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A new analytical RP-HPLC method development and validation for the estimation of Nilutamide in bulk form and marketed pharmaceutical dosage form

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Abstract

A rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validated of Nilutamide, in its pure form as well as in tablet dosage form. Chromatography was carried out on a Symmetry C18 (4.6 x 150 mm, 5 µm) column using a mixture of Methanol and water (45: 55 % v/v) as the mobile phase at a flow rate of 0.8 ml/min, the detection was carried out at 260 nm. The retention time of the Nilutamide was 2.379 ± 0.02 min respectively. The method produce linear responses in the concentration range of 24-120 mg/ml of Nilutamide. The method precision for the determination of assay was below 2.0 % RSD. The method is useful in the quality control of bulk and pharmaceutical formulations. The method was validated for accuracy, precision, linearity, robustness, ruggedness and LOD & LOQ of standard solution. The developed RP-HPLC method was found to be accurate, precise, linear, and robust and was successful applied to a pharmaceutical tablet formulation for qualitative estimation of Nilutamide in Bulk form and Marketed Pharmaceutical Dosage forms.

Keywords: Nilutamide; RP-HPLC; Method Development; Validation; Accuracy

1. Introduction

Nilutamide is an antineoplastic hormonal agent primarily used in the treatment of prostate cancer. Nilutamide is a pure, nonsteroidal anti-androgen with affinity for androgen receptors (but not for progestogen, estrogen, or glucocorticoid receptors). Consequently, Nilutamide blocks the action of androgens of adrenal and testicular origin that stimulate the growth of normal and malignant prostatic tissue. Prostate cancer is mostly androgen-dependent and can be treated with surgical or chemical castration. To date, antiandrogen monotherapy has not consistently been shown to be equivalent to castration. For use in combination with surgical castration for the treatment of metastatic prostate cancer involving distant lymph nodes, bone, or visceral organs (Stage D2). Nilutamide is an antineoplastic hormonal agent primarily used in the treatment of prostate cancer. Nilutamide is a pure, nonsteroidal anti-androgen with affinity for androgen receptors (but not for progestogen, estrogen, or glucocorticoid receptors). Consequently, Nilutamide blocks the action of androgens of adrenal and testicular origin that stimulate the growth of normal and malignant prostatic tissue (Scholars Research Library-Book, 2012). Prostate cancer is mostly androgen-dependent and can be treated with surgical or chemical castration. To date, antiandrogen monotherapy has not consistently been shown to be equivalent to castration. The relative binding affinity of Nilutamide at the androgen receptor is less than that of Bicalutamide, but similar to that of hydroxuflutamide. Nilutamide competes with androgen for the binding of androgen receptors, consequently blocking the action of androgens of adrenal and testicular origin that stimulate the growth of normal and malignant prostatic tissue. This blockade of androgen receptors may result in growth arrest or transient tumor regression through inhibition of androgen-dependent DNA and protein synthesis (FARMACIA, 2009). The IUPAC Name

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of Nilutamide is 5, 5-dimethyl-3-[4-nitro-3-(trifluoro methyl) phenyl] imidazolidine-2, 4-dione. The Chemical Structure of Nilutamide is shown in following fig-1.

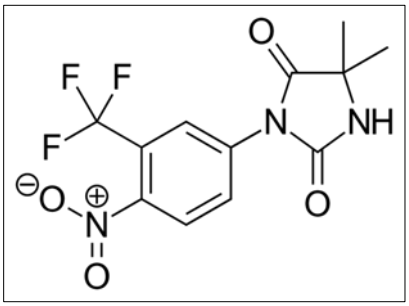

Figure 1 Chemical Structure of Nilutamide

In the literature survey (A. Ramesh Babu, et al, 2014, Husna Kanwal Qureshi, et al, 2023, Poojari Venkatesh, et al, 2022, Anjaneyulu Reddy, et al, 2019) , we found that there are several spectroscopic and liquid chromatographic procedures for the determination of Nilutamide by UPLC, HPLC, HPTLC, and UV and there is no UHPLC method reported. Hence, our proposal involved creating an efficient RP-HPLC technique for concurrently determining Nilutamide in both pure and marketed pharmaceutical formulations. This research is focused on developing and validating a rapid, sensitive RP-HPLC method that offers improved resolution and peak symmetry, adhering to ICH guidelines during validation (Journal of Pharmaceutical and Biomedical Analysis-Book, 1999).

2. Materials and methods

Table 1 Instruments used

S.No.	Instruments and Glasswares	Model
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, PDA 996 Detector.
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital Ultra Sonicator	Labman

2.1. Chemicals used

Table 2 Chemicals Used

S.No.	Chemical	Brand Names
1	Nilutamide (Pure)	Ziluta – 150 Tablet
2	Water and Methanol for HPLC	LICHROSOLV (MERCK)
3	Acetonitrile for HPLC	Merck

2.2. HPLC Method Development

2.2.1. Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Nilutamide working standard into a 10 ml of clean dry volumetric flasks add about 7 ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.72 ml of the above Nilutamide stock solutions into a 10 ml volumetric flask and dilute up to the mark with Methanol.

2.2.2. Procedure

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines (Tropical Journal of Pharmaceutical Research, Book, 2009).

2.2.3. Mobile Phase Optimization

Initially the mobile phase tried was methanol: Water and Acetonitrile: Water with varying proportions. Finally, the mobile phase was optimized to Methanol and Water in proportion 45: 55 v/v respectively.

2.2.4. Optimization of Column

The method was performed with various C18 columns like ODS column, Xterra, and X Bridge C18 column. Symmetry C18 (4.6 x 150 mm, 5 μm) was found to be ideal as it gave good peak shape and resolution at 1 ml/min flow.

2.2.5. Preparation of Mobile Phase

Accurately measured 450 ml (45 %) of HPLC Methanol and 550 ml of HPLC Water (55 %) were mixed and degassed in a digital ultrasonicator for 10 minutes and then filtered through 0.45 μ filter under vacuum filtration.

2.2.6. Diluent Preparation

The Mobile phase was used as the diluent.

2.3. Method Validation Parameters

2.3.1. System Suitability

Accurately weigh and transfer 10 mg of Nilutamide working standard into a 10 ml of clean dry volumetric flasks add about 7 mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Ravi Sankar, et al, 1997, 1999, 2001, 2010). (Stock solution)

Further pipette 0.72 ml of the above Nilutamide stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents.

Procedure

The standard solution was injected for five times and measured the area for all five injections in HPLC. The % RSD for the area of five replicate injections was found to be within the specified limits.

2.3.2. Specificity Study of Drug

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Nilutamide working standard into a 10 ml of clean dry volumetric flasks add about 7 ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.72 ml of the above Nilutamide stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluents (Snyder, et al, 2009).

Preparation of Sample Solution

Take average weight of the Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight of Nilutamide sample into a 10 mL clean dry volumetric flask and add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 0.72 ml of Nilutamide above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

Procedure

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

$$\%\text{ASSAY} = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

2.3.3. Linearity:

Accurately weigh and transfer 10 mg of Nilutamide working standard into a 10 ml of clean dry volumetric flasks add about 7 ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Guidance for Industry-Book, 2000). (Stock solution)

Preparation of Level – I (24 ppm of Nilutamide):

Pipette out 0.24 ml of stock solution in to a 10 ml volumetric flask and make up the volume up to mark by using diluent.

Preparation of Level – II (48 ppm of Nilutamide):

Pipette out 0.48 ml of stock solution in to a 10 ml volumetric flask and make up the volume up to mark by using diluent.

Preparation of Level – III (72 ppm of Nilutamide):

Pipette out 0.72 ml of stock solution in to a 10 ml volumetric flask and make up the volume up to mark by using diluent.

Preparation of Level – IV (96 ppm of Nilutamide):

Pipette out 0.96 ml of stock solution in to a 10 ml volumetric flask and make up the volume up to mark by using diluent.

Preparation of Level – V (120 ppm of Nilutamide):

Pipette out 1.2 ml of stock solution in to a 10 ml volumetric flask and make up the volume up to mark by using diluent.

Procedure

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

2.3.4. Precision

Repeatability

- Preparation of Nilutamide Product Solution for Precision:

Accurately weigh and transfer 10 mg of Nilutamide working standard into a 10 ml of clean dry volumetric flasks add about 7 ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Y. F. Cheng, et al, 2000). (Stock solution)

Further pipette 0.72 ml of the above Nilutamide stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluents.

The standard solution was injected for five times and measured the area for all five injections in HPLC. The % RSD for the area of five replicate injections was found to be within the specified limits.

Intermediate Precision:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions (USP, 2002).

Procedure:

- Day 1

The standard solution was injected for six times and measured the area for all six injections in HPLC. The % RSD for the area of six replicate injections was found to be within the specified limits.

- Day 2

The standard solution was injected for six times and measured the area for all six injections in HPLC. The % RSD for the area of six replicate injections was found to be within the specified limits.

2.3.5. Accuracy

For Preparation of 50 % Standard Stock Solution:

Accurately weigh and transfer 10 mg of Nilutamide working standard into a 10 ml of clean dry volumetric flasks add about 7 mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.36 ml of the above Nilutamide stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents (ICH Q2B, FDA, May-1997).

For Preparation of 100 % Standard Stock Solution:

Accurately weigh and transfer 10 mg of Nilutamide working standard into a 10 ml of clean dry volumetric flasks add about 7 mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.72 ml of the above Nilutamide stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents.

For Preparation of 150 % Standard Stock Solution:

Accurately weigh and transfer 10 mg of Nilutamide working standard into a 10 ml of clean dry volumetric flasks add about 7 mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1.08 ml of the above Nilutamide stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents.

Procedure

Inject the Three replicate injections of individual concentrations (50 %, 100 %, 150 %) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Nilutamide and calculate the individual recovery and mean recovery values.

2.3.6. Robustness

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results (ICH Q2B, FDA, 2003).

For Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Nilutamide working standard into a 10 ml of clean dry volumetric flasks add about 7 mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.72 ml of the above Nilutamide stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents.

Effect of Variation of Flow Conditions

The sample was analyzed at 0.7 ml/min and 0.9 ml/min instead of 0.8 ml/min, remaining conditions are same. 10 µl of the above sample was injected and chromatograms were recorded

Effect of Variation of Mobile Phase Organic Composition

The sample was analyzed by variation of mobile phase i.e. Methanol: Water was taken in the ratio and 40: 60, 50: 50 instead of 45: 55, remaining conditions are same.10 µl of the above sample was injected and chromatograms were recorded (M. V. Gorenstein, et al, 1994).

3. Results and discussion

3.1. Development of a New Analytical Method

3.1.1. Optimized Chromatographic Conditions:

- Mobile phase ratio : Methanol: water (45: 55 % v/v)
- Column : Symmetry ODS C18 (4.6 mm × 150 mm) 5 µm
- Column temperature : Ambient
- Wavelength : 260 nm
- Flow rate : 1.0 ml/min
- Injection volume : 20 µl
- Run time : 6 minutes

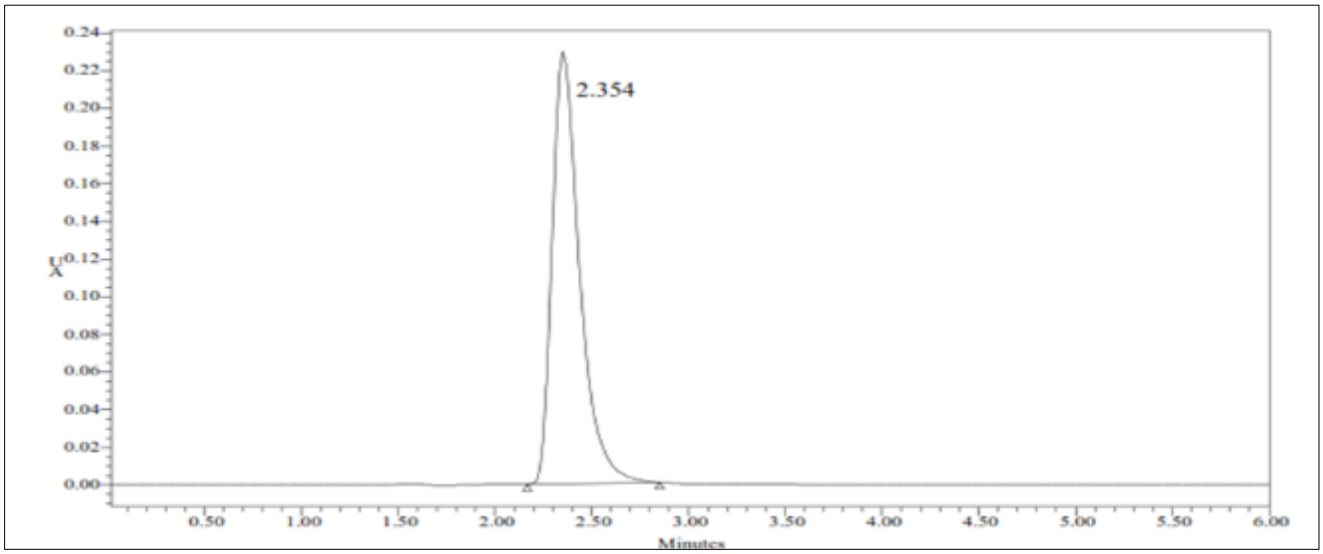

Figure 2 Optimized Chromatographic Condition

Observation: In this trail it shows well peak shape and proper plate count and tailing under limit in the chromatogram. So it's optimized chromatogram.

3.2. Analytical Method Validation

The developed method was validated with respect to system suitability, specificity, linearity, precision, accuracy LOD, LOQ and robustness in the accordance of the ICH Q2 guidelines (P. M. Young, et al, 1994).

3.2.1. System Suitability

System Suitability tests are an integral part of method development and were used to ensure adequate performance of the chromatographic system. Retention Time (RT), tailing factor, peak asymmetry, and theoretical plates (T) were evaluated (W. J. Warren, et al, 1995). The results are shown here in Table 3.

Table 3 Results of System Suitability for Nilutamide

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Nilutamide	2.317	2274631	239458	5728	1.2
2	Nilutamide	2.302	2284721	239582	5093	1.2
3	Nilutamide	2.323	2238127	236493	5391	1.2
4	Nilutamide	2.343	2259349	249482	6139	1.2
5	Nilutamide	2.321	2204850	239452	5281	1.2
Mean			2252336			
Std. Dev.			31827.08			
% RSD			1.41307			

3.2.2. Specificity

The developed method was found to be selective for Nilutamide, since the injection of the blank solution confirmed the absence of interfering peak at RT examined substance at 260 nm. The results obtained demonstrate that there was no interference from other material in the developed method and therefore confirm the specificity of the method (M. E. Swartz, et al, 2005).

$$\%\text{ASSAY} = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Nilutamide in pharmaceutical dosage form was found to be 99.765 %.

3.2.3. Linearity

The preparation of five distinct concentration calibration standards in five replicates allowed for the determination of the method's linearity. Graphs are plotted where the y-axis represents peak areas and the X-axis represents concentrations where the concentration ranges from 24-120 µg/mL for Nilutamide produced the calibration curve plot for Nilutamide (M. Swartz and B. Murphy, et al. 2005). It is desirable for the correlation coefficient to exceed 0.9989.

Table 4 Linearity Data of Nilutamide

Concentration µg/ml	Average Peak Area
24	791554
48	1647073
72	2283804
96	3058339
120	3839630

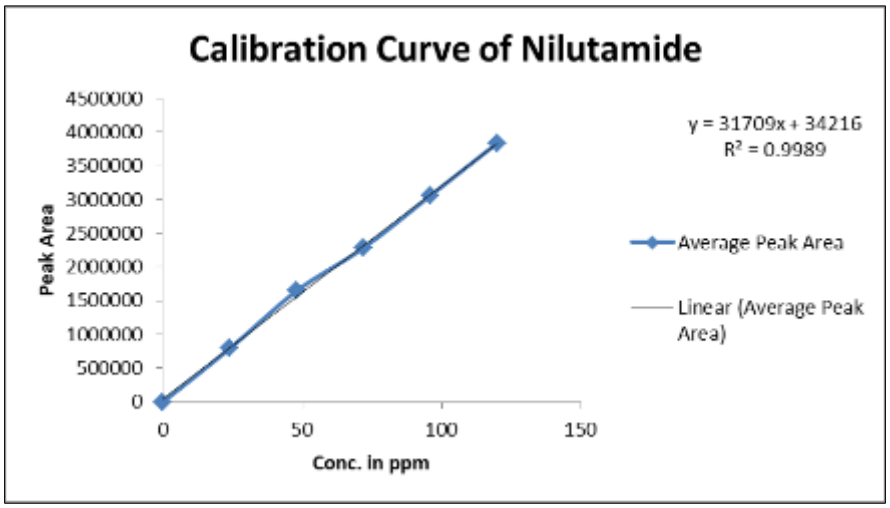

Figure 3 Calibration Curve of Nilutamide

3.2.4. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions (M. Swartz, et al. 2004).

- **Repeatability:** Obtained Six (6) replicates of 100 % accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table 5 Results of Repeatability for Nilutamide

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Nilutamide	2.356	2259464	245362	5938	1.2
2	Nilutamide	2.356	2275915	248293	5827	1.2
3	Nilutamide	2.357	2282117	240795	5032	1.2
4	Nilutamide	2.358	2278675	230139	5978	1.2
5	Nilutamide	2.359	2282448	249605	6183	1.2
6	Nilutamide	2.356	2288967	256831	6257	1.8
Mean			2277931			
Std. Dev			9177.573			
%RSD			0.402891			

Intermediate Precision

- Analyst1:

Table 6 Results of Intermediate Precision for Nilutamide

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USPPlate count	USPTailing
1	Nilutamide	2.380	2236184	202188	5472	1.2
2	Nilutamide	2.383	2238020	201837	6193	1.2
3	Nilutamide	2.385	2239352	201273	5980	1.2

4	Nilutamide	2.385	2242466	203923	7163	1.2
5	Nilutamide	2.389	2244692	202938	6182	1.2
6	Nilutamide	2.389	2247654	201982	7684	1.2
Mean			2241395			
Std. Dev.			4333.851			
% RSD			0.193355			

- Analyst 2

Table 7 Results of Intermediate Precision Analyst 2 for Nilutamide

S. No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USPPlate count	USPTailing
1	Nilutamide	2.380	2236184	217363	5928	1.2
2	Nilutamide	2.383	2238020	218467	6183	1.2
3	Nilutamide	2.385	2239352	218346	5927	1.2
4	Nilutamide	2.385	2242466	221736	5163	1.2
5	Nilutamide	2.389	2244692	228361	4827	1.2
6	Nilutamide	2.346	2263431	217553	5019	1.2
Mean			2244024			
Std. Dev.			9988.458			
% RSD			0.445114			

3.2.5. Accuracy

Recovery experiments involve the addition of measured quantity of pure standard drug to the sample solution and measuring its recovery by assessing the peak areas and were used to evaluate the method's accuracy (Biomedical Chromatography, 2008). Standard was added to the sample at concentrations of 50 %, 100 %, and 150 % of the test. A duplicate assay was performed on the resulting spiked sample. For every level, the recovery percentage should range from 98 % to 102 %.

Table 8 The Accuracy Results for Nilutamide

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50 %	1172485	36	35.8	99.4	99.5 %
100 %	2314753	72	71.6	99.4	
150 %	3480210	108	107.9	99.9	

3.2.6. Limit of Detection for Nilutamide

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

$$\mathbf{LOD = 3.3 \times \sigma / s}$$

Where
σ = Standard deviation of the response
S = Slope of the calibration curve

Result

=5.5 µg/ml

3.2.7. Quantitation Limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined (Journal of Chromatography, 2008).

$$\mathbf{LOQ = 10 \times \sigma/S}$$

Where
σ = Standard deviation of the response
S = Slope of the calibration curve

Result:

=16.7 µg/ml

3.2.8. Robustness

The robustness was performed for the flow rate variations from 0.7 ml/min to 0.9 ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Nilutamide (Kassen Mussen, et al. 2006). The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ± 5 %. The standard and samples of Nilutamide were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Table 9 Results for Robustness

Parameter Used for Sample Analysis	Peak Area	Retention Time	Theoretical Plates	Tailing Factor
Actual Flow rate of 0.8 mL/min	3119086	2.379	5837	1.2
Less Flow rate of 0.7 mL/min	2640811	2.763	5361	1.2
More Flow rate of 0.9 mL/min	2640354	2.234	5231	1.2
Less organic phase	2640758	2.765	4503	1.5
More organic phase	2640125	2.236	4491	1.5

3.3. Stability Studies

Results of Degradation Studies: The results of the strain studies indicated the specificity of the tactic that has been developed. Nilutamide was stable in all stress conditions except thermal stress condition (Matheson A.J, et al. 2000). The results of forced degradation studies are given in the following table-10.

Table 10 Results of Forced Degradation Studies of Nilutamide API

Stress Condition	Time in hrs	Assay of Active Substance	Assay of Degraded Products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24 Hrs.	92.985	7.015	100.0
Basic Hydrolysis (0.1 M NaOH)	24 Hrs.	91.062	8.938	100.0
Wet heat	24 Hrs.	89.749	10.251	100.0
UV (254 nm)	24 Hrs.	95.625	4.375	100.0
3 % Hydrogen peroxide	24 Hrs.	96.548	3.452	100.0

4. Conclusion

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 260 nm and the peak purity was excellent. Injection volume was selected to be 10 µl which gave a good peak area. The column used for study was Symmetry C_{18} because it was giving good peak. 40 º C temperatures was found to be suitable for the nature of drug solution. The flow rate was fixed at 0.8 ml/min because of good peak area and satisfactory retention time. Mobile phase is Methanol: water was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study. Methanol: water was selected because of maximum extraction sonication time was fixed to be 10 min at which all the drug particles were completely soluble and showed good recovery. Run time was selected to be 6 min because analyze gave peak around 2.3 and also to reduce the total run time. The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range. The analytical method was found linearity over the range of 24-120 ppm of the Nilutamide target concentration. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Nilutamide in bulk drug and pharmaceutical dosage forms. This method was simple, since diluted samples are directly used without any preliminary chemical derivatization or purification steps. The % RSD values were within 2 and the method was found to be precise. The results expressed in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods. This method can be used for the routine determination of Nilutamide in bulk drug and in Pharmaceutical dosage forms.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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