IJPSR (2019), Volume 10, Issue 6 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 20 September 2018; received in revised form, 13 December 2018; accepted, 26 February 2019; published 01 June 2019

NOVEL RP-HPLC AND UV METHOD DEVELOPMENT AND VALIDATION FOR INOTUZUMAB OZOGAMICIN IN BULK AND ITS PHARMACEUTICAL DOSAGE FORM

S. K. Mastanamma *, S. K. Reehana and M. Hari

Department of Pharmaceutical Analysis, University College of Pharmaceutical Science, Acharya Nagarjuna University, Nagarjunanagar, Guntur - 522510, Andhra Pradesh, India.

Keywords:

RP HPLC, Inotuzumab Ozogamicin, Stability indicating Method, ICH Guidelines, Degradation products

Correspondence to Author:
Dr. S. K. Mastanamma

M. Pharm, Ph.D
Department of Pharmaceutical Analysis, University College of Pharmaceutical Science, Acharya Nagarjuna University, Nagarjunanagar, Guntur - 522510, Andhra Pradesh, India.

E-mail: masthanamma.sk@gmail.com

ABSTRACT: A simple, Precise, Accurate method was developed for the estimation of Inotuzumab Ozogamicin by RP-HPLC and UV technique. Chromatographic conditions used were stationary phase Discovery c18 150 × 4.6 mm, 5 m. Mobile phase O- phosphoric acid buffer: Acetonitrile in the ratio of 50v:50v and the flow rate were maintained at 1 ml/min, detection wavelength at 240nm, column temperature was set to 30 ºC, and diluents was Acetonitrile: Water (50v:50v), Conditions were finalized as an optimized method. System suitability parameters were studied by five times standard was injected, and results were well under acceptance criteria. Linearity study was carried out between 4.5 µg/ml to 27 µg/ml levels, R^2 value was found to be as 0.999. Precision was observed to be less than 2% for both repeatability and intermediate precision. LOD and LOQ are found to be 0.033 µg/ml and 0.099 µg/ml respectively. The explored HPLC method was fast, simple, sensitive, accurate and precise for the estimation of Inotuzumab Ozogamicin in bulk and its dosage form. The compounds were subjected to forced degradation applying several stress conditions. The proposed method is specific and stability-indicating. Hence, the developed method can be adapted to regular quality control analysis.

INTRODUCTION: Inotuzumab Ozogamicin [1] is an antibody-drug conjugate developed by Pfizer and UCB and approved by EU on June 2017 and FDA on August 2017 for the treatment of an adult with relapsed or refractory B-cell precursor acute lymphoblastic leukemia. The molecular formula of Inotuzumab Ozogamicin $C_{6518}H_{10002}N_{1738}O_{2036}S_{42}$ and molar mass is 1, 50,000 g/mol. The only formulation available with this drug was Response in the form injection.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.10(6).2845-51

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.10(6).2845-51

An exhaustive literature survey revealed there was no analytical method was developed for the estimation of Inotuzumab ozogamicin in bulk and its pharmaceutical dosage form. An attempt has been made to develop an emerging RP-HPLC method with a simple, convenient, rapid, precise, economical and usable method with a simple and easily available mobile phase for the quantitative estimation of Inotuzumab ozogamicin in the injection dosage form. This method was validated as per ICH guidelines. Chemical Structure of Inotuzumab Ozogamicin was shown in **Fig. 1**.

MATERIALS AND METHODS:

Chemicals: Inotuzumab Ozogamicin pure drugs (API), Ivabradine injection (BESPONSA), obtained from Spectrum pharma research solutions,

Hyderabad. Distilled water, Acetonitrile, Phosphate buffer, Methanol, Glacial acetic acid, Potassium dihydrogen orthophosphate buffer, Ortho-phosphoric acid are from Rankem chemicals, Hyderabad, India.

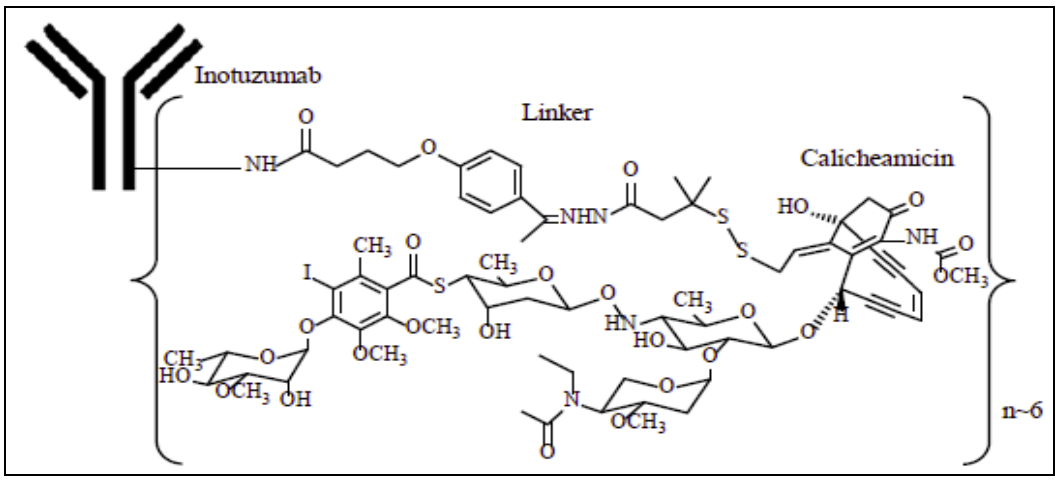

FIG. 1: STRUCTURE OF INOTUZUMAB OZOGAMICIN

Instruments: WATERS HPLC 2965 with PDA Detector using Software Empower, UV-VIS spectrophotometer.

Preparation of Solutions and Reagents:

Preparation of Diluents: A mixture of Acetonitrile and water are taken in the ratio of 50:50 v/v was used as a diluent.

Preparation of 0.1% OPA Buffer: 1 ml of Orthophosphoric acid was pipetted out into a 500 ml of Milli-Q water taken in a 1000 ml Volumetric flask and final volume was made up to the mark with Milli-Q water.

Preparation of Mobile Phase: Mobile phase was prepared by mixing 0.1% OPA and acetonitrile HPLC Grade in the ratio of 50:50 v/v. The prepared mobile phase was sonicated for 15 min and filtered through 0.22 mm membrane filter to remove the impurities which may interfere with the final chromatogram

Preparation of Standard Stock Solution: Accurately weighed 1.8 mg of Inotuzumab Ozogamicin transferred into10 ml volumetric flask, 3/4th of diluents was added and sonicated for 10 min. Flasks were made up with diluents and labeled as Standard stock solution (180 µg/ml of Inotuzumab Ozogamicin).

For UV method: Accurately weighed 10 mg of Inotuzumab Ozogamicin working std in 10 mL volumetric flask containing 5 ml of Acetonitrile shaken for 5 min then remaining volume made up with Acetonitrile. The final concentration obtained was 1000 µg/ml. It was further diluted to get concentration 100 µg/ml was prepared with distilled water. From this, a series of aliquots were prepared to get concentration ranging from 25 to 175 µg/ml in 10 ml vol. flask using distilled water.

Determination of Wavelength Maximum Absorbance: Wavelength maximum absorbance was determined by scanning 25 µg/ml solution using Shimadzu UV-1800 double beam spectrophotometer from 200-400 nm **Fig. 2.**

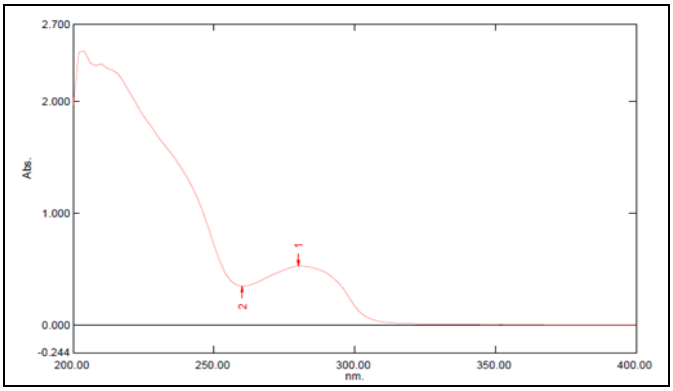

FIG. 2: SPECTRUM OF INOTUZUMAB OZOGAMICIN

Preparation of Sample Stock Solutions: commercially available Inotuzumab Ozogamicin 10 vials were taken Reconstitute vials each vial with 4 ml of Sterile Water for Injection, USP, to obtain a concentration of 0.9 mg/ml of a vial that delivers 3.6 ml (0.09 mg) transferred in to 10 ml of volumetric flask 5 ml diluents was added and

sonicated for 25 min, further volume was made up with diluents and filtered by membrane filters (90 µg/ml Inotuzumab Ozogamicin).

Optimized Chromatographic Conditions: For optimization of HPLC method, different mobile phases were tried, but acceptable retention times, theoretical plates and good resolution were observed with 0.1% OPA and acetonitrile in the ratio of 50:50% v/v. using column Discovery C18 250 × 4.6 mm, 5m. The results were shown in **Fig. 3** and **Table 1**.

Estimation of Inotuzumab Ozogamicin in Injection: For the estimation of Inotuzumab Ozogamicin in the commercial formulations, 10 vials each containing 1 mg of Inotuzumab Ozogamicin was weighed, and the average weight was calculated. For the analysis of drug, the quantity of injection equivalent 10 mg of Inotuzumab Ozogamicin was transferred to 10 ml volumetric flask and dissolved in sufficient quantity of Acetonitrile and volume made up to the mark with Acetonitrile to obtain conc. of 1000 µg/ml of Inotuzumab Ozogamicin. Then the solution was filtered through Whatman filter paper no. 41. Further dilutions of the solution were made in water to get a required concentration of 10 µg/ml. The concentration of Inotuzumab Ozogamicin in the formulation was determined by above-developed methods. The assay procedure was repeated six times (n= 6) for each method.

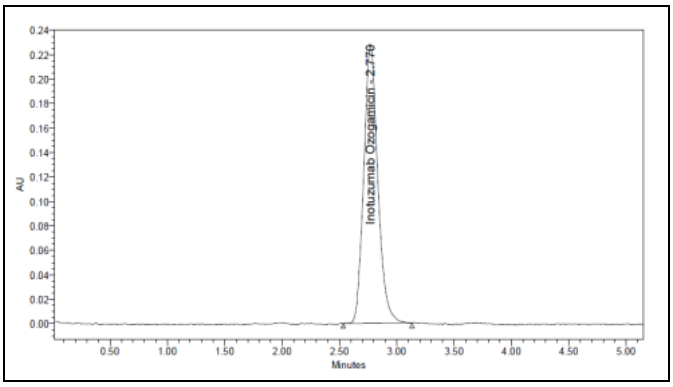

FIG. 3: OPTIMIZED CHROMATOGRAM OF INOTUZUMAB OZOGAMICIN

TABLE 1: OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Column	Discovery C18 (150 × 4.6 mm, 5µm)
Mobile phase	ACN: 0.1% OPA Buffer pH-5 (50:50)
Flow rate	0.8 ml /min
Column temperature	30°C
Injection volume	10 µL
Detection Wavelength	240 nm
Runtime	5 min
Retention time	2.770 min

TABLE 2: RESULTS OF MARKETED FORMULATION ANALYSIS

Compound name	Brand name	Label claim (mg)	Amount took (mg)	Amount found (mg)	% Assay
Inotuzumab Ozogamicin	Besponsa	1	10	10.3	103

Validation of the RP-HPLC Method: Validation of the optimized method was performed according to the ICH guidelines [2].

System Suitability: The system suitability parameters were determined by preparing standard solutions of Inotuzumab Ozogamicin (18 µg/ml).

The solutions were injected six times, and the parameters like peak tailing, resolution and USP plate count were determined. The % RSD for the area of six standard injections results should not be more than 2%. The results were shown in **Table 3**.

TABLE 3: SYSTEM SUITABILITY PARAMETERS

Parameter	Result
Retention Time (min)	2.7
Theoretical Plates (N)	2156
Tailing Factor	1.4
Limit of Detection (µg/ml)	0.033
Limit of Quantification (µg/ml)	0.099

Specificity: The specificity of the method was evaluated about interference due to the presence of any other excipients. The HPLC chromatograms recorded for the drug-matrix (a mixture of the drug and excipients) showed almost no interfering peaks within retention time ranges.

Linearity: The linearity method was determined in the concentration range of 4.5 ppm to 27 ppm of Inotuzumab Ozogamicin. Each solution was injected in triplicate. The average peak area versus concentration data of drug was treated by least squares linear regression analysis, and the results were shown in **Table 4** & **Fig. 4.**

TABLE 4: RESULTS FOR LINEARITY

S. no.	Parameter	Result
1	Concentration range (µg/ml)	4.5-27µg/ml
2	Slope (m)	10345
3	Intercept (b)	1398
4	Correlation coefficient	0.999

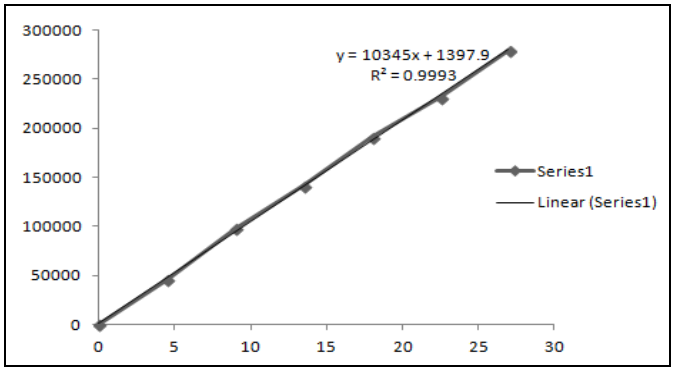

FIG. 4: CALIBRATION CURVE OF INOTUZUMAB OZOGAMICIN

Precision: The repeatability of the method was verified by calculating the % RSD of six replicate injections of 100% concentration (9 ppm Inotuzumab Ozogamicin) on the same day, and for intermediate precision % RSD was calculated from repeated studies on different days. The results were shown in **Table 5**.

LOD and LOQ: Limit of Detection (LOD) and Limit of Quantitation (LOQ): The LOD and LOQ were calculated from the slope(s) of the calibration plot and the standard deviation (SD) of the peak areas using the formulae LOD = 3.3 S/N and LOQ = 10 S/N. The results were shown in **Table 3**.

Accuracy: Accuracy was carried out by % recovery studies of Inotuzumab Ozogamicin at three different concentration levels (50%, 100%, and 150%). Percentage recovery was calculated from the amount added and the amount recovered. The percentage recovery was within the acceptance criteria; this indicates the accuracy of the method. The results were shown in **Table 6**.

TABLE 5: RESULTS OF PRECISION

Concentration of Inotuzumab ozogamicin (µg/ml)	Observed concentration of Inotuzumab ozogamicin (µg/ml)			
	Repeatability		Intermediate	
	Mean (n=3)	RSD%	Mean (n=6)	RSD%
	191409	0.2	188280	0.4

TABLE 6: RESULTS FOR ACCURACY

Spiking levels	Conc. took	Amount Added (µg)	Amount recovered (µg)	% Recovery	Mean recovery
50 %	10µg	9µg	8.917061	99.10	
50 %	10µg	18µg	17.89550	98.16	99.26%
50 %	10µg	27µg	26.83251	99.51	

Robustness: Robustness of the method was verified by altering the chromatographic conditions like flow rate, mobile phase ratio and temperature are made, but there was no recognized change in the result, and all are within range as per ICH guidelines. Robustness conditions like flow minus (0.9 ml/min), flow plus (1.1 ml/min), mobile phase minus 55:45 mobile phase plus, temperature minus (25 °C) and temperature plus (35 °C) were maintained and samples were injected in a duplicate manner. System suitability parameter was passed. % RSD was within the limit. The result was shown in **Table 7**.

TABLE 7: RESULTS FOR ROBUSTNESS

S. no.	Parameter		RT	Avg.	% RSD
1	Flow rate (f1)	0.7mL/m	3.136	2.785	0.6
2	Flow rate (f2)	1.0mL/m	2.435		
3	Temperature (t1)	27 ºC	2.820	2.740	0.3
4	Temperature (t2)	23 ºC	2.660		

Degradation Studies: Forced degradation studies are conducted on the sample using acid, alkaline, peroxide, thermal, UV, water degradations. The sample was exposed to these conditions main peak of the drug was studied for peak purity, that indicating the method effectively separated the degradation products from the pure active ingredient. These results are shown in **Table 8** and **Fig. 5**.

Acid Degradation Studies: Add 1ml of 2N Hydrochloric acid to 1ml of stock solution and refluxed for 30 min at 60 °C, cool to room temperature and add 1 ml of NaOH and make up to final volume with diluents to get concentration of 18 µg/ml this concentration injected 6 times into the column and calculated the % degradation.

TABLE 8: RESULTS FOR STABILITY STUDIES

S. no.	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	4.86	1.241	1.517
2	Alkali	3.79	0.792	1.170
3	Oxidation	3.39	0.952	1.148
4	Thermal	2.77	1.090	1.264
5	UV	1.62	0.964	1.202
6	Water	0.81	0.999	1.246

Alkali Degradation Studies: Add 1 ml of 2N sodium hydroxide to 1 ml of stock solution and refluxed for 30 min at 60 ºC. The resultant solution was cooled and neutralized with HCl and makeup to final volume to obtained solution.

Cool to room temperature and add 1ml of NaOH and makeup to final volume with diluents to get a concentration of 18 µg/ml this concentration injected 6 times to the column and calculated the % degradation.

Oxidation: To 1 ml of stock solution of Inotuzumab ozogamicin, 1 ml of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min at 60 ºC. For HPLC study, the resultant solution was diluted to obtain a solution. Cool the solution to room temperature, makeup to final volume with diluents to get a concentration of 18 µg/ml this concentration injected 6 times to the column and calculated the % degradation.

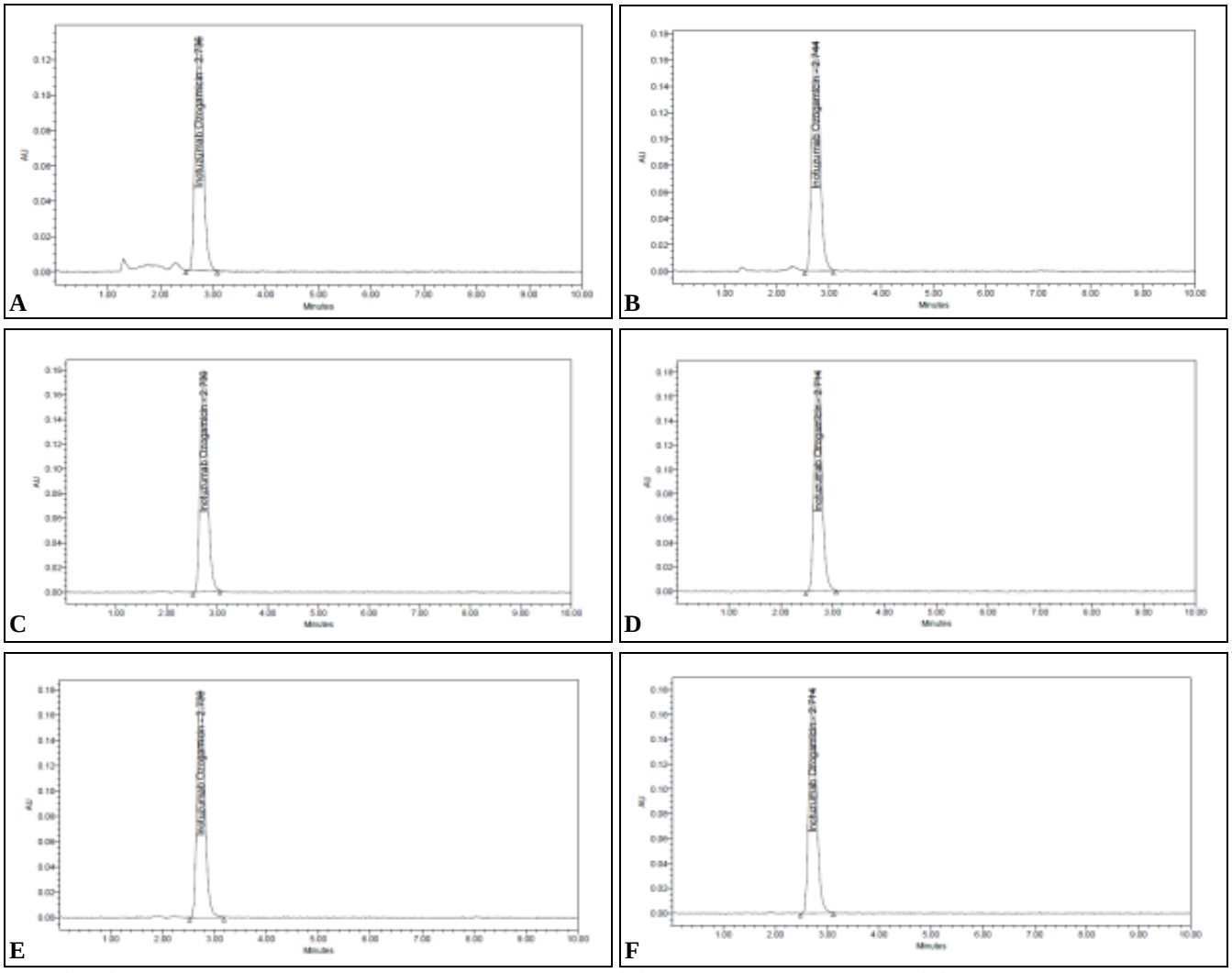

FIG. 5 (A-E): CHROMATOGRAMS OF DEGRADATION (A) ACID DEGRADATION (B) ALKALI DEGRADATION (C) PEROXIDE DEGRADATION (D) THERMAL DEGRADATION (E) PHOTO DEGRADATION (F) HYDROLYTIC DEGRADATION

Dry Heat Degradation Studies: The standard drug solution was subjected to a high temperature of 105 ºC for 6 h. For HPLC study, the resultant solution was making up to final volume to obtain a solution. Cool the solution to room temperature make up to final volume with diluents to get a concentration of 18 µg/ml this concentration injected 6 times to the column and calculate the % degradation.

Photo Stability Studies: The solution is exposed to UV Light by keeping the beaker for 7 days or 200 Watt-hours/m^2 in photostability chamber. For HPLC study, the resultant solution was diluted make up to final volume with diluents to get a concentration of 18 µg/ml and injected 6 times to the column and calculates the % degradation.

Hydrolysis Degradation: To the standard drug solution add 1ml of distilled water and refluxed for 30 min at 60 ºC. Makeup to final volume with diluents to get the concentration of 18 µg/ml this concentration injected 6 times to the column and calculated the % degradation.

Validation of UV Method:

TABLE 9: SUMMARY OF VALIDATION PARAMETERS OF INOTUZUMAB OZOGAMICIN

Parameters	Results
Correlation coefficient	0.999
Accuracy	100.4%
Precision	
Method precision	0.97%
System precision	0.47%
Inter day precision	0.993%
Intraday precision	1.306%
LOD	0.495
LOQ	1.5

RESULTS AND DISCUSSION:

UV Method: The wavelength was selected by preparing a solution of concentration 10µg/ml by diluting the standard solution with distilled water. The solution was scanned in the wavelength range of 200-400 nm using distilled water as a blank. The UV spectrum of Inotuzumab Ozogamicin working showed λ_{max} at 240 nm. The calibration curve was prepared in the concentration range of 25 to 175 µg/ml. Absorbance of each concentration plotted by taking absorbance on y-axis and concentration on the x-axis. The regression equation was calculated, and the results were shown in **Table 9** the regression equation was used to estimate the drug content in the injection dosage form.

Optimization of Chromatographic Conditions: A simple, accurate and precise RP-HPLC method was developed and validated for the simultaneous estimation of Inotuzumab Ozogamicin**.** A mobile phase consisting of 0.1% OPA and acetonitrile in the ratio of 50:50% v/v was set with isocratic programming for 15 min.

Chromatographic conditions were optimized for mobile phase using Discovery C18 250 × 4.6 mm, 5m. Column at a flow rate of 0.8 ml/min. Effluents were detected at 240 nm by variable wavelength PDA detector. Column compartment temperature was in ambient. Chromatogram of Inotuzumab Ozogamicin optimized chromatographic condition was shown in **Fig. 2, Table 1**.

Selectivity: There were no interfering peaks at retention time of Inotuzumab Ozogamicin was observed.

Linearity and Range: The linearity regression coefficient (R^2) values were found to be 0.999 for Inotuzumab Ozogamicin. Regression equation obtained were y = 10345x + 1397. **Fig. 3** and **Table 4**, shows linearity graphs for Inotuzumab Ozogamicin. The method was linear over the range of 4.5 µg/ml to 27 µg/ml of drug.

System Suitability: Six replicates of a standard stock solution containing Inotuzumab Ozogamicin was given to evaluate equipment, electronics, and analytical operations and samples suitability. Parameters calculated for system suitability were some theoretical plates, tailing factor, resolution, retention time, and area. The results as shown in **Table 3** indicates the system is a suitable or proposed method.

LOD and LOQ: The LOD and LOQ were measured, LOD value of Inotuzumab Ozogamicin was found to be 0.033 µg/ml. The LOQ values were found to be 0.099. The statistical data were presented in **Table 3**.

Recovery: The percentage recovery was calculated by preparing standard drug concentrations of Inotuzumab Ozogamicin with concentration levels of 50%, 100%, and 150%. A known amount of the standard drug was added to the pre-analyzed concentration at each level. Good recovery of the spiked drugs was obtained at each added

concentration, and the mean percentage recovery of Inotuzumab Ozogamicin was achieved between 99.29. The results are given in **Table 6**.

Precision: Precision is expressed as the closeness of agreement between a series of measurements obtaining from multiple sampling of the same homogeneous sample. Six replicate injections of a known concentration of Inotuzumab Ozogamicin (9 µg/ml), have been analyzed by injecting them into an HPLC column on the same day and consecutive days. From the results obtained, % RSD was calculated and was found to be within limits <2. The results are given in **Table 5**.

Robustness: Robustness of the proposed analytical method is a measure of its capacity to remain unaffected, and it reflects the reliability of the analysis concerning deliberate changes in the parameters such as flow rate (1.0 ± 0.2 ml/min), column temperature (ambient), and mobile phase ratio. The parameters chosen for the study of robustness is the flow rate and mobile phase composition. From the results obtained, there were no significant changes observed at the end of the study. The results are given in **Table 7**.

Degradation Studies: All the stability studies results were shown in **Table 8** and **Fig. 5 A-E**.

Acid Hydrolysis (Fig. 5A): Upon performance of acid degradation studies 4.86% of Inotuzumab Ozogamicin was degraded.

Base Hydrolysis (Fig. 5B): Upon performance of base degradation studies 3.79% of Inotuzumab Ozogamicin was degraded.

Peroxide Hydrolysis (Fig. 5C): Upon performance of peroxide degradation studies 3.39% of Inotuzumab Ozogamicin was degraded.

Thermal Degradation (Fig. 5D): Upon the performance of Thermal degradation studies 2.77% of Inotuzumab Ozogamicin was degraded.

Photolytic Degradation (Fig. 5E): Upon performance of Photolytic degradation studies 1.62 % of Inotuzumab Ozogamicin was degraded.

Hydrolysis degradation (Fig. 5F): Upon performance of Hydrolysis degradation studies 0.81% of Inotuzumab Ozogamicin was degraded.

CONCLUSION: A simple, fast, accurate and precise stability-indicating UV & RP-HPLC analytical method has been developed and validated for the quantitative analysis of Inotuzumab Ozogamicin in bulk drugs and dosage form. The literature survey revealed that no methods are available for estimation of inotuzumab ozogamicin in bulk drug and dosage form. So, the author attempted to develop a novel method for estimation of inotuzumab ozogamicin. Stress testing is a significant part of drug development process, and the pharmaceutical industry has a lot of interest in this area. The results of stress testing undertake according to the ICH guidelines reveal that the method is specific and stability-indicating. The proposed method can separate this drug from their degradation products in tablet dosage forms and hence can be applied to the analysis of routine quality control samples and samples obtained from stability studies.

ACKNOWLEDGEMENT: Authors are very thankful to the principal, Prof. A. Prameela Rani University College of pharmaceutical sciences, Acharya Nagarjuna University, Guntur, for providing the library facilities for literature survey to carry out entire study. The authors are also thankful to Spectrum Pharma Pvt. Ltd., Hyderabad, India for providing standards and lab facilities.

CONFLICT OF INTEREST: Nil

REFERENCES:

1. www.drugbank.ca/drugs/DB05889
2. ICH guidelines Q3A, Validation of analytical procedures: Definition and terminology Geneva, Switzerland 1995; 68-76.

How to cite this article:
Mastanamma SK, Reehana SK and Hari M: Novel RP-HPLC and UV method development and validation for inotuzumab ozogamicin in bulk and its pharmaceutical dosage form. Int J Pharm Sci & Res 2019; 10(6): 2845-51. doi: 10.13040/IJPSR.0975-8232.10(6).2845-51.