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Short communication

# Determination of 2-hydroxyflutamide in human plasma by liquid chromatography–tandem mass spectrometry (LC–MS/MS): Application to a bioequivalence study on Chinese volunteers

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ABSTRACT

A sensitive, simple and rapid ultra fast liquid chromatography (UFLC)–ESI-MS/MS method was developed for the determination of 2-hydroxyflutamide in human plasma using tegafur as the internal standard. The plasma sample was pretreated with methanol for protein precipitation and the analytes were separated on an Ultimate C18 column (5 μm, 2.1 mm × 50 mm, MD, USA) with the mobile phase consisted of acetonitrile and water (2:1, v/v). Detection was performed on a triple-quadrupole tandem mass spectrometer under a negative multiple reaction-monitoring mode (MRM). The mass transition ion-pair was followed as *m/z* 290.90–204.8 for 2-hydroxyflutamide and 198.9–128.8 for tegafur. Linear calibration curves were obtained in the concentration range of 1.742–1452 ng/ml with a lower limit of quantification of 1.742 ng/ml. The intra- and inter-batch precision values were less than 8.1% and 5.6%, respectively. The established method was successfully applied to a bioequivalence study of two flutamide preparations (250 mg) in 20 healthy male volunteers.



## 1. Introduction

Flutamide, 2-methyl-N-[4-nitro-3-(trifluoromethyl) phenyl] propanamide, is a pure nonsteroidal antiandrogen widely used in the treatment of prostate cancer [1,2]. Owning to its similar structure to testosterone, flutamide works as a competitive antagonist that blocks the attachment of the male hormone at the androgen receptor in the prostate [3,4]. Flutamide is rapidly metabolized to its active metabolite 2-hydroxyflutamide by extensive first-pass metabolism [5–7]. The plasma concentration of flutamide is low and highly variable, which may result in considerable interindividual pharmacokinetic variability. After administration of 250 mg flutamide, 2-hydroxyflutamide reaches much higher blood concentration than the parent drug flutamide [6,8]. And 2-hydroxyflutamide appears to be largely responsible for the therapeutic efficacy [9]. The consideration of active metabolites has been of concern in pharmaceutical development and regulation for many years [10]. Therefore monitoring the plasma levels of 2-hydroxyflutamide is used to evaluate the pharmacokinetics and bioavailability properties of flutamide [11,12].

Several methods for determination of 2-hydroxyflutamide in plasma have been published using gas chromatography with electron-capture detection [5,6] or HPLC with UV detection [11–14]. However, all these reported methods need long analysis time and complicated sample pretreatment procedures. These plasma sample preparation methods are time-consuming or expensive.

In this paper, we develop a sensitive, simple and rapid UFLC–ESI-MS/MS method for determination of 2-hydroxyflutamide in human plasma. The plasma sample was pretreated with methanol for protein precipitation, which is simple and time-saving. Also we improved the LLOQ as low as 1.742 ng/ml. This method was fully validated and successfully applied to the bioequivalence study of two flutamide preparations (250 mg) in 20 healthy male volunteers.

## 2. Experimental

### 2.1. Chemicals and reagents

2-hydroxyflutamide standard (Batch No. Cat#H942475) was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). Reference standard of tegafur (internal standard, I.S.) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China).

The test preparation was flutamide tablets (250 mg, Batch No. AW08007A), which was supplied by S.C. Sindan S.R.L Co. (Bucuresti, Romania). The reference preparation was also flutamide tablets

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(250 mg, Batch No. CA8XCPA02), which was obtained from S-P Canada (Quebec, Canada).

HPLC grade acetonitrile was purchased from Tedia Company Inc. (Fairfield, OH, USA). Methanol of analytical grade was purchased from Tianjin Kermel Chemical Reagent Co. Ltd. (Tianjin, PR China). Deionized water (Millipore, Bedford, MA, USA) was used throughout the entire experiment.

### 2.2. Apparatus and operation conditions

#### 2.2.1. Liquid chromatography

The chromatography was performed on Shimadzu UFLC system (Chiyoda-Ku, Kyoto, Japan) equipped with two LC-20AD pumps, DGU-20A3 on-line degasser, SIL-20ACHT autosampler, CTO-20AC column thermostat.

Chromatographic separation was carried out on an Ultimate C18 column (5 μm, 2.1 × 50 mm, MD, USA) protected by a Phenomenex ODS guard column (5 μm, 4.0mm × 3.0 mm i.d., Torrance, CA, USA). A mobile phase consisting of acetonitrile and water (2:1, v/v) was pumped at a flow rate of 0.2 ml/min. The column temperature was maintained at 30 °C, and the inject volume was set at 5 μl. The total run time of each sample analysis was 4 min.

#### 2.2.2. Mass spectrometry

Mass spectrometric analysis was performed using an API 3200 LC/MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ion source and operated in the negative ionization mode. The spray voltage and source temperature were 4000 V and 500 °C, respectively. The other gas source parameters were set as followings: curtain gas (CUR) 20 psi, GAS1 40 psi, GAS2 40 psi, collision activated dissociation (CAD) 5 psi. Quantification was performed using multiple reaction monitoring (MRM) of the transitions *m*/*z* 290.90–204.8 for 2-hydroxyflutamide and 198.9–128.8 for tegafur, with a dwell time of 200 ms per transition. The declustering potential (DP), collision energy (CE), entrance potential (EP), collision cell exit potential (CXP) were optimized as 40 V, 30 V, 9 V, 2.5 V for 2-hydroxyflutamide; 30 V, 20 V, 9 V, 1.5 V for tegafur. Data acquisition and analysis were achieved using the Analyst 1.5 software (Applied Biosystems, Foster City, CA, USA).

### 2.3. Preparation of stock and working solutions

Primary stock solutions of 2-hydroxyflutamide and tegafur were prepared by dissolving accurately weighed reference substances in methanol. Standard solutions of 2-hydroxyflutamide at concentrations of 17.42 ng/ml, 69.70 ng/ml, 261.36 ng/ml, 871.2 ng/ml, 2613.6 ng/ml, 7260 ng/ml, 14,520 ng/ml were obtained by serial diluting the primary stock solution with methanol. Another set of working solutions of 2-hydroxyflutamide at concentrations of 34.85 ng/ml, 1016.4 ng/ml, 11,616 ng/ml were made for preparation of quality control (QC) samples. The working internal standard solution containing 1 μg/ml tegafur was also prepared in methanol by diluting the stock solution. All solutions were stored at −20 °C when not used.

### 2.4. Plasma sample preparation

200 μl human plasma was spiked with 20 μl methanol and 20 μl I.S. working solution and vortex-mixed. To precipitate plasma proteins, 600 μl methanol was added. After vortex mixing for 60 s and centrifuging at 12,000 rpm for 5 min, 400 μl of supernatant was transferred into autosampler vials and 5 μl was injected into the LC–MS/MS system.

### 2.5. Calibration curves and QC samples

Calibration standards were prepared by spiking 200 μl drug-free human plasma with 20 μl of 2-hydroxyflutamide standard solutions, ranging from 1.742 ng/ml to 1452 ng/ml. These standards were processed as the human plasma samples described above.

The QC samples were prepared in the same way as calibration standards with blank plasma, containing 3.485 ng/ml, 101.64 ng/ml, 1161.6 ng/ml of 2-hydroxyflutamide. The calibration curves and QC samples were freshly prepared and assayed along with each batch of human plasma.

### 2.6. Method validation

#### 2.6.1. Specificity

The specificity of the method was evaluated by analyzing blank plasma samples from six sources to determine the interference with the analyte. The chromatograms were compared with plasma sample spiked with 2-hydroxyflutamide (1.742 ng/ml) and tegafur (100 ng/ml) and plasma sample after oral doses of flutamide tablets.

#### 2.6.2. Linearity and lower limit of quantification (LLOQ)

Linearity of the method was evaluated by analyzing calibration standard plasma samples containing 2-hydroxyflutamide at concentrations of 1.742 ng/ml, 6.97 ng/ml, 26.136 ng/ml, 87.12 ng/ml, 261.36 ng/ml, 726 ng/ml and 1452 ng/ml. The calibration curve was constructed by plotting peak-area ratios of 2-hydroxyflutamide to I.S. against spiked concentration, using weighted ($1/X^2$) least squares linear regression.

The LLOQ was defined as the lowest concentration on the calibration curve, and it was evaluated using six replicates of plasma sample spiked with analyte at a concentration of 1.742 ng/ml. To validate the method, each LLOQ sample should be obtained with an acceptable accuracy (RE) within ±20% and a precision (RSD) not exceed 20% [15].

#### 2.6.3. Precision and accuracy

Intra-batch, inter-batch precision and accuracy were determined by replicate analysis of QC samples at three concentrations ($n = 5$) on three consecutive days. The concentration of each sample was determined using freshly prepared calibration standards. The precision was expressed as the relative standard deviation (RSD) and the accuracy as relative error (RE).

#### 2.6.4. Recovery and matrix effect

The recovery was determined by comparing the peak areas of 2-hydroxyflutamide obtained from five replicates at three QC concentrations with those from samples prepared by spiking deproteinized blank plasma samples with the same amounts of analyte.

The matrix effect was measured by comparing the peak areas of 2-hydroxyflutamide spiked into drug-free plasma after protein precipitation with those of analyte in the same amounts of mobile phase.

#### 2.6.5. Stability

Stability was evaluated by repeated analysis of QC samples at three concentrations of 3.485 ng/ml, 101.64 ng/ml and 1161.6 ng/ml. The autosampler stability was tested by analyzing processed QC samples kept under autosampler condition (4 °C) for 8 h. Room temperature stability and long-term stability were assessed using untreated QC samples kept at room temperature for 8 h and stored at −80 °C for 30 days, respectively. After three freezing-thaw cycles, QC samples were processed and analyzed to determine the freeze-thaw stability. Samples were considered to

be stable if their assay values were within 15% error of the nominal values [15].

### 2.7. Bioequivalence study

The method was applied to two-period crossover bioequivalence study of two kinds of flutamide tablets in 20 healthy male volunteers. The protocol of this study was approved by ethics committee and all subjects gave written informed consent. After an overnight fast, each volunteer was given a test (250 mg) or reference flutamide tablet (250 mg) along with 200 ml water per period. The washout time between two periods was 7 days. Blood samples were collected pre-dose, 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 4 h, 6 h, 8 h, 12 h, 24 h, 36 h and 48 h post-dosing. Samples were centrifuged at 3000 rpm for 10 min and plasma was separated and stored at −80 °C until analysis.

Pharmacokinetic parameters were calculated using DAS program (Vesion 2.0, Sun Ruiyuan, China). The maximum plasma concentrations ($C_{max}$) and the time to reach them ($T_{max}$) were obtained directly from the experimental data. The terminal elimination rate constant ($k_e$) was calculated from the plot of logarithms of plasma concentration against time using least square regression. The area under the plasma concentration–time curve from zero to the last measured concentration ($AUC_{0-t}$) was calculated according the linear trapezoidal rule. $AUC_{0-\infty}$ (area under the plasma concentration–time curve extrapolated to infinity) was calculated using the following formula: $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$, where $C_t$ is the last measurable plasma concentration. The terminal elimination half-life ($t_{1/2}$) was calculated as $0.693/k_e$.

## 3. Results and discussion

### 3.1. Chromatography and mass spectrum

Tandem mass parameters were optimized for best response by directly infusing standard solutions of 2-hydroxyflutamide and I.S. at a flow rate of 10 μl/min via a syringe pump. MS scan was carried out both in positive and negative ion mode, but the result showed that negative ion mode produced higher sensitivity and fewer fragments. This is in agreement with other published literature [4]. From the product ion scan (MS2), it could be identified that the fragment ions with $m/z$ 204.8 for 2-hydroxyflutamide and $m/z$ 128.8 for I.S. were of most abundant. Thus, the transitions $m/z$ 290.90–204.8 for 2-hydroxyflutamide and 198.9–128.8 for tegafur were selected for determination.

Acetonitrile, methanol and water were mixed at different ratios to decide the most suitable mobile phase. Addition of ammonium acetate into the mobile phase was also tested. The result revealed that a mixture of acetonitrile and water at ratio of 2:1 (v/v) could achieve high sensitivity, sharp peak shape and short analytical time. This binary mobile phase is simpler than that reported in the literature [4]. Different types of column (Ultimate C18, Ultimate XB-CN, Lichrospher C18) were evaluated and the Ultimate C18 column provided the best chromatographic separation.

### 3.2. Selection of I.S.

The use of internal standard can minimize any variation produced in the analytical process. In this study, we have tried acyclovir, tegafur and lisinopril for I.S. selection, which can be easily obtained in our laboratory. Tegafur was selected as I.S. for its high sensitivity, similar chromatographic and mass spectrometric behavior to the analyte. Under the selected chromatographic conditions, both 2-hydroxyflutamide and I.S. were well analyzed with retention times 1.95 min and 1.24 min, respectively.

### 3.3. Pretreatment of plasma samples

Many liquid–liquid extraction methods were reported using extraction solvents such as mixture of cyclohexane and diethyl ether [7], dichloromethane [11,13] and ethyl acetate [12]. Leibinger and Kapas [14] developed a solid-phase extraction (SPE) method for plasma sample pretreatment. Protein precipitation was applied to prepare plasma samples because it was simple to operate and economical. Common solvents such as acetonitrile, methanol and trichloroacetic acid were tested as protein precipitation reagents. In the end, methanol was chosen as the precipitation reagent, considering that it can provide high recovery and efficiency of protein precipitation. Liquid–liquid extraction was also evaluated in our study. Although it can improve the sensitivity to 0.1 ng/ml, it was poor in reproducible and recovery.

### 3.4. Method validation

#### 3.4.1. Specificity

The specificity was examined by analyzing six different human blank plasma samples and plasma samples at the LLOQ. Typical chromatograms of a blank plasma sample, a blank plasma spiked with I.S. and 2-hydroxyflutamide at the concentration of 1.742 ng/ml, and a plasma sample obtained from a volunteer 0.5 h after a single oral administration of 250 mg flutamide are shown in Fig. 1. As shown in the figure, no interfering peaks from endogenous compounds were observed at the retention time of analyte and I.S. The retention time of 2-hydroxyflutamide and I.S. were about 1.95 min and 1.24 min, respectively. The total run time was 4 min.

#### 3.4.2. Calibration curves and LLOQ

Five calibration curves were performed during the method validation and showed good linearity over the concentration range of 1.742–1452 ng/ml, which could span all the clinical concentrations in this study. A typical linear equation of the calibration curves was: $y = 0.0463x + 0.013$, $r = 0.9989$, where $y$ was peak-area ratio of 2-hydroxyflutamide to I.S. and $x$ was the plasma concentration of 2-hydroxyflutamide.

The lower limit of quantification (LLOQ) of 2-hydroxyflutamide was defined as the lowest concentration of the calibration curve and it was detected to be 1.742 ng/ml in human plasma using six independent plasma samples. The LLOQ in our paper is much lower than the reported methods, in which the LLOQ were 10–25 ng/ml [12–14]. The mean precision and accuracy at LLOQ were 10.7% and 10.4%, respectively. With the LLOQ of 1.742 ng/ml, this method was sensitive enough to determine the concentration of 2-hydroxyflutamide in human plasma 48 h after a single oral administration of 250 mg flutamide in our bioequivalence study.

#### 3.4.3. Precision and accuracy

Intra-batch, inter-batch precision and accuracy were evaluated by analyzing five replicate quality control samples at three concentration levels of 3.485 ng/ml, 101.64 ng/ml, and 1161.6 ng/ml over three validation days. The intra-batch and inter-batch precision ranged from 4.5% to 8.1% and 4.6% to 5.6%, respectively. The accuracy was determined by calculating RE and the results were within −6.0% to 7.0%. As shown in Table 1, the precision and accuracy were with the acceptable criteria where the precision at each concentration level should not exceed 15% of the coefficient of variation (CV) and accuracy within ±15% of the actual value [15].

#### 3.4.4. Recovery and matrix effect

The mean extraction recoveries of 2-hydroxyflutamide were 100.5 ± 3.2%, 96.2 ± 3.2%, 95.9 ± 6.1% at concentrations of 3.485, 101.64, 1161.6 ng/ml, respectively. The mean matrix effect values calculated in this assay were 98.6 ± 1.5%, 105.4 ± 6.5% and

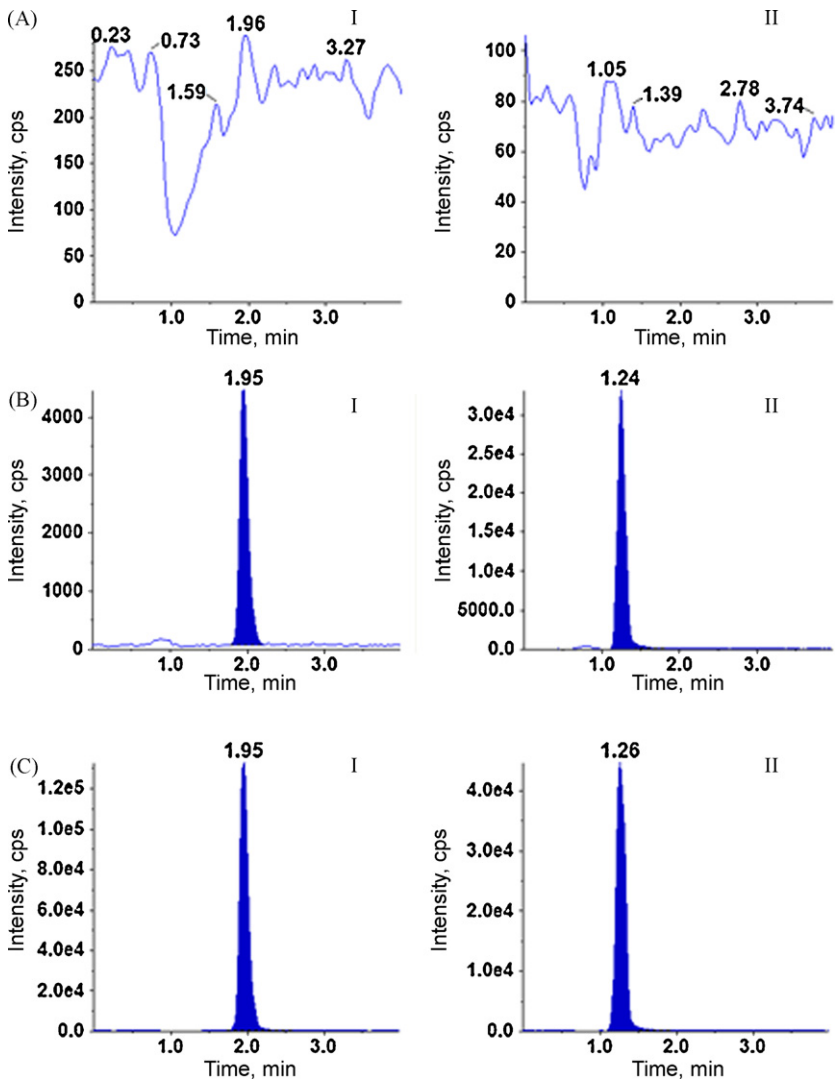


**Fig. 1.** Typical MRM chromatograms of (A) blank human plasma; (B) blank plasma spiked with 2-hydroxyflutamide at LLOQ (1.742 ng/ml) and I.S. (100 ng/ml); (C) volunteer plasma after 36 h oral dose of 250 mg flutamide. Peak I: 2-hydroxyflutamide; Peak II: I.S.

100.9 ± 3.3% at low, middle, high QC levels, which indicated no co-eluting endogenous substances interfering with the ionization of the analytes.

### 3.4.5. Stability

The stability of 2-hydroxyflutamide under different storage and handling conditions was fully evaluated by analyzing QC samples. Stability was expressed as a percentage of nominal concentration. The results are summarized in Table 2 which showed a good stability of 2-hydroxyflutamide over all steps of the determination.

## 3.5. Application to a bioequivalence study in healthy subjects

The validated method was successfully applied to a bioequivalence study of two flutamide formulations. The mean concentration versus time curves in 20 healthy volunteers after administration of reference and test preparations are shown in Fig. 2. The main pharmacokinetic parameters are summarized in Table 3.

The bioequivalence of two preparations was determined on basis of $C_{max}$, $AUC_{0-t}$ and $AUC_{0-\infty}$ by analysis of variance (ANOVA) and two one-side *t*-test and $T_{max}$ by Wilcoxon test. The 90% confidence intervals (90% CI) of the test/reference percent ratio were 94.3–115.3% for $C_{max}$, 103.0–122.2% for $AUC_{0-48}$ and 102.8–122.1% for $AUC_{0-\infty}$, respectively. In case of $T_{max}$, the result showed that there was no significant difference between the two preparations. The intra-subject variability (CV%) was 14.0% ± 11.1% for $C_{max}$, 14.6 ± 8.8% for $AUC_{0-48}$ and 14.5% ± 8.9% for $AUC_{0-\infty}$, respectively. It could be concluded that the two flutamide preparations were bioequivalent in their rate and extent of absorption.

**Table 1**
Precision and accuracy for 2-hydroxyflutamide in plasma (three batches, five replicates per batch).

| Nominal concentration (ng/ml) | RSD (%) | | RE (%) | |
|---|---|---|---|---|
| | Intra-batch | Inter-batch | Intra-batch | Inter-batch |
| 3.485 | 5.2 | 5.5 | 6.7 | 7.0 |
| 101.64 | 4.5 | 4.6 | −4.7 | −5.6 |
| 1161.6 | 8.1 | 5.6 | −2.1 | −6.0 |

**Table 2**
Stability of 2-hydroxyflutamide under various storage conditions ($n = 3$).

| Storage conditions | Nominal concentration (ng/ml) | Mean measured concentration (ng/ml) | RSD (%) | RE (%) |
|---|---|---|---|---|
| Autosampler for 8 h | 3.485 | 3.720 ± 0.270 | 7.2 | 7.1 |
| | 101.64 | 96.97 ± 2.60 | 2.7 | −2.2 |
| | 1161.6 | 1165.2 ± 117.2 | 10.1 | 1.3 |
| Room temperature for 8 h | 3.485 | 3.552 ± 0.260 | 7.2 | 2.3 |
| | 101.64 | 99.16 ± 6.20 | 6.3 | 0.0 |
| | 1161.6 | 1095.5 ± 50.9 | 4.6 | −4.7 |
| 3rd freeze and thaw | 3.485 | 3.786 ± 0.170 | 4.5 | 9.0 |
| | 101.64 | 99.22 ± 1.77 | 1.8 | 0.1 |
| | 1161.6 | 1124.7 ± 68.2 | 6.1 | −2.2 |
| −80 °C for 30 days | 3.485 | 3.537 ± 0.180 | 5.1 | 1.8 |
| | 101.64 | 90.43 ± 2.95 | 3.3 | −8.8 |
| | 1161.6 | 1104.8 ± 54.9 | 5.0 | −3.9 |

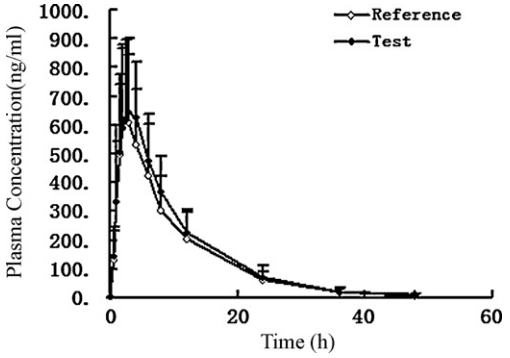


**Fig. 2.** Mean plasma concentration–time profiles of 2-hydroxyflutamide in 20 volunteers after oral dose of test and reference preparations.

**Table 3**
Mean pharmacokinetic parameters in 20 volunteers after oral administration of test and reference preparations at the flutamide dose of 500 mg.

| Parameters | Test tablet | Reference tablet |
|---|---|---|
| $t_{1/2}$ (h) | 6.6 ± 1.1 | 6.8 ± 1.4 |
| $C_{max}$ (ng/ml) | 724.8 ± 237.8 | 706.0 ± 255.3 |
| $T_{max}$ (h) | 2.9 ± 1.1 | 2.8 ± 1.0 |
| $AUC_{0-48}$ (ng h/ml) | 7440.7 ± 2471.6 | 6626.9 ± 2252.9 |
| $AUC_{0-\infty}$ (ng h/ml) | 7508.2 ± 2503.7 | 6695.2 ± 2276.4 |

## 4. Conclusion

A sensitive, simple and rapid UFLC–ESI-MS/MS method for the determination of 2-hydroxyflutamide in human plasma was developed. The described method showed good specificity, precision, accuracy and linearity over the range of 1.741–1452 ng/ml. This method provided a sample preparation by protein precipitation with methanol which could save considerable time and simplify the operating process. The established LLOQ of 1.742 ng/ml was adequate to determine the concentration of 2-hydroxyflutamide in human plasma 48 h after oral administration of 250 mg flutamide. No significant interferences caused by endogenous compounds were observed. The method was successfully applied to demonstrate the bioequivalence of test and reference preparation.

## References

[1] R. Anahara, Y. Toyama, C. Mori, Reprod. Toxicol. 25 (2008) 139.
[2] S. Budavari, M.J. O'Neil, A. Smith, P.E. Heckelman, Merck Index, 11th edn., Merck & Co Inc., Rathway, NJ, USA, 1989, p. 658.
[3] P.D. Tzanavaras, D.G. Themelis, J. Pharm. Biomed. Anal. 43 (2007) 1820.
[4] A. Tevell, H. Lennernas, M. Jonsson, M. Norlin, B. Lennernas, U. Bondesson, M. Hedeland, Drug Metab. Dispos. 34 (2006) 984.
[5] M. Schulz, A. Schmoldt, F. Donn, H. Becker, Eur. J. Clin. Pharmacol. 34 (1988) 633.
[6] E. Radwanski, G. Perentesis, S. Symchowicz, N. Zampaglione, J. Clin. Pharmacol. 29 (1989) 554.
[7] C.J. Xu, D. Li, Zhongguo Yao Li Xue Bao 19 (1998) 39.
[8] A. Belanger, M. Giasson, J. Couture, A. Dupont, L. Cusan, F. Labrie, Prostate 12 (1988) 79.
[9] R.N. Brogden, P. Chrisp, Drugs Aging 1 (1991) 104.
[10] S.C. Gad, Curr. Opin. Pharmacol. 3 (2003) 98.
[11] R.H. Asade, L. Prizont, J.P. Muino, J. Tessler, Cancer Chemother. Pharmacol. 27 (1991) 401.
[12] I. Niopas, A.C. Daftsios, J. Chromatogr. B 759 (2001) 179.
[13] H. Jalalizadeh, E. Souri, H.R.J. Ghanami, A. Almasirad, A. Foroumadi, Intl. J. Pharmacol. 2 (2006) 221.
[14] J. Leibinger, M. Kapas, J. Pharm. Biomed. Anal. 14 (1996) 1377.
[15] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), 2001.