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Bioanalytical method development, validation and quantification of flutamide in spiked rat plasma by using high-performance liquid chromatography

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Abstract

Background A quick, low cost and precise method was developed and validated for flutamide in the rat plasma using a high-performance liquid chromatography. Acetanilide was used as internal standard in the study. Flutamide was extracted from rat plasma using methanol. The chromatographic analysis was carried out on Cromasil C18 column at 227 nm using methanol–water mixture as the mobile phase and flow rate of 0.8 ml/min.

Results The developed method demonstrated good linearity over the 100–1000 ng/ml concentration range with a regression coefficient of 0.9947. The accuracy of the developed method was found to be 97–101%, while its precision was found to be less than 5%. Limits of detection and quantitation were determined to be 2.52 ng/ml and 7.66 ng/ml, respectively. Flutamide showed recovery of about 96–100% and the developed method was further used to investigate the pharmacokinetics of flutamide in the marketed formulation.

Conclusions From the results, it can be concluded that the developed method has the potential to successfully determine flutamide in rat plasma and establish the pharmacokinetic parameters of other flutamide-containing formulations.

Keywords Flutamide, HPLC, Plasma, Validation, Bioanalytical

Background

Prostate cancer is one of the most prevalent malignancies and the leading cause of malignancy in males over 50 [1]. It is associated with the uncontrolled growth of cells in the prostate gland which over the time may spread to other body areas. The cells will initially develop slowly and remain isolated, but they begin to spread to other organs via the lymphatic system and bloodstream. Radical prostatectomy is a treatment option for organ-confined cancers. The primary cause of the high mortality rate is the lack of curative treatment for prostate cancer that has spread to other organs [2] and locally generated androgens in the prostatic tissues are the primary treatment challenge for prostate cancer [3, 4]. Compared to steroidal antiandrogens, which have dramatically increased androgen receptor selectivity, nonsteroidal antiandrogens such as bicalutamide and flutamide are more potent and safer [5]. Chemically, flutamide is 2-methyl-*N*-[4-nitro-3-(trifluoromethyl) phenyl] propanamide. Flutamide is an anti-androgenic and nonsteroidal drug used in the treatment of advanced prostate cancer [6]. It has a half-life of around 6 h and is available in the form of a crystalline pale yellow powder with acicular-shaped particles. It belongs to BCS class

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II and is completely soluble in methanol and ethanol, but insoluble in water [7]. It competes with testosterone and its active metabolite for binding to androgen receptors in the prostate gland by acting as an antagonist of the androgen receptor, preventing them from activating prostate cancer cell growth [8].

Flutamide is included in the British Pharmacopoeia (BP) [9] and United States Pharmacopoeia (USP) [10]. Flutamide was assessed in both pure form and capsules using an RP-HPLC method utilizing C18 column in USP, whereas it was determined by spectrophotometric method in BP. Other techniques such as electrochemical [11–13], gas–liquid chromatography (GLC) and polarography [14] have been documented for the detection of flutamide. The HPLC methods are effective in determination of active ingredient in pharmaceutical dosage form and biological materials. The stability of flutamide in the solid form and in aqueous solution was also investigated using HPLC [15].

The literature survey reveals that available methods for analysis of flutamide usually suffer from long analysis time or involve equipment not commonly available. Therefore, the goal of the present research work was to develop the HPLC technique with UV detection that is sensitive, low cost and time-effective, fully validated and may be used in future to measure flutamide levels in rat plasma.

Methods

Materials

Flutamide and acetanilide (internal standard) were gifted by Cipla Pvt. Ltd. and CTX Lifesciences Pvt. Ltd., India. Methanol and water of HPLC grade were procured from Loba Chemie Pvt. Ltd., Mumbai, India. Analytical grade ethylenediaminetetraacetic acid disodium and orthophosphoric acid were obtained from Merck India Ltd.

Animals

Healthy male rats (Sprague–Dawley) were approved for bioanalytical and pharmacokinetic studies (protocol no. DBCOP/IAEC/1426/21-22/P6). The animals were kept in a controlled environment (25 °C, 60% RH and 12-h cycle of darkness and light) and fed standard laboratory pellet diet along with water as needed. The animals were fasted overnight before the study.

Instrumentation

The chromatographic analysis was carried out on the HPLC binary gradient system (HPLC 3000 series, Analytical Technologies Ltd.) with a reciprocating pump (P-3000-M) operating at pressure of 40 MPA. HPLC workstation software was used to process the data, and analysis was performed at the temperature of 40 °C in the column oven. The chromatographic separation of flutamide and IS on C18 column was performed at the flow rate of 0.8 ml/min. The 20-µl sample was injected into the HPLC system and analyzed at 227 nm for 11.34 min.

Chromatographic condition

On the RP-C18 column (250 mm × 4.6 mm) with particle size 5 µm, chromatographic separation was accomplished. In order to get the best flutamide detection, various mobile phase compositions were tested. Prior to use, the degassed mobile phase was filtered through 0.45 µm membrane filter. The mobile phase consisting of methanol and water were employed for the chromatographic analysis of the flutamide and the pH was maintained at 3.0 throughout the analysis by adding orthophosphoric acid in the ratio 85:15 (v/v). The mobile phase was used to dilute the standard and sample solutions.

Extraction procedure

The collected blood samples were taken and subjected to centrifugation at 2000 rpm for 10 min. The quantity of stock solution was added to 2 ml of plasma and vortexed for 3–4 min to achieve the required concentration. Then, to the above solution, 2 ml of ethyl acetate was added as an extracting solvent. The mixture was vortexed once more for 3–4 min and subjected to centrifugation. The extracted material was isolated from the clear supernatant of non-polar solvent. After evaporation of the non-polar solvent (ethyl acetate), the remaining sample was mixed with mobile phase and the resulting solution was injected for additional testing [16].

Stock solution preparation

Flutamide and acetanilide primary stock solutions were prepared separately by dissolving 10 mg of drug in 10 ml of mobile phase to get 1000 ppm concentration. The working standard solutions of suitable concentrations were prepared using stock solutions and mobile phase. To prepare quality control samples in rat plasma and to plot the calibration curve, diluting working solutions were used [16].

Quality control samples and calibration standards preparation

Blank plasma was tested for the presence of endogenous compounds at the flutamide and acetanilide retention times prior to spiking. Flutamide was measured at seven different concentrations ranging from 100 to 1000 ng/ml by spiking the blank rat plasma with each flutamide working solution (i.e., 100, 200, 400, 500, 600, 750 and 1000 ng/ml). The low, medium and high concentrations

(100, 600 and 1000 ng/ml of flutamide) of the QC working solutions were added in proper quantities to blank rat plasma. In all the samples, acetanilide (IS) concentration was kept as 500 ng/ml.

HPLC method validation

Following optimization of the chromatographic method based on separation of flutamide, acceptable shape of peak and high resolution, the proposed method was validated using International Conference on Harmonization (ICH) standards. For the developed method, validation factors such as selectivity, sensitivity, linearity range, accuracy and precision were all assessed [17–24]. The method validation was carried out to ensure that the established method can produce repeatable and dependable results when analyzed in various laboratories.

Selectivity

Six distinct blank plasma samples collected from six different rats were injected to assess the method's selectivity in order to find any potential endogenous chemicals that might interfere with the effects of flutamide and acetanilide. The retention times of flutamide and acetanilide chromatographic peaks were investigated.

Specificity

The specificity of method was assessed by injecting both blank and flutamide-spiked plasma. The chromatograms were examined for any undesirable elements that would affect the flutamide and internal standard acetanilide peaks. Parameters such as retention time, resolution and tailing factor were determined to demonstrate the specificity of method.

Linearity

Linearity was assessed by spiking plasma with known concentration of flutamide solution over the concentration range 100–1000 ng/ml. The peak area ratio of flutamide/IS versus flutamide concentrations was plotted to establish the regression equation. By subtracting the flutamide concentrations from the regression equation, it was possible to determine the precision and accuracy.

Precision and accuracy

The developed method was assessed for intra- and inter-day precision and accuracy by spiking flutamide in rat plasma. The samples were spiked at concentrations of 100, 600 and 1000 ng/ml to assess intra-day precision and accuracy. The same concentrations were used to measure the inter-day precision and accuracy for three consecutive days. The % relative error was calculated to measure accuracy, while the coefficient of variation was used to determine precision (CV).

Recovery

QC (LQC, MQC and HQC) standards were used to evaluate recovery of flutamide from rat plasma. The recovery of flutamide was calculated by comparing the peak areas of each extracted QC standard to the peak areas of unextracted standard solutions containing the appropriate concentrations of flutamide in the mobile phase. Recovery was determined in triplicate.

LOD and LOQ

The limit of detection (LOD) determines the lowest concentration in sample that can be distinguished from background noise but not quantitated. LOD was calculated using signal-to-noise ratio (S/N) of 3:1 by comparing test results from samples with known flutamide concentrations to blank samples. The lowest concentration of analyte that can be determined with acceptable precision and accuracy is defined as the limit of quantitation (LOQ). The LOQ was determined by collecting mobile phase and plasma standards with known flutamide concentrations.

Stability study

The stability of the flutamide should be evaluated during sample collection, handling as well as after short-term and long-term storage. Freeze–thaw stability was ascertained by testing the QC samples (LQC, MQC and HQC) in triplicate across three freeze–thaw cycles. Each concentration was divided into three aliquots, which were then frozen for 24 h at −40 °C and thawed unassisted at room temperature. Following complete thaw, the samples were refrozen for another 24 h in the same manner; the freeze–thaw cycle was then repeated twice more and the drug stability in plasma was investigated. Under these conditions, three aliquots of each QC concentration were stored. At room temperature, four aliquots of each concentration were stored for 1 and 8 h. For assessment of long-term stability, four aliquots of the low, medium and high concentrations in the plasma were kept at 40 °C for 21 days and the concentration of analyte present in the sample was analyzed.

Application of method

The study used six healthy male Sprague–Dawley rats weighing between 200 and 250 g and was allowed to adapt to the housing environment for at least 1 week. Using oral gavage, rats were given single dose of the marketed formulation Cytomid® (manufactured by Cipla Ltd.) equivalent to 25 mg/kg of flutamide orally. At intervals of 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h, blood samples were taken from the retro orbital plexuses. The collected plasma samples were centrifuged at 5000 rpm for 15 min

to extract plasma. The bioanalytical technique stated above was used to process the plasma samples and to obtain pharmacokinetic parameters.

Results

Selectivity

The selectivity of method was assessed by examining blank rat plasma ($n=6$). The outcome of one sample of blank plasma is presented in Fig. 1, and the absence of interference is comparable to that of other samples that were also examined. The absence of any interference of endogenous compounds of blank plasma was observed at the retention period of 7.03 min of the flutamide.

Specificity

The flutamide eluted quickly with good resolution within 10 min. The flutamide peaks were not hampered by the plasma matrix components (Fig. 2). Flutamide eluted at 7.03 min and IS eluted at 4.65 min. The method's specificity against endogenous chemicals and other related compounds could be seen as no interfering endogenous peaks were seen. It was found that the peak shape and retention period were identical to those of pure standards.

Linearity

The calibration curve of flutamide was linear and ranged from 100 to 1000 ng/ml, as demonstrated by a linearity equation of $y=0.6824x-0.0629$ and correlation coefficient of 0.9947 (Fig. 3). The lower limit of quantitation (LLOQ) for flutamide was found to be 50 ng/ml. In light of this, the developed method showed good linearity. A standard curve was found to be validated if coefficient of correlation should be near to one.

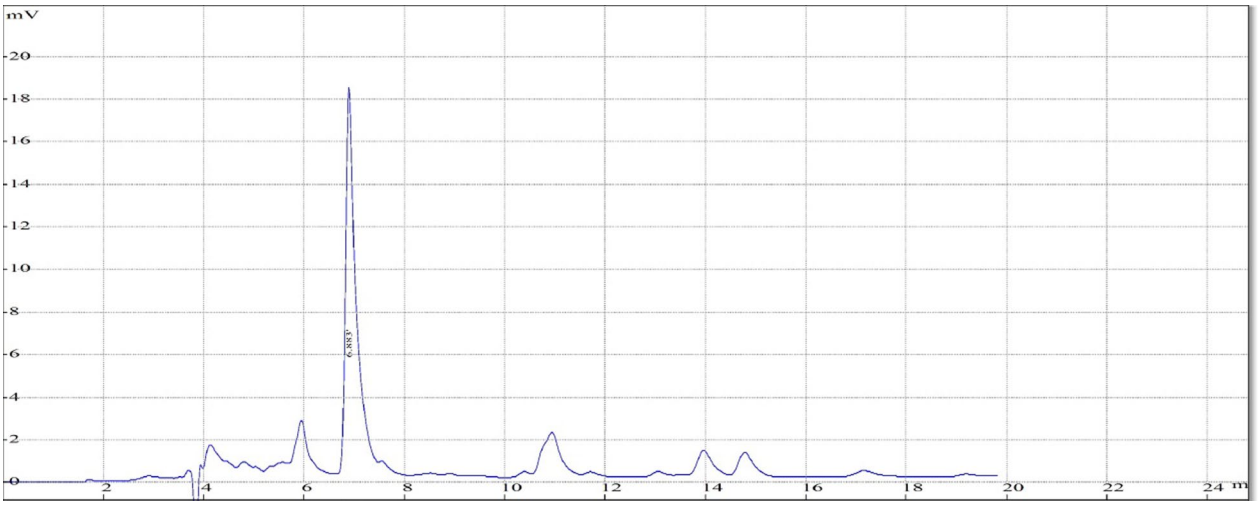

Fig. 1 Representative chromatogram of flutamide in blank plasma

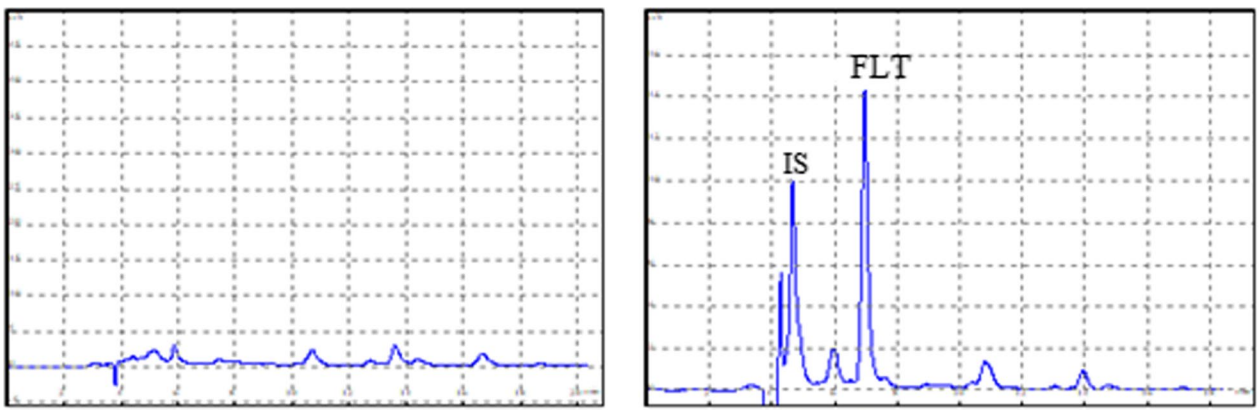

Fig. 2 Chromatogram of plasma spiked with flutamide (FLT) and internal standard (IS) acetanilide

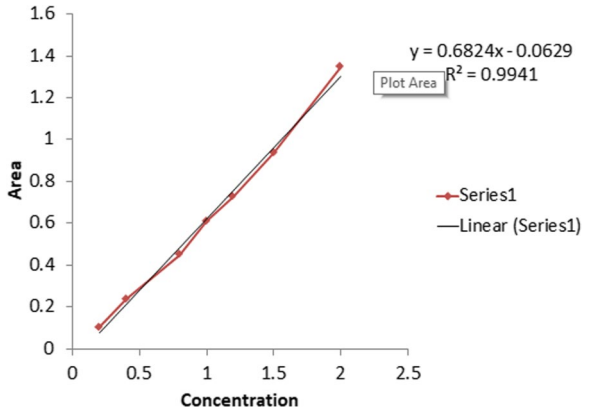

Fig. 3 Calibration curve of flutamide spiked in rat plasma

Table 1 Flutamide determination in rat plasma: intra-day precision and accuracy

Theoretical conc. (ng/ml)	Calculated conc. (ng/ml)	Accuracy % recovery	Intra-day precision % RSD
100	101.19 ± 3.0	101.19	2.99
600	600.86 ± 8.9	100.14	1.48
1000	1010.65 ± 5.3	101.06	0.52

Table 2 Flutamide determination in rat plasma: inter-day precision and accuracy

Theoretical conc. (ng/ml)	Calculated conc. (ng/ml)	Accuracy % recovery	Inter-day precision % RSD
100	97.89 ± 1.4	97.89	1.43
600	595.86 ± 7.41	99.31	1.24
1000	989.77 ± 10.05	98.97	1.05

Precision and accuracy

Table 1 provides data of the precision and accuracy for each day. The intra-day precision was determined by computing the % CV of peak area ratio for three replicates at concentration levels of 100, 600 and 1000 ng/ml evaluated on the same day. The % CV was between 0.55 and 2.99%. The mean percentage recoveries ranging from 101.06 to 101.19% were observed in the case of intra-day accuracy. Similar measurements of intra-day precision and accuracy were taken for three replicates of various flutamide plasma concentrations over the course of 3 days. The accuracy varied from 97.89 to 99.31%, and the precision represented as CV% ranged from 1.05 to 1.43% (Table 2). Accuracy and precision were confirmed by the acceptance criteria of RSD ≤ 15%, except for LLOQ (RSD ≤ 20% for LLOQ).

Table 3 Extraction efficiency of flutamide in rat plasma

Concentration (ng/ml)	%Recovery ($n = 3$)	% CV
100	96.81 ± 3.31	4.96
500	98.12 ± 6.56	5.71
1000	99.35 ± 5.22	6.01

Table 4 Analytical data on stability study of flutamide

Concentration (ng/ml)	Recoveries ($n = 3$)		
	− 40 °C	Room temperature	40 °C
100	96.55 ± 0.88	100.12 ± 1.25	100.51 ± 0.85
500	98.36 ± 1.72	99.98 ± 2.55	100.05 ± 1.41
1000	99.53 ± 2.36	100.01 ± 3.12	99.61 ± 2.11

Recovery

The effectiveness of the developed method to extract the flutamide was assessed, and the results are presented in Table 3. The average recovery of flutamide from plasma ranged from 95.81 to 99.35% across the three QC levels, demonstrating the effectiveness of the developed method.

Limit of detection and quantification

The limit of detection was measured to know the ability of the instrument to detect flutamide even at low concentration. The injection of 5 ng/ml of the flutamide concentration resulted in LOD of 2.52 ng/ml and S/N values between 3 and 5. The lowest concentration of 7.66 ng/ml was found to demonstrate the limit of quantification for this developed method.

Stability study

Throughout whole period of sample storage, processing and analysis, there was no detectable degradation of flutamide (losses were under 4.0%). Table 4 displays the stability evaluation results.

Pharmacokinetic studies

The pharmacokinetic parameters of flutamide were determined using the optimized method after single-dose oral administration (Table 5). The pharmacokinetic parameters were computed using pksolver software. In order to analyze samples from the pharmacokinetic study done on Sprague–Dawley rats, the optimized method has been effectively used. Figure 4 depicts the pharmacokinetic profile of flutamide in rat plasma after single-dose oral administration of a marketed formulation to six rats.

Table 5 Pharmacokinetic parameters of marketed formulation of flutamide on Sprague–Dawley rats ($n = 6$)

Parameter	Unit	Value
$t_{1/2}$	H	10.64
T_{max}	H	4.00
C_{max}	µg/ml	202.23
AUC 0-t	µg/ml*h	2562.73
MRT 0-inf_obs	H	15.53

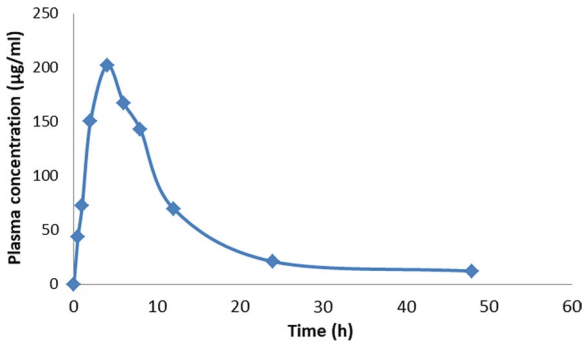

Fig. 4 Plasma concentration of flutamide at different time intervals after oral administration of the marketed formulation

The C_{max} was found to be 202.23 ± 0.73 ng/ml and half-life ($t_{1/2}$) was 10.6 ± 0.05 h. The parameters are consistent with those previously reported.

Discussion

A quick and precise high-performance liquid chromatography (HPLC) was developed and validated for pure drug flutamide. The developed method further was applied on marketed formulation for the evaluation of pharmacokinetic parameters. Acetanilide was used as the internal standard (IS) in the study. Methanol and water mixture (80:20) was used as mobile phase with an injection volume of 20 µl at 0.8 ml/min flow rate. The method was validated for the different parameters such as specificity, selectivity, linearity, accuracy, precision, LOD, LOQ, recovery and stability. The pharmacokinetic parameters evaluated using developed method showed satisfactory results with the reported values. Hence, the developed method can be successfully used for the determination of flutamide in different biological fluids.

Conclusion

The appropriate and straightforward new bioanalytical approach for flutamide was established using acetanilide as an internal standard with advantages in terms of selectivity, sensitivity and speedy analysis. The developed method was validated in accordance with the standards of the International Conference on Harmonization. It was observed that the developed method complied with all validation parameters within the specified limitations. From the results, the method was found to be accurate and precise. The recovery of the developed method proved the applicability to assess flutamide in biological matrices including plasma, serum and urine.

Abbreviations

HPLC	High-performance liquid chromatography
IS	Internal standard
LOD	Limit of detection
LOQ	Limit of quantitation
BP	British Pharmacopoeia
USP	United States Pharmacopoeia
GLC	Gas–liquid chromatography
RP	Reverse phase
QC	Quality control
LQC	Lower quality control
MQC	Medium quality control
HQC	Higher quality control
RSD	Relative standard deviation
LLOQ	Lower limit of quantitation

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Author contributions

VP, MJ and PS were involved in conceptualization of the research work. MJ performed the experiments and analyzed the data. VP and PS were involved in supervision. All authors drafted and reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data will be made available on request.

Declarations

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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