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A validated specific stability-indicating RP-HPLC method for altretamine and its process related impurities

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ABSTRACT

A Validated Specific Stability indicating Reversed-phase Liquid chromatographic method was developed for the quantitative determination of Altretamine and its related substances in bulk samples, pharmaceutical dosage forms in presence of degradation products. Forced degradation studies were performed on bulk sample of Altretamine as per ICH prescribed stress conditions using acid, base, oxidative, water hydrolysis, thermal stress and photolytic degradation to show the stability indicating power of the LC method. Significant degradation in oxidative hydrolysis was observed and no degradation was observed in other stress conditions. The chromatographic method was optimized using the samples generated from forced degradation studies and the impurity spiked solution. Good resolution between the process related impurities and degradation products from the analyte was achieved on Zorbax SB-C18 column using the mobile phase consists a mixture of 10mM ammonium carbonate in water pH adjusted to 8.0 with ammonium hydroxide solution and Methanol in the ratio of 35:65 v/v at detection of 227 nm wavelength. The limit of detection and the limit of quantitation for Altretamine and its process related impurities were established. The stressed test solutions were assayed against the qualified standard of Altretamine and the mass balance in each case was in between 98.7%-99.8% indicating that the developed LC method was Stability-indicating. Validation of the developed LC method was carried out as per ICH requirements. The developed method was found to be suitable to check the quality of bulk samples of Altretamine at the time of batch release and also during its storage and stability studies.

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KEYWORDS

Reversed phase liquid chromatography;
Forced degradation;
RSD and validation;
Altretamine.

INTRODUCTION

Altretamine is described chemically as: N², N², N⁴, N⁴, N⁶, N⁶-hexamethyl-1,3,5-triazine-2, 4, 6-triamine. It is white crystalline powder soluble in chloroform and

insoluble in water. The drug is an anti-neoplastic agent; it is used to treat refractory ovarian cancer^[1]. It is not considered a first-line treatment, but it can be useful as salvage therapy. It also has the advantage of being less toxic than other drugs used for treating refractory ova-

rian cancer^[2,3]. The precise mechanism by which altretamine exerts its anti-cancer effect is unknown but it is classified by MeSH as an alkylating antineoplastic agent^[4]. In one of publication, Determination of altretamine in human plasma with high-performance liquid chromatography^[5] was reported. Based on literature survey the following methods were available, High-Pressure Liquid Chromatography Separation of Potential Impurities of Altretamine^[6], Minicolumn centrifugation-HPLC determination of entrapment efficiency for liposomal formulation of altretamine^[7]. So far all these articles were published on analytical method of Altretamine and its metabolites in biological fluids. Most of the reported methods involve troublesome mobile phase (buffers) and difficult detection methods (fluorescence or mass detectors). Based on the literature survey, there were no stability indicating LC methods published for the determination of Altretamine in bulk sample in the presence degradation products and its process related impurities. The present research work was to develop a suitable stability indicating single LC method for the determination of Altretamine as well its related substances and the developed method was validated with respect to specificity, LOD, LOQ, linearity, precision, accuracy and robustness to show the stability indicating power of the LC method and also to ensure the compliance in accordance with ICH Guidelines^[8].

EXPERIMENTAL

Chemicals and reagents

Samples of Altretamine and its two process impurities (Figure 1) were received from Bulk Actives, Unit-II of Cheminova Laboratories, Hyderabad, India.

HPLC grade Methanol was purchased from Rankem, Mumbai, India.

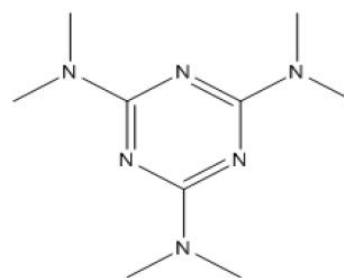
Ammonium carbonate was purchased from Sd. Fine Chemicals, Mumbai, India.

Ammonium hydroxide was purchased from Loba Chemie Mumbai, India.

High pure water was prepared by using Millipore Milli Q plus purification system.

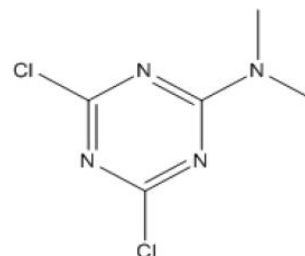
Equipment

The LC method development, validation and forced



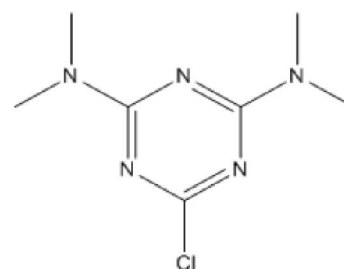
Altretamine

N^2,N^2,N^4,N^4,N^6,N^6 -hexamethyl-1,3,5-triazine-2,4,6-triamine
(Mol. Wt.: 210..28)



Impurity-1

4,6-dichloro-N, N-dimethyl-1,3,5-triazin-2-amine (Mol. Wt.:
193.03)



Impurity-2

6-Chloro- N^2, N^2, N^4, N^4 -tetramethyl-1,3,5-triazine-2,4-diamine (Mol. Wt.: 201.66)

Figure 1 : Chemical structures of altretamine and its process related impurities

degradation studies were done using Waters (Milford, U.S) HPLC system and Shimadzu (Japan) HPLC system with diode array detector. The data were collected and the peak purity of the Altretamine peak was checked using Shimadzu LC solutions software. Stability studies were carried out in a humidity chamber (Thermo Lab, India) and photo stability studies were carried out in a photo stability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (MACK Pharmatech, Hyderabad, India).

Chromatographic conditions

The chromatographic separations were achieved on Zorbax SB-C18 column 250 mm length X 4.6 mm

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ID with 5 μm particle size using the mobile phase: 10 mM ammonium carbonate pH adjusted to 8.0 with ammonium hydroxide solution and Methanol mixed in the ratio of 35:65 v/v. The column temperature was maintained at 25°C and the detection was carried out at 227 nm. The test concentration was about 250 $\mu\text{g mL}^{-1}$ and the injection volume was 10 μL . A degassed mixture of water and methanol in the ratio of 20:80 v/v was used as diluent during the standard and test samples preparations.

Preparation of standard solutions

A working solution of 250 $\mu\text{g mL}^{-1}$ was prepared for the determination of assay and related substances analysis. A stock solution of impurities (impurity-1 and impurity-2) at 100 $\mu\text{g mL}^{-1}$ was also prepared in diluent.

Method validation

Stress studies/specificity

Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule. Specificity is the ability of the method to measure unequivocally the analyte response in the presence of its potential impurities^[9]. All stress degradation studies were performed at initial drug concentration of 250 $\mu\text{g mL}^{-1}$. Acid hydrolysis was performed in 0.5 N HCl at 70°C for 5 days. The study in basic solution was carried out in 0.5 N NaOH at 70°C for 5 days. For study in neutral solution, the drug dissolved in water and was heated at 70°C for 5 days. Oxidation studies were carried out at ambient temperature in 3.0% hydrogen peroxide for 3 days. Photo degradation studies were carried out according to Option 2 of Q1B in ICH guidelines^[10]. The drug sample was exposed to light for and overall illumination of 1.2 million lux hours and an integrated near ultraviolet energy of 200 W h m². The drug sample was exposed to dry heat at 100 °C for 5 days. Samples were withdrawn at appropriate times and subjected to LC analysis after suitable dilution (250 $\mu\text{g mL}^{-1}$) to evaluate the ability of the proposed method to separate Altretamine from its degradation products. Photodiode array detector was employed to check and to ensure the homogeneity and purity of Altretamine peak in all the stressed sample solutions. Assessment of mass balance in the degraded

samples was carried out to confirm the amount of impurities detected in stressed samples matches with the amount present before the stress was applied. Quantitative determination of Altretamine was carried out in all the stressed samples against qualified working standard and the mass balance (% assay + % sum of all impurities + % sum of all degradation products) was tabulated in TABLE 1.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for impurity-1 and impurity-2 were established at a signal-to-noise ratio of 3:1 and 10:1, respectively^[11,12] by injecting a series of diluted solutions with known concentration. Precision study was also carried at the LOQ level by injecting six individual preparations of impurity-1, impurity-2 and calculating the RSD percentage of the area for each impurity.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of the analyte in the sample^[13]. Linearity test solutions for the assay method were prepared from 50 to 150% of with respect to analyte concentration 250 $\mu\text{g mL}^{-1}$ (i.e. 125, 188, 250, 313 and 375 $\mu\text{g mL}^{-1}$) respectively. The peak area versus concentration data was performed by least-squares linear regression analysis. Linearity test solutions for related substance method were prepared by diluting the impurity stock solution to the required concentrations. The solutions were prepared at six concentration levels from LOQ to 200% with respect to the impurities specification level of 0.10% (i.e. LOQ, 0.05, 0.075, 0.10, 0.125, 0.15 and 0.2%). The calibration curve was drawn by plotting the peak areas of impurity-1 and impurity-2 versus its corresponding concentration. Linearity test was performed for two consecutive days in the same concentration range for both assay and related substance method. The correlation coefficient of the calibration curve was calculated.

Precision

Assay method precision was evaluated by carrying out six independent assays of test sample of Altretamine against qualified working standard and calculated the percentage of RSD. The precision of the related sub-

stance method was checked by injecting six individual preparations of Altretamine spiked with 0.10% level of impurity-1 and impurity-2 with respect to target analyte concentration (i.e. 250 $\mu\text{g mL}^{-1}$). RSD percentage of area for each impurity-1 and impurity-2 was calculated. The intermediate precision of the method was also verified using different analyst, different day and different make instrument in the same laboratory.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found^[14]. The accuracy of the assay method was evaluated in triplicate at three concentration levels i.e. 125, 250 and 375 $\mu\text{g mL}^{-1}$ in bulk drug samples. The percentage recoveries were calculated from the slope and Y-intercept of the calibration curve. Standard addition and recovery experiments were conducted to determine accuracy of the related substance method for the quantification of two impurities in bulk drug samples. The study was carried out in triplicate by spiking each impurity at 0.05, 0.10 and 0.15% in bulk drug sample solution (250 $\mu\text{g mL}^{-1}$). The percentage recoveries for impurity-1 and impurity-2 were calculated from the slope and Y-intercept of the calibration curve

Selectivity

The selectivity of the method was established from the resolution of the drug peak from the nearest peak and also among all the other peaks. All the degradants and impurities were separated amongst as well as from analyte with a resolution greater than 3.9 show the selectivity of the method.

Solution stability and mobile phase stability

The solution stability of Altretamine was carried out by leaving the test solution and the test solution spiking with the impurities at 0.10% level with respect to analyte concentration (250 $\mu\text{g mL}^{-1}$) in a tightly capped volumetric flask at room temperature for 48 h. The solution was assayed at 6 h intervals to the end of the study period, using a freshly prepared standard solution of Altretamine for comparison each time. The mobile phase stability was also investigated by assaying the freshly prepared sample solutions against freshly prepared stan-

dard solutions at 6 h intervals up to 24 h and after at intervals of 24 h up to 5 days. Mobile phase composition and preparation was kept constant during the study period. The % RSD of the assay of Altretamine and impurities was calculated during the time of the mobile phase and solution stability experiments.

Robustness

To evaluate the robustness of the developed LC method, the chromatographic conditions were deliberately altered, System suitability parameters like tailing factor, theoretical plates and the resolution between the impurity-1, impurity-2 and with the analyte peak was evaluated. To study the effect of flow rate on the resolution, the flow rate was altered by 0.2 units i.e. 1.5 and 1.9 mL min^{-1} from the actual flow 1.7 mL min^{-1} . The effect of column temperature on resolution was studied at 20°C and 30°C instead of 25°C. The effect of change in the mobile phase composition by changing the % methanol to 60 and 70 from the actual value 65%. The effect of pH was studied by changing pH by 0.5 units from the actual value 8.0 keeping remaining method conditions were kept constant.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The main target of the chromatographic method is to get the separation of impurity-1 and impurity-2 and the degradation products generated during stress studies from analyte peak. Impurities were co-eluted by using different stationary phases like C8, Cyno, XTerra and Phenyl and different mobile phases containing buffers like Phosphate, Sulphate and acetate with different pH (4–10) and using organic modifiers like Acetonitrile, Methanol and THF in the mobile phase. Apart from the co-elution of impurities, poor peak shapes for some impurities and degradation products were also noticed. Ammonium carbonate buffer pH 8.0 adjusted with ammonium hydroxide solution mixed with Methanol in the ratio of 50:50v/v at 1.0 mL min^{-1} was chosen for initial trial with a 250 mm length X 4.6 mm ID column and 5 μm particle size C18 stationary phase. When impurity spiked sample was injected the resolution between two impurities and analyte was poor and all peaks are eluting at greater retention times (about 30 min) with

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more tailing. To increase the resolution between impurities and to reduce the retention of the analyte peak the % of methanol was increased to 70% from initial value of 50% at this ratio the retention time of analyte was showing about 20 minutes and the impurities slightly separated. Still to reduce the retention time the flow rate of the mobile phase was increased to 1.7 mL min^{-1} in this condition the retention time of analyte was about 12 minutes. At these conditions all the stressed solutions were injected, only degradation observed in oxidative condition and the degradation product formed in this condition was no well separated from the two impurities. To increase the resolution between impurities and degradation product the % of methanol was reduced to 65% from 70%. At these chromatographic conditions all the stressed solutions and impurity spiked solution were injected, all the impurities and degradants were well separated amongst and also from Altretamine. The effect of buffer pH was also studied under the above conditions and it was found that at higher pH the tailing of the Altretamine peak was more and also resolution was poor between impurities and degradants and also from the analyte. The results clearly indicated that on Zorbax SB-C18 column 250 mm length X 4.6 mm ID with $5 \mu\text{m}$ particle size and isocratic mobile phase Ammonium carbonate buffer pH 8.0 adjusted with ammonium hydroxide solution, mixed with Methanol in the ratio of 35:65v/v with run time of 30 minutes at detection wavelength 227 nm, flow rate of 1.7 mL min^{-1} was successful in separation of drug from its impurities and degradation products. Under the above conditions, results were as follows, retention time of Altretamine was around 9.7 min, with a tailing factor of 1.0, number of theoretical plates (N) for the Altretamine peak was 80856 and % RSD for 5 replicate injections was 0.1%. the typical retention times of impurity-1 and impurity-2 were about 3.7 and 5.4 minutes respectively (Figure 2). Peak purity of stressed samples of Altretamine was checked by using a photodiode array detector on Shimadzu LC Solutions, the peak purity index was greater than the threshold limit in all the stressed samples, demonstrating the homogeneity of analyte peak. Accelerated and Long term stability study results as per ICH Q1A (R2) for Altretamine were generated for 12 months by using the developed LC method and the results were well within the limits this further confirms the

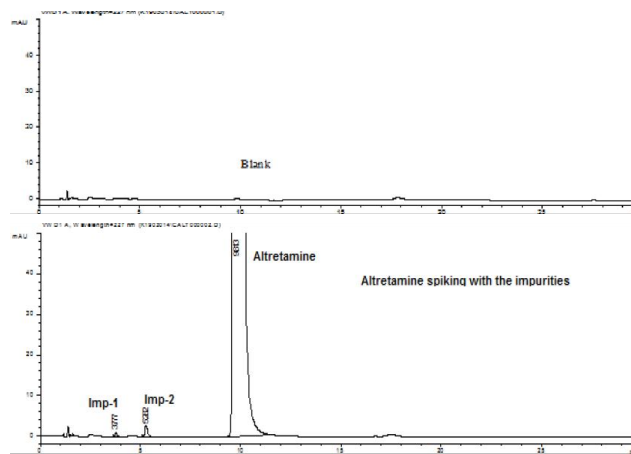


Figure 2: Typical HPLC chromatogram of blank, altretamine spiked with known impurities

stability indicating of the developed LC method.

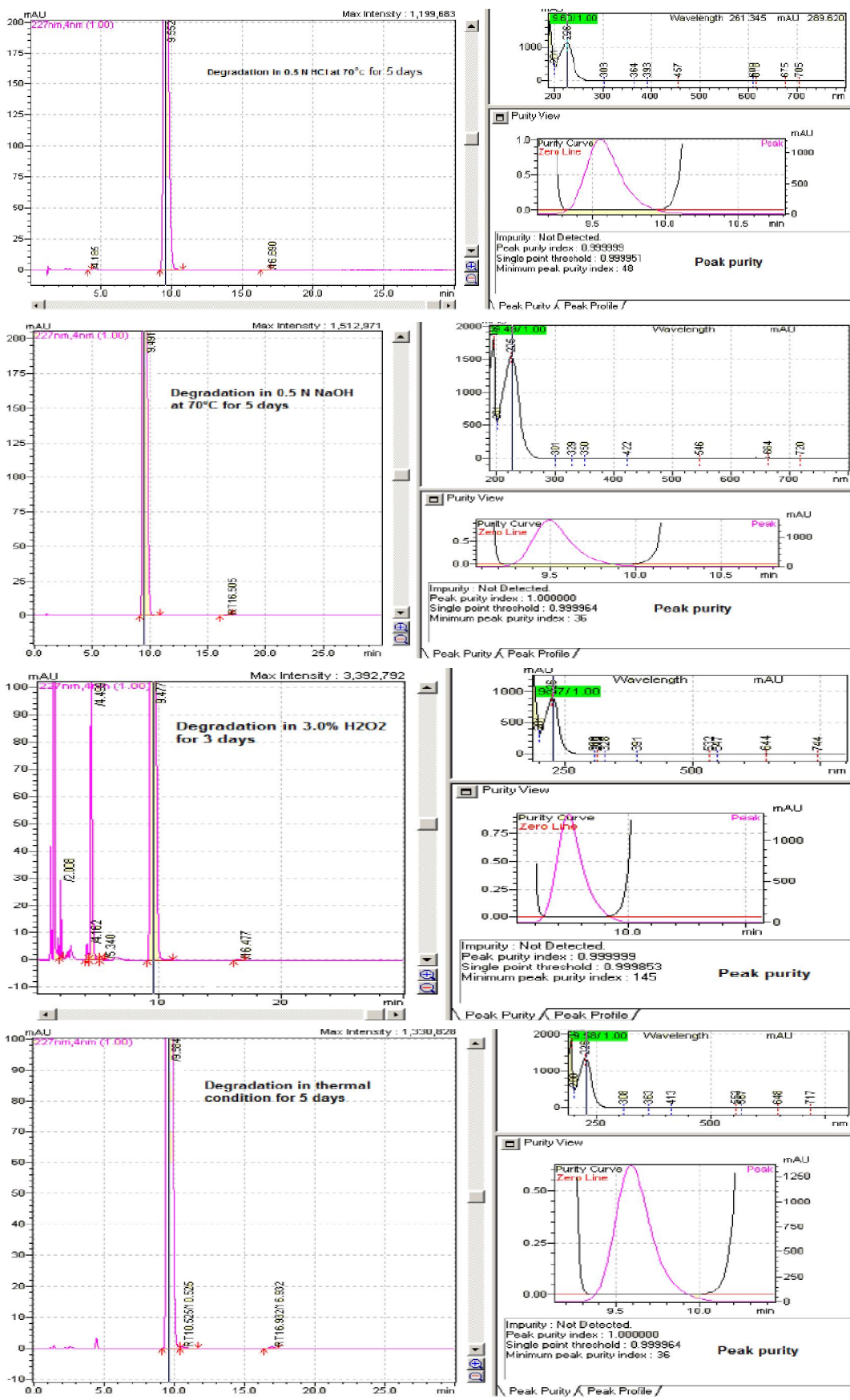
Results of forced degradation

Altretamine was stable under stress conditions such as photolytic stress, acid hydrolysis, basic hydrolysis, water hydrolysis and thermal conditions. While significant degradation of the drug substance was observed under oxidative hydrolysis. In oxidative stress one major unknown degradation peak at 0.47 RRT along with some small degradation products (Figure 3). Peak purity test results obtained from PDA confirm that the Altretamine peak is homogeneous and pure in all the stress samples analyzed. The mass balance is a process of adding together the assay value and the levels of degradation products to see how closely these add up to 100% of initial value with due consideration of the margin of analytical error¹⁵. The mass balance of stressed samples was close to 98.7%-99.8% (TABLE 1). The assay of Altretamine was unaffected by the presence of impurity-1, impurity-2 and degradation products thus confirms the Stability-indicating power of the developed LC method.

Results of method validation

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD of impurity-1 and impurity-2 were 0.004, and $0.009 \mu\text{g mL}^{-1}$ respectively (of analyte concentration $250 \mu\text{g mL}^{-1}$) for $10 \mu\text{L}$ injection volume. The LOQ of impurity-1 and impurity-2 were 0.012 and $0.031 \mu\text{g mL}^{-1}$ respectively (of analyte concentration $250 \mu\text{g mL}^{-1}$) for $10 \mu\text{L}$ injection volume



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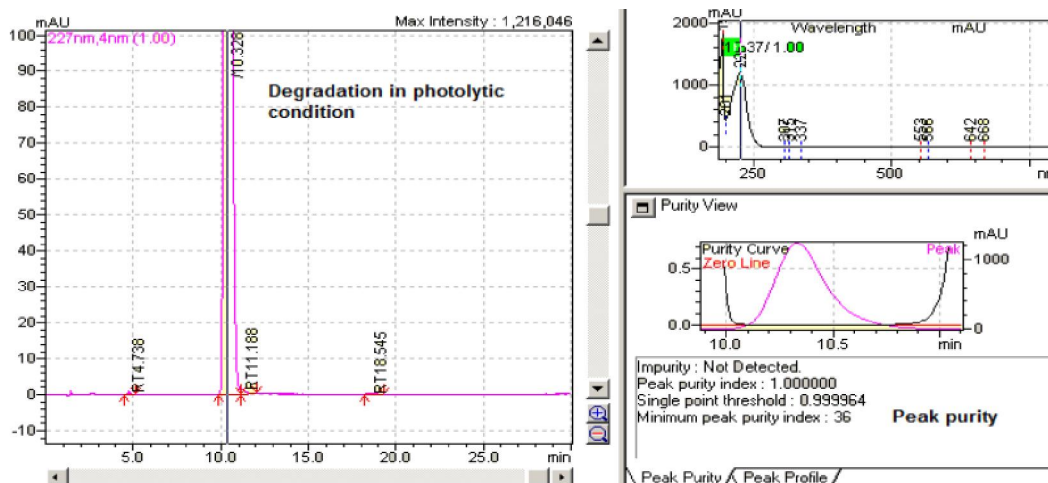


Figure 3 : Typical peak purity HPLC chromatograms of altretamine under various stress conditions (acidic, basic, oxidative, thermal and photolytic) respectively during forced degradation study

TABLE 1 : Results of stress studies

Stress condition	Period of study	Percentage assay of Altretamine	Mass balance (% assay +% sum of all impurities +% sum of all degradants)	Remarks
Acid hydrolysis(0.5 N HCl at 70°C)	5 days	99.2	99.1	No degradation products formed
Base hydrolysis (0.5 N NaOH at 70 °C)	5 days	99.5	99.6	No degradation products formed
Oxidation (3.0% H ₂ O ₂ at ambient temperature)	3 days	92.9	98.7	One major Unknown degradation product formed
Water hydrolysis at 70°C	5 days	99.1	99.2	No degradation observed
Thermal degradation at 100 °C	5 days	98.8	98.8	No degradation products formed
Photolytic degradation	11 days	99.7	99.8	No degradation products formed

Linearity

Linear calibration plot for the assay method was obtained over the calibration ranges tested, i.e. 50 to 150% of assay analyte concentration and the correlation coefficient obtained was greater than 0.9992. Linearity was checked for the assay method over the same concentration range for two consecutive days. The results show that an excellent correlation existed between the peak area and concentration of the analyte. Linear calibration plot for the related substance method was obtained over the calibration ranges tested, i.e. LOQ to 0.20% for impurity-1 and impurity-2. The correlation coefficient obtained was greater than 0.991. Linearity was checked for the related substance method over the same concentration range for two consecutive days. The results demonstrate that an excellent correlation existed between the peak area and concentration of impurity-1 and impurity-2.

Precision

The RSD percentage of assay of Altretamine during assay method precision study was well within 1.0% and the RSD percentage of area of impurity-1 and impurity-2 in related substance method precision study was within 4.5%. The RSD percentage of assay results obtained in the intermediate precision study was within 1.0% and the RSD percentage of area of impurity-1 and impurity-2 were within 6.8%, confirming the good precision of the developed LC method. The method precision for impurity-1 and impurity-2 at LOQ level was below 6.3% RSD.

Accuracy

The percentage recovery of Altretamine in bulk drug samples ranged from 99.3 to 100.2. The percentage recovery of impurity-1 and impurity-2 in bulk drugs samples ranged from 96.7 to 103.7.

TABLE 2 : Results of stability samples (Long term and accelerated conditions) of altretamine (B.No: ALT-004-C07)

Storage condition	Period	Description	Water Content by KF	Related substances by HPLC		Any Unknown impurity	Total impurities	Assay on Anhydrous basis
				Impurity-1	Impurity-2			
				Long term condition				
Temperature 25°C±2°C, Relative humidity 60±5%	Initial	White color crystalline powder	0.7	0.05	0.04	0.02	0.11	99.6
	1 st month	White color crystalline powder	0.4	0.04	0.02	0.02	0.08	100.2
	2 nd month	White color crystalline powder	0.8	0.06	0.04	0.03	0.13	99.3
	3 rd month	White color crystalline powder	0.6	0.05	0.06	0.01	0.12	99.8
	6 th month	White color crystalline powder	0.5	0.07	0.03	0.02	0.12	99.3
	9 th month	White color crystalline powder	0.8	0.04	0.02	0.04	0.10	99.5
	12 th month	White color crystalline powder	0.6	0.06	0.04	0.04	0.14	99.1
	Accelerated condition							
	Temperature 40°C±2°C, Relative humidity 75±5%	Initial	White color crystalline powder	0.7	0.05	0.04	0.02	0.11
1 st month		White color crystalline powder	0.5	0.07	0.03	0.03	0.13	99.3
2 nd month		White color crystalline powder	0.6	0.04	0.06	0.02	0.12	100.1
3 rd month		White color crystalline powder	0.8	0.07	0.03	0.04	0.14	99.1
6 th month		White color crystalline powder	0.7	0.04	0.05	0.03	0.12	99.3

TABLE 3 : Results of assay and related substances for altretamine of three production batches

Batch No:	Description	Water Content by KF	Related substances by HPLC		Any Unknown impurity	Total impurities	Assay on Anhydrous basis
			Impurity-1	Impurity-2			
ALT-076-A12	White color crystalline powder	0.5	0.05	0.03	0.04	0.12	99.3
ALT-111-G01	White color crystalline powder	0.3	0.04	0.06	0.03	0.13	99.4
ALT-142-K17	White color crystalline powder	0.7	0.08	0.03	0.05	0.15	99.0

Robustness

In all the deliberate varied chromatographic conditions (flow rate, column temperature, pH variation) the resolution between Altretamine and impurities as well as degradation products was not significantly affected hence the developed LC method was robust for the determination of Altretamine in bulk samples.

Application of the developed LC method to stability samples and quality monitoring of altretamine

Accelerated and Long term stability studies are carried out to establish retest period or a shelf life of

drug product, to know the effect of storage conditions at different atmospheric conditions and to show the stability indicating of the method^[16]. Altretamine samples stored at Long term condition (Temperature: 25°C±2°C, Relative humidity 60±5%) Accelerated (Temperature: 40°C±2°C, Relative humidity 75±5%) were analyzed by using the developed LC method for period of one year at different intervals i.e; Initial, 1, 2, 3, 6, 9, and 12 months (TABLE 2). And also by using the developed LC method quality of the Altretamine was monitored during production

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of three batches (TABLE 3). The Results clearly indicates that the drug was stable under Long term and Accelerated conditions and there were no interference of the impurities for Altretamine which demonstrates that developed LC method was stability indicating and well applied for drug stability study as well as to quality monitoring of Altretamine.

CONCLUSION

In this paper a specific, validated and well-defined single Stability-indicating LC method for the quantitative determination of Altretamine as well as its related substances was described and the behavior of Altretamine under various stress conditions was studied and presented. All the degradation products formed during stress conditions and process impurities were well separated from the drug substance indicates the developed LC method was a simple accurate, specific and stability indicating. The less concentration (10mM) of buffer in the mobile phase is volatile hence can be easily used to run the samples in LC-MS. The information presented here in could be very useful for quality monitoring of bulk samples and as well employed to check the quality of drug during stability studies.

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