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Development and Validation of RP-HPLC Method for Determination of Related Substances of Medetomidine in Bulk Drug

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ABSTRACT

A rapid, reverse phase HPLC method has been developed for the determination of medetomidine and its related impurities. These impurities were isolated from crude sample of Medetomidine using reverse phase HPLC. The IUPAC names of impurities were Impurity-A is 4,5-dihydro-4-(1-o-tolylethyl)-1H-imidazole Impurity-B is 4-(2,3-dimethylbenzyl)-4,5-dihydro-1H-imdazole.The effective separation was achieved on an X-terra RP-18(250X4.6) 5Mm column using a gradient mode using two mobile phases A and B. 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 retention times of Medetomidine and its impurities are 18.57, 7.26, and 21.45 minutes respectively. The developed method was validated in terms of system suitability, specificity, linearity range, precision, accuracy, limits of detection and quantification for the impurities following the ICH guidelines. Therefore, the proposed method is suitable for the simultaneous determination of medetomidine and its two related impurities.

INTRODUCTION

Medetomidine ((RS)-4-[1-(2, 3-dimethylphenyl) ethyl]-3H-imidazole) is a synthetic drug used as both a surgical anesthetic and analgesic often used in the form of hydrochloride salt as Medetomidine hydrochloride. It is a crystalline white α_2 adrenergic agonist that can be administered as an intravenous drug solution with sterile water. It is currently approved for dogs in the Untied states and distributed in the United States by Pfizer Animal Health and by Novartis Animal Health in Canada under the product name Domitor. The marketed product is a racemic mixture of 2 stereoisomers; dexmedetomidine is the compound with more useful effects and is now marketed as Dexdomitor. The free base form of Medetomidine is distributed by the Swedish company I-Tech AB under the product name selektope for use as an antifouling substance in marine paints.

There were studies reported in the literature relating to metabolic studies for the Medetomidine in bulk drug. However, no stability-indicating RP - HPLC method for the quantitative estimation of Medetomidine in bulk drug sample along with its potential impurities was reported. The purpose of 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 [1-7]. The development and validation of RP-HPLC method for the determination of Medetomidine and its related impurities are as per ICH guidelines [8, 9, 10].The chemical structure of Medetomidine is shown in the Fig.1

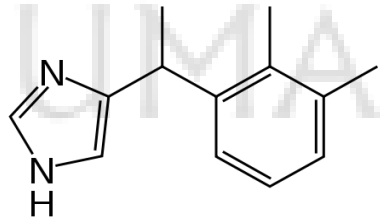


Fig.1. Medetomidine

MATERIALS AND METHODS

Instrumentation and software

SHIMADZU 2010 series prominence High performance liquid chromatograph with binary pumping, PDA system, with LC Solution software was used for the studies.

Chemicals and reagents

All the reagents were of analytical reagent grade unless stated otherwise. Distilled and deionized HPLC –grade water, HPLC-grade acetonitrile, ammonium chloride, ammonia and methanol were purchased from Merck, Mumbai. Samples of Medetomidine and its impurities are gift sample of Shakhty chemicals labs, Hyderabad, India

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 gradient program is given in Table - 1

Table: 1. Gradient program

Time(Minutes)	Solution A (%)	Solution B (%)
0.01	65	35
40	65	35
45	80	20
55	80	20
56	65	35
60	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 1.0 ml of this solution to 100.0 ml with diluent. Further, dilute 1.0 ml of this solution to 10.0 ml with diluents.

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).

Method validation

Validation of the developed method for the determination of medetomidine and the two impurities was performed according to the ICH guidelines with standards and bulk drug. Thus, system suitability along with method selectivity, specificity, linearity, range, precision (repeatability and intermediate precision), accuracy, limits of detection and quantification for the impurities are established as follows.

System suitability

The system suitability was conducted using diluted standard preparation and evaluated by injecting three replicate injections

Specificity

Specificity is the ability of analytical method to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, and matrix components. The specificity parameter of the method was performed by injecting diluent, standard preparation, sample preparation, sample spiked with impurities (impurity-A and impurity-B) into the chromatographic system by making three replicate injections.

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.

Precision

The precision of analytical method is usually expressed as the standard deviation or relative standard deviation (Coefficient of variation) of series measurements. The system precision was conducted using all the impurities spiked to Medetomidine and evaluated by making three replicate injections.

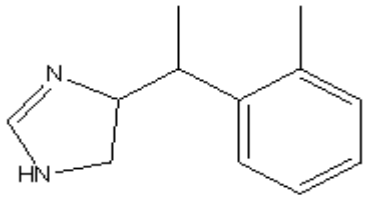
Accuracy

The accuracy of the method was determined by analyzing medetomidine sample solutions spiked with each impurity at three different concentration levels ranging from 50% 100% and 150%.

LOD and LOQ

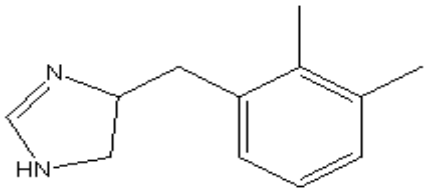
The LOD and LOQ were determined for medetomidine and for each of the impurities based on the standard deviation of (SD) of the response and slope (S) of the regression line as per ICH guidelines.

Impurity - A



4, 5-dihydro-4-(1-o-tolylethyl)-1H-imidazole

Impurity - B



4-(2, 3-dimethylbenzyl)-4, 5-dihydro-1H-imdazole

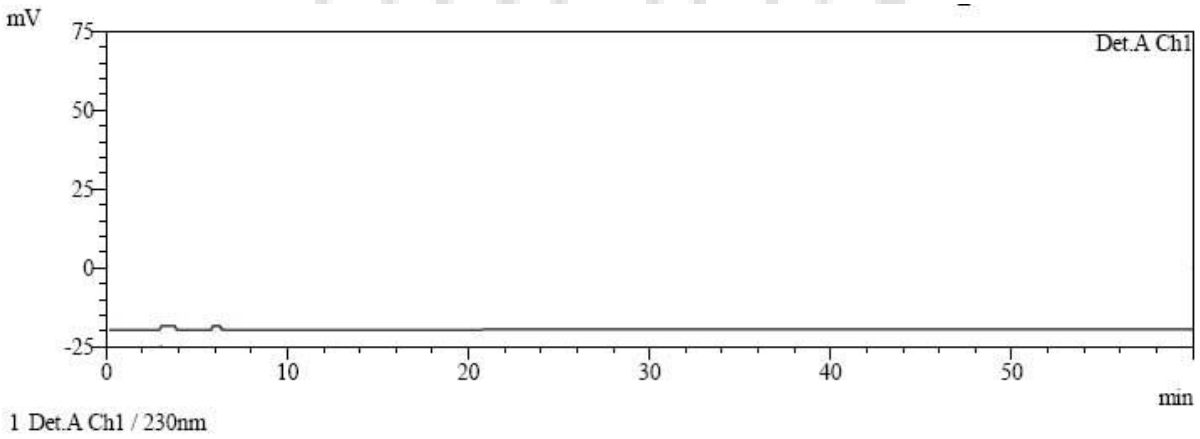


Fig.2 A Blank chromatogram of the Medetomidine.

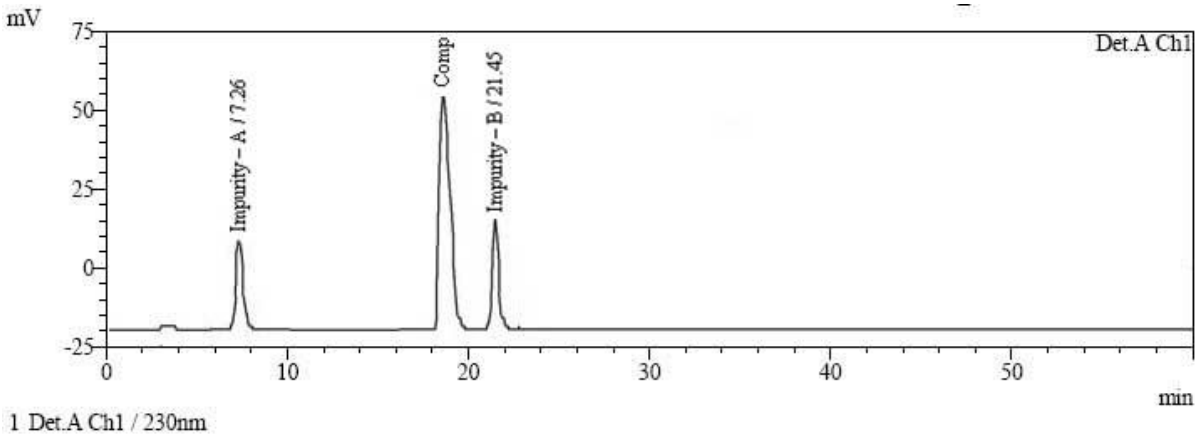


Fig.3 A chromatogram of the Medetomidine for selectivity

Table – 2. Summary of Relative Retention time of Impurities

S.No	Compound	RT (in minutes)	RRT
1	Medetomidine	18.57	1.00
2	impurity - A	7.26	0.39
3	impurity - B	21.45	1.15

RESULTS AND DISCUSSION

Optimization of chromatographic conditions:

The main target for the development of chromatographic method was to get the reliable method for the bulk drug and which will be also applicable to products. Initially, we took the effort for the development of HPLC method quantification of medetomidine from bulk. For this purpose we have used in ertsil ODS (250X4.6) mm, 5µ and unison (250x4.6) mm, 5µ column but peak shape was not good. Severe tailing was observed. Then we used X-terra RP-18 (250X4.6)5 µm column with mobile phase combination of 10 mm ammonium chloride and pH adjusted to 9.0 with ammonia and the organic modifier was acetonitrile. Peak shape is good but peaks of impurity-B and the main compound were merged. For this, we changed the organic modifier to a mixer of acetonitrile and methanol (50:50). Impurity -B peak was separated from the major peak but the peak shape was not good. Again we changed the organic modifier to mixer of acetonitrile: methanol (65:35). All impurities were separated from the major analyte peak and peak shape of the Medetomidine was slightly fronting. Because of this we again increased the strength of buffer to 10 mm of ammonium chloride

and pH was adjusted to 9.2.Then the peak shape was so good and all impurities were well resolved from the major analyte peak. Finally, the method was optimized in X-terra RP-18 (250x4.6) 5µ column with buffer of 10 mm ammonium chloride and pH was adjusted to 9.2 with ammonia. The organic modifier was mixer of acetonitrile: methanol (65:35). The gradient elution programme was 0.01/35,40/35,45/80,55/80,56/35,60/35 stop and flow rate was 1.5 ml/min.

Method validation

System suitability

The system suitability was performed by analyzing three replicate injections of a standard solution at 100% of the specifics limit with respect to the working strength of API. Results of peak area response and resolution for impurities are summarized in Table - 3

Table – 3. Summary of peak area response for impurity – A, impurity – B and Medetomidine

Sr.No	Area response		
	Impurity - A	**Medetomidine**	**Impurity - B**
1	58426	36684	124061
2	58115	35940	120985
3	58223	36335	120938
Average	58255	36355	121995
%RSD	158	374	1790
Retention time	7028	19.03	21.58
Resolution	0.0	24.82	3.86

Specificity

Each known impurity solution was prepared individually at the specification limit with respect to Medetomidine working concentration. Individual and combination solution of the impurities were analyzed to verify the retention times and specificity. Table - 4 summarizes the retention time and the resolution values obtained for all the impurities. The study showed that all the impurities were adequately resolved. Therefore the method is selected for the determination of impurity – A, impurity – B in Medetomidine.

Table – 4. Summary of retention time and the resolution values

Impurities	Retention time		Resolution
	Individual	Mixed	
Impurity - A	7.27	7.26	0.00
Medetomidine	--	18.57	24.59
Impurity - B	21.45	21.45	4.00

Limit of detection

The limit of detection (LOD) is defined as the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated. The limit of detection was determined as the lowest concentration for which the response is approximately three times greater than the baseline noise. The result obtained for each individual component (impurities) is summarized in Table - 5

Table -5. Summary of LOD data

Impurities	LOD With respect to slope conc.mg/mL	S/N Ratio
Impurity - A	0.00050028	232.1
Medetomidine	0.000164175	52.7
Impurity - B	0.0004917	181.8

Limit of quantification

Based on the limit of detection roughly three folds of detection solution was prepared and analyzed, the results are summarized in Table – 6

Table – 6. Summary of LOQ data

Impurities	LOQ With respect to slope conc.mg/mL	S/N Ratio
Impurity - A	0.001516	1143.8
Medetomidine	0.0004975	267.0
Impurity - B	0.00149	856.1

Linearity and range

Solution containing impurity – A, Medetomidine and impurity – B at concentration ranging from LOQ to about 150% of their specification value were prepared and analyzed as described in the validation protocol. The concentration and the peak area response obtained for each solvent are summarized in the following Tables 7.1, 7.2 and 7.3 and Figs. 4, 5 to 6 show the line of best fit peak area ratio verses concentration of each impurity.

Table – 7.1. Impurity – A

Sr.No	Concentration (% of level)	Concentration mg/mL	Average area response
1	LOQ	0.001516	34603
2	50%	0.001516	33193
3	75%	0.002274	47610
4	100%	0.003032	65170
5	150%	0.004548	97388
Slope			21008339.616
Intercept			1450.107
Correlation			0.999
R^2			0.998

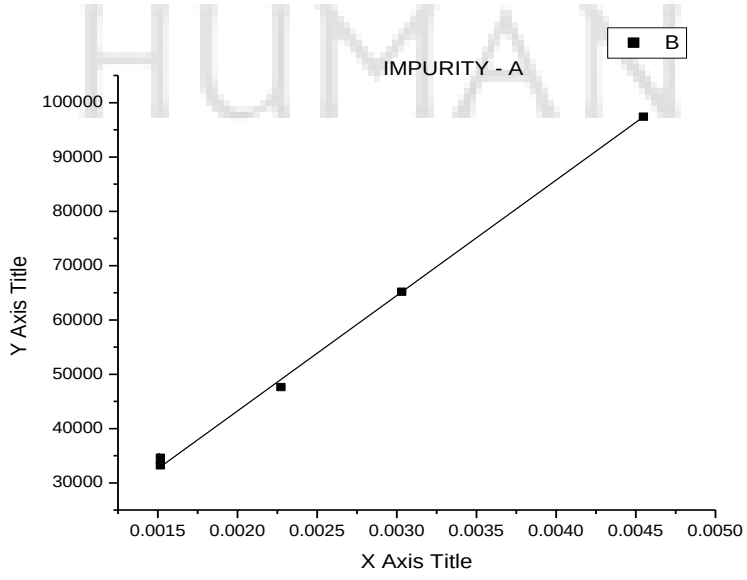


Fig. 4. Linearity curve graph: impurity – A

Table – 7.2. Medetomidine

Sr.No	Concentration (% of level)	Concentration mg/mL	Average area response
1	LOQ	0.0004975	19492
2	50%	0.0004975	19854
3	75%	0.00074625	28200
4	100%	0.000995	37958
5	150%	0.0014925	58357
Slope			38815003.589
Intercept			-55.589
Correlation			0.999
R^2			0.0099

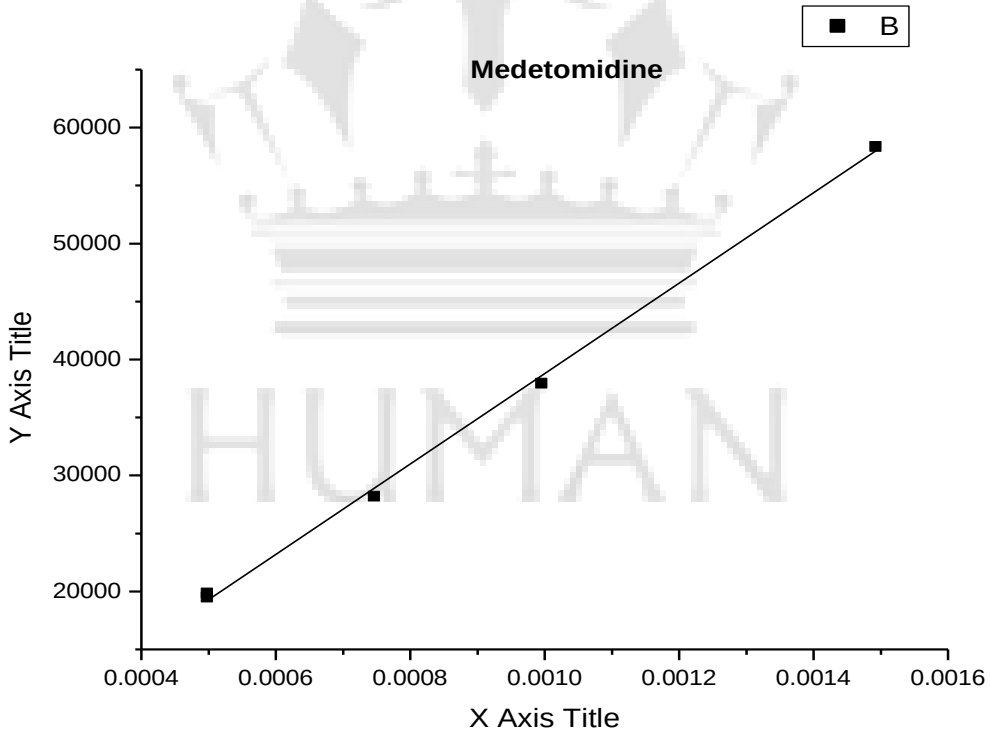


Fig. 5. Linearity curve graph: Medetomidine

Table – 7.3. Impurity - B

Sr.No	Concentration (% of level)	Concentration mg/mL	Average area response
1	LOQ	0.00149	64745
2	50%	0.001490	62112
3	75%	0.002235	91933
4	100%	0.00298	123873
5	150%	0.00447	185972
Slope			41644669.223
Intercept			1456.893
Correlation			1.000
R^2			0.999

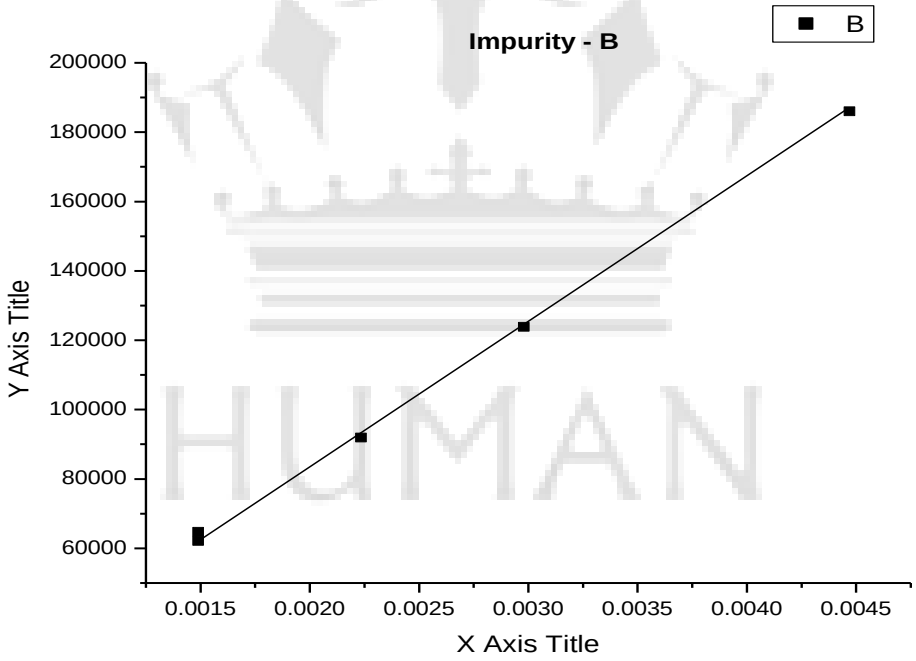


Fig. 6. Linearity curve graph: impurity – A

Accuracy

The accuracy of the method was determined using three solutions containing medetomidine sample spiked with the impurity – A, impurity – B at approximately 50% of the specification limit. Each solution was analyzed in triplicate. The percentage recovery obtained for each impurity is listed in Tables – 8.1 and 8.2.

Table – 8.1. Summary of % recoveries for impurity – A

Sr.No	Level	Theoretical Con. in mg/ml	Measured Conc. in mg/ml	% Recovery	Avg.% Recovery	%RSD
1	50%	0.001556	0.001437	92.4	92.4	0.08
		0.001556	0.011439	92.5		
		0.001556	0.001438	92.4		
2	100%	0.003112	0.002821	91.7	92.3	0.08
		0.003112	0.002873	92.3		
		0.003122	0.002876	92.4		
3	150%	0.004668	0.004537	97.2	97.3	0.14
		0.004688	0.004549	97.5		
		0.004688	0.004543	97.3		

Table – 8.2. Summary of % recoveries for impurity – B

S .No	Level	Theoretical Con. in mg/ml	Measured Conc. in mg/ml	% Recovery	Avg.% Recovery	%RSD
1	50%	0.00157	0.00145	92.2	92.3	0.41
		0.00157	0.00145	92.7		
		0.00157	0.00144	91.9		
2	100%	0.00314	0.00311	99.0	99.0	0.02
		0.00314	0.00311	99.0		
		0.00314	0.00311	99.0		
3	150%	0.00471	0.00471	100.1	99.5	0.82
		0.00471	0.00471	98.5		
		0.00471	0.00470	99.8		

Precision

System precision

The system precision was performed by six replicate injections of a standard solution at 100% of the specified limit with respect to the working strength of peak area of each impurity are summarized in Table - 9.

Table – 9. Summary of peak area response for impurity – A Medetomidine and impurity – B

Sr.No	Area response		
	impurity – A	**Medetomidine**	**impurity – B**
1	64559	39967	124275
2	64680	42513	124180
3	64602	42041	124214
4	64582	40314	124149
5	64645	41681	124265
6	64473	42306	124206
Average	64590	41470	124215
SD	72.1	1072.6	48.5
%RSD	0.11	2.59	0.04

Method precision

The method precision was performed by analyzing a sample solution of medetomidine at working concentration six times (six replicate sample preparation). Results of area response for each of the impurities are summarized in Table - 10

Table – 10. Summary of peak area response for impurity – A Medetomidine and impurity – B

Sr. No	Area response		
	impurity – A	**Medetomidine**	**impurity – B**
1	39695	40174996	114275
2	39667	38540610	114180
3	34179	38210182	114014
4	39767	40120825	114149
5	39436	38223725	114265
6	33919	38163147	124206
Average	37777	38905581	115848
SD	2891	971715	41.7
%RSD	7.65	2.50	0.03

Robustness

Table - 11 show the parameters of the method that were altered to test the robustness of the method. System suitability solution was analyzed to assess if these changes had any significant effect on the chromatography and the results. Results of RT, RRT for each impurity are summarized in below Table 11.1, 11.2, 11.3 and 11.4

Table – 11. Parameters of the method that were altered to test the robustness

Sr. No	Flow rate	Column temperature	Mobile phase – B (ACN:MeOH)	pH variation
Actual	1.00mL/mg	40^0C	700:300	9.20
Low	0.90mL/mg	38^0C	680:320	9.10
High	1.10mL/mg	42^0C	720:280	9.30

Table – 11.1. Summary of the results of flow rates

Flow rates	Impurities	RT	RRT	Resolution
0.90mL/mg	impurity – A	8.00	0.39	0.00
	Medetomidine	20.74	1.00	24.96
	impurity – B	23.95	1.05	4.35
1.10mL/mg	impurity – A	6.56	0.38	0.00
	Medetomidine	17.04	1.00	23.80
	impurity – B	19.69	4.20	4.20

Table – 11.2. Summary of the results of Column temperature

Column temperature	Impurities	RT	RRT	Resolution
38^0C	impurity – A	7.33	0.39	0.00
	Medetomidine	19.21	1.00	24.29
	impurity – B	22.23	1.16	4.30
42^0C	impurity – A	7.33	0.38	0.00
	Medetomidine	19.21	1.00	24.29
	impurity – B	22.23	1.16	4.30

Table – 11.3. Summary of the results of Mobile phase – B

Mobile phase – B	Impurities	RT	RRT	Resolution
680:320	impurity – A	7.47	0.38	0.00
	Medetomidine	19.84	1.00	25.33
	impurity – B	22.97	1.16	4.41
720:280	impurity – A	7.16	0.39	0.00
	Medetomidine	18.35	1.00	24.63
	impurity – B	21.18	1.15	4.38

Table – 11.3. Summary of the results of pH variation

pH variation	Impurities	RT	RRT	Resolution
9.10	impurity – A	6.95	0.40	0.00
	Medetomidine	17.52	1.00	23.46
	impurity – B	21.18	1.15	4.18
9.30	impurity – A	7.16	0.39	0.00
	Medetomidine	18.48	1.00	23.96
	impurity – B	21.30	1.15	4.17

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 deviations 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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