± 9.8, suggesting that the recoveries
were acceptable for this method.

3.5. Matrix effect
The matrix effects of QC samples at three different concen-
trations in rat plasma are presented in Table 1, ranged
from 87.6 ± 5.1 to 91.8 ± 4.3. Meanwhile, the matrix effect
of IS at 2,000 ng mL^{-1} was 89.4 ± 5.8, suggesting that the
matrix effects were acceptable for this method.

![Fig. 1. Extracted ion chromatograms (XIC) for the analysis of BCL (left panels) and tolbutamide (IS, right panels). (A) Blank rat plasma. (B) Standard solution of IS and BCL at the LLOQ. (C) Blank rat plasma spiked with IS and BCL at LLOQ concentration](image)
3.6. Stabilities

The stability tests for QC samples at three different concentrations in rat plasma are presented in Table 2. In all cases, the RSD values were less than 15%, and RE values were within 85–115%, which could meet the requirements of plasma sample analysis.

3.7. Pharmacokinetic study

The mean concentration-time curves of BCL after single administration (BCL) and combination administration (BCL + VD) are presented in Fig. 2. The major pharmacokinetic parameters of each group are presented in Table 3.

Compared with the single administration group, the AUC(0-t), AUC(0-∞), and Cmax of BCL in combination administration group were increased by 31.53%, 30.47%, and 40.05% respectively (P < 0.01), and the peak time (Tmax) of BCL in rat plasma was prolonged for 2 h (increased from 6 to 8 h), suggesting that vitamin D enhances the absorption degree and relatively slows the absorption rate of BCL.

The Vz/F of BCL in single administration group was 4.746 ± 1.007 L kg⁻¹, suggesting that BCL is mainly distributed in blood with a high binding rate of plasma protein. BCL is highly bound to plasma protein (approximately 96% in plasma of rat and human) [43, 44], and a minor reduction in the binding rate of plasma protein will dramatically alter the free drug concentration in plasma. In combination administration group, the Vz/F of BCL was 2.78 ± 0.515 L kg⁻¹ (P < 0.01), suggesting that the significant improvement of BCL concentration in plasma possibly due to altered plasma protein binding.

While some significant difference also can be seen in the pharmacokinetic parameters of t1/2, MRT(0-t), MRT(0-∞) for BCL between the two groups. The t1/2 decreased from 28.681 ± 5.86 to 21.88 ± 3.648 h (P < 0.05), the MRT(0-t) decreased from 35.031 ± 2.614 to 31.004 ± 2.073 h (P < 0.05), and the MRT(0-∞) decreased from 38.52 ± 3.919 to 31.991 ± 2.170 h (P < 0.05), suggesting that vitamin D accelerates the metabolism of BCL. After oral dosed, BCL is extensively metabolized via phase I (oxidation by CYP 3A4) and phase II (glucuronidation) transformations in the liver [43]. On the other hand, VDR is found binding to the promoter of CYP 3A genes, and ligands like 1,25-(OH)₂D₃, up-regulates the transcription of CYP 3A in different species [45–49]. VDR also participates in the regulation of phase II metabolic enzymes, such as UDP-glucuronosyl transferases: UGT2B15/2B17, UGT2A1/2 and sulfotransferase 2A1 (SULT2A1) [45, 50]. Nevertheless, the induction of CYP 3A by VDR is species-specific and tissue-specific [47], and the role in human subjects especially in Pca patients remains to be studied.

The calculated relative oral clearance (CLz/F) of BCL was significantly decreased from 0.115 ± 0.008 to 0.088 ± 0.006 L h⁻¹ kg⁻¹ (P < 0.01), which may be caused by a large reduction in the Vz/F.

| Table 1. Precision, accuracy, absolute recovery and matrix effect of BCL in rat plasma (n = 6) |
|-----------------------------------------------|----------------|----------------|----------------|
| Analyte | Concentration (ng mL⁻¹) | Precision (RSD%) | Absolute recovery (%) | Matrix effect (%) |
|         | Intra-day | Inter-day |         |                  |
| BCL     | 10       | 11.4     | 5.3    | 97.8 ± 5.7       | 90.3 ± 6.2       |
|         | 150      | 7.6      | 13.7   | 94.3 ± 6.5       | 87.6 ± 5.1       |
|         | 1,500    | 4.9      | 8.1    | 86.8 ± 3.5       | 91.8 ± 4.3       |
| IS      | 2,000    | -        | -      | 89.1 ± 9.8       | 89.4 ± 5.8       |

| Table 2. Stabilities of BCL in rat plasma (n = 3) |
|-----------------------------------------------|----------------|----------------|
| Concentration (ng mL⁻¹) | Short-term | Long-term | Freeze-thaw | Post-preparation |
|                          | RE% | RSD% | RE% | RSD% | RE% | RSD% | RE% | RSD% |
| 10                       | -5.3 | 10.4 | 9.2 | 12.5 | 9.6 | 8.0 | 10.8 | 4.7  |
| 150                      | 6.1  | 4.6  | -4.9 | 5.8  | -4.3 | 4.5 | 9.6  | 8.9  |
| 1,500                    | 5.9  | 7.1  | 12.5 | 7.5  | -9.5 | 9.2 | -11.8 | 7.4  |

Fig. 2. Mean concentration–time curves of BCL in rat plasma after oral administration of BCL tablet with and without co-administration of vitamin D capsule (n = 6)
Table 3. Pharmacokinetic parameters of BCL in rat plasma (n = 6)

| Parameters          | Unit        | BCL                  | BCL + vitamin D          |
|---------------------|-------------|----------------------|--------------------------|
| AUC(0-∞)            | ng mL⁻¹ h   | 3882.07 ± 2702.826   | 5106.58 ± 3908.792**     |
| AUC(0-∞)            | ng mL⁻¹ h   | 3936.22 ± 2702.136   | 5136.24 ± 4010.415**     |
| t1/2a               | h           | 28.681 ± 5.86        | 21.88 ± 3.648            |
| Tmax                | h           | 6                    | 8                        |
| CLz/F               | Lh⁻¹ kg⁻¹   | 0.115 ± 0.008        | 0.088 ± 0.006**          |
| MRT(0-∞)            | h           | 35.031 ± 2.614       | 31.004 ± 2.073           |
| MRT(0-∞)            | h           | 38.52 ± 3.919        | 31.991 ± 2.170**         |
| Cmax                | ng mL⁻¹     | 1110.172 ± 223.762   | 1554.771 ± 139.511**     |
| Vz/F                | L kg⁻¹      | 4.746 ± 1.007        | 2.78 ± 0.515**           |

**P < 0.01, *P < 0.05.

4. CONCLUSION

A specific, sensitive and rapid UHPLC–MS/MS method was established to determine BCL in rat plasma, and was successfully applied to compare the pharmacokinetic behaviours after BCL single administration and combination with vitamin D. Our study preliminarily proved the pharmacokinetic behaviours of BCL with and without the combined use of vitamin D for the first time. Additional validation studies, involving larger group of animals, especially in humans, are also needed in the future.

The results demonstrated that the pharmacokinetic property of BCL is significantly affected by combined use of vitamin D. This pharmacokinetic information in our study about BCL might provide useful evidence for the clinical therapy and further pharmacokinetic study.

Conflicts of interest: The authors declare no conflict of interest.

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