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RP–HPLC Method Validation and Forced Degradation Studies for the Determination of Belinostat in Bulk and its Pharmaceutical Dosage Form

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ABSTRACT

A reverse phase high performance liquid chromatographic method was developed for the determination of Belinostat in bulk and pharmaceutical dosage form. The separation was affected on a Altima ODS C_{18} column (150 mm x 4.6 mm,5μ) using a mobile phase mixture of buffer and acetonitrile in a ratio of 40:60 v/v at a flow rate of 1.0mL/min. The detection was made at wave length of 210 nm. Calibration curve was linear over the concentration range of 25 – 150 μg/mL of Belinostat. The proposed method was validated as per International Conference on Harmonization (ICH) guidelines. The method was accurate, precise, specific , rapid and found to be suitable for the quantitative analysis of the drug and dosage forms. Forced degradation studies have been carried out under different conditions.

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Introduction

Belinostat (PXD101, trade name Beleodaq) is a drug under development by TopoTarget for the treatment of hematological malignancies and solid tumors. It is a histone deacetylase inhibitor1. The chemical name is (2E)-N-Hydroxy-3-[3-(phenyl sulfamoyl)phenyl] prop-2-enamide.The molecular formula is $C_{15}H_{14}N_2O_4S$ and the molecular weight is 318.35 g/mol. Belinostat along with Panobinostat is used in vitro and in vivo studies in thyroid cancer2. The literature survey reveals that the Belinostat is used for the treatment of Peripheral T-Cell Lymphomas (PTCL) 3. Belinostat in combination with cyclophosphamide (PAC) has shown activity in thymic malignancies with a predicable adverse events profile4. LC-MS/MS assay for the quantitation of the HDAC inhibitor Belinostat and five major metabolites in human plasma was reported[5]. Liquid chromatography–tandem mass spectrometric method for determination of belinostat in plasma from liver cancer patients has been reported[6]. The present investigation describes a rapid, accurate, precise and forced degradation studies RP–HPLC method validation for the determination of Belinostat in bulk and its pharmaceutical dosage forms. The detector responses were linear in the concentration range of 25μg/mL – 150 μg/mL of drug. The method was validated as per ICH guidelines. The molecular structure of Belinostat was shown in Fig 1.

Materials and methods

Chemicals and reagents

All experiments were performed with pharmaceutical Belinostat. HPLC grade Merck make Ortho Phosphoric Acid(OPA), aceto nitrile were used. All dilutions were performed in Standard Class-A, Volumetric glassware. For the estimation of commercial formulation, Beleodaq tablets having 500mg Belinostat were procured from M/s SPECTRUM LABS,

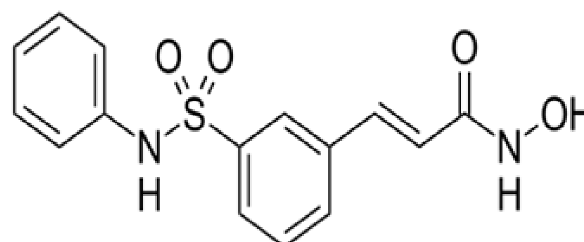

Fig 1. Structure of Belinostat

Hyderabad, Telangana State, India. HPLC grade Milli-Q water was used throughout the analysis. Solvents were filtered through 0.45μ nylon membrane filters. All dilutions were performed in Standard Volumetric flasks.

Instrumentation

The determination of Belinostat was carried out on waters HPLC model 2695 equipped with UV- Visible detector using data handling system-water alliance empower two Software. Chromatographic separation was carried out on a C_{18}Column [Altima ODS,150mm×4.6mm,5 μ]. Sartorious electronic balance was used for weighing the samples. Ultra-Sonic bath Sonicator was used for degassing and mixing of the mobile phase.

Experimental

Chromatographic Conditions

The column used in the development for determination of Belinostat was Altima ODS C_{18} column (150mm × 4.6mm, 5 μ). The detector wavelength was set at 210 nm. A flow rate of 1.0mL/min was used for the determination of Belinostat.

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A freshly prepared buffer solution, acetonitrile in a ratio of 40:60 (v/v) were used. HPLC grade acetonitrile and double distilled water were purchased from Merck, Mumbai, India, orthophosphoric acid AR grade was purchased from SD Fine Chem, Mumbai, India. The mobile phase was delivered at a flow rate of 1.0mL/min in isocratic conditions. PDA detector was used for detecting the separated components. The data was analysed on Empower-2 Software version. Before analyzing, the mobile phase was degassed by use of a Sonicator and filtered through a 0.45 µm filter.

Drug Samples

The Belinostat reference and branded formulation was supplied by M/s SPECTRUM LABS, Hyderabad, Telangana State, India.

Mobile phase

Measured accurately 1mL orthophosphoric acid and dissolved in a 1000 mL of water. The solution was filtered through 0.45µm membrane filter and was degassed. A freshly prepared buffer solution and acetonitrile in a ratio of 40:60 (v/v) was filtered through 0.05µm membrane filter and Sonicated by using power Sonicator (Model No. 405, Hwashin Technology, Korea) before use. The flow rate of the mobile phase was maintained at1.0mL/min. The column temperature was maintained at 30ºC and the detection of the Belinostat drug was carried out at wave length of 210 nm.

Method Development

Preparation of Standard Stock Solution

Weighed accurately about 10 mg of Belinostat and transferred into 10 mL clean, dry volumetric flask, added 7 mL of diluent and the solution was sonicated for 30 minutes and make upto the final volume with diluents to prepare a 100 µg/mL stock solution. From the above stock solution, 1mL of standard stock solution was pipetted out into 10mL volumetric flask and make upto the final volume with diluents. The solution was filtered through 0.45µ nylon membrane filter paper. The solution was preserved as was used as standard stock solution for the preparation of calibration curve of Belinostat.

Method Validation

The developed HPLC method for the determination of Belinostat was validated for accuracy, precision, reproducibility, specificity, robustness and limit of detection (LOD) and limit of quantification (LOQ), in accordance with ICH guidelines.

System suitability and system precision

System suitability for chromatographic separation was checked on each day of validation to evaluate the components of the analytical system inorder to show the performance of the system, meet the standards required by the method. System suitability parameters established for the developed method include number of theoretical plates (efficiency), tailing factor. TheHPLC system was equilibrated using the initial mobile phase composition, followed by 3 injections of the standard solution of 100% concentration containing Belinostat. These 3 consecutive injections were used to evaluate the system suitability on each day of method validation. The result was given in the Table 1.

Specificity

Blank interference

Specificity studies include application of the proposed method for blank, placebo solution, sample solution and standard solution. A study to establish the interference of blank was conducted. Diluent was injected into the chromatograph in the above defined chromatographic conditions and the blank chromatogram was recorded.

Chromatogram of blank solution (Fig 2) showed no peaks at the retention time of Belinostat peak. This indicates that the diluent solution used in sample preparation do not interfere in estimation of Belinostat in Beleodaq tablets. Similarly typical representative chromatogram of standard was also shown in Fig 3.

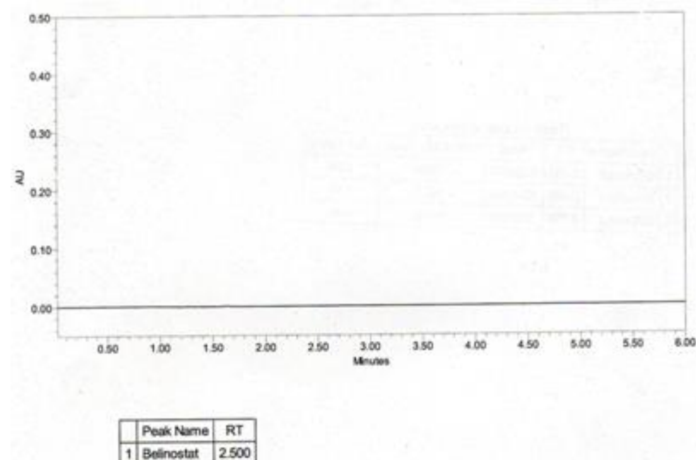

Fig 2. Chromatogram of Blank Solution

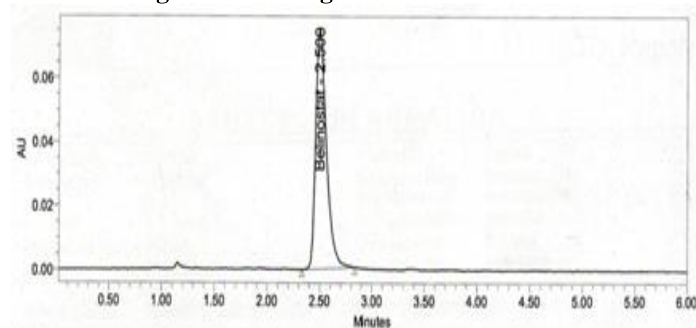

Fig 3. Chromatogram of Standard

Peak Name: Belinostat

	Peak Name	RT	Area	USP Plate Count	USP Tailing
1	Belinostat	2.494	533278	3033	1.58
2	Belinostat	2.498	532497	2993	1.53
3	Belinostat	2.500	532082	2968	1.53
4	Belinostat	2.502	536483	2974	1.51
5	Belinostat	2.503	541907	2942	1.54
6	Belinostat	2.509	535396	3006	1.56
Mean			535274		
Std. Dev.			3670.4		
% RSD			0.7		

Forced Degradation Studies

Forced degradation or accelerated degradation is a process whereby the natural degradation rate of a product or material is increased by the application of additional stress.

Forced degradation or stress test was undertaken to demonstrate specificity when developing stability- indicating methods, particularly when little information is available about potential degradation products. These studies also provide information about the degradation pathways and degradation products that could form during storage. Forced degradation studies may help to facilitate pharmaceutical development as well as in areas such as formulation development, manufacturing and packaging in which knowledge of chemical behavior can be used to improve a drug product.

Forced Degradation study was carried out by treating the sample under the following conditions. One vial each 30 mL clear vial contains sterile, lyophilized powder equivalent to 500 mg Belinostat was transferred into 500 mL volumetric flask.

Table 1. System suitability parameters for Belinostat by proposed method

Name of the compound	Retention time	Area	USP Plate count	USP Tailing
Belinostat	2.512	540497	2932	1.49

Table 2. Linearity data for Belinostat

Concentration of (µg/mL)	peak area(µV^2 Sec)	
25	134626	Concentration range (µg/mL) = 25 - 150 Correlation coefficient (r^2) = 0.9999 Slope (m) = 5315.2 Intercept (b) = 240.57
50	265766	
75	405790	
100	539844	
125	662127	
150	807198	

Table 3. Accuracy Data for Belinostat

	Accuracy-- 50%	Accuracy-- 100%	Accuracy-- 150%
S No	Area(µV^2 Sec)	Area(µV^2 Sec)	Area(µV^2 Sec)
Injection-1	805318	1069775	1335990
Injection-2	803176	1074375	1340552
Injection-3	802025	1081995	1350896
Average	803506	1075381	1342479
*Amount added (µg/mL)	50	100	150
*Amount Recovered(µg/mL)	49.88	100.63	150.48
* % Recovered	99.93	100.66	100.23

Table 4. Forced Degradation for Belinostat

Condition	Time (hours)	Retention time (min)	Area	Retention time of additional degradation peak (min)	%Degradation	% of Active drug Present after Degradation
Acid Degradation	02	2.656	508515	2715	8.69	91.31
Alkaline Degradation	02	2.563	526853	1619	0.60	99.40
Thermal Degradation	06	2.589	516161	-----	---	100
PhotolyticDegradation	168	2.563	529578	-----	---	100
Peroxide Degradation	02	2.589	506494	408	0.15	99.85

Table 5. Analysis of marketed formulation (Assay) data for Belinostat

Drug	Quantity claim (mg/vial)	*Quantity found (mg/vial)	* % Assay found
Belinostat	500	503.5	100.7

*Each value is a mean of three readings

Table 6. Robustness data for Belinostat

Variations	Chromatographic parameters			
	Retention time (min)	Area (µV^2 Sec)	Theoretical plates	Asymmetry
Change in Column temperature at ± 5				
1. Column temperature at 25^{0}C	2.309	493317	2630	1.29
2. Column temperature at 35^{0}C	2.458	546361	2719	1.32
Change in flow rate at ± 0.2mL/min				
1.flow rate at 0.8mL/min	2.768	594122	2932	1.39
2.flow rate at 1.2mL/min	2.309	500329	2617	1.31
Change in Buffer variation (±5%)				
1. Buffer at 35:65	2.458	546361	2719	1.32
2. Buffer at 45:55	2.582	554213	2715	1.31

The contents of the flask were sonicated for about 25 minutes for complete solubility of the drug and the volume was made upto 500 mL with mobile phase. Then the mixture was filtered through a 0.45µ membrane filter. Transfer 1 mL of standard stock solution into 10 mL volumetric flask and dilute to volume with diluents.

Oxidation Studies: To 1 mL of stock solution of Belinostat, 1 mL of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 2 hours at 60^0C. For HPLC study, the resultant solution was diluted to obtain 100µg/mL solution and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of sample (Fig 4).

Acid Degradation Studies: To 1mL of stock solution of Belinostat, 1mL of 2N Hydrochloric acid was added and refluxed for 2hours at 60^0C .The resultant solution was diluted to obtain 100µg/mL solution and 10 µL solutions were injected into the system and the chromatograms were recorded to assess the stability of sample (Fig 5).

Alkali Degradation Studies: To 1 mL of stock solution of Belinostat, 1 mL of 2 N sodium hydroxide was added and refluxed for 2hours at 60^0C. The resultant solution was diluted to obtain 100µg/mL solution and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of sample (Fig 6).

Dry Heat Degradation Studies: The standard drug solution was placed in oven at 105^0C for 6 hours to study dry heat degradation. For HPLC study, the resultant solution was diluted to 100µg/mL solution and 10µL were injected into the system and the chromatograms were recorded to assess the stability of the sample (Fig 7).

Photo Stability Studies: The photochemical stability of the drug was also studied by exposing the 120µg/mL solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 100µg/mL solutions and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of sample (Fig 8).

Neutral Degradation Studies: Stress testing under neutral conditions was studied by refluxing the drug in water for 6hours at a temperature of 60ºC. For HPLC study, the resultant solution was diluted to 100µg/mL solution and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of the sample (Fig 9).

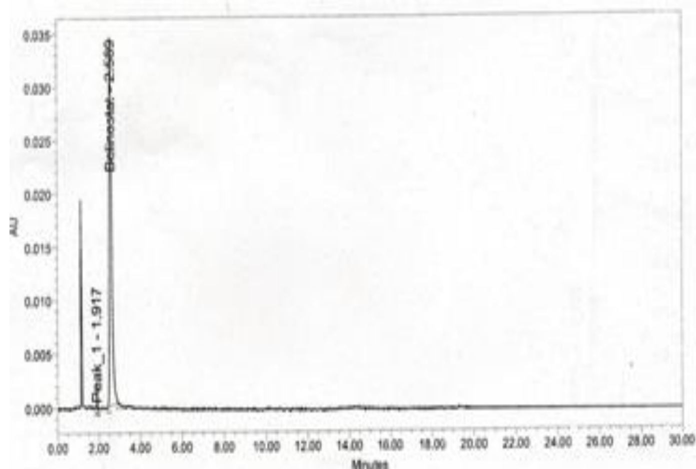

Fig 4. Chromatogram of Peroxide

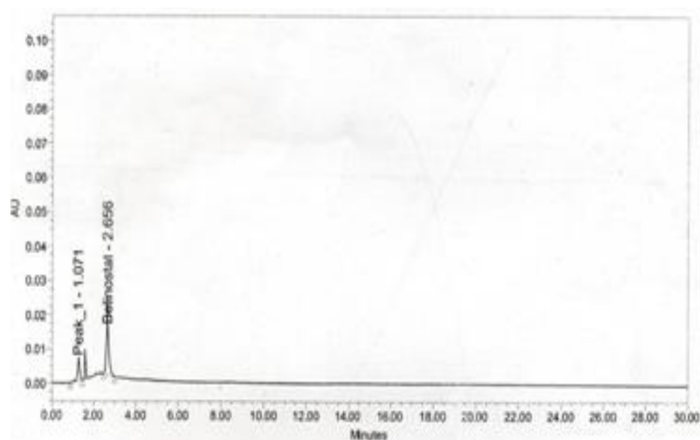

Fig 5. Chromatogram of acid

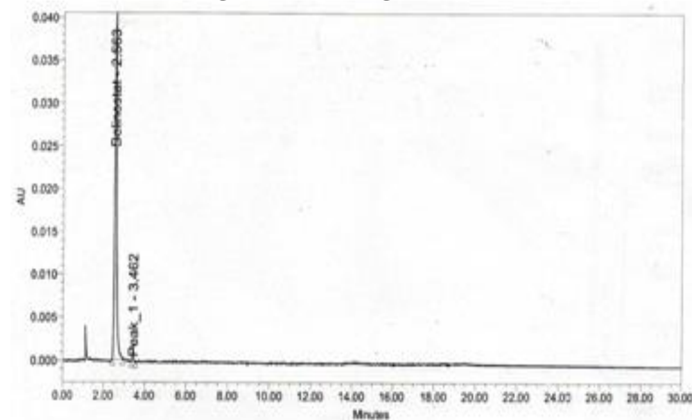

Fig 6. Chromatogram of Alkali

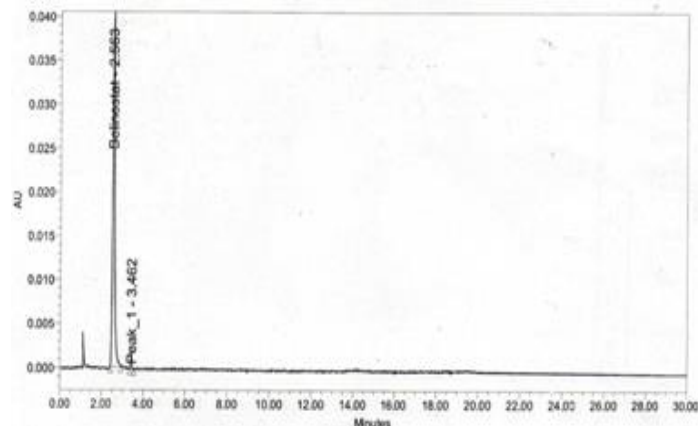

Fig 7. Chromatogram of Thermal Studies

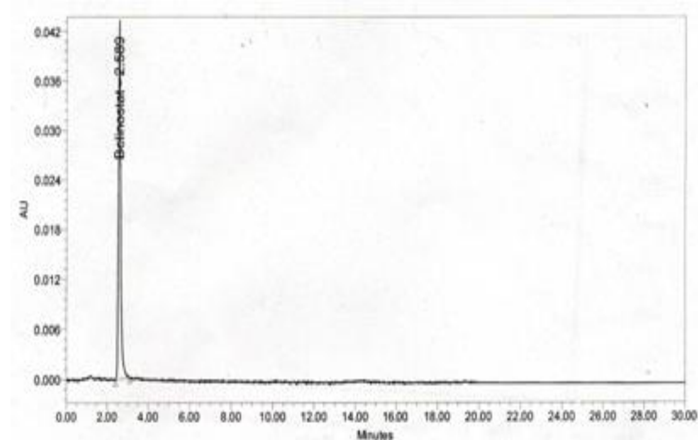

Fig 8. Chromatogram of UV sample

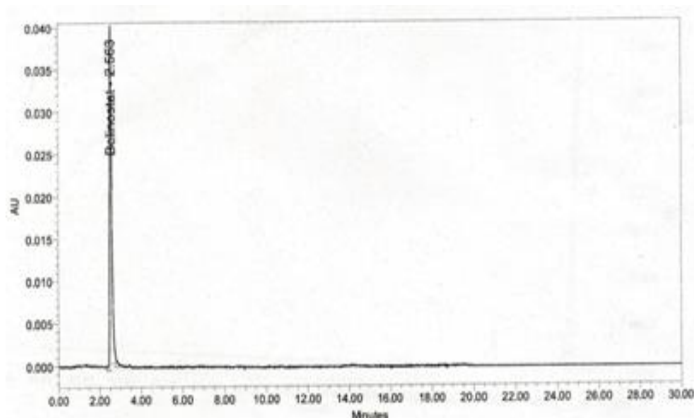

Fig 9. Chromatogram of Neutral Degradation

Robustness

To evaluate the robustness, the following small deliberate variations were made in the method and analysed the sample in triplicate. The system suitability was evaluated in each condition and compared the results with method precision results. The method is robust for change in flow rate, buffer variation and Column temperature.

Results and Discussion

The goal of this study was aimed at developing a sensitive, precise and accurate RP- HPLC method for the analysis of Belinostat in bulk drug and in pharmaceutical dosage form and also for forced degradation studies. In order to achieve optimum separation of the component peaks, mixtures of buffer and Acetonitrile in different combinations were tested as mobile phase on an Altima ODS C_{18} column (150 mm x 4.6 mm, 5µ) stationary phase. A mixture of buffer and Acetonitrile in a proportion of 40:60 (v/v) was selected as the chromatographic peaks, were well defined and resolved with no tailing. To validate the RP-HPLC method, a series of tests were made using the most promising conditions. For linearity, a Calibration curve was made and concentrations examined within the detection range of 25µg/mL - 150µg/mL for Belinostat, the correlation coefficient was found to be 0.9999.The regression curve was constructed by linear regression fitting and its mathematical expression was Y = 5315.2X-240.57 (Where Y gives peak area and X is the concentration of the drug). The regression characteristics were given in Table 2. The system precision was established by six replicate injections of the standard solutions containing analytes of interest. The value of relative standard deviation was found to be 0.7 indicating the injection repeatability of the method. The method precision was established by carrying out the analyte six times using the proposed method. The LOD and LOQ values as obtained by the proposed method were found to be 0.05069 and 0.15362 µg/mL respectively indicates the sensitivity of the method. The relative standard deviation was found to be 0.90 indicating the injection repeatability of the method. For accuracy determination, three different concentrations were prepared separately i.e.50%, 100%, and 150% of analyte and the chromatograms were recorded for the same. The results obtained for recovery were found to be within the limits. Hence the proposed method was found to be accurate and precise summarized in the Table 3. The absence of additional peaks indicated non-interference of common excipients used in the tablets. The stability of sample was checked by forced degradation in different conditions and the percentage of degradation was calculated. When it was subjected to forced degradation as per ICH guidelines which was carried out with acidic, alkaline, photolytic, peroxide and thermal degradation at 105°C.The results of specificity data for degradation study were given in Table 4. The following values indicate that the any other impurity was not merged with the main peak. The system suitability was evaluated in each condition and compared the results with method precision results. The method is robust for change in flow rate, buffer variation and Column temperature summarized in the Table 6. The amount of Belinostat present in per vial was found to be 503.5 mg/vial. Total label claim for Beleodaq formulation was 500 mg/vial summarized in the Table 5.

Linearity and Construction of Calibration Curve

The quantitative determination of the drug was accomplished by a standard method. The column was equilibrated with the mobile phase for at least 30 minutes prior to the injection of the drug solution. Linearity of the peak area response was determined by taking measurement at Six concentrations of Belinostat working dilution in the range of 25µg/mL - 150µg/mL, were prepared by taking suitable aliquots of working standard solution in different 10 mL volumetric flasks and diluting up to the mark with the mobile phase. 20µL quantity of the dilution was injected each time in to the column at a flow rate 1.0 mL/min. The detector wavelength was monitored at 210 nm. To assess the linearity of the proposed method slope, intercept and correlation coefficient (r^2) of standard curve was calculated and was given in Fig 11. The results were given in the Table 2. From the data obtained (for Belinostat), the method was found to be linear within the proposed range. The linearity chromatograms were given in Fig 10.

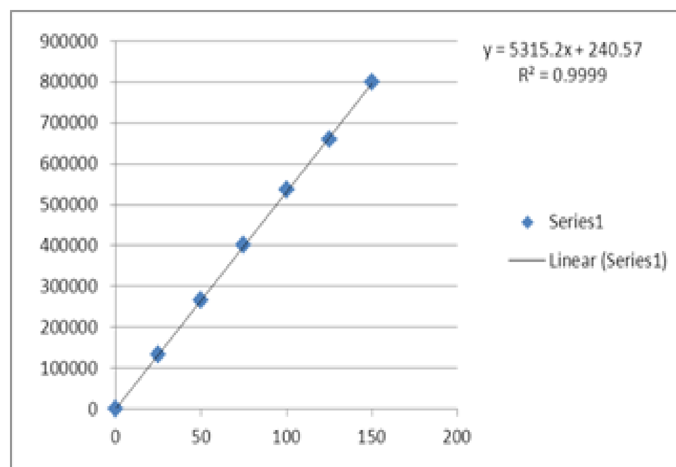

Fig 11. Calibration curve of Belinostat

Analysis of Tablet

One vial each 30 mL clear vial contains sterile, lyophilized powder equivalent to 500 mg Belinostat was transferred into 500mL volumetric flask. The contents of the flask were sonicated for about 25 minutes for complete solubility of the drug and the volume was made up to 500 mL with mobile phase.

Then the mixture was filtered through a 0.45µ membrane filter. Transfer 1 mL of standard stock solution into 10 mL volumetric flask and dilute to volume with diluent. Inject 20 µL of blank solution, placebo solution, standard solution, Disregard peaks due to blank and placebo (500 mg, lyophilized powder in single-use vial for reconstitution) Beleodaq (Belinostat) for injection was supplied in single vial cartons; each 30 mL clear vial contains sterile, lyophilized powder equivalent to 500 mg Belinostat. NDC 68152-108-09: Individual carton of Beleodaq 30 mL single-use vial containing 500 mg Belinostat.

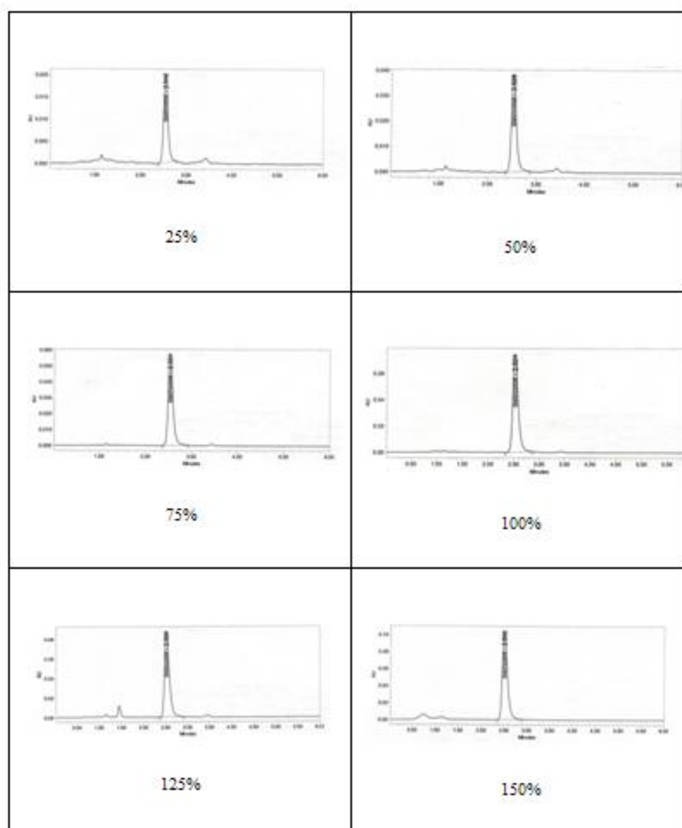

Fig 10. Linearity Chromatograms of Belinostat

Conclusion

The proposed method was completely validated as per ICH guidelines and found to be precise and accurate, as depicted by the statistical data of analysis. High values of correlation coefficients and small values of intercepts validated the linearity of the calibration plots and obedience to Beer's laws. The RSD values, the slopes and intercepts of the calibration graphs indicate the high reproducibility of the proposed method. The low values of LOD and LOQ indicate that the method can be employed over a wide concentration range for linearity. The stability indicating nature of the proposed method was established by performing forced degradation, which provided degradation behavior of Belinostat under various conditions.

RP-HPLC method was developed for the determination of Belinostat tablets which was simple, quick, specific and reliable. The results indicate that the described method can be used for quantitative analysis of the compound with short analysis time.

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