RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF NILUTAMIDE IN BULK FORM AND MARKETED PHARMACEUTICAL DOSAGE FORM

Husna Kanwal Qureshi, Ashfeen Begum, Syeda Ayesha Fatima, Mohammed Ibaduddin, Zainab Khatoon*

Deccan School of Pharmacy, Goshamahal, Aghapura, Goshamahal Rd, near Nampally, Hyderabad, Telangana 500001

ABSTRACT: ***The present work includes a simple, economic, rapid, accurate and precise isocratic RP-HPLC method development for estimation of Nilutamide in bulk form and its marketed formulation. Estimation was done at 286nm which was found to be λmax of Nilutamide. The simple, selective, isocratic RP-HPLC method for Nilutamide was developed on Phenomenex Luna (C_{18}) RP Column; 250 mm x 4.6 mm, 5µm with a mobile phase of Phosphate Buffer (pH-4.6) and Methanol were taken in the ratio of 65:35% v/v at a flow rate of 1.0 ml/min and detection wavelength 286nm. The developed method was validated successfully according to ICH Q2 (R1) guidelines. The chromatographic methods showed a good linear response with r2 values of 0.9995. The percentage relative standard deviation for method was found to be less than two, indicating that the methods were precise. The mean percentage recovery was for RP-HPLC method was 100.437%. From the results it could be concluded that both the developed method was specific, selective and robust. The method could be successfully applied for analysis of Bulk form and Marketed formulation of Nilutamide.***

Key Words: Nilutamide, RP-HPLC, Method Development, Validation, ICH Guidelines.

I. INTRODUCTION

Nilutamide is an imidazolidinone, a member of (trifluoromethyl) benzenes, and a C-nitro compound. It has a role as an antineoplastic agent and an androgen antagonist. Nilutamide[1] is an antineoplastic hormonal agent primarily used in the treatment of prostate cancer. Nilutamide is a pure, nonsteroidal anti-androgen with an 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. Nilutamide[2] is an Androgen Receptor Inhibitor. The mechanism of action of Nilutamide is as an Androgen Receptor Antagonist. 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. 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[3] at the androgen receptor is less than that of bicalutamide, but similar to that of hydroxuflutamide. The IUPAC Name of Nilutamide is 5, 5-dimethyl-3-[4-nitro-3-(trifluoromethyl) phenyl] imidazolidine-2, 4-dione. The Chemical Structure of Nilutamide is as follows

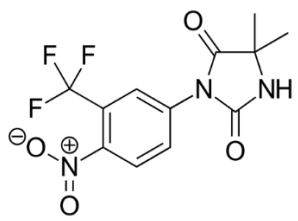

Fig.1. Chemical Structure of Nilutamide

The review of the literature[32-35] regarding the quantitative analysis of Nilutamide revealed that the attempts were

made to develop analytical methods for estimation of Nilutamide by spectrometric methods and LC methods the estimation individually and simultaneously. The chemical structure of Nilutamide is shown in (Fig-1).
UV spectrophotometric methods and high-performance liquid chromatography (HPLC) methods for estimation of Nilutamide various dosage forms are also reported.
The objective of this analytical method development was to develop, optimize, and validate a rapid, specific, and economic and simple reversed phase-HPLC (RP-HPLC) method for the estimation of Nilutamide in bulk and pharmaceutical dosage form on isocratic mode offering better separation of peak of interest as well as impurities so that it is useful even for studying degradation impurities when compared to isocratic mode analytical methods.

II. MATERIALS AND METHODS

Table-1: List of Instrument Used

S. No.	Instruments/Equipments/Apparatus
1.	**HPLC** with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2.	**ELICO SL-159** UV – Vis spectrophotometer
3.	Electronic Balance **(SHIMADZU ATY224)**
4.	Ultra Sonicator **(Wensar wuc-2L)**
5.	Thermal Oven
6.	Symmetry ODS RP C_{18},5µm, 15mm x 4.6mm i.d.
7.	P^H Analyzer **(ELICO)**
8.	Vacuum filtration kit **(BOROSIL)**

Table-2: List of Chemicals Used

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	HPLC Grade Water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
3.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
4.	Hydrochloric Acid	99.9	A.R.	Sd fine-Chem ltd; Mumbai
5.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
6.	Sodium Hydroxide	99.9	A.R.	Sd fine-Chem ltd; Mumbai
7.	Ethanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai
8.	Octanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai

Selection of Wavelength:

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[4] in the range of 200 to 400nm. This has been performed to know the maxima of Nilutamide, so that the same wave number can be utilized in HPLC UV detector for estimating the Nilutamide. The scanned UV spectrum is attached in the following page,

Sample & Standard Preparation for the UV-Spectrophotometer Analysis:

25 mg of Nilutamide standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 1ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

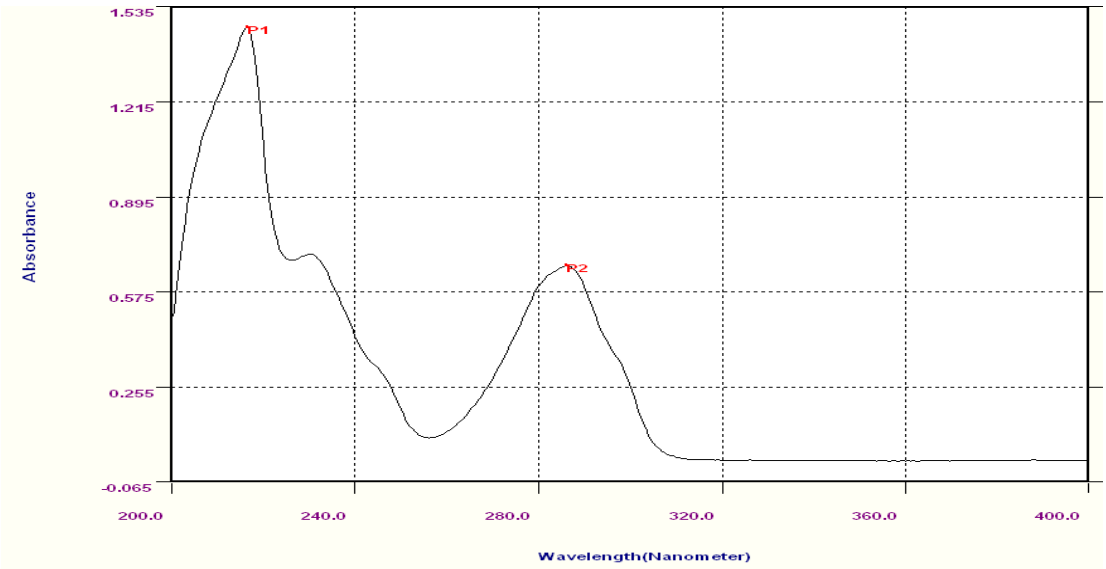

Fig.2. UV Spectrum for Nilutamide

Observation: While scanning the Nilutamide solution we observed the maxima at 286nm. The UV spectrum has been recorded on ELICO SL-159 make UV – Vis spectrophotometer model UV-2450.

Preparation of Mobile Phase:

600ml of prepared Methanol and 400ml of HPLC Grade Acetonitrile were mixed well and degassed in ultrasonic water bath[5] for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration.

III. RESULTS AND DISCUSSION

Method Development:

Summary of Optimized Chromatographic Conditions: The Optimum Chromatographic conditions[6] obtained from experiments can be summarized as below:

Table-3: Summary of Optimized Chromatographic Conditions

Mobile phase	Methanol: Acetonitrile (60:40) v/v
Column	Symmetry C_{18} (4.6mm×250mm) 5µm
Column Temperature	Ambient
Detection Wavelength	236 nm
Flow rate	1.0 ml/ min.
Run time	10 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	20µl

Type of Elution	Isocratic
Retention time	4.778 minutes

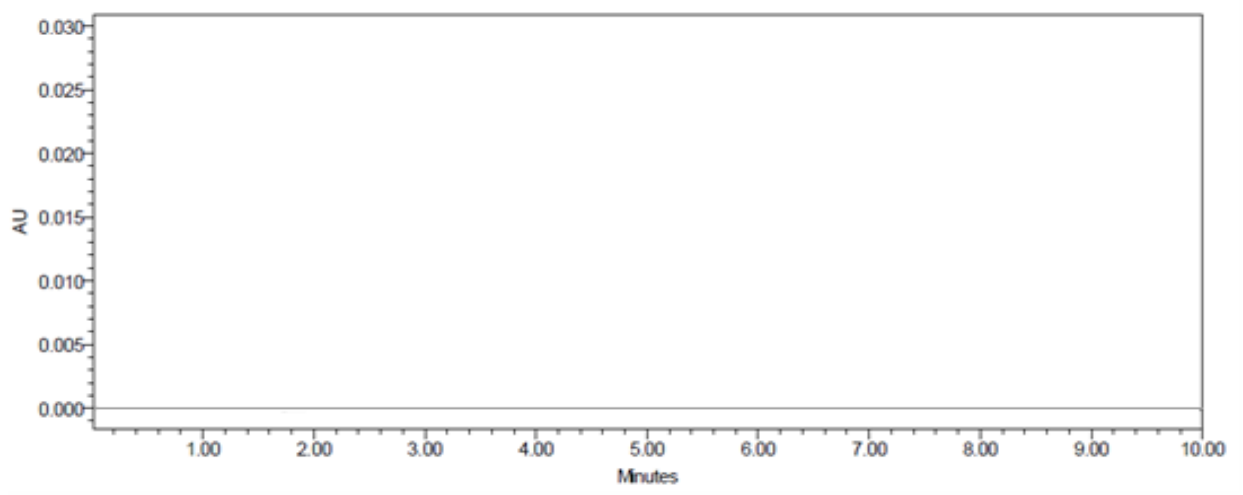

Fig.3. Chromatogram for Blank Solution

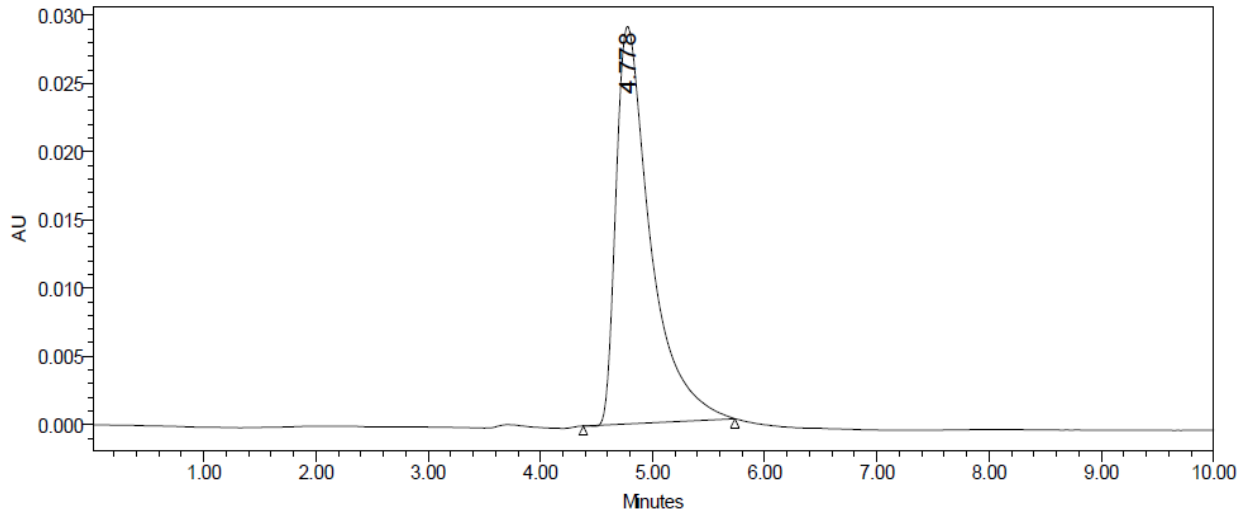

Fig.4.Chromatogram of Nilutamide in Optimized Condition

Observation: The selected and optimized mobile phase was Methanol: Acetonitrile in the ratio of 60:40% v/v and conditions optimized were flow rate (1.0 ml/minute), wavelength (236nm), Run time was 10 mins. Here the peaks were separated and showed better resolution, theoretical plate count and symmetry[7]. The proposed chromatographic conditions were found appropriate for the quantitative determination of the drug.

Method Validation

1. System Suitability Test

System suitability testing[8-10] 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 analysed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table-4 & 5.

Table-4: Data of System Suitability Test

S.No.	Injection No.	RT	Area	USP Plate Count	USP Tailing
1	Injection 1	4.817	745236	6986	1.39
2	Injection 2	4.783	743652	6857	1.37
3	Injection 3	4.840	742587	6856	1.36
4	Injection 4	4.783	742946	6847	1.39
5	Injection 5	4.817	743654	6896	1.38
6	Injection 6	4.778	741698	6874	1.37
Mean			**743295.5**	**6886**	**1.37666**
S.D			**1199.773604**		
%RSD			**0.161412736**		

Table-5: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Retention Time	RT > 2	Nilutamide= 4.778
2	Asymmetry	T ≤ 2	Nilutamide= 1.35
3	Theoretical plate	N > 2000	Nilutamide= 6859
4	Tailing Factor	T<2	Nilutamide= 1.37

2. Linearity:

To evaluate the linearity[11], serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 60-140µg/ml. The prepared solutions were sonicated. From these solutions, 10µl injections of each concentration were injected into the HPLC system[12] and chromatographed under the optimized conditions. Calibration curve[13] was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

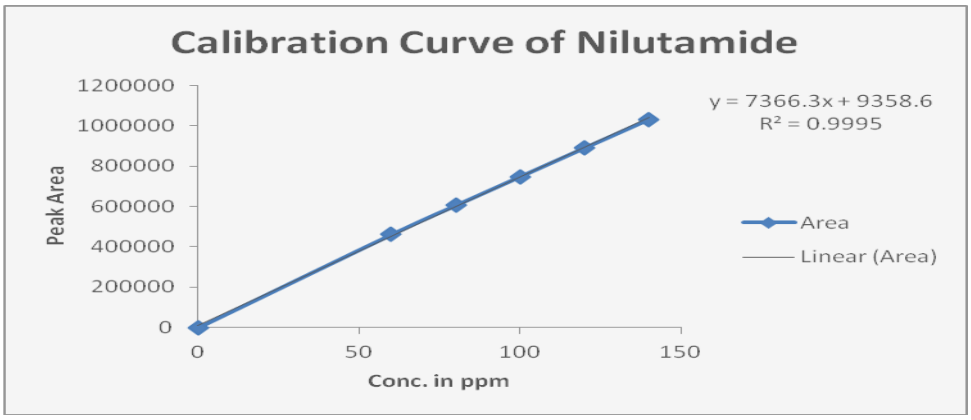

Fig.5. Calibration Curve of Nilutamide

Table-6: Linearity Data for Nilutamide

Conc. (µg/ml)	Area
0	0
60	461404
80	606157
100	748506
120	891041
140	1032196

3. Accuracy: The accuracy[14] of the method was determined by recovery studies and the percentage recovery[15] was calculated. The recoveries of Nilutamide were found to be in the range of 99-102%. The proposed Liquid Chromatographic method was applied to the determination of Nilutamide. The results for Nilutamide comparable with the corresponding labeled amounts.

Table-7: Shown Accuracy Observation of Nilutamide

Accuracy	Amount Added	Amount Recovered	Peak Area	% Recovery	Mean Recovery
80%	80	80.798	604517	100.997	100.437%
	80	80.673	603598	100.841	
	80	80.756	604213	100.945	
100%	100	99.933	745471	99.933	
	100	100.083	746574	100.083	
	100	100.365	748652	100.365	
120%	120	120.290	895415	100.241	
	120	120.201	894762	100.167	
	120	120.442	896541	100.368	

4. Precision:

Repeatability: The precision[16] of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Nilutamide (API). The percent relative standard deviation[17] was calculated for Nilutamide are presented in the table-8.

Table-8: Repeatability Data for Nilutamide

S. No.	INJECTION	PEAK AREA
1	Injection 1	743826
2	Injection 2	745277
3	Injection 3	742506
4	Injection 4	747576
5	Injection 5	746715
6	Injection 6	741278
7	**Average**	**744529.6667**
8	**SD**	**2440.4116**
9	**% RSD**	**0.32777**

Intermediate Precision:

The Intermediate Precision[18-20] consists of two methods:-

Intra Day: In Intra Day process, the 80%, 100% and 120% concentration are injected at different intervals of time in same day.

Inter Day: In Inter Day process, the 80%, 100% and 120% concentration are injected at same intervals of time in different days.

Table-9: Results of Intra-Assay & Inter-Assay

Conc. of Nilutamide (API) (µg/ml)	Observed Conc. of Nilutamide (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
80	80.096	0.487	79.685	0.688
100	100.074	0.968	100.057	0.789
120	120.056	0.847	120.016	0.698

Observations: The intra & inter day variation of the method was carried out for standard deviation & % RSD (% RSD < 2%) within a day & day to day variations[21] for Nilutamide revealed that the proposed method is precise.

5. Specificity:

Specificity[22] can be determined by comparing the chromatograms obtained from the drugs with the chromatogram obtained from the blank solution. Blank solution was prepared by mixing the excipients in the mobile phase without drug. Drug solutions were prepared individually and the sample containing one drug was also prepared. Now these mixtures were filtered by passing through 0.45 µ membrane filter before the analysis. In this observation no excipient peaks were obtained near the drug in the study run time[23]. This indicates that the proposed method was specific.

The chromatograms representing the peaks of blank, Nilutamide and the sample containing the one drug was shown in following figures respectively.

Observation: In this test method blank, standard solutions were analyzed individually to examine the interference. The above chromatograms show that the active ingredient was well separated from blank and their excipients and there was no interference of blank with the principal peak. Hence the method is specific.

6. Limit of Detection (LOD) and Limit of Quantification (LOQ):

The LOD[24] and LOQ parameter was evaluated by mistreatment the slope of line and variance obtained from accuracy studies.

The detection limit (LOD) and quantization limit (LOQ) may be expressed as:

$$\textbf{L.O.D. = 3.3(SD/S).}$$

$$\textbf{L.O.Q. = 10(SD/S)}$$

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified[25] (LOQ) were found to be 1.469 & 4.454µg/ml respectively.

7. Method Robustness: Influence of small changes in chromatographic conditions[26-28] such as change in flow rate 1.0 ml (± 0.1ml/min), Wavelength of detection 236 (±2nm) & organic phase content in mobile phase (±5%) studied to determine the robustness of the method are also in favour of (Table-10, % RSD < 2%) the developed RP-HPLC method for the analysis of Nilutamide (API).

Effect of Variation of Flow Conditions:

The sample was analyzed at 0.9ml/min and 1.1ml/min instead of 1ml/min, remaining conditions are same. 10µl 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: Phosphate Buffer was taken in the ratio and 40:60, 30:70 instead of 35:65, remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded.

Table-10: Results for Robustness

Parameter Used for Sample Analysis	Peak Area	Retention Time	Theoretical Plates	Tailing Factor
Actual Flow rate of 1.0 mL/min	742946	4.778	1.37	2896
Less Flow rate of 0.9 mL/min	698965	4.783	1.39	2986
More Flow rate of 1.1 mL/min	786598	4.817	1.42	2985
Less organic phase	732642	4.842	1.29	3102
More organic phase	702546	4.773	1.37	3247

8. Estimation of Nilutamide in Pharmaceutical Dosage Form

Label Claim: 150mg

Each tablet contains: 150mg

Twenty Tablets were taken and the I.P. method was followed to determine the average weight[29]. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 25 mg of drugs were transferred to 25 ml volumetric flask, make and solution was sonicated for 15 minutes, there after volume was made up to 25 ml with same solvent. Then 10 ml of the above solution was diluted to 100 ml with mobile phase. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. The solution prepared was injected in five replicates into the HPLC system and the observations were recorded. The data[30-31] is shown in Table-11.

ASSAY:

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg Wt.} = \text{mg}$$

Where:

AT = Peak Area of drug obtained with test preparation

AS = Peak Area of drug obtained with standard preparation

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard

Table-11: Recovery Data for estimation Nilutamide in Ziluta – 150 Tablet

Brand name of Nilutamide	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Ziluta – 150 Tablets (Zee Laboratories Ltd.)	150mg	149.695 (± 0.389)	99.749 (± 0.698)

Result & Discussion: The amount of drug in Ziluta – 150 Tablet was found to be 149.695 (± 0.4389) mg/tab for Nilutamide & % Purity was 99.749%.

IV. SUMMARY

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Nilutamide, different chromatographic conditions were applied & the results observed are presented in previous chapters.

Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So,

it was preferred for the current study over gradient elution.

In case of RP-HPLC various columns are available, but here Phenomenex Luna (C18) RP Column, 250 mm x 4.6 mm, 5µm Column was preferred because using this column peak shape, resolution and absorbance were good.

Mobile phase & diluent for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, Acetonitrile, water, 0.1N NaOH, 0.1NHCl).

Nilutamide was found to be soluble in organic solvents such as ethanol, DMSO, and dimethyl formamide (DMF), sparingly soluble in aqueous buffers, almost insoluble in water, slightly soluble in methanol, freely soluble in acetone, soluble in ethyl acetate, sparingly soluble in water. Using these solvents with appropriate composition newer methods can be developed and validated.

Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Nilutamide it is evident that most of the HPLC work can be accomplished in the wavelength range of 286 nm conveniently. Further, a flow rate of 1.0 ml/min & an injection volume of 20µl were found to be the best analysis.

The result shows the developed method is yet another suitable method for assay and stability related impurity studies which can help in the analysis of Nilutamide in different formulations.

V. CONCLUSION

A sensitive & selective stability indicting RP-HPLC method has been developed & validated for the analysis of Nilutamide in bulk and pharmaceutical dosage form.

Based on peak purity results, obtained from the analysis of samples using described method, it can be concluded that the absence of co-eluting peak along with the main peak of Nilutamide indicated that the developed method is specific for the simultaneous estimation of Nilutamide in the bulk and pharmaceutical dosage forms.

Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility. The specific Retention time for Nilutamide are found to be 4.778min. The tailing factor was found to be 1.37 with theoretical plates 6859 for Nilutamide. The %Recoveries was determined as 100.437% for Nilutamide in Accuracy. The %RSD in Repeatability is 0.327 with Intermediate Precision (Intra & Inter Day) are 0.767 & 0.725 for Nilutamide in Precision respectively. In Linearity, the correlation coefficient was found to be 0.9995 for Nilutamide. The LOD for Nilutamide was 1.469 and LOQ for Nilutamide are 4.454 respectively.

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