A validated stability-indicating RP-HPLC assay method for amsacrine and its related substances

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Received: 14th July, 2010 ; Accepted: 24th July, 2010

ABSTRACT

A validated specific stability indicating reversed-phase HPLC method was developed for the quantitative determination of Amsacrine as well as its related substances determination in bulk samples, in presence of degradation products and its process related impurities. Forced degradation studies were performed on bulk sample of Amsacrine as per ICH prescribed stress conditions using acid, base, oxidative, thermal stress and photolytic degradation to show the stability indicating power of the method. Significant degradation was observed during basic hydrolysis, slight degradation was observed in oxidative and thermal stress 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 peaks corresponds to process related impurities and degradation products from the analyte were achieved on Inertsil ODS column using the mobile phase consists a mixture of 1.0% Triethyl amine in 20 mM Potassium dihydrogen Orthophosphate pH adjusted to 6.5 with ortho phosphoric acid in water and Acetonitrile using a simple linear gradient. The detection was carried out at wavelength 248 nm. The mass balance in each case was in between 99.4% to 99.9% 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 Amsacrine at the time of batch release and also during its stability studies.   © 2011 Trade Science Inc. - INDIA

INTRODUCTION

Amsacrine is described chemically as: N-(4-(acridin-9-ylamino)-3-methoxyphenyl)methanesul-fonamide. The drug is an antineoplastic agent which inhibits and combats the development of tumors. Its planar fused ring system can intercalate into the DNA of tumor cells, thereby altering the major and minor groove

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proportions. Amsacrine also inhibits topoisomerase II activity lead to S phase and G2 arrest[1,2] and may exert an effect on cell membranes[3]. This drug also possesses immunosuppressive and antiviral properties[1] and is a chemotherapy drug used to treat some types of cancers including acute adult leukaemia[4].

According to current good manufacturing practices all drugs must be tested with a stability indicating assay method before release. Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability-indicating power of the analytical procedures used. An LC method has been developed for the determination of amsacrine (AMSA in human nucleated hematopoietic cells was available in literature[5,6]. To our knowledge, there was no stability indicating HPLC methods for quantification of Amsacrine is available in the literature. The present research work was to develop a suitable stability indicating LC method for the determination of Amsacrine 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 method and also to ensure the compliance in accordance with ICH Guidelines[7]. The developed method was applied for analysis of drug stored at long term and accelerated stability studies to show stability indicating of the method.

EXPERIMENTAL

Chemicals and reagents

Samples of Amsacrine and its three process impurities (Figure 1) were received from Bulk Actives, Aurbind pharmaceuticals Ltd, Hyderabad, India.

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

Potassium dihydrogen orthophosphate was purchased from Qualigens Fine Chemicals, Mumbai, India.

Triethylamine was purchased from Loba Chemie Mumbai, India.

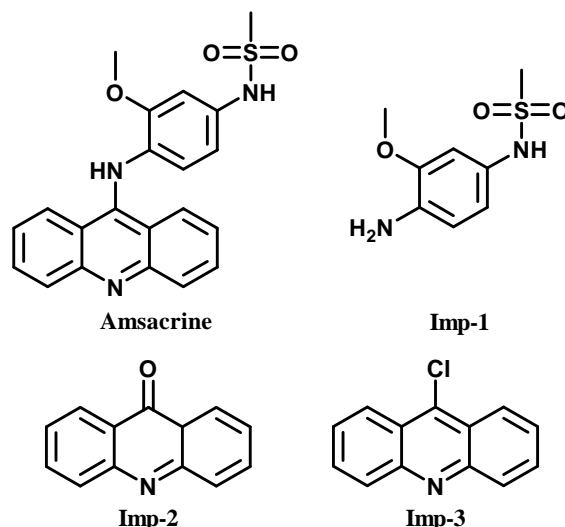High pure water was prepared by using Millipore Milli Q plus purification system.

Chemical names

i) **Amsacrine:** N-(4-(acridin-9-ylamino)-3-methoxyphenyl) methane sulfonamide (Mol.Wt.: 393.12)

ii) **Imp-1:** N-(4-amino-3-methoxyphenyl) methane sulfonamide (Mol. Wt.: 216.22)

iii) **Imp-2:** Acridin-9(8aH)-one (Mol. Wt.: 195.11)

iv) **Imp-3:** 9-chloroacridine (Mol. Wt.: 213.03)

Figure 1 : Chemical structures of amsacrine and its process related impurities.

Equipment

The LC method development, validation and forced degradation studies were done using Waters HPLC system with diode array detector. The data were collected and the peak purity of the Amsacrine peak was checked using millennium 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 Inertsil ODS column 150 mm length X 4.6 mm ID with 5 μm particle size using the solvent A as 20 mM Potassium dihydrogen Orthophosphate with 1.0 % Triethyl amine pH adjusted to 6.5 with ortho phosphoric acid in water and ACN in the ratio of 90:10 % v/v and solvent B as Acetonitrile with a gradient programme: Time (min)/% solvent B, 0/15, 15/30, 30/80, 35/80 with a post run time of 5 minutes. The column temperature was maintained at 25°C and the detection was carried out at 248 nm. The test concentration was about 300 μg mL^{-1} and the injection volume was 20 μL for related substances and 10 μL for assay determination.

A degassed mixture of water and acetonitrile in the ratio of 50:50 % v/v was used as diluent during the standard and test samples preparations.

Preparation of standard solutions

Standard and sample solutions of Amsacrine were prepared separately at about 300 µg mL^{-1} in diluent for the determination of assay and related substances analysis. A stock solution of impurities (imp-1, imp-2 and imp-3) at 300 µg mL^{-1} concentration was 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 the analyte response in the presence of its potential impurities/degradation products[8]. All stress degradation studies were performed at an initial drug concentration of 300 µg mL^{-1}. Acidic stress was performed in 0.5 N HCl at 60°C for 5 days.

The study in basic solution was carried out in 0.001 N NaOH at ambient temperature for 10 hours. Oxidation studies were carried out at ambient temperature in 3 % hydrogen peroxide for 3 days. Photo degradation studies were carried out according to Option 2 of Q1B in ICH guidelines[9]. 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 80 °C for 10 days. Samples were withdrawn at appropriate times and subjected to LC analysis after suitable dilution (300 µg mL^{-1}) to evaluate the ability of the proposed method to separate Amsacrine from its degradation products. Photodiode array detector was employed to check, to ensure the homogeneity and purity of Amsacrine 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 Amsacrine 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.

TABLE 1 : Results of stress studies

Stress condition	Period of Study	Percentage assay Amsacrine	Mass balance (% assay+% mpurities +%degradnats)	Remarks
0.5 N HCl (acidic)	5 days	99.8	99.7	No degradation products formed
0.001 N NaOH (Basic)	10 hours	76.4	99.4	One Major degradation product formed at 1.22 RRT*
3% H2O2 (Oxidative)	3 days	97.4	99.8	Two Major degradation Products formed at 0.42, 0.74 RRT
Thermal degradation at 80 °C	10 days	99.1	99.5	Mild degradation was observed
Photolytic Degradation	11 days	99.8	99.9	No degradation products formed

*RRT-relative retention time

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

The LOD and LOQ for imp-1, imp-2 and imp-3 were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively,[10,11] 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 imp-1, imp-2 and imp-3 and calculating the RSD percentage of the area.

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[12]. Linearity test solutions for the assay method were prepared from 50 to 150% of with respect to analyte concentration 300 µg mL^{-1} (i.e. 150, 225, 300, 375 and 450 µg 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 cali-

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bration curve was drawn by plotting the peak areas of imp-1, imp-2 and imp-3 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 Amsacrine against qualified working standard and calculated the percentage of RSD.The precision of the related substance method was checked by injecting six individual preparations of Amsacrine spiked with 0.10% level of imp- 1, imp-2 and imp-3 with respect to target analyte concentration (i.e. 300 µg mL^{-1}). RSD percentage of area for each imp-1, imp-2 and imp-3 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[13]. The accuracy of the assay method was evaluated in triplicate at three concentration levels i.e. 150, 300 and 450 µg mL^{-1} in bulk drug samples. The percentage recoveries were calculated from the slope and Y-intercept of the calibration curve. The bulk samples available for development work, does not show the presence of imp-1, imp-2 and imp-3. Standard addition and recovery experiments were conducted to determine accuracy of the related substance method for the quantification of all three 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 (300 µg mL^{-1}). The percentage recoveries for imp-1, imp-2 and imp-3 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 Amsacrine 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 12 show the selectivity of the method.

Solution stability and mobile phase stability

The solution stability of Amsacrine was carried out by leaving the test solution 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 Amsacrine for comparison each time. The mobile phase stability was also investigated by assaying the freshly prepared sample solutions against freshly prepared standard solutions at 6 h intervals up to 24 h. Mobile phase composition and preparation was kept constant during the study period. The % RSD of the assay of Amsacrine was calculated during the duration of the mobile phase and solution stability experiments.

Robustness

To evaluate the robustness of the developed method, the chromatographic conditions were deliberately altered and the resolution between closely eluting impurity i.e; imp-2 and Amsacrine was evaluated.

To study the effect of flow rate on the resolution, the flow rate was altered by 0.2 units i.e. 0.8 and 1.2 mL min^{-1} from the actual flow 1.0 mL min^{-1}. The effect of column temperature on resolution was studied at 20°C and 30°C instead of 25°C. All the other mobile phase components were held constant as described above. The effect of pH was studied by changing pH by 0.5 units from the value 6.5 keeping remaining method conditions were constant.

RESULTS AND DISCUSSION

Development and optimization of HPLC method

The main target of the chromatographic method is to get the separation of imp-1, imp-2, imp-3 and the degradation products generated during stress studies from analyte peak. As the pKa value of Amsacrine was 7.4, Potassium dihydrogen Orthophosphate buffer with pH 6.5 adjusted with KOH as solvent A and Acetonitrile as solvent B at 1.0 mL min^{-1} was chosen for initial trail with a 150 mm length X 4.6 mm ID column and 5 µm particle size C18 stationary phase. When impurity spiked sample solution was injected the resolution between impurities and analyte was poor and the retention of Amsacrine was very less. To get the good resolution of impurities from analyte and to increase the retention, in the phosphate buffer 1.0 mL of Triethyl amine added and pH adjusted to 6.5 using phosphoric acid.

At these conditions the impurities were separated and stressed solutions were injected .The degradation product formed at 0.74 RRT in oxidative hydrolysis was not well separated from imp-2 at RRT 0.70 although it was separated from main analyte.Separation of imp-2(0.70 RRT) and degradation product (0.74 RRT) was achieved when solvent A was mixed with Acetonitrile in the ratio of 90:10 v/v with some change in gradient programme. At these chromatographic conditions all the impurities and degradants were well separated amongst and also from Amsacrine.The effect of buffer pH was also studied under the above conditions and it was found that at higher and lower pH the tailing of the Amsacrine peak was more and also resolution was poor between impurities and degradants and also from the analyte.The results clearly indicated that on Inertsil ODS 150 mm length X 4.6 mm ID with 5 µm particle size and solvent A as 20 mM Potassium dihydrogen Orthophosphate with 1.0 % Triethyl amine pH adjusted to 6.5 with phosphoric acid in water and Acetonitrile in the ratio of 90:10 % v/v and solvent B as Acetonitrile with a gradient programme: Time (min)/% solvent B, 0/15, 15/30, 30/80, 35/80 with a post run time of 5 minutes at detection wavelength 248 nm was successful in separation of Amsacrine from its impurities and degradation products. Under the above conditions, results were as follows, retention time of Amsacrine was around 16.9 min with a tailing factor of 1.02, number of theoretical plates (N) for the Amsacrine peak was 45256, % RSD for 5 replicate injections was 0.2.and all the impurities were well separated with a resolution greater then 14 from the Amsacrine peak (Figure 2, II). Peak purity of stressed samples of Amsacrine was checked by using a photodiode array detector of waters with millennium software, the purity threshold is greater than purity angle in all the stress samples, demonstrating the homogeneity of analyte peak. Accelerated and long term stability study results as per ICH Q1A (R2) for Amsacrine were generated for 6 months by using the developed LC method and the results were well within the limits this further confirms the stability indicating of the developed LC method.

Results of forced degradation

Amsacrine was stable under stress conditions such as photolytic stress, acid stress conditions While significant degradation of the drug substance was observed under basic hydrolysis leads to formation of one major

degradation product at 1.22 RRT and oxidative hydrolysis leads to the formation of two major unknown degradation peaks at 0.42 RRT, at 0.74 RRT along with some small degradation products (Figure 2 III, IV). Slight degradation was observed in thermal stress conditions. From the peak purity test results obtained in the test samples, the purity threshold is greater than purity angle; confirms that the Amsacrine 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 error[14]. The mass bal-
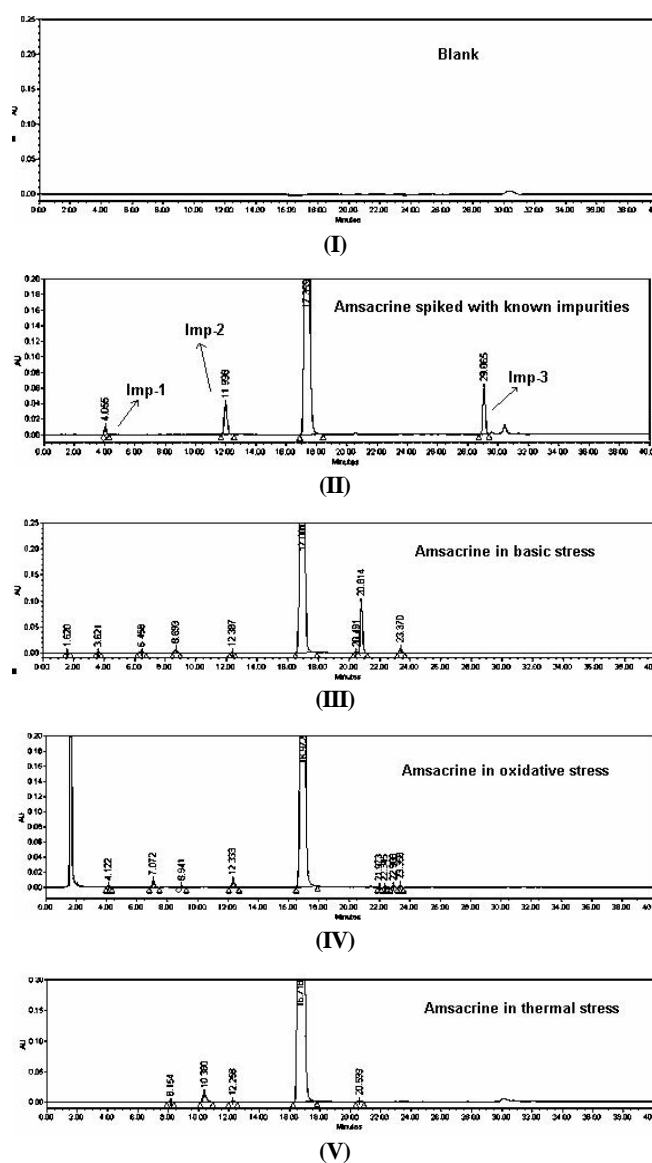

Figure 2 : Typical HPLC chromatograms of blank (I), amsacrine spiked with impurities (II), amsacrine in basic stress (III), oxidative stress (IV) and thermal stress (V).

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ance of stressed samples was in between close to 99.4% to 99.9% (TABLE 1). The assay of Amsacrine was unaffected by the presence of imp-1, imp-2 and imp-3 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 quantitation (LOQ)

The LOD of imp-1, imp-2 and imp-3 were 0.05, 0.06 and 0.04 µg mL^{-1} respectively (of analyte concentration i.e.300 µg mL^{-1}) for 20 µL injection volume. The LOQ of imp-1, imp-2 and imp-3 were 0.15, 0.19 and 0.12 µg mL^{-1} respectively (of analyte concentration. 300 µg mL^{-1}) for 20 µL injection volume.

Linearity

Linearity was established by least squares regression analysis of the calibration curve .Calibration curve for the assay method was obtained over the calibration ranges tested, i.e. 150 µg mL^{-1} to 450 µg mL^{-1} of assay analyte concentration and the correlation coefficient(r) obtained was greater than 0.9998. 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 0.6 µg mL^{-1} for imp-1, imp-2 and imp-3. The correlation coefficient(r) obtained was greater than 0.9988. 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 imp-1, imp-2 and imp-3.The regression equations were as follows

y = 188117x+80.146, r = 0.9998 for Amsacrine
y = 68457x+30.675, r = 0.9997 for imp-1
y = 134757x-127.68, r = 0.9988 for imp-2
y = 687561x-246.84, r = 0.9989 for imp-3

Precision

The RSD percentage of assay of Amsacrine during assay method precision study was well within 1.0% and the RSD percentage of area of imp-1, imp-2 and imp-3 in related substance method precision study was within 11%. The RSD percentage of assay results

obtained in the intermediate precision study was within 1.0% and the RSD percentage of area of imp-1, imp-2 and imp-3 were within 8%, confirming the good precision of the developed method. The method precision for imp-1, imp-2 and imp-3 at LOQ level was below 12% RSD.

Accuracy

The percentage recovery of Amsacrine in bulk drug samples ranged from 99.4 to 100.5%. The percentage recovery of imp-1, imp-2 and imp-3 in bulk drugs

TABLE 2 : Results of accuracy for related substances and assay

Compound	Level %	Spiked quantity (µg mL^{-1})	Recovered quantity (µg mL^{-1})	%Recovery
Amsacrine	50	155	154	99.4
	100	306	308	100.7
	150	459	458	99.8
Imp-1	0.05	0.149	0.147	98.7
	0.10	0.305	0.307	100.7
	0.15	0.454	0.435	95.8
Imp-2	0.05	0.151	0.153	101.3
	0.10	0.301	0.295	98.0
	0.15	0.447	0.427	95.5
Imp-2	0.05	0.152	0.144	94.7
	0.10	0.304	0.299	98.4
	0.15	0.468	0.458	97.9

samples ranged from 93.7 to 102.9% and the results are tabulated in TABLE 2.

Robustness

In all the deliberate varied chromatographic conditions (flow rate, column temperature, pH variation) the

TABLE 3 : Results of robustness of the method

Parameter	Modification	Resolution between Imp-2 and Amsacrine	USP tailing factor for Amsacrine	% Recovery for Amsacrine
pH	6.3	13.5	1.21	99.6
	6.5	14.2	1.02	99.8
	6.7	12.5	1.13	100.1
Buffer	15	11.5	1.3	99.8
	20	14.9	1.01	100.6
	25	13.3	1.1	100.2
Flow	0.9	15.3	1.25	99.2
	1.0	14.7	1.03	100.3
	1.1	12.8	0.98	99.5

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resolution between Amsacrine and imp-2 as well as with other impurities was not significantly affected hence the developed LC method was robust for the determination of Amsacrine in bulk samples. The results tabulated in TABLE 3.

APPLICATION OF THE DEVELOPED HPLC METHOD TO STABILITY SAMPLES AND QUALITY MONITORING OF AMSACRINE

Accelerated and Long term stability studies are carried out to establish retest period or a shelf life of drug product[15]. Amsacrine samples stored at Long term condition (Temperature:30°C±2°C,Relative humidity 60±5%) Accelerated condition (Temperature: 40°C±2°C, Relative humidity 75±5%) were analyzed by using the developed LC method for period of 6 months at different intervals i.e; Initial, 1, 2, 3, and 6 months. And also by using the developed LC method quality of the Amsacrine was monitored during production of three batches. The Results of formal stability studies clearly indicates that the drug was stable under Long term and Accelerated conditions and there were no interference of the impurities for Amsacrine which demonstrates that developed LC method was stability indicating and well applied for drug stability study as well as to quality monitoring of Amsacrine.

CONCLUSIONS

In this paper a simple validated and well-defined specific stability indicating HPLC method for the determination of Amsacrine as well as its related substances was described and the behavior of Amsacrine under various stress conditions was studied and presented. All the degradation products and process impurities were well separated from the drug Amsacrine demonstrates the stability indicating of the method. 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.

ACKNOWLEDGMENTS

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