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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR THE ASSAY OF MEDETOMIDINE

Nuzhath Fathima*[1], P. Raveendra Reddy[2], M. Brahmaiah[2] and G. Abdul Huq[1]

*[1]Department of Chemistry, Rayalaseema University, Kurnool-518 007, AP, India.

[2]Department of Chemistry, Sri Krishnadevaraya University, Anantapur-515003, AP, India.

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***Corresponding Author'**
Nuzhath Fathima
Department of Chemistry,
Rayalaseema University,
Kurnool-518 007, AP, India.

ABSTRACT

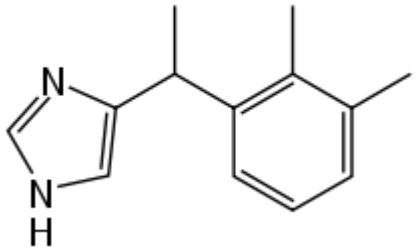
A simple and rapid RP-HPLC method is developed for the determination and assay of medetomidine. The IUPAC names of medetomidine is (*RS*)-4-[1-(2, 3-dimethylphenyl) ethyl]-3*H*-imidazole. Due to high efficiency and suitability for polar moieties an X-terra RP-18(250X4.6) 5 µm column is selected for chromatographic separation using gradient program. The flow rate of the mobile phase was 1.5 ml/min, column is maintained at 40^0C and the wavelength detection is 220 nm. The injection volume is 20 µl and the data acquisition time is 14 min. The typical retention time of Medetomidine is 7.53 minutes The developed method is validated in terms of system suitability, specificity, linearity range, precision, accuracy, robustness, limits of detection (LOD) and quantification (LOQ) for medetomidine following the ICH guidelines. The proposed method is therefore suitable for the determination and assay of medetomidine by RP-HPLC method.

KEYWORDS: Medetomidine, Method development and validation, X-terra RP-18, RP-HPLC.

INTRODUCTION

Medetomidine is a synthetic drug as both a surgical anesthetic and synthetic analgesic often used as the hydrochloride salt.[1] It is chemically (*RS*)-4-[1-(2, 3-dimethylphenyl) ethyl]-3*H*-imidazole, its empirical formula is $C_{13}H_{16}N_2$.HCl, with molecular weight of 200.27954. It is a crystalline white alpha-two adrenergic agonist that can be administered as an intravenous drug solution with sterile water Medetomidine hydrochloride is a potent and highly selective

α2-AR adrenoceptor agonist (K_i values are 1.08 and 1750 nM for α2- and α1-adrenoceptors respectively).



It shows greater selectivity over α1-adrenoceptors than clonidine and UK 14,304 (1620-, 220- and 300-fold respectively). Medetomidine is freely soluble in methanol, acetonitrile, water (100 mM), and slightly soluble in ether. Medetomidine is desiccated at room temperature. It is available as both crystalline and amorphous forms. Amorphous forms are generally more unstable than crystalline forms.[2,3] Medetomidine hydrochloride is an alpha-two adrenergic agonist used as both a surgical anesthetic and analgesic in small animal practice (dogs). The drug was developed by Orion Pharma and is currently approved for dogs in the United States, and distributed in the United States by Pfizer Animal Health and by Novartis Animal Health in Canada under the product name Domitor. Medetomidine hydrochloride is a racemic mixture, with one of the enantiomers, dexmedetomidine has been shown to have more useful effects, and is now marketed as Dexdomitor.[4]

Medetomidine can be used as an antifouling substance in marine paint. It is mainly effective against barnacles, but has also shown effect on other hard fouling like tube worms. When the barnacle cyprid larva encounters a surface containing medetomidine the molecule enters the octopamine receptor in the larva. This makes the larva legs start kicking and it cannot settle to the painted surface. When the larva swims away from the surface, the effect disappears (reversible effect). The larva regains its function and can settle somewhere else.

Literature survey revealed, LC-MS method has been reported for the quantitative determination of medetomidine.[5] No methods are reported for the quantification of medetomidine in pharmaceutical preparations. UV-spectrophotometry, HPLC methods are reported for enantimeric separation of medetomidine.[6] So far no method is reported for determination of medetomidine. The reported methods of medetomidine are not quantifying medetomidine without interference from the other impurities. The present research work is to develop a single stability indicating HPLC method, validated with respect to specificity,

LOD, LOQ, linearity, precision, accuracy and robustness.[7-13] The present paper reports the various aspects relating to the development and validation of stability indicating HPLC method for assay of Medetomidine as per ICH guidelines.[14-16] However, the present authors have already reported the Development and Validation of RP-HPLC method for determination of related substances of Medetomidine in bulk form.[17]

MATERIALS AND METHODS

Instrumentation and software

SHIMADZU 2010 series prominence High Performance Liquid Chromatography with binary pumping, PDA system, with LC Solution software is used for the studies.

Chemicals and Reagents

All the reagents used are of analytical reagent grade unless stated otherwise. Distilled and deionized HPLC grade water, HPLC grade acetonitrile, ammonium chloride, ammonia, NaOH, HCl, H_2O_2 and methanol are obtained from Merck, Mumbai. A sample of Medetomidine is gift sample of Shakhty chemical labs, Hyderabad, India. High purity water is prepared by using Millipore milli Q plus purification system.

The suitable wavelength for the determination of Medetmidine in diluent is identified by scanning over the range 200–400 nm with a UV – 160 Perkin Elmer double beam spectrophotometer with UV detector.

Chromatographic conditions

The effective separation was achieved on an X-terra RP-18(250X4.6)5µm column using a gradient mode by the mobile phase A: 10mL/molar ammonium chloride and pH adjusted to pH = 9.2 with ammonia and mobile phase B: acetonitrile: methanol (65:35).The flow rate of the mobile phase was 1.5 mL /min and the total elution time including the column equilibration was approximately 60.01 minutes. The UV detection was carried at wavelength 220nm and experiments were conducted at 40^0 C. the ejection volume is 20µl. The gradient program is given in Table 1.

Table 1: Gradient program.

Time (Minutes)	Solution A (%)	Solution B (%)
0.01	65	35
05	65	35
7	80	20
10	80	20
15	60	35

Preparation of standard solutions

Weigh and transfer 10.0 mg of Medetomidine standard into a 10mL of volumetric flask and dissolve with diluents (Acetonitrile: Methanol (65:35). Dilute 1.0mL of this solution to 100.0mL with diluent. Further, dilute 1.0 mL of this solution to 10.0 mL with diluents. The Specimen overlay chromatogram of diluent and standard is shown in Fig. 1.

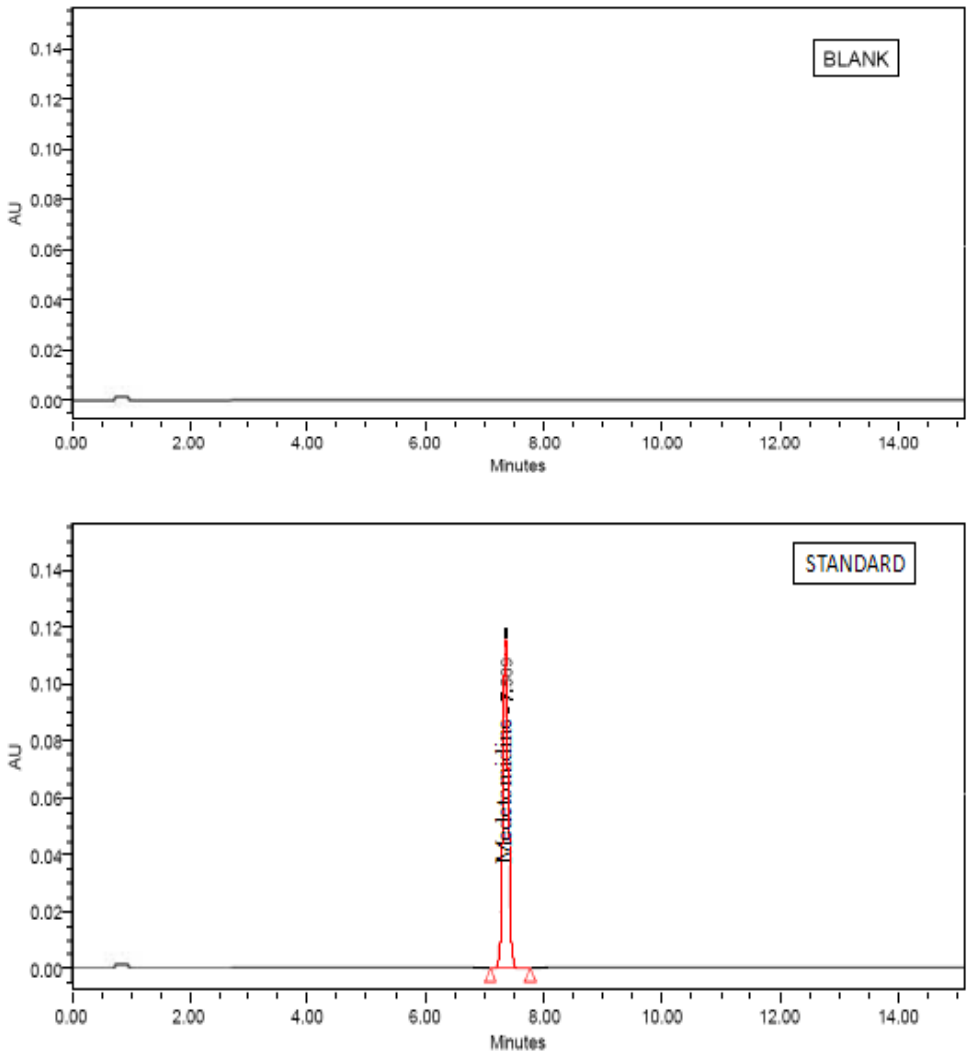


Fig. 1: Specimen chromatogram of diluent and Medetomidine standard.

Preparation of sample solutions

Weigh and transfer 10.0 mg of Medetomidine standard into a 10 mL volumetric flask and dissolve with diluents (Acetonitrile: Methanol (65:35).The Specimen overlay chromatogram of placebo and test samples is shown in Fig. 2.

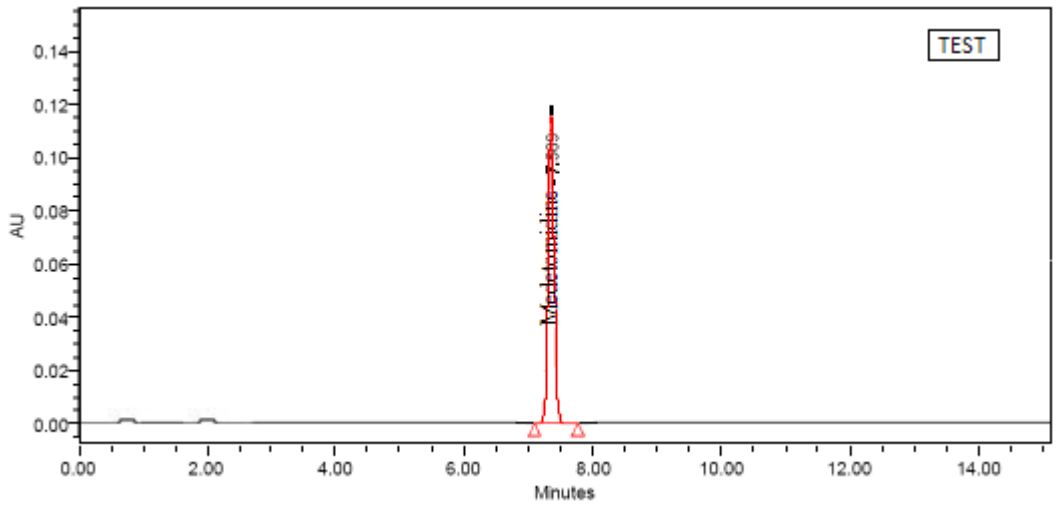


Fig 2: Specimen chromatogram of Medetomidine test solution

Specificity

Regulatory guidance in ICH Q2A, Q2B, Q3B and FDA 21 CFR section 211, require the development and validation of stability-indicating potency of assays. However, the current guidance documents do not indicate detailed degradation conditions in stress testing. The forced degradation conditions, stress agent concentration and time of stress, are found to effect the % degradation. Preferably not more than 20% is recommended for active materials to make the right assessment of stability indicating nature of the chromatographic methods. The optimization of such stress conditions which can yield not more than 20% degradation is based on experimental studies. Chromatographic runs of placebo solution and samples subjected to force degradation are performed in order to provide an indication of the stability indicating properties and specificity of the method. The stress conditions employed are acid, base, neutral and oxidant media, moisture, heat and light. After the degradation is completed, the samples are allowed to equilibrate to room temperature, neutralized with acid or base (as necessary), and diluted with diluent. The samples are analyzed against a freshly prepared control sample (with no degradation treatment) by using photo diode array detector. Specific conditions are described below.

Preparation of test solution for degradation with acid

2 mg ml^{-1} Medetomidine in a round bottom flask added 5 ml of 0.1N HCl and fixed a condenser. Heated to 60^0C and maintained between 60 - 65^0C under reflex for 12 hours. Cool and room temperature and transferred the solution to 100 ml volumetric flask dissolved and made up with the diluent up to mark and filtered by using 0.45 µm nylon 66 membrane filter.

Preparation of test solution for degradation with Alkali

2 mg ml^{-1} Medetomidine in a round bottom flask added 5 ml of 0.1N NaOH and fixed a condenser. Heated to 60^0C and maintained between 60 - 65^0C under reflex for 12 hours. Cool and room temperature and transferred the solution to 100 ml volumetric flask dissolved and made up with the diluent up to mark and filtered by using 0.45 µm nylon 66 membrane filter.

Preparation of test solution for degradation with Hydrogen Peroxide

2 mg ml^{-1} Medetomidine in a round bottom flask added 5 ml of 3%H_2O_2 and fixed a condenser. Heated to 60^0C and maintained between 60 - 65^0C under reflex for 12 hours. Cool and room temperature and transferred the solution to 100 ml volumetric flask dissolved and made up with the diluent up to mark and filtered by using 0.45 µm nylon 66 membrane filter.

Preparation of test solution for degradation with Heat

5gms of Medetomidine sample in a cleaned petri dish and kept the dish in a humidity chamber which is at a temperature of 40^0C and a RH of 75%. Then, the samples are prepared in diluent as described in the test preparation.

Preparation of test solution for degradation with UV and visible light

5gms of Medetomidine sample in a cleaned petri dish and kept the dish under the UV light for 6 hours. After exposure the samples are prepared in diluent as described in test preparation.

METHOD VALIDATION

Precision

Precision (intra-day precision) of the assay method is evaluated by carrying out six independent assay test sample of Medetomidine capsules against qualified reference standard. The % of RSD of six assays obtained is calculated. The intermediate precision (inter-day precision) of the method is also evaluated using two different HPLC systems and different HPLC columns in different days in the same laboratory.

Linearity and range

The linearity of Medetomidine impurities was also studied by preparing standard solutions at 16 different levels. The linearity of an analytical method is its ability to elicit test results that are directly, or by a well defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. The linearity was verified with Medetomidine standard and an impurity in the range of LOQ to 150% of specification limit. The area response for each level was recorded and the slope, intercept & correlation coefficient were calculated. These were evaluated by injecting three replicate injections.

Accuracy

A study of recovery of Medetomidine from spiked placebo is conducted. Samples are prepared by mixing placebo with Medetomidine equivalent to 80%, 100% and 120% of the assay of highest test concentration. Sample solutions are prepared in triplicate for each spike level as described in the test preparation. The % recovery is calculated.

Robustness

To determine the robustness of the developed method, experimental conditions are purposely altered one after the other to estimate their effect. Five replicate injections of standard solution are injected under each parameter change. The effect of flow rate, column temperature and organic phase composition (acetonitrile) in mobile phase is studied by verifying tailing factor and %RSD for peak areas of replicate injections of standard. The flow rates of 1.0 mL/min and 1.6 mL/min, column temperatures of 38ºC and 42ºC, pH of the buffer 9.1and 9.3, and organic phase compositions (acetonitrile) in mobile phase at $\pm$ 10% along with the method conditions of 1.5 mL/min, 40ºC and 100% organic phase composition is studied.

Solution stability and mobile phase stability

The solution stability of test and reference standard is established by allowing solutions on bench top in a tightly capped volumetric flasks at controlled room temperature for 48 hours. The assay is determined for both test and reference standard solutions by using freshly prepared reference standard at 24 hours interval up to study period.

RESULTS AND DISCUSSION

Determination of suitable wavelength

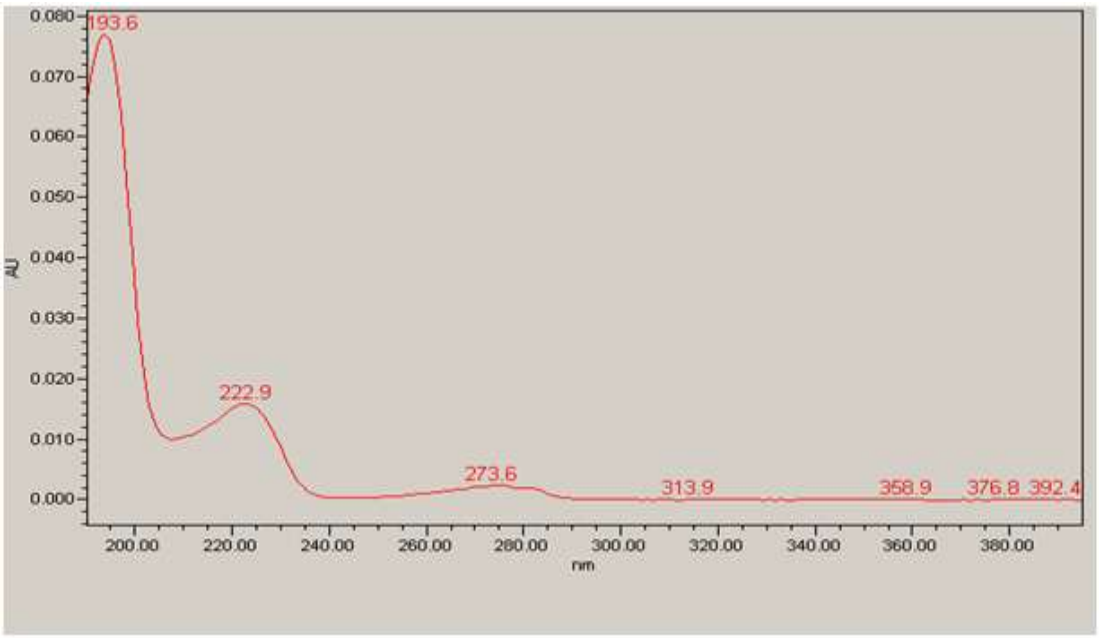


Fig. 3: UV Spectra of Medetomidine.

Optimization of chromatographic conditions

Assay method plays a major role in dosage form, to quantify the amount of analyte. The main target of the chromatographic method is to get the separation of all degradants of medetomidine without interfering with the main analyte peaks in single chromatographic conditions. An X-terra RP-18 (250X4.6)5 µm column is selected due to its high efficiency and suitability for polar moieties compared with other commercially available octadecyl silanized silica packed columns. Lower particle size column is used to achieve the better resolution. Key parameters like selection of buffer solution and its pH, organic modifiers in mobile phase are selected based on chemical characteristic of active moieties. Different mobile phase buffers are tried by changing different gradient programs. The chromatographic separation is achieved on X-terra RP-18 (250X4.6)5 µm column by following gradient program Time (min)/%B; 0/35, 5/35, 7/20, 10/20 and 14/35 using mobile phase combination of 10 mM ammonium chloride and pH adjusted to 9.0 with ammonia and the organic modifier was acetonitrile. The flow rate of the mobile phase is 1.5 mL/min, column is maintained at 40°C and wavelength detection is 220 nm. The injection volume is 20 µL and the data acquisition time 14 min. The typical retention time of medetomidine is 7.53 min. This method is capable to separate all degradants from its analyte peak within 15 min.

Method validation

Precision

Method repeatability (intra-day precision) is evaluated by assaying six samples, prepared as described in the sample preparation. The mean % assay value of Medetomidine is found to be 99.9, % RSD for assay values of medetomidine found to 0.51. These values are within the acceptable limits of between 97.0% -103.0% and %RSD not more than 2.0. The intermediate precision (inter day precision) is performed by assaying six samples on different HPLC systems and different HPLC columns in different days as described in the sample preparation. The precision and intermediate precision results are summarized below in Table 2.

Table 2: Result of precision of test method.

S.NO.	%Assay of Medetomidine	
	Intra - day Precision	Inter - day Precision
1.	100.1	102.4
2.	99.3	101.7
3.	100.1	102.0
4.	100.7	102.3
5.	100.0	102.1
6.	99.3	102.3
Average	99.9	102.1
% RSD	0.5	0.3

Linearity

A linear calibration plot for assay of Medeomidine is obtained over the calibration range of 80% - 120% of assay concentration and the correlation co-efficient is found to be 0.998 for Medetomidie. The graphical plots shown in Fig 4 indicates that a good correlation exists between the peak area and concentration of the analyte.

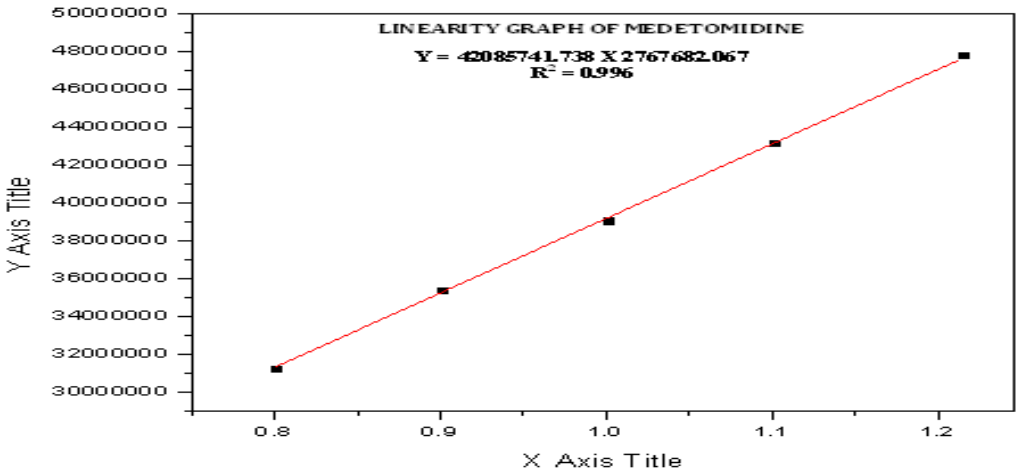


Fig. 4: Linearity graph of Medetomidine

Accuracy

The percentage recovery of Medetomidine in pharmaceutical dosage form shown in table 3 ranged from 98.8 to 99.7for Medetomidine which indicates the high accuracy of the method.

Table 3: Recovery results of Medeomidine

Spike level (%)	Average 'mg' added	Average 'mg' found	% Recovery	%Mean recovery	%RSD
80%	0.79656	0.7874	98.8	98.8	0.04
	0.79656	0.7868	98.8		
	0.79656	0.7868	98.8		
100%	0.9957	0.9916	99.6	99.7	0.11
	0.9957	0.9923	99.7		
	0.9957	0.9938	99.8		
120%	1.19484	1.1883	99.5	99.5	0.14
	1.19484	1.1872	99.4		
	1.19484	1.1905	99.6		

Robustness

In all the deliberately varied chromatographic conditions studied (flow rate, column temperature and ratio of mobile phase), the tailing factor and the % RSD for the Medetomidine peak areas for five replicate injections of standard is found to be within the acceptable limits of not more than 2 for tailing factor and not more than 2 for %RSD, the results are summarized in Table 4.

Table 4: Results of Robustness study

Parameter	Observed value		
	Variation	Tailing factor	% RSD for five injections of standard
Flow rate	0.9	1.2	0.17
	1.1	1.1	0.20
	1.5	1.1	0.26
Column temperature	38	1.2	0.35
	42	1.1	0.12
	40	1.1	0.31
pH	9.2	1.2	0.17
	9.3	1.1	0.18
	9.1	1.0	0.17

Solution stability and mobile phase stability

The difference in % assay of test and standard preparations upon storage on bench top is found to be less than 1.0 up to 48 hours. Mobile phase stability experiments showed that tailing factor and % RSD are less than 1.6 and 0.7 respectively up to 48 hours. The solution

stability and mobile phase stability experimental data confirmed that sample solutions and mobile phase used during assay determination are stable up to 48 hours.

Results of specificity studies

Placebo and stressed samples solutions are injected into the HPLC system with photodiode array detector as per the described chromatographic conditions. Chromatograms of placebo solutions have shown no peaks at the retention time of Medetomidine peak. This indicates that the excipients used in the formulation do not interfere in estimation of Medetomidine in capsules.

All degradant peaks are well resolved from Medetomidine peak in the chromatograms of all stressed samples. The chromatograms of the stressed samples are evaluated for peak purity for Medetomidine peak using LC Solution Networking software. For all forced degradation samples, the purity angle is found to be less than threshold angle for Medetomidine peak (Table 5). This indicates that there is no interference from degradants in quantification of Medetomidine in capsules. Thus, this method is considered "Stability indicating". The typical chromatogram and purity plots of all stressed samples are shown in Fig 5 to 9.

Table 5 : Specificity study results

Stress Conditions	Details of Stressed Drug product		
	% degradation	Purity Angle	Purity Threshold
Treated with 0.1 N HCI solution for 30 minutes at 60 °C	1.2	0.068	6.127
Treated with 0.1 N NaOH solution for 30 min at 60 °C	0.7	0.068	5.516
Treated with 3% H_2O_2 solution for 30 minutes at 60 °C	2.2	0.063	10.178
Exposed UV light (200 W h m^{-2})	0.6	0.055	5.834
Exposed to Heat for 15 hour at 105 °C	0.9	0.096	6.503

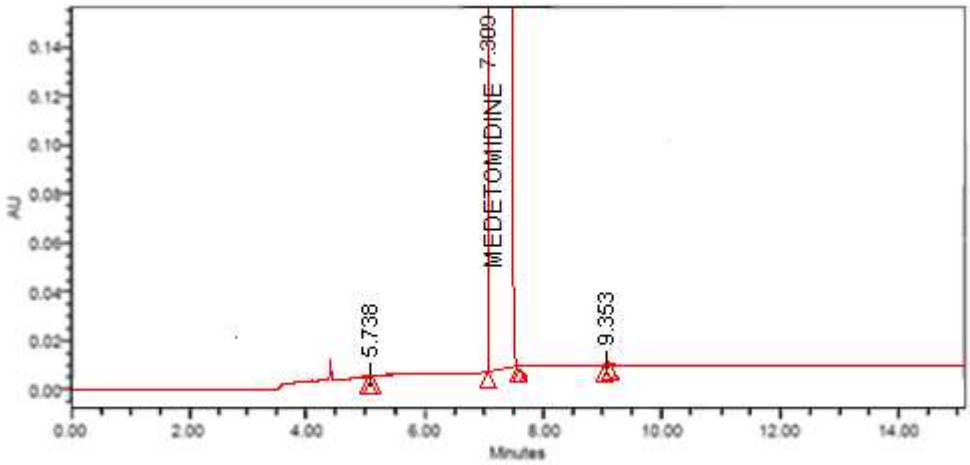


Fig. 5: A chromatogram of Medetomidine in Alkali degradation.

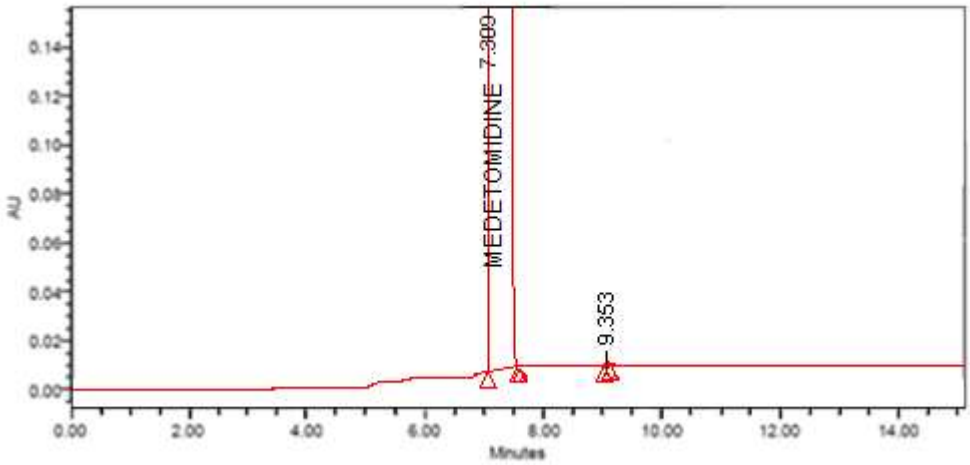


Fig. 6: A chromatogram of Medetomidine in Acid hydrolysis degradation.

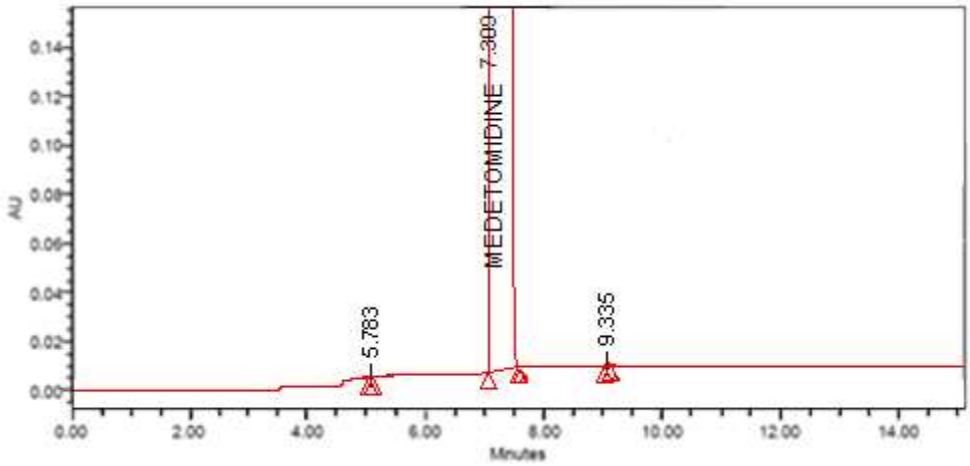


Fig. 7: A chromatogram of Medetomidine in Peroxide degradation

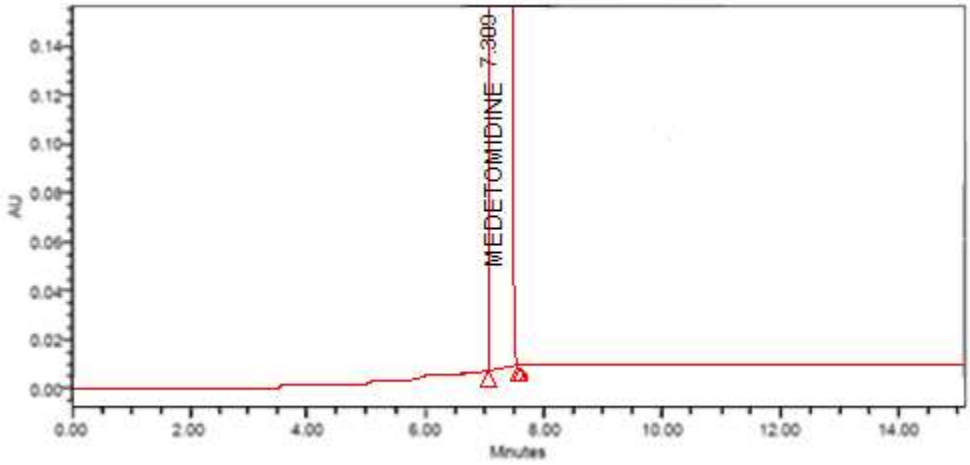


Fig. 8: A chromatogram of Medetomidine under heating.

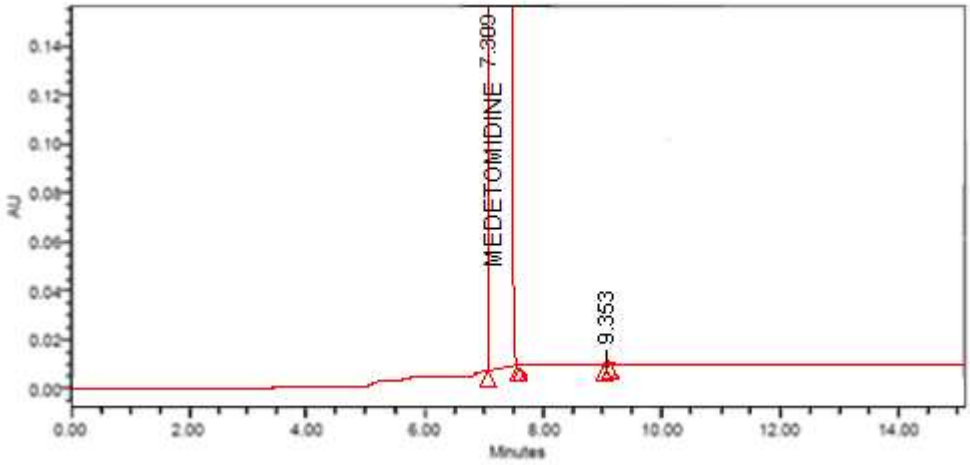


Fig. 9: A chromatogram of Medetomidine under Photolytic degradation

CONCLUSION

The validation study has been carried out as per the protocol. A review of the data compiled for various parameters shows that all the laid down acceptance criteria have been met. The method is specific, linear, accurate and precise over the range studied. No devation is observed during the complete validation activity. This method can be considered as validated and put to use for routine analysis of Medetomidine by RP- HPLC.

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