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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING ASSAY DEVELOPMENT FOR ENZALUTAMIDE IN STANDARD AND DOSAGE FORM BY RP-HPLC METHOD

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ABSTRACT

A simple, rapid, accurate and economic stability indicating method has been developed for estimation of Enzalutamide from bulk and pharmaceutical formulation. In RP-HPLC method, chromatographic separation was achieved on Phenomenax, C18 (150mm×4.6mm×5m) column using Acetonitrile: Water (65:35 v/v) as the mobile phase with detection at 236 nm. The drug was subjected to acidic, alkali, oxidative, thermal and photolytic stress conditions whereas Capsule was subjected to thermal and photolytic stress conditions. The drug follows linearity in the concentration range 10-50 µg/mL with correlation coefficient value 0.9995. The proposed method was applied to pharmaceutical formulation and % amount of drug estimated 101.09% was found in good agreement with the label claim. The accuracy of the method was checked by recovery experiment performed at three different levels i.e., 80%, 100% and 120%. The % recovery was found to be in the range 99.45-100.4%. The low values of %R.S.D. are indicative of the accuracy and reproducibility of the method. The precision of the method was studied as an intra-day, inter-day variations and repeatability. The %R.S.D. value less than 2 indicates that the method is precise. Ruggedness of the proposed method was studied with the help of two analysts. The above method was a rapid and cost-effective quality-control tool for routine analysis of Enzalutamide in bulk and in pharmaceutical dosage form.

Keywords: Enzalutamide; Validation; Stability indicating, RP-HPLC, Quantitative determination, Methanol

INTRODUCTION

Enzalutamide chemically is 4-{3-[4-cyano-3-(trifluoromethyl)phenyl]-5,5-dimethyl-4-oxo-2-sulfanylideneimidazolidin-1-yl}-2-fluoro-N-methylbenzamide [5-6}prostate cancer is the typical cause of cancer-related death; Although most patients initially respond to androgen-deprivation therapy, prostate cancer eventually progresses to castration-resistant prostate cancer (CRPC). Effective treatment options for Metastatic castration-resistant prostate cancer are lacking, and the median survival for men with mCRPC is <2 years **[1]**. The androgen receptor (AR) is central to the biology of metastatic castration resistant prostate cancer. The mechanisms that explain resistance to traditional androgen deprivation therapy include AR amplification or point mutations, ligand-independent activation of the AR, and alternative signaling pathways that no longer involve the AR. Androgen antagonists, including Enzalutamide, have been shown to exhibit agonist activity when AR is overexpressed **[2]**.

ENZ is a second generation androgen receptor inhibitor. It competitively inhibits androgen binding to androgen receptors and inhibits androgen receptor nuclear translocation and interaction with DNA. Enzalutamide is androgen receptor inhibitor with clinically significant clinical activity in patients with metastatic castration-resistant prostate cancer following docetaxel based chemotherapy **[3, 4] (Figure 1)**.

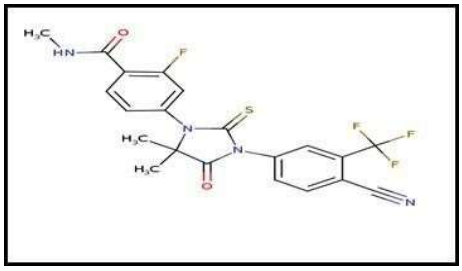

Figure 1: Structural formula of ENZ

Literature survey revealed that Enzalutamide is not official in any pharmacopoeia but determined by various methods including Simple UV spectrophotometry and LC-MS- MS. Literature survey reveals that only LC-MS methods were reported for the determination of enzalutamide in Animal plasma & Brain homogenates and in human plasma **[5, 6, 7]**. This study presents Stability Indicating RP-HPLC method for the determination of Enzalutamide in bulk and Pharmaceutical formulations. Accordingly, the objective of this study was to develop and validate Stability Indicating RP-HPLC method for

the estimation of Enzalutamide in bulk and pharmaceutical formulation as per ICH guidelines **[8]**.

Chemicals, Reagents and Solutions:

- Enzalutamide, Capsule formulation (Xtadi), Label claim: Enzalutamide - 40 mg,
- AR grade methanol (Finar Chemical Ltd., Ahmedabad, India), Acetonitrile, Water (HPLC grade-FINAR),
- Analytical grade Hydrochloric acid (HCl),
- Analytical grade sodium hydroxide (NaOH), and hydrogen peroxide (H_2O_2) were purchased from S D FineChem. Ltd. Mumbai, India.
- A 50mM phosphate buffer was prepared by accurately weighed 3.4 g of potassium dihydrogen phosphate dilute upto 500ml with HPLC grade water and adjusts the pH 6.5 with 0.1N NaOH and filtered through 0.45µ pore size vaccum filteration.
- Electronic analytical balance – Acculab ALC 210.4,
- Ultra sonicator (EN 30 US, Enertech Fast Clean, Mumbai, India,) HPLC LC2010CHT (Shimadzu) (LC_2010 CHT) [software LC Solution],
- Hot air oven (TO- 90S, Thermolab),
- Stability chamber(TH-90S, Thermolab, Mumbai, India),
- pH meter (Thermo Electron Crop., Pune, India).

Instrumentation and Chromatographic Conditions:

HPLC-UV

- Analytical Chromatography was performed on Shimadzu (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with HPLC LC2010CHT (Shimadzu) (LC_2010 CHT) [software LC Solution, Equipped with U.V. detector]
- Samples were injected through an Auto-sampler with fixed loop at 10 µl.
- Chromatographic separation of EVZA was achieved at ambient temperature using Phenomenex C18 (150×4.6 mm, 5 µm particle size) with mobile phase composition of Acetonitrile: Water (65:35 v/v).
- The flow rate was at 1.0 ml min-1 at ambient column temperature and effluent was detected at 236 nm. Before use, the mobile phase was filtered through a 0.2 µ nylon membrane filter and sonicated by Sonicator (EN 30 US, Enertech

Fast Clean, Mumbai, India) for 5 min.

Preparation of Standard Stock Solution of Enzalutamide (1000 µg/mL):

Accurately weighed quantity of 50 mg of ENZ was transferred into 50 mL volumetric flask, dissolved and diluted up to mark with HPLC Grade Methanol. This will give a stock solution having strength of 1000 µg/mL of Enzalutamide.

Preparation of Working Standard Solution Containing Enzalutamide (30 µg/mL):

From above standard stock solution (1000 µg/mL of Enzalutamide) 0.3 mL was taken into 10 mL volumetric flask to get 30 µg/mL of Enzalutamide.

Test Sample Preparation:

Twenty Xtadi Cap were accurately weighed, calculate average weight. An amount of Capsule equivalent to 40 mg ENZ was weighed and transferred into a 50 mL volumetric flask and 30 mL of methanol was added into it. The contents of the flask were sonicated for 15 min to dissolve the active ingredients completely. The solution was then diluted to 40 mL with methanol and solution was filtered through whatman filter paper .Take 0.3 mL aliquot from the filtered solution was transferred into a 10 mL volumetric flask to get concentration 30 µg/mL ENZ was then used as working sample solution.

Forced Degradation Study

In order to prove the selectivity of analytical method, the API of ENZ was studied under various stressed conditions to perform forced degradation study. Stress study was carried out under the condition of acid hydrolysis, base hydrolysis, oxidation, thermal and photolytic condition, as mentioned in ICH Q1A (R2).

Preparation of Samples for Force degradation study:

Acid Hydrolysis: Forced degradation in acidic media was performed by taking 3 mL stock solution of ENZ was transferred to 10 mL volumetric flask. Add 3 mL of 1 N HCl in volumetric flask and kept at 90ºC for 2 hr. Then neutralized it with 1 N NaOH and diluted up to the mark with mobile phase. Solution has strength of 30 µg/mL of ENZ.

Alkali Hydrolysis: Forced degradation in Alkali media was performed by taking 3 mL stock solution of ENZ was transferred to 10 mL volumetric flask. Add 3 mL of 1 N NaOH in volumetric flask and kept at 90ºC for 1 hr. Then neutralized it with 1 N HCl and diluted up to the mark with mobile phase. Solution has strength of 30 µg/mL of ENZ.

Oxidative degradation: Forced degradation was performed by taking 3 mL stock solution of ENZ, was transferred to 10 mL volumetric flask. Add 3 mL of 12% H2O2 in volumetric flask and kept at

room temperature for 4 hrs. and diluted up to the mark with mobile phase. Solution has strength of 30 µg/mL of ENZ.

Thermal degradation: Individually 30 mg accurately weighed amount of ENZ Powder and Formulation were exposed to 60º C for 6 days. After this exposure, the drug powder was transferred in to 10 mL volumetric flask, dissolved in methanol and diluted up to mark with diluent. Final dilution was done with sample diluent to make final concentration of 30 µg/mL ENZ.

Photolytic degradation: Individually 30 mg accurately weighed amount of ENZ Powder and Formulation were exposed to 236 nm for 6 days. After this exposure, the drug powder was transferred in to 10 mL volumetric flask, dissolved in methanol and diluted up to mark with diluent. Final dilution was done with sample diluent to make final concentration of 500 µg/mL ENZ.

Table 1: Force Degradation conditions for ENZ

Sr. No.	Stress Type	Stress Condition
1	Acid hydrolysis	1 N HCl at 90°C for2 hrs
2	Alkali hydrolysis	1 N NaOH at 90°C for 1 hrs
3	Oxidation	12% H2O2 at R.T. for 4 hrs
4	Thermal Degradation	At 60°C for 6 days
5	Photolytic	UV 236 nm for 6days

METHOD VALIDATION:

System suitability test parameters

System suitability tests are used to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters used in this test were the chromatographic peak resolution, theoretical plate number and tailing factor. The repeatability of these parameters was checked by injecting six solutions of Enzalutamide **(Table 2)**.

Table 2: Value of System suitability Parameter

Sr. No.	Parameters	ENZ ± S.D, (n=6)
1	Retention Time	5.480 ± 0.011
2	Theoretical Plates	2941.136 ± 12.04
3	Tailing Factor	1.022 ± 0.007
4	Resolution	3.608 ± 0.018
5	Capacity Factor	3.737 ± 0.016

Linearity and Range

Aliquots working std. solution (1, 2, 3, 4 and 5mL) were transferred into series of 10 mL volumetric flask and diluted up to mark with Methanol. This yielded solution of 10, 20, 30, 40 and 50 µg/mL ENZ. An aliquot of 10 µL of each solution was injected under operating chromatographic condition. Plot the calibration curve of Area of the ENZ peak versus respective concentration of ENZ. Find out correlation coefficient and regression line equation for ENZ. Each response was an average of three determinations.

Precision

a) Repeatability (Intra-day Precision)

Intra-day precision was determined by analyzing of ENZ standard solutions in the range 20, 30, and 40 µg/mL in triplicate on thesame day. Calculate % RSD for ENZ.

b) Inter-day Precision

Inter-day precision was determined by analyzing of ENZ standard solutions in the range 20, 30, and 40 µg/mL in triplicate on three consecutive days. Calculate % RSD for ENZ.

Accuracy

Accuracy was determined by calculating recovery of ENZ by the standard addition method. Known amounts of standard solutions of ENZ 24, 30 and 36 µg/mL were added to a prequantified test solutions of ENZ (30 µg/mL). Each solution was injected in triplicate, and the recovery was calculated by measuring peak areas and fitting these values into the regression equation of the calibration curve by peak areas.

Limit of Detection and Limit of Quantitation

LOD and LOQ of the drug were calculated using following equations according to ICH guideline.

$$\boldsymbol{LOD} = \mathbf{3.3} \times \sigma/\mathbf{S}$$

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

Where σ is the SD of the response

S is the slope of the calibration curve.

Robustness

The robustness study was performed to evaluate the influence of small but deliberate variation in the chromatographic condition. The robustness was checked by changing three small changes.

1) Mobile phase flow rate (± 0.1 mL/min)
2) pH (± 0.2 units)
3) Column temperature (40 ± 2 ºC)

After each changes sample solution was injected and system suitability parameters were checked.

ANALYSIS OF MARKET FORMULATION

Twenty Xtadi Cap were accurately weighed, calculate average weight. An amount of Capsule equivalent to 40 mg ENZ was weighed and transferred into a 50 mL volumetric flask and 30 mL of methanol was added into it. The contents of the flask were sonicated for 15 min to dissolve the active ingredients completely. The solution was then diluted to 40 mL with methanol and solution was filtered through whatman filter paper. Take 3 mL aliquot from the filtered solution was transferred into a 10 mL volumetric flask to get concentration 30 µg/mL ENZ. From this solution, 10 µL was injected into HPLC column and same procedure was followed as described in linearity.

RESULT AND DISCUSSION

Method Development and Optimization

A detection wavelength 236 nm was selected from the full range UV spectral data due to its high sensitivity for all degradation products and minimal difference in response factors. Isocratic run

was accessed using mobile phase. ACN: MeOH (65:35 v/v) on C18 columns.

Forced Degradation Study

The results from the stress testing studies indicate the method is highly specific for ENZA. Degradation products were completely distinguishable from the parent compound. The drug undergoes significant degradation under acid, alkaline, oxidative and photolytic condition. Acidic degradation was faster than basic, oxidative and photolytic degradation. Enzalutamide was significantly degraded in acidic and Oxidative and marginally in alkali and photolytic and stable in thermal conditions. The typical chromatograms from assay of stressed samples are shown in **Figure 3**. In acidic hydrolysis degradants peaks were obtained at RT of 1.54, 1.81 min. In basic hydrolysis, oxidative and photolytic additional degradants peaks were obtained at RT of 1.49, and 1.13, 1.27 and 0.76 min apart from drug peak. Overall ENZA degradants peaks were observed at RT of 1.54, 1.81, 1.49, 1.13, 1.27, 0.76. Results of forced degradation study are summarized in **Table 3**.

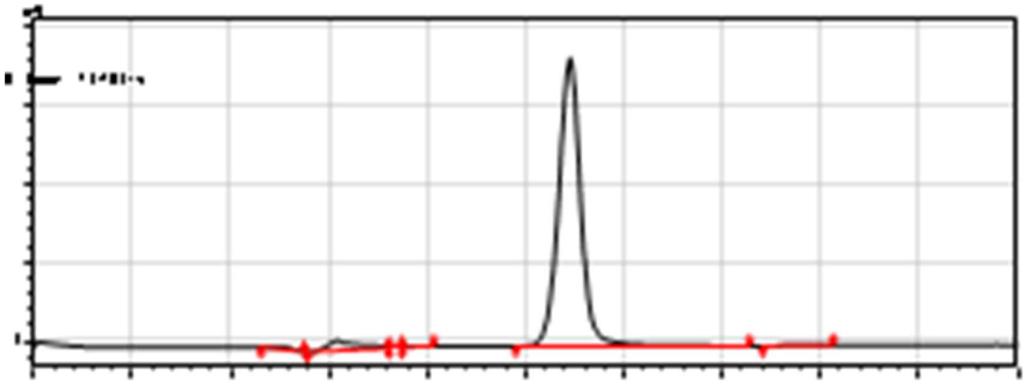

Figure 2: Chromatogram of ENZ in Acetonitrile: Water (65:35 v/v)

a) Acid Hydrolysis

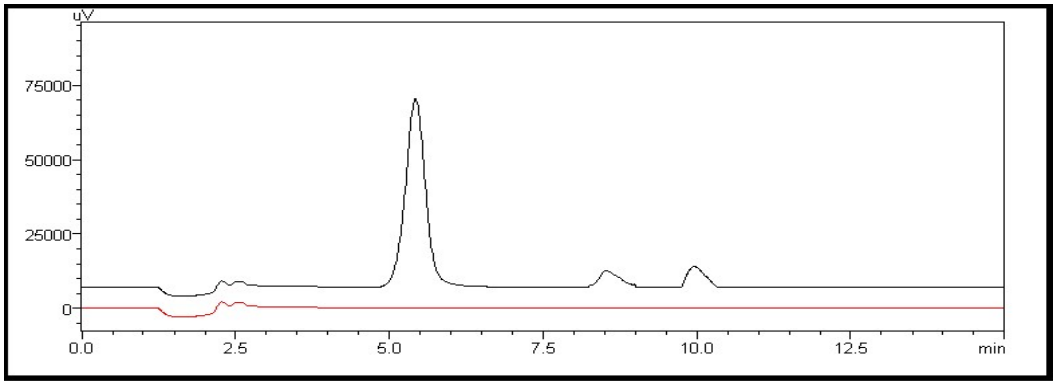

Figure 3(a): Chromatogram of ENZ in 1 N HCl for 2 hrs.

b) Alkali Hydrolysis

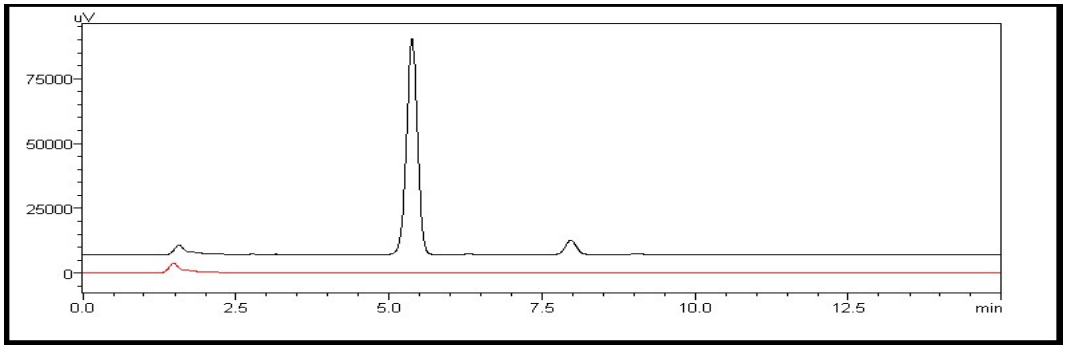

Figure 3(b): Chromatogram of ENZ in 1 N NaOH for 1 hrs

c) Oxidative Degradation

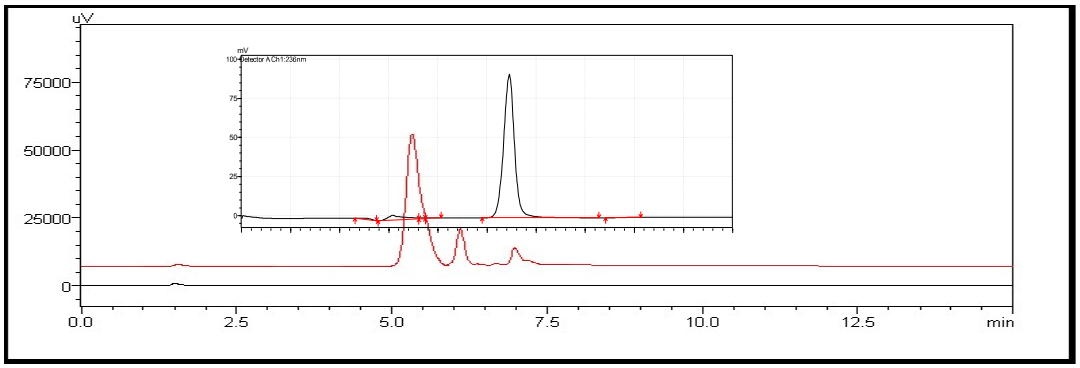

Figure 3(c): Chromatogram of ENZ in 12% H2O2 at R.T. for4 hrs

d) Photolytic Degradation

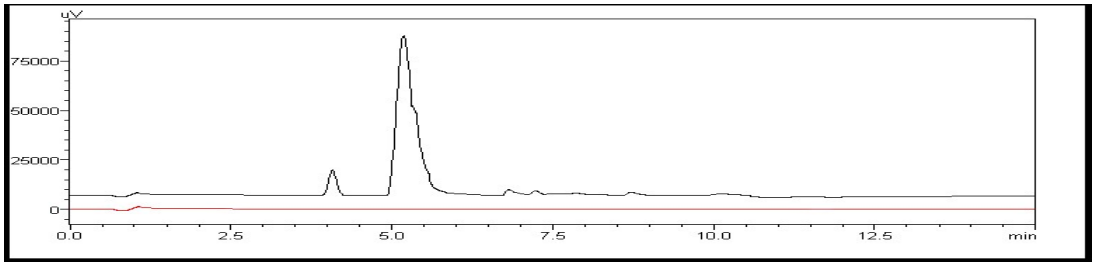

Figure 3(h): Chromatogram of ENZ under UV light at 236nm for 6 days

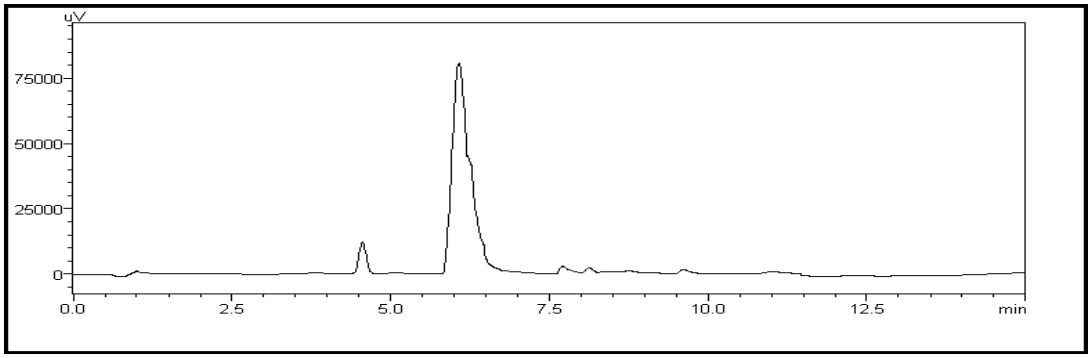

Figure 3(i): Chromatogram of ENZ tablet under UV lightat 236 nm for 6 days

e) Thermal Degradation

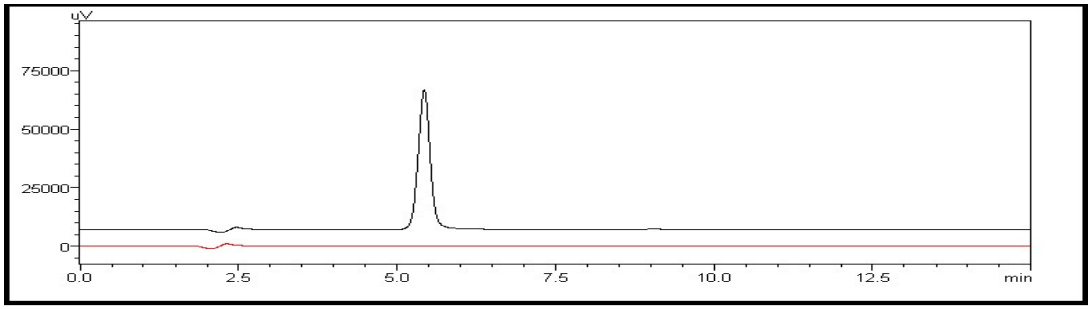

Figure 3(f): Chromatogram of ENZ tablet in dry heat at 60°C for 6 days

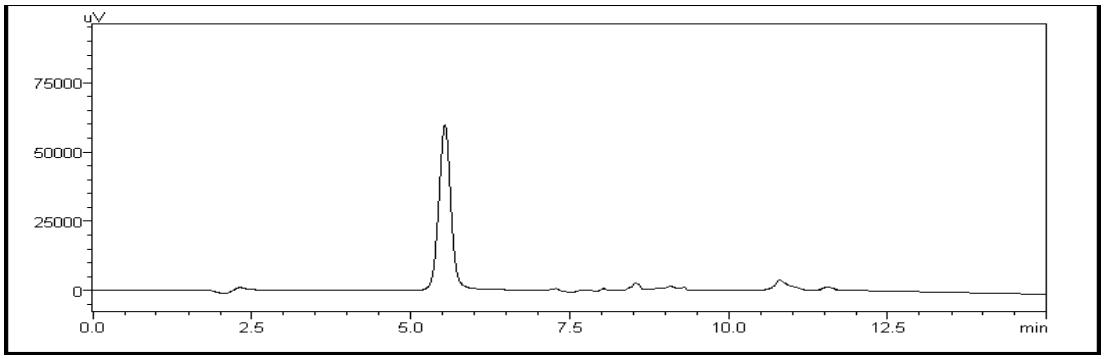

Figure 3(g): Chromatogram of ENZ tablet in dry heat at60°C for 6 days

Table 3: Result of forced degradationstudies of ENZ

StressType	tR (min) ENZ	No. of Degraded peaks	RelativeRetention Time	%Degradation
Acid	**5.504**	**2**	**1.54, 1.81**	**22.48**
Alkali	**5.367**	**1**	**1.49**	**7.11**
Oxidation	**5.483**	**2**	**1.13, 1.27**	**18.03**
Photolytic	**5.378**	**1**	**0.76**	**9.37**
Thermal	**5.517**	**0**	**-**	**-**

Method Validation Linearity and range

The linearity of ENZ was found between 10- 50 µg/mL. The calibration data is presented in **Table 4** and correlation coefficient and regression line equation analysis presented in **Figure 5**. The overlain chromatogram of standard ENZ chromatogram is presented in **Figure 4**.

Precision

a) Repeatability (Intra-day precision)

The data for Intra-day precision for ENZ isshown in **Table 5**. The % RSD for Intra-day precision was found to be 0.31-0.47 % for ENZ.

c) Inter-day precision

The data for Intra-day precision for ENZ isshown in **Table 6**. The % RSD for Inter-day precision was found to be 0.52-0.65 % for ENZ.

Accuracy

Accuracy of the method was confirmed by recovery study from marketed formulation

at three level of standard addition. Percentage recovery was found to be in range, for

ENZ 99.85-101.13 %, as shown in **Tables 7**.

LOD and LOQ

The Limit of detection (LOD) was found to be 0.28µg/mL; while the Limit of quantification (LOQ) was found to be 0.86 µg/mL, as shown in **Table 8**.

Analysis of marketed formulation

Applicability of proposed method was tested by analysing the commercially available XNTADI Capsule. The results are shown in **Table 9**.

Robustness

The typical variations studied under these parameters are flow rate, change in pH and temp, the results are shown in **Table 10**. Variation seen was within the acceptable range respect to peak asymmetry and theoretical plates, so the method was found to be robust.

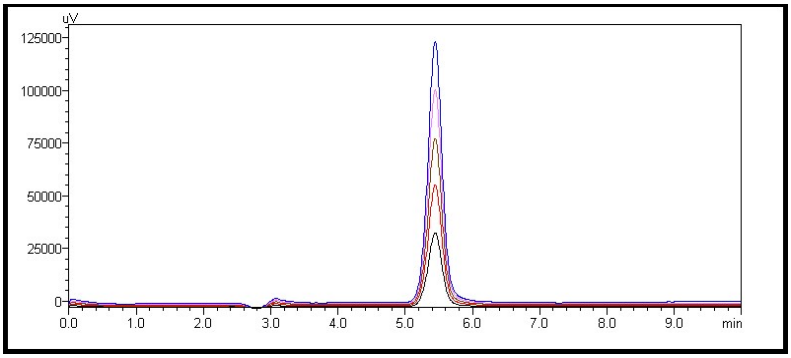

Figure 4: Overlain Chromatogram of ENZ

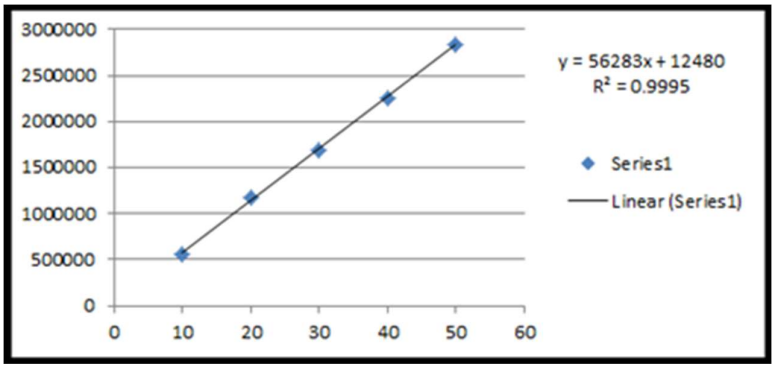

Figure 5: Calibration Curve of ENZ

Table 4: Linearity data of ENZ

Concentration (µg/mL)	Mean Peak Area* ± S.DENZ	% RSD
10	555320±4840.49	0.87
20	1170640±9130.187	0.79
30	1695900±10916.58	0.64
40	2256348±8948.801	0.39
50	2826604±8229.906	0.21

*=Average of three determination

Table 5: Intra-day precision data forestimation of ENZ

Concentration(µg/mL)	Mean Peak Area* ±S.D	% RSD
20	1170640± 5613.223	0.47
30	1695900± 5125.058	0.31
40	2256348± 10445.13	0.46

*=Average of three determination

Table 6: Inter-day precision data forestimation of ENZ

Concentration (µg/mL)	Mean PeakArea* ± S.D	% RSD
20	1178123±7070.821	0.65
30	1695781±8933.957	0.57
40	2258763±11660.49	0.52

*=Average of three determination

Table 7: % Recovery data of ENZ

Amountof Test Solution(µg/mL)	Amountof Std added (µg/mL)	Amount found (µg/mL)	%Recovery±RSD
30	24	23.97	99.87 ±0.9
30	30	30.12	100.4 ±0.99
30	36	35.8	99.45 ±0.61

*=Average of three determination

Table 8: Limit of Detection and Limit ofQuantitation

ENZ	
LOD	LOQ
0.2838 µg/mL	0.86 µg/mL

Table 9: Analysis of market formulation

Drug	Amount of drug (mg)		%Assay(n=6)	% RSD
	Labelled	Estimated		
Enzalutamide	40	40.7	101.94	1.03

Table 10: Robustness

Condition	Variations	% Assay	% RSD
Temp. (40±2°C)	42°C	101.56	0.92
	38°C	101.12	
Flow Rate(1.0 ± 0.1 mL/min)	1.1	101.79	
	0.9	99.56	
pH (6.5 ± 0.2)	6.3	100.98	
	6.7	102.32	

Table 11: Summary of validationparameters for HPLC method

Sr.no	Parameters		Enzalutamide
1	Linearity		10-50 µg/mL
2	Regression line equation		y = 56283x+12480
3	Co-relation coefficient (R2)		0.9995
4	Precision (% RSD)	Intraday	0.41
		Interday	0.58
5	Accuracy (%Recovery)		99.45-100.4
6	% Assay		101.9%
7	Limit of Detection		0.2838 µg/mL
8	Limit of Quantification		0.86 µg/mL

CONCLUSION

The developed RP-HPLC method adequately separated the drugs from the degradation products proving the specificity of method than other reported method. Enzalutamide was significantly degraded in acidic and Oxidative and marginally in alkali and photolytic and stable in thermal conditions. It gives symmetric peak shape, good resolution and

reasonable retention for Enzalutamide. Hence, the methods can be successfully used for the estimation of Enzalutamide in Capsule dosage form in quality control laboratory.

REFERENCES

[1] Rang HP, Dale MM, Pitter JM. And Moore PK. In Pharmacology; 15th Edition; Elesvier, New Delhi, 2005, pp 698-706.

[2] Goyal RK. Mehta AA., Balaraman R. and Burande MD. In Elements of Pharmacology; 16th Edition; B.S. Shah Prakashan, Ahmedabad, 2006, pp 548-550.

[3] "Cancer", September 2016, http://en.wikipedia.org/wiki/cancer.

[4] Joel GH. And Lee EL. In Goodman and Gilman´s The Pharmacological basic of Therapeutics; 9th Edition McGraw Hill Publishers, New York, 1996, pp 1225-1232.

[5] Mendham J, Denney RC, Baraes JD and Thomas MJ, Vogel's Textbook of Quantitative Chemical Analysis; 5th Edition; Pearson Education , London ,1991, pp 5.

[6] Sharma PP, How to Practice GMPs and Good Manufacturing Practices; 3rdEdition; Vandana Publication Pvt Ltd, Pune, 2001, pp 214.

[7] Ewing G W, Instrumental Methods of Chemical Analysis; 5th Edition; McGraw- Hill Book Company, New York, 1985, pp 1.

[8] Mendham J, Denney RC, Barnes JD, and Thomas MJ, Vogel's Text Book of Quantitative Analysis; 6th Edition; Pearson Education Limited, London, 2004, pp 1.

[9] Skoog DA, Holler FJ and Nieman TA, Principle of Instrumentation Analysis; 5th Edn; Thomas Asia Pvt Ltd, 2005, pp 580.

[10] Snyder LR, Kirkland JL, and Glajch JL, Practical HPLC Method Development; 2nd Edition; Wiley Interscience, New York, 1977, pp 26.

[11] Achrya MM, "Pharmaceuticals Stability Testing and Studies: An Overview", the Eastern Pharmacist, 1999, 42(497), 31-33.

[12] Singh SS, Bakshi M, "Development of Validated Stability Indicating Assay Methods: Critical Review",J.Pharm. Biomed. Anal. 2002, 28, 1011- 1040.

[13] WHO TRS 937, 40th report, Appendix 4, Analytical Method Validation, 2006, 136.

[14] Ravichandran VS, Sundram KM and Harish R, "Validation of analytical methods– strategies & importance", Int. J. Pharm. Pharm. Sci. 2010, 2(3), 18-22.

[15] Pubchem, sept 2016, https://pubchem.ncbi.nlm.nih.gov/compound/ Enzalutamide.

[16] Selleck.com, sept 2016, www.selleckchem.com/products/MDV3100.html

[17] Zamir GK, Shwetal SP, Prashant KD and Praveen OP, "Validated UV spectroscopic method for enzalutamide in pure and pharmaceutical dasage form" Analytical Chemistry: An IJ Research, 2016, 16 , 1-8.

[18] Yoshiaki O, Dhiren RT, Jacqueline AG, Kenjiro T , Shohe O, Hiroshi A "Determination of the Androgen Receptor Inhibitor Enzalutamide and its Metabolites in Animal Plasma and Brain Homogenates Using LC-MS/MS and its Application to Pharmacokinetic Studies" Chromatography, 2015, 36, 115-122.

[19] Daniel B, Jacqueline AG, Roelof Mol, Yoshiaki O & Clark W "Validation of a method for quantifying enzalutamide and its major metabolites in human plasma by LC– MS/MS" Bioanalysis , 2013, 4, 1-9.

[20] The Indian Pharmacopoiea 2010; Vol 1; The Indian Pharmacopoeia Commission, Ghaziabad, 2010, pp 147.

[21] Singh S and Bakshi M, "Guidance on conduct of stress tests to determine inherent stability of drugs." Pharm. Tech., 2000, 24, 1-14. (google scholar)

[22] Reynolds DW, "Available Guidance and Best Practices for Conducting Forced Degradation Studies." Pharm. Tech., 2002, 26(2), 48-56.

[23] Alsante KM, Martin L and Baertschi SW, "A Stress Testing Benchmarking Study."Pharm. Tech., 2003, 27(2), 60–72.