Volume 3 (2), Suppl. 2015, Page-15-21
CODEN (USA)-IJPRUR, e-ISSN: 2348-6465

PHS Scientific House
PHARMAHEALTHSCIENCES

International Journal of Pharma Research and Health Sciences

Available online at www.pharmahealthsciences.net

Original Article

UPLC Method Development and Validation for Butoconazole in Active Ingredient

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ARTICLE INFO

Received: 12 Apr 2015
Accepted: 20 May 2015

ABSTRACT

The objective of the study was to develop UPLC method for the determination of purity of Butoconazole in API and its validation. Ultra performance liquid chromatoghaphy is a better technique than HPLC in terms of performance and speed, so it was selected. The method was developed using Acetonitrile and Sodium dihydrogen ortho phosphate and Acquity BEH C18 (50×2.1mm, 1.7µ) as a stationary phase at a flow rate of 0.25ml/min. Validation was done by linearity, precision, and robustness studies. The precision was found to be within the limits. The linearity studies indicated the drug obeys Beer's law and revealed the specified range of linearity for drug was between 80µg/ml and 120µg/ml. The robustness was observed from the insignificant variation in the analysis by changes in flow rate, mobile phase ratio, wavelength, column oven temperature and pH. Forced Degradation study revealed the drug degraded initially by the fluence of acid, alkali, and peroxide. Solution stability study showed the drug was not stable for more than 2 h at 25˚C but stable at 5˚C.

Key words: Butoconazole , Antifungal activity, Acquity BEH C 18, method development and validation

1. INTRODUCTION

High performance liquid chromatography (HPLC) has proven to be the predominant technology used in laboratories worldwide during the past 30 plus years. Waters Corporation has taken the principles of HPLC and further adapted them to create Ultra Performance Liquid Chromatography (UPLC™), a new separation technique with increased speed, sensitivity and resolution. The performance of a column can be measured in terms of the height equivalent to the

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theoretical plates (HETP) which is calculated from the column length (L) and the column efficiency, or number of theoretical plates (N). N is calculated from an analyte retention time (tR) and the standard deviation of the peak (s). H = L/N. UPLC instrumentation involves a Binary solvent manager, sample manager, detector. The types of UPLC techniques include Normal phase chromatography (NP-UPLC), Reverse phase chromatography (RP-UPLC), Size exclusion chromatography, Ion exchange chromatography and Bio-affinity chromatography . Chromatographic methods are commonly used for the quantitative and qualitative analysis of raw materials, drug substances, drug products and compounds in biological fluids. The objective of a test method is to generate reliable and accurate data regardless of whether it is for acceptance, release, stability or pharmacokinetics study. The various steps to be performed for UPLC method development involve solubility studies to establish the solubility of the API in a number of aqueous and organic solvents, selection of the mobile phase, selection of the detector and detector wavelength, and selection of isocratic or gradient mode of elution. For UPLC method development optimization of some critical parameters is required. They include selection of the buffer, pH of the buffer and the mobile phase, column, column temperature, test concentration and injection volume. Validation of a method is the process by which a method is tested by the developer or user for reliability, accuracy and preciseness of its intended purpose. Methods validation should not be a one-time situation to fulfill agency filing requirements, but the methods should be validated and also designed by the developer or user to ensure ruggedness or robustness. There is no single validation approach that must always be employed for a new method. Validation approaches include zero-blind method, single-blind method, doubleblind method and inter-laboratory collaborative study. The parameters involved for validation of UPLC methods include precision, accuracy, Linit of Detection (LOD), Limit of Quantitation (LOQ), specificity, inearity, ruggedness, robustness, solution stability, and system suitability(capacity factor, resolution, tailing factor, theoretical plate number. The acceptance criteria for the different characteristics of validation are mentioned in ICH Q2A guidelines. The drug used in the present study is Cefditoren Pivoxil which is a cephalosporin category antibiotic. It is used to treat uncomplicated skin and skin structure infections, community-acquired pneumonia, acute bacterial exacerbation of chronic bronchitis, pharyngitis, and tonsillitis. Thus the objective of the present study is to develop UPLC method for the determination of purity of Cefditoren Pivoxil in API and validation of the same. There are very few works that has been done on this drug by HPLC but no method has been mentioned by UPLC technique. [1]

2. MATERIALS & METHODS

2.1 Method Development of Butoconazole

Materials and Instruments:

The fast liquid chromatography was performed using waters UPLC system with uv detector. Chromatogram and data were recorded by means of Empower 2 software .The chromatographic system was performed using an Acquity BEH C18 (50 × 2.1 mm) id., 1.7 μm column. Separation was achieved using a mobile phase consisted of Sodium dihydrogen ortho phosphate : acetonitrile in the ratio of 50: 50 , adjusted to pH 2.5 adjust with orthophosphoric acid , at a flow rate of 0.4 mL/min in only 4 minute runtime , injection volume of 1 μL . The column temperature was maintained at 40∘C, injection volume was 2 μL, and detection wavelength was set at 294 nm for determination of Butoconazole. [2]

Table 1: Solubility study

Reagents	Solubility
Water	Higly hSoluble

Ethanol	Soluble
1 (M) HCl	Soluble
1(M) NaOH	Insoluble
Methanol	Very slightly Soluble

UPLC Instrumental Condition:

The UPLC system enrolled was **Waters Acquity** with Empower -2 Software with Isocratic which is associated with UV-Visible detector (L-2400).

2.2 Standard & sample preparation for UV-spectrophotometer analysis:

50 mg of Butoconazole standard was transferred into 250 ml volumetric flask, dissolved in mobile phase & make up to volume with mobile phase. Further dilution was done by transferring 0.1 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

The standard & sample stock solution:

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent.(After optimization of all conditions) for UV analysis. It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Butoconazole, so that the same wave number can be utilized in UPLC UV detector for estimating the Butoconazole. While scanning the Butoconazole solution we observed two absorption peaks at 280. [3] The absorption maxima were at 266 nm. The UV spectrum has been recorded on UPLC make UV – Vis spectophotometer model UV-2450. The scanned UV spectrum is attached in the following page

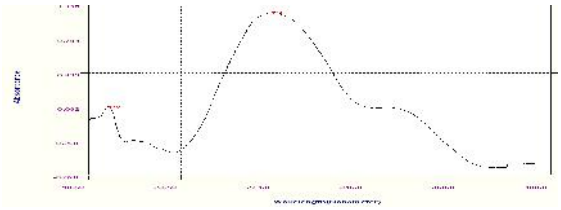


Fig 1: UV-Spectrum for Butoconazole

Mobile phase preparation:

The mobile phase used in this analysis consists of a mixture of Buffer (0.05 M Sodium dihydrogen ortho phosphate, pH adjusted to 3.1) & Acetonitrile in ratio 50: 50.

Preparation of solutions:

Preparation of Standard solution:

Working concentration should be around 10 µg/ml. Accurately weighed around 25mg of Butoconazole working standard, taken into a 25 ml volumetric flask, then dissolved in mobile phase and diluted to volume with the mobile phase to obtain a solution having a known concentration of about 1000 mcg/ml. Further dilutions have been made to get the final concentration of 10 µg/ml [4].

Preparation of Test solution:

Diluted quantitatively an accurately measured volume of label claim solution with diluents to obtain a solution containing about a linear range. [5-7]

Optimized Chromatographic Conditions:

Column : Acquity BEH C-18, 50: 2.1mm, 1.7µm

Mobile Phase : ACN: phosphate buffer (pH 2.5) = 60:40

Flow Rate : 0.25 ml/minute

Wave length : 280 nm

Injection volume : 5 µL

Run time : 5 minutes

Column temperature : Ambient

Sampler cooler : Ambient

Different Trials for Chromatographic Conditions

Table 2: The various parameter for method development.

Parameters	Method 1	Method 2	Method 3	Method 4	Method 5
Column	Acquity BEH C_{18}, 50* 2.1 mm , 1.7 µm	Acquity BEH C_{18}, 50* 2.1 mm , 1.7 µm	Acquity BEH C_{18}, 50* 2.1 mm , 1.7 µm	Acquity BEH C_{18}, 50* 2.1 mm , 1.7 µm	Acquity BEH C_{18}, 50* 2.1 mm , 1.7 µm
Mobile Phase	ACN:Water = 20 : 80	ACN: Water = 30 : 70	ACN: Water = 35 : 65	ACN: phosphate buffer = 40:60	ACN: phosphate buffer (pH=2.5) = 60:40
Flow rate	0.25 ml/min	0.25 ml/min	0.25 ml/min	0.25 ml/min	0.25 ml/min
Run Time	5 min	5 min	5 min	5 min	5 min
Detection	296 nm	296 nm	296 nm	296 nm	296 nm

Column Temp.	40 ^{0}C	40 ^{0}C	40 ^{0}C	40 ^{0}C	40 ^{0}C
Injection volume	5 µL	5 µL	5 µL	5 µL	5 µL
Observation	Peak broken	Very low response	Tailing peak	Broad Peak	Nice peak
Result	Method Rejected	Method Rejected	Method Rejected	Method Rejected	Method Accepted

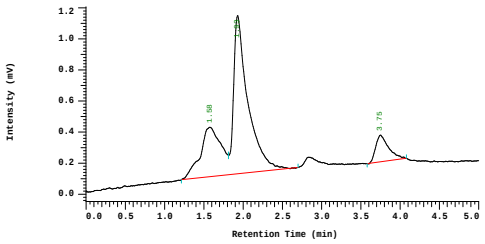


Fig 2: Chromatogram for Blank

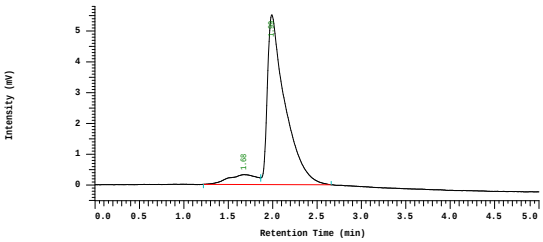


Fig 3: Chromatogram for Butoconazole (Rt1.93)

2.3 Stability/Forced degradation studies:

Following protocol was strictly adhered to for forced degradation of Butoconazole Active Pharmaceutical Ingredient (API). The API (Butoconazole) was subjected to stress conditions in various ways to observe the rate and extent of degradation that is likely to occur in the course of storage and/or after administration to body. This is one type of accelerated stability studies that helps us determining the fate of the drug that is likely to happen after along time storage, within a very short time as compare to the real time or long term stability testing. The various degradation pathways studied are acid hydrolysis, basic hydrolysis, thermal degradation. [9]

2.3.1 Acid Hydrolysis :

An accurately weighed 25 mg. of pure drug was transferred to a clean & dry 25 ml volumetric flask. To which 0.1 N Hydrochloric acid was added & make up to the mark & kept for 24 hrs. from that 4 ml was taken in to a 10 ml volumetric flask & make up to the mark with mobile phase, then injected into the HPLC system against a blank of HCl . [8]

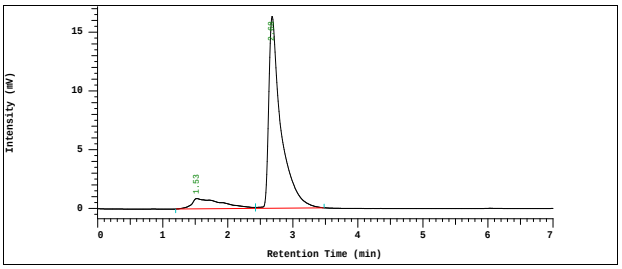


Fig 4: Chromatogram showing degradation of Butoconazole in 0.1 N HCl

2.3.2 Basic Hydrolysis

An accurately weighed 10 mg. of pure drug was transferred to a clean & dry 10 ml volumetric flask. To which 0.1 N Sodium hydroxide was added & make up to the mark & kept for 24 hrs. from that 4s ml was taken in to a 10 ml volumetric flask & make up to the mark with mobile phase, then injected into the HPLC system against a blank of . NaOH (after all optimized conditions)

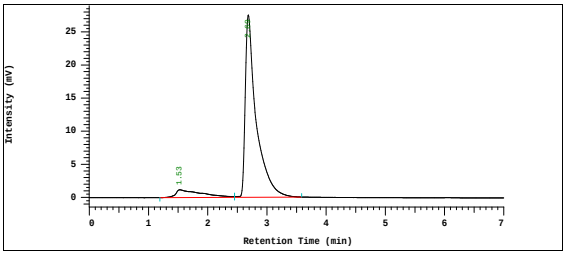


Fig 5: Chromatogram showing degradation peak of Butoconazole in 0.1 N NaOH

2.3.3 Thermal Degradation

An accurately weighed 10 mg. of pure drug was transferred to a clean & dry 100 ml volumetric flask, make up to the mark with mobile phase & was maintained at 50 ^{0}C. for 24 hrs. then injected into the HPLC system against a blank of mobile phase (after all optimized conditions)

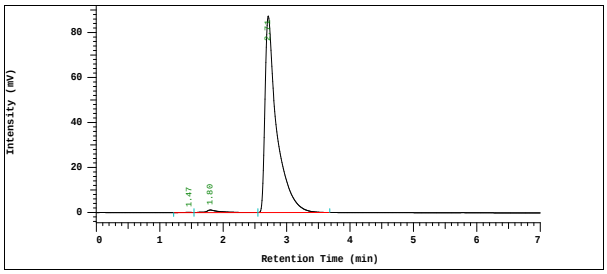


Fig 6:Chromatogram showing thermal degradation related impurity

3. RESULT & DISCUSSION

3.1 Results of degradation studies:

The results of the stress studies indicated the **specificity** of the method that has been developed. Butoconazole was degraded in all conditions except temperature stress conditions. The result of forced degradation studies are given in the following table.

Table 3: Results of force degradation studies of Butoconazole API.

Stress condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	31.25	68.36	99.61
Basic Hydrolysis (0.I M NaOH)	24Hrs.	30.41	68.32	98.73
Thermal Degradation (50 ^{0}C)	24Hrs.	98.36	----------	98.29
3% H_2O_2	24Hrs.	33.94	65.79	99.73

3.2 Method Validation

3.2.1 Accuracy : Recovery study:

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of BUTOCONAZOLE were taken and added to the pre-analyzed formulation of concentration 10µg/ml. From that percentage recovery values were calculated. The results were shown in table-4.

Table.4: Accuracy Readings

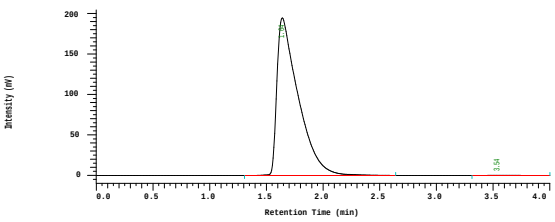
Level of Conc	Conc. Injected	AUC	Conc. Found	% Recovery
75	30	955630	30.662	102.2067
75	30	945214	30.31	101.0333
75	30	946534	30.35	101.1667
100	40	1248995	40.49	101.225
100	40	1249430	40.51	101.275
100	40	1248253	40.47	101.175
125	50	1562536	51.01	102.02
125	50	1562861	51.02	102.04
125	50	1553408	50.7	101.4
			Avg	101.5046
			SD	0.451657
			% RSD	0.444961

3.2.2 Precision: Repeatability

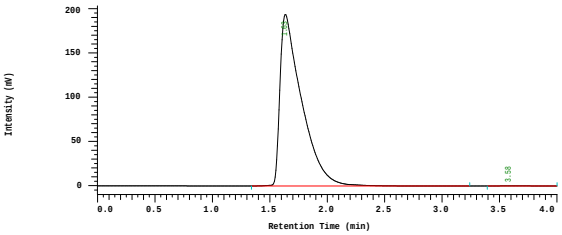
The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of five replicates of a fixed amount of drug. BUTOCONAZOLE (API). The percent relative standard deviation were calculated for BUTOCONAZOLE are presented in the table-5. [10]

Table 5: standard deviation were calculated for Butoconazole

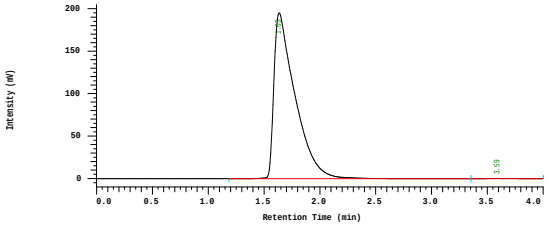
HPLC Injection Replicates of Butoconazole	Area	Retention Time
Replicate – 1	1248623	2.71
Replicate – 2	1267192	2.69
Replicate – 3	1248613	2.69
Replicate – 4	1258741	2.71
Replicate – 5	1276495	2.71
Average	1259933	2.702
Standard Deviation	12087.93	0.010954
% RSD	0.959411	0.40542



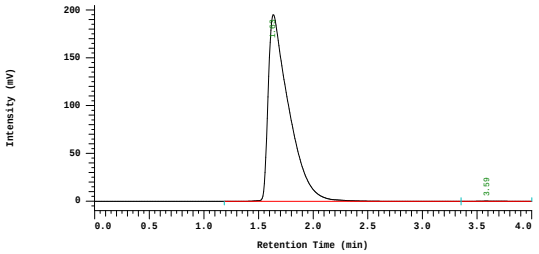
Replicate – 1



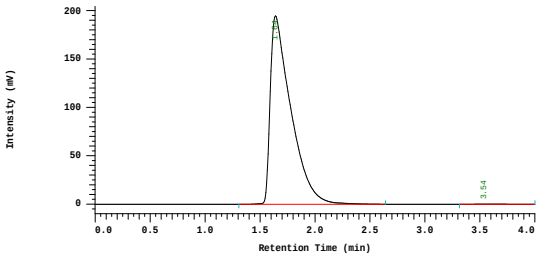
Replicate – 2



Replicate – 3



Replicate – 4



Replicate – 5

3.2.3 Linearity & Range **:** The calibration curve showed good linearity in the range of 0-80 µg/ml, for Butoconazole (API) with correlation coefficient (r^2) of 0.998 (Fig. 4). A typical calibration curve has the regression equation of y = 29826x + 41097 for Butoconazole .

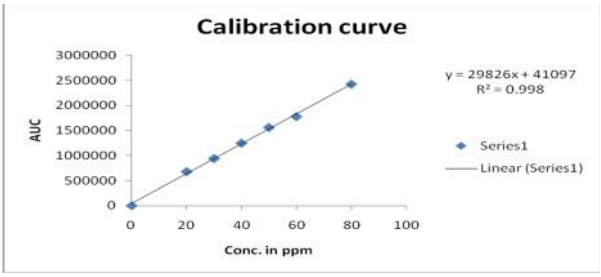


Fig 7 : Calibration curve of .Butoconazole (API).

3.2.4. Method Robustness:

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-4, % RSD < 2%) the developed RP-HPLC method for the analysis of Butoconazole (API).

Table 6: Result of method robustness test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.25
Flow (0.9 ml/min)	1.87
Temperature (27^{0}C)	1.42
Temperature (23^{0}C)	0.36
Wavelength of Detection (223 nm)	1.05
Wavelength of detection (229 nm)	1.04

3.2.5 LOD & LOQ:

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.01 & 0.03 µg/ml respectively.

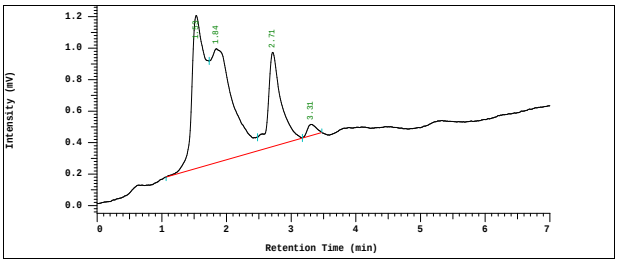


Fig 8: LOD

3.3 System Suitability Parameter

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table 7.

Table 7: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs > 2	9.15
2	Asymmetry	T ≤ 2	Butoconazole=0.12
3	Theoretical plate	N > 2000	Butoconazole=3246

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